Review



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*Review*

# Applications of Electrochemical Biosensors in the Analysis of Biomarkers

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**Abstract**

Biomarkers are objective medical signals and can facilitate the diagnosis and monitoring of a multitude of diseases, including those whose symptomatology is largely subjective.Electrochemical biosensors offer an ideal platform for the application of emerging knowledge resulting from biomarker research. They combine the sensitivity of electrochemical detection techniques with the specificity of a biochemical reaction.In this review article, an introductory reference was made to the usefulness of biomarkers and the importance of their validation, to the problems encountered in the diagnosis of diseases, as well as to the structural characteristics and role of biosensors.ubsequently, applications of electrochemical biosensors for the determination of biomarkers from recent literature were presented, which were classified based on the biological mechanism of recognition of the sensor into enzymatic, immunochemical, DNA, other (biomimetic) and multipotent.

**Keywords:** electrochemical biosensor; biomarkers;point of care;detection;diagnosis

## Introduction

*Biomarkers*

Biomarkers serve as indicators of a medical condition, a term that encompasses processes related to the biology and physiology of organisms, irrespective of whether they are pathological or not. This includes both pathogenic processes and pharmacological responses. They must be measurable in a manner that is accurate, reproducible, and, most importantly, objective. This objectivity, along with their quantifiable nature, helps to distinguish them conceptually from the mere symptoms of a disease, as evidenced by their characterization as "medical signals" [1,2].

A diverse array of indicators is classified as biomarkers, ranging from fundamental chemical parameters to intricate mathematical models that link a medical condition with one or more measurable characteristics. Initially, they can be categorized into molecular, cellular, and imaging biomarkers [3]. Molecular biomarkers are those that are detected or measured in biological samples through methods that rely on their molecular properties. These can be further subdivided into those related to gene mutations or polymorphisms (i.e., nucleic acids) and those that arise from gene expression (such as peptides, lipids, and metabolites) [4]. In contrast to the aforementioned, imaging biomarkers do not necessitate direct sampling and instead assess alterations in the structure or function of systems (with the nervous system being a primary example) or tissues, employing techniques such as Positron Emission Tomography (PET), Computed Tomography (CT), and Magnetic Resonance Imaging (MRI) [2]. An alternative classification may be predicated on their biological characteristics: this encompasses molecular biomarkers, those associated with physiology (such as heart rate and blood pressure), and those pertaining to morphology (for instance, the shape and size of a tumor) [1,5]. Additionally, biomarkers can be categorized into two broader groups based on their intended application: either for evaluating the risk linked to exposure, whether acute or

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chronic (for example, the adverse effects of a drug), or for a specific disease, particularly in terms of its diagnosis, screening, and monitoring.

Biomarkers may indicate the pathophysiology and the mechanisms through which a disease progresses, either in part or throughout its entire range. They offer insights into disease etiology, help define and characterize disease stages, assist in diagnosis, forecast the progression of a disease regardless of the treatment administered, and evaluate treatment efficacy or possible adverse effects, as well as establish dose–response relationships for a therapy or pharmaceutical product. Certain biomarkers also facilitate the exclusion of diseases with similar clinical manifestations during the initial diagnostic phase. Additionally, they are utilized in monitoring and ultimately confirming disease remission [2,5,6]. Furthermore, they can aid in optimizing drug development, acting as endpoints in clinical trials, which in turn reduces costs and enhances efficiency [4].

Biomarkers can be assessed repeatedly, often, and typically through relatively straightforward and cost-effective methods [6]. In spite of the numerous potential biomarkers identified through proteomics, genomics, and other systems biology methodologies (including metabolomics, glycomics, and lipidomics), only a limited number have demonstrated true utility in wider clinical applications. The effectiveness of a biomarker is largely contingent upon the strength of its correlation with clinical outcomes. Ideally, this correlation should be articulated through a scale defined by reproducible cut-off points that correspond to various risk categories. At this juncture, variability should not be overlooked as a representation of disease heterogeneity across diverse populations, as such variation frequently constrains the generalized application of biomarkers for diagnosis or risk assessment.

This association is frequently founded on a computational model that may not accurately represent the underlying pathophysiological mechanism. In such instances, a biomarker can be regarded as a surrogate endpoint, offering enhanced certainty about the existence of a disease or the probability of its emergence. Conversely, a biomarker is deemed validated when its biological connection with the clinical outcome has been substantiated and mechanistically comprehended.

The extensive application of tests that have not been sufficiently assessed and validated can lead to significant repercussions, ranging from the squandering of healthcare resources to severe outcomes, such as erroneous diagnoses that may result in invasive monitoring of healthy individuals or patients with alternative conditions, as well as an unnecessary rise in morbidity due to unjustified drug treatments and therapeutic interventions [7,8]. In research environments or for internal decision-making within the pharmaceutical sector, the implications are typically less severe, and regulatory validation standards are less stringent. This has resulted in the development of the concept of “fit-for-purpose” validation [4,5]. Although this level of validation is comparatively less rigorous and less expensive, it must still guarantee the reliability and efficacy of the involved procedures, as inconsistent results may hinder comparisons across studies or, at later stages, their applicability to the wider patient population [7].The MarkerDB database is a remarkable effort to gather all known biomarkers, whether they are validated or in the experimental stage. Within it, biomarkers are categorized as diagnostic, prognostic, preventive, and exposure biomarkers, in addition to being classified as chemical, genetic, protein-based, or karyotype biomarkers.

## Gender Bias in Diagnosis

Gender bias in medicine can be defined as the systematic neglect of patients belonging to one sex, of parameters influenced by sex, or even of diseases that affect exclusively one sex, and it has direct consequences for diagnosis and treatment [10]. To describe bias in favor of men, for example in what are considered normal values of clinical endpoints as well as in subjective symptoms such as pain, the term *andronormativity* is also used [11]. A characteristic example of this is the use of male bodies as “neutral” models in anatomy textbook illustrations, since historically anatomical science itself has been based on dissections of male bodies [12].

