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Review

Advances and Challenges in Nanomaterial-Based Electrochemical Immunosensors for Small Cell Lung Cancer Biomarker Neuron-Specific Enolase

Daisy Mehta, Divyani Gupta, Alankar Kafle, Sukhjot Kaur, and Tharamani C. Nagaiah*

Cite This: *ACS Omega* 2024, 9, 33−51

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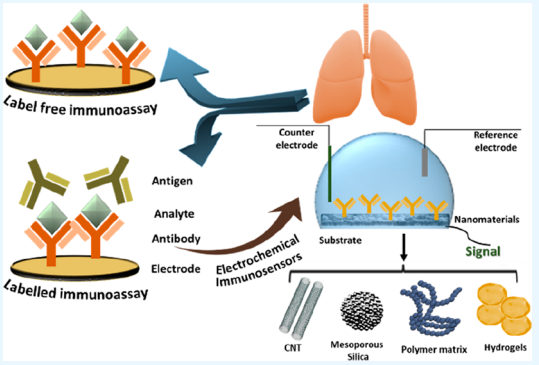
ABSTRACT: Early and rapid detection of neuron-specific enolase (NSE) is highly significant, as it is putative biomarker for small-cell lung cancer as well as COVID-19. Electrochemical techniques have attracted substantial attention for the early detection of cancer biomarkers due to the important properties of simplicity, high sensitivity, specificity, low cost, and point-of-care detection. This work reviews the clinically relevant labeled and label-free electrochemical immunosensors developed so far for the analysis of NSE. The prevailing role of nanostructured materials as electrode matrices is thoroughly discussed. Subsequently, the key performances of various immunoassays are critically evaluated in terms of limit of detection, linear ranges, and incubation time for clinical translation. Electrochemical techniques coupled with screen-printed electrodes developing market level commercialization of NSE sensors is also discussed. Finally, the review concludes with the current challenges associated with available methods and provides a future outlook toward commercialization opportunities for easy detection of NSE.

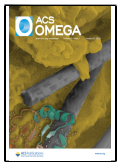


INTRODUCTION

Globally, cancer is one of the most intricate and ubiquitous diseases which arises due to rapid and unregulated cell proliferation or division.[1] It exists in more than 200 different types and affects people of all ages and over 60 human organs.[2] It is a fatal disease with the highest mortality rate around the globe, accounting for more than 8.2 million deaths each year, and is anticipated to cross 13 million by 2030.[3] Of the different kinds of cancer, the most lethal and frequently diagnosed cancer types include breast, prostate, lung, and colon cancer. Lung cancer, i.e., pulmonary carcinoma, is a ubiquitous global health concern of the 21st century, with high mortality and incidence rates (11.6% of the total cases) in comparison to breast and prostate cancers.[4,5] Lung cancer accounts for approximately 25% of all cancer deaths (1.5 million) altogether.[6−8] Human lung cancer can further be grouped into two major categories: small-cell lung carcinoma (SCLC) and nonsmall-cell lung carcinoma (NSCLC). Nonsmall cell lung cancer comprises squamous cell carcinoma, adenocarcinoma, large cell carcinoma, and mixtures while SCLC can be categorized into small-cell carcinoma, mixed small-cell/large-cell carcinoma, and combined small-cell carcinoma. SCLC is one of the most malignant and deadly subtypes of lung cancer, which represents roughly 15−20% of all lung cancers and causes 30,000 deaths in the United States per year.[8−10] For this reason, early stage small-cell lung cancer detection has gained rapid prominence over the past decades and has been the subject of deep scientific research to avoid its occurrence, recurrence after treatment, high morbidity, and mortality due to late diagnosis. SCLC is often suppressed or is symptomless in its early stages, due to which the early diagnosis of lung cancer remains a significant challenge for oncologists.[11] Presently, a number of diagnostic tools are applied for the determination of SCLC, including noninvasive techniques such as chest X-ray, computerized axial tomography (CAT) scan, low-dose helical CT scan, magnetic resonance imaging (MRI), and positron emission tomography (PET) imaging and invasive methods such as sputum cytology, bronchoscopy, and biopsy.[12,13] However, these conventional methods are associated with a number of bottlenecks, due to which they are inefficient for early-stage cancer detection, which are described as follows:

Patients suffering from SCLC are often misdiagnosed as suffering from pulmonary tuberculosis when using conventional diagnostics for the following reasons:

Received: August 27, 2023
Revised: November 5, 2023
Accepted: November 30, 2023
Published: December 25, 2023



(1) Noninvasive methods depend on the phenotype properties of cancer and exhibit low resolution detection for sub millimeter (mm) sized tumors.

(2) Invasive methods have sampling complexity and high resolution errors; in addition, they are very painful.

(3) The conventional methods are time-consuming and labor intensive and require sophisticated apparatus; quite a lot of diagnostic tests are necessary, which are expensive and associated with low resolution and poor reliability.

A dilemma results due to the issues allied with delayed SCLC detection and diagnosis; given the seriousness of lung cancer, there is a great need to diagnose the disease at an early stage by means of more sensitive and rapid methods. Despite technological advances, SCLC remains a prominent reason for global deaths due to late diagnosis. As per the statistics, the 5-year survival rate for patients with SCLC (17% at present) can be improved significantly from 50% for stage IA to 2% for stage IV if diagnosed early.[14,15] Unfortunately, only one-third of the patients are diagnosed at an early stage, entailing a burden on both the health care system and the individual. With the advances and improved understanding of SCLC at molecular levels, a link has been established between molecular and tissue level changes during cancer, thus enabling sensitive and specific biomarker analysis in blood or other body fluids to diagnose SCLC at early stages and avoid false positives. Biomarkers play a decisive role in identifying whether the cancer is present or absent, as they are secreted by the cancerous tumor, and these molecules undergo variations in their levels during the initial progression states of cancer and during treatment. Biomarkers can be nucleic acids, proteins, peptides, enzymes, antibodies, lipids, and even carbohydrates and can be investigated by noninvasive techniques or minimally invasive methods.[16,17] A highly specific and sensitive detection of biomarkers associated with SCLC from blood samples using a noninvasive technique may provide both early and easy detection without causing any pain or discomfort. Biomarkers can be classified in two categories, i.e., genetic and protein, where the genetic biomarker techniques have gained massive recognition but are limited by the fact that they may not detect post-translational modifications in genes.[18,19] In this regard, protein biomarkers allow the detection of these modifications and are more sensitive toward detection even if present in a lesser amount. Thus, protein biomarkers can assist in the early diagnosis of SCLC and monitor the treatment response and recurrence of SCLC after treatment. Presently, various protein biomarkers are used for the diagnosis of small-cell lung cancer such as carcinoembryonic antigen (CEA), cytokeratin fragment (CYFRA), neuron-specific enolase (NSE), alpha fetoprotein (AFP), carcinoembryonic alpha enolase (ENO1, ENO2), etc.[20] Among which, NSE is a highly specific and sensitive marker with elevated blood concentrations in body fluids of patients with SCLC due to malignant proliferation. It is a specific marker for neurons and peripheral neuroendocrine cells and so the most reliable tumor marker in the diagnosis, prognosis, and post-treatment of small-cell lung cancer. NSE being a human brain protein is present in about 0.4−2.2% (>5 ng mL^{-1})[21] of total soluble protein of the brain, and excessive increase in such levels indicates a metastatic disease such as melanoma, seminoma, renal cell carcinoma, carcinoid tumors, Guillain-Barre syndrome, Creutzfeldt-Jakob disease, and so on.[22,23] The amount of NSE present in the brain predicts quantifiable measures of brain damage, which lead to strokes, hemorrhage, seizures, etc.[24] Recently, reports have published sufficient data of COVID-19 positive patients showing the elevated level of NSE, signifying the role of NSE as a potential clinical biomarker for COVID-19 because it primarily targets human respiratory and neurological systems.[25,26]

Although the detection of protein cancer biomarkers is advantageous, it also possesses certain shortcomings. For example, proteins cannot be amplified, i.e., they cannot increase their concentration like nucleic acids during detection; they are highly sensitive to temperature, pH, etc.; and the presence of high background of other proteins in high amount.[27] On the other hand, the utilization of conventional techniques like surface plasmon resonance (SPR), surface-enhanced Raman scattering (SERS), fluorescence, enzyme-linked immunosorbent assay (ELISA), chemiluminescence, and electrophoresis involves complex multistep procedures, requires a large volume of samples, and is time-consuming.[28−30] For this reason, there is an urgent need to develop a rapid, simplified, reliable, and economical method for detection of NSE biomarker to diagnose SCLC at early stages. A likely and potent substitute for the conventional biosensors and optical immunoassay techniques for the protein cancer biomarkers is an electrochemical immunosensor, which possesses high sensitivity and selectivity due to strong binding forces and a stable complex formation between antibody and antigen giving an electrochemical response. Electrochemical immunosensors are based on the measurement of an electrical signal produced on an electrochemical transducer due to the binding between the antibody and antigen. Electrochemical detection of protein biomarkers provides a simplified, specific, portable, as well as in situ automated detection technique. Moreover, the electrochemical techniques offer good stability, broad detection range, easy miniaturization, and small sample volume requirement; thus, they hold immense significance.[31−35] The electrochemical immunosensing can be categorized into two types: (a) labeled (sandwich-type antibody−antigen interaction) and (b) label-free (simple antibody−antigen interaction).[36] Among which, tremendous attention has been paid to label-free electrochemical NSE detection owing to the direct interaction between antibody and antigen without the requirement of any labeling agent and secondary antibody.[37,38] The elimination of the use of secondary antibody is beneficial for the rapid response, reduced amount of analyte, decreased false positives due to nonspecific interactions, *in situ* NSE detection, and simplified fabrication procedures and operation in comparison to the labeled techniques.[39,40] The most crucial step in the development of electrochemical immunosensors is the antibody immobilization over the electrode surface to promote the efficacy of immunosensors toward biomarker detection. Nanomaterials have opened up new prospects for the boosted activity of electrochemical immunosensors due to their high surface area, enhanced electrochemical activity, improved electron transfer ability, and high conductivity.[41] Besides these, the nanomaterial-based electrochemical immunosensors for NSE detection would most likely result in the sensitive transduction of biomolecules, short response times, and improved stability and lifetime of the immunosensor.[42,43] The rising interest toward nanomaterials as electrode materials for electrochemical immunosensors for the detection of NSE demands the understanding of the reported literature. In this

https://doi.org/10.1021/acsomega.3c06388
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review, we have focused on the recent advances in the labeled and label-free nanomaterial-based electrochemical immunosensors for detection of the SCLC biomarker NSE. Herein, we focus on the different conventional and electrochemical methods for NSE detection along with their advantages and shortcomings, and the significance of electrochemical immunosensing is emphasized for future development in this field.

