T.C.

BİRUNİ UNIVERSITY

INSTITUTE OF GRADUATE EDUCATION DEPARTMENT OF MOLECULAR BIOLOGY AND GENETICS

MOLECULAR AND MEDICAL GENETICS GRADUATE PROGRAM

GENETICALLY DESIGN AND SYNTHESIS OF MULTIFUNCTIONAL FUSION PROTEINS FOR BIOSENSOR APPLICATIONS

Sena ŞİMŞEK

Thesis Advisor

Asst. Prof. Banu TAKTAK KARACA

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DECLARATION

I declare that I am the author of this thesis, that I have not engaged in any unethical behavior at any stage, that I have obtained all the information contained in it in accordance with academic and ethical standards, that I have cited all the information I have used as a source and included these sources in the list of references, and that I have obtained patents and copyrights during the execution and writing of this thesis. I affirm that I have not violated their rights in any way.

SENA ŞİMŞEK

To humanity

ACKNOWLEDGEMENT

My special thanks to my thesis advisor, Assistant Professor Banu TAKTAK KARACA, for her support and efforts. She was a consistent source of support throughout the most stressful and tough stages of my academic career, assisting me in finding solutions to my problems and providing me with encouragement when I was feeling down.

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JUNE 2022 Sena ŞİMŞEK

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ABBREVIATIONS

Anti-MBP : Antibody against MBP
APS : Alkaline phosphatase buffer
BSA : Bovine Serum Albumin
BWA : Bio-Warfare Agents
CBB : Coomassie Brilliant Blue
CBQCA : 3-(4- carboxybenzoyl) quinoline-2-carboxaldehyde
D (Asp) **:** Aspartic acid
FM : Fluorescence microscopy
H (His) : Histidine
IPTG : Isopropyl β-D-1-thiogalactopyranoside
kDa : Kilo Dalton
L (Leu) : Leucine
LB : Luria-Bertani
MALDI : Matrix-Assisted Laser Desorption/Ionization
MBP : Maltose Biding Protein
MS : Mass spectrometry
P (Pro) : Proline
PBS : Phosphate buffered saline
PDMS : Polydimethylsiloxane
Q (Gln) **:** Glutamine
SAM : Self-Assembly Monolayer
SDS – PAGE: Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
SDS :Sodium Dodecyl Sulfate
SiBP : Silica Binding Peptide
TEMED : N,N,N',N'-Tetramethyl ethylenediamine
W (Trp) : Tryptophan
Y (Tyr) : Tyrosine
µCP : Microcontact printing

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ÖZET

Son yıllarda önem kazanan ve kullanım alanları genişleyen biyosensörlerin geliştirilmesinin anahtarı, uygun sensör performansı için gerekli moleküllerin biyolojik aktivitesini koruyarak biyo-uyumlu bir biyosensör ara yüzü oluşturmaktır. Ara yüz oluşturmak için kullanılan biyo moleküllerin özellikle fonksiyonel proteinlerden olan enzimlerin hassas yapıları ve hızlı denatüre olma eğilimleri sebebiyle, elektrotların veya diğer inorganik malzemelerin yüzeyine immobilizasyonu zordur. Proteinin immobilizasyonu, serbest protein kullanımına kıyasla üstün stabilite, ayırma ve yeniden kullanılabilirlik sağlar. Ancak, protein immobilizasyonu için kullanılan klasik yöntemlerin, proteinlerin işlevi üzerinde olumsuz etkileri vardır. Bu çalışmada, protein immobilizasyonunun istenmeyen etkilerini ortadan kaldırmak için silika yüzeye tam olarak tutunan peptit kullanılarak tek aşamalı, kolay, kontrollü ve yönlendirilmiş immobilizasyon gerçekleştirildi. Moleküler klonlama yöntemleriyle tasarlanan çok fonksiyonlu proteinin (MBP-SiBP) Maltoz Bağlayan Protein (MBP) içeren ifade vektörü aracılığıyla bakteride sentezlenerek affinite kromatografisi ile saflaştırıldı. İleri analiz yöntemleri olan protein tayin yöntemleri, elektroforez teknikleri ve kütle spektrofotometresi ile tasarlanan protein karakterize edildi. Kontrol olarak peptit içermeyen MBP (wt-MBP) kullanılarak MBP-SiBP çok fonksiyonlu proteinin birbiriyle fonksiyonel açıdan kıyaslandı. Silikaya bağlanan peptidin kendi kendine montajlama (self-assembly) özelliği sayesinde, MBP-SiBP proteinin herhangi bir kimyasal veya fiziksel modifikasyona ihtiyaç duymadan kontrollü immobilizasyon sağladığı, mikro baskılama yöntemi ile kanıtlandı. MBP-SiBP çok fonksiyonlu proteinin florosan bağlı antikor ve maltoz ile aktif olarak etkileşime geçmesiyle, immobilizasyon sırasında fonksiyonlarını kaybetmediği gözlemlendi.

Anahtar Kelimeler: Tek Adımlı İmmobilizasyon, Biyosensör Tasarımı, Yüzey spesifik Bağlanan Peptitler, Çok Fonksiyonlu Protein

ABSTRACT

The key to the development of biosensors, which have gained importance in recent years, is to create a biocompatible biosensor interface by preserving the biological activity of the molecules required for proper sensor performance. The biomolecules used to form the interface are difficult to immobilize on the surface of electrodes or other inorganic materials, especially due to the sensitive nature of enzymes, which are functional proteins, and their tendency to denature quickly. The immobilization of the protein provides superior stability, separation and reusability compared to the use of free protein. However, classical methods for protein immobilization have adverse effects on the function of proteins. In this study, a one-step, easy, controlled, oriented and directed immobilization was performed using the peptide that fully adheres to the silica surface to eliminate the undesirable effects of protein immobilization. Multifunctional protein (MBP-SiBP) designed by molecular cloning methods was synthesized in bacteria by expression vector containing Maltose Binding Protein (MBP) and purified by affinity chromatography. The designed protein was characterized by advanced analysis methods such as protein determination methods, electrophoresis techniques and mass spectrophotometer. Functional comparison of MBP-SiBP multifunctional protein with each other was performed using peptide-free MBP (wt-MBP) as a control. Due to the self-assembly feature of the silica-binding peptide, it has been proven by Micro-contact printing method that MBP-SiBP protein provides controlled immobilization without the need for any chemical or physical modification. It was observed that MBP-SiBP multifunctional protein did not lose its functions during immobilization as it interacted actively with fluorescent bound antibody and maltose.

Keywords: One-Step Immobilization, Biosensor Design, Surface-specific Binding Peptides, Multifunctional Protein

1. INTRODUCTION and PURPOSE

The integration of biology and materials requires the ability to design, create, manage and fabricate interfaces at the biological interface of materials, as they are essential for the application of nanotechnology, the invention of new materials and processes in molecular engineering, and the development of bio-nanotechnology. Through peptides and proteins, which are molecular communication agents, biology also regulates all interfaces between molecular materials, tissues, and organs. Proteins are, in a sense, the workforce in biology, performing chemical, physical, and biological activities. Similar to biology, we are able to choose peptides that can attach to inorganic materials to create a new essential building block that will genetically fuse biological and synthetic organizations in engineering and technological systems.

In recent years, as it has been more apparent that peptide molecules play a crucial role in influencing a broad variety of biological activities. Their usage in biotechnology and bioengineering has grown rapidly because of the easy with which they can be synthesized and manipulated. Owing to their propensity to self-assemble into higher-order structures, peptides serve as adaptable building blocks for supramolecular and flexible scaffolds. Moreover, peptides are capable of folding into mono- and bilayers, fibers, micelles, tubes, and compact structural motifs that form nanoized structures due to their modifiable physiochemical properties. Through molecular recognition during self-assembly, peptides are guided by non-covalent intermolecular interactions (electrostatic, hydrogen bonding, hydrophobic, and van der Waals). In addition, they are biocompatible, making them ideal for stabilizing unstable macromolecules such as enzymes, antibodies, and nucleic acids used in biosensors and bio-nanodevices. Peptide-based biosensors have been developed for the detection of diverse analytes, including cells, proteins, ions, and tiny molecules, (Puiu & Bala, 2018).

Biosensors are very sensitive and target-specific device, with a rapid response time, making them excellent for real-time detection. Some are reusable and transportable for investigation on-site. Currently, biosensors are used in several industries, including health, food safety, environmental monitoring, and the military (S. Li et al., 2012).

Due to their biocompatibility, electrical, optical, and mechanical characteristics, silica based materials offer the most technological advancement potential for biosensor applications. In addition, they are non-toxic, a crucial need for biomedical and biological applications. Despite their insulating and electro inactive qualities, materials based on silica are commonly employed in electrochemistry. Silica-modified electrodes have intriguing properties, such as a porous, robust inorganic framework that enables rapid mass transport in relation to material properties, the possibility of covalent bonding of functional groups to tune reactivity and recognition ability, and the ability to provide a surface for active biomolecules.

Bioavailable linkers are essential for Biosensor applications as they are adaptable and genetically modifiable. Traditional chemical methods may be replaced here by the use of linker peptides as molecular binding agents. When proteins bond to a hard surface in bionanotechnology applications such as biosensors and chips, the term "immobilization" comes to mind. Various physical and chemical processes, like adsorption, encapsulation, cross-linking, and covalent bonding, are used to achieve immobilization. Increased stability, easy separation from the reaction mixture, and the flexibility to reuse the process are all benefits of immobilizing the protein rather than using free protein. Because of the traditional non-specific adsorption immobilization techniques, proteins might end up with a random orientation, which has a considerable impact on how they function and operate (J. K. Kim, Abdelhamid, & Pack, 2019). Chemical and biological binding agents have been utilized to sustain protein function in solid matrices to this point. We used a silica-binding peptide as a binding agent in this study, and several techniques were used to demonstrate the immobilization. Additionally, we were able to avoid the drawbacks of physical or

chemical immobilization approaches while yet ensuring a precise and directional binding to the silica surface.

The overall goal of this thesis is to propose a one-step immobilization method related to the construction of stable biosensor interfaces in material recognition. Firstly, a multifunctional protein is constructed using molecular cloning techniques. Multifunctional proteins are proteins with two or more biological functions and are produced through genetic engineering or protein engineering. Due to the self-assembly ability of the peptides attached to the inorganic surface, the engineered protein is easily attached to the silica surface through a peptide without any chemical modifications. This ensures that the protein used is actively immobilized without compromising functionality, eliminating the traditional difficulties associated with immobilization.

2. GENERAL INFORMATION

2.1 Genetically Engineered Multifunctional Proteins for Biosensor

Fusion proteins are a type of unique biomolecules that have been generated as an outcome of the recombinant DNA technology. These proteins have the ability to perform many functions. The fusion protein product that results from genetically fusing two or more protein domains together may acquire several different functionalities, each of which are derived from one of the component moieties of the original proteins (H. Li, Cao, Lou, Zhang, & Liu, 2019).

Component proteins and linkers are required for the synthesis of a recombinant fusion protein, properly and both of these parts are essential to the process. The selection of the component proteins for a fusion protein product is, in most instances, a very easy process since it is determined by the required functionalities of the fusion protein product.

MBP is one of the fusion partners that have been employed for the manufacture of recombinant proteins in bacterial cells. This process involves the use of bacterial cells to produce the proteins. In addition to that, it is well recognized as a leading partner in fusion. Moreover, because of its role as a receptor for chemotaxis and transcription factors, it is a component of the maltose/maltodextrin system that is found in *E. coli.* MBP is made by E. coli as a byproduct of the maltose/maltodextrin system, which is controlled by the malE gene. One of the advantages of using Maltose Binding Protein is that it allows MBP to be produced in bacterial cells, often in a form that is secreted as well as in a form that is not secreted. Nevertheless, fusing the protein of interest to the released form of MBP causes the complex to be transported into the periplasm, which may facilitate the folding of proteins that contain disulfide linkages. When the protein is synthesized in the cytoplasm, expression levels are increased.

MBP improves the solubility of its fusion partner while simultaneously increasing the amount of synthesis that the partner is capable of producing. The exact mechanism by which this improvement takes place is not yet completely known.

Regardless of the fact that it places a significant strain on the metabolic processes of the host cell due to the substantial size of the protein, MBP is currently considered to be one of the top options for avoiding difficulties associated with heterologous expression. This is despite the fact that MBP is presently considered as one of the top options for avoiding difficulties involved with heterologous expression (about 42 kDa). MBP is better suited for the tagging of small proteins since the bacterial process is less efficient at creating large proteins (over 90 kDa), and translation may result in low productivity and partially truncated protein forms (up to 40 kDa). A protein that has been tagged with MBP can be isolated and purified with the assistance of an affinity column, which is not only user-friendly but also very inexpensive. After a single stage of capture, this kind of column may generate tagged protein that is between 70 and 90 % pure (Lebendiker & Danieli, 2017) .

Fusion systems such as the MBP allow for better expression, greater solubility and easier purification, as well as mild elution conditions. Recombinant protein expression benefits greatly from MBP as a fusion partner because of the high efficiency and wide applicability of MBP purification methods. As a result, MBP is a one of the best alternative (Lebendiker & Danieli, 2017). In contrast, the selection of an appropriate linker to unite the protein domains may be difficult and is often overlooked in the construction of fusion proteins. Direct fusing of functional domains without a linker may result in a number of unfavorable effects, such as misfolding of the fusion proteins, poor yield in protein synthesis, and diminished bioactivity. In recombinant fusion protein technology, the selection or rational design of a linker to combine fusion protein domains is crucial (Chen, Zaro, & Shen, 2013; Silver, Leonard, Gould, & Spangler, 2021)

The most fundamental function of linkers in recombinant fusion proteins is to covalently bind the functional domains (such as flexible or rigid linkers) or to release them under certain circumstances. Linkers may also play several derived roles in the design of protein drugs, such as enhancing biological activities, increasing production, and providing regulated or targeted drug delivery (Chen et al., 2013; Huang, Zhang, & Xing, 2021).

The use of inorganic binding peptides as molecular linkers in the display or immobilization of essential biomolecules through directed assembly procedures is an example of a possible use for these peptides. The simplicity with which these peptides may be coupled with functional proteins via the application of site-directed genetic recombination is one of the distinguishing aspects of these peptides. Once a comprehensive collection of peptides that can attach to inorganic surfaces has been assembled, these peptides may be used to improve the properties inorganic surfaces by functioning as specific surface-binding ligands. Surface-specific binding abilities may lead to the formation of self-forming bimolecular layers on the surface of inorganic materials.

Inorganic binding peptides have both the requisite material selectivity and a high binding affinity for the substances to which they bind, as indicated by dissociation constant (K_D) values within the range of nM to μM. Additionally, it has been shown that various distinct inorganic binding peptides may form densely packed monolayers, which is beneficial for the application of surface engineering and modification. Peptide sequences may be genetically inserted into a protein both at one of its terminals (C- or N-terminus) or at a permissive spot that has been found on the protein. Following the cloning of fusion proteins, the proteins are produced and purified using the best suitable expression techniques, which may be based on bacteria or yeast. Numerous model fusion proteins, including the discovered maltose-binding protein, DNA-binding protein and green fluorescent protein have been developed for a variety of engineering applications. The design methodologies of these fusion proteins include the controlled hierarchical assembly of hybrid nanostructures, the templating of nanoparticle synthesis, shape, and decoration, and the site-directed assembly of proteins for enhanced biosensing signaling pathways and recognition. The capacity of fusion proteins to undertake self- assembly makes all of these applications feasible. In recent years, it has been shown that engineered fusion proteins may boost the site-directed display of functional protein and enzyme motifs on a variety of metal surfaces by using specific inorganic binding tags (Akagawa, 2018; Karaca, Hnilova, & Tamerler, 2014).

2.2 Molecular Recognition and Immobilization of Biomolecules

Biosensors typically detect analytes by turning a biological reaction into an electrical or optical signal using immobilized biomolecules such as antibodies, nucleic acids, or enzymes. The initial step in the manufacture of biosensors is the creation of functional surfaces for the immobilization of just the necessary biomolecules. Physical adsorption, including electrostatic and hydrophobic contacts, covalent bonding, and specialized interactions such as DNA hybridization, antibody-antigen interaction, and biotin-avidin, provides appropriate platforms for the immobilization of desired biomolecules on functional surfaces. Immobilization of biomolecules through molecular recognition and specific interactions on surfaces, in particular, can result in good orientation and stability of the immobilized biomolecules, resulting in high functionality within the biosensor production process (Kahn & Plaxco, 2010; D. C. Kim & Kang, 2008; Seitz, Grenier, Csoros, Yang, & Ren, 2021).

Recent breakthroughs in nanobiotechnology have enabled the manufacturing of innovative nanoscale biosensors. The well-controlled immobilization of functional biomolecules on nanomaterials or nanopatterned surfaces via molecular recognition and particular interactions might improve biosensor sensitivity and specificity. Biomolecular nanopatterning has been accomplished via molecular self-assembly through molecular recognition and specific interactions in conjunction with various lithographic techniques (e.g., soft lithography, and electron beam lithography) and the synthesis of nanomaterials such as nanorods, nanoparticles, nanowires, and nanotubes (Parisi et al., 2022; Seitz et al., 2021).

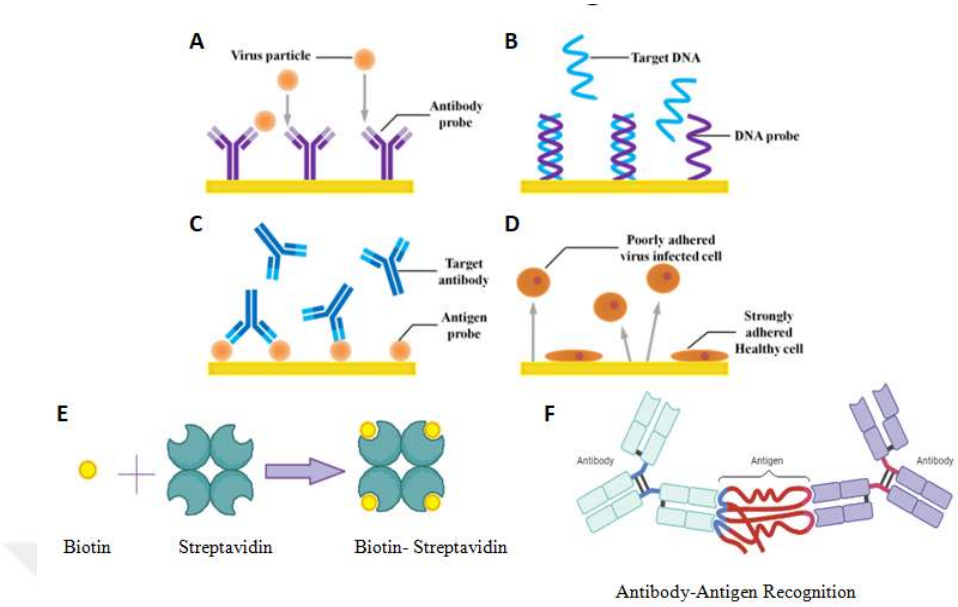

Figure 2.1: Examples of Molecular Recognition (Cheng & Toh, 2013)

2.2.1 Biorecognition Elements

A biorecognition element, or bioreceptor, is an important component of a biosensor since it enables the selective measurement of an analyte. In theory, any biomolecules or molecular assembly capable of recognizing a target analyte can be employed as a biorecognition element to provide specific and selective binding of the target analyte to the sensor surface (Bazin, Tria, Hayat, & Marty, 2017). According to their operating method, biorecognition components can be classified as catalytic or affinity. In the first example, a biological element, often an enzyme catalyzes a reaction, resulting in the biosensor response. As bioreceptor, affinity biosensors comprise antibodies, nucleic acids, and aptamer, and the analytical signal is based on the binding process of the analyte to the biorecognition element (Cesewski & Johnson, 2020; Dincer et al., 2019; Mercante et al., 2021).