Uterine fibroids, polycystic ovary syndrome (PCOS), and endometriosis are among gynecological diseases that, despite their severe impact on patients’ quality of life, have been actively

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and systematically ignored and underestimated [13]. For these conditions, uncertainty and lack of consensus prevail regarding pathogenesis and symptomatology, while known clinical indications are often overlooked, leading to misdiagnosis or delayed diagnosis [13–15]. In a relevant study on polycystic ovary syndrome involving women from North America, Europe, and other regions of the world, 47.1% of women had to consult three or more physicians, while 33.6% had to wait more than two years from the initial onset of symptoms to receive a diagnosis [16]. A corresponding study on endometriosis showed that diagnosis required an average of twelve years, with two-thirds of gynecologists citing the lack of a non-invasive test as the main reason for the delay [14]. It has also been observed that the likelihood of receiving a diagnosis increases when fertility problems arise, compared to other symptoms such as pelvic pain [17].

Pain as a subjective symptom is often questioned in women. Women are frequently perceived as exaggerating or fabricating their pain, or as being unreliable in their descriptions. The psychologization of pain and, in particular, the perception of pelvic pain as a liminal condition intrinsically linked to menstruation, fertility, and sexual health ("pain without an external cause is something natural for the female body" [11]) constitute significant reasons why related conditions remain insufficiently explained and why research into their diagnosis and treatment has been largely underfunded [11,13,15,18]. Indicatively, in the case of endometriosis, its characterization as a condition primarily affecting "career women" (i.e., women who do not prioritize motherhood), stemming from observations by Meigs in the mid-20th century, prevailed in the media and public perception at least until the early years of the new millennium, despite the existence of clinical and histological data demonstrating that this association does not hold [19].

Another issue that has emerged is the need for self-diagnostic tests in cases where the invasiveness of diagnosis may act as a deterrent for certain populations of women. Such tests must be accessible and available to women from all socioeconomic backgrounds, accompanied by clear instructions and appropriate counseling support. Their results must be scientifically valid, and their quality as medical devices must be assured [15].

The gaps identified in the diagnosis of gynecological medical conditions can therefore be summarized as the need for objective medical signals and reliable non-invasive diagnostic tests. These gaps can be addressed by investing in research on biomarkers associated with these conditions.

For endometriosis, blood biomarkers such as miRNAs, growth factors, and hormones are currently under investigation, with none having yet been validated. Due to the heterogeneity of the disease, the use of biomarker panels may facilitate diagnosis and allow for the determination of disease types and stages [14,20–22].

Uric acid has also been identified as a potential biomarker of dysfunctions of the female reproductive system: elevated levels are observed in individuals with polycystic ovary syndrome, gestational hypertension, preeclampsia, and chronic hypertension with superimposed preeclampsia. Serum uric acid levels above 393 μmol/L may predict pregnancy complications in women with preeclampsia or eclampsia, such as preterm birth, perinatal asphyxia, and intrauterine growth restriction [23]. Corominas *et al.* have proposed a uric acid ratio greater than 1.5 as indicative of an increased likelihood of developing preeclampsia [24].

For breast cancer, the proteomic markers CA125 and HER2 have been approved by the Food and Drug Administration (FDA) [25]. The cancer antigen CA125 is also widely used in the diagnosis of ovarian cancer; however, due to its lack of specificity, it is considered that in combination with other markers, such as follicle-stimulating hormone (FSH), it may yield more clinically useful results [26].

Finally, in the MarkerDB database, variants in the BRCA1 and BRCA2 genes are listed as biomarkers of hereditary breast and ovarian cancers, while for polycystic ovary syndrome the biomarkers include FSH (≤ 1.4 IU/L), sex hormone-binding globulin (SHBG; < 1.8 nmol/L), and anti-Müllerian hormone (AMH; > 3.8 ng/L) [9].

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*Biosensors*

Structure of Biosensors

Biosensors are devices that, as their name implies, utilize a highly selective biochemical mechanism toward a target molecule as their sensing element [27,28]. The recognition of the target molecule is based on this element, which consists of two parts: the molecular units that function as recognition receptors (which, according to the IUPAC definition [29], include isolated enzymes, immunosystems, tissues, organelles, or even whole cells) and the interfacial material where the biological event takes place [27,30].

Depending on the nature of the biological recognition, biosensors can be divided into two broad categories [31]:

1. **Affinity biosensors** (including antibodies, membrane receptors, and oligonucleotides), in which the receptors bind the analyte. The measurement is based on the thermodynamics of the interaction.
2. **Biocatalytic sensors** (mainly enzymes, cells, and tissues), which catalyze selective reactions leading to a measurable change in the amount of a product or reactant, or in another property of the solution, such as conductivity. The measurement is based on reaction kinetics.

Another classification of receptors can be made depending on whether they are natural (such as enzymes and antibodies), semi-synthetic (nucleic acids, aptamers), or synthetic (molecularly imprinted polymers) [32].

The chemical information is "translated" into a measurable signal by the signal transducer. Transduction involves the interaction of the analyte with the sensing system in a way that alters a physical property, which may be thermal, mechanical, optical, or electrochemical. It may also involve changes in concentration (signal amplification) and in the physicochemical properties (usually through labeling) of the analyte (or of another component, if the relationship between their quantities is well defined), so that it becomes detectable and quantifiable [27,28].

Labeling, as a practice, improves the sensitivity of a biosensor and leads to lower detection limits. However, it is more complex than direct recognition, slower, and often more costly. In addition, label molecules may be unstable (affecting the repeatability and lifetime of the biosensor) or, due to their structure, may create steric hindrance in certain reactions [33].

Another important structural parameter is the immobilization of the biorecognition element on the surface of the biosensor. This can be achieved through physical methods, such as adsorption onto insoluble surfaces via ionic interactions, hydrophobic forces, and van der Waals interactions, or through entrapment within vesicles, hydrogels, sol–gel matrices, polymeric micelles, and polyelectrolytes. This type of immobilization is advantageous due to the mild conditions applied and allows the preservation of biomolecular integrity in terms of both structure and functionality [30,34].

When stronger binding is required, immobilization can be achieved chemically through the formation of covalent bonds between functional groups of the biomolecule and those of the surface, through the use of a cross-linking agent such as glutaraldehyde, or through the formation of self-assembled monolayers (SAMs) [35].

