

Detection of *Candida albicans* Pathogen by Electrochemical Methods with Metal Nanoparticle Modified Molecular Imprinted Polymer

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in Nanoscience & Nanotechnology

by

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Detection of *Candida albicans* Pathogen by Electrochemical Methods with Metal Nanoparticle Modified Molecular Imprinted Polymer

Abstract

Candida albicans is a type of fungus commonly found in the human body, especially in the mouth, throat, intestine, and genito-urinary system. It is responsible for a wide range of infections, from mucosal infections to genito-urinary system infections, and can cause highly fatal invasive infections. The current methods for detecting *Candida albicans* include microscopic examination, culture methods, and molecular biological methods. These methods are time-consuming, costly in terms of equipment and consumables, and require specialized personnel. They also generally rely on visual examination, which makes quick and accurate diagnosis challenging, thereby increasing the error rate. However, these methods lack selectivity and require pre-preparation steps, despite their speed. Therefore, it is crucial to develop a fast, sensitive, easy-to-use, and economical analysis method for detecting *Candida albicans* to promptly start the treatment process.

First, the graphite electrodes were modified with nickel ferrite nanoparticles (NiFeNP). Next, the pyrrole monomers and *C. albicans* were electropolymerized onto the electrodes using Cyclic Voltammetry (CV), resulting in molecularly imprinted polymers on the electrode. Then, *C. albicans* was removed from the electrode surface using a surfactant, creating highly specific cavities on the electrode for *C. albicans*. Various electrochemical methods such as CV, Differential Pulse Voltammetry (DPV), and Electrochemical Impedance Spectroscopy (EIS) were used to optimize the experimental steps.

In this thesis, molecularly imprinted polymers were used for the first time to electrochemically detect the *C. albicans* pathogen, with the electrodes modified using nickel ferrite nanoparticles. The use of molecularly imprinted polymers made the detection system highly selective for the *C. albicans*, while the modification of the electrodes with NiFeNP increased the system's sensitivity. The use of pencil graphite electrodes, molecularly imprinted polymers, NiFeNP and electrochemical detection methods in the detection system made it fast, sensitive, easy to use, and economical.

Keywords: Electrochemical Biosensor, *C. albicans*, Molecularly Imprinted Polymers, Nickel Ferrite Nanoparticles, Electropolymerization.

Candida albicans Patojeninin Metal Nanopartikül Modifiye Moleküler Baskılı Polimer ile Elektrokimyasal Yöntemlerle Tespiti

ÖZ

Candida albicans, ağız, boğaz, bağırsak ve genito-üriner sistemde yaygın olarak bulunan bir mantar türüdür. Mukoza enfeksiyonlarından genito-üriner sistem enfeksiyonlarına kadar geniş bir enfeksiyon yelpazesinden sorumludur ve oldukça ölümcül invaziv enfeksiyonlara neden olabilir. Bu patojeni tespit etmek için mevcut yöntemler arasında mikroskopik inceleme, kültür yöntemleri ve moleküler biyolojik yöntemler bulunmaktadır. Bu yöntemler zaman alıcı, ekipman ve sarf malzemeleri açısından maliyetli ve uzman personel gerektirmektedir. Ayrıca, bu patojenin tespiti genellikle görsel incelemeye dayanır, bu da hızlı ve doğru teşhis yapmayı zorlaştırır ve hata oranını artırır. Bu nedenle, *Candida albicans*'ın hızlı, hassas, kullanımı kolay ve ekonomik bir analiz yöntemi ile tespit edilmesi ve tedavi sürecine hızlı bir şekilde başlanması önemlidir.

Bu çalışmada, ilk olarak grafit elektrotlar nikel ferrit nanopartikülleri ile modifiye edilmiştir. Ardından, pirol monomerleri ve *C. albicans*, Dönüşümlü Voltametri (CV) kullanılarak elektropolimerize edilmiş ve elektrot üzerinde moleküler baskılı polimerler oluşturulmuştur. Daha sonra, yüzey aktif bir madde kullanılarak *C. albicans* elektrot yüzeyinden uzaklaştırılmış ve elektrot üzerinde *C. albicans* için spesifik boşluklar oluşturulmuştur. Deneysel adımları optimize etmek için CV, Diferansiyel Puls Voltametrisi (DPV) ve Elektrokimyasal İmpedans Spektroskopisi (EIS) gibi çeşitli elektrokimyasal yöntemler kullanılmıştır.

Bu tezde, moleküler baskılı polimerler, *C. albicans* patojenini elektrokimyasal olarak tespit etmek için ilk kez kullanılmış ve elektrotlar nikel ferrit nanopartikülleri ile modifiye edilmiştir. Moleküler baskılı polimerlerin kullanımı, *C. albicans* için tespit sistemini son derece seçici hale getirirken, elektrotların nikel ferrit nanopartikülleri ile modifiye edilmesi sistemin hassasiyetini artırmıştır.

Tespit sisteminde kurşun grafit elektrotlar, moleküler baskılı polimerler, nikel ferrit nanopartikülleri ve elektrokimyasal tespit yöntemlerinin kullanılması, sistemi hızlı, hassas, kullanımı kolay ve ekonomik hale getirmiştir.

Anahtar Kelimeler: Elektrokimyasal Biyosensör, *C.albicans*, Moleküler Baskılı Polimerler, Nikel Ferrit Nanopartiküller, Elektropolimerizasyon.



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List of Abbreviations

ACB	Acetate Buffer
AC	Alternating Current
AE	Auxiliary Electrode
Ag/AgCl	Silver/Silver Chloride
AIDS	Acquired Immunodeficiency Syndrome
API	Analytical Profile Index
<i>C. albicans</i>	<i>Candida albicans</i>
<i>C. parapsilosis</i>	<i>Candida parapsilosis</i>
<i>C. tropicalis</i>	<i>Candida tropicalis</i>
CDC	Centers for Disease Control and Prevention
CE	Counter Electrode
CFU	Colony-Forming Unit
CFU/mL	Colony-Forming Units per milliliter
CH ₃ COOH	Acetic Acid
CH ₃ COONa	Sodium Acetate
CoFe ₂ O ₄	Cobalt Ferrite
CV	Cyclic Voltammetry
DNA	Deoxyribonucleic Acid
DPV	Differential Pulse Voltammetry
<i>E. coli</i>	<i>Escherichia coli</i>
EIS	Electrochemical Impedance Spectroscopy
ELISA	Enzyme-Linked Immunosorbent Assay
Fe ₃ O ₄	Iron(II,III) oxide (Magnetite)
Fe ²⁺	Ferrous Ion
Fe ³⁺	Ferric Ion

GCE	Glassy Carbon Electrode
GO	Graphene oxide
HAc	Acetic acid
H7N9	Influenza Virus
HIV	Human Immunodeficiency Virus
K ₃ [Fe(CN) ₆]	Potassium ferricyanide
K ₄ [Fe(CN) ₆]	Potassium ferrocyanide
KCl	Potassium chloride
KH ₂ PO ₄	Monopotassium phosphate
K ₂ HPO ₄	Dipotassium phosphate
LOD	Limit of Detection
LOQ	Limit of Quantification
MALDI-TOF MS [®]	Matrix-Assisted Laser Desorption/Ionization Time of Flight Mass Spectrometry
MFe ₂ O ₄	Metal Ferrite (General Formula)
MEMS	Microelectromechanical Systems
MHz	Megahertz
MIP	Molecularly Imprinted Polymer
MIP/CIP	Molecularly Imprinted Polymer / Competitive Imprinted Polymer
mHz	Millihertz
mV/s	Millivolts per Second (Scan Rate)
NaCl	Sodium chloride
NanoMIP	Molecularly Imprinted Polymers coated with Nanoparticles
NanoNIP	Non-Imprinted Polymers coated with Nanoparticles
Ni	Nickel
NiFe ₂ O ₄	Nickel Ferrite
NiFeNP	Nickel Ferrite Nanoparticles
NIP	Non-Imprinted Polymer

PBS	Phosphate-Buffered Saline
<i>P. aeruginosa</i>	<i>Pseudomonas aeruginosa</i>
PCR:	Polymerase Chain Reaction
PGE	Pencil Graphite Electrode
R_{ct}	Charge Transfer Resistance
RE	Reference Electrode
RNA	Ribonucleic Acid
RSD	Relative Standard Deviation
RPM	Revolutions Per Minute
RPMI	Roswell Park Memorial Institute
SDS	Sodium dodecyl sulfate
SEM	Scanning Electron Microscope
SYBR	SYBR Green (a dye used in molecular biology)
TEM	Transmission Electron Microscope
TE	Tris-EDTA
UPW	Ultra-Pure Water
USA	United States of America
UV	Ultraviolet
V	Volt
WE	Working Electrode

List of Symbols

μA	Microampere
R_{ct}	Charge Transfer Resistance
ΔR_{ct}	Change in Charge Transfer Resistance
$\Delta R_{ct}\%$	Percentage Change in Charge Transfer Resistance
Z	Impedance
Z'	Real part of Impedance
Z''	Imaginary part of Impedance
V	Potential
S	Slope of the calibration curve
σ	Standard deviation of the response
Ω	Ohm

Chapter 1

1.1 Introduction

Fungal infections have recently become one of the most current topics in infectious diseases and microbiology due to their increasing incidence and the emergence of treatment-resistant isolates (Muzahed et al., 2022). Although various fungal species cause this problem, affecting millions of people each year, *Candida spp.* is the most frequently isolated fungal pathogen worldwide (Evren et al., 2022). In the past, *Candida spp.* being members of the human microbiota, were not considered a significant threat. However, it is now reported that they directly cause more than 700,000 infections annually (Bongomin et al., 2017). Therefore, *Candida* species are listed in the "Antimicrobial Resistance Threat Report" regularly published by the Centers for Disease Control and Prevention (CDC) as urgent and serious threats due to the increasing number of cases and their natural or acquired antifungal resistance, posing the highest risk to public health (Centers for Disease Control, n.d.). This family of fungi, which can cause a wide range of symptoms from mucosal infections and genitourinary system infections to invasive infections with high mortality, is predominantly responsible for a large portion of infections caused by *Candida albicans* (Neves SOUZA et al., n.d.). However, recent studies have identified that the incidence of *C. glabrata*, *C. parapsilosis*, *C. tropicalis*, and *C. krusei* species is increasing, and they are responsible for a significant number of cases (Deorukhkar, 2018). The intrinsic (natural-chromosomal) antifungal resistance of some of these pathogens within the *Candida* species presents a major challenge in treatment (Magalhães et al., 2022).

Antifungal drugs are medications designed to treat fungal infections by targeting the cell membrane or cell wall of fungi, thereby inhibiting their growth or killing them. These drugs are particularly important for immunocompromised individuals, including those with HIV/AIDS, cancer patients undergoing chemotherapy, organ transplant recipients, and people with chronic conditions like diabetes, who are more susceptible to severe fungal infections.

Antifungal drugs help prevent fungal infections in hospital settings, reducing morbidity and mortality rates associated with nosocomial infections. Therefore, selecting the right molecule and achieving a rapid therapeutic response depend on the accurate and swift identification of the causative species.

Fungal identification methods range from traditional to advanced molecular techniques. Traditional methods include microscopy, where light microscopy and special stains help visualize fungal elements, and culture techniques that grow fungi on specific media to observe colony morphology. In most healthcare institutions, phenotypic methods are routinely used for the identification of yeast species isolated from clinical samples. Accurate identification involves the incubation of the causative agent in suitable media, the evaluation of the shape, size, and color of the growing fungal colonies (macroscopy), and the microscopic examination of samples prepared from clinical specimens or produced colonies. These steps are usually followed by an additional incubation on chromogenic agar for species determination (Clancy & Nguyen, 2013). While these traditional methods are useful in diagnosis, their success relies on the cultivation of the agent and visual inspection. This method not only makes rapid identification difficult but also has a high error rate due to the reliance on visual examination. Biochemical tests, such as carbohydrate assimilation and enzyme activity assays, further aid identification. Advanced molecular methods include Polymerase Chain Reaction (PCR), which amplifies fungal DNA for detection, and DNA sequencing, particularly of the internal transcribed spacer region, which offers precise identification. These methods combined provide accurate, rapid, and comprehensive identification of fungal species, crucial for effective diagnosis and treatment (Madhavan et al., 2011). Although these methods provide high sensitivity detection, their application in routine diagnostic laboratories is hindered by the need for numerous solutions, kits, enzymes, and devices, as well as multiple sequential steps. In recent years, automated systems developed for use in clinical microbiology laboratories have made it possible to diagnose *Candida* within hours (Sudhan et al., 2016). Although devices based on different operating principles, such as API®, VITEK®, and MALDI-TOF MS®, successfully perform identification processes, their consumable costs make it impractical for every routine diagnostic laboratory (Cassagne et al., 2013). Therefore, there is still a need for applications that can detect the presence and species differences of *Candida* with minimal procedures.

