



To my mommy

**GENETICALLY DESIGNED MICROBES FOR BIOIMAGING
AND BIOSENSING**

**A DISSERTATION SUBMITTED TO
THE GRADUATE SCHOOL OF ENGINEERING AND SCIENCE
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DOCTOR OF PHILOSOPHY**

**IN
MATERIALS SCIENCE AND NANOTECHNOLOGY**

By

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GENETICALLY DESIGNED MICROBES FOR BIOIMAGING AND BIOSENSING

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September 2024

We certify that we have read this dissertation and that in our opinion it is fully adequate,
in scope and in quality, as a thesis for the degree of Doctor of Philosophy.

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ABSTRACT

**GENETICALLY DESIGNED MICROBES FOR BIOIMAGING
AND BIOSENSING**

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PhD in Materials Science and Nanotechnology

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The advantageous approach to the utilization of the microbes for bioimaging and biosensing underlies under their active motility and self-propulsion characteristics besides their easy bioengineering feature to gain multi-functional activities. The emerging developments make use of microorganisms as therapeutic agents in disease diagnosis and treatment. The dynamic nature of the habitat forces the microorganisms to acclimate themselves to changing living conditions via evolving exclusive bio-functionalities for their survival. Therefore, the living microorganisms producing functional materials serve as a biohybrid system with unprecedented potential for enhancing the detection of a disease biomarker molecule or meeting the great need in cancer diagnosis. The synthetic biology approach, a multidisciplinary field of science, gives the ability to engineer and modulate the microorganisms to redesign existing natural pathways, resulting in the gain of the desired function. Inspiring from nature, the biomineralization of iron-oxide materials is demanding for their potential usage in antitumor effect due to their easy modulation, stability, and magnetic properties.

Furthermore, the certain respiratory capacities of electrochemically active microbes enable the respiration of diverse inorganic and organic molecules for their survival in redox-stratified environments. The ability of exchanging electrons with electrodes possesses several diverse biotechnological applications like the construction of microbial fuel cells, electro-fermentation, and electro-genetics. In this thesis, the microbes were engineered for their utilization in bioimaging and biosensing applications. Firstly, intracellular and extracellular magnetite accumulating *Escherichia coli* bacterial cell machineries were constructed as contrast agents for the MRI scanning, promising for a cancer diagnostic. Secondly, the intracellular magnetite accumulating bacterial cells, possessing all the redox reactions that readily take place in their cytoplasm via synthetically produced proteins, were further engineered to improve their targeting capability for breast cancer tumor cells by displaying a certain nanobody on the cell surface. Thirdly, electronic sentinel bacterial cells were designed utilizing the electron transfer modules for extracellular electron consumption by targeted acceptors for their wireless biomonitoring applications upon detecting a disease molecule. The methodologies described in this thesis are envisioned as promising tools for diagnostic applications.

Keywords: synthetic biology, biomaterial production, magnetic bacteria, living electronic sensors, contrast agents

ÖZET

BİYOĞÖRÜNTÜLEME VE BİYOSENSİNG İÇİN GENETİK OLARAK TASARLANMIŞ MİKROPLAR

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Mikroorganizmaların aktif hareketliliği ve kendiliğinden ilerleme özelliklerinin yanı sıra, çok işlevli aktiviteler kazanabilmeleri biyomühendislik ile kolayca yapılabileceğinden biyo-görüntüleme ve biyosenör uygulamalarında kullanılması avantajlı bir yaklaşım sunmaktadır. Ortaya çıkan gelişmeler, mikroorganizmaların, hastalık teşhis ve tedavisinde terapötik ajanlar olarak kullanımını içermektedir. Habitatın dinamik yapısı, mikroorganizmaları hayatta kalmaları için özel biyo-işlevsellikler geliştirerek değişen yaşam koşullarına uyum sağlamaya zorlar. Bu nedenle, fonksiyonel materyaller üreten canlı mikroorganizmalar, bir hastalık biyobelirteci molekülünün tespiti ve kanser teşhisindeki büyük ihtiyacı karşılamak üzere benzersiz potansiyele sahip biyo-hibrit bir sistem olarak görev görmektedir. Disiplinlerarası bir bilim alanı olan sentetik biyoloji yaklaşımı, mikroorganizmaları mevcut doğal yolları yeniden tasarlamak ve düzenlemek için olanak vererek istenilen işlevin kazanılmasını sağlar. Demir oksit materyallerinin doğadaki biyomineralizasyon yolağından ilham alarak, anti-tümör etkisindeki potansiyeli göz

önünde bulundurulduğunda, kolay modifikasyon, stabilite ve manyetik özellikleri nedeniyle talep görmektedir. Ayrıca, belirli solunum mekanizmalarına sahip elektrokimyasal olarak aktif mikropların, redoks tabakalı ortamlarda hayatta kalmaları için çeşitli inorganik ve organik moleküllerin solunumunu mümkün kılar. Elektrotlarla elektron alışverişi yapma yeteneği, mikrobiyal yakıt hücreleri oluşturma, elektrofermantasyon ve elektro-genetik gibi çeşitli biyoteknolojik uygulamalara olanak sağlar. Bu tezde, mikroplar biyo-görüntüleme ve biyosensör uygulamalarında kullanılmak üzere tasarlanmıştır. İlk olarak, MRI taramasında kontrast ajanı olarak kullanılmak üzere hücre içinde ve hücre dışında manyetit biriktiren, kanser teşhisi için umut vadeden *Escherichia coli* bakteriyel hücre makineleri oluşturulmuştur. İkinci olarak, sentetik olarak üretilen proteinler aracılığı ile sitoplazmalarında gerçekleşen tüm redoks reaksiyonlarına sahip hücre içi manyetit biriktiren bakteri hücreleri, hücre yüzeyinde belirli bir nanobody proteini göstererek meme kanseri tümör hücrelerine yönelik hedefleme davranışlarını geliştirmek amacıyla tasarlanmıştır. Üçüncü olarak, hedeflenen alıcılar tarafından kablosuz biyoizleme uygulamaları için bir hastalık molekülünü tespit ettiklerinde elektron transfer modüllerini kullanarak hücre dışı elektron tüketimi yapan bakteri hücreleri tasarlanmıştır. Bu tezde anlatılan metodolojiler, teşhis uygulamaları için umut vadeden araçlar olarak öngörülmektedir.

Anahtar kelimeler: sentetik biyoloji, biyomalzeme üretimi, manyetik bakteriler, canlı elektronik sensörler, kontrast maddeler

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CHAPTER 1 : INTRODUCTION

1.1 The Bionanotechnology and Biomaterials

In nature, inorganic materials are formed in certain organisms via their complex biological operations. The involvement of biomolecules in these microorganisms, like proteins and metabolites, enables the synthesis of the natural biomaterials. The interface of biomineralization and bionanotechnology gives scientists a crucial potential for nature-inspired innovation. The biomineralization is a natural process where the creation and deposition of minerals are performed by living beings. The bionanotechnology field also serves as a source of inspiration on which biological systems are used for the fabrication of nanomaterials.

Biomineralization is fundamentally the exact regulation and control of the mineralization process by living organisms. For instance, corals, and some bacteria strains generate complex and resilient mineral formations with extraordinary characteristics through well-controlled metabolic pathways. The biominerals inspire the creation of synthetic materials that have outperforming characteristics in terms of biocompatibility, hardness, strength, and flexibility. [1] The production of numerous organic/ inorganic hybrid materials inspired by biomineralization has a wide range of applications in fields like biomedicine. [1] The utilized proteins and macromolecules in synthetic hybrid materials provide control of the crystal structure, morphology, size and composition with enhanced functionalities like mechanical strength and structural hierarchy. [1]

The biological processes, especially biomineralization, are widely acquired to produce desired nanoscale material hybrids and agents in the field of bionanotechnology. The

natural inspiration for utilizing components of life, such as DNA and proteins, has revealed accurate and well-functioning nanostructures. Nature sheds light on developing nanoscale system serving for various functions and having numerous application fields according to the need. By taking inspiration from biomineralization organisms as models, various number of investigations have been made for creating synthetic nanomaterials with enhanced characteristics and multi-functionality depending on the therapeutic action. The microorganisms are being investigated as cargo delivery systems to transport the therapeutic agent into the targeted cells. There are many application areas, like energy storage and biomedical usage for bio-inspired nanomaterials to be utilized.