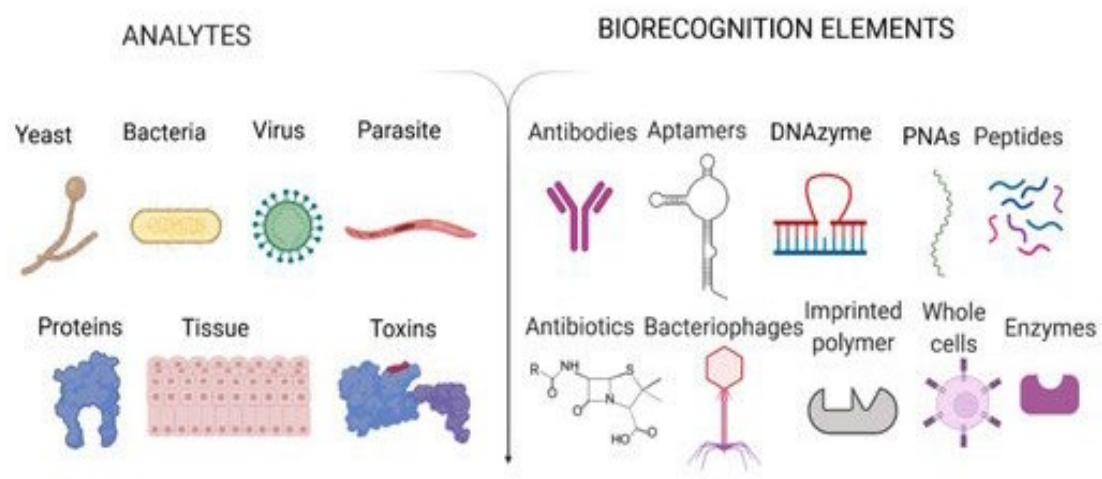

Figure 2.2: Examples of analytes and biorecognition elements. Created in BioRender.

The essential guidelines for selecting the best recognition element for a biosensing assay are described in the following:

- Identify the analyte of interest (metabolites and drugs are examples of huge entities or tiny compounds).
- Define the biosensing application's criteria, such as experimental circumstances and measurement type,
- Summarize the characteristics of all potential recognition components in order to pick appropriate candidates and evaluate their performance in order to find the best solution.

2.3 High Specific Detection Devices: Biosensors

Biosensors are analytical devices that use specific biochemical reactions mediated by cells, organelles, tissues, enzymes, or immune systems to detect chemical compounds, usually by electrical, thermal, or optical signals. Also, they measure biological or chemical reactions by generating signals proportional to the concentration of an analyte in the reaction (Banerjee, Maity, & Mastrangelo, 2021; Bhalla, Jolly, Formisano, & Estrela, 2016; Tetyana, Morgan Shumbula, & Njengele-Tetyana, 2021).

Although the concept of biosensors has been popular in recent years, it has a history dating back to 1906. The history of biosensors, which began with M. Cremer's demonstration that the acid concentration in a liquid is proportional to the electrical potential arising between portions of the liquid on opposite sides of a glass membrane, led to the development of the concept of pH (hydrogen ion concentration) in 1909 by Soren Father Lauritz Sorensen. Then, in 1922, to measure this pH, an electrode was developed by W.S. Hughes. In 1916, Edward Griffin and J.M. Nelson (Nelson & Griffin, 1916) demonstrated for the first time the immobilization of the enzyme invertase on charcoal and aluminum hydroxide (Bhalla et al., 2016; Naresh & Lee, 2021; Tetyana et al., 2021).

The "Clark Electrode" was developed by Leland C. Clark, Jr. in 1959. It is a sensor developed to detect glucose in biological samples using a glucose oxidase electrode that detects the presence of hydrogen peroxide or oxygen. Known as the "father of biosensors," Leland Clark was a pioneer in the development of the "first true biosensor." Based on this work, Guilbault and Montalvo, Jr. developed a biosensor that detects urea in 1969 (Guilbault & Montalvo, 1969). The first commercial biosensor was developed by Yellow Spring Instruments (YSI) in 1975 (Bhalla et al., 2016; Tetyana et al., 2021). Since then, significant progress has been made in the construction of extremely sensitive and selective biosensors.

2.3.1 The Basic Concept of Biosensors

A biosensor combines a biological element with an electrical component to produce a quantifiable signal. The electronic component detects, records, and communicates information on physiological changes in the environment, as well as the presence of various chemical or biological elements.

Biosensors are available in a variety of sizes and forms, and they can detect and quantify low concentrations of pathogens, harmful chemicals, and pH values. An analyte, a bioreceptor, a transducer, electronics, and a display are all components of a conventional biosensor (Bhalla et al., 2016; Naresh & Lee, 2021; Sawant & Shiralkar, 2022; Tetyana et al., 2021).

- *Analyte: A* compound of interest whose contents are being detected or recognized (maltose is an analyte in this research).
- *Bioreceptor:* A biological element or molecule that can identify or detect the analyte (MBP-QBP Fusion Protein).
    - ***Biorecognition*** refers to the process of signal generation (pH, heat, change of mass or charge, etc.) that occurs during the contact of a bioreceptor and an analyte.
- *Transducer:* A device that transforms the energy into another energy. The transducer is an important component of a biosensor. It turns the biorecognition event into a measurable (electrical) signal that corresponds to the quantity or presence of a chemical or biological target. This energy conversion process is called signalization**.**

The number of analyte–bioreceptor interactions is proportional to the number of optical or electrical signals generated by transducers. Transducers are classified as electrochemical, optical, thermal, electronic, and gravimetric transducers based on their functioning principles.

- *Electronics:* The converted signal is processed and ready for display. The transducer's electrical impulses are amplified and transformed into digital form. The display unit quantifies the processed signals.

- *Display:* The display unit is made up of a user interpretation system, such as a computer or a printer, which provides the output so that the user may read and comprehend the appropriate answer. The output might be in the form of a tabular, numerical, graphical value, or a figure, depending on the end-user requirement.

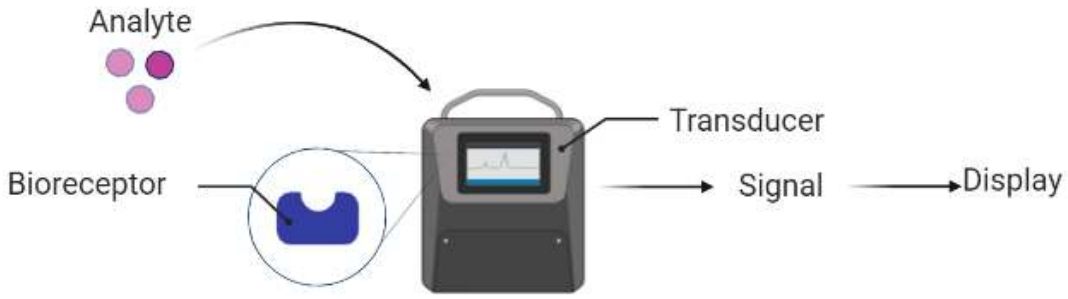

Figure 2.3: Schematic representation of Biosensor's Components

To summarize the working principle of the above-mentioned biosensor components: At first, the relevant molecule in the test sample specifically binds or interacts with the biological receptor, causing a physiological change. Thus, it changes the physicochemical properties of the transducer close to the biological receptor and leads to a change in the electronic or optical characteristics of the transducer, which is converted into a detectable electrical signal.

2.3.2 Biosensors with Silica

Due to their unique features, recent approaches in the creation of biosensors have resulted in the utilization of silica or glass materials. Because of its biocompatibility, abundance, electrical, optical, and mechanical features, silicon nanoparticles offer the greatest promise for technical improvements in biosensor applications. Moreover, silica-based materials are non-toxic, which is a critical need for biological and biomedical applications (Vigneshvar, Sudhakumari, Senthilkumaran, & Prakash, 2016).

Silica-based materials are commonly employed in electrochemistry considering their insulating and electro-inactive characteristics. Silica-modified electrodes have intriguing qualities such as a porous, robust inorganic structure that allows for rapid

mass transport in relation to material properties; the possibility of covalent bonding of organo-functional groups to optimize reactivity and identification ability; and the provision of a surface for nano-objects or active biomolecules. Silica-based materials have been utilized in electrochemical applications as nanoparticles distributed in conductive composite carbon matrices, coated as nano-labels on electrode surfaces, produced like metal or carbon-ceramic composites, or deposited as thin or thick films on solid electrodes (Vigneshvar et al., 2016; Walcarius, 2018).

Considering the unique features, silica-based materials have several uses, including bioimaging, biosensing, and cancer treatment. Several novel biosensors were developed with high-end technology for enhancing biomedicine technology, taking into account the unique properties of silica, quartz, or glass materials, although cost-effectiveness and biosafety demand attention. Silica-based materials can be prepared as biocompatible scaffolds to immobilize or protect biological objects, especially inorganic-organic biohybrids or conductive biocomposite-based electrodes. For electrochemical biosensors, specially designed nanoparticles on the electrode and porous silica thin films oriented on the electrode have gained importance in the last few years. Examples of biosensors created using a glass surface are measuring the presence of bacteria in the food industry (Massad-Ivanir et al., 2016) (Massad-Ivanir et al., 2016), and detecting glucose levels in diabetes patients (Sönmez et al., 2018), and the design of optical fiber biosensors (Wu, 2015).

2.3.3 Applications of biosensors

Typical 'off-site' analysis necessitates sending samples to a laboratory for examination. These procedures provide the best measurement precision and lowest detection limits. However, they are costly, time-intensive, and necessitate the employment of highly trained staff. Because of the aforementioned drawbacks, biosensor technology is gaining popularity. In recent years, there has been amazing progress in the field of biosensor development, with applications developing in a wide range of fields. Biosensors offer a wide range of applications that aim to improve people's quality of life. This category includes applications in disease

diagnosis, environmental monitoring, drug development, food safety, and other areas.

One of the most common uses of biosensors is the detection of biomolecules that are disease markers or therapeutic targets. Electrochemical biosensing methods, for example, can be utilized as clinical tools to identify protein cancer biomarkers (Formisano et al., 2015; Jolly, Formisano, & Estrela, 2015; Jolly, Formisano, Tkáč, et al., 2015). Biosensors may also be used to track the traceability, quality, safety, and nutritional content of food (Sharma, Ramanathan, Rakwal, Agrawal, & Bansal, 2015; Van Dorst et al., 2010). These applications are classified as "one-shot" analysis tools because they need cost-effective and biodegradable detection systems. A biosensor, on the other hand, is required to work for a few hours to a few days in an application such as pollution monitoring (Gavrilescu, Demnerová, Aamand, Agathos, & Fava, 2015; Van Dorst et al., 2010).

These biosensors might be referred to as "long-term monitoring" analytical instruments. Biosensors are used as technologically advanced devices, whether for long-term monitoring or single-shot analysis, in both limited resource and complex medical settings: for example, in drug discovery (Bhalla et al., 2015; Sönmez et al., 2018); for the detection of a variety of chemical and biological agents regarded as to be defensive toxic materials; for use in artificial implantable medical devices (Grayson et al., 2004) and other prosthetic devices. A number of electrochemical, optical, and acoustic sensing methods, as well as their incorporation into analytical equipment) has been employed for a wide range of applications (Bhalla et al., 2016).

The quality, safety, care, and processing of food items are a difficult challenges in the food processing sector. Cost-effective food verification and monitoring solutions with consistent and accurate measurement of food items are desirable for the food business. The presence of E. coli in vegetables is an indicator of fecal contamination in foods. E. coli was detected utilizing potentiometric alternative biosensing devices to detect pH fluctuations induced by ammonia. (Torun, Hakkı Boyacı, Temür, & Tamer, 2012). Another industry where biosensors are favored is the dairy business. A biosensor with a display carbon electrode is being developed to detect three

organophosphate insecticides in milk (Mishra, Dominguez, Bhand, Muñoz, & Marty, 2012).

One of the popular food additives widely used today is sweeteners that cause unwanted diseases such as dental caries, cardiovascular diseases, obesity, and type 2 diabetes. Traditional methods for the detection of artificial sweeteners are ion chromatographic methods, which are complex and laborious. A more efficient method combining lipid films as biosensors with electrochemical techniques for the fast and sensitive detection of sweeteners has been discovered by the multi-channel biosensor. Also, biosensors can be used for military purposes during biological attacks. The main motive of such biosensors is to sensitively and selectively identify organisms posing threats in virtually real-time called biowarfare agents (BWAs), namely, bacteria (vegetative and spores), toxins, and viruses. Several attempts to develop devices such as biosensors have been made using molecular techniques which are able to recognize the chemical markers of BWAs (Bhalla et al., 2016; Naresh & Lee, 2021; Song et al., 2021).

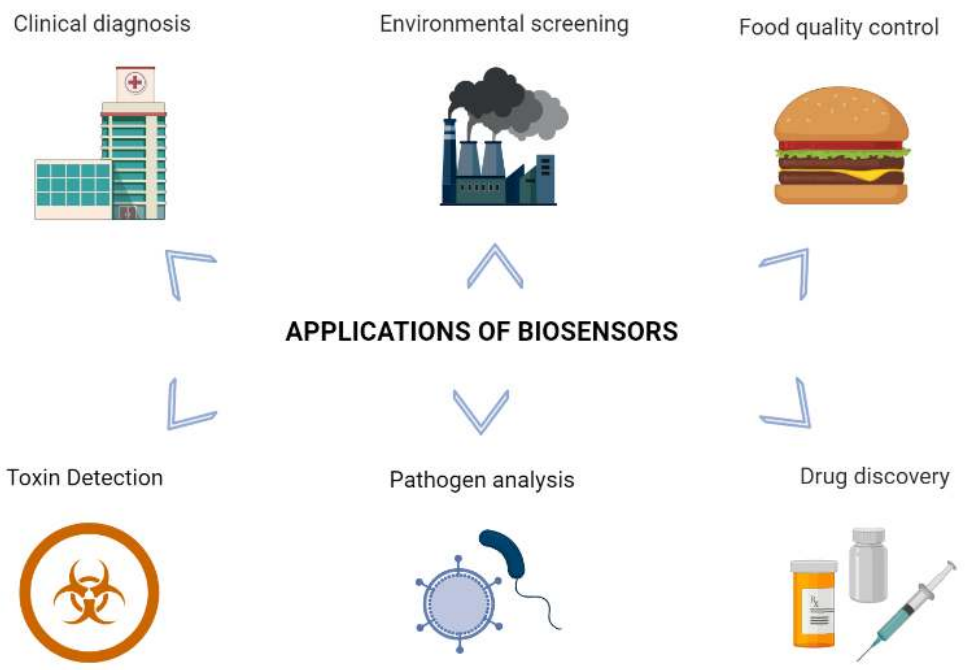

Figure 2.4: Applications of biosensors (Tetyana et al., 2021)

2.4 Interface of Biosensors

One of the most difficult tasks in biosensor design is combining the bionic system to build a functioning interface. The immobilization of biomolecules at the sensor surface is a critical stage in the process. However, because biomolecules are very sensitive to their surroundings and can denature quickly as a result of changes in their surroundings, they cannot be directly fixed on the surface of electrodes or other inorganic solids. To maintain the biological activity of molecules, which is required for normal sensor operation, a biocompatible interface layer should be present between the receptors or sensing elements and the electrode surface.

The layer is referred to as a biosensor interface in this context. The stability of the interface influences the interaction of analytes on it, which in turn influences the sensing performance of the biosensor. As a result, designing a robust interface in biosensor applications is critical (Song et al., 2021).

One of the most essential components in the manufacture and manipulation of biosensors is the design of sophisticated surfaces that provide regulated interaction with biomolecules for analyte recognition.

The sensing surface can be functionalized by attaching organic molecules by physical adsorption, covalent bonding, or biological interaction, among other methods. Undesired interactions of nonspecific molecules with the biosensor must be avoided in order to immobilize only the target biomolecules on the surface of the biosensor for detection of the desired molecule. As a result, surface engineering to manage the interface between the conjugated biomolecules and the surface is a critical precondition for effective biosensor applications. Surface chemistry and physics advancements have resulted in the construction of highly selective and more efficient surfaces at the nanoscale scale, resulting in the development of new biosensors with great sensitivity and selectivity (Dincer et al., 2019; Movilli & Huskens, 2020).

2.4.1 Biointerface Engineering Approach: Immobilization

Protein immobilization refers to the binding of proteins to a solid or hard surface and has recently emerged as a critical issue in bionanotechnology applications. One of the most important aspects of sensor preparation is the selection of the appropriate immobilization technique. This is because there is a possibility that the biorecognition element or molecule may become inactivated or leaches out after immobilization. Biomolecules should be able to maintain their structure, function, and biological activities during the use of biosensors. This immobilization is accomplished using various physical and chemical processes such as adsorption, encapsulation, cross-linking, and covalent bonding. In general, immobilization of protein improves stability, ease of separation from the reaction mixture, and process reusability compared to using free protein. The choice of an appropriate technique depends on the selected biorecognition element, transducer and physical-chemical environment, and the properties of the analyte. The immobilization process should cover the following criteria (J. K. Kim et al., 2019; Mercante et al., 2021; Naresh & Lee, 2021):

- It should guarantee that the target molecules have easy access to the active regions of the biorecognition element.
- It must provide the biomolecules with a suitable and inert environment.
- It must avoid substantial alterations to the natural structure of biomolecules, which may harm their biological capabilities and limit their utility

2.4.1.1 Physical Immobilization

This method involves binding enzymes to the surface of the transducer without any formation of chemical bonds. Physical immobilization methods include physical adsorption, and physical entrapment (microencapsulation, sol-gel technique, and electropolymerization).

