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INVITED REVIEW

Nucleic acid-based electrochemical biosensors for rapid clinical diagnosis: advances, challenges, and opportunities

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ABSTRACT

Clinical diagnostic tests should be quick, reliable, simple to perform, and affordable for diagnosis and treatment of diseases. In this regard, owing to their novel properties, biosensors have attracted the attention of scientists as well as end-users. They are efficient, stable, and relatively cheap. Biosensors have broad applications in medical diagnosis, including point-of-care (POC) monitoring, forensics, and biomedical research. The electrochemical nucleic acid (NA) biosensor, the latest invention in this field, combines the sensitivity of electroanalytical methods with the inherent bioselectivity of deoxyribonucleic acid (DNA) and ribonucleic acid (RNA). The NA biosensor exploits the affinity of single-stranded DNA/RNA for its complementary strand and is used to detect complementary sequences of NA based on hybridization. After the NA component in the sensor detects the analyte, a catalytic reaction or binding event that generates an electrical signal in the transducer ensues. Since 2000, much progress has been made in this field, but there are still numerous challenges. This critical review describes the advances, challenges, and prospects of NA-based electrochemical biosensors for clinical diagnosis. It includes the basic principles, classification, sensing enhancement strategies, and applications of biosensors as well as their advantages, limitations, and future prospects, and thus it should be useful to academics as well as industry in the improvement and application of EC NA biosensors.

Abbreviations: AuNP: gold nanoparticle; AuNR: gold nanorod; CFU: colony forming unit; CNT: carbon nanotube; DNA: deoxyribonucleic acid; EC: electrochemical; EIS: electrochemical impedance spectroscopy; MWCNT: multi-walled carbon nanotube; NA: nucleic acid; NP: nanoparticle; PCR: polymerase chain reaction; PNA: peptide nucleic acid; POC: point-of-care; RNA: ribonucleic acid; SELEX: systemic evolution of ligands by exponential enrichment; ssDNA: single-stranded DNA

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Introduction

Diagnosis is the process of finding the causes and nature of the illness. With a correct diagnosis, the doctor can treat the patient, prescribe medication and decide if surgery is needed. Health programs are aimed at diagnosing, monitoring, and treating diseases effectively. Diagnostic tests play an important role in this and in preventing or reducing the prevalence of diseases before they become a public health problem [1]. Rapid and early diagnosis can prevent unnecessary complications for patients [2]. Undiagnosed and asymptomatic patients may spread diseases without being aware of them, as is the case for COVID-19. Early diagnosis may help in rapid recovery as well as in preventing outbreaks of infectious disease [3]. Accurate and timely diagnosis allows doctors to institute the treatment that can reduce hospitalization and length of stay, increase survival, and lower costs [4,5] as well as reduce treatment-related complications such as the side effects of chemotherapy, thereby increasing the patient's quality of life [6–8].

The world is facing an increased risk of outbreaks of infectious diseases due to emerging and reemerging pathogens. Such outbreaks contribute to morbidity and death and require rapid diagnosis. Otherwise, the healthcare sector may be caught unaware, and hospitals may not have adequate resources to treat the

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disease [9]. Existing diagnostic systems, therefore, need to be more robust.

The recent outbreak of COVID-19, a highly infectious and pathogenic virus, has shocked the world, led to societal lockdowns, and slowed economic output. It continues to spread at an alarming rate, in many cases causing serious illness and rapid death. Many who survive to suffer from long COVID (COVID-related complications for a long period of time after recovery from the illness). This virus spreads when air-borne particles or droplets of saliva or mucus from an infected individual who is coughing or sneezing diffuse into the air or onto surfaces, and the next person becomes infected when they inhale them through their nose, when droplets enter their eyes or mouth, or when they contact contaminated surfaces or objects [10–12].

Rapid diagnostic tools like biosensors could play a vital role in diagnosing disease as they could be used as point of care (POC) diagnostic devices or for home applications [13]. Hence, there has been a steady increase in demand for high-performing devices for rapid detection and quantification of specific molecules in biological samples for clinical diagnosis and monitoring.

A variety of techniques, such as Gram-stain, culture and microscopy, biochemical tests, and serological methods such as enzyme-linked immunosorbent assay, enzyme immunoassay, agglutination, and immunomagnetic separation, are available for clinical diagnosis [14,15]. In addition, there are various molecular-based techniques such as polymerase chain reaction (PCR), real-time PCR, restriction fragment length polymorphism, nucleic acid (NA) sequence-based amplification, and NA microarray, among others [14–16].

Although traditional diagnostic approaches detect single or multiple analytes, several crucial challenges hamper their use in the field [17,18]; they are often laborious, may have low sensitivity and specificity, are costly, and are not amenable for POC diagnosis [19–22]. NA electrochemical (EC) biosensor approaches, on the other hand, may be able to meet the growing demands for rapid diagnosis, affordability, robustness, sensitivity and selectivity. These approaches also require minimal sample preparation [23,24] and they apply to POC tests. Therefore, EC biosensors are increasingly being seen as a viable and appropriate alternative to traditional laboratory methods as the devices are simple and user-friendly, specific for the target analyte, capable of continual monitoring, provide quick results, and are portable [25–27].

Biosensors are analytical devices that integrate a biologically active component with a suitable physical transducer to produce a quantifiable signal that is proportional to the concentration of analyte in the sample [28]. Over the last three decades, biosensors have been shown to be a promising and reliable substitute to conventional techniques owing to these features [29]. EC biosensors whose detection material or target analytes are NA are known as NA-based biosensors. NA-based biosensors use primarily deoxyribonucleic acid (DNA) and aptamers (both DNA and ribonucleic acid (RNA)). Peptide nucleic acid (PNA) is also used as an oligonucleotide probe [30]. The low production cost of microelectronic circuits, their ease of interfacing with standard electronic read-outs [31], and the ease of fabrication of NA on electrode surfaces with dropped nanomaterials that increase their sensitivity are additional advantages.

The spread of nanoscience and nanotechnology has impacted the development of EC biosensor technology [32]. The application of nanotechnology in biosensors has enabled the monitoring of trace amounts of analytes with a current signal level between 10^{-12} and 10^{-7} ampers [33]. The high sensitivity of biosensors along with their compatibility with current microfabrication technologies have made them ideal candidates for a wide variety of applications in clinical diagnosis [34]. Because nucleotide strands have strong bonds between the bases and their complementary sequences, biosensors based on NA are very sensitive tools [35]. The nanoparticle (NP)-based EC DNA biosensor can identify specific DNA sequences or mutated genes in human disease with high sensitivity and selectivity [36,37]. Thus, the introduction of the EC NA biosensor technique could potentially have a large positive impact on healthcare.

This review summarizes the findings of recent research on EC NA biosensors for clinical diagnosis and discusses strategies for improving their performance. The final section presents limitations as well as prospects for future application. The review should be a useful reference for both academics and industrialists in the development of advanced EC NA biosensors for clinical diagnosis.

Principles and design strategies of electrochemical nucleic acid biosensors

NA hybridization with capture probe or analyte tethering through aptamers is fundamental for the development of NA-based biosensor devices. In the NA hybridization process, single-stranded DNA (ssDNA) probes that are immobilized on a transducer surface recognize and bind the complementary target DNA/

RNA. For hybridization-based NA biosensors, specificity is attained by this exclusive recognition of the immobilized NA probe for its complementary target sequence [38–40]. The hybridization event is converted into a quantifiable electrical signal by the transducer in the EC NA biosensor [41]. A suitable NA probe immobilization procedure promotes high reactivity and appropriate alignment of the immobilized NA probe so that it hybridizes with its corresponding NA target [41]. Several immobilization techniques, such as physical adsorption, covalent immobilization, EC adsorption, biotin-avidin coupling, and EC entrapment, have been used to immobilize ssDNA probes onto transducer matrices. Biotin-avidin and covalent immobilization are the most common methods to fabricate the NA hybridization electrodes [42]. These fabrication processes result in high sensitivity and specificity, minimize nonspecific adsorption, and stabilize the immobilized DNA probes. For accurate biosensors, the control of this step is vital to ensure the orientation, accessibility, optimal reactivity, and stability of the surface-confined probe and to avoid nonspecific attachment [43].