A multi-disciplinary field called synthetic biology utilizes biological and engineering aspects for developing innovative biological moieties to obtain maximum performance from living organisms to synthesize materials. The living materials enable the researchers to control the characteristics, morphology, and size of the materials synthesized by acquiring mineral depositing behavior via integrated synthetic genetic circuitries. Hybrid living materials are dynamic structures with self-healing, self-propelled characteristics that can be designed to sense an extracellular stimulus. They have promising potential to be used in disease diagnosis and treatment in the field of biomedical applications. They can be utilized in tissue engineering by producing scaffolds and enabling tissue regeneration. For example, silica biomineralization stimulating osteoblastic transformation has been implemented for human dental pulp stem cells, and the cells possess calcium crystal accumulation in their surroundings. [2] Moreover, the crystal formation of a major biomineral of the bone called hydroxyapatite (HAP) has been analyzed for obtaining enhanced mammalian hard

tissue environment via HAP-interacting proteins for bone biomineralization applications in regenerative medicine. [3]

Cancer targeting is one of the most outstanding needs in the field of medicine. The direct targeting of malignant cells has a crucial role in cancer treatment, and it should not be limited as severe effects might be seen in healthy tissues. Precise detection and targeting of the cancer cells have been leveraged via living materials capable of delivering therapeutic molecules to the targeted area. The efficacy of the cancer treatment increases with minimized side effects, so the patient's quality of life is improved. The combinatorial effect of bacteriotherapy and multi-functional nanoplateforms have been investigated as prominent theranostic platforms. [4] The nano-bacteria hybrids are applicable to chemodynamic therapy, phototherapy, drug delivery, and bio-imaging with distinct characteristics like enhanced penetration, release of extracellular anti-cancer molecules, hypoxia targeting, tunable physiochemical properties, and harboring multifunctional nanoparticles. [4]–[6]

1.2 Developments of Microbe-Material Hybrids

Microbe-material hybrids possess a wide range of applications in biomedical fields, like bioimaging and anti-cancer therapy. [7] The microorganisms can be engineered to be hybridized with materials via various ways like biomineralization, biosynthesis, covalent conjugation, and electrostatic interaction. The bioengineered system promises enhanced drug delivery and less side effects by overcoming the challenges faced due to complexity and heterogeneity of disease areas. The material hybridized microbes give a dynamic platform to offer improved disease therapy by specifically targeting a disease via hypoxia, light, or magnetic targeting. For cancer, the commonly used chemotherapy causes unintended damage to the healthy cells. Targeted therapy

increases therapeutic success, and hurdles drug resistance, so it plays a role in personalized medicine. The microorganisms can be designed to target specific characteristics. For instance, a bacterial strain has been modified to target hypoxic regions of tumors without affecting the non-diseased cells. [7]

1.2.1 Importance of Magnetite in Biomedical Applications

Magnetic targeting also has advantages for precision therapy. Biomimetic mineralization is the concept where the natural biomineralization process, the synthesis of minerals from biomacromolecules via microorganisms, led researchers to utilize the material formation for therapeutic applications. Living biomaterials can be designed to obtain enhanced material properties in terms of unique morphology or desired crystal size via control over the synthesis conditions. [7]

In nature, magnetotactic bacteria (MTB) AMB-1 synthesize magnetic crystals in their specialized organelles called magnetosomes. [8], [9], [18], [10]–[17] They utilize magnetic characteristics in their survival and sensing Earth's geomagnetic field. [11] The magnetosomes act as a magnetic sensor for enabling microbial magnetoreception. [19] The crystals in magnetosomes align as chain-like structures. There are magnetic nanocrystals inside the lipid bilayer membrane. The crystals are mostly magnetite (Fe_3O_4) and greigite (Fe_3S_4). (**Figure 1.1**)

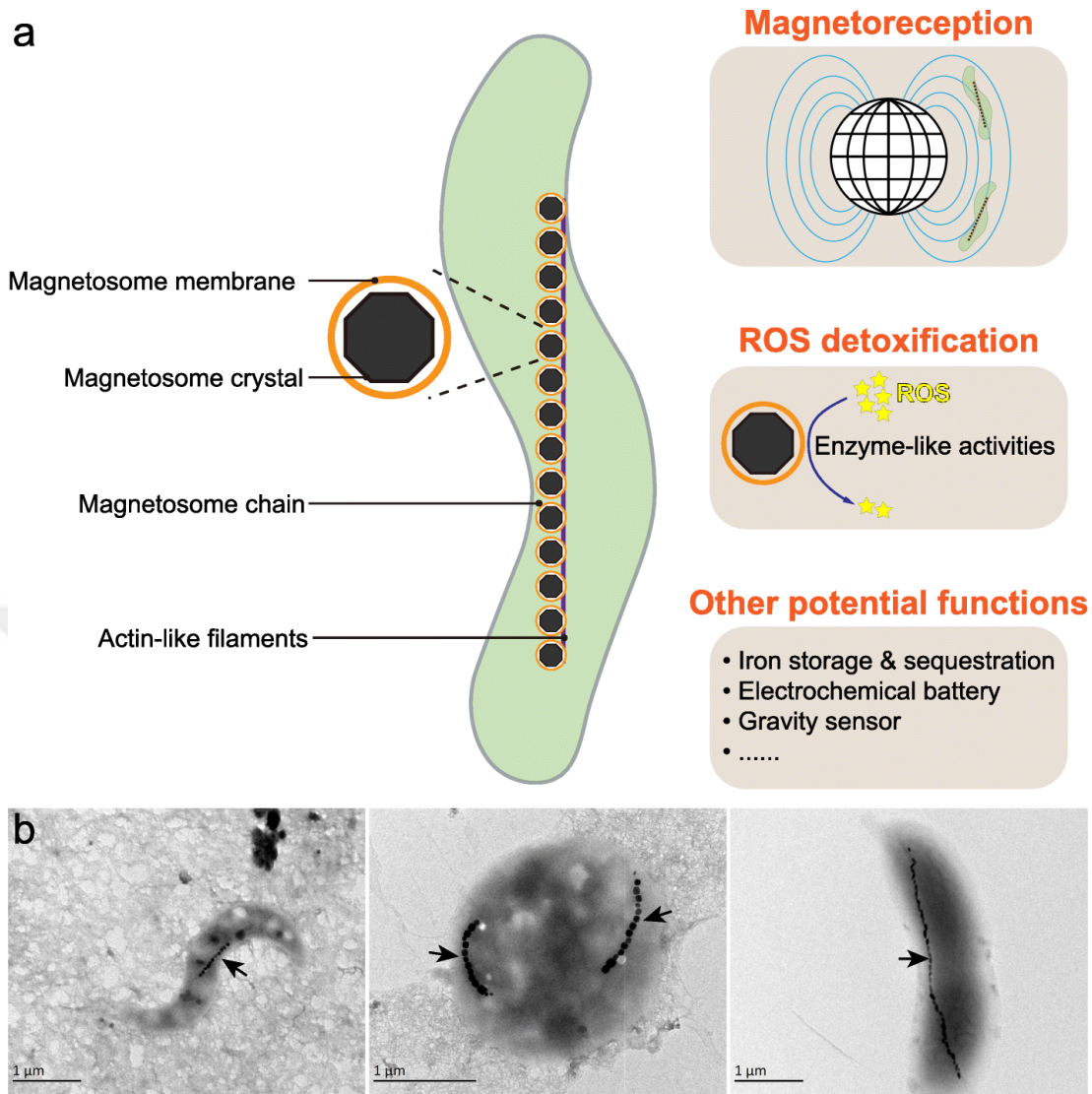


Figure 1.1 The specialized organelle magnetosome in magnetotactic bacteria (MTB) serve for biomineralization of magnetic crystals. a) Intracellular magnetic crystals align into chain-like filaments for optimization of magnetic dipole moment of the cell. They function for magnetoreception, iron sequestration and ROS detoxification. b) The chain-like structures of magnetosomes in MTB are visible via electron microscopy imaging. Reprinted with permission from reference [19]

Magnetosomes possess biocompatible characteristics, and due to their enhanced magnetic properties, they have been considered for usage in magnetic resonance imaging (MRI) and drug transportation. [20], [21] The facultative anaerobe nature of magnetotactic bacteria serves significant potential for them to be used as hypoxia-driven cancer targeting in the field of biomedical applications. There are various proteins involved in magnetic nanocrystal synthesis to control their size, morphology, and crystalline structure. [11] There are gene clusters called mam and mms in the specialized organelle. [1], [9], [13]–[15], [17], [22], [23] A number of investigations have demonstrated that for controlling the assembly of bacterial magnetic nanocrystal chains, MamJ proteins take a role, whereas nucleating from the long filament is done via MamK protein. Similarly, the efflux and influx of iron are provided by the MagA protein. [23] **(Figure 1.2)** Mms16 and MpsA proteins take place in vesicle formation by initiating invagination from the inner membrane. After the formation of vesicles, they align into a linear chain via cytoskeletal filaments. Iron transporters enable the ferrous iron ions to be taken to the cytoplasm, and the accumulation of irons results in crystal formation. [1]