1. METHODS FOR EARLY DETECTION OF NSE

The late diagnosis of SCLC and increased mortality rate associated with it increase the demand for the early-stage detection of cancer, where the detection of the NSE biomarker is significant. A number of methods have been developed for SCLC detection, including computed tomography, radiography, magnetic resonance imaging, positron emission tomography, and biopsy. Unfortunately, the poor sensitivity, expensive nature, and physical as well as chemical damage to the electrode obstruct its application for early detection of SCLC. Until now, a widespread family of analytical detection methods have been developed for the sensitive and effective detection of NSE (Table 1). Immunosensors convert and detect biochemical signals depending on the interaction between antibody and antigen over a transducing material. An immunosensor comprises three components, i.e., a biomarker, a bioreceptor, and a transducer, and can be classified into the following categories:

- Optical immunosensors, based on light absorption, emission, fluorescence, reflection, etc.[44]
- Traditional immunoassays such as ELISA, radioimmunoassay.[45]
- Piezoelectric immunosensors, based on transduction of electron interference into frequency[46]
- Electrochemical immunosensors, based on electron transfer and the diffusion of ions[47]

These methodologies for NSE detection are discussed in detail in the following sections (Figure 1).

1.1. Optical Techniques. Optical immunosensors are most widely used to detect tumor markers depending on the variation in optical signal upon reaction between the analyte under study and detection reagents. An optical immunosensor consists of a sensing layer with optical signal conversion and amplification processing where in the interaction between the bioanalyte and optical field takes place, and then the optical signal is converted into an electrical signal to detect the analyte under study. Based on this principle, the optical methods for biomarker detection can be further categorized into techniques stated as follows:

- Fluorescent immunosensing involves the use of immune and fluorescent reagents which act as recognition sites and labels, respectively, to detect the presence of a specific antigen or antibody via reaction between the antigen and antibody.[48,49] One of the promising approaches is to use fluorescent nanoparticle-based probes with high stability, e.g., quantum dots (QDs), metal nanoparticles and composites (particularly gold nanoparticles (Au NPs)), polymer dots (PDs), upconversion nanoparticles (UCNPs), etc., for the fluorescence biosensors.[50−53] However, the limited fluorescence lifetime, poor sensitivity, and photo degradation restrict their use to the laboratory scale.

Table 1. Different Immunoassay Techniques

Immunoassay Techniques	Working Principle	Advantages	Disadvantages
Enzyme-linked immunoassay (ELISA)	Enzyme as labeled followed by change in color after antigen−antibody interactions	Quick response, high sensitivity, low-cost, automated	High probability of false negative results, time-consuming, not suitable for POCT
Lateral flow assays	Based on fluid transport by capillary action after antigen−angtigen action	Only one-step assay, user-friendly, long-term stability, suitable for onsite monitoring	Less sensitive, not automated, no high-throughput, low repeatability, sample matrix effect
Radio immunoassay	Radioactive molecules are used as labels for the formation of immunocomplex	Detect trace amounts of analyte, resistance to matrix effect, highly specific	Requires trained personnel and license, short half-life of isotopes, disposal of radioactive labels
Chemiluminescence	Chemical reaction results in change of electronic excited state after the antigen−antibody interactions	Highly sensitive, less time-consuming, detect very small amount of analyte	False results due to interference of background signal, consume large amount of sample, less selective
Fluorescence immunoassay	Fluorescent probes are used as labels, and the measurements are based on change in fluorescent intensity	Simple assay technique, highly sensitive, rapid response	Nonlinearity in results, short half-life of labeled antibodies, suitable only for fluorescent active molecules
Flow injection immunoassay	Based on diffusion of analyte molecule from the solution	No separation required, highly sensitive, fast detection, reusable	Very short time, chances of reproducibility is less, short reaction time, unstable signal
Electrochemical immunosensors	Based on interaction between substrate material and antibody resulting change in electrical signal in the form of current, potential, and impedance	Rapid, highly sensitive and selective, cost-effective, reusable, portable, onsite monitoring, suitable for multiple sample detection	Selection of suitable substrate material for immobilization of protein molecules, optimization electrochemical parameters such as pH, incubation period, etc.

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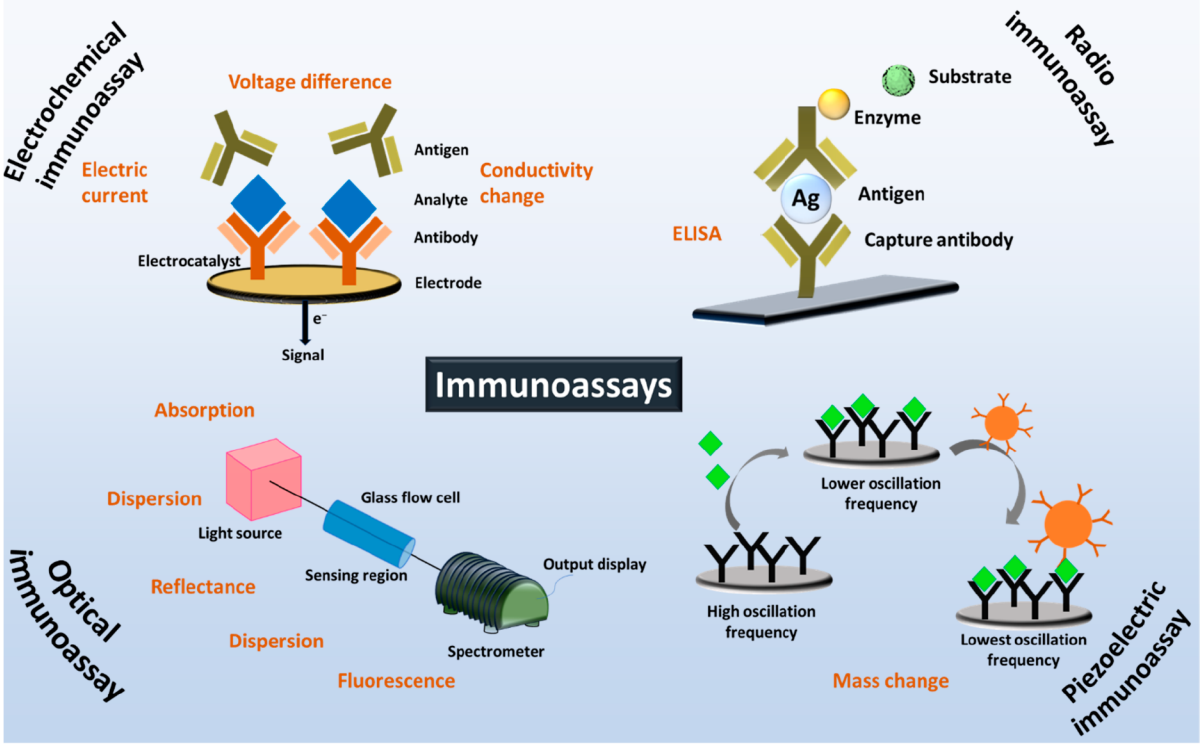

Figure 1. Schematic representation of various types of immunoassays.

- In the chemiluminescence (CL) based immunoassays, the emission of visible light takes place as a result of a chemical reaction between two reagents which generates a high-energy intermediate and releases visible light upon returning back to a lower-energy state.[54,55] The most promising development in CL-based immunosensors is electrochemiluminescence (ECL) immunosensors, which are a combination of electrochemical excitation and visible light.[56,57] In the recent past various ECL-based immunosensors have been explored for NSE using diverse materials.[58,59] Although ECL-based immunosensors are advantageous for early detection of cancer since they do not require any external light source, but the poor sensitivity of the same restricts its application for real-time monitoring.[60]
- In surface plasma resonance (SPR) immunosensors a noble metal surface is irradiated via visible or near-infrared light due to which the free electrons in metal conduction band get excited, forming an electric field.[60] Briefly, the antigens which are immobilized onto the noble metal surface undergo interaction with the antibodies, resulting in the variation in oscillation frequency of free electrons in the metal-conduction band. This variation in conduction band is then utilized to detect the analyte by the subsequent altered intensity of the reflected light. A few reports of SPR immunosensors have been published recently, presenting superior performance in terms of detection range, LOD, and sensitivity.[61−63]
- Surface-enhanced Raman spectroscopy (SERS) utilizes metal plasma nanostructures to enhance the Raman signal up to 10^6−10^{12} times via electromagnetic and chemical enhancements.[64] This electromagnetic and chemical enhancement takes place due to electron transfer between metal nanoparticles and the target molecules.[65] The SERS technique allows the detection of low-concentration analyte as well and can be classified into two forms: intrinsic and extrinsic SERS.[66] The SERS immunosensors exhibited superior performance as compared to the ELISA method in terms of sensitivity and limit of detection (LOD). However, the application of SERS as a point-of-care (POC) tool for monitoring protein biomarkers in real-world blood samples is still a challenging task.[67]

1.2. Traditional Immunoassay-Based Techniques. Apart from the optical immunosensing techniques discussed in the previous section, there are few traditional immunoassay techniques for the specific detection of NSE in view of rapid and early diagnosis of SCLC. The most common ones include ELISA and radioimmunoassay.

1.2.1. Enzyme-Linked Immunosorbent Assay. ELISA is one of the most commonly used immunosensing techniques based on the use of antibodies for detection of biomarkers via 96-well plates in which the antibodies are coated via physical adsorption. The antigen from the desired analyte makes a complex with the antibody in the well upon incubation, which is then incubated with the enzyme substrate. This enzymatic reaction leads to the change in the color of dye added as an indicator, which is used to detect the concentration of the biomarker.[68] The ELISA-based immunoassay technique offers reproducible, sensitive, and specific detection of biomarkers for diagnosis of cancer. A number of ELISA kits, such as Elabscience immunoassay kit, Simplex Kit by Thermofisher, NSE ELISA Assay Kit by Eagle Biosciences, etc., are available in the market for detection of NSE; however, the testing procedure is tedious and time-consuming, requires centralized lab equipment and high sample volume, and is expensive as well.[69] Yet another drawback is the poor detection limit of the same (>1 pM, less than the nanomolar concentration) which is

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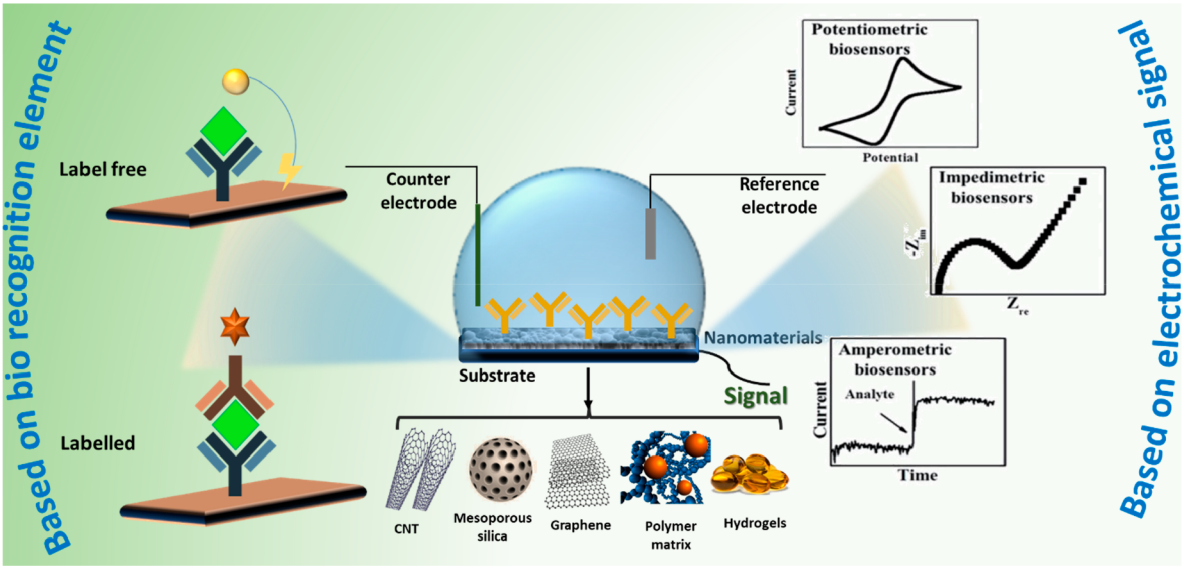

Figure 2. Scheme showing the categories of electrochemical immunosensors.

not sufficient to detect the concentration of biomarkers present in very low amounts, since the concentrations in serum can be as low as 100 aM to 1 pM during early stages. Consequently, the early diagnosis of cancer is challenging and critical by ELISA techniques. In order to tackle the issues related to optical ELISA, recently, an electrochemical ELISA immunoassay technique has been developed which combines the pros of traditional ELISA such as sensitivity, selectivity, and multiplexing with the benefits allied with electrochemical assay, such as simplified operation, rapid detection, less sample volume, low cost, portable equipment, etc.

