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DNA Polymerases in DNA Biosensors

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INTRODUCTION

Biosensors have recently undergone significant developments in their application and specificity, making them a valuable tool in fields such as the analysis of biochemicals, environmental monitoring, drug development, and medical diagnosis. Biosensors are analytical tools that use biological materials, biologically derived materials, or biomimics as recognition molecules. These materials are either incorporated into or closely related to physicochemical transducers or transducing microsystems. Typically, the goal is to provide a digital electrical signal proportionate to the analyte or set of analytes' concentration (Figure 1). The activity of the sensing molecules may cause a change in the proton concentration, the uptake or release of gases like oxygen and ammonia, light emission, reflectance or absorption, heat emission, or other mechanisms that result in this signal. The transducer can then transform this signal into a response that can be measured [1]

The enzymes that catalyze biological reactions are essential to the effectiveness of biosensors, with DNA polymerases being key components in many sensing devices. An essential component of this investigation is comprehending their composition, capabilities, and incorporation into biosensor platforms. The methods for examining DNA polymerase mechanisms are described in detail in the paper, providing a foundation for their easy incorporation into biosensor structures.

The tactics for improving the sensitivity and reliability of biosensors are evolving along with the technology. Techniques for signal amplification, which include a variety of methods such as enzyme-catalyzed processes and amplification based on nanomaterials, have become essential for accomplishing these objectives. We reveal the subtleties of these approaches in a variety of sensing modalities, from electrochemical to fluorescence-based detection systems, by closely examining them.

The investigation goes beyond conventional biomolecular recognition components to include non-imprinted polymers (NIP) and molecularly imprinted polymers (MIP), providing insights into their function in selective molecular recognition. Additionally, the study explores the principles and uses of Electrochemical Impedance Spectroscopy (EIS). The recent progress in biosensor technology has been concentrated on improving the amplification and detection of signals. Researchers have developed biosensor platforms with previously unheard-of levels of sensitivity and specificity by taking advantage of the special qualities of nanomaterials. Furthermore, enzyme-based amplification techniques have shown to be effective methods for raising detection limits and signal-to-noise ratios. Techniques for surface modification are essential for customizing biosensor surfaces for intended uses. Researchers can improve the stability and selectivity of biosensor platforms by functionalizing surfaces with MIPs or NIPs. This will allow for the accurate detection of target analytes in complicated biological samples. Furthermore, the use of sensing technologies, such as Electrochemical Impedance Spectroscopy (EIS) offers important insights into the thermodynamics and kinetics of biomolecular interactions.

Aim:

To evaluate the effectiveness of molecularly imprinted polymers in enhancing the immobilization of Proteinase K on gold surfaces for biosensing applications.

Objectives

1) Investigate the electrochemical properties of modified surfaces: conduct cyclic voltammetry (CV) experiments to compare the electrochemical behaviour with surfaces modified by NIP, MIP, and unmodified gold-based electrodes in PBS solutions containing pyrrole polymer and enzyme.
2) Characterize the electrical properties of modified surfaces: measure and compare the resistance of electric charge and capacitance of the double layer of surfaces modified with NIP, MIP, and unmodified gold-based electrodes to determine the impact of surface modifications on electrical properties.
3) Evaluate enzyme immobilization efficiency: assess the efficiency of proteinase K immobilization on surfaces modified with MIP and NIP.

1. FUNDAMENTALS OF BIOSENSORS

1.1 Principles

A biosensor is a device that uses signals proportionate to the concentration of an analyte in a reaction to quantify biological or chemical responses. Applications for biosensors include illness monitoring, drug discovery, and the identification of contaminants, pathogen-causing microorganisms, and disease-indicating markers in physiological fluids (blood, urine, saliva, sweat).

Conventional methods are widely used to immobilise biological materials like certain enzymes in place for different applications. These techniques include membrane entrapment, in which the biological material is embedded within a semi-permeable membrane; physical entrapment, in which the biological material is physically confined within a matrix or structure; and binding techniques, which can be either covalent (permanent bonding) or non-covalent (temporary bonding).

After the organic material is rendered immobile, it is directly touched by a sensor or transducer. Through this interaction, the sensor is able to track alterations in biological material brought about by the presence of the substance being measured or detected, or the analyte.

The analyte forms what is known as a bound analyte when it binds to the immobilized biological material. The sensor has a measurable electrical reaction as a result of this binding event. Different products may be produced depending on the precise contact or reaction that takes place between the biological material and the analyte. These products may include, among other things, heat, gas (like oxygen), electrons, or hydrogen ions. The sensor has the ability to transform the alterations linked to the creation of these compounds into electrical signals. The physical or chemical changes are usually converted into electrical impulses during this process, which the sensor subsequently amplifies and measures.

A typical biosensor consists of four main parts as mentioned above. The mechanism of a biosensor can be seen in Figure 1. The parts are as follows:

Analyte: analyte is a substance of interest that needs detection. For instance, in a biosensor used to identify glucose, glucose serves as an "analyte." There is a wide range of analytes that can be analysed with a biosensor.

Biological molecules: proteins [2], enzymes [3], antibodies [3], antigens [3], nucleic acids (DNA, RNA) [4], hormones [2], and neurotransmitters [2] are examples of biological molecules. Biosensors are widely utilized in environmental monitoring, food safety testing, and medical diagnostics because of their ability to detect living substances.

Chemical molecules: glucose [5], cholesterol [3], ethanol [3], pesticides [5], heavy metals, and medications (pharmaceuticals) are just a few of the chemical molecules that biosensors can identify. These biosensors are used in drug screening, environmental analysis, and clinical diagnosis.

Pathogens: bacteria, viruses, and fungi are examples of pathogens that biosensors can identify. They are employed in food and water quality monitoring, as well as in the diagnosis of infectious diseases in medicine [4].

Toxic Substances: microorganism-produced poisons, pollutants, and dangerous chemicals found in food and the environment can all be detected by biosensors. These biosensors are useful instruments for industrial safety and environmental monitoring [4].

Biological Activities: a number of biosensors are intended to track biological processes, including cell metabolism, enzyme activity, and signalling inside cells. In biomedical research, these biosensors are used to examine medication interactions and biological processes [6].

Physical factors: Biosensors are capable of measuring physical factors including temperature, pH, pressure, and gas concentration in addition to detecting biological and chemical analytes. Numerous industrial and environmental applications make use of these biosensors [7]

Bioreceptor: a bioreceptor is a type of molecule that can identify the analyte with precision. Bioreceptors include things like enzymes, cells, aptamers, deoxyribonucleic acid (DNA), and antibodies [8]. Bio-recognition is the process of generating a signal (such as light, heat, pH, charge or mass shift, etc.) when the bioreceptor and analyte interact.

Transducers: an element that transforms one type of energy into another is called a transducer. The transducer's job in a biosensor is to transform the bio-recognition event into a signal that can be measured. Signalization is the term used to describe this energy conversion process. Analyte–bioreceptor interactions are typically proportionate to the optical or electrical signals produced by most transducers.

Electronics and display: this part of the biosensor is in charge of transduced signal processing and presentation preparation. It uses complex electronic circuitry to perform signal conditioning functions, such as amplification and analog-to-digital conversion of signals. The display unit of the biosensor then quantifies the signals that have been processed.

In contrast, the display unit makes it easier for users to interpret the results by displaying them in an understandable manner. Depending on the end user's choices, it usually uses technologies like liquid crystal displays or direct printers to produce numerical numbers, graphical representations, tabular data, or images. This component typically consists of a hardware and software combination intended to provide biosensor results in an intuitive way [9]

For the analyte to identify the substrate, size and form are crucial. This is depicted in Figure 1 [10]

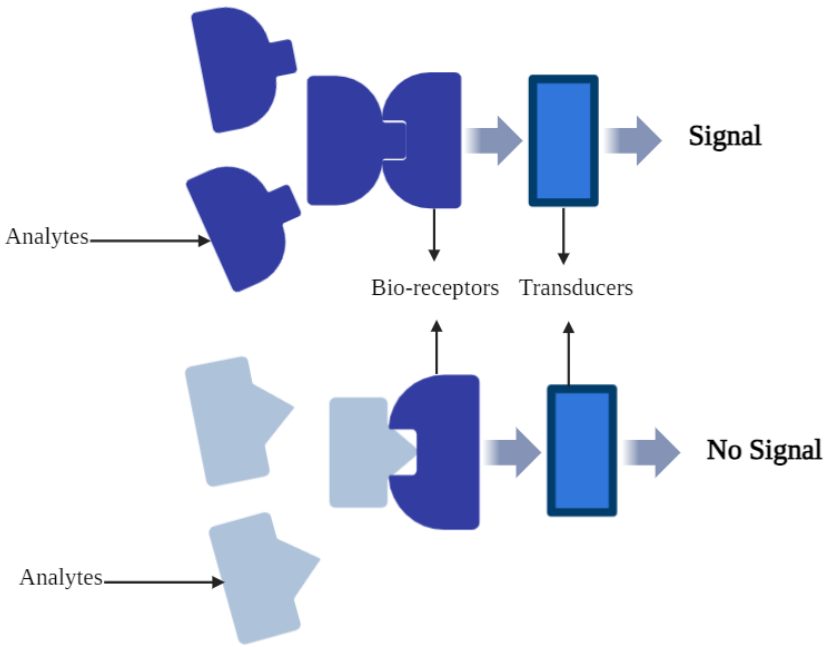


Figure 1. The working principle of a biosensor

Equipment behaviour and characteristics are to be ascertained through the use of characterization methods [11], which includes repeatability, reproducibility, and linear measurement range.

1.2 Types of Biosensors

Biosensors can be classified based on the type of analytes used and the results needed. Figure 2 shows the classification of some commonly used biosensor types.

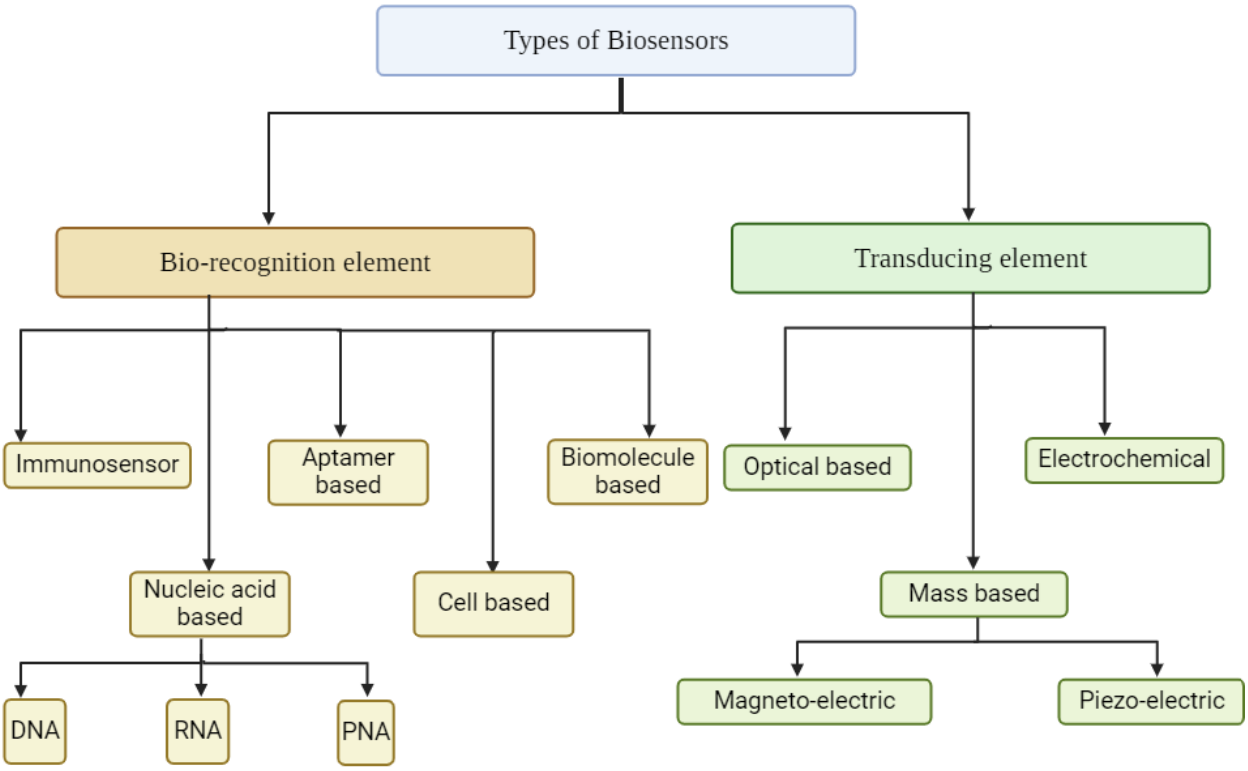


Figure 2. Classifications of different types of biosensors.

Biosensor classification takes place depending on the relationship that develops between transmission and measurement systems. Biocomponent-based biosensors use nucleus acids, enzymes, cell organelles, microbials, tissues, etc, while transducing elements are divided into optical, electrochemical and mass-based biosensors[10]. The research is based on DNA Biosensors which come under the nucleic acid-based biosensor.

1.3 DNA-Biosensors: an overview

DNA biosensors hold significant promise for the monitoring of various diseases. DNA biosensors have several advantages over traditional biosensors, including high specificity, efficiency, and the ability to detect a wider range of analytes. DNA- biosensors have customisable biosensing functions which makes them a better candidate for next-generation biomedical detections. It has been noted that DNA probes with improved heat stability, such as aptamer, can be obtained by manual screening and modification[12,13][13]. The application of DNA and its assembly structure for the detection of particular targets, such as nucleic acids, proteins, metal ions, and small biological

molecules, is well known[14,15,16]. DNA is a good candidate for intelligent biosensing because of its amazing addressability, tunable rigidity, and more persistent biological activity when compared to routinely utilized bioprobes[17]. Given the enormous promise of DNA biosensors, many studies have examined the fundamental ideas and most recent developments in biosensors based on DNA origami [18,19], DNA hairpins[20], DNA aptamers[21,22,23], DNA enzyme[24], and DNA tiles[25]. DNA biosensors also referred to as applicable biosensors, are essential for a variety of fields, including forensics, biomedical research, drug discovery, food control, and environmental monitoring[26]. DNA Biosensors can be further classified into DNA template-based biosensors, DNA Hybridisation biosensors and Functional DNA-strands-based biosensors.

1.3.1 DNA Template based biosensors

DNA tile self-assembly is a potent method that builds sophisticated nanoarchitectures with complicated patterns and capabilities by taking advantage of the programmability of DNA interactions[27]. DNA tiles, which are monomeric units with sticky ends that establish reversible bonds to matching sticky ends on other tiles, are the fundamental component[27,28]. By adjusting the sticky end sequences, the assembly rules may be programmed. Complex algorithmic growth patterns are made possible by cooperative attachment, in which a tile connects only when it is able to produce a specific pair of binding contacts [28]

Significant advancements have been made through technique improvements and a deeper comprehension of the underlying thermodynamics and kinetics of tile attachment and detachment in solution, allowing the construction of increasingly complex structures, even though experimental implementation still faces obstacles like spurious nucleation and growth errors[28,29]. In order to develop nano architectures with programmable patterns and scaffolds for guided self-assembly of nanomaterials, DNA tile assembly offers an appealing method. These nanoarchitectures could be used to build drug delivery systems, protein/ligand nanoarrays, and nanoelectronic/photonic devices[27,28]. With further study advancing our comprehension of the underlying physical principles and resolving practical obstacles, DNA tile self-assembly presents a highly promising bottom-up method for precisely atomically constructing complex nanostructures[28].

1.3.2 Functional DNA Strands-Based Biosensors

Over the past few decades, oligonucleotides, enzymes, and antibodies have all been widely used as biosensor probes with specific recognition roles[2,30]. Because of their stable chemistry, DNA probe-based biosensors have the advantages of high heat tolerance, ease of modification, and effective surface regeneration compared to enzyme or antibody-based biosensors. DNA probes with different affinity for target analytes can be obtained through targeted screening of DNA libraries. DNA aptamer biosensors are one such kind of functional DNA strand biosensors.

Aptamers are synthetic, short, single-stranded oligonucleotides that bind selectively to a wide range of molecular targets, including proteins, tissues, cells, and small molecules. Aptamers have the advantage of being highly specific, relatively small in size, and non-immunogenic[31]. The word "aptamer," which refers to the lock and key interaction between aptamers and their binding targets, is derived from the Latin word *aptus*, which means "to fit“[32]. Systematic evolution of ligands by exponential enrichment (SELEX) is the process used to create aptamers based on nucleic

acids[33]. The aim of SELEX is to find aptamers that bind to a target molecule strongly through a series of iterative steps that include selection, elution, and amplification.