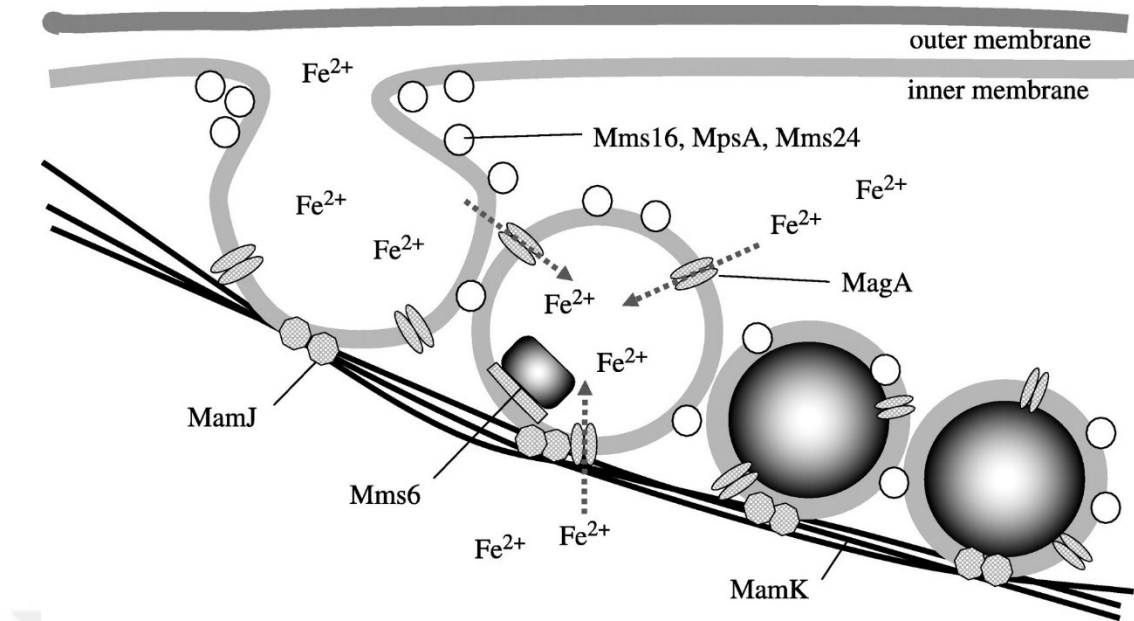


Figure 1.2 The iron oxide biomineralization in nature serves for the synthesis of bacterial magnetic nanoparticles in specialized lipid bilayers called magnetosomes. Reprinted with permission from reference [23]

The AMB-1 bacterial cells have been examined for their usage in magnetic resonance imaging (MRI) and hyperthermia treatment via their hypoxia targeting capabilities. [11] Their selective migration to hypoxic regions of the tumor resulted in deep tumor penetrability because of their anaerobic nature. The use of an external magnetic field serves for the precise delivery of payloads and the generation of heat by alternating magnetic field application in order to cause thermal ablation to the solid tumor cells. [7] The tumor microenvironment (TME) is preferable for microbes to colonize due to their nutrient-rich surroundings.

Biohybrid microsystems have been developed consisting of both motile microorganisms and functional materials for promising applications in biomedical and bioengineering. [24], [25], [34]–[40], [26]–[33] Microrobot materials have been designed to be self-directed and self-propelled to serve as multifunctional

microswimmers in a way that is minimally invasive therapeutics. This way, the modules, for example, can be triggered by the pH gradient of the tumor microenvironment for on-demand drug delivery. The flagellated bacteria are utilized as an actuator or sensor to give the system high motility and detection ability that can be controlled externally via magnetically for targeted localization and stimulated drug release. *Escherichia coli* bacterial cells have been hybridized with magnetic nanoparticles (MNPs) and nanoliposomes for therapeutic agent loading. The bacterial system can be localized to tumor cells via magnetic field application and can release anti-tumor agents upon near-infrared (NIR) stimulus. [24]

Magnetic nanoparticles (MNPs) can be integrated into bacterial cells for various applications. For example, the chemical bonding of MNPs to the microorganisms has been done via amide-carboxylic acid bonding for controllable disease treatment purposes. The remote propelling activity of bacterial cells serves as an advantage for obtaining desired therapeutic effects via biohybrid microrobots. The probiotic *Escherichia coli* Nissle 1917 has been engineered and utilized for magnetic and hypoxic targeting of cancer cells. In addition, the cells have been designed as a therapeutic agent by sensing increased heat via an integrated thermal-logic circuit and synthetically synthesizing an enzyme to induce reactive oxygen species (ROS) formation when an alternating magnetic field (**Figure 1.3**). [41] The combinatorial effect of both the heat generated via external magnetic field application and the produced ROS provides enhanced anticancer treatment efficiency. [41]–[44]

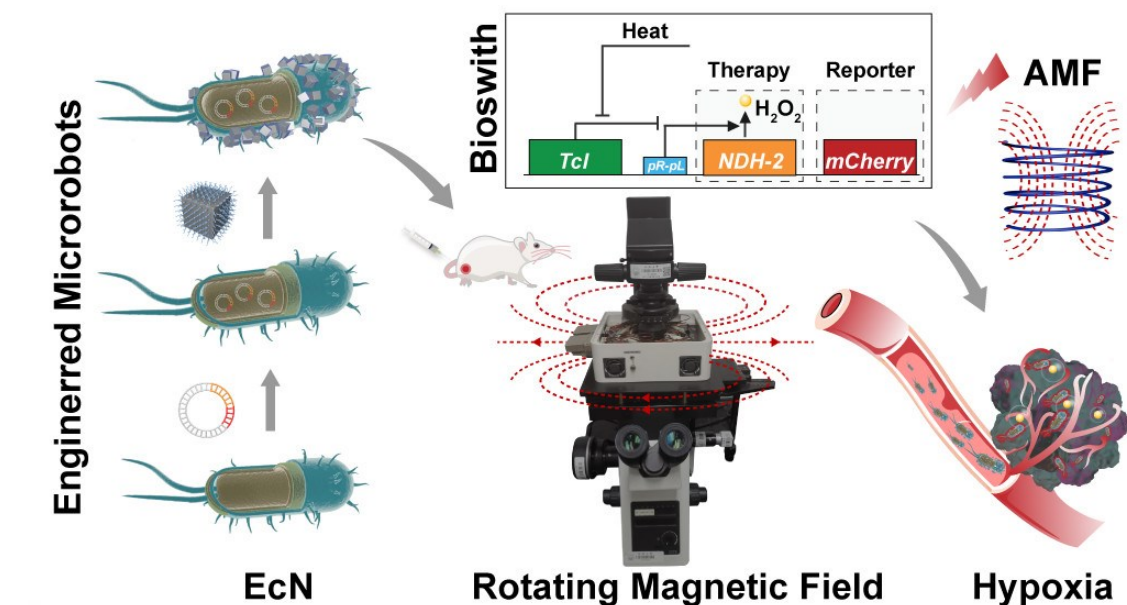


Figure 1.3 The designed *EcN* bacteria cells harboring targeting capability via hypoxia and magnetic manipulation synthetically express an enzyme to trigger reactive oxygen species formation that enhances the therapeutic effect of the system. Reprinted with permission from reference [41]

Bioimaging is a crucial step in the diagnosis and assessment of cancer progress. As a non-invasive imaging technique, magnetic resonance imaging (MRI) serves to acquire comprehensive information about the tissue and provide detailed anatomical images. The microbe-material hybrid can be utilized for bioimaging of tumor tissues. The intracellular magnetosomes can be produced via magnetotactic bacteria that can serve as MRI contrast agents with the help of their biocompatible and superparamagnetic characteristics. [7] Moreover, MNPs have been dip-coated on a surface of biodegradable microalgae that can be remotely navigated and utilized as MRI contrast agents. [45]

Microbe-material hybrids can be utilized for tumor therapy, like via chemodynamic therapy. The therapy utilizes Fenton-like reactions to damage cancer cells via ROS

synthesis. The generation of hydroxyl radicals causes reactions with organic and inorganic molecules to take place, therefore causing severe damage to the cells. The increase in the concentration of hydrogen peroxide around the tumor surrounding and the effective catalytic performance of Fenton-like agents improves the efficiency of chemodynamic treatment. [7] *E. coli* bacterial cells have been designed to express the NDH-2 enzyme as a therapeutic agent. In addition, the bacterial cells have been coated with MNPs via amine reaction, which allocates the cells as tumor targeting activity. The enzyme catalyzes the oxygen abundant in tumor cells into hydrogen peroxide formation. In Fenton-like reactions, the ferric ions give reactions with hydrogen peroxide and result in ferrous ion production that later causes the formation of hydroxyl radical production. The oxidative stress leads to the death of tumor cells. [42]

