Regular Article

ANALYTICAL
AND BIOANALYTICAL
CHEMISTRY
RESEARCH

Published by the
Iranian Chemical Society

***Anal. Bioanal. Chem. Res.**, Vol. 13, No. 1, 119-132, January 2026.*

Antibody-based Electrochemical Biosensors

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(Received 11 June 2025, Accepted 23 November 2025, Available Online 24 November 2025)

Electrochemical immunosensors are a type of biosensor that combines the principles of electrochemistry and immunology to detect specific biomarkers or analytes in a sample. These sensors typically consist of a transducer, a recognition element, and a signal processing unit. The role of the transducer is to transform the biochemical reaction occurring between the analyte and the recognition component into an electrical output. This can be achieved through various electrochemical techniques, such as amperometry, potentiometry, Voltammetry or conductometry. The recognition element is usually an antibody or an antigen that is immobilized on the surface of the transducer. When the analyte binds to the recognition element, it triggers an alteration in the electrical properties of the transducer, which is then measured as a signal. The signal processing unit is responsible for amplifying and processing the electrical signal to produce a measurable output. This output can be in the form of a current, voltage, or resistance, and can be used to quantify. The level of the analyte present in the sample. Biosensors based on Electrochemical Immunoassays have been widely used for the detection of various biomarkers, including proteins, hormones, and antibodies. They offer several advantages over traditional methods, including high sensitivity, specificity, and speed.

Keywords: Biosensor, Electrochemical, Immunosensor, Amperometry, Conductometry, Potentiometry

INTRODUCTION

The concept of ‘biosensor’ is derived from the fusion of two words: ‘bio,’ which pertains to biology or living organisms, and ‘sensor,’ which refers to a device or system that detects and reacts to stimuli. A biosensor is a complex analytical instrument designed to identify subtle changes within complex biological processes and convert these minor variations into recognizable electrical signals. This sophisticated device combines a biological sensing component that can interact with a range of biological materials like enzymes, tissues, microorganisms, cells, and acids, along with a cleverly engineered transducer. At the heart of the biosensor lies the cooperative function between this biological sensing component and the transducer, which plays a crucial role in converting biological information into measurable electrical signals [1]. Biosensors are analytical instruments used to identify and measure biological substances. By converting physical or chemical impulses into optical or electrical signals, which can then be examined to ascertain the analyte's existence and concentration, they accomplish biomolecule detection. A biosensor aims to deliver fast, precise, real-time, and trustworthy information regarding the target analyte. Additionally, biosensors can be designed to be extremely specific to a certain analyte, allowing for accurate detection, generally without interference from other substances present in the sample [2]. In a biosensor, a bioreceptor selectively recognizes and binds to the target analyte, subsequently interacting with it through a process known as biorecognition [3]. The primary elements of a biosensor system include the transducer and the bioreceptor [4]. Recent advancements in biosensor technology have predominantly centered around the immune response. The immune system functions by generating antibodies in response to foreign entities referred to as antigens. Due to their high specificity, antibodies are an

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excellent option for integration into biosensors. The role of antibody-based biosensors, commonly referred to as immunosensors, has been explored, highlighting their use in diagnostics, drug safety assessments, food safety monitoring, and environmental management. In summary, immunosensors play a crucial role in the food and healthcare sectors, thanks to their exceptional selectivity and sensitivity, which are utilized for various applications [3]. The development of biosensors is pivotal in accelerating clinical diagnosis, notably for disease detection [4]. Antibody-antigen interactions serve as the foundation for electrochemical immunosensors. In the construction of electrochemical immunosensors, immobilizing antigens on a transducer as a biorecognition element is crucial. Among the mentioned approaches, electrochemical deposition is a desirable and exciting method [5]. Electrochemical biosensors are the most developed presently and dominate the commercial market, where they have been applied in clinical and other fields [4]. Electrochemical sensors are primarily composed of three key components: the area for sample introduction, the transducer section, and the signal processing unit. The fundamental principles behind these sensors are based on conductometric, amperometric, and potentiometric measurements. To enhance sensitivity, electrochemical sensors are engineered by altering electrodes made from various materials. Typically, working electrodes incorporate materials such as gold, platinum, or glassy carbon [6,7]. In the construction of electrochemical biosensors, the non-conductive polymer layer serves a dual purpose, acting both as a substrate for immobilization and as a medium for transducing elements. Apart from the transducer layer, the selection of an appropriate bioreceptor is equally important for obtaining sensitive and effective biosensor operation, and conventionally, antibodies are employed due to their specific high-affinity binding. Electrochemical biosensing is the most abundant approach compared to the other biosensor categories, with amperometric biosensors being the most prominent technique, whilst voltammetric, impedimetric, capacitive, and potentiometric measurements are less frequently used [4]. It can be found that it is important to adopt a cheaper and more convenient testing technique, especially for use in developing countries, such as electrochemical methods. Electrochemical immunoassay is widely used in the field of disease diagnosis. In recent years, researchers have been focused on the development of electrochemical equipment with excellent performance and portability that can be used for on-site detection [8]. Due to their higher sensitivity and selectivity, electrochemical biosensors have lately emerged as a promising technique in the field of clinical diagnosis. They have also shown potential in developing a fast detection system for early diagnosis of diseases [6]. This paper explores the realm of electrochemical biosensors, with a specific focus on those employing antibodies as their foundational recognition element.

TYPES OF ELECTROCHEMICAL SENSORS

Amperometric Immunosensor

Amperometric immunosensors depend on the precise binding between an antigen and its matching antibody, making them highly effective tools for diagnostic analysis [9]. Amperometric immunosensors are bioelectronic devices that utilize antibodies or antigens as bioreceptors to detect target analytes at a specific potential by measuring changes in current. These changes are related to the interaction between the antigen and antibody, which is captured by an electrochemical transducer [10]. In amperometric immunosensors, the current density is measured by assessing the electrochemical reactions at a constant voltage [11]. Electrochemical immunosensors measure capacitance, potential difference (potentiometric), or current (amperometric). The most popular and practical technique for detecting biomolecules is current measurement [12]. Amperometric measurements are commonly used as an analytical method with high accuracy and sensitivity, where the applied voltage acts as a driving force for electrocatalytic redox reactions. These reactions produce electrical currents that are proportional to the concentration of the analyte [13]. Amperometric biosensors require a minimal amount of analyte, making them suitable for monitoring various analytes [11]. The majority of electrochemical immunosensors generate a current by the electrochemical oxidation or reduction of an electroactive molecule by keeping a constant voltage at the working electrode in relation to the reference electrode. This allows for amperometric measurements [14]. A controlled-potential system is essential for fundamental instrumentation in electrochemistry. This system typically consists of an electrochemical cell made up of two electrodes submerged in an appropriately composed electrolyte. A more advanced and