Electrochemical biosensors are frequently used in the healthcare field due to their ability to provide rapid and sensitive results in the detection of pathogens (Menon et al., 2020). These biosensors can be designed for use in hospitals and laboratories or for home use (Chen et al., 2020). Due to their high accuracy in detecting pathogens, electrochemical biosensors play a crucial role in identifying bacterial, viral, or fungal diseases (Li et al., 2021). In recent years, the integration of **Molecularly Imprinted Polymers (MIP)** into biosensors has garnered significant interest. MIP are synthetic polymers designed to specifically recognize and bind to a particular target molecule, such as a drug, hormone, pathogen, or contaminant. Typically, MIP are created through a process called "molecular imprinting," where a template molecule (the target molecule) is mixed with a polymerizable monomer and a cross-linker. In the second stage, the polymerization of the monomer-template complex is carried out with cross-linkers, porogen, or initiators under photo-/thermal/electric conditions. During polymerization, the template molecule forms specific binding sites within the MIP, allowing the MIP to recognize and bind to the template molecule when it is present in a sample (Gui et al., 2017). In the final stage, the template molecule is removed from the structure to reveal binding microcavities that are highly specific to the template molecule. A **non-imprinted polymer (NIP)** is a polymer that is synthesized in a similar way to a MIP but without the presence of the template molecule during the polymerization process. While MIPs have specific cavities or binding sites that are complementary in shape and functional groups to a target molecule (template), NIPs do not have these specific binding sites. Instead, NIPs serve as control materials to demonstrate that the enhanced recognition and binding properties of MIPs are due to the presence of the template during polymerization.

There are two main methods for preparing MIP: covalent and non-covalent imprinting. Covalent imprinting involves forming reversible covalent bonds between the template and functional monomers. Non-covalent imprinting, on the other hand, relies on weaker interactions such as hydrogen bonds, van der Waals forces, or ionic interactions to form the recognition sites around the template molecule.

Covalent imprinting is a method for creating MIPs where the template molecule forms a covalent bond with a functional monomer, followed by polymerization with a cross-linker to form a rigid matrix. The template is then removed by breaking the covalent bond, leaving behind highly specific binding sites. This method offers high specificity, stable binding sites, and reduced non-specific binding, making it ideal for applications requiring precise molecular recognition. However, the synthesis process is more complex and time-consuming, and not all templates can form covalent bonds with monomers. Non-covalent imprinting is a widely used method for creating MIPs based on non-covalent interactions like hydrogen bonds, van der Waals forces, ionic interactions, and hydrophobic effects between the template molecule and functional monomers. After forming a pre-polymerization complex, polymerization with a cross-linker creates a rigid matrix, and the template is removed, leaving specific binding sites. This method is simpler, faster, and more versatile than covalent imprinting, accommodating a broader range of templates. However, it may offer lower specificity and be sensitive to environmental conditions, with potential issues of template leaching. It's popular in sensors, drug delivery, and separation processes.

The detection of various pathogens using electrochemical biosensors with the molecular imprinting polymer technique has gained importance and is an increasingly popular application in recent years. MIPs have a range of potential applications, including the detection and quantification of drugs, hormones, pathogens, and contaminants in various samples such as blood, urine, and environmental samples. They have also been used in the development of biosensors, chromatographic columns, and drug delivery systems (Suzaei et al., 2022). The most common method for improving the performance of MIP sensors is modifying the electrode surface (Crapnell et al., 2019). Zaidi and colleagues developed an adrenaline sensor using nicotinamide as the functional monomer, modifying the GCE surface with graphene oxide (GO). GO provides a very large surface area, low noise, and excellent electrical and thermal conductivity. By using this MIP-based platform with GO, they achieved a detection limit (LOD) of 3 nM, which compared favorably with other adrenaline sensors reported in the literature (Zaidi, 2018). Yola and colleagues, using white graphene and graphene quantum dots together, successfully detected serotonin with a low detection limit of 0.2 pM through electropolymerized polyphenol (Yola & Atar, 2018).

Nanoparticles are a broad class of materials that include particulate matter with at least one dimension smaller than 100 nm (Laurent et al., 2009). It has been reported that size can affect the physicochemical properties of a substance, particularly optical properties (Khan et al., 2019). The advantages of using nanoparticles in biosensors include enhancing the stability of biomaterials and electrodes, increasing the sensitivity of the analysis, accelerating catalysis during the process, increasing the likelihood of reactions occurring at lower potentials, speeding up electron transfer at the electrode surface, and improving the conductivity and electroactive surface area of the electrode (X. Huang et al., 2021; Luo et al., 2006). The primary functions of nanoparticles in electrochemical analyses include immobilization of biomolecules, catalysis of electrochemical reactions, enhancement of electron transfer, labeling of biomolecules, and use as reactants (Luo, Morrin, Killard, & Smyth, 2006). The modification of electrode surfaces with nanoparticles particularly enhances the sensitivity of (bio)sensors due to the high surface-to-volume ratio (X. Huang et al., 2021). Nanoparticles are generally categorized based on their morphology, size, and chemical properties. These categories primarily include carbon-based, metal, ceramic, semiconductor, polymeric, and lipid-based nanoparticles (Khan et al., 2019). Among them, the metal nanoparticles are the most common nanoparticles. The size and shape of metal nanoparticles play a crucial role in enhancing electrocatalytic activity, selectivity in biomolecule binding, and metal-electrode/metal-support interactions (H. Ma et al., 2020).

Examples of metal nanoparticles include gold, silver, and platinum nanoparticles, metal ferrite nanoparticles, etc. (Islam et al., 2020). Metal nanoparticles are frequently used for the detection of biomolecules such as genes, proteins, and enzymes, as well as for the detection of biomarkers for diseases and various pathogens such as viruses, bacteria, and fungi (Islam et al., 2020). Their unique optical, electrical, and catalytic properties enhance the sensitivity and specificity of biosensors, making them invaluable in medical diagnostics and environmental monitoring.

As an example, for metal nanoparticle use, a nanobioconjugate was formed using gold nanoparticles and a single-stranded Zika virus DNA probe for the detection of Zika virus DNA. Using a voltammetric method, measurements were done before and after hybridization of the nanobioconjugate with the target Zika virus DNA, allowing for the detection of the virus DNA. It was determined that the nanobioconjugate formed with gold nanoparticles and the DNA probe was more stable and provided high sensitivity and selectivity (Cajigas et al., 2020). Ferrites, a type of metal nanoparticle composed of iron oxides and ferromagnetic compounds, can be chemically mixed with other metals.

Ferrite-based magnetic metal oxides such as Fe_3O_4 , CoFe_2O_4 , NiFe_2O_4 , and ZnFe_2O_4 have great potential due to their unique electrical, magnetic, and semiconductor properties, and are used in applications such as catalysts (Kombaiah et al., 2019), magnetic recording, controlled drug release (Fang et al., 2020), and photovoltaic cells (Alsabbagh et al., 2019). Nanometer-sized ferrites have the formula MFe_2O_4 , where M corresponds to divalent metal ions such as Ni, Zn, Co, Mn, and Cu. Among these nanoparticles, nickel ferrite (NiFe_2O_4), a soft ferrite material and one of the most important spinel ferrites, is widely used due to its high frequency and electrical conductivity, permeability, mechanical and electrochemical stability, and resistance to corrosion (Majid et al., 2021). The use of NiFe_2O_4 nanoparticles in biosensor applications is highly preferred because of their excellent conductive properties (Nouri et al., 2020; Zabihpour et al., 2020), ability to allow target molecules to easily bind to the relevant surface (Devi et al., 2020), facilitation of faster mass transfer (Kesavan et al., 2020), and being an economical and environmentally friendly nanoparticle.

There are several potential benefits of integrating MIPs with nanoparticles. MIPs are highly specific for their target molecules but can be sensitive to temperature, pH, and other environmental conditions. By integrating MIPs with various nanoparticles, such as metal and polymeric nanoparticles, carbon nanotubes, or zeolite nanoparticles, MIPs can be protected from environmental degradation, leading to enhanced stability and sensitivity (Zhao et al., 2020). MIPs can recognize and bind to their target molecules with high affinity and specificity, but they can also bind to other molecules with similar structures.

By integrating MIPs with nanoparticles, MIPs can be selectively activated based on the size, shape, or charge of the nanoparticles, leading to higher selectivity (Y. Huang et al., 2019). They can also produce a stronger or more easily detectable signal, such as a change in the optical, electrical, or magnetic properties of the nanoparticles (Sharma et al., n.d.). MIPs can be toxic or immunogenic, which can limit their use in some applications. By integrating MIPs with nanoparticles, MIPs can become more biologically compatible, making them more suitable for use in medical or biological applications (Luliński, 2017).

In this thesis, we developed a rapid, sensitive, and selective detection of *Candida albicans* by integrating electrochemical detection methods with nickel ferrite (NiFe_2O_4) nanoparticles and the molecular imprinting technique. *Candida albicans*, a common fungal pathogen, poses significant health risks, necessitating prompt and accurate detection methods to facilitate timely treatment. Traditional detection methods, although effective, are often hampered by their time-consuming nature, high costs, and the requirement for specialized personnel. To address these limitations, we aimed to create an electrochemical method capable of detecting *Candida albicans* quickly and accurately.

Our biosensor integrated the unique properties of MIP and nickel ferrite nanoparticles (NiFeNP) to enhance performance. Initially, the graphite electrodes were modified with NiFe_2O_4 nanoparticles to increase the electrode's surface area and conductivity, which is crucial for improving the sensitivity of the detection system. The molecular imprinting technique was employed to introduce high specificity to the biosensor. This process involved electropolymerizing pyrrole monomers in the presence of *Candida albicans* on the electrode surface, forming a polymer matrix with specific binding sites for the target pathogen. After polymerization, the *Candida albicans* were removed, leaving behind imprinted cavities on the electrode surface that could rebind to *Candida albicans* in subsequent analyses. We used Cyclic Voltammetry (CV) and Electrochemical Impedance Spectroscopy (EIS) to characterize and optimize the biosensor. These techniques allowed us to monitor the modifications on the electrode surface and measure the changes in impedance as *Candida albicans* bound to the imprinted sites.

In conclusion, the integration of NiFe₂O₄ nanoparticles and the molecular imprinting technique provided a robust platform for the development of a rapid, sensitive, and selective detection for *Candida albicans*. The resulting design demonstrated excellent sensitivity and was capable of detecting *Candida albicans* at various concentrations with high precision. This method offers a significant advancement over traditional detection methods, providing a cost-effective and user-friendly solution for clinical diagnostics.

1.1.1 Biosensors

The first biosensor system, a glucose biosensor utilizing an enzyme electrode, was introduced by Clarks & Lyons in 1962. Since then, biosensor research has seen significant growth. Collings & Caruso (1997) describe a biosensor as “*an analytical device that utilizes a biological detection or recognition system for a target molecule or macromolecule, along with a physicochemical transducer that converts the biological recognition event into a usable output signal*”.

Biosensors can be defined by three main components. The biorecognition element, such as DNA, antibodies, enzymes, cells, or bacteria, is responsible for specifically recognizing and binding the target biological element, crucial for the biosensor's specificity and sensitivity. The transducer detects this biorecognition event and converts the biological interaction into a measurable physical or chemical signal, such as electrical, optical, or thermal, allowing the biosensor to generate a detectable output. Finally, the signal processing and output system processes and amplifies the signal from the transducer, converting it into a readable format through amplification, filtering, and visualization, enabling clear interpretation of the results. Depending on the biorecognition element, biosensor detectors can be classified into several categories: catalytic (enzymes, organelles, tissues), affinity-based (antibody-antigen), and hybrid receptors (DNA). Various physical and chemical transducers have been employed to monitor these biological recognition events.

Biosensors can be also classified based on their measurement methods into electrical, optical, thermal, chemical, and mechanical categories, and based on their measured variables into biochemical, physiological, and environmental categories (Monošík et al., n.d.). The operation principle of mechanical transducers, such as piezoelectric and Microelectromechanical Systems (MEMS) devices, is based on changes in the mechanical properties of a thin layer adhering to the transducer surface due to the biorecognition reaction. Optical transducers are devices that convert light or optical signals into another form of signal, such as electrical, mechanical, or chemical. They are used in various applications including optical sensors, displays, communication systems, and imaging devices. These methods are among the most widely reported in the literature for biosensing applications. Electrochemical transducers are devices that convert a chemical signal into an electrical signal or vice versa. They typically involve electrochemical reactions at an interface between an electrode and an electrolyte solution. These transducers are widely used in biosensors and chemical sensors for detecting analytes such as glucose, DNA, and various ions (Ronkainen et al., 2010). Electrochemical transducers are known for their sensitivity, selectivity, and ability to provide real-time measurements.