*Electrochemical Biosensors*

In electrochemical biosensors, the signal transducers (usually the working electrodes) enable the electrochemical detection of the analytes. These systems ideally combine the high sensitivity of electroanalytical techniques with the specificity of the biorecognition element. They typically employ two or three electrodes [31,32]:

- **Working electrode (WE):** The site where the analyte reacts and is recognized.
- **Counter electrode (CE):** The electrode at which the current—usually corresponding to the signal—is measured. It is also in contact with the electrolyte, thereby completing the circuit.

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- **Reference electrode (RE):** Provides a stable and reproducible potential.

Since the reactions are detected at the electrode interface, construction parameters that significantly affect detection performance include [30]:

1. The electrode material
2. The electrode dimensions
3. The surface morphology or modification

Amperometric biosensors are commonly preferred due to their high sensitivity and wide linear range. They reach a steady state rather than thermodynamic equilibrium, and the rate-limiting step of the reaction is critical for measurement. They detect changes in analyte concentration near the electrode surface. Working electrodes are typically made of metals (gold, platinum, indium tin oxide) or carbon-based materials, often chemically modified. The presence of electroactive species in the sample matrix may lead to false-positive signals, requiring sample dilution or matrix modification. Other common strategies include the use of suitable electron transfer mediators and coating with conductive polymers.

Potentiometric biosensors operate under equilibrium conditions, and key parameters affecting the measurement include reaction kinetics and the magnitude of the equilibrium constant (k). Continuous response is required; therefore, the addition of supplementary reagents is often necessary. They can also function as affinity biosensors by detecting the formation of a biocomplex. These systems employ glass electrodes, gas-sensing electrodes, metal electrodes, and ion-selective electrodes.

Voltammetric biosensors are also widely used, measuring current as a function of the applied potential. An important advantage is their ability to simultaneously detect multiple analytes, which makes them suitable for multiplex biosensing applications [36].

Other measurement principles applied in electrochemical transducers include conductometry, electrochemical impedance spectroscopy, and field-effect transistor-based sensors, such as ion-selective field-effect transistors (ISFETs) and metal–oxide–semiconductor field-effect transistors (MOSFETs) [28,32,37].

*Applications of Biosensors in Diagnosis*

Many of the characteristics of electrochemical biosensors make them ideal platforms for the determination of biomarkers in disease prediction and diagnosis, as well as for monitoring therapeutic response and even the physiological functions of an individual:

- **High sensitivity** allows the detection of biomarkers at trace or ultra-trace levels, which is essential for the early diagnosis of diseases such as cancer.
- **High selectivity** toward a specific biomolecule, in this case the biomarker. In the case of macromolecules such as nucleic acids and proteins, certain issues may arise due to non-specific adsorption; however, these can be minimized through appropriate surface blocking or coating strategies (e.g., with albumin, casein, or other blocking agents).
- **Rapid detection**, often achieved in a single step (i.e., without sample pretreatment, washing steps, or additional reagents), and operation that does not require supervision by specialized personnel. Even more complex measurement protocols can be integrated, through appropriate automation, into compact and user-friendly devices.
- **Multiplex capability**, as advanced systems incorporating multiple working electrodes and different biorecognition elements can be used for the simultaneous determination of biomarker panels.
- **Miniaturization of analytical devices**, achieved through the use of capillary sampling systems and advances in nanotechnology and screen-printing techniques for the fabrication of innovative electrodes. These developments enhance device portability and biocompatibility while also improving the signal-to-noise ratio.

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Thanks to these characteristics, electrochemical biosensors are suitable for decentralized clinical applications such as self-testing devices, implantable systems, home-care devices, and, more generally, point-of-care (POC) measurements.

A characteristic historical example of such an application is the enzymatic Clark electrode for monitoring blood glucose levels in diabetic patients, invented in 1962. Its subsequent developments continue to dominate—if not research (since it is now a highly mature technology), then certainly the personal-use biosensor market [37–41].

An electrochemical biosensor must be sensitive and accurate, with wide linear ranges and low detection limits. To ensure selectivity, the influence of interfering substances present in the sample must be minimized through appropriate potential control, the use of electron transfer mediators, and surface modification or coating strategies that prevent non-specific binding. The device must remain stable both during operation and throughout storage periods. From one measurement to another, biosensors should exhibit minimal variability, provide repeatable and reproducible results, and allow surface regeneration for multiple uses.

For user-friendly operation without the need for specialized personnel, measurement protocols should be simple, as independent as possible from environmental conditions, and should not require complex sample pretreatment. For this purpose, many required cofactors should ideally be immobilized on the electrode together with the sensing element. In addition, analysis should be performed in real time, meaning that the response time (defined from the moment the sample is introduced into the device) should be short. Finally, the devices should be cost-effective and portable.

When clinical diagnosis requires invasive use of biosensors, they must be biocompatible, small in size, and free of toxic effects. If diagnosis involves the determination of biomarker panels, effective peak separation and high sensitivity toward each individual analyte are of critical importance.

Additional factors that make biosensors a reliable investment relate to the nature of the disease itself, particularly its chronic character and the need for regular monitoring of associated biomarkers [30–32,36,38].

*Enzymatic Biosensors*

Yang et al. immobilized uric acid oxidase on a cobalt-based metal-organic framework (UOx@MOF) for the electrochemical and ratiometric determination of uric acid (UA) [42]. Such sensors utilize the ratio of two signals (in this case, doxorubicin as the reference signal and uric acid as the response) for the quantitative determination of the target component, featuring built-in correction for non-specific interferences and providing accurate measurements [43]. The UOx@MOF was electrodeposited onto doxorubicin-loaded boron nanosheets to create a nanohybrid-modified glassy carbon electrode (GCE with UOx@MOF/BNSs-DOX). The encapsulation, which preceded the electrodeposition, was carried out via in situ co-polymerization of ZIF-67 MOF and UOx. Comparison of the cyclic voltammograms of the modified electrode versus the bare electrode revealed higher cathodic peaks for the former, highlighting the excellent electroactive activity of the boron nanosheets (Figure 2). The optimal conditions for obtaining square wave voltammograms (SWV) with high peak currents and high $I_{UA}/I_{DOX}$ ratios involved continuous incubation in phosphate buffer solutions (PBS) (1 mM, pH 7.4) containing the target for 20 minutes at 37 °C. Over a concentration range of 0.1–200 μM, a low limit of detection (LOD) of 0.025 μM, a good linear correlation between $I_{UA}/I_{DOX}$ and [UA] (Figure 3), and a low relative standard deviation (RSD) of 2.2% were obtained, confirming the selectivity, sensitivity, and stability of the biosensor.