widely used design features a three-electrode cell, where one of the electrodes serves as a reference electrode. In this context, the working electrode is defined as the electrode at which the reaction of interest takes place. Conversely, the reference electrode such as Ag/AgCl or Hg/Hg_2Cl_2 is responsible for maintaining a constant potential in comparison to the working electrode [13]. In amperometry, a reducing or oxidizing potential is applied to the working electrode, and the concentration of the reduced or oxidized substances is directly proportional to the measured current [11]. The materials used include platinum, gold, glassy carbon, epoxy graphite, carbon, and carbon paste. Among these, gold is the most commonly used because it is a relatively inert metal that does not react with atmospheric oxygen. Additionally, gold is non-toxic and well-suited to cellular structures and biomolecules [10]. This method has biosensing characteristics comparable to other techniques, including response time, dynamic range, and sensitivity [11]. Only a limited number of label-free immunosensors have been created as amperometric devices, mainly because most antibodies and antigens lack electroactivity and therefore do not generate an amperometric signal. Therefore, in order to effectively detect biomarkers, the majority of amperometric immunosensors rely on electroactive labels or mediators [14]. The detection of various biomarkers is important for diagnosing different medical conditions. For example, recognizing the cancer biomarker known as vascular endothelial growth factor (VEGF) is essential [15]. The assessment of penicillin G and other beta-lactam antimicrobial agents. Detecting antigens for Mycobacterium tuberculosis is vital for tuberculosis diagnosis [16]. Additionally, measuring thyroid-stimulating hormone (TSH) levels is essential for assessing thyroid function [17]. Screening for biomarkers is also important for the early detection of heart failure, as well as for the determination of hepatitis B (HBV) and hepatitis C (HCV) infections [9]. The immunosensor was constructed by incorporating antibodies or antigens into a polymer-based membrane [16]. Immunosensors can be categorized as electrochemical, optical, or piezoelectric depending on the kind of transduction detecting method they employ. Direct and indirect are the two primary categories into which amperometric immunosensors fall. The only basis for the detecting mechanism in direct immunosensors is the interaction between antigens (Ag) and antibodies (Ab). On the other hand, indirect immunosensors utilize an additional tag, such as an enzyme or fluorescent molecule, to show whether a binding event has occurred. Proteins with electroactive amino acids, including tyrosine (Tyr) and tryptophan (Trp), or metalloproteins proteins with a metal core with reversible redox activity, are the main targets of direct immunosensors [12]. The amperometric immunosensor has both advantages and disadvantages. A major disadvantage is its dependence on an indirect detection method. Nevertheless, this drawback is compensated by its high sensitivity, demonstrated by a linear correlation between the immunosensor's signal and the analyte (antigen) concentration. In contrast, potentiometric systems exhibit a logarithmic relationship in their responses [14]. Many studies have demonstrated the effectiveness of amperometric immunosensors using antibody-electroactive probe conjugates in a sandwich assay format. These sensors typically involve an immobilized capture antibody that interacts with the target antigen, while antibodies conjugated with electroactive labels bind to distinct regions on the antigen. The oxidation and reduction changes associated with these labels facilitate precise quantification of the antigen concentration. Ferrocene stands out as a preferred electroactive label for antibody conjugation due to its rapid and reversible redox properties.

The amperometric immunosensor generally includes the following models: 1) the sandwich model and 2) the direct signal model, as outlined below.

1) Here, we will discuss specific examples of immunosensors that utilize a sandwich configuration in amperometric detection (as illustrated in Fig. 1).

In a significant instance of an immunosensor sandwich, DNA nano-pyramids were secured to the surface of a gold electrode. The ferrocene moiety within the Ab1-Atg-(FeC-Ab2) complex possesses enough flexibility to interact effectively with the electrode surface, thereby maintaining a strong current signal. In this system, the diagnostic agent is the anti-IgG antibody. A notable example of a sandwich immunosensor involves DNA nano pyramids immobilized on a gold electrode surface, which act as a scaffold for capturing anti-IgG antibodies. Upon binding to immunoglobulin G, the ferritin-labeled anti-IgG antibody produces significant electrochemical signals. These DNA pyramids provide attachment sites for capture anti-IgG antibodies *via* free-standing carboxyl groups. Furthermore, ferrocene-labeled anti-IgG antibodies elicit notable electrochemical responses upon interaction with IgG. In a

separate investigation, researchers developed a boronic acid derivative that spontaneously organizes on a gold electrode created using screen printing. This innovation was designed to detect anti-glycated hemoglobin by forming diol bonds. To enhance detection, antibodies targeting glycated hemoglobin were tagged with electroactive ferrocene and used in an immunoassay setup resembling a sandwich format. The electrochemical properties of three distinct ferrocene compounds were examined: Fc (1), which is ferrocene monocarboxylic acid, Fc (2), known as β-ferrocenyl-propenoic acid, and Fc (3) or 1,10-ferrocenedicarboxylic acid. Among these variants, Fc (3) emerged as the superior label due to its increased availability of carboxylic acid (COOH) groups for labeling purposes [15].

2) Various investigations have focused on developing electrochemical immunosensors that utilize amperometric detection by integrating antibody-electroactive probe conjugates in a simple signal output format to identify vascular endothelial growth factor (VEGF), a key biomarker linked to cancer. These sensors typically involve immobilizing VEGF-specific antibodies on electrode surfaces, enabling sensitive and specific electrochemical responses upon VEGF binding, which facilitates rapid and accurate detection even in complex biological samples. Ferrocene's oxidation current, which is used to gauge VEGF concentrations, decreases as a result of the interaction between VEGF and ferrocene-modified anti-VEGF antibodies. This direct electrochemical process, occurring at nearly 0.2 volts in phosphate-buffered saline (PBS), also facilitates the analysis of cardiac indicators like human cytokine interleukin 10 (IL-10) and creatinine kinase (CK). Another commonly used electroactive label for signal probing in immunosensors is the iron within the heme group of horseradish peroxidase (HRP). When antibodies are tagged with HRP, they can be immobilized directly onto the electrode surface, enabling effective redox reactions involving the iron center of the heme moiety. This iron undergoes cyclic oxidation and reduction, facilitating electron transfer processes that enhance the electrochemical signal and improve detection sensitivity (Fig. 2) [15].