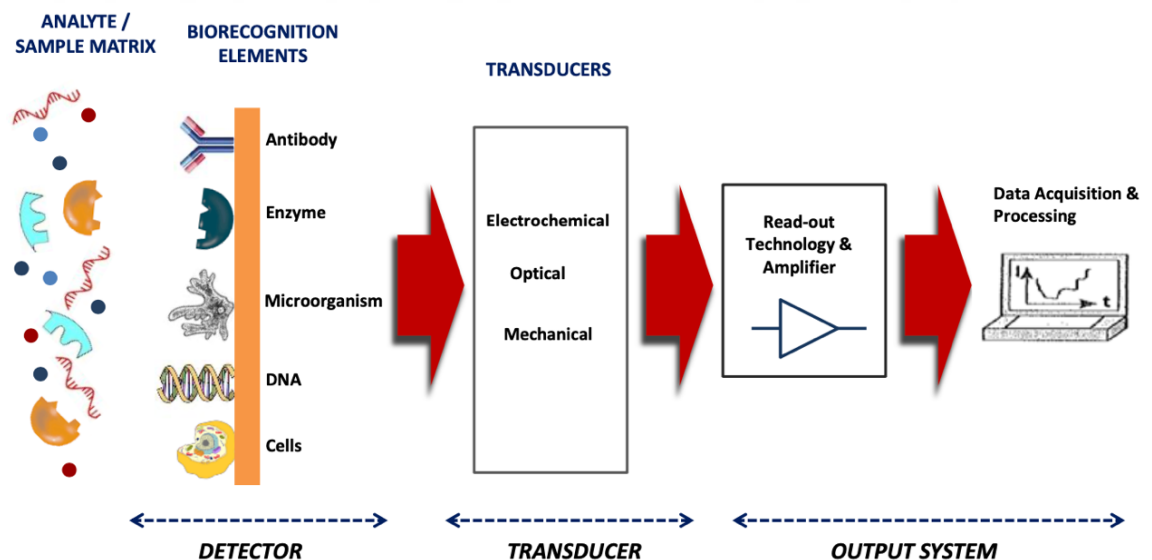


Figure 1: Typical structure of a biosensor consisting of an analyte, bioreceptor, and transducer part (Mohd Said, 2014).

Biosensors are widely used in medical and clinical diagnostics, agricultural testing (such as food pathogen and food safety testing), environmental monitoring, and defense (Figure 2). Biosensors are used to diagnose, monitor, and measure medical conditions (such as glucose, cholesterol, and hormones) (Singh et al., 2020). Additionally, there are biosensor systems designed to monitor environmental pollutants such as heavy metals and pesticides in air, water, and soil (Gavrilaş et al., 2022). Another application of biosensors is in monitoring and controlling industrial processes, such as the fermentation of biofuels and pharmaceutical production (Mehrotra, 2016). In recent years, the use of biosensors to ensure food safety by detecting foodborne pathogens such as *E. coli* and *Salmonella* in food products has gained increasing importance (Yahaya et al., 2021). Biosensors are used in the military and security sectors to detect and identify biological agents such as toxins and pathogens that could be used as biological or chemical weapons (Yahaya et al., 2021).

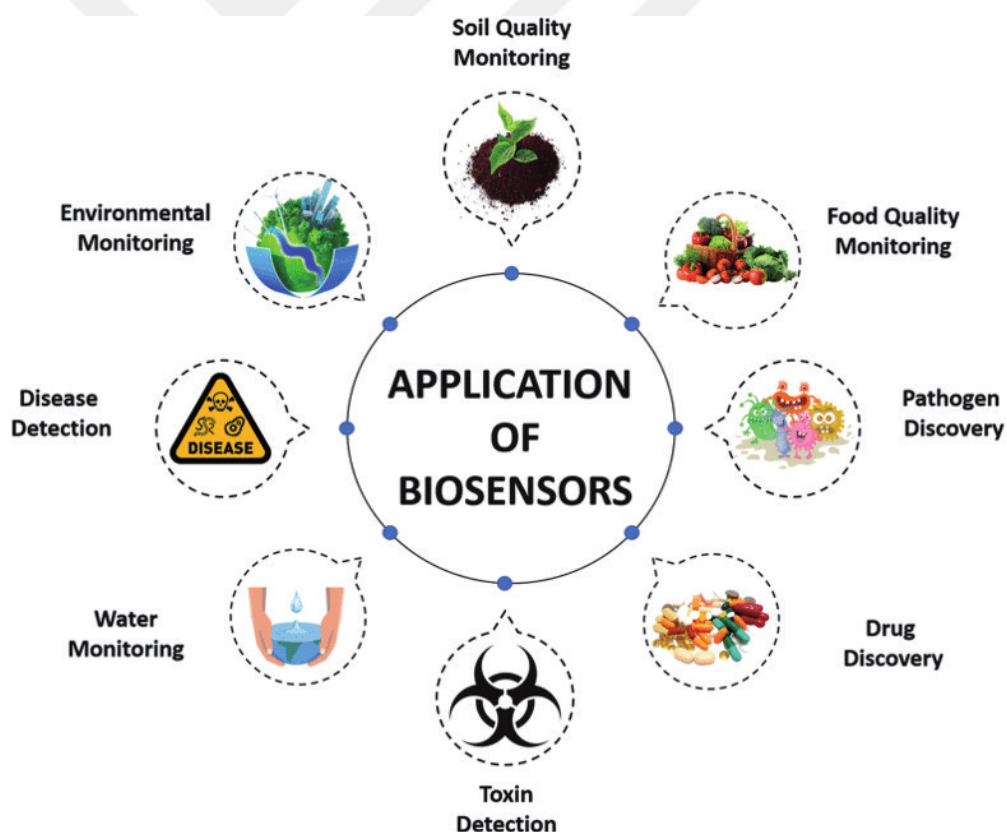


Figure 2: Schematic presentetation of application of biosensors (Tetyana et al., 2021).

1.1.1.1 Electrochemical Biosensors

Electrochemical sensors are miniaturized systems and/or devices that generate selective and reversible electrical signals based on the concentration of chemical substances, drug molecules, or ions. Electrochemical biosensors are formed by incorporating biological materials such as enzymes, cells, tissues, antibodies, and nucleic acids into the structure of an electrochemical sensor (Kaur & Bhosale, 2018). In electrochemical biosensors, detection can be performed with high sensitivity even in the presence of trace amounts of analytes. They produce an electrical signal corresponding to the concentration of the analyte being examined. In electrochemical biosensors, the electrode is chemically modified and typically coated with a biochemical component (Grieshaber et al., 2008). Electrochemical biosensors can be used for the detection of different types of analytes. Examples of these analytes include antigen/antibody, enzyme/substrate, DNA/RNA, pathogens, etc. (Cho et al., 2020). There are various types of electrochemical-based biosensors used for pathogen detection (Figure 3). Examples of these types of biosensors include:

Immunosensors: These biosensors use antibodies or antigens to detect specific pathogens. For example, an immunosensor can use an antibody that binds to a specific protein on the surface of a pathogen like the Ebola virus and produces a signal when the pathogen is present in a sample (Brazaca et al., 2021).

DNA biosensors: These biosensors use DNA probes or primers to detect specific pathogens based on their genetic material. For example, a DNA biosensor can use a probe that specifically binds to the DNA of a pathogen like the H7N9 influenza virus and produces a signal when the pathogen is present in a sample (Ozer et al., 2020).

Bacteriophage-based biosensors: These biosensors use bacteriophages (viruses that infect bacteria) to detect specific bacterial pathogens. For example, a bacteriophage-based biosensor can use a bacteriophage that specifically infects *Escherichia coli* (*E. coli*) and produces a signal when the bacteria are present in a sample (Hinkley et al., n.d.).

Microorganism-based biosensors: These biosensors use living microorganisms such as bacteria or yeast to detect specific pathogens. For example, a microorganism-based biosensor can use a bacterial species that produces a specific enzyme in the presence of a pathogen like Salmonella and generates a signal when the pathogen is present in a sample (Mahari & Gandhi, 2022)).

Enzyme-linked immunosorbent assay (ELISA)-based biosensors: These biosensors use the ELISA method, which involves the binding of an antibody to a specific pathogen and then using an enzyme to produce a signal for the detection of specific pathogens. For example, an ELISA-based biosensor can use an antibody that specifically binds to the protein coat of the HIV virus and an enzyme that produces a signal when the virus is present in a sample (Mahmudunnabi et al., 2020).

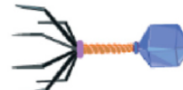
Analyte	Biorecognition Element
Protozoa (cyst)  ~10 μm	Antibodies 
Bacteria  ~1 μm	Proteins 
Mycoplasma  ~200 nm	Oligonucleotides (DNA/RNA) 
Virus  ~100 nm	Phages 
	Aptamers 
	MIP/CIP 

Figure 3: Electrochemical detection strategies of microorganisms (Cesewski & Johnson, 2020).

Chapter 2

2.1 Materials and Methods

2.1.1 Chemicals and Reagents

The buffer solutions, culture media, and their contents used during the experiments are shown in Table 1.

Table 1: Buffer solutions, culture media, and their contents.

	Solution	Contents	Purpose
1	Sodium Acetate Buffer (ACB) pH: 4.8	Acetic Acid (CH_3COOH), Sodium acetate (CH_3COONa), and Sodium chloride (NaCl)	Buffer
2	Phosphate Buffered Saline (PBS) pH: 7.4	Dipotassium phosphate (K_2HPO_4), Monopotassium phosphate (KH_2PO_4), and Sodium chloride (NaCl)	Buffer
3	Resolving Agent	Sodium dodecyl sulfate (SDS) (10% w/v), Acetic Acid (10% v/v)	Removing agent
4	Pyrrole Solution	Pyrrole (50 mM) in Potassium chloride (KCl) (0.1 M)	Electropolymerization
5	Redox Solution	Potassium ferricyanide ($\text{K}_3[\text{Fe}(\text{CN})_6]$), Potassium ferrocyanide ($\text{K}_4[\text{Fe}(\text{CN})_6]$) in Potassium chloride (KCl)	Redox Indicator
6	Nickel Ferrite Solution	Nickel Ferrite (NiFe_2O_4), Ethanol, Ultra Pure Water	Nanomaterial solution
7	RPMI 1640 culture medium	Containing glutamine but not sodium bicarbonate.	Fungi culture
8	Lysis Buffer	Lysis Buffer consists of the following components: Triton (2% v/v), SDS (10%) (1% w/v), NaCl 5M (100 mM), Tris-Cl (1M, pH: 8) (10 mM), and EDTA (0.5M, pH: 8) (1mM).	Removing agent

Ferricyanide/Ferrocyanide ($\text{Fe}(\text{CN})_6^{3-}/\text{Fe}(\text{CN})_6^{4-}$): This redox couple was used in electrochemical studies. Ferricyanide is in the ferric (Fe^{3+}) form, while ferrocyanide is in the ferrous (Fe^{2+}) form. This system serves as an indicator in both CV and EIS, aiding in the examination of redox reactions (Peto-Gutiérrez et al., 2023).

Culture: *C. albicans* fungus was first cultured in a growth medium. *C. albicans* was incubated overnight at 37°C in RPMI 1640 medium using a shaker. Following incubation, the fungal cells were centrifuged at 6000 rpm for approximately 5 minutes, and the supernatant was discarded. The cell pellet was then resuspended in PBS and centrifuged again. This process yielded a clean fungal suspension in the medium. The fungal samples obtained were measured for absorbance at 600 nm using a UV-visible spectrophotometer to determine bacterial concentrations. The results of these measurements were correlated with the colony counts of *C. albicans*.

The morphological analysis of *Candida albicans* and *Candida tropicalis* using both SEM and light microscopy provides valuable insights into their structural characteristics. Figure 4 and Figure 5 highlight the distinct cell morphology and surface cavities that are crucial for identifying and differentiating between these fungal pathogens. The surface cavities, in particular, influence the specificity and sensitivity of the biosensors, making it possible to target specific fungal species accurately. The light microscope images (Figure 4) show the overall cell arrangement and morphology while the SEM images (Figure 5) reveal detailed surface structures.