1.2.2. Radioimmunoassay. Another widely adopted traditional immunoassay technique for detection of cancer biomarkers is radioimmunoassay (RIA), which is based on the principle of binding of a radioactive labeled antigen (Ag*) and an unlabeled test antigen (Ag) with a specific antibody (Ab). Upon addition of another Ab (specific to Ag*), this results in the formation of a complex that precipitates out in the solution and is then analyzed for the concentration of target Ag by the amount of the Ag*−Ab complex. RIA is a highly sensitive, specific, and easy to operate technique with a low detection limit of even a few picograms of analyte.[70]

1.3. Electrochemical Techniques. The traditional immunoassay methods and optical methods for detection of low-concentration NSE biomarker in the early stages of SCLC necessitate efficient and trained professionals as well as furnished laboratories; thus, they are are labor-intensive and costly.[64] In this context, electrochemical immunosensors come across as a potential and competent tool for rapid and early diagnosis of SCLC via detection of NSE. Electrochemical immunosensing is based on the principle of variation in electrical signal due to immune reaction between Ag and Ab. It allows simplified, inexpensive, rapid, reproducible, biocompatible, and convenient detection of biomarkers with high sensitivity for low concentrations of analyte even at a miniaturized level.

2. TYPES OF ELECTROCHEMICAL IMMUNOSENSORS

The electrochemical immunosensors can be classified into two categories depending on the electrochemical signal used to detect the target analyte and based on the type of biorecognition molecule used during immunosensing. These categories are discussed in detail in the following sections.

2.1. Based on Electrochemical Signal. Under this category of electrochemical immunosensing, the classification is based on the variation of physical quantities during the measurement of electrochemical signals, which can be potential, current, impedance, as well as conductance (Figure 2).

2.1.1. Potentiometric Immunosensors. As the name suggests, this technique involves the measurement of a potential difference by a voltammeter due to oxidation and reduction of analyte species in a sample solution. The potential difference is measured between working and reference electrodes when no current is flowing between them. During the measurements, the working electrode undergoes variation in potential due to alteration in the concentration of the analyte, while the potential of the reference electrode remains as such. In this way, potentiometric immunosensors hold the advantage of detecting a low concentration of biomarker with a minimal sample volume requirement.[71]

2.1.2. Amperometric Immunosensors. Amperometric immunosensors are based on the principle of variation in current due to electrochemical oxidation and reduction of electroactive species at the electrode surface via the bioaffinity interactions between the target analyte and antibodies on the surface of a working electrode. A number of techniques, including cyclic voltammetry, linear sweep voltammetry, differential pulse voltammetry, square wave voltammetry, etc., can be utilized to measure the currents between the electrodes in a three-electrode system equipped with working, reference, and counter electrodes. This assembly is miniaturized over a substrate made up of glass, silicon, or even a printed circuit board to detect the response of target analyte upon passage of current.[72]

2.1.3. Impedimetric Immunosensors. Impedimetric immunosensors detect the concentration of the target analyte as a function of frequency via measurement of impedance under a perturbed alternating current. The impedance is analyzed by means of electrochemical impedance spectroscopy (EIS) at the working electrode, which monitors the interfacial properties and electron transfer reaction at the working electrode when

https://doi.org/10.1021/acsomega.3c06388
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the analyte is adsorbed onto its surface and formation of immunocomplex has taken place. The formation of immunocomplex results in the increased resistance at the electrode−electrolyte interface and thus obstructs the diffusion of electrons toward the electrode surface. EIS measurements can be utilized to calculate the limit of detection and linear range for the biomarker concentration. Impedimetric immunosensors generally require a redox-active compound in the electrolyte solution to study the interaction between antibody and antigen. Recently, label-free impedimetric immunosensors have also been developed which can even operate without the use of redox-active species; instead, they use surface-confined redox groups to generate the electrochemical signal.[73]

2.1.4. Conductometric Immunosensors. Conductometric immunosensors are based on the reaction between the biorecognition species and antigen, which leads to the alteration in the conductivity of the solution because of the change in the concentration of ions. The conductance between two metal electrodes separated from each other is measured using a multimeter as a consequence of a change in the conductivity of the solution. This altered conductivity is due to the conjugation of enzyme-labeled antibodies with antigens present in the solution. Conductometric immunosensing provides low driving voltage and can be scaled up and miniaturized for point-of-care diagnostics.[74]

2.2. Based on Immunosensing Strategy. The electrochemical immunosensors can be classified into labeled (sandwiched), and label-free immunosensors (Figure 2) depending upon the analytical sensing strategy that can be employed for selective sensing of tumor antigens. All of these are briefly discussed below.

2.2.1. Labeled Immunosensors. This is one of the approaches of immunosensing in which the target antigen present as the analyte in the electrolytic solution selectively attaches to their primary antibodies on a solid surface and then to the secondary antibodies, enzymes, or nanoparticles which act as labels. The Ab−Ag complex formation takes place at the immune electrode surface, and the signal is obtained in conjunction with them. The use of these labels, which are commonly classified as radioactive, fluorescent or chemiluminescence, and electrochemical signal based, is time-consuming and laborious because it requires an additional step. More importantly, it is thought that in this case, the affinity between the biorecognition element and the analyte may be adversely affected.[75] To eliminate these limiting factors, label-free detection systems have become highly preferred in recent years.

2.2.2. Label-Free Immunosensors. Label-free electrochemical immunosensors particularly involve the direct measurement of physical or change induced by the formation of an antigen−antibody complex on the electrode surface. It is a major analytical sensing strategy employed for the sensitive and selective detection of tumor antigens. Label-free techniques avoid interference due to the tagging molecules and determine reaction kinetics of biomolecular interactions in real-time analysis. During the recent past, various nonlabeled or label-free electrochemical immunosensors have been reported for highly sensitive determination of tumor markers. Label-free electrochemical immunosensors have a high capability of being adapted into point-of-care (POC) systems that can be beneficial for easily accessible healthcare services. In POC testing, microfluidic devices have achieved great attention for effective and accurate cancer diagnosis owing to their ability to separate analytes at a good resolution in a rapid reaction time and to minimize the handling errors and costs.[76] As a result, promising detection systems with high performance are acquired with the elimination of the need for trained personnel.

3. CHALLENGES OF ELECTROCHEMICAL IMMUNOSENSORS

When electrochemical techniques are compared to each other, it is observed that each of them has limitations in different aspects. For example, the sensitivity of the potentiometric method depending on the environment factors such as temperature, humidity, pH, etc. is an important limitation. Also, redox elements are needed in the amperometric technique, whereas EIS requires theoretical simulation for data analysis.[77] Various recently developed materials and protocols have been used to overcome this problem. Besides this, choosing an appropriate sensing technique for analyte detection can minimize the limitations. Additionally, parameters such as pretreatments applied to the working electrode and the functionality of the electrodes can have a great impact on the precise and effective determination. It is particularly important to focus on and discuss these limitations to put the developed technologies into clinical practice. Reducing or overcoming all of the disadvantages could help to develop more accurate and sensitive electrochemical cancer biosensors. More effective platforms for early diagnosis can be created with a multidisciplinary study. It is expected that the label-free electrochemical methods will increase in reliability by overcoming the above-mentioned difficulties so that they can be used in clinical practice.

For this, development of novel and advanced electrochemical cancer immunosensors with different perspectives is a current need. The key challenges associated with electrochemical immunosensing that need to be addressed are described in the following sections.

3.1. Orientation of Antibodies. Orientation of antibodies refers to the attachment of antibodies to the heterogeneous transducer substrate. The orientation of immobilized antibodies on electrode surfaces strongly influences the recognition ability to the relevant antigen in the analyte solution, which in turn affects the detection limit, sensitivity, and overall performance of the immunosensor.[78] The immunoglobulin (IgG) of the antibody possesses a three-lobe (Y) structure with one Fc region called a constant region (which attaches to the electrode surface) and two Fab regions (where antigen binding takes place). The Fc region of the antibodies attaches to the surface *via* four different orientations, i.e., flat-on (all three lobes attached to surface), side-on (Fc and one Fab at substrate), head-on (Fc-up and both Fab attached to surface), and tail-on (Fc at surfaces and both Fab-up) resulting in different access to binding sites and antigen binding efficiency (Figure 3). The tail-on orientation of antibodies is highly

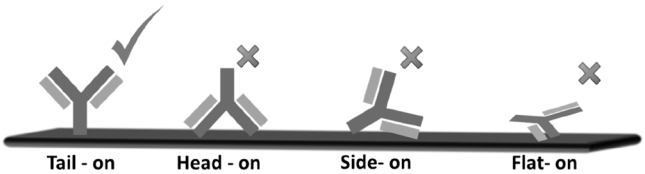

Figure 3. Schematic representation of various kinds of antibody orientation.

https://doi.org/10.1021/acsomega.3c06388
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recommended to achieve the maximum recognition capacity of immunosensor and long-term stability of antibodies.[79] Therefore, the immobilization strategy should be chosen in such a way that antibodies are attached firmly to the surface with the least possibility of leaching during the course of long-term biomarker detection in order to avoid false negative results. In this regard, the covalent binding strategy supports the correct site-specific orientation of immobilized antibodies *via* covalent bonding between the Fc region of the antibody and substrate surface, but the tedious fabrication procedure and high cost of the coupling agents used in the same limit the performance of an immunosensor.[80] Therefore, choosing the material for site-specific orientation of antibodies over the substrate surface without altering their specificity as well as immunological activity is one of the most critical steps to develop high-performance immunosensors.

3.2. Nonspecific Interactions. Binding of the analyte NSE antigens with other sites instead of antibodies is considered a nonspecific interaction. These interactions occur by adsorption of molecules on the exposed area of the substrate, where the protein molecules are not available. These nonspecific interactions impose major hitches toward the affinity of the immunosensors by reducing the number of available active sites which in turn reduces the sensitivity of the immunosensor and gives rise to erroneous measurements.[81] The nonspecific bindings in electrochemical immunosensors can be suppressed by adopting different immobilization strategies based on physical and chemical modification of antibodies. The physical modification involves the attachment of blocking agents (bovin serum albumin, avidin, streptavidin, etc.) directly to the surface or by forming a complex on the electrode surface. On the other hand, the chemical modification takes place through the chemical reaction between interacting molecules with different functional groups like thiols, carboxylic acids, amine, etc.[82] Although efforts are continuously being made in the direction of suppressing the nonspecific bindings, the most essential prerequisite is to develop the immunosensor which is capable of measuring the smallest possible amount of the target analyte present in a real sample.