The process flow of SELEX process is shown in Figure 3 [34]. The process starts with the creation of a diverse library of nucleic acid molecules, often up to approximately 1014 different molecules[34]. These molecules are usually single-stranded DNA or RNA with random sequences that cover a broad range of potentialities. The target molecule is then combined with this first library, and the nucleic acid sequences that are unbound and those that have interaction with the target are separated. After the latter group is eluted and amplified, many copies of sequences that have shown an affinity for the target are produced using polymerase chain reaction (PCR) for DNA libraries or reverse transcription PCR for RNA libraries. The following phase of selection is based on these amplified sequences, and this process is continued through a number of rounds, often between six and fifteen. Following several rounds of selection, the pool of sequences that remain is analyzed and sequenced; those that show the highest levels of specificity and affinity for the target are identified and considered to be possible aptamers[33].

It is important to note that as recognition elements, aptamers which are short, single-stranded DNA or RNA molecules chosen to bind to particular target molecules are used by both DNA aptamer biosensors and aptamer-based biosensors (aptabiosensors)[35]. Their functions and designs, however, differ from one another.

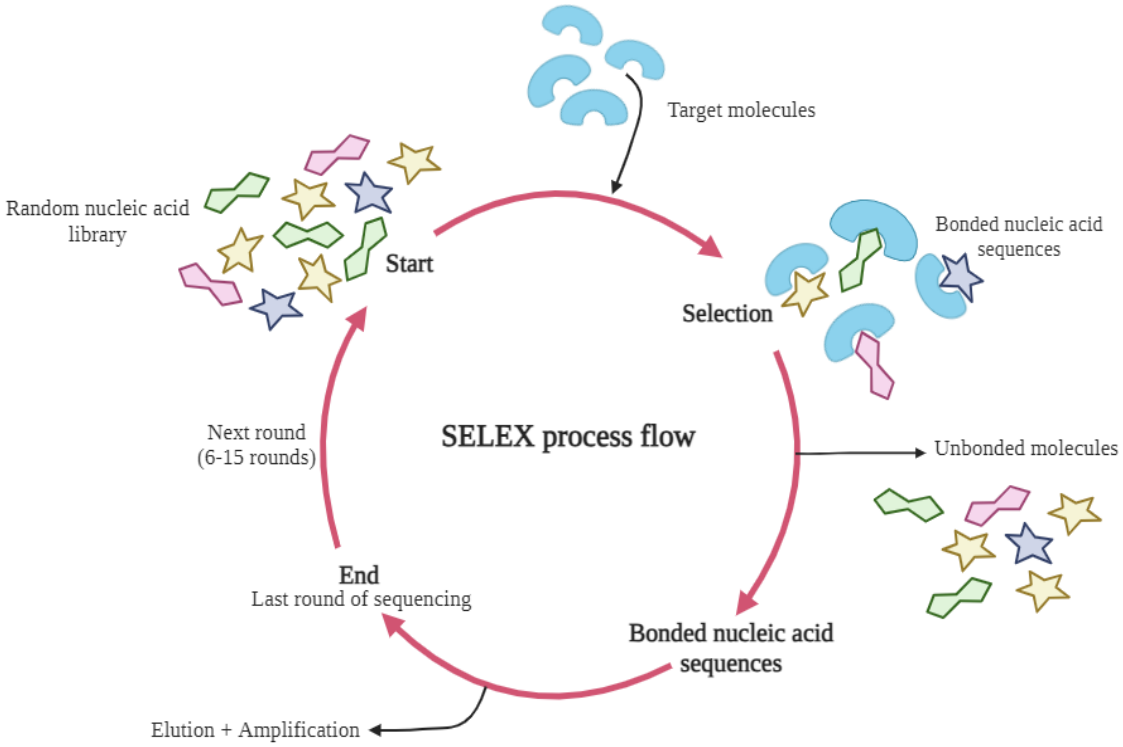


Figure 3. SELEX process flow includes the selection, elution and amplification of nuclei acid sequences.

The most used strategy to detect a biotarget with a DNA aptamer is to functionalize the DNA aptamer with a report molecule (ferrocene, methylene blue) and an immobilization molecule (alkane thiol, alkane amino, streptavidin, and hydrazoate) at the 5′ end and 3′ end of the DNA strand, respectively[17]. Detecting the electrochemical change on the electrode surface can reveal the alteration in DNA aptamer construction. Liu et al. created an electrochemical sensor for interferon-

gamma (IFN-γ) detection based on a 34-mer IFN-binding aptamer[36]. A covalent reaction between alkyl mercaptan and gold was used in their investigation to fix the suggested DNA aptamer on the electrode's surface. A secondary spatial loop structure was formed by the DNA aptamer self-folding in the absence of a specific target, causing the reporting molecules to come into contact with the sensor electrode surface. The distance between the electrode surface and the reporting molecule increased as a result of the conformational change of the DNA aptamer caused by the combination of the target. The reporting molecules and the electrode surfaces experienced a shift in electronic transfer efficiency as a result. There was an increase in the linear detection range to 10 nM and a limit of detection (LOD) to 0.06 nM. While functional DNA aptamer biosensors have many benefits, immobilizing the aptamers firmly onto the sensor surface requires a complicated modification process.

DNA Enzyme-based sensors is another type of functional DNA Strands-Based biosensors. Due to their great catalytic efficiency, enzymes are frequently employed in biosensing[37]. However, a variety of environmental conditions can impact enzymatic activity, which restricts its use in biosensors[38]. It has been shown that some manually screened DNA strands with enzymatic activity are also more environmentally friendly and resistant to nuclease degradation. These DNA strands, known as DNA Enzyme-based sensors offer a great deal of promise as effective biometric probes. The nucleic acid cleavage function and the catalytic function of peroxides are two DNA Enzyme-based sensors with distinct functions that are frequently employed in biosensing[17].

The catalytic domain of the DNA Enzyme-based sensors with nucleic acid cleavage function is formed like a loop and is surrounded by two substrate-recognition domains. By using the Watson-Crick model, the substrate-recognition domains are able to ensnare the substrate strand. In the absence of the catalytic core, the cleavage will not occur. The cleavage process is triggered when the external catalytic core integrates into the DNA Enzyme-based sensors' catalytic domain. Then, when the melting temperature drops, the substrate strand is severed and split free from the substrate-recognition domains[39,40]. Only specific tiny molecules can function as the matching DNA Enzyme-based sensors' catalytic core during this process. As a result, the cleavage process is highly specifically stimulated, which can be useful for small-molecule identification.

1.3.3 DNA Hybridization-Based Biosensors

Currently, DNA chip-based microarrays, enzyme-linked immunosorbent assays (ELISA), polymerase chain reaction (PCR), and loop-mediated thermal amplification (LAMP) are the most used nucleic acid detection techniques. But these techniques, call for sophisticated testing equipment, trained personnel, extended incubation periods, and complicated manufacturing methods. These drawbacks restrict the use of these techniques in towns and cities, train stations, airports, etc. With its high detection speed, sensitivity, and stability, the DNA hybridization amplification detection technology has significant potential to overcome the challenges.

DNA hairpin biosensors are a subset of biosensors that identify target molecules or sequences by using DNA probes shaped like hairpins.

Nucleic acid can be detected electrochemically without the need for labels (label-free) or enzymes by immobilizing hairpin DNA probes on electrodes[41,42]. Nanostructures, cascade catalysis, target recycling, and hairpin assembly have all been used to create ultrasensitive electrochemical biosensors for microRNAs and other analytes[42]. For surface-immobilized DNA hybridization research, Fang et al. suggested a type of molecular beacon. As fluorescence–quenching pairings, tetramethylrhodamine (TMR) and dimethylaminoazobenzen aminoexal-3-acryinido (DBCAL) were added to the hairpin in their study. The rise in the fluorescence signal was caused by the target opening its hairpin, which separated TMR and DBCAL. This sensitivity might be as little as a nanometer[43]. Jiahao et al. described DNA hairpin probes (HDPs) as adaptable instruments for developing biosensors on both liquid and solid-state platforms. Targets include proteins, metal ions,

nucleic acids, and tiny molecules that can be detected with great specificity and sensitivity thanks to these biosensors. Molecular beacon (MB)-based sensors for homogeneous systems and other HDP-based solid-state sensors are the two primary categories into which HDP-based biosensors are divided in this article. They describe the latest developments in each category as well as the architecture of MBs with various signaling pairs. Examples include the detection of Hg^{2+} and Zr^{4+} ions, high-throughput screening of DNA 3'-phosphatases, and real-time monitoring of nucleic acid ligation [44].

To further enhance performance, recent developments include cascaded reactions, localized HCR/CHA, and branched HCR/CHA[45]. Nevertheless, there are still issues to be resolved, including strong background signals, less amplification when compared to enzyme-based methods, sluggish kinetics, low stability, and trouble internalizing cells. Immobilizing hairpin DNA probes on electrodes allows label-free, enzyme-free electrochemical detection of nucleic acids[42].

1.4 Applications of DNA Biosensors

Reliable results, quick tests, and low cost are the main advantages of DNA biosensors. DNA biosensors are not without issues, though. Materials used in DNA-based biosensors often need to interact with DNA at the atomic level. Identifying new nanomaterials that may interact with DNA is therefore the primary step in the creation of DNA biosensors. In actuality, under uncontrolled circumstances, nanomaterials must form a tight bond with DNA. Finding these nanoparticles is therefore an unavoidable challenge in the use of DNA biosensors.

1.4.1 Food analysis

Products from agriculture are frequently traded across nations. In this sense, screening for safety, quality, and authenticity requires a dependable, relevant, and accurate approach. Monitoring food safety is a critical component of responding to hazards to public health and welfare.

Food contamination is often monitored for pathogens and toxins. For the purpose of guaranteeing food safety, security, and public health, the detection of tainted food by pathogenic microorganisms is crucial. 90% of all food-borne infections are caused by pathogenic bacteria such as *Salmonella typhimurium*, *Bacillus cereus*, *Staphylococcus aureus*, and *Escherichia coli* [46,47]. These bacteria can contaminate food and cause a variety of food-borne illnesses.

Conventional methods for identifying bacteria often need 2-4 days and a variety of time-consuming growing techniques as well as biochemical assays[48]. Nonetheless, in a short amount of time, electrochemical biosensors have produced a dependable method for pathogen screening. For instance, it was reported that *Staphylococcus aureus* was found when single-walled carbon nanotubes were examined in pig skin[47]. Furthermore, a DNA-based electrochemical biosensor for Bacillus cereus in milk and baby formula was reported by [46]. It should be mentioned that gold nanoparticles and carbon nanotubes were used to detect *Salmonella typhimurium*[49]. Biosensors are extensively employed in the identification of biological toxins that are tiny molecules, particularly mycotoxin and neurotoxic.

A variety of marine algae species and contaminated shellfish create neurotoxins, which are among the most poisonous non-protein chemicals known. Mycotoxins are a broad class of organic compounds produced by fungus species. Conventional techniques including fluorescence detection (FLD) and high-performance liquid chromatography (HPLC) with ultraviolet (UV) have been used in the past[50]. These methods are not without issues, though. For instance, they require a great deal

of work and are not ideal for screening a large number of samples for fieldwork. Additionally, they require highly skilled users and take too much time[51].

1.4.2 Environmental monitoring

The spread of numerous pollution types into the environment has been attributed to the development of various industries, farming, and the pace of population expansion worldwide. It takes a large-scale screening technique to find and eliminate the pollutant chemicals. Numerous studies have demonstrated the use of biosensors in environmental monitoring. The use of an electrochemical DNA biosensor for environmental monitoring was explained by Chiti et al [52].

They described how an electrochemical DNA biosensor can be used to identify harmful aromatic amines. Furthermore, it was noted that carbon electrodes modified with DNA may be used to monitor the interaction of low molecular weight priority pollutants with DNA, and that DNA hybridization sensors might be used to detect sequences linked to microbial or viral pollutions. Remarkably, a biomonitoring instrument in the form of a DNA biosensor was created in fish bile in a significant study. It was demonstrated that the electrochemical DNA biosensor is suggested as a screening tool for the quick identification of fish bile samples containing metabolites of polycyclic aromatic hydrocarbons (PAHs). Because of their durability and capacity for accumulation, PAH have detrimental impacts on aquatic biota.

1.4.3 Medical diagnostics and research

DNA biosensors offer enormous potential as effective diagnostic instruments for identifying a wide range of illnesses and ailments[53]. When a target DNA biomarker is complementary to one strand (the probe), they measure the binding between the two strands of DNA. A semiconductor device such as a field-effect transistor (FET) can detect the voltage shift caused by the target DNA binding to the probe.

Furthermore, as noted in a NIST News article, a recent advancement in DNA biosensors by NIST scientists, Brown University, and CEA-Leti presents a unique biosensor chip design that provides precise and affordable diagnostics for medical research. With the use of a field-effect transistor (FET), this biosensor chip detects voltage variations brought on by DNA binding events, making it possible to precisely and highly sensitively identify biomarkers. The biosensor shows promise in advancing medical diagnostics and research; potential uses in clinical diagnostics include identifying genetic material linked to viruses and disorders like COVID-19 [54].

For example, one of the primary components of cancer treatment is an accurate and timely diagnosis. The most sensitive instruments are DNA biosensors, which offer data to help doctors decide on the best course of action and raise patient survival rates[55]. Senel et al. demonstrated the use of an electrochemical DNA detection technique for the identification of a gene linked to breast cancer in a significant study. The findings demonstrated the great sensitivity and selectivity of the investigated biosensor in identifying the target DNA. The findings are also very encouraging for the investigation of DNA biosensing technologies in the diagnosis of breast cancer brought on by BRCA1 gene mutations[56].

2. DNA POLYMERASES: STRUCTURE AND FUNCTION

DNA polymerases have a structure resembling a right hand [57]. There are three primary subdomains within them: "palm," "fingers," and "thumb." Within the cavity that these subdomains form, DNA binding takes place. The palm subdomain's conserved amino acid residues serve as the foundation for the catalytic core. While the thumb binds DNA, the "fingers" place a template in the active site and bind dNTPs. At the enzyme's N terminus is a distinct domain that carries out exonuclease activity. The palm is relatively conserved among all polymerase groups, although the thumb and fingers can have different structural variations [58]. DNA polymerases have been divided into seven families—A, B, C, D, X, Y, and RT—based on evolutionary analysis and similarity of primary protein sequences, despite structural similarity [59]. This has been shown in Table 1. There is considerable diversity among DNA polymerases and their functions are not all identical [59].

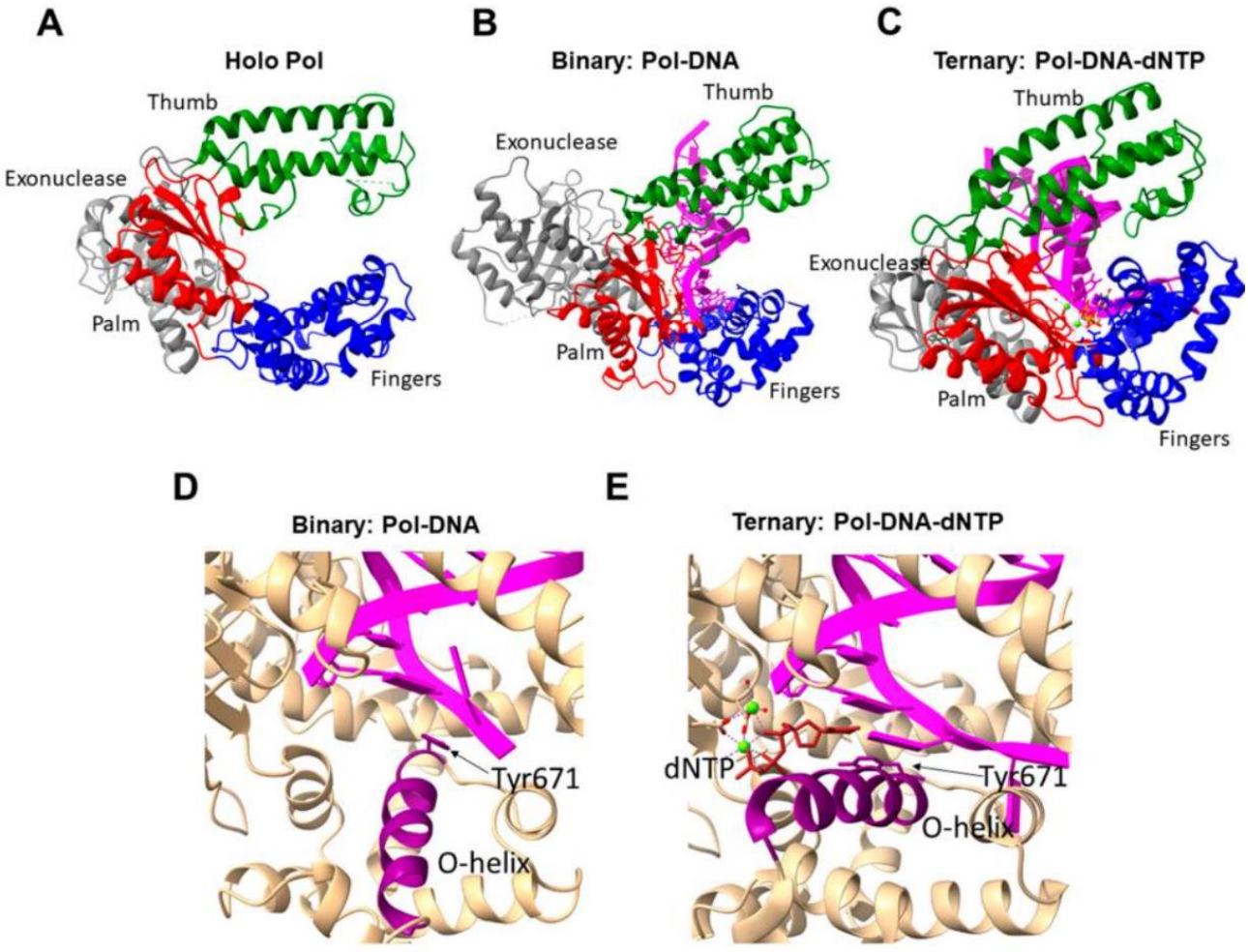


Figure 4. Three-dimensional configuration of E. Coli DNA polymerase I: (A) ternary Pol-DNA-dNTP (PDB ID 1qtm); (B) binary Pol-DNA (PDB ID 4ktq); (C) holo enzyme (PDB ID 1ktq). The subdomains labelled "thumb" and "fingers" have green and blue hues, respectively. The active-site areas in the binary and ternary complexes are carefully compared in (D, E). The "palm" subdomain is red, the "exonuclease" subdomain is grey, and the DNA is magenta. The O-helix is depicted in purple in each instance, while the protein is displayed in beige. The arriving dNTP displays a brown color. [57] Copyright MDPI, Basel, Switzerland.

