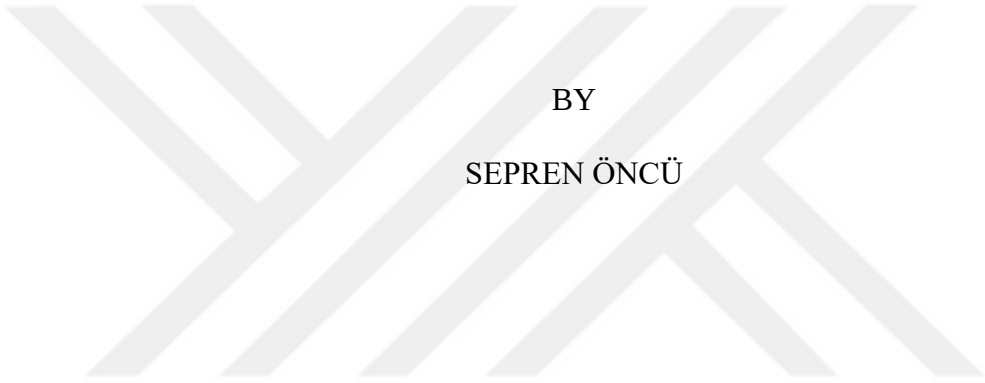


NANOMATERIAL BASED ELECTROCHEMICAL SENSING SYSTEMS
TOWARD CATECHOL DETECTION

A THESIS SUBMITTED TO
THE GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES
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IN
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Approval of the thesis:

**NANOMATERIAL BASED ELECTROCHEMICAL SENSING SYSTEMS
TOWARD CATECHOL DETECTION**

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ABSTRACT

NANOMATERIAL BASED ELECTROCHEMICAL SENSING SYSTEMS TOWARD CATECHOL DETECTION

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Catechol, also known as pyrocatechol, is an organic phenolic compound. Monitoring catechol is crucial due to its low degradability and toxicity. Various methods, including liquid and gas chromatography and spectrophotometry, have been used to detect catechol. The impracticality of real-time monitoring is due to high cost, time consumption, and simple pretreatment required by these classical methods. Laccase-based biosensors have become popular due to their ability to detect highly toxic environmental substances. This thesis aimed to fabricate two different nanomaterial-based laccase biosensors for the determination of catechol.

In the first study, a new nanocomposite called Chit-AgNPs was synthesized using a chemical reduction method. This nanocomposite was applied as a coating on the graphite electrode surface, serving as a matrix to immobilize the Lac enzyme. Then, amperometric measurements conducted resulted in with LOD of 3.85 μM , LOQ of 12.71 μM with LDR of 5-250 μM for the GE/Chit-AgNPs/Lac biosensors. Results showed that the biosensor is able to detect catechol in tap water and wine samples.

In the second study, newly synthesized conjugated polymer nanoparticles (P-PBF NP) were used as a matrix for the Lac enzyme via the reprecipitation method. The nanoparticles were successfully modified on graphite electrodes, followed by laccase immobilization. Amperometric measurements conducted resulted in with LOD of 0.49 μM , LOQ of 1.48 μM with LDR of 0.5-25 μM for the GE/P-PBF NP/Lac biosensors. The biosensor demonstrated good sensitivity in detecting catechol in tap water and green tea samples.

Keywords: Electrochemical Laccase Biosensors, Conjugated Polymer Nanoparticles, Chitosan, Silver Nanoparticles, Catechol

ÖZ

KATEKOL TESPİTİNE YÖNELİK NANOMALZEME TEMELLİ ELEKTROKİMYASAL SENSÖR SİSTEMLERİ

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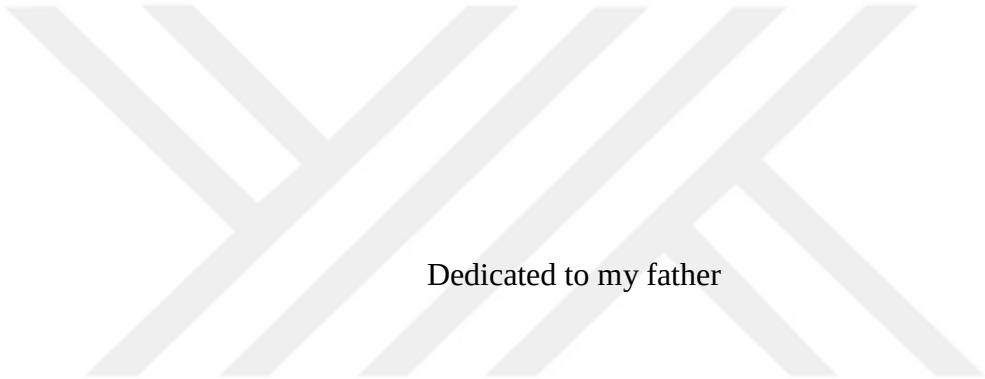
Haziran 2024, 159 sayfa

Katekol, aynı zamanda pirokatekol olarak da bilinen, organik bir fenolik bileşiktir. Katekolün tespiti, düşük bozunabilirliği ve toksisitesi nedeniyle çok önemlidir. Katekol tespiti için sıvı ve gaz kromatografisi ve spektrofotometri dahil olmak üzere çeşitli yöntemler kullanılmaktadır. Gerçek zamanlı izlemenin pratik olmaması, bu klasik yöntemlerin yüksek maliyetli, zaman alıcı ve basit ön işlem gerektirmesinden kaynaklanmaktadır. Lakkaz temelli biyosensörler, yüksek toksisiteye sahip çevresel maddeleri tespit etme özellikleri nedeniyle popüler hale gelmiştir. Bu tez, katekolün belirlenmesi için iki farklı nanomalzeme bazlı lakkaz biyosensörün üretilmesini amaçlamaktadır.

İlk çalışmada, kimyasal indirgeme yöntemi kullanılarak Chit-AgNPs adı verilen yeni bir nanokompozit sentezlenmiştir. Bu nanokompozit, Lac enzimini immobilize etmek için matris görevi görerek grafit elektrot yüzeyine kaplama olarak uygulanmıştır. Ardından yapılan amperometrik ölçümler, GE/Chit-AgNPs/Lac biyosensörleri için LOD 3.85 μ M, LOQ 12.71 μ M LOQ ve LDR 5-250 μ M LDR olarak hesaplanmıştır. Sonuçlar, biyosensörün musluk suyu ve şarap örneklerinde katekol tespit edebildiğini göstermiştir.

İkinci çalışmada, çökelme yöntemi ile Lak enzimi için matris olarak yeni sentezlenmiş konjuge polimer nanoparçacıkları (P-PBF NP) kullanılmıştır. Nanoparçacıklar, grafit elektrotlar üzerinde başarılı bir şekilde modifiye edilerek ardından lakkaz immobilizasyonu gerçekleştirilmiştir. Yapılan amperometrik ölçümler, GE/P-PBF NP/Lac biyosensörleri için LOD 0.49 μ M, LOQ 1.48 μ M ve LDR 0.5-25 μ M olarak hesaplanmıştır. Biyosensör, musluk suyu ve yeşil çay örneklerinde katekol tespitinde iyi bir seçicilik göstermiştir.

Anahtar Kelimeler: Elektrokimyasal Lakkaz Biyosensörleri, Konjuge Polimer Nanoparçacıklar, Kitosan, Gümüş Nanoparçacıklar, Katekol



Dedicated to my father

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LIST OF ABBREVIATIONS

ABBREVIATIONS

AA	Ascorbic acid
Ag	Silver
AgNPs	Silver Nanoparticles
APAP	Paracetamol
CC	Catechol
CE	Counter Electrode
CA	Citric acid
CPs	Conjugated Polymes
CPNs	Conjugated Polymers Nanoparticles
CV	Cyclic Voltammetry
Et	Ethanol
FA	Folic acid
FE-SEM	Field Emission Scanning Electron Microscopy
GA	Glutaraldehyde
GE	Graphite Electrode
Glu	Glucose
KCl	Potassium chloride
K _m	Michaelis constant
Lac	Laccase

LOD	Limit of Detection
NaCl	Sodium chloride
NPs	Nanoparticles
PBS	Phosphate Buffer Saline
Ph	Phenol
PL	Photoluminescence Spectroscopy
Pt	Platinum
RE	Reference Electrode
RSD	Relative Standard Deviation
SD	Standard Deviation
TEM	Transmission Electron Microscopy
WE	Working Electrode
UA	Uric acid
Ur	Urea
UV-Vis	Ultraviolet-Visible
$V_{\max}$	Maximum velocity

CHAPTER 1

INTRODUCTION

1.1 Biosensors

Up to date, several definitions are given for biosensors. “A biosensor is defined as an analytical device that converts the chemical reaction to useful analytical signal”¹. A biosensor can also be defined as a combined receptor-transducer tool that can transform biological responses into measurable electrical signals². Biosensors can be applied in many areas, such as environmental monitoring (toxic pollutants), medicine (glucose in blood, cholesterol), food (quality and safety), and biodefence (biological warfare).

A biosensor consists of four components which are receptor, transducer, signal processor (transforming electronic signal to a desired signal) and display (Figure 1.1.).

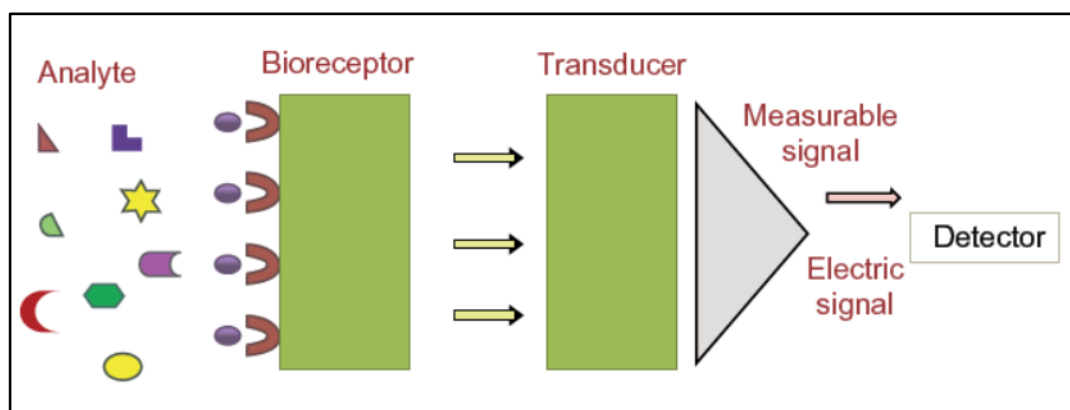


Figure 1.1. Schematic representation of a biosensor¹

The history of biosensor is summarized below:

- Clark and Lyons developed the first glucose sensor³.
- The oxygen background was redesigned by utilizing two working oxygen electrodes. While one measured the differential current, the other electrode had the enzyme⁴.
- In 1969, the first enzyme-based potentiometric biosensor was developed via immobilization of urease on ammonia electrode to detect urea⁵.
- In order to detect glucose in biological samples, especially in blood, an amperometric enzyme electrode was fabricated by Guiboult and Lubrano in 1973. This method was based on a direct amperometric assessment of the liberated hydrogen peroxide⁶.

Characteristics of a biosensor are listed below⁷:

- Sensitivity is defined as the limit of detection (LOD), the lowest amount of analyte a biosensor can monitor.
- Selectivity is known as a bioreceptor's capability to identify a particular analyte within a sample that involves various other mixtures and impurities.
- Stability is defined as the level of vulnerability to environmental disturbances in and around the biosensor.
- Linearity is known as the indication of the accuracy of the measured response.
- Reproducibility is defined as the capacity to yield consistent responses when the experiment is repeated.

1.2 Biosensor Classification

The most widely used biosensor classification is mainly based on the principal components of biosensors, which are biorecognition elements and transducers⁸.

1.2.1 Biorecognition Elements

Biorecognition elements can be divided into two subcategories, which are biocatalytic (whole cell and enzyme) and affinity (antibody and nucleic acids) (Figure 1.2.)⁹.

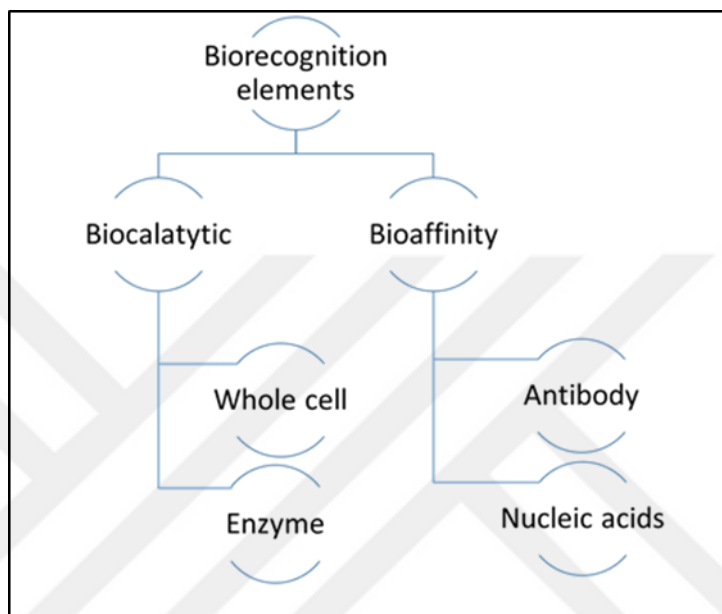


Figure 1.2 Classification of biosensors according to biorecognition elements

1.2.1.1 Enzymes

Most enzymes are globular proteins composed primarily of 20 natural amino acids. Their primary function is to catalyze biochemical reactions and enhance reaction rates significantly compared to non-catalyzed responses. The interaction between enzymes and substrates can be described through kinetic studies¹⁰.

Due to their selectivity and catalytic capabilities for forming electroactive products, enzymes are one of the preferred biorecognition elements for constructing biosensors. They are the most essential components of enzyme electrodes. The enzyme activity depends on immobilization techniques, solution parameters, and

biosensor design. Besides, the lifespan of the developed biosensor is directly related to the stability and shelf life of the immobilized enzymes¹¹.

1.2.1.1.1 Enzyme Classification

Enzymes are identified using a distinct four-digit code known as the Enzyme Commission (EC) number. The six categories outlined in Table 1.1 were established in the initial Enzyme List and remained unaltered until 2018, when 'Translocases' were included as a new class (EC 7)¹².

In enzyme classification, the Enzyme Commission assigns each enzyme a four-digit code. The first digit of this code represents the enzyme class or the type of reaction. The second digit represents the donor's nature. The third digit represents the acceptor's nature. The fourth digit represents the substrate acting as the reaction's acceptor (Table 1.1).

An enzyme like laccase, which bears the EC number 1.10.3.2, falls under the category of oxidoreductases (as indicated by the first digit), functions with diphenols and similar compounds as donors (as noted in the second digit), utilizes oxygen as the acceptor (third digit) and is the second enzyme classified within this particular group (fourth digit)¹².

Table 1.1 Main classes of enzymes according to the type of reaction catalyzed

First EC digit	Enzyme Class	Reaction type
1.	Oxidoreductases	Oxidation/reduction
2.	Transferases	Atom/group transfer
3.	Hydrolases	Hydrolysis
4.	Lyases	adding or removing groups to double bonds
5.	Isomerases	Isomerization
6.	Ligases	Formation of C-C, C-S, C-O and C-N bonds
7.	Translocases	The movement of ions or molecules across membranes, or their division within membranes

1.2.1.2 Whole Cells

Whole-cell biosensors (WCB) are catalytic-based biosensors that are alternative tools to classical biosensors. These biosensors use whole eukaryotic (e.g., yeast, microalgae, protozoa, fungi, tissue culture cells) or prokaryotic cells (bacteria) as biorecognition elements¹³.

1.2.1.3 Nucleic Acids

Nucleic acid biosensors are becoming increasingly popular due to the specificity and variability of their sequences. Oligonucleotides, fragments of DNA or RNA, are the biorecognition elements in these biosensing platforms. These biosensors typically consist of single-stranded DNA (ssDNA) molecules that can hybridize with other DNA or RNA molecules. They are widely used in food and environmental analysis, health monitoring, and diagnostics. DNA biosensors, also known as DNA-based biosensors or genosensors, offer several advantages over traditional nucleic acid assays, including fast detection, simplicity, sensitivity, selectivity, and low cost^{14–18}.

1.2.1.4 Antibody

Immunosensors are affinity-based biosensing devices that monitor the specific interactions between an antigen and an antibody immobilized on the transducer surface. In order to design immunosensors, several transducers can be used involving electrochemical, optical, and piezoelectric. Electrochemical immunosensors have been used to detect numerous analytes. They are known for their rapid response, cost-effectiveness, and ease of use¹⁹.

1.2.2 Transducers

According to the transducer, biosensors can be classified into electrochemical, optical, thermal, and piezoelectric ⁸(Figure 1.3.).

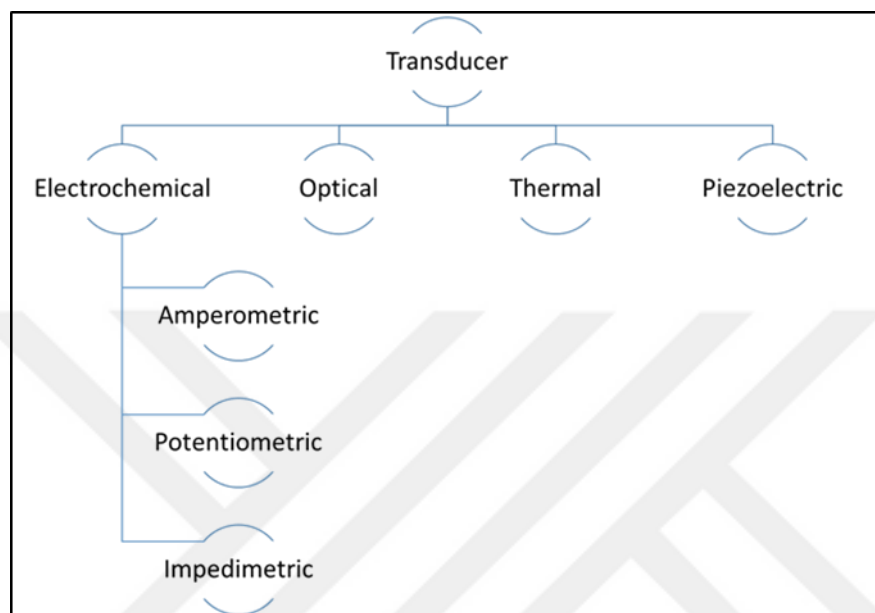


Figure 1.3. Classification of biosensors according to the transducer

1.2.2.1 Optical Biosensors

Optical biosensors can monitor changes resulting from interactions between a biorecognition element and analyte regarding the optical field's phase, polarization, or frequency. According to the transduction mechanism used the optical biosensors can be divided into absorption, fluorescence, luminescence, and colorimetric biosensors ²⁰. Surface plasmon resonance (SPR) biosensors are the most common and widely used among optical biosensors. Several biorecognition elements, including antibodies, antigens, receptors, enzymes, tissues, and whole cells, can be used in order to develop optical biosensing platforms ²¹.

1.2.2.2 Thermal Biosensors

Thermal biosensors (thermometric biosensors) can monitor temperature changes due to heat production or absorption during biological reactions. The heat generated by absorption correlates directly to the molar enthalpy and the total quantity of product molecules formed due to biological responses. An enzyme thermistor is a type of thermal biosensor that relies on an immobilized enzyme column or reactor (IMER) placed in a flow microcalorimeter, acting as a flow-injection analyzer. Enzyme thermistors can detect various analytes, such as ascorbic acid, cholesterol, ethanol, fructose, glucose, lactose, urea, and uric acid²².

1.2.2.3 Piezoelectric Biosensors

Piezoelectric biosensors are analytical devices that monitor affinity reactions. Piezoelectric biosensors are advantageous due to real-time and label-free transduction. Therefore, they have been used to analyze several biomolecular interactions. In particular, immunosensors use an antibody as a biorecognition element, making their specificity dependent on the antibody's specificity. Immunosensors can be used for cancer marker and microbial detection in clinical assays^{23–25}.

1.2.2.4 Electrochemical Biosensors

Electrochemical biosensors are based on monitoring changes in current, potential or conductance due to interactions between biorecognition element and target analyte. Compared to other biosensors, electrochemical biosensors offer several advantages, including robustness, sensitivity, selectivity. Electrochemical biosensors are categorized into four types, based on the transducer: amperometry, voltammetry, potentiometry, and conductometry²⁰.

Voltammetry is a process that involves measuring the electric current that arises due to applying a specific potential. However, amperometry is based on using constant potential; the current is monitored as a function of time. On the other hand, potentiometry is based on the potential difference under no current flow. Potential difference is related with change in free energy. Impedometry includes subjecting the cell to a low-intensity alternating potential and observing how the system responds to the perturbation when it reaches a stable state²⁶.

1.2.2.4.1 Amperometric Biosensors

An amperometric biosensor is characterized by monitoring the current resulting from electrochemical oxidation or reduction of electroactive species at a constant applied potential. Current response during electrochemical reactions is directly proportional to the concentration of electroactive species²⁶.

Amperometric measurements are performed in a cell with a three-electrode system, including a working electrode, reference electrode, and counter/auxiliary electrode. The working electrode is the main electrode that is constructed by conductive materials. The electron transfer takes place on the surface of the working electrode, and the working electrode acts as the transducing element. Selecting the appropriate electrode is crucial for successful surface modification in electrochemical sensing platforms since surface modification is performed on this working electrode. The selection of a working electrode is related to the redox behavior of the target analyte and the background current over the potential region. The reference electrode (e.g. Ag wire, Ag/AgCl or saturated calomel electrode) is used to provide reproducible and stable potential in order to be compared with the working electrode potential. The counter/auxiliary electrode maintains stability. In these three electrode systems, the potential applied is controlled by a reference electrode, and the current flow through the solution between the working and counter electrodes is determined²⁷.

Amperometric biosensors are widely studied due to providing high sensitivity as well as wide linear range²⁸.

Amperometric-based biosensors are classified into three groups, which are first-generation, second-generation, and third-generation biosensors, depending on the electron transfer technique utilized²⁹.

First-generation biosensors (called mediatorless amperometric biosensors) determine the concentrations of analytes produced by enzymatic reactions that pass through the transducer and have electrical signals (Figure 1.4.) Pretreatment of the electrode is necessary to obtain reproducible surface and sensor response^{30,31}.

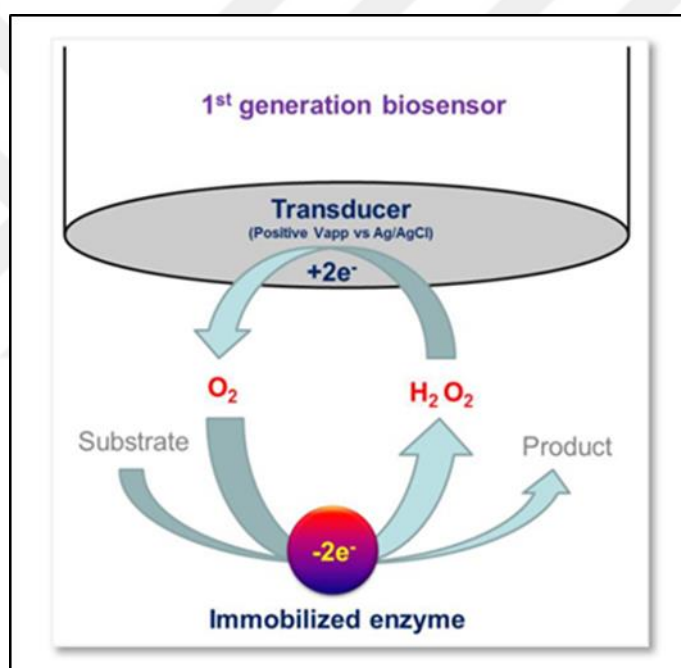


Figure 1.4. Schematic representation of a first generation biosensor³¹

Second-generation biosensors (also called mediator amperometric biosensors) (Figure 1.5.) are needed to utilize mediators such as oxidizing agents in order to act as electron transporters³². This method enables studying at low potential, preventing the effect of interfering substances and O₂ dependence. While ferrocene and ferricyanide are well-known mediators, others are methylene blue, phenazines, alizarin yellow, Prussian blue, and toluidine blue.

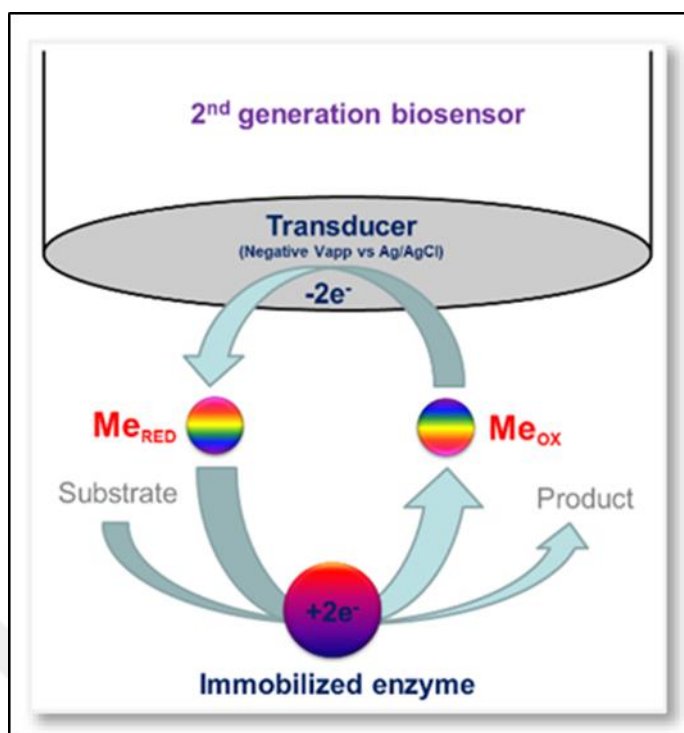


Figure 1.5. Schematic representation of a second generation biosensor³¹

Third-generation biosensors are based on direct electron transfer between the electrode and the enzyme (Figure 1.6.). Electrode, enzyme (as a biorecognition element), and redox polymer (for signal amplification) are the components of a third-generation biosensor³³.

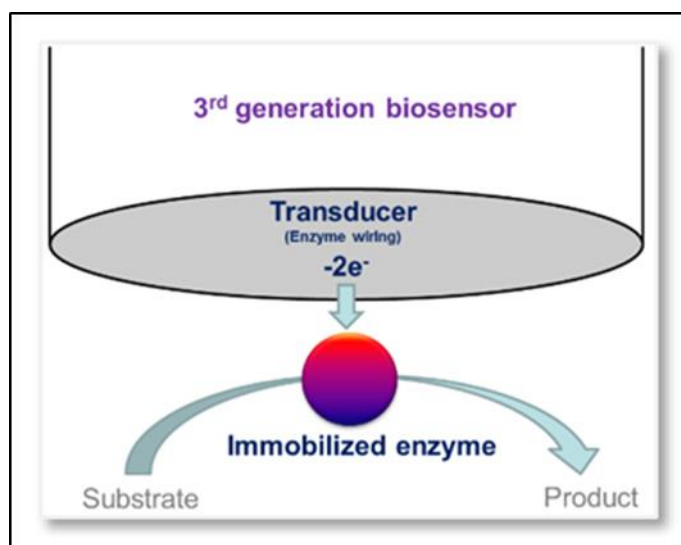


Figure 1.6. Schematic representation of a third generation biosensor³¹

1.3 Enzyme Immobilization Methods

Immobilization is a method that enzymes are attached onto or within solid supports to form an enzyme immobilization system³⁴. Enzyme immobilization can be defined as the localization of an enzyme in a certain area while preserving its catalytic properties. Enzymes, matrices, and types of attachments are the components of the immobilized enzyme systems. Enzyme immobilization offers some benefits. One of their main advantage is to provide enzyme stability. More importantly, the immobilized enzymes can be removed from the reaction mixture and reused repeatedly. Enzyme immobilization also decreases the expenses related to the enzyme and the products resulting from enzymatic reactions³⁵.

Commercially, immobilized enzymes are preferred due to their ease of reuse, which provides a distinct physical advantage. This is mainly because of the efficient immobilization of the enzymes, which makes the system more favorable for commercial use¹⁰.

Immobilization may result in biochemical changes that cause induced enzyme stability, which can be observed as follows:

- A higher speed of catalysis
- Longer catalytic duration
- Better operational stability, even under extreme conditions such as pH, temperature, etc.

Enzyme immobilization can be categorized into two main groups: Reversible (entrapment, covalent binding, and crosslinking) and irreversible (physical adsorption) (Figure 1.7.). Enzyme immobilization is also divided as physical, where weak intermolecular interactions form between the solid support and the enzyme, as chemical where covalent bonds are formed between the enzyme and solid support³⁴.

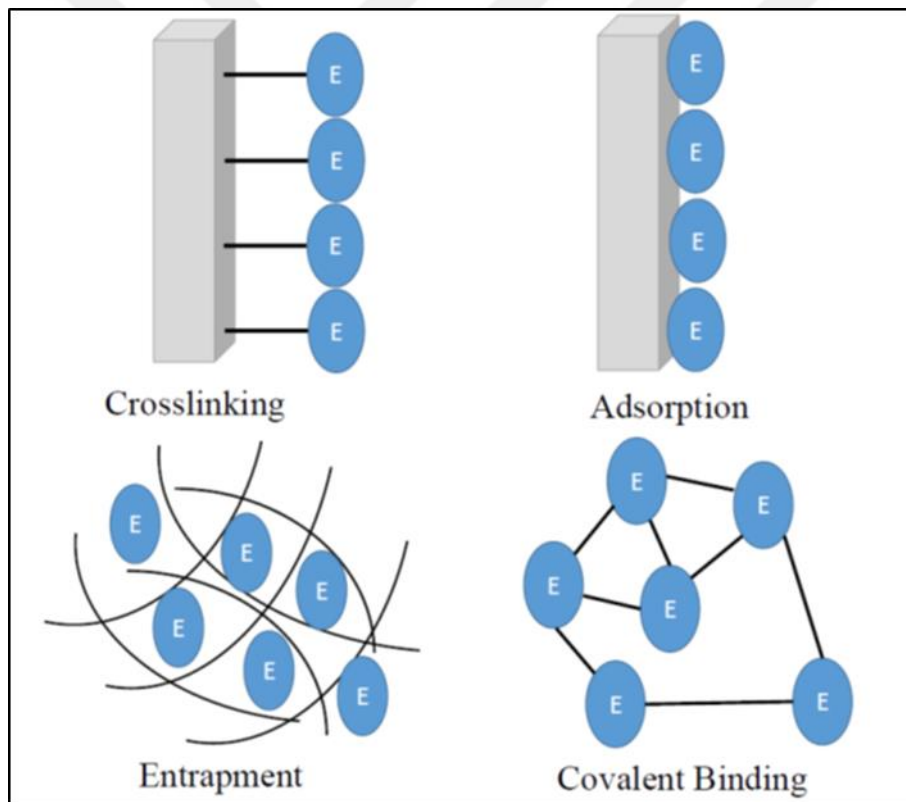


Figure 1.7. Schematic representation of enzyme immobilization methods

1.3.1 Adsorption

As a reversible immobilization technique, adsorption provides that the enzyme is attached to the surface of the material via non-specific forces (van der Waals, hydrogen bonds, or ionic interactions). Since this method provides weak interactions, immobilized enzymes can be susceptible to leaching. This method includes immersing the support into the enzyme solution and giving time for physical adsorption to take place. Being a relatively chemical-free method, physical adsorption is simple, fast, and cheap^{36,37}.

