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MOLECULARLY IMPRINTED POLYMER BASED BIOSENSOR SYSTEM FOR A PEPTIDE BIOMARKER DETECTION

M.Sc. Thesis by

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M.Sc THESIS EXAMINATION RESULT FORM

We have read the thesis entitled "**MOLECULARLY IMPRINTED POLYMER BASED BIOSENSOR SYSTEM FOR A PEPTIDE BIOMARKER DETECTION**" completed by **FATIH TÜRK** under supervision of **Asst.Prof.Dr. NIMET YILDIRIM TİRGİL** and we certify that in our opinion it is fully adequate, in scope and in quality, as a thesis for the degree of Master of Science.

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MOLECULARLY IMPRINTED POLYMER BASED BIOSENSOR SYSTEM FOR PEPTIDE BIOMARKER DETECTION

ABSTRACT

Biosensors for a range of targets would be more crucial as indicators of disease, health status, environmental monitoring, food quality, fermentation control, and civil defense applications grow more widespread. One of the approaches being researched to enhance their unique recognition properties is molecularly imprinted polymers (MIPs). With exceptional sensitivity and selectivity, MIPs mimics the structure and binding principle of antibodies and biological receptors. MIP is a key component in constructing polymeric sensors and can be employed in various signal amplification or transmission systems.

Molecularly imprinted polymers have generally been used for small molecules, but have limited uses for large molecules such as peptides. Detection and early diagnosis of peptide biomarkers is important in medicine and many other fields in order to prevent peptide-related diseases such as thrombin deficiency, which is an extremely rare bleeding disorder. In this study, the detection of various peptide molecules was made using MIP, and it was proven once again that molecular imprinted polymers are useful for large molecules such as peptide molecules.

In this study, MIPs were created and studied using different polymers for a peptide biomarker. Based on the results of the experiments without aptamer, it was concluded that the studies using polydopamine were more stable and more selective, and the system was developed as a dual hybrid based on MWCNTs/Aptamer/MIP. During the MIP preparation steps, several monomers were used to test the detection of thrombin, an exemplary peptide biomarker. Electrochemical methods such as CV, DPV, EIS were used to evaluate the effectiveness of different polymers and the results were evaluated. And again, cyclic voltammetry, electrochemical impedance spectroscopy and differential pulse voltammetry were used to characterize the MIP-based sensor system. The MIP-based biosensor device for thrombin has a detection limit of 2.5 pg/mL and a robust linear response from 10 pg/mL to 0.5 ng/mL.

Keywords: Biosensor, Polymer, Molecular imprinted polymers, Electropolymerization, MWCNTs, Peptide, Thrombin, Dopamine, Ethanolamine, Thionine, Aptamer

PEPTİT BİYOBELİRTEÇ TESPİTİ İÇİN MOLEKÜLER BASKILI POLİMER BAZLI BİYOSENSÖR SISTEMİ

ÖZ

Hastalık, sağlık durumu, çevresel izleme, gıda kalitesi, fermantasyon kontrolü ve sivil savunma uygulamaları daha yaygın hale geldikçe, bir dizi hedef için biyosensörler daha önemli olacaktır. Eşsiz tanıma özelliklerini geliştirmek için araştırılan yaklaşımlardan biri de moleküler baskılı polimerlerdir (MIP'ler). Moleküler Baskılanmış Polimer (MIP), olağanüstü hassasiyet ve seçicilik ile antikorların ve biyolojik reseptörlerin yapısını ve işleyişini taklit eder. MIP, polimerik sensörlerin yapımında önemli bir bileşendir ve çeşitli sinyal yükseltme veya iletim sistemlerinde kullanılabilir.

Moleküler baskılı polimerler genellikle küçük moleküller için kullanılmıştır fakat peptitler gibi büyük moleküller için sınırlı sayıda kullanımı bulunmaktadır. Son derece nadir bir kanama bozukluğu olan trombin eksikliği gibi peptitlere bağlı hastalıkların önüne geçmek için peptit biyobelirteçlerinin tespiti ve erken teşhisi tıp alanında ve bir çok alanda önem arz etmektedir. Bu çalışmada çeşitli peptit moleküllerinin tespiti MIP kullanılarak yapılmış olup, moleküler baskılı polimerlerin peptit molekülleri gibi büyük moleküller içinde kullanışlığı olduğu bir kez daha kanıtlanmıştır.

Bu çalışmada, bir peptit biyobelirteç için farklı polimerler kullanılarak MIP'ler oluşturulmuş ve incelenmiştir. Aptamersiz deneylerin sonuçlarından yola çıkarak polidopamin kullanılarak yapılan çalışmaların daha kararlı daha seçici olduğu kanısına varılıp sistem dual hibrit olarak MWCNTs/Aptamer/MIP bazlı olarak geliştirildi. MIP hazırlama aşamaları sırasında, örnek bir peptit biyobelirteç olan trombinin saptanmasını test etmek için birkaç monomer kullanıldı. Farklı polimerlerin etkinliğini değerlendirmek için CV, DPV, EIS gibi elektrokimyasal yöntemler kullanılıp sonuçlar değerlendirildi. Ve yine MIP bazlı sensör sistemini karakterize etmek için döngüsel voltametri, elektrokimyasal empedans spektroskopisi ve diferansiyel darbe voltametrisi kullanıldı. Trombin için MIP tabanlı biyosensör cihazı, 2,5 pg/mL'lik bir algılama sınırına ve 10 pg/mL ila 0,5 ng/mL arasında sağlam bir doğrusal yanıta sahiptir.

Anahtar Kelimeler: Biyosensör, Polimer, Moleküler baskılı polimerler, Elektropolimerizasyon, MWCNT'ler, Peptit, Trombin, Dopamin, Etanolamin, Tiyonin, Aptamer

CONTENTS

NOMENCLATURE

Acronyms

MIP	Molecularly Imprınted Polymer
AFM	Atomic Force Microscope
CV	Cyclic Voltammetry
DPV	Differential Pulse Voltammetry
EIS	Electrochemical Impedance Spectroscopy
TEM	Transmission Electron Microscope
SEM	Scanning Electron Microscope
DTA	Differential Thermal Analysis
DSC	Differential Scanning Calorimetry
TGA	Thermogravimetric Analysis
AIBN	Azobisisobutylonitrile
BPO	Benzoyl peroxide
TNP	Trinitrophenol
TNT	Trinitrotolune
FITC	Fluorescein isothiocanate
RLS	Resodance Light Scattering
QCM	Quartz Crystal Microbalances
SAW	Surface Acoustic Waves
PBS	Phosphate Buffered Saline
DA	Dopamine
EA	Ethanolamine
BSA	Bovine serum albumin
CNT	Carbon Nanotube
MWCNT	Multi Walled Carbon Nanotube
Thr	Thrombin

LIST OF TABLES

LIST OF FIGURES

CHAPTER 1

INTRODUCTION

Biosensors are analytical tools that incorporate biological diagnostic material to help identify analytes and convert the product created by the interaction of the analyte and the biological diagnostic material into a detectable signal with the aid of a physicochemical converter. Fundamentally, biosensors need to be extremely precise and reusable regardless of environmental factors like pH and temperature. According to their mechanisms, the materials employed in biosensors are split into three groups: a biocatalytic group made up of enzymes, a bioaffinity group made up of antibodies and nucleic acids, and a microbe-based group made up of microorganisms. The bioaffinity group includes biosensor systems with bioidentification components made of synthetic materials (such MIP or aptamer) [1].

Biosensors are very significant in many industrial fields, including automation, quality control, and energy storage, as well as in medical, agriculture, food, pharmacy, environmental pollution, and defense. Foods, metabolites, antibiotics, medicines, some inorganic molecules, and organic substances including enzymes, viruses, and microbes can all be detected with biosensors.

Large-molecular-weight molecules called polymers are created from monomers, or single molecules joined together by covalent bonds. Polymerization reactions are those in which monomers interact chemically to produce polymer molecules. The degree of polymerization refers to the quantity of repeating units in the polymer molecule (Pn). One of the factors affecting a polymer's qualities is the degree of polymerization. One type of monomer or several types of monomers can make up polymers. Homopolymers are polymers made up of just one type of monomer, while copolymers are made up of multiple types of monomers [2].

The glass transition temperature is the most significant characteristic of polymers (Tg). A specific melting point cannot be given since polymers have enormous molecular weights and are made up of chains with various molecular weights. It is possible to

determine the regions and use characteristics of polymers using the glass transition temperature. Below the glass transition temperature, polymers are brittle and rigid, while beyond it, they are rubbery and soft [3].

There are natural polymers found in nature in addition to synthetic polymers produced in a lab. Starch, cellulose, enzymes, deoxyribonucleic acid (DNA), cotton, wool, natural rubber, and other similarly related biological macromolecules are examples of natural polymers [9]. These organic substances exhibit unique and outstanding qualities because they have large molecular weights. The creation of superior and high-quality synthetic polymers is the result of rapidly evolving technology and intensifying competition. Polymers are frequently used in industry due to their high elasticity, viscosity, heat and corrosion resistance, and ease of formability [3].

Molecularly imprinted polymers (MIPs), biological fluids, and environmental samples are examples of complex samples that can be distinguished from and analyzed using polymeric matrices produced using imprinting technology. These matrices are strong molecular recognition components that can mimic natural recognition entities such as antibodies and biological receptors. The process of creating synthetic recognition sites in polymeric matrices is known as molecular printing. These artificial recognition sites ought to complement the template in terms of functional group size, form, and spatial organization. The template molecule and functional monomers can interact in covalent, non-covalent, or semi-covalent ways to achieve these spatial configurations. Next, the pre-complex and template molecule are created by copolymerizing crosslinking and functional monomers. The template molecule is eliminated after polymerization, leaving behind complementary gaps that can be realized as synthetic recognition sites [4].

MIPs provide bioanalysis systems with a number of exceptional benefits. They can be manufactured far more quickly and cheaply than enzymes or antibodies, and the synthesis stage doesn't require any biological material. After usage, they are simple to regenerate and exhibit stability in both storage and function. Depending on how well they interact with the target molecule, selectivity and binding strengths can be quite high. The ability of MIPs to change either the entire polymer structure or only its functional regions while utilizing various monomers/functional monomers in various

ratios is another impressive feature [4]. So even when exposed to high pH, organic medium, or autoclave conditions, MIPs can have great stability. They are extensively employed for small compounds with chemical structures despite their limited application in protein/peptide detection due to their superior stability, selectivity, and facile binding capabilities.

Additionally, the narrative that began with the block polymerization of MIPs has evolved into one that focuses mostly on smaller particles. They can be created as micro/nanoparticles with greater specific surfaces, which enhances their binding capabilities. MIPs are employed in a variety of processes, including purification, isolation, biosensors, catalysis, and chiral separation, thanks to all of their benefits [4;5].

Aptamers have recently become the preferred alternative biological identification molecules because of their advantages over antibodies. Single-stranded DNA, RNA, or modified nucleic acids, called aptamers, can be folded into a three-dimensional shape and attached to specific molecules, including cells, proteins, and small compounds [6;7]. A procedure known as the in vitro selection process (SELEX) can be used to rapidly obtain aptamers from a library (1012-16 units) containing a substantial amount of random nucleotide chains [7]. Aptamers have several important benefits over antibodies. 1) Aptamers, although much smaller than antibodies, can bind to detection surfaces to form higher density layers; 2) Aptamers facilitate molecules with different functional groups (such as various fluorescent and redox compounds, biotin or thiol molecules) to replace them; 3) aptamers are also more stable than antibodies under harsh operating conditions (such as high pH, high temperature, samples containing a wide variety of molecules), which extends their shelf life. In addition, aptamers can be produced from proteins, peptides, cells, bacteria or viruses, drugs, carbohydrates and other aptamers selected in vitro for any target molecule such as small organic and inorganic compounds [6;8;9].

It is possible to develop a better biosensor system by utilizing both molecules by combining the MIP and aptamer principles in a biosensor system and applying them for peptide-based biomarker detection. In our proposed effort, similar systems that have been examined in a relatively small number in the literature will be tested by

pairing various monomers and various peptide molecules, and the most suited system will be selected.

1.1 Aim of the Thesis

In the molecularly imprinted polymer-based biosensor system, which was first developed using different polymers, the detection of the peptide biomarker was aimed to be a highly sensitive and selective sensor for the target molecule. The MIP system, which gave the best results by comparing the effects of the monomers used in the structure of the MIP-based biosensor system developed later, such as polymerization, binding and selectivity sensitivity for the target molecule on the electrode surface used, was used for another sensor development: a biosensor system that was two times more sensitive and developed as a dual hybrid. It was aimed to increase and improve the binding capacity and selectivity properties of the system as a dual hybrid by using MWCNTs and aptamers.

Development of MWCNTs/Aptamer/MIP based biosensor system; In addition to the limited number of studies for thrombin peptide biomarker in the literature or to be detected by different methods, it was aimed to develop a more selective and sensitive sensor system by electrochemical methods. It is aimed that this biosensor system, which was developed using electrochemical methods, contributes to the literature in terms of selectivity, rapid detection, easy use and reusability for thrombin peptide biomarker detection of MWCNTs/Aptamers with different methods and molecularly imprinted polymers.

CHAPTER 2

LITERATURE REVIEW

2.1 Introduction to Polymers

Monomers, single molecules joined by covalent bonds, create polymers, which have a significant molecular weight. Polymerization reactions are those in which monomers combine with chemical bonds to generate polymer molecules. The polymerization degree is the number of repeating units in a polymer molecule (P_n). One of the characteristics that determines the properties of polymers is polymerization degree. Polymers can be made up of a single type of monomer or several types of monomers. Homopolymers are polymers made up of only one type of monomer, while copolymers are polymers made up of more than one type of monomer [10].

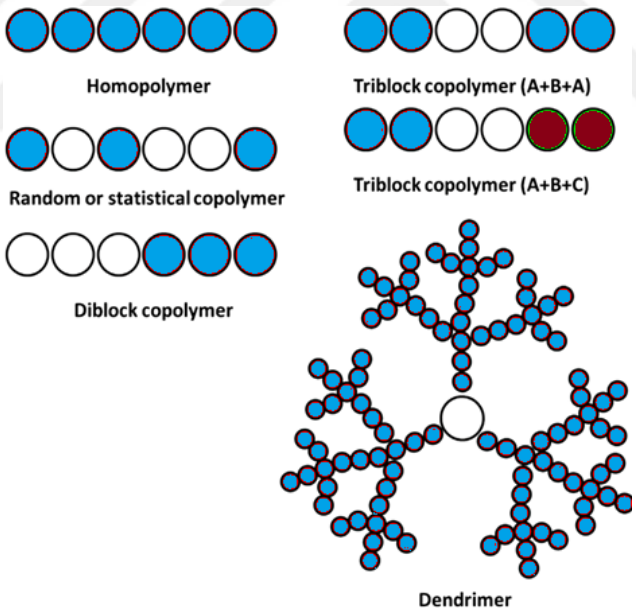


Figure 2.1 A linear homopolymer, a diblock, an A+ B+A and A+B+ C triblock copolymer, a random or statistical copolymer, and a dendrimer are all examples of block copolymers [11]

2.1.1 Polymer Types

Thermoplastics, thermosets, and elastomers are the three main categories of polymers. The best way to differentiate between these types is by how they behave when heated.

2.1.1.1 Thermoplastics

Amorphous or semi-crystalline structures can be seen in thermoplastics. Long-chain molecules exist in thermoplastics in linear bonding, but they are held together by secondary Van Der Waals forces (secondary bonds). A form of plastic polymer material known as a thermoplastic solidifies after cooling and is moldable only at specific high temperatures. Thermoplastics have a significantly lower degree of elasticity than elastomers and are readily irreversibly deformed [12].

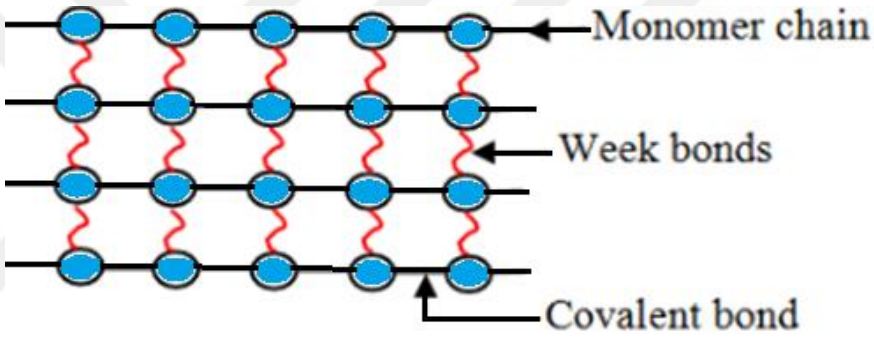


Figure 2.2 Molecular structure of thermoplastics black bonds are Linear Bonding and red bonds are Van Der Waals Forces

2.1.1.2 Thermosets

A thermosetting polymer is a polymer that is formed by permanently hardening ("curing") a soft solid or viscous liquid prepolymer, also known as a thermoset in materials science.Curing is the process of forming these cross-links. Because cross-linking locks the molecular chains in place, a thermoset plastic cannot be remelted and instead decomposes when heated over the Tg. The thermoset polymers are solely in the amorphous form because crosslinking prevents the molecular arrangement with a controlled crystal structure.

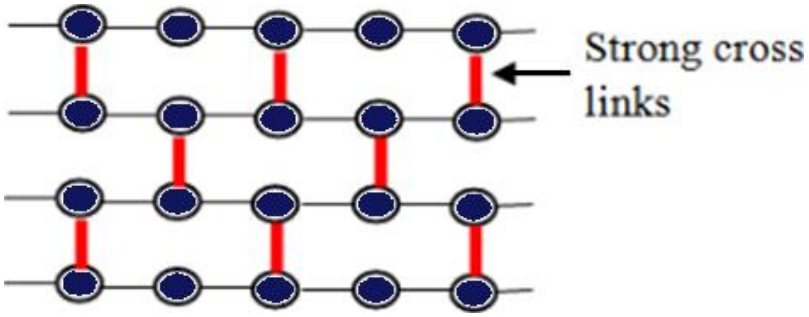


Figure 2.3 Molecular structure of thermosets

2.1.1.3 Elastomers

Elastomers are a type of flexible polymeric materials. Elastomers can elongate to great lengths because of their low intermolecular forces. Some crystalline areas inside the elastomeric structure appear when elastomers are stretched. After the imposed stress—dynamic or static—is removed, the cross-links within the elastomers provide for the ability to restore the original form [12].