Family	A	B	C	D	X	Y	RT
Taxon	Eukaryota Bacteria Viruses	Eukaryota Bacteria Archea Viruses	Bacteria Cryptic phages Plasmids	Archea	Bacteria Archea Eukaryota Viruses	Eukaryota Bacteria Archea Plasmids	Eukaryota Viruses
Associated Activity	3’–5’ Exonuclease	3’–5’ Exonuclease	3’–5’ Exonuclease	3’–5’ Exonuclease	5' phosphatase (β)		
DNA Polymerases	Pol γ, Pol θ, and Pol ν, Pol I T7 DNA Pol	DNA pol B T4 DNA Pol Pol II Pol ζ, Pol α, Pol δ, Pol ε	Pol III	Pol D	Pol β, Pol σ, Pol λ, Pol μ, TdT Pol X ASFV DNA Pol	Rev1, Pol ι, Pol κ, and Pol η Dpo4 DNA Pol	Telomerase Reverse transcriptase

Table 1. DNA polymerase families including A, B, C,D,X,Y and RT. [57,59]

DNA polymerases of family A have replication and repair activities. A corrective function is carried out by the activity of 3′ → 5′ exonuclease, i. e. removal of an incorporated noncomplementary nucleotide in the event of an error, improving the fidelity of DNA synthesis. On the other hand, ribonucleotide primers required for the replication of the lagging strand of DNA are physiologically eliminated by 5′ → 3′ exonuclease activity. This family of polymerases is present in bacteria (Pol I), viruses (T7 DNA Pol), and eukaryotes (Pol γ, Pol θ, and Pol ν) as shown in Table 1.

PolBs are the most abundant replicative polymerases, present in all eukaryotic domains as well as multiple DNA virus lineages[60]. The majority of these polymerases, which are present in eukaryotes, also lack the domain that is accountable for the 3′ → 5′ exonuclease activity and do not have 5′ → 3′ exonuclease activity as well. However, 3′ → 5′ exonuclease activity in other taxa is 1000 times greater than that of Escherichia coli Pol I [57]. PolBs can be classified into three primary, seemingly monophyletic groups according to the primers they employ. Selfish mobile genetic elements and tiny linear genomes of viruses are replicated by protein-primed PolBs (pPolBs) [60]. Eukaryotes (Pol ζ, Pol α, Pol δ, and Pol ε), bacteria (Pol II), archaea (DNA polymerase B), and viruses (DNA polymerase T4) have all been reported to have family B polymerases as shown in Table 1.

The C family of polymerases, which has no sequence homology with any other DNA polymerase family, includes the DNA polymerases that duplicate bacterial chromosomes [61]. The C family is the last polymerase family to be structurally examined, despite the fact that the first member was found almost 40 years ago [61,62]. The primary proteins in charge of chromosomal replication are the enzymes that make up the C family of polymerases [57]. C family polymerases are quite large multidomain proteins, larger than polymerases of other families, which may explain why structural representatives of this class have been solved only recently [61]. These are holoenzymes that require interaction with at least ten other proteins to form a sizable multisubunit complex in order to function [57]. These are found in Bacteria Cryptic phages, Plasmids, an example is Pol III. Its α-subunit is coupled to the ε-subunit, which possesses 3′ → 5′ exonuclease activity and has DNA polymerase activity [63]. Euryarchaeota contains family D polymerases, of which Pol D is an example [64]. It is a heterodimer, this enzyme. While the big component DP2 has polymerase activity, the tiny subunit

DP1 is in charge of 3′ → 5′ exonuclease activity. Low yet significant homology exists between the DP1 subunit and eukaryotic DNA polymerase δ [57,65].

Small monomeric proteins are family X polymerases. These enzymes are a component of the cell's repair system. They are essential to nonhomologous end-joining (NHEJ) and base excision repair mechanisms, among other repair processes. These enzymes have been identified in viruses (DNA polymerase of African swine fever virus), bacteria (Pol X), archaea (Pol X), and eukaryotes (e.g., Pol β, Pol σ, Pol λ, and Pol μ)[63]. Only Pol β and Pol λ have an active 8 kDa domain, but members of this family share a structurally similar 8 kDa domain that is responsible for dRP lyase activity[57].

The translation synthesis that Y-Family DNA polymerases are known for allows them to avoid damaged bases that would impede replication fork progression normally. Because of their distinct structural characteristics, Y-Family polymerases are able to bind broken DNA and guide nucleotide incorporation using a modified template base[66]. Every Y-family polymerase has two functional regions: a regulatory area that ranges in length from 10 residues (found in DinB, Dbh, and Dpo4) to 600 residues. The polymerase catalytic region is composed of 350–500 residues.

They are represented by E. coli pol IV (known as DinB) and pol V (of which UmuC is the catalytic subunit) and four human enzymes, pol η, ι, κ, and Rev1[66]. Compared to replicative polymerases, the fingers subdomain of family Y polymerases is smaller. Furthermore, every member of the Y family has an extra subdomain known as "little fingers" that interacts with the primary groove of DNA to facilitate DNA synthesis even in the presence of damage[57]. The ability to pass through thymidine dimers is primarily due to the finger's subdomain's smaller size since a thymidine dimer can fit inside the cavity created by three catalytic domains [66]

DNA polymerases known as reverse transcriptases (RT) are able to synthesise DNA using RNA as a template. As a result, they catalyze transcription in reverse[67]. Reverse transcriptases from retroviruses interact with different nucleic acids during the process of reverse transcription, resulting in the conversion of a single-stranded viral RNA genome into double-stranded pro-viral DNA (duplexes RNA/RNA, DNA/RNA, RNA/DNA, or DNA/DNA). Certain retroviral reverse transcriptases, like those from HIV-1 and HIV-2, are dimers, whereas others, like the reverse transcriptase of the Moloney murine leukemia virus (MoMLV), are monomeric[57]. However, for the purpose of cleaving viral RNA during DNA synthesis, both kinds contain polymerase and RNase domains[68]. Since telomerases use integral RNA as a template for telomere synthesis, they are also members of the reverse transcriptase family[69].

2.1 Methods for DNA Polymerase Mechanism Analysis

Understanding the inner workings of these complex molecular machinery has been the focus of much research in the fifty years since the first DNA polymerase was discovered. A thorough explanation of the fundamental steps in the reaction route, including the structural changes that occur and the impact of reaction rates on the flow of substrates through the pathway, is the ultimate aim of DNA polymerase enzymology. A multitude of cocrystal structures that most likely correspond to intermediates along the reaction pathway have been produced by X-ray crystallography[70] ; the situation is like having an amazing collection of movie stills without knowing for sure which ones to view first or even if they are all from the same film.

The understanding of enzymatic-catalysis mechanisms is greatly enhanced by structural approaches; nevertheless, these methods are limited to providing information on a certain fixed state of the enzyme and substrate at a particular moment in the enzymatic process, such as in a catalytic

complex. Only a crystal of the free enzyme and a crystal of the enzyme complex with a reaction product may be obtained from catalytically active enzymes. The small number of potential states of the enzyme-substrate complex that can be registered using such approaches is a significant drawback of static structural data. Additionally, under equilibrium circumstances in solution, X-ray diffraction measurements are not always verified, particularly when covalent crosslinks between complicated components are added to produce complex crystals. The sequence of these events is also unknown, even though the entirety of the processes required for the assembly of a catalytic complex is known. These include the interactions that occur during the initial stages of substrate recognition, the formation of contacts that allow for the discrimination between a substrate and a "nonsubstrate," and the interactions that guarantee the specificity of the enzyme.

Nuclear magnetic resonance (NMR) spectroscopy, a "crystal-less" method of identifying structure based on 15N, 13C, and 2H atoms, has advanced significantly [71]. Near-atomic resolution structural characterization of macromolecular structures has greatly benefited from cryo-electron microscopy (cryo-EM). When biological samples are cryogenically heated and flash-frozen in their natural habitat, this imaging method is frequently employed [72].

An essential technique for comprehending the intricate processes by which DNA polymerases catalyze the creation of DNA is enzyme kinetics. Researchers can learn more about how DNA polymerases interact with substrates, integrate nucleotides, and replicate DNA with high fidelity by observing the rates of enzymatic reactions under varied conditions. Steady-state kinetics and pre-steady-state kinetics are a few such methods. After an initial transient phase, when the production and breakdown of enzyme-substrate complexes have achieved a steady state, the reaction rate is evaluated in steady-state kinetics. The Michaelis-Menten equation is used to model these reactions:

$$\mathrm{v} = \frac{V_{max}[S]}{K_M + [S]} \qquad (1)$$

where v is the reaction velocity and [S] is the substrate concentration, K_M is Michaelis-Menten constant and V_{max} is the maximum velocity.

The quick, transient stages of the reaction that take place prior to the steady state being established are captured by pre-steady-state kinetics. Methods like stopped-flow and quick quench-flow enable the examination of distinct stages within the catalytic cycle. One benefit of using steady-state measurements of the DNA polymerase reaction is that they are easy to do; on the other hand, the kinetic parameters that are produced are often difficult to interpret or don't reveal anything about the relatively uninteresting steps in the process. The practical benefits come from the steady-state requirement that there be a significant excess of DNA substrate over the polymerase; as a result, they preserve valuable material and do not require rapid kinetics instrumentation because many enzyme turnovers are required to convert a significant portion of substrate into product.

Table 2. A comparison of kinetic parameters of various polymerases.

Polymerase	**Family**	**KdDNA, (nM)**	**KddNTP, (μM) Correct N**	**kpol, (s−1)**	**koff, (s−1)**	**Ref.**
Pol I (Klenow)	A	5	5.5	50	0.2	[73]
DNA polymerase T7	A	23	18	287	0.2	[74]

Pol T4	A	70	20	400	6	[75]
Human Pol α	B	58	9.2	26.8	7	[76]
E. coli Pol II	B	21	4.4	13.1	0.05	[77]
Mammalian Pol δ	B	300	0.93	13	ND	[78]
Yeast Pol δ	B	30	24	0.93	0.03	[79]
Human mitochondrial large subunit Pol γ Holo Pol γ	B	39 9.9	14 0.78	3.5 45	0.03 0.02	[80,81]
Vent Pol B	B		70	66	1.1	[82]
RB69	B		69	200	0.35	[83]
Sau-PolC-ΔNΔExo	C	390	4	180	150	[84]
Pol D	D		0.9–2.5	1.8–3.1	0.4	[85]
Pol β	X	49	110	10	0.3	[86]
Pol λ	X	0.15	1.1–2.4	3.0–6.0	ND	[87,88]
Pol μ	X		0.35–1.8	0.006–0.076	ND	[89]
E. coli Pol IV	Y	50	441	12	0.18	[90]
Sulfolobus solfataricus Dbh	Y	60	600	0.64–5.6	ND	[91]
S. solfataricus Dpo4	Y	10–40	70–230	7.6–16.1	ND	[88]
Yeast Pol η	Y	16	6.8–15	3.9–15.6	ND	[92]
RT	RT	4.7	4–14	33–74	0.16	[93]

Fluorescence-based methods include methods like Fluorescent nucleotide analogues, which when incorporated into DNA release fluorescence, allowing for real-time monitoring of DNA synthesis. Furthermore, variations in the distance between the fluorophores on the DNA and the polymerase are detected by Förster Resonance Energy Transfer (FRET), which sheds light on the conformational dynamics of catalysis [94]. Large-scale conformational changes in enzyme–DNA complexes and functional coordination are studied using single-molecule Förster resonance energy transfer (smFRET) microscopy[57,94].

Radiolabeled nucleotides are used in incorporation assays, which quantify the incorporation rate by looking for radioactivity in the produced DNA. This process is known as radioactive labeling. When it comes to measuring nucleotide incorporation, this technique is incredibly exact and sensitive.

Primer Extension Assays, a technique used in gel electrophoresis, monitor the extension of fluorescently or radiolabeled primers on a DNA template. Gel electrophoresis is used to separate the products, and polymerase activity is shown by primer extension length.

3. INTEGRATION OF DNA POLYMERASES IN BIOSENSOR SYSTEMS

The most important stage in creating an electrochemical DNA sensor is immobilizing DNA probes on the working electrode surface so that they can hybridize to recognize their complementary DNA target[95]. High reactivity and proper alignment of the immobilized DNA probe to hybridize with its complementary DNA target would be promoted by an effective DNA probe immobilization approach. Methods such as adsorption, covalent bonding [96] and active site masking are a few of the commonly used strategies for immobilising DNA polymerases to be used in DNA biosensors.

3.1 Adsorption methods

Adsorption, which involves weak connections like Van der Waals forces, electrostatic contacts, and hydrophobic interactions, is a popular technique for immobilizing enzymes. Even though they are straightforward and reasonably priced, immobilized enzymes by adsorption may be susceptible to variations in experimental setup and non-specific adsorption of other substrates, which could result in contamination and signal interference.

The enzyme is dissolved in a solution, and under ideal circumstances, the solid support is in contact with the enzyme solution for a certain amount of time to maintain enzyme activity. The enzyme molecules that have not been adsorbed are subsequently washed from the surface using a buffer. Adsorption immobilization is a straightforward, affordable method that doesn't require the use of any reagents, is inexpensive and typically doesn't harm enzyme performance because it doesn't require functionalizing the support.

However, there are disadvantages to this method: because the enzymes have weak physical bonds holding them to the support, variations in temperature, pH, or ionic strength may cause the enzymes to desorb or leach. Furthermore, in addition to enzyme leaching, non-specific adsorption of other proteins or chemicals on the transducer surface may result in contamination and signal interference, which makes biosensors based on adsorbed enzymes unstable during operation and storage. Immobilization by adsorption is commonly classified into physical adsorption, electrostatic binding, and hydrophobic adsorption.

3.1.1 Physical adsorption

Enzymatic biosensors have extensively employed this immobilization technique. In order for physical adsorption to take place, the support must be soaked in an enzyme solution and left to incubate for a predetermined amount of time (hours). An alternative method involves letting the enzyme solution dry on the electrode surfaces and then washing the enzymes that haven't been adsorbed away [97]. Although this process is time- and reagent-consuming, it offers simplicity, surface regeneration, and cost-saving capabilities. Furthermore, the immobilized enzyme layer that was formed lacks homogeneity in the orientation of bound molecules, which may impede substrate binding to the active sites of the enzyme.

3.1.2 Electrostatic binding

The pH of the reaction solution and the enzyme's isoelectric point are the two factors that must be taken into account in order to use electrostatic force to immobilize an enzyme. Enzyme molecules can have a positive or negative charge on their surface based on the relative difference between the enzyme's isoelectric point and the pH of the solution. This allows the enzyme to be immobilized via ionic and strongly polar interactions onto the oppositely charged surface. Layer-by-layer deposition and electrochemical doping are two popular electrostatic adsorption immobilization methods that have been used extensively in the development of enzyme biosensors.

Layer-by-layer (LBL) deposition, is a thin-film fabrication technique where materials and enzymes with opposing charge layers are alternately created on top of one another on a solid support with wash processes in between. A cationic/anionic charged substrate is simply dipped into an aqueous solution of anionic/cationic polyelectrolyte, or vice versa, to initiate the deposition process. After that, the coated substrate is cleaned and submerged in a cationic/anionic enzyme solution. Until the required number of layers is achieved, these alternate deposition operations are repeated.

Electrostatic contacts, hydrogen bonding, coordination bonding, charge transfer, molecular recognition, hydrophobic interactions, or a combination of these are used to produce multilayers of oppositely charged layers. Basically, the application guidelines for the LBL assembly approach can change based on the kind of material that deposits on the enzyme layer or film. Examples of such materials include biomacromolecules , polyelectrolytes (conductive polymers) and particles, dyes, and dendrimers [98]. By electrochemically doping the polymer during its oxidation or reduction, enzymes can also be immobilized within the conductive polymer film.