1.3.2 Entrapment

The entrapment technique relies on encasing an enzyme inside a polymeric structure that permits the passage of substrates and products while keeping the enzyme intact. For this purpose, different methods are used to entrap enzymes, such as fiber entrapping, gel, or micro-encapsulation. This method may lead to the limitation of mass transfer through gels or membranes³⁸.

1.3.3 Covalent Binding

In this technique, the enzyme is covalently attached to polymeric support. The enzyme is attached via its functional groups that are not affecting the catalytic activity³⁹.

1.3.4 Crosslinking

Called as also carrier-free immobilization, crosslinking is another irreversible technique. This method does not need any support. It is based on the formation of cross-linkages between enzyme and crosslinker. Glutaraldehyde is a commonly used crosslinking agent since it is economical and easily obtainable. By using this

immobilization method, the resulting enzyme complex becomes resistant to harsh pH and temperature conditions³⁶. Each immobilization method includes advantages, and disadvantages as summarized in Table 1.2.



Table 1.2 Comparison of immobilization methods

Immobilization Methods	Advantages	Disadvantages
Adsorption	Simple, easy, cheap Little conformational change of enzyme No reagents needed	Weak interactions Desorption of enzymes
Entrapment	Simple and easy No chemical change Wide applicability Mild conditions needed	Loss in enzyme activity Poor stability Enzyme leakage
Covalent Binding	Wide applicability Stable No leakage or desorption problem Short response time	Formation of toxic products Major loss of enzyme activity
Crosslinking	Cost-effective, simple Controllable enzyme amount	Lead to chemical change Loss of activity

1.4 Laccase

Laccases (benzenediol: oxygen oxidoreductase, EC 1.10.3.2), a versatile enzyme, have been widely utilized for different areas like bioremediation processes in the textile, food, and paper industry^{37,40,41}. Fungus, insects, plants and bacterias are main laccase sources.

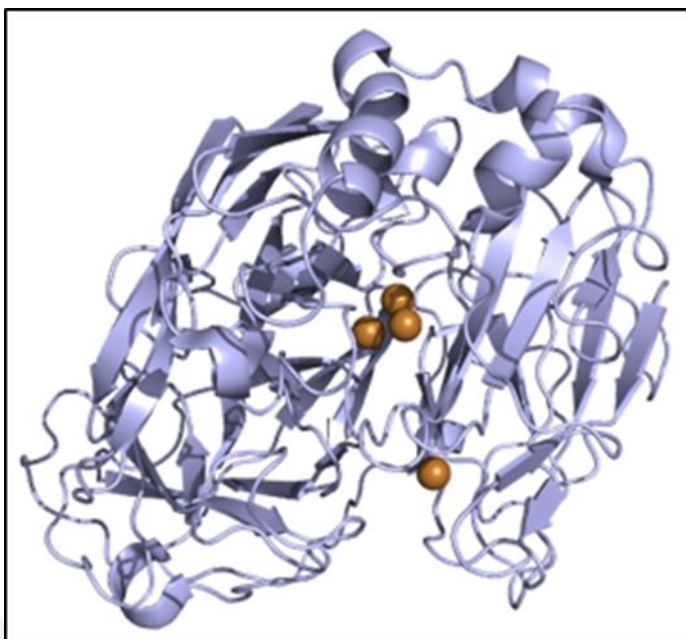


Figure 1.8. Crystal structure of laccase (https://www.creative-enzymes.com/similar/laccase_388.html)

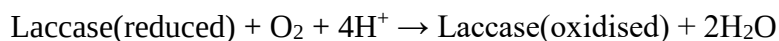
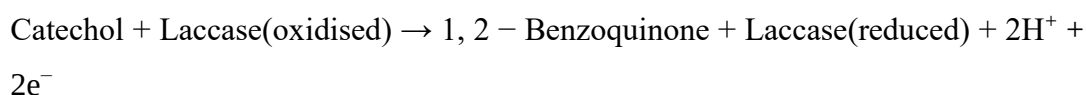
As an extracellular multicopper enzyme, laccases belong to blue multi copper oxidases family^{42,43}. Crystal structure of laccase is shown in Figure 1.8. Laccases include three types of copper ions which are type-1 (T1), type-2 (T2) and type-3 (T3). T1 oxidize some compounds including phenolic compounds and trinuclear cluster (T2/T3), composed of one type-2 (T2) and two type-3 (T3), catalyze the reduction of molecular oxygen to water⁴⁴.

Laccases can perform single-electron oxidation of phenolic compounds while simultaneously reducing molecular oxygen to water. Typically, they exhibit a limited substrate selectivity⁴⁵, which allows for the oxidation of various substrates, such as o- and p-diphenols, polyphenols, and phenols with methoxy substitution⁴⁶. Furthermore, laccases can catalyze when electron transfer mediator molecules are present^{47,48}. Depending on the source of laccase enzyme and substrate, optimum temperature and pH differ from 20 to 60 °C and 3 to 9, respectively⁴⁹.

1.4.1 Laccase Based Biosensors

Evaluation of phenolic compounds in environmental samples, the food industry, and medical applications has become important in recent years. Conventional techniques to detect these compounds, such as spectrophotometry⁴¹ and chromatography^{50,51} are time-consuming and expensive. Portable and cost-effective analytical devices such as enzymatic biosensors offer fast and cost-effective detection of phenolic compounds³⁷. Enzyme based electrochemical biosensors emerge as a promising alternative in the realm of analytical methods. To detect phenolic compounds, enzymes such as tyrosinase^{52,53} and horseradish peroxidase^{54,55} can be utilized for biosensor construction. However, these two enzymes have some drawbacks. The presence of hydrogen peroxide is necessary for the completion of the catalytic function of horseradish peroxidase, while tyrosinase has low stability and can be inhibited by reaction products. Compared to these enzymes, laccase offers some specific advantages, such as good stability and the ability to catalyze electron transfer reactions without the need for cofactors and oxidize o, m, p-benzenediol, and phenolic compounds such as catechol when molecular oxygen is present (Figure 1.9.)³⁷.

The mechanism between laccase and its substrates in determining catechol involves converting catechol to 1,2-benzoquinone and electrocatalytically reducing oxygen to water at the electrode surface.



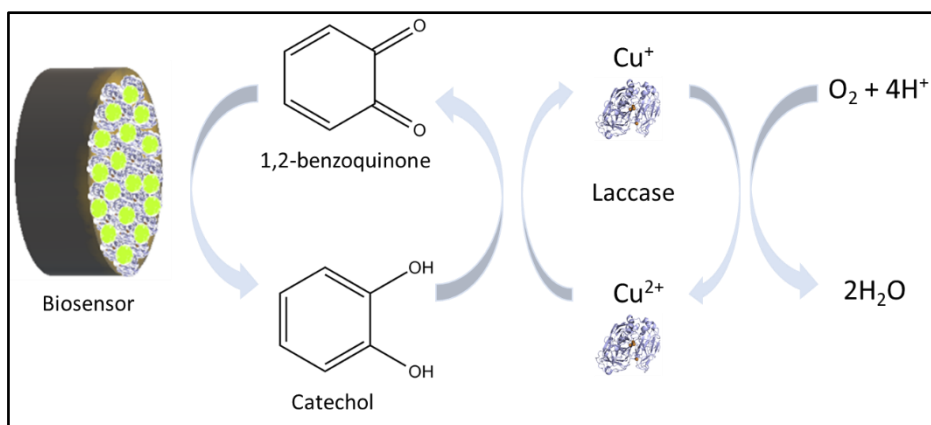


Figure 1.9. Schematic representation of catechol determination by a laccase biosensor

1.5 Conjugated Polymers

Conjugated polymers (CPs) have emerged as a promising material for various electronic applications since the late 1970s when high electrical conductivity was discovered in doped poly(acetylene) (PA)⁵⁶. This groundbreaking discovery earned Alan Heeger, Alan MacDiarmid, and Hideki Shirakawa the Nobel Prize in Chemistry in 2000 and paved the way for the field of "plastic electronics." Unlike traditional polymers, polymer plastic contains alternating single and double bonds along the polymer chain, which prevents the electrons on the polymer backbone from carrying an electric current. However, when strong electron acceptors are introduced, the material becomes conductive. Because PA is sensitive to air, new CPs have been developed over the past few decades, leading to exciting new uses for these materials in modern science⁵⁷.

Conjugated polymers possess alternating σ and π bonds along their backbone, with a delocalized π -electron cloud over the chain responsible for their attractive optical and electrochemical properties. Commonly used CPs are given in Figure 1.10. Researchers have been highly interested in these polymers for the past thirty years due to their vast potential for technological applications, such as solar cells⁵⁸, organic

electrochemical transistors (OECTs)⁵⁹, light-emitting diodes (LEDs)⁶⁰, energy storage systems⁶¹, organic field-effect transistors (OFETs)⁶², and chemical sensors⁶³ and biosensors⁶⁴.

Chemical or electrochemical techniques are typically utilized to synthesize CPs. Condensation and addition polymerization are the two main methods used in traditional chemical synthesis, which offer many effective and accessible routes to generate various CPs. Electrochemical synthesis employs a three-electrode system comprising working, counter, and reference electrodes submerged in a mixed monomer, solvent, and electrolyte solution. By passing an electrical current through the solution, CPs are deposited onto the working electrode. Electrochemical synthesis is a favored alternative to traditional chemical synthesis for producing CPs due to its reproducibility, simplicity, and ability to produce several novel CPs with modified monomers or complex structures^{65,66}.

Due to their immiscibility, conjugated polymers have limited applications in water. Moreover, their processing often involves hazardous organic solvents that can threaten the environment and human health. To overcome these challenges, researchers have been exploring ways to convert these polymers into nanoparticle dispersions in alcohol or water. This approach eliminates the need for toxic solvents and improves their practicality.

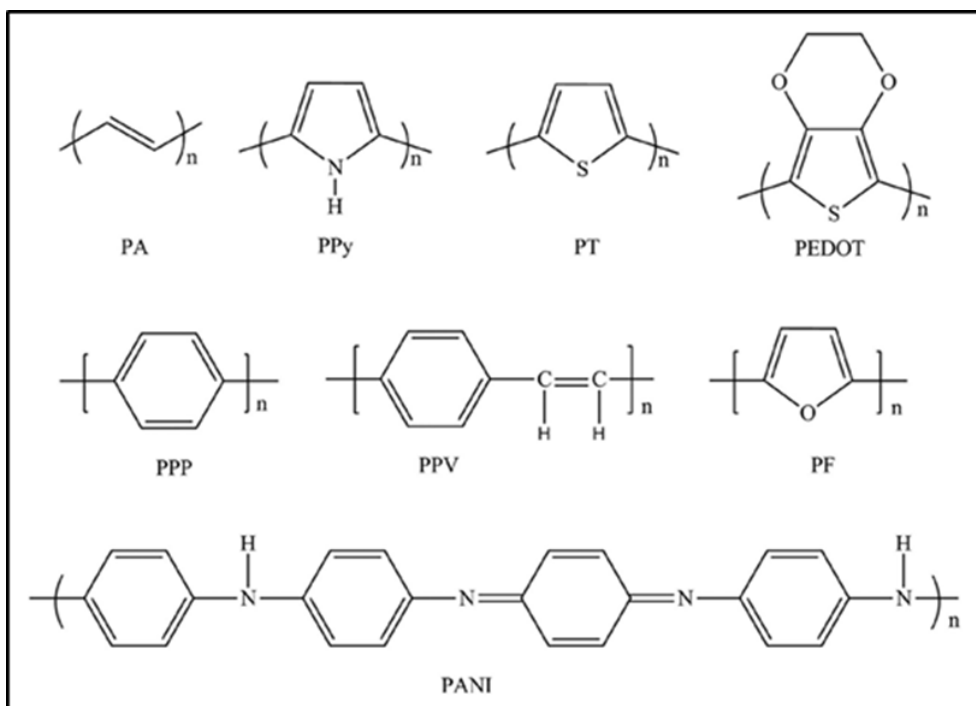


Figure 1.10. Chemical structures of conjugated polymers (polyacetylene (PA), polypyrrole (PPy), polythiophene (PT), poly(phenylenevinylene) (PPV), poly(3,4-ethylenedioxythiophene) (PEDOT), poly(phenylenevinylene) (PPV), polyfluorene (PF), polyaniline (PANI))⁶⁷

In the second chapter of this study, repeating units of synthesized polymers were selected as benzodithiophene (BDT), fluorene and pyrazine to obtain a novel conjugated polymer named P-PBF. BDT has been extensively studied as a donor unit for D-A-type conjugated polymers. It is a common organic semiconducting unit with a flat conjugated structure and high resistance to oxidation due to the fusion of a benzene group with two flanking thiophene units^{68–70}. Additionally, various substituents can be added to the central benzene core of BDT to adjust the energy levels, thermal stability, absorption spectra, and solubility of BDT-based conjugated polymers^{71–73}.

Polyfluorenes are the most appealing blue-emitting materials due to their high efficiency, charge transport properties, excellent solubility and film-forming ability, good chemical and thermal stability, and adjustable properties through chemical

modifications and copolymerization⁷⁴. Pyrazine is well-suited for efficient transport due to its ability to provide high hole mobility, intermolecular enhancement, and cost-effectiveness⁷⁵. Moon and colleagues were the first to study polymer donors with a pyrazine core⁷⁶. They showed that integrating the pyrazine unit into the backbone of polymer donors with a wide band gap could increase their flatness and enhance their optoelectronic characteristics. This was achieved by controlling the alkyl side chains.

1.5.1 Conjugated Polymers in Biosensor Applications

Foulds and Lowe pioneered the development of a polypyrrole (PPy) film for detecting glucose, a biosensing material used for various applications of CPs⁷⁷. Since then, several types of CPs have been utilized to develop multiple biosensors. The main effects of CPs on biological recognition and signal response can be summarized as follows:

- During construction of biosensors, CPs provide reproducibility, sensitivity and simplicity^{8,78}.
- Biomolecules' electrocatalytic properties are induced in biosensor construction through CPs. This is because the conjugated π electron backbones of CPs allow for the free movement of electrons⁷⁹. As a result, fast electron transfer and direct interaction between the biomolecule and transducer are achieved^{80,81}.
- Effective immobilization of enzyme molecules onto a substrate is essential in enzymatic biosensors to maintain stability and prevent activity loss over time. CPs are excellent materials due to their unique structure, electronic character, and compatibility. For this reason, CPs can serve as immobilization platforms that provide a stable matrix to enzymes located on the electrode surface⁸².

By integrating enzymes with conjugated polymers, biosensors can detect various analytes, such as ethanol, catechol, glucose, paraoxon, and others, developing advanced biosensors for the next generation (Table 1.3).



Table 1.3 Conjugated polymers in biosensor applications

Modified Electrode	Analyte	LOD (mM)	Linear range (mM)	Sensitivity $\mu\text{A}/(\text{mM}\cdot\text{cm}^2)$	Real Sample	Reference
GE/poly(EDOT-TPD)/GOx	glucose	0.018	0.1-0.5	65.765	Coke-Zero Sugar	⁸³
GE/poly[BDT-alt-(TP;BO)]C ₆₀ /Tyr	indo	0.11x10 ⁻³	0.0005-0.0625	2820	-	⁸⁴
P(DTFBT)/GE/Lac	catechol	0.014	0.025-0.4	166.74	-	⁸⁵
F-HS-Ni/GE	Ach	8.1x10 ⁻³	0.02-6	250x10 ³	Human serum	⁸⁶
Au-XOR/fMWCNT-PEDOT/GCE	xanthine	0.1x10 ⁻³ -1x10 ⁻²	5.45x10 ⁻⁵	16.075x10 ³	fish, meat, blood, urine	⁸⁷
P(BDIT-BDA)/GOx	glucose	17.2x10 ⁻³	0.10-1.0	20.31	Human serum	⁸⁸
PM1/Lac	catechol	9.36x10 ⁻³	0.005-0.175	153.6	Tap water	⁸⁹
Poly(BT)/AChE	paraoxon	0.015	0.025-4	19.12	Tap water, milk	⁹⁰

AChE: Acetylcholinesterase, ACh: acetylcholine, Au: gold, C₆₀: Fullerene, EDOT: 3,4-ethylenedioxythiophene, fMWCNTs: functionalized multiwalled carbon nanotube, GCE: Glassy carbon electrode, GE: Graphite electrode, GOx: Glucose oxidase, Indo: indomethacin, Lac: Laccase, DTFBT: 5-fluoro-4,7-bis (4-hexylthio-phen-2 yl) benzo [c][1,2,5]thiadiazole, F-HS-Ni: Nickel ions embedded fullerenzyme-based catalyst, PEDOT: poly(3,4-ethylenedioxythiophene), Poly(BT): poly-2,2'-(9,9-dioctyl-9 h-fluorene-2,7-diyl)bisthiophene, poly[BDT-alt-(TP;BO)]: benzoxadiazole, thienopyrroledione, and benzodithiophene containing conjugated polymer, P(BDIT-BDA): poly(benzenediamine-bis(2-ethylhexyl)oxy)benzodithiophene, Tyr: Tyrosinase, TPD: thieno[3,4-c]pyrrole-4,6-dione, XOR: xanthine oxidoreductase

1.6 Nanomaterials Used in Biosensors

The use of nanomaterials (NMs) for designing biosensors has gained attention due to their unique chemical and physical features. Catalytic activity, electrical conductivity, and magnetic properties of nanomaterials make them an important candidate for biosensor applications⁹¹. They can be utilized to enhance the analytical properties of biosensors, such as linear range, detection limit, sensitivity, stability, reproducibility, response rate and selectivity⁹². The surface of nanomaterials can be modified by using different chemical groups^{93,94}.

Reasons for use of NMs for biosensor design are listed below^{92,95}:

- NMs can be used to enhance electron transport during enzymatic reaction, which occurs between the enzyme and the surface of the transducer.
- NMs can be used to enhance the analytical features of biosensors such as sensitivity, limit of detection (LOD), dynamic linear range, response time, etc.
- NMs can be used to increase the surface area of transducers which causes an increase in immobilized enzyme amount.

According to their chemical structure, nanomaterials can be categorized into two main groups, which are organic and inorganic nanomaterials. Metal nanoparticles and their oxides, quantum dots, are inorganic nanomaterials, while organic nanomaterials include carbon-based structures such as graphene, graphene oxide, fullerene, and carbon nanotubes⁹².

1.6.1 Conjugated Polymer Nanoparticles

Conjugated polymer nanoparticles (CPNs), also called semiconducting polymer nanoparticles or polymer dots (Pdots), have been successfully produced and used for various biological purposes. Fluorescence imaging with CPNs has gained significant attention due to their excellent photostability, high fluorescence brightness, low

cytotoxicity, and ease of modification and these include imaging (*in vitro*⁹⁶ and *in vivo*⁹⁷). In addition to fluorescence imaging, CPNs have demonstrated promising outcomes in detecting and treating various health conditions, including cancer, microbial infections, and cardiac diseases. Many CPNs and nanohybrids have been used effectively as photosensitizers, photothermal agents, and efficient carriers of drugs or genes for photodynamic therapy-PDT⁹⁸, photothermal therapy-PTT⁹⁹, drug delivery¹⁰⁰ and gene therapy¹⁰¹ A summary of these applications is provided in Table 1.4.



Table 1.4 Conjugated polymer nanoparticles for biological applications

Conjugated polymer	Matrix	Size	Application	Reference
MEH-PPV	MEH-PPV/m-THPC	~50 nm	<i>in vitro</i> imaging	96
PFBT	PFBT/PS-PEG-COOH/IONPs	130–140 nm	<i>in vivo</i> imaging	97
PTB7	PTB7@F127	~190 nm	Photodynamic therapy	98
PPV-ST	PPV-ST/PSMA	80 nm	Photothermal therapy	99
DPP-BDP	DPP-BDP/FA/F127	200 nm	Drug delivery	100

DPP-BDP: poly(2,5-bis(2-octyldodecyl)-3,6-di(thiophen-2-yl)-2,5-dihydropyrrolo[3,4-c] pyrrole-, 4-dione-alt-1, 5-didodecanoyl-3, 7-diphenylpyrrolo [2, 3-f] indole-2,6 (1H,5H)-dione), F127: Pluronic F-127, IONPs: Iron oxide nanoparticles, MEH-PPV: poly[2-methoxy-5-((2-ethylhexyl)oxy)-p phenylenevinylene], m-THPC: meta tetra(hydroxyphenyl)-chlorin, PFBT: poly(9,9-dioctylfluorene-alt-benzothiadiazole PPV-ST: poly(p-phenylene vinylene) functionalized with photo-responsive DASA and folic acid unit, PS-EG-COOH: polystyrene grafted with ethylene oxide functionalized with carboxyl groups, PSMA: poly(styrene-co-maleic anhydride), PTB7: (poly({4,8-bis[(2-ethylhexyl)oxy]benzo[1,2-b:4,5-b']dithiophene-2,6-diyl}{3-fluoro-2-[(2-ethylhexyl)carbonyl]- thieno[3,4-b]thiophenediyl}))

Due to their prominent properties, such as excellent photostability, high brightness, low cytotoxicity, good biocompatibility, small particle size, and excellent ability to sensitize the production of reactive oxygen species, CPNs are emerging as a promising candidate in the biological field¹⁰². In addition, these materials are highly attractive due to their water dispersibility, simple production process and easy separation steps.

CPNs possess unique signal amplification properties, high photostability, sensitive fluorescence quantum yield, and low toxicity. As a result, they are regarded as one of the most promising candidates for fluorometric detection. CPNs employ various signal transduction processes to detect desired analytes easily. Common detection strategies include disruption of Förster resonance energy transfer (FRET), photo-induced electron transfer (PET), aggregation-induced FRET, charge transfer, perturbation of conjugation, and aggregation-caused quenching (ACQ) mechanism. These techniques usually require favorable interactions between the analytes and CPNs to alter the signal effectively. CPNs used for biosensing applications toward several analytes including CD 44, hyaluronidase, glutathione S-transferase, alkaline phosphatase, glutathione, tyrosinase, biogenic amines, thiol, dopamine, hypochlorous acid, Fe(III) are summarized in Table 1.5.

Table 1.5 Conjugated polymer nanoparticles for biosensing applications

Conjugated polymer	Matrix	Size	Analyte	LOD	Reference
PFEP	PFEP/FA-HA	200–350 nm	CD 44	2.3×10^{-8} g/mL	¹⁰³
PFEP	PFEP/FA-Dox	30–50 nm	Hyaluronidase	0.075 U/mL	¹⁰⁴
PFBT	PFBT/PSMA/ GSH	27 nm	Glutathione S- transferase	1.03 ng/mL	¹⁰⁵
PFBT	PFBT/PSMA	17 nm	Alkaline phosphatase	0.01 μ U/mL	¹⁰⁶
PBEC	PBEC/PSMA	28.53 nm	Glutathione	20.4 nM	¹⁰⁷
PFO	PFO/CN-PPV	32 nm	Tyrosinase	1.1 U/L	¹⁰⁸
PFTBT-COOH	PFTBT-COOH/ CS-graft-OA	100–120 nm	Biogenic amines	10 μ M	¹⁰⁹
PF-DBT-PEG	PF-DBT-PEG NPs	24 nm	Thiol	2.56 μ g/mL	¹¹⁰
PFPBA	PFPBA NPs	120 nm	Dopamine	38.8 nM	¹¹¹
PFAN	PFAN NPs	200 nm	Hypochlorous acid	0.5 mM	¹¹²
PFP-PEG	PFP-PEG NPs	75 nm	Fe(III)	10^{-7} mol/L	¹¹³

CN-PPV: poly-[2-methoxy-5-(2-ethylhexyloxy)-1,4-(1-cyanovinylene-1,4-phenylene)], CS: Chitosan, DBT: 1,4-dithienyl benzothiadiazole, Dox: Doxorubicin, FA-HA: Fluoresceinamine-hyaluronan, GSH: glutathione, OA: Oleic acid, PBEC: poly[3-{2-[2,5-bis-(2-ethyl-hexyloxy)-4-propenyl-phenyl]-vinyl}-9-octyl-6-methyl-9H-carbazole], PEG: poly(ethylene glycol), PF: Polyfluorene, PFAN: poly[2,7-(9,9'-dioctylfluorene)-co-alt-2,5-phenylamine], PFBT: poly[9,9-dioctylfluorenyl-2,7-diyl]-alt-co-1,4-benzo-{2,1'-3}-thiadiazole], PFEP: poly{[9,9-bis(6'-(N,N,N-diethylmethylammonium)hexyl)-2,7-fluorenylene ethynylene]-alt-co-[2,5-bis(3'-(N,N,N-diethylmethylammonium)-1'-oxapropyl)-1,4-phenylene]} tetraiodide, PFO: poly(9,9-dioctylfluorenyl-2,7-diyl), PFP: conjugated polymer, PFPBA: conjugated polymer, PSMA: Poly(styrene-co-maleic anhydride)

CPNs can be produced using different techniques to achieve desired features such as size, strength, structure, and stability. Although mini-emulsion and nanoprecipitation are commonly used methods for generating CPNs, newer techniques such as the microfluidic approach and self-assembly have also been developed as shown in Figure 1.12.

1.6.1.1 Nanoprecipitation method

The nanoprecipitation method involves dissolving CPs in an organic solvent, which is then quickly injected into water and subjected to ultrasonication. After removing the organic solvents, nanoparticles with uncertain colloidal stability are obtained. Nanoprecipitation is similar to reprecipitation, but stabilizers are added to the water-miscible solvent to produce colloidal stable nanoparticles. The solvent's polarity change causes the CPs to aggregate and form corresponding nanoparticles, and these CPNs' size depends on the solution's polymer concentration^{102,114}.

The reprecipitation technique is based on the hydrophobic effect, which reduces the solvent's hydrophobicity. To carry out this method, the polymer is dissolved in a solvent like tetrahydrofuran (THF) and then rapidly injected into excess water, where the polymer has poor solubility. This causes the polymer chains to collapse due to the reduced contact area with solvated molecules. By adjusting the polymer's concentration, chemical structure, and molecular weight, this method can produce spherical nanoparticles ranging from 3 to 150 nm in size^{115,116}. Our study selected the reprecipitation method to produce conjugated polymer nanoparticles. (Figure 1.11.).

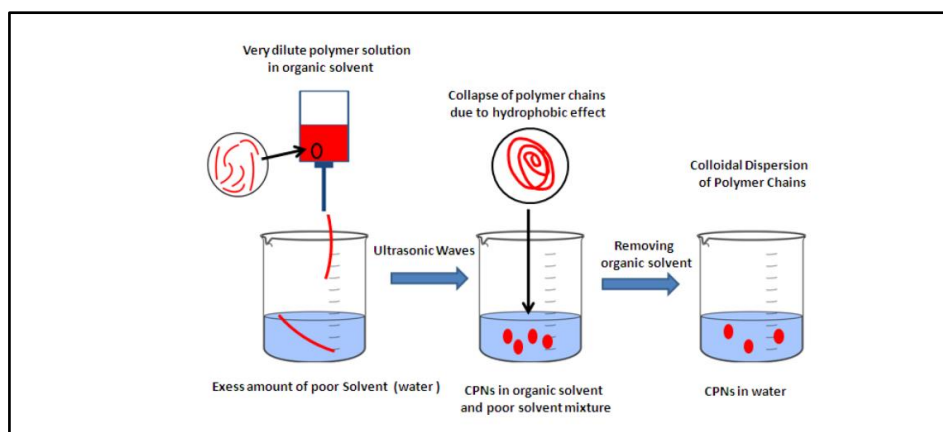


Figure 1.11. Schematic diagram of production of conjugated polymer nanoparticles via reprecipitation¹¹⁵

1.6.1.2 Mini-emulsion method

The mini-emulsion method is used to prepare CPNs by utilizing surfactants to prevent emulsion droplet aggregation. Firstly, CPs are dissolved in an apolar solvent, such as dichloromethane (DCM), and then introduced into an aqueous solution containing surfactant molecules. Next, ultrasonic treatment is used to uniformly disperse the organic phase within the water phase to form a homogeneous emulsion. Finally, CPNs are obtained by evaporating the organic solvent. This method has made it easier and less time-consuming to chemically modify nanoparticles with specific functional groups compared to before its development¹¹⁴.

1.6.1.3 Microfluidic approach

The method of microfluidics has garnered significant interest from researchers due to its ability to adjust the size of nanoparticles by altering the flow rate of the polymer and the volume of poor solvent. This high-throughput technique can regulate the morphology of the CPs by utilizing the tiny volume of fluid in the microscale fluid channel, producing spherical or linear CPNs. Preparing CPNs using a microfluidic system principally involves the creation of the nucleus and the crystal fiber. The

polymer solution is cooled and nucleated under flow conditions. Upon UV light irradiation, the degree of order and planarization between the main chains of polymerization is enhanced, and the CPs aggregate, decreasing solubility and forming highly ordered crystal fibers¹¹⁴.

1.6.1.4 Self-assembly method

Self-assembly is a novel approach for creating CPNs that has gained popularity recently. This method involves the spontaneous construction of polymer molecules into nanoparticles with distinct shapes and structures through different interactions, such as electrostatic, hydrogen bonding, van der Waals forces, and hydrophobic interaction.

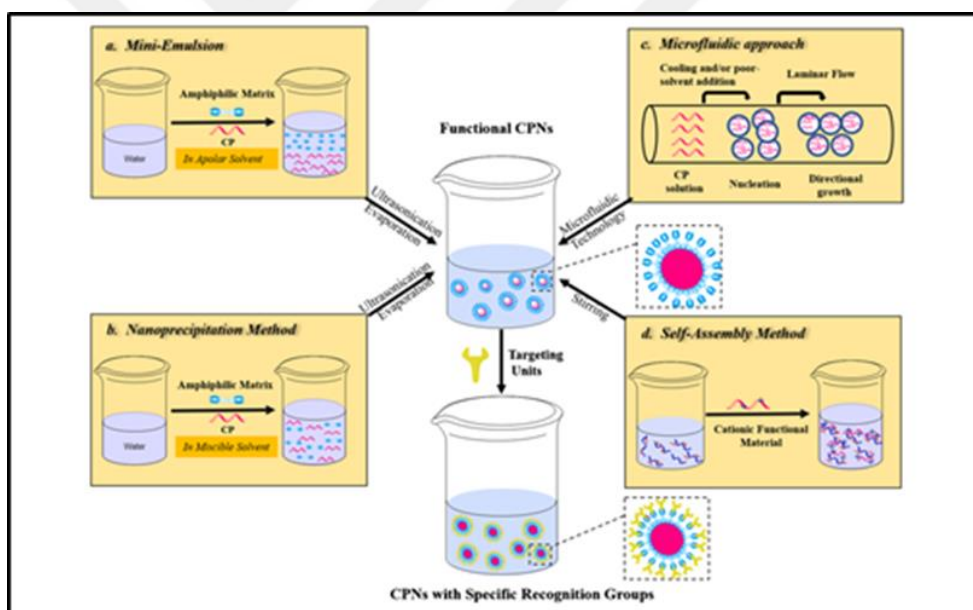


Figure 1.12. Fabrication methods of CPNs (a) Mini-Emulsion (b) Nanoprecipitation (c) Microfluidic approach (d) Self-Assembly¹¹⁴

1.6.2 Metal Nanoparticles

Nanoparticles (NPs) have been integrated into electrochemical sensors in order to enhance the performance of the electrode. This is accomplished via increased surface area, improved immobilization efficiency, strengthened molecular recognition, and amplified transduction signals.