Figure 4 : Light microscope images of *Candida albicans* (Aa), *Candida tropicalis* (Ba), and a combination of both (Ca). These images illustrate the cell morphology and surface cavities of the fungal cells under light microscopy. (S. Ma et al., 2014).

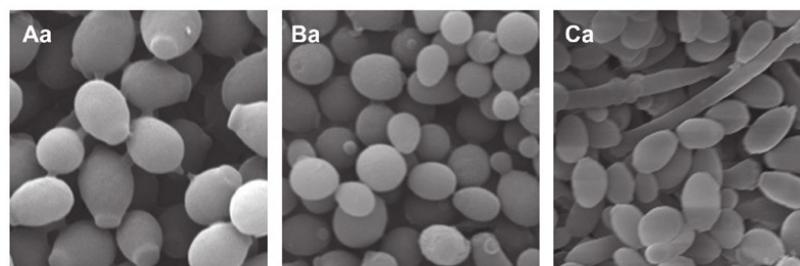


Figure 5 : Scanning electron microscope (SEM) images of *Candida albicans* (Aa), *Candida tropicalis* (Ba), and a combination of both (Ca). These images illustrate the cell morphology and surface cavities of the fungal cells. (S. Ma et al., 2014)

2.1.2 Apparatus

In electrochemical biosensor systems, a three-electrode configuration is standard, comprising a working electrode (WE), reference electrode (RE), and counter electrode (CE or AE). The working electrode facilitates the specific biochemical reaction with the analyte present in the electrolytic solution, generating an electrical signal proportional to the analyte concentration. The reference electrode provides a stable potential against which the working electrode's potential is measured, ensuring accurate voltage readings. Meanwhile, the counter electrode completes the electrical circuit by balancing the current generated during the analyte reaction at the working electrode, maintaining electrical neutrality in the solution. This setup allows precise control and measurement of electrochemical processes, essential for sensitive and reliable detection in applications ranging from medical diagnostics to environmental monitoring and beyond.

In this study, a graphite electrode was used as the working electrode, an Ag/AgCl electrode as the reference electrode, and a platinum wire as the counter electrode. The graphite electrode was a Tombow HB pencil lead placed inside a Rotring pencil. This three-electrode system was connected to a potentiostat device. An AUTOLAB 204.FRA32M potentiostat and NOVA 2.1.5 software were used.

2.1.3 Methods

Cyclic Voltammetry (CV), an electrochemical technique used to study the redox properties of analytes in solution, was employed as a voltammetric method; it involves sweeping the potential applied to a working electrode linearly versus time while measuring the resulting current. The potential is typically scanned forward and then backward in a cyclic manner, forming a characteristic voltammogram that plots current against applied potential. During the forward scan, oxidation or reduction reactions occur at the electrode surface, generating a current that is recorded. In the reverse scan, the potential is returned to its initial value, causing the reverse of the previous reactions (Rafiee et al., 2024). CV provides information about the redox behavior, electrochemical reversibility, and kinetic parameters of analytes (Gulaboski, 2024).

Electrochemical Impedance Spectroscopy (EIS) is a powerful electrochemical technique used to study the electrical properties of interfaces and analyze electrochemical systems. It involves applying a small amplitude alternating current (AC) signal across an electrochemical cell over a range of frequencies, typically from mHz to MHz. During EIS measurements, the impedance (Z) of the system, which is the ratio of the applied AC voltage to the resulting AC current, is measured and analyzed. EIS can successfully monitor the changes occurring at each modification step on the electrode (Rafiee et al., 2024).

Differential Pulse Voltammetry (DPV) was also used as a voltammetric method for electrode activation. In DPV, the excitation signals are generated by creating periodic pulses during a linear scan, and constant magnitude pulses, adjusted according to a linear potential increase, are applied to the working electrode for a certain period. At the end of this period, the current values obtained in response to the applied potential are measured, and the analysis is conducted accordingly.

The experimental steps were shown in Figure 6 and explained in below:

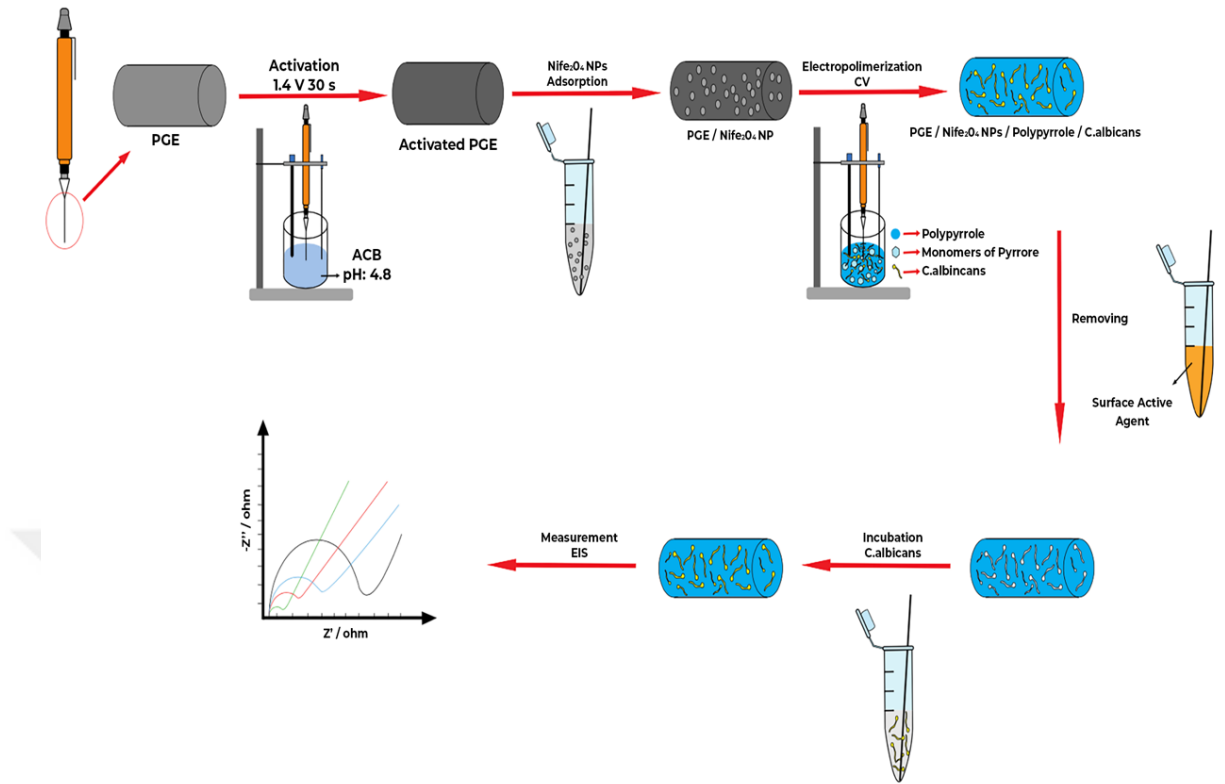


Figure 6: Schematic representation of the electrode modification and detection process.

Figure 6 illustrates the step-by-step procedure for modifying the pencil graphite electrode (PGE) and the subsequent pathogen detection process. Initially, the PGE is activated by applying a potential of +1.4 V for 30 seconds in ACB. The activation process increases the electrode's surface area and forms surface-active -COOH functional groups, making the surface more reactive for further modifications. This step is crucial as it enhances the adsorption capacity of the electrode surface, allowing for more effective modification and subsequent polymerization (Subak, 2022). The electropolymerization step involves subjecting the NiFeNP coated electrodes to a solution containing pyrrole monomers and *Candida albicans*. This process forms a polypyrrole layer on the electrode surface, embedding the *C. albicans* cells within the polymer matrix. After electropolymerization, the *C. albicans* are removed using a surface-active agent, leaving behind specific cavities in the polypyrrole matrix that can selectively rebind to *C. albicans* during detection.

The modified electrode is then incubated in a solution containing the target pathogen, allowing the cells to bind to the specific cavities formed during the electropolymerization process. Finally, EIS is employed to measure the changes in impedance, indicating the presence and concentration of the target pathogen.

2.1.3.1 Preparation of NiFeNP Solution and Electrode Surface

Modification

In this study, to activate the electrodes, a potential of +1.4 V applied to the graphite pencil working electrodes for 30 seconds. To facilitate the activation of the working electrode, a measurement cell filled with 5 mL of ACB. Subsequently, the working graphite electrode connected to the working electrode, and both the reference and counter electrodes immersed in the measurement cell. After the activation process, DPV measurements performed to check for any contamination on the electrodes. If a current signal exceeding 10^{-7} μA is observed at any voltage value during the DPV measurement, it indicates the presence of impurities on the electrodes.

NiFeNP solution prepared by adding equal volumes of ethanol and UPW, followed by ultrasonic mixing. Subsequently, this stock solution diluted with ACB. A volume of 100 μL of this diluted solution added to PCR tubes containing activated graphite electrodes. These PCR tubes placed in a thermal shaker set to 10 min, 60°C and agitated at 500 rpm. After this process, the electrodes washed once with ACB to remove any unbound NiFeNP from the surface. The NiFeNP modified electrodes then dried at 60°C for 5 minutes. This completes the modification of the graphite electrodes with NiFeNP (Topkaya et al., 2022).

2.1.3.2 Creating of MIP and NIP Structures

In this thesis, the abbreviations **NIP**, **MIP**, **nanoNIP**, and **nanoMIP** are used to describe different types of electrode surface designs and their specific applications:

NIP (Non-Imprinted Polymers): These are surfaces that are synthesized without any specific target molecule, resulting in a surface that does not have any selective cavities for particular molecules. They serve as a baseline to understand the non-specific interactions on the surface.

MIP (Molecularly Imprinted Polymers): These are electrode surfaces synthesized in the presence of a target molecule, such as *Candida albicans*, which is then removed, leaving behind specific cavities that can selectively rebind the target molecule. This imprinting process enhances the surface's selectivity and sensitivity for the target molecule.

NanoNIP (Non-Imprinted Polymers with Nanoparticles): These are non-imprinted surfaces that include nanoparticles, specifically NiFeNP, combined with polypyrrole. The inclusion of nanoparticles aims to enhance the surface's properties, such as conductivity and surface area, while maintaining a non-specific binding characteristic.

NanoMIP (Molecularly Imprinted Polymers with Nanoparticles): These are molecularly imprinted electrode surfaces that also incorporate nanoparticles, specifically NiFeNP, combined with polypyrrole and imprinted with a target molecule like *Candida albicans*. After the target molecule is removed, the surface has specific cavities that enhance selectivity and sensitivity for the target molecule due to the combined effects of molecular imprinting and nanoparticle inclusion.

Initially, a pyrrole monomer solution was prepared. A solution containing 50 mM pyrrole monomer and 0.1 M KCl in 5 mL was prepared (Ait Lahcen et al., 2018). This solution was filled into a measurement cell, and a pre-activated and NiFeNP coated graphite electrode was immersed in this cell. Using the CV method, a potential range from 0 V to +0.95 V with a scan rate of 50 mV/s was applied for 11 cycles, allowing polymer formation on the electrode surface. Subsequently, a 10 mM $K_3/K_4[Fe(CN)_6]$ solution in 0.1 M KCl was prepared. The bare electrodes, the electrodes covered with polypyrrole, and the electrodes covered with NiFeNP+polypyrrole were compared using CV (Kilic et al., 2022).

For obtaining the MIP structure, a pyrrole monomer solution containing 10^7 CFU/mL *C. albicans* was prepared. The electropolymerization process was performed using pre-activated electrodes under optimal conditions. The signals obtained from CV measurements of the prepared electrodes (*C. albicans* + polymer) in a solution containing 10 mM $K_3/K_4[Fe(CN)_6]$ in 0.1 M KCl were compared with those of electrodes containing only polymer (without *C. albicans*) and bare electrodes. This comparison was used to characterize the prepared electrodes.

2.1.3.3 Validation and Statical Analysis

After determining the optimal conditions through optimization processes, the system was validated. For intra-day precision studies, five measurements were performed at the experimentally determined optimal fungal concentration. The precision results are presented as % Relative Standard Deviation (RSD). A concentration study was conducted to obtain the calibration graph, from which the slope will be calculated. Limit of Detection (LOD) indicates the smallest concentration value that the biosensor can measure. LOQ represents the lowest concentration that can be quantitatively determined with acceptable precision and accuracy, not falling within the linearity limits or forming the lowest concentration of the linearity. The sensitivity of the method was demonstrated by calculating the LOD and Limit of Quantification (LOQ): LOQ values using the formulas $LOD = 3s/m$ and $LOQ = 10s/m$ (where s is the standard deviation of the smallest value on the calibration curve, and m is the average slope of the calibration curves).