The polymer gets positively or negatively charged throughout the oxidation or reduction process, allowing charged enzymes to be added to the conductive polymer in the appropriate amounts. For galactose monitoring, for instance, a biosensor based on electrochemical doping immobilization of galactose oxidase was created [99].

3.1.3 Hydrophobic adsorption

Using hydrophobic interactions between the support and enzyme molecules is another method of immobilization. The interaction is created during immobilization as a result of entropy gain by the displacement of water molecules from support surface material and the surface of enzyme molecules [100].

The hydrophobicity of the adsorbent and the enzyme both have a significant impact on the strength of the interaction. The hydrophobic interactions between the enzyme and support can be controlled by adjusting experimental variables like pH, temperature, and salt concentration during enzyme immobilization, or by adjusting the size of the hydrophobic ligand molecule and the degree of support replacement [101].

The ease of use and efficiency of hydrophobic adsorption in maintaining the structure and activity of enzymes is one of its key benefits. By protecting the immobilized enzymes against aggregation and proteolysis, their stability and activity can be increased. Furthermore, the immobilized enzymes can be easily recovered and reused due to the non-covalent nature of the interactions. The adsorption of enzymes on gold electrodes is mostly dependent on hydrophobic interactions [102,103]. The adsorption process is controlled by the distribution of hydrophobic amino acid residues on the surface of the enzyme [103]. Nanoporous gold (np-Au) electrodes have been explored for enzyme immobilization due to their high surface area and ability to enhance the loading

and wiring of enzymes. The porous structure can provide a suitable environment for maintaining the activity and stability of the immobilized enzymes.

3.2 Covalent Bonding

One of the most popular techniques is covalent binding for enzyme immobilization, which creates stable complexes between the functional groups on the molecules of the enzyme and a support matrix. The functional group that an enzyme possesses, which allows for the establishment of a covalent bond with support, should not be necessary for the activity of the enzyme.

Typically, this involves binding via the side chains of amino acids (lysine), carboxylic acids (cysteine), and aspartic and glutamic acids. Enzyme binding to a solid support typically occurs in two steps: (1) surface activation via linker molecules like glutaraldehyde or carbodiimide, and (2) covalent attachment of the enzyme to the activated support. Covalent coupling can be facilitated by the following functional groups of enzymes: amino groups, carboxylic groups, phenolic groups, sulfhydryl groups, thiol groups, imidazole groups, indole groups, and hydroxyl groups [104]. Through covalent bonding, linker molecules, such as glutaraldehyde or carbamidimide, serve as a bridge between the surface and the enzyme. The second group attaches to preactivated support and subsequently forms a covalent link with the enzyme, while the first group matches the immobilization surface and forms a self-assembled monolayer (SAM). Different linkers are utilized for different immobilization techniques (either directly onto the transducer surface or onto a thin membrane attached to the transducer) and surfaces (inorganic material, natural or synthetic polymer, membranes). Covalent bonding necessitates a longer incubation period than adsorption because it takes several hours for the SAM to develop and the enzymes to bind to it. To attain great homogeneity in the SAM, a more sophisticated procedure necessitates careful attention to chemical purity [105].

Carbodiimides are functional groups that have the formula RN=C=NR. They let an enzyme's amino functionalities (-NH2) attach more easily to a support's carboxyl groups (-COOH). N-hydroxysuccinimide (NHS) can be used with carbodiimides prior to the enzyme covalent coupling phase to improve immobilization efficiency. By strengthening the bond between the enzyme and the support, this combination serves to improve the immobilization process as a whole.

Additionally, carbodiimides can help an amine-functionalized support and a carboxyl-functionalized enzyme link together. Another technique is to use glutaraldehyde as the activator. An aldehyde group of glutaraldehyde and the amine-functionalized support engage in a Schiff-base reaction throughout this procedure. Then, an amine-functionalized enzyme and glutaraldehyde's second aldehyde group bind covalently. The strong immobilization of the enzyme onto the support is ensured by this two-step procedure.

Thiol groups (-SH) and gold substrates (Au) have a strong affinity and semi-covalent bonding that is essential for chemisorption. Enzymes with thiol groups can immobilize on gold surfaces. Examples of these enzymes are oxidoreductases and isomerases, which have cysteine residues in their double-catalytic sites. These enzymes can be genetically engineered to contain reactive thiol groups, or they can be altered chemically or otherwise. Specific literature contains detailed methods for immobilizing enzymes through their thiol groups. As an alternative, thiol-containing enzymes can be fastened onto supports by immobilizing them with disulfide oxides or reactive disulfides.

A thiol-containing bifunctional linker is used to do this. On one end, it creates disulfide bonds (S–S) with the surface, while on the other, it gives N-hydroxysuccinimide (NHS) groups, which can react with the enzyme's free amino groups. This process guarantees that the enzyme will adhere to

the support in a stable and targeted manner. Immunosensors frequently use covalent immobilization of other biomolecules, such as DNA and aptamers, to trap particular antigens.

3.3 Cross-linking

Cross-linked enzyme aggregates (CLEA) are one of the carrier-free immobilization methods that have been widely characterized for a wide range of different enzymes. Enzyme immobilization through cross-linking is an irreversible technique wherein covalent connections are established between the molecules of the enzyme to generate intermolecular crosslinks. In order to unite enzyme molecules into three-dimensional cross-linked aggregates, a multifunctional reagent serves as a linker during the process. The immobilized enzyme is not attached to any support and is present in the reaction mixture. Cross linking enzyme aggregate (CLEA) and cross linking enzyme crystal (CLEC) are the two methods used in cross linking immobilization.

In order to cross-link enzyme molecules via the interactions of the free amino groups of lysine residues on the reactive site of nearby enzyme molecules, both approaches require the application of a cross-linking agent, such as glutaraldehyde. Glutaraldehyde is introduced to cross-link enzyme crystals in the CLEC-based technique following crystallization. The mechanical characteristics of enzymes immobilized by CLECs are typically significantly improved; as a result, immobilized enzymes in CLECs are typically more stable and efficient than their untreated counterparts. The CLEA-based approach produces aggregates of enzymes that maintain their catalytic qualities through the addition of salts, organic solvents, or non-ionic polymers[106]. By using cross-linking, as opposed to immobilization on a carrier, the support material does not dilute the enzyme activity. Additionally, it gets beyond the drawbacks of enzyme crystallization (CLECs), which needs highly pure enzymes.

3.4 Entrapment

Enzyme diffusion is limited in entrapment immobilization because the enzyme is not directly bonded to the support surface but is instead trapped inside a polymeric network that only permits substrate and product passage while holding onto the enzyme. Two phases are involved in the entrapment immobilization process: (1) incorporating an enzyme into a monomer solution; and (2) causing the monomer solution to polymerize through a chemical reaction or by altering the experimental conditions. An enzyme does not chemically interact with the entrapping polymer since it is physically contained within a polymer lattice network.

As a result, the technique may increase enzyme stability while reducing denaturation and leaching. The technique also has the benefit of optimizing the enzyme's microenvironment by changing the encapsulating material to have the ideal pH, polarity, or amphilicity. The mass transfer resistance that results from polymerization extension tends to thicken the gel matrix; as a result, the substrate is unable to diffuse deeply into the gel matrix to reach the active site of the enzyme, which is a restriction of the approach. Moreover, if the support matrix's pores are too big, the enzymes that are trapped may experience leakage.

Additionally, the process has a low capacity for loading enzymes, and the polymerization process may contaminate the support material. Depending on the type of entrapment, several techniques are employed for immobilization, including electropolymerization, photopolymerization, the sol-gel method for lattice or fiber type, and microencapsulation for microcapsule type.

3.4.1 Electrochemical polymerization

The process of electrochemical polymerization, also known as electropolymerization, is a straightforward one that involves adding the right amount of current or potential to a solution that contains monomer and enzyme molecules. Reactive radical species can then be produced by the oxidation or reduction of monomers in the solution at the electrode surface. These species then combine to create an adhering polymer at the electrode surface. As the polymerization process proceeds, enzyme molecules that are present in the solution near the electrode surface become caught inside the expanding polymer network. The oxidation of the monomer to produce a radical cation is the first stage in the polymerization process. This radical can then react with another comparable radical to form a dimer or with a neutral monomer.

Following additional oxidation and coupling events, the generated dimers yield oligomers and, in the end, an insoluble polymer that is deposited on the electrode surface. Electrochemically conducting polymers such as polyaniline, polypyrrole or polythiophene, pyrroles, thiophenes, and polyindole make up the majority of electropolymerized films used for enzyme immobilization [107].

The homogeneity and thickness of the polymer film can be better controlled by electrochemical deposition by mean of polymerization than by manual deposition because the former can be assessed by looking at the formation of a diffusion barrier over the film, while the latter can be determined by looking at the charge transferred during film formation.

The choice of solvent, counter-ion, and other factors utilized in the electrochemical polymerization process, such as temperature, monomer concentration, and the length of the polymer's electrolyte chain, could also have an impact on the characteristics and shape of the polymer film. With just one easy step and easy potential control, homogenous films can be produced using electrochemical polymerization.

3.4.2 Photopolymerization

Liquid, photopolymers (radiation-curable resins), and enzyme solutions are needed for the process-based immobilization of enzymes through photopolymerization. The photopolymers are subjected to light in the visible or ultraviolet regions of the electromagnetic spectrum, which starts the chain-growth polymerization events known as photopolymerization reactions. These photopolymers perform chemical processes that cause the molecules to cross-link when exposed to light, hardening the material as a result. Light can be directly absorbed by the reactant monomer or indirectly through the transmission of energy from a photosensitizer.

Since its initial synthesis, the polymerization technique has been employed to entrap enzymes for poly(vinyl alcohol)-bearing styrylpyridinium groups (PVASbQ), a soluble pre-polymer containing photocrosslinkable groups. Light irradiation is necessary for the propagation of photopolymerization events because it initiates more cross-linking reactions between monomers, forming oligomers, and ultimately producing an insoluble polymer.

3.4.3 Sol-gel process

The foundation of the sol-gel technique is the capacity to create matrices of silica, metaloxide, and organosiloxane with specified porosity through the reaction of organic precursors at ambient temperature. Depending on the kind of precursors (beginning materials) utilized, there are two general approaches for the sol-gel technique: the polymeric (or alkoxide) route and the colloidal way. The

latter technique is frequently used in enzyme immobilization. The two steps on the journey are as follows: (1) The metal alkoxide precursor(s), such as methyltrimethoxysilane or tetramethoxysilane, should be suspended or dissolved in an appropriate liquid (pH that is acidic when water is present) in order to facilitate hydrolysis and create silanol (Si-OH) groups. (2) A basic, such as potassium hydroxide, is then added to the hydrolyzed precursor to activate it and start condensation processes between the silanol moieties that create siloxane (SiO-Si) polymers. The liquid's viscosity rises exponentially with the network's age and growth in relation to temperature and time until gelation happens.

Consequently, the network creates a matrix in which the enzyme molecules are encased [108]. Popular immobilization technique sol-gel creation produces a stable nanoporous material with high encapsulation concentration and gentle immobilization conditions that preserve enzyme function and increase biosensor sensitivity.

3.4.4 Micro-encapsulation

An entrapment technique known as "immobilization by encapsulation" involves encasing enzymes in a spherical semipermeable membrane. The type of membrane can be non-ionic, lipoidal, polymeric, or based on lipoproteins. Generally speaking, there are two ways to microencapsulate: (1) coacervation (also known as phase separation), which involves separating out enzyme microdroplets in a solvent that is impervious to water, and (2) interfacial polymerization, which involves forcing a monomer to polymerize at the interface of two immiscible substances (a hydrophobic monomer and another monomer that is dispersed in a solvent that is impervious to water).

The basis of the immobilization principle is the size difference between the molecules of the substrate or product and the enzyme when compared to the membrane pore size. The barrier prevents bigger enzyme molecules from entering while allowing smaller molecules, such as substrate and product, to permeate in and out of it. Enzyme molecules are therefore free to float inside the capsule while being restricted by the membrane. Since enzymes are shielded from the environment by the encapsulation process, there is little chance of enzyme leakage, maintaining the integrity of enzyme structure and activity. Additionally, by enclosing multiple enzymes within the membrane, a multi-enzyme system could be created [109].

The method's demand for precise membrane pore size management in order to prevent enzyme leakage is one of its drawbacks. Because the enzyme and substrate molecules have varying sizes, the membrane porosity needs to be precisely controlled. As a result, this method cannot be used in reactions where the diameters of the substrate and the enzyme molecules are similar, nor can it be used in reactions where the substrate molecules are too big.

4. AMPLIFICATION STRATEGIES IN BIOSENSOR DESIGN

4.1 Signal Amplification Strategies Used in electrochemical sensing

It is critical to increase the sensitivity of biosensors since some clinical analyses and disease diagnoses are becoming more stringent when it comes to the trace detection of target analytes. Numerous signal amplification techniques are employed based on the benefits of electrochemical sensors in order to boost the electrochemical output signals and increase the sensitivity of electrochemical biosensors. The electrochemical biosensor is a metrological tool that operates by utilizing the electrochemical characteristics of electrolyte solutions and the law of charge change of materials on electrode surfaces. The analytical technique used to quantify various components is known as electroanalytical chemistry. It is based on the metrological relationship between electrical quantities, such as current, potential, conductance, and specific quantities of the material to be tested. The method of electrochemical analysis is very selective, highly sensitive, and highly accurate. Voltammetry, electrochemical impedance spectroscopy (EIS), the time-current curve approach, and other commonly used electrochemical analytical techniques are utilized in the field of electrochemical sensors. A popular electrochemical technique is voltammetry. A popular electrochemical technique is voltammetry.

A new electrochemical immunosensor for the detection of m6A was built by Xie et al.[110] First, the m6A-DNA probe was used as a signal molecule to compete with m6A-RNA for antibody binding, based on the fact that anti-m6A-antibodies (anti-m6A-Ab) can recognize both m6A-RNA and m6A-DNA. Next, ribonuclease A (RNase A) hydrolyzes the RNA that binds to the antibody, and lastly, the electrode hydrolysis detects the EIS signal. The amount of m6A-RNA in the sample is inversely correlated with the signal strength; the detection limit was 0.016 nM [110].

4.1.1 Nanomaterial-Based Signal Amplification

Materials of at least one nanometer size in the 1–100 nm range in three dimensions that, as a result of their physical characteristics, have favourable electrochemical properties are referred to as nanomaterials. They can be used for their good electrical conductivity, large specific surface area, and strong catalytic ability, and apply them to improve the performance of electrochemical sensors to achieve the purpose of improving sensor sensitivity [111]. Nanomaterials such as Au nanoparticles (Au NPs), Pt nanoparticles (Pt NPs), graphene oxide (GO), quantum dots (QDs), or metal–organic frameworks (MOFs) are frequently employed in electrochemical sensors to enhance signal strength.

In order to detect miRNA with extreme sensitivity, Dong et al. suggested a proportional electrochemical sensing method based on catalytic hairpin assembly target recovery, which activates dual signal output. In order to implement a ratio dual signalling technique, graphene aerogel (GA) pores were filled with the electrochemical indicator methylene blue (MB), and metal–organic framework (MOF) composites and FeMOFs with different redox potential separation were chosen as the other electrical signal probes. Target miRNAs can initiate the CHA process and introduce the signal probe to the electrode surface, resulting in the production of a large amount of double-stranded H1-H2@FeMOFs-NH2. Finally, measurement and analysis were performed using differential pulse voltammetry (DPV). The findings demonstrated that the concentration of miRNA may be precisely represented by the peak current ratio of IFe–MOFs/IMB [112].

Based on anti-m6A-Ab and PtCo mesoporous nanospheres (MPN), Xie et al. established a highly sensitive electrochemical immunosensor for m6A detection [113]. The m6A-DNA is coupled with PtCo MPN to form an M6A-DNA-PtCo probe, which can be employed as a signal probe to compete with the target m6A-RNA for binding to the antibody on the electrode surface because the anti-m6A antibody can recognize both m6A-RNA and m6A-DNA. PtCo MPN has the ability to catalyze H2O2 to produce electrochemical signals, and then it can conduct DPV scanning to detect m6A with a high degree of sensitivity.

4.1.2 Nucleic Acid-Based Signal Amplification

Due to its benefits of simplicity, affordability, and high sensitivity, nucleic acid signal amplification technology has emerged as one of the most successful signal amplification techniques. Through the integration of biosensing and nucleic acid amplification technologies, researchers have developed a range of highly sensitive and selective biosensors. In electrochemical biosensors, nucleic acid signal amplification techniques including catalytic hairpin assembly (CHA), rolling circle amplification (RCA), and hybridization chain reaction (HCR) are frequently employed.

Long double-stranded DNA (DsDNA) with many repeating units is formed in the HCR system via a sequence of nano-cascade self-assembly amplifications that are continually triggered by the target DNA or RNA. Because of its advantages—simple operation, high assembly specificity, constant temperature reaction, and lack of enzyme involvement—HCR has been frequently used in the design of electrochemical biosensing. Zhao et al. applied Au NPs to the gold electrode's surface in order to enhance the electrical activity and immobilize the capture probe. To increase the reactivity, the TDN probe served as the foundation. The presence of miRNA-122 then initiates the HCR response. Ultimately, a significant quantity of HRP was altered on the electrode surface by the particular combination of biotin and streptavidin, and HRP catalyzed H2O2 to produce an enhanced electrical signal with a lower limit detection of 0.74 aM. They presented an electrochemical biosensor utilizing horseradish peroxidase catalysis (HPEC), tetrahedral DNA nanostructured (TDN) probes, and HCR.