.A portable, low-cost uricometer for the non-invasive monitoring of uric acid concentration was developed by Xing et al [44]. Monitoring was performed indirectly based on the measurement of the produced hydrogen peroxide and relied on the activity of the UOx enzyme (and the reduction of $Ca^{2+}$ ions which served as charge mediators), using an immobilization strategy in nanoflower structures (NFs). This strategy was shown to enhance stability (with a sensor lifetime of at least half a year), reusability, and specificity compared to the free enzyme. The UOx@NFs were directly drop-casted onto a gold nanoparticle-modified screen-printed carbon electrode (UOx@NFs/AuNPs/SPCE). Both

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amperometric and cyclic voltammetry determinations were studied. Cyclic voltammetry experiments were conducted at a scan rate of 50 mV/s, within a potential range of -0.2 V to 0.6 V. Amperometric detection was performed at +0.5 V vs. Ag/AgCl with a 300 s scan duration. Urine samples were collected in a centrifuge tube into which the uricometer was inserted, and the exact current value at 20 s was selected for quantitative analysis. Under optimal conditions, LODs of 0.82 μM and 8.87 μM were obtained for cyclic voltammetric and amperometric measurements, respectively, while the RSD did not exceed 7% for artificial samples and 10% for real samples.

Cancelliere et al. [45] compared the analytical characteristics and electrochemical properties of five amperometric uric acid biosensors based on the same enzymatic mechanism (using Prussian Blue as a charge mediator for the hydrogen peroxide oxidation reaction) and employing screen-printed electrochemical cells (graphite ink was used for the working and counter electrodes, and silver ink for the reference electrode). The differentiation between them involved the nanomaterials used for the modification of the working electrode, which were graphene nanoplatelets (GNPs) and multi-walled carbon nanotubes (pristine MWNTs, MWNT-COOH, -$NH_2$, and -OH). The modified electrodes exhibited peak currents two to seven times higher compared to the bare electrodes. Correspondingly, the chronoamperometric and cyclic voltammetric LODs were 420 nM and 3.4 μM, 213 nM and 2.2 μM, 2.2 nM and 1.2 μM, 8.3 nM and 0.9 μM, and 3.8 nM and 1.1 μM, respectively. Furthermore, the biosensors demonstrated satisfactory selectivity against interferents found in real urine samples, such as glucose and ascorbic acid.

Tvorynska et al. [46] developed innovative flow-injection amperometric biosensors featuring an enzymatic mini-reactor placed before a tubular detector of polished silver solid amalgam (TD-p-AgSA), with the latter serving as the working electrode (Figure 4). This approach was adopted to address capacity and stability issues and to eliminate potential interferences by separating the enzymatically catalyzed reaction from the electrochemical detection. Uric acid oxidases from Bacillus fastidiosus (UOx(b)) and Candida (UOx(c)) were covalently bonded to mesoporous silica powder in three different ways (via simple bonding to −$NH_2$ functional groups using glutaraldehyde as an activator, via EDC/NHS as a coupling reagent, and onto epoxy resin functional groups) and via physical adsorption onto four types of carbon powder (glassy carbon, graphite, mesoporous carbons $C_{mes}$–300C and $C_{mes}$–800) within the flow-cell reactor. In all cases, the measurement was based on oxygen reduction at a highly negative potential (-1100 mV vs. SCE), involving a four-electron transfer (two electrons in two steps) from the working electrode. The best performance was achieved by the first reactor, with an LOD of 18.5 μM, reusability, and stability lasting at least one year.

## DNA Biosensors

*Aptamers*

Khoshroo et al. [47] developed a paper-based aptasensor for the detection of breast cancer cells (MCF-7). A three-electrode system made of graphite ink was manually printed on wax paper using a patterned sticker, without the need for complex or expensive instrumentation (Figure 5). The ink was prepared by adding 2 g of cellulose acetate to 0.9 g of graphite powder and mixing until homogeneous. The working and reference electrodes were modified with gold nanoparticles (Au/P-apt) and silver ink, respectively. Recognition was based on the binding of mucin on the surface of the cancer cells to the thiol groups of the synthetic aptamers (5′-GCA GTT GAT CCT TTG GAT ACC CTG G-SH-3′) on the working electrode. As the number of cells increases, and consequently the size of the resulting complex, the peak currents of the differential pulse voltammograms decrease. Ultimately, an LOD of 7 cells·$mL^{-1}$ was obtained, along with a linear correlation between the peak currents and the logarithm of the MCF-7 cell count for a concentration range of 20 – $10^6$ cells·$mL^{-1}$, and recoveries in real samples ranging from 95.4% to 104.2%. Regarding selectivity, it was found to be good in the presence of HepG2, HeLa, and SKBR-3 cells. To evaluate reproducibility, measurements were performed with 7 aptasensors, and the RSD was acceptable (3.1 – 3.3%). The

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stability of the biosensor was also tested by storing it at 4 °C in a refrigerator for 21 days and was found to be acceptable, retaining 96.3% of its initial response.

Aptamers (conjugated via a thiol group at the 5′ end to gold nanocubes) were incorporated into a chitosan and hydroxyethyl cellulose hydrogel to create a biosensor for progesterone detection in blood samples [48]. The use of a hydrogel provides a suitable platform for the integration of biorecognition molecules and acts as a protective membrane, significantly limiting non-specific binding due to its hydrophilic properties. As shown by the cyclic voltammograms, the electron transfer of the redox probe decreases sequentially during modification with gold nanocubes and subsequently with the hydrogel. The biosensor's excellent selectivity for progesterone was demonstrated in samples containing structurally similar testosterone and cortisol. During square wave voltammetry experiments with a response time of 5 min, the limit of detection (LOD) was calculated at 1 ng·mL$^{-1}$, while the sensitivity was 0.78 μA·ng$^{-1}$·mL$^{-1}$. The decrease in response was indicative of the increasing progesterone concentration in 1000-fold diluted spiked blood samples.