NPs can be combined with enzymes in order to enhance the sensitivity of the sensor, induce the conductivity and increase the surface area¹¹⁷.

For biosensor fabrication, metal nanoparticles such as silver (Ag), gold (Au), platinum (Pt), and palladium (Pd) are the most studied NPs candidates. AuNPs and AgNPs both have good electronic properties and conductivity¹¹⁷. However, since gold is relatively expensive, silver is preferred for the construction of enzyme-based biosensors. In addition to having relatively good conductivity, Pd and Pt also offer catalytic properties^{117,118}.

1.6.2.1 Silver Nanoparticles

Chemical, physical, or biological methods can be used to synthesize the silver nanoparticles. Chemical and biological techniques are based on the reduction of Ag^+ to Ag^0 by utilizing appropriate agents¹¹⁹. Due to its elemental properties, silver nanoparticles (AgNPs) have higher activity than gold nanoparticles (AuNPs). In addition, AgNPs have higher conductivity than that of AuNPs. Therefore, AgNPs based biosensors generally provide better response time¹²⁰.

1.6.2.1.1 Chemical Methods

Chemical methods for nanoparticle synthesis can be subdivided into chemical reduction¹²¹, electrochemical methods¹²², pyrolysis¹²³, and irradiation-assisted chemical techniques¹²⁴. Its convenience and simplicity are the benefits of chemical reduction.

Chemical synthesis of AgNPs in a solution includes three main components:

- metal precursors (commonly silver nitrate or silver sulfadiazine)
- capping/stabilizing agents (chitosan, polyethylene glycol, polyvinyl alcohol, bovine serum albumin, ethylene diamine tetra acetic acid etc.)
- reduction agents (sodium borohydride, hydrazine, hydrogen, natural such as chitosan and cellulose, or synthetic polymers such as natural rubber and co-polymers etc.)

The production of colloidal nanoparticle solutions from the reduction of silver salts occurs in two main stages, which are nucleation and subsequent growth. The shape and size of the produced nanoparticles are related to these stages. These stages can be controlled by adjusting the experimental conditions such as the pH of the solution, temperature, concentration of Ag precursor, and reducing and stabilizing agents^{125,126}. In the first chapter, Chit-AgNPs were synthesized using the chemical reduction method.

1.6.2.1.2 Biological Methods

In biological synthesis, polysaccharides, microorganisms, plant extracts and proteins are preferred as nontoxic components instead of toxic reducing agents. Biological approaches based on microorganisms such as yeast¹²⁷, bacteria¹²⁸, and fungi¹²⁹ have been reported in the literature. This technique can be considered as a green method compared to chemical and physical approaches. Although it has a low cost it is challenging to produce a large quantity of AgNPs via a biological approach¹³⁰.

1.6.2.1.3 Physical Methods

Physical methods include arc-discharge¹³¹, ball milling technique¹³², magnetron sputtering¹³³ and physical vapor deposition¹³⁴. In contrast to chemical reduction

methods, physical methods don't contain toxic materials. They are usually fast processing methods¹²⁶.

1.6.2.1.3.1 Silver nanoparticles for biosensor applications

AgNPs modified electrodes provide increased surface area and superior conductivity compared to unmodified electrodes. These electrodes can detect several types of analytes such as glucose^{135,136}, ABTS¹³⁷, H₂O₂¹³⁸, and urea¹³⁹ (Table 1.6).



Table 1.6 AgNPs including electrochemical enzyme-based biosensors for detection of several analytes

Modified Electrode	Analyte	LOD	Real Sample	Reference
Ag-HPB NPs/AChE/SPE	TCF	2.3 pg/mL	Apple samples	140
WT-AgNPs/MCPE	Glucose	5x10 ⁻³ μm	Fruit juice	141
rGO@AgCys FASOx/GCE	Sulfite	0.007 mg/L	Fruit samples	142
PCT/Ab/AgNP/SLG@ITO:	PCT	0.55 ng/mL	NR	143
AgNPs-Pdop@Gr/GCE	Adenine	2.0 nm	fish sperm	144
	Guanine	4.0 nm	fish sperm	
ss-DNA/Agnano/PPAA/	Adriamycin	3.2x10 ⁻³ nm	NR	145
MWCNTs-COOH/GCE				
AChE/Ag@Ti ₃ C ₂ T _x /GCE	ATCl	3.27x10 ⁻³ nm	NR	146
f-MWCNT/AgNP/CE	Dopamine	0.27 μm	Human urine	147

AChE: acetylcholinesterase, Ag-HPB NPs: Hollow Prussian blue with ultrafine silver nanoparticle, AgNPs: silver nanoparticles, ATCl: Acetylthiocholine chloride, Cys-FA: Cysteine and folic acid functionalization, f-MWCNT: functionalized multi-walled carbon nanotube, GCE: Glassy carbon electrode, ITO: Indium tin oxide, MCPE: modified carbon paste electrode, MWCNTs-COOH: Multiwalled carbon nanotubes with carboxyl groups, PPAA: poly(trans-3-(3-pyridyl) acrylic acid, Pdop@Gr: polydopamine (Pdop)@graphene (Gr), PCT: Procalcitonin, rGO: Reduced graphene oxide, SOx: Sulfite oxide, SPE: Screen-printing electrode, TCF: trichlorfon, Ti₃C₂T_x: transition metal carbides (MXenes), WT-AgNPs: green synthesized waste tea based silver nanoparticles

Naghieb et al. designed glucose biosensing platform by using a polyvinylalcohol/graphene oxide/silver nanocomposite. Under optimized conditions LOD values and linear range were evaluated. The developed biosensor indicated improved linear range and LOD values 150 nM-10 mM and 25 nM, respectively¹³⁶.

In another work, a laccase-based amperometric biosensor based on ureasil–chalcogenide glass composite, including silver nanoparticles, was fabricated to detect waste water pollution. It was concluded that the efficacy of the created biosensor was positively influenced by interactions between ureasil–chalcogenide glass composite and silver nanoparticles¹³⁷.

Wang and Zheng developed a non-enzymatic hydrogen peroxide biosensor via electrodeposition of silver nanoparticles onto zinc oxide-modified glassy carbon electrode to enhance the sensitivity. Sensitivity increased 3-fold with this biosensor in comparison to only silver nanoparticle-modified biosensor¹³⁸.

Crespilho et al. constructed an amperometric urease biosensor. For this purpose, indium tin oxide electrode was modified with an electroactive membrane where polyvinyl alcohol and silver nanoparticles stabilized in polyaniline¹³⁹.

1.7 Chitosan

Chitosan (linear β -1,4-linked polysaccharide) is an essential natural polymer used in several biomedical applications since it has many advantages, such as biocompatibility, biodegradability, and nontoxicity. Chitosan can be obtained from the deacetylation of chitin (Figure 1.13.). Chitosan offers different chemical, biological, and physical properties. It can be utilized in the form of membranes, films, and three dimensional structures^{148,149}. Chitosan possesses excellent film-forming ability as well as good adhesion that makes it suitable matrix for electrochemical biosensor applications. Also, chitosan can be utilized to provide solvent resistance and enzyme immobilization matrix¹⁵⁰.

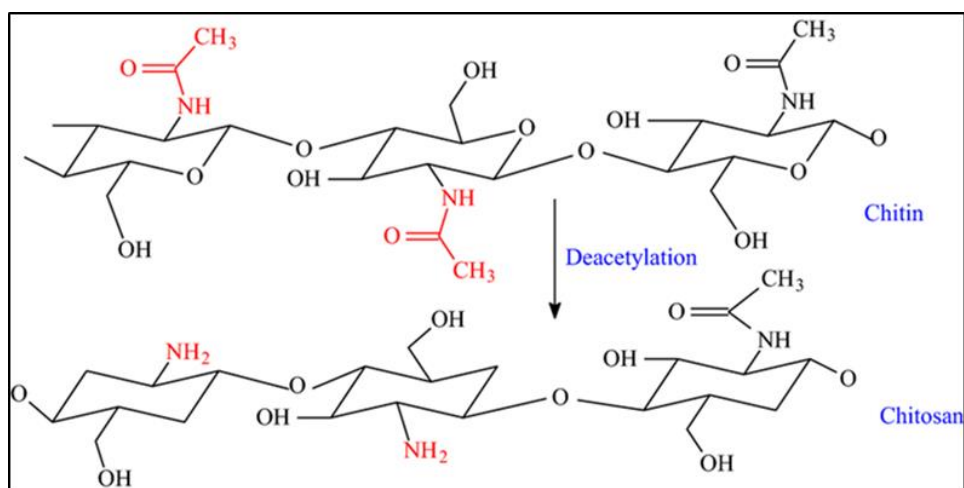


Figure 1.13. Formation of chitosan from chitin¹⁵¹

According to the abovementioned reasons, chitosan is widely used to fabricate biosensor toward detecting for several analytes such as glucose^{152,153}, drug¹⁵⁴, nucleic acids¹⁵⁵, phenolic compounds^{156,157}, pesticides¹⁵⁸ and xanthine¹⁵⁹, etc (Table 1.7).

Artigues et al. studied glucose biosensors with Ti/TiO₂NTAs/ppHEMA-co-EGDA/pgPFM/GOx/Chit. The electrochemical platform was fabricated by attaching PFM (pentafluorophenyl methacrylate) onto a customized plasma polymerized hydrogel made of HEMA-co-EGDA (ppHEMA-co-EGDA), which forms a layer over a collection of titanium dioxide nanotubes (TiO₂NTAs) and chitosan film was used to increase the long-term stability of the biosensor¹⁵³.

Another amperometric glucose biosensor was fabricated to test the direct electron transfer of glucose oxidase. A glassy carbon electrode was modified by entrapment of glucose oxidase into carbon nanotube-chitosan composite without the additional use of a crosslinker¹⁵².

In order to detect phenolic compounds, an electrochemical biosensor was constructed via entrapment of polyphenol oxidase into chitosan solution and then it was immobilized onto surface of polyaniline-porous polyacrylonitrile-graphene nanostructure modified electrode¹⁵⁶.

Narang et al. developed a nanocomposite-based electrochemical biosensor for monitoring acetaminophen (an analgesic drug). Au electrode was modified via electrodeposition chitosan film containing core-shell $\text{ZrO@Fe}_3\text{O}_4$ nanoparticles. Chitosan film was preferred to enhance the binding performance of horseradish peroxidase¹⁵⁴.

An electrochemical biosensor based on immobilized horseradish peroxidase onto poly(aniline-co-o-aminobenzoic acid) was fabricated and coated by chitosan film to determine hydrogen peroxide¹⁶⁰.

In order to detect xanthine in fresh meat extract, an amperometric xanthine biosensor modified with gold coated iron nanoparticles/chitosan/xanthine oxidase was constructed by Devi et al.¹⁵⁹

In another study conducted by Diaconu et al. an electrochemical laccase biosensor for monitoring polyphenols in cultivated plants was fabricated. Enzyme immobilization was performed via entrapment into MWCNT-chitosan nanocomposite as an effective method¹⁵⁷.

An amperometric tyrosinase biosensor based on chitosan-quantum dots films was fabricated to detect catechol. These nanocomposite films demonstrated many advantages in immobilizing enzymes, including film-forming capacity, biocompatibility, and hydrophilicity. Therefore, the modified electrode has grand potential for other biosensor platforms¹⁶¹.

The amperometric Pt/ZnO/AChE/Chit electrode was developed to monitor both melamine and urea in cow milk. Detection limits were obtained as 3 pM and 1 pM for melamine and urea respectively¹⁶².

To determine maltose, bi-enzyme based electrochemical biosensor was developed via co-immobilization of α -glucosidase and pyranose oxidase. Immobilization was performed by crosslinking these two enzymes in chitosan and carbon nanotubes via glutaraldehyde. Real sample analysis was carried out in beer sample. It was

concluded that fabricated biosensor could be used during the beer fermentation process¹⁶³.



Table 1.7 Chitosan including electrochemical enzyme based biosensor toward different types of analytes

Modified Electrode	Analyte	Real Sample	Reference
Ti/TiO ₂ NTAs/ppHEMA-co-EGDA/pgPFM/GOx/Chit	Glucose	Food samples	153
GRA/Pani-Pan/Chit-PPO	Phenolic compounds	Sea water Fresh water	156
Pt/ZnO/AChE/Chit	Melamine Urea	Cow milk sample	162
Au/Chit/ZrO@Fe ₃ O ₄ NP/HRP	Acetaminophen	Paracetamol	154
GCE/Tyr/Chit/QDs	Catechol	Water samples	161
GCE/HRP-p(Ani-co-o-Aba)/Chit	H ₂ O ₂	NR	160
PGE/Fe-NPs@Au/Chit/XOD	Xanthine	Fish meat extract	159
GE/AG/PyOx/Chit-CNT	Maltose	Beer sample	163
Au/Chit-MWCNT/Lac	Phenolic compounds	Total polyphenolic content (<i>S. officinalis</i> and <i>M. piperita</i> extracts)	157
GCE/Chit/CNTs/GOx	Glucose	NR	152

AChE: acetylcholinesterase, AG: α -glucosidase, Au: gold, Chit: chitosan, CNT: carbon nanotube, Fe-NPs@Au: gold coated iron nanoparticles, GCE: glassy carbon electrode, GE: Graphite electrode, GOx: glucose oxidase, GRA: graphene, HRP: horseradish peroxidase, MWCNT: multi-wall carbon nanotubes p(Ani-co-o-Aba): poly(aniline-co-o-aminobenzoic acid), Pani-Pan: Polyaniline-polyacrylonitrile ppHEMA-co-EGDA: plasma polymerized HEMA-co-EGDA, PGE: pencil graphite electrode, pgPFM: plasma-grafted pentafluorophenyl methacrylate, PPO: polyphenol oxidase, Pt: platinum, PyOx: pyranose oxidase, QDs: quantum dots, Ti: titanium, TiO₂NTAs: titanium dioxide nanotubes array, Tyr: tyrosinase, XOD: xanthine oxidase, ZnO: zinc oxide, ZrO@Fe₃O₄NP: zirconia coated iron oxide nanoparticles

Chitosan can be utilized for the formation and functionalization processes of metal nanoparticles as a stabilizer, as a reducing agent, as a shape-directing agent and as a size-controllable agent¹⁶⁴. For biomedical applications, chitosan is utilized as a well-known stabilizer for metal nanoparticles^{164,165}. Having important biological and physicochemical properties, chitosan has been widely utilized as a surface coating material for the production of metal nanoparticles. Coating metal nanoparticles with chitosan resulted in improved biocompatibility of these nanoparticles, and they can be used as carriers for drugs¹⁶⁶.

Chitosan as a stabilizer

Due to being positively charged, chitosan can be used as a good stabilizer for synthesizing metal nanoparticles. Because of Van der Waals interactions, metal nanoparticles can aggregate in a solution. However, chitosan can form a steric barrier around the production of metal nanoparticles, preventing aggregation through charge repulsions which is depicted in Figure 1.14. Several studies have reported the performance of chitosan as a stabilizer for the synthesis of AgNPs¹⁶⁷ and AuNPs^{168,169}.

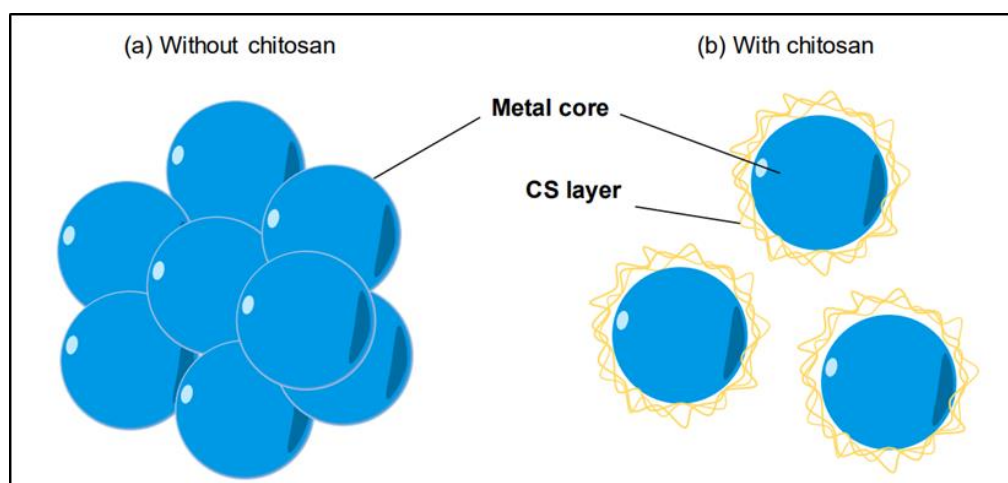


Figure 1.14. The effect of chitosan as a stabilizer for synthesizing metal nanoparticles a) without chitosan, b) with chitosan¹⁶⁴.

Chitosan as a reducing agent

Hazardous reducing agents could be used to produce nanoparticles via chemical methods. Due to toxic reducing agents, these synthesized nanoparticles could not be directly utilized for biomedical purposes. Therefore, green methods with green materials become essential for nanoparticle synthesis. Chitosan has been reported as both a green reducing agent and a stabilizing agent for the green production of AgNPs^{170,171}, AuNPs^{170,172,173} and CuNPs¹⁷⁴.

1.7.1 Chitosan-Capped Silver Nanoparticles

Silver nanoparticles can be coated with different polymers to improve the properties such as physicochemical properties, cellular uptake, and cellular interactions^{170,175,176}. It was reported that chitosan-functionalized silver nanoparticles were synthesized and utilized as an antibacterial agent in essential oils. In this study, chitosan was selected to stabilize silver nanoparticles as well as improve their biocompatibility of these nanoparticles¹⁷⁷.

In order to be used in both antibacterial and anti-lung cancer applications, chitosan-capped silver nanoparticles were produced via biological method (green production method) using *Sida acuta*. While the antibacterial properties of these nanoparticles were tested against both *K. pneumonia* and *S. aureus*, their anti-cancer activity was also studied against the human lung cancer cell line (A549)¹⁷⁸.

Wei et al. studied the antimicrobial activity of chitosan-capped silver nanoparticles toward both Gram negative (*E. coli*) and Gram-positive bacteria (*S.aureus* and *B. subtilis*). Silver nitrate salts reduced by chitosan showed both the bactericidal effect of silver nanoparticles and the cationic effect of chitosan¹⁷⁹.

1.8 Phenolic Compounds

Phenolic compounds are widely used for industrial purposes such as paint, medical industry, pharmaceuticals, textiles etc ^{180,181}. These compounds are toxic and cause environmental pollution even at low concentrations. Some of the phenolic compounds with their molecular formulas are shown in Figure 1.15. Phenolic compounds have toxicity the range of 9-25 mg/L while US Environmental Protection Agency (US EPA) has prescribed 1.0 ppb phenol as a threshold for water purity¹⁸¹. According to the European Commission, 0.001 mg/L was defined as a concentration limit for phenolic compounds. High concentration of phenolic compounds in drinking water may cause long-term health problems including skin burns, liver damage or kidney problems etc.¹⁸².

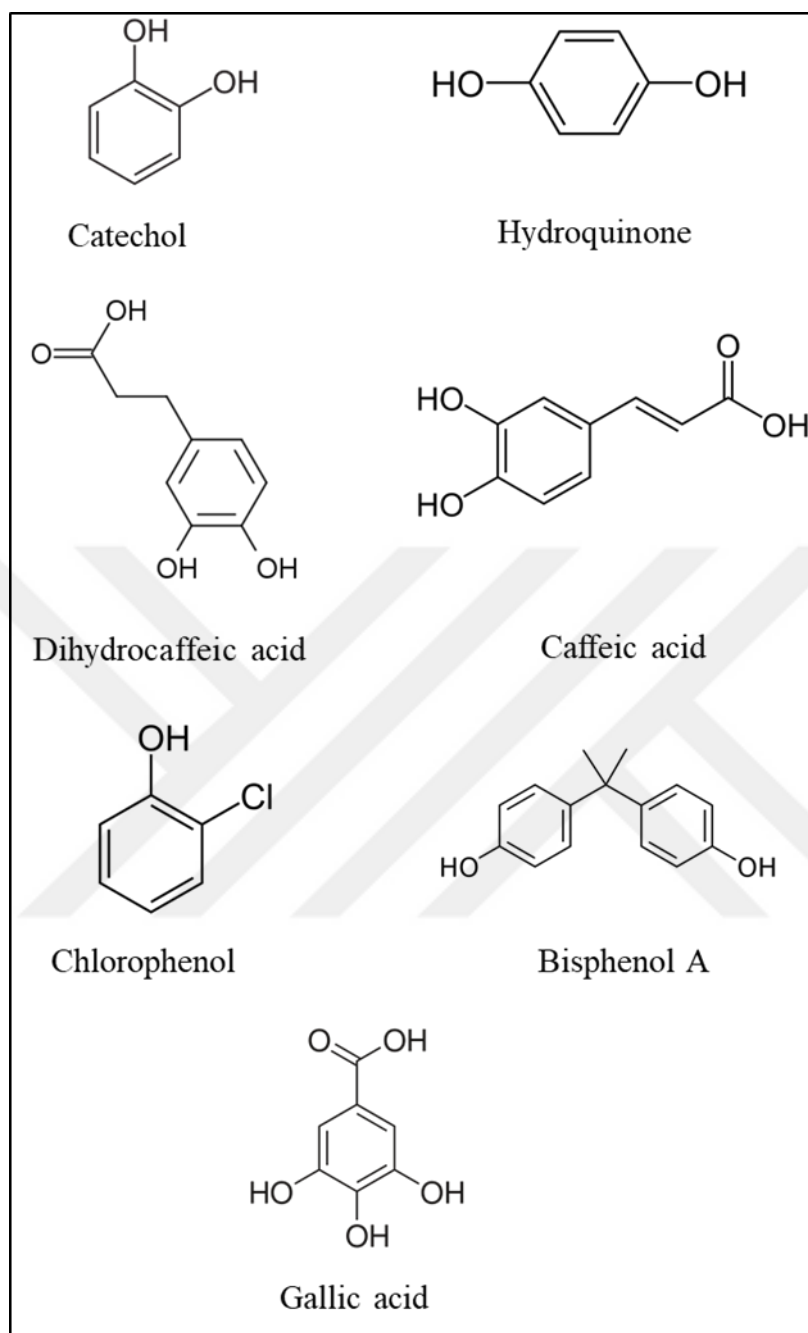


Figure 1.15. Widely used phenolic compounds

1.8.1 Catechol

Catechol, also known as pyrocatechol and 1,2-dihydroxybenzene, is an organic phenolic compound (Figure 1.16.). Catechol can be found in fruits, vegetables, tobacco, plants and beverages such as wine, tea, coffee, beer^{183,184}. Catechol is used as a reagent in many fields, including the pharmaceutical industry, photography, plastics, rubber, textile, pesticides, cosmetics^{184–186}. Catechol has a low degradation rate and high toxicity^{187,188}. According to the US Environmental Protection Agency (US EPA), the International Agency for Research on Cancer (IARC) and European Union (EU) catechol can be classified as a human carcinogen (Group 2B) and an environmental pollutant^{189–192}. Low amounts of catechol can cause several health problems including high blood pressure, retinal damage etc. Moreover, excessive amount catechol may cause formation nervous disorder, Parkinson's disease, Alzheimer's disease and damage to organs^{187,193}. As a result, it is important to develop sensitive, fast and cheap systems to detect catechol.

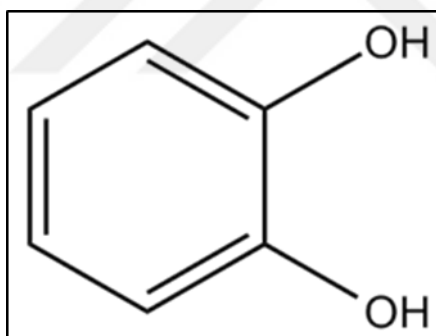


Figure 1.16. Chemical structure of catechol

Several analytical methods are applied to detect catechol including high-performance chromatography^{50,51}, fluorosence⁵⁰, gas chromatography-mass spectrometry¹⁹⁴, chemiluminescence^{195,196}. However, these methods are expensive, time-consuming, and require special equipment. In comparison to other methods, enzyme-based electrochemical biosensors offer sensitivity, selectivity, ease of use, and cost-effectiveness. Analytical properties of electrochemical laccase biosensing systems for catechol determination are listed in Table 1.8.

Table 1.8 Electrochemical laccase biosensor for catechol determination

Modified Electrode	Detection Method	LOD (μM)	Linear Range (μM)	Reference
GE/P(DTFBT)/Lac	A	14	25-400	85
GCE/M1/Lac	A	9.86	5-175	89
CPE-Lac-a-Fe ₂ O ₃ NC	A	4.28	8-800	197
GCE/Chit/AP-rGO/Lac	A	7.0	15-700	198
MWCNTSs-PVA-AuNPs-Lac	A	5	10-200	199
GCE/GO,chit/ZnO-Al ₂ O ₃ /AuNPs/Lac	DPV	3.1	0.5-40	200
GCE/PANI/GA/Lac	CV	2.07	3.2-19.6	201
GCE/Nafion/Lac/CFNs/Cu	A	1.18	9.95-9760	202
CB-SPE/e-Lac	A	2.0	2.5-50	203

A: Amperometry, CV: Cyclic voltammetry, DPV: Differential pulse voltammetry

Considering the analytical properties of laccase biosensors listed in Table 1.8, the thesis aimed to develop biosensors with lower LOD values and a more comprehensive working range for detecting catechol. This research has the potential to inspire future advancements in biosensor technology. Additionally, advanced materials are gaining traction in addressing challenges related to the close relationship between the element and its support. Researchers are also examining innovative immobilization techniques to improve the biosensor's usability and storage stability. Within the thesis framework, two promising nanomaterials were developed as support materials for immobilization, and their potential as laccase biosensors was investigated.

1.9 Scope of the Thesis

The selection of suitable nanomaterials greatly enhances the fundamental analytical characteristics of biosensors, including sensitivity, the limit of detection (LOD), and stability. Additionally, techniques for immobilizing enzymes improve the effectiveness of electron transfer between the enzyme's redox-active site and the electrode. The aim of the thesis is to develop simple, selective, and sensitive electrochemical laccase biosensors for the determination of catechol. This research examined the effectiveness of Chit-AgNPs nanocomposite and P-PBF NP (conjugated polymer nanoparticles) as individual support materials for laccase immobilization in conjunction with a transducer for electrochemical biosensors. Therefore, two types of biosensors were developed, characterized, and tested: a nanocomposite-based amperometric laccase biosensor and a conjugated polymer nanoparticle-based amperometric laccase biosensor.

In the first study, Chit-AgNPs nanocomposite was synthesized by using a chemical reduction method and then characterized. The chemical reduction technique is a well-defined, the most common method due to its convenience and simplicity. In addition, high yields can be obtained by this method. Ag is one of the best conductor among the metals. Therefore, AgNPs have been widely used in biosensing applications. However, they have some drawbacks, such as instability and less straightforward surface chemistry. Chitosan was used to stabilize these nanoparticles and overcome these disadvantages. Chitosan is the one of the most studied natural polymer. In addition, chitosan is advantageous for improving electron transfer and electric conductivity. A new laccase biosensor was developed by modifying graphite electrodes with Chit-AgNPs nanocomposite. To our knowledge, the Chit-AgNPs nanocomposite has been utilized for the first time to fabricate amperometric laccase biosensors for catechol detection. Catechol was used as a target analyte. Optimization studies were performed to improve the performance of biosensors. Following these studies, the developed biosensor was tested regarding analytical merits, selectivity, sensitivity, reproducibility, and stability. In the last step, the

proposed biosensor was compared with other laccase-based catechol detection platforms reported in the literature.

In the second study, a novel conjugated polymer named as P-PBF was synthesized and characterized. A novel conjugated polymer nanoparticle was produced via reprecipitation using this novel conjugated polymer. To our knowledge, there is no research about conjugated polymer nanoparticle-based electrochemical biosensor constructions. The produced conjugated polymer nanoparticle was characterized. A novel laccase biosensor was constructed by modifying graphite electrodes with the conjugate polymer nanoparticles. Optimization studies were performed to improve the performance of biosensors. Following these studies, the developed biosensor was tested regarding analytical merits, selectivity, sensitivity, reproducibility, and stability. In the last step, the proposed biosensor was compared with other laccase-based catechol detection platforms reported in the literature.

CHAPTER 2

GE/Chit-AgNPs/Lac BIOSENSOR

Selecting the appropriate matrix and immobilization method is crucial in developing biosensors. The first part of the discussion focuses on creating a laccase biosensor with high sensitivity and specificity by immobilizing laccase on a chitosan-silver nanocomposite to detect catechol, which is a significant achievement. The study also investigates the role of the nanocomposite-modified electrode in enzyme immobilization. The chitosan-silver nanocomposite was synthesized using a chemical reduction technique. The study involved optimizing the quantity of chitosan-silver nanocomposite and laccase pH to ensure consistent outcomes. Electrochemical techniques such as FE-SEM and CV were employed to examine the electrode surface. Amperometry was utilized to measure catechol levels based on enzyme activity, and a comprehensive analysis of the biosensor's characteristics was conducted.

2.1 Materials and Methods

Chemical used for experiments are listed in Table 2.1.

Table 2.1 Chemicals used in the study

Chemicals	Supplier
Acetic acid, glacial	Labkim (Turkey)
Ascorbic acid	Sigma-Aldrich (USA)
Chitosan, low viscous	Fluka-BioChemica (Israel)
Citric acid	Sigma-Aldrich (USA)
Ethanol	Merck (Darmstadt, Germany)
Ferrocyanide	Fisher Scientific Company (USA)
Ferricyanide	Sigma-Aldrich (USA)
Glucose	Sigma-Aldrich (USA)
Glutaraldehyde (50% wt in H ₂ O),	Sigma-Aldrich (USA)
Hydrochloric acid fuming, 37%	Merck (Darmstadt, Germany)
Laccase (Lac, EC 1.10.3.2. from <i>Trametes versicolor</i> , 21.8 U/mg)	Sigma-Aldrich (USA)
Phenol	ATABAY's chemicals (Turkey)
Folic acid	Acros Organics (USA)
Pyrocatechol	Sigma-Aldrich (USA)
Silver nitrate	Kimetsan (Turkey)
Sodium acetate	Sigma-Aldrich (USA)
Sodium dihydrogen phosphate	Sigma-Aldrich (USA)
Sodium hydroxide, minimum 98%	Sigma-Aldrich (USA)
Sodium hydrogen phosphate dihydrate	Merck (Darmstadt, Germany)

2.2 Instrumentation

PalmSense potentiostat (EmStat 3, Netherlands) with a three-electrode system was utilized for amperometry measurements. During experiments, graphite electrode (Ringsdorff Werke GmbH, Bonn, Germany, typeRW001, 3.05 mm diameter, and 13% porosity) was used as the working electrode; platinum (Pt) wire electrode was used as the counter (auxiliary) electrode, and silver (Ag) wire electrode was used as the reference electrode (Figure 2.1.). The reference electrode measures the potential on the working electrode. The counter electrode is responsible for balancing the current. All measurements were carried out in ambient conditions.