A signal amplification technique called RCA is used to amplify nucleic acids in isothermal environments [114]. The detection signal can be amplified by combining the long-chain DNA generated by RCA with additional signal probes. Nucleic acid lock (NAL) nanostructure was created by Wang et al., and NAL was combined with RCA technology to create an electrochemical biosensor that is extremely sensitive for miRNA-21 detection.

Because of its effective enzyme-free signal amplification qualities, CHA is another isothermal enzyme-free signal amplification technique that has garnered a lot of interest [115]. Initially, two DNA hairpin probes were created for the CHA amplification reaction. These probes are stable and do not react with one another. The hairpin probe can be made to open the hairpin structure when the target is present, hybridize to create a stable double-stranded DNA structure, and simultaneously move the target for the subsequent cycle.

4.1.3 Enzyme-Catalyze Signal Amplification

Because of the benefits of enzyme-catalyzed reactions, including high catalytic efficiency, excellent specificity, and gentle reaction conditions, enzyme-catalyzed signal amplification is one of the most often employed strategies in signal amplification strategy. By attaching the enzyme to the electrode surface and using its catalytic activity to catalyze the matching enzyme substrate, this method produces signal molecules and amplifies the signal. Horseradish peroxidase (HRP), glucose oxidase (GOD), and alkaline phosphatase (ALP) are examples of natural enzymes that are frequently utilized in electrochemistry.

For the very sensitive and precise detection of miRNAs, Guo et al. developed an electrochemical biosensor platform based on the enzyme-catalyzed deposition of DNA conducting molecules (polyaniline) and DNA framework electrochemical background signal conversion technique (etFNA) [47]. Target miRNAs enable base stacking effects between tFNA probes and their partially complementary botanized DNA to build a sandwich structure. Next, PANI deposition was catalyzed by HRP in the presence of aniline and H2O2. Using an electroactive PANI/DNA combination, TFNA efficiently transforms the "background" capture probe into a detectable electrical output.

To achieve ultra-sensitive and precise detection of miRNAs, this signal amplification technique combines the advantages of 3D tFNA in interface regulation and noise signal conversion with the signal augmentation of enzyme catalysis [116]. For the sensitive measurement of miRNA-21, Su et al. developed an electrochemical technology based on tiptoe-mediated DNA substitution and bimetallic nanozyme [51]. Good peroxidase characteristics are exhibited by AuPt nanoparticles (AuPt NPs), which may catalyze H2O2 to generate detectable electrochemical signals. A novel DNA replacement technique mediated by pedicle points was employed to further improve the detection signal. Particular DNA strand sequences were altered on the nano-enzyme and electrode surface, respectively. Continuous cyclic chain replacement is possible in the presence of miRNA-21, which leads to a significant buildup of AuPt NPs on the electrode surface. After catalyzing H2O2, a much stronger electrical signal was produced, with a detection limit of just 84.1 fM [117].

4.2 Signal Amplification Strategies Used in PEC Sensing

Under light irradiation, excited-state photomaterials undergo electron excitation and charge transfer, changing the photocurrent. This process is known as photoelectrochemistry. The foundation of PEC biosensor construction is photoelectrochemistry. PEC (Photoelectrochemical Biosensor) biosensors are inexpensive, simple to miniaturize, and highly sensitive for the detection of compounds related to life, including proteins, nucleic acid molecules, cells, tiny molecules, and ions. They also don't require complicated instruments or equipment. The PEC biosensor is made up of a signal converter, signal output device, photoelectric active material, light source, and object identification unit. PEC biosensors use photoelectric active electrode materials that are excited by a suitable light source.

After redox reactions between the electrode materials and electrolyte produce corresponding photocurrent or photovoltage signals, the electrode materials absorb photons under light irradiation to produce electron excitation and charge transfer, which ultimately realizes the conversion from light energy to electrical energy. The PEC biosensor works on the following detecting principle: when exposed to light, the sensor can induce the associated photoelectric material's photoelectric performance, producing a photocurrent signal. The concentration of the target substance and the

change value of the photoelectric signal have a specific functional relationship that exists within a given range, and it is this relationship that allows for the quantitative detection of the target substance.

4.2.1 Enzyme-Catalyze Signal Amplification:

Due to their high substrate selectivity and catalytic efficiency, enzymes are frequently utilized for PEC biosensor signal amplification, including HRP, GOD, ALP, and others [118]. Numerous artificial enzymes with superior qualities have been created with the swift advancement of nanotechnology and biomimetic materials, such as hemin, metal oxides, and porphyrins [https://pubs.acs.org/doi/10.1021/acs.chemrev.8b00672]. A cascaded amplification PEC sensing approach for the highly sensitive detection of miRNA-21 was described by Li et al. [77]. Using an enzyme cleavage reaction, the DNA probe and target in this sensing technique build G-quadruplex (G4) forming sequences. The released target simultaneously starts the succeeding cycle process, which can produce a huge number of G4-forming sequences [119].

In order to enable the sensitive detection of miRNA-141, Wang et al. synthesized a novel AuPt nanodendrites (NDs) as a nanoenzyme and built a PEC biosensing platform based on the sensitive heterogeneous structure BiVO4/CoPi [78]. When the target RNA is present, the AuPt NDs-labeled DNA probe (pDNA) can be attached to the electrode surface. When 3-amino-9-ethylcarbazole (AEC) is present, the AuPt NDs nanoenzyme can efficiently catalyze its oxidation to create a precipitate on the photoanode in the presence of H2O2. According to the detection results, the constructed sensor's photocurrent sharply declined upon the addition of AuPt NDs tagged DNA probe (pDNA), which ultimately enabled the sensitive detection of miRNA-141 [120].

4.2.2 Nanomaterial-Enhanced Signal Amplification:

Because of its distinct electrical and optical benefits, the application of nanomaterial-enhanced signal amplification in PEC sensing analysis is becoming more and more common. Several types of photoelectric composite materials, including semiconductor–metal composites and semiconductor–carbon composites, have been created in an effort to increase the immobilization of biomolecules and further improve the photoelectric conversion efficiency of photoactive materials. For the ultrasensitive detection of miRNA-141, Li et al. presented a PEC biosensor based on CdTe quantum dot-CeO2 complexes (CdTe QDs-CeO2 complex) and a DNA super-sandwich structure dual signal amplification method [79]. The CdTe QDs-CeO2 complex can drastically increase the sensor's photoelectric conversion efficiency while lowering energy loss. To accomplish the goal of converting a little amount of target RNA into a big amount of DNA, a targeted conversion amplification technique based on DNA Walker is also utilized. Binding proteins help establish a DNA-protein complex that effectively blocks electron transport, which in turn causes a dramatic lowering of the PEC signal output [121].

4.2.3 Nucleic Acid-Based Signal Amplification

Because of its high selectivity and the benefits of cascade amplification, nucleic acid amplification signal amplification technology has emerged as one of the most efficient methods for enhancing the detection of RNA output signals from PEC biosensors.

The PEC sensor has a very high detection sensitivity thanks to RCA, an enzyme-assisted constant temperature nucleic acid amplification technique that can also be used to amplify the target nucleic acid's signal and create long-chain DNA with multiple sets of repeated sequences.

In order to accomplish sensitive and precise detection of miRNA-141, Deng's team designed a photoelectrochemical and electrochemical dual-mode biosensor that can produce remarkably strong PEC signals and clear ECL signals [81]. The RCA reaction is started by first producing a significant amount of DNA through the cleavage cycle of the target's ternary Y structure. The DNA is then immobilized on a TiO2 substrate. Then, the RCA products created multifunctional DNA nanospheres with the help of PDA+ and magnesium ions. As a result of the DNA nanospheres' close proximity to the TiO2 substrate, a PDA+-TiO2 sensitized structure developed that, when combined with ascorbic acid (AA), was able to produce a potent PEC signal [122].

Two hairpin DNAs continually hybridize with one another in an isothermal, enzyme-free environment to generate a long double-stranded DNA. This process is known as strand displacement reaction, which powers HCR.

CHA is a method of DNA-hairpin self-assembly that does not require enzyme involvement. In PEC biosensing, it is a frequently employed nucleic acid signal amplification technique because of its advantages of high sensitivity, great specificity, and simple and flexible operation. For the ultrasensitive detection of miRNA-21, Zhao et al. described a synergistic approach based on nucleic acid signal amplification, enzyme-catalyzed amplification, and chemical signal amplification [84]. Carbon cloth (CC) enhanced with CdS NPs on polyimide (PI) film is used in this PEC biosensor as a photoelectric material to function as a photoelectrode. The nucleic acid signal amplification processes (CHA and HCR) are initiated in the presence of the target miRNA-21, resulting in the production of lengthy dsDNA tagged with biotin.

The ultra-sensitive detection of miRNA is then achieved by the enzyme-catalyzed process, in which ALP bound to the DNA strand catalyzes the synthesis of ascorbic acid (AA), which ultimately produces a strong photoelectric signal.

4.3 Signal Amplification Strategies Used in Fluorescence sensing

Fluorescence biosensors are a type of optical biosensor that can analyze biological samples both qualitatively and quantitatively. They work by using biomolecules as recognition elements, fluorescence signal changes brought on by fluorescence resonance energy transfer or electron transfer as detection signals. Fluorescent biosensors have a higher selectivity than traditional biosensors and also combine the benefits of fluorescence analysis technology, including low cost, fast reaction times, simple equipment, and high analytical sensitivity. These biosensors have a wide range of applications, including food testing, environmental monitoring, clinical disease diagnosis, and more [123]. Reducing the reaction system's background signal and increasing the reaction system's signal output through the use of nucleic acid molecular signal amplification technology are the two common strategies for increasing the sensitivity of fluorescent biosensing platforms.

4.3.1 Enzyme-Based Nucleic Acid Signal Amplification:

DNA polymerase, endonuclease, and exonuclease are often utilized enzymes in enzyme-assisted nucleic acid signal amplification technique. These days, strand displacement amplification (SDA), polymerase chain reaction (PCR), the RCA reaction [124], and others are frequently utilized amplification techniques. Guo's team revealed a fluorescent biosensor that uses a self-sufficient fuel amplification method (SFAS) to detect miRNA-21 with great sensitivity and without the need for a label. This sensing method initially establishes a series connection between two SDA processes to enable cascaded amplification of single-stranded DNA. DNA polymerase can cause single-stranded DNA to partially hybridize with the hairpin template and stretch along the template, destroying the hairpin shape. Since AgNCs are altered on the hairpin template and their fluorescence is dependent on the wrapping of the hairpin structure, the breakdown of the hairpin structure causes AgNCs' fluorescence to be quenched, which in turn weakens the fluorescence of miRNA-21 [125].

Based onPstIendonuclease cleavage (PEC) and RCA, Lee et al. suggested a fluorescence resonance energy transfer (FRET) approach for the very sensitive detection of miRNA-21. Long hairpin DNA (HD) structures were formed by the self-assembly of the produced RCA products, which were particularly triggered by the miRNA-21. The PstI endonuclease may uniquely detect and cleave the structure when the PstI enzyme is present. This will produce a lot of small single-stranded DNA fragments, which open the stem-loop and diminish FRET efficiency by complementarily hybridizing with HD probes tagged with FAM on one end and BHQ-1 on the other. For miRNA-21, the detection limit was 39.7 aM (attomoles) [126].

4.3.2 Enzyme-Free Nucleic Acid Signal Amplification:

One benefit of enzyme-free nucleic acid signal amplification is that it's easy to use, which makes it a good option for creating innovative fluorescence biosensors with a straightforward layout and excellent sensitivity. Non-enzyme-assisted signal amplification techniques that are frequently employed are the CHA reaction, the HCR reaction [127] etc. A straightforward miRNAs detection and imaging method based on Mo2B nanosheets (NSs) and hybrid chain reaction (HCR) was presented by Zada et al. In addition to offering a sizable surface area loading for ssDNA loops and hairpin probes (HPs), Mo2B NSs also exhibit superior multifluorescent dye quenching capabilities for ultra-low background signals. Following transfection, HPs identify particular target miRNAs and cause HCR to form a significant amount of DNA-miRNA double helices. These helices separate from the Mo2B NS surface and produce powerful fluorescent signals that aid in the detection of miRNAs. Ultimately, it was possible to identify and image miRNAs in various cells [128].

The CHA reaction's triggering mechanism is comparable to the HCR reaction's, with the exception that in the former, the trigger chain is not released after it has been joined with the hairpin probe, but in the latter, the trigger probe can be released to take part in the subsequent cycle. Li et al. created a highly selective detection system (GO-CHA-HCR) based on the interaction of graphene oxide (GO), CHA, and HCR for the imaging of circRNA and CIRC-Foxo3 in living cells. Four specially made hairpin probes are included with the system; one of them has a fluorescent group labeled on it. Adsorbed on the GO nanosheet surface, these cause the fluorescence to be quenched by GO, which serves as a quencher.

5. MOLECULARLY IMPRINTED POLYMERS (MIP) AND NON-IMPRINTED POLYMERS (NIP)

5.1 Molecularly imprinted polymers (MIP)

Molecular imprinting is the process of template-induced formation of specific recognition sites in a polymer. Synthetic receptors prepared using molecular imprinting possess a unique combination of properties such as robustness, high affinity, specificity, and low-cost production, which makes them attractive alternatives to natural receptors. Improvements in polymer science and nanotechnology have contributed to enhanced performance of molecularly imprinted polymer (MIP) sensor . The pharmaceutical and biomedical sectors have been faced with recent challenges that necessitate the creation of novel analytical techniques [129].

Molecularly imprinted polymers (MIPs) have become the subject of pharmaceutical research due to their favorable properties and wide range of applications in biomedicine. The versatility of MIPs makes them useful as diagnostic or imaging tools, drug delivery systems (DDS) or biosensing elements [130]. MIPs, also known as artificial antibodies, have drawn the attention of the research community due to their numerous advantages, such as selective binding of target molecules, increased drug loading capacities, biocompatibility and biodegradability, reduced toxicity, low-cost synthesis, and various routes of administration.

Microscopy techniques, such as scanning electron microscopy (SEM) to photograph polymer macropores and light microscopy to confirm the inherent integrity of polymer beads, can be used to adequately explore the morphological properties of MIPs. Furthermore, the primary method for determining the specific surface area, specific pore volume, pore size distribution, and average pore diameter values of the polymer particles is nitrogen sorption porosimetry using BET (Brunauer, Emmett, and Teller) analysis, whereas mercury intrusion porosimetry is occasionally employed and is better suited for characterizing larger pores.

In the fabrication process of MIPs, a certain template molecule interacts with one or multiple functional monomers via covalent or non-covalent bonds, followed by polymerization in the presence of a cross-linking agent. Upon template removal, specific binding cavities are left behind that are complementary to the molecule in terms of shape, size, and functionality [130]. MIPs have become popular for drug delivery due to their versatility in terms of administration routes, high loading capacity, and (stereo)selectivity for the desired target.

Selectivity for a different molecule but structurally related to the template appears to be an even better MIP evaluation than imprinting factor because the presence of the template during polymerization synthesis frequently results in general changes in the polymer's morphology, such as porosity and surface area, which are confirmed by binding differences compared with the polymer control and may not be solely attributable to the presence of imprinted sites. However, the imprinting factor must be taken into account in addition to selectivity results because a compound's ability to be retained longer than another may simply be a function of its unique physicochemical characteristics rather than particular imprinted sites.

Nonetheless, a crucial aspect of MIP characterization pertains to their molecular recognition behavior, specifically their binding capacity. To assess binding capacity and selectivity, batch rebinding is a highly effective technique.

Their superior drug loading, compared to conventional non-imprinted polymeric systems, leads to lower dosages and, thus, to a reduced susceptibility to adverse reactions and better safety profiles [130]. Despite their low immunogenic characteristics, MIPs biocompatibility is still a controversial

subject. It is generally accepted that MIPs offer excellent biocompatibility, however, their long-term effects on living organisms have not been thoroughly investigated yet [130].

5.2 Non-imprinted polymers (NIP)

Non-imprinted polymers (NIPs), also known as blank polymers, are synthetic polymers that are similar in composition to molecularly imprinted polymers (MIPs) but lack the selective binding sites for specific target molecules. Unlike MIPs, NIPs are prepared through the same polymerization process but without the presence of a template molecule. As a result, NIPs do not possess the specific recognition capability exhibited by MIPs [131,132].

While MIPs are designed to exhibit selective binding towards target molecules, NIPs serve as control materials in drug delivery systems and electrochemical studies. NIPs are used as a comparison or reference group to evaluate the specific binding and release properties of MIPs.