Aptamers consist of single-stranded NAs that are designed using a combinatorial assortment method known as systemic evolution of ligands by exponential enrichment (SELEX) [44]. Classically, an aptamer is 100 base pairs in a span that contains a 20–70 randomized base pair binding zone in the center and has primer binding areas at the two terminals [38,44]. Aptamers are designed in a hairpin structure that intercalates their target molecules. When an aptamer is immobilized on a conductive support, the single-stranded chains of NA enable tethering, and it folds into a three-dimensional shape upon binding its target. The intercalation of the target releases the hairpin structure and subsequently changes the EC signal [30].

Peptide nucleic acid (PNA) is a modified DNA or DNA analog with an uncharged N-(2-aminoethyl) glycine-based pseudo peptide backbone that has been shown to form complementary duplexes with normal DNA or RNA in the same way that a DNA probe does. PNA hybrids are more thermally stable than DNA duplexes and can be formed at low ionic strengths. PNA also has better specificity for DNA and RNA sequence recognition, allowing the use of shorter probes [45]. The capacity to detect target NA at ultra-low concentrations along with the ability to detect single-base mutations is one of the most striking characteristics of this detection method [46]. Standard peptide solid-phase synthetic procedures are used to make PNAs [47]. PNA oligomers are purified by reverse-phase high-performance liquid chromatography and are characterized by mass spectrometry after being cleaved off their solid-state support using conventional chemical techniques. PNAs are also commercially available, but they are still more expensive than DNA oligonucleotides. Pure PNAs are generally neutral molecules with characteristics of self-aggregation and low water solubility [46]. As a result, the use of PNAs in an EC NA biosensor is limited.

DNA dendrimers can be utilized to make more sensitive NA biosensors. The single-stranded arms of these tree-like structures hybridize with their complementary DNA/RNA sequence. Immobilization of these dendritic NAs onto the physical transducer results in a significantly higher hybridization capacity and as a result, a magnified response [48]. This concept may be realized in an EC NA biosensor for clinical diagnosis in the future.

The biosensor is composed of three parts: (a) the detector/transducer element that allows fabrication and decoding of the biological signal into a readable output; (b) a bio-recognition element such as NA or DNA, which serves as a mediator; and (c) the signal processor, which displays the user-friendly version of the transduced signal [49] (Figure 1). The most common transducers include gold electrodes, screen-printed electrodes, pencil graphite electrodes, glassy carbon electrodes, and carbon ionic liquid electrodes. The interaction between the bioreceptor and target analyte takes place on the surface of the electrode, generating electrons or ions, and this alters the electric potential, current, or other electrical parameters of the solution.

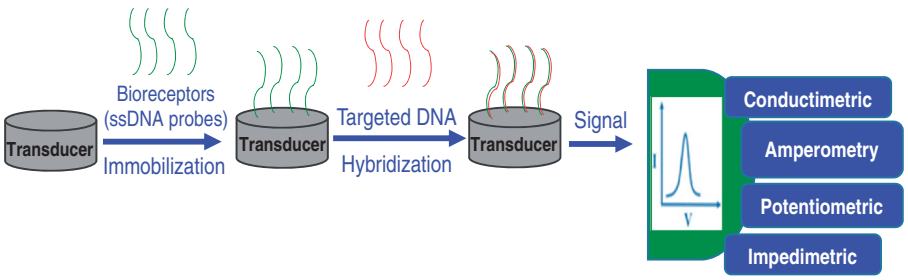

Figure 1. Schematic diagram of electrochemical DNA biosensors with classification.

The biological signal is transformed by the transducer into a measurable electrical signal that is proportional to the concentration of the analyte and that is displayed on a monitor after internal processing [24].

The EC NA biosensor is based on the attraction of ssDNA for its complementary strand to identify target sequences [50]. The electrode modified with bio-capture NA is placed in the buffer solution containing the target analytes. A hybrid duplex is formed between the bio-capture probe and the target NA at the electrode surface, or its hairpin structure is reformed or released. After hybridization, the electrical properties of the biosensor system are altered, and these changes are measured directly as current/resistance by the signal processor. Redox indicator compounds such as methylene blue are used to increase the signal. Such indicator chemicals are intercalated or electrochemically bound to hybridized molecules, resulting in enhanced electrochemical signals and allowing more sensitive and precise detection. The preparation of effective bio-capture modified electrodes along with the design of specific bio-capture probe sequences are important in developing the EC NA biosensor [31,51].

The performance of the EC NA biosensor relies on the method of immobilization of the ssDNA onto the transducer surface. Therefore, the surface modification technique has to be compatible with the related sensing technology [52]. Several approaches have been established to fabricate the NA probe onto the surface of biosensors: self-assembling monolayers on gold electrodes [53,54], biotinylated NA probes fabrication *via* biotin-avidin interaction on the electrode surface [55] or electro-polymerization that generates probes of different lengths [56]. The features of EC NA biosensors, such as specificity, sensitivity and lifespan, rely mostly on the immobilization of bioreceptors on the electrode surface. Consequently, the most significant requirement for the immobilization technique is that it should not destroy the biological activity of the bioreceptor or affect its interaction with the analyte of interest.

Types of electrochemical biosensors

Different types of EC DNA biosensors are used in clinical diagnostic applications. Each class of biosensor has its uses based on requirements. Biosensors are categorized as conductometric, amperometric, potentiometric, and impedimetric, depending on the type of EC parameter measured. A typical EC NA biosensor with its fundamental elements is shown in Figure 1.

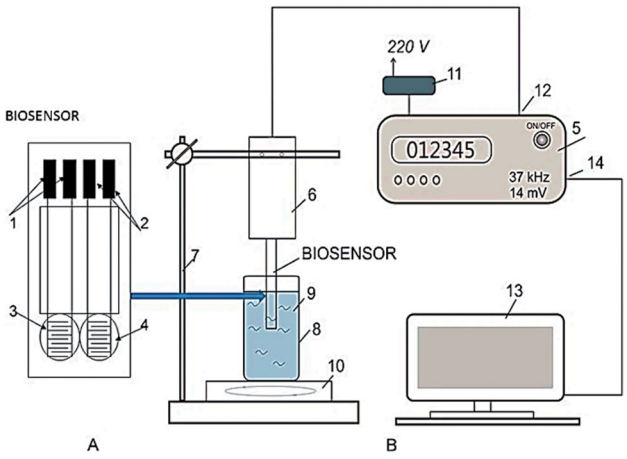

Figure 2. Arrangement of a conductometric biosensor (A) and conductometric set-up (B): two pairs of electrodes (1) and (2) were used; electrode pair (1) held the working membrane (3) containing the patulin-selective enzyme, urease; electrode pair (2) held the reference membrane (4); measuring device (5); holder (6); stand (7); working cell (8); testing solution (9); magnetic stirrer (10); system adapter (11); holder connection (12); computer (13); computer connection (14). Reprinted from Ref. [59] with permission. Copy right (2017) Elsevier.

Conductometric biosensor

Conductometry measures the change in conductance of a medium. In conductometric biosensors, charged species are either formed or consumed in the presence of the analyte, which changes the conductivity of the solution. This change is proportional to the concentration of the analyte being measured. Conductometric biosensors have significant advantages over other types as there is no need for a reference electrode; they operate at low-amplitude AC voltage, thus averting Faraday processes on the electrodes; they are insensitive to light; and they can be miniaturized and combined with cheap standard thin-film technology [57]. However, the use of these biosensors is limited because they are non-specific, and they have a poor signal-to-noise ratio [58]. Conductance measurements are comparatively less sensitive than other biosensor methods because sensitivity is interfered with by the parallel conductance of the solution [37]. A conductometric biosensor is described in Figure 2. The experimental setup developed by Soldatkin et al. for measurement of patulin examined changes in conductivity in a near-electrode layer of the conductometric biosensor [59].

Amperometric biosensor

The amperometric biosensor generates a current when a potential is applied between the reference and working electrodes. It is a self-reliant integrated device based on the quantification of the current generated

from the oxidation or reduction of an electroactive biological component [60]. The current produced is directly related to the amount of the electroactive component or its generation or feeding rate within the adjacent biocatalytic layer [61]. This biosensor is rapid, highly sensitive, specific, and precise compared with the potentiometric biosensor; it does not need thermodynamic equilibrium and its response is proportional to the amount of the analyte. The signal-to-noise ratio can be improved by increasing the amount of electroactive measurable analyte-related species [60]. However, the redox potential of other electroactive species in the sample controls the selectivity of the amperometric devices. Thus, the current measured by the device can contain the contributions of species other than the target analyte [58].