2.4.1.1.1 Physical Adsorption

A biorecognition element is immobilized on the outer surface of an inert solid substance by mild attractive forces such as ionic bonding, Van der Waal's force,

hydrogen bonding, or electrostatic force in the adsorption approach. In the case of enzyme biosensors, this strategy is used. Physical adsorption has the advantage of being a simple and inexpensive procedure that is non-destructive to bioreceptor activity and does not require the alteration of biological materials or the formation of a matrix. The downsides of this approach include immobilization being sensitive to changes in temperature, pH, and ionic strength, being linked by weak interactions; and having poor operational and storage stability (Arya, Gangwar, & Kumar, 2019; Sneha, Beulah, & Murthy, 2019)

Biorecognition components are physically entrapped inside the 3D matrices through covalent or non-covalent bonds in this technique. The entrapment immobilization procedure can take two routes: the enzyme is combined into a monomer solution, which is then polymerized by a chemical reaction or by altering experimental circumstances. The biorecognition components are embedded inside a three-dimensional matrix of inorganic or organic materials. Activated carbon and porous ceramic materials are the inorganic elements employed; while gelatin, acetate, phthalate cellulose, modified polypropylene, polydimethylsiloxane, polyacrylamide, photopolymer, and alginate are the organic materials. Microencapsulation, electro polymerization, and sol-gel processes are generally used in this process.

The microencapsulation method is a technique for enclosing biorecognition components (enzymes) in a spherical semi-permeable membrane. Lipoprotein-based, lipoidal, polymeric, or non-ionic membranes are all possible. Two approaches are chosen for microencapsulation: polymerization of a monomer at the interface of two immiscible substances (interfacial polymerization), and phase separation of enzyme micro-droplets in water-immiscible liquid phase (Arya et al., 2019; Naresh & Lee, 2021; Nguyen, Lee, Lee, Fermin, & Kim, 2019; Sneha et al., 2019)

Electropolymerization is a basic procedure that involves applying a current or potential to an aqueous solution or electrolyte that contains both biomolecules and monomer molecules. On the surface of the electrode in the electrolyte, either reduction or oxidation of the monomer occurs, generating reactive radical species that participate together and lead to the creation of a polymer, which traps the

biorecognition components (enzymes) that are close to the electrode in the solvent. Thiophene, pyrrole, and aniline electro-polymerized films are employed for enzyme immobilization (Naresh & Lee, 2021; Nguyen et al., 2019).

The Sol-gel procedure is the most frequently utilized technology for enzyme trapping at low temperatures. The hydrolysis and condensation of metal alkoxides result in the formation of a nanoporous substance. The bio-elements are enclosed in a 3D matrix as the network expands with time and temperature. Chemical and thermal stability, ease of production, and the capability to encapsulate large quantities of biomolecules under moderate immobilizing conditions are all advantages of this approach (Naresh & Lee, 2021; Nguyen et al., 2019; Sneha et al., 2019).

2.4.1.2 Chemical or Irreversible Immobilization

This approach is concerned with the development of strong chemical interactions, such as covalent binding or covalent linking, between the functional groups of biorecognition elements and the transducer surface. Chemical immobilization techniques are classified into two types based on chemical bonding: covalent cross-linking and direct covalent binding.

2.4.1.2.1 Cross-linking

Cross-linking occurs by the formation of intermolecular covalent cross-linkages between biorecognition elements and functionally inactive proteins or biorecognition elements (enzymes). This procedure is carried out with the assistance of multi-functional chemicals that serve as linkers to join enzyme molecules in 3D cross-linked clusters to the transducer surface. The ideal cross-linking conditions include ionic strength, temperature and pH. It enables stronger adhesion, better enzyme catalytic activity, and faster response time.

Stronger Chemical binding, less enzyme leakage, and the ability to adapt to the environment for the bioreceptor use of suitable stabilizing agents are the advantages. However, the formation of covalent cross-links between protein molecules rather than matrix and protein, as well as denaturation of this partial protein structure, is a disadvantage that limits the application of crosslink immobilization.

2.4.1.2.2 Covalent binding

The most common method of enzyme immobilization is direct covalent binding, wherein the biorecognition element is securely attached to either the inert matrix of the membrane or the electrode/transducer surface. The immobilization technique via the membrane matrix consists of two steps: functional polymer synthesis and covalent immobilization. The binding process is based on the interaction of the biorecognition element with functional protein groups (often amino acid side chains) and reactive groups on the transducer/membrane matrix interface. Direct covalent binding has several benefits, including high tolerance to environmental changes, limited leakage of the biorecognition element, and strong bond formation between the matrix and biorecognition element. The primary drawbacks of this technology are the usage of harsh chemicals and the inability to replenish the created matrix after it has been utilized.

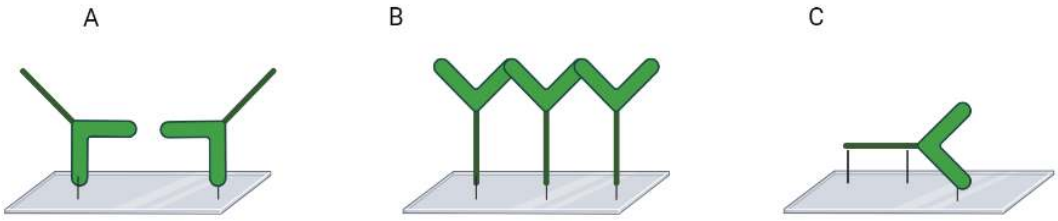

Figure 2.5: Potential immobilization problems (A) improper orientation, (B) Binding site blocking, (C) multi-site attachment (Hage, 1999)

2.4.2 New Approach for Immobilization: Peptide

Unfortunately, traditional protein immobilization approaches based on non-specific adsorption frequently result in random protein orientation, which significantly affects protein functionality or activity. One method for overcoming these constraints is to place a linker between the protein and solid matrices. To keep protein functioning on solid matrices, several chemical and biological linkers have been used. Biological linkers, such as peptides and proteins, are thought to be more effective instruments for direct immobilization of functional proteins on solid substrates than chemical linkers, which need harsh and non-eco friendly conditions (J. K. Kim et al., 2019).

These biological linkers are termed solid-binding peptides or proteins. Ceramics such as Titania and silica have been the most commonly utilized solid supports for protein immobilization among solid inorganic materials. Immobilization on hydrophobic surfaces has been shown to cause conformational changes in immobilized proteins. Immobilization of target protein on hydrophilic surfaces was found to better preserve natural protein characteristics.

Direct interactions between the surface and the protein, on the other hand, may result in undesired conformational consequences and steric hindrance. As a result, various solid-binding peptides and proteins were employed to cost-effectively guide protein immobilization onto unmodified solid matrices without compromising the natural protein characteristics.

In this study, immobilization was achieved by using a peptide bound to silica as the binding agent. Thus, the mentioned negative effects were eliminated, and a fusion protein was formed without harming the functionality of the protein. Until now, various chemical and biochemical binders have been used to preserve protein functionality in solid matrices. Biological binders such as peptides and proteins are considered effective tools for the direct immobilization of functional proteins on solid supports compared to chemical binders that require harsh and environmentally unfriendly conditions. Among solid inorganic materials, ceramics such as silica and titania have been the most commonly used solid supports for protein immobilization. It has been determined that immobilization on hydrophobic surfaces can cause conformational changes of immobilized proteins. In contrast, immobilization of the target protein on hydrophilic surfaces such as silica and titania was found to better preserve the native protein properties (Gori & Longhi, 2016; Kruis, Löwik, Boelens, Van Hest, & Pruijn, 2016). One study demonstrated the effect of a site-specific bioconjugation strategy by selectively immobilizing maltose binding protein (MBP) via the copper-catalysis variant (CuACC) on a glass surface and evaluating its binding activity compared to randomly bound MBP. The results clearly demonstrated that immobilized MBP retained much higher binding activity than randomly paired MBP (Lin et al., 2006). In another study, functionalization of a Si-SiO2 substrate was provided using a clickable polymeric coating to enable binding of

azido-modified peptides to demonstrate the advantages of directed immobilization, so that the correct orientation of the peptidic probes was shown to be significantly effective for optimal ligand-target antibody interaction (Zilio et al., 2015).

2.5 Self-Assembly Monolayers (SAM)

Layer-by-layer (LBL) self-assembly, self-assembly monolayer (SAM) technique, and Langmuir–Blodgett (LB) approach have received the most attention in studies on monolayer membranes. On the biofunctionalized surface, these approaches may be employed to generating an almost complete monolayer with an extremely dense molecularly organized structure. The surface loading of biomolecules is relatively high, which might be advantageous in the construction of highly selective and sensitive sensors. The careful tuning of sensor surfaces by LB/SAM/LBL provides great opportunities for the creation of novel biosensor surfaces. Herein, SAM is presented in detail since it is one of the most often used manufacturing processes in biosensor interfaces (Song et al., 2021).

Self-assembly refers to the spontaneous production of ordered structures through a stochastic process that incorporates pre-existing components, is reversible, and can be regulated by suitable design of the components, the driving force, and the environment. The chemical adsorption of molecules at the liquid-solid interface results in the formation of a self-assembled monolayer (SAM). The usual technique of preparation is to immerse an appropriate solid support in a solution containing self-assembling molecules. Adsorption occurs before molecular organization during SAM growth (Koutsopoulos, 2018).

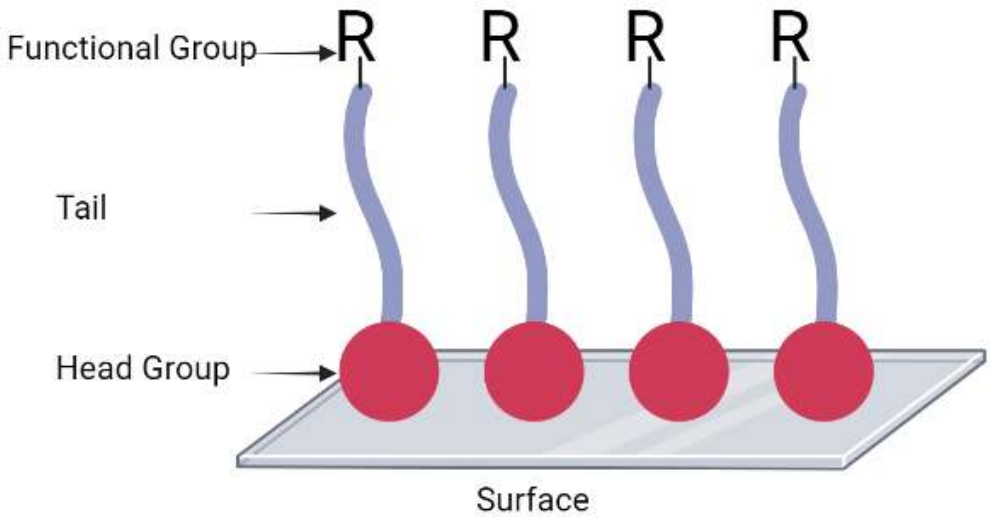

Figure 2.6: Schematic representation of SAM

When generating functional bio-scaffolds using PDMS stamping, genetically designed peptides may be employed as inks instead of traditional inks. Quartz surfaces, which are often treated with silane chemistry in order to facilitate facile covalent bonding, are good candidates for applying this. Techniques that are now considered to be state-of-the-art make use of genetically modified peptides in order to define peptide affinity to certain surfaces in order to employ such surfaces as biosensors.

For the development of innovative chemo sensors and biosensors, hybrid materials that are formed of nanostructures of noble metals and are functionalized with protein receptor molecules (such as antibodies and enzymes) have garnered a lot of attention in recent years. In various biosensor applications, the assembly of the recognition layer has been accomplished by a variety of methodological techniques. The first method involves immobilizing the proteins via a process known as physical adsorption. This method does not include the addition of any tags or chemical groups. The fact that the protein is immobilized in an unoriented form is the most significant limitation of this technique. This results in a decrease in the activity of the protein that is adsorbed on the surface. This is because the adsorption of the protein's active site might be triggered by uncontrolled and unoriented adsorption, which

causes the active site to be adsorbed. Using links that are based on thiols or silanes is another strategy, which may be used to create an active chemical layer on the surface of interest. After the surface was prepared, other protein groups, such as amines or carboxyl, were used to immobilize the proteins on the newly created chemically active surfaces. On the other hand, the immobilization of protein on a target surface is performed in a way that is completely at random. Because of this, the use of inorganic binding peptides may be a particularly useful tool for the assembly of biomolecules on any inorganic surface of choice, which can then be put to use in a wide variety of applications, such as sensors and medical equipment.

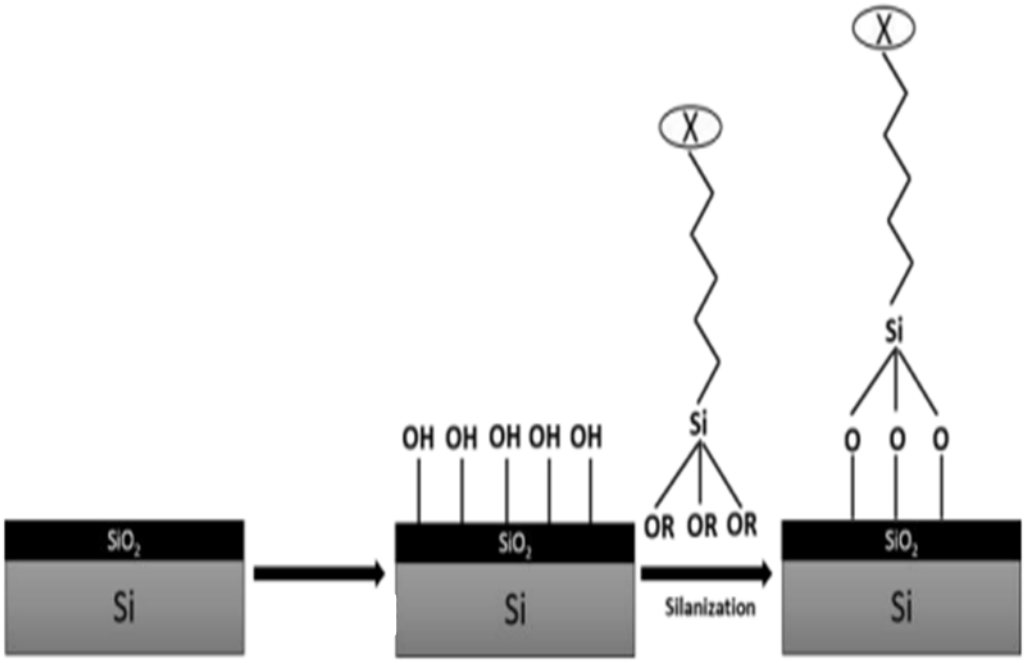

Figure 2.7: SAM Examples for silane with silica surface (Singh, Kaur, & Comini, 2020)

2.6 High Specific Detection Devices: Biosensors

Biosensors are analytical devices that use specific biochemical reactions mediated by cells, organelles, tissues, enzymes, or immune systems to detect chemical compounds, usually by electrical, thermal, or optical signals. Also, they measure biological or chemical reactions by generating signals proportional to the

concentration of an analyte in the reaction (Banerjee et al., 2021; Bhalla et al., 2016; Tetyana et al., 2021).

Although the concept of biosensors has been popular in recent years, it has a history dating back to 1906. The history of biosensors, which began with M. Cremer's demonstration that the acid concentration in a liquid is proportional to the electrical potential arising between portions of the liquid on opposite sides of a glass membrane, led to the development of the concept of pH (hydrogen ion concentration) in 1909 by Soren Father Lauritz Sorensen. Then, in 1922, to measure this pH, an electrode was developed by W.S. Hughes. In 1916, Edward Griffin and J.M. Nelson (Nelson & Griffin, 1916) demonstrated for the first time the immobilization of the enzyme invertase on charcoal and aluminum hydroxide (Bhalla et al., 2016; Naresh & Lee, 2021; Tetyana et al., 2021).

The "Clark Electrode" was developed by Leland C. Clark, Jr. in 1959. It is a sensor developed to detect glucose in biological samples using a glucose oxidase electrode that detects the presence of hydrogen peroxide or oxygen. Known as the "father of biosensors," Leland Clark was a pioneer in the development of the "first true biosensor." Based on this work, Guilbault and Montalvo, Jr. developed a biosensor that detects urea in 1969 (Guilbault & Montalvo, 1969). The first commercial biosensor was developed by Yellow Spring Instruments (YSI) in 1975 (Bhalla et al., 2016; Tetyana et al., 2021). Since then, significant progress has been made in the construction of extremely sensitive and selective biosensors.

3. Materials & Methods

3.1 Materials

Materials are shown in appendices.

3.1.1 Bioengineering of MBP-SiBP Fusion Protein

In this thesis, it is shown that the self-assembly ability of the silica-binding peptide SiBP is very efficient in the immobilization of fusion molecules and nanostructures on the silica surface. It is feasible to create it as an MBP-SiBP by separately fusing the C-terminus of the maltose-binding protein (MBP) with the genetically distinct SiBP peptide tag.

Cloning techniques were used to link the two functional domains, SiBP and MBP.. In this particular configuration, two separate spacers were inserted between MBP and SiBP to account for the effects of molecular linkers on the fusion protein function. Two different linkers were used to combine the functional domains. One of the linkers is flexible, SGGG, and the other is rigid, PGPGPG. MBP-S$(G)_3$-SiBP and MBP-$(PG)_3$-SiBP encoded by generated pMAL-SiBP vectors (Figure 7.1) were expressed in *Escherichia coli (E. coli)* ER 2507 cells as well as wild-type MBP. The pmal-c2X-linker-SiBP expression vector was generated by my supervisor of this thesis, Assist. Prof. Dr. Banu TAKTAK KARACA

Table 3.1: Sequence of Silica Binding Peptide

Silica Binding Peptide Sequence	LPDWWPPPQLYH

3.1.1.1 Expression Vector for Wt-MBP expression

The design vector in this thesis was provided by Dr. Banu Taktak Karaca, the supervisor of this thesis shown in Figure 3.1. The pMAL™-2 vectors allow for the expression and purification of proteins derived from cloned genes. The cloned gene

is inserted downstream from *malE* gene of *E. coli*, which codes for a maltose-binding protein (MBP), resulting in the production of an MBP fusion protein.