3.3. Incubation Time of Antibodies. The incubation period for the formation of the immune complex is the time taken by antibodies to get immobilized over the electrode surface. Incubation time depends upon the type of bonding affinity and specific binding of antibodies with the substrate material. In view of achieving a high performance of an immunosensor, the incubation time is obliged to be less. Moreover, the incubation of immobilization of antibodies over the electrode surface defines the complexity of the fabrication procedure.[83] The greater the incubation time, the more complex the fabrication procedure. Therefore, optimization of incubation time of both antigen and antibodies plays a very key role in developing an electrochemical immunosensor of NSE.

3.4. Need for Redox Probe. Redox mediators are electrochemically active species commonly used in electrochemical sensors to enable or enhance the electron transfer between the electrode and electrolyte interface. The choice of a redox probe plays a very important role for fabrication of immunosensors. The redox probes ferrocyanide/ferricyanide ($Fe(CN)_6^{3-/4-}$), hexaammineruthenium ($Ru(NH_3)_6^{3+/2+}$), and ferrocene derivatives are very common because of their stable oxidized/reduced state, good biocompatibility, and absence of irreversible deposition or corrosion on the surface of the electrode during the redox process. Some reports have chosen the electrochemically redox active species like quiniline, glucose, dopamine, ascorbic acid, and H_2O_2 as a redox probe for the determination of NSE.[84−86] However, the polarity of the redox probe sometimes dramatically affects the adsorption and orientation of protein molecules onto the electrode surface and thus does not facilitate electron transfer processes and impedes the sensing ability of the immunosensor.[75,87] Therefore, the electrochemical approaches that are based on label-free, reagent-less, redox, capacitive transducers are more readily translated into POC formats.

3.5. Low Sensitivity. The sensitivity of an electrochemical immunosensor depends on immunoassay strategy, orientation of antibodies, and their affinity toward the sensor substrate.[88] An immunosensor is known to be highly sensitive if it can detect up to femtomolar, nanomolar, or micromolar of the targeted analyte antigen. To be more specific, for the highly sensitive detection of NSE by an electrochemical immunosensor, the most crucial factor is the transducing material required for immobilization of antibodies. The transducing material should possess high electrochemical stability, large surface area, high porosity, and variable functionality, which is promising enough to enhance the sensitivity of the immunosensor at the signal transduction step by multiplying the signal-to-recognition ratio. Therefore, high sensitivity can be achieved by choosing a suitable immobilization strategy based on different transducing materials, which are discussed later in this review.

3.6. Other Challenges.

(1) Many immunosensors have attained impressive sensitivity as well as much shorter incubation time under optimal conditions in the laboratory but face difficulty when applied to real biological samples due to complications allied with stability and reproducibility.

(2) Determining a single analyte can sometimes give false result because a single tumor marker is not enough to meet the strict diagnostic standard. In this context multiple antigen detection techniques should be developed to increase the diagnostic accuracy and efficiency of the immunosensor.

(3) Various sensors may use the same material in different ways, which leads to fluctuations in the electrical and mechanical properties of materials and difficulty in maintaining the originality of the material, which can affect the analytical performance of the sensor.

4. IMMOBILIZATION STRATEGIES FOR SURFACE MODIFICATION

In order to have efficient and effective immobilization of antibodies on the substrate material and significant amplification in electrochemical signal, the assay techniques are mainly focused on the electrode materials for immobilization of antibodies to achieve the high performance of both labeled and label-free electrochemical immunosensors. The choice of a suitable immobilization approach plays a key role for the occurrence of a specific immunoreaction between antibody and antigen. An appropriate strategy is that, first, it should preserve the biological activity of the bioelement (antibody or aptamer), and second, it should afford an orientation for exposed binding sites toward the intended analyte with proper density.[89] Emerging nanotechnology has opened up new doors

https://doi.org/10.1021/acsomega.3c06388
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Table 2. Various Electrochemical Immunosensors for Labelled Technique

Electrode Material	Electrochemical Technique	LOD	Linear Range	Immunocomplex incubation Time	RSD	ref
AP-anti-IgG/AuNP/SWNT	CV, DPV	0.033 ng/mL	0.1 ng/mL to 2 μg/mL	60 min	1%−6.7%	97
Liposomes contained AA&UA	LSV		5.0 to 100 ng/mL			93
$Au/Cu_xO@CeO_2$	Chronoamperometries, EIS	31.3 fg/mL	50 fg/mL to 100 ng/mL	50 min	2.18%−4.14%	110
Antibody modification by disulfide	EIS, DPV	4.6 ng/mL	0−25 ng/mL	60 min		98
OMCSi−Au	LSV, EIS	0.008 pg/mL	0.02 pg/mL to 35 ng/mL	40 min	4%	99
PtCu nanoprobe	CV, SWV	52.14 fg/mL	0.0001−100 ng/mL	40 min	2.37%	103
MnO_2 UNs/Au@PdPt NCs	CV, SWV, EIS	4.17 fg/mL	10 fg/mL to 100 ng/mL		2.43%	106
HP−Ag/Pt/NGR	CV, EIS	18.5 fg/mL	50 fg/mL to 100 ng/mL	45 min	2.07%	113
Cu-MOFs-Au/Fc-L-Cy	CV, DPV, EIS	0.011 pg/mL.	1 pg/mL to 1 μg/mL		>5%	107
$AuNPs@MoS_2/rGO$	EIS, CV, DPV	3.00 fg/mL	0.01−1.00 pg/mL		0.69%	104
ZnO/CdSe	EIS	34 fg/mL	0.10 pg/mL to 100 ng/mL		2.3%	114

concerning the use of nanomaterials as labels and surface modifiers. Various nanostructured materials and their composites have demonstrated a key role in electrochemical fabrication and operational immobilization of antibodies due to their striking chemical and physical properties.[90] Also, their straightforward, easy synthesis technique and excellent biocompatibility with appropriate ligands have become a developing platform for substrate fabrication. The current scenario demonstrates inclusion of various nanomaterials in different labeled and label-free immunosensors with exceptionally amplified current signals. An effective functionalization technique should not only introduce multipurpose and consistent substrates but also curtail its impact on the properties of the substrate material. Major immobilization strategies and an overview of the application of nanoparticles in different electrochemical investigation protocols for NSE are discussed in this section.

4.1. Physical Adsorption. Physical adsorption is the most common strategy for immobilization of protein molecules over the electrode surface. The antibodies can be adsorbed on the surface of the transducing elements *via* intermolecular forces, for instance, ionic bonds, electrostatic interactions, hydrogen bonding, Van der Waals interactions, and hydrophobic and polar interactions. Physisorption is a simple and cost-effective approach, which does not require any coupling agents for surface functionalization. It is usually done by immersing the electrode surface in a biomolecule solution for a fixed time of incubation. The downsides of physical immobilization are random orientation and weak attachment, resulting in desorption of the protein molecules from the electrode surface. Moreover, the background current and transport effects originating from nonspecific interactions can result in false kinetics during real-time analysis. Moreover, they may undergo conformational changes of biomolecules.

4.2. Trapping of Antibodies. Antibody entrapment approach is an irreversible approach focused on the encapsulation of antibodies within a confined space, either in polymer network, sol−gel matrices, and molecular imprinted polymers (MIP) based templates. This technique exhibits more stability as compared to the physical adsorption due to a number of factors like unpretentious procedure, insignificant leaching of substrate material, high chemical and thermal stability, tunable porosity, and mechanical strength. The efficacy of this technique depends on certain physicochemical properties such as pH of electrolyte medium and diffusion of analyte toward the entrapped bioreceptor. Although the entrapment procedure increases the sensitivity of the immunosensor, it suffers from nonspecific binding and low stability. Additionally, the variation of the ratio of particle size restricts the usability of the probe matrix. To overcome this, the covalent immobilization of antibodies over the porous materials is preferred due to improved stability of bioreceptor inside the matrix and can be utilized for several measurements after repeated washing processes.

4.3. Covalent Bonding. The most frequently used binding strategy for immobilization of antibodies is based on covalent interactions between the modified electrode surface and the functional groups of antibodies or aptamers. Covalent bonding can be achieved either by introduction of the functional groups, *viz.* carboxylate, amine or sulfhydryl groups, which can react with the corresponding functional groups present in the structure of antibodies or it can be achieved through the use of coupling agents. *N*-Ethyl-*N*′-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) and *N*-hydroxy succinimide (NHS) or Sulfo-NHS are the most commonly used coupling agents. EDC reacts with carboxyl groups to form o-acylisourea ester intermediate, which can be easily displaced by primary amine, and an amide bond is formed between the carboxyl group and the primary amine, which is often used to facilitate the coupling reaction. The EDC−NHS coupling method forms NHS ester which is more stable than the intermediate o-acylisourea ester because o-acylisourea ester is unstable in aqueous solution.[91] Functional groups of antibodies for covalent binding can include amine, carboxyl, carbohydrate, and thiol moieties. The covalent strategy of immobilization depends on numerous physical and chemical conditions such as pH, temperature, and degree of conjugation. Although covalent binding gives a stable and well-oriented immobilization of antibodies, the immobilization procedure is a bit complex and tedious.

Therefore, the choice of the substrate material for any of the immobilization strategies plays a very crucial role in developing highly sensitive immunosensors. In this respect, nanomaterials have turned out to be the focus of technical as well as scientific research. The emergence of nanotechnology has opened up new horizons for real-time diagnostics owing to their favorable multivalent affinity interactions with proteins through hydrophobic or $\pi-\pi$ stacking interactions and electrostatic

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Table 3. Comparison of Various Label-Free Electrochemical Immunosensors

Electrode Material	Electrochemical Technique	Linear range	LOD	Immunocomplex incubation time	RSD	ref.
PtNF	CV, EIS	0.05 to 150 ng mL	104.15 ng mL			118
CSnanoAu/APTES/PB-SiO_2	CV	0.25−0.75 ng/mL	0.08 ng/mL			110
NH_2-G/Thi/AuNPs	DPV	10 pg/mL	1−500 ng/mL		2.8%	111
NiHCFNPs	CV, EIS	0.3 pg/mL	0.001−100 ng/mL	30 min	4%	79
AuNPs/TCCR	SWV	0.08 ng/mL	0.2 to 25 ng/mL	30 min	7.3%	129
AuPd-MWCNT	SWV, EIS	0.483 pg/mL	1 pg/mL to 100 ng/mL	50 min	4.1%	121
1,3,5-benzenetricarboxylic acid and Fe^{3+}	SWV, EIS	0.26 pg/mL	1 pg/mL to 200 ng/mL	45 min	4.52%	114
epoxy-substituted poly(pyrrole) polymer	CV, EIS	6.1 fg/mL	0.02−7.5 pg/mL		4.72%	119
3D M rGO/PANI	CV, DPV	0.1 pg/mL	0.5 pg/Ml to 10.0 ng/mL	40 min	3.7%	118
Polyresorcinol-Au/Pt nanocomposite	SWV	7.8 pg/mL	10 pg mL to 100 ng/mL	50 min	5%	117
polypyrrole-polythionine-gold	SWV	0.65 pg/mL	100 pg/mL to 100 ng/mL	50 min	8%	122
r-GO/Thi/AuNPs	SWV	10 pg/mL	0.01−100 ng/mL			130
Zr-TAPP Complex	DPV, EIS	7.1 fg/mL	10.0 fg/mL to 2.0 ng/mL	40 min		120
Au−MoS2/MOF	CV, EIS	0.37 pg/mL and 0.52 pg/mL	1.00 pg/mL to 100 ng/mL		5.4%	127
rGO/Cu_8Ni_2	Chronoamperometry, CV, EIS	137 fg/mL	500 fg/mL to 50 ng/mL		3.92%	131

interactions, which are usually exploited as carriers for receptor molecules such as antigen−antibody for amplified immunoreactions. Currently, the nanostructured materials and their implementation in immunosensors for detection of various tumor markers have increased tremendously owing to their extraordinary properties, such as large surface area to support high loading capacity and mass transport for reaction molecules, suitable electron transfer ability which results in synergic contribution toward signal amplification, and excellent biocompatibility with biological molecules such as capturing of antibodies.The following section is entirely focused on the use of various functional nanomaterials as substrate materials for labeled and label-free electrochemical immunosensors for sensitive and selective detection of NSE biomarker in view of rapid and early diagnosis of SCLC.