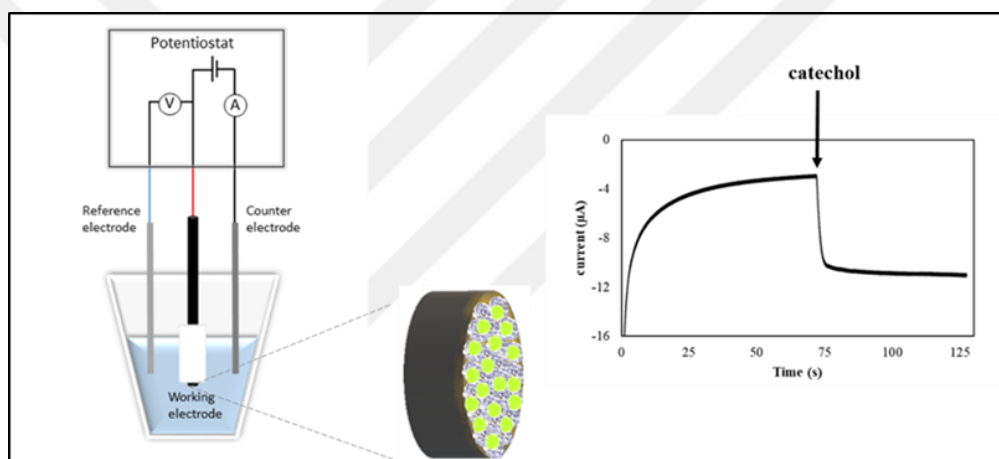


Figure 2.1. Amperometric measurement with a three electrode system

A UV visible spectrometer (Jasco V-730, Easton, MD, USA) was used to analyze the spectral properties of synthesized nanocomposites (Chit-AgNPs) by scanning over a wavelength ranging from 300 to 700 nm.

An inductively coupled plasma-optical emission spectrometer (ICP-OES) (Perkin Elmer Optima 4300 DV) was used to determine Ag concentration in the nanocomposite.

Transmission Electron Microscope (TEM) micrographs were obtained using Tecnai G2 Spirit BioTwin CTEM microscope (FEI, BOTWIN, USA) to analyze the morphology of the nanocomposite.

The nanocomposite's average hydrodynamic diameter and polydispersity index (PDI) were measured by dynamic light scattering technique using a zeta sizer Nano-ZS 90 analyzer (Malvern Instruments Ltd, Malvern, UK). The zeta potential value was obtained by a zetasizer Nano-ZS 90 analyzer (Malvern Instruments Ltd, Malvern, UK).

Surface characterization of the modified electrodes was carried out by field emission scanning electron microscopy (FESEM) (FEI, QUANTA 400F).

2.3 Experimental

2.3.1 Synthesis of Chit-AgNPs Nanocomposite

For the synthesis of Chit-AgNPs nanocomposites, the chemical reduction method described in the literature²⁰⁴ was applied with a slight modification in which concentrations of NaOH and AgNO₃ were slightly altered. Amount of chitosan, NaOH, and AgNO₃ are given in Table 2.2.

Table 2.2 Amount of chitosan , NaOH and AgNO₃ used in the synthesis of chitosan silver nanocomposites

	Chitosan	NaOH	AgNO ₃
NC ₁	0.2 g chit+100 ml (1% HAc)	3.35 ml 1.0 M NaOH	3.0 mL 0.1 M AgNO ₃
NC ₂	0.2 g chit+100 ml (1% HAc)	2.0 ml 3.0 M NaOH	3.0 mL 0.1 M AgNO ₃
NC ₃	0.2g chit+100 ml (1% HAc)	2.0 ml 5 M NaOH	3.0 mL 0.2 M AgNO ₃

Briefly, 0.2 g chitosan was dissolved in 100 mL (1% HAc) and stirred vigorously for 30 min. Then, the desired amount of NaOH (Table 2.2) solutions were independently added to the chitosan solution to obtain NC and stirred for 30 min. The solution was

heated up to boiling under constant stirring. 3.0 mL AgNO_3 (0.1 or 0.2 M) solution was added into the resultant solution to obtain NC_3 . The colorless solution gradually turned into light yellow and yellowish brown after the addition of AgNO_3 , indicating the formation of nanocomposite (Figure 2.2.).

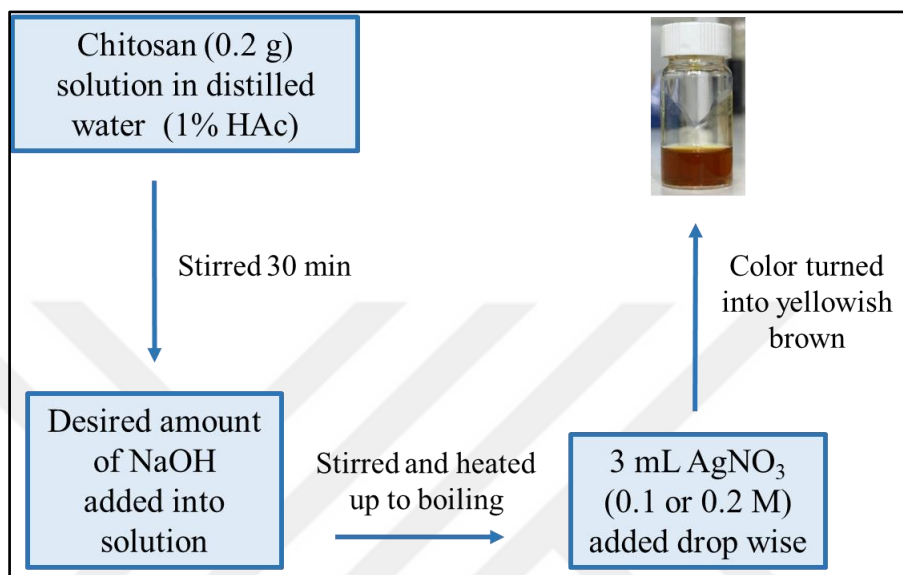


Figure 2.2. Schematic representation of synthesis of Chit-AgNPs nanocomposite

The formation of Chit-AgNPs nanocomposites (NC_1 , NC_2 , and NC_3) was analyzed and confirmed by a UV-visible spectrometer. ICP-OES was used to determine the concentration of elemental silver in the synthesized nanocomposite in solution. The dimensions and structure of the fabricated silver-chitosan nanocomposite (NC_3) were examined using a Transmission Electron Microscope (TEM) (Tecnai G2 Spirit BioTwin CTEM microscope (FEI, BOTWIN, USA)) at an accelerating voltage ranging from 20 to 120 kV. Ten microliters of the Chit-AgNPs solution (diluted 1:3 in PBS) were dispensed onto the grids using a micropipette for the TEM samples prepared with the utmost care. The solution was then left to air-dry at room temperature for 30 minutes.

2.3.2 Construction of Biosensor (GE/Chit-AgNPs/Lac)

Graphite electrodes were polished with emery paper to develop the modified electrode, followed by rinsing with distilled water and subsequent drying. The construction of the GE/Chit-AgNPs/Lac electrode proceeded as follows: Initially, a Chit-AgNPs solution was prepared through a 1:3 dilution of the stock solution in PBS (50 mM, pH: 7.0). Subsequently, 5.0 μL of this solution was carefully applied to the surface of the graphite electrode, and the modified electrode was allowed to air-dry at room temperature for 1 hour. For the immobilization of the enzyme, 5.0 μL of a laccase solution (10.9 U) and a glutaraldehyde solution (GA) (5 μL , 1% in H_2O) were evenly deposited onto the surface of the modified electrode. The modified electrode was then allowed to dry for an additional 2 h at room temperature. Before conducting amperometric measurements, the electrodes were thoroughly washed with distilled water to eliminate any unbound enzyme molecules (Figure 2.3.).

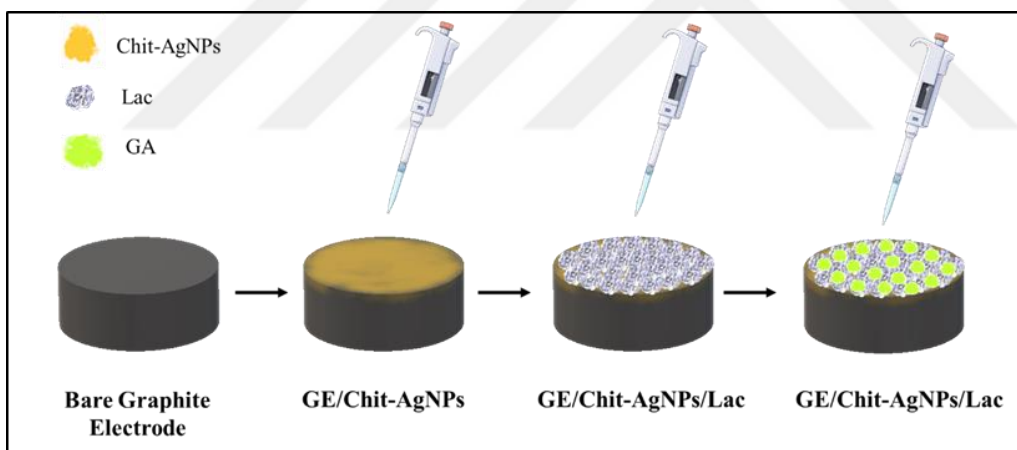


Figure 2.3. Schematic representation of GE/Chit-AgNPs/Lac biosensor

2.3.3 Amperometric Measurements for Detection of Catechol

Amperometric measurements were carried out in a reaction cell containing 10 mL sodium acetate buffer (50 mM, pH: 5.5). The electrodes were placed into the reaction medium that connected to a potentiostat, and the response of the modified biosensor was monitored by applying a constant potential (-0.3 V). The response signal is based

on oxygen consumption from the oxidation of pyrocatechol by laccase. After each measurement, the reaction cell solution was refreshed, and each electrode was washed with distilled water.

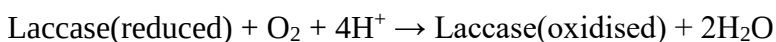
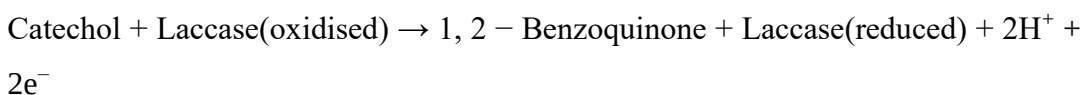
During amperometric measurements, catechol solution (250 μM) was added to the reaction cell medium after the background current reached a stable value. The current immediately decreased after catechol addition and then reached a steady state. ΔI represented the resulting current difference recorded. The calibration curve graph, a precise representation, was constructed by plotting ΔI (current difference) against analyte concentration, ensuring the accuracy of our results.

$$\Delta I = I_2 - I_1$$

I_1 = Before catechol injection

I_2 = After catechol injection

The mechanism between laccase and its substrates in determining catechol involves converting catechol to 1,2-benzoquinone and electrocatalytically reducing oxygen to water at the electrode surface²⁰⁵.



2.3.4 Optimization Studies of Constructed Biosensor

2.3.4.1 Optimization of Nanocomposite Amount

The Chit-AgNPs solution was diluted in PBS, and a 1:3 solution was obtained. 1:3 solution was prepared by diluting the Chit-AgNPs stock solution with twice the volume of PBS. Then, different diluted amounts of Chit-AgNPs solutions (3.0 μL , 5.0 μL , 7.0 μL , 10.0 μL and 15.0 μL) were immobilized onto five graphite electrode

surfaces, and these electrodes were air-dried for 1 h at room temperature. Then, 5.0 μL laccase enzyme solution (0.3 mg-6.54 U prepared in 50 mM acetate buffer, pH: 5.5) and 5.0 μL glutaraldehyde solution (1% w/w in dH_2O) and modified electrodes were air-dried for 2 h at room temperature. Before amperometric measurements, modified electrodes were washed with dH_2O . All amperometric measurements were recorded in a reaction cell, including 50 mM acetate buffer solution (pH 5.5) at room temperature in the presence of 250 μM catechol. The applied potential was -0.3 V.

2.3.4.2 Optimization of Enzyme Amount

Following a systematic approach, we prepared 6 different laccase enzyme solutions in 50 mM acetate buffer, pH: 5.5 as follows: 0.05 mg-1.09 U, 0.1 mg-2.18 U, 0.3 mg-6.54 U, 0.5 mg-10.9 U, 0.75 mg-16.35 U, and 1 mg-21.8 U. 5.0 μL Chit-AgNPs solutions (1:3 diluted in PBS) were immobilized onto six graphite electrode surfaces, these electrodes were air-dried for 1 h at room temperature. Then, 5.0 μL laccase enzyme solution and 5.0 μL glutaraldehyde solution (1% w/w in dH_2O) and modified electrodes were air-dried for 2 h at room temperature. Before amperometric measurements, modified electrodes were washed with dH_2O . All amperometric measurements were recorded in a reaction cell, including 50 mM acetate buffer solution (pH 5.5) at room temperature in the presence of 250 μM catechol. The applied potential was -0.3 V.

2.3.4.3 Optimization of pH

For pH optimization studies 50 mM buffer solutions were prepared in the pH range of 4.5-7.5 (NaOAc buffer at pH 4.5, 5.5, and 6.5, PBS buffer at pH 7.0 and 7.5), and the responses of the constructed biosensor were recorded at solutions with these five different pH values. 5.0 μL Chit-AgNPs solutions (1:3 diluted in PBS) were immobilized onto six graphite electrode surfaces, these electrodes were air-dried for 1 h at room temperature. Then, 5.0 μL laccase enzyme solution (0.5 mg-10.9 U

prepared in 50 mM acetate buffer, pH: 5.5) and 5.0 μL glutaraldehyde solution (1% w/w in dH_2O) and modified electrodes were air-dried for 2 h at room temperature. Before amperometric measurements, modified electrodes were washed with dH_2O . Amperometric measurements were conducted in different buffer solutions in the pH range of 4.5-7.5 at room temperature in the presence of 250 μM catechol. The applied potential was -0.3 V.

2.3.5 Characterization of the Constructed Biosensor

2.3.5.1 Analytical Characterization

After all optimization studies, the GE/Chit-AgNPs/Lac electrode was constructed by modifying graphite electrodes with the following solutions: 5.0 μL Chit-AgNPs solutions (1:3 diluted in PBS), 5.0 μL laccase enzyme solution (0.5 mg-10.9 U prepared in 50 mM acetate buffer, pH: 5.5) and 5.0 μL glutaraldehyde solution (1% w/w in dH_2O). A calibration curve was constructed using the modified electrode to demonstrate the relationship between the catechol concentration and the current change in the 5 - 250 μM catechol concentration range. Analytical parameters, including a limit of detection (LOD), linear range, and sensitivity, were calculated using the calibration curve.

Limit of detection (LOD) equation is as follows²⁰⁶:

$$\text{LOD} = \frac{3.3\sigma}{S}$$

σ : the standard deviation of the response

S: the slope of the calibration curve

Limit of quantitation (LOQ) equation is as follows²⁰⁶:

$$\text{LOQ} = \frac{10\sigma}{S}$$

σ : the standard deviation of the response

S: the slope of the calibration curve

Sensitivity equation is as follows²⁰⁷:

$$\text{Sensitivity} = \frac{S}{\text{Electrode area}}$$

S: the slope of the calibration curve

2.3.5.2 Determination of Kinetic Parameters

Our study has revealed the significant linear operational range of the GE/Chit AgNPs/Lac biosensor created with the best composition for detecting catechol. This was determined by examining the amperometric current responses of these biosensors to catechol at various concentrations under optimal working conditions (see Figure 2.14 and Figure 2.15). The working range was determined to be 5-250 μM from the linear section of the calibration plot. Analytical parameters such as LOD, LOQ, and sensitivity were derived from this critical biosensor linear range, highlighting the significance of our discoveries.

According to the Michaelis-Menten equilibrium model, substrate binding occurs at a quicker pace than the breakdown of the enzyme-substrate complex. This leads to the reaction reaching an equilibrium state, where the value of the equilibrium constant (K_M^{app}) signifies the enzyme's attraction toward its substrate at half the maximum velocity ($1/V_{\text{max}}$)^{208,209}.

The kinetic parameters of the proposed biosensor such as K_M^{app} and I_{max} (instead of V_{max} for amperometric biosensors) were determined from the Lineweaver–Burk plot under optimized conditions (Figure 2.4.)²¹⁰

When working with amperometric biosensors, the Lineweaver-Burk Plot (Figure 2.4.) can be used to calculate K_M^{app} and I_{max} . K_M^{app} serves as a crucial parameter that shows the enzyme's substrate affinity and signifies the concentration value at which I_{max} is achieved. In the case of a small K_M^{app} , the enzyme needs a small amount of

substrate in order to be saturated^{208,209}. Lineweaver-Burk Plot involves plotting “1/current response” vs. “1/substrate concentration” and performing a linear regression of the resulting graph, which yields an equation in the form of $y=Bx+A$ ^{210,211}. By taking reciprocal of A, $I_{\max}$ can be determined when x is zero. Similarly, K_M^{app} can be calculated as $B(A)^{-1}$ when y reaches zero at a specific point.

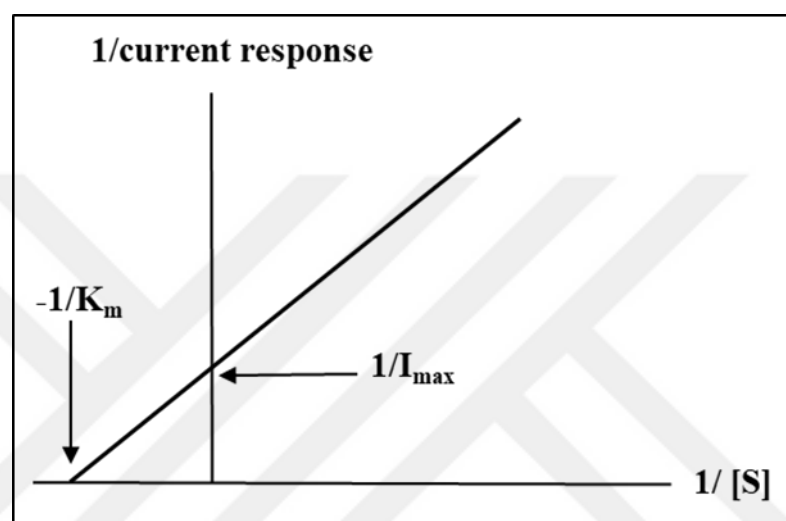


Figure 2.4. A typical Lineweaver-Burk plot

2.3.5.3 Operational Stability

Under optimum operating conditions, the GE/Chit AgNPs/Lac biosensor was constructed. Before amperometric measurements, the modified electrodes were washed with dH₂O to remove unbound enzyme molecules. Ten sequential amperometric measurements were recorded to thoroughly evaluate the operational stability of the fabricated biosensor in a reaction cell, including 50 mM acetate buffer solution (pH 5.5) at room temperature in the presence of 250 μ M catechol at -0.3 V. According to these measurements, the standard deviation was calculated.

2.3.6 Electrochemical and Surface Characterization

2.3.6.1 Electrochemical Characterization

To examine the electroactive surface area of electrodes, a cyclic voltammetry technique was carried out. Cyclic voltammetry was conducted in a buffer solution including 5.0 mM $\text{Fe}(\text{CN})_6^{3-/4-}$, 0.1 M KCl, and 50 mM at pH 5.5 acetate buffer. The Randles-Sevcik equation was used to calculate the electroactive surface areas of each surface electrode. Randles-Sevcik equation is as follows²¹²:

$$I_p = 2.69 \times 10^5 A D^{1/2} n^{3/2} V^{1/2} C \quad (3)$$

I_p : peak current (A)

A: area of the electrode (cm^2)

D: diffusion coefficient of the molecule in solution (cm^2/s)

n: the number of electrons involved in the redox reaction

V: scan rate (V/s)

C: the concentration of the probe molecule in the bulk solution (mol/cm^3)

2.3.6.2 Surface Characterization

To analyze the surface morphology of the electrodes, the following three electrodes were prepared under optimized conditions:

- a bare graphite electrode (GE)
- Chit-AgNPs modified graphite electrodes (GE/Chit-AgNPs)
- Chit-AgNPs modified graphite electrodes immobilized with laccase (GE/Chit-AgNPs/Lac)

Each of the three electrodes was coated with 3 nm of Au-Pd before FE-SEM analysis. FE-SEM (FEI, QUANTA 400F) monitored the surface morphology of the three electrodes prepared at the same magnifications (20.000X).

2.3.7 Investigation of Interferents

To confirm the selectivity of the developed biosensor, it is essential to examine the interference effects of different substrates on the constructed biosensor. For this purpose, 0.05 M citric acid, phenol, ethanol, glucose, ascorbic acid, and folic acid were prepared. 50 μ L of these substrates were injected into 10 mL sodium acetate buffer (50 mM, pH 5.5), and amperometric measurements were conducted with these analytes.

2.3.8 Sample Application

In order to evaluate the analytical applicability of the biosensor, the spike recovery method was selected. In this method, known concentrations of catechol (such as 250 μ M-high, 100 μ M-medium, and 5 μ M-low) were injected into both tap water and wine solutions, and recovery values of catechol were calculated using a calibration curve as the standard^{213–215}. The following equation was used to calculate recovery values.

$$\% \text{Recovery} = \frac{(\text{Observed})}{(\text{Expected})} \times 100 \quad (5)$$

2.4 Results and Discussion

2.4.1 Characterization of Synthesized Nanocomposite

2.4.1.1 UV Visible Spectrometer

Catechol is an organic phenolic compound utilized as a reagent in various industries such as pharmaceuticals, photography, plastics, rubber, textiles, pesticides, and cosmetics. It has a slow degradation rate and is highly toxic, even in small quantities, leading to various health issues. As a result, it is crucial to create affordable, rapid, and sensitive detection systems for catechol^{85,216,217}. For this purpose, three chitosan-silver nanocomposites were synthesized through chemical reduction. Silver nanoparticles were selected for their ability to enhance electrode surface area and conductivity. Chitosan served as both a stabilizing and reducing agent in the synthesis. Firstly these three nanocomposites were characterized by UV-visible analysis.

A UV visible spectrometer confirmed the formation of Chit-AgNPs nanocomposites by chemical reduction of different concentrations of AgNO₃ and NaOH. While stock solutions for NC₁ and NC₂ were used for UV analysis, NC₃ was diluted in PBS (1:3). Due to the higher concentration of Ag in the NC₃ a dilution was done in a 1:3 volume ratio with PBS to achieve the desired solution concentration. The final characterization was performed using UV-vis spectroscopy, TEM, DLS, and zeta potential.

Photographs of the synthesized nanocomposites (NC₁, NC₂, NC₃) are demonstrated in Figure 2.5. The presence of Chit-AgNPs nanocomposite can be confirmed during synthesis via visual inspection through a colour transition from yellow to yellow-brown. This colour change indicates the formation of silver nanoparticles²¹⁸⁻²²². This characteristic colour of colloidal Chit-AgNPs solution was related to surface plasmon resonance^{223,224}.

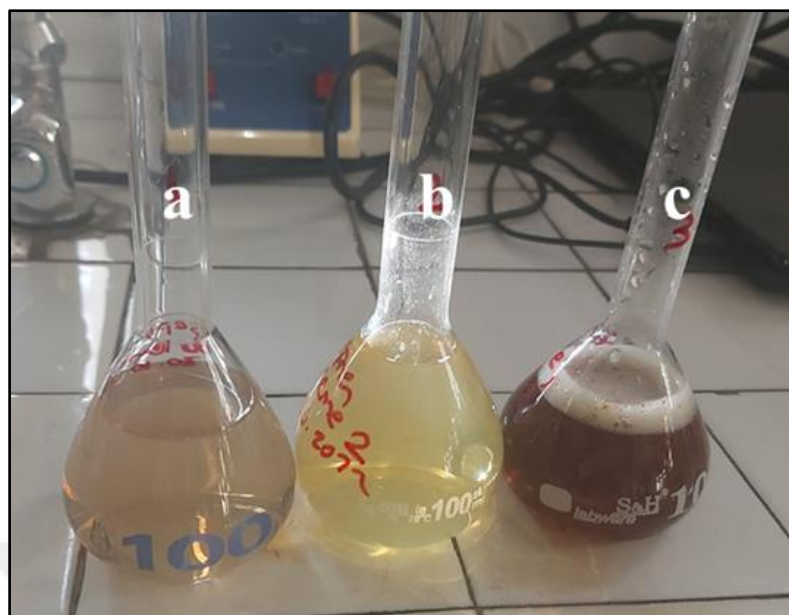


Figure 2.5. Photographs of synthesized Chit-AgNPs nanocomposites a) NC₁, b) NC₂, c) NC₃

The UV visible absorption of three nanocomposites (NC₁, NC₂, NC₃) are depicted in Figure 2.6. It can be seen that NC₁ displays an absorption maximum peak at 410 nm that gives a yellow colour to the solution (Figure 2.6 (a)), NC₂ shows a maximum absorption at 410 nm that gives a yellow colour to the solution (Figure 2.6 (b)), and NC₃ displays an absorption peak at 400 nm that gives a yellowish brown colour to the solution (Figure 2.6 (c)). The results are in agreement with those of the previous studies^{204,225,226}.

One study produced a chitosan-silver nanocomposite material by combining AgNO₃ and NaOH with the chitosan solution. They made different concentrations (0.02 M, 0.04 M, and 0.06 M) of AgNO₃. The study found that the highest absorption peak occurred at around 413 nm. Additionally, it was noted that the intensity of the absorption peak of Ag nanoparticles increased as the concentration of AgNO₃ used to produce the chitosan-silver nanocomposite was increased²⁰⁴. In another study, chitosan-silver nanocomposites were synthesized using chemical reduction methods such as chitosan, AgNO₃, and NaOH. Another study involved synthesizing chitosan-silver nanocomposites using chemical reduction methods with chitosan, AgNO₃, and

NaOH. The formation of AgNP was achieved by reducing different concentrations of AgNO₃ in the chitosan-dilute acetic acid solution at 95°C, which was monitored using UV-Vis spectroscopy. All spectra showed the characteristic surface plasmon resonance (SPR) absorption maximum between 412 nm and 416 nm, indicating the formation of spherical silver nanoparticles²²⁶.

NC₃ exhibited a strong, narrow band peak with an absorption maximum peak at 400 nm compared to NC₁ and NC₂. Therefore, NC₃ was selected for further studies.



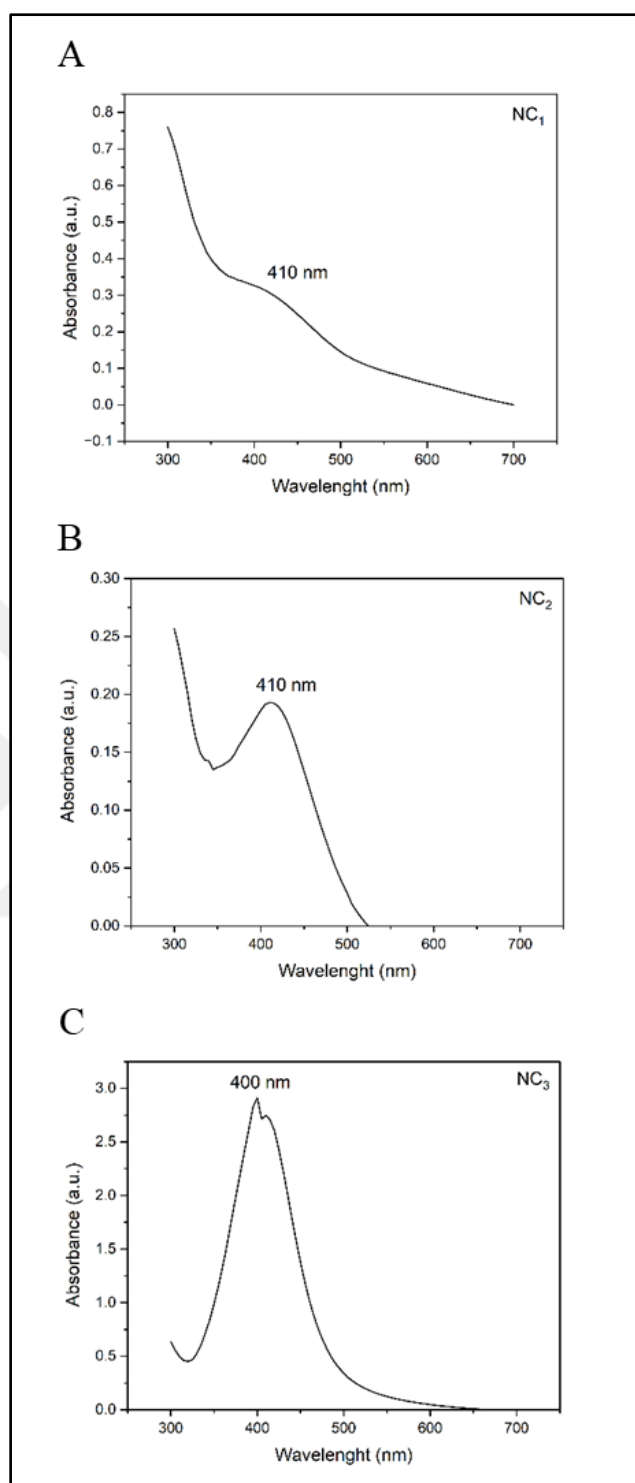


Figure 2.6. UV visible synthesized Chit-AgNPs nanocomposites (A) NC₁, (B) NC₂, (C) NC₃

2.4.1.2 Transmission Electron Microscopy

Transmission electron microscopy (TEM) and dynamic light scattering (DLS) were used to analyze the dimensional structure of the synthesized Chit-AgNPs nanocomposites. Figure 2.7 shows the TEM micrographs of Chit-AgNPs nanocomposites. The image demonstrates that the synthesized nanocomposite is predominantly spherical and has a well-dispersed structure.

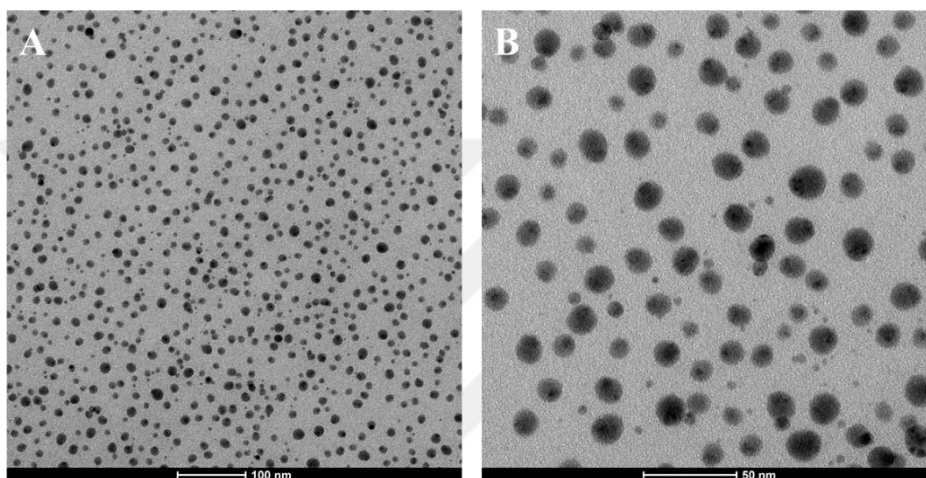


Figure 2.7. TEM micrographs of Chit-AgNPs nanocomposite.