Biosensors are classified based on the electroactivity of the bio-receptor substrate or product (first generation), the use of redox mediators (second generation), or direct electron transfer between the redox-active biomolecule and the electrode surface (third-generation) [62]. In first-generation biosensors, either the concentration of redox enzymatic reaction products or the reduced concentration of the substrate is measured. Generally, this type of biosensor measures hydrogen peroxide or oxygen reduction; thus, dehydrogenase or oxidase enzymes are used. In second-generation biosensors, a mediator that transports electrons between the redox-active center of the enzyme and the working electrode surface is introduced into the system. The most common mediator is ferrocene. Third-generation biosensors transfer electrons directly from the active center of the enzyme to the electrode surface, avoiding the necessity for exterior mediators. Although electron transfer without a mediator is challenging, direct electron transfer has been attained for numerous redox proteins and enzymes by various immobilization methods, electrode surface modification, and protein engineering to obtain optimal enzyme orientation [60]. The scheme of a NA based amperometric detection process is described in Figure 3.

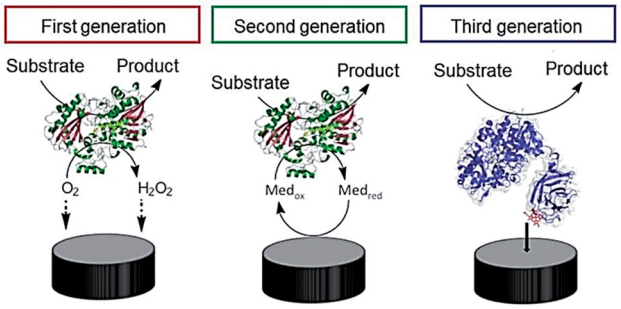

Figure 3. First, second and third-generation amperometric enzymatic biosensors. Reprinted from Ref. [62] under Creative Common (CC BY) license.

Potentiometric biosensor

Potentiometry is currently the main method used in clinical diagnostic applications. In a potentiometric biosensor, a biological recognition element is integrated into an EC transducer [63–65]. In response to the analyte, the detection element produces a biochemical signal which is transformed by the transducer to a measurable potential [66]. The signal is measured as the potential difference between the working electrode and the reference electrode, which relates to the amount of analyte present in the working electrode. An advantage of potentiometric biosensors is that a redox probe is not essential to assess the interaction between the target analyte and biorecognition element [67]. Additionally, such biosensors are very sensitive and selective in the presence of a stable and accurate reference electrode [68]. Goda et al. [69] reported on a PNA-based label-free potentiometric biosensor for detecting DNA hybridization. Although it is not based on NA, the glucose meter used for the quantification of blood glucose levels is a good example of an EC biosensor based on potentiometry. A potentiometric biosensor is described in Figure 4; details of a DNA-based multi-spotlight-addressable potentiometric sensor setup with layer structure and measuring setup are described [70].

Impedimetric biosensor

The impedimetric biosensor, which is fabricated by immobilizing the NA probe onto the transducer surface, uses impedance as the transduction principle. Impedimetric biosensors measure the variation in charge capacitance and conductance of the electrode surface as determined by the variation in the activity of the electrons or ions generated during the conjugation of receptors and target analytes [71]. Generally, an electrochemical impedance spectroscopy (EIS) technique is used to measure the changes in impedance [72] generated due to hybridization or coupling of analytes with designed probes or aptamers. EIS is a sensitive indicator for diverse chemical and physical properties [73–75]. It may also be designed without the need for indicator molecules, that is, for label-free detection. This non-destructive technique could be used as a tool for investigating biorecognition events at the electrode surface [76]. However, it is time-consuming compared with amperometry or potentiometry, and may not be appropriate for POC applications. An example of an impedimetric biosensor is shown in Figure 5 [77].

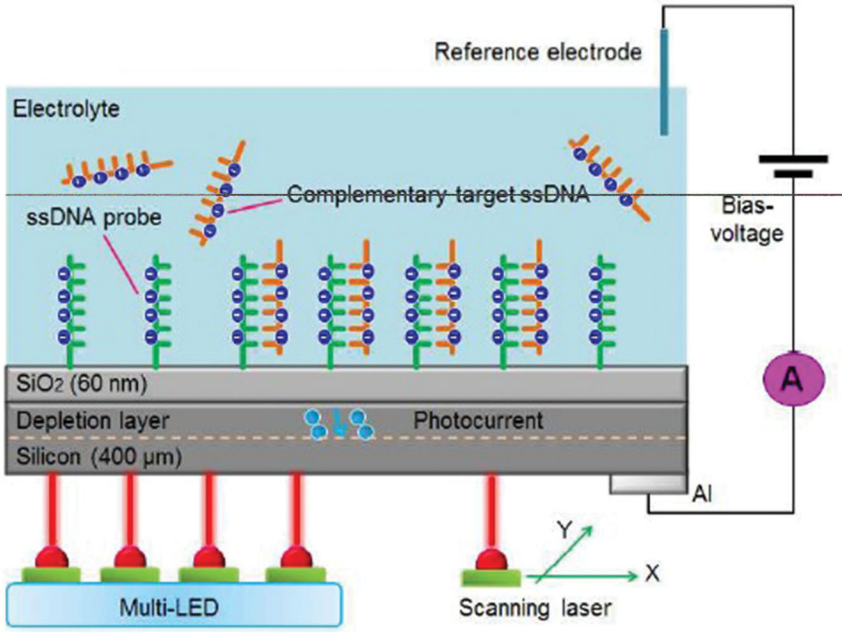

Figure 4. A schematic illustration of light-addressable potentiometric sensors (LAPS). The thiol-modified ssDNA was immobilised on the gold (Au) surface via gold-thiol bond. Covalent immobilisation of amino-modified ssDNA onto the SiO_2 surface functionalised with 3-aminopropyltriethoxysilane (APTES). Layer-by-layer adsorption of negatively charged ssDNA on a positively charged poly (allylamine hydrochloride) (PAH) and hybridised with complementary DNA. Reprinted from Ref. [70] with permission. Copyright (2014) Elsevier.

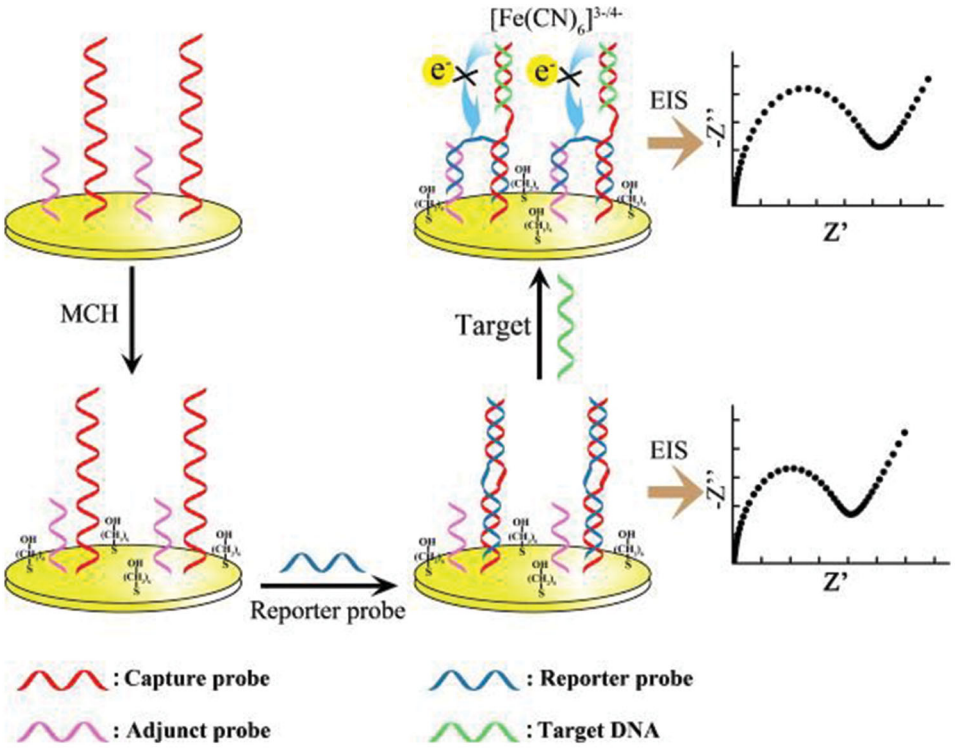

Figure 5. A schematic illustration of an impedance biosensor using an adjunct probe for sensitive and label-free electrochemical detection of DNA sequence. This shows the step-by-step fabrication of capture probe ssDNA, reporter probe and complementary DNA hybridisation, and electrochemical measurement. Reprinted from Ref. [77] with permission. Copyright (2013) Elsevier..