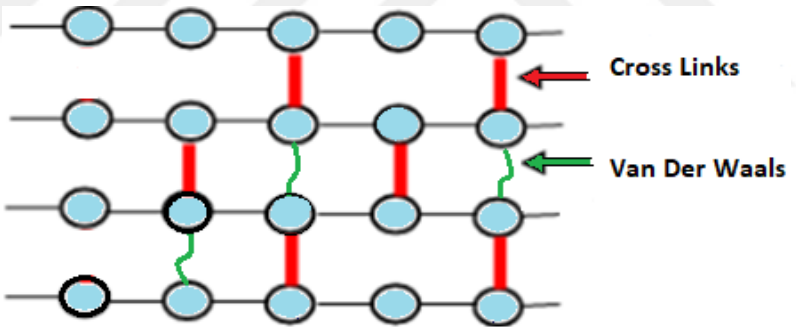


Figure 2.4 Structure of elastomers

2.1.2 Basic Properties of Polymers

High strength or modulus to weight ratios (lightweight yet relatively stiff and strong), toughness, resilience, resistance to corrosion, lack of conductivity (heat and electrical), color, transparency, processing, and low cost are a few advantageous characteristics of various engineering polymers [12].

There are natural polymers in nature, in addition to synthetic polymers created in a lab. Starch, cellulose, enzymes, deoxyribonucleic acid (DNA), cotton, wool, natural

rubber, and other biologically derived macromolecules are examples of natural polymers. These natural chemicals have different and superior properties due to their large molecular weights. Rapid technological advancements and increased competition have resulted in the development of more superior and certified synthetic polymers. Polymers are frequently utilized in İndustry because of properties such as strong elasticity, viscosity, heat and corrosion resistance, and ease of shape [13].

2.1.3 Polymerization Techniques in Biosensor Systems

Polymers have been utilized since the beginning of humanity, yet the term "polymer" was not yet used. Polymers became commonplace after the 19th century. Polymers, particularly synthetic polymers, are also ubiquitous nowadays [14]. Polymers are utilized to enhance selectivity, ease of operation, and high affinity in biosensor technology. Various polymerization processes have been explored in recent studies for biosensors, particularly for molecularly imprinted polymers. Bulk polymerization, electropolymerization, and precipitation polymerization are some of the most commonly utilized processes. For biosensors to be reused, material recovery is critical. [15] Bulk polymerization has a high rate of recovery. The electrical polymerization process also allows for precise control of layer thickness and shape. Like paper-based biosensors, these two features have an impact on biosensor performance [16]. Different types of polymerization techniques are used for different biosensor applications, and these different types of polymerization techniques may affect the selectivity, stability, affinity, reusability, and cost of molecularly imprinted polymer-based biosensors, which is another important parameter. Bulk, Electro, Emulsion, Precipitation, and Free Radical Polymerization techniques are discussed in this section.

2.1.3.1 Bulk Polymerization

Bulk polymerization is a solvent-free technique that does not require the use of dispersants. Among various polymerization procedures, the procedure is straightforward and widely used. Bulk polymerization is favored, especially for MIP synthesis. Operating expenses are inexpensive, and because there is no solvent, environmental problems like pollution and emission are minimized [17]. Monomers,

initiators, and chain transfer agents are all included in the reaction mixture in bulk polymerization [18]. The molecular weight is controlled by the chain transfer agent. Heat must be used in order to complete the polymerization process. The mixture is heated while being stirred regularly. When the reaction starts, the heating process is also stopped, and eventually, viscosity increases as a result of polymerization. Therefore the agitation rate must be constantly monitored [15].

2.1.3.2 Electropolymerization

Electropolymerization is a polymer fabrication technology based on electrosynthesis. It is a process of deposition. As a result, on an electrode, a molecularly imprinted polymer layer or a coated polymer can form [16]. The principle is similar to that of metal coating. A solution employs three electrode systems (reference electrode, working electrode, and counter electrode). Monomers, solvents, and electrolytes are all present in this solution. The system is subjected to potential and current, which must be properly managed. The appropriate potential value is required for the oxidation of monomers, and a high potential results in metal dissolution, which is unfavorable for the polymerization process. The polymerization of monomers occurs after the oxidation process. An electrochemical cell is used to carry out the procedure. This approach comes in a variety of forms.

The current is kept constant in galvanostatic electropolymerization, and polymerization happens at the same pace. A cyclic and regular potential change is used to stabilize the growth of a polymer film in potentiodynamic electropolymerization. The potential is constant in potentiostatic polymerization. The polymerization rate is controlled by this constant potential. When tiny areas, like biosensors, are involved, electropolymerization is used. The final product is determined by the voltage, current, solvent, electrolyte, and monomer type used. It is a suitable method for molecularly imprinted biosensors when a polymer layer is needed for the application [19].

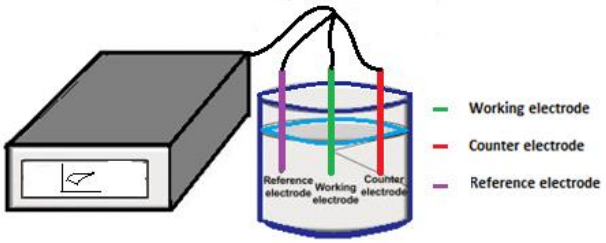


Figure 2.5 Electropolymerization; three electrode system [20]

2.1.3.3 Precipitation Polymerization

Precipitation polymerization is a straightforward procedure. The polymerization reactions do not have any stabilizers. This method produces uniform, homogeneous particle sizes. Forming oligomers from monomers is the initial stage. The oligomers are then cross-linked. Particles begin to develop after these processes. From the solution, new polymers are precipitated. Precipitation speed, monomer, and cross-linker selection must all be regulated to get uniform and homogeneous particles. The ultimate application's performance is determined by particle homogeneity. Precipitation polymerization is a good option for applications that need precise and homogeneous particle sizes, such as microspheres and clean polymers [21].

2.1.3.4 Emulsion Polymerization

Emulsion polymerization is a good way to get a lot of surface area out of a small amount of material. Emulsion polymerization can be used to create 2D layers. An organic phase with functional monomer, cross-linking agent, surfactant, and the initiator is created, as well as an aqueous phase. The organic phase is ultrasonicated. Both the organic and aqueous phases are combined. A homogenizer agitates the mixture after it has been mixed. Polymerization occurs as a result of agitation. Finally, the polymer is obtained when the cleaning process is completed. High molecular weight polymers can be generated in a short amount of time. The polymer is simple to clean [22]. If reproducibility is important in biosensor applications, emulsion polymerization is the way to go. The elution of molecularly imprinted polymers is simple, and biosensors can be reused multiple times [23].

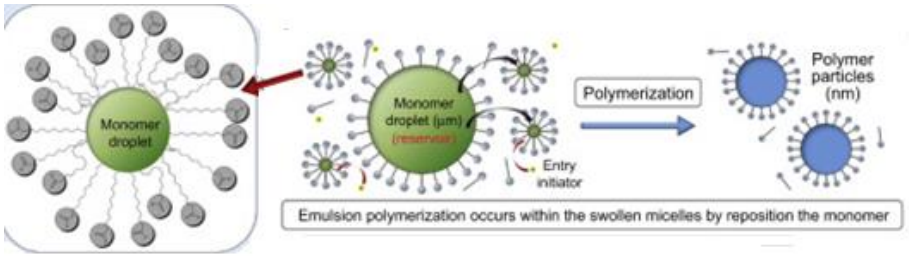


Figure 2.6 The radical initiators and their entry mechanism are depicted in this diagram of a classic emulsion polymerization process. Diffusion of monomers into micelles and the micellar structure are depicted as a self-organized process containing monomer droplets (head as the hydrophilic part and tail as the hydrophobic part) with a surfactant concentration greater than the critical micelle concentration [24]

2.1.3.5 Free Radical Polymerization

Hydrogels are often made by free radical polymerization. Free radical polymerization provides ideal operating conditions and properties for gel applications, particularly in biotechnology. Polymerization takes place quickly. Polymerization is a multi-step process. 1) Initiation, 2) Propagation, and 3) Termination are the three stages. Initiators react with monomers to produce free radicals in the initiation stage. Redox agents, thermal, or visible lights can all be used as initiators. During the propagation phase, newly formed free radicals interact with monomers. Polymer chains begin to form as a result of propagation. Chains are bound together in the termination stage. The radicals are removed from the chains, which are then joined together via covalent bonds. The polymer is then created [25].

2.1.4 Conductive Polymers

An electric current result of the orderly movement of charges in a material as a response of forces that act on them, when a voltage is applied. Negative charges move in the inverse direction from positive charges, which flow in the direction of the applied electric field. Most materials produce a current as a result of an electron flux, or electric conduction.

Conjugated chains, or an alternation of single and double bonds between the atoms, make up the structure of these materials. Due to these conjugated links, doping

conductive polymers becomes simpler. The polymeric chain develops flaws and deformations throughout this process. Polarons are a type of electron-deformation pair or electron-phonon cloud pair that are important for the conductivity of polymers. Other types of quasi-particles, such as bipolarons and solitons, also take part in the conductivity mechanism. The dopant employed determines the kind of soliton, bipolaron, or polaron that is generated. Their significance and the underlying physics are outside the scope of this discussion.

Due to its conductivity properties as a metal, while retaining its relatively inert and high mechanical capabilities, this type of material has generated interest in many fields of study. These materials have a wide range of uses in electronics, including batteries, sensors, and microelectronic devices. The conductive polymers can be employed in the medical industry to create biosensors, controlled-release medications, and artificial muscles. [26]

As was mentioned, conductive polymers are widely used, and this has greatly increased the demand for them as biosensor materials. Sensors utilizing these polymers and MIPs are possible. The use of MIPs in conjunction with conductive polymers is one way to overcome this connection (CPs). While CPs offer great sensitivity, strong adhesion, real-time detection, and direct electrical contact between analytes and electrodes, MIPs have high specificity towards a desired molecular target, good stability, and durability under challenging circumstances [27]. Direct deposition of molecularly imprinted conductive polymers on electrode surfaces is possible through chemical or electrochemical polymerization. The latter technique offers good film thickness control and reproducibility [28]. It is usually possible to establish a connection between the concentration of a molecule and a measured electrical property because the electrical behavior of molecularly imprinted conductive polymers changes upon attaching the molecule of interest. To make conductometric, voltammetric/amperometric, potentiometric, and impedimetric sensors, respectively, one can choose between conductivity, current, potential, and capacitance. Potentiometric measurements have been used to identify various chemical compounds, such as melamine in milk, utilizing sensors based on molecular imprinting conductive polymers [29]. By measuring capacitance using electrochemical impedance

spectroscopy (EIS), theophylline and the gp51 protein were identified [30;31]. In addition, amperometric sensors are particularly successful in detecting electroactive molecules [32;33].

2.1.5 Usage Areas of Polymers

Information technology, medicine, nourishment, communications, transportation, irrigation, containers, clothing, registration histories, buildings, freeways, and other areas directly responsible for life itself are all heavily reliant on natural and manufactured polymers. Without synthetic and natural polymers, human society's imagination is limited. Polymers are typically made at a low cost. Because of their light and adaptable construction, they can be used in a variety of industries. From the space sector to the aircraft industry, polymers are gradually replacing metals in numerous high-tech fields [34].

2.2 Biosensor Systems

While clinical electrochemistry can be used to make sensors that just detect anions and cations, biomaterial can be added to the system to detect a variety of other organic and biological molecules. As a result, the prepared analytical systems are referred to as biosensors [35]. Biosensors are analytical instruments that contain biological diagnostic material and use a physicochemical converter to convert the product generated by the interaction of the analyte and the biological diagnostic material into a quantifiable signal [36]. Enzyme immobilization at electrochemical detectors was first described by Leland C. Clark Jr. in 1962. Enzyme electrodes are obtained as a result of this research, and these enzyme electrodes extend the range of the analyte-based sensor. These concepts are regarded as the forerunners of the contemporary biosensor notion [37]. Analytical gadgets that turn a biological response into an electrical signal are known as biosensors. Biosensors must, in essence, be very specific and reusable, regardless of physical conditions like pH and temperature. According to their mechanism, biosensor materials are categorized into three groups: biocatalytic, which includes enzymes, bioaffinity, which includes antibodies and nucleic acids, and microbe-based, which includes microorganisms [38].

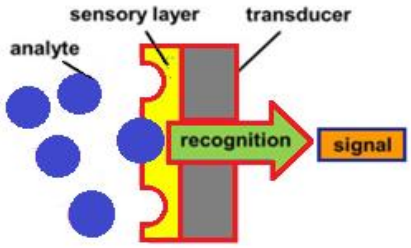


Figure 2.7 Simple Biosensor system

2.2.1 Working Principle of Biosensor

Three crucial components make up the biosensor system. These are the biomolecule with the selective recognition mechanism, as well as the "converter" and "electronic" parts that convert the physicochemical signals created by this biomolecule's interaction with the substance under inquiry into electronic signals. The most essential of these elements are highly selective yet reversibly sensitive biomolecules against the chemical under investigation. Enzymes, microbes, organelles, tissue sections, antibodies, nucleic acids, and chemical receptors embedded in biological membranes can all be employed as biomolecules in biosensors. Receptors are biomolecules that play a role in the structure of biosensors. The substance to be studied is transformed by bioreceptors, and the signal converter detects the changes that occur as a result of this transformation. Because of their high specificity, enzymes are the most often employed bioreceptors. When an appropriate enzyme cannot be discovered or the enzyme is unstable, cell systems or microorganisms are used to identify several compounds. The biomolecule should have a physicochemical effect in the presence of the drug under inquiry, which can be measured by a transducer [39].

Transducers are devices that transform chemical energy into electrical energy so they can detect electrical signals, or they can use colorimetry to detect different analytes' wavelengths. They transform bioactivity into a measurable signal. Electric potential, electric current, intensity, mass, temperature, and other types of transducers exist. Transducers and amplifiers can be used together to boost and clarify the signal [40].

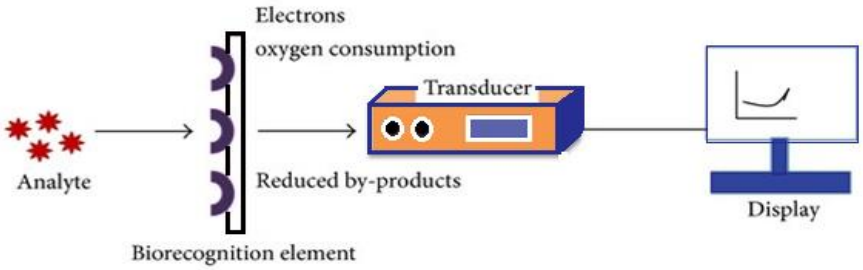


Figure 2.8 Working principle of biosensors

2.2.2 Application Areas of Biosensors

Biosensors are widely used in health, agriculture, food, pharmacy, environmental pollution, military, and a wide range of industrial applications, particularly in automation, quality control, and energy storage. Organic substances such as meals, metabolites, antibiotics, medicines, some inorganic compounds, enzymes, viruses, and microbes can all be detected with biosensors. The biomedical sector is without a doubt the best market for biosensors. The first biosensors to find use in this industry are enzyme sensors. The glucose oxidase electrode was the first commercially available biosensor, and it allows for glucose measurement in blood and urine to detect diabetes [41].

2.3 Polymers in Biosensors

Polymers are widely used in energy applications, everyday life, and medicine. Not only are new conductive polymers employed in biosensor technologies, but they are also used in lithium batteries. New polymeric materials are used in new medication delivery methods. Polymers, on the other hand, have become inseparable from biosensor systems. Because of their properties such as flexibility, ease of use and low cost polymers are being used as a major material in biosensor systems [42].

The term "molecular imprinted polymer" (MIP) refers to polymeric substances exhibiting antibody-like recognition characteristics. A molecularly imprinted polymer in terms of a specific target molecule is created via the molecular imprinting technique. A specific "Template" molecule imprinted on a polymer matrix. A template molecule is frequently used to start the polymerization of the monomers, and it is afterwards

removed, leaving behind complementary vacancies. These polymers are utilized in applications like chemical separations, catalysis, or molecular sensors because they are near to the original molecule. Similar to a molecular lock and key, molecularly imprinted compounds are described (the so-called template molecule) [43].

The use of Molecular Imprinted Polymers (MIPs) for the separation and purification of certain biological or chemical components, such as amino acids and proteins, nucleotide derivatives, contaminants, medicines, and food products, is widespread. Applications include drug delivery, biological antibodies, receptor systems, chemical sensors, separation and purification, and frequently biosensor systems [44;45].

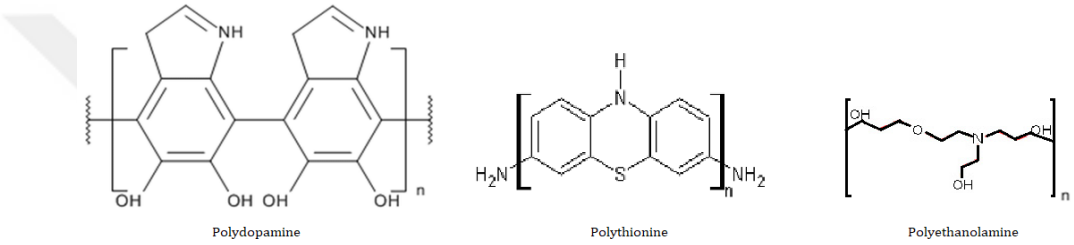


Figure 2.9 Polymeric structures of used polymers in developed MIP based sensor system

2.4 Molecularly Imprinted Polymers (MIPs)

Molecularly Imprinted Polymers (MIPs) are robust molecular recognition components capable of mimicking natural recognition entities such as antibodies and biological receptors that are useful for distinguishing and analyzing complex samples such as biological fluids and environmental samples obtained using imprinting technology. The process of molecular printing is utilized to construct artificial recognition sites in polymeric matrices. In terms of size, form, and spatial arrangement of functional groups, these synthetic recognition sites should be complementary to the template. Covalent, non-covalent, and semi-covalent interactions can be used to achieve these spatial configurations between the template molecule and functional monomers. The cross-linking monomers and functional monomers that constitute the pre-complex and the template molecule are then co-polymerized. The template molecule is removed after polymerization, leaving complementary gaps that can be used as false recognition sites [46].