Innovative DNA Sensor Architectures

Aptasensors combining vanadium disulfide ($VS_2$) nanosheets with hybrid cross-shaped and arched DNA nanostructures have been developed for the detection of MCF7 breast cancer cells [49]. A horseradish peroxidase (HRP)-based labeling strategy was employed, in which target cells competitively bind to the arched DNA, displacing the cross-shaped DNA and thereby suppressing the activity of biotinylated HRP. HRP catalyzes the reaction between hydrogen peroxide ($H_2O_2$) and hydroquinone (HQ), which generates the electrochemical signal. Consequently, increasing concentrations of cancer cells result in a progressive decrease in the electrochemical response.

Under optimized differential pulse voltammetry (DPV) conditions (pulse period: 0.2 s, pulse width: 50 ms, pulse amplitude: 50 mV), analysis of spiked PBS samples using an AuNPs/$VS_2$/GCE electrode achieved a limit of detection (LOD) of 5 cells mL$^{-1}$ and recoveries ranging from 90 to 103.2%. In real whole-blood samples diluted tenfold, recoveries decreased to 77.8–84.1%, likely due to interference from erythrocytes, leukocytes, and high protein content. The sensor exhibited good selectivity in the presence of SGC-7901, HeLa, A549, and L02 cells, as well as satisfactory reproducibility (six independently prepared sensors) and stability (storage at 4 °C for 10 days). Cytotoxicity was also evaluated by incubating cells with various concentrations of cross-shaped and arched DNA, followed by a CCK-8 assay and optical density measurements at 450 nm to assess cell viability.

A biosensor for the early diagnosis and monitoring of cervical cancer was reported based on recognition of the HPV16 oncogene using a Y-shaped DNA nanostructure [50]. Initially, a thiolated hairpin probe (H1) was self-assembled onto the electrode surface via Au–S bonds, and unoccupied sites were subsequently blocked with mercaptohexanol (MCH). Upon introduction of a sample containing HPV16, specific hybridization at the stem region of H1 induced hairpin opening. Sequential addition of hairpin DNAs H2 and H3 led to the formation of a Y-shaped nanostructure, enabling signal amplification through continuous catalytic elongation of the 3′ ends of the two arms. As a result, the peak current increased approximately sevenfold compared to that obtained with the MCH–H1/GE electrode.

Template-free elongation required the presence of a reaction mixture containing terminal deoxynucleotidyl transferase (TdT, 5 U μL$^{-1}$), dGTP (5 mM), and dNTPs (1 mM). Methylene blue, which preferentially binds to single-stranded DNA regions, was employed as the electroactive indicator in DPV measurements. Experiments were performed in 10 mM Tris–HCl buffer over a potential range of −0.5 to 0.1 V (pulse width: 0.2 s, pulse period: 0.5 s, pulse amplitude: 50 mV). Under optimized conditions, an LOD of 0.19 fM was achieved. For real-sample analysis, serum from healthy volunteers was treated with perchloric acid, centrifuged, diluted with buffer, and spiked with increasing concentrations of HPV16, yielding recoveries between 93.5 and 105.5%.

Figure 7 Schematic illustration of the development of the electrochemical biosensor for HPV16 oncogene detection.

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Malecka *et al.* combined aptamers and antibodies for the detection of the HER/neu protein complex, a well-established biomarker of breast cancer [51]. Target complexes were captured in a sandwich-type configuration using magnetic beads functionalized with either antibodies or aptamers and antibodies labeled with biotinylated cellulase. The magnetic beads were magnetically concentrated onto a graphite electrode coated with a nitrocellulose film and modified with methylene blue. Quantification was performed by chronocoulometry and relied on pore formation within the nitrocellulose film due to the hydrolytic activity of cellulase, leading to a decrease in electrode capacitance. The extent of film degradation correlated with HER/neu concentration over the range $10^{-15}$–$10^{-10}$ M. Detection down to 1 fM was achieved in both buffer and serum samples, with high sensitivity and minimal cross-reactivity.

HPV-16 detection was also achieved using a CRISPR–Cas-based electrochemical biosensing platform [52]. RNA-guided CRISPR–Cas proteins enable highly specific and sensitive recognition of target sequences, typically through collateral cleavage of nonspecific single-stranded DNA within a multidimensional DNA architecture. A 16-channel printed carbon electrode array was fabricated and modified with gold nanoflowers (NFs) to enable parallel sample analysis. Tetrahedral framework nucleic acids (FNAs), bearing three thiol groups, an extended HPV-16-complementary sequence, and a biotin label at the 5′ end, were immobilized via Au–S bonding. The ordered arrangement of tetrahedra minimized steric hindrance and electrostatic interactions, thereby reducing nonspecific adsorption. Additional blocking with casein and bovine serum albumin (BSA) further improved selectivity.

Two cleavage strategies were evaluated: sequential addition of target DNA and Cas12a/crRNA to the electrode, favoring cis cleavage, and premixing prior to electrode exposure, resulting in both cis and trans cleavage. The latter approach was selected due to its stronger amperometric response and simplified workflow. In the presence of single-stranded HPV-16, hybridization with crRNA triggered cleavage of the biotin-labeled extended arm of the tetrahedron. In the absence of the target, the intact biotinylated arm bound avidin–HRP, producing a strong current signal. Consequently, decreases in chronoamperometric peak currents were used for quantitative analysis.

Figure 8 (Left) Immobilization of biotin-labeled DNA tetrahedra and surface blocking with casein and BSA. (Right) cis and trans cleavage pathways of biotin-labeled DNA tetrahedra in the presence of Cas12a/crRNA [52].