5. MATERIALS FOR LABELED IMMUNOSENSORS

The commonly used sandwich technique is enzyme-linked immunoassay (ELISA), typically based on the transduction of an optical signal. Electrochemical immunosensors are analogues to ELISA which use biorecognition elements and target analyte to produce signals by employing diverse electrochemical techniques. In this technique, two antibodies called primary antibody (Ab_1) and secondary antibody (Ab_2) are involved.[92] The primary antibody is used to interact with the antigen while the secondary antibody is labeled with different enzymes such as horseradish peroxidase, alkaline phosphates (ALP), and different nanomaterials to catalyze the reduction of substrate material in the presence or absence of redox mediator for the generation of an electrochemical signal.[93] This section will highlight different recently reported nanostructured material-based labeled and label-free electrochemical immunosensors for NSE, highlighting the achievements in limit of detection (LOD), linear range, and incubation time (Tables 2 and 3).

5.1. Carbon-Based Labeled Immunosensors. Carbonaceous materials (carbon nanotubes, graphene oxide, etc.) are of tremendous attraction for electrochemical immunosensing due to their key properties, such as high electrical conductivity, biocompatibility, and high active surface area.[94,95] Additionally, uniform distribution of carbonaceous nanomaterials on the surface of an electrode holds the analytes or bioreceptors strongly and enhances the stability, sensitivity, and long-range linearity of the immunosensors.[96] This section highlights the reports using carbon and its composites as substrate materials for labeled immunosensors. For instance, Yu *et al.* designed an immunosensor by modifying the glassy carbon electrode with covalently functionalized with single-walled carbon nanotubes (SWCNTs). SWCNTs along with signal amplification provide numerous domains for competitive recognition of NSE. Gold nanoprobe AP-Anti-IgG was designed by using alkaline phosphate conjugates as labels. The AP-anti-IgG/AuNPs exhibited high catalytic activity toward hydrolysis of apha-naphthyl phosphate (alpha-NP), leading to a dual signal amplification of SWNTs and gold nanoprobe for detection of a low-concentration of target. The incubation time for the formation of the immunocomplex was found to be 60 min. Moreover, the designed immunosensor provided a pragmatic tool for convenient detection of tumor markers in clinical diagnosis with the wider linear range of 0.01 ng mL^{-1} to 2 μg mL^{-1} and LOD of 0.033 ng mL^{-1}.[97] This range is an unbeatable linear range to date for electrochemical detection of NSE. Likewise, Acero Sánchez *et al.* described the introduction of disulfide linkage as anchor sites into immunoglobin structure for covalent self-assembly of antibody onto the bare gold surface. Disulfide moieties were introduced *via* primary amines, carboxylic acid, and carbohydrates present in the structure. They have compared all the strategies using SPR (surface plasma resonance) and concluded that carbohydrates give the best performance in terms of analytical response as the sugar moieties in the carbohydrates are located on the specific sites on the immunoglobin structure. Further, the ability of carbohydrate strategy was investigated by EIS and DPV and exhibited linear range and sensitivity. They have shown

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ACS Omega 2024, 9, 33−51

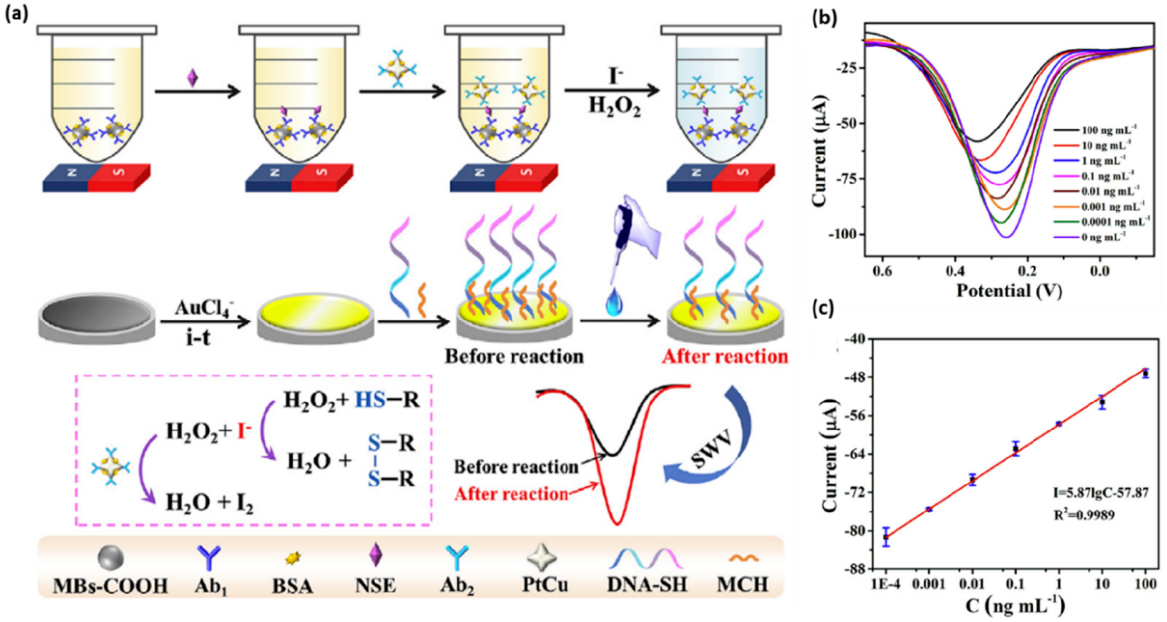

Figure 4. (a) Preparation process of MnO_2 UNs/Au@Pd^Pt NCs-Ab_2 and fabrication procedure of the immunosensor (b) DPV curves of the designed immunosensor for detecting various NSE concentrations from 0.0001 ng L^{-1} to 100 ngmL^{-1}. (c) Fitted curve of detecting various NSE concentrations (SE, n = 5). Reprinted with permission from *Biosens. Bioelectron* **2019**, *143*, 111612, Copyright, 2017, Elsevier.[23]

remarkable results in terms of linear range and LOD. The sensor exhibited the detection range of 0−25 ng $mL^{-1}$and LOD of 4.666 ng mL^{-1}.[98] Fang and co-workers designed a novel immunosensor with triple signal amplification strategy based on 3D graphene fabricated labeled immunosensor in which ordered mesoporous carbon OMC-Si/Au was used as a label for secondary antibody while the gold nanoparticle AuNP induced the silver deposition on the electrode surface. They concluded that the immunosensor does not require any specific conditions or pretreatment of the metals. The proposed immunosensor has shown amazing results with the linear range of 0.02 pg mL^{-1} to 35 ng mL^{-1} and LOD of 0.008 pg mL^{-1}. The immunosensor has reached the incubation time of 40 min as compared to other reports. Moreover, the immunosensor has shown relative error of 4% as compared with ELISA for two methods, proving that the established strategy has good potential for clinical analysis and diagnosis.[99] Progastrin peptide (Pro-GRP) is another promising marker which is utilized in conjunction with NSE in monitoring SCLC with improved sensitivity and specificity. Zhong *et al.* published a report based on multiwalled carbon nanotubes (MWCNTs) for simultaneous determination of NSE and PRO-GRP. Progastrin releasing-peptide (Pro-GRP) is another promising tumor marker for SCLC because of its high diagnostic sensitivity. They have used liposomes encapsulated with electrochemically active biomolecules such as uric acid (UA) and ascorbic acid (AA) as immune labels on MWCNTs on glassy carbon electrodes. The immunosensor has shown the wider linear range for NSE and Pro-GRP of 50−1000 pg mL^{-1} and 5−100 ng mL^{-1} and incubation time of 30 min, respectively. Moreover, a 3.4% difference was shown after addition of interfering agents. The proposed immunosensor has shown parallel results with single-analyte detection with shorter immunoassay time.[100]

5.2. Metal/Metal−Carbon Composites. At present, metal-based electrochemical immunosensors, such as metal oxides, metal nanoparticles, metal−carbon composite nanomaterials, etc., have gained substantial interest.[101] Compared with single metals, the bimetallic compounds are more beneficial to enhance the superior electrochemical performance due to synergistic changes in electronic and physicochemical properties between the bimetallic components.[102] For example, a bimetallic Pt/Cu nanoparticle initiated the cascade reaction of oxidation of iodide to iodine in the presence of H_2O_2 which was used for conjugation with Anti NSE(Ab_2) to trigger the same reaction. This strategy has achieved an ultralow detection limit of 52.14 fg mL^{-1} and wider linear range from 0.0001 to 100 ng mL^{-1} with good selectivity and repeatability (Figure 4).[103] Similarly, Karaman *et al.* designed a sandwich immunosensor utilizing gold nanoparticle-modified molybdenum disulfide and reduced graphene oxide (AuNPs@MoS_2/rGO) as the electrode platform and $CoFe_2O_4$@Ag nanocomposite for the signal amplification. The primary anti-NSE (Ab_1) was captured and immobilized on the AuNPs@MoS_2/rGO modified electrode surface by amino-gold affinity, and the conjugation of anti-NSE secondary antibody (Ab_2) on $CoFe_2O_4$@Ag nanocomposite was successfully completed by the strong esterification reaction. The proposed immunosensor offered a highly sensitive determination of antigen NSE with a wide linearity from 0.01 to 1.00 pg mL^{-1} and a LOD of 3.00 fg mL^{-1}, demonstrating the utility of the immunosensor in the early stage determination of lung cancer.[104] Zhou *et al.* published a new report based on host−guest chemistry and biomimetic nanoenzymes which have achieved success in robust immobilization of signal molecules by host−guest molecular recognition and sensitive catalytic amplification of electrochemical signals. Water-soluble pillar arene functionalized PdPt porous core−shell octahedral nanodendrites have been synthesized and used for fabrication of an NSE immunosensor. The addition of Pd and WP6@PdPt altered the electronic structure, accelerated the electron transport, and promoted the synergic catalytic effect. This new type of immunosensor achieved a wide linear range from 0.0003 to 100 ng mL^{-1} and lowest detection limit of 0.095 pg mL^{-1},