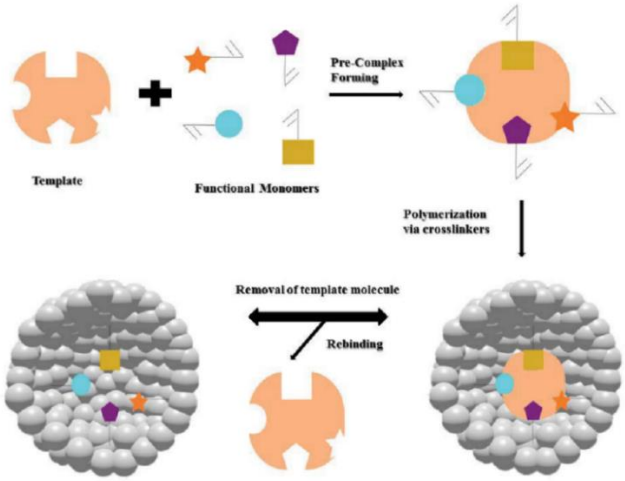


Figure 2.10 Schematic representation of MIP synthesis: a) forming pre-complex with template and functional monomers; b) polymerization around pre-complex via cross-linkers c) removing the template from the polymer and rebinding of the template [46]

2.4.1 History of Molecular Imprinting

Modern theories of molecular imprinting started to emerge in 1894, when Fischer's renowned "Key-Lock" model for the first time revealed the interaction between the enzyme and substrate. In the future, in 1931, Polyakov produced a rigid matrix by drying the gel-like silica polymer using an acidic sodium silicate solution [47]. In his later work, Polyakov investigated selective molecular recognition and asserted that selectivity results in structural modifications because of the chemical make up of the additive.

Synthetic organic polymers, the first examples of molecular imprinting, were independently presented in 1972 by Takagashi, Klotz, Wulff, and Sarhan. Wulff was the one who originally suggested using covalent imprinting [48;49]. Mosbach later devised a non-covalent suppression technique based on secondary contacts that is simpler than covalent suppression [50]. S. Hjerten and J.L. Positive findings were observed when myoglobin-suppressed polymers were used as column filler in protein adsorption in the study by Liao et al [51]. MIPs, a model lectin in Concanavalin A (Con A) adsorption, uses glycoproteins produced by Nagahori and S. Nishimura as monomers [52]. Methacrylate-based MIPs were created using amino acid derivatives in a 1990 study by K. Mosbach et al., and successful results for their application in

HPLC were attained [53]. Figure 2.11 depicts studies on molecular imprinting conducted over the years [54].

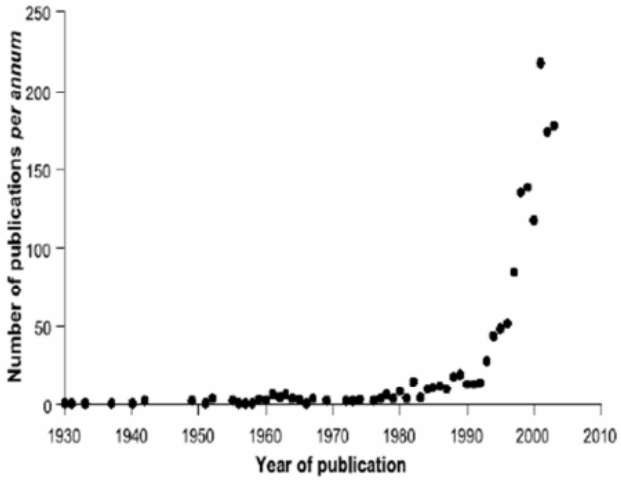


Figure 2.11 The annual number of papers published in the field of molecular imprinting research and technology from 1931 to 2011 [54]

2.4.2 Basic Principles in Molecular Imprinting

In summary, molecular imprinting is the process of generating polymers with centers that have properties that recognize template molecules. For synthesis, there are two main prerequisites: 1) The template molecule, or the molecule to be imprinted; and 2) a functional monomer that can interact with the template molecule through covalent or non-covalent interaction. There are supplementary requirements as well as these fundamental ones, like solvent and cross-linker.

Three phases make up the molecular imprinting procedure, as shown in Figure 2.10. The functional monomer(s) (a) and the imprintable molecule (c) combine to form a complex in the first stage. This intricate structure is polymerized over the useful monomer in the subsequent step (b). Following polymerization, washing is used to remove the imprinted molecule from the polymeric structure. As a result, binding sites are imprinted on a polymer. Now, this polymer has a high affinity for the template molecule and selectivity for it. For instance, when a mixture containing a template molecule interacts with an imprinted polymer, the template molecule will only be recognized and bound to the imprinted polymer due to the binding sites it has. As a

result, the template molecule will be purified by being removed from the mixture medium.

The functional monomer and template molecule can be joined covalently or through noncovalent interactions in the first step. The molecular imprinting process can be divided into three categories in this regard: covalent, non-covalent, and semi-covalent molecular imprinting, which combines the benefits of the two.

2.4.2.1 Covalent Imprinting

Covalent bonds are used in covalent imprinting to connect the functional monomer with the template molecule before the polymerization process. Covalent bonds are broken and removed from the polymer after the polymerization process in order to create a mold. When the template molecule interacts with the imprinted polymers, the same covalent link is recreated [55;56].

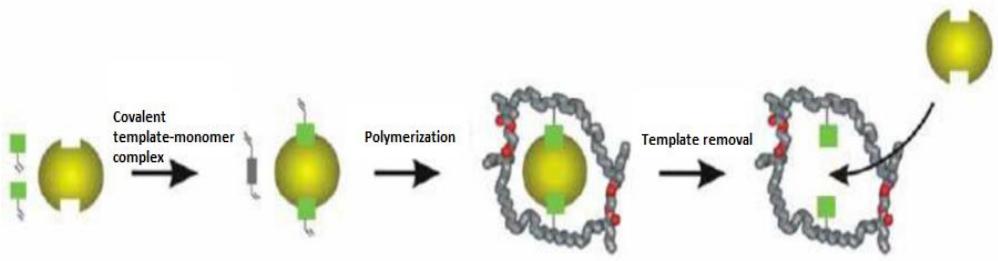


Figure 2.12 Schematic representation of covalent imprinting

2.4.2.1.1 Advantages of Covalent Imprinting

The monomer-pattern molecule complex in covalent imprinting is extremely stable, occurs in stoichiometric ratios, and results in a homogenous distribution. Due to the conjugates' covalent bonding and great stability, polymerization conditions can be applied for high temperature, high or low pH, and polar solvents [57].

2.4.2.1.2 Disadvantages of Covalent Imprinting

Due to the several drawbacks of covalent imprinting, the application of this method is more constrained. The number of times the template molecule can attach to the

polymer reversibly is constrained [58;59]. Additionally, because the covalent bond formation between the template and the polymer usually occurs in a slow step during the pattern recognition step using the polymer, and because the polymer stationary phase and the analyte interact quickly during chromatographic separations, poor chromatographic results are obtained. Covalent bond formation is the cause of the slow binding kinetics [60;61].

2.4.2.2 Non-Covalent Imprinting

Noncovalent interactions are used in non-covalent imprinting to bind the functional monomer to the template molecule (such as hydrogen bonding, electrostatic interactions, and coordination bond formation). Following polymerization, the template molecule is appropriately extracted from the polymer using solvents. Non-covalent interactions link template molecules and imprinted polymers [62;63].

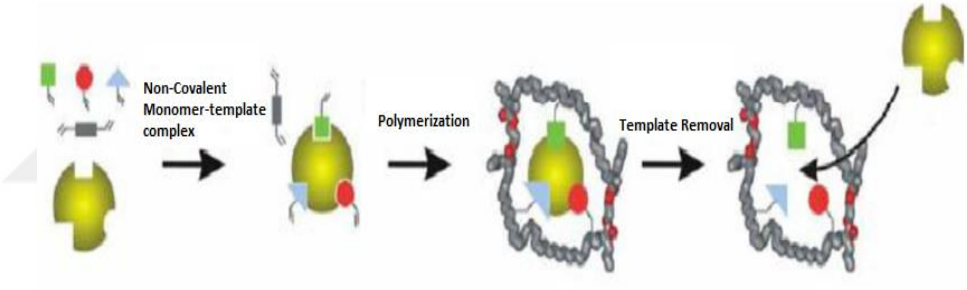


Figure 2.13 Schematic representation of non-covalent imprinting

2.4.2.2.1 Advantages of Non-Covalent Imprinting

Today, non-covalent imprinting is preferred by many scientists when creating MIP [59;66]. The non-covalent method allows for the non-covalent placement of functional monomers around the template molecule prior to polymerization, making it straightforward and unproblematic. The covalent polymeric conjugate doesn't need to be made; instead, the template monomer complex can be formed through a variety of binding interactions. When compared to covalent binding, the kinetics of non-covalent binding are similar to that of enzyme-substrate binding [59]. Because non-covalent interactions are weaker after polymerization, the template molecule can be easily taken out of the polymer. It takes only a little time for the template molecule to bind. It is

possible to create imprinted polymers without having a thorough understanding of the structure and reactivity of the template molecule.

2.4.2.2.2 Disadvantages of Non-Covalent Imprinting

Non-covalent imprinting restricts polymerization conditions to boost non-covalent interactions. In an effort to boost bond formation stability, functional monomers are frequently utilized, which could lead to the development of non-specific binding sites. In non-covalent polymers, the distribution of binding sites is heterogeneous. As a result, weak molecules may be recognized by the template molecule and non-specific binding may result [67;68]. Peak widening may occur if the synthesized polymers are employed in chromatographic experiments [69;70]. Both approaches offer advantages over one another, as can be shown. The kind and structure of the template molecule, the level of selectivity, the amount of time required, and the cost all affect the method choice.

2.4.2.3 Semi-Covalent Imprinting

This method combines the advantages of covalent and non-covalent imprinting. The template molecule, which can form covalent or partially covalent bonds, polymerizes by forming non-covalent bonds with the combination of monomers. Hydrolysis removes the template molecule from the surrounding area. The functional regions are evenly distributed throughout the polymer once the template molecule is taken out. The template molecule is backlinked through noncovalent interactions, and the only kinetic barrier is diffusion. Some compounds (sterol acrylate template molecules, for example) are difficult for hydrolysis to remove from the environment. In this instance, more $LiAlH_4$ is introduced into the medium [71;72]. Examples of experiments using this technique include polymers of some phenolic chemicals and testosterone methacrylate imprinted in ester form [73;74;75,57;58].

Intermediary groups that linger between the template molecule and the functional molecule and move away with the molecule can be employed to get around the restrictions of semi-covalent imprinting. These groups guarantee that the monomer template molecule will connect to the polymer during polymerization and remove

steric interference during non-covalent backlinking. The first intermediate group utilized for this is the carbonyl group of the carbonate ester. Examples of molecules that the carbonyl group inhibits include urea, urethane, and cholesterol [76;77]. Studies on propofol and nandrolone polymers have also recently been conducted [78;79]. The intermediate group might also be salicylate (2-hydroxybenzoate). The intramolecular hydrogen link between the amide hydrogen and the ester oxygen boosts the bonding effectiveness in salicylate-mediated repression, making the removal of the phenyl methacrylate ester easier than with other groups [80]. Aqueous suspension polymerization and emulsion polymerization both employ semi-covalent imprinting.

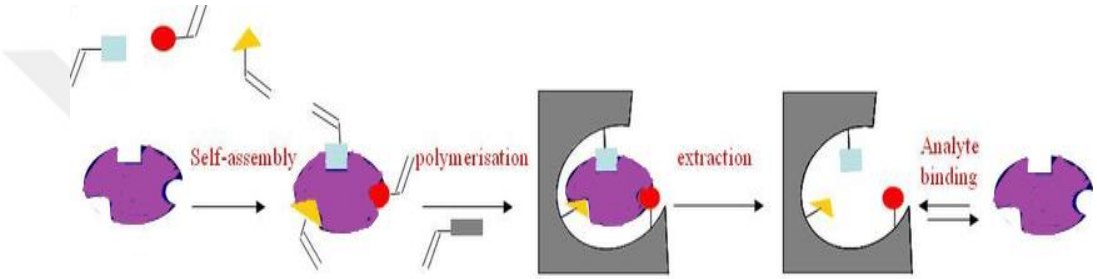


Figure 2.7 Schematic representation of Semi-Covalent Imprinting

2.4.3 Fundamental Components of Molecular Imprinting

The polymerization components, including the template molecule, functional monomer(s), crosslinker, and appropriate solvent, must be identified during the design and preparation of the molecularly imprinted polymer. The most crucial element in ensuring the appropriateness and usability of the synthesized polymer is the selection of appropriate components. The elements' broad properties are outlined below along with their components.

2.4.3.1 Template Molecule

The template molecule is crucial to the success of molecular imprinting in all processes. The attached functional monomers' functional groups communicate with the template molecule. For a variety of reasons, not all template molecules, however, are suited for direct templates. To avoid interfering with the polymerization recipe, the template molecule must be chemically inert under the polymerization circumstances.

In situations when the template molecule goes through different processes or is unstable under polymerization conditions for some reason, different imprinting procedures can be necessary. Because the recognition centers created in this situation won't be as anticipated, molecular imprinting won't achieve the desired results. When looking at a template molecule, the following inquiries ought to be made.

- Does the template molecule include polymerization-capable groups?
- Does the template molecule include any functional groups that will prevent or postpone polymerization, such as thiol groups?
- Can the template molecule maintain its stability under harsh environmental circumstances, such as UV light, or at high temperatures where polymerization occurs? For instance, when AIBN is used as an initiator, the temperature is around 60°C [81].

The size of the template molecule is another drawback. Since very large molecules will be challenging to extract from the cross-links of the polymer, small molecules are often preferred as templates in the molecular imprinting process. However, freshly created techniques can imprint it in huge molecules. As shown in Table 2.1, the suppression process involved the use of a sizable number of molecules, including medications, hormones, proteins, pesticides, amino acids, and carbohydrates.

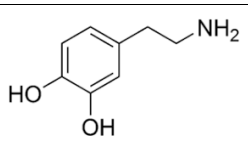
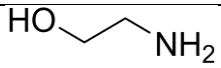
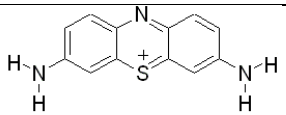
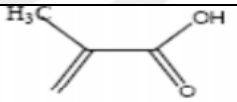
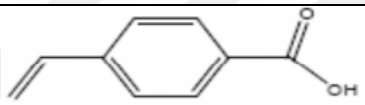
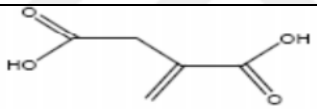
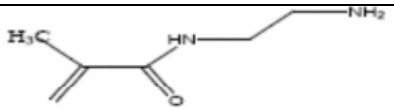
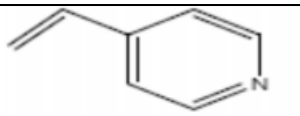
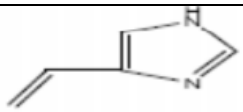
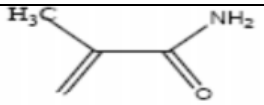
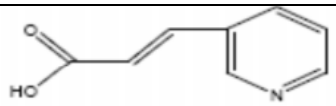
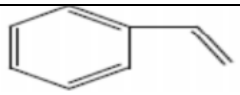
Table 2.1 Major template molecules and their application areas

CLASS	COMPOUND	APPROACH	APPLİCATİON
Medicines	Timolol	Non-Covalent	Chiral separation
	Theophylline	Non-Covalent	Immunoassay
	Diazepam	Non-Covalent	Immunoassay
	Morphine	Non-Covalent	Immunoassay
	Naproxen	Non-Covalent	Chiral separation
	Ephedrine	Non-Covalent	Chiral separation
	Pentamidine	Non-Covalent	Separation
Hormones	Enkephalin	Non-covalent	Immunoassay
Pesticides	Atrazine	Non-covalent	Immunoassay/separation
Proteins	Thrombin	Covalent	Separation
	Transferrin	Non-Covalent	Separation
	RNase A	Non-Covalent	Separation
	Urease		
Amino Acids	Amino Acid Derivatives	Non-Covalent	Chiral Separation
	Dansyl-Phe-OH	Non-Covalent	Sensor
Peptides	Ac-Trp-Phe-Ome	Non-Covalent	Chiral Separation
Peptides	Phe-Gly-anilide	Non-Covalent	Chiral Separation
Carbohydrates	Galactose derivatives	Non-Covalent	Chiral separation
	Glucose derivatives	Non-Covalent	Chiral Separation
	Fructose derivatives	Non-Covalent	Catalyst
Coenzymes	Pyridoxal derivatives	Non-covalent	Separation
Nucleotides	NAD+	Covalent	Separation
Steroids	Cholesterol	Covalent	Separation
Metal ions	Ca(II)	Metal	Sensor
	Cu(II)	Metal	Separation

2.4.3.2 Functional Monomer

The binding interactions at imprinted recognition centers are caused by functional monomers. An excessive amount of functional monomer is utilized in non-covalent bonding techniques to improve the functional monomer's interaction with the template molecule. (The ratio of the template to functional monomer may be as high as 1:4) The compatibility of the template molecule's and the functional monomer's functions is a further significant consideration. This compatibility promotes the complex formation and, thus, the effect of suppression (for instance, hydrogen bond donor, hydrogen bond acceptor). It is important to make sure that the reactivity ratios of the monomers enable copolymerization if more than one monomer is utilized together. Typically, carboxylic acids, sulfonic acids, and heteroarmic bases are utilized as functional monomers in molecular imprinting [82].

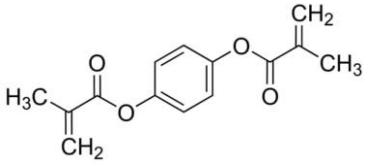
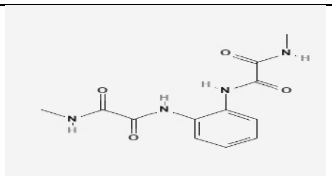
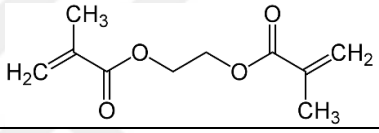
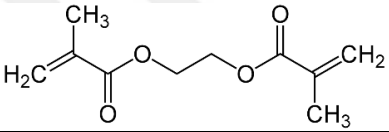
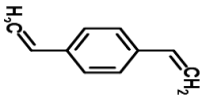
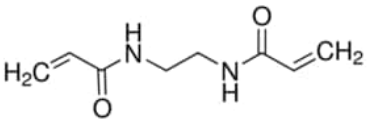
Table 2.2 Major functional monomers used in molecular imprinting

Functional Monomer	Open Structure Formulas of Monomer	Acidic,Basic and Neutral Character
Dopamine (DA)		Basic
Ethanolamine (EA)		Basic
Thionine (TH)		Basic
Methacrylic Acid (MAA)		Acidic
p-vinylbenzoic acid		Acidic
Itaconic Acid		Acidic
N-(2-aminoethyl)-methacrylamide		Basic
4-vinyl pyridine(4-VP)		Basic
4-(5)-vinylimidazole		Basic
Methacrylamide		Neutral
Trans-3-(3-pyridyl)-acrylic acid		Neutral
Styrene		Neutral

2.4.3.3 Crosslinker

In imprinted polymers, the crosslinker serves three basic purposes. The matrix structure of the polymer is controlled by the crosslinker, allowing the polymer to be in gel, macroporous, or microgel powder form. The repressed binding center's stability is the second purpose. The stability of the polymer matrix is ultimately determined by the crosslinker. It is typically chosen in molecular imprinting techniques in order to produce macroporous materials with high mechanical strength and to increase the stability of the recognition centers of imprinted polymers that are heavily crosslinked. As a result, molecularly imprinted polymers often have a crosslinker content of more than 80%. The reactivity ratio of the crosslinker and the reactivity ratio of the monomers must be compatible when more than one monomer is utilized in order to achieve the aforementioned qualities. If not, the crosslinking agent or functional monomer may polymerize more strongly and the copolymerization process may fail [83]. For random copolymerization to be successful, functional ends must form and be distributed uniformly throughout the polymeric structure. Another crucial factor is the crosslinking agent's mole percent to functional monomer ratio. The recognition centers cannot work independently of one another if the ratio is too tiny because they are too close together. A chemical may suppress several nearby binding sites when it binds to a recognition site, for instance. The effect of molecular imprinting may be diminished at excessively high mole ratios because the crosslinking agent may interact in non-covalent interactions with the functional monomers or template molecule [81].