Improving sensing ability and specificity of biosensors

The conversion of biological information to an easily processed electronic signal is challenging due to the difficulty of linking an electronic system to a biological setting. The EC NA biosensor provides an attractive process to analyze the content of a biological sample by direct transformation of a biological event to an electronic signal [31]. Compared with traditional antigen-antibody reactions, NAs have many exceptional characteristics, such as ease of synthesis, stability on storage, high specificity, and broad applicability [78,79].

Sensitivity is a critical parameter for the EC NA biosensor. Detecting low concentrations of an analyte may be difficult due to the background signal. Therefore, researchers have integrated nanotechnology and molecular biology in EC NA biosensors to augment the sensitivity and enhance their performance. Nanomaterials have emerged as popular candidates for biosensing because of their excellent physico-chemical features, large surface-to-volume ratio, and great electronic conductivity. These have been applied effectively to sensing approaches to increase the sensitivity of EC NA biosensors [5,24]. A large number of nanomaterials have been developed for immobilization of NA, for example, noble metal NPs (such as Au, Ag, CdS), nanowires, nanotubes, nanocomposites [39,80–82].

A gold nanoparticle (AuNP) or gold nanorod (AuNR) modified conducting polymer is applied as a fabricating material on the electrode [83,84]. Methylene blue, ruthenium complexes, ferrocene, ferrocene-bearing polymers and $Fe(CN)_6^{4-/3-}$ are used as electroactive reporters for signal transduction [85–89].

One-dimensional (single-walled) nanostructure materials have been broadly studied as resources for immobilization of the NA sensor due to their high conductivity, rich surface chemistry, large surface-to-volume ratio and exceptional electrocatalytic activity [90–93]. For example, one-dimensional nanomaterials like nanowires, nanotubes and nanorods have found a wide variety of applications, including biosensing [94]. Another approach is to use bimetallic nanowires with nano-porous and dendritic structures that enhance surface roughness and thus enhance electrocatalytic properties [95].

Carbon nanotubes (CNTs) are another material for the enhancement of electrical conductivity in the EC biosensor. CNTs with sp2 hybridized carbon atoms consist of single or multiple layers that are folded to form cylinders termed nano-cylinders [96]. CNTs have been hugely popular and widely used because of their unique features [97]. The two major categories of CNT are single-walled carbon nanotubes and multi-walled carbon nanotubes (MWCNTs) [98]. The MWCNTs have received more attention from researchers than single-walled carbon nanotubes due to their unique characteristics that include large surface area, good adsorption capacity, improved chemical stability, significant electrical conductivity, sharp EC response, and higher tensile strength [99]. Typically, carbon is insoluble in water, and its incorporation in functionalized-MWCNTs to electrodes offers substantial performance improvements [100].

In some cases, NPs enhance the electron exchange between the conjugated biomolecules and electrodes functioning as electron transfer mediators or electrical wires due to their high conductivity [101]. A prerequisite for biosensors for clinical diagnosis is accuracy. In biosensor design, it is necessary to consider that only the target analyte should interact with the bioreceptor, so that other compounds do not interfere with the sensor's accuracy [102]. Several homolog/analog compounds may interact nonspecifically with DNA aptamers, for example, by attaching to the sugar-phosphate spine of the DNA, and so competitively hinder the conjugation of the target analytes. In addition, non-target NA in biological fluids may hybridize with the NA probe, altering the biosensor's structure and preventing it from binding to its target [39].

As a result, a research focus is to identify specialized bioreceptors. The target should be designed and compared with nucleotide sequences of other genes or species so that the target probe can distinguish it from possible interferences. For the design of a perfect probe, relevant nucleotide sequences can be retrieved from the National Center for Biotechnology Information (NCBI) (https://www.ncbi.nlm.nih.gov). The retrieved sequences are aligned using bioinformatics tools such as MEGA software to identify intra-species conserved and inter-species variable regions so the probe sequence that is designed matches completely with target species but has numerous mismatches with non-target species. Finally, the selected probe is checked for specificity between the closely related and distant species using the online Basic Local Alignment Search Tool (BLAST) in the NCBI database (http://blast. ncbi.nlm.nih.gov/Blast.cgi) so that the designed probe will be unique and hybridize with only target sequences. Repeated cycles are carried out until the specified sequence increases exponentially and low-affinity sequences are eliminated. Aptamers are capable of recognizing both small molecules and high-molecular-weight substances as well as complete cells. However, it is worth noting that aptamer commercialization is still in its early stages [103]. Thus, future improvements include developing

novel bioreceptors and upgrading existing bioreceptors to cater to commercial needs.

NA probes with sufficient and effectively immobilized DNA and with minimum nonspecific binding on the electrode surface can result in better accessibility of complementary DNA targets, which can influence the sensitivity, selectivity, accuracy, and life of the sensor. Appropriate immobilization methods must be used to ensure that the immobilized DNA probe monolayer is stabilized on the working electrode surface and does not desorb [41].

Sensitivity and specificity are important aspects of the performance of biosensors. Sensitivity refers to the smallest amount of analyte that it can detect. Biosensors are required in medical applications to detect analyte concentrations as low as ng/mL or even fg/mL to confirm the presence of trace analytes in a sample. Specificity, on the other hand, is possibly the most significant characteristic of a biosensor. Specificity refers to a bioreceptor's ability to detect a specific analyte in the presence of various admixtures and contaminants, which is critical for real-time sample analysis. As a result, sensitivity and specificity are crucial properties of a biosensor [38,104,105].

Applications of EC biosensors in clinical diagnosis

There is a growing demand for the development of more sensitive, reliable, rapid, and cost-effective techniques to analyze clinical samples. Conventional immunological or NA-based techniques, as well as microbial culture-based techniques, are time-consuming and expensive. Biosensor technology has the potential to solve such problems [106]. Ongoing research focuses on developing devices with these qualities to identify diseases, support early and POC diagnosis, as well as offer personalized medicine. In this regard, the EC NA biosensor possesses the ability to fulfill these requirements due to its wide spectrum of targets, which include peptides, proteins, metabolites, biomarkers, drugs, cells, pathogens (virus, bacteria, etc.), and genetic markers [107]. EC biosensors offer an attractive means to quantitate analytes in biological samples [31].

Application of EC NA biosensors for detection of disease biomarkers

Biomarkers are indicator molecules used to assess the normal physiological state, disease state, or response to therapy. Identification and measurement techniques, especially for protein biomarkers, have become increasingly important for diagnosis, monitoring of treatment, and prediction of outcome [108–112], and their very low concentrations may be a challenge to measure [113,114]. Numerous EC NA biosensors have been designed to detect biomarkers in cancers and other diseases. Recent innovations and applications of these biosensors are summarized in Table 1.

Gold electrodes [118,142,143] or AuNPs [117,118,121] are commonly used as a sensing platform to facilitate the fabrication of thiol modified aptamers or DNA probes with few exceptions. Other nanomaterials or nanocomposites are used to enhance sensitivity and lower the detection limit. Among them, carbon nanotubes, semiconductor quantum dots, nanodiamonds, polymer NPs, and graphene have been the most extensively studied [144]. Electrochemical biosensors can detect biomarkers with different ranges of sensitivity. For example, a thrombin biomarker-based device was reported to have a sensitivity of 200 fmol/L [118]. Similarly, lysozyme, troponin I, lipopolysaccharide, vascular endothelial growth factor and carcinoembryonic antigen biomarker-based biosensors have limits of detection of 2.09×10^4 molecules/mL [143], 8.0 pg/mL [120], 0.33 fg/mL [121], 30 nmol/L [122], and 80 ag/mL [123], respectively.

Cancer is a devastating and potentially fatal disease. There are many different types of cancer including breast, ovarian, lung, prostate, hematological, skin, and colon. The incidence of cancer has grown among all races and age groups according to national vital statistics reports [145,146]. Because timely treatment can increase survival rates, early detection is vital. In this regard, biosensors may be useful for diagnosis and monitoring treatment [147]. Biosensors can detect and measure the levels of specific proteins that are expressed and/or secreted by cancer cells [146,147]. An EC aptamer-based biosensor for the detection of cancer biomarkers is shown in Figure 6.