2.4.1.3 Dynamic Light Scattering and Zeta Potential

The DLS data in Figure 2.8A revealed that the hydrodynamic diameter was 106.0 ± 41.05 nm. TEM and DLS data vary because the hydrodynamic diameter incorporates the core size and the swollen stabilizing polymer shell when immersed in water. The synthesized nanocomposite comprises a silver nanoparticle core and chitosan shell, which act as stabilizers or capping agents. However, detecting the chitosan layer of the synthesized nanocomposites may not be possible using TEM micrographs. The TEM imaging showed only the AgNP particles because the sample was dried before imaging^{227,228}. Moreover, they were reasonably uniform since the polydispersity index (PDI) (0.368) was lower than 0.4.

The zeta potential, a measure of electric charge between particles, is crucial to their stability. Higher zeta potential values discourage particle merging, while lower values can lead to clumping. Our research revealed that the Chit-AgNPs had a zeta potential of 38.7 ± 5.51 mV, a value that is considered highly stable (Figure 2.8B). According to the literature, nanoparticles with a zeta potential above +30 mV or below -30 mV are also considered stable in the dispersion medium²²⁹.

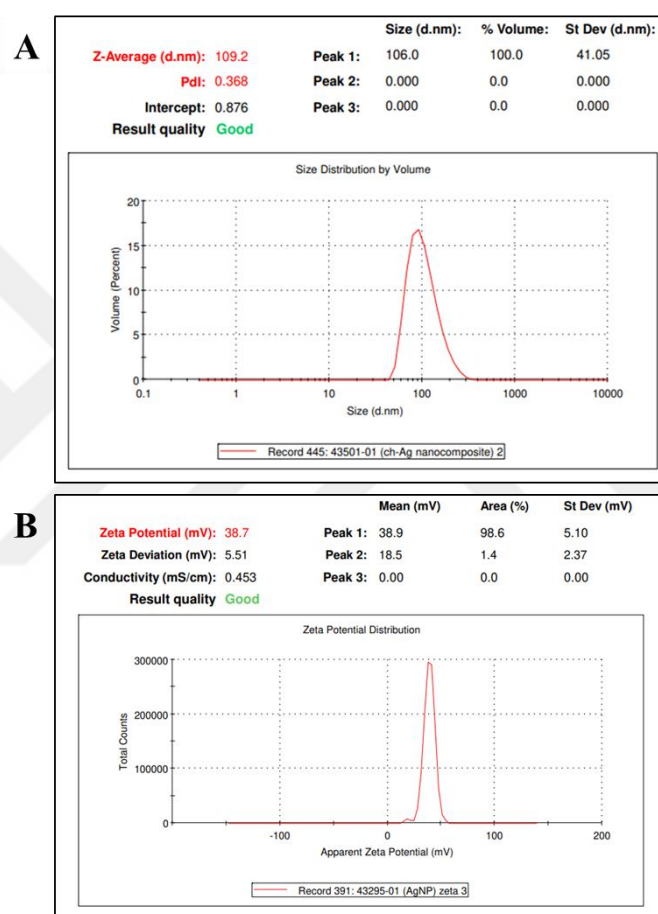


Figure 2.8. Particle size (A) and zeta potential distribution (B) Chit-AgNPs nanocomposite (NC₃)

2.4.1.4 ICP-OES

To determine the silver (Ag) content within the nanocomposite (NC₃), Inductively Coupled Plasma Optical Emission Spectroscopy (ICP-OES) was used. The analysis yielded an Ag content of 0.70 ± 0.01 g/L. Qasim et al. synthesized chitosan-derived silver nanoparticles via a chemical reduction method similar to ours. During synthesis, the amount of chitosan, NaOH and AgNO₃ used to synthesize silver chitosan nanocomposites was lower than that we used. ICP-OES analysis of these nanoparticles yielded an Ag content of 0.66 mg/L²³⁰ lower than our results.

2.4.2 Optimization Studies

The proposed GE/Chit-AgNPs/Lac biosensor was optimized regarding the nanocomposite amount, enzyme amount, and pH of the working medium to obtain a sensitive biosensor. The amperometric responses of the fabricated GE/Chit-AgNPs/Lac biosensor toward catechol was monitored at -0.3V. Optimization studies were performed in the presence of 250 μ M catechol at room temperature.

2.4.2.1 Optimization of Nanocomposite Amount

The application of Chit-AgNPs nanocomposite was aimed at improving the electrical conductivity of the proposed biosensor. Therefore, the amount of nanocomposite plays a vital role in the biosensor's performance. For this purpose, the nanocomposite amount used for the construction of the biosensors was optimized by preparing the following five Chit-AgNPs solutions: 3.0 μ L, 5.0 μ L, 7.0 μ L, 10.0 μ L, and 15.0 μ L. These solutions were cast to five electrodes while other parameters were kept constant. Different nanocomposite amounts (3.0 μ L, 5.0 μ L, 7.0 μ L, 10.0 μ L, and 15.0 μ L) and % biosensor response were used to draw a graph for the created lac biosensor, given in Figure 2.9. The optimum amount of Chit-AgNPs, which yielded the highest current response, was determined to be 5 μ L. The nanocomposite

amount was kept constant at 5.0 μL in further optimization studies. 3.0 μL nanocomposite solution did not cover the surface of the electrode. On the other hand, 7.0 μL , 10 μL , and 15 μL nanocomposite solutions might have caused thicker layers on the electrode's surface. Therefore, a high amount of nanocomposite may hinder the electron transfer⁸⁸.

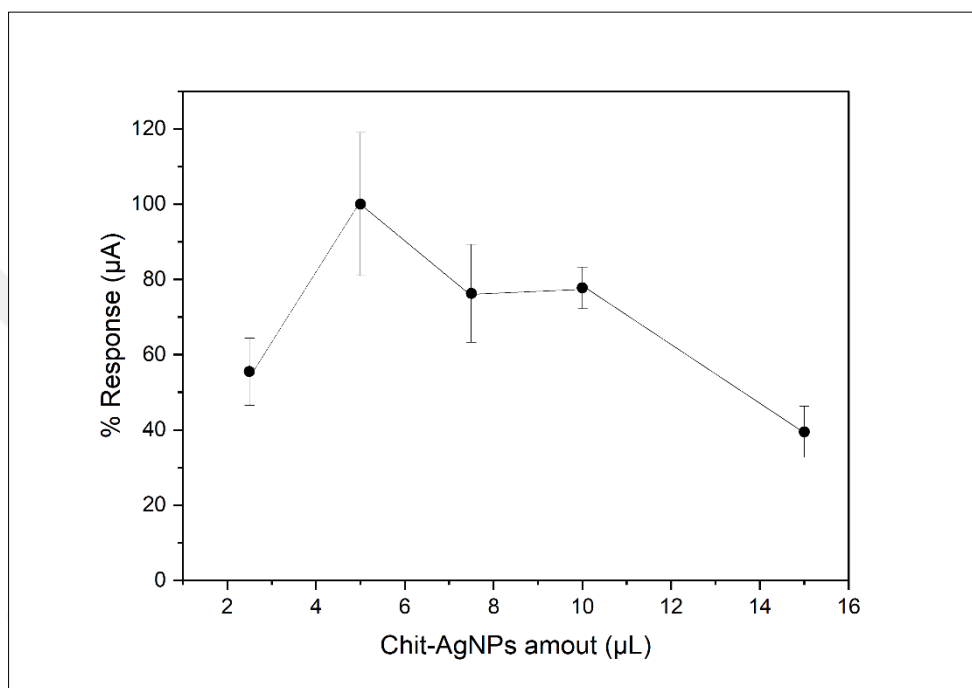


Figure 2.9. Effect of nanocomposite amount on the response of the laccase biosensor (in 50 mM acetate buffer pH 5.5, 25 °C). Error bars represent standard deviation of three measurements

2.4.2.2 Optimization of Enzyme Amount

The amount of enzyme immobilized onto the surface of the electrode is an essential parameter for the performance of biosensors. Excess immobilization of enzymes may result in leaching out from the surface. On the other hand, desired biosensor responses may not be obtained due to insufficient enzyme amounts^{85,88}. Therefore, the laccase (Lac) amount was also optimized to determine the effect of the enzyme on the biosensor's performance. To investigate the optimum enzyme amount,

different amounts of laccase (0.05 mg-1.09 U, 0.1 mg-2.18 U, 0.3 mg-6.54 U, 0.5 mg-10.9 U, 0.75 mg-16.35 U, and 1 mg-21.8 U) were prepared in acetate buffer solution (50 mM, pH: 5.5). Cross-linking agent (glutaraldehyde) was kept constant (1% w/w) for each enzyme amount. Based on the results shown in Figure 2.10, the highest current response from the biosensor was recorded using enzymatic biosensors containing 10.9 U (0.5 mg) of Lac. Consequently, further studies used biosensors containing 0.5 mg of Lac.

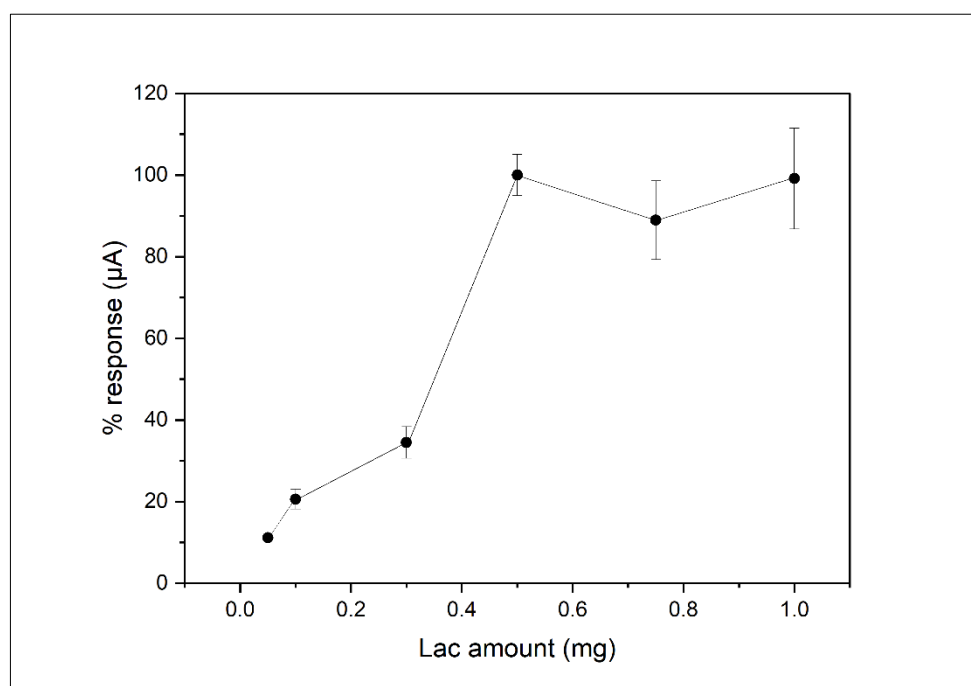


Figure 2.10. Effect of enzyme amount on the response of the laccase biosensor (in 50 mM acetate buffer pH 5.5, 25 °C). Error bars represent standard deviation of three measurements

2.4.2.3 Optimization of pH

It is known that the performance of enzyme-based electrochemical biosensors is pH-dependent²³¹. Since pH changes in the working medium may cause denaturation of the enzyme, the optimum pH value should be determined. For this purpose, 50 mM

buffer solutions were prepared in the pH range of 4.5-7.5 (NaOAc buffer at pH 4.5, 5.5, and 6.5, PBS buffer at pH 7.0 and 7.5), and the responses of the constructed biosensor were recorded at solutions with these five different pH values. Figure 2.11 demonstrates that the highest current was recorded at pH 5.5, therefore the medium pH was chosen as 5.5 to use further experiments.

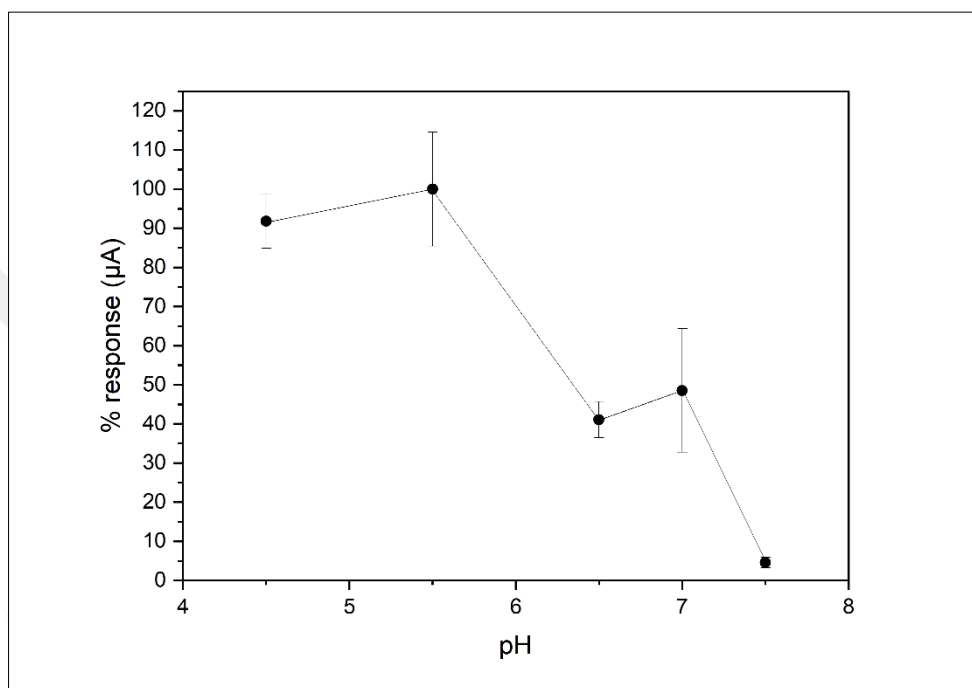


Figure 2.11. Effect of pH on the response of the laccase biosensor. Error bars represent standard deviation of three measurements

2.4.3 Characterization of Constructed Biosensor

2.4.3.1 Electrochemical and Surface Characterization

The electroactive surface areas of both the bare electrode and modified electrodes were characterized through cyclic voltammetry (CV) analysis. The CV experiments were carried out in a solution containing 5.0 mM $[\text{Fe}(\text{CN})_6]^{3-}$ and $[\text{Fe}(\text{CN})_6]^{4-}$ in a 50.0 mM phosphate buffer solution (pH 7.0) with the addition of 0.1 M KCl. The

potential range covered was from -0.4 V to 0.8 V, and a scan rate of 100 mV/s was employed.

Figure 2.12. shows the cyclic voltammograms obtained for three electrode configurations: the bare graphite electrode (GE), the modified electrode (GE/Chit-AgNPs), and the modified electrode incorporating laccase (GE/Chit-AgNPs/Lac), all under ambient conditions. The Randles-Sevcik equation defines the relationship between the electroactive surface area and the peak current.

The peak current for the bare graphite electrode was measured at 381 μA . Upon modification with Chit-AgNPs, the peak current notably increased to 576 μA . This enhancement in peak current provides evidence of the significant influence of charge transfer on the effective surface area of the modified GE/Chit-AgNPs electrode.. According to the current responses, the effective surface areas were estimated as 0.34 cm^2 for bare GE, 0.51 cm^2 for GE/Chit-AgNPs electrode. When the laccase enzyme was immobilized onto the GE/Chit-AgNPs/Lac electrode, the peak disappeared, which is related to the reduction of conductivity after enzyme immobilization. It can be concluded that enzyme immobilization onto a modified electrode was successfully achieved²¹⁶.

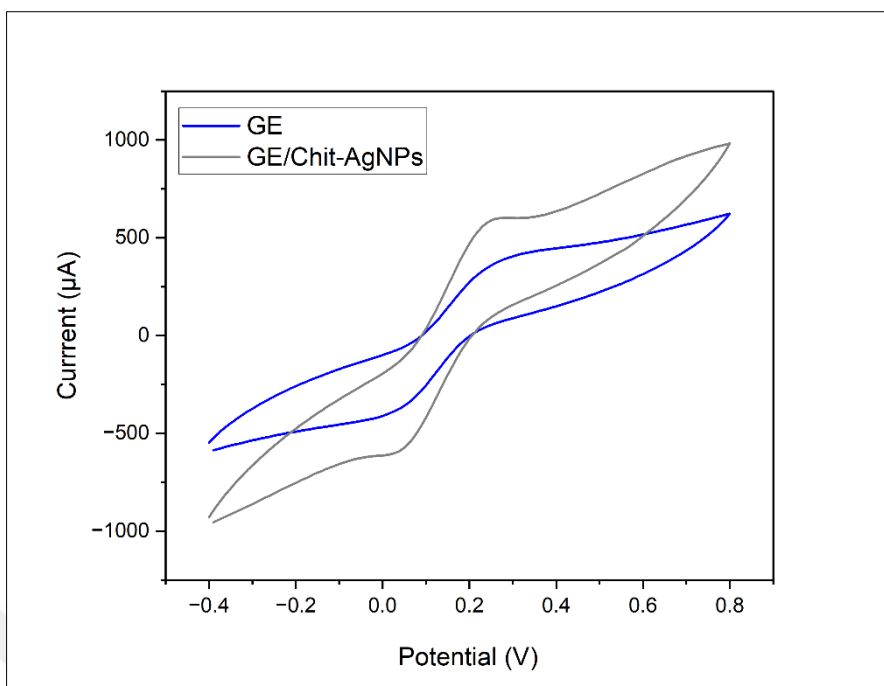


Figure 2.12. Cyclic voltammograms of GE (black) and GE/Chit-AgNPs (blue) electrodes

The surface characteristics of three electrodes, namely bare GE, GE/Chit-AgNPs, and GE/Chit-AgNPs/Lac, were examined using Scanning Electron Microscopy (SEM), as shown in Figure 2.13 (a-c).

To gain more information about the constructed electrodes, the surface morphology at each step was investigated. Figure 2.13 shows SEM micrographs of the bare GE and Chit-AgNPs modified GE (a) and (b), respectively.

Figure 2.13. (b) illustrates the successful modification of the graphite electrode with Chit-AgNPs, as evidenced by the altered surface morphology. The bare GE displayed a relatively smooth topology before nanocomposite modification. Modifying bare GE with Chit-AgNPs resulted in homogenous dispersion on the surface of the electrode (Figure 2.13. (b)).

Furthermore, the surface morphology was compared before and after laccase immobilization, as shown in Figure 2.13. (b-c). A stark comparison of SEM

micrographs of GE/Chit-AgNPs and GE/Chit-AgNPs/Lac indicated the clear and significant difference caused by the immobilization of laccase. Post-laccase immobilization, the GE/Chit-AgNPs/Lac electrode surface exhibited enzyme coverage (Figure 2.13. (c)), affirming the profound impact of laccase deposition.



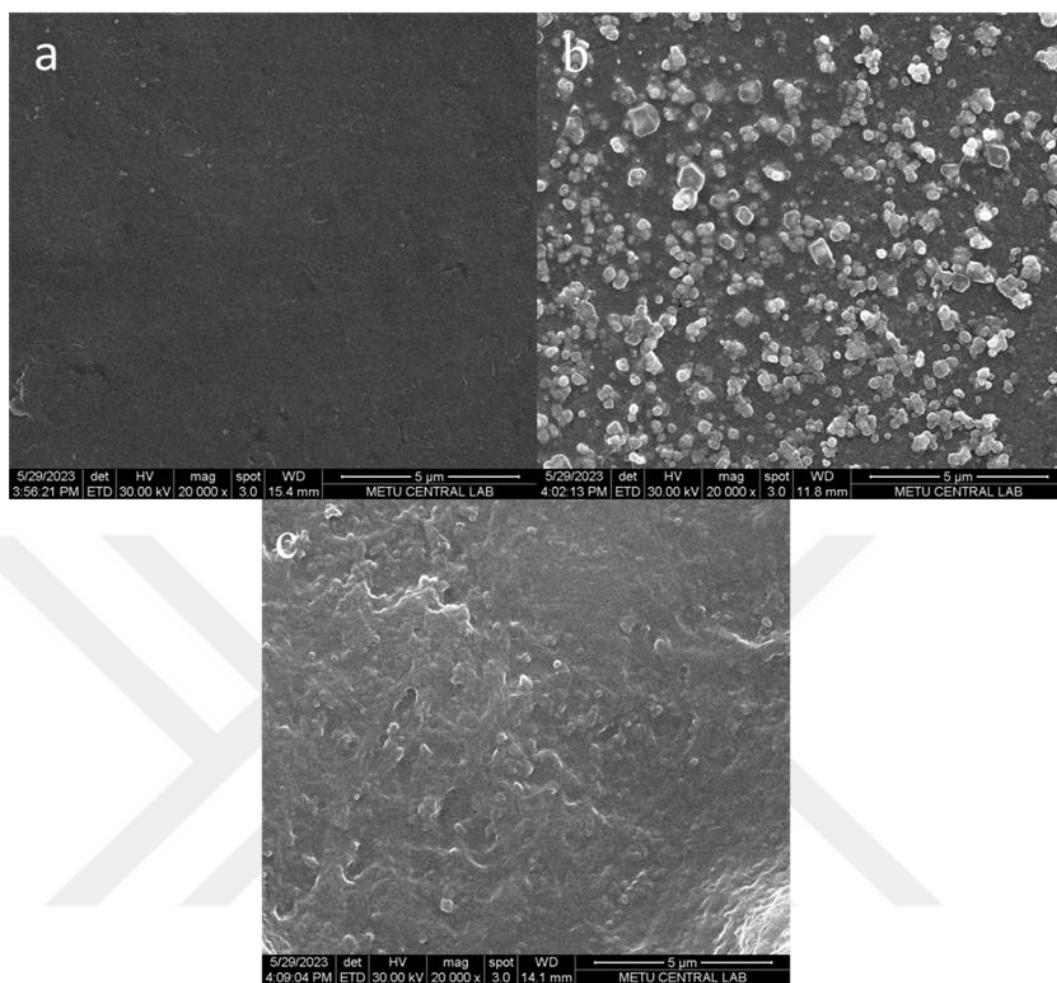


Figure 2.13. SEM images of electrodes a) bare GE, b) GE/Chit-AgNPs, c) GE/Chit-AgNPs/Lac

2.4.3.2 Analytical Characterization

Under the optimized conditions, the fabricated biosensor was characterized analytically at -0.3 V, and amperometric responses toward catechol are shown in Figure 2.14. A plateau occurred after 250 μM catechol concentration, and the enzyme was saturated.

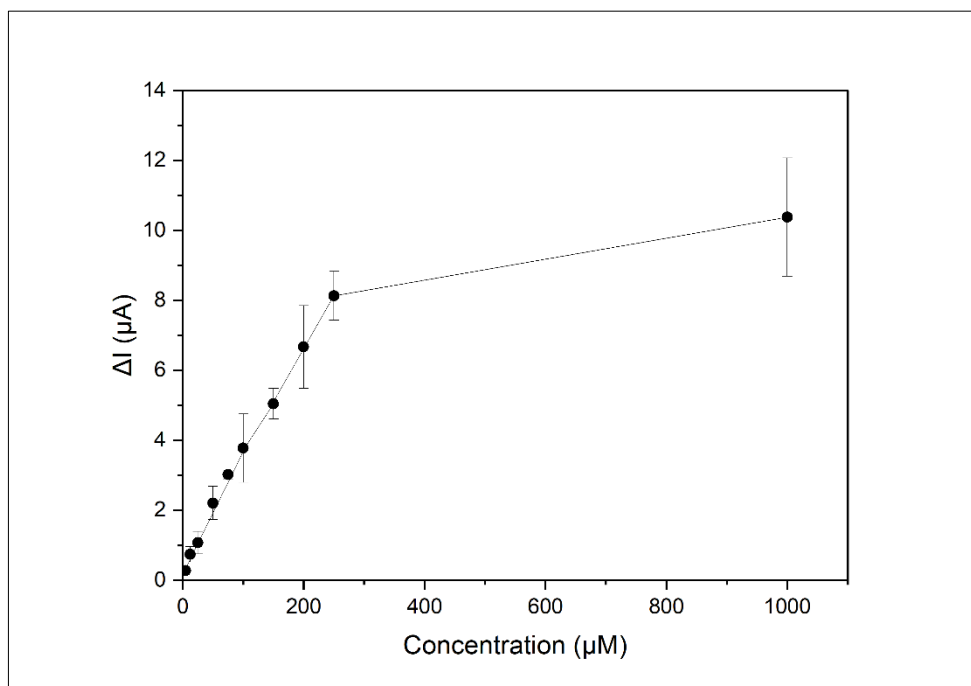


Figure 2.14. Amperometric response of the fabricated biosensor (GE/Chit-AgNPs/Lac) toward catechol detection (whole range) (in 50.0 mM acetate buffer pH 5.5, 25 °C). Error bars represent standard deviation of three measurements

The calibration equation of the GE/Chit-AgNPs/Lac biosensor in the range of 5-250 μM catechol concentration was estimated $y=0.0314x+0.4129$ ($R^2=0.99$) (Figure 2.15.). GE/Chit-AgNPs/Lac biosensor exhibited a larger linear range compared to other previous studies reported in the literature^{199,232}.

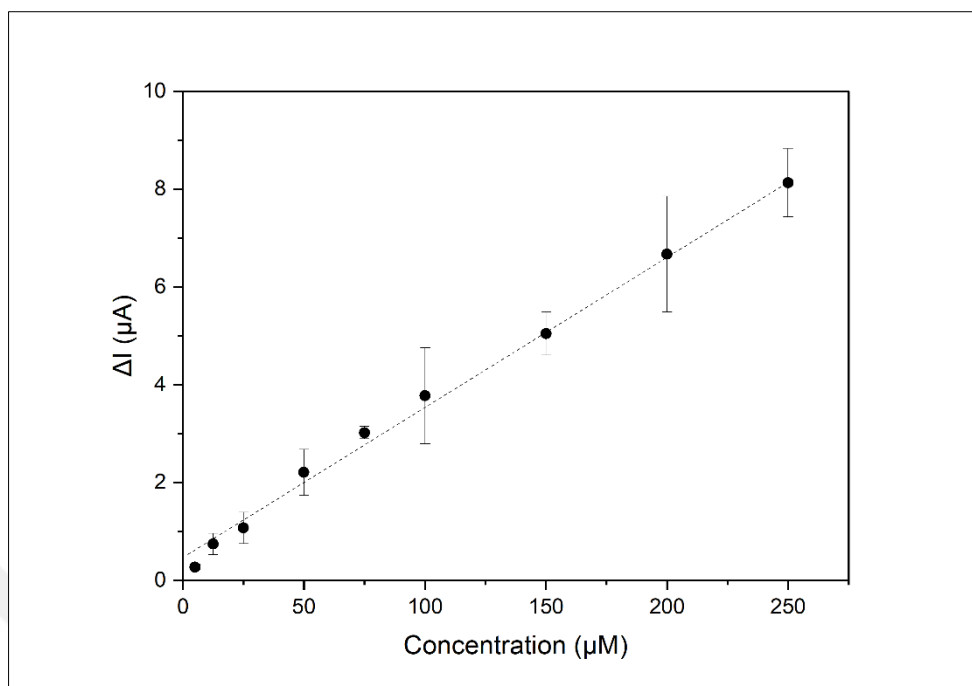


Figure 2.15. Linear range obtained for the fabricated biosensor (GE/Chit-AgNPs/Lac) toward catechol detection (linear range) (in 50.0 mM acetate buffer pH 5.5, 25 °C). Error bars represent standard deviation of three measurements

The limit of detection (LOD) and limit of quantitation (LOQ) values were calculated as 3.85 μM and 12.83 μM for catechol respectively. LOD value the proposed biosensor is lower than the other reported laccase biosensors in literature MWCNTs-PVA-AuNPs-Lac (5 μM)¹⁹⁹, GE/P(DTFBT)/Lac (14 μM)⁸⁵, GE/SiNPs/PBSeThTh/Lac (7.2 μM)²¹⁶, GCE/M1/Lac (9.86 μM)⁸⁹, CPE-Lac-a-Fe₂O₃NC (4.28 μM)¹⁹⁷, GCE/Chit/AP-rGO/Lac (7.0 μM)¹⁹⁸.

The calibration curve known as the Lineweaver Burk plot (Figure 2.16) was utilized for the study of enzyme kinetics. By plotting $1/I$ (μA⁻¹) against $1/[S]$ (μM⁻¹) essential parameters such K_M^{app} and I_{max} values could be calculated^{210,211}. Sensitivity, I_{max} , and K_M^{app} values were calculated as 0.444 μA/μM.cm², 11.9 μA, and 12.3 μM, respectively (Table 2.3).

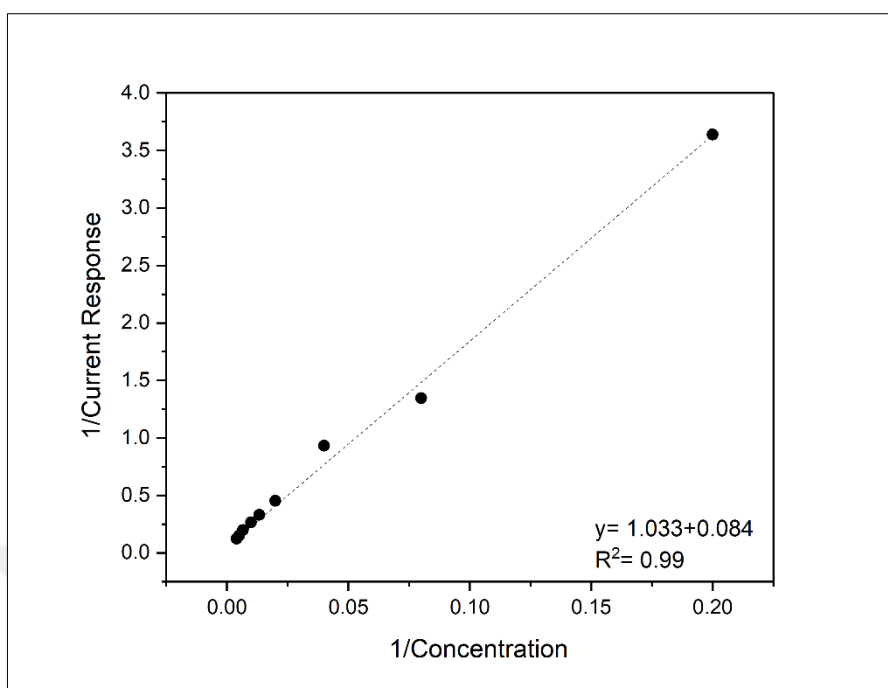


Figure 2.16. Lineweaver-Burk Plot of GE/Chit-AgNPs/Lac biosensor

K_M^{app} is commonly used as a measure of the enzyme's substrate affinity, An enzyme with a low K_M^{app} value requires only a small amount of substrate to become saturated, resulting in the maximum velocity being reached at relatively low substrate concentrations. On the other hand, an enzyme with a high K_M^{app} value needs high substrate concentrations to achieve maximum reaction velocity¹². The K_M^{app} of the fabricated biosensor was lower than that of other laccase biosensors compared to the studies reported in the literature: GCE/PANI/GA/Lac (95.9 μM)²⁰¹, GCE/GC-rGO/Lac (93.4 μM)²³³, GCE/Chit/AP-rGO/Lac (300 μM)¹⁹⁸, MB-MCM-41/PVA/Lac (256 μM)²³⁴, Au/Chit/Lac/Cu-OMC (40.2 μM)²³⁵.