Table 2.3 Major crosslinkers used in molecular imprinting

Crosslinker	Open molecular structure of the Crosslinker
Iso propylene bis (1,4-phenylene) dimethacrylate	
N,N'-1,3-phenylene bis (2-methyl -2-propenamide (PDBMP)	
Ethylene glycol dimethacrylate (EDMA)	
Tetra methylene dimethacrylate (TMDMA)	
p-divinylbenzene (DVB)	
N,N' Ethylene bis methacrylamide	

2.4.3.4 Porogen

A solvent is a crucial agent in getting the template molecule, functional monomers, crosslinker, initiator, and all other components to combine in a single phase. It also performs the pore-formation process, which is essential in the production of macroporous materials. Because of this, the term "porogen" is frequently used in place of "solvent." Solvent molecules are distributed throughout the polymer structure

during polymerization, and as the solvent is withdrawn, pores start to form in the polymer structure. The shape of the polymer and the overall pore volume can be controlled throughout the creation of the macroporous polymeric structure using the characteristics of the porogen. When using solvents with good thermodynamic properties, homogeneous pore formation and high specific surface area are produced, but when using solvents with bad thermodynamic properties, uneven pore formation and low specific surface area are produced. The pore volume grows together with the solvent volume. In order to restore particular binding and remove the template molecule from the structure, the pores generated are remarkably effective. The type of printing affects the solvent choice as well. If they dissolve the components as needed, a variety of solvents can be utilized in covalent imprinting. The interaction between the solvent, template, and functional monomer, however, is crucial to the creation of complexes in non-covalent imprinting methods. It is expected that the solvent will facilitate this formation and hence contribute to the enhancement of the inhibitory effect. Low dielectric constant solvents, such as toluene and dichloromethane, perform well. The recognition capacity will decrease as a result of the use of polar solvents, which will lessen the forces of interaction between the template molecule and the functional monomer. On the other hand, the kind of solvent also affects the average pore diameter of the produced polymer. Acetonitrile, for instance, has a dielectric constant of $\varepsilon=36$ and is a strongly polar solvent. As a result, when acetonitrile is present, more macroporous polymers ($\varepsilon=5$) are produced than chloroform. "Molecular recognition" will decline if surface area and macroporosity are reduced because fewer locations are accessible [84].

2.4.3.5 Temperature

The environment's temperature has an impact on how the mold-monomer complex forms. Numerous research have examined the impact of polymerization temperature on MIP performance [85;86;87]. In contrast to MIPs generated at high temperatures, low temperature MIPs exhibit remarkable selectivity. The process of photochemical polymerization requires a low temperature.

L-phenylalanine Mosbach et al. conducted research on the enantioselectivity of anilide imprinted polymers. The initial temperature is set at 60, 40, and 0 ℃. In this work, it was found that specified polymers prepared thermally had lower selectivity than low-temperature-made polymers [85].

By using chromatographic tests, Piletsky et al. investigated the impact of polymerization temperature on the affinity and specificity of MIP. The MIP was designed through a computational approach. Computer modeling was utilized to develop polymers imprinted on microcystin-LR, a cyanobacterial toxin found in aquatic microorganisms, by consulting a database of the most frequently used functional monomers [88]. At -30, -20, -10, and 80 ℃, (-) ephedrine was reduced using the same monomer combination (MAA/EGDMA). According to experimental findings, MIP components like the polymerization solvent and the template molecule can also be stored depending on variables like temperature. Although low starting temperatures can sometimes help with MIP preparation, the general consensus from observations is that the temperature inside the monolith is higher than the surrounding air. This is because the polymerization process is exothermic. At -10, 0, 20, 30, 40, and 50 ℃, the affinities of the ephedrine enantiomers were investigated. It was shown that when the polymerization temperature dropped, the affinity and specificity of the polymers increased [88].

2.4.4 Creating a Successful Imprinted Polymer

A good MIP is formed as a result of several factors, including:

a) the monomer-to-die ratio
b) the functional monomer's acidic or basic character
c) the porogen (solvent) select
d) the polymerization conditions
e) the conformation of the template molecule and polymer
f) the amount of crosslinker

The functional monomer-pattern molecule ratio is the most important element during polymerization. Only a very little quantity of monomer complexes with the template

molecule and creates appropriate binding sites, whereas unwanted non-specific interactions grow when there is a concentration of monomers in the environment that is higher than the concentration of the template molecule. Trial and error or computer modeling techniques can be used to find the ideal functional monomer template molecule ratio. The capacity factor is only impacted by the polarity of the solvent, the make-up of the mobile phase, temperature, and the removal of the template molecule from the polymer. Stabilizing the template molecule-monomer interaction is another technique for regulating the polymerization temperature. Typically, thermal or photochemical initiators are used at low temperatures to produce well-imprinted polymers. Free radical polymerization happens at low temperatures because it's an exothermic reaction. The polymerization reaction medium's ambient temperature in the studies was 80°C, whereas the reaction medium's temperature was recorded at 187°C [89]. Table 2.4 lists the characteristics that a successful MIP should have. Accordingly, the physical characteristics of the polymer, the binding capabilities of the template molecule, and the synthesis method are the characteristics of a good MIP.

Table 2.4 The features that the ideal MIP should have;

Synthesis Method	• Polymerization in one or a few easy steps, • Dynamic suppression,which reduces the chance of mistakes forming during polymerization, • The potential polymer's capabilities
Physical Properties	• It has a polymer with soluble or insoluble qualities. • High reusability, • Spectral approaches for identifying binding locations, • Presence of homogeneous printed regions with high stability.
Binding Characteristics	• Being able to bind the molecule with as high affinity as possible, • Rebinding of the template molecule as highly selective as possible, • Fast binding kinetics.

2.4.5 Classification of MIP Based Biosensors Based on Their Transducers

Biosensors are divided into two categories: bioactive layers and transducer kinds. Based on biomolecules like enzymes, antibodies, receptors, and nucleic acids, it is possible to characterize them as more stable synthetic structures as well as types of bioactive layers and MIPs (aptamer, DNA, etc.). If we need to categorize it using the most often used transducers [90], we can utilize electrochemical, optical, and mass sensitive transducers [91].

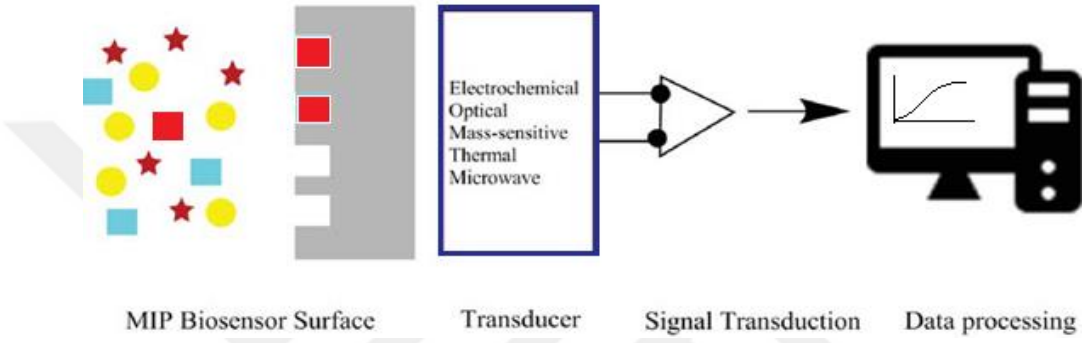


Figure 2.8 Typical biosensor systems with different transducer types

2.4.5.1 Electrochemical MIP Biosensors

Electrochemical biosensors measure the electrical changes in a solution, such as the electric potential or current, that occur as electrons or ions are produced or consumed. When the target molecule chemically interacts with certain MIP cavities, the biosensor's bioactive layer, these changes occur in electrochemical MIP biosensors. An electrochemical sensor is a device that turns an analyte's contact with a receptor on the electrode's surface into a useful analytical signal [92]. Chemical interactions are converted into electrical signals using techniques such as potentiometry, voltammetry, conductometry, and impedimetry. Microparticles and nanoparticles (NPs) or nano-structured coatings are another trend in the development of electrochemical MIP sensors.

By accelerating electrochemical reactions, gold NPs (AuNPs) enhance the electrode surface, improve the electron transfer process between electroactive species, and raise the sensitivity of electrochemical sensors, drawing attention as an appealing technique.

The voltammetric MIP sensor for adenine based on AuNPs covered with 3-thiophene acetic acid, which results in a conductive 3D mesh composite film after polymerization, is another example of nano-engineering (Figure 2.16). With a 0.99 nM LOD (detection limit), this form of sensor construction has improved selectivity and sensitivity for adenine detection [93].

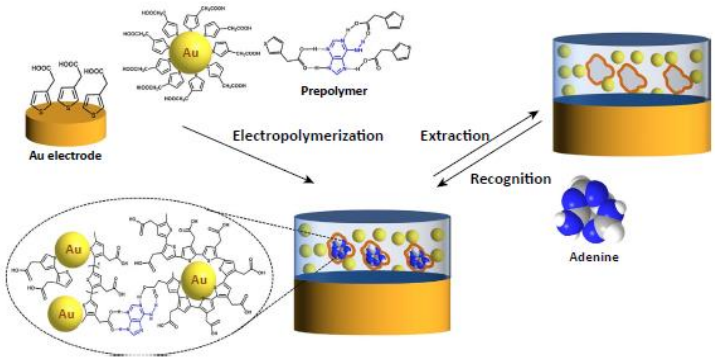


Figure 2.9 Fabrication of a Voltammetric Sensor for Adenine Based on a Molecularly Imprinted Polymer (MIP) Conductive 3D Network Composite [85]

2.4.5.2 Optical MIP Biosensor

Optical sensors convert chemical interactions into measurable signals using light. The analyte can be examined with the biosensor using an optical transducer if the system's optical properties change with the binding of the specific analyte of the MIP, which is the biosensor's bioactive layer. SPR, RLS, fluorescence, luminescence, and Raman spectroscopy are only a few examples of optical-based assay procedures [90].

2.4.5.3 SPR Based MIP Biosensor

An optical method for determining the refractive index changes at a metal surface is called surface plasmon resonance (SPR), and it is used to assess affinity interactions between biomolecules. Due to their distinctive characteristics, such as real-time measurement, high sensitivity, specificity, reproducibility, and no requirement for labeling, SPR-based biosensors have the potential to be used in bioscience, food analysis, and environmental analysis [94]. A very accurate real-time sensing system with quick response times, simplicity of use, online monitoring, and remote sensing is

also offered by optical fiber surface plasmon or localized surface plasmon resonance sensors [95]. Additionally, there are benefits such as simple fabrication, low cost, and stability for molecularly imprinted polymers having selective binding sites for the target molecule in SPR sensors [96].

The SPR chip's mechanism is shown in Figure 2.17 and was created by Shaikh et al. in 2015. They proposed a study for the creation of an SPR sensor using a synthetic poly(ethylene glycol dimethacrylate-N-methacryloyl-L phenylalaninevinyl imidazole) [poly(EGDMA-MAPA-VI)] film imprinted with Bisphenol A (BPA) and radical polymerized under UV light. FTIR-ATR, atomic force microscopy, and ellipsometry were used to characterize the SPR sensor. The SPR sensor was created to find BPA in Milli Q water, tap water, and artificial wastewater. BPA may be detected at concentrations between 0.08 and 10 mg/L. The sensor's LOD and LOQ values for Milli Q water were 0.02 and 0.08 mg/L, tap water was 0.06 and 0.2 mg/L, and synthetic wastewater was 0.08 and 0.3 mg/L. The Langmuir adsorption model can be used with the adsorption system. The molecularly imprinted film favoured BPA over 4-nitrophenol, hydroquinone, phenol, and 8-hydroxy quinoline, with relative selectivity coefficients of 2.5, 2.6, 2.7, and 2.5, respectively, according to investigations on selectivity using competitive detection of these substances.

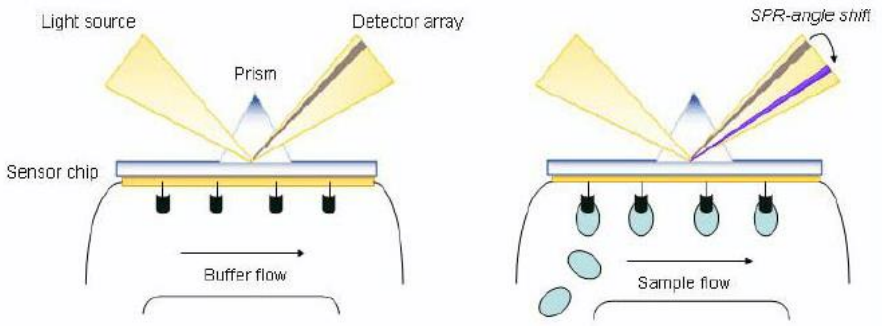


Figure 2.10 Principle of a biosensor using surface plasmon resonance (SPR). The SPR angle shifts as a result of biomolecule attachment to the sensor surface, which raises the refractive index. The mass increase and the shift are inversely related [96]

2.4.5.4 Fluorescent Based MIP Biosensor

The benefits of fluorescence detection are its high sensitivity, ease, and broad applicability range. The benefits of high selectivity MIP recognition and high sensitivity fluorescence detection are combined in MIP-based fluorescence sensors. Direct and indirect applications for MIP-based fluorescence detection are possible. Due to variations in the fluorescence intensity of the MIPs, the analyte is active during direct fluorescence detection to change the signals for quantitative and qualitative analysis. When using non-fluorescent analytes for indirect fluorescence detection, the fluorescent functional monomer's fluorescence effect is measured by observing the shift in the fluorescence spectrum of the mixture of analytes and a fluorescent functional monomer [97].

Mesoporous structured MIPs@CDs fluorescence sensor for very sensitive trinitrotoluene (TNT) detection was studied by Xu et al. TNT is poisonous to all living things and crucial for world safety in particular. Thus, it is very important to detect TNT in a focused and sensitive manner. Mesoporous structured molecularly imprinted polymers with carbon dot caps (M-MIPs@CDs) were created with this objective in mind. Figure 2.18 below shows each stage in detail. Trinitrophenol (TNP) served as a fake pattern for imprinting while amino-CDs were functional monomers. TEM, SEM, and fluorescence measurements were used to export characterization studies. The detection threshold of the M-MIPs@CDs sensor is 17 nM. The sensor remained stable ten times without a drop in yield. With 88.6-95.7% recoveries, the enhanced sensor was realized in soil and water samples [98].

For more effective and quick detection of -cyhalothrin in environmental water, molecularly imprinted fluorescent empty nanoparticles were developed [99]. Insecticide LC-cyhalothrin is poisonous. SiO_2 nanoparticles were produced by a Stöver process for the rapid and effective detection of -cyhalothrin in water samples. After then, fluorescein isothiocyanate was added to them (FITC). Fluorescent empty nanoparticles with LC imprints were created. TEM, SEM, differential scanning calorimetry (DSC), and thermogravimetric analysis (TGA). The range for fluorescence density detection is 0–1000. The fast detection rate was 8 minutes, and the detection limit was 10.26 nM. 8 cycling operations with satisfactory fluorescence characteristics

were achieved. With good recovery, this enhanced sensor was used to identify -cyhalothrin in actual water samples from the Beijing-Hangzhou Grand Canal.

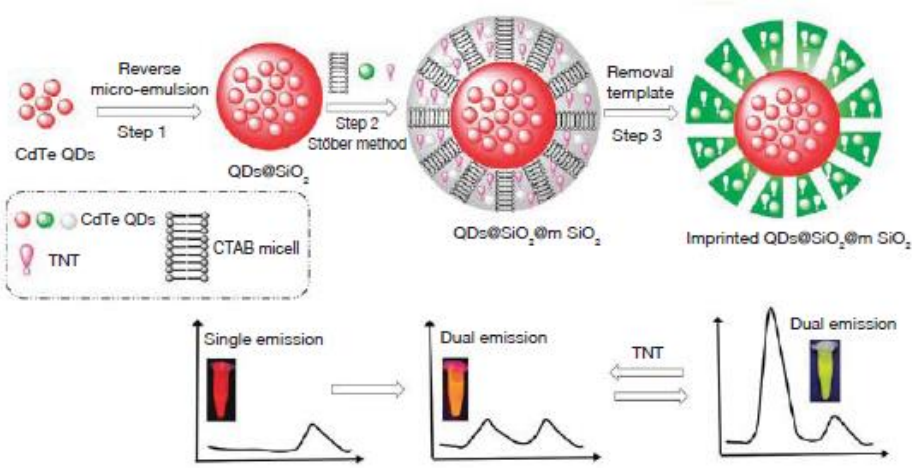


Figure 2.11 Mesoporous structured MIP@CDs ratiometric fluorescent probes fabrication and TNT detecting processes are shown schematically in [99]

2.4.6 Advantages of MIPs

MIPs provide a variety of difficult benefits. They are far simpler to make than enzymes or antibodies, and the manufacturing procedure is quite inexpensive. They exhibit both storage and operational stability, as well as excellent regeneration after use. They exhibit selectivity and binding power in particular. MIPs are referred to as plastic antibodies or plastic bodies since they exhibit exceptional sensitivity and selectivity and perform even better than commercial antibodies. The flexibility to change the entire polymer structure or only the functional sections of MIPs using various monomers and functional monomers in various ratios is another intriguing feature of MIPs. So even when exposed to high pH, organic medium, or autoclave conditions, MIPs can exhibit good stability [100].