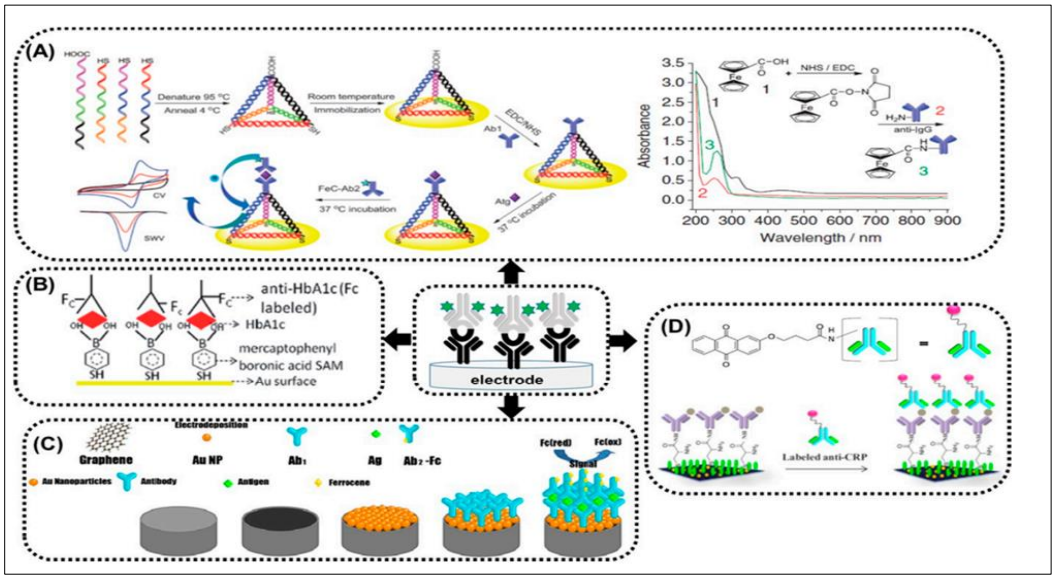

Fig. 1. Here are several instances of sandwich-type immunosensors that incorporate electroactive tag conjugates. (A) A schematic representation depicting the alteration of the gold electrode with DNA nano-pyramids (DPs) and ferrocene (fc)-conjugated anti-IgG, alongside the UV-Vis spectra for ferrocene carboxylic acid and anti-IgG, denoted as FeC-anti-IgG (Reprinted with permission from Yuan *et al.*). (B) A modification diagram for a gold screen-printed sensor that employs self-assembled monolayers of mercaptophenyl boronic acid in conjunction with ferrocene-labeled anti-glycated hemoglobin antibodies (reproduced with permission from Chopra *et al.*). (C) The construction diagram of the sandwich-type immunosensor features a composite of graphene and gold nanoparticles along with ferrocene-labeled secondary antibodies (Reprinted with permission from Wang *et al.*). (D) A schematic diagram illustrating the modification of screen-printed graphene electrodes utilizing anthraquinone-labeled antibodies specific to C-reactive protein (Reprinted with permission from Jampasa *et al.*) [15,18].

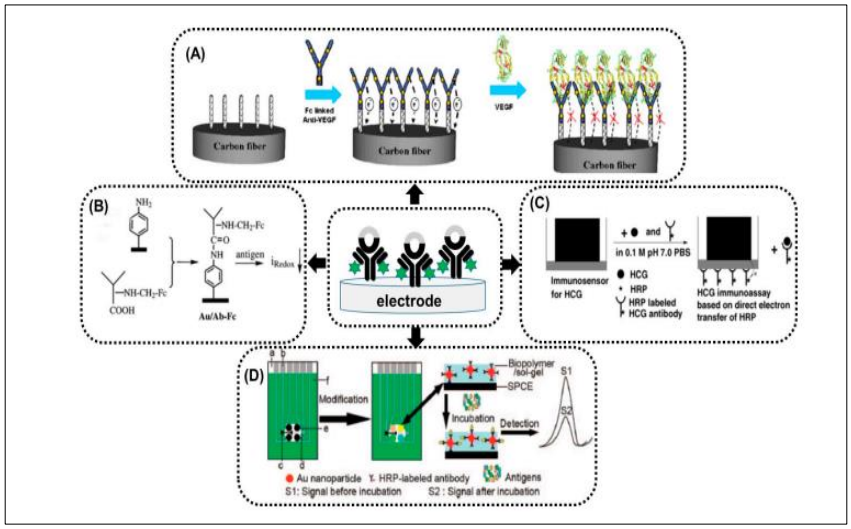

Fig. 2. Instances of immunosensors that employ direct signaling through electroactive label conjugates are illustrated as follows. (A) A simplified diagram illustrates the construction of an immunosensor where anti-VEGF antibodies tagged with ferrocene monocarboxylic acid are attached to carbon fiber microelectrodes using a Jeffamine cross-linker. This setup secures the antibodies on the electrode surface, with ferrocene serving as an electrochemical label for detecting VEGF. (Reprinted with permission from Prabhulkar *et al*.). (B) The method for creating an immunosensor featuring A gold substrate coated with a layer of polyaniline polymer and covalently attached ferrocenyl-labeled antibodies. (C) An immunosensor design featuring a glassy carbon electrode modified with a titania sol-gel, combined with an anti-HCG antibody tagged with horseradish peroxidase. (Reprinted with permission from Chen *et al*.). (D) A diagram illustrating an immunosensor array made of a biopolymer/sol-gel membrane combined with gold nanoparticles functionalized with horseradish peroxidase (HRP)-tagged antibodies. (Reprinted with permission from Wu *et al*.) [15,19].