1.2.2 Importance of Sentinel Bacterial Cells as Therapeutic Tools

Certain microorganisms utilize electron movements naturally in several processes for their survival, for instance, respiration, communication, and sensing. [46] In nature, specific organisms like electroactive bacteria harbor the extracellular electron transfer (EET) system in their metabolic routine through redox-active proteins for electricity production or consumption interacting with an extracellular acceptor or donor, respectively. [46]–[49] This natural process has been used for CO₂ reduction into organic molecules by consuming electrons from an extracellular electrode. Yet, the efficacy and the rate of the reduction have not met the need for an overall economic contribution to society. [50] The physiological limitations slow down the extracellular electron transfer (EET) pathway in which the natural electroactive bacteria grow anaerobically. [46]

Shewanella oneidensis can utilize electron acceptors or solid metals for their respiratory activities. The phenomenon is also called a metal reduction pathway (Mtr), where protein complexes aid the electron transfer throughout the cells and through the outside of the cell. The first electron transfer occurs from intracellular electron donors, like pyruvate and lactate, are metabolically processed. The electrons in the inner membrane are transferred to the outer membrane via periplasmic proteins as a shuttle mechanism. The outer membrane cytochromes deliver the electrons to the external electron acceptors, like metal oxides. The anaerobic respiration in the bacterial strain can be utilized for current consumption or production involving an electrode, metal synthesis, and medical field. [7] The second well-known electroactive bacteria model is *Geobacter sulfurreducens*. Both of the biofilm-forming gram-negative bacterial strains have been investigated to reduce iron and manganese in their habitat to make connections with extracellular electrodes via biofilm. [46] Direct electron transfer occurs as the quinol oxides transfer electrons to periplasmic c-type cytochromes that move electrons from the inner membrane to the outer membrane. Lastly, the protein complexes in the outer membrane release the electron to extracellular space. [51], [52] The mechanism of electron transfer in *S. oneidensis* starts with the membrane-bound cytochrome protein CymA carrying electrons from menaquinol (MQ) to periplasmic cytochromes called fumarate reductase (FccA) and tetraheme cytochrome (CctA) (**Figure 1.4A**). [53]–[57] On the other hand, in *G. sulfurreducens*, the quinol is oxidized by inner membrane proteins called ImcH and CbcL, and then certain periplasmic cytochrome proteins give response to the external conditions according to the reduction potential of it (**Figure 1.4B**). [46], [58], [59] In addition, the outer membrane possibly forms an extension carrying the Mtr complex in *S. oneidensis* (**Figure 1.4C**). According to the conditions, there might be a need for organisms to

use a soluble molecule to interact with a distant electrode (**Figure 1.4D**). One example of these redox-active shuttles is called flavins, which facilitate electron transfer. [46]

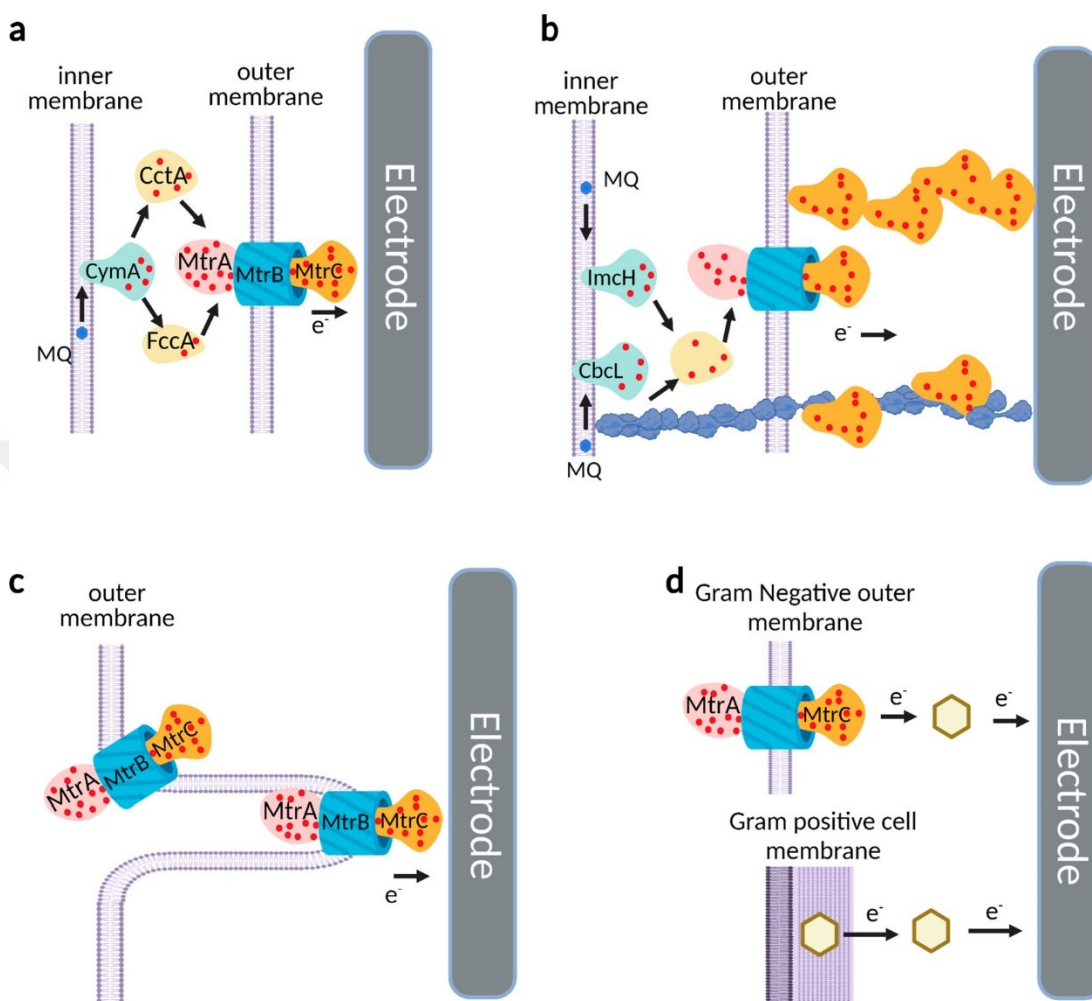


Figure 1.4 The extracellular electron transfer (EET) systems are provided. a) Outer membrane c-type cytochrome proteins enable the direct electron transfer in *Shewanella*. b) The direct transfer of electrons in *Geobacter* from inner membrane proteins to the periplasm and outer membrane, and lastly, the electrode sequentially is connected through nanowires. c) The EET in *S. oneidensis* might involve the outer membrane extensions, including redox-active cytochromes conduits. d) A soluble redox-active shuttle can be utilized for EET. Reprinted with permission from reference [46]

A non-native host, the *E. coli* strain, has been modified to sense and transfer electrons for transforming functionality among native and non-native hosts in order to obtain enhanced electrical current biosynthesis and develop bioelectronic sensors. The necessary conditions, such as appropriate temperature, pH, and oxygen amount, have to be considered when acquiring the EET system in a non-native host. [46]

1.2.3 The Biosafety of the Biohybrid Materials

The developments in bioengineering have revealed the usage of biohybrid materials in theranostic applications. The combination of living and nonliving moieties improves the effectiveness of biomaterials in biomedical usage. On the other hand, micro-material hybrids have to possess biocompatibility features so that they do not cause a systemic effect in patients and are biosafe for clinical applications. [60] The hybrid material has to be subjected to detailed examination for its efficacy, possible side effects, and duration of action. The administration way of the therapeutic agent is crucial to determining according to the disease progression. The external guidance of the biohybrid material is also an option to regulate the spatial distribution of bacteria and minimize the possible side effects on the non-diseased cells. Moreover, the pathogenicity genes can be eliminated from the designed bacteria without affecting the therapeutic activity. [61], [62] Usage of probiotic strains in microbe-assisted nanomedicine is also a safe alternative that does not cause any septic shock in the human body and can be loaded with therapeutic molecules. [7], [63]–[65]