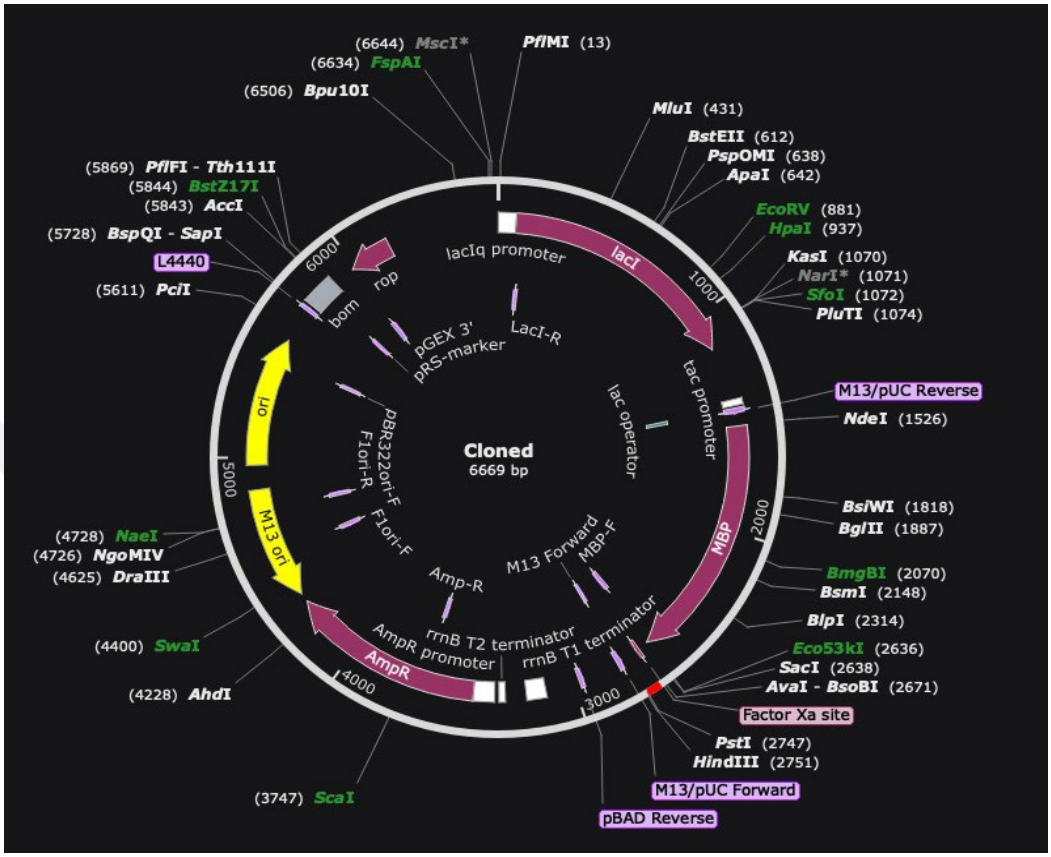

Figure 3.1: Expression Vector of wt-MBP

Recombinant vectors of MBP-Linker-SiBP are shown in Figure 3.2 and Figure 3.3. DNA sequence of both MBP-Linker- SiBP are shown in red, protein sequence are shown in yellow.

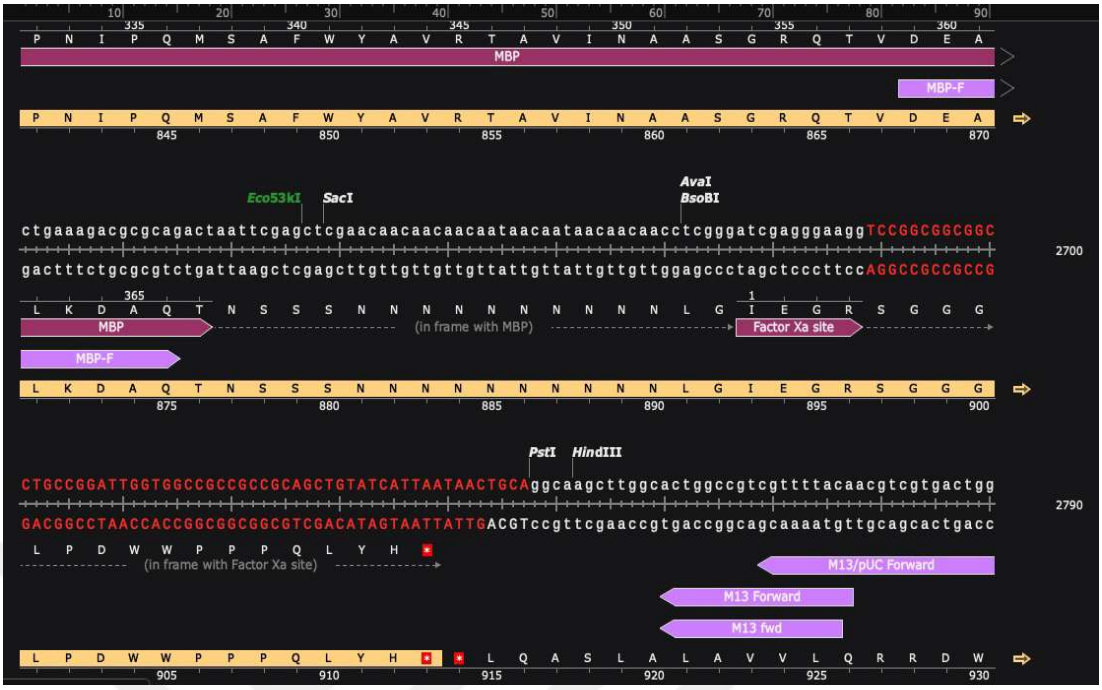

Figure 3.2: Recombinant Vector of MBP-S(G)$_3$- SiBP Vector

Figure 3.3 : Recombinant Vector of MBP-(PG)$_3$-SiBP

3.2 Methods

3.2.1 Activation of Cells in Glycerol Stock (-80 °C)

All cells are provided as glycerol stock by Dr. Banu Taktak Karaca Normally, glycerol stock cells are stored in the freezer at -80 °C degrees for years. In order to express protein in bacteria the cell should be activated and grow at 37 °C.

3.2.2 Preparing LB Agar Plate

To successfully cultivate bacteria, they must be grown in the appropriate medium. LB, also known as Luria-Bertani Broth, is a nutrient-dense broth that is the industry standard for cultivating Escherichia coli because it provides quick growth and high yields. In addition, the addition of agar to LB broth produces a gel for bacterial growth, which is utilized for plating bacterial cultures on Petri plates.(Sezonov, Le Joseleau-Petit, & Ari, 2007)

10 g tryptone, 5 g NaCl,5 g yeast extract, and 15 g LB agar were dissolved in 1 L distilled water, mixture was distributed to the Erlenmeyer as 100 ml and sterilized in an autoclave for 15 min at 121 °C. The molten agar mix was retrieved from the autoclave and 100 µl of Ampicillin was added, allowing it to cool down enough to handle. LB Agar medium was quickly and evenly poured into the bottom of Petri dishes, capped, and allowed to cool and solidify completely. For each 3 Petri dishes were labeled as wt-MBP, MBP-$(PG)_3$-SiBP, and MBP-$SG_{(3)}$-SiBP.

Then, the lab bench was sterilized with 70% ethanol and sterility was maintained by working near a Bunsen burner. A wire loop was sterilized by passing it through a flame and waiting for to cool before touching bacteria. Each bacteria was picked up from glycerol stock and the streak-plate procedure was performed. Plates were incubated overnight at 37 °C.

3.2.3 Protein Expression Studies

Wild type (Wt) and recombinant proteins were expressed in E.coli bacteria. First a small-scale experiment was carried out to determine the behavior of a specific Wt-MBP and fusion proteins (recombinant proteins) to optimize expression level and purification steps. During the expression process, a single colony carrying just pMALc2X-S$(G)_3$-SiBP and pMALc2X- $(PG)_3$-SiBP was chosen with a sterile toothpick from each LB agar plate of the ER2507 strain and cultured in 5 ml LB broth with Ampicillin at 100 µg/ml overnight to evaluate target protein synthesis.

IPTG Optimization

Bacteria containing genes of recombinant proteins were grown and expressed in 5 ml LB broth to determine optimum IPTG concentration. To do this, the different concentrations of the inductor (IPTG) varied from 0.1 to 0.4 mM at 37 °C were tried and the best expression level was achieved with 0.3 mM IPTG in Figure 4.1. The post-induction time of protein expression was tried for 3h as the master thesis of Dr. Banu Taktak Karaca.

An overnight cell culture containing 100 µg/ml of Ampicillin, 2% glucose, and 0.8 ml of fusion plasmid was inoculated with LB with 80 ml of rich broth. The bacterial culture was grown on a shaker at 200 rpm at 37°C until the optical density OD600 was ~0.5. Before induction with IPTG, a 1 ml sample was taken to analyze the uninduced cell by SDS-PAGE and centrifuged for 2 minutes (sample 1: uninduced cells).The supernatant was separated and the cells were suspended in 50 µl of 2X SDS-PAGE Sample Buffer by vortex and frozen at –20°C.

Then IPTG was added to the remaining culture until a final concentration of 0.3 mM was reached. The induced cells were incubated at 37°C for 3 hours. To analyze whether the induced cells expressed protein or not, a 0.5 ml sample was taken and centrifuged for two minutes (sample 2: induced cells). The supernatant was discarded and the cells were resuspended in 100 µl of 2X SDS-PAGE sample buffer, vortexed and frozen at –20°C. Additional time intervals of 1 and 4 hours are useful to determine when to harvest cells for large-scale preparations. Cells were harvested by centrifugation at 4000 x g for 10 minutes. The supernatant was removed and the

pellet was frozen overnight at -20°C.While cells were being disrupted, the frozen pellet was suspended in 5 ml of column buffer and thawed on ice. The cells were placed on ice and then sonicated in 10-second pulses on and 10-secondpulses off at amplitude of %30. To monitor the released protein after disruption of cells and to determine the amount of sonication, Bradford assay was applied to the sonicated samples, each time after the application with ultrasound. 10 µl of the sonicated sample was added to 1.5 ml of Bradford reagent and mixed,, then the formation of blue color was observed. The dark blue color means that more protein is present. Sonication was continued until the amount of protein released reached a maximum (usually about 2 minutes). The sonicated cells were centrifuged at 9,000 x g at 4°C for 20 minutes. The supernatant was poured and saved on ice. The pellet was resuspended in a 5 ml Column Buffer. This is an insoluble material suspension. 5 µl of 2X SDS-PAGE Sample Buffer was added to 5 µl of the crude extract and insoluble matter fractions (samples 3 and 4, in order).

In a microfuge tube, approximately 200 µl of amylose resin was put in and briefly spun in a micro centrifuge. Aspiration was used to remove and discard the supernatant. The resin was suspended in a 1.5 ml column buffer and the supernatant was removed after brief centrifugation. The resin was resuspended in 200 µl of Column Buffer once again. 50 µl of the crude extract was combined with 50 µl of amylose resin slurry.

The mixture was incubated on ice for 15 minutes before being centrifuged for 1 minute, and the supernatant was discarded. After washing the pellet with 1 ml of Column Buffer and centrifuging for 1 minute; the resin was resuspended in 50 µl of SDS-PAGE Sample Buffer (sample 5: protein bound to amylose).

3.3 Characterization of MBP-SiBP Multifunctional Protein

3.3.1 The Bradford Assay for Protein Determination

The concentration of protein was determined according to the Bradford Assay to analyze the sonication efficiency and to measure the amount of the fusion protein in

total cell protein. (Catalog number B 6916) As a standard, bovine serum albumin (Sigma) was utilized, and Bradford reagent was purchased from Sigma.

3.3.2 Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) was carried out utilizing a vertical gel electrophoresis technique, substantially as reported by Laemmli (Laemmli et al., 1970). Proteins were resolved on 12% (w/v) acrylamide slab gels in the presence of 10% SDS. Proteins were dyed with CBB-G250 and then stained for 30 minutes in the stain solution on a rocker at 15.000 rpm at room temperature. In a destain solution, proteins were destined for overnight on a rocker at 15 rpm at room temperature. A 30% acrylamide mix was prepared by dissolving acrylamide and N, N'-Methylenebisacrylamide in ddH2O. After that, the mixture was filtered by using a 0.45 μm cellulose acetate membrane filter.

Solutions for preparing SDS-PAGE gels

To generate 12% resolving gels for SDS-PAGE, all of the solutions in Table 3.1 were mixed and to prepare a %4 stacking gel for SDS PAGE, all ingredients in Table 3.2 were mixed.

Table 3.1: Ingredients of 12% of separating gel.

Components	Volume
ddH2O	1.6 ml
30% acrylamide mix	2.0 ml
1.5 M Tris Buffer, pH 8.3	1.3 ml
10% SDS	50 µl
10% APS (fresh)	40 µl
TEMED	10 µl

Table 3.2: Ingredients of 4% of separating gel.

Components	Volume
ddH2O	1.36 ml
30% acrylamide mix	0.34 ml
1 M Tris Buffer, pH 6.8	0.26 ml
10% SDS	20 µl
10% APS (fresh)	20 µl
TEMED	2 µl

CBB-G250 Stain Solution

All components given in Table 3.3 were mixed, and then filtered through chromatography paper.

Table 3.3: Ingredients of CBB-G250 Stain Solution

Components	Volume
dH2O	650 ml
Isopropanol	250 ml
Acetic acid glacial	100 ml
CBB-G250	2.5 g
Total	1000 ml

Destain Solution

All components given in Table 3.4 were mixed to prepare the destain solution.

Table 3.4: Ingredients of destain solution

Components	Volume
dH2O	650 ml
Methanol	250 ml
Acetic acid glacial	50 ml
Ethanol	200 ml

Components	Volume
Total	1000 ml

3.3.2.1 MALDI-TOF Mass Spectrometry

Mass spectrometry (MS) was used with the purified wt MBP and the MBP-SiBP fusion proteins to validate the purity and identify them as separately produced proteins. To achieve a strong signal, desalted samples were diluted in a matrix solution; 5 mg/ml Sinapinic acid (SA) (Sigma) in water, acetonitrile solution (50%: 50% vol: vol), and spotted onto the MALDI sample plate using 2 µl/well. The droplets were dried before being placed in the mass spectrometer.

3.3.3 Protein Expression and Purification on a Large Scale

The method used in this study was designed for the purification of a soluble fusion protein expressed in the cytoplasm of a pMALc2X vector. The method was as follows:

1 L rich broth containing 2% glucose and Ampicillin of 100 µg/ml with 10 ml of an overnight culture of cells containing the fusion plasmid was inoculated. For 3 hours, the cells were grown until they reached 0.5 of OD_{600}, 2 x 108 cells/ml. IPTG was added to a final concentration of 0.3 mM. The cells were incubated for 3 h at 37◦C.

Then, the cells were harvested by centrifugation for 20 min at 4000 x g and the supernatant was discarded. Harvested cells were frozen overnight at –20 ◦C and thawed on ice, and then cells were suspended with 50 ml column buffer containing 2-mercaptoethanol. The sample was placed on ice and sonicated in short pulses of 10 seconds, and 10 seconds stopped in 1.5 minutes. The sonicated cells were centrifuged for 30 minutes at 9,000 x g and the supernatant, called crude extract, was saved. The crude extract was diluted at a ratio of 1:4 with column buffer. The amylose resin column was packed when the entire cell protein analysis was considered. 8 column volumes of Column Buffer were used to wash the column. The diluted crude extract was loaded at a flow rate of 1 ml/min for a 2.5 cm column. The

loaded column was washed with 12 column volumes of Column Buffer. The fusion protein was eluted by using Column Buffer and 10 mM maltose as the elution buffer, and the fractions were identified using UV absorbance at 280 nm and SDS PAGE.

The protein-containing fractions were pooled and concentrated in an Amicon® Ultra 15 Centrifugal Filter with a 30,000 NMWL membrane (Millipore, Catalog # UFC9 030 24). The protein buffer was replaced with water, and the protein in water was lyophilized for long-term storage and potential applications.

3.3.4 Immobilization of Multifunctional Protein

3.3.4.1 Self-assembly of Multifunctional Protein (MBP-Linker-SiBP) on Silica Surface

100 µL of reaction solution containing 50 µM of multifunctional protein was applied to the patterned side of the PDMS stamp and incubated at room temperature for 1 hour. The protein solution was carefully withdrawn off the surface of the stamp by using a pipette and then dried with nitrogen after a quick wash with DI water. Isopropanol and ethanol were used to fully rinse silica substrates before drying with inert gas. The clean substrate was then placed into contact with the surface of the stamp and gently pushed for 10 seconds, before being held on the stamp surface for up to 10 minutes.

The substrate was detached from the stamp, and the side that had contact with the stamp was washed for 2 minutes with DI water before being dried under nitrogen flow. Fluorescence microscopy (Nikon, Yokohama, Japan) and Metamorph Software were used to characterize the patterned silica substrate (Universal Imaging LLC, Burbank, CA).

3.3.4.2 Micro-contact Printing µCP) of Multifunctional Protein on Silica surface

The silica surface (1cm X 1cm) was then ultrasonically cleaned for 5 minutes in a cleaning solution (methanol/acetone (1:1), isopropyl alcohol, and DI water), followed by nitrogen gas drying. Stamps made of polydimethylsiloxane (PDMS)

were ultrasonically cleaned in ethanol and DI water for 20 minutes before being dried with nitrogen gas. For 10 minutes, 100 µl of 20 µM MBP-SiBP constructions and wt-MBP solutions were incubated on the patterned side of a clean PDMS stamp. The surplus protein solution was carefully removed from the surface of the stamp using a pipette, and the stamp was dried with nitrogen gas after a quick wash with DI water.

Silica substrates were cleaned completely with isopropanol and ethanol before being dried with inert gas. One clean silica surface was placed on the protein-loaded stamp, lightly pressed for 10 seconds, and remained on the stamp surface for up to 10 minutes. The substrate was detached from the stamp, and the side that had contact with the stamp was washed for 1 minute with DI water before drying under a nitrogen gas flow. The unreacted silica surface was blocked for 1 hour with 1% BSA in PBS solution, followed by a 1 minute wash and dried with nitrogen gas. After 30 minutes of incubation with fluorescently labeled anti-MBP (New England Biolabs, Catalog # E8032S), the immobilized MBP fusion proteins (multifunctional protein) were observed using the Zenon-Alexa-488 labeling kits (Life Technologies, Catalog # Z-25004 or Z-25102) as described in the following: 2 µL of anti-MBP solution were combined with 5 µL of Zenon-Alexa-488 labeling dye and 8 µL of PBS and shaken on ice for 30 minutes at a slow pace.

The mixture was diluted to 500 µl with reaction buffer (PBS) and applied to the protein-stamped silica surface for 1 hour at room temperature. Finally, the substrate was gently rinsed with DI water for 1 minute, dried, and visualized with a fluorescence microscope (Nikon, Yokohama, Japan) and Metamorph Software (Universal Imaging LLC, Burbank, CA).