An amperometric immunoassay sensor was developed for the discovery of thyroid-stimulating hormone (TSH). This immunosensor utilized an electrode made of glassy carbon (GCE) that has been altered with an electroactive azo compound. The azo compound served dual functions: it acted as a platform for the immobilization of anti-TSH antibodies and also functioned as a redox probe.Anti-TSH antibodies were attached to the azo platform using EDC/NHS (1-ethyl-3-(3-dimethylaminopropyl)carbodiimide/N-hydroxy-succinimide) chemistry. An amide bond was formed between the amino group of the antibody and the carboxyl group of the azo dye. In the presence of the TSH antigen, a film of the azo compound was deposited on the surface of the GCE, leading to a decrease in the electrochemical signal. This decrease was proportional to the amount of TSH antigen present in the serum sample. The biosensor showed a low detection limit of 0.04 μIU ml^{-1} and a broad linear detection range of 0.2 to 20.0 μIU ml^{-1} [17]. The antibodies possess regions with varying affinities that must be appropriately positioned to keep the Fab (fragment antigen-binding) area accessible for antigen recognition [10]. A poly(o-phenylenediamine) film, gold nanoparticle amplification, and ferrocene monocarboxylic acid as a label were combined to create a very sensitive immunoassay technique. Immunoglobulin G (IgG) is the model analyte used in this procedure. The method effectively measured human chorionic gonadotropin levels in clinical samples through the oxidation of ferrocene monocarboxylic acid attached to a secondary antibody. Additionally, employing a sophisticated flow-based immunoassay method, ferrocene monocarboxylic acid was essential in determining hemoglobin A1c and human chorionic gonadotropin [12].

Voltammetry Immunosensors

Voltammetric immunosensors are advanced analytical devices that utilize electrochemical techniques for the detection and quantification of specific biomolecules, such as cancer biomarkers and viral proteins. These sensors are gaining traction due to their high sensitivity, rapid response times, and potential for point-of-care testing [20].

Voltammetric immunosensors typically operate by measuring the current response generated during an electrochemical reaction that occurs when an analyte binds to a specific antibody immobilized on an electrode surface. The variation in current is directly related to the concentration of the target substance. Commonly utilized materials include screen-printed electrodes (SPE), glassy carbon electrodes modified with nanomaterials (*e.g.*, gold nanoparticles, graphene), and other conductive materials that enhance electron transfer and sensitivity. Antibodies or antigens that are tailored to the specific target analyte are fixed onto the surface of the electrode. This level of detail enables accurate identification of biomarkers within intricate biological specimens [21]. Recent research has highlighted the application of voltammetric immunosensors in identifying breast cancer biomarkers, including CA 15-3. These sensors utilize electrochemical techniques to enhance the detection capabilities for specific cancer-related proteins, facilitating Prompt diagnosis and ongoing surveillance of the illness. The sensitivity of these sensors is remarkable, achieving detection limits of 0.56 U ml^{-1} in both human serum and saliva specimens. The binding affinity between anti-CA 15-3 antibodies and the CA 15-3 protein is used by immunosensors designed to measure CA 15-3 quantities. Many electrochemical immunosensors designed to identify CA 15-3 have been documented in scientific journals throughout the last ten years. The transducer has undergone a number of changes to improve stability, conductivity, and overall electrochemical functioning. Among other things, these enhancements include the use of nanoparticles, mercaptosuccinic acid, nanocomposites, nitrogen-doped graphene sheets, poly (glutamic acid), and composites composed of copper sulfide and reduced graphene oxide. Redox-active species that contribute to the electrochemical signal generation, such as potassium ferrocyanide, potassium ferricyanide, ferrocene, and catechol, are typically involved in the detection of CA 15-3 [22].

Construction of the Immunosensor and Fabrication of the SPE

Following established protocols, graphite and silver inks were used to manufacture the screen-printed electrode (SPE). The Turkevich method was used to create gold nanoparticles (AuNPs), which were identified by a color shift from bright yellow to red. 30 µl of the produced AuNPs were then applied to the SPE surface and allowed to dry. After six hours of antibody incubation (2.0 µg ml^{-1}), the platform was treated for one hour with bovine serum albumin (BSA) to prevent non-specific binding. Phosphate-buffered saline (PBS) was used to wash and dry the sensor after each procedure. A solution of Cancer Antigen 15-3 (CA 15-3) was used to incubate the resultant immunosensor (SPE/AuNP/Ab/BSA). Differential pulse voltammetry (DPV) was used for electrochemical measurements in order to ascertain the correlation between the electrochemical response and the concentration of CA 15-3 (Fig. 3) [23,24].

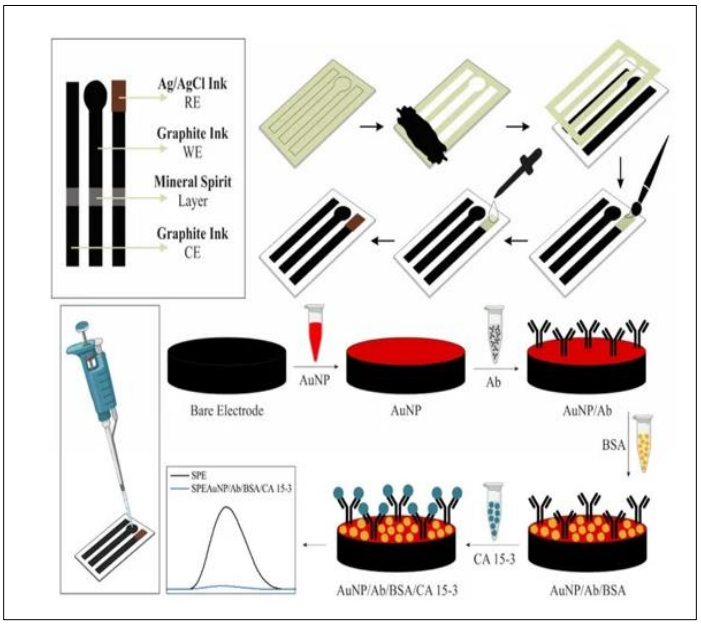

Fig. 3. (A) Fabrication and (B) modification. The study involved modifying a screen-printed electrode (SPE) with gold nanoparticles (AuNPs). A monoclonal antibody targeting CA 15-3, along with bovine serum albumin (BSA), was utilized to develop an electrochemical biosensor for detecting CA 15-3. The surface modification involved applying these modifiers to the electrode using a micropipette. After exposure to CA 15-3, a notable reduction in the electrochemical response was observed, as evident from the recorded voltammogram [22].