1.3 Significance of Synthetic Biology in Biomaterial Hybrid Systems

Synthetic biology tools facilitate the engineering of microbes for specific disease targeting. [66] The integration of genetic circuitries to bacterial cells enables them to

colonize tumor regions, and it is also designed to deliver anticancer agents at the same time. The developed therapeutic strategies via genetic engineering enhanced the efficacy and safety of the detection and treatment process. The bacterial cells provide the control in spatiotemporal activity of therapeutic effect. The release of payload at the desired area and at the purposed time decreases the possible adverse effects on the human body. [66]–[68] The colonization to tumor cells can also be achieved by utilizing a probiotic bacterium called *Escherichia coli* Nissle 1917 bacterial strain that harbors intrinsic features to localize to cancer cells. [69]–[73] The colorectal cancer (CRC) detection and treatment is aimed to be actuated via EcN cells that showed specific colonization of colorectal adenomas upon oral administration to engineered murine and orthotopic models of CRC. The bacterial cells producing salicylate have been delivered orally, and the adenoma-bearing mouse showed elevated levels of salicylate in urine which serves as a lesion detection tool. Moreover, EcN cells are engineered to release a cytokine locally to block nanobodies at the neoplastic site as a therapeutic action. [69] In addition to therapeutic activity, the bacterial system harboring payloads can serve in imaging modalities like magnetic resonance imaging. [66], [74]

The behavior of bacteria can be manipulated to plan the release of therapeutic time and location via external forces like ultrasound or magnetic field applications. [66] The combination of living and nonliving modalities enhances the visualization of tumors via, for example, positron emission tomography (PET) or MRI. Biomarker detection via engineered bacterial cells would serve as a minimally invasive detection method for even screening microscopic tumors and metastasis. [66], [73], [75]–[77] Bacteria might also serve as an adjuvant in order to address the challenges faced in the immunosuppressive feature of the tumor microenvironment (TME). [78] Bacteria

capture and adsorb the tumor antigens via cationic polymer nanoparticle coating on the surface of the bacteria upon radiotherapy or chemotherapy applications. Therefore, the immune system stimulation is enabled when the bacteria transport the tumor antigens to antigen-presenting cells in the tumor surrounding. For this purpose, the *Salmonella* VNP20009 attenuated strain was utilized for its high motility characteristic and engineered for absorbing tumor antigen and improving immunogenicity via its binding ability to Toll-like receptors (TLRs). The intratumorally injected bacteria transport the antigens to marginal dendritic cells (DCs), surpassing the immunosuppressive nature of the TME. Acting as an anti-tumor agent in the tumor periphery, this strategy serves as bacteria-based cancer therapy (BCT), harboring potential for in situ cancer vaccination. [78]

The magnetic field is used as a safe tool to control the bacterial cells' motion into the desired direction. A magnetic torque-driven control mechanism enables the wireless actuation of engineered cells toward hypoxic tumor cores. [79], [80] The hybrid material containing magnetotactic bacteria (MTB) conjugated to liposomes is utilized for enhanced payload transport. The bacterial microrobots have been loaded with liposomal cargo and gave rotational motion in response to the rotating magnetic field both in the vascular endothelium model and tumor spheroid model where the bacterial machineries show response as rotating individually. The conjugated system is able to function to migrate into tumor cells controllably through a blood vessel's openings that promise on-target activity, minimizing off-tumor toxicity. [79]

E. coli strain makes it easy to transform cells with foreign DNA and express recombinant proteins, enabling smooth manipulation. The strains have been engineered to express CymA and MtrCAB complex for insoluble iron reduction, which

gives opportunities to use them in energy metabolism. [81] The cytochrome c maturation (ccm) pathway is required to be expressed without any toxicity and in a correct assembly to be functional to optimize electron transfer in *E. coli*. [46], [82] The term electrogenetics means that gene expression is controlled by electronic signals via redox molecules, and indirect electronic control takes place. In another word, the bacterial cells are able to sense the extracellular redox active molecule and express genes of interest. [83] Via synthetic biology implementation, the electrochemical reactions can be tightly controlled in host bacteria, harboring EET pathways for enhancing microbial fuel cells, developing living electronic sensors, and microbial electrosynthesis coupled bioproduction. [46] A smart bioreactor has been developed by combining an anti-cancer drug-loaded nanocarrier with *Shewanella oneidensis* MR-1 strain in order to enhance the chemotherapeutic effect. [84] The living material is able to carry out cyclic lactate metabolism by itself and serves as a sensing and responding module for the lactate-rich tumor microenvironment. Hypoxia targeting leveraged by the anaerobic nature of bacterial strain plays a role in minimizing adverse effects on healthy tissues. In addition, the electron transfer pathway naturally taking place in the bacterial strain enables the continuous catabolism of intratumoral lactate that is coupled with ferric iron reduction into ferrous iron ions. The developed strategy possesses enhanced tumor targeting capability and improved chemotherapeutic effect as a theranostic agent. [84]

1.4 Overview of Current Challenges in Cancer Diagnostics and Treatment

A severe global disease, cancer, is a collection of diseases from diverse cell types and occurs via over-proliferation and aberrant cell differentiation. The tumor

microenvironment possesses unique characteristics like a hypoxic tumor center, acidic surroundings, and immunosuppressive features. All the characteristics make it hard for them to be reached and cured. [85] Low oxygen concentration causes anaerobic glycolysis to take place, which leads to neovascularization factor production and lactic acid synthesis, suppressing chemotherapeutic treatment efficiency. [86], [87] In addition, the high production of vascular endothelial growth factor (VEGF), like in hematopoietic tumor cells or breast carcinoma cells, hyper-permeabilize the vasculature. [86], [87] If early detection is achieved, surgical removal of tumor tissue might be performed for solid tumors, like breast or colon, according to the cancer screening for vital signs, so that complete removal is not guaranteed and might reoccur. [88] Several therapeutic methods are available, like chemotherapy and radiotherapy. On the other hand, radiotherapy might cause damage to DNA and lead to necrosis, in addition to therapy resistance due to mutations. [88] Chemotherapy acts on tumors by affecting metabolism, cell division, and biosynthesis that might get failure due to multi-drug resistance. [88] New strategies are needed to target the cancer cells specifically and show therapeutic activity for the regression of solid tumors. [85]

1.5 Scope and Structure of the Study

In this thesis, the bioimaging and biosensing harboring microbe utilization is provided as they offer advantageous approaches with their actively motile nature and self-propulsion behavior in addition to their easy genetic engineering characteristics to perform multi-functional activities. The living microorganisms synthesizing functional materials, called biohybrid systems, serve for promising potential in enhanced detection of a disease biomarker or in satisfying the need of cancer diagnosis.

In Chapter 2, *E. coli* bacterial cells have been engineered to synthesize magnetic nanoparticles (MNPs) either inside the cellular machineries or on the cell surface in order to serve as an MRI contrast agent. [74] For the accumulation of magnetite intracellularly, the iron-binding peptide was combined with a ferroxidase enzyme, material-binding peptide, and an iron transporter protein in order to enable the bacteria to perform all the redox reactions required to synthesize iron oxide nanoparticles inside the bacterial cells. For the construction of the extracellular magnetite accumulating bacteria system, the iron-binding peptide was fused to truncated autotransporter protein in order to display the iron-binding peptide on the bacterial cell surface for enabling and facilitating the magnetic nanoparticle synthesis under proper conditions. The developed bacterial systems were performed with enhanced MRI contrast capability, which showed a promising alternative for cancer diagnostic agents.

In Chapter 3, the intracellular magnetite accumulating bacteria (ICMAB) system was developed further for its possible usage in biomedical applications. The system provides a ready-to-use agent where all the redox reactions take place inside the bacterial cells themselves. Therefore, they were aimed at being integrated with a nanobody to specifically target cancer cells. The nanobody expression on the ICMAB cell surface serves to adhere the bacterial cells to the breast cancer cells, therefore decreasing the possible side effects of the theranostic agent on the healthy tissues. The designed system showed a dual-functional performance where it can both synthesize magnetites intracellularly and attach to specific mammalian cells. Also, a higher MRI contrast capability was shown for the cells after infecting the breast cancer cells, which implies a promising tool for the detection of cancer.

In Chapter 4, *E. coli* bacterial cells were utilized for developing electronically active cellular machineries. The cells harboring electron transfer modules serve as an electron releasing moieties for an extracellular acceptor. The iron or magnesium metal coated surfaces was incubated with proper cell groups to observe the metal dissolving behavior of the cells. The cells hold a great promise to track a disease molecule and provide a wireless biomonitoring application.

In the final chapter, the studies performed in this thesis are summarized, and their future perfectives are provided as promising tools for diagnostic applications.