3.3.5 Protein-surface Interactions Characterization

3.3.5.1 Fluorescence Microscopy (FM)

The dried substrates were taped on glass slides and viewed with a Nikon Eclipse TE-2000U Optical Microscope (Nikon, Japan) with a Hamamatsu ORCA-ER cooled charge-coupled device (CCD) camera. Nikon FITC (exciter 460–500, dichroic 505,

emitter 510–560). Nikon's 50X and 100X DF lenses, as well as a DF filter set, kept the camera in dark-field mode. The light source was a mercury illumination (X-cite 120, EXFO, USA). Metamorph Software is used to capture the photos (Universal Imaging, USA).

3.3.5.2 Depletion Assay

In this section, to quantitation of our multifunctional proteins via depletion assay was performed by using CBQCA Protein Quantitation Kit (Invitrogen™) according to manufacturer's protocol. The interaction of the 3-(4- carboxybenzoyl)quinoline-2-carboxaldehyde (CBQCA) dye with the main amines in protein is used in the fluorometric CBQCA technique (You, Haugland, Ryan, & Haugland, 1997) .

In order to reach the desired final volume of 135 µL, the protein samples were prepared in a buffer containing 0.1 M sodium borate and pH 9.3. After thoroughly combining the 5 µL of 20 mM KCN that had been added to each protein sample, a 40 mM ATTO-TAG CBQCA DMSO stock solution was then allowed thawing at room temperature. After diluting in the stock solution, which was a 0.1 M sodium borate buffer with a pH of 9.3, a working solution of 5 mM was obtained. Each sample of KCN-protein had 10 µL of the aqueous working solution of CBQCA added to it, and the mixture was well combined. The reactions were first covered with aluminum foil to shield them from the light, and then they were incubated at room temperature while being shaken for at least an hour. Following the duration of incubation, the fluorescence emission was measured at a wavelength of 550 nm, whereas the excitation wavelength was 465 nm.

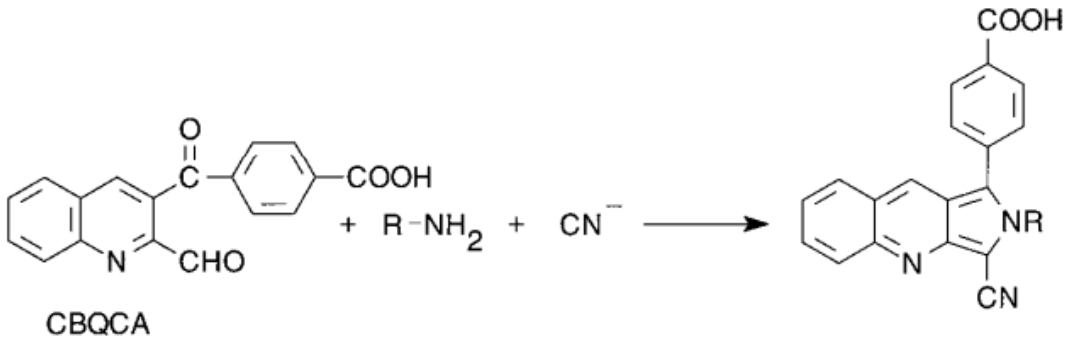

Figure 3.4: Reaction of protein with CBQCA (You et al., 1997)

3.3.5.3 Cytation Hybrid Multi-Mode Reader

Cytation 3 is a multi-mode micro plate reader for cell imaging. This system includes automated digital microscopy as well as traditional micro plate detection. Furthermore, for unrivaled adaptability and performance, Cytation 3 has both high sensitivity filter-based detection and a versatile quadruple monochromatic-based system. This system's upgradable fluorescent cellular imaging module allows for extensive cellular imaging analysis without the complexity. Fluorescence microscopy is an effective method for observing biological responses in order to better understand protein expression, cell proliferation, other expressions and cytotoxicity. It can do typical quantitative fluorescence measurements as well as cell imaging with orbital shaking control systems, gas such as CO_2/O_2, and temperature. Cytation Hybrid is compatible with Gen5™ Microplate Reader and Imager Software (BioTek), which is particularly developed to facilitate plate reading and picture recording.

4. RESULTS

4.1 Producing MBP-SiBP Fusion Proteins through Genetic Engineering

Fusion protein derivatives with specified SiBP tags and MBP were genetically generated by applying two structurally unique spacing motifs between the peptide and protein to show that the concept described in Section 3.2.1 was applicable. This was done in order to genetically engineer fusion proteins with specific SiBP tags. In order to demonstrate that the hypothesis has a chance of being correct, this task was carried out. It has been shown that the creation of linkers that are capable of physically separating two functional domains is of the utmost importance for the productive production and fabrication of hetero-functional protein units (Chen et al., 2013; Silver et al., 2021).

In specifically, MBP-SiBP fusion constructs that either contained a flexible $S(G)_3$ or rigid $(PG)_3$ linker that separated the SiBP from the MBP-tag were developed. These linkers were used to generate the fusion protein.

4.2 Expression Studies and Purification Analysis of MBP and MBP-SiBP multifunctional protein

In order to create different protein versions of MBP- $S(G)_3$ -SiBP and MBP$(PG)_3$ -SiBP, bacterial host cells of the strain *E.coli* ER 2507 were employed. It has been shown that the bacteria *E.coli* is capable of producing these proteins in certain circumstances.

This strain was selected because the *malE* gene, which is responsible for encoding maltose-binding protein, is deleted together with the *malB* gene as part of the *malB* deletion. Along with the *malB* gene, the *malE* gene was also removed from this strain in order to prevent it from generating MBP. Because of this, purifying only the fusion proteins may be a very beneficial process. During the process of expressing

and purifying the wild-type MBP protein, the *E. coli* TB1 strain was used as a bacterial host organism. Since the hsdR gene in the JM83 strain has been changed, there is no question that it is genetically connected to the TB1 strain. Due to the presence of the *lacZ-M15* allele, complementation is a distinct possibility. *E. coli* is the host organism preferred for the specific expression of desired recombinant proteins, regardless of the original source. Because it can produce high amounts of recombinant proteins in controllable mean, the inducible T7 RNA polymerase is one of the most popular *E. coli* expression systems. Using IPTG, the expression of the target protein can be inducted efficiently. By varying inducer concentration, these inducible expression systems are able to control the exact growth and express the desired protein amount (Briand et al., 2016; Marbach & Bettenbrock, 2012).

The concentration of IPTG was optimized before the protein extraction step in order to reach the high level of protein. Preparing stock solutions of varying concentrations (0.1-0.4 mM) allowed for the accurate final IPTG concentration. The optimum IPTG concentration at 37 °C was determined as 0.3 mM for protein expression according to results of optimization experiments (Figure 4.1).

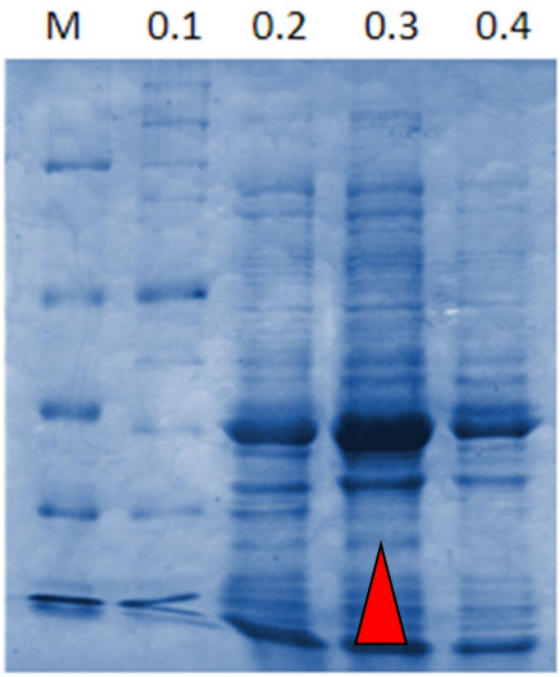

Figure 4.1: Optimization of IPTG Concentration

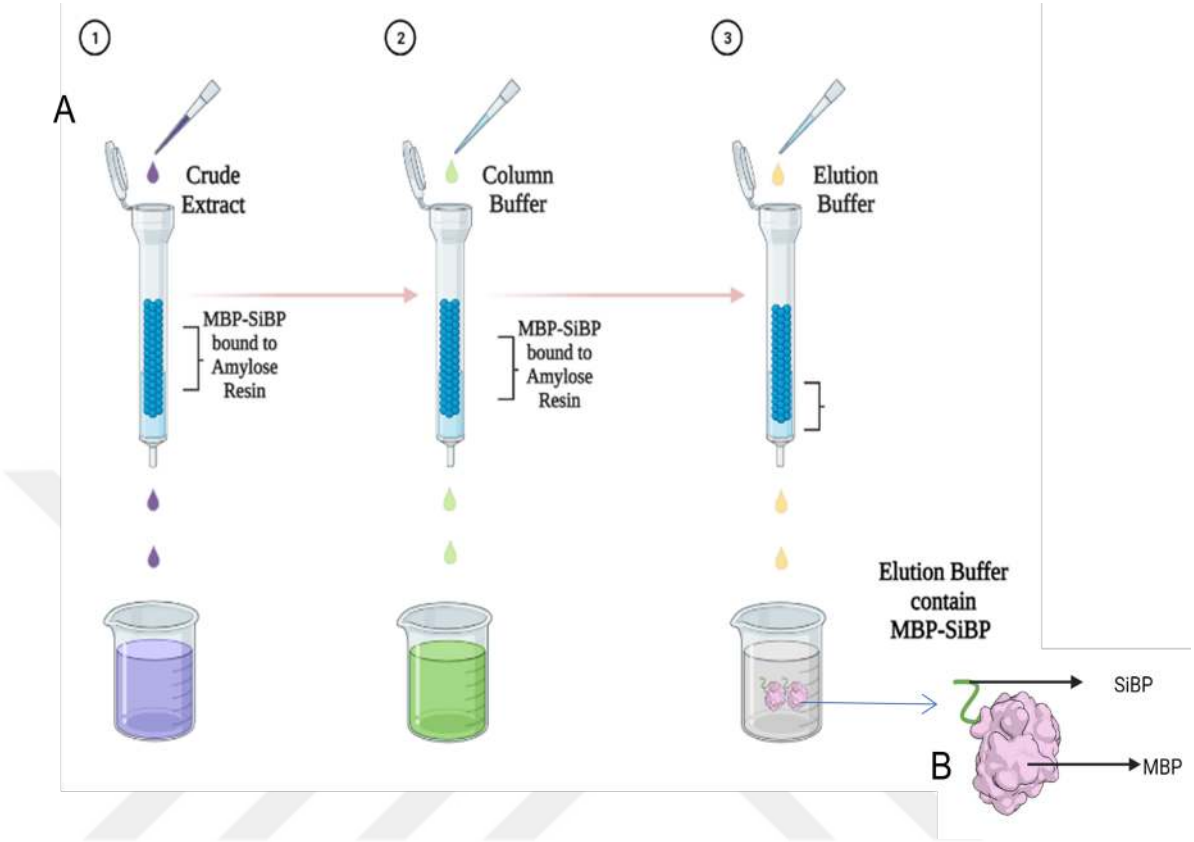

Figure 4.2: A) Schematic representation of purification of MBP-SiBP multifunctional protein. (B) Illustration of MBP (PDB:1URG)– SiBP multifunctional protein.

The affinity chromatography method that was utilized to purify the proteins that were generated is shown in Figure 4.2 (Mahmoodi, Pourhassan-Moghaddam, Wood, Majdi, & Zarghami, 2019).

Both wild-type MBP and MBP-SiBP were purified using an altered version of a protocol developed by New England Biolabs (Figure 4.2).

4.3 Characterization of MBP-SiBP Multifunctional Protein

After completing the purification of the multifunctional protein (MBP-SiBP), a series of tests were carried out to determine the concentration and molecular mass of the protein. These tests, along with their explanations, are included in the following section.

For the purpose of protein quantification, dye-based protein assays see widespread use. The Bradford test relies on the binding of the Coomassie Brilliant Blue G-250 dye, and the dye causes a change in the absorbance when attached to a protein, which may be used to determine the concentration of the protein by measuring the absorbance at 595 nm (Khramtsov et al., 2021).

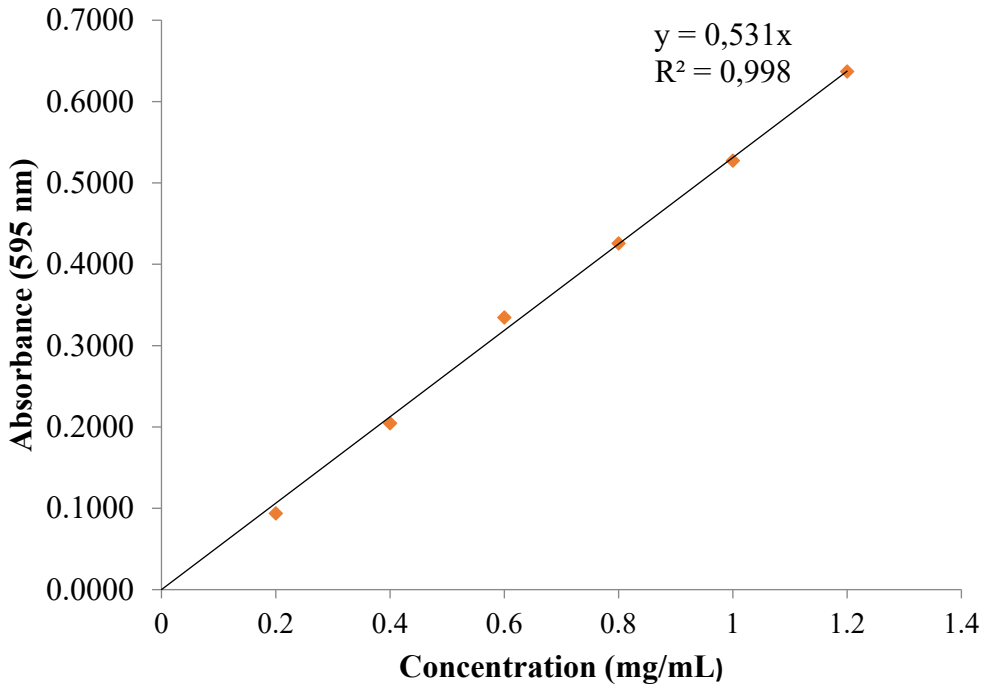

Figure 4.3: Standard curve to calculate concentration of wt-MBP

The y and R^2 value were calculated according to Table 4.1., and slope of standard curve (y=0,531x) used to calculate total protein concentration of wt-MBP (Table 4.2 and 4.3)

Table 4.1: BSA Standards to calculate y and R^2 value for wt-MBP

BSA STDs	Absorbance (595 nm)			AVG	X-Blank
0	0,3933	0,4021	0,4060	0,4005	0,0000
0,2	0,4832	0,5064	0,4931	0,4942	0,0937
0,4	0,5883	0,6177	0,6089	0,6050	0,2045
0,6	0,7386	0,7300	0,7363	0,7350	0,3345
0,8	0,8140	0,8280	0,8361	0,8260	0,4255
1	0,9433	0,9284	0,9119	0,9279	0,5274
1,2	1,0632	1,0261	1,0231	1,0375	0,6370

To calculate total protein concentration of wt-MBP ;

1. Taking the average of absorbance values = **AVG**
2. **AVG** – AVG of BSA STDs "0" = **X-Blank**
3. **X-Blank** / 0,5311 (coefficient of x) = **mg/ml**
4. **mg/ml*** Dilution Factor = **Net mg/ml**

} **Table 4.2**

Table 4.2. Total protein concentration of wt-MBP

	Absorbance (595 nm)			AVG	X-Blank	mg/ml	Dilution Factor
MBP	1,0312	1,0017	0,9653	0,9994	0,5989	1,12766	1
MBP	0,6551	0,6611	0,6482	0,6548	0,2543	0,47882	3
MBP	0,6689	0,6057	0,5619	0,6122	0,2117	0,39854	5

Table 4.3 Continue of Table 4.2

	Net mg/ml	Volume	AVG	Total Protein
MBP	1,12766			
MBP 1:3	1,43645	100	1,518943911	151,8943911
MBP 1:5	1,99272			

5. Taking the average of **Net mg/ml = AVG**
6. **AVG***Volume = Total Protein

} **Table 4.3**

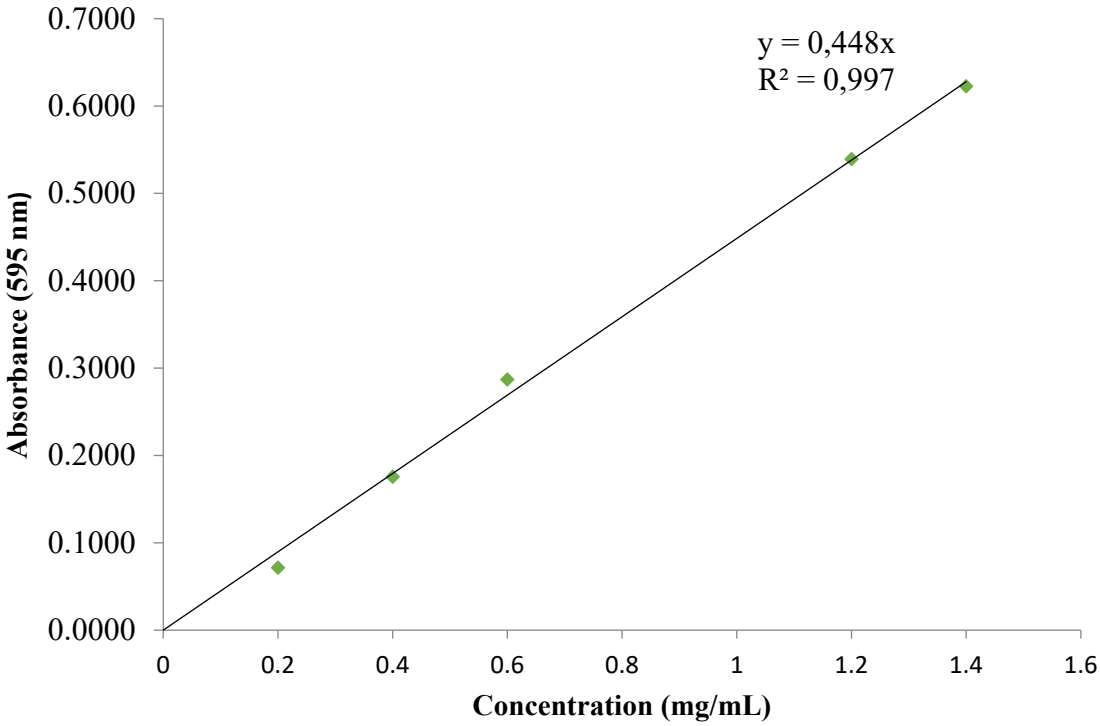

Figure 4.4: Standard curve to calculate concentration of MBP-SiBP

The y and R^2 value were calculated according to Table 4.4., and slope of standard curve (y=0,448x) used to calculate total protein concentration of MBP-SiBP Protein (Table 4.5 and 4.6).