Voltammetric immunosensors have also been developed for detecting COVID-19 spike proteins, achieving detection limits from 0.20 to 0.54 ng ml^{-1} across different sample types (saliva, urine, serum) [25].

Point-of-Care Testing (POCT)

The integration of microfluidic platforms with voltammetric immunosensors enhances their applicability in POCT, allowing for simultaneous detection of multiple analytes with minimal sample volume [20,26].

Conductometric Immunosensor

Conductometric sensors operate on the principle that many biochemical reactions in solution cause changes in electrical resistance (or reciprocal conductance) due to variations in ionic compounds. Conductance measurements typically involve determining the resistance of a sample solution between two parallel electrodes. Biosensors that utilize the conductometric principle appear to offer several advantages in various aspects: 1) Noble metals can be swapped out for less expensive alternatives, and thin-film electrodes are well-suited for mass production and downsizing through economical means. 2) They have no light sensitivity, don't need a reference electrode, and can use a low operating voltage to significantly reduce power usage. 3) A wide range of different analytes can be determined through various reactions and mechanisms. Conductometric immunosensors measure electrical conductivity at a constant voltage. They assess the current generated by the ion exchange that occurs during specific reactions, such as enzyme reactions [27]. Conductometric biosensors leverage the relationship between conductivity and the biorecognition phenomenon. Two metal electrodes at a certain distance apart make up a conductometric biosensor. Silver and platinum are common materials used to build electrodes. The continuous flow of electrical current between these electrodes is made possible by applying an alternating voltage to them. The ionic composition shifts during a biorecognition event, and the conductivity between metallic electrodes is measured by an ohmmeter (or multimeter). This technique's limited sensitivity is a major disadvantage when compared to other electrochemical approaches [28,29].

Detection Mechanism

Conductometric immunosensors work on the basis of identifying alterations brought about by a target antigen binding to its complementary antibody that is fixed on the sensor's interface. A recent investigation developed a conductometric immunosensor designed to detect Escherichia coli O157:H7 by utilizing a nanocomposite film composed of polyaniline and zinc oxide (PANI/ZnO) applied onto a gold electrode. This sensor demonstrated a detection range of 10 to 10^4 CFU ml^{-1}, with a minimum sensitivity of 4.8 CFU ml^{-1} in peptone water. Electrochemical biosensors have gained popularity for E. coli O157:H7 detection, offering advantages like increased specificity, reasonable detection limits, affordability, portability, rapid response, simplicity, and the option for nanomaterial modifications [30]. Conducting polymers, particularly polyaniline (PANI), are frequently used in electrochemical sensors due to their electroactivity and conductivity, which amplify sensitivity. PANI also stands out for its compatibility with biological molecules, eco-friendliness, and ease of preparation.

Enhancing PANI's performance is achievable by incorporating metal oxides like gold (Au), silver (Ag), titanium dioxide (TiO_2), zinc oxide (ZnO), or carbon nanostructures, which improve electrical affinity and conductivity while reducing the composite's ionization potential. Chowdhury *et al.* Using the unique interaction between antibodies and antigens, a label-free impedimetric sensor based on Au/PANI was created to detect E. coli O157:H7. Nevertheless, this sensor demonstrated a detection threshold of 102 CFU ml^{-1}. Additionally, Pangajam, Theyagarajan, and Dinakaran advanced the field by creating an electrochemical sensor aimed at identifying E. coli O157:H7 DNA in water samples [31]. This sensor achieves a detection limit of 1.3×10^{-18} M by using a surface-printed carbon electrode (SPCE) modified with a composite of carbon dots (CD), ZnO nanorods, and PANI. The screen-printed carbon electrode's (SPCE) improved electrical conductivity helps explain why this sensor is more sensitive than existing electrochemical DNA sensors. Within 70 min, Setterington and Alocilja were able to identify as few as 7 CFU of E. coli O157:H7 in phosphate-buffered saline (PBS). Their approach combined biofunctionalized magnetic nanoparticles (MNPs) coated with polyaniline (PANI) and immunomagnetic separation (IMS) with an SPCE sensor. The linear detection range of the sensor in PBS was 10^1 to 10^5 CFU ml^{-1}. Nevertheless, the study did not test the sensor's performance in food matrices and lacked specificity evaluations, including mixed bacterial populations. Zinc oxide (ZnO) nanostructures have attracted considerable interest for biosensor applications because of their distinct optical, piezoelectric, and semiconducting characteristics, as well as their capacity to bind various biomolecules. Moreover, ZnO nanostructures demonstrate excellent stability at physiological pH, ensuring their compatibility for

in vivo use. Nevertheless, ZnO nanoparticles exhibit notable antimicrobial properties by releasing Zn^{2+} ions that generate reactive oxygen species (ROS), which can hinder their effectiveness in capturing bacterial cells. Recent advancements in nanotechnology have enabled the creation of nanocomposites consisting of metal oxides within polymeric materials to form new chemical bonds that minimize interference with bacterial cells [32-34].

The morphological characteristics of nanomaterials were examined through scanning electron microscopy (SEM). The ZnO nanoparticles had an average diameter of 100 nm, as seen by the SEM images in Fig. 4a. On the other hand, the PANI/ZnO nanocomposite's SEM images, which exhibit bigger particles, are shown in Fig. 4b. This observation implies that polyaniline (PANI) has entirely encased the ZnO nanoparticles, producing ovoid-shaped particles with a diameter of about 200 nm (Fig. 4).

XRD Analysis

The diffraction profile obtained from X-ray analysis (XRD) of ZnO shows narrow and sharp peaks at 2θ values of 31.44, 34.10, 35.94, 47.22, 56.28, 62.54, 66.10, 67.64, and 68.78 degrees. The presence of these peaks reveals that ZnO has a hexagonal wurtzite configuration and verifies its crystalline nature. The X-ray diffraction (XRD) analysis of polyaniline (PANI) reveals broad and diffuse peaks at approximately 2θ values of 18.8° and 25°, indicating its amorphous characteristics. In contrast, the PANI/ZnO nanocomposite's XRD pattern resembles that of PANI with slight variations and heightened intensity at 2θ values of 20.4° and 25.56°. This alteration is attributed to the presence of ZnO nanoparticles, which are present in quantities below the detection threshold of the diffractometer (Fig. 5a) [36,37].