1.5.1 Potential impact of bionanomaterials on the field of nanotechnology and medicine

The utilization of bacteria as a therapeutic agent is favorable as they can be designed to diagnose and treat a disease with remarkable spatiotemporal control of action. The payload can be released upon stimulation or sensing of a biomarker at the desired area and at the planned time. The on-target functionality of bacteria minimizes the off-target side effects and toxicity in the human body. [66], [79] The biocompatibility and safety concerns can be overcome via several strategies with the help of synthetic biology tools like attenuation of a strain and probiotic bacterial strain usage. [7], [66]

CHAPTER 2 : NANOMAGNET ACCUMULATING BACTERIAL SYSTEMS AS MRI CONTRAST AGENTS

The work described in this section is partially published in the following study [74]:

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2.1 Objective of the Study

With the rising cancer rates, the demand for highly efficient cancer diagnostic tools is increasing alongside. Furthermore, there is an urgent need for novel cancer therapies that do not possess the side effects associated with conventional treatments. Developments in this aspect bring the use of magnetic nanoparticles (MNPs) in biomedical applications due to their stability, biocompatibility, and magnetic properties. Certain organisms, including many bacteria, can synthesize magnetic nanocrystals that aid in their spatial orientation and survival by sensing the earth's geomagnetic field. This study intends to modify *Escherichia coli* to accumulate magnetite, which can be coupled with drug delivery modules. The authors use genetic circuitries to create magnetite-accumulating bacterial machineries that employ Mms6, a protein that binds iron and is crucial for magnetite crystal production. The study demonstrates that the combinatorial effect of Mms6 with ferroxidase, iron transporter protein, and material-binding peptide enhances the paramagnetic behavior of the cells in magnetic resonance imaging (MRI) measurements. Additionally, cellular machines are engineered to display the Mms6 peptide on the cell surface via an autotransporter protein, resulting in augmented MRI performance. The findings are promising for developing a probiotic bacterium capable of accumulating magnetite both intracellularly and extracellularly, serving as a theranostic agent for cancer diagnostics via MRI scanning and hyperthermia treatment.

2.2 Introduction

Early detection of a disease is essential for a successful intervention and treatment. Although many advancements have been occurred in the medical field over the last century, imaging techniques remain among the most widely used methods for obtaining anatomical or physiological information about a dysfunctional body part. In addition, noninvasive imaging is particularly preferred because it assesses the condition without affecting healthy tissues. Noninvasive imaging is one of the most important tools for cancer diagnosis and prognosis, offering real-time monitoring of applied therapy and patient responsiveness. Therefore, this approach is vital to obtain time- and cost-effective treatments. [89] Magnetic resonance imaging (MRI), a noninvasive imaging technique, enables tissue density differentiation at high contrast, in addition to impeccable spatial resolution and penetration depth. The working principle of MRI is measuring the changes in hydrogen magnetization, which can be enhanced via the use of contrast agents. [90] The magnetic behavior of the contrast agents affects the T1 (longitudinal) and T2 (transverse) relaxation time measurements. MRI contrast agents can be categorized into two groups based on their magnetic properties: paramagnetic contrast agents, such as gadolinium and manganese-based agents, and superparamagnetic contrast agents, such as iron oxide and iron platinum agents. [91] Iron oxide nanoparticles (IONPs) are especially used as functional contrast enhancement agents for cancer diagnosis via MRI, due to their biocompatibility and magnetic properties. [90], [92] According to synthesis conditions, the size of IONPs can be controlled which allows them to be coupled with biological moieties, such as bacteria, in the desired size. [93]

Utilizing bacterial cells in cancer therapies offers the construction of a multi-functional system that may serve to sense and respond to the environment and can be engineered to produce an anti-cancer agent simultaneously. Certain types of bacterial strains possess a distinguished characteristic, that is specific colonization on tumors and selective replication in tumor tissue. [94]–[97] Also, bacteriabots have a critical role in cancer therapies in a way that stimulates them externally via acquired nanoparticles. [98] Thus, bacteria are promising tools for tumor therapeutics as certain strains have the ability to specifically target cancerous tissues due to the weakened immune system and nutrient-rich tumor microenvironment (TME). [94], [99] By using live-cell imaging, recording information about the dynamic changes in cells is enabled [100], which is crucial to follow up the progression of tumor tissues. MRI visualization might be enhanced by using magnetic nanoparticles (MNPs) bearing living cells that can specifically colonize to the tumor sites and be externally controlled via a magnetic field.

Certain biomineralization proteins control the biomaterial nucleation and synthesis process in nature. The bio-inspired synthesis of organic/ inorganic hybrid materials can be governed by the biomineralization proteins used to design enhanced materials. [1] By utilizing synthetic biology tools, the bacterial cells can be engineered for protein-based nanomaterial synthesis. In an earlier study by Olmez et al., a biomaterial synthesizing cellular system including a combined sensory and material nucleation module in *Escherichia coli* was constructed successfully. [101] Similarly, silica biomineralization proteins fused to fluorescent proteins are performed for the synthesis of fluorescent silica nanoparticles with low toxicity levels and good optical properties. [102] Silica itself can be coated on IONPs' surface to enhance the stability of the

particles by preventing aggregation; therefore, composite nanomaterials are obtained for possible biomedical applications. [18]

In a similar strategy, taking inspiration from natural magnetotactic bacteria (MTB), which have certain proteins and organelles for iron biomineralization, peptide-based nanomaterials can be constructed as tumor-specific contrast agents. Schuerle et al. engineered *Magnetospirillum magneticum* AMB-1 strain for displaying specific peptides on the surface of magnetosomes' surface in order to target TME acidity and integrin-dependent binding of the magnetosomes selectively on tumors. Therefore, surface-functionalized iron biomineralization cores as an end product serve potential usage in clinical applications as T2- weighted imaging probes. [92] As an alternative, due to the challenges in obtaining metal nanoparticles with well-controlled properties in nature, the *M. magneticum* AMB-1 might be genetically engineered to tune the proteins' expression, taking a role in the chain length, size, and morphology of the IONPs by modifying ribosome binding site (RBS) and promoter for acquiring desired IONP properties. [18] Moreover, the *E. coli* MG1655 strain has been engineered to overexpress specific enzymes in a way to bacteria act as a fenton-like bioreactor covalently linked with MNPs and simultaneously synthesizing hydrogen peroxide around colonized TME. The role of MNPs as a catalyst in Fenton-like reactions causes the production of toxic hydroxyl radicals, giving the engineered bacteria anti-cancer activity in a potential treatment. [103]

Here, developed systems offer a promising noninvasive contrast agent which possess the potential to be selective to hypoxic regions of tumors and potent anti-cancer activity. In addition, biocompatible bacterial systems minimize the therapies' toxicity to healthy tissues and harbor a potential for theranostic applications for cancer. To this

end, previously, researchers engineered cells for expressing Mms6 protein as a primary moiety, which serves a role in iron-oxide nucleation [9] for nanomaterials' synthesis inside the cell or on the cells' surface to design bacterial cells as a contrast agent. In the extracellular magnetite accumulating bacteria (ECMAB) system, the Mms6 peptide was displayed on the bacterial cell surface via an autotransporter protein Ag43 that enables nanoparticle production on the surface. For constructing the intracellular magnetite accumulating bacteria (ICMAB) system, Mms6 peptide was combined with ferroxidase (EncFtn) enzyme derived from *Rhodospirillum rubrum*, [104] material binding peptide (MBP), [101] and iron transporter protein (EfeU) from *E. coli* Nissle 1917 for improving the nanomaterial production. [105] The selected peptides give the system the ability of performing required functions by itself and provide ready-to-use cellular contrast agents when supplemented with corresponding ions. It is shown that the ECMAB and ICMAB systems give higher r_2 relaxivity and reduced T2 relaxation time compared to control groups in MRI measurements, respectively. Consequently, the engineered cells possess enhanced MRI contrast performance, having potential usage as a diagnostic agent for cancer diagnosis.

2.3 Materials and Methods

2.3.1 Cell Strains, Cell Growth, Cell Maintenance, Chemical Competent Cell Preparation and Transformation

E. coli DH5 α and *E. coli* BL21 (DE3) strains were used for cloning and protein expression experiments, respectively. The cells' long-term storage condition was at -80°C in a freezing medium containing 25% glycerol in Lysogeny Broth (LB) media. *E. coli* BL21 (DE3) strain encodes the T7 polymerase gene under the control of Lac promoter. The strain lacks proteases, and it was used for protein expression and

purification. Both *E. coli* strains were inoculated in LB medium and incubated on LB agar at 30 or 37°C.

The uptake of plasmids into bacterial cells was done via chemical transformation. For the preparation of chemically competent cells, the bacterial cells were inoculated in LB medium containing proper antibiotic from -80°C cell stock overnight and diluted in fresh LB at 1:100 ratio on the next day morning. The bacterial cells were allowed to grow until OD₆₀₀ of 0.2-0.5 and cooled on ice for 10 min. The cells were collected via centrifugation at 3000 rpm for 10 minutes at +4°C. The cell pellets were resuspended in 1:10 culture volume of TSS (Transformation and Storage Solution) buffer and aliquoted 100 µl into chilled microcentrifuge tubes that were stored at -80°C.