Table 4.4 BSA Standards to calculate y and R^2 value for MBP-SiBP Proteins

BSA STDs	Absorbance (595 nm)			AVG	X-Blank
0	0,4307	0,4189	0,4376	0,4291	0,0000
0,2	0,5035	0,4949	0,5035	0,5006	0,0715
0,4	0,6106	0,6085	0,5951	0,6047	0,1756
0,6	0,7353	0,7166	0,6964	0,7161	0,2870
1,2	0,9316	0,9446	1,0292	0,9685	0,5394

To calculate total protein concentration of wt-MBP ;

1. Taking the average of absorbance values = **AVG**
2. **AVG** – AVG of BSA STDs "0" = **X-Blank**
3. **X-Blank** / 0,448 (coefficient of x) = **mg/ml**
4. **mg/ml*** Dilution Factor = **Net mg/ml**

} **Table 4.5**

Table 4.5 Total protein concentration of MBP-SiBP Proteins

	Absorbance (595 nm)		AVG	X-Blank	mg/ml	Dilution Factor
MBP-$(PG)_3$-SiBP	0,8821	0,8578	0,8700	0,4409	0,9831	3
MBP-$S(G)_3$-SiBP	0,8972	0,8514	1,1884	0,7593	1,6934	3

Table 4.6 Continue of Table 4.5

	Net mg/ml	Volume	Total Protein
MBP-$(PG)_3$-SiBP	2,9494	30	88,4846
MBP-$S(G)_3$-SiBP	2,9785	50	148,93

5. **Net mg/ml** * Volume = **Total protein** } **Table 4.6**

Quantitative investigations in molecular biology, biophysics, biochemistry and structural biology absolutely need an accurate estimation of protein concentration. One way that is especially effective is the absorbance at 280 nm (Figure 4.4 and 4.5), which gives a high level of specificity (Reinmuth-Selzle et al., 2022).

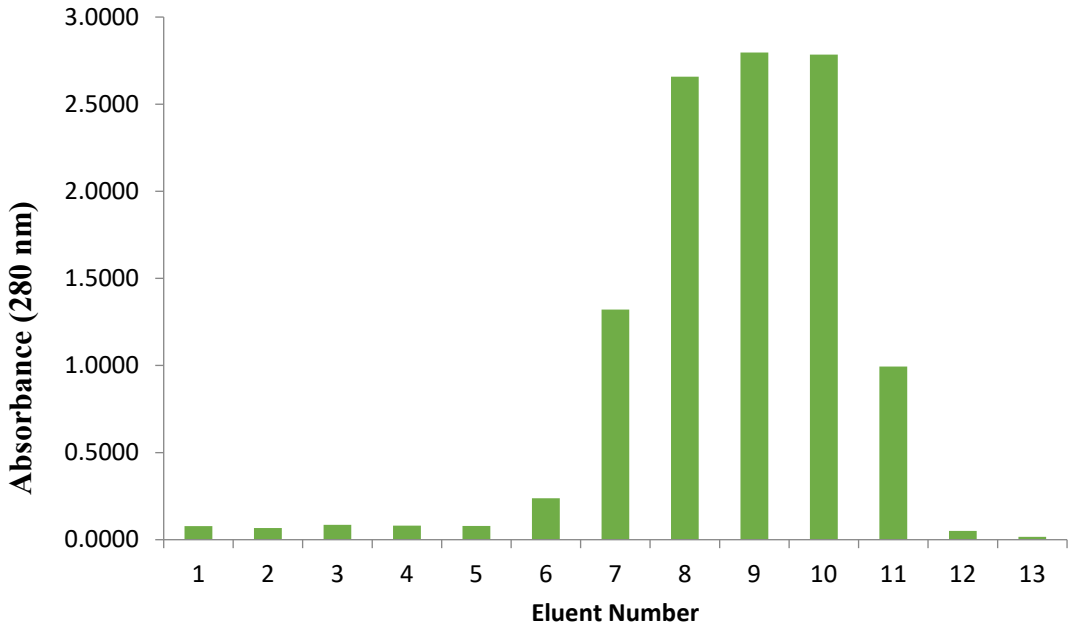

Figure 4.5: Quantitation of purified MBP-$S(G)_3$-SiBP

The purified eluents from Figure 4.2-(3) was put through an analysis and selection process under UV light with a wavelength of 280 nm, which results in the Figure 4.5 and 4.6.

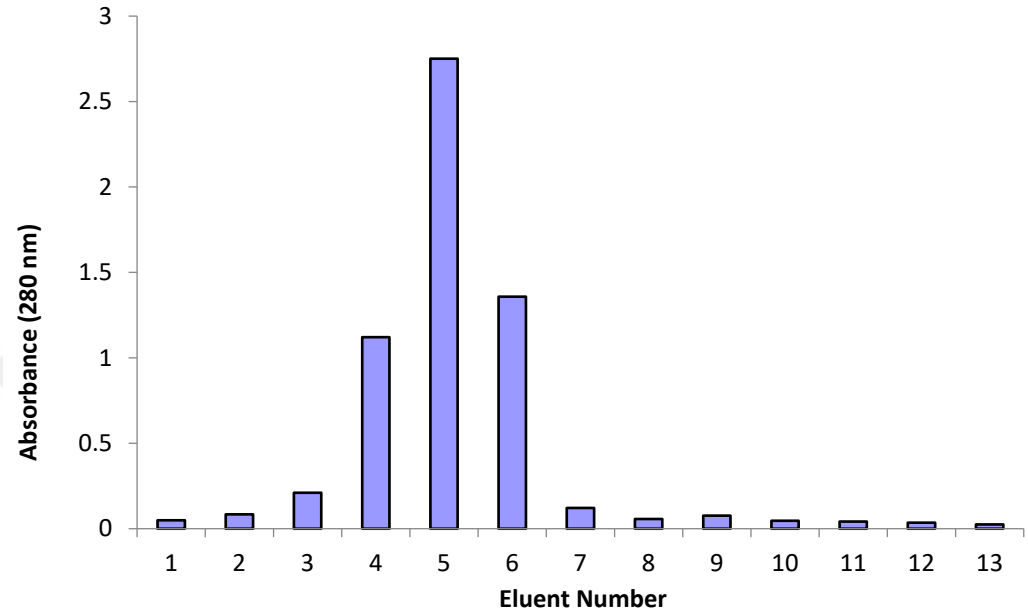

Figure 4.6: Quantitation of purified MBP-$(PG)_3$-SiBP eluents

Figure 4.6 is the proof of controlled expression of our multifunctional protein. After purification, SDS PAGE was performed to observe molecular weight of MBP-SiBP protein. In Figure 4.7, 5-6-7 refers to 4-5-6 in Figure 4.6, respectively.

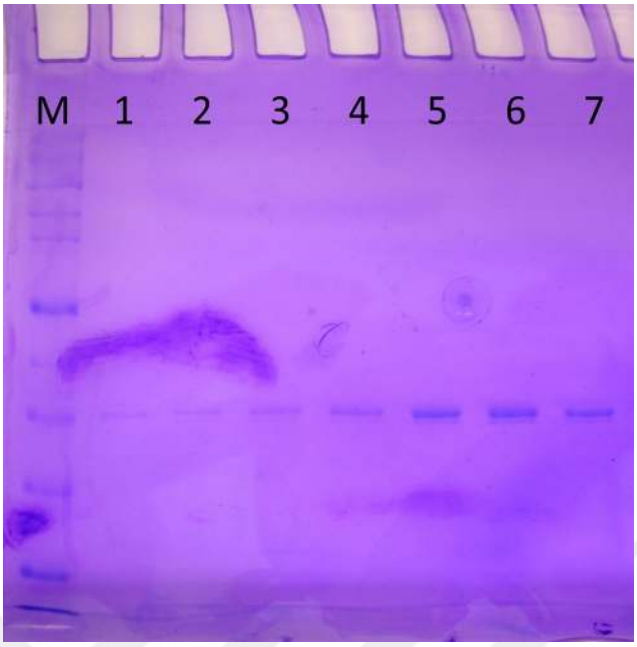

Figure 4.7: M is marker (Appendices A). SDS-PAGE Analysis of purified eluents (shown Figure 4.6).

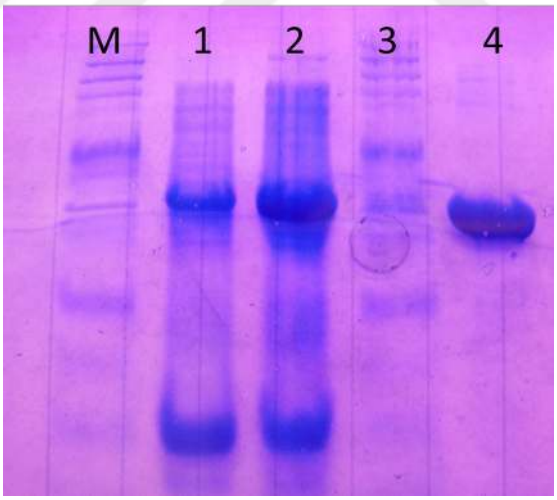

Figure 4.8: Effect of induction with IPTG. M represents marker. 1: MBP-$(PG)_3$-SiBP induced (IPTG), 2: MBP-S$(G)_3$-SiBP induced (IPTG), 3: Uninduced cell, 4: Pooled Eluent

Purified wild-type MBP and the MBP-SiBP multifunctional proteins were analyzed by mass spectrometry (MS) to verify the quality of the purified proteins, as well as their identities and digestion reactions.

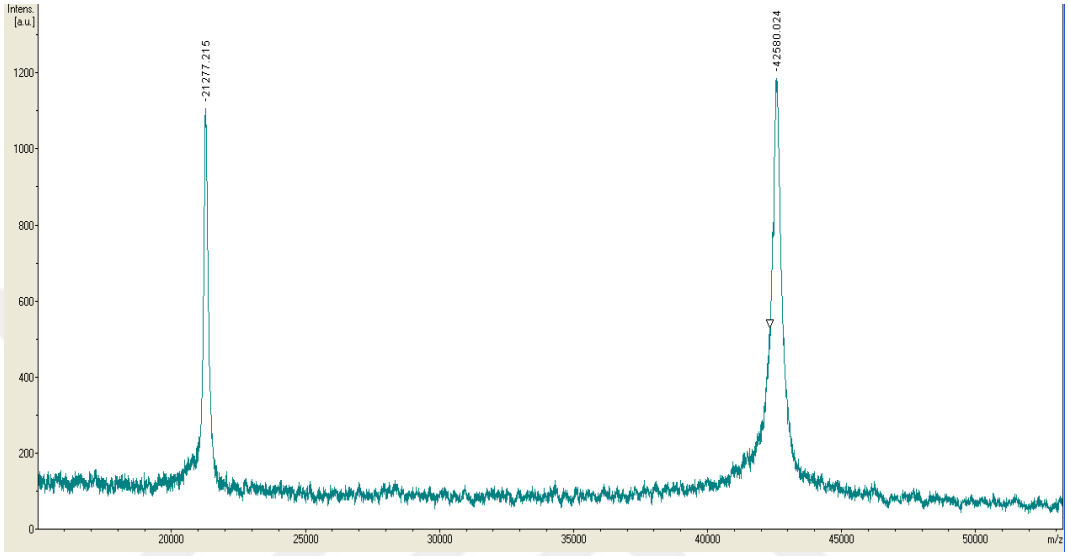

Figure 4.9: Spectra of wt-MBP

Respectively, Figure 4.9, 4.10 and 4.11 shows the spectra of the MBP protein and the MBP-SiBP proteins. The highest-intensity peaks have m/z values that match to the expected single-charged species of several proteins.

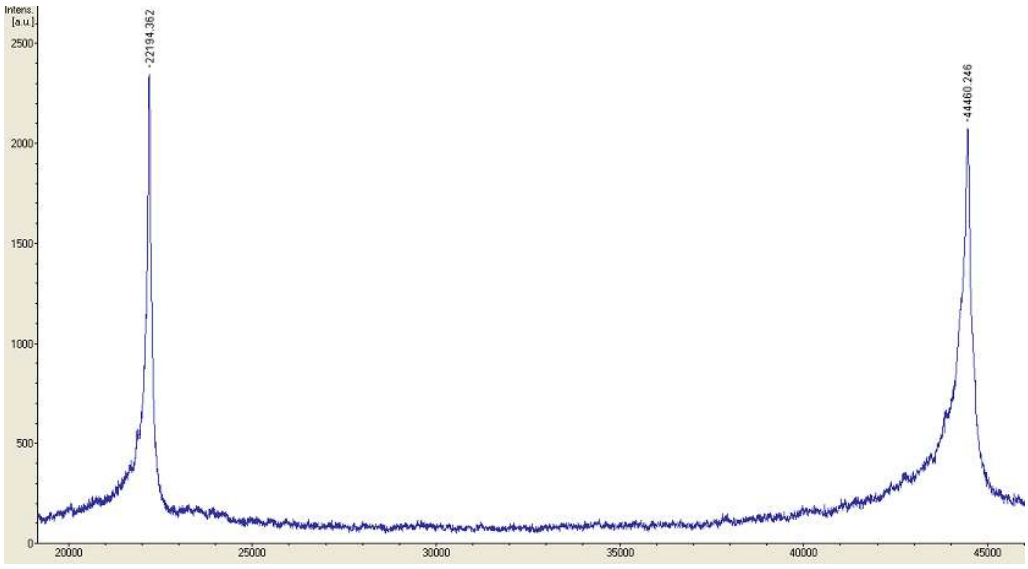

Figure 4.10: Spectra of MBP-$(PG)_3$-SiBP

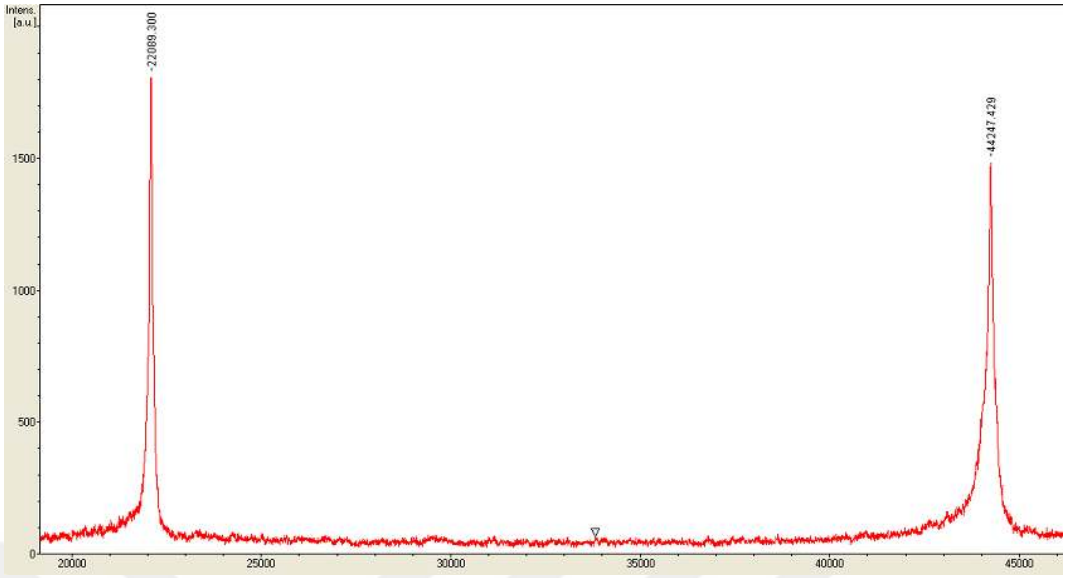

Figure 4.11: Spectra of MBP-S(G)$_3$-SiBP

The molecular masses in Table 4.2 are shown for both the measured values and the predicted values. For the individual proteins, the most intense peaks correspond to predicted m/z values for singly charged species.

Table 4.7: Measured and predicted molecular masses of wt-MBP and MBP-SiBP Multifunctional Proteins

Constructs	Total Protein Yield	Multifunctional Protein Yield	Predicted Molecular Mass of Protein	Measured Molecular Mass of Protein
wt-MBP	180 mg/L culture	40 mg/L culture	42.481 kDa	42.580 kDa
MBP-S(G)$_3$-SiBP	150 mg/L culture	60 mg/L culture	44.270 kDa	44.247 kDa
MBP-(PG)$_3$-SiBP	90 mg/L culture	30 mg/L culture	44.475 kDa	44.460 kDa

4.4 Self Assembly of Multifunctional Protein onto Silica Surface

Purification of the MBP protein fused with the inorganic binding peptide was followed by a number of procedures to test their binding properties, which will be explained in the next section.

4.4.1 Micro-contact Printing (μCP) of Multifunctional Protein on Silica surface

Printing proteins onto patterned self-assembled monolayers (SAMs) via micro-contact printing has been presented as a way to manage their concentration and spatial distribution. It is a member of the soft lithography family of surface modification methods, which is one of the most widely employed in biomedical applications. Primary uses of μCP in surface manipulation are the creation of precise geometric patterns and the patterning of two-dimensional surfaces. Microcontact printing has a distinct advantage over other surface modification methods because of its ability to create two-dimensional patterns on the surface. Polymers, proteins, and biological signals may be formed or transferred onto a substrate surface in precisely specified patterns thanks to this technique (Qiu et al., 2021).

The peptide tag of SiBP has a strong silica-binding affinity and combined with the high efficiency of standard micro-contact printing technology (μCP) allows the creation of protein arrays on silica surfaces with increased molecular density in only one reaction step.