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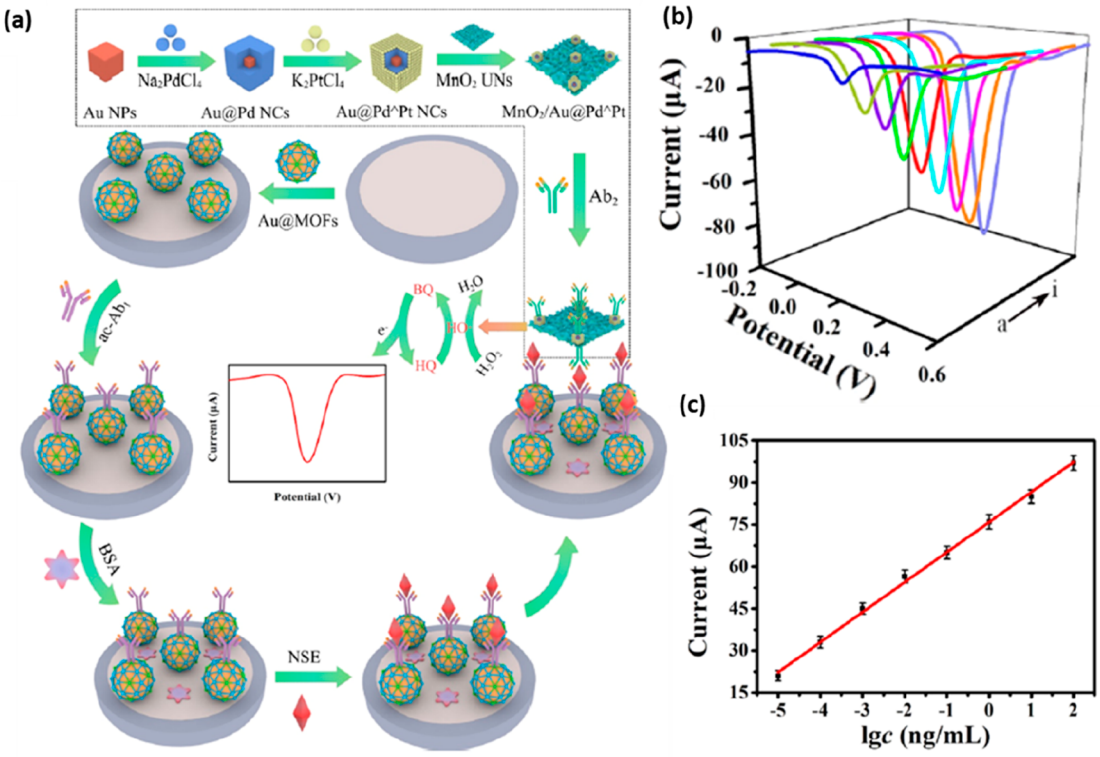

Figure 5. (a) Schematic diagram of sandwich-type immune process of PtCu/thiol-modified DNA (DNA-SH) and 6-mercaptohexanol (MCH) based immunosensor. (b) SWV responses of the improved electrochemical biosensor toward different NSE concentrations from blank to 100 ng mL^{-1} in 0.01 M PBS (pH 7.4) containing 5 mM $[Fe(CN)_6]^{3-/4-}$. (c) Calibration curve between current values of SWV and the different concentrations of NSE. Reprinted with permission from *ACS Biomater. Sci. Eng.* **2020**, *6* (3), 1418−1427. Copyright, 2020, American Chemical Society.[106]

which is highly comparable with sandwich immunoassay-based detection methods.[105] Likewise, Wang *et al.* have reported an article based on core−shell Au nanoparticles embedded Zn based metal organic framework (MOF). It was prepared as a substrate material for primary antibody immobilization. Due to the presence of large functionalities, it could increase primary antibody immobilization through covalent linkage. On the other hand, Au@PdPt NCs loaded on ultrathin MnO_2 nanosheets acted as labels for secondary antibody. The immunosensor exhibited a low detection limit (4.17 fg mL^{-1}) and broad linear range 10 fg mL^{-1} to 100 ng mL^{-1} under the optimal conditions (Figure 5).[106] The immunosensor gave satisfactory results in human serum samples also. Another work based on Cu-MOF and Fc-L-Cys was reported by Huang and co-workers. They employed a ratiometric electrochemical immunosensor for quantitative analysis of NSE. In this work Cu-MOFs-Au is employed as the electrode sensing surface and Fc-L-Cys as the label of Ab_2 in which Cu-MOFs served as a redox mediator for the signal where its large specific surface area could provide more sites for the placement of Au nanoparticles. Additionally, cysteine (L-Cys) could avoid a large amount of Fc-COOH leakage so that Fc^+ can stably provide the required signal. With the beefing up of NSE concentration, the redox peak of Cu-MOFs-Au decreased and that of Fc-L-Cys increased. Cu-MOFs showed a good redox activity at −0.2 V. After modification with Au nanoparticles, the increase in electrochemical signal was because of the increased conductivity of Cu-MOF due to incorporation of Au nanoparticles increasing the binding sites for Ab_1 so that Fc-L-Cys was chosen to label Ab_2. The immunosensor showed excellent performance in the concentration range of 1 pg mL^{-1} to 1 μg mL^{-1}, and the detection limit was 0.011 pg mL^{-1}.[107] The mesoporous silica nanoparticles (MSNs) with controllable pore diameters have also been used to fabricate an electrochemical immunosensor with antibodies confined to the pore channels. Due to poor conductivity and hydrophobicity of silica, it leads to weak electrical signals along with undesirable and poor detection limit. In this context, Wang *et al.* published a report regarding the synthesis of mesoporous silica with the introduction of gold nanoparticles to the inner walls of silica. The aforementioned AuNP/MSN based sensor has shown desirable linear relationship with the concentration of 0.1−2000 ng mL^{-1} and a detection limit of 0.05 ng mL^{-1}.[108] Later, Soomro *et al.* reported the development of a highly sensitive photoelectrochemical (PEC) immuno-biosensor based on highly photoelectroactive $NiWO_4$ nanostructures, grown directly (*in situ*) over a conductive substrate Indium Titanium Oxide (ITO) using a template-controlled low-temperature coprecipitation approach. They have demonstrated the photocatalytic activity of $NiWO_4$ toward uric acid (UA) which served as the base for the electrochemical-mechanism (EC) based PEC inhibition sensing. This approach enabled highly sensitive detection of NSE within the analytical range of 75 to 723 ng mL^{-1} with signal sensitivity measurable up to LOD of 0.12 ng mL^{-1}.[109] Yu *et al.* designed a sandwich-type electrochemical immunosensor for ultrasensitive detection of NSE using $Au/Cu_xO@CeO_2$ as the label and AuPt NSNs as the substrate. Therein, $Cu_xO@CeO_2$ showed favorable catalytic activity toward hydrogen peroxide (H_2O_2) in PBS buffer, pH 7.38. AuPt NSNs due to the rugged and spiny

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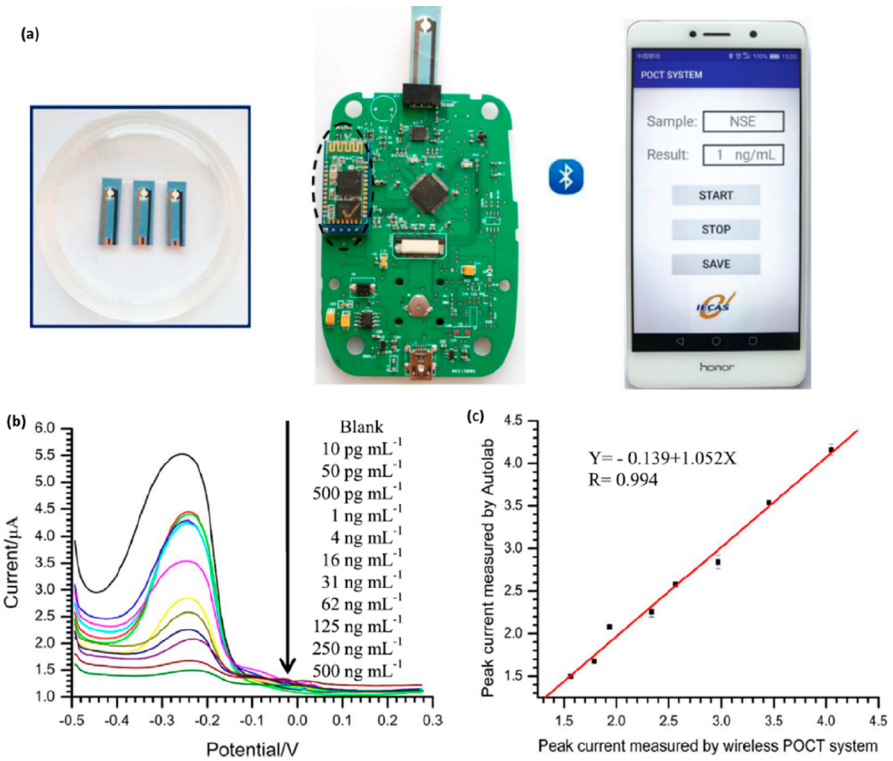

Figure 6. (a) The prototype of the wireless POCT system (Photograph courtesy of Yan Fan, Copyright 2017). (b) The DPV responses of NSE assay in the concentration range from 10 pg mL^{-1} to 500 ng mL^{-1} using the wireless POCT system. (c) The linear correlation of DPV peak currents measured between the wireless POCT system and Autolab. Reprinted with permission from *Biosens. Bioelectron.* **2017** *95*, 60−66. Copyright 2017, Elsevier.[117]

morphology of the nanocomposites, the specific surface area was increased that could increase the immobilization amount of Ab_1, and Cu_2O, CuO, and CeO_2 amplified the current signal by promoting the catalytic efficiency toward H_2O_2. The constructed immunosensor showed a low detection limit (31.3 fg mL^{-1}); a broad linear range (50 fg mL^{-1} to 100 ng mL^{-1}); and great performance in terms of sensitivity, specificity, and stability.[110] Furthermore, anchoring metal alloys onto graphene oxide (GO) has been considered an ideal strategy for immobilization of antibodies. Larger surface area provides more active regions and can load more active probes bound to biomolecules. Moreover, the heteroatom-doped graphene (GR) with larger functional surface area and higher number of active sites can be utilized for immoblization of metal atoms.[111]

5.4. Polymer and Its Composites. Various conducting and nonconducting polymers attract scientific interest for their role as support matrices for the immobilization of biomolecules. Polymers and their composites have been widely used as the substrate material for immoblization of antibodies in electrochemical immunosensors.[112] Polypyrrolepoly(3,4-ethylenedioxythiophene) (PPy-PEDOT) nanotubes were synthesized by Tang *et al. via* a novel chemical polymerization route, which effectively increased the interfacial electron transfer rate. Coupling of Au nanoparticles (AuNPs) with PPy-PEDOT resulted in an excellent immune response under optimal conditions. The developed immunosensor showed wide detection range (50 fg mL^{-1} to 100 ng mL^{-1}), low detection limit (18.5 fg mL^{-1}), good stability, and reproducibility in real sample analysis also.[113] Fan *et al.* recently reported a photoelectrochemical immunosensor (PEC) based on a ZnO/CdSe semiconductor composite. They have chosen an antifouling interface composed of poly(acrylic acid) (PAA) and polyethylene glycol (PEG) that can prevent nonspecific proteins from adhering to the electrode surface. The immunosensor was constructed with $Rh_{0.6}Ru_{0.4}$ as the core and the outer layer of polydopamine-coated $Rh_{0.6}Ru_{0.4}$@PDA as the second antibody marker. Due to its light absorption characteristics, the photocurrent of the system is significantly reduced, so the sandwich photochemical sensor can be constructed. The sensor achieved a linear range of 0.1 pg mL^{-1} to 100 ng mL^{-1} and good stability and reproducibility with the standard deviation of 2.3%.[114] The sandwich immunosensors are the most reliable and frequently employed tools for selective and specific monitoring of antigens. These assays have demonstrated the lowest detection limit as low of 16 fg mL^{-1} and wider linear ranges for NSE. Undoubtedly, labeled immunoassays are the most successful strategies and an excellent platform for immunosensing of NSE to date; however, the multiple operational and washing steps have reduced their reproducibility and repeatability. Moreover, the labeled immunoassays have many other disadvantages, such as sensitivity toward pH and temperature, and time-consuming preparation of immunosensors, etc., which limit the application of these assays for POC detection.