Additionally, the history of MIPs, which began with block polymerization, has largely changed to become the history of smaller particles in modern times. With better binding characteristics, they can be created as micro or nanoparticles with higher specific surface areas. With all of their benefits, MIPs are used in several applications in purification, isolation, biosensors, catalysis, and chiral separation [101].

2.4.7 Importance and Usability of MIP Biosensors

The production of MIPs directly on the electrode surface is one typical approach of using MIPs in biosensors, if that is even necessary to mention. In addition to this MIP, micro/nanoparticles can be produced independently and immobilized on the electrode surface via co-polymerization or combining different interactions. For instance, when correctly designed and produced, nano-sized MIPs can compete with antibodies. The quantity of cross-links in the polymer structure plays a key role in this by influencing the MIP's stability. Furthermore, despite the fact that MIP is not a biological component, MIP-based sensors are frequently referred to as "biosensors." The usage of MIPs as solid-phase extraction supports is one of its uses in analytical procedures. Here, the MIPs are exposed to an analyte-containing solution before being washed to produce a concentrated fraction for analysis. Additionally, a variety of MIP biosensors can be created using the surface printing technique [100].

CHAPTER 3

EXPERIMENTAL

3.1 Materials and Methods

3.1.1 Reagents

The chemicals used in the studies are as follows; Monomers (Dopamine, Ethanolamine, Thionine) used in MIP preparation stages, target peptide molecules (Inosine, Glucose, Albumin, Bovine Serum Albumin (BSA) used for the sensor system, and Thrombin peptide molecule that we use as the main target molecule in the sensor system. Carbon nanotubes and aptamer used in the MWCNTs/aptamer/MIP based sensor system prepared by injecting carbon nanotubes onto the electrode surface Phosphate buffered saline (PBS) used in the prepared sensor systems and, ethanol+acetic acid solution used for the elution process of the system, electropolymerization and chemicals such as $[Fe(CN)_6]^{3-/4-}$ solution, which is used as electrolyte in characterization processes such as CV, DPV and EIS were actively used in these studies, and their preparation rates and stages are specified in the preparation sections.

3.1.2 Instruments and Apparatus

Screen printed electrodes (Au SPE) (DRP-210 model) were purchased from DropSens^{DS} (Spain). They have deposited three electrode system with carbon working, Ag/AgCl reference and Au counter electrodes on a durable substrate were used in the electrocehemical works. Electrochemical techniques (Cyclic voltammetry; CV, differential pulse voltammetry; DPV and Electrochemical Impedance Spectroscopy; EIS) were carried out using a CHI potentiostat (CH Instruments, Inc., Austin, USA).

Each step of MIP based electrochemical biosensor preparation were imaged with atomic force microscopy (AFM) using a Bruker Multimode microscope, equipped with the Nanoscope V controller. Phosphorus-doped Si tip and cantilevers of 376 kHz resonant frequency, and k=60 N/m, were used for imagining in Tapping Mode™. For

measurement of the film thickness, some parts of the film were carefully removed with a Teflon spatula, under an optical microscope. Film was scratched at a few places for measurement of average thickness. For roughness measurements four points of the sample were imaged at (5 × 5) μm^2. Then average roughness was calculated from the results obtained for each image. Roughness calculations of single image were performed by using NanoScope Analysis v 1.2 software of Bruker.

3.2 MIP Preparation Using Polydopamine Polymer

0.5 mM and 3 ml of dopamine in PBS solution were prepared for electropolymerization step during the MIP preparation. The target thrombin (Thr) molecule for the study was produced as 2 μg/ml concentration in PBS solution. Pure forms of acetic acid and ethanol were combined at a 50% concentration to create the elution solution. For cyclic voltammetry (CV) and differential pulse voltammetry (DPV) measurements in $[Fe(CN)_6]^{4-/3-}$, the electrode was utilized. $[Fe(CN)_6]^{4-/3-}$ was made by adjusting and preparing ferrate (III) 0.16g + ferrate (II) 0.211 g and filtered water 5ml.

First, 2 μg/ml of thrombin was drip-applied to the carbon screen-printed electrode (SPE), and incubated at room temperature for 45 minutes till drying. Then, electropolymerization was carried out 10 cycle in a 0.5 mM dopamine solution between -0.6 and 1.0 V. Following polymerization, CV and DPV measurements were performed for surface characterization, then elution was conducted for 30 minutes in ethanol + acetic acid. Elution was followed by additional CV and DPV measurements, and data was collected to compare with those made after polymerization. Following the addition of the Thr target molecule to the system in various concentrations (2pg/ml, 10pg/ml, 50pg/ml, 100pg/ml, and 250pg/ml), CV and DPV measurements were made, and the impact of the different concentration of Thr rebinding onto the MIP was observed and visualized using linear calibration curves.

3.3 MIP Preparation Using Polythionine Polymer

For the polythionine tested, a 5 mM of thionine monomer in PBS (phosphate buffered saline) solution was created. In addition to these substances, the target thrombin (Thr)

molecule for the investigation was generated at a concentration of 2 µg/ml. The elution solution was prepared by mixing pure acetic acid and ethanol to a 50% concentration. The electrode was used to measure the cyclic voltammetry (CV) and differential pulse voltammetry (DPV) in $[Fe(CN)_6]^{4-/3-}$. By regulating and combining ferrate (III) 0.16 g, ferrate (II) 0.211 g, and filtered water 5 ml, $[Fe(CN)_6]^{4-/3-}$ was created. The carbon screen-printed electrode (SPE) was first drip-applied with thrombin at a concentration of 2 µg /ml while waited. Then, in a 5 mM thionine solution, 15 cycles of electropolymerization were performed between (-0.4V and 0.4V). After measuring the CV and DPV after polymerization, elution was carried out for 30 minutes in ethanol + acetic acid. Additional CV and DPV measurements were performed after elution, and data was gathered to contrast with results obtained after polymerization. The focused on the targeting Thr was sequentially administered to the system at different concentrations (5 pg/ml, 25 pg/ml, 50 pg/ml, 100 pg/ml and 200 pg/ml), CV and DPV measurements were taken, and the effects of the different concentrations on the system were seen and graphically represented.

3.4 MIP Preparation Using Polyethanolamine Polymer

It was prepared for electropolymerization by dilution with 10 mM Ethanolamine PBS, followed by the addition of 2 µg/ml thrombin and a 45-minute wait before the addition of the produced ethanolamine, which was electropolymerized at 10 cycles (-0.6V and 0.8V). The molecularly imprinted polymer-based biosensor system was produced using these methods and substances. After the ethanolamine monomer needed to produce the MIP system was polymerized, the MIP system was established, and control methods such as CV and DPV were employed. After using acetic acid and ethanol to elute the system, CV DPV operations were repeated. The response of the system to the target molecule was successfully assessed using CV and DPV methods by adding the target molecule to the sensor system at varying concentrations (5pg/ml, 25pg/ml, 50pg/ml, 100pg/ml, and 200pg/ml), and the measurement graphics are shown in the results section.

3.5 Biosensor Development

It was aimed to develop a MIP-based electrochemical sensor with highly selective properties prepared with various monomers given above. In the developed sensor system, MIPs were prepared by electropolymerization method on the system in order to improve both selectivity, fast bonding and reusability by adding the thrombin target molecule to the surface. After these preparatory steps, the system was ready to bind the target molecule, i.e. peptide biomarkers. The binding and rebinding effect of the target molecule was observed by CV, DPV and EIS methods.

To verify the thrombin detection capability of the MIP-based system prepared with different monomers, different thrombin concentrations such as 5 pg/ml, 25 pg/ml, 50 pg/ml, 100 pg/ml and 200 pg/ml were loaded on the electrode surface. Electrochemical signal was recorded by DPV method in solution containing 5.0 mM $[Fe(CN)_6]^{3-/4-}$ and 0.1 M KCl solution. The schematic representation for biosensor system fabrication and thrombin detection is shown below.

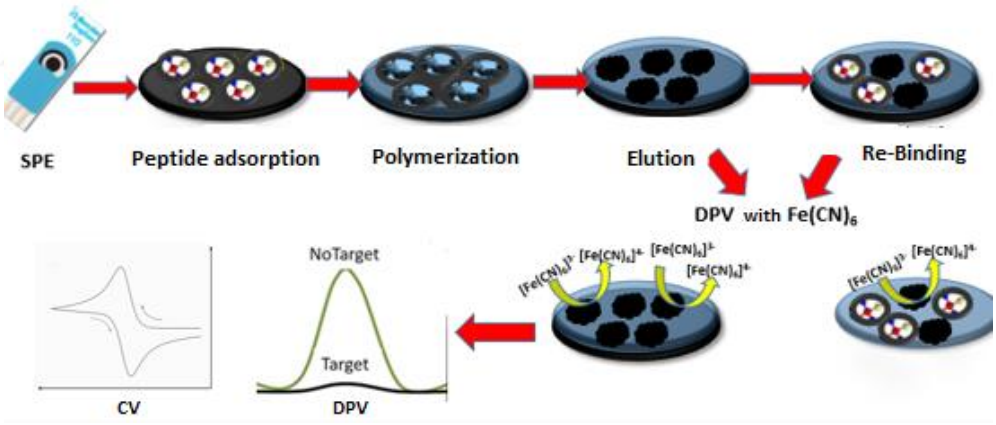


Figure 3.1 Developed MIP based biosensor system; First, peptide adsorption on screen-printed electrodes was performed to increase the properties of the system such as selectivity, then MIP was created by performing electropolymerization process, after the MIP formed, the system was eluted and the target peptide molecule was rebound at different concentrations, and finally the results were observed with DPV and CV processes

3.6 Preparation of Aptamer/MIPs

The preparation process of molecular imprinting membrane was shown in Figure 3.1. Firstly, thrombin aptamers were attached onto the MWCNTs modified electrode surface by EDC/NHS linkers. Carboxylic acid modified CNTs were incubated for 30 minutes at 37°C with solution containing 15 mM EDC and 22.5 mM NHS prepared in 10mM MES buffer (pH 5). At this stage, the surfaces of the CNTs were activated suitable for binding of aptamers modified with amino groups. After the carboxyl group was activated, the electrode was rinsed with PBS solution (pH 7.4). After that, 5 µl of 1 µM thrombin aptamer was injected onto the electrode surface and incubated at 37 ˚C for 3 h. Finally, 2 µg/ml thrombin was added dropwise to the surface of the electrode, and reacted at 37 ˚C for 1 h to obtain the thrombin-bound electrode surface, which was ready to prepare MIP.

Figure 3.2 Thrombin (Thr) aptamer immobilization onto MWCNTs surface by EDC/NHS crosslinkers and Thr binding to the aptamer

The thrombin modified electrode was immersed in 5 mM dopamine solution (DA solution, pH 7.0, containing 0.1 M KH_2PO_4, 0.1 M K_2HPO_4, 0.01 M NaCl and 0.01 M KCl), and the cyclic voltammetry (CV) method (- 0.6 to 0.5 V, 100 mV/s, 20 cycles) was used to polymerize DA on the electrode surface to form polydopamine (PDA) membrane, which was coated on thrombin.

The electrode with PDA membrane was immersed in ethanol/acetic acid (1:1) solution, and the PDA membrane was subjected to template removal of thrombin. Finally, the electrode was placed in PBS solution at pH 7.4 for further use.

3.6.1 Surface Modification with MWCNTs

Drop casting is a quick and low-cost way to make a large surface area of CNT films thickness from a few hundred nanometers to a few micrometers. 10 µg/ml

concentration of MWCNTs were first dissolved in NaCl solution using an ultrasonic stirrer. After using the ultrasonic mixer, the dissolved carbon nanotubes were dropped on the surface of the electrodes, and the CNTs film was made ready for other processes after waiting for a day at room temperature to dry.

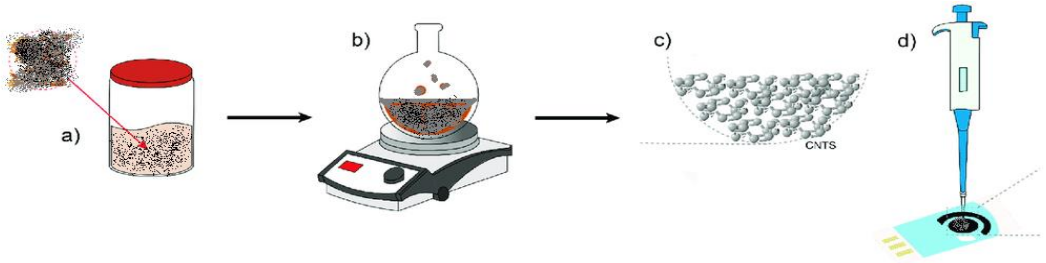


Figure 3.3 Schematic representation of drop casting method for MWCNTs modification onto SPE surface, a) dissolving of MWCNTs in 0.1 M NaCl solution, b) mixing the NaCl solution containing MWCNTs with an ultrasonic mixer, c) dissolved MWCCNTs, d) injecting of MWCNTs to SPE working electrode surface

3.6.2 Biosensor Development with MWCNTs/Aptamer/MIP

We have aimed to develop an electrochemical sensor with highly selective properties based on MWCNTs, aptamer and MIPs. First of all, in the developed sensor system, by injecting the previously prepared carbon nanotubes which were prepared by using drop casting method onto the SPE (Figure 3.3), the detection capacity was increased by increasing the conductivity and surface area. Then, by adding aptamer to the surface, features such as selectivity and binding capacity of the sensor system were improved. In addition, in order to improve both selectivity, fast binding and reusability, MIPs was prepared on the system by electropolymerization method. After these preparation steps, the system was ready to bind the target molecule, i.e. peptide biomarkers. The binding and rebinding action of the target molecule was observed with CV, DPV and EIS methods.

In order to validate the capability of MWCNTs/aptamer/MIP based system for thrombin detection, electrode surface was loaded with different concnetrations of thrombin such as 5pg/ml, 25pg/ml, 50pg/ml, 100pg/ml and 200pg/ml. The electrochemical signal was recorded by DPV method in the solution containing 5.0

mM [Fe $(CN)_6]^{3-/4-}$ and 0.1 M KCl solution. The schematic representation for surface modification, MWCNTs/Aptamer/MIPs biosensor system fabrication and thrombin detection are shown in Figure 3.4.

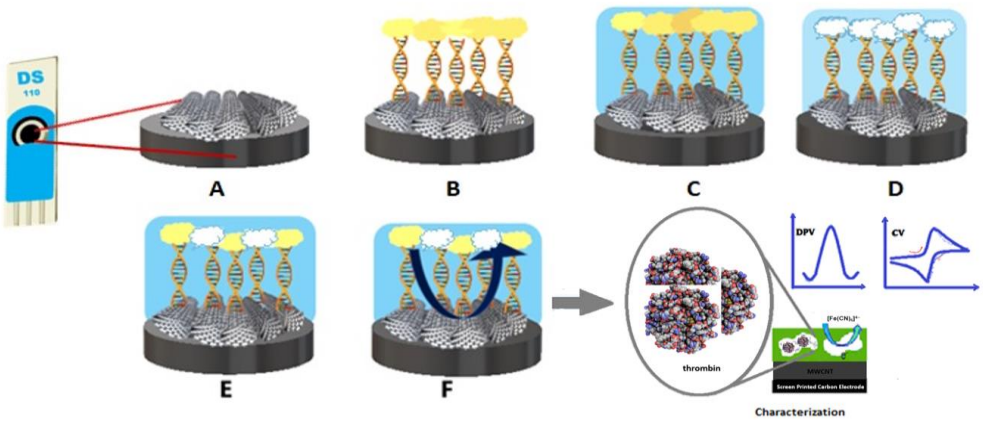


Figure 3.4 Schematic representation of the developed selective and sensitive MWCNTs/MIP/Aptamer based biosensor system, A) addition of MWCNTs by drop casting method on carbon SPE, B) covalent aptamer binding onto the MWCNTs\ and specific tamplate binding onto the aptamers, C) performing dopamine electropolymerization for MIPs formation, D) elution of the template in ethanol+acetic acid solution, E) rebinding of the target molecule, (F) monitoring binding and rebinding of the target molecule by using CV and DPV

Based on the result of DPV measurements for the developed sensor system, $\Delta I/I_0$ values were calculated by measuring the peak heights for the template removed stage and target molecule bounded stage and all graphs were drawn by using these values. While calculating this value;

- I_0: The peak height obtained from the DPV graphs before the addition of thrombin to the electrode (template eluted stage),
- I_1: The peak heights obtained from the DPV measurements taken after the addition of the thrombin target molecule,
- ΔI: $\Delta I=I_0-I_1$, Signal change after thrombin binding onto the MIPs surface,
- $\Delta I/I_0$: The relative signal changes for each concentration of thrombin.

3.7 Biosensor Performance Optimization

The biosensor was employed to determine thrombin by voltammetric measurements (DPV) at the applied potential range of -0.4 V to 0.8 V in the steady-state condition

with different amounts of thrombin (5pg/ml, 25pg/ml, 50pg/ml, 100pg/ml and 200pg/ml) respectively in the PBS (pH 7.5). For selectivity studies, 100 pg/ml of different peptides such as human albumin, BSA, human IgG, and glucose (used as negative control) and were prepared in the PBS solution and tested with developed MWCNTs/Aptamer/MIP biosensor system. The stability of the developed system was also tested by storing prepared and ready to use electrodes at 4 °C for 45 days.

After optimizing the working conditions of the developed biosensor system, to estimate the matrix effect of the real samples on the biosensor performance, we assessed commercial serum samples that spiked with different thrombin concentrations.

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Characterization of Polydopamine Used MIP Based Biosensor System

The electrode surface formed using dopamine monomer and treated with MIP was analyzed by EIS, CV and DPV methods. Step-by-step surface modifications were compared in CV, DPV and EIS experiments using $[Fe(CN)_6]^{4-/3-}$ redox couple. As seen in the Figure 4.1, after the MIPs were formed, both the anodic and cathodic peak currents increased sharply on the electrode surface and the peak heights were higher than the empty electrode. After elution of the template molecule (Thr), the redox peak current increased significantly. The peak-to-peak distinctions between the cathodic and anodic peaks became smaller as seen in Figure 4.2. This behavior was attributed to the formation of voids in the MIP film by the removal of thrombin molecules, which could accelerate electron transfer between the poly-DA and the basal carbon SPE electrode.