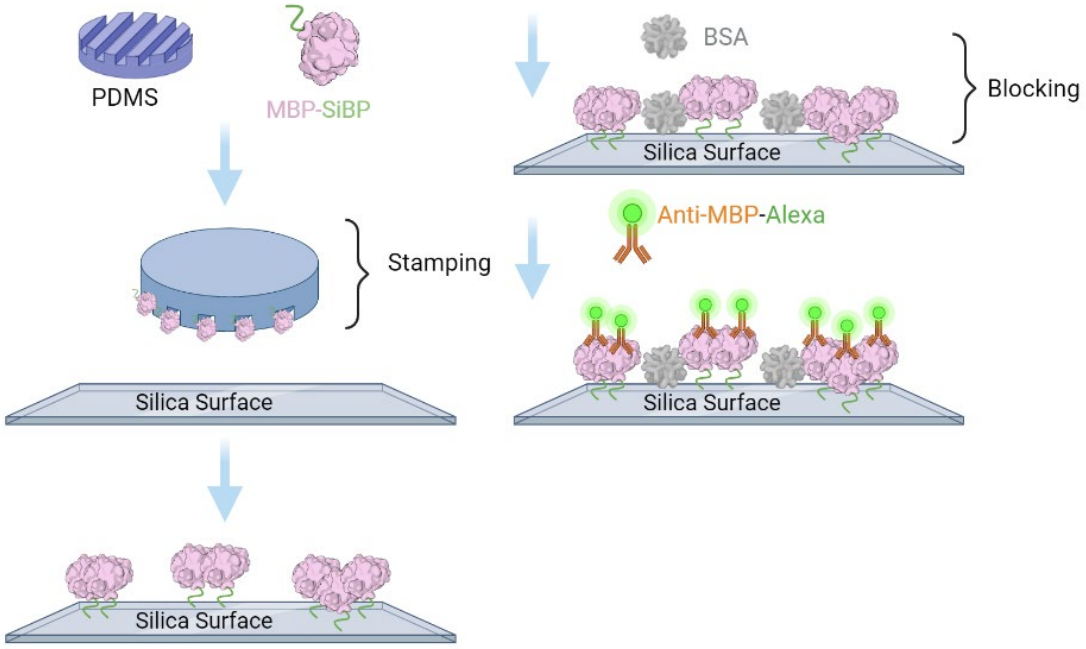

Figure 4.12: Controlled protein patterning on flat silica surface via micro contact printing technique.

MBP-SiBP fusion protein arrays immunodetected using a particular anti-MBP-Alexa combination (Figure 4.12) showed improved binding of both fusion proteins when compared to wt-MBP alone as demonstrated in the findings.

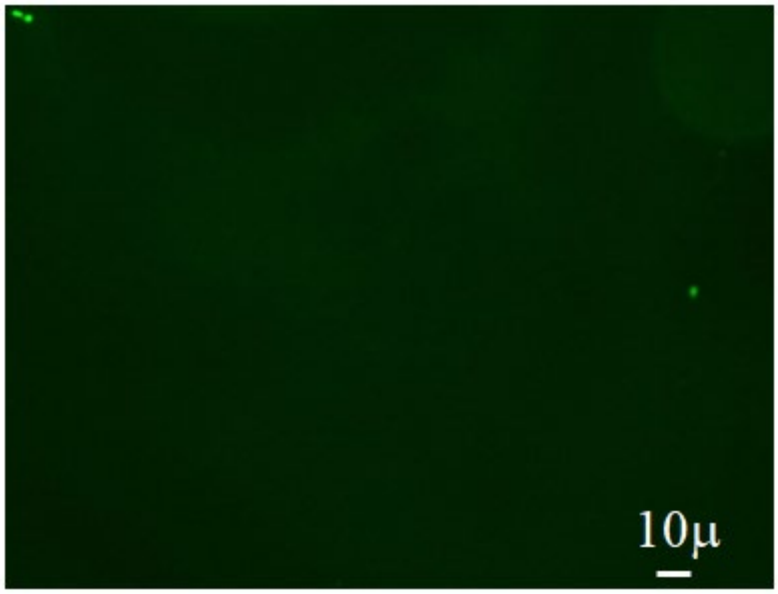

Figure 4.13: Negative Control (BSA blocked)

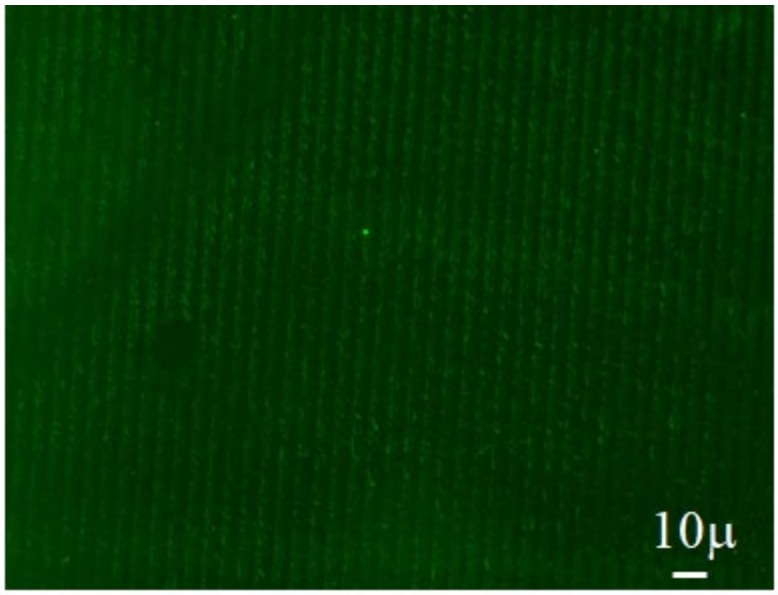

Figure 4.14: wt- MBP (BSA Blocked)

Antibodies to MBP-SiBP proteins show clear, well-defined patterns, indicating that the proteins assemble on the silica surface with excellent molecular packing density and pattering efficiency (Figure 4.13 and 4.14). An efficient interface for protein–surface interactions has been shown to be a consequence of the unique SiBP tags found in MBP-SiBP fused proteins.

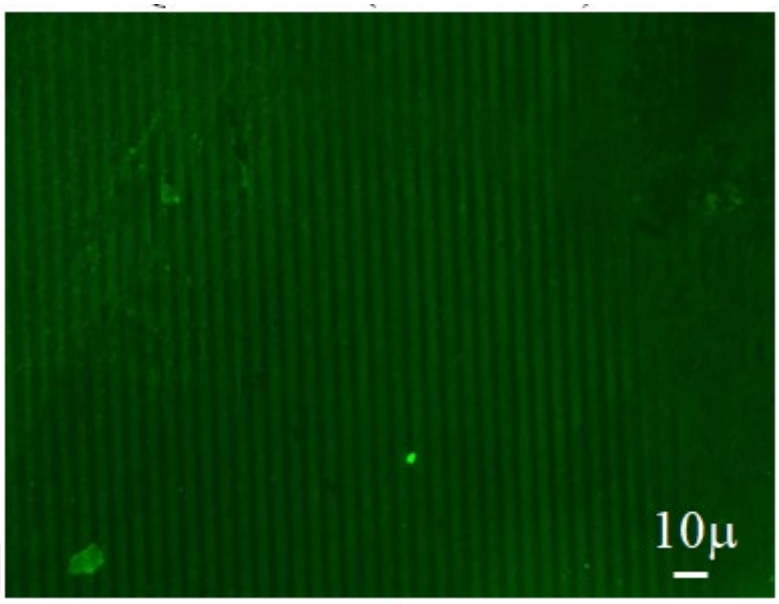

Figure 4.15: MBP-PGPGPG-SiBP (BSA Blocked)

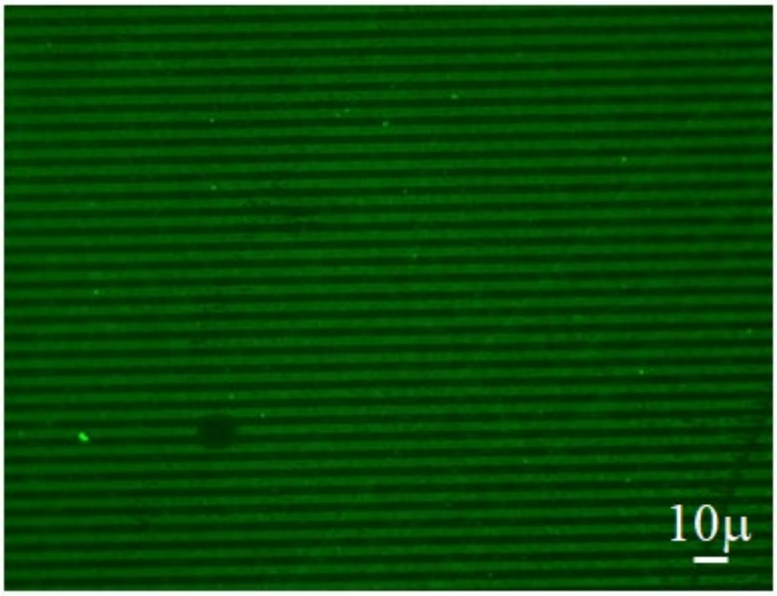

Figure 4.16 : MBP-SGGG-SiBP (BSA Blocked)

Figure 4.12 illustrates micro-contact printing and protein labeling; fluorescent pictures of anti-MBP-Alexa-488 antibody immobilized on a flat silica surface using micro-contact printing are shown for 20 mM wt-MBP and MBP-$(PG)_3$-SiBP and MBP-$S(G)_3$-SiBP proteins. Images were taken using a fluorescence microscope (20X) with the matching fluorescent filter to see the protein-arrayed silica surfaces.

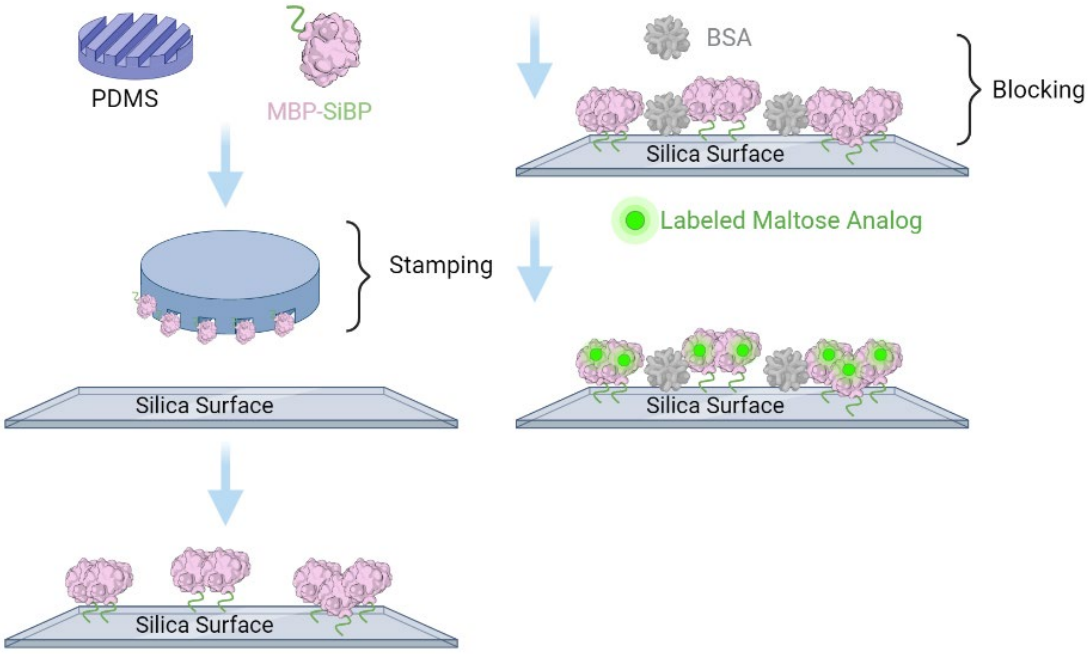

Figure 4.17: Labeled Maltose Detection via immobilized MMBP-SiBP

4.4.2 Depletion Assay

Depletion assay was performed to quantitate concentration range of the purified wt-MBP and MBP-SiBP multifunctional proteins via CBQCA dye. In Figure 3.1, binding strategy of CBQCA and proteins was illustrated. Herein, fumed silica (SiO_2) was used as binding surface, and Langmuir isotherm was used to calculate K_D value, which is the constant related to the affinity of the binding sites, of each protein.

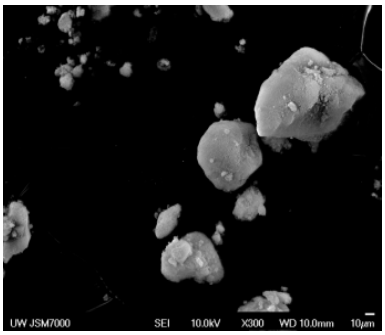

Figure 4.18: Fumed silica particles (SiO_2)

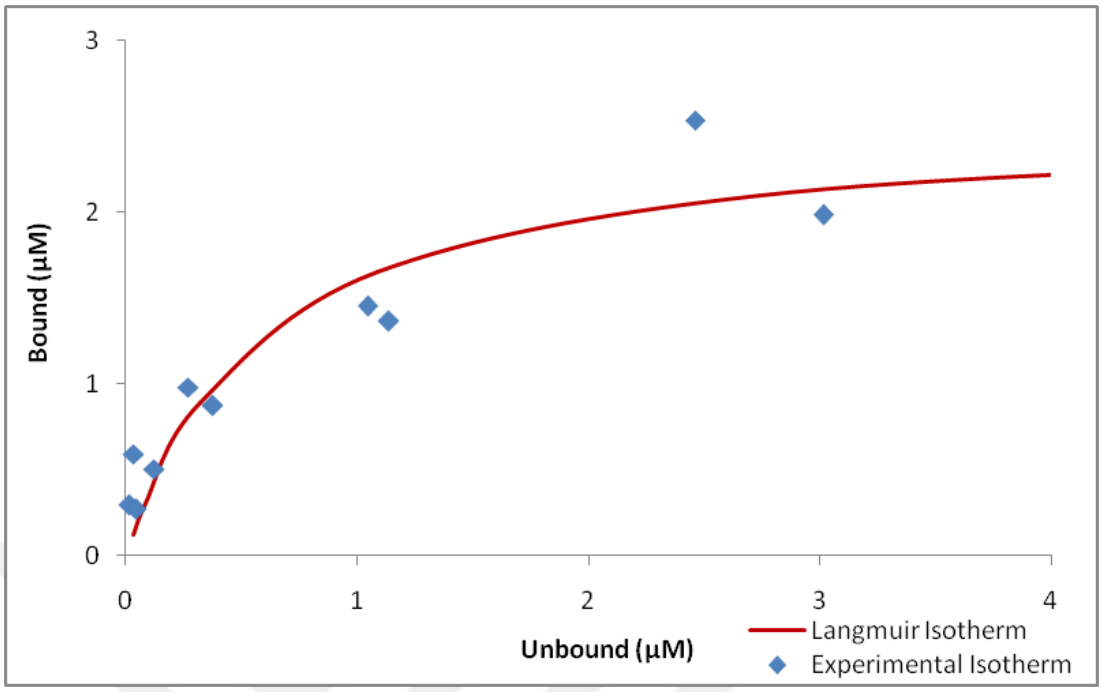

Figure 4.19: wt- MBP

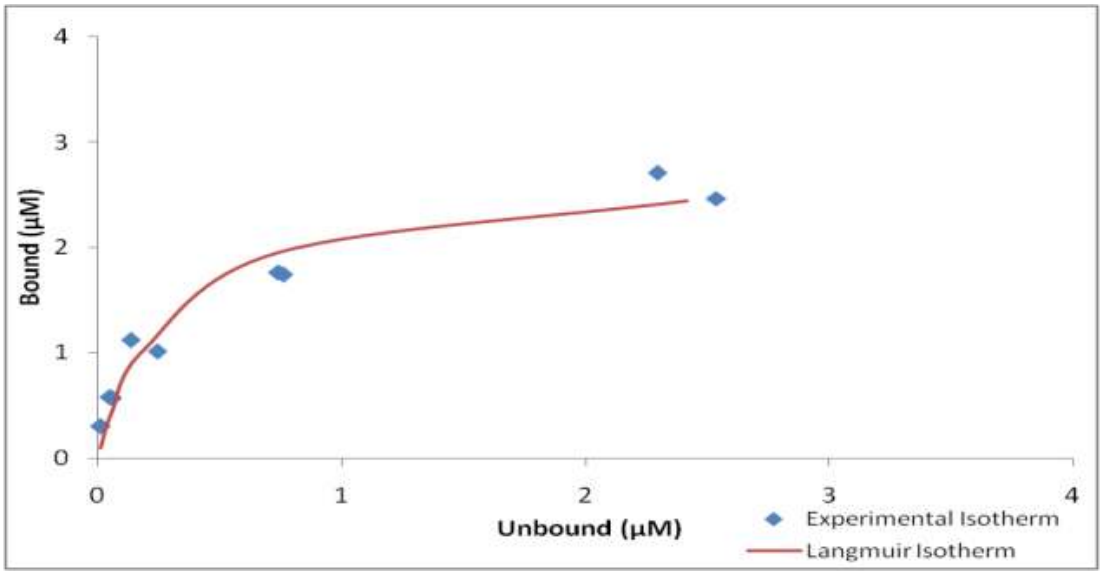

Figure 4.20: MBP-S(G)$_3$-SiBP

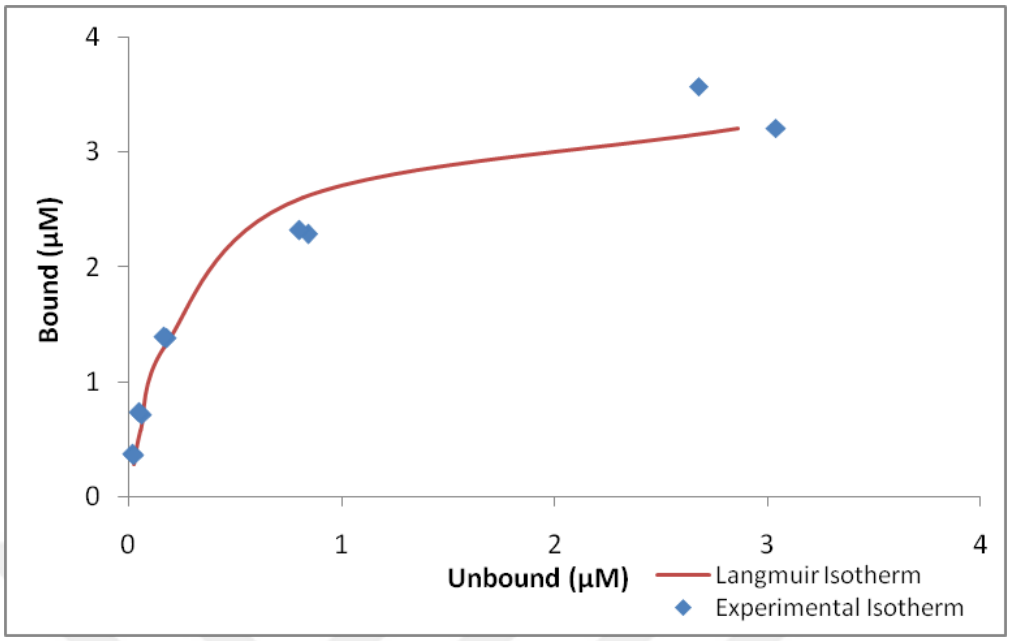

Figure 4.21: MBP-$(PG)_3$-SiBP

Thus, the calculated K_D values of both MBP-SiBP proteins were lower than those of the control wt-MBP (Table 4.2).