No single oncogene or tumor suppressor gene correlates with all types of cancers. In addition to genomic mutations, other complex molecular changes, such as protein over/under-expression, can result during oncogenesis [148]. A variety of molecular biomarkers, such as platelet-derived growth factor [124], vascular endothelial growth factor) [130], epidermal growth factor receptor [139], exosome [125,126], MUC1 [137], osteopontin [128,129], TNF-α [131], CT26 cell [134], MCF-7 cell [135], carcinoembryonic antigen [136), L-tryptophan [140], and p53 [141], can be linked with cancer classification and disease progression. Each biomarker has different biomolecular characteristics, which makes it difficult to include several of them in a common

Table 1. Electrochemical nucleic acid biosensor for the detection of disease biomarkers.

Analyte biomarker	Biosensor used	Measurement method	LOD	Indicator	Medical implication of biomarker	References
Common disease biomarkers						
Thrombin	Laser-scribed graphene/1-pyrenebutyric acid (TBA)/aptamer	DPV	1 pmol/L	K3[Fe(CN)6]	Principal enzyme of hemostasis; catalyzes conversion of fibrinogen to fibrin and stimulates blood clotting.	[115]
Thrombin	AuNPs/thrombin (TB)/MCH/TBA1/AuNPs/WSe2/GCE	DPV	190 fg/mL	SA-ALP/DNA linked	Principal enzyme of hemostasis; catalyzes conversion of fibrinogen to fibrin and stimulates blood clotting.	[116]
Thrombin	Poly(methyl methacrylate) (PMMA)/poly (diallyldimethylammonium chloride) (PDDA)/AuNPs/SH-aptamer	DPV	1 pmol/L	—	Principal enzyme of hemostasis; catalyzes conversion of fibrinogen to fibrin and stimulates blood clotting.	[117]
Thrombin	Au electrode/GPD*/AuNPs/thrombin aptamer/hexanethiol	CV	200 fmol/L	Label free	Principal enzyme of hemostasis; catalyzes conversion of fibrinogen to fibrin and stimulates blood clotting.	[118]
Lysozyme	Aptamer/MWCNT/SPCE	EIS	90 ng/mL	Methylene blue	Glycoside hydrolase; ubiquitous protein	[119]
Troponin I	76-mer TnI aptamer/gold nanodumbbells	DPV	8.0 pg/mL	6-Mercapto-1-hexanol	Cardiac muscle protein, marker of heart attack.	[120]
Lipopolysaccharide	Hairpin probes (HP1)HP2/HP3/AuNPs/Cu-based metal organic frameworks (Cu-MOFs)	CV and EIS	0.33 fg/mL	Ferrocene	Glycolipids found in the outer membrane of some types of Gram-negative bacteria	[121]
Vascular endothelial growth factor	6-mercapto-1-hexanol/spacer thiol/thio-DNA aptamer/Au-plated screen-printed carbon electrode	DPV	30 nmol/L	Streptavidin-alkaline phosphatase	Key regulator of both physiological and pathological angiogenesis; serum biomarker for diseases, including cancer	[122]
Carcinoembryonic antigen	Hairpin DNA/graphene/glassy carbon electrode	DPV/EIS	80 ag/mL	Methylene blue	Cancer biomarker	[123]
Cancer biomarkers						
Platelet-derived growth factor	3D-4MgCO3·Mg(OH)2·4H2O-Au NPs	Amperometry	1.2 fmol/L	Pt-Au NPs	Tumor growth, cancer marker	[124]
Exosomes	Aptamer/DNA nanotetrahedron (NTH)/SPAuE	SWV	2.09×10^4/mL	$[Fe(CN)_6]^{3-/4-}$	Extracellular vesicles, facilitating tumorigenesis by regulating angiogenesis, immunity, and metastasis	[125]
Exosomes	CD63-aptamer/Au electrode/glass surface	SWV	1×10^6/mL	Methylene blue	Extracellular vesicles, facilitating tumorigenesis by regulating angiogenesis, immunity, and metastasis	[126]
MUC1 (breast cancer biomarker)	Aptamer/carbon nanotubes (CNTs)/SPCE	EIS	0.02 U/mL	$[Fe(CN)_6]^{4-/3-}$	Monitor cancer therapy at all (I–IV) stages	[127]
Osteopontin	FAM-RNA aptamer/microelectrode gold surface	CV and SWV	3.7 ± 0.6 nmol/L	Fluorophore 6-carboxyfluorescein (FAM)	Protein present in several body fluids, associated with breast cancer progression and metastasis	[128]
Osteopontin	DNA aptamer/streptavidin-biotin/microelectrode gold surface	CV and SWV	1.4 ± 0.4 nmol/L	$[Fe(CN)_6]^{3-/4-}$	Protein present in several body fluids, associated with breast cancer progression and metastasis	[129]
Vascular endothelial growth factor (VEGF)	DNA aptamer/Au electrode	CV and EIS	0.82 pg/mL	6-Mercapto-1-hexanol	Key mediator of angiogenesis in cancer; up regulated by oncogene expression	[130]
Tumor necrosis factor-α	TNF-α DNA aptamer/Au electrode	SWV	58 pmol/L	Methylene blue	Key inflammatory cytokine	[131]
MUC1 and vascular endothelial growth factor ($VEGF_{165}$)	Aptamer/Fc-cDNA/Au electrode	SWV	0.33 nmol/L	Ferrocene (Fc)	Breast cancer marker	[132]
Human papilloma virus	Peptide aptamers/polyaniline-multiwalled carbon nanotube film (PANi-MWCNT)/platinum electrode	SWV	490 pmol/L	Antigen-antibody	Marker of cervical cancer precursor lesions and invasive carcinoma	[133]
CT26 cell	SBA-15-3-aminopropyltriethoxysilane (SBA-15-pr-NH2)/Au nanoparticles (AuNPs)/graphite screen printed electrode (GSPE)	CV	2 cells/mL	$Fe(CN)_6^{3-/4-}$	Colorectal cancer	[134]

(*continued*)

Table 1. Continued.

Analyte biomarker	Biosensor used	Measurement method	LOD	Indicator	Medical implication of biomarker	References
	or Apt/cell/Apt/AuNps/SBA-15-pr-NH2/SPE					
MCF-7 cell	DNA Walker/D-RNA (chimeric DNA/RNA oligonucleotide with a cleavage point rArU)/Au electrode	EIS	47 cells/mL	6-Mercapto-1-hexanol	Breast cancer	[135]
Carcinoembryonic antigen	Fc-AuNPs-Apt2/CEA/Apt1/Au	DPV	0.5 ng/mL	6-Ferrocenyl hexanethiol (Fc)	Glycosylated protein over-expressed on breast, colon, and other cancer cells	[136]
MUC1	GCE/MWCNT/streptavidin/HO-AuNP-HRP	DPV	2.2 nmol/L	Streptavidin	Breast, gastric, colorectal, lung, prostate, ovarian, pancreatic, and bladder carcinomas	[137]
Vascular endothelial growth factor ($VEGF_{165}$)	Anti- VEGF/aptamer/OMC-Au nano/SPE	EIS	1.0 pg/mL	$[Fe(CN)_6^{3-/4-}]$	Lung cancer marker	[138]
Epidermal growth factor receptor	SPE/ordered mesoporous carbon-gold nanocomposite (OMC-Au_{nano}/aptamer	EIS	1.0 pg/mL	$[Fe(CN)_6^{3-/4-}]$	Cancer biomarker	[139]
L-tryptophan	Apt-MWCNT-AuE	CV, LSV	6.4×10^{-11} mol/L	Ethidium bromide (EtBr)	Cancer biomarker	[140]
p53	AuE/PNA SAM/MCH/DNA	DPV	6.82×10^{-10} mol/L	Methylene blue	p53 tumor suppressor gene	[141]

CV: cyclic voltammetry; DPV: differential pulse voltammetry; EIS: electrochemical impedance spectroscopy; LOD: limit of detection; LSV: linear sweep voltammetry; SPCE: skin printed carbon electrodes; SPE: screen printed electrode; SWV: square wave voltammetry.

diagnostic platform. Therefore, a separate method is needed for each biomarker. The limit of detection of biosensors also differs owing to different methodologies, as described in Table 1.

Application of EC NA biosensor for detection of pathogens

Microbial diseases caused by bacteria, viruses, and other organisms are leading causes of death in developing countries [149]. Therefore, the detection of pathogens is significant for the identification and prevention of diseases. Culture and colony counting methods, immunochemistry, and PCR are the most common techniques used for pathogen detection. Although these techniques often produce reliable and robust outcomes, their limitations include lack of sensitivity and specificity as well as being time-consuming [150]. Typical standard bacteria detection approaches may take around a week. Thus, researchers have focused on the development of rapid methods like EC NA biosensors to meet the demand [150].