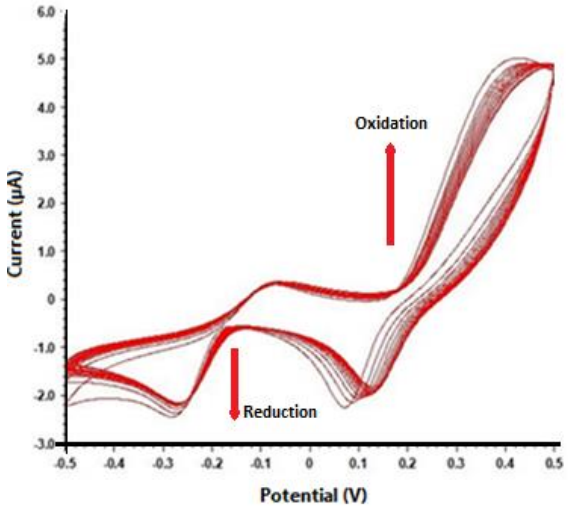


Figure 4.1 DA electropolymerization onto MWCNTs/Aptamer modified SPE surface via CV methods (- 0.6 to 0.5 V, 100 mV/s, 20 cycles) in the electrolyte solution (0.1 M KH_2PO_4, 0.1 M K_2HPO_4, 0.01 M NaCl and 0.01 M KCl)

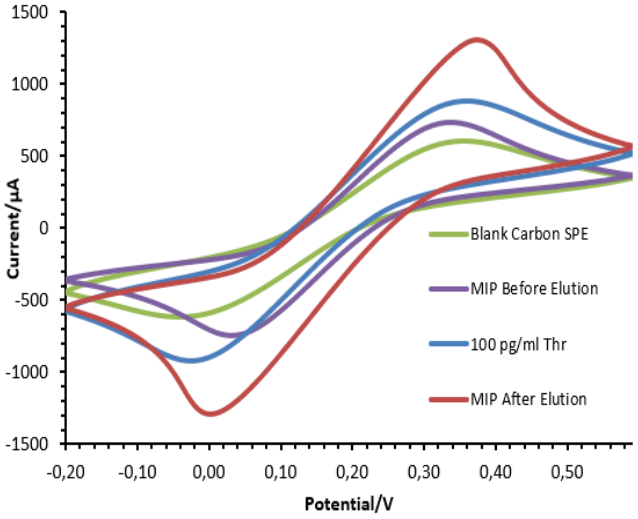


Figure 4.2 The CV measurements of polydopamine used MIP based sensor system; The blank electrode that appears in the middle of the graph and the surface increase with MIP formation, the increase following the addition of the target molecule to the system, and the maximum increase was seen after elution. The cause of this was interpreted as an increase in the surface area of the structures known as cavities, which are created by eluting the template molecule from the system

After the addition of the thrombin target molecule to the system, the increase in the surface area of the system by performing the electropolymerization process was observed with cyclic voltammetry measurements, and the properties such as surface conductance and resistance of the MIP system formed on the electrode surface were checked with EIS measurements. How non-conductive structures such as the peptide molecule added to the system decrease the system conductance and increase the resistance and since there was conductive polydopamine on the surface in the next step after the blank carbon screen printed electrode, there was a decrease in resistance and an increase in conductance in the system made with MIP, it is observed in the Figure 4.3 that the conductivity increases again and the resistance decreases with the elution.

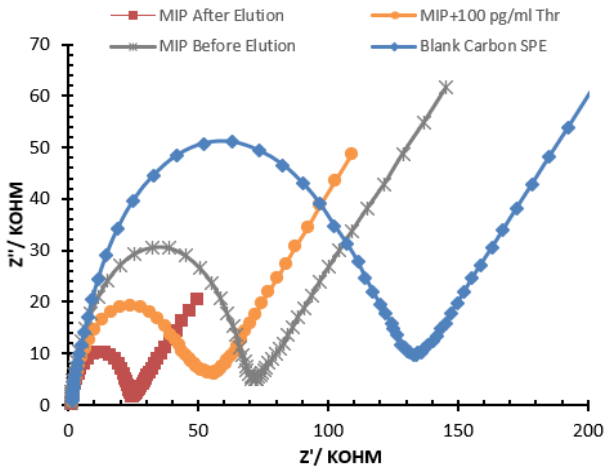


Figure 4.3 Electrochemical Impedance Spectroscopy measurements of poly-DA used MIP based electrochemical biosensor system and its plots of blank carbon SPE, after Thr binding and mip formation, after elution and 100 pg/ml Thr addition steps

Following the addition of the thrombin target molecule at various concentrations to the system and the elution of the sensor system, the influence of the electrode surface's conductivity on the system was detected using DPV measurements. It was assumed that the conductivity of the system surface had decreased when the target molecule concentration was increased because it produced diminishing results in the graph. A calibration graph was made based on the previously described DI/I0 values utilizing DPV measurements, with the calibration graph changing when more of the thrombin target molecule was added to the system and we got 0.95 R^2 linearity as it seen in Figure 4.4.

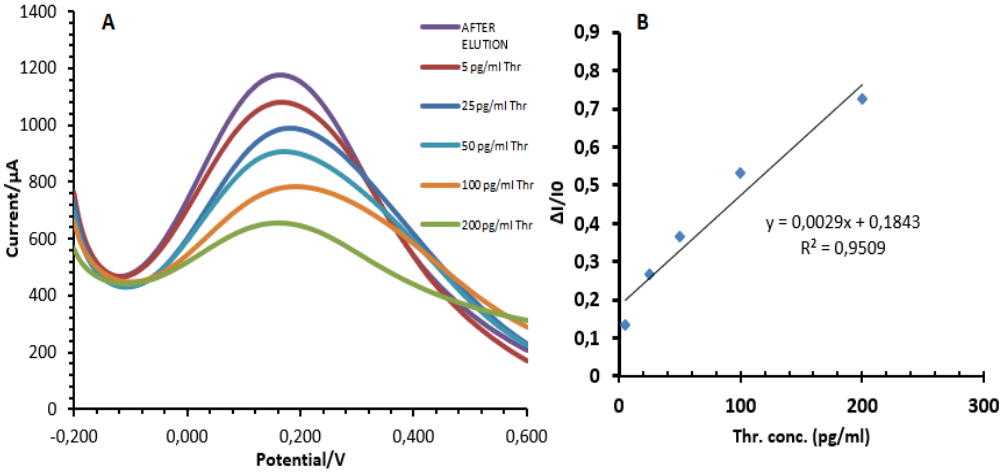


Figure 4.4 A) Differential pulse voltammetry results for each concentration thrombin combining with blank experiment , B) Calibration curve dopamine used mip based biosensor system with different concentrations of thrombin

AFM measurements (Figure 4.5) were made to observe the MIP system formed on the electrode surface and the experimental stages as elution. In the 3D AFM measurements taken as a result of the measurements, A) blank carbon screen printed electrode, B) molecular imprinted polymer formed on the surface was observed, and in part C, structures called cavity formed on the surface after elution could be clearly observed. By looking at the 3D AFM measurements, the surface thickness after MIP formation was greater than the blank electrode surface thickness, and in the image taken after elution, it was observed that the thickness decreased with the removal of the template molecule from the system.

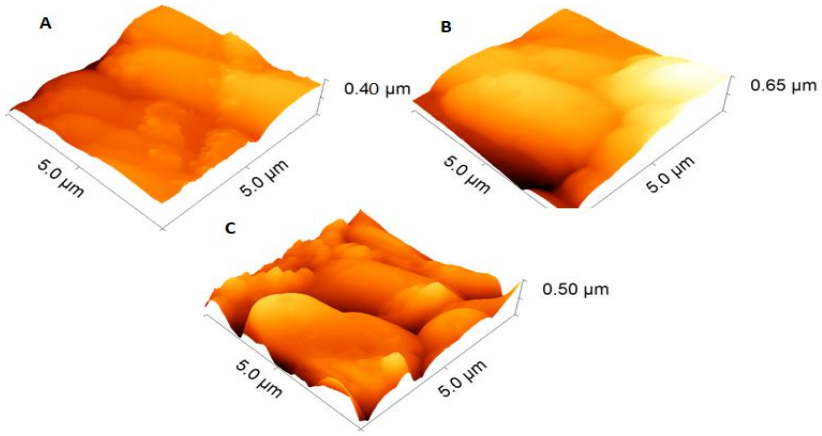


Figure 4.5 3D AFM measurements of dopamine used biosensor preparation steps; A) blank Carbon SPE, B) MIP formation onto the Carbon SPE surface, C) template removal after elution step

4.2 Characterization of Polythionine Used MIP Based Biosensor System

By using EIS, CV, and DPV techniques, the electrode surface created with thionine monomer and treated with MIP was examined. Using the $[Fe(CN)_6]^{4-/3-}$ redox pair, successive surface modifications were compared in the CV, DPV, and EIS tests. The anodic and cathodic peak currents on the electrode surface increased significantly after the MIPs were produced, as shown in Figure 4.6, and their peak heights were higher than those of the empty electrode. The model molecule (Thrombin) was isolated, resulting in a significant increase in redox peak current. The differences between cathodic and anodic peaks decreased from peak to peak. This phenomenon was attributed to thrombin molecules extracted from the MIP film, which caused the appearance of voids. These gaps can accelerate electron transfer between the polythionine and the basal carbon SPE electrode and these were clearly observed by CV measurements in Figure 4.7.

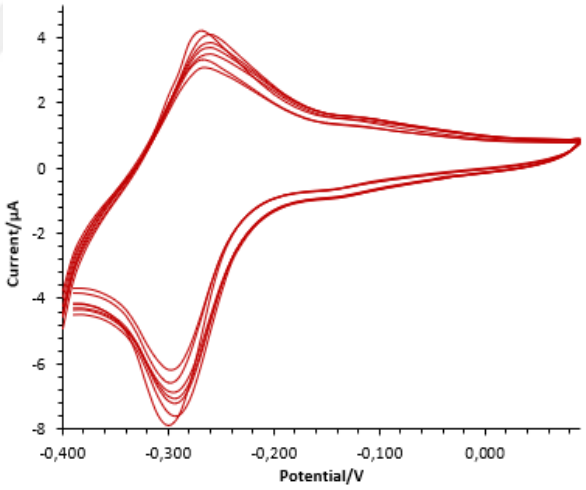


Figure 4.6 Thionine electropolymerization onto SPE surface via CV methods (-0.4 to 0.5 V, 100 mV/s, 15 cycles) in the electrolyte solution (0.1 M KH_2PO_4, 0.1 M K_2HPO_4, 0.01 M NaCl and 0.01 M KCl)

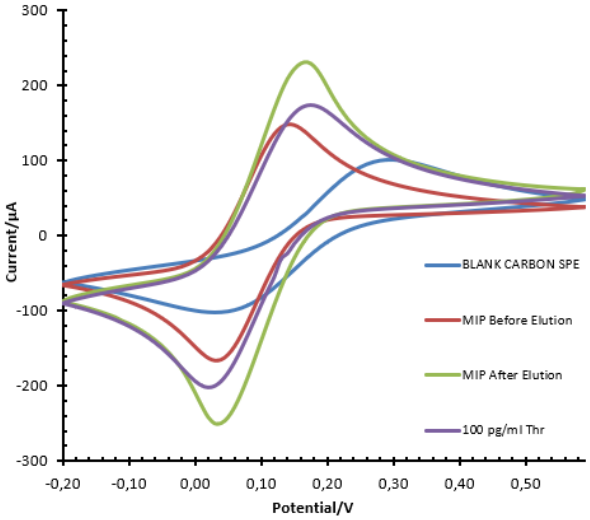


Figure 4.7 CV measurements of polythionine used sensor system; the middle-of-the-graph blank electrode and the surface increased with MIP creation, increased again after the target molecule was added to the system, and increased most after elution. This was explained by the increase in surface area of the structures known as cavities, which are created by eluding the template molecule from the system

EIS measurements were used to examine the parameters of the MIP system generated on the electrode surface, including surface conductance and resistance. As seen in Figure 4.8, the system conductivity improves once more and the resistance falls with elution, demonstrating how non-conductive materials like the peptide molecule added to the system decrease the system conductance and raise the system resistance.

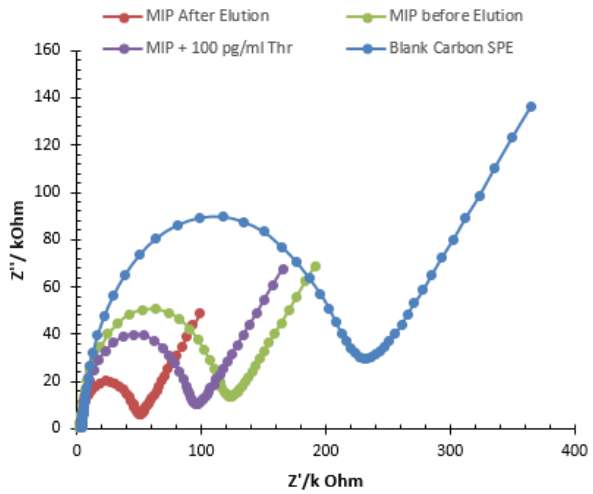


Figure 4.8 Electrochemical Impedance Spectroscopy measurements of Thionine used MIP based electrochemical biosensor system and its plots of blank carbon SPE, after Thr binding and mıp formation, after elution and 100pg/ml Thr addition steps

With DPV measurements, the effect of the conductivity of the electrode surface on the system was observed after the elution of the sensor system of the thrombin target molecule at different concentrations added to the system. The fact that increasing target molecule concentration gave decreasing results in the graph was interpreted as decreasing the conductivity of the system surface. By calculating the previously mentioned DI/I0 values using DPV measurements, a calibration graph was created depending on the increasing concentration of thrombin target molecule added to the system, we got 0.9485 R^2 and linearity as it seen in Figure 4.9.

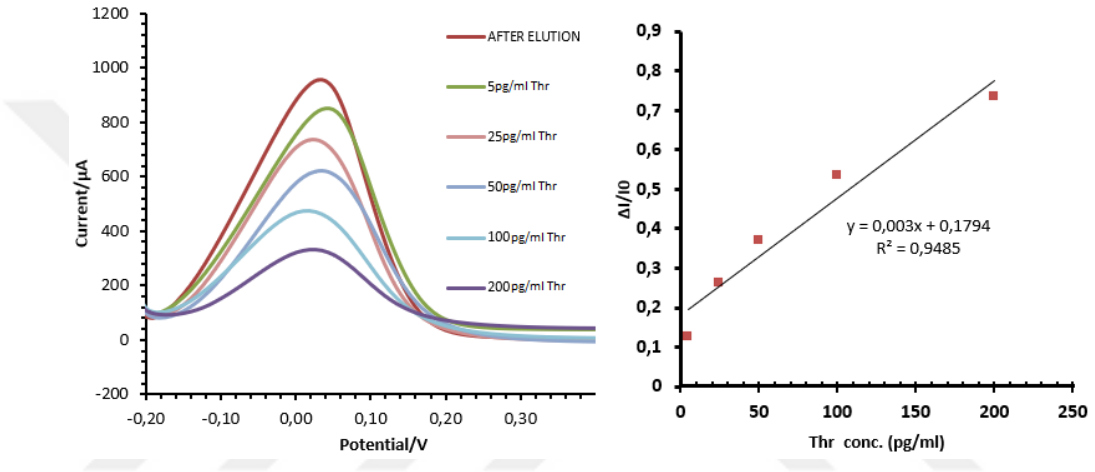


Figure 4.9 A) Differential pulse voltammetry results for each concentration thrombin combining with blank experiment , B) Calibration curve of thionine used mip based biosensor system with different concentrations of thrombin

The experimental stages as well as the MIP system that had formed on the electrode surface were observed using AFM measurements as it seen in Figure 4.10. In the 3D AFM measurements made as a result of the measurements, A) a blank carbon screen printed electrode, B) a molecular imprinted polymer created on the surface, and in part C), structures termed cavities generated on the surface after elution could be clearly seen. According to the 3D AFM measurements, the surface thickness after MIP formation was more than the surface thickness of the blank electrode, and it was found in the picture captured after elution that the thickness reduced as the template molecule was eliminated from the system.

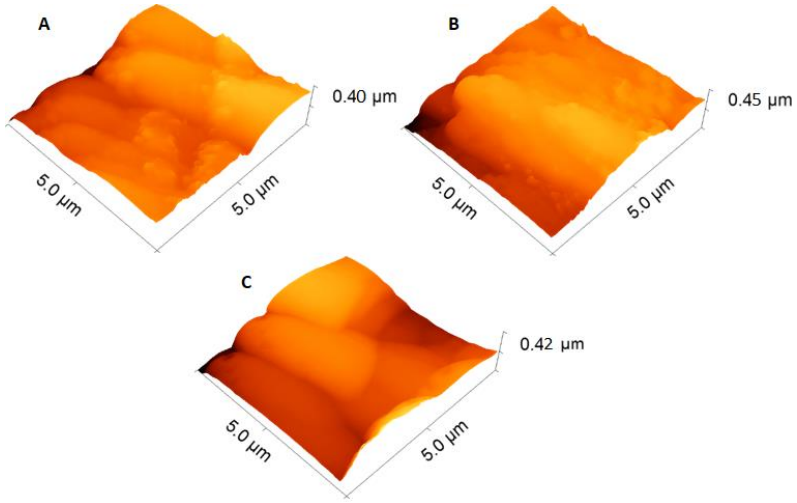


Figure 4.10 3D AFM measurements of thionine used biosensor preparation steps; A) blank Carbon SPE, B) MIP formation onto the Carbon SPE surface, C) template removal after elution step

4.3 Characterization of Polyethanolamine Used MIP Based Biosensor System

The electrode surface formed with polyethanolamine and modified with MIP was examined using the EIS, CV, and DPV procedures. The CV, DPV, and EIS experiments were used to compare subsequent surface changes using $[Fe(CN)_6]^{4-/3-}$ redox pair. After the MIPs were created, the anodic and cathodic peak currents on the electrode surface rapidly rose and reached peak heights that were higher than those of the empty electrode, as shown in Figure 4.11. The template molecule (Thr) was eluted, which caused the redox peak current to increase significantly. From peak to peak, there was a decreasing difference between the cathodic and anodic peaks. This occurrence was attributed to the removal of the thrombin molecules from the MIP film, which led to the emergence of voids. The movement of electrons between the poly-EA and the basal carbon SPE electrode may be sped up by these spaces and these changes observed by CV measurement as is seen in Figure 4.12.