By comparing the performance of MIPs and NIPs, the selective binding and release capabilities of MIPs can be better understood and assessed. The main difference between MIPs and NIPs is related to differences in the magnitude of the binding affinity rather than to the presence or absence of specific recognition sites [131]. In drug delivery systems, NIPs can be used as control carriers or matrices to evaluate the impact of specific binding sites provided by MIPs. The drug loading and release behavior of NIPs can be compared to that of MIPs to determine the contribution of the molecular recognition sites in achieving controlled and targeted drug release. This comparative analysis helps validate the effectiveness of MIP-based drug delivery systems and provides insights into the role of selective binding in enhancing therapeutic outcomes.

NIPs have been tested as a replacement for conventional techniques such powdered activated carbon in the treatment of water and wastewater in order to remove heavy metals and micropollutants [133]. Because NIPs are non-selective, they can adsorb a variety of pollutants, which makes them useful for handling complicated mixtures. Research has contrasted the efficacy of NIPs with materials such as wastewater and humic acid in terms of their ability to eliminate pollutants [134].

The potential of NIPs to adsorb contaminants from soil and groundwater in environmental remediation applications has also been studied. Their adaptability to many environmental cleanup settings stems from their ability to capture a wide range of contaminants without requiring specialized binding sites [133]

NIPs are being used in medicine delivery systems in addition to environmental remediation and water purification. NIPs can release the active ingredients in a regulated way by adding medications into the polymer matrix. NIPs' non-specific binding capabilities make them a viable platform for targeted dr delivery since they enable the encapsulation and release of a variety of medications [135].

NIPs have many benefits, but there are also issues that must be resolved. The possible toxicity of NIPs and their effects on the environment and human health are important factors to take into account. To guarantee NIPs are used safely and effectively in water treatment processes, more study is required to determine the best ways to incorporate them into treatment plants.

6. CYCLIC VOLTAMMETRY

A strong and popular electrochemical method for examining the reduction and oxidation processes of molecular species is cyclic voltammetry (CV). The study of chemical reactions involving electron transfer, such as catalysis, is greatly aided by CV [136].

An electrolysis cell, a potentiostat, a current-to-voltage converter, and a data gathering system make up a CV system. A working electrode, counter electrode, reference electrode, and electrolytic solution make up the electrolysis cell. The reference electrode keeps its potential constant, but the working electrode's potential varies linearly with time. Electricity is transferred from the signal source to the working electrode via the counter electrode. Ions are supplied to the electrodes during oxidation and reduction by the electrolytic solution. A potentiostat is an electrical device that permits tiny currents to be taken into the system without altering the voltage by using a DC power source to create a potential that can be maintained and precisely measured. The resultant current is measured by the current-to-voltage converter, and the voltammogram is generated by the data acquisition system.

When studying qualitative data regarding electrochemical processes under different circumstances, such as the existence of intermediates in oxidation-reduction reactions or the reversibility of a reaction, cyclic voltammetry can be employed. The formal reduction potential, which can be used as an identification tool, the diffusion coefficient of an analyte, and the electron stoichiometry of a system can all be found using CV. Furthermore, by creating a calibration curve of current vs. concentration, one can ascertain the concentration of an unknown solution since in a reversible, Nernstian system, concentration is proportionate to current. Measuring the current at the working electrode while doing potential scans yields a cyclic voltammogram. Figure 6. demonstrates the cyclic voltammogram that is produced when one electron is reduced and then oxidized. For example, the reversible reaction that follows:

$$M^{+} + e^{-} \rightleftharpoons M$$

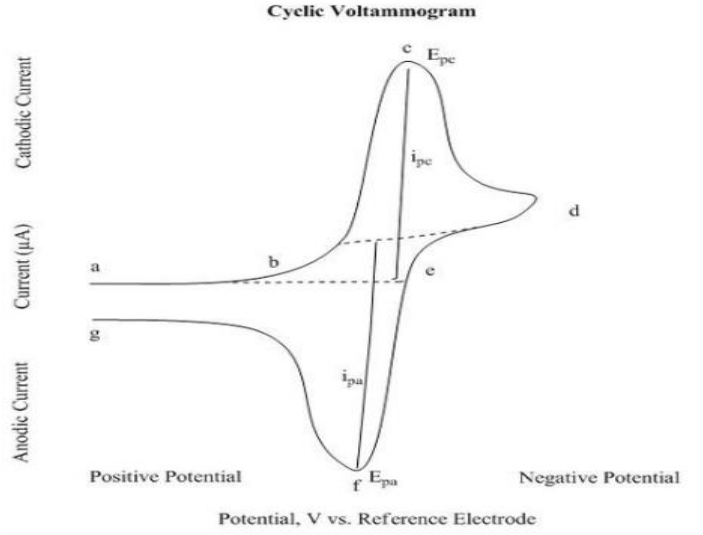


Figure 7. From (a) the starting potential to (d) the switching potential, the reduction process takes place. The potential is negatively scanned in this area, leading to a decline. The term cathodic current (ipc) refers to the resultant current. The cathodic peak potential (E_{pc}), which is the equivalent peak potential, is located at (c). When all of the substrate at the electrode's surface has been reduced, the Epc is reached. The potential scans favourably from (d) to (g) after reaching the switching potential (d). Anodic current (i_{pa}) and oxidation are the outcomes of this. The anodic peak potential (E_{pa}), which

is the peak potential at (f), is attained when all of the substrate at the electrode's surface has been oxidized [137].

The electrochemical event of interest is carried out by the working electrode. The applied potential of the working electrode is regulated by a potentiostat in relation to the potential of the reference electrode. The fact that the working electrode is made of redox-inert material within the potential range of interest is its most crucial feature. From experiment to experiment, the type of working electrode can be changed to offer various potential windows or to lessen or increase the species of interest's surface adsorption. The working electrode surface must be incredibly clean and have a well-defined surface area because here is where the electrochemical event of interest takes place. To get rid of any adsorbed species that were left over from the polishing process, it is frequently also required to carry out multiple CV scans in simple electrolyte over a large potential window. Until no peaks are seen and the scans overlap, this can be done again.

A reference electrode's equilibrium potential is steady and well-defined. In an electrochemical cell, it serves as a benchmark for measuring the potential of other electrodes. For this reason, the applied potential is usually expressed as "vs" a particular reference. Standard hydrogen electrode (SHE), AgCl/Ag electrode, and saturated calomel electrode (SCE) are a few examples of reference electrodes that are frequently employed in aqueous media. Usually, a porous frit keeps these reference electrodes apart from the fluid. By using the same solvent and electrolyte in the reference compartment as in the experiment, junction potentials can be reduced as much as possible.

Current flows when the working electrode is subjected to a voltage that allows the analyte to be reduced or oxidized. The counter electrode's function is to finish the electrical circuit. As electrons move between the WE and CE, current is measured. The surface area of the counter electrode is larger than the surface area of the working electrode in order to guarantee that the kinetics of the reaction occurring there do not impede those occurring at the working electrode.Depending on the experiment, counter electrodes may occasionally be separated from the rest of the system by a fritted compartment since they can produce byproducts.

A potential forms at the electrodes once the analyte has been introduced and the cell has been formed. For instance, the potential of the electrode equilibrates with the solution (such that no current flows) when Fc+ is introduced to a cell. The open circuit potential (OCP) is the potential that is measured when there is no current flowing. When a solution contains a mixture, the OCP can provide information about the concentration of several species as well as the redox state of the species in the bulk solution.

7. ELECTROCHEMICAL IMPEDANCE SPECTROSCOPY (EIS)

The concentration of electroactive species, charge and mass transfers from the bulk solution to the electrode surface, and electrolyte resistance are examples of matter–(redox species)–electrode interactions in a standard electrochemical cell. An electrical circuit made up of resistances, capacitors, or constant phase components connected in series or parallel to create an equivalent circuit characterizes each of these qualities. Therefore, mass-transfer, charge-transfer, and diffusion processes could all be investigated using the EIS. Thus, the EIS can investigate inherent material characteristics or particular mechanisms that may affect an electrochemical system's conductance, resistance, or capacitance.

Since resistance seen in DC circuits directly obeys Ohm's Law, impedance is different from resistance. The impedance response is measured using a little signal stimulation. When a current is delivered at a frequency that produces a sinusoid response to a sinusoidal potential, the electrochemical cell response is pseudo-linear, resulting in the acquisition of a phase-shift. As a result, Equation presents the excitation signal as a function of time:

$$E_t = E_0 \cdot \sin(\omega t) \qquad (3)$$

where E_0 is the signal's amplitude, ω is its radial frequency, and E_t is the potential at time t.

There are two parts to the impedance expression: the real and imaginary parts. A "Nyquist Plot" is created when the imaginary part (Zimag) is plotted on the Y-axis and the real part (Zreal) is plotted on the X-axis. The Zimag is negative, and each point on the Nyquist plot represents an impedance value at a frequency point. Low-frequency impedance is conducted at the right side of the plot along the X-axis, whereas high frequency impedance is formed at the left side. Furthermore, impedance can be shown as a vector (arrow) of length |Z| on a Nyquist plot. The "phase angle" is the angle formed by this arrow and the X-axis. An alternative method of expressing the impedance data is the Bode plot, which is more popular in the engineering community than the Nyquist plot. The Bode plot consists of two distinct logarithmic plots: phase vs. frequency and magnitude vs. frequency. In practical terms, impedance is determined by recording the current wave that results from applying a potential wave to the working electrode. Z, Φ, Zreal, and Zimag are extracted and sketched from these two waves. By measuring these properties for hypothetical waves with various frequency, the spectrum is produced.

The total resistance to the flow of current in an electrical circuit made up of resistors (R), capacitors (C), and inductors (L) is known as impedance. The impedance of the electrical circuit under test will vary depending on the various passive elements (R, C, and L) present in the circuit and how they are connected to one another.

EIS comes in two forms: non-Faradaic and Faradaic. Redox processes in the former result in impedance, whereas double layer capacitance in the latter produces its electrical properties and is a DC-based impedance. The Faradaic current is a measure of electron transfer over electrode surfaces and is used in quantitative analysis. A Bode plot is created when the frequency is plotted against phase angle.. Generally speaking, resistive processes are analyzed using Nyquist charts, whereas capacitive systems are evaluated using Bode plots [138].

The total impedance of the entire circuit that is subject to analysis is the sum of the impedances of its constituents. The total of each element's impedance is then used to calculate the overall impedance of a circuit with many components using Ohm's law:

$$Z_{total} = Z_1 + Z_2 + Z_3 + ... + Z_x \quad (4)$$

Conversely, redox species or molecules diffusing can produce a second resistance called the Warburg impedance (W). The frequency depends on this impedance. Consequently, the Warburg impedance is low at high frequencies because the diffusing reactants do not need to go very far. The redox molecules have the ability to diffuse at low frequencies, which raises the Warburg resistance. The infinite Warburg impedance appears as a 45° slanted line on the Nyquist plot. Conversely, a 45° phase shift related to the Warburg effect is shown on the Bode plot.

A biosensor is made up of a recognition element that recognizes one or more molecular components in the sample under study. Subsequently, a variety of transducers (colorimetric, optical, electrochemical, or mass change) are used to detect the recognition event. These transducers gather particular signals that are then processed and magnified in order to analyze the data. Biosensors have become indispensable diagnostic instruments because of their tiny sample requirements, good selectivity, repeatability, quick detection, and high sensitivity. Remember that specific sensor configurations and sensing algorithms must be established for each target (analyte).

Analyzing EIS data typically involves fitting them to a comparable electrical circuit model. Common electrical components including resistors, capacitors, and inductors make up the majority of the circuit elements in the model. The model's components must be grounded in the system's physical electrochemistry in order to be effective.

One equivalent circuit element can be used to model very few electrochemical cells. Rather, EIS models typically comprise several components within a network, like both parallel and serial element combination

In EIS-immunosensors, the kinetic interaction of antibodies and their antigens at the sensor surface produces a variation in the electrical signal [138]. Electron transfer/charge transfer resistance, which indicates the number of bound molecules, is thus generated. Therefore, the EIS biosensors allow for the direct determination of biomolecular recognition actions as a label-free detection. Because of its easy manipulation, quick reaction, ability to be miniaturized, and readiness for low-cost lab-on-a-chip integration with online measurement to detect extremely low concentrations, EIS biosensors have become much more common.

Additionally, EIS has been used to investigate the processes and kinetics of interactions between the enzyme and the substrate in enzyme-based biosensors [139]. Enhancing the efficacy of enzyme-based biosensors requires an understanding of the catalytic activity, inhibition, and stability of the immobilized enzymes, which can be obtained by monitoring the changes in the electrical characteristics of the sensor surface during enzymatic processess.

EIS biosensors have been developed using nanomaterials, including metals, metal oxides, carbon, nanowires, nanocomposite, nanopores, nanochannel arrays, and nanogap species. By using these nanomaterials, analytical properties were improved in a number of ways, such as by increasing the surface area of the sensor, enhancing its sensitivity and selectivity, intensifying its electrochemical signals, and quickening its reaction time [138].

8. METHODOLOGY

8.1 Materials

Protienase K, 1mL, >600 U/mL (20 mg/mL) from Thermo Fisher Scientific, T7 DNA Polymerase (10 U/mL, EP0081), 10X phosphate buffered saline (PBS) (pH 7.4) (AM9625), 0.05 M/L Polypyrrole, 98% purity, Nuclease free water, Gold electrode, Metrohm DropSens potentiostat, Gamry Reference 600 potenstiostat.

8.2 Experimental 1 – Immobilisation of Proteinase K

Proteinase K was immobilized on a gold electrode as part of the study's approach to assess its electrochemical characteristics. Initially, the buffer solution used for the entire experiment was 10X phosphate-buffered saline (PBS). The 98% pure pyrrole was dissolved in PBS to create a 0.05 M/L polypyrrole (Ppy) solution. To make sure the gold electrode was well cleaned, it was initially put through cyclic voltammetry (CV) with just PBS present. To get rid of any contaminants and prepare the electrode surface for later changes, this first cleaning procedure was essential.

After the gold electrode was cleaned, a Ppy film was deposited it. The homogeneous deposition of the Ppy layer onto the electrode surface was done by doing 10 cycles of cyclic voltammetry at +0.5 V to +1.0 V potential. Setting up the electrode with the Ppy film was a crucial step in creating an environment that was favorable for the immobilization of the enzyme. Cleaning the electrode is crucial because it guarantees that any remaining impurities—such as organic or inorganic substances—that could obstruct the electrochemical processes are eliminated. The uniform and active region that a clean electrode surface offers for the deposition of polypyrrole and the ensuing immobilization of the enzyme improves the repeatability and dependability of the experimental outcomes [140].

After the Ppy film was successfully deposited, 30 µL of Proteinase K enzyme, PBS, and Ppy were combined to create a combination. After that, this mixture underwent ten further cycles of cyclic voltammetry. This procedure was done to make sure the enzyme was properly integrated into the Ppy matrix on the gold electrode. The electrode containing the enzyme combination was incubated for two hours after the CV runs. Electrochemical measurements were made every 30 minutes, 1 hour, and 2 hours throughout this incubation time to track the enzyme's immobilization and stability on the electrode surface.

The electrode was tested once more with PBS alone after the 2-hour incubation period, and then with PBS that contained the enzyme. To compare the data sets and assess any alterations in the electrode's electrochemical characteristics brought about by the enzyme's presence, these experiments were carried out independently. The usefulness of the immobilization procedure and the functional stability of the enzyme on the electrode were both revealed by this comparative investigation. By methodically preparing and functionalizing the gold electrode with Proteinase K, this methodological approach made it possible to evaluate the electrochemical behaviour of the electrode under controlled circumstances. To guarantee the accuracy and repeatability of the findings, every step was carried out with great care, offering a solid foundation for comprehending the immobilization of the enzyme on a conductive surface.

8.3 Experimental 3 - Immobilisation of Proteinase K with EIS system with Non-Imprinted Polymers (NIP)

Materials:

Protienase K, 1mL, >600 U/mL (20 mg/mL) from Thermo Fisher Scientific, 10X phosphate buffered saline (PBS) (pH 7.4) (AM9625) from Thermo Fisher Scietific, 0.05 M/L Polypyrrole, 98% purity, Gold electrode, Gamry potentiostat.

Procedure:

The system was first prepared by cleaning gold (Au) electrode with 2 steps. 1) Manual cleaning with sand paper and polishing for a smooth mirror like surface 2) Electrochemical cleaning with CV in 10X PBS (pH 7.4) at a scan rate of 100mV per second for 5 cycles, between -0.2 V to +1.2 V. This was done to ensure the electrode was rid of organic and inorganic components that might interrupt or produce unreliable results. The potentiostat was used for this purpose with CV through computer command. After running and cleaning with CV, EIS was done in 10X PBS in the frequency range of 20kHz to 1Hz with 0 V potential. The impedance was recorded with this system for comparison after the enzyme immobilization system.
Formation of Non-imprinted polymer (NIP) was the next step, which was done by using the solution of 0.05 M/L Pyrrole for forming a flat surface of PPY deposition on the Gold electrode with electrochemical deposition with CV at a scan rate of 100mV per second for 10 cycles, between -0.2 V to +0.9 V. Prior to CV, EIS measurement was taken with the frequency range of 20kHz to 1Hz and 0 V potential. After CV, PPY layer was formed on the Au electrode, EIS was again performed with the same frequency and potential mentioned above.
After NIP was formed the solution was changed to pure PBS and EIS was once again measured. Solution with PBS and Proteinase K with 20ml 10X PBS and 30 µL was made with final concentration calculated to be 0.04 M/L. The Gold electrode was immersed in this solution and measured with EIS system with frequency range of 20kHz to 1Hz and 0 V potential to detect changes in impedance from the surface. The results are described in the next chapter.