For the transformation of a plasmid into chemically competent cells, the cell stock was thawed on ice for 20 minutes, and a plasmid or reaction mixture was added onto the cell. After 20 minutes of incubation on ice, the heat shock was performed at 42°C for 45 seconds for plasmid uptake into the cells. The cells were incubated on ice for 2 minutes, and 250 µl LB was added onto the cells for growth at 37°C 200 rpm for 45 minutes. The cells were centrifuged after incubation, and the excess supernatant was removed. The remaining supernatant was used to resuspend the cell pellet, and all suspension was spread on an LB-agar plate containing the appropriate antibiotic. The plate was placed in at 37°C stationary incubator for overnight growth.

2.3.2 Construction of Plasmid Maps and Cloning

For constructing an inducible expression cassette of Mms6, the pET22b vector was restriction digested with KpnI-HF (NEB) and XhoI (NEB) enzymes at 37°C for 3 h.

The Mms6 gene fragment was synthesized (Genewiz, USA), and truncated Mms6 was constructed by eliminating the first 24 amino acids, no specific task-assigned region, from the original sequence in order to ease the workload of bacterial cells. The gene fragment was amplified with P1 and P2 primers (given in Appendix B, Table B1.) in order to obtain KpnI and XhoI restriction cut sites containing truncated Mms6. The amplicon was restriction digested, and the purified DNA products were ligated via a T4 ligation reaction based on the suggested protocol for obtaining the pET22b 6H Mms6 vector.

In order to clone Mms6 in pET22b PelB 6H 160N Ag43 backbone, the vector was restriction digested with BamHI-HF (NEB) and AflIII (NEB) enzymes. For the addition of the specified cut sites at the end of the gene, P3 and P4 primers were used for PCR amplification. The restriction-digested gene and vector were isolated for ligation reaction via T4 enzyme according to the manufacturer's instructions.

For pET22b EncFtn 6H Mms6 MBP EfeU vector construction, there was a sequential assembly of all genes and the backbone. P5 and P6 primers were used to amplify the 6H Mms6 T7 Terminator region from the pET22b 6H Mms6 vector. The PCR product was assembled with BamHI-HF (NEB) and XhoI (NEB) digested pZE PROD backbone via Gibson assembly protocol (NEB). P7 and P8 primers were used to amplify the pZE PROD 6H Mms6; P5 and P9 primers were also used to amplify the EfeU T7 terminator gene fragment (IDT). Then, both PCR products were assembled via the Gibson assembly protocol. P10 and P11 primers were used to amplify the 6H Mms6 EfeU region that later assembled with KpnI-HF (NEB) and HindIII-HF (NEB) digested pZs mPROD backbone. P12 and P13 primers were used to amplify the EncFtn gene fragment (IDT); additionally, P14 and P15 primers were utilized for pZs mPROD

6H Mms6 EfeU amplification. Later, both PCR products were assembled via Gibson assembly. P16 and P17 primers were utilized for EncFtn 6H Mms6 EfeU gene part amplification, which was later assembled with KpnI-HF (NEB) and XhoI (NEB) digested pET22b backbone via Gibson assembly. In order to clone MBP in pET22b EncFtn 6H Mms6 EfeU vector, the backbone was amplified with two separate PCR reactions with P18-P19 and P20-P21 primer pairs. Later, both PCR products were assembled via the Gibson assembly protocol.

2.3.3 Sequence Alignments

The genetic circuitries were constructed via an online tool, Benchling.com. The plasmid maps were exported as .gb files. The sequencing process was obtained from Genewiz company as Sanger Sequencing or from InterGen company as Next Generation Sequencing (NGS). The Geneious® 9.05 software or Benchling alignment option was used to import both the vector sequence and sequence results as .ab1 files. For analysis, standard parameters were used for pairwise alignment of the sequence to the reference, and gaps or distinction reads were determined.

2.3.4 Expression of Recombinant Proteins in *E. Coli*

E. coli BL21 (DE3) strain was used for protein expression from sequence verified construct. For N-terminal hexa-histidine tagged Mms6 protein expression, pET22b 6H Mms6 plasmid was transformed into competent cells via chemical transformation. The expression of Mms6 peptide is controlled by T7 promoter-lac operon system. After transformation, single colonies were inoculated into 3ml of LB medium containing proper antibiotic and incubated overnight. The cells were diluted 1:100 into a fresh LB media supplemented with antibiotic in the morning and allowed to grow approximately

OD₆₀₀ of 0.45, corresponding to the mid-log phase of growth. After several optimizations, the cells were induced with 1 mM Isopropyl β -D-1-thiogalactopyranoside (IPTG) at 30°C, 200 rpm for 6 hours. After induction, the cells were harvested at 8000 rpm, +4°C for 5 minutes.

For protein expression from Extracellular Magnetite Accumulating Bacteria (ECMAB), the sequence verified pET22b PelB 6H Mms6 160N Ag43 plasmid was transformed into chemical *E. coli* BL21 (DE3) competent cells. After the growth of a single colony overnight, the cells were diluted into fresh LB media containing proper antibiotic and 1% (w/v) glucose at 1:100 ratio. When they reached OD₆₀₀ around 0.4, they were induced with 1mM IPTG via T7-lacO for 24 hours at 18°C, 200 rpm.

For protein expression from Intracellular Magnetite Accumulating Bacteria (ICMAB), the verified pET22b EncFtn 6H Mms6 MBP6 EfeU construct was transformed into chemical *E. coli* BL21 (DE3) competent cells, and single colonies from the plate were inoculated into LB media supplemented with proper antibiotic for overnight growth. The cells were sub-cultured at 1:50 ratio into fresh 1× M9 minimal media that is supplemented with proper antibiotic as well as 0.4% (w/v) glucose, 0.2% (w/v) casamino acid, 2 mM MgCl₂, and 0.1 mM CaCl₂. When the OD₆₀₀ of cells reached around 0.1, they were induced with 0.1 mM IPTG and 1 mM ammonium ferrous sulfate hexahydrate [105] at for 24 hours at 30°C, 200 rpm.

2.3.5 Verification of Protein Expression

For verification of recombinant protein expression, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)/ Western Blotting analysis was performed. For sample preparation, a certain amount of induced cells (5×10^7) were

collected, and the final concentration of $1\times$ SDS loading dye was added. After boiling at 95°C for 5 min for denaturation of cells, the samples were loaded on 15% SDS-polyacrylamide gel and ran at 120V for around 2 hours. For Western Blotting, the gel was transferred to a pre-activated PDVF membrane with methanol treatment via Trans-Blot Turbo (BioRad) utilizing the standard miniblott protocol. The membrane was blocked against non-specific bindings by incubating in 5% skim-milk dissolved in TBS-T buffer at room temperature for 2 hours on the rotator. Subsequently, the membrane was transferred to the 1: 10000 primary anti-his mouse antibody (Abcam ab18184-100 μg) in 5% skim milk in TBS-T solution at 4°C overnight on the rotator to enable binding of primary antibody to the His-tagged proteins on the membrane. The membrane was washed three times in TBS-T with shaking in order to get rid of excess primary antibody. After washing, the membrane was incubated in 1: 10000 horseradish peroxidase (HRP) conjugated goat anti-mouse secondary antibody (Abcam ab205719-500 μg) in 5% skim milk in TBS-T solution at room temperature for 1 hour. The excess secondary antibody solution was removed via TBS-T washing three times on the rotator at room temperature. ECL substrate (Biorad 170-5061) was used for HRP to give a reaction with His-tagged proteins on the membrane so that the protein bands can be visualized via the gel imaging system (Vilber Fusion Solo S).

2.3.6 Purification of Recombinant Protein via Nickel Column

The functionality of the Mms6 peptide was planned to be ensured via magnetite crystal formation *ex vivo*. For this purpose, the protein was purified under denaturing conditions due to its tendency to oligomerize and form aggregates. The cells were induced with 1 mM IPTG at 30°C , 200 rpm for 6 hours, and pellets were resuspended in binding buffer (6 M GuHCl 10 mM imidazole $1\times$ PBS). After vortex, 0.2 % tween

was added to the sample and incubated for 2 hours at room temperature. Then, the sample was sonicated on ice at 35% amplitude for 15 cycles of 10 sec ON/ 10 sec OFF. The cell lysate was centrifuged at 13000 g for 1 hour in order to separate the insoluble part from supernatant, which is then filtered with a 0.45 μ m syringe filter.

The HisTrap nickel column (GE Life Sciences 17524701) was used for the protein purification. The column equilibration was performed with the binding buffer; then, the filtered sample was loaded on the column using a syringe pump at a 1 ml/min rate. The column was washed with a ten-column volume of binding buffer, and the protein was eluted with an elution buffer (6 M GuHCl 500 mM imidazole 1 $\times$ PBS). The refolding of Mms6 protein was performed via resuspending in an equal volume of refolding buffer (50 mM Tris, 1 mM EDTA, 10% (v/v) glycerol, pH 8), and buffer exchanging into 1 $\times$ TBS for renaturation of the protein [22] via centrifugation in 3 kDa cut-off (Millipore centrifugal filtration). The His-tagged purified protein was analyzed via Western Blotting.