## Immunosensors

Kalyani *et al.* [53] developed an electrochemical immunosensor for the determination of CA19-9, a potential biomarker of endometriosis. Magnetite nanoparticles and chitosan were incorporated into a dispersion of multi-walled carbon nanotubes (MWCNTs) and drop-cast onto a glassy carbon electrode (CS–MWCNT–$Fe_3O_4$/GCE). Cyclic voltammetry demonstrated enhanced electron-transfer capability of the modified electrode compared to bare GCE. Antibodies were immobilized onto chitosan via glutaraldehyde-assisted crosslinking, exploiting the reaction between the aldehyde groups of glutaraldehyde and the amino groups of chitosan. Detection was performed at 35 °C with an incubation time of 30 min, while the PBS pH was adjusted to 7.4 to ensure optimal antibody stability. Square-wave voltammetry over a concentration range of 0.001–100 ng $mL^{-1}$ revealed a concentration-dependent decrease in peak current, yielding an LOD of 0.163 pg $mL^{-1}$. The sensor exhibited satisfactory selectivity in the presence of CA125, AFP, and CEA, good reproducibility (four parallel electrodes), and stability over 15 days when stored at 4 °C in PBS. Analysis of real samples was carried out using serum from patients without recent pharmacological or hormonal treatment and no prior gynecological surgery.

In a subsequent study, the same research group [54] fabricated a GCE-based immunosensor by immobilizing glycodelin antibodies through Schiff base formation between lysine residues of the antibodies and glutaraldehyde, followed by crosslinking onto self-assembled monolayers of cysteamine. This biosensor was developed for the diagnosis and monitoring of endometriosis. Square-wave voltammetry revealed an inverse linear relationship between the logarithm of

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glycodelin concentration (1–1000 ng $mL^{-1}$) and the logarithm of peak current, with an LOD of 0.43 ng $mL^{-1}$. The sensor demonstrated effective regeneration for at least four cycles, satisfactory selectivity in the presence of CA125, CA19-9, and IL-10, good reproducibility (two parallel electrodes), and long-term stability for one month at 4 °C. In both immunosensors, $[Fe(CN)_6]^{3-/4-}$ was used as a redox probe, and results were validated against the ELISA reference method.

A gold electrode modified with copper, iron, and graphene oxide nanoparticles was developed for the detection of sex hormone-binding globulin (SHBG), a biomarker of polycystic ovary syndrome (PCOS) [55]. In PCOS, reduced SHBG levels increase testosterone bioavailability, elevating the risk of comorbidities. HRP-labeled SHBG antibodies were immobilized within a chitosan matrix. In the presence of $H_2O_2$ and hydroquinone as a redox mediator, HRP catalysis resulted in enhanced current signals. Amperometric measurements conducted in sodium phosphate buffer (pH 5.5) at 30 °C and a constant potential of 0.6 V yielded an LOD of 0.01 nM over a linear range of 0.01–250 nM. Analysis of serum samples revealed significantly lower SHBG concentrations in patients (100.01–190.03 μM) compared to healthy volunteers (410.01–1910.01 μM). The sensor exhibited rapid response (2 s), strong correlation with ELISA, and operational stability for up to 180 days when stored at 4 °C.

Bahavarnia *et al.* [56] designed a flexible immunosensor on photographic paper using silver nanoparticle/reduced graphene oxide (Ag–rGO) ink. Various patterns with different ink thicknesses were evaluated for conductivity, elasticity, and durability using an LED–battery setup, with a spiral design selected as optimal. After drying and hydrazine vapor treatment, gold nanoparticles and cysteamine were introduced. Cysteamine formed Au–S bonds with gold nanoparticles and interacted with Ag–rGO via its amino groups. The platform was applied to the detection of the ovarian cancer biomarker CA125 in human plasma. CA125 antibodies were immobilized using NHS/EDC chemistry, and nonspecific binding sites were blocked with albumin. Chronoamperometric detection in the presence of $[Fe(CN)_6]^{3-/4-}$ showed increasing current with increasing antigen concentration, yielding a low LLOQ of 0.78 U $mL^{-1}$ over a wide linear range (0.78–400 U $mL^{-1}$). However, limited sensor stability was identified as a drawback.

Biswas *et al.* [57] also targeted CA125 using a biosensor based on the *in situ* synthesis of a biocompatible zirconium–trimesic acid metal–organic framework (MOF-808) on activated carbon nanotubes. A GCE modified with the MOF-808/CNT nanocomposite served as a highly protein-affinitive platform for antibody immobilization, aided by streptavidin and chitosan. Differential pulse voltammetry revealed a concentration-dependent decrease in oxidation peak current of the $[Fe(CN)_6]^{3-/4-}$ redox probe due to mass transfer hindrance upon antigen–antibody binding. Two linear ranges were observed (0.001–0.1 and 0.1–30 ng $mL^{-1}$), with an LOD of 0.5 pg $mL^{-1}$. The sensor demonstrated good reproducibility, selectivity, and stability, and clinical applicability was validated using diluted serum samples, yielding recoveries of 97–103% and agreement within 10% compared to electrochemiluminescence immunoassay.

*Other Types of Electrochemical Biosensors*

Molecularly Imprinted Polymers (MIPs)

A molecularly imprinted polymer targeting CA125 was fabricated via electrochemical polymerization of pyrrole and applied as a highly selective biomimetic electrochemical sensor for ovarian epithelial cancer diagnosis and monitoring [58]. A gold printed electrode was first modified with a cysteamine self-assembled monolayer, followed by adsorption of CA125 as the template molecule and formation of a thin polypyrrole film using cyclic voltammetry. Subsequent removal of the template and excess monomer generated selective binding cavities. Square-wave voltammetry in the presence of $[Fe(CN)_6]^{3-/4-}$ enabled CA125 detection in synthetic serum with an LOD of 0.1 U $mL^{-1}$ and a linear relationship between current and the logarithm of concentration over the range 0.01–500 U $mL^{-1}$. The sensor exhibited good selectivity against CA153; however, limited reusability (two cycles) was noted, a common limitation of MIP-based sensors.

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Pareek *et al.* [59] developed a molecularly imprinted electrochemical biosensor for follicle-stimulating hormone (FSH) detection in serum for PCOS diagnosis. The MIP was synthesized on an ITO electrode modified with $NiCo_2O_4$/rGO by electrochemical polymerization of methyl methacrylate in the presence of FSH and ethylene glycol dimethacrylate as a crosslinker. After template removal, the resulting thin film contained recognition cavities complementary to FSH. Detection was performed by electrochemical impedance spectroscopy using Ag/AgCl and Pt as reference and counter electrodes, respectively. The biosensor exhibited high recoveries, minimal sample volume requirements (5 μL of blood), and a low LOD of approximately 0.1 pM.