The fabricated biosensor exhibited the highest sensitivity with a value of 444 $\mu\text{A/mMcm}^2$ compared to other constructed biosensors GCE/Chit/AP-rGO/Lac (15.79 $\mu\text{A/mMcm}^2$)²³⁶; MWCNTs-PVA-AuNPs-Lac (51 $\mu\text{A/mMcm}^2$)¹⁹⁹; CPE-Lac-a-Fe₂O₃NC (78.56 $\mu\text{A/mMcm}^2$)¹⁹⁷; GE/SiNPs/PBSeThTh/Lac (100.8 $\mu\text{A/mMcm}^2$)²¹⁶, GCE/M1/Lac (153.6 $\mu\text{A/mMcm}^2$)⁸⁹ and GE/P(DTFBT)/Lac (166.74 $\mu\text{A/mMcm}^2$)⁸⁵.

The reproducibility of GE/Chit-AgNPs/Lac biosensor toward catechol detection was determined by ten sequential measurements under the optimized conditions in the presence of 250 μM . The standard deviation of these ten measurements was found as 7.049 ± 0.419 .

Table 2.3 Analytical performance values of GE/Chit-AgNPs/Lac biosensor

Parameters	
K_M^{app}	12.3 μM
I_{max}	11.9 μA
Linear range	5-250 μM
Sensitivity	0.444 $\mu\text{A}/\mu\text{M}\cdot\text{cm}^2$
LOD	3.85 μM
LOQ	12.71 μM

The literature has discussed various electrochemical laccase-based biosensors for the detection of catechol. A comparison of the analytical parameters of the GE/Chit-AgNPs/Lac biosensor was made with previously reported literature, which is presented in Table 2.4. Udum et al. utilized cyclic voltammetry to coat graphite electrodes with a novel conducting polymer called poly(DTFBT), which then acted as a platform to immobilize laccase⁸⁵. Deniz et al, created amperometric catechol biosensors by modifying graphite rod electrodes with SiNPs, a novel conducting polymer (PBSeThTh), and MWCNTs, respectively, where laccase was immobilized²¹⁶. Electrochemically polymerized conjugated polymer was used to fabricate another laccase-based electrochemical sensing platform onto the surface of the graphite electrode⁸⁹. Lastly, a chronoamperometric laccase biosensor was developed by impairing the laccase onto a 1-aminopyrene (1-AP) functionalized reduced graphene oxide (rGOs) modified glassy carbon electrode²³⁶. Favero et al. created a biosensor for detecting catechol by modifying a screen-printed electrode with gold nanoparticles (AuNPs) and multi-wall carbon nanotubes (MWCNTs)¹⁹⁹. In a similar fashion, Castrovilli et al. created a sensing platform using carbon-black

nanomodified electrodes with laccase immobilization through electrospray deposition²⁰³. Nazari et al. also constructed an electrochemical laccase biosensor based on ZnO-Al₂O₃ ceramic nanofibers and AuNPs²⁰⁰. Finally, a catechol biosensor was designed based on α -Fe₂O₃ nanocrystals (NCs) incorporated into a carbon-paste electrode, followed by laccase immobilization¹⁹⁷. These abovementioned biosensors all demonstrated good analytical features as given in Table 2.4. However, they mostly required more modification steps during construction compared to ours (Table 2.4.).

On the other hand, the proposed biosensor was simple, sensitive, and low-cost compared with these biosensors. In addition, the use of Chit-AgNPs nanocomposite in construction of an electrochemical laccase biosensor was reported for the first time in here. Chit-AgNPs served as an immobilization platform and provided stability (Table 2.3).

Table 2.4 Comparison of analytical parameters of laccase biosensors toward catechol detection in literature

Modified Electrode	Detection Method	LOD (μM)	Linear Range (μM)	Sensitivity ($\mu\text{A}/\mu\text{Mcm}^2$)	Reference
GE/P(DTFBT)/Lac	A	14	25-400	0.166	85
GE/SiNPs/PBSeThTh/Lac	A	7.2	50-350	0.100	216
GCE/M1/Lac	A	9.86	5-175	0.153	89
CB-SPE/e-Lac	A	2.0	2.5-50	NR	203
GCE/AuNPs/ZnO/Al ₂ O ₃ /GO,Chit/Lac	DPV	3.1	0.5-40	NR	200
CPE-Lac-a-Fe ₂ O ₃ NC	A	4.28	8-800	0.078	197
GCE/Chit/AP-rGO/Lac	A	7.0	15-700	0.015	236
MWCNTs-PVA-AuNPs-Lac	A	5	10-200	0.051	199
GE/Chit-AgNPs/Lac	A	3.85	5-250	0.444	This work

A: Amperometry, Al₂O₃: Aluminium(III) oxide, AP: 1-Aminopyrene, AuNPs: Gold nanoparticles, CB-SPE: Carbon black modified screen-printed electrode, Chit: Chitosan, CPE: Carbon paste electrode, DPV: Differential pulse voltammetry, Fe₂O₃NC: Haematite nanocrystal, GA: Gluteraldehyde, GCE: Glassy carbon electrode, GE: Graphite electrode, GO: Graphene oxide, Lac: Laccase, M1:(6,9-bis(4-hexylthiophen-2-yl)-11H-indeno[2,1-b]quinoxalin-11-one), MWCNTs: Multi-walled carbon nanotubes, PBSeThTh: A type of conducting polymer, P(DTFBT):poly(5-fluoro-4,7-bis (4-hexylthiophen-2 yl) benzo [c][1,2,5]thiadiazole, PEDOT: Poly(3,4-ethylenedioxythiophene), PSSLi: poly(4-lithium styrenesulfonic acid), PVA: Poly(vinylalcohol), rGO: Reduced graphene oxide, SiNPs: Silica nanoparticles, ZnO: Zinc oxide

2.4.4 Investigation of Interference

One vital property of the constructed biosensor was its specificity toward catechol. For this reason, the selectivity of the biosensor was tested in the presence of possible interferents such as phenol, ethanol, glucose, citric acid, ascorbic acid, and folic acid via amperometry because these substrates can be easily oxidized on bare electrodes.

A volume of these substrates (0.05 M, 50.0 μ L) was injected into the reaction medium (50.0 mM acetate buffer, pH 5.5 instead of catechol). The results indicated that the current signal for catechol was significantly higher than that of the possible interferents, which confirmed the biosensor's selectivity toward catechol. (Figure 2.17.). Therefore, it can be concluded that the constructed biosensor was very suitable for catechol detection in real samples.

Substances like paracetamol, ascorbic acid, or uric acid can cause electrochemical interference, disrupting the accuracy of measurements when a biosensor is used at high applied potentials²³⁷. Enzymatic electrodes may provide inaccurate results when used with food products despite their enzyme specificity. Complex media may contain electroactive species that can be oxidized on the electrode, impacting the results. Additionally, certain compounds can serve as inhibitors, activators, or even secondary substrates for the enzyme. For example, glucose and ascorbic acid can interfere with determining polyphenols and potentially affect the biosensor signal. Ascorbic acid, in particular, can be electrochemically oxidized at low potential and react with the added H_2O_2 , decreasing biosensor response²³⁸.

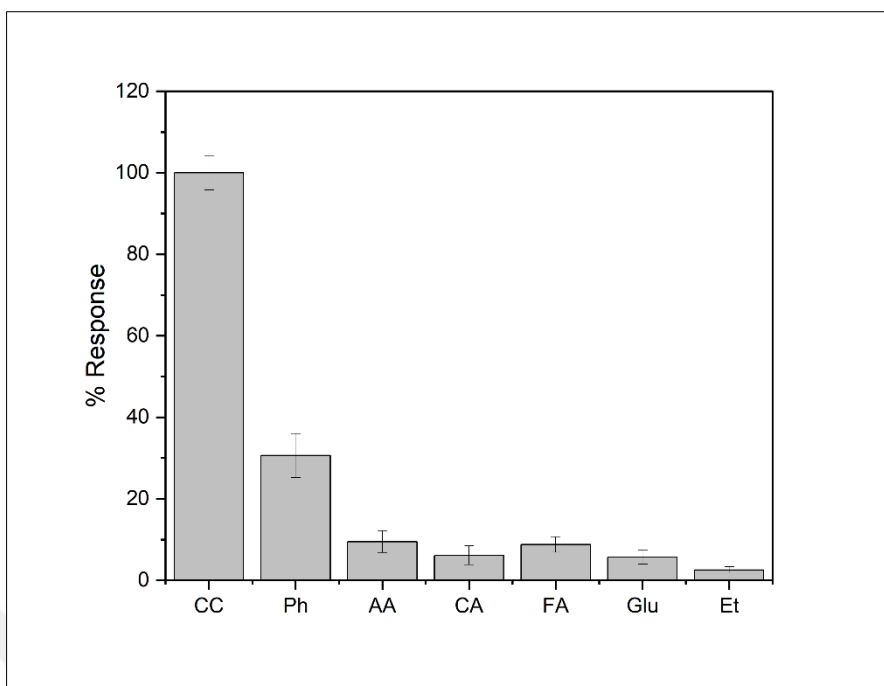


Figure 2.17. The response of the fabricated biosensor toward different substrates including catechol 250 μ M catechol (in 50.0 mM acetate buffer pH 5.5, 25 $^{\circ}$ C) at - 0.3 V.

2.4.5 Sample Application

The applicability of the fabricated biosensor was assessed using tap water^{85,89,216,217} and wine²³⁹ as real samples. Recoveries were achieved by determining catechol in the tap water and wine after spiking them with the known catechol concentrations. Three different concentrations (250 μ M, high, 100 μ M, medium, and 12.5 μ M, low) of catechol were chosen for the spiking analysis. All experiments were performed under optimized conditions at room temperature. The calibration curve graph (Figure 2.15.) was utilized to calculate the concentration of samples.

The quantitative recoveries were found to be 91, 103, and 94% for the spiked catechol concentration in tap water of 250 μ M, 100 μ M, and 12.5 μ M, respectively. The quantitative recoveries were 98, 107, and 94% for the spiked catechol concentration in wine of 250 μ M, 100 μ M, and 12.5 μ M, respectively. As

summarized in Table 2.5, the results are in good agreement, indicating the applicability and reliability of the suggested biosensor. It can be concluded that the GE/Chit-AgNPs/Lac biosensor is an accurate and effective system for the detection of catechol in real samples.

Table 2.5 Determination of catechol in tap water and wine

Sample			
Tap water	Spiked with Catechol (μM)	Found with the Biosensor (μM)	Recovery (%)
	12.5	12 $\pm$ 2	94
	100	103 $\pm$ 9	103
	250	228 $\pm$ 23	91
Wine	Spiked with Catechol (μM)	Found with the Biosensor (μM)	Recovery (%)
	12.5	12 $\pm$ 3	94
	100	107 $\pm$ 17	107
	250	244 $\pm$ 19	98

2.5 Conclusion

Phenol and its derivatives are frequently found as human-made water contaminants²⁴⁰. They are produced when organic substances used in making dyes, pesticides, and insecticides break down. Catechol, a phenolic compound, is highly toxic and breaks down slowly in the environment²⁴¹. Additionally, catechol can induce nervous system alterations^{187,193}. Because of this, researchers are focusing on finding ways to remove catechol from water and accurately measure its levels.

This section presents a new method for detecting catechol using a Chit-AgNPs nanocomposite-based amperometric laccase biosensor. AgNPs have been widely used in biosensing platforms. Although they provide increased surface area and conductivity, these nanoparticles are unstable. Chitosan was used as both a stabilizing and reducing agent¹⁶⁷. The chemical reduction method was used to synthesize the chitosan-silver nanocomposite and characterized.

The bare graphite electrode surface was coated with the nanocomposite, and then the laccase was immobilized on the layer using glutaraldehyde. Chit-AgNPs nanocomposite was used to improve electron transfer. The amperometric biosensor response was tested at -0.3 V in a buffer solution with a pH of 5.5 to test the biosensor response. The biosensor's response was then monitored regarding the current change produced by the enzymatic reaction between laccase and catechol. The parameters that affect the biosensor's response were optimized to achieve a high biosensor response, and then a calibration curve was plotted for catechol detection under the optimized conditions. The biosensor was subjected to analytical and electrochemical characterizations. The analytical features of the developed biosensors were found to be good, with a LOD of 3.85 μM , a linear range of 5-250 μM , and a sensitivity of 0.444 $\mu\text{A}/\mu\text{M}\cdot\text{cm}^2$. The biosensor had good analytical properties, a more comprehensive dynamic linear range^{199,242}, and a lower detection limit^{85,89,197–199,216} than similar biosensing platforms reported in the literature. Various interferents were used to test the prepared biosensor's selectivity, and the biosensor demonstrated high specificity for detecting catechol. This biosensing platform can be effectively used to detect catechol.

In this chapter, Chit-AgNPs were synthesized using the chemical reduction method and then characterized. Chemical reduction is a cheap and simple method. This is the first study in which a chitosan-silver nanocomposite was investigated to fabricate an electrochemical biosensing platform for catechol detection. Due to the aforementioned analytical features of the developer's biosensors, it can be inferred that the nanocomposite effectively modified the electrode and served as a suitable matrix for immobilization. Notably, the Chit-AgNPs nanocomposite shows potential for use in other enzyme-based biosensing platforms, opening up new avenues for biosensing research.



CHAPTER 3

GE/P-PBF NP/Lac BIOSENSOR

Conjugated polymers, with their unique structure of alternating σ and π bonds along their backbone, have long fascinated researchers. This structure creates a delocalized π -electron cloud, endowing these polymers with appealing optical and electrochemical properties²⁴³. Over the past three decades, these polymers have been explored for a wide range of applications, including solar cells, organic electrochemical transistors (OECTs)⁵⁹, light-emitting diodes (LEDs)⁶⁰, energy storage systems⁶¹, organic field-effect transistors (OFETs)⁶², chemical sensors⁶³, and biosensors⁶⁴. However, their limited water usability and reliance on hazardous organic solvents for processing have posed significant environmental and health risks. To overcome these challenges, researchers have been investigating methods to convert these polymers into nanoparticle dispersions in alcohol or water, thereby eliminating the need for toxic solvents and improving their practicality. This approach has successfully produced and utilized conjugated polymer nanoparticles (CPNs), also known as semiconducting polymer nanoparticles or polymer dots (Pdots), for various biological purposes, underscoring the immense potential of these polymers¹¹⁶. In this chapter, a new conjugated polymer called P-PBF was created and examined. Using this newly developed conjugated polymer, a novel conjugated polymer nanoparticle was generated through reprecipitation. As far as we know, research is currently needed on constructing electrochemical biosensors based on conjugated polymer nanoparticles. The produced conjugated polymer nanoparticle was meticulously examined, instilling confidence in its reliability. Furthermore, a new laccase biosensor was developed by modifying graphite electrodes with the conjugated polymer nanoparticles. We conducted comprehensive optimization studies to enhance the biosensor's performance, providing reassurance about its

effectiveness. After these studies, the developed biosensor underwent testing for analytical properties, selectivity, sensitivity, reproducibility, and stability. In the final phase, the proposed biosensor was compared with other laccase-based catechol detection platforms documented in the literature.

3.1 Materials

Chemicals used for experiments are listed in Table 3.1.



Table 3.1 Chemicals used in the study

Chemicals	Supplier
Ascorbic acid	Sigma-Aldrich (USA)
Acetone	Merck (Darmstadt, Germany)
Chlorofom	Merck (Darmstadt, Germany)
Citric acid	Sigma-Aldrich (USA)
Ferrocyanide	Fisher Scientific Company (USA)
Ferricyanide	Sigma-Aldrich (USA)
Glucose	Sigma-Aldrich (USA)
Glutaraldehyde (50% wt in H ₂ O)	Sigma-Aldrich (USA)
Hexane	Merck (Darmstadt, Germany)
Laccase (Lac, EC 1.10.3.2. from <i>Trametes versicolor</i> , 21.8 U/mg)	Sigma-Aldrich (USA)
Methanol	Merck (Darmstadt, Germany)
Palladium scavenger	Sigma-Aldrich (USA)
Paracetamol	Sigma-Aldrich (USA)
Potassium chloride	Sigma-Aldrich (USA)
Pyrocatechol	Sigma-Aldrich (USA)
Sodium acetate	Sigma-Aldrich (USA)
Sodium chloride	Sigma-Aldrich (USA)
Sodium dihydrogen phosphate	Sigma-Aldrich (USA)
Sodium hydroxide, minimum 98%	Sigma-Aldrich (USA)
Sodium hydrogen phosphate dihydrate	Merck (Darmstadt, Germany)
Tetrakis(triphenylphosphine)palladium(0)	Merck (Darmstadt, Germany)
Toluen	Merck (Darmstadt, Germany)
Urea	Sigma-Aldrich (USA)
Uric acid	Sigma-Aldrich (USA)
2,5-dibromopyrazine	Sigma-Aldrich (USA)
4,8-bis((2-ethylhexyl)oxy) benzo[1,2-b:4,5-b']dithiophene-2,6-diylbis(trimethylstannane)]	Sigma-Aldrich (USA)
2,7-dibromo-9,9-dihexyl-9H-fluorene	Sigma-Aldrich (USA)

3.2 Instrumentation

A structural analysis of the P-PBF was performed using a nuclear magnetic resonance (NMR) (Bruker Spectrospin Avance DPX-400 Spectrometer (400 MHz)). Gel permeation chromatography (GPC) (Shimadzu RID-20A with polystyrene standard) was used for the molecular weight analysis.

The optical properties of P-PBF and P-PBF NP were analyzed using a UV-Vis-NIR spectrophotometer (Shimadzu UV-3600) and photoluminescence spectroscopy (PL) (Agilent Technologies, USA). The UV-visible spectra of P-PBF and P-PBF NP were measured using a quartz cell from 350 to 700 nm. The emission spectra of P-PBF and P-PBF NP were captured from 500 to 800 nm with excitation at 290 nm.

P-PBF conjugated polymer nanoparticles (P-PBF NP) were characterized by using High Contrast Transmission Electron Microscopy (CTEM) (FEI Tecnai G2 Spirit BioTwin), Dynamic Light Scattering (DLS) spectrometer (Malvern CGS-3), Zeta potential analysis was performed vis ZetaSizer (MALVERN Nano ZS90).

The surface morphology of fabricated biosensors was analyzed by Field Emission Scanning Electron Microscopy (FESEM) (Quanta 400F Field Emission). Energy-dispersive X-ray spectrum (EDS) was used to identify the elemental spectrum of the P-PBF conjugated polymer nanoparticles.

The electrochemical experiments, including CV and EIS, were conducted using a conventional three-electrode cell configuration with the GAMRY Instruments Potentiostat/Galvanostat (Interface 1010 E, USA).

A PalmSense potentiostat (PalmSense 4, Netherlands) was employed with a three-electrode system for amperometry measurements. The working electrode used during the experiments was a graphite electrode (Ringsdorff Werke GmbH, Bonn, Germany, type RW001, 3.05 mm diameter, and 13% porosity). The counter (auxiliary) electrode was a platinum (Pt) wire electrode, while a silver (Ag) wire electrode was used as the reference electrode.

3.3 Experimental

3.3.1 Synthesis of P-PBF Conjugated Polymer

P-PBF conjugated polymer was synthesized by Çırpan Research Group via Stille cross-coupling reaction.

2,5-dibromopyrazine (**M1**) (38.5 mg, 161.85 μmol , 1.0 eq.), 4,8-bis((2-ethylhexyl)oxy)benzo[1,2-b:4,5-b']dithiophene-2,6-diyl)bis(trimethylstannane) (**M2**) (250.0 mg, 323.7 μmol , 2.0 eq.), and 2,7-dibromo-9,9-dihexyl-9H-fluorene (**M3**) (79.68 mg, 161.85 μmol , 1.0 eq.) were placed in a 50.0 mL of schlenck tube. To remove possible residual air and humidity, the vacuum was applied for 10 minutes; then, the Schlenck tube was purged with nitrogen gas (N_2 (g)). This process was repeated three times. After completion of the process, dry toluene (8.0 mL) and tetrakis(triphenylphosphine)palladium(0) ($\text{C}_{72}\text{H}_{60}\text{P}_4\text{Pd}$) (18.7 mg, 16.18 μmol , 0.1 eq.) were introduced to the reaction medium, and the mixture was degassed for 30 minutes using N_2 (g). The reaction temperature was set to 100°C and stirred for 30 hours. Cold methanol (CH_3OH) was added into the reaction medium to quench the polymerization reaction. After that, palladium scavenger was added onto the polymer and methanol suspension and stirred for 1 hour. The crude polymer was then filtered and purified by Soxhlet extraction with methanol, acetone, hexane, and chloroform as washing solvents in order (2h for each extraction). The chloroform fragment was collected, and the solvent was evaporated under low pressure. The cherry-red polymer **P-PBF** (214 mg, 82.42% yield) was obtained.

According to molecular weight analysis (GPC), the results were as follows: $M_n = 5.5$ kDa, $M_w = 11.0$ kDa, $\text{PDI} = 2.0$. ^1H NMR (400 MHz, CDCl_3 , δ : ppm) 8.10 ~ 7.30 (aromatic region), 4.50 ~ 4.00 (O- CH_2), 2.20 ~ 0.50 (aliphatic region). ^1H NMR spectrum of P-PBF is given in Appendices A.

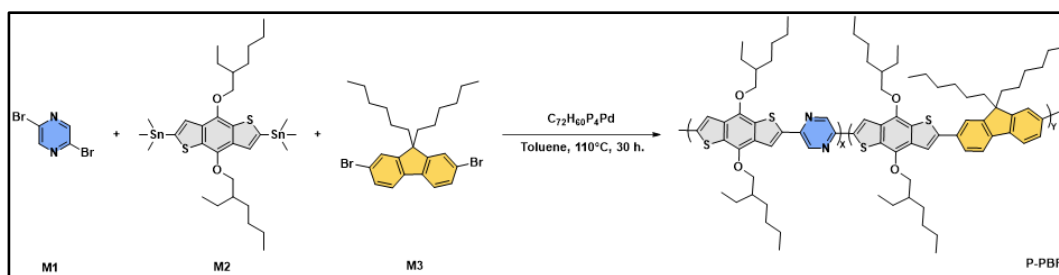


Figure 3.1. Synthetic pathway to obtain P-PBF

3.3.2 Synthesis of P-PBF Conjugated Polymer Nanoparticles

P-PBF conjugated polymer nanoparticles were synthesized by Söylemez Research Group via preprecipitation method¹¹⁶

3.0 mg of P-PBF conjugated polymer was dissolved in 1 ml of tetrahydrofuran (THF). 10 ml of ultrapure water was transferred into a glass bottle and placed into the ultrasonic bath. Under sonication, 1 ml of the conjugated polymer solution (3.0 mg/ml) was added dropwise via syringe, and the solution was sonicated for 10 minutes. Subsequently, the bottle was left inside the hood for three days to allow THF to evaporate. The solution was then filtered via a 4.0 μm syringe, a crucial step for removing large particles and aggregates. Produced conjugated polymer nanoparticles were stored at room temperature.

3.3.3 Construction of Biosensor

The graphite electrode's surface was polished using emery paper and then washed with distilled water before any modifications were made. 6.0 μL of conjugated polymer nanoparticles were then deposited onto the surface, and a specific amount of laccase (5.0 μL Lac 6.54 Units) was applied to the working electrode area. Once the enzyme was immobilized, 5.0 μL of 1% GA solution was added as a crosslinking agent to the modified electrode. The biosensor was left to dry at room temperature for 2 hours, and then the biosensor surface was rinsed with distilled water to remove any additional enzyme residues (Figure 3.2.).

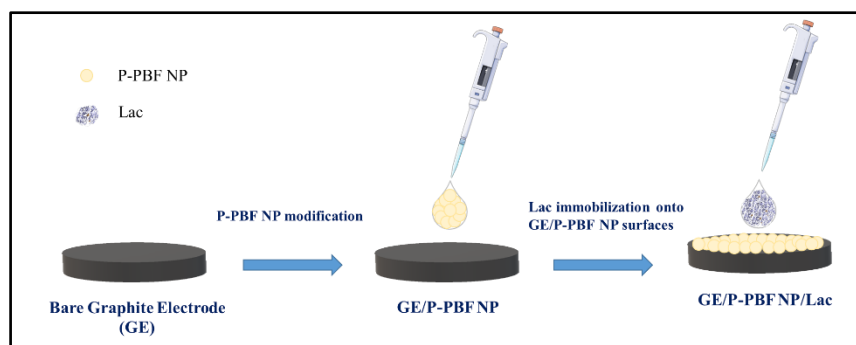


Figure 3.2. Schematic representation of the preparation steps of the designed GE/P-PBF NP/Lac biosensor

3.3.4 Amperometric Measurements for Detection of Catechol

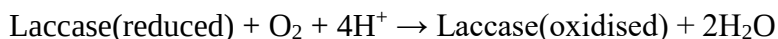
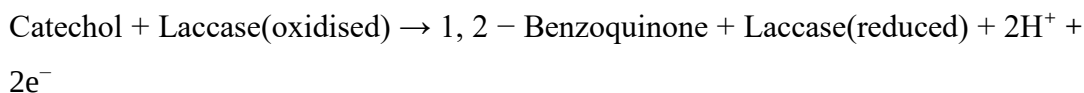
The current measurements for the enzymatic reaction were carefully conducted in a buffer solution at room temperature. Catechol (10 μM) was added as the substrate, and the response was recorded after reaching a stable baseline (50 s). The difference in current between the response before and after the substrate addition was named current difference. The calibration curve graph, a precise representation, was constructed by plotting ΔI (current difference) against analyte concentration, ensuring the accuracy of our results. After each measurement, a standard cleaning process was applied to the working cell and electrodes. The biosensor response was determined by monitoring the formation of hydroquinone resulting from the enzymatic hydrolysis of catechol. The detection of catechol was achieved by oxidation at -0.3 V compared to Ag wire.

$$\Delta I = I_2 - I_1$$

I_1 = Before catechol injection

I_2 = After catechol injection

The mechanism between laccase and its substrates in determining catechol involves converting catechol to 1,2-benzoquinone and electrocatalytically reducing oxygen to water at the electrode surface²⁰⁵.



3.3.5 Optimization Studies of Constructed Biosensor

Parameters that affect the performance of the GE/P-PBF NP/Lac biosensor should be optimized to develop a stable and sensitive biosensor. Therefore, the response of the constructed biosensor was evaluated in terms of the amount of the conjugated polymer nanoparticles, the amount of enzyme, and pH.

3.3.5.1 Optimization of PBF NP amount

For optimization of the conjugated polymer nanoparticles, different amounts of P-PBF NP (3.0 μL , 5.0 μL , 6.0 μL , 7.0 μL , or 8.0 μL) were immobilized onto five graphite electrode surfaces, and these electrodes were air-dried for 1 h at room temperature. Then, 5.0 μL laccase enzyme solution (0.3 mg-6.54 U prepared in 50.0 mM acetate buffer, pH: 5.5) and 5.0 μL glutaraldehyde solution (in 50.0 mM PBS pH: 7.00) and modified electrodes were air-dried for 2 h at room temperature. Before amperometric measurements, modified electrodes were washed with dH_2O . All amperometric measurements were recorded in a reaction cell, including 50.0 mM acetate buffer solution (pH 5.5) at room temperature in the presence of 10 μM catechol. The applied potential was -0.3 V.

3.3.5.2 Optimization of Enzyme Amount

For enzyme optimization, we prepared five different laccase enzyme solutions in 50.0 mM acetate buffer, pH: 5.5 as follows: 0.1 mg-2.18 U, 0.2 mg-4.36 U, 0.3 mg-6.54 U, 0.4 mg-8.72 U, 0.5 mg-10.9 U). 6.0 μL P-PBF NP solutions were immobilized onto five graphite electrode surfaces, these electrodes were air-dried for

1 h at room temperature. Then, 5.0 μ L laccase enzyme solution and glutaraldehyde solution (in 50.0 mM PBS pH: 7.00) and modified electrodes were air-dried for 2 h at room temperature. Before amperometric measurements, modified electrodes were washed with dH₂O. All amperometric measurements were recorded in a reaction cell, including 50.0 mM acetate buffer solution (pH 5.5) at room temperature in the presence of 10 μ M catechol. The applied potential was -0.3 V.

3.3.5.3 Optimization of pH

For pH optimization studies 50.0 mM buffer solutions were prepared in the pH range of 4.5-7.0 (NaOAc buffer at pH 4.5, 5.0, and 5.5, PBS buffer at pH 6.5 and 7.0), and the responses of the constructed biosensor were recorded at solutions with these five different pH values. 6.0 μ L P-PBF NP solutions were immobilized onto five graphite electrode surfaces, these electrodes were air-dried for 1 h at room temperature. Then, 5.0 μ L laccase enzyme solution (0.3 mg-6.54 U prepared in 50.0 mM acetate buffer, pH: 5.5) and 5.0 μ L glutaraldehyde solution (in 50.0 mM PBS pH: 7.00) and modified electrodes were air-dried for 2 h at room temperature. Before amperometric measurements, modified electrodes were washed with dH₂O. Amperometric measurements were conducted in different buffer solutions in the pH range of 4.5-7.0 at room temperature in the presence of 10 μ M catechol. The applied potential was -0.3 V.

3.3.6 Characterization

3.3.6.1 Electrochemical and Surface Characterization

Electrochemical and surface characterization were performed as mentioned in Section 2.2.6 using P-PBF NP instead of Chit-AgNPs.

Electrochemical impedance spectroscopy (EIS) was used to examine the interfacial properties of bare, GE modified with P-PBF NP, GE modified with P-PBF NP and

Lac. The experiments were conducted in a solution containing 5.0 mM $\text{Fe}(\text{CN})_6^{3-/4-}$ and 0.1 M KCl.

3.3.6.2 Analytical Characterization

Analytical characterization of GE/P-PBF NP/Lac biosensor, including LOD, LOQ and sensitivity, was estimated using the same methods and equations mentioned in section 2.3.5.1.

3.3.6.3 Determination of Kinetic Parameters

Kinetic parameters of GE/P-PBF NP/Lac biosensor was determined as mentioned mentioned in section 2.3.5.2.

3.3.7 Investigation of Interferents

Accurate measurement can be disrupted by electrochemical interference caused by certain substances such as ascorbic acid, uric acid, or paracetamol³⁷. For a biosensor to work successfully on multiple samples, it must be free of any interference²⁴⁴. To ensure this, studies were conducted to investigate the effects of different materials as interferents. Specifically, glucose, urea, citric acid, ascorbic acid, uric acid, paracetamol, NaCl, and KCl were studied as possible interferences for our bisensing system.