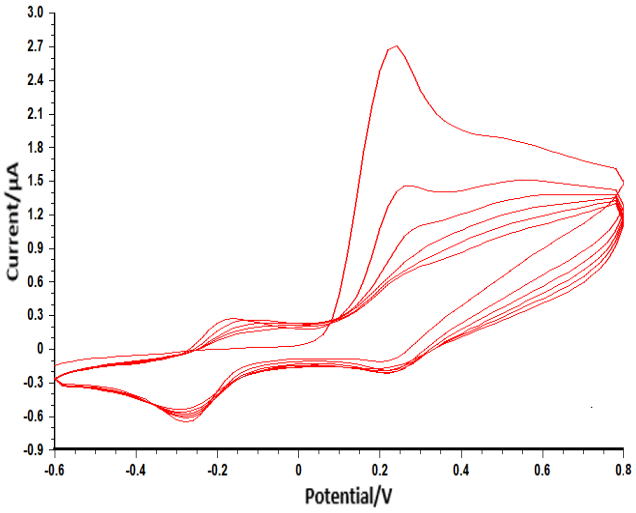


Figure 4.11 Ethanolamine electropolymerization onto SPE surface via CV methods (-0.6 to 0.8 V, 100 mV/s, 15 cycles) in the electrolyte solution (0.1 M KH_2PO_4, 0.1 M K_2HPO_4, 0.01 M NaCl and 0.01 M KCl)

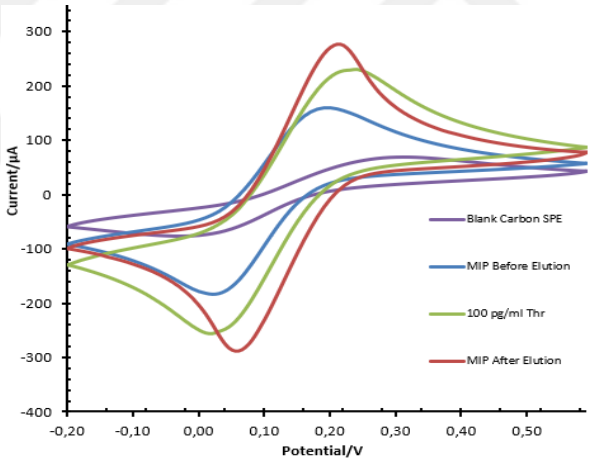


Figure 4.12 CV measurements of Thionine monomer used sensor system; the blank electrode observed in the middle of the graph and the surface increase with the MIP formation, the increase after the addition of the target molecule to the system, and the highest increase was observed after elution. The reason for this was interpreted as increasing the surface area of the structures called cavities, which are formed by removing the template molecule from the system with elution

DPV measurements were used to determine the effects of the electrode surface's conductivity on the system after the addition of the thrombin target molecule at various concentrations to the system and the elution of the sensor system. Because the target molecule concentration produced diminishing results in the graph, it was presumed

that the conductivity of the system surface had reduced when the concentration of the target molecule was increased. DPV measurements were used to create a calibration graph based on the previously disclosed DI/I0 values, with the calibration graph changing when more of the thrombin target molecule was added to the system, and we got 0.9565 R^2 and linearity as it seen in Figure 4.13.

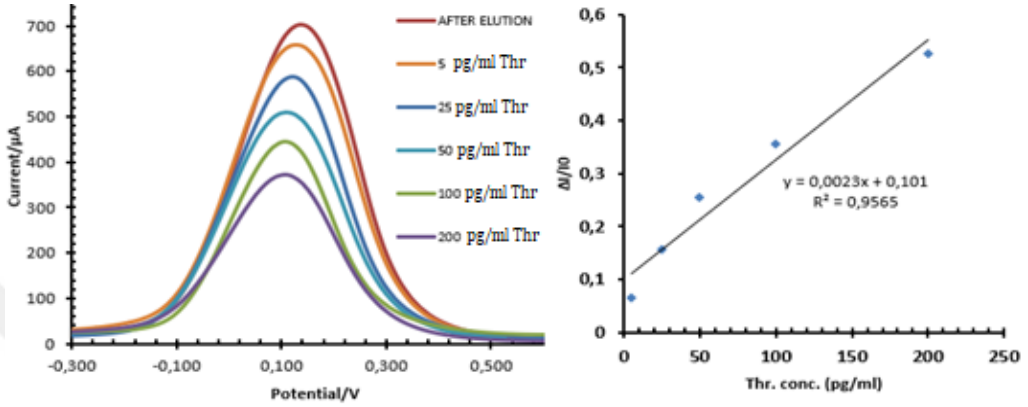


Figure 4.13 A) Differential pulse voltammetry results for each concentration thrombin combining with blank experiment , B) Calibration curve ethanolamine used mip based biosensor system with different concentrations of thrombin

MIP system formation on the electrode surface and the experimental stages that were eluted were observed using AFM measurements as shown in Figure below. A blank carbon screen-printed electrode, a molecular imprinted polymer that had formed on the surface, and in part C, structures called cavities that had formed on the surface following elution, could all be clearly seen in the 3D AFM measurements that were acquired as a result of the measurements. In the images taken after elution, it was noticed that the thickness decreased with the removal of the template molecule from the system. Based on 3D AFM measurements, it was shown that the surface thickness after MIP synthesis was larger than the blank electrode surface thickness.

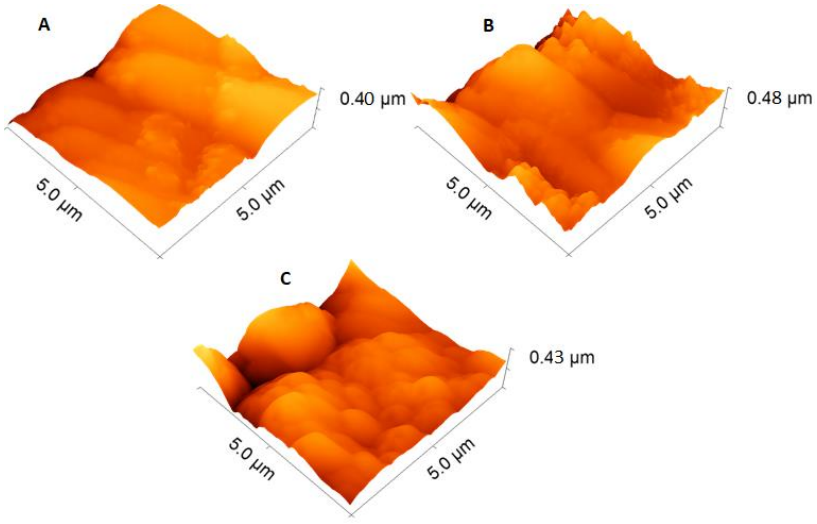


Figure 4.14 3D AFM measurements of ethanolamine used biosensor preparation steps; A) blank Carbon SPE, B) MIP formation onto the Carbon SPE surface, C) template removal after elution step

4.4 Comparison of Developed MIP-Based Sensor Systems in Case of The Polymers Used

After using three different polymers in the MIP-based electrochemical sensor system, these three separate sensor systems were compared in terms of the polymers used, and the following results were obtained: The most stable and best results were obtained for dopamine electropolymerization. Better AFM results (Figure 4.5) were obtained in the sensor system using dopamine.

The results before and after elution and after the formation of the molecularly imprinted polymer were better than the other results (Figure 4.4). In the biosensor system using dopamine, by looking at the oxidation peak height differences after MIP formation and after elution in CV measurements (Figure 4.2); The difference between the peak heights was calculated for all monomers and it was determined that the experiment using dopamine was 72% ahead of the experiment using thionine and 59% ahead of the experiment using ethanolamine, and it was concluded that better results were obtained. Based on such results, MWCNTs/Aptamer/MIP based sensor system was developed to improve the properties of this sensor system such as selectivity and sensitivity by using polydopamine, and to increase the properties such as increasing the system surface and binding capacity.

4.5 Characterization of MIP/MWCNTs/Aptamer Based Biosensor System

The aim of this experiment, which was developed using dopamine, was to develop the system further and be more sensitive and selective. Accordingly, the system; surface modified by drop casting of MWCNTs and processed with MIP was analyzed with both EIS and CV methods. Step by step surface modifications were compared in CV and EIS experiments performed with using $[Fe(CN)_6]^{4-/3-}$ redox couple. As it is seen in Figure 4.15, after MWCNTs drop casting onto the electrode surface both anodic and cathodic peak currents increased sharply, and the peak heights were larger than the blank electrode. After the electropolymerization of dopamine(DA) with thrombin as the template molecule, the increased oxidation and reduction peaks of electroactive DA appeared at the MIP/MWCNTs/Carbon SPE before extracting thrombin. After the elution of template molecule thrombin, the redox peaks current increased significantly and the peak-to-peak separations between cathodic and anodic peaks became smaller. This behavior was attributed to the fact that cavities were formed in the MIP film by extracting the thrombin molecules, which could accelerate the electron transfer between poly-DA and basal carbon SPE electrode.

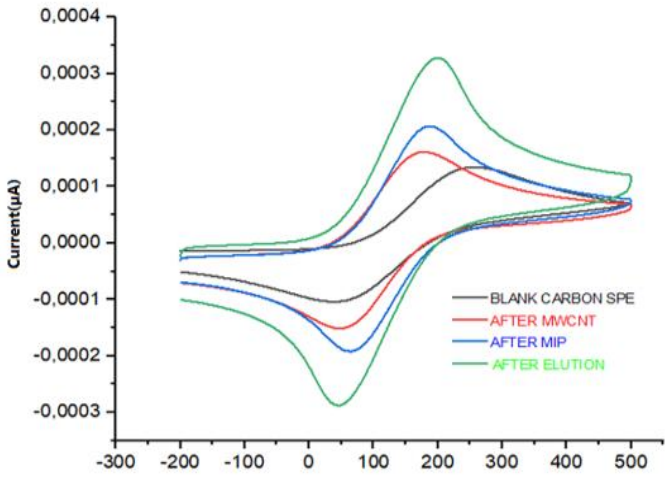


Figure 4.15 The MWCNT, aptamer and molecularly imprinted polymer effect on carbon screen printed electrode

EIS can sensitively monitor the interface properties of the modified electrode during the modification process, which was measured using $[Fe(CN)_6]^{4-/3-}$ as the redox probe. Electrochemical impedance spectrum (EIS) is an important method to show the process of the sensor construction. As shown in Figure 4.16, the charge transfer resistance (Rct) value of the bare Carbon SPE was about 300 ohms. And the impedance value of MWCNTs modified electrode was reduced to 40 ohms, which indicated that carbon nanotubes had excellent conductivity and could accelerate the electronic transmission rate. When aptamers and thrombin were bounded on the modified electrode, their impedances increased respectively. This was attributed to the non-electroactive molecules were bounded successfully onto the electrode surface which can hinder the electronic transmission. However, after the poly-DA was electropolymerized on the electrode surface the Rct value decreased compared to the thrombin bounded step. This indicated that the poly-DA film had better electrochemical activity and a faster electron transfer rate. After elution of thrombin from the MIP developed electrode surface, holes that represented the template molecule spaces were formed, and the Rct value was greatly reduced. It indicated that $[Fe(CN)_6]^{4-/3-}$ can easily pass through the MIP cavities. While after hatched in 50 pg/ml thrombin the Rct of the MIP electrode raised which indicated that the recombining of the target molecule thrombin into the imprinted cavities can block the penetration of the $[Fe(CN)_6]^{4-/3-}$ to electrode surface.

In Figure 4.16, we can observe the linear increase of the Rct values form EIS tests with increasing thrombin concentrations (0 pg/ml to 200 pg/ml). in the inner figure the linear calibration curve with different thrombin concentration depends on the Rct values also could be observed with 0.99 R^2 value. That indicates the successful template removal and thrombin rebinding onto the MWCNTs/Aptamer/MIPs surface.

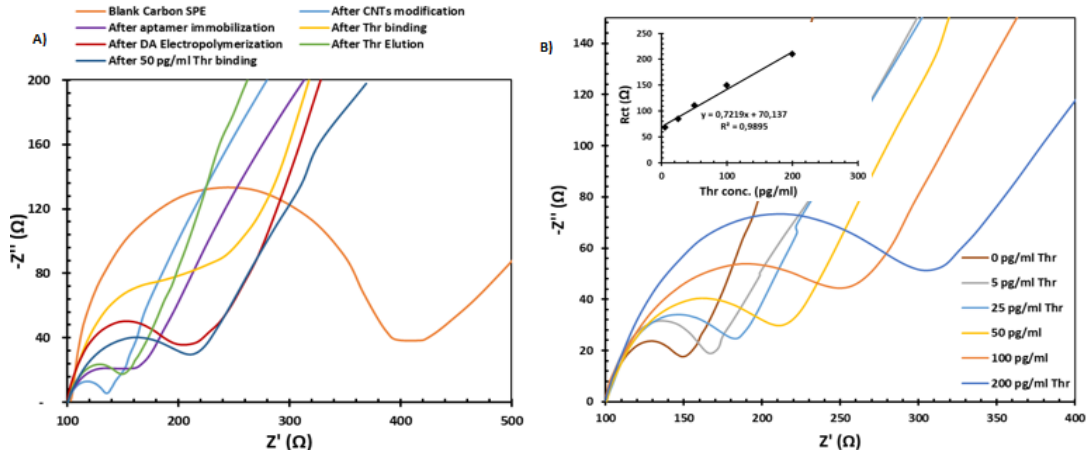


Figure 4.16 A) Electrochemical impedance spectroscopy (EIS) measurement for the steps of preparation of DA used MIP based biosensor system, B) EIS graphs for different concentrations of thrombin injection onto the biosensor system and their Rct values represented as a calibration graph in the inner figure

Morphology of MWCNTs/Aptamer/MIPs biosensor preparation steps were studied with AFM (Figure 4.17). AFM imaging helped to determine three-dimensional morphology of each prepared electrode surfaces. The MWCNTs deposited surface shows granules (Figure 4.17 B) bigger compared to those in the blank carbon SPE electrode surface (Figure 4.17 A). After Aptamer/MIPs formation the surface filling with higher thickness were also could be observed from both Figure 4.17 and 4.18. For template removal, MIPs prepared surface were washed with 1:1 Ethanol and Acetic acid. This treatment decreased surface thickness and also the composed cavities from template removal could be observed from both figures respectively.

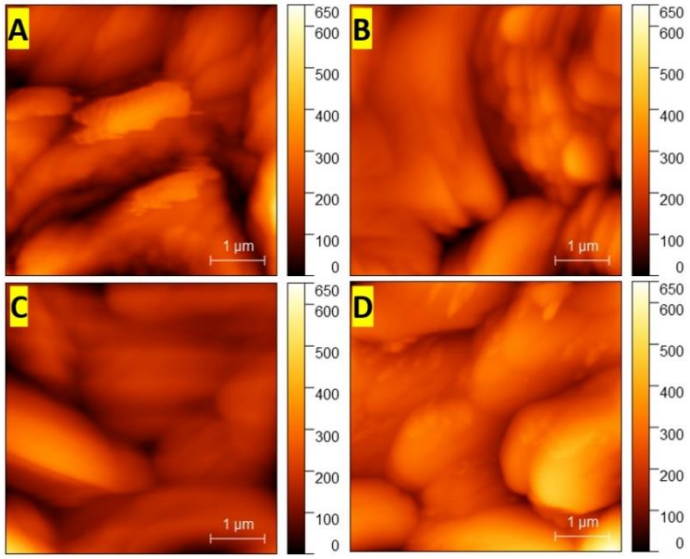


Figure 4.17 2D AFM measurements of MWCNTs/Aptamer/MIPs biosensor preparation steps; A) blank Carbon SPE, B) MWCNTs attached onto the Carbon SPE surface, C) Aptamer/MIPs formation onto the surface, D) template removal after elution step

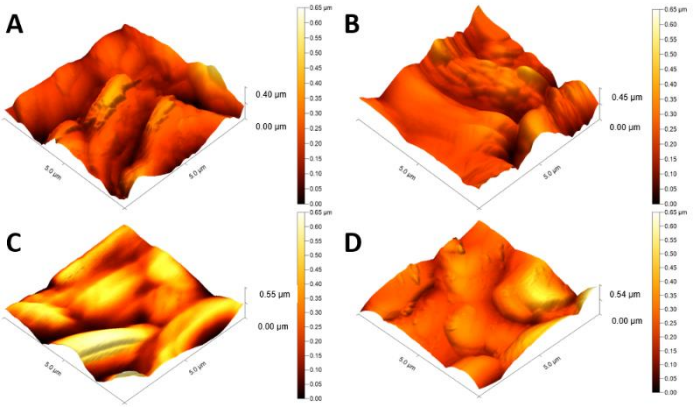


Figure 4.18 3D AFM measurements of MWCNTs/Aptamer/MIPs biosensor preparation steps; A) blank Carbon SPE, B) MWCNTs attached onto the Carbon SPE surface, C) Aptamer/MIPs formation onto the surface, D) template removal after elution step

4.6 Biosensor Performance Assessment

While performing the performance evaluation of the developed MWCNTs/Aptamer/MIPs biosensor system, the target molecule (thrombin) of the system was injected onto the prepared electrode surface with different concentrations. The target molecule bounded onto the template whole in the MIPs surface and DPV

method was performed to measure and calculate $\Delta I/I_0$ values for each concentration. After each concentration of thrombin injection onto the electrode surface, waited 15 minutes at room temperature for the rebinding of thrombin to the MIPs cavities. Before and after the injection DPV measurements were performed with 5 mM $[Fe(CN)_6]^{4-/3-}$ in 0.1 M KCl solution. The original DPV graphs were plotted and overlayed in Figure 4.19.B which showed the correlated decrease of the peak height after increasing concentrations of thrombin injection onto the MIPs surface. When we plotted the $\Delta I/I_0$ values and different thrombin concentrations, we got the linear calibration curve with 0.96 R^2 value. The limit of detection of the developed MWCNTs/Aptamer/MIPs biosensor system was also calculated as equal to 1.4 pg/ml (S/N=3).

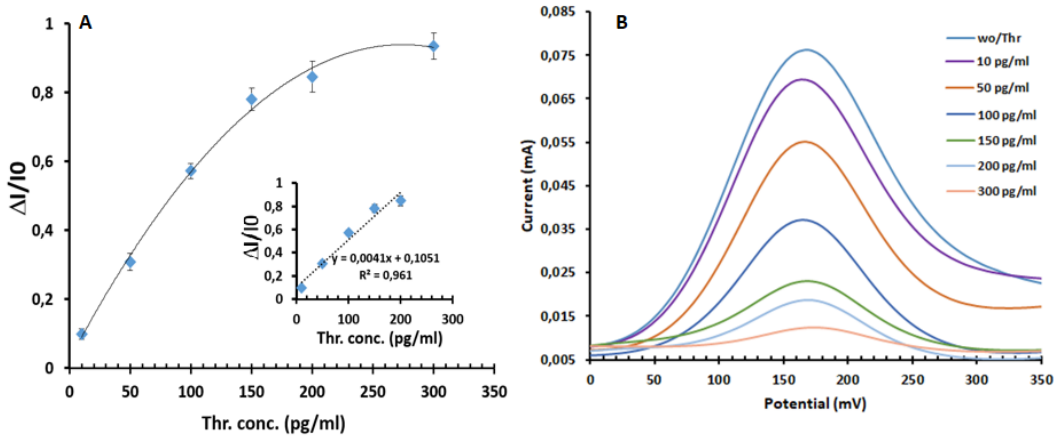


Figure 4.19 A) Calibration curve of MWCNTs/Aptamer/MIPs biosensor system with different concentrations of Thrombin, B) Differential pulse voltammetry results for each concentration Thrombin combining with blank experiment (biosensor system without thrombin injection)

To confirm the presence of selective molecular cavities in the developed MIP system, the selectivity in the presence of peptide molecules such as albumin, IgG, BSA, and glucose were tested parallel to thrombin. Selectivity study was focused on interferences analytes that are commonly found in blood serum. $\Delta I/I_0$ values of DPV measurements for 100 pg/ml of each peptide molecules (Figure 4.20) were calculated and plotted on the selectivity graph to compare with thrombin results. The other molecules gave 18% to 22% of $\Delta I/I_0$ results depend on the thrombin results.