6. MATERIALS FOR LABEL-FREE IMMUNOSENSORS

A direct or label-free strategy gives pronounced results due to the direct interaction between Ab-Ag without the use of any labeling agent as well as secondary antibody. Eradication of the secondary antibody in label-free immunosensors offers various advantages, such as

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- Increased immune speed
- Reduced reagent amount
- Decreased false positive signals related with the nonspecific bindings
- Simplified immunoassay procedure
- *In situ* analysis of NSE

The label-free technique also wipes out the role of interfering agents and resolves the issue of multiple labeling, and most importantly, it provides ease of measuring the reaction kinetics of a biological systems.[115] Different protocols based on various substrate materials employed for label-free electrochemical detection of NSE are discussed in this section.

6.1. Carbon/Metal−Carbon Composite Based. Zhong *et al.* introduced an immunosensor based on a Prussian blue (PB-SiO_2) nanocomposite using a microemulsion method. PB acts as a redox mediator, and SiO_2 provides a biocompatible environment for the immobilization of the antibody. Also, PB-SiO_2 shows high catalytic activity toward H_2O_2. To improve the antibody loading, APTES (3-aminopropyltriethoxysilane) was prepared by self-assembly, followed by attachment of chitosan/AuNP to the entire surface. The resulting CS-nanoAu/APTES/PB-SiO_2 based immunosensor showed remarkable sensitivity with the linear range of 0.25−5.0 and 5.0−75 ng mL^{-1} and a limit of detection of 0.08 ng mL^{-1} along with the longer lifetime of 20 days.[116] Although the immunosensor showed a longer lifetime, the sensitivity of the proposed immunosensor needed improvement. In this context, Fan *et al.* reported a wireless POCT system consisting of microfluidic paper-based analytical devices (μPADs), electrochemical detector, and an Android smartphone. They modified the μPADs with nanocomposites synthesized by Amino functional graphene, thionine, and gold nanoparticles (NH_2-G/Thi/AuNPs) as a substrate material for NSE detection (Figure 6a). Combined with μPADs, the performance of the wireless POCT system was evaluated using the differential pulse voltammetry (DPV) technique. The immunosensor was able to achieve wider detection range of 1−500 ng mL^{-1} with LOD of 10 pg mL^{-1} (Figure 6b,c). The detection results were automatically stored in the EEPROM memory and could be displayed on the Android smartphone through Bluetooth in real time, and these results were comparable with the commercial workstation results.[117] Later Jing Han *et al.* investigated, a novel label-free electrochemical immunosensor based on nickel hexacyanoferrate nanoparticles (NiHCFNP) assembly over gold nanosheets (AuNC) in the presence of DA where further coating of AuNP functionalized Graphene nano sheets over NiHCFNP/AuNC film increased the Anti NSE loading and enhanced the electrocatalytic activity of NiHCFNP toward the electrochemical catalysis of DA because of the large surface area and high conductivity of AuNP. The proposed immunosensor Au−Gra/NiHCFNPs/AuNCs/GCE exhibited a linear range of 0.001−100 ng mL^{-1} with the lowest detection limit of 0.3 pg mL^{-1}.[85] Fu *et al.* highlighted the role of inorganic Pt nano flowers as labels for a highly efficient enzyme-free electrochemical immunoassay for NSE. Pt nanoflowers based immunosensor achieved the detection range 0.05 to 150 ng mL^{-1} and LOD of 104.15 ng mL^{-1}. To investigate the accuracy of the developed immunoassay, the standard samples were spiked into blank NSE human serum samples, and the recovery was found to be between 87% and 121.1%.[118] Later, Li *et al.* reported another enzyme-free strategy of bioelectrocatalytic reaction of the most oxidizable base guanine on nanostructured graphene in the presence of $Ru(ppy)^{3+}$. They aimed to develop an *in situ* amplified immunoassay without the participation of enzymes. Also, this methodology has avoided the use of two working electrodes or multiple enzymes for signal amplification and resulted in the system having an unbeatable shorter incubation time of 25 min. The developed immunosensor showed a wide linear range from 1.0×10^{-11} to 1.0×10^{-5} mg mL^{-1} with a low detection limit of 1.0×10^{-11} mg mL^{-1} for NSE.[119] A novel conductive hydrogel was prepared by a cross-linking method using 1,3,5-benzene tricarboxylic acid as the ligand and Fe^{3+} as the metal ion and was fabricated on the glassy carbon electrode using the drop coating method. Gold nanoparticles were deposited over the hydrogel by electrodeposition. The immunosensor displays a linear relationship with NSE concentration with the wider linear detection range of 1 pg mL^{-1} to 200 ng mL^{-1} and improved lowest detection limit of 0.26 pg mL^{-1}. The authors have also demonstrated the specificity of the immunosensor in the presence of an excess amount of interferents like DA, UA, AA, AFP, BSA, CEA, IgG, and PSA.[120] However, the NSE response was found to be the same regardless of the presence or absence of the interferents, exhibiting the remarkable superiority of the immunosensor. Likewise, Yin and co-workers published an article based on the naturally occurring polymer chitosan. Chitosan is highly biodegradable and biocompatible and holds the ability to form a hydrogel. The catalytic signal amplification toward H_2O_2 was realized in the following main steps: First, the large amine groups on condensed ferrocene (Fc-CHO) by covalent linkage (−C=N−). Subsequently, the Fc^+/Fc redox couple catalyzed the formation of H_2O_2 with signal amplification. In the same context, they have developed a noble bimetallic nanoparticle-based immunosensor for NSE by using Au-PD/MWCNT/CS-Fc hydrogel. Remarkably, AuNP/MWCNT composite enhanced peroxidase like catalytic activity toward H_2O_2 to achieve further signal amplification. The proposed immunosensor showed a sensitivity of 7.22 μA with the lowest detection limit of 0.483 pg mL^{-1}. Electrocatalytic performance was investigated with Au-Pd/CS-FC. The current response for the same was lesser than that of Au-PD/MWCNT/CS-FC, displaying the superiority of MWCNTs.[121] The fabricated immunosensor showed a good correlation with electrochemiluminescence, proving the reliability of the immunosensor. Considering the outstanding performance of the polymer toward NSE, the same group has synthesized multifunctional conductive hydrogel polypyrrole-polythionine-gold with glucose oxidase via a one-pot method using pyrrole, thionine as monomer, $HAuCl_4$ as coexisting agent, and glucose oxidase as doping agent. The hydrogel exhibited strong electrochemical response toward NSE at 0 V (vs Ag/AgCl), eliminating the role of redox mediator. They introduced a new cascade reaction signal amplification strategy to achieve good analytical performance. Although the immunoassay time was 50 min, a limit of detection 0.65 pg mL^{-1} was achieved, which is lower than that in previously reported literature.[122]

6.2. Polymer and Its Composites. Wang *et al.* explored a novel electrochemical redox-active polyresorcinol/Au/Pt nanocomposite using resorcinol as monomer and $HAuCl_4$ and H_2PtCl_6 as coexisting agents. The analytical performance of the immunosensor was investigated in 0.1 M phosphate buffer (PBS) (pH 6.5). The role of multifunctional polyresorcinol/Au/Pt nanocomposite substrate as redox mediator has been highlighted showing the redox signal at 0.92 V, which unveils

https://doi.org/10.1021/acsomega.3c06388
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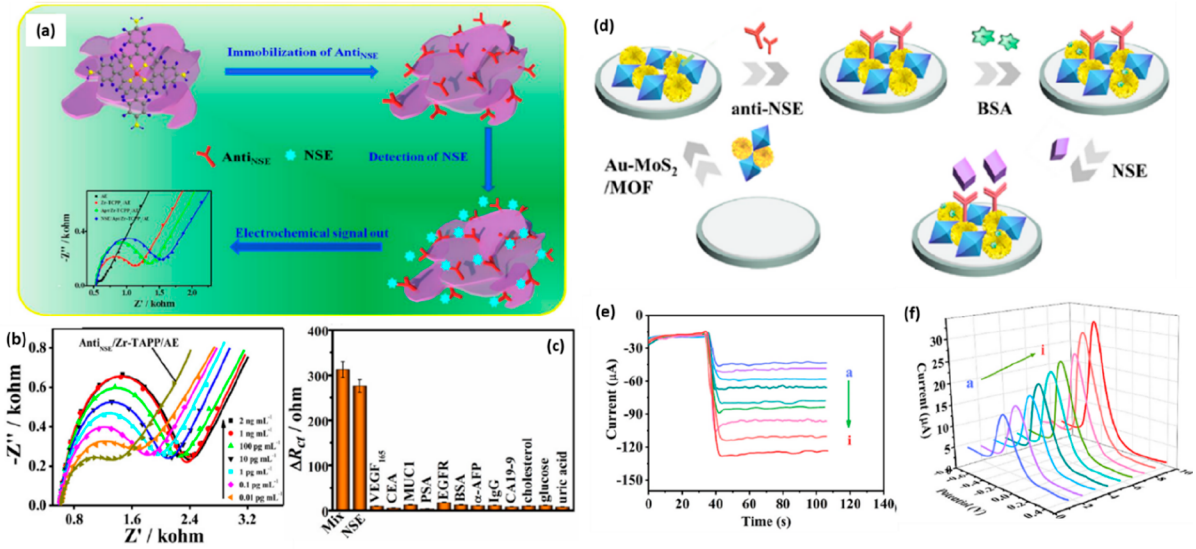