8.4 Experimental 4 - Immobilisation of Proteinase K with EIS system with Molecularly Imprinted Polymers (MIP)

Materials:

Protienase K, 1mL, >600 U/mL (20 mg/mL) from Thermo Fisher Scientific, 10X phosphate buffered saline (PBS) (pH 7.4) (AM9625) from Thermo Fisher Scietific, 0.05 M/L Polypyrrole, 98% purity, Gold electrode, Gamry potentiostat

Procedure:

The Gold electrode was once again cleaned manually with sand paper and polishing; then electrochemically cleaned with CV in 10X PBS (pH 7.4) at a scan rate of 100mV per second for 5

cycles, between -0.2 V to +1.5 V. Once again EIS system was used after cleaning with CV system, to ensure the surface was cleaned and for comparison after MIP formation.

Formation of Non-imprinted polymer (NIP) was the next step, which was done by using the solution of 0.05 M/L Pyrrole for forming a flat surface of PPY deposition on the Gold electrode with electrochemical deposition with CV at a scan rate of 100mV per second for 10 cycles, between -0.2 V to +0.9 V. Prior to CV, EIS measurement was taken with the frequency range of 20kHz to 1Hz and 0 V potential. After CV, PPY layer was formed on the Au electrode, EIS was again performed with the same frequency and potential mentioned above.

In the subsequent steps, MIP was formed with 0.05 m/l Pyrrole (98% purity) and Proteinase K was added together in this step contrary to NIP formation, to from cavities in the surface of Gold electrode for enzymes to lock into. The clean Au electrode was first measured in EIS system. After EIS with frequency range of 20kHz to 1Hz and 0 V potential, the electrode was run in CV at a scan rate of 100mV per second for 10 cycles, between -0.2 V to +0.9 V. In this step, MIP was formed on the gold electrode with PPY deposition and enzyme deposition. This was measured with EIS system once again with frequency range of 20kHz to 1Hz and 0 V potential.

Solution was changed to pure PBS and EIS system was measured on the electrode with MIP formation on Gold electrode, then, the solution was changed to PBS and enzyme (30 µL) and measured with EIS at 0mins, 5mins, 30mins and 1 hour respectively and measurements were recorded.

9. RESULTS

9.1 Experimental 1 – Immobilisation of Proteinase K

Proteinase K was to be immobilized on a gold electrode in this investigation, and several important discoveries were made throughout the course of the experiment. First, using cyclic voltammetry (CV) in a 10X phosphate-buffered saline (PBS) solution, the gold electrode was carefully cleaned. This cleaning process ensured a spotless surface for the changes that followed by successfully eliminating any impurities. After cleaning, ten cycles of CV were used to deposit a polypyrrole (Ppy) layer onto the gold electrode within the potential range of +0.5 V to +1.0 V. Pyrrole polymerization equation is as follows:

$$nC_4H_5N \rightarrow (C_4H_3N)\,n + 2nH + 2ne$$

This process was essential for generating a homogenous Ppy layer that provided Proteinase K with an ideal immobilization environment. The constant electrochemical signals seen during the CV cycles verified that the generated Ppy layer had a homogeneous distribution on the electrode surface. The results before and during PPY deposition, during and after enzyme immobilization were measured using Metrohm DropSens potentiostat with Cyclic Voltammetry and results and observations are presented in graphs below.

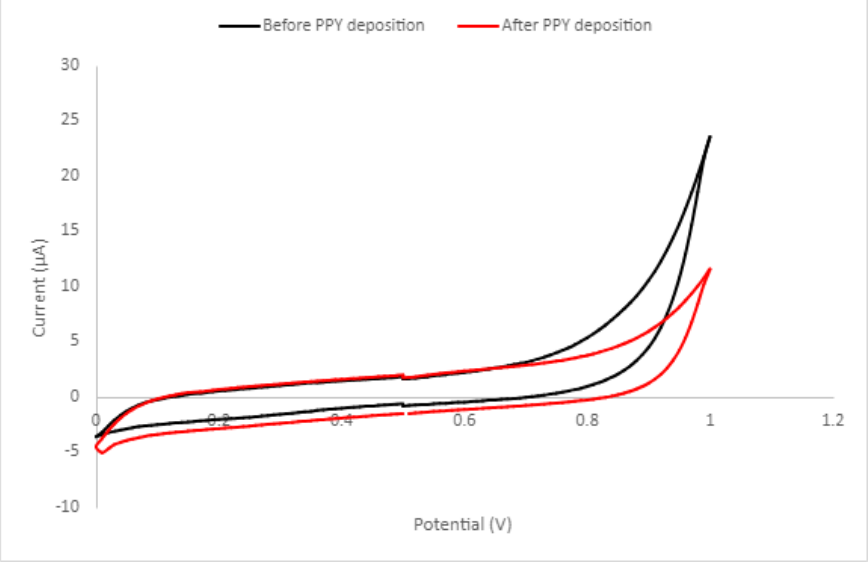


Figure 8. The figure shows the CV (Cyclic Voltammetry) measurements of the 0.05 M/L Pyrrole, in 10 cycles of cyclic voltammetry at +0.5 V to +1.0 V potential, which was used for PPY film deposition (in black)) and measurement after the deposition of PPY film on the electrode with 10X PBS (in red). From 10 cycles, the second cycle scan of each of the different solutions has been shown here for clear data representation. The measurement was the Current (µm) change against Potential (V). A decrease in current (µA) can be observed after the Ppy deposition, which shows the decreased transfer of electrons due to the deposited layer of Ppy. The Ppy layer was also seen visually after deposition, showing a thin layer of brown deposits on the electrode surface.

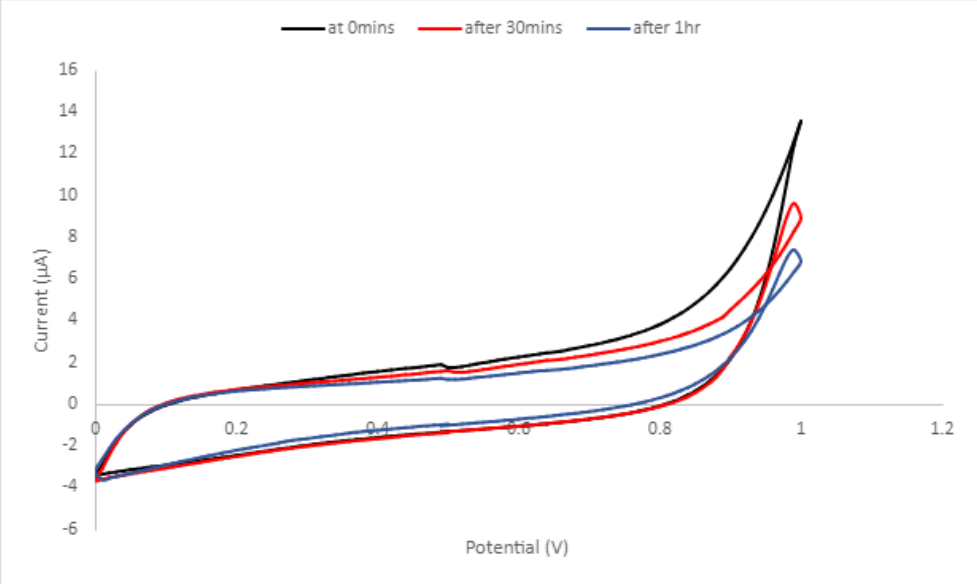


Figure 9. The figure shows the CV (Cyclic Voltammetry) measurements of 0.05 M/L of Pyrrolein 30 µl of Proteinase K enzyme after 10 cycles of Cyclic Voltammetry at +0.5 V to +1.0 V potential at 0mins (in blue), 30mins (in red) and at 1 hour (in yellow) respectively.

The measurement was the Current (µA) change against Potential (V). Decrease on current (µA) can be observed as time increases, which might be due to few of speculated reasons. **1)** the current might be decreasing due to the lack of electron transfer as enzymes blocks the surface. **2)** Polymer Insulation: Polypyrrole is a conductive polymer, although the degree of polymerization and the deposition circumstances can affect how conductive it is. The polypyrrole coating may grow more insulating over time or as a result of external factors, which would lower the current. During repeated CV cycles, the polypyrrole film may deteriorate or experience structural changes that impact its conductivity and consequently the current [141]. **4)** Biofouling: The electrode surface may become coated with proteins or other biomolecules from the enzyme solution, causing fouling. Current may drop as a result of this biofouling layer obstructing electron transport between the electrode and the solution. Polypyrrole fouling: Biomolecules or other contaminants may be trapped by the polypyrrole itself, decreasing its effective conductivity and raising resistance. [142] **5)** Electrochemical Deactivation Enzyme Inactivation: Over time, denaturation or other deactivation processes may cause kinase K to lose its activity, which would reduce its catalytic contribution to the current. Electrochemical Side Reactions: An accidental reaction during a CV measurement could change the enzyme or the electrode surface, which would change the total current.

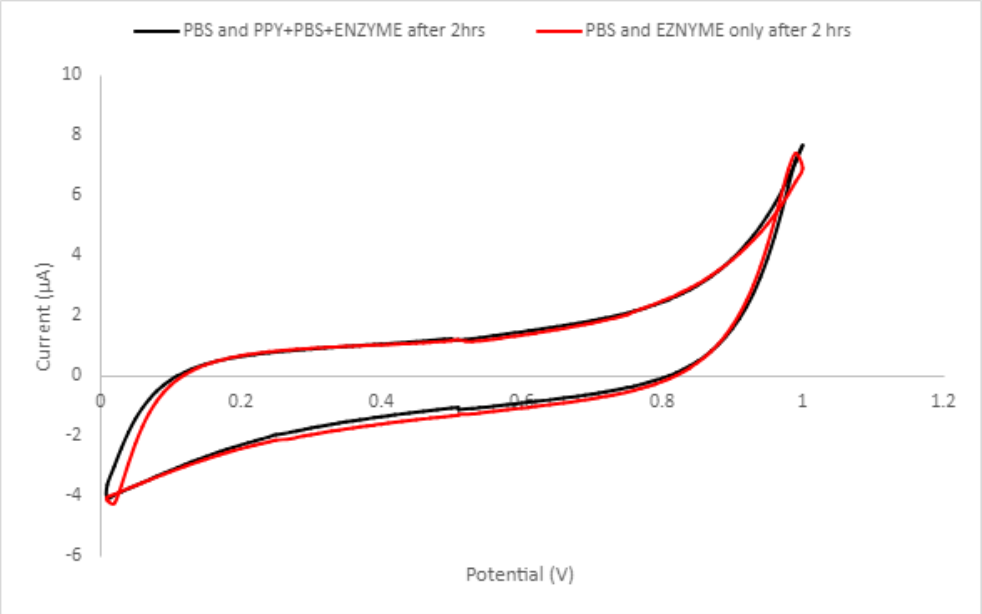


Figure 10. The figure shows the CV (Cyclic Voltammetry) measurements 0.05M/L Pyrrole and 30µl Proteinase K enzyme in 20ml 10X PBS after 10 cycles; 2 hours of incubation (in blue) and measurements 0.05M/L Pyrrole and 30µl Proteinase K enzyme in PBS and 30 µl enzyme mixture. The measurement was the Current (µA) change against Potential (V). Both measurements are almost similar with no specific shifts in signal and no major change in area was observed.

This experiment focused on using only CV measurements for immobilization of Proteinase K, however from the obtained data, the effectiveness of the immobilization and path of immobilization can only be speculated based on the data obtained. The following experiments was conducted to make sure that the proteinase K was immobilized for biosensing system and which system would be much more compatible if the enzyme is successfully immobilized.

9.2 Experimental 3- Immobilisation of Proteinase K with EIS system with Non-Imprinted Polymers (NIP)

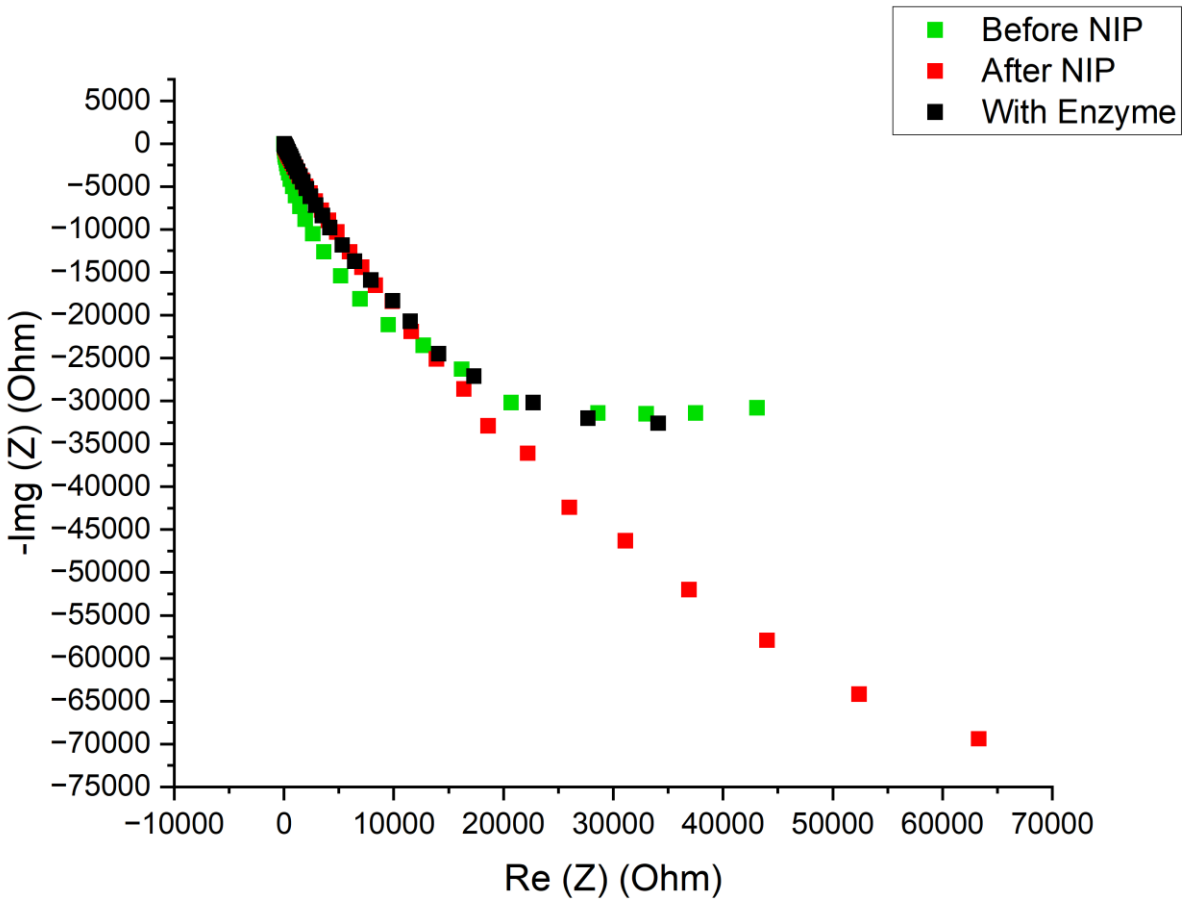


Figure 12. The figure shows Nyquist plot of the EIS measurements of gold electrode before NIP formation, after NIP formation and NIP with enzyme. Green plots: The solutions used before NIP were 20ml 0.05M/L Pyrrole and clean Au surface. Red plots: After NIP the same solution of 0.05M/L Pyrrole but with polymerized Pyrrole ie., PPY surface. Black plots: The solution used was 20ml 10X PBS and 30µl Proteinase K in which NIP formed on Au surface was measured in EIS. The dotted lines indicate the imaginary points (Z''/Ohm) against real points (Z'/Ohm).