Additionally, thrombin response was measured in $\Delta I/I_0$ values presence of each non-specific targets for the developed system (Figure 4.21). A slight increase in $\Delta I/I_0$ values (between 5-12%) was obtained in the presence of 100 pg/ml of each non-specific targets with 100 pg/ml thrombin while testing via developed biosensor system. Apparently, the designed MWCNTs/Aptamer/MIPs system was selective for thrombin detection.

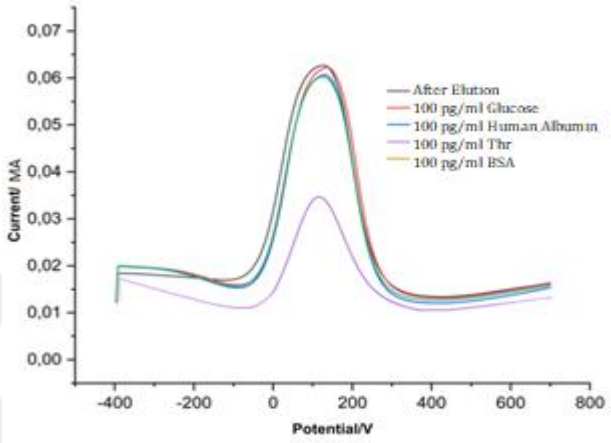


Figure 4.20 A) Schematic illustration of DPV graph of BSA(Bovine serum albumin), IgG (Immunoglobulin) , Glucose, and albumin were detected in addition to the target molecule, the selectivity of the sensor system to the thrombin target molecule is noticeable compared to other molecules

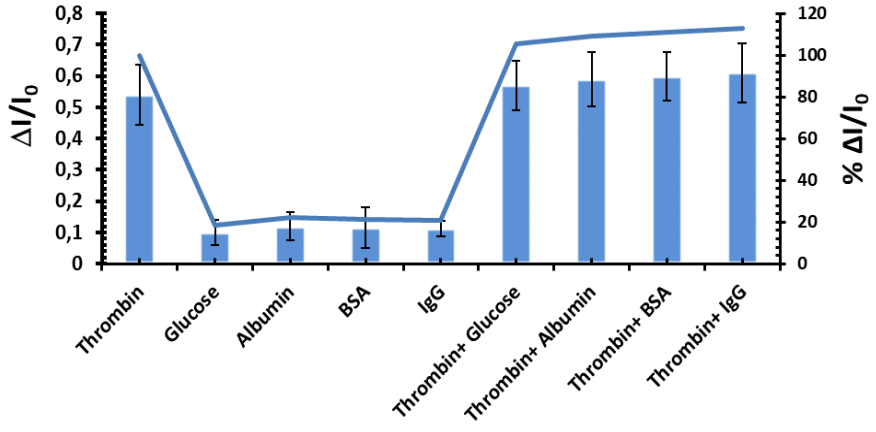


Figure 4.21 Comparison of the developed biosensor system with thrombin and different molecules at the same concentration; $\Delta I/I_0$ values for each molecule and % of $\Delta I/I_0$ values depends on thrombin results were presented

Biosensor systems prepared with MWCNTs/Aptmer/MIPs were stored at 4 °C for a period of 30 days and tested with 25 pg/ml thrombin at regular intervals. The stability of the systems was determined by comparing the results obtained depending on the days with the results obtained on the first day. As seen in Figure 4.22, the stability of the results obtained with the developed biosensor system was 85% at the end of 30 days.

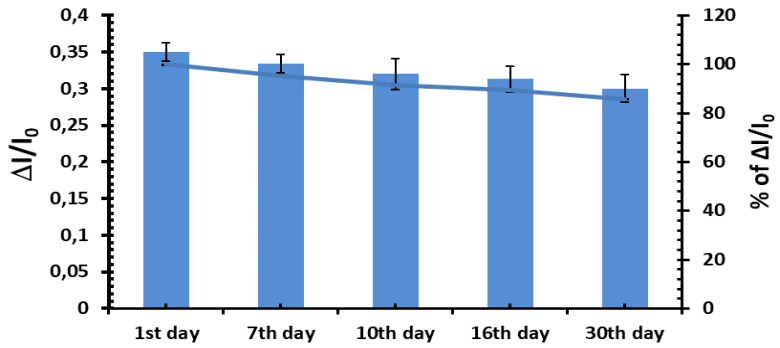


Figure 4.22 Schematic representation of the stability graph of the target molecule thrombin with its measurements on different days

4.7 Real Serum Sample Analysis

As a result of the experiments, real serum analyzes were performed in the prepared MWCNTs/Aptamer/MIP biosensor system. 1:5 ratio diluted commercial serum samples were spiked with at 5pg/ml , 50pg/ml and 100 pg/ml concentrations of thrombin and tested via developed system. As mentioned biosensor performance testing step, DVP measurements were performed for each spiked sample respectively, and $\Delta I/I_0$ values were calculated. Using the calibration curve that was drawn previously, the thrombin amount in each spiked sample were calculated. Spiked and calculated thrombin amounts for each sample were compared, and % of recovery values were calculated. Accuracy (% of recovery) values of all measured samples were observed between 89% to 114% respectively with 0,43 to 6,1 SD results.

Table 4.1 Spiked Thr concentrations after testing real serum samples

SPIKED THR CONC. (PG/ML)	CALCULATED THR CONC. (PG/ML)	%RECOVERY	SD
5	4,8	97,0	0,4
50	44,0	88,1	3,8
100	103,2	103,2	6,1

4.8 Comparison with Similar Studies

There have been numerous investigations into peptide biomarker detection using biosensor systems. Twelve different research are analyzed and summarized separately from this thesis in the table below. Each study found different values for the detection limit, detection range, repeatability, and stability. Similar to this, detection limits were calculated for real sample tests in related investigations.

According to the table below, the first study described a hybrid recognition sensor based on reduced graphene oxide (rGO) and gold nanoparticles termed a molecularly imprinted polymer (MIP)-aptamer (Apt) (AuNPs). rGO and AuNPs were used to initially modify the glassy carbon electrode (GCE). After that, thrombin-Apt composites were created by incubating template thrombin with thiolated thrombin aptamer. The Au-S bond between the AuNPs and the thiol group at the aptamer terminus was subsequently used to immobilize the thrombin-aptamer composites to the AuNPs/rGO/GCE. Finally, MIP-Apt hybrid recognition sensor was prepared by electropolymerization of thionine. The MIP-Apt sensor exhibited linear response from 2.5×10^{-9} mg/mL to 1.3×10^{-6} mg/mL for thrombin with a detection limit of 1.6×10^{-10} mg/mL and stability of 4.6% SD in a weekly experiments.

In a different research, a voltammetric biosensor was created to measure thrombin. The bioconjugates are immobilized on the surface of the modified glassy carbon electrode via a sandwich process in the presence of thrombin. The electrochemical reaction of poly(o-phenylenediamine) in the presence of H_2O_2 is effectively catalyzed by HRP, AuPd nanoparticles, and G-quadruple/hemin as peroxidase mimics. Poly(o-phenylenediamine) also serves as a redox mediator. As a result, triple catalytic

amplification produces an electrochemical signal that is noticeably magnified. Finally, the developed sensor system has 20 fmol/L detection limit.

In another study, a label-free and amplified electrochemical impedimetric aptasensor based on functionalized graphene nanocomposites (rGO-AuNPs) was developed for the detection of thrombin. Thiolated aptamer and dithiothreitol (TBA15-DTT) were first immobilized on the gold electrode to capture thrombin molecules, and then aptamer functionalized graphene nanocomposites (rGO-TBA29) were used to fabricate a sandwich sensing platform to amplify impedimetric signals. The Rct increase was linearly proportional to the thrombin concentration from 0.3 to 50 nM, and the 0.01 nM thrombin detection limit was reached.

The next research found a dual improved signal enhancement approach based on the integration of in situ DNA polymerization caused by terminal deoxynucleotidyl transferase (TdT) and CHA for the electrochemical detection of thrombin in human serum samples. The sensor's linear range is 0.5pM–10.0nM, and its thrombin detection limit is 0.12pM.

In a different study, gold nanoparticles (AuNP-SN-GO) were added to graphene oxide that had been doped with nitrogen and sulfur and used as a substrate to alter a glassy carbon electrode (GCE). On the altered GCE, an aptamer against the model protein thrombin was self-assembled before being exposed to thrombin. Biotin-labeled DNA and aptamer(2) are immobilized on another AuNP-SN-GO hybrid and then reacted with the thrombin/AuNP-SN-GO/GCE to produce a sandwich after the aptamer-thrombin interaction. The electrode was then coated with the enzyme label horseradish peroxidase (HRP) using a biotin-avidin reaction. Hydroquinone is oxidized by hydrogen peroxide thanks to HRP. In the range of 1.0×10^{-13} M to 1.0×10^{-8} M with a detection limit of 2.5×10^{-14} M (S/N = 3), this produces an electrochemical signal that rises linearly with the logarithm of the thrombin concentration.

Another work described the development of an electrochemical aptasensor for thrombin based on the conjugation of an aptamer with gold nanoparticles and horseradish peroxidase (aptamer-AuNPs-HRP). Aptamer1 (Apt1), which was immobilized on core/shell Fe(3)O(4)/Au magnetic nanoparticles (AuMNPs) for this

electrochemical procedure, acted as the capture probe. As a detection probe, Aptamer2 (Apt2) was double-labeled with AuNPs and HRP. The sandwich shape of AuMNPs-Apt1/thrombin/Apt2-AuNPs-HRP was created in the presence of thrombin. With the suggested approach, a linear response to thrombin concentration in the range of 0.1-60 pM and a reduced detection limit down to 30 fM (S/N=3) were obtained.

On another study the detection of thrombin using an aptamer-based electrochemiluminescent (ECL) method is discussed. A glassy carbon electrode (GCE) was coated with three-dimensional nitrogen-doped graphene oxide (3D-NGO) to create an electrode surface that exhibits outstanding electrical conductivity and functions as a potent ECL emitter. Further chitosan coating was applied to the modified electrode using electrodeposition. The modified GCE was then used to immobilize the amino-modified aptamer. ECL is reduced as a result of the interaction between thrombin and the aptamer. The assay responds linearly in the thrombin concentration range of 1 fM to 1 nM and has a lower detection limit of 0.25 fM (at a S/N ratio of 3).

In another study; Thrombin detection strategy based on fluorescent resonance energy transfer (FRET) from the cationic conjugated polymer (CCP) to the Ir(III) complex has been developed. The energy donor (CCP) and acceptor (Ir(III) complex) were brought into close proximity leading to the formation of FRET via π-π stacking interaction and electrostatic interaction. Subsequently, the re-emergence of the FRET phenomenon was confirmed by the specific binding interaction between aptamer and thrombin, thereby enabling the quantitative detection of thrombin. In the study, it was able to detect thrombin at a concentration of about 0.05 pM.

In another study, DNA aptamers were used as molecular recognition components in biosensors with SWNT-FET architectures for the real-time detection of proteins. Using CDI-Tween connecting molecules, anti-thrombin aptamers that are particular to the serine protein thrombin were immobilized on the sidewall of a SWNT-FET. The negatively charged backbone of the DNA aptamers is probably what triggered the binding of thrombin aptamers to SWNT-FETs, which resulted in a rightward change of the threshold gate voltages. While the conductance of the thrombin aptamer immobilized SWNT-FET abruptly decreases with the addition of thrombin solution, no discernible change was seen with elastase. As a result of the studies, the detection

limit was calculated as 7.5×10^{-15} to 2.5×10^{-10} mol/L and <3.2% RSD at the end of 30 days.

In a different study, they present a one-step detection technique using a nanofibrous sensing platform that combines proximity-induced DNA strand displacement (PiDSD), catalytic hairpin assembly (CHA), and thioflavin T (ThT) binding. To advance interface reaction kinetics and thermodynamics, the interface behaviors on the nanofibrous membrane were investigated. The nanofibrous sensing technology was employed with thrombin as a model biomarker, and it produced results with a limit of detection as low as 1.0 pM and a broad linear range of 50 pM to 5 nM.

In our study, molecular imprinted polymers were formed using different monomers, and thrombin was detected electrochemically. As a result of the experiments, a biosensor system based on MWCNTs/MIP/Aptamer was developed with the aim of more selective, faster and more accurate target detection with dopamine monomer. The developed sensor system is highly selective for the target peptide molecule, and the calculated values such as detection limit selectivity are given in the table below together with the experimental results.

Table 4.2 Comparison tabe of current biosensor methods and this work

Biosensor Method	Detection limit (LOD)	Linear range	Repeatability	Stability	Real Sample testing
Electrochemical Molecularly Imprinted Polymer-Aptamer based Voltammetric biosensor system for thrombin detection [102]	1.6×10^{-10} mg/mL	2.5×10^{-9} mg/mL to 1.3×10^{-6} mg/mL	10 times with 5.3 %sd	In weekly experiments, the response to Thrombin was 4.6% SD	Bovine blood,plasma %4.7 reflex sympathetic dystrophy RSD, 90.6% Recovery
Graphite modified surfaces, electrochemical sensor system for thrombin detection [103]	20 fmol/L	100 fmol/L~20 nmol/L	-	-	-
Label-free aptamer biosensor for thrombin detection using EIS method, and based on functionalized graphene nanocomposites [104]	0.01 nmol/L	0.3 nmol/L~50 nmol/L	-	-	1% fetal calf serum, 30 R/kΩ
Target-triggered catalytic hairpin assembly and TdT-catalyzed DNA polymerization for amplified electronic detection of thrombin in human serums [105]	1.2×10^{-13} mol/L	5.0×10^{-13} to 1.0×10^{-8} mol/L	-	-	Human serums, rate of recovery 96.6-104.1%, 2.7% RSD
Sandwich electrochemical thrombin assay using a glassy carbon electrode modified with nitrogen- and sulfur-doped graphene oxide and gold nanoparticles [106]	25 fmol/L	0.1 pmol/L~10 nmol/L	From five parallel prepared electrode under same experimental conditions SD of 2.4%	4 °C for two weeks, and 90.1% of the initial electrochemical response to 1 × 10−13 M thrombin	Serum samples, The recoveries are 96.0%–106.1% and the RSDs are 3.2%–4.9%
Electrochemical aptasensor for thrombin based on the amplification of aptamer–AuNPs–HRP conjugates [107]	30 fM	0.1– 60pM	20 pM thrombin gave an average peak current of 82 mA with a relative standard deviation of 4.3%	-	-
Electrochemiluminescent aptasensor for thrombin using nitrogen-doped graphene quantum dots [108]	2.5×10^{-13} mol/L	1.0×10^{-12} to 1.0×10^{-9} mol/L	Under the same experimental conditions, RSD of 2.4%	For 10 cycles, RSD of less than 2.8%	Human serum, 94% to 105% recovery and 2.4% to 2.8% RSD
Competition-derived FRET-switching cationic conjugated polymer-Ir(III) complex probe for thrombin detection [109]	5.0×10^{-14} mol/L	1.0×10^{-14} to 2.0×10^{-10} mol/L	-	-	In urine, spit and serum sample, the recoveries are between 95% and 105% in human urine, between 93% and 104% in human spit, and between 92% and 108% in human serum
Novel chemiluminescence sensor for thrombin detection based on dual-aptamer biorecognition and mesoporous silica encapsulated with iron porphyrin [110]	2.2×10^{-15} mol/L	7.5×10^{-15} to 2.5×10^{-10} mol/L	6 samples and different days, RSD of < 2.8%	30 days, <3.2% RSD	Bovine serum, 2.83% of RSD
Label-free aptamer biosensor for selective detection of thrombin [111]	0.15 nM	1–10.5 nM	-	-	Human blood samples, < 3.4% RSD, the range of 101–104% recovery
One-step sensitive thrombin detection based on a nanofibrous sensing platform, point-to-care testing [112]	1.0×10^{-12} mol/L	5.0×10^{-11} to 5.0×10^{-9} mol/L	-	After 51 days, fluorescent intensity of nanofibrous membrane 7.3%	Human serum samples, 2.83%RSD and 102.9 recovery
Multi-DNAzymes-functionalized gold nanoparticles for ultrasensitive chemiluminescence detection of thrombin on microchip [113]	5.5×10^{-13} mol/L	1.0×10^{-12} to 2.5×10^{-11} mol/L	-	-	Human serum, 90% to 104% recovery range and 5.6% to 14.1% RSD
This Work	0.5 pg/ml	2-40 pg/ml	10 times with 0.4%SD	30 days with %85 stability	Human serum, with 3.43% SD and 81.1% to 114.4% recovery

CHAPTER 5

CONCLUSION

When we look at the experiments in which all different polymers are used as a result of the experiments without aptamer; It has been concluded that studies using dopamine monomer are more stable, fast and long-lasting, and this study was developed with aptamer and carbon nanotubes using dopamine monomer. In this study, a sensitive pair recognition approach was successfully established to identify a peptide-structured biomarker by combining the concepts of aptamer and MIP. In this electrochemical sensing procedure, the electrode surface was replaced with MWCNTs, which increased its surface area and conductivity. Under optimum conditions, this dual recognition approach represented a dynamic range of up to 300 pg/ml with a low detection limit of 1.4 pg/ml. The developed system achieved good selectivity for the target peptide biomarker and gave good accuracy in serum sample testing. The study's findings suggested that a rapid approach could be developed for the accurate detection of biomolecular peptide compounds by combining the benefits of molecular imprinting, MWCNTs and aptamer MIP as electron probes.

In summary, molecularly imprinted polymers in a biosensor are an alternative to biological recognition elements. The use of biological recognition elements requires the extraction of biological molecules from animals, raising ethical issues. You do not need to use animals when using MIP. However, there are difficulties such as selectivity, sensitivity. These problems can be solved with new technologies such as interactions between functional monomers and template molecules, use of aptamer to increase selectivity and fast binding, MWCNTs and aptamers can also be used to increase binding and improve electrochemical activity in light of this work.

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