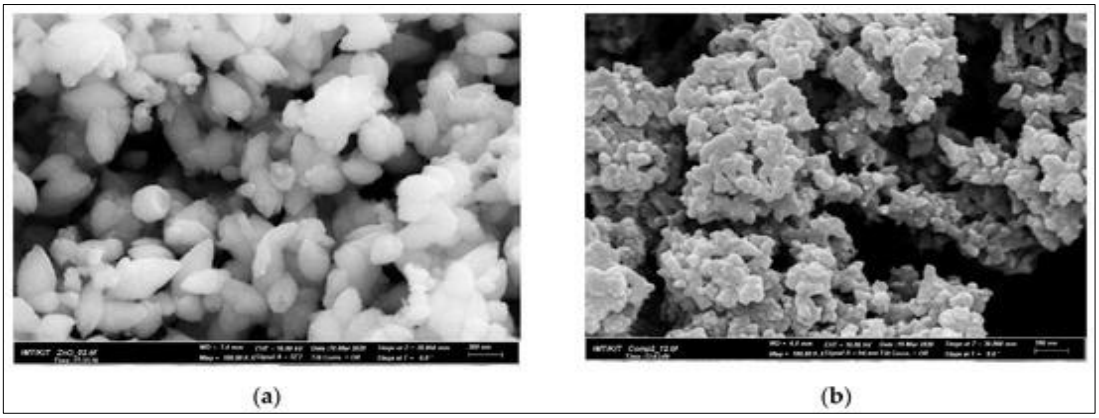

Fig. 4. SEM pictures of ZnO nanoparticles (a) and PANI/ZnO nanocomposite (b) [35].

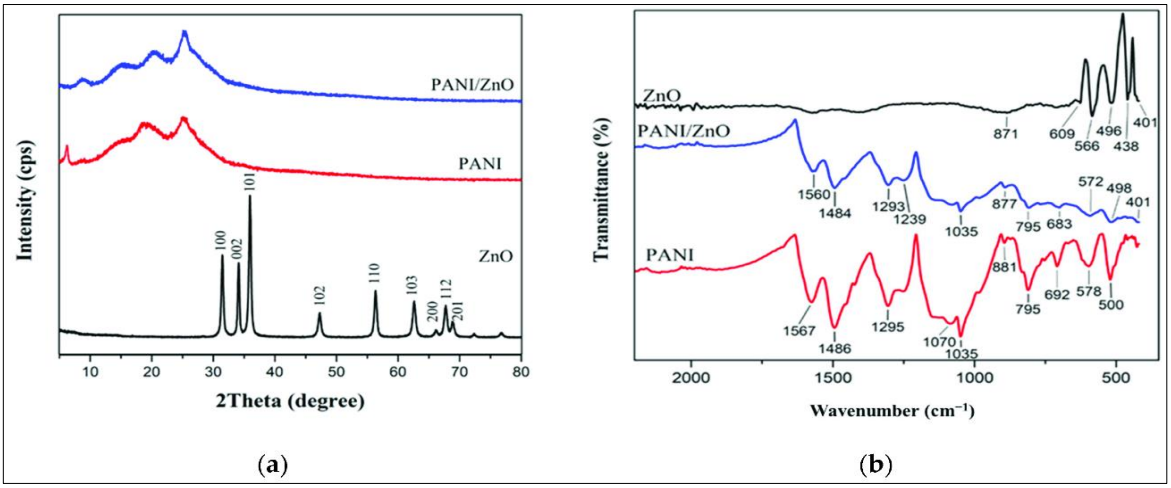

Fig. 5. The ZnO nanoparticle, PANI/ZnO nanocomposite, and PANI XRD patterns are displayed in (a), along with the FTIR spectra of ZnO nanoparticles, PANI, and PANI/ZnO nanocomposite (b) [38].

Figure 5b shows Fourier-transform infrared spectroscopy (FTIR) spectra obtained for ZnO nanoparticles (NPs), polyaniline (PANI) nanostructures, and the PANI/ZnO nanocomposite within a wavenumber range of 400-4000 cm^{-1}. The PANI spectrum showed key absorption peaks at 1567 cm^{-1} and 1486 cm^{-1}, attributed to C=N and C=C stretching vibrations, respectively. Additional peaks at 1295 cm^{-1} (C–N bond stretching), 1070 and 1035 cm^{-1} (O=S=O bond stretching), and 881 cm^{-1} (C–H bending) were also observed. The ZnO spectrum displayed absorption bands from 871 to 401 cm^{-1}, indicative of Zn–O–Zn and Zn–O stretching vibrations. These shifts suggest an interaction between the H–N groups in PANI and O (oxygen) in ZnO. The disappearance of the ZnO peak at 871 cm^{-1} indicates that PANI may have fully covered the ZnO, suggesting a core/shell structure [39-41].

Performance Characteristics

In the PANI/ZnO composite, slight shifts in the distinctive absorption bands of both PANI (1560, 1484, 1035, and 877 cm^{-1}) and ZnO were observed.

The construction of these sensors often involves nanomaterials that enhance sensitivity and specificity. A PANI/ZnO nanocomposite thin film was deposited via spin-coating onto a gold electrode. The resulting modified electrode surface was functionalized with monoclonal antibodies targeting E. coli O157:H7. Other materials, such as phthalocyanine thin films, have also been explored for their excellent sensitivity in different biosensing applications .Conductometric immunosensors are noted for their rapid analysis times, often providing results within 30 minutes. They can operate effectively in complex matrices like food products, maintaining high specificity and reliability.

These sensors are particularly useful for detecting foodborne pathogens, allergens, and toxins in various food matrices. Their rapid response and high sensitivity make them suitable for real-time monitoring in food quality control settings [42].

Potentiometric Immunosensors

When an immunocomplex forms at the sensing device's interface, potentiometric immunosensors rely on changes in surface charge or potential. The ionic structure of the solution and the isoelectric points of the antigens and antibodies in an aqueous solution determine their net electrical charge. The electrical charge of the complex that results from an antibody's interaction with an antigen is different from the charge of the antibody alone. Potentiometric techniques can be used to measure this change by immersing a reference electrode in the same solution.