3.3.8 Sample Application

The performance and reliability of biosensors are vital factors to consider. The constructed biosensor was used to test catechol in tap water^{85,89,216,217} and green tea²⁴⁵ samples to test these factors. In order to evaluate the analytical applicability of the GE/PBF NP/Lac biosensor, the spike recovery method was used. In this method, the

known concentrations of catechol (5.0 μ M and 10 μ M). The target sample was injected into the reaction cell without pretreatment, and the catechol concentration was calculated using the calibration curve.

3.4 Results and Discussion

3.4.1 Characterization of Conjugated Polymer and Conjugated Polymer Nanoparticles

Conjugated polymers have attracted considerable attention globally due to their potential for use in designing biosensors. They can provide a framework for biological molecules, allowing for creating sensitive and dependable sensor designs. However, their limited compatibility with water and the use of harmful organic solvents in their processing have restricted their applications. Nonetheless, their cost-effective production and diverse uses in biosensors, imaging, drug delivery systems, and other fields have made fluorescent conjugated polymers the focus of extensive research in recent years^{102,114,116,246}. This chapter explores the production and characterization of conjugated polymer (P-PBF) and conjugated polymer nanoparticles (P-PBF NP) using the Stille cross-coupling reaction and reprecipitation method, respectively.

The ultraviolet-visible (UV-Vis) absorption and photoluminescence (PL) emission spectra of P-PBF were recorded to investigate its optical properties, as depicted in Figure 3.3A and Figure 3.3B. Due to their low-cost synthesis and wide range of applications, such as biosensors, imaging, drug delivery carriers, and others, fluorescent conjugated polymers have received much attention in the last few decades. Luminescence is defined as emitting optical light that exceeds the thermal radiation emitted by the substance²⁴⁷. UV-VIS spectrophotometry determines the excitation wavelength. The specific optical absorption of an organic semiconductor is critical to the performance of organic optoelectronic devices²⁴⁷. In conjugated polymers, absorption results in the generation of an exciton. If the diffusion length

is sufficiently long so that the exciton meets an internal field, hole and electron separation occurs, and these charges are collected at their respective electrodes²⁴⁸. The photophysical properties of the P-PBF polymer are analyzed using these two methods in both nanoparticle and solution forms. In UV-VIS spectrophotometry, the maximum absorption was measured at 492 nm in solution, implying that the polymer absorbs greenish blue. The FWHM range is relatively high, indicating a high absorption range²⁴⁹. When the polymer is excited at 492 nm, there is emission at ~600 nm. This shows an orange-yellow emission during the excitation process²⁴⁹. In nanoparticle form, absorbance and emission shifted blue. Hence, it is deduced that P-PBF is probably partly amorphous in the NPs and partly aggregated, but not really crystallized as in the solid film²⁴⁹. Moreover, full width at half maximum (FWHD) increased for absorbance and decreased for emission. This implies a higher absorbance range and more pure color emission²⁵⁰. Maximum absorption was measured at ~420 nm in NP, implying NP absorbs blue light. When excited at 420 nm, NP emits light at ~535 nm. This shows a green light emission during excitation. Upon a thorough comparison of the UV-vis and PL spectra results of P-PBF conjugated polymer and P-PBF NP (Figures 3.3a and 3.3b), we can conclude that the structure of P-PBF NP is distinct from that of P-PBF conjugated polymer.

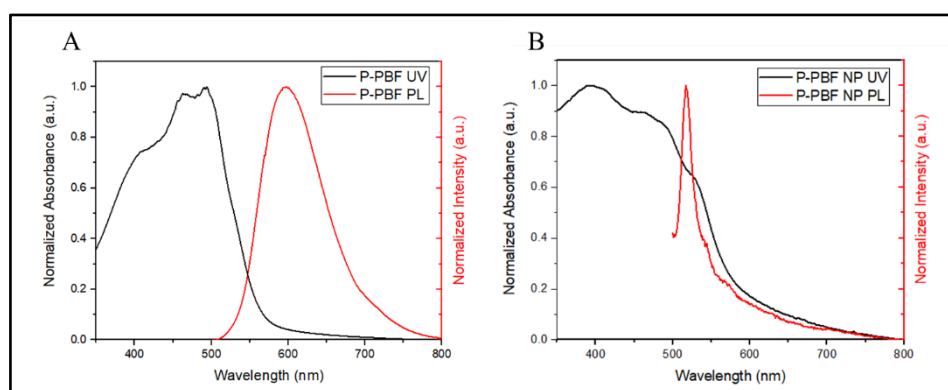


Figure 3.3. UV-VIS and PL spectra of P-PBF a-) in solution form b-) in nanoparticle form

The DLS data in Figure 3.4A revealed that the hydrodynamic diameter was 92.28 ± 4.26 nm. Moreover, the polymer was reasonably uniform since the polydispersity index (PDI) (0.213) was lower than 0.4. The measurement of zeta potential is crucial in determining colloidal dispersions' stability. Particles with a strong positive or negative charge repel each other, resulting in stable colloidal solutions with minimal tendencies to form agglomerates²⁵¹. The charge of P-PBF NP was -33.1 ± 14.8 mV, which indicates that these conjugated polymer nanoparticles do not tend to agglomerate (Figure 3.4B). Colloids that exhibit zeta potential values within the range of ± 30 -30 mV or beyond are generally considered highly stable²⁵¹.

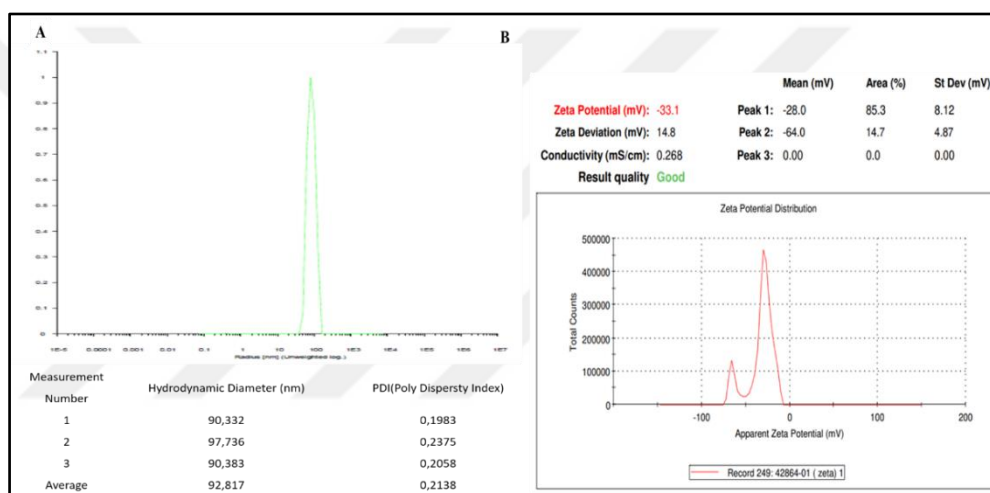


Figure 3.4. Particle size (A) and zeta potential distribution (B) of P-PBF NP

3.4.2 Optimization Studies

The optimization stages of the developed biosensor are crucial for significantly enhancing its response and efficiency^{217,244}. The GE/P-PBF NP/Lac biosensing platform must be optimized to ensure consistent measurements and significantly improved performance⁸⁸. Within the scope of the study, the amount of P-PBF NP, laccase enzyme, and pH of the working medium were optimized, respectively. In each optimization study, one parameter is changed while the other two parameters are kept constant. In all optimization studies, 10 μ M catechol solution was used to show the change in current.

3.4.2.1 Optimization of P-PBF NP amount

To optimize the conjugated polymer nanoparticles used in the biosensor, different amounts of P-PBF NP (3.0 μL , 5.0 μL , 6.0 μL , 7.0 μL , or 8.0 μL) were cast on five separate electrodes, and the response of the electrode was measured. All other parameters were kept constant during the measurements. Casting 6.0 μL of the P-PBF NP solution provided the highest signal. Using higher (7.0 μL and 8.0 μL) or lower (3.0 μL and 5.0 μL) amounts of conjugated polymer nanoparticles resulted in a lower response. This was due to the unfavourable redox performance caused by conjugated polymer nanoparticle coatings that were either too thick or too thin, which hindered the interaction between the enzyme's active site and the electrode surface (Figure 3.5.). Udum et al. developed a laccase biosensor for determining catechol using a conjugated polymer⁸⁵. Similar to our findings, they observed that improper enzyme immobilization, resulting from excessively thin or thick polymer films, can impede diffusion between the polymer-coated electrode and the laccase enzyme biomolecule.

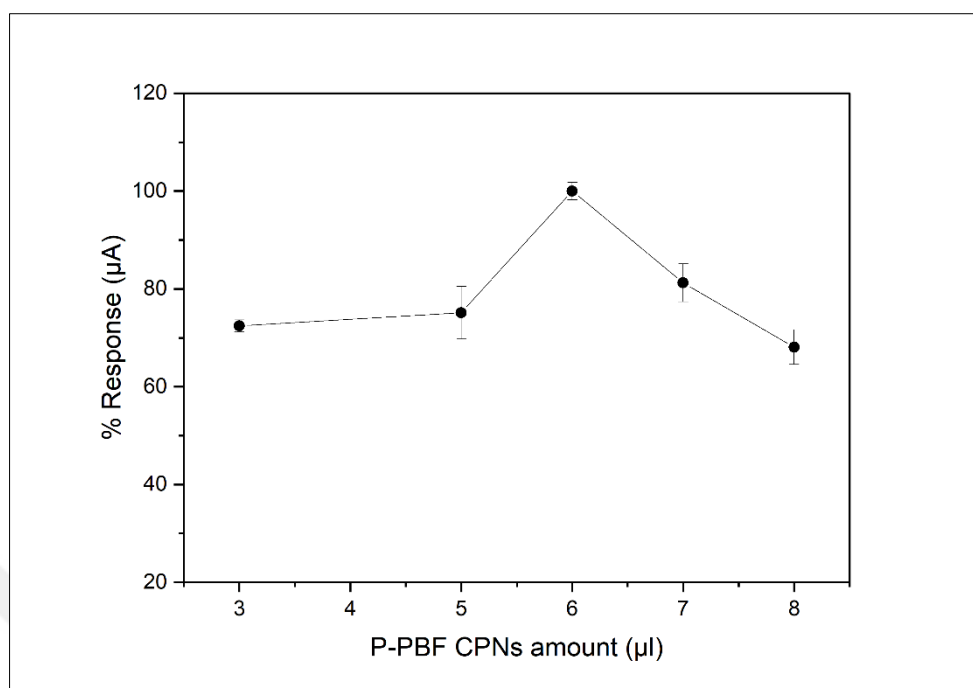


Figure 3.5. Effect of P-PBF NP amount on the response of the biosensor. Error bars represent standard deviation of three measurements

3.4.2.2 Optimization of enzyme amount

In order to optimize the performance of the sensor, it is essential to use the right amount of Laccase (Lac). Too much enzyme can exceed the enzyme-loading capacity, while too little can cause low diffusion in the enzymatic surface^{83,88,90}. Different electrodes were prepared to determine the ideal amount of enzyme by varying the concentration of enzyme (ranging from 0.1 mg-2.18 U to 0.5 mg-10.9 U of Lac) while keeping other parameters constant. After conducting amperometric tests, it was found that 0.3 mg (6.45 U) of Lac provided the highest response, as shown in Figure 3.6. The signal was highest in the graphite electrode with 0.3 mg of laccase enzyme. The enzyme units of 2.18 U and 4.36 U may not be enough for proper enzyme immobilization. In comparison, at enzyme units of 8.72 U and 10.9 U, the enzyme's active site cannot interact appropriately with catechol due to steric hindrance, leading to a lower response. Umut et al. designed a glucose biosensing

platform using a conjugated polymer and conducted experiments to optimize the amount of conjugated polymer, enzyme, and pH⁸⁸. Their findings, which underscore the critical role of enzyme concentration in the biosensing platform, revealed that lower enzyme concentrations might not effectively immobilize the enzyme. In comparison, higher concentrations could lead to less effective interaction between the enzyme's active site and glucose due to steric hindrance, resulting in a reduced response.

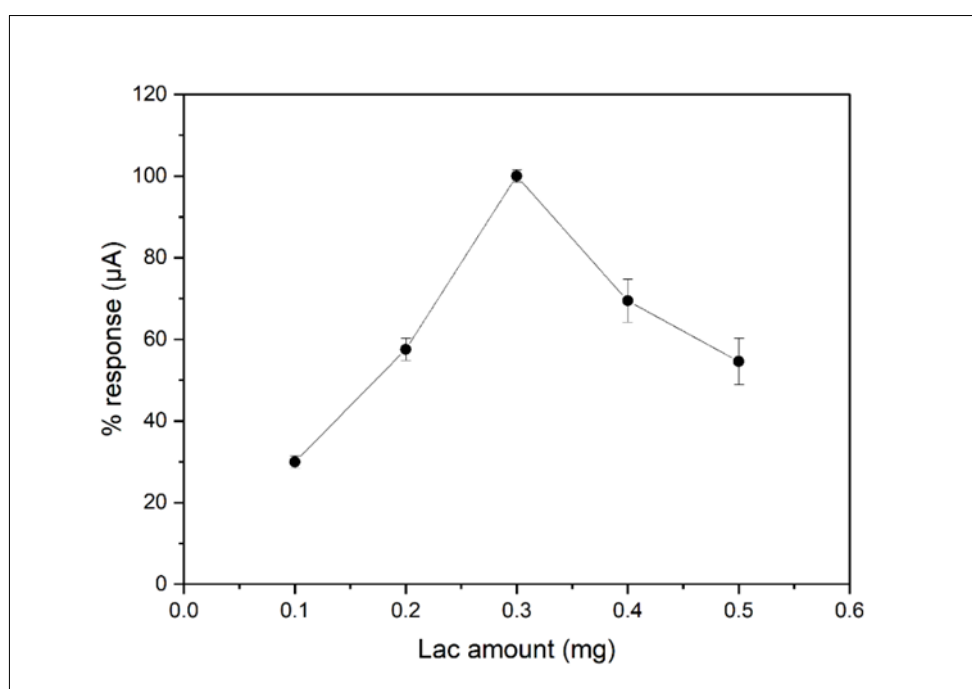


Figure 3.6. Effect of the enzyme amount on the response of the biosensor. Error bars represent standard deviation of three measurements

To obtain the most sensitive and stable system, the pH of the working medium for the fabricated biosensor was optimized. For this purpose, 50.0 mM buffer solutions were prepared in the pH range of 4.5 –7.0 using NaOAc buffer at pH 4.5, 5.0, and 5.5 and PBS buffer at pH 6.5 and 7.0. The sensor response was recorded at these five different pH solutions. The results, shown in Figure 3.8, indicate that the maximum and most stable current was obtained at pH 5.5 medium.

The experiment's findings underscore the potential impact of pH changes on enzyme activity and the shape of the active site. The enzyme's response increased with pH from 4.5 to 5.5 but decreased at higher pH levels (6.5 and 7.5), possibly leading to enzyme denaturation⁸⁸. This decline could be due to alterations in the shape of the enzyme's active site, making it less favourable to the reaction with catechol highlighting, the importance of maintaining optimal pH conditions for enzyme activity (Figure 3.7).

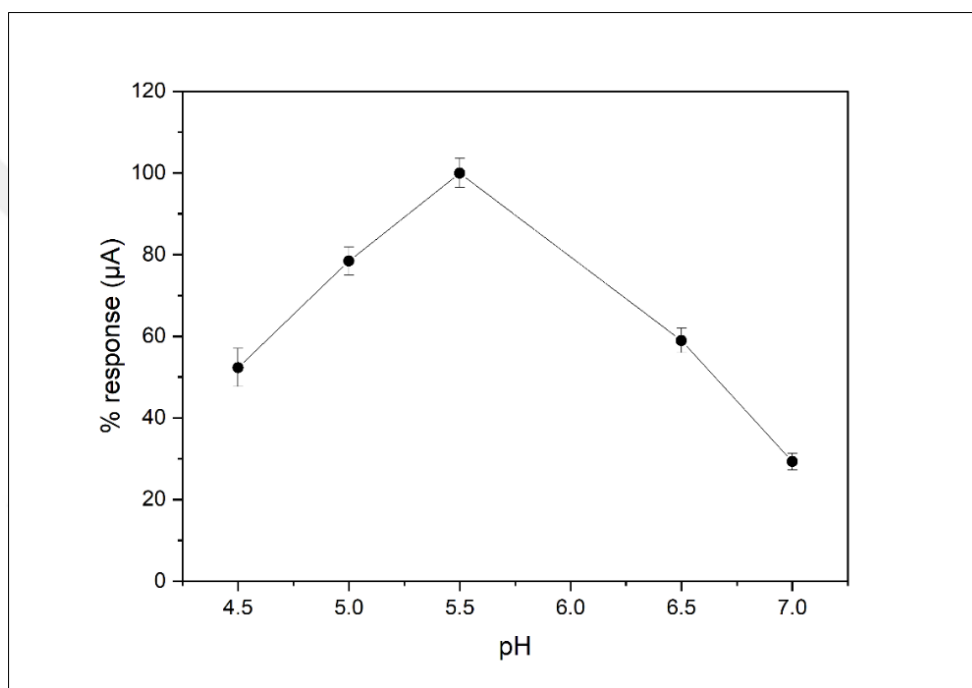


Figure 3.7. Effect of pH on the response of the biosensor. Error bars represent standard deviation of three measurements

3.4.3 Characterization of Constructed Biosensor

3.4.3.1 Electrochemical and Surface Characterization

Both cyclic voltammetry (CV) and electrochemical impedance spectroscopy (EIS) were carried out to confirm the sequential electrode modification process. The surface characterization and charge transfer mechanisms of P-PBF NP and P-PBF

NP/Lac modified electrodes were analyzed using CV under optimized conditions. CV experiments were also conducted to compare the modified electrodes with the unmodified ones. The electron transfer mechanism between the species and the electrode surface in the solution was examined using 5mM $\text{Fe}(\text{CN})_6^{3-/4-}$. The results revealed the oxidation-reduction peaks for bare GE, GE/P-PBF NP, and GE/P-PBF NP/Lac electrodes, as illustrated in Figure 3.8. Upon analyzing the CV (cyclic voltammetry) values in the presence of a redox mediator, it was observed that the bare electrode displays sharp reversible oxidation and reduction peaks. However, upon comparison with the electrode modified with P-PBF NPs (conjugated polymer nanoparticles), a decrease in oxidation current is noticed due to adding a layer on the electrode surface. Coating the electrode surface with conjugated polymer nanoparticles has formed distinct structures on the electrode surface. Subsequently, there is a significant reduction in peak currents upon modifying the electrode surface with P-PBF NP/Lac. The presence of the enzyme molecule on the electrode surface has caused a decrease in conductivity. The reduction in peak current is a clear indicator of the successful immobilization of the enzyme on the graphite electrode surface. According to the current responses, the effective surface areas were estimated as follows: 0.10 cm² for bare GE, 0.07 cm² for GE/P-PBF NP electrode and 0.04 cm² for GE/P-PBF NP/Lac electrode²⁵².

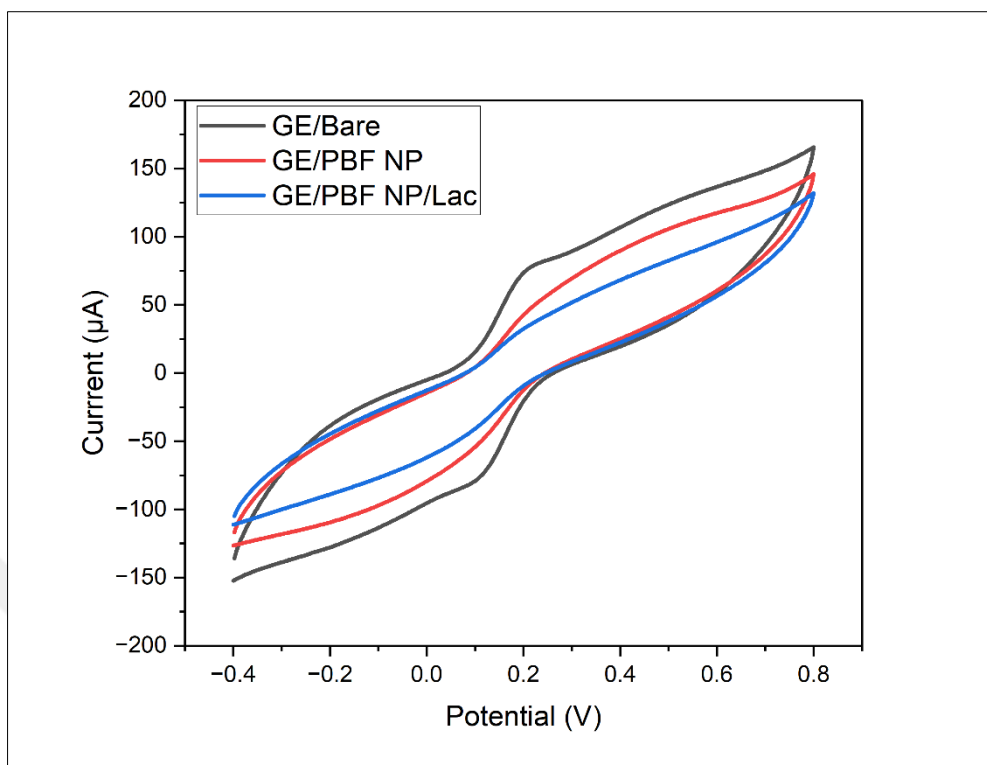


Figure 3.8. Cyclic voltammograms of unmodified graphite electrode, GE/P-PBF NP and GE/P-PBF NP/Lac in 5.0 mM $\text{Fe}(\text{CN})_6^{3-/4-}$ containing 0.1M KCl and pH 5.5 acetate buffer at 50 mV/s.

An effective way to evaluate the relationship between graphite electrodes and conjugated polymer nanoparticles, with Lac modification, is electrochemical impedance spectroscopy (EIS). The EIS experiments used a 5.0 mM $\text{Fe}(\text{CN})_6^{3-/4-}$ a solution containing 0.1M KCl and a pH 5.5 acetate buffer. Figure 3.9. displays the impedance spectra of the bare GE, GE/P-PBF NP, and GE/P-PBF NP/Lac configurations. Introducing P-PBF NP onto the bare GE altered the electron transfer resistance, potentially due to the nanoparticle's hindrance of electron exchange between the redox probe and the electrode surface²¹¹. The subsequent application of Lac onto the GE/P-PBF NP electrode further increased the charge transfer resistance, indicating successful enzyme immobilization. The results obtained from CV and EIS experiments were consistent and collectively confirmed the effectiveness of the electrode surface modifications

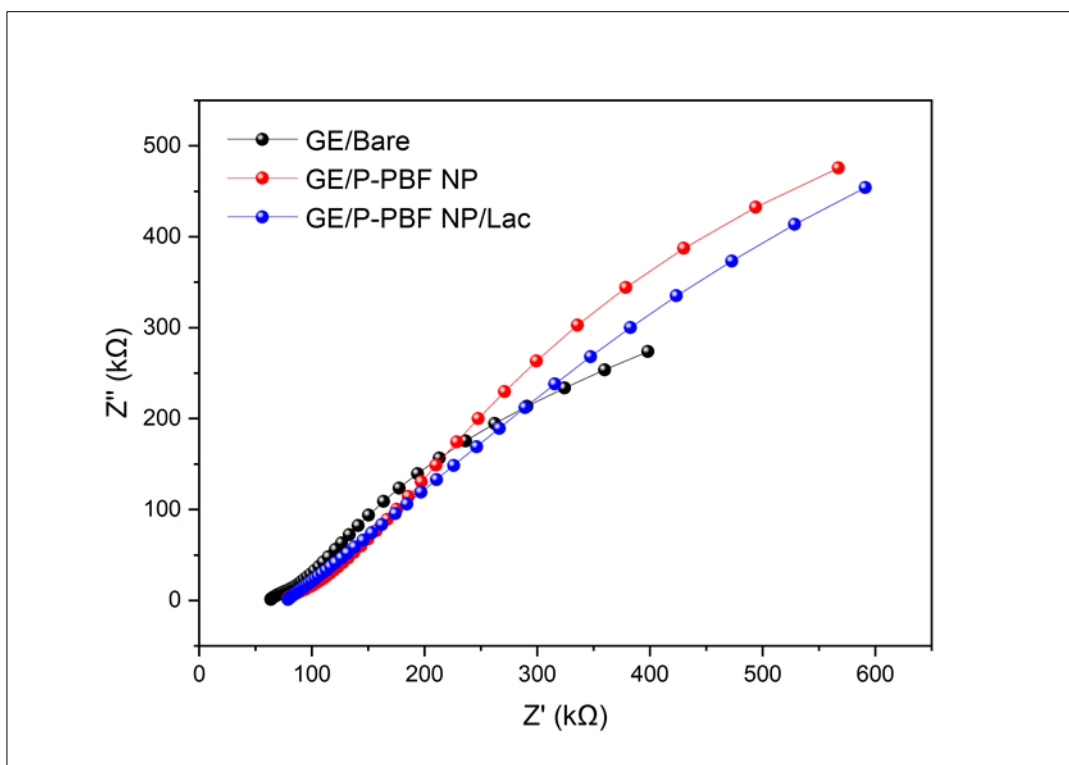


Figure 3.9. EIS of unmodified graphite electrode, GE/P-PBF NP and GE/P-PBF NP/Lac in 5 mM $\text{Fe}(\text{CN})_6^{3-/4-}$ containing 0.1M KCl and pH 5.5 acetate buffer.

FE-SEM analysis was performed to analyze the surface morphology of the P-PBF NP and P-PBF NP/Lac modified graphite electrodes. FE-SEM images of GE/P-PBF NP (Figure 3.10A-B) and GE/P-PBF NP/Lac (Figure 3.10C-D) at different magnifications As depicted in Figure 3.10, the conjugated polymer nanoparticles (P-PBF NP) cover the surface of the graphite electrode homogeneously. A significant change was observed after the Lac enzyme was immobilized onto the GE/P-PBF NP electrode's surface. The enzymes formed a second layer covering the surface (Figure 3.10C-D). The GE/P-PBF NP/Lac electrode surface was an enzyme deposition anchor.

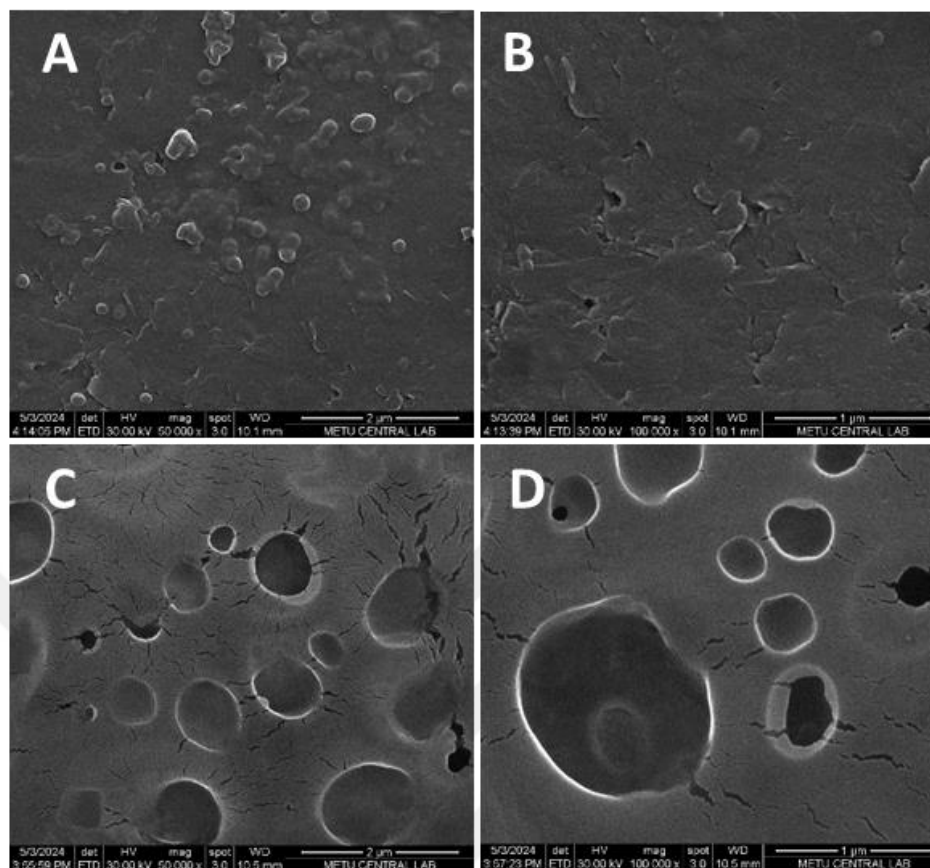


Figure 3.10. FE-SEM images of the modified electrodes; A and B GE/P-PBF NP (at 50.000x and 100.000x magnification), C and D GE/P-PBF NP/Lac (at 50.000x and 100.000x magnification)

3.4.3.2 Analytical Characterization

Under our study's specific experimental conditions, a perfect linear relationship between current signals and catechol concentration was observed in the GE/P-PBF NP/Lac biosensor. This relationship, as shown in Figure 3.11, was obtained at room temperature for catechol concentrations ranging from 0.5 to 25 μM in a 50.0 mM sodium acetate buffer with a pH of 5.5. The GE/P-PBF NP/Lac biosensor calibration equation in the 0.5–25 μM catechol concentration range was determined as $y=0.0793x+0.093$ ($R^2=0.99$). Our biosensor features a broader linear range than

other laccase biosensors documented in existing literature GCE/GO,chit/ZnO-Al₂O₃/AuNPs/Lac (0.5-40 μ M)²⁵³, GCE/PANI/GA/Lac (3.2-19.6 μ M)²⁰¹, GCE/GC-rGO/Lac (0.2-15 μ M)²³³, GCE/GO,chit/ZnO-Al₂O₃/AuNPs/Lac (0.5-40 μ M)²⁰⁰, CB-SPE/e-Lac (2.5-50 μ M)²⁰³.

Moreover, the K_M^{app} and I_{max} values, significant indicators of the biosensor's performance, were calculated using the Lineweaver-Burk Plot (Figure 3.12.) and found to be 49.01 μ M and 5.47 μ A, respectively. When the K_M^{app} value was obtained within the scope of this work, it was lower compared to the literature examples: GCE/PANI/GA/Lac (K_M^{app} : 95.9 μ M)²⁰¹, GCE/GC-rGO/Lac (K_M^{app} : 93.4 μ M)²³³, GCE/Chit/AP-rGO/Lac (K_M^{app} : 256 μ M)¹⁹⁸, the mesoporous silica sieve MCM-41 containing methylene blue and polyvinyl alcohol modified Au electrode (K_M^{app} : 500 μ M)²³⁴. It is worth mentioning that low values for an enzyme indicate a high affinity toward its substrate.