Most EC NA biosensors for pathogens detection have been based on aptamers. Aptamers can capture a variety of NA as well as non-NA targets with high affinity and specificity. They can accurately distinguish bacterial and viral strains without prior knowledge of the membrane-bound antigenic determinants or molecular biomarkers existing in microorganisms [151]. In contrast to conventional antibody-antigen-based detection, aptamers can be adapted quickly to detect evolving virus strains and they have a high discriminating capacity for specific virus serotypes. Aptamers are ideal molecules for diagnosis and chemical sensing applications owing to their stability and ease of modification [152]. Figure 7 demonstrated a DNA aptamer immobilized hybrid nanomaterial-modified electrode (MWNT/PPNWs/GNPs), a sensitive electrochemical approach for the detection of avian influenza virus H5N1 gene sequences. The porous structure of the hybrid nanomaterial has a large effective surface area, excellent electrocatalytic activity, and electronic conductivity [153].

In the case of virus detection, EC NA biosensors have been used for the detection of influenza viruses [34,153–157], human immune deficiency virus [158–160], hepatitis C virus [161,162], vaccinia virus [163], and oncolytic virus [164]. A variety of electrodes, such as glassy carbon electrodes, gold electrodes, platinum electrodes as well as skin printed carbon electrodes have been used as a basic platform for the fabrication of designed target DNA sequences known as capture probes. In addition to the basic platform

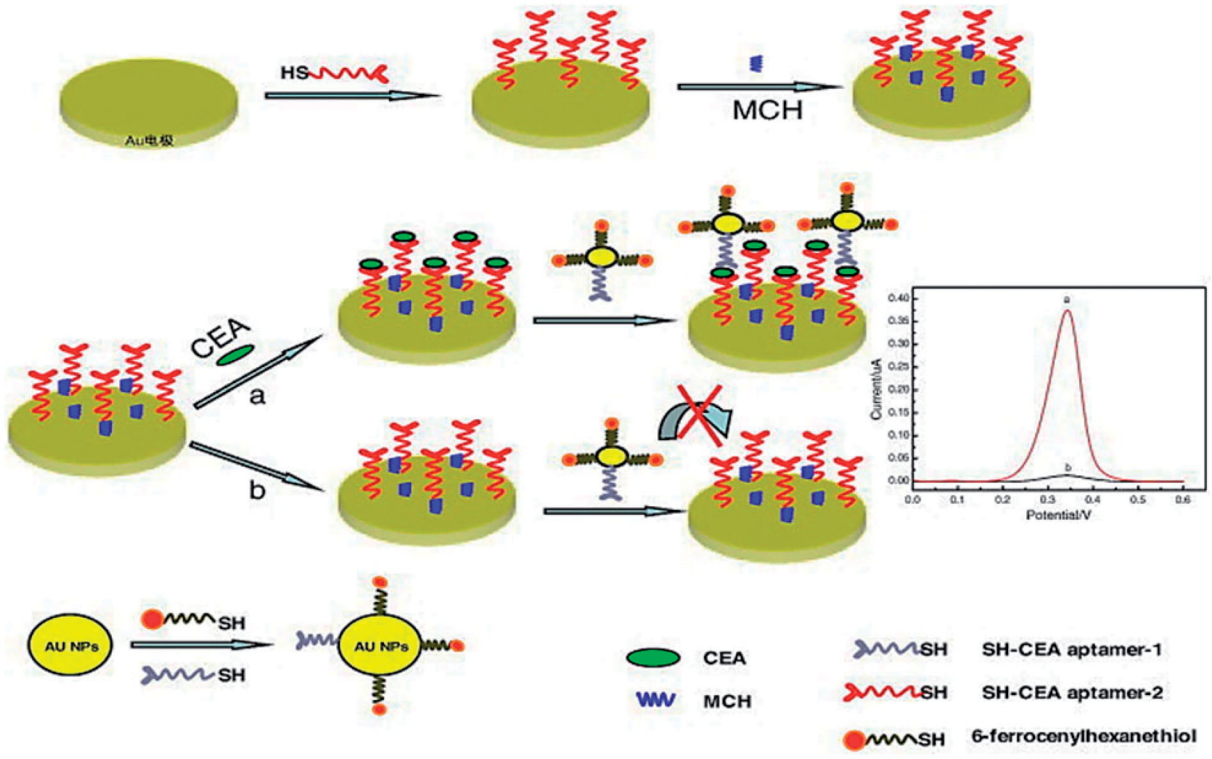

Figure 6. Schematic representation of the electrochemical aptamer biosensor based on AuNPs signal amplification for the detection of carcinoembryonic antigen. The Au electrode was incubated in aptamer 1 solution and after washing, submerged in 6-ferrocenyl hexanethiol to obtain a well-aligned DNA monolayer. It was then immersed in carcinoembryonic antigen solution and Fc capped AuNPs–aptamer 2 conjugate. The electrode was scanned for differential pulse voltammetry signals in phosphate-buffered saline. Reprinted from Ref. [136] with permission. Copyright (2013) Elsevier.

mentioned, some researchers have used NPs (AuNPs, silica NPs, quantum dots, and magnetic NPs) and nanocomposites (AuNPs on graphene oxide, chitosan/carbon nanotube, and nanopolymers) to facilitate the immobilization of capture probes as well as to enhance EC detection at lower concentrations. For the detection of viruses, capturing probe sequences mostly target toxic gene sequences (H5N1 hemagglutinin gene, human immunodeficiency virus-1 gag gene, etc.) related to the virulence of that particular virus, with a few exceptions where the targets are specific viral antigens [154,156,159,161,162]. The limit of detection of analytes varies from strain to strain due to the choice of fabrication methods, target analytes, EC measurement methods, and use of leveling indicators, as shown in Table 2. Because the isolation of viral DNA/RNA is easy, the engagement of biosensors for virus detection is encouraging. However, each capturing probe is unique to its specific viral sequence, which limits the universal application of biosensors for other virus types.

EC NA biosensors have not been widely used to detect bacteria except for *Salmonella* and *Staphylococcus* species. As with virus detection, electrodes such as glassy carbon electrodes [165], AuNPs [166], and screen-printed electrodes [167] are used. Capturing probe sequences have been designed by targeting cell surface components of the bacteria. Some bacteria may share common pathogenic genes, which could make it challenging to design a species-specific nucleotide probe. Here, aptamers capture the whole cell rather than hybridize with complementary NA sequences [165–169]. NPs and nanocomposites are also used to modify the electrode surface to enhance sensitivity. For example, a very low limit of detection (3 CFU/mL) was observed in *Salmonella* detection using graphene oxide-AuNPs composite modified glassy carbon electrodes [165]. EC NA biosensors to detect bacteria are reported in Table 3. Figure 8 depicts an electrochemical biosensor for the detection of *Salmonella*. To make the aptamer-based electrochemical biosensor, the graphene oxide-AuNP modified glassy carbon electrode was incubated with the *Salmonella*-specific recognition aptamer. EIS was used to measure the change in impedance [165].

Application of EC NA biosensors for detection of genetic disorders

Genes, segments of DNA passed from parent to offspring, are the building blocks of inheritance for all