Ion-selective electrodes (ISEs), field-effect transistor (FET) sensors, and light-addressable potentiometric sensors (LAPS) are the three main types of potentiometric immunosensors. FET sensors are an adaptation of the ISE design, where the FET transducer is used in place of the electrode. These kinds of sensors are usually thought to be the best option for immunosensing applications. Among the most popular potentiometric sensors are ion-selective electrodes (ISEs).

Ion transport or exchange at a selective membrane results in a change in membrane potential. In actuality, pH and electrolyte concentrations have been measured using ion-selective electrodes (ISEs). However, the range of analytes that these electrodes can detect has expanded due to advancements in membrane technology [43,44]. For example, a recent study used a silver ion-selective electrode to measure the potential change associated with the virus concentration in a sample in order to detect enterovirus 71 (EV71). Potentiometry is an electrochemical method that measures the voltage across an interface between a working electrode and a reference electrode while allowing a negligible bias current to flow. This method's simplicity, portability, and low power consumption make it useful. Additionally, compared to voltammetric or amperometric sensors, the approach should be more resilient to interference effects and ohmic drop considerations due to the small current flow [45,46]. The low operating current and potential measurement in the operating mode make potentiometry much more robust to interference performance [47]. Lastly, it has been demonstrated that potentiometry is mostly insensitive to electrode size, allowing for shrinking without sacrificing sensitivity. One of the intrinsic benefits of potentiometry is its low initial power requirements. For target analytes, potentiometric immunosensors can achieve excellent selectivity and low detection limits. For instance, the previously mentioned sensor exhibited a detection threshold of 0.058 ng ml^{-1} for EV71 (see Fig. 6). 50 µl of

either clinical samples or EV71 standards were first added to the microplate, and it was then continuously mixed end over end for 45 min at 37 °C in order to detect the target analyte. 50 µl of the made pAb-AgCl suspension was added to each well after a thorough PBS wash at pH 7.4, and the wells were then gently stirred for an additional 45 min at 37 °C. After that, the microplate was cleaned once more. To release Ag^+ from the trapped AgCl nanospheres, 50 µl of 0.1 M $NH_3 \cdot H_2O$ was then added to each well and allowed to react for 8 min at room temperature. Given that the detection limit of the purchased Ag-ISE was 10 ppb, the resultant alkaline solution containing the liberated silver ions was transferred into 2 ml of an aqueous 1.0 mM $NaNO_3$ solution with 10.25 ppb Ag^+. A digital ion analyzer with a two-electrode system the Ag-ISE acting as the working electrode and the Na-ISE as the reference electrode was used to measure the potential. To minimize potential discrepancies caused by varying sample additions, the signal for each measurement was recorded starting 30 s after sample addition until equilibrium was achieved (defined as a change of less than 1.0 mV min^{-1}). The potential shift with respect to the background signal was used to define the response. The sensing signal that corresponded to the concentration of EV71 was recorded as the resultant signal. Every experiment was carried out at room temperature (25 ± 1.0 ºC), and three parallel measurements were used to gather all of the data [45,46].

Applications

These sensors can provide quick results, making them suitable for clinical diagnostics and food safety applications. New approaches have emerged, such as disposable paper-based potentiometric immunosensors designed for real-time detection of pathogens like Salmonella typhimurium, which offer practical advantages in terms of cost and ease of use [48]. Research has also focused on using artificial antibodies based on molecularly imprinted polymers, enhancing the versatility and applicability of potentiometric immunosensors across various fields. Potentiometric immunosensors are utilized in various domains, including clinical diagnostics (*e.g.*, detecting cancer markers like alpha-fetoprotein), environmental monitoring, and food safety testing. Their ability to function without complex sample preparation makes them particularly attractive for on-site testing. In conclusion, potentiometric immunosensors, which combine the sensitivity of electrochemical measurements with the specificity of immunological techniques, constitute a substantial breakthrough in biosensing technology. Their ongoing development promises to enhance diagnostic capabilities across multiple sectors [46,49].

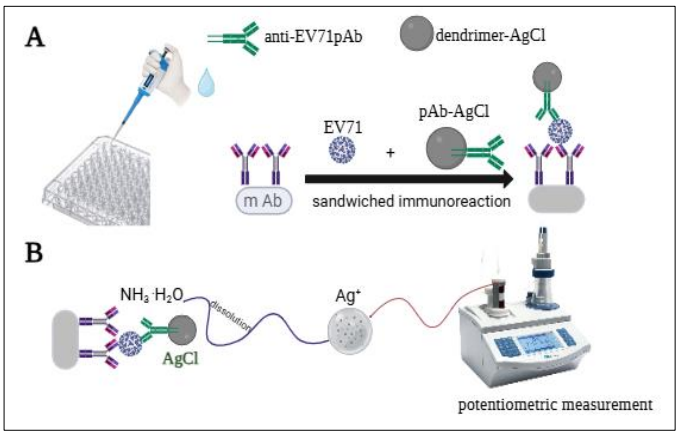

Fig. 6. A diagrammatic representation of the potentiometric Immunosensor designed for detecting enterovirus 71 (EV71): (A) The immunological reaction occurs on a microplate coated with monoclonal anti-EV71 antibodies (mAb) using bis-MPA-COOH dendrimer-infused AgCl nanospheres tagged with polyclonal anti-EV71 antibodies (pAb AgCl) as the detection antibody; and (B) the potentiometric evaluation is performed using an Ag+ ion-selective electrode, with the Na^+ ISE serving as the reference, after the AgCl nanospheres have dissolved in $NH_3 \cdot H_2O$ (ammonia aqueous solution) [45].

KEY NANOMATERIAL PROPERTIES GOVERNING LOW LIMITS OF DETECTION (LOD)

Electrochemical immunosensors achieve very low LODs when the sensor interface maximizes bioreceptor loading while maintaining fast and low-noise electron transfer. In practice, the following nanomaterial properties are consistently associated with superior LODs [50]:

• Very high electroactive surface area (*e.g.*, porous, wrinkled, or 3D architectures such as CNT/graphene foams) to increase the surface density of immobilized receptor antibodies/antigens without steric crowding [51].