*An Application in Human Milk*

A biosensor for monitoring changes in the oligosaccharide composition of human milk was developed based on the affinity of *Ulex europaeus* agglutinin I (UEA) for α-1,2-glycosidic linkages, primarily present in 2′-fucosyllactose (2′-FL) [60]. UEA was immobilized onto a gold working electrode via cysteamine. Electrochemical impedance spectroscopy was employed using Ag/AgCl as the reference electrode and Pt wire as the counter electrode. Real milk samples, initially diluted 1000-fold, were classified into secretors and non-secretors using a cutoff value of 410 μM 2′-FL. Secretor samples were further diluted tenfold for quantification. Although the analysis time (35 min) was four times shorter and significantly more cost-effective than HPLC, strong correlation between the two methods was observed. The sensor also demonstrated good reproducibility, stability, and a low LOD of 330.7 nM.

*Multianalyte Electrochemical Biosensors*

A label-free multianalyte immunosensor was developed for simultaneous determination of breast cancer-related antigens (CEA, CA125, and CA153) in serum [61]. Antibodies were immobilized within a chitosan matrix incorporating methylene blue as a redox mediator. The resulting cryogel was used to coat graphene-modified indium tin oxide electrodes (WE1, WE2, WE3). The biomarkers were successfully quantified with recoveries exceeding 91% using linear sweep voltammetry after a 15 min incubation period.

Pothipor *et al.* [62] designed a dual-function biosensor for the detection of CA153 and microRNA-21 for breast cancer diagnosis. Two printed carbon electrodes modified with poly(3-aminobenzylamine)/two-dimensional molybdenum selenide/graphene oxide were functionalized with gold nanoparticle dyes: 2,3-diaminophenazine for antibody immobilization and toluidine blue for DNA probe attachment. BSA and mercaptohexanol were employed to suppress nonspecific binding. Differential pulse voltammetry enabled quantification over a wide range, yielding LODs of 0.14 U $mL^{-1}$ and 1.2 fM for CA153 and miRNA-21, respectively, with recoveries above 100% and low RSD values. In both cases, increasing target concentration resulted in a decrease in peak current, with linear correlation between signal response and logarithmic analyte concentration.

Kuntamung *et al.* [63] reported a label-free multianalyte immunosensor for simultaneous detection of MUC1, CA153, and HER2 breast cancer biomarkers. Signal differentiation was achieved using three redox-active species—anthraquinone-2-carboxylic acid, thionine chloride, and $Ag^+$—conjugated to PEI-AuNPs corresponding to each biomarker. Square-wave voltammetry measurements revealed oxidation peak decreases upon antigen–antibody binding, yielding LODs well below clinically relevant levels.

Finally, Khanwalker *et al.* [64] characterized antibodies targeting fertility-related hormones (estradiol, FSH, and progesterone) to design a biosensor for hormonal monitoring during the menstrual cycle. Antibodies were immobilized on a gold disk electrode via 16-MHDA self-assembled monolayers activated with EDC–NHS chemistry. Quantification was performed using electrochemical impedance spectroscopy across a frequency range of 0.1 Hz–100 kHz, with optimal coupling frequencies identified for each hormone–antibody pair. Distinct frequency responses enabled multiplexed hormone detection. While FSH exhibited the broadest linear range, selectivity

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issues were observed in highly diluted whole blood samples, suggesting the use of Nafion membranes in future studies to reduce protein interference.

**Table 1.** Summary of the characteristics of the electrochemical biosensors under review, where: CV: Cyclic voltammetry, SWV: Square wave voltammetry, DPV: Differential pulse voltammetry, LSV: Linear sweep voltammetry, EIS: Electrochemical impedance spectroscopy.

| Analyte | Substrate | Mechanism | Electrochemical technique | Working electrode | LOD | $t_{απόκριση}$ | Ref |
|---|---|---|---|---|---|---|---|
| Uric acid | Blood serum and urine | Enzymic | SWV | UOx@MOF/BNSs-DOX/GCE | 0.025 μM | 20 min | [42] |
| Uric acid | Urine | Enzymic | Amperometry/CV | UOx@NFs/AuNPs/SPCE | 0.82 μM / 8.87 μM | 20 s | [44] |
| Uric acid | Urine | Enzymic | Amperometry/CV | UOx@GNP/SPE | 0.42 μM / 3.4 μM | 5 min | [45] |
| Uric acid | Urine | Enzymic | Amperometry/CV | UOx@MWNT/SPE | 0.213 μM / 2.2 μM | 5 min | [45] |
| Uric acid | Urine | Enzymic | Amperometry/CV | UOx@MWNT-$CO_2H$/SPE | 0.002 μM / 1.2 μM | 5 min | [45] |
| Uric acid | Urine | Enzymic | Amperometry/CV | UOx@MWNT-$NH_2$/SPE | 0.008 μM / 0.9 μM | 5 min | [45] |

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| | | | | | | | |
|---|---|---|---|---|---|---|---|
| Uric acid | Urine | Enzymic | Amperometry/CV | UOx@MWNT-OH/SPE | 0.004 μM / 1.1 μM | 5 min | [45] |
| Uric acid | Urine | Enzymic | Amperometry | UOx(b)@MCM41$_{(GA)}$ mini reactor coupled with TD-p-AgSA | 18.5 μM | 5 min | [46] |
| Uric acid | Urine | Enzymic | Amperometry | UOx(b)@GC$_{(phys.ads.)}$ mini reactor coupled with TD-p-AgSA | 13.7 μM | 5 min | [46] |
| Neoplastic cells MCF-7 | Blood serum | DNA aptamer | DPV | Apt@AuNPs/p-CE(ink) | 7 ± 1 cells.mL−1 | 3 h | [47] |
| Progesterone | Whole blood | DNA aptamer | SWV | Apt@AuNCs/CS-g-HEC$_{(hybrid\ hydrogel)}$/Au electrode | 1 ng mL$^{-1}$ | 5 min | [48] |
| MCF-7 | Whole blood | DNA aptamer | DPV | HRP/target cell/cruciform DNA/MCH/arch-DNA structure/AuNPs/VS2/GCE | 5 cells.mL$^{-1}$ | > 2 h | [49] |
| HPV16 | Blood serum | DNA (Υ σχήματος φουρκέτα) | DPV | Y-shape nDNA/Au electrode | 0.19 fM | > 80 min | [50] |
| HER-2/neu | Blood serum | Sandwich (antibody +antibody) / | Chronocoulometry | Ab $_{sandwich}$@MBs/Nit/Gr electrode | 1 fM | < 3h | [51] |