Figure 7. (a) Schematic diagram of the construction of Zr-TAPP-based aptsensors for the detection of NSE. (b) EIS responses of Zr-TAPP-based immunosensor for the detection of different NSE solutions with the series of concentrations (0.01, 0.1, 1.0, 10, 100, 1000, and 2000 pg mL^{-1}). (c) Selectivity of the proposed biosensor toward NSE (n = 3) (10 fg mL^{-1}). Reprinted with permission from *Sens. Actuators B Chem.* **2020** *314*, 128090. Copyright, 2020, Elsevier.[126] (d) Stepwise fabrication of the Au−MoS_2/MOF based immunosensing interface. (e) Amperometric i−t results and (f) SWV results of electrochemical immunosensor at different concentrations of NSE. Reprinted with permission from *Biosens. Bioelectron.* **2022**, 195, 113648. Copyright, 2022, Elsevier.[127]

the signal amplification ability by catalyzing H_2O_2 through electrochemical electron transfer. The immune electrode has a linear response in the 10 pg mL^{-1} to 100 ng mL^{-1} NSE concentration range and a 7.8 pg mL^{-1} detection limit (at an SNR of 3).[123] Zhang *et al.* for the first time introduced an immunosensor based on 3D rGO/PANI by the electrodeposition method. 3D rGO/PANI showed a well-defined cross-linked porous structure and large accessible surface area for antibody immobilization. The streptavidin−biotin complex also increased the biocompatibility for antibody loading on the electrode surface, and EDC/NHS was used as coupling agent for covalent interactions of antibody with the surface. The key factor in the development of this immunosensor lies in its stability as the immunosensor retained 91% and 83% of its initial response after 15 and 30 days of storage, respectively. Under the optimal conditions, a linear current response of PANI to NSE concentration was obtained over 0.5 pg mL^{-1} to 10.0 ng mL^{-1} with a detection limit of 0.1 pg mL^{-1}. Moreover, the immunosensor showed excellent selectivity, good stability, and satisfactory reproducibility and regeneration and was employed to detect NSE in clinical serum specimens.[124] Recently, Aydin *et al.* reported the first biosensor fabricated by utilizing epoxy substituted polypyrrole polymer on an indium tin oxide electrode as a platform for label-free immunosensing of NSE. Signal frequency impedance (SFI) was performed to characterize the binding interactions, while the changes in surface morphology were investigated by scanning electrochemical microscopy and atomic force microscopy. The proposed immunosensor showed a linear range of 0.02−7.5 pg mL^{-1} having an ultralow limit of detection of 6.1 fg mL^{-1}. More importantly, the incubation time was reduced to just 15 min.[125] Li *et al.* demonstrated a novel impedimetric immunosensor based on a novel metal−ligand complex strategy using a zirconium-porphyrin complex for a label-free immunosensor for sensitive detection of NSE. The efficient coordination of the Zr(III) and four N atoms on porphyrin rings of 5, 10, 15, and 20-tetra(4-aminophenyl) porphyrin (TAPP) (denoted as Zr-TAPP) exhibited a 2D nanostructure, rich N-related groups, and high hydrophobicity, as well as strong π−π* stacking that provided a strong adsorption ability toward AntiNSE and good stabilization ability for the antibody−antigen complex (Figure 7a). The as-developed immunosensor displayed superior sensing performance for the NSE detection, thereby resulting in an extremely low detection limit of 7.1 fg mL^{-1} within a wide linear range of 10.0 fg mL^{-1} to 2.0 ng mL^{-1} and a long-term storage stability of 15 days (Figure 7b,c).[126] Another report based on the same M-ligand charge transfer was reported by Liu *et al.* for label-free electrochemical immunosensing based on the Au−MoS_2/MOF catalyst for the detection of NSE (Figure 7d−f). The immunosensor displayed a wide linear range from 1.00 pg mL^{-1} to 100 ng mL^{-1} with a limit of detection of 0.37 pg mL^{-1} and 0.52 pg mL^{-1}. The real sample analysis obtained good recovery results in the range of 99.00−105.20%.[127] Label-free immunoassays have overcome the shortcomings of labeled immunoassays in reducing the analysis time, cutting down the complex multistep procedure to a single step, and applicability toward in situ analysis. However, it still possesses certain drawbacks such as the nonspecific adsorption on the electrode surface which sometimes produces different signals that are problematic to decouple with the generated signal.

Development of label-free detection tools is undoubtedly a great benefit for the large-scale study of protein−protein interactions. However, sensitivity and specificity often become major concerns for such label-free techniques during the handling of very complex samples. The marriage of microarrays and label-free techniques is gaining popularity; the scientific community is still waiting for successful transformation of these label-free principles to large microarray surfaces.

With the ongoing active research efforts, it is expected that the field of label-free protein microarrays will become more robust, sensitive, reliable, rapid, cost-effective, and user-friendly in the near future.

https://doi.org/10.1021/acsomega.3c06388
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7. COMMERCIALIZATION

Despite numerous research efforts and innovations, the bottleneck toward commercialization of these sensors lies in the gap between industry and academia. The pathway from a scientific breakthrough originating from fundamental research to a marketable product, procedure, or service is extensive and fraught with substantial challenges. However, the collaboration between industrial and academic research is expected to jump-start the commercialization process and develop cost-effective and sustainable biosensors. A few commercial kits are now available in the market, as explained below.

In the current market, cancer diagnostics is a process of discovering biomarkers, proteins, and other indicators that lead to the detection of a cancerous tumor. Diagnostic testing is used to confirm or rule out the presence of sickness, track disease progression, and schedule and analyze treatment outcomes. The method of identifying cancer entails the use of specific technologies and gadgets designed for cancer diagnostics. This review has demonstrated an overview which provides an outlook for the development of early diagnostic techniques and development of electrochemical immunosensors for detection of NSE biomarker for early diagnosis of small-cell lung cancer. According to National Cancer Institute estimates, about 29.5 million cancer cases are expected to rise every year and the diagnostic market needs a worldwide increase. The major countries for the growth of cancer diagnostic market include Japan followed by USA and Germany. Along with government initiatives, heavy investment by the private sector in the diagnostic centers is also considered to be one of the enlightening aspects. The development of biomarkers has boosted the immune assay-based test cancer kits. The cancer market is prevailing with ELISA kits. A global market analysis of the growth of the US$ 19.63 billion in 2022 reported an expected revenue compound annual growth rate (CAGR) of 3.9%, resulting in US$ 27.74 billion by 2031.[128] The key companies for the manufacturing of rapid lung cancer detection kits are Wuhan EIAab Sci. Co., Ltd. (Cat# E0537h, China); Diagnostic Automation, Inc. (Cat# 6334Z, USA); Alpha Diagnostic Intl. (Cat# 0050, USA); and USCN Life Sci. Inc. (Cat# E90537Hu, USA). Although the ELISA-based immunoassay techniques have shown excellent evolution, the development of electrochemical immunosensors for NSE detection and their commercialization still remain challenging. Technical hurdles need to be overcome to lift up the commercialization of electrochemical immunosensors. The productive collaboration between educational research institutes may lead to the innovative research growth in the sector of development of simpler, cheap, automated, and fast immunosensors to meet the market demand.

8. FUTURE PERSPECTIVES AND SUMMARY

This review conferred the trends of labeled and label-free immunosensors for the electrochemical detection of NSE. In recent years, research efforts have been made toward improving the sensitivity, specificity, LOD, and fabrication procedure of immunosensors for clinical analysis applications. Although the desirable advantages in current electrochemical immunosensors for NSE are noticeable, to explore immunosensors for real-time analysis, there are still some significant challenges and obstacles in this field. Other aspects are associated with more recent challenges.

The development of flexible substrate-based immunosensors with long-term storage stability and biocompatibility is necessary. The sensors based on screen-printed electrodes have emerged as a boom for clinical analysis of various diseases because of their disposable nature, low cost, and more importantly on-site detection (POC).[132] The advancement in electrochemical techniques coupled with screen-printed electrode technology has given a new direction to electroanalytical techniques.[133,134] Electrochemical screen-printed electrodes fabricated with different materials have also been reported in the past few years for detection of NSE. Although the evolved techniques are highly sensitive, they still require a lot of improvements in terms of flexible substrates for real-time analysis of clinical samples.

To ensure excellent enzyme stability and activity, the orientation and three-dimensional arrangement of antibodies over the substrate material are highly required. Most of the immobilization strategies do not actively elaborate the controlled antibody orientations over the electrode surface, resulting in the inaccessibility of antibody activity as well as increased chances of nonspecific interactions. Therefore, surface orientation, functionality, and homogeneity on the surface coverage play a key role in developing the electrocatalyst for fabrication of precise and accurate immunosensors. Analytical techniques like SEM, TEM, and XPS just provide two-dimensional and three-dimensional structural projections of synthesized materials. Other powerful surface techniques, such as atomic force microscopy (AFM), surface plasma resonance (SPR), scanning electrochemical microscopy (SECM), electrochemical quartz microscopy (EQCM), and time-of-flight secondary ion mass spectroscopy (TOF-SIMS), that feature the surface characterizations, such as orientation, composition, and spatial distribution of the molecules, are required. Nonetheless, excellent evolution has occurred in the field of electrochemical immunosensing, but the future of electrochemical immunosensors remains challenging. Therefore, the development of new analytical techniques can be applied to optimize the procedures of immunosensor functionalization for various applications. The identification of the dominant orientation (tail-on vs head-on) and surface density values of immobilized antibodies enhances the practical applicability of NSE electrochemical immunosensors for point-of-care diagnosis.

In addition, the automation of electrochemical immunosensors by rechargeable devices and self-powered systems is crucial. The integrated and compact electrochemical sensors are a primary requirement for the development of portable sensing devices to overcome the limitations associated with the large-scale production and commercialization of electrochemical immunosensors. Furthermore, the flexible and sensors combined with automated wireless data communication systems present a significant step toward new market potential as point-of-care devices. Moreover, wearable sensors have seen growth in real-time monitoring for a wide range of biomedical, sport, and military scenarios owing to their peculiar features. To date, self-powered sensors have found their place in the analysis of metabolites, and the expansion to other fields, such as food analysis, medicine, and healthcare, is still under consideration. The ultimate goal is to meet the challenge of reliability and robust self-powered electrochemical immunosensors which can replace the use of high power consumption instruments like potentiostat/galvanostat and can be utilized as point-of-care detection devices at low cost.

https://doi.org/10.1021/acsomega.3c06388
ACS Omega 2024, 9, 33−51

CONCLUSION

Herein, various aspects of immunosensors for the detection of NSE are discussed in detail. The main features and recent advances of various electroanalytical techniques are compared in Table 1. The performance of nanomaterials to enhance the analytical characteristics of immunosensors is discussed in detail, along with the advantages and limitations of immunoassay techniques with an emphasis on the commercialization of screen-printed electrodes in clinical diagnosis for future developments in this field. We predict a bright future for NSE immunosensors based on different immunoassay techniques. However, to date, very little work has been done on NSE immunosensing for perspective commercialization. Through constant efforts to minimize the drawbacks associated with available techniques, one can expect the extensive commercialization of immunosensors for health monitoring applications, especially in remote areas.

AUTHOR INFORMATION

Corresponding Author

Tharamani C. Nagaiah − *Department of Chemistry, Indian Institute of Technology Ropar, Rupnagar, Punjab 140001, India;* orcid.org/0000-0003-3545-6668; Email: tharamani@iitrpr.ac.in

Authors

Daisy Mehta − *Department of Chemistry, Indian Institute of Technology Ropar, Rupnagar, Punjab 140001, India*

Divyani Gupta − *Department of Chemistry, Indian Institute of Technology Ropar, Rupnagar, Punjab 140001, India*

Alankar Kafle − *Department of Chemistry, Indian Institute of Technology Ropar, Rupnagar, Punjab 140001, India*

Sukhjot Kaur − *Department of Chemistry, Indian Institute of Technology Ropar, Rupnagar, Punjab 140001, India*

Complete contact information is available at: https://pubs.acs.org/10.1021/acsomega.3c06388

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

T. C. Nagaiah thanks DST-Nanomission (SR/NM/NS-1376/2014) and the Science and Engineering Research Board (SPG/2021/002415).

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