All quantitative experiments were conducted with at least three replicates, and at least five samples from each experimental group will be tested ($n=5$). The numerical evaluations of the obtained results were reported as mean values and standard deviations.

Chapter 3

3.1 Results and Discussion

The synthesis of imprinted and non-imprinted polymers for the *Candida albicans* pathogen is a crucial step for fungal detection. This step aims to successfully create imprinted and non-imprinted forms through electropolymerization. After forming the imprinted structure, it is essential to completely remove the embedded fungus for analyte detection, which is achieved using surfactants. The goal is to ensure the complete removal of the fungus from the surface. Demonstrating that the surface properties of electrodes modified with the polymer differ from unmodified electrodes is another important step, proving the successful integration of the imprinted polymer onto the surface. This was verified using electrochemical methods such as voltammetry and impedance-based techniques. Achieving detection of the target fungus, *Candida albicans*, over a wide linear range and with a low LOD in artificial urine samples is a significant success for the study. The analytical performance of the developed biosensor system was also evaluated using validation parameters. The detection of *Candida albicans* using imprinted polymer is expected to be significantly more selective than with non-imprinted polymer. The results for the detection of *Candida albicans* using the MIP structure are discussed in detail below.

3.1.1 Preparation of NiFeNP Solution and Electrode Surface Modification

The effect of NiFeNP and polypyrrole on the conductivity of the electrodes are demonstrated in Figure 7.

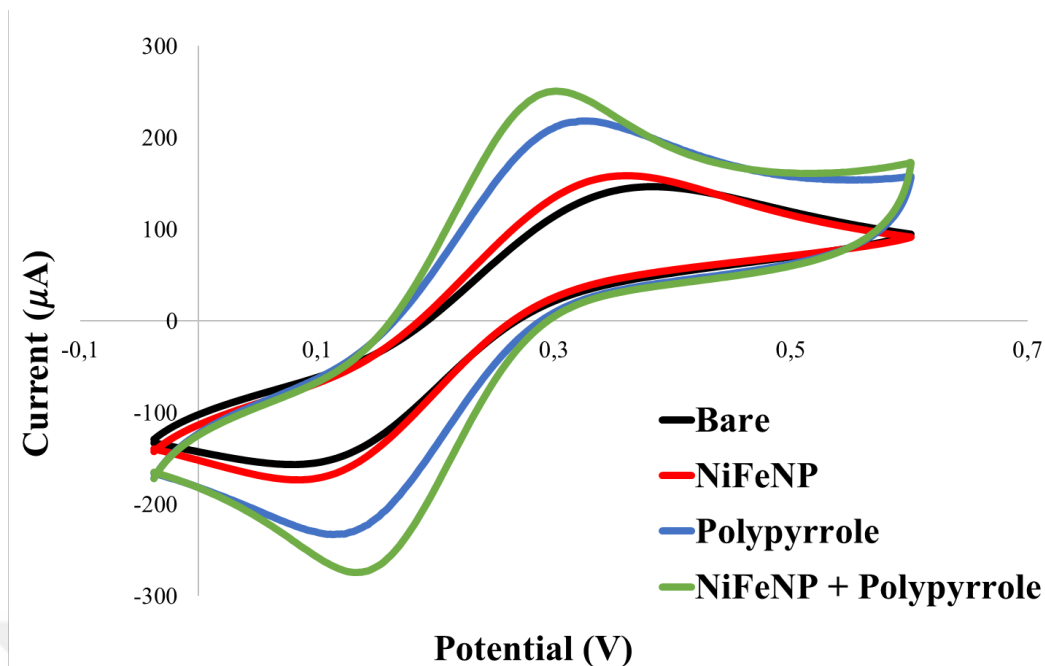


Figure 7: Cyclic voltammograms of bare, NiFeNP, Polypyrrole and NiFeNP + Polypyrrole coated electrodes measured in 10 mM $K_{3/4}[Fe(CN)_6]$ solution. Data represent an average of five repetitions (n:5).

The CV data compares the current signals of blank electrodes with those modified by NiFeNP, polypyrrole, and a combination of both (Figure 7). The blank electrode showed minimal electrochemical activity with low current values (129.3 μA , black line). In contrast, the NiFeNP-modified electrode exhibited higher current values, indicating enhanced electrochemical activity and better electron transfer facilitated by the nickel-iron nanoparticles (150.0 μA , red line). The polypyrrole-modified electrode displayed even higher current values, suggesting that polypyrrole significantly boosts electrochemical performance (216.5 μA , blue line). It was previously demonstrated the positive effect of polypyrrole on conductivity in earlier studies (S. Ma et al., 2014). The electrode modified with both NiFeNP and polypyrrole showed the highest current signals, highlighting the synergistic effect of combining these materials, which substantially improves the electrochemical activity compared to the individual modifications (223.8 μA , green line).

In Figure 8, the different concentrations of *Candida albicans* were mixed with pyrrole and NiFeNP and subject to electropolymerization for the formation of MIP structure.

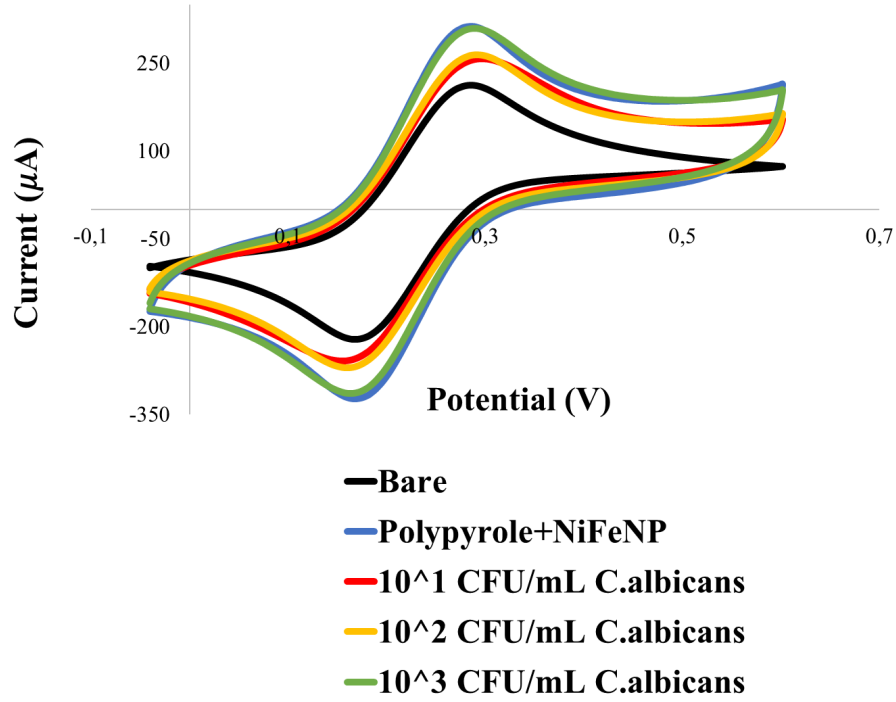


Figure 8: Cyclic voltammograms of bare electrodes (black line) and electrodes modified with polypyrrole+NiFeNP (blue). Pyrrole and NiFeNP were mixed with various concentrations of *Candida albicans*, and the currents were measured in a 10 mM K₃[Fe(CN)₆]/K₄[Fe(CN)₆] solution. Data represent an average of five repetitions (n:5).

As seen in Figure 8, the bare electrodes (black line) had the lowest conductivity in CV, with a current value of 218.6 μA. When the electrodes were coated with polypyrrole and NiFeNP together (blue line) through electropolymerization, the conductivity increased significantly, reaching 260.0 μA. However, when the electrodes were coated with both pyrrole+NiFeNP and 10¹ CFU/mL *Candida albicans* (red line), the conductivity decreased to 232.6 μA, suggesting that *C. albicans* blocks electron transfer by adhering to the surface along with polypyrrole. Electrodes coated with 10² CFU/mL *Candida albicans* also showed similarly low conductivity, with a value of 232.9 μA.

In contrast, electrodes coated with solutions containing *Candida albicans* at concentrations of 10^3 CFU/mL (green line) and 10^4 CFU/mL (data not shown, measuring 260.4 μ A) exhibited conductivity levels nearly identical to those of the polypyrrole-coated electrodes, measuring 259.2 μ A. This indicates that the large structure of fungal cells at very high concentrations leads to inefficiency in forming the MIP structure. Consequently, it can be concluded that the presence of large fungal cells at high concentrations hampers the proper formation and functionality of the MIP structure, rendering it ineffective for reliable conductivity measurements. This underscores the importance of optimizing cell concentrations to ensure the successful application of MIP-based sensors in detecting specific analytes. Therefore, a concentration of 10^1 CFU/mL *C. albicans* was chosen for electropolymerization to create the MIP structure.

3.1.2 Extraction of *Candida albicans* from MIP Structure

Using a removal agent in MIPs is essential for extracting the template molecule from the polymer matrix post-polymerization, creating specific recognition sites for target analytes. This process enhances the specificity and selectivity of the MIP, crucial for applications in sensing, separation, and catalysis. Effective template removal improves binding performance, reduces non-specific binding, and ensures the availability of active sites, enhancing sensitivity and accuracy. Additionally, it supports the reusability of MIPs, making the process economically and environmentally sustainable. For the successful formation of the MIP structure, *C. albicans* cells adhering to the electrode surface with polypyrrole after polymerization must be removed using surfactants. This leaves specific gaps on the electrode surface, allowing *C. albicans* to reattach, thus enabling selective detection. The removal agents, lysis buffer and SDS, were tested and compared in terms of efficiency.

Figure 9 examines the effects of different removal agents to remove *C. albicans* from the surface using both histograms and DPV graphs.

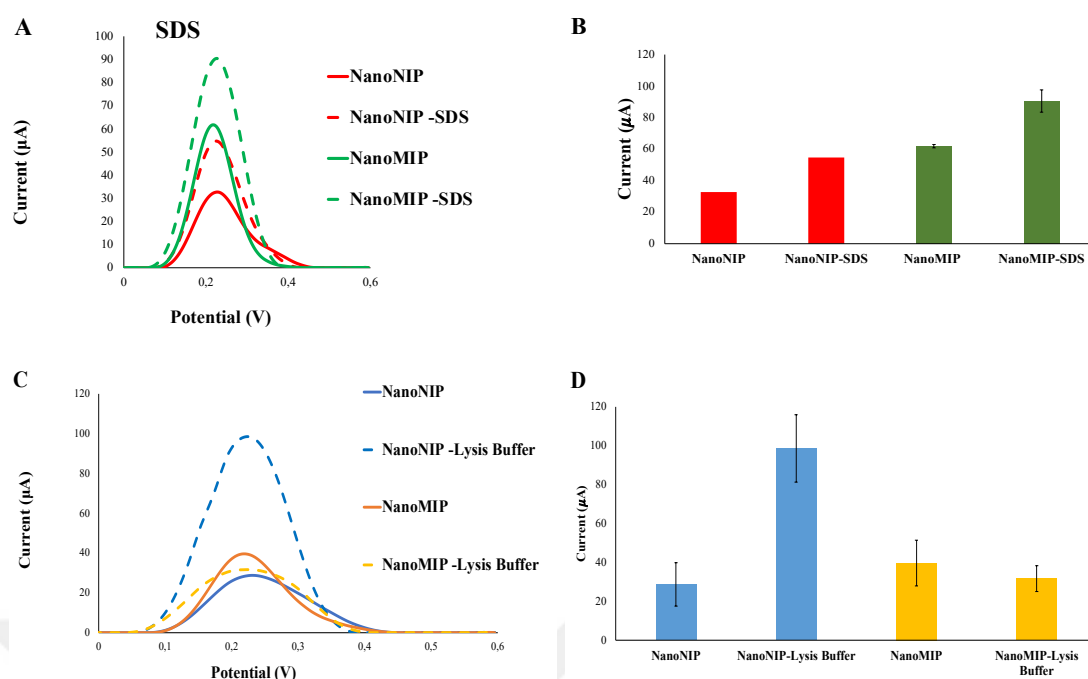


Figure 9 : The current signals of redox indicator obtained from DPV for different modified electrodes. NanoMIP and NanoMIP were treated with SDS (A) and Lysis Buffer (C) as the removal agents. The histograms of the obtained data from DPV measurements for SDS (B) and Lysis Buffer (D). Data represent an average of five repetitions (n:5).