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| | | | | | | | |
|---|---|---|---|---|---|---|---|
| | | (aptamer+ antibody) | | | | | |
| HPV-16 | Blood serum | DNA | Chronoamperometry | SA-HRP/FNAs@AuNFs/(16-channel)SPCE | 100 fM | 3h | [52] |
| CA199 | Blood serum | Immunochemical | SWV | $Ab_{CA199}$@CS/MWCNT/$Fe_3O_4$/GCE | 0.163 pg $mL^{-1}$ | 30 min | [53] |
| Γλυκοδελίνη | Blood serum | Immunochemical | SWV | $Ab_{GLY}$@GHY/CYS/GDE | 0.43 ng $mL^{-1}$ | 30 min | [54] |
| SHBG | Blood serum | Immunochemical | Amperometry | $Ab_{SHBG}$@CHIT/CuNPs/$Fe_3O_4$NPs/GrONPs/Au electrode | 0.01 nM | 2 s | [55] |
| CA125 | Blood plasma | Immunochemical | Chronoamperometry | $Ab_{CA125}$/AuNP/CysA /p-Ag-rGO | 0.78 U $mL^{-1}$ | 1 h | [56] |
| CA125 | Blood serum | Immunochemical | DPV | $Ab_{CA125}$@SA-CHIT/MOF-808/CNT/GCE | 0.5 pg $mL^{-1}$ | 25 min | [57] |
| CA125 | Synthetic blood serum | MIP | SWV | $MIP_{CA125}$@Au-SPE | 0.1 U $mL^{-1}$ | – | [58] |
| FSH | Blood serum | MIP | EIS | $MIP_{FSH}$@$NiCo_2O_4$/rGO/ITO | 0.1 pM | – | [59] |

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| | | | | | | | |
|---|---|---|---|---|---|---|---|
| 2′–FL | Μητρικό γάλα | Συγγένειας (λεκτίνη) | EIS | UEA@CYS/Au electrode | 330.7 nM | 35 min | [60] |
| CEA, CA125, CA153 | Blood serum | Immunochemical | LSV | Ab/MB-CHI/GR/ITO | 0.04 pg $mL^{-1}$, 0.00004 U $mL^{-1}$, 0.00004 U $mL^{-1}$ | 15 min | [61] |
| CA153, miRNA-21 | Blood serum | Immunochemical | DPV | $Ab_{CA153}$@DAP-AuNPs/P3ABA/2D-$MoSe_2$/GO/SPCE, DNA@TB-AuNPs/P3ABA/2D-$MoSe_2$/GO/SPCE | 0.14 U $mL^{-1}$, 1.2 fM | 30 min | [62] |
| MUC1, CA153, HER2 | Blood serum | Immunochemical | SWV | AQ-$Ab_{MUC1}$/PEI-AuNPs/SPCE, TH-$Ab_{CA153}$/PEI-AuNPs/SPCE, $Ag^{+}$-$Ab_{HER}$/PEI-AuNPs/SPCE | 0.21 U $mL^{-1}$, 0.53 ng $mL^{-1}$ 0.50 ng $mL^{-1}$ | – | [63] |
| FSH, Estradiol , progesterone | Whole blood | Immunochemical | EIS | Ab@SAMs(16-MHDA)/GDE | 4.02 pg $mL^{-1}$ 2.35 pg $mL^{-1}$ 8.22 pg $mL^{-1}$ | – | [64] |

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## Conclusions

In the present article, the gaps that have been identified in the diagnosis of gynecological diseases were highlighted, which can be addressed by emerging biomarkers. It was found that electrochemical biosensors constitute an ideal platform for biomarker detection due to their combined sensitivity and selectivity, as well as their particular advantages, such as the ability to determine panels of biomarkers using multiplex biosensor configurations.

These studies investigated applications of electrochemical biosensors for the diagnosis and monitoring of conditions such as endometriosis, polycystic ovary syndrome, and cancers of the breast, ovaries, and cervix, as well as for monitoring the menstrual cycle through the determination of fertility hormones and the potential secretion of nutrients in breast milk. With regard to the determination of uric acid, this was not performed with the specific aim of monitoring conditions such as preeclampsia; that is, the proposed threshold concentration values and the components that may coexist in urine samples of affected individuals were not taken into account. This could constitute a subject for future research.

In the majority of the reports, biosensor development was based on nanomaterials [65–68]. In addition, innovative metal–organic frameworks, molecularly imprinted polymers, and paper-based lithographic electrodes were employed. Immobilization practices were followed to ensure the stability and functionality of the recognition biomolecules, such as immobilization in hydrogels, cross-linking on self-assembled monolayers (SAMs), and entrapment via copolymerization.

Regarding the electrochemical techniques applied for detection and measurement, in the case of enzymatic biosensors there was a clear preference for amperometric determination, which is based on changes in concentration near the electrode surface. An exception was one application in which the signal of a second component was also measured in order to correct for non-specific binding interferences, using square-wave voltammetry.

For the same reason—namely, the discrimination between signals from more than one component—in multiplex biosensors detection was carried out exclusively using voltammetric techniques and electrochemical impedance spectroscopy. As for their electrode configurations, discrete working electrodes were mainly selected, shaped according to the biomolecules to be immobilized. In another case, three different antibodies were immobilized on a single electrode surface via a self-assembled monolayer.

Overall, low limits of detection and wide linear ranges were obtained. In many cases, the selectivity of the devices (through interference studies), reproducibility (via the development of parallel sensors), and stability (after storage for a given number of days) were validated.

Some analyses were performed very rapidly, while others lasted several hours. Given that response time is defined from the moment the sample is introduced into the sensor, this is justified, since the latter category includes many cases in which the sample was added at an intermediate stage of biosensor development

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