• High electrical conductivity and fast charge transfer kinetics (graphene, CNTs, doped carbons, Au/Ag/Cu nanostructures, conducting polymers), which increase the signal-to-noise ratio by reducing the surface resistance [52].
• Intrinsic (or catalyst-tagged) electrocatalytic activity to enhance redox cycling (*e.g.*, AuNPs, Pt composites, MOF@Au, and enzyme-mimicking nanozymes) [53,54].
• Tunable porosity/roughness on the 5-100 nm scale to balance mass transfer and receptor accessibility [55].
• Dense, directional, and stable bioconjugation chemistry (*e.g.*, $–NH_2$/–COOH/–SH, protein A/G, site-directed binding) to maintain affinity and reduce binding heterogeneity [56].
• Antifouling properties in complex matrices (zwitterionic/pegylated brush, hydrophilic conducting polymers) to suppress nonspecific adsorption and open drift [57,58].
• Composite or doped hybrids (rGO-Au) that simultaneously enhance conductivity, surface area, and chemical functionality [59].
• Mechanical/chemical stability (resistance to aggregation, corrosion resistance) to maintain long-term calibration and reproducibility [60].

Also for biomarkers with low abundance in blood/serum, 3D carbon-metal hybrids with antifouling polymer layers, coupled with redox labeling or enzyme amplification, often offer sub-ng/ml LODs while keeping assay times short [50].

BIOLOGICAL INTERACTIONS THAT AFFECT SIGNALS IN THE DETECTION

In electrochemical biosensors, various drugs and metabolites may induce false positive or false negative signals. Compounds such as acetaminophen and uric acid undergo direct oxidation at the electrode potential, leading to increased current and thus false positives. Vitamin C can act as an antioxidant at low concentrations, reducing the signal (false negative), whereas at higher concentrations it is oxidized, generating excess current (false positive) [61].

In neurotransmitter sensors, L-DOPA oxidizes at a potential similar to dopamine, causing a false positive response [62]. Drugs that alter the physiological environment often result in false negatives: for example, diuretics (*e.g.*, furosemide) change electrolyte levels and reduce conductivity, while proton pump inhibitors (*e.g.*, omeprazole) modify pH and decrease enzyme activity [63].

Additionally, drugs with high protein-binding affinity, such as warfarin, lower the availability of the analyte, leading to reduced signals (false negatives) [64]. Surface-active antibiotics (*e.g.*, aminoglycosides) may block electron transfer by covering the electrode, also producing false negatives [65]. Conversely, compounds like caffeine and nicotine increase background current, creating false positive signals even in the absence of the target analyte [66].

In electrochemical biosensors, several blood-derived factors can interfere with signal detection and contribute to false positive or false negative outcomes. Endogenous electroactive molecules such as uric acid, ascorbic acid may undergo oxidation or reduction at electrode surfaces, producing current responses independent of the target analyte and thus leading to false positives [67]. On the other hand, serum proteins (*e.g.*, albumin, globulins) and lipids can adsorb onto the electrode or bind to the analyte, reducing its bioavailability and electron transfer, thereby causing false negatives. In addition, fluctuations in electrolyte concentrations (Na^+, K^+, Ca^{2+}, $Cl^–$) or changes in blood pH may alter enzyme activity and conductivity, further affecting biosensor accuracy. These interferences highlight the importance of considering blood composition when interpreting biosensor signals in clinical applications [68].

PERFORMANCE-BASED CRITICAL ANALYSIS OF REPORTED ELECTROCHEMICAL IMMUNOSENSORS

In a critical comparison of the reported studies, it is evident that some electrochemical immunosensor designs outperform others based on key merit criteria such as sensitivity, limit of detection (LOD), and operational stability. For instance, the sandwich-type amperometric immunosensor developed by Yang Y. *et al.* (reference 8) using Au@Ag-Cu_2O nanoparticles demonstrated exceptional sensitivity and stability, enabling detection of prostate cancer biomarkers at ultralow concentrations. Similarly, the voltammetric immunosensor designed by Oliveira A.E.F. *et al.* (reference 22) for breast cancer biomarker CA 15-3 achieved a remarkably low detection limit of 0.56 U ml^{-1} with high reproducibility. By contrast, conductometric

Table 1. Figure-of-merit Comparison of Representative Electrochemical Immunosensors Based on their Reported Limits of Detection (LOD). Values are Taken from the Cited Papers

Analyte/Target	Transduction	Nanomaterial(s)	LOD (units)	Ref.
CA 15-3 (breast cancer)	Voltammetric (DPV)	AuNPs	0.56 U ml^{-1}	[22]
Enterovirus-71 antigen	Potentiometric (ion-selective)	Au-coated Si chip	0.058 ng ml^{-1}	[45]
TSH (thyroid-stimulating hormone)	Amperometric	Gold	0.04 μIU ml^{-1}	[8]
E. coli O157:H7	Conductometric	PANi/ZnO nanocomposite	4.8 CFU ml^{-1} (peptone)	[30]

immunosensors such as the Escherichia coli O157:H7 sensor by Uda M.N.A. *et al.* (reference30), though cost-effective and rapid, still suffer from lower sensitivity and limited applicability in complex biological matrices. Potentiometric immunosensors, exemplified by the Enterovirus 71 sensor by Sun A.-L. (reference 45), showed good selectivity and low power consumption, but their LOD values remain higher than those of amperometric and voltammetric approaches. Therefore, among the reviewed studies, the voltammetric and sandwich-type amperometric immunosensors incorporating advanced nanomaterials (*e.g.*, gold nanoparticles, graphene composites) can be considered the most promising designs based on merit metrics such as LOD, sensitivity, and stability. This comparison highlights the importance of nanomaterial engineering in pushing the performance boundaries of electrochemical immunosensors.

CONCLUSION

The conclusion for electrochemical immunosensors is that they have shown significant potential in recent years due to their high sensitivity, selectivity, and simplicity. These sensors are extensively employed to identify a range of biomarkers, such as antibodies. The use of electrochemical immunosensors has been explored in various fields, including clinical diagnostics.The development of electrochemical immunosensors has been driven by advances in nanotechnology, materials science, and electrochemistry. The integration of nanomaterials, such as graphene and metal nanoparticles, has improved the sensitivity and stability of these sensors. The use of advanced electrochemical techniques, such as chronoamperometry and cyclic voltammetry, has also enabled the detection of biomarkers at very low concentrations. However, there are still challenges to be addressed in the development of electrochemical immunosensors, including the need for improved specificity and the development of more robust and stable sensors. Overall, electrochemical immunosensors have shown great promise in recent years, and their continued development and improvement are expected to have a significant impact on various fields.

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