For the removal agent to work effectively, there should be no significant difference in the DPV results between the NanoNIP and the NanoNIP treated with the removal agent. Conversely, a significant difference should be observed between the NanoMIP and the NanoMIP treated with the removal agent. This would indicate the successful removal of the pathogen from the electrode surface and the formation of selective cavities capable of capturing *C. albicans* on the electrode surface.

The effect of SDS (70% ethanol+10%SDS) on the currents of the different electrodes was analyzed using DPV and the results were shown in Figure 9A. The histogram observed from the DPV were shown in Figure 9B. In the experiment using SDS, half of the groups were immersed in SDS while the remaining half were kept in UPW. This procedure aimed to effectively remove *Candida albicans* from the electrode surface. Furthermore, the addition of SDS enhances the performance of NanoNIP and NanoMIP groups, but the enhancement is more pronounced in NanoMIP, suggesting

that SDS not only improves particle dispersion and stability but also maximizes the efficacy of the molecular imprinting technique.

The effect of Lysis Buffer on the currents of the different electrodes was analyzed using DPV and the results were shown in Figure 9C. The histogram observed from the DPV were shown in Figure 9D. Figures 9C and 9D showed that Lysis Buffer causes significant differences between NanoNIP groups, indicating that it damages the polymer coating on the electrode surface. Additionally, it was observed that the fungus was not successfully removed from NanoMIP groups. In conclusion, SDS (an anionic surfactant) and Lysis Buffer were tested as removal agents damaged the electrode surface and failed to effectively remove *C. albicans* fungus from the surface. This finding highlights the challenge of developing suitable removal agents that can effectively eliminate fungal cells without compromising the integrity of the electrode surface. Further research is needed to identify or develop more effective and less damaging removal methods to maintain electrode performance and longevity. Further research and testing of alternative removal agents or their combinations may be necessary to achieve optimal results.

In our study, we observed that after the initial removal attempts, the rebinding capacity of the MIP for *Candida albicans* was not optimal, suggesting that the removal process needs further optimization. For this reason, we tried SDS and acetic acid mixture as a removal agent and Figure 10 shows the impedance data of the results obtained.

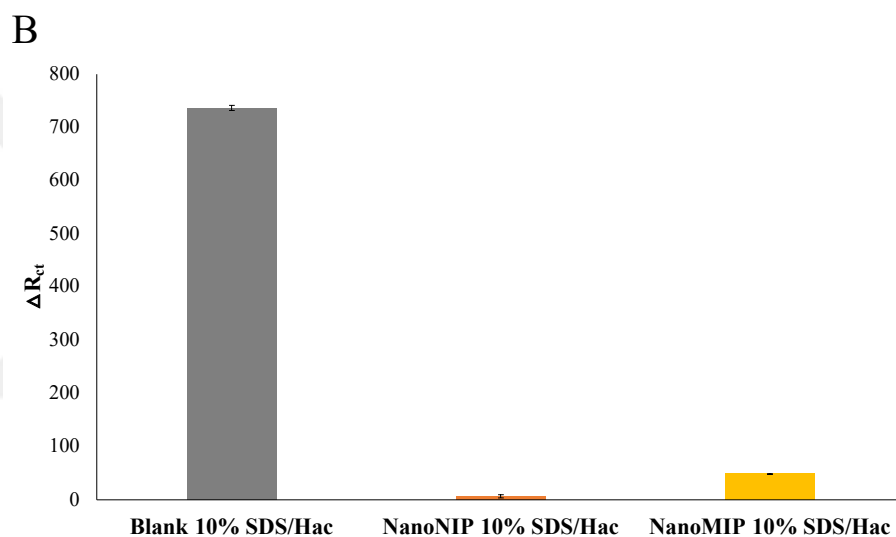
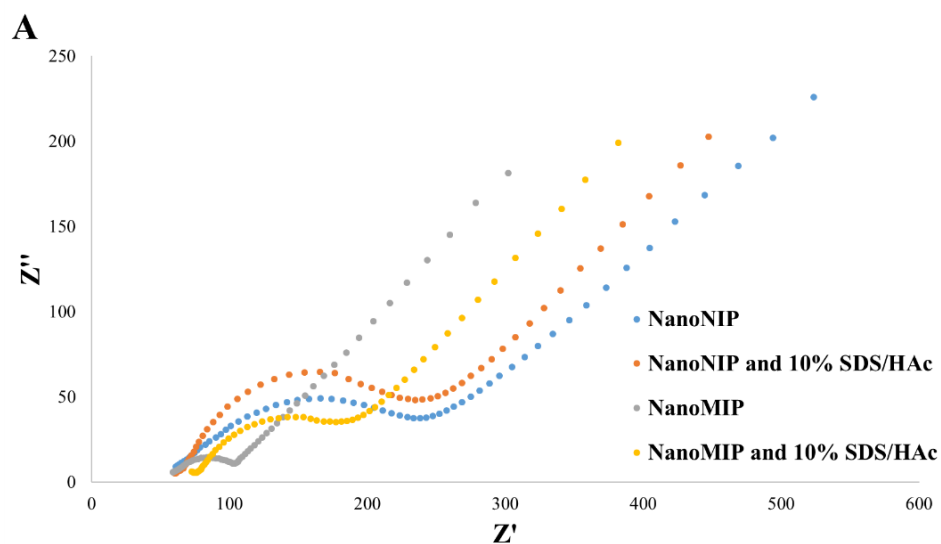


Figure 10: Nyquist plots for NanoNIP, NanoNIP treated with 10% SDS/HAc, NanoMIP, and NanoMIP treated with 10% SDS/HAc. Bare electrode data were excluded from the Nyquist plot due to extremely high R_{ct} values making the plot difficult to interpret (A). Histogram of ΔR_{ct} values for electrodes in the bare, NanoNIP, and NanoMIP treated with 10% SDS/HAc for 2 h compared to their controls (B). Data represent an average of five repetitions (n:5).

Figure 10A shows Nyquist plots for NanoNIP, NanoNIP treated with 10% SDS/HAc, NanoMIP, and NanoMIP treated with 10% SDS/HAc. In this experiment, the removal agent was applied to the electrodes for 2 hours. The NanoNIP electrodes exhibited electron transfer resistance (R_{ct} : 140.57 Ω) very close to their controls (R_{ct} : 134.59 Ω). UPW was used as the control group. This clearly emphasizes that the extraction agent does not compromise the conductivity of the electrode surface. The NanoMIP electrodes (R_{ct} : 35.15 Ω), shows a significantly different electron transfer resistance compared to the NanoMIP treated with 10% SDS/HAc group (R_{ct} : 83.57 Ω).

Figure 10B shows that the histogram obtained from EIS measurements. The bare electrodes treated with 10% SDS/HAc group exhibited the highest ΔR_{ct} values (ΔR_{ct} : 735.79 Ω); This can be attributed to the direct contact of the removal agent with the electrode surface, since the electrode surface does not undergo any electrode surface modification. ΔR_{ct} is the change in charge transfer resistance on the electrode surface. High ΔR_{ct} values support the differentiation between the compared groups. Here, the low ΔR_{ct} between the NanoNIP treated with 10% SDS/HAc and the NanoNIP control indicates that the removal agent does not deform the modified electrode surface. Additionally, the high ΔR_{ct} between the NanoMIP treated with 10% SDS/HAc and the NanoMIP control demonstrates that the process of removing *Candida albicans* fungus from the electrode surface is highly efficient, resulting in the formation of a clear MIP structure.

ΔR_{ct} values for the NanoNIP 10% SDS/HAc group were the lowest (ΔR_{ct} : 5.98 Ω) among SDS/HAc-treated samples; This indicates that NiFeNP and polypyrrole behave more stable towards the removal agent after coating on the electrode surface. In contrast, the NanoMIP 10% SDS/HAc group exhibited higher (ΔR_{ct} : 48.42 Ω) ΔR_{ct} values than the NanoNIP 10% SDS/HAc group; This indicates that the polymer on the electrode surface is molecularly imprinted, providing specific binding sites. This indicates that the use of 10% SDS/HAc as the elicitation agent is more effective in recognizing and binding to *Candida albicans* by forming the MIP structure. The use of 10% SDS/HAc as a fungal extraction agent has proven to be effective, as shown in Figure 10. Based on these results, further studies were conducted to optimize the concentration (Figure 11) and treatment duration (Figure 12) of SDS/HAc.

Figure 11 shows the effects of different concentrations of the SDS/HAc (10%, 20%, 30% SDS/HAc) removal agent on the electrode surface and the efficiency of removing *C. albicans* fungus from the surface.

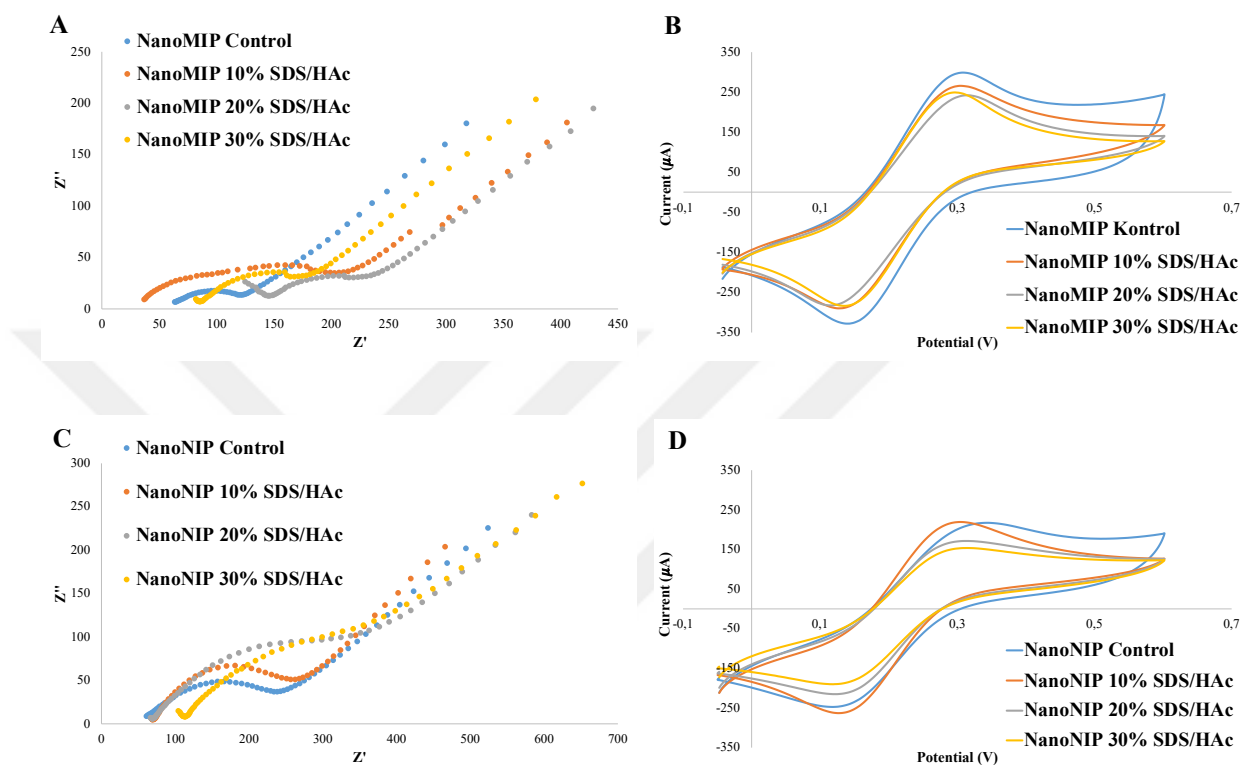


Figure 11 : Impedance spectra (A, C) and cyclic voltammograms (B, D) for NanoMIP and NanoNIP structures treated with different concentrations of the removing agent in 10 mM $K_3/K_4[Fe(CN)_6]$. Blank electrode data were excluded from the Nyquist plot due to extremely high R_{ct} values making the plot difficult to interpret. Data represent an average of five repetitions (n:5).

In Figures 11A (EIS) and 11B (CV), the effects of different concentrations of the removal agent on the NanoMIP structures were evaluated. Meanwhile, in Figures 11C (EIS) and 11D (CV), the effects on the NanoNIP structures were assessed.