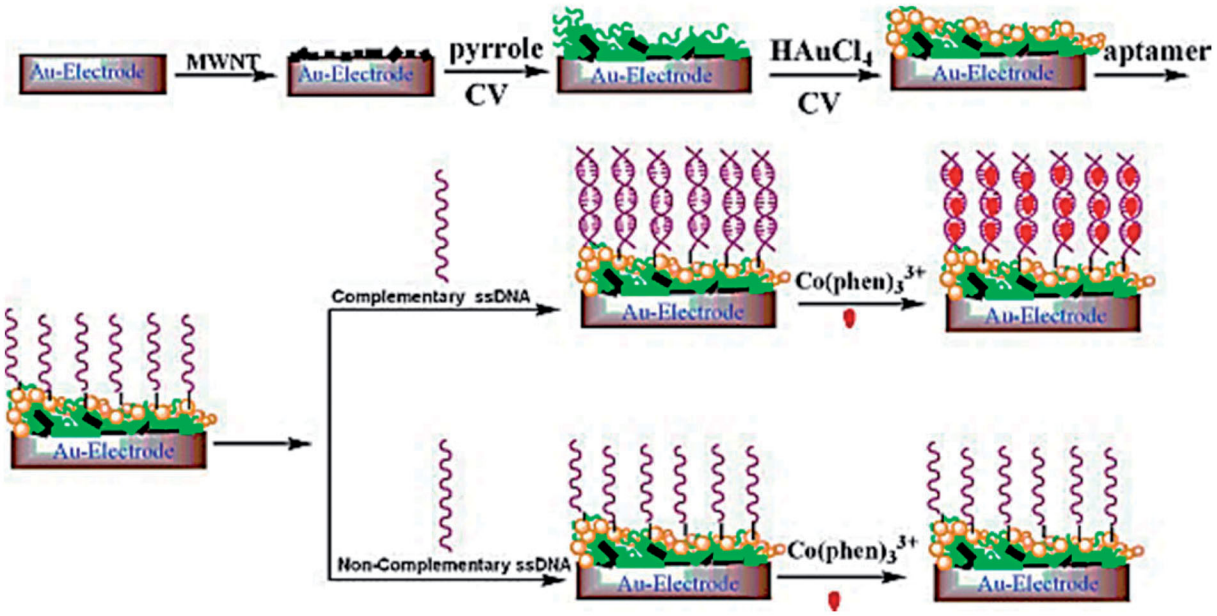

Figure 7. A schematic representation depicting the experimental procedures for the detection of avian influenza virus (AIV) H5N1 using an electrochemical nucleic acid biosensor. Reprinted from Ref. [153] with permission. Copyright (2011) Elsevier.

living beings. Proteins made from genes perform most of the functions in cells. Mutations cause alterations in the nucleotide sequences of genes, which may cause abnormal proteins to be made that do not function correctly, or the protein may be completely missing. Then a genetic disorder may occur [170,171]. The most common mutations are base substitutions, deletions, and insertions.

In general, three types of genetic disorders are identified in humans: single-gene disorders (mutation affecting one gene), chromosomal disorders (part of a chromosome is missing or altered), and complex disorders (mutation in two or more genes) [171]. In diagnosing genetic disorders, specific DNA sequences and the base-pair composition are both important [50]. Disorders such as Down syndrome, thalassemia, cystic fibrosis, Tay-Sachs disease, and sickle cell anemia, are among the commonest genetic disorders [172]. It is important to identify genetic disorders early for optimal management. NA biosensors have been used to detect such disorders but in a very limited way. Table 4 summarizes EC NA biosensors that have been used to detect genetic disorders. A gold electrode is used as a basic platform for the fabrication of designed probe DNA (capturing probe) in most cases. The capturing probe DNA is modified with a thiol group to facilitate conjugation on the gold electrode surface. The target analytes are detected based on the hybridization dynamics of the capturing probe, and the presence of the electroactive compound enables EC detection. These methods are very simple and easy to operate, but the design of a suitable probe and optimization of hybridization conditions along with assay parameters may be challenging.

Limitations of EC NA biosensors

The EC NA biosensors are promising tools in clinical applications. The development of highly sensitive and precise devices for the diagnosis of specific biomolecules is important for detecting biomarkers and pathogens [177]. However, there are obstacles to the application of biosensors in clinical diagnosis. A key challenge is the sample preparation step, which makes it difficult to apply EC NA biosensors in the analysis of clinical samples [178]. The target biomarkers are present in a complex mixture and pathogens may reside within the cells. Therefore, analysis of the target biomarkers may require the isolation of NA from the samples. In addition, the possibility of hybridization between the capture probe and the isolated DNA/RNA may be lower because the length of the isolated DNA/RNA must be longer than that of the probe. Furthermore, EC experiments are carried out at ambient temperature, but the target biomarkers (e.g. NA or DNA) exist as double-stranded molecules at this temperature and cannot hybridize with the immobilized single-stranded captured probe. Therefore, denaturation of target biomarkers to the single-stranded form before hybridization is vital, but that demands extra energy and precautions. Moreover, non-specific hybridization may occur with non-targets at

Table 2. Application of electrochemical nucleic acid biosensor for the detection of pathogenic viruses.

Target virus	Biosensor used	Target analyte	Measurement method	LOD	Indicator	Medical implication of target virus	References
Influenza virus (type A-H5N1)	10 nm Ti/200n Pt/silicon dioxide (SiO2)/ DNA aptamer	DNA	Conductivity	0.5 nmo/L	Level free	Serious public health threat	[34]
Avian influenza A virus	Gold/single-stranded DNA oligonucleotide probes	Hemagglutinin gene	EIS	30 and 100 fmol	6-Mercapto-1-hexanol	Serious public health threat	[154]
Avian influenza virus	GCE/multi-walled carbon nanotubescobalt phthalocyanine (MWNTs–CoPc)/poly (amidoamine) (PAMAM) dendrimer/ DNA probes	H5N1 hemagglutinin gene	DPV	1.0 pg/mL	G4 PAMAM dendrimer	Serious public health threat	[155]
Avian influenza virus H5N1	Au nanoparticles (AuNPs)/concanavalin A (ConA)/glucose oxidase (GOx)/ H5N1 aptamer	Virus particle	EIS	8×10^{-4} HAU	GOx	Serious public health threat	[156]
Avian influenza virus H5N1	AuNPs/MWCNTs/polypyrrole nanowires (PPNWs)/DNA aptamer DNA aptam/MWNT/PPNWs/AuNPs	H5N1 specific sequence	CV and DPV	4.3×10^{-13} mol/L	$Co(phen)_3^{3+}$	Serious public health threat	[153]
H5N1	Gold interdigitated microelectrode/ streptavidin/biotin labeled H5N1 aptamer/ polyethylene glycol	H5N1 specific RNA	Impedance	0.25 HAU	Thiocyanuric acid & AuNPs	Serious public health threat	[157]
HIV	Au electrode/probe/MCH/target DNA/ auxiliary probe 1/auxiliary probe 2	HIV-1 U5 LTR	DPV	5 amol/L	$[Ru(NH3)_6]^{3+}$ *or* RuHex	Immune deficiency disease	[158]
HIV-1	Molecular beacon (CAs-MB)/nafion graphene/SPCE	HIV-1 gag gene	CV and DPV	5 nmol/L	Becon on/off	Immune deficiency disease	[159]
HIV-1	Diamond-FET/RNA aptamer/	HIV-1 trans-activator transcription (HIV-1 Tat)	CV	100 pmol/L	—	Immune deficiency disease	[160]
Hepatitis C virus	GCE/graphene quantum dots (GQD)/aptamer	Core antigen	EIS, CV and DPV	3.3 pg/mL	BSA	Infects hepatocytes in the liver	[161]
Hepatitis C virus	AgNPs/GQD-SH/anti HCV	Hepatitis C virus core antigen	EIS, CV and DPV	3 fg/mL	BSA	Infects hepatocytes in the liver	[162]
Vaccinia virus (VACV)	GNPs-SPCE/VACV-specific aptamer	VACV-DNA	Square wave voltammetry	60 PFU in 30 mL	–	Oncolytic agent that can replicate in and kill tumor cells	[163]
Oncolytic viruses	Au electrode/thiol-ssDNA primer/Anti VSV aptamer/VSV/Anti VSV antibody	Vesicular stomatitis virus (VSV)	EIS	600 PFU	Antigen- antibody	Selectively replicate in and kill tumor cells	[164]

CV: cyclic voltammetry; DPV: differential pulse voltammetry; EIS: electrochemical impedance spectroscopy; HIV: human immunodeficiency virus; LOD: limit of detection; PFU: plaque-forming unit; SPCE: skin printed carbon electrodes.

Table 3. Application of electrochemical nucleic acid biosensor for the detection of pathogenic bacteria.

Target bacteria	Used biosensor	Target analyte	Measurement method	LOD	Indicator	Medical implication of target bacteria	References
Salmonella	GCE/GO/AuNPs/ aptamer ssDNA	Whole cell	EIS	3 CFU/mL	—	Most common causes of food-associated disease	[165]
Staphylococcus aureus (S. aureus)	Electrode/SWCNTs/ anti-S. aureus aptamer/	Whole cell	EMF	8×10^2 CFU/mL	—	Common Gram-positive food poisoning pathogen and other life-threatening infections	[168]
Salmonella enteritidis	AuNPs-SPCE/ thiolated S. enteritidis specific DNA aptamer	Whole cell	EIS	600 CFU/mL	Label-free	Release of proinflammatory cytokines which induce inflammatory reaction	[166]
Salmonella typhimurium	AuNP-SPCE/ thiolated *S. typhimurium* specific aptamer	Whole cell	EIS	600 CFU/mL	Label-free	Salmonellosis	[169]
Salmonella typhimurium	SPEs/ adiazonium/ aminated-aptamer	Whole cell	EIS	6 CFU/mL	Label-free	Salmonellosis	[167]

EIS: electrochemical impedance spectroscopy; EMF: potentiometry; LOD: limit of detection; SPCE: skin printed carbon electrodes; SPE: screen printed electrode.