9.3 Experimental 4- Immobilisation of Proteinase K with EIS system with Molecularly Imprinted Polymers (MIP)

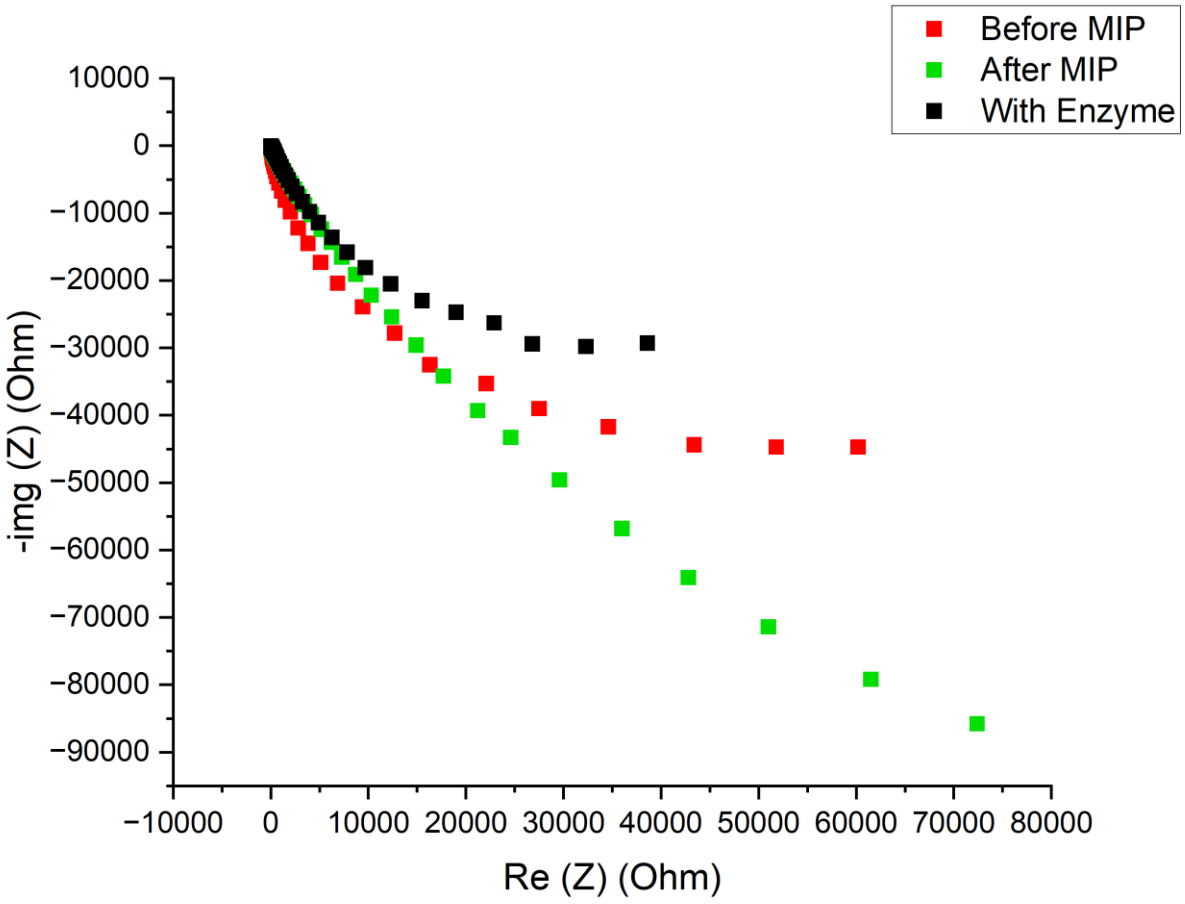


Figure 13. The figure shows Nyquist plot of the EIS measurements of gold electrode before MIP formation, after MIP formation and MIP with enzyme. Green plots: The solutions used before MIP were 20ml 0.05M/L Pyrrole and 30µl enzyme with clean Au surface. Red plots: After MIP the same solution of 20ml 0.05M/L Pyrrole and 30µl enzyme were used but with polymerized Pyrrole ie., PPY surface. Black plots: The solution used was 20ml 10X PBS and 30µl Proteinase K in which MIP formed on Au surface and measured with EIS. The dotted lines indicate the imaginary points (Z''/Ohm) against real points (Z'/Ohm).

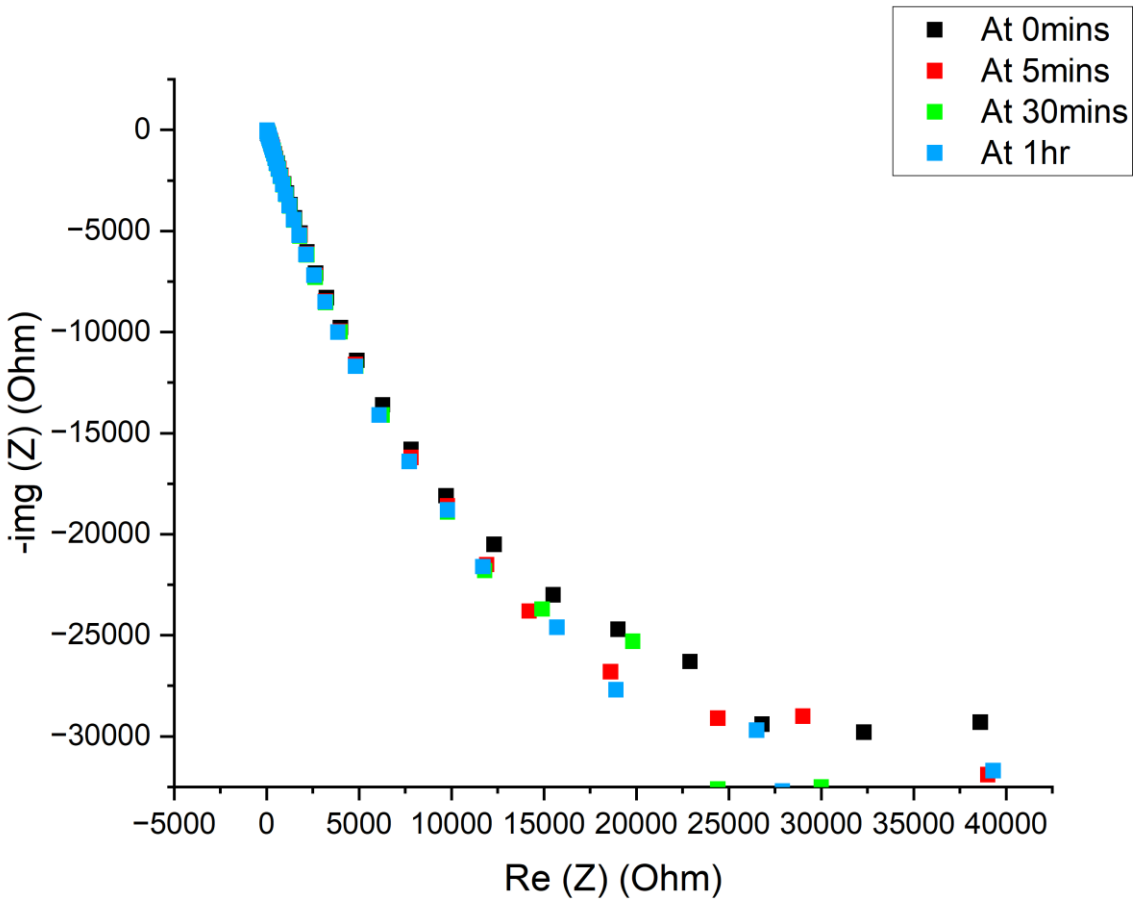
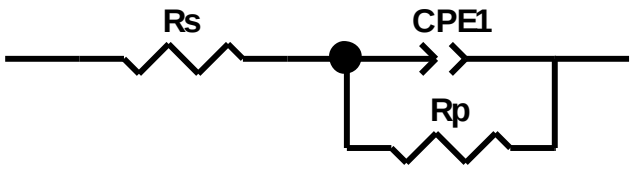


Figure 14. The figure shows Nyquist plot of the EIS measurements of gold electrode in MIP surface after forming cavities with MIP (during CV in solution of 20 ml 0.05M/L Pyrrole and 30 µl enzyme) and in the solution of 20ml 10X PBS and 30µl enzyme. The plots at 0mins, 5mins, 30mins and 1hr are plotted respectively. The dotted lines indicate the imaginary points (Z''/Ohm) against real points (Z'/Ohm).

The fitting curves were plotted using Zview 4 software and Nyquist plot was plotted using OriginPro 2024b. The fitting model for all the EIS measurements was the following model:



with R_s being Ohmic drop (RΩ), CPE1 being Double layer capacitance (Cdl) and Rp being Charge transfer resistance (Rct).

Table 3. Fitting values of different electrode surfaces in different solutions has been shown. CPE refers to the double-layer capacitance and Rp refers to the resistance between electrode and solution.

Solution	Surface	CPE	Error %	CPE - α	Error %	Rp	Error %
20ml 10X PBS	Clean Au	6.75E-06	0.84	0.97	0.13	64402	6.50
20ml 10X PBS	Au with NIP	4.19E-06	2.11	0.78	0.36	122730	11.42
20ml 10X PBS	Au with MIP	4.00E-06	3.28	0.79	0.56	171110	24.51
20ml 0.05M/L Pyrrole	Clean Au	6.75E-06	0.85	0.97	0.13	64379	6.55
20ml 0.05M/L Pyrrole + 30µl enzyme	Clean Au	1.32E-06	1.55	0.97	0.21	81970	3.42
20ml 10X PBS + 30µl enzyme	Au with NIP	4.57E-06	2.05	0.78	0.35	165990	16.27
20ml 10X PBS +30µl enzyme	Au with MIP	3.59E-06	2.85	0.80	0.48	114240	13.62
20ml 0.05M/L Pyrrole	Au with NIP	1.57E-06	4.56	0.82	0.74	136480	11.75
20ml 0.05M/L Pyrrole + 30µl enzyme	Au with MIP	1.31E-06	3.73	0.83	0.59	167980	10.39

These results of Electrochemical Impedance Spectroscopy (EIS) shed important light on the behavior of double layer capacitance in relation to Proteinase K immobilization on gold (Au) electrodes. This work examined the effects of surface modifications involving Proteinase K in different solutions, non-imprinted polymers (NIP), and molecularly imprinted polymers (MIP).

As shown in Table 3.:

NIP Modified Au: Au with NIP in 10X PBS + 30µl enzyme showed a higher Rp (165,990 Ω) compared to Au with NIP in 10X PBS alone (122,730 Ω), indicating that Proteinase K immobilization increases charge transfer resistance on NIP-modified surfaces. The CPE value showed a slight increase (from 4.19E-06 to 4.57E-06), suggesting a modest impact on double layer capacitance. However, NIP-modified surfaces showed an increase in Rp upon enzyme immobilization (165,990 Ω) but with higher error percentages for CPE (2.05%) and Rp (16.27%), suggesting more variability in the measurements.

MIP Modified Au: For Au with MIP in 0.05M/L Pyrrole + 30µl enzyme, the CPE value was significantly reduced (1.31E-06) compared to Au with MIP in 10X PBS alone (4.00E-06). This indicates an effective reduction in double layer capacitance due to the immobilized Proteinase K. The increase in Rp (167,980 Ω) compared to Au with MIP in 10X PBS alone (171,110 Ω) confirms the higher charge transfer resistance introduced by the enzyme layer. The significant increase in Rp (167,980 Ω) with a moderate error percentage for Rp (10.39%) and the above mentioned reduction in

CPE value indicates that MIP-modified surfaces provide the most substantial reduction in double layer capacitance and a notable increase in resistance, demonstrating effective and consistent immobilization of Proteinase K.

Also, for clean Au with 0.05M/L Pyrrole + 30µl enzyme, the increase in Rp (81,970 Ω) compared to clean Au in 10X PBS alone (64,402 Ω) further supports the conclusion that Proteinase K immobilization leads to increased resistance, likely due to the protein layer hindering charge transfer.

9.4 Comparison of different surfaces

The comparison of different surfaces was conducted, comparing clean Au surface, NIP and MIP surface in CV and EIS systems.

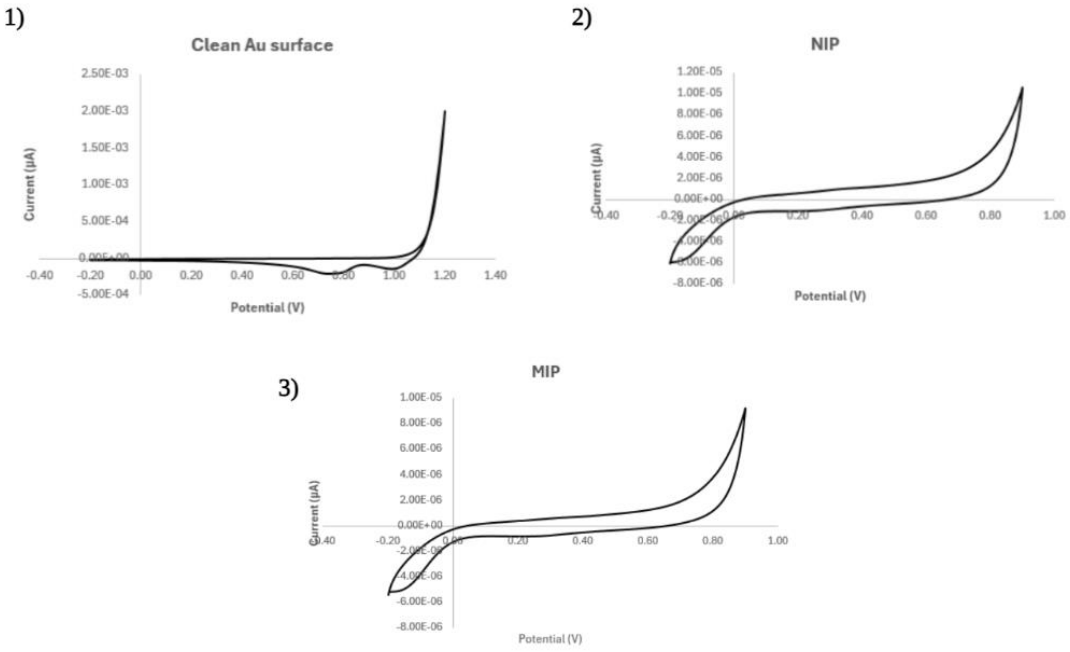


Figure 15. The figure shows cyclic voltammetry plot of gold electrode in a clean gold surface, MIP surface and NIP surface. 1) Shows the clean Au surface in Pure PBS solution. 2) Shows the polymerization of Pyrrole to form NIP in the solution of PBS and Pyrrole. 3) Shows the polymerization of pyrrole to form cavities in MIP in the solution of PBS, Pyrrole and Enzyme. The plot shows the oxidation and reduction peaks.

In clean Au Surface; because it is conductive and may take part in oxidation-reduction reactions, it shows a high current responsiveness. While, NIP shows a decreased current response, which suggests a lack of particular binding sites and low conductivity. MIP indicated an intermediate current response, indicating the presence of particular binding sites that facilitate electron transfer and confirm the polymer's successful imprinting.

CONCLUSIONS

1) A decrease in current (µm) in CV measurements as time increased after polypyrrole (Ppy) formation in the solution containing PBS, pyrrole, and enzyme. This decrease could be attributed to the insulating properties or surface attachment of either the enzyme, pyrrole, or both. These are the hypothesized mechanisms.
2) Surfaces modified with NIP or MIP exhibit higher resistance and lower capacitance due to surface alterations, whereas unmodified Au (gold-based electrodes) surfaces show lower resistance and higher capacitance.
3) The NIP-modified surface shows increased resistance and decreased capacitance in EIS upon the addition of pyrrole to the PBS solution, whereas the clean Au surface maintains high capacitance and moderate resistance.
4) Surfaces treated with MIP demonstrate enhanced resistance and efficient enzyme integration, while surfaces modified with NIP show moderate resistance and efficient enzyme integration in the PBS solution containing the enzyme.
5) In the presence of pyrrole and enzyme in solution, the capacity of the Au electrode is lowest. In contrast, the MIP-modified surface shows the lowest capacitance and highest resistance, suggesting it is the most promising configuration.
6) The MIP-modified Au surface with 0.05M/L Pyrole + 30µl enzyme configuration seems to be the most efficient for Proteinase K immobilization, according to the EIS results. This surface has the lowest CPE value, which indicates the largest decrease in double layer capacitance, and a notably higher Rp, which indicates that the enzyme layer is effectively preventing charge transfer.

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SUMMARY

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DNA Polymerase in DNA Biosensors

Biosensors have recently undergone significant developments in their application and specificity, making them a valuable tool in fields such as the analysis of biochemicals, environmental monitoring, drug development, and medical diagnosis. DNA biosensors have several advantages over traditional biosensors, including high specificity, efficiency, and the ability to detect a wider range of analytes. DNA- biosensors have customisable biosensing functions which makes it a better candidate for next generation biomedical detections. Due to their great catalytic efficiency, enzymes are frequently employed in biosensing. While DNA biosensors are generally less expensive to produce than traditional biosensors, the cost of enzymes used in the analytical processes can be high. This research focused on immobilising proteinase K enzyme on different electrode surfaces namely MIP (Molecularly imprinted polymers) with cavities and NIP (Non-imprinted polymers) and compared with clean Gold (Au) surface. To achieve this, Proteinase K enzyme was immobilised on the above mentioend surfaces, enabling the detection of signals EIS (Electrochemical impedance spectroscopy) and CV (Cyclic Voltammetry systems). The results showed that MIP-modified Au surfaces are better for applications requiring efficient and consistent Proteinase K immobilization, optimizing electrode performance for bioelectronic applications such as biosensing and bio-catalysis. Conclusively, successful immobilisation was performed and the results were assessed leading to this conclusion. Further studies must be done to explore the mechanistic interactions and stability of Proteinase K immobilized on these surfaces to enhance their application potential.
Key words: Biosensors, DNA polymerases, Electrochemical impedance spectroscopy, Cyclic Voltammetry

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