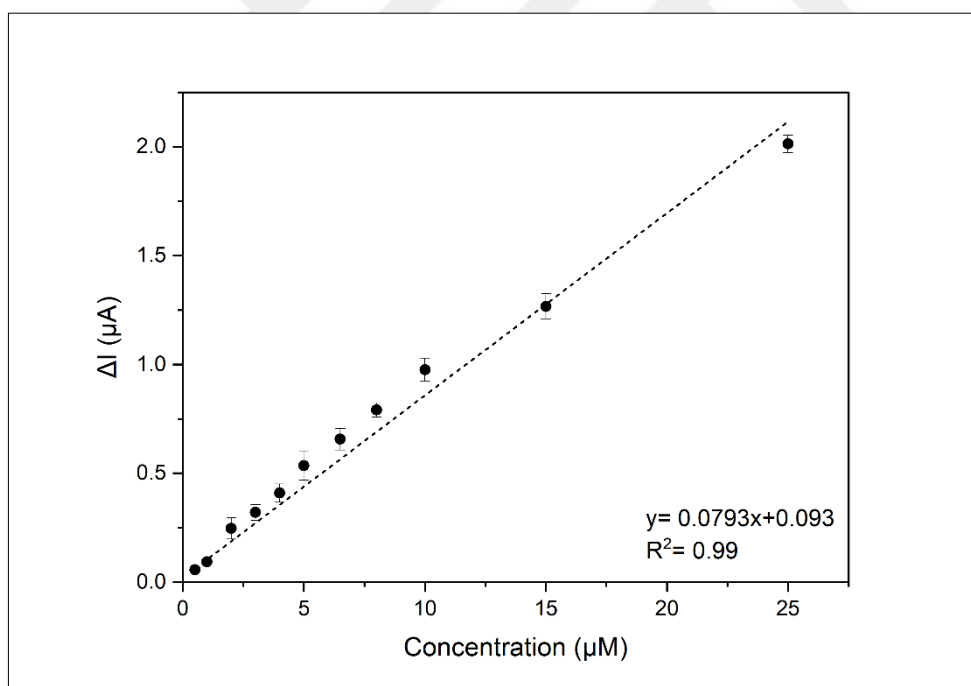


Figure 3.11. Amperometric current-time response curve of GE/P-PBF NP/Lac

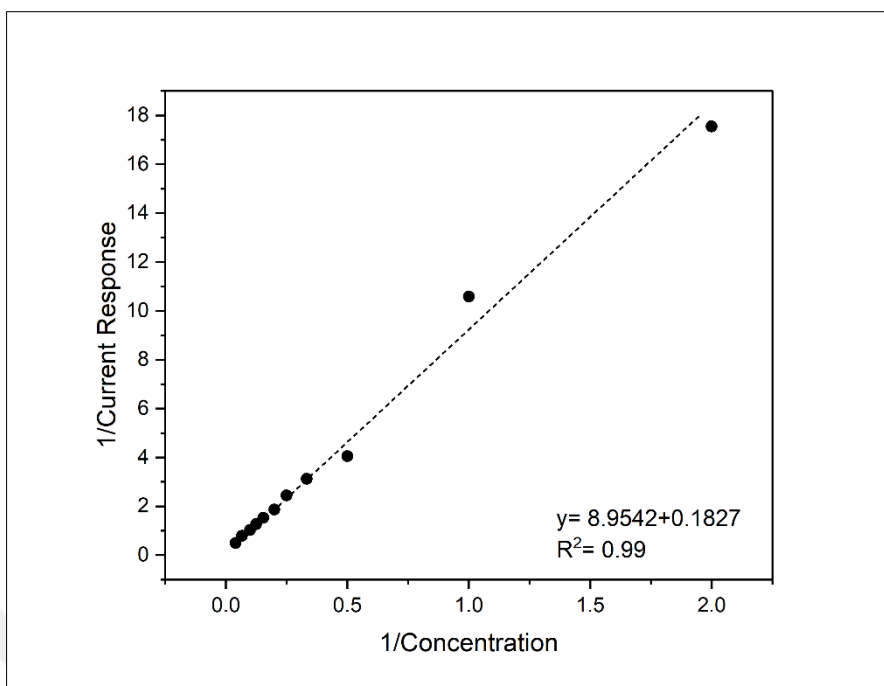


Figure 3.12. Lineweaver-Burk Plot of GE/P-PBF NP/Lac biosensor

In addition to the linear range and linearity equation, limit of detection (LOD) values were determined under optimal operational conditions. Analytical properties values of GE/P-PBF NP/Lac biosensors are listed in Table 3.2. The $3s/m$ relation was used to determine the LOD value, where "s" represents the standard deviation of the lowest concentration and "m" represents the calibration slope. The LOD value of the GE/P-PBF NP/Lac biosensor was calculated as $0.49 \mu\text{M}$. Moreover, the LOQ value of the proposed biosensor was calculated at $1.48 \mu\text{M}$. The proposed biosensor's detection limit is lower than other laccase biosensors reported in the existing literature: NiO/MCPE/Lac ($0.95 \mu\text{M}$)²⁵⁴, CB-SPE/e-Lac ($2.0 \mu\text{M}$)²⁰³, GCE/Nafion/Lac/CNFs/Cu ($1.18 \mu\text{M}$)²⁰², GCE/PANI/GA/Lac ($2.07 \mu\text{M}$)²⁰¹, GCE/GO,chit/ZnO-Al₂O₃/AuNPs/Lac ($3.1 \mu\text{M}$)²⁰⁰, CPE/Chit/Lac/PANI/Fe₃O₄ ($0.4 \mu\text{M}$)²⁵⁵.

Table 3.2 Analytical properties values of GE/P-PBF NP/Lac biosensor

Parameters	
K_M^{app}	49.01 μ M
I_{max}	5.47 μ A
Linear range	0.5-25 μ M
Sensitivity	1.229 μ A/ μ Mcm ²
LOD	0.49 μ M
LOQ	1.48 μ M

A comparison for analytical parameters of the GE/P-PBF NP/Lac biosensor was made with previously reported in the literature is presented in Table 3.3. The designed biosensor had the highest value of 1.229 μ A/ μ Mcm² compared to other laccase biosensors previously reported in the literature N-OMC/PVA/Lac/Au (0.29 μ A/ μ Mcm²)²⁵³, GCE/MWCNT_2/75_Lac (0.56 μ A/ μ Mcm²)²⁵⁶, GCE/PANI/GA/Lac (0.707 μ A/ μ Mcm²)²⁰¹, GCE/Nafion/Lac/CNFs/Cu (0.033 μ A/ μ Mcm²)²⁰², SPCE/Lac/GR-CMF (0.932 μ A/ μ Mcm²)²⁵⁷, GCE/GC-rGO/Lac (0.093 μ A/ μ Mcm²)²³³, CPE/Chit/Lac/PANI/ Fe₃O₄ (0.126 μ A/ μ Mcm²)²⁵⁵.

After comparing the analytical properties of the GE/P-PBF NP/Lac electrode with those of the GE/Chit-AgNPs/Lac electrode (Table 4.1), it is evident that the GE/P-PBF NP/Lac electrode is eight times more sensitive. Additionally, the GE/P-PBF NP/Lac biosensor displays lower LOD (limit of detection) and LOQ (limit of quantification) values (0.49 μ M, 1.48 μ M) compared to the GE/P-PBF NP/Lac biosensor (3.85 μ M, 12.71 μ M), with the former showing eightfold and ninefold lower values, respectively. K_M^{app} measures an enzyme's affinity for its substrate, and a lower K_M^{app} value signifies better substrate binding and higher catalytic activity.

Table 3.3 Comparison of analytical performances of electrochemical laccase biosensors for catechol detection

Modified Electrode	Detection Method	LOD (μM)	Linear Range (μM)	Sensitivity ($\mu\text{A}/\mu\text{M.cm}^2$)	Reference
GCE/GO, chit/ZnO- Al ₂ O ₃ /AuNPs/Lac	DPV	3.1	0.5-40	NR	200
N-OMC/PVA/Lac/ Au	A	0.31	0.39-8.98	0.29	253
GCE/MWCNT_2/7 5_Lac	DPV	0.028	0.1-57	0.56	256
GCE/PANI/GA/Lac	CV	2.07	3.2-19.6	0.767	201
GCE/Nafion/Lac/C NFs/Cu	A	1.18	9.95-9760	0.033	202
SPCE/Lac/GR- CMF	A	0.085	0.2-209.7	0.932	257
CB-SPE/e-Lac	A	2.0	2.5-50	NR	203
NiO/MCPE/Lac	A	0.95	2-160	1.415	254
GCE/GC-rGO/Lac	A	0.076	0.2-15	0.093	233
CPE/Chit/Lac/PANI /Fe ₃ O ₄	A	0.4	0.5-80	0.126	255
GE/Chit- AgNPs/Lac	A	3.85	5-250	0.444	Chapter 2
GE/P-PBF NP/Lac	A	0.49	0.5-25	1.229	This work

A: Amperometry, Al₂O₃: Aluminum oxide, Au: Gold, AuNPs: Gold nanoparticles, Chit: Chitosan, CNFs: Carbon nanofibers, CV: Cyclic voltammetry, Cu: Copper, CPE: Carbon paste electrode, DPV: Differential pulse voltammetry, Fe₃O₄: Iron(II,III) oxide, GA: Glutaraldehyde, GCE: Glassy carbon electrode, GC: Glycol chitosan, GO: Graphene oxide, rGO: Reduced graphene oxide, GR-CMF: Graphene-cellulose microfibers, Lac: Laccase, MCPE: Modified carbon paste electrode, MWCNT: Multi-walled carbon nanotubes, N-OMC: nitrogen-containing ordered mesoporous carbon, SPCE: Screen printed carbon electrode, PANI: Polyaniline, PVA: Polyvinyl alcohol, ZnO: Zinc oxide

The performance of the GE/PBF NPs/Lac biosensor was assessed through reproducibility tests and shelf-life experiments. To evaluate reproducibility, twelve amperometric signals were recorded toward 10 μM catechol, and the results are presented in Figure 3.13. The analysis showed a low percentage relative standard deviation (% RSD) of 2.94, indicating excellent reproducibility and stability of the biosensor.

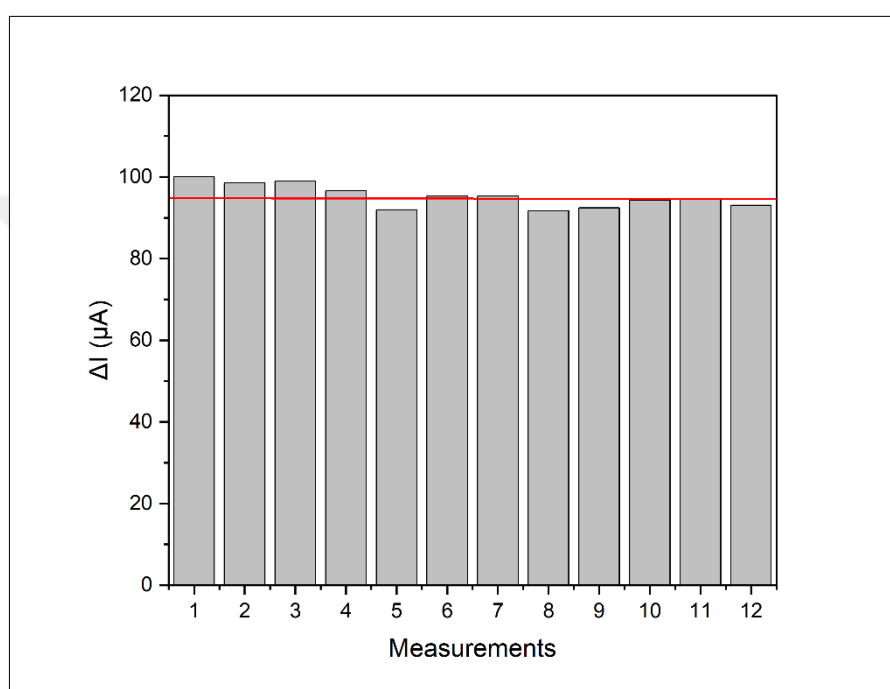


Figure 3.13. Reproducibility results of the GE/P-PBF NP/Lac biosensor

The storage duration of the developed biosensor was evaluated over 30 days. As part of the investigation, a 10 μM catechol solution was introduced into the electrochemical measurement cell, which contained 10 mL of buffer solution at a pH of 5.5. Figure 3.14. illustrates the results, indicating that the biosensor maintained 56% of its initial response after being stored at 4°C for 30 days.

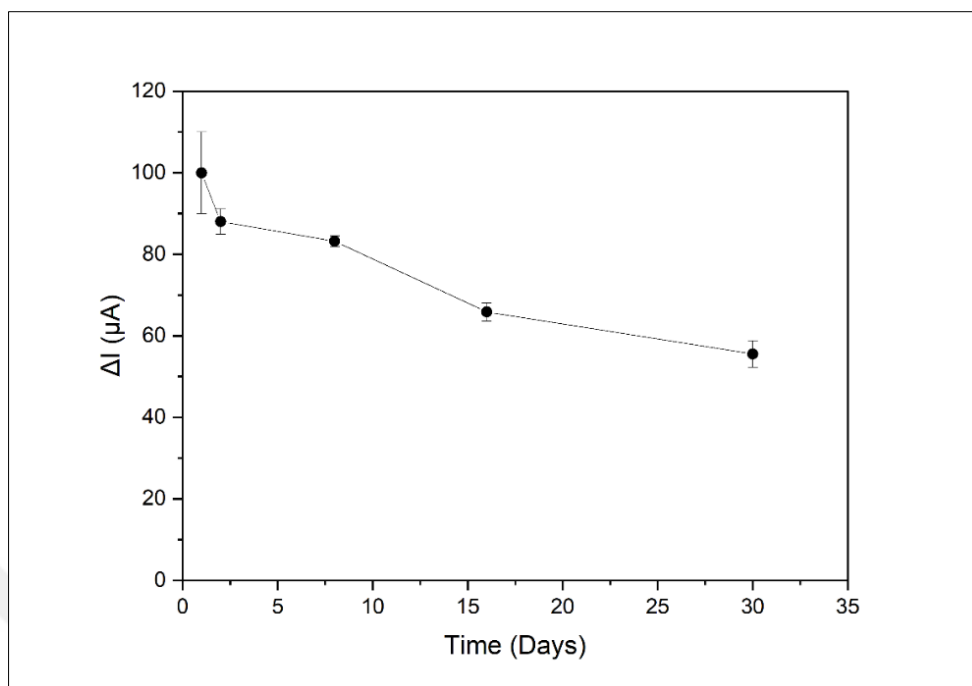


Figure 3.14. Shelf-life results of the GE/P-PBF NP/Lac biosensor

3.4.4 Investigation of Interference

Detecting target analytes and distinguishing them from other interfering chemicals is crucial for the practical biosensor system's reliability. Our study examined several substances' impact on the electrochemical detection of the target analytes using amperometric measurement under optimized conditions. The effects of glucose, ascorbic acid, citric acid, urea, uric acid, paracetamol, NaCl, and KCl were evaluated because these analytes may interfere with the system during amperometric measurements^{85,89,216,217,244}. To test the biosensor's selectivity, the amperometric responses were measured for all these interfering chemicals (10 μM) in a 50.0 mM pH 5.5 sodium acetate buffer at -0.3 V. The results indicated that the current signal for catechol was significantly higher than the responses of any other interfering chemicals (Figure 3.15). These findings suggest that the developed biosensor has excellent selectivity and potential for practical applications.

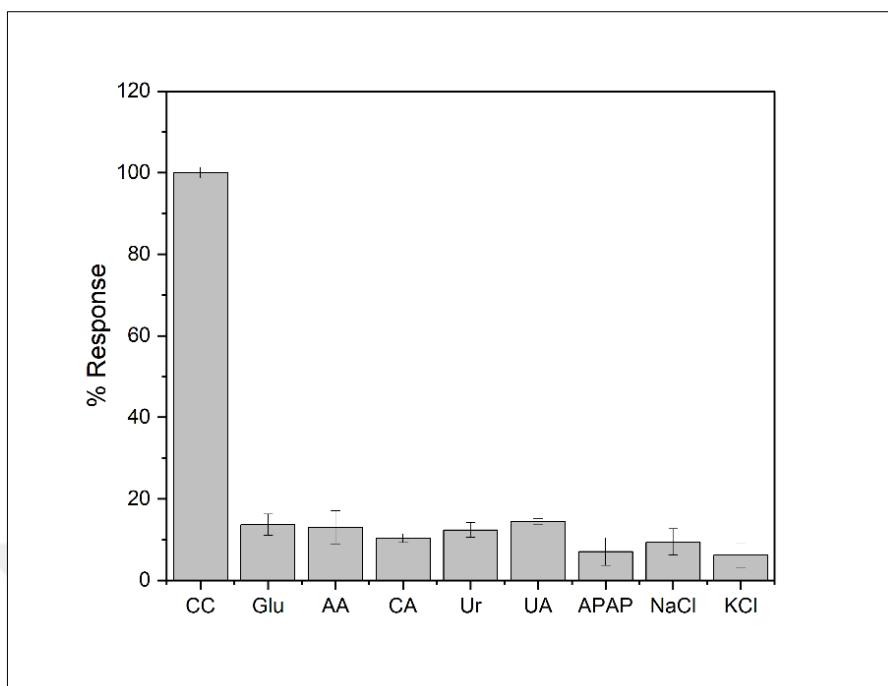


Figure 3.15. Interference results of the GE/P-PBF NP/Lac biosensor

3.4.5 Sample Application

During the recovery test, the effectiveness of the GE/P-PBF NP/Lac biosensor was evaluated for analyzing samples. The test involved adding spiked catechol to tap water and green tea. The results indicated amperometric responses when the samples were spiked with different catechol concentrations, precisely at 5.0 and 10 μM . Amperometry was used to determine the response of tap water samples spiked with 5.0 and 10 μM catechol, and the estimated % recovery values were 104.2 and 143.0, respectively. Similarly, amperometry was used to test the green tea samples spiked with 5.0 and 10 μM catechol. The estimated % recovery values for the green tea samples were 81.4 and 90.7, respectively (Table 3.4).

Table 3.4 Catechol analysis in tap water and green tea with the GE/P-PBF NP/Lac biosensor

Sample			
Tap water	Spiked with Catechol (μM)	Found with the Biosensor (μM)	Recovery (%)
	5.0	5.209 ± 0.417	104.2
	10.0	14.303 ± 1.583	143.0
Green tea	Spiked with Catechol (μM)	Found with the Biosensor (μM)	Recovery (%)
	5.0	4.069 ± 0.829	81.4
	10.0	9.068 ± 0.427	90.7

3.5 Conclusions

Organic compounds used in industrial processes such as dyes, pesticides, and insecticides can decompose and release pollutants such as phenol and its derivatives into the environment. Catechol, a type of phenol, is highly toxic and degrades slowly in the environment^{187,188}. It can cause harmful symptoms like liver and kidney damage and nervous system changes and can enter the human body through the skin and mucous membranes^{187,193}. While instrumental methods can be used to determine the presence of catechol, such as gas and liquid chromatography, fluorescence, chemiluminescence, and spectrophotometry, their practical use is limited due to high costs²⁵⁸. To address this, low-cost and highly sensitive electrochemical-based biosensors are often used to determine catechol in environmental samples simultaneously quantitatively.

In the context of this chapter, a new type of polymer named P-PBF and its nanoparticles, P-PBF NP, were synthesized. P-PBF conjugated polymer was synthesized by Stille cross-coupling reaction for biosensor design. Three monomers were selected for this purpose as follows: Benzodithiophene, fluorene and pyrazine. The BDT monomer, short for benzodithiophene, is an organic semiconductor with flat conjugated structures. It has excellent resistance to oxidation due to the

combination of a benzene group with two adjacent thiophene units^{68–70}. Additionally, various substituents can be added to the central benzene core of BDT to adjust the energy levels, absorption spectra, thermal stability, and solubility of polymers based on BDT^{71–73}. Polyfluorenes are one the most attractive blue-emitting materials due to their high efficiency, charge transport properties, excellent solubility and film-forming ability, good chemical and thermal stability, and tunable properties via chemical modifications and copolymerization⁷⁴. Pyrazine is well-suited for efficient transport due to its ability to provide high hole mobility, intermolecular enhancement, and cost-effectiveness⁷⁵. Then, the surface of a graphite electrode was modified with P-PBF NP and the enzyme Lac was immobilized to create a biosensing platform for detecting catechol.

The biosensor surface was characterized through cyclic voltammetry (CV) and scanning electron microscopy (SEM) techniques. Amperometric responses were recorded in 10 mL sodium acetate buffer (50.0 mM, pH: 5.5) at -0.3 V in the presence of catechol for analytical characterization of GE/P-PBF NP/Lac biosensor. Conjugated polymer nanoparticles were used to cover the electrode surface to determine the optimal amount of P-PBF NP. A biosensor coated with 6.0 μ L P-PBF NP resulted in the highest responses. For enzyme optimization, different amounts were prepared, and the best amount was 0.3 mg. pH optimization was done in a wide pH range of 4.5 to 7.0, and it was discovered that the GE/P-PBF NP/Lac biosensor had the highest responses at pH 5.5. After optimization, analytical characterization of the biosensor was performed. The GE/P-PBF NP/Lac biosensor calibration equation was $y=0.0793x+0.093$ ($R^2=0.99$) in the range of 0.5–25 μ M. Furthermore, K_M^{app} and I_{max} values were calculated to be 49.01 μ M and 5.74 μ A, respectively. The LOD and sensitivity values of the GE/P-PBF NP/Lac were calculated to be 0.49 μ M and 1.229 μ A/ μ M cm^2 . An experiment was conducted to examine the effects of interference on the GE/P-PBF NP biosensor. The test involved adding 10 μ M of different substrates, such as glucose, ascorbic acid, citric acid, urea, uric acid, paracetamol, NaCl, and KCl separately in the reaction cell. None of these chemical compounds caused any signal changes when compared to catechol. Therefore, it can

be concluded that none of these relevant chemical compounds have any interference effects on the GE/P-PBF NP biosensor.

In this chapter, novel conjugated polymer and conjugated polymer nanoparticles were synthesized. This is the first study in which conjugated polymer nanoparticles were investigated to fabricate an electrochemical biosensing platform for catechol detection. The biosensor showed a wide linear range, low limit of detection value, and high sensitivity compared to other laccase biosensor studies. Moreover, it showed good reproducibility and stability. Based on the findings, it can be concluded that P-PBF NP was effectively utilized to coat the electrode and served as a suitable matrix for immobilization. These findings inspire further exploration of the potential of conjugated polymer nanoparticles in electrochemical enzyme biosensors.



CHAPTER 4

CONCLUSIONS

Nanomaterials, with their specific designs, are tailored for various commercial products and processes. Their small size grants them unique characteristics, making them ideal for specific applications²⁵⁹. One area where nanomaterials have seen increased use is in the development of biosensor devices²⁶⁰. Due to their large surface areas and inherent properties, nanomaterials enhance the immobilization of biomolecules, thereby improving biosensor characteristics²⁶¹.

Catechol is utilized in various items, such as dyes, anti-ageing beauty products, medications, plastics, insecticides, and photographic supplies^{185,186}. It decomposes slowly and is highly toxic^{187,188}. Even small quantities of catechol can lead to various issues. Hence, developing affordable, rapid, and highly sensitive detection methods for catechol is crucial.

The first laccase biosensor has been developed to detect catechol using Chit-AgNPs nanocomposite. While AgNPs are commonly used in biosensing applications, they have drawbacks, particularly instability²⁶². To address this, chitosan, a popular biopolymer, is often employed to improve the stability of silver nanoparticles²⁶³. Chitosan, with its amino and hydroxyl functional groups, is considered a highly effective immobilization matrix²²⁵. The nanocomposite was synthesized via the green chemistry route, and chitosan was used as a reducing and stabilizing agent. The biosensor was constructed using a crosslinking technique, and the surface morphology was confirmed using SEM images. The CV technique characterized the electrochemical property. The fabricated biosensor demonstrated good analytical properties (Figure 4.1). The optimized biosensor was tested on tap water and wine

and yielded satisfactory results. This biosensor has the potential for real-time detection and analysis of catechol.

The properties of conjugated polymers have piqued the interest of the global scientific community. Their enhanced sensitivity, speed, and stability make them excellent materials for building biosensors and serving as suitable matrices for biological molecules^{8,78–80}. However, their limited applications in water due to immiscibility present a challenge. This issue can be resolved by converting conjugated polymers into water-dispersible nanoparticles, a solution that we, as a global community, are working towards. Conjugated polymer nanoparticles, also called semiconducting polymer nanoparticles, have been extensively manufactured and studied. Their affordable production and diverse range of uses, including biosensors, imaging, drug delivery systems, and more, have made fluorescent conjugated polymers a subject of great interest in the past few decades¹¹⁶. In the second of the thesis a novel conjugated polymer (P-PBF) and novel conjugated polymer nanoparticles (P-PBF NP) produced and characterized. A second laccase biosensor that utilizes laccase was developed, and it included a new nanomaterial of modifying the graphite electrode's surface using conjugated polymer nanoparticles called P-PBF NP. The reprecipitation technique resulted in conjugated polymers that could disperse in water. The biosensor was created using a crosslinking technique, and SEM images were used to confirm its surface morphology. Electrochemical characterization was performed using both CV and EIS analyses, and the proposed biosensor demonstrates satisfactory kinetic parameters, including a low LOD and high sensitivity, as seen in Figure 4.1. Notably, the biosensor was tested successfully in tap water and green tea samples, proving its effectiveness.

Table 4.1 compares the analytical capabilities of GE/P-PBF NP/Lac and GE/Chit AgNPs/Lac. The comparison includes parameters such as K_M^{app} , I_{max} , linear response range, sensitivity, LOD, and LOQ values. The GE/Chit AgNPs/Lac biosensor has a lower K_M^{app} value than the GE/P-PBF NP/Lac biosensor. K_M^{app} measures an enzyme's affinity for its substrate, and a lower K_M^{app} value denotes better substrate

binding and higher catalytic activity. Hence, the GE/Chit-AgNPs/Lac biosensor is more efficient than the GE/P-PBF NP/Lac biosensor. The $I_{\max}$ value, the maximum current measured under a saturated substrate condition, is more significant in the GE/P-PBF NP/Lac biosensor than in the GE/Chit-AgNPs/Lac biosensor. In the linear range of response, the GE/P-PBF NP/Lac biosensor has lower values than GE/Chit-AgNPs/Lac. The data in Table 4.1 reveals a striking difference in sensitivity between the GE/P-PBF NP/Lac biosensor, which is approximately eight times more sensitive ($1.229 \mu\text{A}/\mu\text{Mcm}^2$), and the GE/Chit AgNPs/Lac biosensor ($0.444 \mu\text{A}/\mu\text{Mcm}^2$). The GE/P-PBF NP/Lac biosensor exhibits lower LOD and LOQ values ($0.49 \mu\text{M}$, $1.48 \mu\text{M}$) than the GE/P-PBF NP/Lac biosensor ($3.85 \mu\text{M}$, $12.71 \mu\text{M}$), with the former having eightfold and ninefold lower values, respectively.

Table 4.1 Comparison of analytical parameters of two designed biosensors

	GE/P-PBF NP/Lac	GE/Chit-AgNPs/Lac
K_M^{app}	49.01 μM	12.3 μM
$I_{\max}$	5.47 μA	11.9 μA
Linear range	0.5-25 μM	5-250 μM
Sensitivity	1.229 $\mu\text{A}/\mu\text{M.cm}^2$	0.444 $\mu\text{A}/\mu\text{M.cm}^2$
LOD	0.49 μM	3.85 μM
LOQ	1.48 μM	12.71 μM

The analysis of phenolic compounds such as catechol has become an exciting subject, and various reliable, fast, and susceptible methods are being used for this purpose. However, some commercial techniques have limitations, such as being non-selective, taking too long to analyze, being expensive, and requiring sample pretreatment. The thesis proposes creating alternative nanomaterial-based matrices to develop better-sensing devices complementary to conventional chromatographic

and spectrometric sensing techniques. Within the scope of this thesis, two types of amperometric laccase biosensors were designed and tested. This biosensor has the potential for environmental monitoring and health safety measures. Additionally, newly synthesized nanocomposite (Chit-AgNPs) and conjugated polymer nanoparticles (P-PBF NP) may be used to design innovative biosensing platforms for other target analytes.



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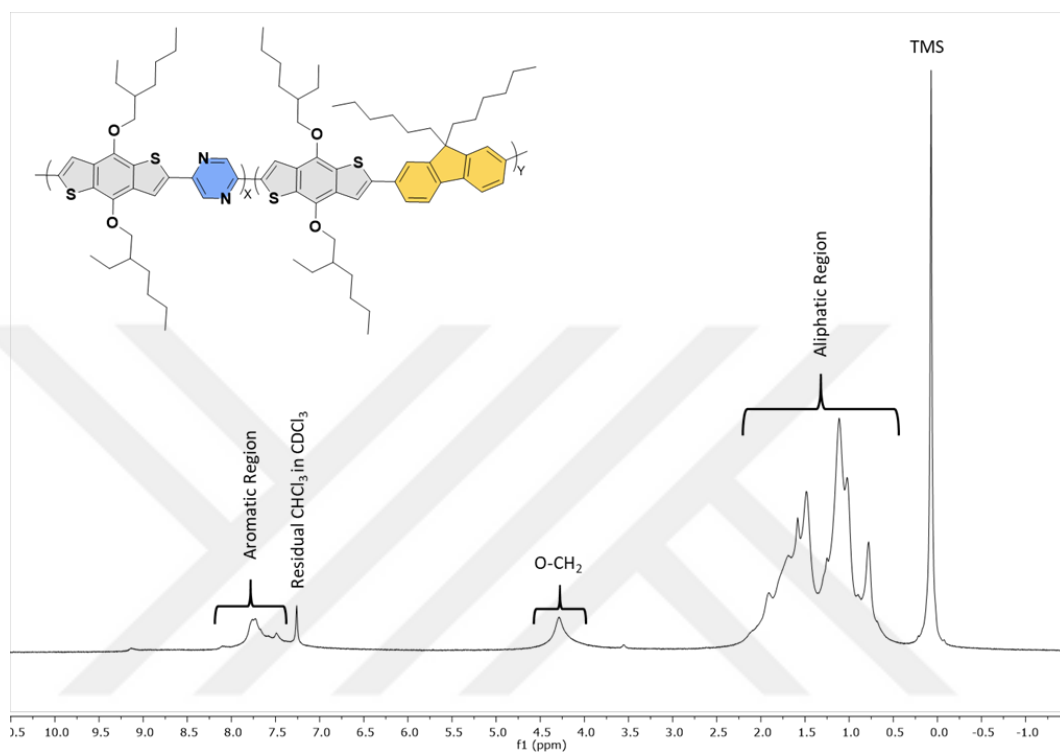
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APPENDICES

A. ^1H NMR spectrum of P-PBF





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CONFERENCE PROCEEDINGS

1. S.Oncu, B.Yedekci, AZ. Alshemary, A. Tezcaner, D. Keskin, Z. Evis. Microstructure Analysis of $\text{Cu}^{2+}/\text{SiO}_4^{4-}$ Co-doped Nanohydroxyapatite. (Poster Presentation), 22th International Biomedical Science and Technology Symposium (BIOMED), Ankara, Turkey, 12-14 October 2017.
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