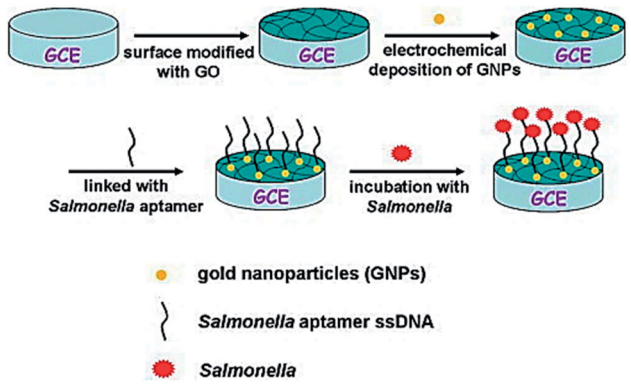

Figure 8. Diagrammatic illustration for the aptamer-based electrochemical recognition of *Salmonella*. Reprinted from Ref. [165] with permission. Copyright (2014) Elsevier.

ambient temperature, which may interfere with the results. In the case of biomarker-based disease diagnosis, the detection process relies mainly on aptamers. However, the selection process of the aptamer for a specific biomarker through SELEX is tedious and time-consuming, and it may lead to nonspecific capture if the assortment is not perfect. Another concern is the lack of a reliable method to produce aptamers to be used specifically in EC biosensors. The process has to be repeated as many times as necessary (usually 8–15 rounds) until aptamers with the desired characteristics are obtained, which is not a simple procedure [179]. Aptamers and NA may need to be modified with an anchoring chemical group for surface immobilization and a redox-active dye to produce a quantifiable signal. Both modifications are not compatible with the SELEX process. The DNA sequence [180] and length [181] are important parameters and add complications to the immobilization procedure because sequence and length determine the structural flexibility of the aptamer [182]. In a study that experimented with different lengths of ssDNA probes containing a terminal 5′ hexanethiol anchoring group, immobilization was found to be reduced for long probes compared with shorter ones [181].

In the case of pathogen detection, most biosensing methods target pathogenic genes, but some pathogenic genes are common in several organisms. If these common genes are used as targets, the chances of obtaining accurate information about the exact pathogen are reduced. All of the processes described above, such as the time-consuming SELEX procedure for aptamer selection, the presence of other sensing targets in complex specimens, the complex and time-consuming NA isolation procedure, and the inability of double-stranded NA to hybridize at ambient temperature, may make electrochemical NA biosensors more challenging to implement in POC applications.

The presence of nucleases in biological samples also needs to be considered for the success of EC NA biosensors for clinical diagnosis. Preventing nuclease contamination is a persistent challenge for molecular biologists dealing with nucleic acids, particularly RNA. As a precaution, it is necessary to always wear gloves and to ensure that the working bench, equipment, and reagents are DNase-free during DNA isolation, and RNase-free during RNA isolation. RNases are used

Table 4. Application of electrochemical nucleic acid biosensor for the detection of genetic disorders.

Disease	Analyte	Used biosensor	Measurement method	LOD	Indicator	Medical implication of the disease	References
β-Thalassemia	Oligonucleotides	Au electrode/ oligonucleotide probe (25-mer)	Piezoelectric	1 ppm	–	Genetic disorder caused by mutations in β-globin gene of adult hemoglobin, leading to severe anemia	[173]
Cystic fibrosis	DF508 mutation	Au electrode/ DNA probe	EIS and DPV	2 μmol/L	$[Fe(CN)_6]^{3-/4-}$ for EIS and methylene blue for DPV	Hereditary disease affecting lungs and digestive system with thick mucus, leading to shorter life span	[174]
Cystic fibrosis	DF508 mutation	Au electrode/ molecular beacon	DPV	0.5 μmol/L	6-Mercapto-1-hexanol	Hereditary disease affecting lungs and digestive system with thick mucus, leading to shorter life span	[175]
Sickle cell anemia	Mutant gene	Au electrode/capture sequence/ 2-mercaptoethanol	EIS	7.0 nmol/L	2-Mercaptoethanol	Genetic condition in which patients produce abnormal form of hemoglobin	[176]

DPV: differential pulse voltammetry; EIS: electrochemical impedance spectroscopy; LOD: limit of detection.

to remove RNA from isolated DNA, and DNases are used to eliminate DNA from isolated RNA. Additional measures must be taken when handling RNA because RNA is much less stable than DNA. Consequently, RNA samples need to be frozen and thawed as few times as possible [183–185].

Biosensor development has advanced dramatically in recent years, although their use in clinical diagnosis is still uncommon except for glucose biosensors. Interferences with unwanted molecules during measurements with clinical samples are the most serious issue. Because treatment is typically based on quantitative clinical indicators, high selectivity and precision are essential [186]. Despite the benefits of NA biosensors or aptasensors over antibodies or other approaches, more work is needed before electrochemical aptamer/NA-based biosensor technology is ready for widespread use in clinical diagnostics [39].

Conclusion and recommendations

This review has discussed recent developments and applications of EC NA biosensors and biosensing techniques, including key advances made in this field, biomolecular sensing strategies, and nanotechnological approaches to clinical diagnosis. Biosensors were first developed almost three decades ago and their combination with electrochemistry opened up exciting opportunities in various fields. Biosensors have great potential to be useful in biomedical diagnosis, monitoring of disease progression, forensic investigations, drug discovery, and biomedical research. The coupling of NA with other high-affinity biomolecules could facilitate the sensitive and selective diagnosis of a range of analytes. Furthermore, advances in bio-nanotechnology and nano-diagnostics based on EC NA nano biosensors could enable impressive developments in the field of diagnostics in the future.

Conjugation of NA with biocatalysts, various EC indicator molecules, and diverse nanomaterials have led to many innovations in this field. Researchers are discovering innovative tools to create artificial receptors that could widen their applicability to almost any analyte. The flexibility of NA biosensors and their ease of chemical alteration have permitted the exploitation of various approaches to biosensor immobilization as well as the application of many techniques for biosensor-analyte identification.

Currently, the EC NA biosensor application is a single target-oriented approach, and the modified transducer is not reusable in general. Hence, concurrent monitoring of multiple targets with the reusability of devices may open new opportunities. The application of NA biosensors in EC sensing is still in the near middle phase of progress. Several difficulties need to be overcome to make them applicable for monitoring multiple targets in complex samples. Some molecules having structural homology/analogy with the desired target molecules may interact with immobilized bio-capture probes nonspecifically, like the binding of a compound to the sugar-phosphate backbone of the probe DNA where the target can bind and competitively inhibit the binding of the target. The basic prerequisite for developing biosensors for clinical diagnosis is accuracy. Small amounts of non-target NA in the sample may hybridize with the immobilized probe and change its

configuration, thus preventing it from binding appropriately to its target and causing false results. Therefore, during the design of the biosensor platform, emphasis should be placed on its attributes so that conjugates are unique to target analytes in a real sample. In addition, lack of contact between the bioreceptor and its target should be taken into account because it will impair the detection result's accuracy. As a result, future research needs to focus on identifying more specific bioreceptors.

The development of more efficient biosensors with high sensitivity, specificity, and portability as well as greater cost-effectiveness should be the focus of future research. A wide variety of technologies are available for the development of robust EC NA biosensors. Among those, coupling with high-affinity biomolecules permits the sensitive and selective diagnosis of a range of analytes. Additionally, operating technologies like microfluidic chip technology and an easily available operating platform like smartphones and other electronic devices may add more value to meet a universal demand for POC applications. Because of its high-quality camera, practicality, and portability, the smartphone has tremendous development potential. Furthermore, cell phones do not require a dedicated reading device as reading tools. The general cost of an EC NA biosensor is nevertheless high, limiting its widespread use, particularly in underdeveloped nations. As a result, the most pressing challenge for detection commercialization has always been to lower detection costs. Due to their low cost, simple product process, and portability, paper-based biosensors may become a viable alternative to conventional electrodes to overcome this problem. Strategies, techniques, fabrication processes, and sensitivity enhancement processes summarized in this review suggest ways to widen the application of EC NA biosensors in POC as well as self-monitoring. This could have a significant outcome in minimizing the outbreak of deadly epidemics like COVID-19.

Disclosure statement

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