Table 4.8: Concentration range and KD values of MBP and MBP-SiBP

Protein/Peptide	Binding Surface	Quantitation Method	Conc. Range (µM)	K_d (µM)
MBP-wild type	Silica (SiO_2); fumed;	CBQCA dye (Molecular Probes)	0.3125-10µM	0.60 0.40
MBP-SG_3-SiBP	Silica (SiO_2); fumed;	CBQCA dye (Molecular Probes)	0.3125-10µM	0.30 0.30
MBP-$(PG)_3$-SiBP	Silica (SiO_2); fumed;	CBQCA dye (Molecular Probes)	0.3125-10µM	0.30 0.24

5. DISCUSSION, CONCLUSION and RECOMMENDATIONS

5.1 Discussion

Creating a functional interface from the bionic system is one of the most challenging aspects of biosensor design. Key to the development of biosensors is the immobilization of biomolecules on solid substrates or the fabrication of interfaces that, when exposed to a specific target, may produce a measurable signal. Receptor biolayer functionality and quality are essential to a biosensor's ability to collect analytes (Sharafeldin & Davis, 2022). However, biomolecules cannot be directly affixed to the surface of electrodes or other inorganic materials because they are very sensitive to their environment and denature fast in response to environmental changes. Between both the receptors or sensing elements and the electrode surface, a biocompatible interface layer is required to preserve the biological activity of molecules required for proper sensor performance. In context, this layer is referred to as the biosensor interface. The stability of the interface influences the interaction of analytes with it, which in turn impacts the detection capability of the biosensor. In conclusion, it is essential to build a dependable interface for biosensor applications (Sharafeldin & Davis, 2022).

Recent advancements in nanobiotechnology have made it feasible to create novel nanoscale biosensors. Through molecular recognition and particular interactions, biosensor sensitivity and specificity may be enhanced by immobilizing functional biomolecules on nanomaterials or nanopatterned surfaces in a regulated manner. (D. C. Kim & Kang, 2008; Parisi et al., 2022)

Biomolecular nanopatterning has been accomplished by molecular self-assembly by means of molecular recognition and particular interactions connected with a variety of lithographic approaches (eg, soft lithography and electron beam lithography, etc.). Immobilization of protein provides superior stability, separation, and reusability as

compared to the usage of free protein. Classical methods for protein immobilization based on non-specific adsorption, on the other hand, often have a deleterious impact on protein functioning. (D. C. Kim & Kang, 2008; Movilli & Huskens, 2020; Parisi et al., 2022)

Using peptides that precisely attach to the surface, a one-step, easy, controlled, orientated, and directed immobilization was done in order to remove these undesired effects in the biosensor design, which is one of the most prevalent problems in the scientific literature.

In this study, we demonstrate the utility of combinatorially selected silica-binding peptide (SiBP) as an excellent alternative binder that enables the immobilization of various fusion molecules and nanostructures on the silica interface, particularly when self-assembly and soft lithography processes are combined. Using this technique, we genetically fused the SiBP peptide tag to the C-terminus of maltose binding protein (MBP). MBP may fuse at either the N- or C-terminus of the protein, however its N-terminal position can decrease translation efficiency. One of the obvious reasons for selecting MBP as the fusion partner was its capacity to withstand C-terminal genetic fusion and its capacity to assist high protein production when synthesized in bacterial cells. The development of linkers effective of physically separating two domains has been shown to be crucial for the fabrication and manufacture of heterofunctional protein units (Chen et al., 2013; Silver et al., 2021).

In particular, MBP-SiBP fusion constructs with a flexible $S(G)_3$ or rigid $(PG)_3$ linker that isolates SiBP from the MBP tag have been created. The fusion protein was constructed using these linkers. The synthesized MBP-SiBP multifunctional protein was purified in a single step by affinity chromatography. To improve the overall quantity of protein produced, optimization tests were conducted first. During purification, the quantity of amylose resin was tuned based on the dimensions of the column and the buffer components (both in buffers; column and elution). 3 mg of MBP-labeled protein may be bound by 1 ml of amylose resin bed volume.

There is a chance that the MBP fusion protein binds poorly to the amylose column because the extracts include components that either interfere with the binding

process or have low intrinsic affinity. Lysozyme and sonication are examples of alternate lysis procedures that result in the release of components in the crude extract that may interfere with binding and cellular components. Significant quantities of amylase, which hinders the binding process, are produced by cells grown in LB. When glucose was added to the medium to inhibit amylase activity, the issue was resolved. With each usage of the column, the binding capacity of a crude extract derived from one liter of cells cultured in LB containing 0.2% glucose should drop by one to three percent. Normal deterioration is accountable for this loss of capability. Given these parameters, the yield of 40 mg of fusion protein is predicted to begin to decrease between three and five rounds at the earliest. With the use of column buffer, the amylose column was equilibrated after being meticulously packed (Appendix A). In this phase of the purification procedure, the column solution was the first item to be modified. To prevent undesired proteases from functioning, protease inhibitors are introduced. To prevent the formation of interchain disulfide bonds, we lowered the concentration of β -mercaptoethanol in the column buffer to 1mM, which is 10 times less than the conventional technique. Because the amylose column could not be decreased, proteins were eluted from it without the need for an additional buffer.

The Bradford assay is dependent on the binding of the Coomassie Brilliant Blue G-250 dye to particular residues on histidine, tryptophan, arginine, tyrosine, and phenylalanine, as well as the hydrophobic interactions between the dye and protein. When linked to a protein, the dye generates a shift in absorbance that may be used to quantify the concentration of the protein by measuring absorbance at 595 nm (Khramtsov et al., 2021).

There is a wide variety of spectrophotometric techniques that may be implemented. The absorption of ultraviolet (UV) light by intrinsic chromophores is a frequently used technique. Absorbance at 280 nm, which arises solely from tyrosine and tryptophan residues, provides a high degree of specificity and is one of the most successful methods (Reinmuth-Selzle et al., 2022).

Using Bradford test, SDS-PAGE, and mass spectrometry, the multifunctionality of purified proteins was determined. During the purification stage, the elution buffer was passed across the column (Figure 4.1) and 10-20 3 ml fractions were collected. The fusion protein often starts to elute during the first 10 fractions, therefore we may conclude that any protein or chemical remaining in the column, with the exception of MBP-SiBP protein, happens within the first 10 elution (Figures 4.4 and 4.5). Using this method, we may simultaneously choose the fractions that will be combined. Based on the SDS-PAGE analysis of the eluents obtained after purification (Figure 4.6), we can conclude that the purification step of our multifunctional proteins was successful as anticipated, and the molecular weight of the pooled MBP-SiBP protein eluents displayed comparable band formation to the IPTG-induced proteins before purification (Figure 4.7). Nonetheless, mass spectrometry tests revealed that the estimated and measured molecular weights were quite near (Table 4.2).

In addition, Microcontact printing directs the assembly of protein samples on the surface, and fluorescent labeling enables the viewing of the formed proteins using a fluorescence microscope. Micro-contact printing of multifunctional proteins onto patterned self-assembled monolayers (SAMs) is a crucial step in our research (Figure 4.11). Compared to wt-MBP, the MBP-SiBP multifunctional protein resulted in a significant fluorescence intensity on the surfaces, showing that the silica-binding peptide is crucial for the assembly process (Figures 4.12, 4.13, 4.14,4.15). In addition, both can detect fluorescently labeled maltose; This is a proof-of-concept since it proves that the silica-binding peptide and MBP form a multifunctional protein, with no loss of function, and capable of controlled, directed, and oriented immobilization due to the self-assembly property of surface-specifically designed Silica-Binding peptides (SiBP).

5.2 CONCLUSION

As a prerequisite for the development of novel molecular engineering materials and procedures as well as bionanotechnology, combining biology and materials necessitates the capacity to create, construct, and regulate interfaces at the biological intersection of materials. Peptides and proteins, which are molecular communication agents, may also be used by biology to govern interfaces. The proteins of an organism are, in a sense, its labor force. They carry out the cellular chemistry, physics, and biology. Using peptides that are capable of binding to inorganic materials, we may design a new basic building block for engineering and technology systems, one that genetically combines biological and synthetic entities.

The MBP protein and a highly specific SiBP peptide tag are combined to form a hetero-functional protein unit, which we explain in this paper. There were two physically distinct spacers between the SiBP tag and MBP protein in this configuration. Both a flexible (SGGG) and a rigid (PGPGPG) linker were utilized to examine the influence of spacers on the fusion protein's functioning.

As a consequence of these findings, the selective SiBP peptide tag has been shown to be useful in improving the immobilization affinity and orientation of MBP-SiBP structures on diverse silica surfaces. It was shown that both of the designed MBP-SiBP proteins displayed much better binding affinities to diverse silica surfaces, as reflected by almost an order of magnitude greater binding parameters for MBP-SiBP fusion protein.

For high-throughput biosensors, an efficient and regulated technique for protein immobilization on micro and nano-patterned surfaces may be applied. Protein immobilization on solid surfaces may be controlled and improved by using these unique peptide tags, as shown in this study. The robust peptide-based strategy reported here, in contrast to standard chemical and physical approaches for protein adsorption, is very exciting since it is easy, physiologically friendly, and ecologically friendly, and does not need harsh chemical or physical conditions.

Through Micro-contact printing and depletion assay proved that the aim of the study was carried out. Due to the self-assembly property of SiBP; controlled and oriented immobilization of multifunctional protein was achieved on the silica surface. Besides, it is proven that MBP has no loss of function and is still active via binding antibody and maltose.

As a whole, the findings show that tailored peptides may be used to build diverse protein and hybrid nanostructure assemblies with highly adjustable design and organization.

5.3 RECOMMENDATIONS

In future work, due to successfully result of this study, we will work on biosensor interface design based on impedance measurement. Apart from that, we will try immobilization on the new surface-specific binding peptides to detect pathogens or diseases, because this thesis proved that combining desired protein with surface specific, such as gold or silver, binding peptides can be used to creating new protein micro arrays, chip and biosensors.

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7. APPENDICES

APPENDIX A: Materials

Bacterial Strains

Escherichia coli K12 TB1 strain

Escherichia coli **K12 TB1 strain** is used in this thesis. The strain is a derivative of JM83 that has been comprised hsdR. The genotype of this strain is F^{-}*ara*Δ*(lac-proAB) [Φ80dlac* Δ*(lacZ)M15] rpsL*(Str^{R})*thihsdR.* (New England Biolabs, Catalog # E4122S).

Escherichia coli K12 ER2507

Escherichia coli strain K12 ER2507 [F^{-} *ara-14 leuB6 fhuA2 (argF-lac)U169 lacY1 glnV44 galK2 rpsL20 xyl-5 mtl-5 (malB) zjc::Tn*5(Kan^{R})*(mcrC-mrr)*$_{HB101}$] was supplied from New England Biolabs (Catalog # E4121S) and used for the design of the expression vector.

pMAL™-2 Vectors

The vector in this thesis was provided by Dr. Banu Taktak Karaca, the supervisor of this thesis. The pMAL™-2 vectors allow for the expression and purification of proteins derived from cloned genes. The cloned gene is inserted downstream from *malE* gene of *E. coli*, which codes for a maltose-binding protein (MBP), resulting in the production of an MBP fusion protein.

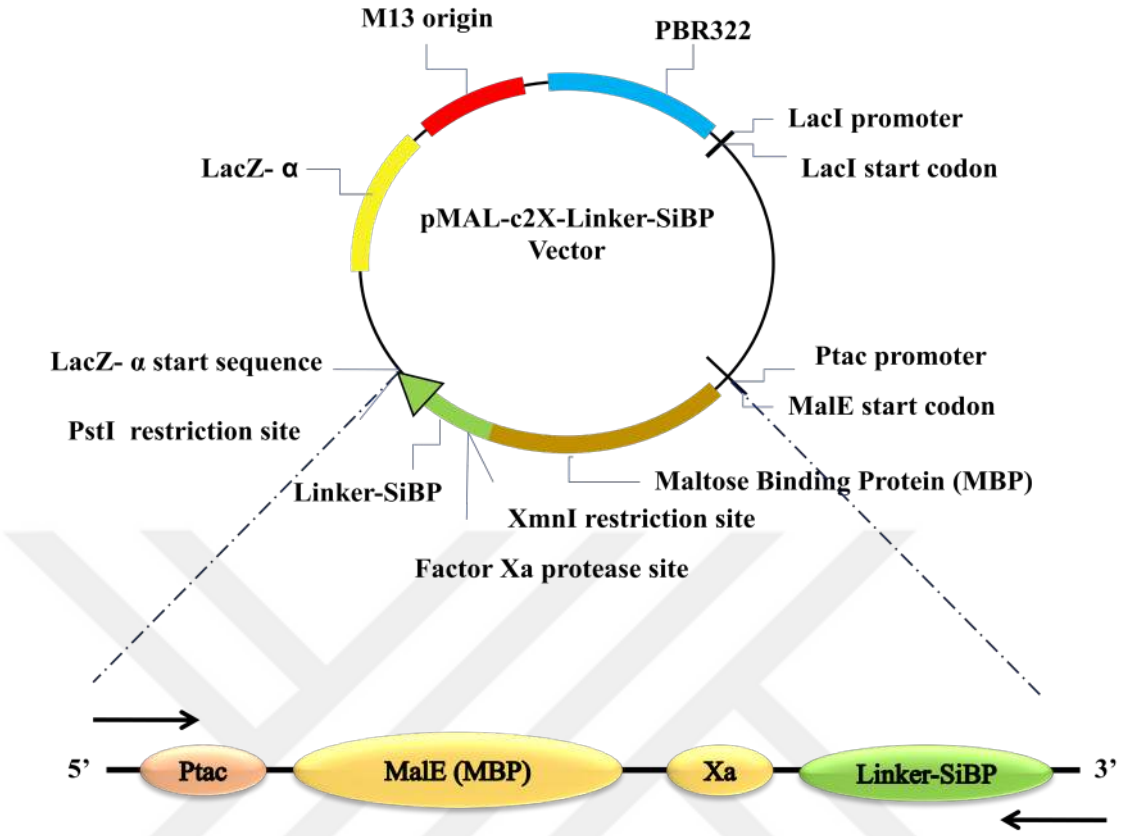

Figure7.1: Schematic representation of Pmal-C2x-Linker-SiBP Vector, provided from thesis advisor Banu TAKTAK KARACA

Protein Molecular Weight (MW) Markers

Unstained Protein Molecular Weight Marker from Fermentas (Catalog # SM0431) (shown in Appendix B) is used for protein detection.

Lysozyme (EC 3.2.1.17)

50,000 units/mg of lyophilized powder containing sodium chloride and sodium acetate buffer salts were purchased from Sigma (Catalog # L7001). It was dissolved in deionized water to achieve a concentration of 10-20 mg/ml.

Bacterial Culture Media

LB Medium

Table 6.1. 20 g LB Medium in 1 Liter

Components	Volume
Tryptone	10 g
Yeast Extract	5 g
NaCl	5 g

Table 6.2 35 g LB Agar Medium in 1 Liter

Components	Volume
Tryptone	10 g
Yeast Extract	5 g
NaCl	5 g
Agar	15 g

LB Medium in 1 Liter (containing 2% glucose)

Components	Volume
Tryptone	10 g
Yeast Extract	5 g
NaCl	5 g
Glucose	2 g

Buffers

Sodium Acetate Buffer

3 M of Na-Ac (Riedel-de-Haen) was dissolved in 65 ml distilled water. pH was adjusted to 5.2 and distilled water was added up to 100 ml.

10X TBE Buffer (1000ml)

108 g of Tris base, 55 g boric acid and 20 ml 0.5 M EDTA at pH 8.0 were dissolved in 1 liter deionized water, its pH was titrated to 8.3 and sterilized for 15 minutes under 1.5 Atm at 121oC.

Column Buffer (1000ml)

20 ml 1.0 M Tris-HCl, pH 7.4, and 2.0 ml 0.5 M EDTA were mixed and 11.7 g NaCl was dissolved this solution. Optionally, 1.0 ml 1M sodium azide and 0.7 ml β−mercaptoethanol were added into this solution

Elution Buffer (1000ml)

4 g Maltose was dissolved in 1 liter column buffer without beta-mercaptoethanol.

Stock Solutions

1000x Ampicillin (Sodium Salt)

1 g Ampicillin (Sigma) was dissolved in 10 ml deionized water, and sterilized by filtration using 0.22 µm filter. This is a 1000x stock solution and stored at -20ºC. Its working strength is 100 µg/ml.

Glycerol Stock Solution

80 ml glycerol (Riedel-de-Haen) and 20 ml distilled water were mixed to get 80% (w/v) solution. It was sterilized under 1.5 Atm at 121ºC for 15 minutes.

1 M IPTG Stock Solution

2.38 g IPTG was dissolved in 10 ml deionized water, sterilized by filtration using 0.22 µm filter and stored at -20ºC

Protein Analysis

CBQCA Protein Quantitation Kit (Cat #C-6667) was purchased from Invitrogen™.

Alexa Fluor™ 488 Protein Labeling Kit (# A10235) was purchased from Invitrogen™.

Chemicals

CaCl2, NH4Cl, NaCl, glycerol, isopropanol, acetic acid (glacial) , absolute methanol, and absolute ethanol were purchased from Fluka.

Amylose resin in 20% Ethanol was purchased from New England Biolabs (Catalog # E8021).

N,N'-Methylenebisacrylamide , APS, Maltose, beta-mercaptoethanol, electrophoresis grade glycine, acrylamide, NaN_3,and $RbCl_2$ were purchased from Sigma.

SDS, CBB-G250, Tris, EDTA, glacial acetic acid, bromophenol blue, NaH_2PO_4, glucose, $MgCl_2$, HCl, TEMED, and NaOH were purchased from Merck.

APPENDIX B

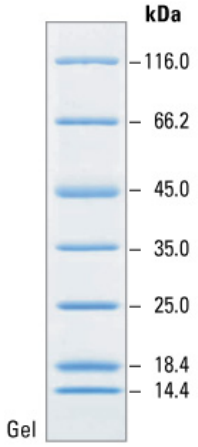

Figure 7.2 Unstained Protein MW Marker. 12% Tris-glycine gel (SDS-PAGE) stained with Coomassie blue gel stain.

APPENDIX C

Computer Software

SnapGene, pDRAW , BioRender, Microsoft Office

Instruments

Amicon® Ultra-15 Centrifugal Filter with a 30,000 NMWL membrane (Millipore, Catalog#UFC90302

8. PLAGIARISM REPORT

GENETICALLY DESIGN AND SYNTHESIS OF MULTIFUNCTIONAL FUSION PROTEINS FOR BIOSENSOR APPLICATIONS

Yazar Sena Şi□mşek

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