When examining the Nyquist plots in Figure 11A and Cyclic voltammograms in Figure 11B, significant differences were observed compared to NanoMIP and the control groups after the application of 10% SDS/HAc. These differences in both impedance and voltammetry results indicated that *Candida albicans* was effectively removed from the surface. However, the values obtained at 20% and 30% SDS/HAc concentrations might be misleading as these concentrations could have caused deformation on the electrode surface. Therefore, 10%SDS/HAc concentration was determined to be the most suitable concentration that does not damage the electrode surface with NanoMIP. The use of 10% SDS/HAc was identified as the most suitable concentration for effectively removing the fungus. For this reason, this concentration was chosen for further studies.

Figure 12 illustrates the optimization study of the removal time for *C. albicans* fungus from the electrode surface.

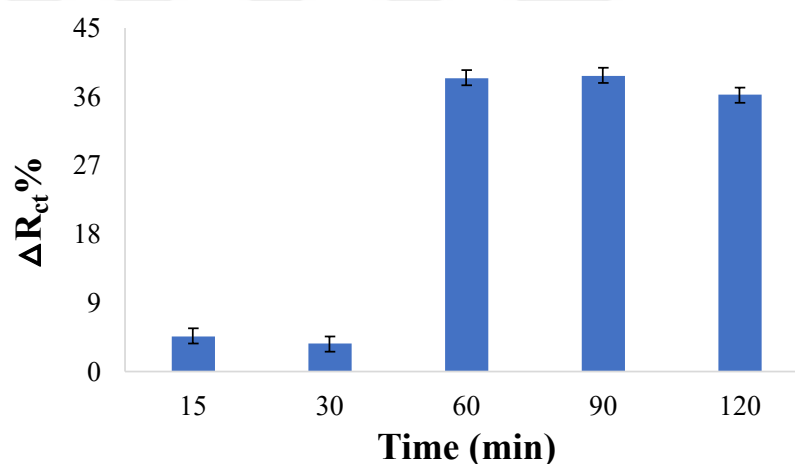


Figure 12 : Histogram showing the change in R_{ct} value as a result of treating Nano MIP-coated electrodes with 10% SDS/HAc for 15-120 minutes. Data represent an average of five repetitions (n:5).

In our previous experiments, the removal agent was applied for 2 hours, which was considered too long. Therefore, experiments were conducted to determine whether the fungus could be efficiently removed in shorter times of 15, 30, 60, 90, and 120 minutes.

The results show that after 60 minutes, the $\Delta R_{ct}\%$ value increased significantly, reaching the highest levels ($\Delta R_{ct}\%$: 38.5). This indicates that treatment with SDS/HAc for 60 minutes is sufficient for the efficient removal of the fungus from the surface. At 90 minutes, the $\Delta R_{ct}\%$ value remained close to the peak value observed at 60 minutes ($\Delta R_{ct}\%$: 38.8), demonstrating that SDS/HAc is also effective at 90 minutes but does not provide much additional benefit compared to 60 minutes. At 120 minutes, the $\Delta R_{ct}\%$ value remained similar to the values at 60 and 90 minutes ($\Delta R_{ct}\%$: 36.3), and even slightly lower. This suggests that longer treatment times do not enhance the efficiency of fungus removal and lead to unnecessary time consumption.

In conclusion, treatment with SDS/HAc for 60 minutes has been identified as the optimal and most efficient duration for the removal of *C. albicans* fungus from the electrode surface, successfully forming the NanoMIP structure.

3.1.3 Rebinding of *Candida albicans* to MIP

The rebinding studies of *Candida albicans* to the MIP demonstrate the selective recognition capability of the MIP structures. The MIPs were specifically designed to have cavities complementary in shape and functional groups to *Candida albicans*, enabling selective rebinding. However, the efficiency of rebinding is highly dependent on the successful removal of the template during the imprinting process. Ineffective removing step, can leave residual templates in the MIP cavities, thereby reducing the number of available binding sites and compromising the selectivity and sensitivity of the MIP. The initial step in creating effective MIP structures involves the complete removal of the template molecules from the polymer matrix, which is critical as any remaining template molecules can block the binding sites and hinder rebinding efficiency. The rebinding process should be conducted under optimal conditions to promote the selective interaction between the MIP and the target analyte. Continuous optimization of the imprinting, removal, and rebinding steps is necessary to improve the performance of MIP-based sensors, and validation against control structures such as NanoNIP ensures reliability and specificity.

Successful rebinding confirms the MIP's performance by assessing binding affinity, capacity, and selectivity, which are essential for practical applications such as biosensors, drug delivery, and environmental monitoring. This step also allows for the optimization of synthesis conditions and ensures consistent quality across batches. Additionally, comparing rebinding results with NIPs or other controls ensures that the binding is due to specific imprinted sites rather than non-specific interactions, proving the selectivity and specificity of the MIP and confirming its readiness for real-world use.

Figure 13 shows the Nyquist plots (Z' vs. Z'') (Figure 13A) and cyclic voltammograms (Figure 13B) of NanoMIP and NanoNIP-coated electrodes tested in the presence and absence of *C. albicans*.

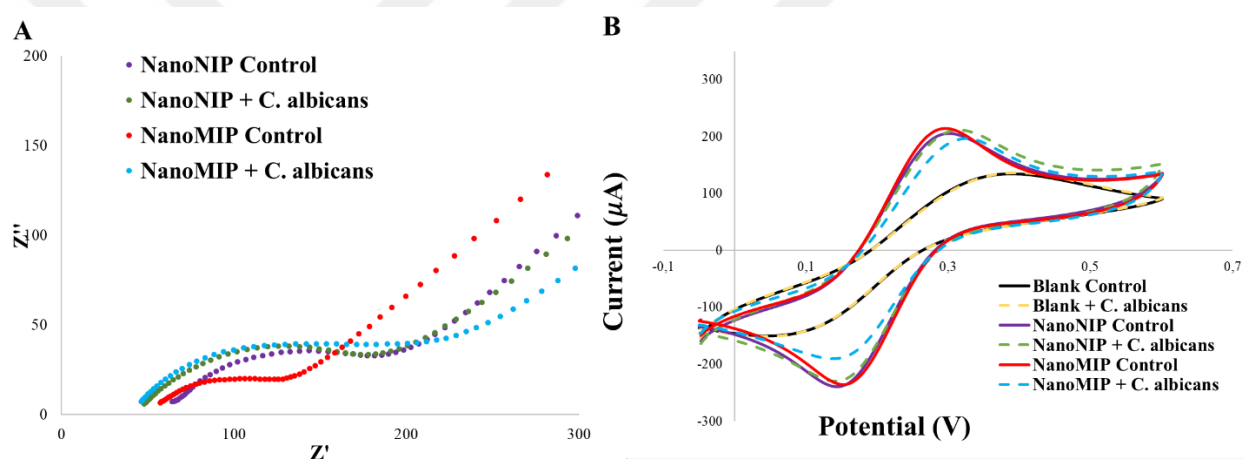


Figure 13 : (A) Nyquist plots (Z' vs. Z'') and (B) cyclic voltammograms of NanoMIP and NanoNIP-coated electrodes. The electrodes were tested in the presence and absence of *C. albicans*. (Blank electrode data were excluded from the Nyquist plot due to extremely high R_{ct} values making the plot difficult to interpret). Data represent an average of five repetitions (n:5).

In Figure 13A, there is no significant difference between the NanoNIP control electrodes and the NanoNIP electrode treated with *C. albicans*. This indicates that *C. albicans* does not adhere to the NanoNIP surface. This is expected because there are no specific cavities for *C. albicans* in the NanoNIP structures. On the other hand, there is a significant difference in the R_{ct} values between the NanoMIP control electrode and the NanoMIP electrode treated with *C. albicans*. NanoMIP electrodes have specific cavities for *C. albicans* on their surfaces.

When NanoMIP structures are treated with *C. albicans*, it is expected that *C. albicans* will adhere to the surface. In Figure 13A, the NanoMIP electrodes treated with *C. albicans* exhibit higher impedance (Z''). This indicates that the fungus successfully adheres to the NanoMIP surface and increases the surface impedance.

In Figure 13B, there is no difference between the blank control and the blank electrode treated with *C. albicans*. This indicates that the *C. albicans* does not adhere to the bare electrode surface. Similarly, there is no significant difference between the NanoNIP control and the NanoNIP electrodes treated with *C. albicans*. This shows that the *C. albicans* does not adhere to the NanoNIP surface. However, there is a significant difference between the NanoMIP control and the NanoMIP electrodes treated with *C. albicans*, which supports the observation in Figure 13A. When the fungus adheres to the NanoMIP surface, the current values change. This demonstrates that the fungus successfully adheres to the surface.

Figure 14 shows the effects of different concentrations of *C. albicans* (10^4 , 10^5 , 10^6 , and 10^7 CFU/mL) on NanoMIP-coated electrodes and the relationship of these effects with impedance spectra and changes in charge transfer resistance (ΔR_{ct}).

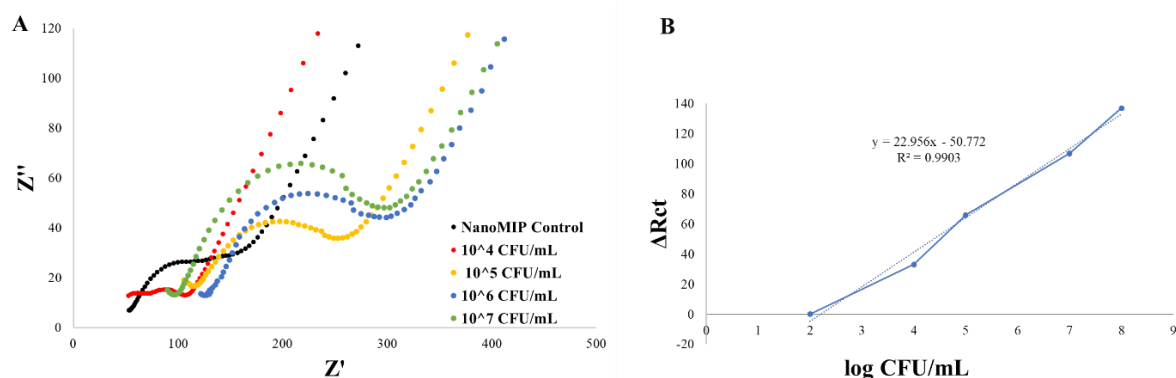


Figure 14 : (A) Nyquist plots (Z' vs. Z'') showing the impedance spectra of nanoMIP-coated electrodes with different concentrations of *C. albicans* (10^4 , 10^5 , 10^6 , and 10^7 CFU/mL). The control is represented by the nanoMIP-coated electrodes without *C. albicans*. (B) The relationship between the change in charge transfer resistance (ΔR_{ct}) and the logarithm of *C. albicans* concentration ($\log \text{CFU/mL}$). The linear fit equation and the R^2 value are indicated on the plot. Blank electrode data were excluded from the Nyquist plot. Data represent an average of five repetitions (n:5).

Figure 14A presents the impedance spectra of NanoMIP electrodes exposed to different concentrations of *C. albicans* (10^4 , 10^5 , 10^6 , and 10^7 CFU/mL). The control group consists of NanoMIP electrodes treated with PBS, the buffer used to prepare the *C. albicans* solution. As shown in Figure 14A, the NanoMIP electrodes exhibit increasing impedance values with increasing concentrations of *C. albicans*. As the concentration of *C. albicans* increases, there is an observed increase in Z'' (imaginary impedance) values. This indicates that *C. albicans* successfully adheres to the surface and increases surface impedance as the concentration increases. The graph does not include concentrations up to 10^7 CFU/mL because, beyond this concentration, there was no significant increase in R_{ct} values. This saturation effect indicates that the binding sites on the NanoMIP surface become fully occupied at higher concentrations, and further increases in *C. albicans* concentration do not result in additional impedance changes. Therefore, the graph includes concentrations up to 10^7 CFU/mL.

Figure 14B shows the relationship between the change in charge transfer resistance (ΔR_{ct}) and the logarithm of *C. albicans* concentration ($\log$ CFU/mL). The linear fit equation and the R^2 value are indicated on the plot. The data in the graph show a high linear correlation between ΔR_{ct} and $\log$ CFU/mL ($R^2 = 0.9986$). This indicates a significant increase in charge transfer resistance (R_{ct}) with increasing concentrations of *C. albicans*. The linear fit equation is given as $\Delta R_{ct} = 25.128 * \log(\text{CFU/mL}) - 88.859$. This result demonstrates that both the impedance spectra and charge transfer resistance increase linearly with the concentration of *C. albicans* on the electrode surface.

The Limit of Detection (LOD) is calculated (Equation 1.1) as 2.8 CFU/mL, and the Limit of Quantification (LOQ) is calculated (Equation 1.2) as 32.3 CFU/mL. These values were determined based on the standard deviation of the response and the slope of the calibration curve as per the following formulas:

$$LOD = \frac{3.3 \times \sigma}{S} \quad (\text{Equation 1.1})$$

$$LOQ = \frac{10 \times \sigma}{S} \quad (\text{Equation 1.2})$$

where σ is the standard deviation of the response and S is the slope of the calibration curve.

Traditional methods for detecting *C. albicans*, such as culture-based techniques and PCR, typically have higher LOD and LOQ values. For instance, culture methods often have LOD values in the range of 10^2 to 10^3 CFU/mL and require longer processing times. PCR-based methods, while faster, still have LOD values around 10 CFU/mL but require complex and costly equipment (Felix et al., 2023). The NanoMIP-based electrochemical method presented here demonstrates a significantly lower LOD and LOQ, highlighting its superior sensitivity and potential for rapid detection of *C. albicans* in various samples. This improved sensitivity can be particularly beneficial in clinical diagnostics and environmental monitoring where early and accurate detection of pathogens is crucial (Krohn et al., 2018).

3.1.4 Selectivity of MIP Structure

The selectivity of MIP structures is of paramount importance because it determines the polymer's ability to distinguish and bind specific target molecules amidst a complex mixture of similar compounds. High selectivity ensures that the MIP can effectively capture and isolate the desired analyte, which is crucial for applications in biosensing, drug delivery, environmental monitoring, and diagnostics. Selectivity enhances the accuracy and reliability of detection methods, reducing false positives and negatives. It also improves the efficiency of separation processes and increases the sensitivity of analytical techniques.

Figure 15 shows the interaction of NanoMIP-coated electrodes with different microorganisms (*C. albicans*, *P. aeruginosa*, *S. aureus*, *C. parapsilosis*, and *C. tropicalis*) and these interactions are depicted through Nyquist plots (Z' vs. Z'').

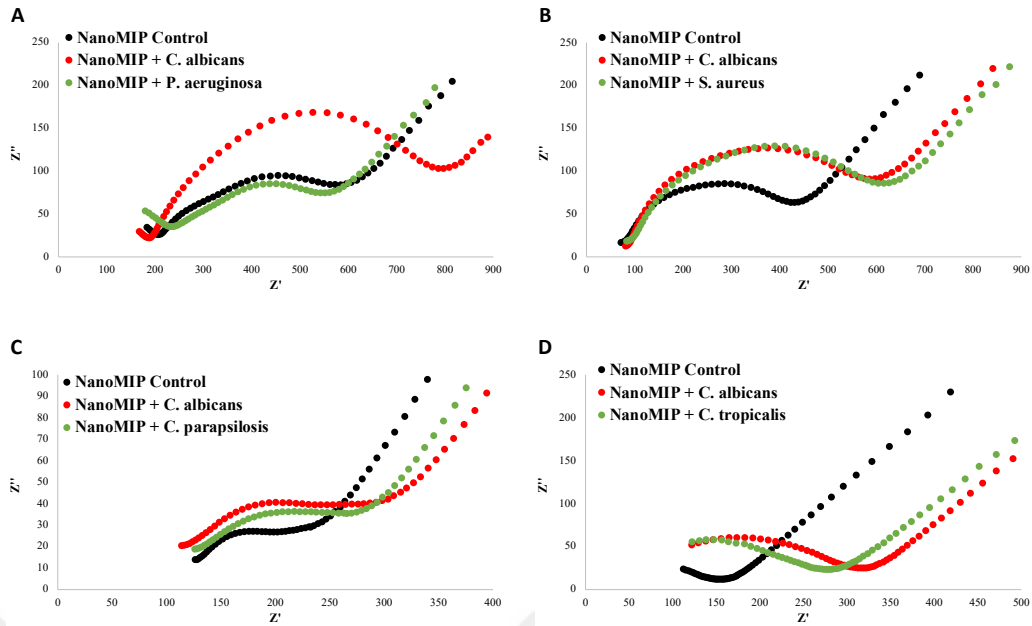


Figure 15 : Nyquist plots (Z' vs. Z'') showing the interactions of NanoMIP-coated electrodes with various microorganisms. (A) *C. albicans* and *P. aeruginosa*, (B) *C. albicans* and *S. aureus*, (C) *C. albicans* and *C. parapsilosis*, (D) *C. albicans* and *C. tropicalis*. Data represent an average of five repetitions (n:5).

Figure 15A presents the interactions of NanoMIP electrodes with *C. albicans* and *P. aeruginosa*. The Nyquist plots compare the control group (black dots) with electrodes treated with *C. albicans* (red dots) and *P. aeruginosa* (green dots). Electrodes treated with *C. albicans* show higher impedance values compared to the control group, indicating successful adhesion of the fungus to the surface. Electrodes treated with *P. aeruginosa*, however, show impedance values quite similar to the control, indicating low adhesion efficiency of the bacteria to the surface.

Figure 15B shows the interactions of NanoMIP electrodes with *C. albicans* and *S. aureus*. Electrodes treated with *C. albicans* show higher impedance values compared to the control group, indicating successful adhesion of the fungus to the surface. However, electrodes treated with *S. aureus* also show impedance values similar to those treated with *C. albicans*, indicating high adhesion efficiency of the coccus-shaped bacteria to the surface. This result suggests that *C. albicans* does not have a selective binding mechanism to the NanoMIP surface, possibly due to dimensional and shape factors.

Figure 15C shows the interactions of NanoMIP electrodes with *C. albicans* and *C. parapsilosis*. Electrodes treated with *C. albicans* show higher impedance values compared to the control group, indicating successful adhesion of the fungus to the surface. Similarly, electrodes treated with *C. parapsilosis* also show high impedance values, indicating high adhesion efficiency of the *C. parapsilosis* fungus to the surface. This result suggests that the designed biosensor is not selective within the *Candida* family.

Figure 15D presents the interactions of MIP-coated electrodes with *C. albicans* and *C. tropicalis*. To better understand whether the biosensor can distinguish between *Candida* species, it was tested in this manner once more. As in Figures A, B, and C, electrodes treated with *C. albicans* show higher impedance values compared to the control group, indicating successful adhesion of the fungus to the surface. Electrodes treated with *C. tropicalis* also show similarly high impedance values, indicating that the designed biosensor is not selective among *Candida* species.

Figure 15 demonstrates that the designed NanoMIP-coated electrodes are selective for *C. albicans*, showing successful adhesion of this microorganism to the surface. However, the NanoMIP structure is selective against the bacillus-shaped *P. aeruginosa* bacteria, but not against the coccus-shaped *S. aureus* bacteria. Moreover, the biosensor could not differentiate between *C. parapsilosis* and *C. tropicalis*, failing to distinguish between *Candida* species. These results indicate that the NanoMIP structures do not possess high sensitivity for specific microorganism detection.

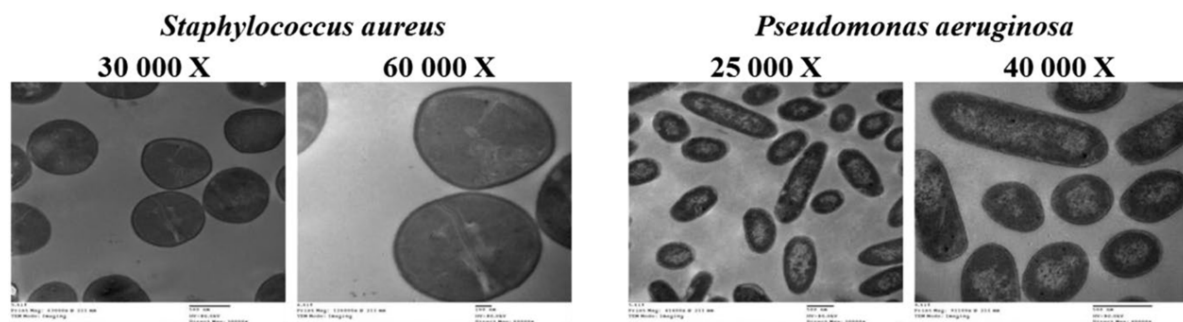


Figure 16 : TEM images of *Staphylococcus aureus* and *Pseudomonas aeruginosa* at various magnifications. *Staphylococcus aureus* shown at 30,000x and 60,000x (left) demonstrates its coccus shape, while *Pseudomonas aeruginosa* shown at 25,000x and 40,000x (right) exhibits its bacillus shape (Abdel-Shafi et al., 2016).

In Figure 15, it was found that the selective binding properties of the NanoMIP structure depend on the compatibility of its surface cavities with the shape, size, and surface characteristics of microorganisms. As seen in Figure 16, the rod shape and surface properties of *P. aeruginosa* did not align with the NanoMIP cavities, preventing adhesion to the NanoMIP structure. In contrast, the round and clustered shape of *S. aureus* facilitated better compatibility and adhesion. These results highlight the selective binding mechanisms of NanoMIP structures based on morphological compatibility and their effects on different microorganisms.

Chapter 4

4.1 Conclusion

Upon reviewing previous classical and electrochemical studies aimed at detecting *C. albicans* fungus in the literature, the following observations were made: Classical methods involve complex, multi-step, and time-consuming sample preparation procedures, or require expensive and non-portable equipment with advanced infrastructure. Systems that present a low limit of detection often require complex sample preparation protocols. There is a need to improve the accuracy and reliability of these methods, make them faster and more practical, and reduce their cost.

This study aimed to address these deficiencies through innovative methods. In this context, we designed an electrochemical detection system for the detection of the *C. albicans* pathogen using, for the first time, molecularly imprinted polymers and pencil graphite electrodes modified with NiFeNP. Electrochemical biosensor systems using electrodes modified with NiFeNP and molecularly imprinted polymers have not previously been used to detect the *C. albicans* pathogen. In this study, graphite electrodes were first modified with NiFeNP. Then, pyrrole monomers and *C. albicans* were electropolymerized using CV to create molecularly imprinted polymers on the electrode surface. After polymerization, *C. albicans* was removed using a surfactant to leave specific cavities on the electrode surface. Various electrochemical methods such as CV, DPV, and EIS were used to optimize the experimental steps.

As a result, the integration of NiFe₂O₄ nanoparticles and the molecular imprinting technique provided a robust platform for developing a rapid, sensitive, and selective detection method for *Candida albicans*. The resulting design demonstrated excellent sensitivity and accurately detected *Candida albicans* at various concentrations. This method offers significant advancement over traditional detection methods, providing an economical and user-friendly solution for clinical diagnoses.

The use of 10% SDS/HAc as a fungal extraction agent proved effective, as evidenced by the Nyquist plots and cyclic voltammograms. The concentration optimization study determined that 10% SDS/HAc does not damage the electrode surface and effectively removes the fungus. This concentration was identified as the most suitable for maintaining electrode integrity while ensuring efficient fungus removal.

Furthermore, LOD and LOQ for the developed biosensor were calculated to be 2.8 CFU/mL and 32.3 CFU/mL, respectively. Compared to traditional methods, where culture-based techniques typically have LOD values ranging from 10^2 to 10^3 CFU/mL and PCR-based methods have LOD values around 10 CFU/mL, the biosensor demonstrated significantly lower detection limits, indicating superior sensitivity and practicality for clinical diagnostics. It was found that the selective binding properties of the NanoMIP structure depend on the compatibility of its surface cavities with the shape, size, and surface characteristics of microorganisms. The rod shape and surface properties of *P. aeruginosa* did not align with the NanoMIP cavities, preventing adhesion to the NanoMIP structure, while the round and clustered shape of *S. aureus* facilitated better compatibility and adhesion. *C. albicans* was successfully bound to the NanoMIP surface. *C. tropicalis* and *C. parapsilosis* fungi exhibited similar impedance values due to their similar surface and morphological characteristics, indicating that the NanoMIP structure is not selective among *Candida* species but has a high binding capacity for *Candida* fungi in general. These results highlight the morphological compatibility-based selective binding mechanisms of the NanoMIP structure and their effects on different microorganisms.

In summary, the electrochemical biosensor system developed with NiFeNP and molecularly imprinted polymers offers a rapid and sensitive method for detecting *Candida albicans*, overcoming many limitations of traditional detection methods, and providing a promising tool for future clinical and environmental applications.

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Appendix B

Publications from the Thesis

Conference Papers

1. BİLSEL INTERNATIONAL KIBYRA SCIENTIFIC RESEARCHES
CONGRESS

Projects

1. TUSEB 2022-A2-YL

Proje No: 31174

Proje Name: Detection of Candida albicans Pathogen by Electrochemical Methods
with Metal Nanoparticle Modified Molecular Imprinted Polymer