2.3.7 Protein Quantitation

The standards were prepared by making serial dilutions of BSA (bovine serum albumin, 2 mg/mL, Pierce 23209) and used for obtaining a standard curve. The protein concentration was calculated via Bradford Assay (Sigma-Aldrich) by taking measurements in SpectraMax M5 spectrophotometer (Molecular Devices) at 595 nm absorbance and using SoftmaxPro software.

2.3.8 Iron Oxide Biomineralization in the Presence of Recombinant Protein

The purified Mms6 peptide was used for magnetic nanoparticle synthesis via the room temperature co-precipitation (RTCP) method. The magnetosome's nature has a

ferrous-rich environment [106], and the synthesis conditions were kept at 1:2 ratio of ferric and ferrous iron ions in order to mimic the organelle of magnetotactic bacteria. For preventing possible oxidation, the ferric and ferrous iron ion solutions were prepared in anoxic water that was treated with nitrogen gas beforehand. 6.6 mM $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ and 3.4 mM $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ solutions were mixed in a glass tube, owing to the three-point openings of the lid, to enable the addition of other requirements. The nitrogen gas purging was utilized for 10 minutes at 400 rpm and throughout the experiment to remove dissolved oxygen and provide the anoxic synthesis conditions. The pure Mms6 protein resuspended in TBS was added to the mixture at a final concentration of approximately 20 $\mu\text{g/ml}$. After a brief nitrogen gas sparging, a dropwise addition of 0.1 M NaOH in anoxic water was performed at a constant rate, 0.63 ml/min, via a syringe pump for allowing oxidation-reduction reactions to take place for magnetic nanoparticles (MNPs) synthesis. The nitrogen purging was utilized for 30 minutes at 400 rpm, and the end product was collected magnetically for washing with anoxic water, and sonication was performed for resuspension.

2.3.9 Characterization of Synthesized MNPs *ex vivo* via X-ray Diffraction

X-ray Diffraction (XRD) analysis was performed from the powder samples of MNPs produced in the absence and presence of pure Mms6 protein via Malvern Panalytical X'Pert Pro Multi-Purpose Diffractometer. The X-rays were produced via a ceramic X-ray tube with Cu $K\alpha$ anode at 45 kV. Intensities were collected between $2\theta = 10^\circ$ and 80° at 0.02 step size on The PIXcel detector.

2.3.10 Verification of Surface Displayed Mms6 protein

An immunocytochemistry (ICC) assay was utilized to label and ensure the display of the Mms6 peptide on the cell surface of the ECMAB system. After induction of the cells, a certain amount of cells (5×10^8) were collected and washed with $1 \times$ PBS. For blocking, the cells were resuspended in 1% BSA in $1 \times$ PBS solution for 2 hours at room temperature on a rocker. The cells' pellet was resuspended and incubated in 1: 250 anti-his primary antibody in 1% BSA in $1 \times$ PBS solution for overnight at $+4^\circ\text{C}$ on the rocker. The cells were washed with $1 \times$ PBS in order to remove the primary antibody that were later incubated in 1: 250 DyLight550 secondary antibody for 1.5 hours in the dark at room temperature on the rocker. The cells were washed with $1 \times$ PBS and diluted at 1:10 ratio in $1 \times$ PBS. For imaging under a fluorescence microscope (Scope A1, Zeiss), the cells were mounted on the slide via a drop of mounting media and a coverslip was sealed to the slide.

2.3.11 Extracellular Magnetite Crystal Formation

After the Extracellular Magnetite Accumulating Bacteria (ECMAB) *E. coli* BL21 (DE3) cells were induced under optimized conditions, the specified amount of cells (5×10^8) were collected and resuspended in 200 μl of $1 \times$ PBS. Similar to *ex vivo* magnetite crystal formation, the room-temperature co-precipitation (RTCP) method was utilized for extracellular magnetite formation. The iron solutions, 1mM $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ and 1mM $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$, were prepared in anoxic water. A glass tube having three-openings cover was used for the reaction to take place. The iron solutions were mixed at 1:1 ratio in the tube, and nitrogen gas purging was applied at 300 rpm for 10 minutes. The cells resuspended in $1 \times$ PBS were added in the tube and purged for a few minutes. The 0.1M NaOH solution in anoxic water was added dropwise into

mixture by utilizing a syringe pump at a rate of 0.21ml/min to allow the magnetite synthesis to begin. For the material synthesis to be completed, the purging was kept at 300 rpm for 30 minutes. After that, the cells and magnetic nanoparticles were centrifuged at 9000 rpm for 4 minutes. The washing was performed with 1× PBS once, and the sample was resuspended in the equivalent collection volume of 1× PBS. For the forthcoming experiments, the sample was kept at +4°C.

2.3.12 Intracellular Magnetite Crystal Formation

The genetic construct was designed to build intracellular magnetite accumulation via combinatorial effect creation in a way that the Mms6 gene was combined with the genes encoding ferroxidase (EncFtn), material binding peptide (MBP), and iron transporter protein (EfeU). After the induction of Intracellular Magnetite Accumulating Bacteria (ICMAB) *E. coli* BL21 (DE3) cells under optimized conditions in minimal media, the cells were collected at 3500 g, +4°C for 10 minutes. The washing step was done with a culture volume of 1× PBS + 2mM EDTA solution in order to pull out of extracellular iron ions. The cells were centrifuged and resuspended in a culture volume of 1× M9 media supplemented with 2 mM MgCl₂ and 0.1 mM CaCl₂ for maintenance at +4°C.

2.3.13 Quantification of Intracellular Iron Content

The quantification of iron ions in the bacterial cells was performed via a colorimetric ferrozine assay. [105] The cells were washed and resuspended in cell maintenance media at OD₆₀₀ of 80. The standards were prepared with ferrous ammonium sulfate hexahydrate at the concentration range from 0.1 mg/ml to 20 mg/ml. For ensuring the dissolution of intracellular iron ions, all bacterial cell groups suspended in maintenance

media, standard solutions, and media control itself were diluted in 65% nitric acid at 1:10 ratio and boiled at 56°C for approximately three hours. After allowing the samples to cool down to room temperature, serial dilution was performed in sterile ddH₂O for all samples. The ferrozine buffer was prepared freshly in the dark consisting of 6.5 mM ferrozine, 2.5 M ammonium acetate, 1 M ascorbic acid at pH 10, and added on samples to permit the complex formation between ferrozine anion and ferrous ions. [107] For absorbance measurements, 200 µl aliquots of samples after 30 min incubation in the dark at room temperature were placed in a 96-well plate. The spectrophotometry measurements were done at 562 nm via SpectraMax Microplate Reader. The constructed standard curve was used to determine the intracellular iron concentration of bacterial cells. All reactions were performed in triplicate for statistical analysis. For obtaining the iron content of cells, the specified serial dilutions were spread on LB agar plates containing proper antibiotics, and then the following day, the colony numbers were acquired. For the calculation of iron content per cell, the iron concentration was divided by the total colony-forming unit.

2.3.14 Determination of Growth Curve and Cell Motility

In order to further characterize the ICMAB cells, the growth curves of the cells were constructed. For that purpose, induced and uninduced cells were collected at 0, 2, 4, 6, 12, and 24 h in triplicate, and their absorbance was measured at OD₆₀₀. Cells picked from single colonies on an agar plate were grown overnight to determine the growth curve. After diluting in a fresh media, they were induced at around 0.1 OD₆₀₀ value under optimized conditions. For performing motility test, the soft agar method was utilized. For that aim, 10µl of induced and overnight cells were dropped and grown on

soft agar for 2 days, and the diameter was measured. Plates were visualized via Vilber imaging system.

2.3.15 Characterization of the Iron Oxide Biomineralization via Electron Microscopy Imaging

The scanning electron microscope (SEM) and transmission electron microscope (TEM) were utilized to characterize iron oxide nanoparticle formation. For SEM imaging, silicon wafers were used that were cut into small pieces, and then washed with ethanol and isopropanol prior to sample preparation. 10 μ l samples were dropped on the silica wafers and allowed to dry overnight. The wafers were coated with 8/10 nm Au-Pd via a precision etching coating system (PECS, Gatan 682) to obtain conductivity for the specimens. The visualization was performed via E-SEM microscope (FEI-Quanta 200F) at 15kV electron acceleration. A representative image was shown in the figures for all groups. Energy-dispersive X-ray spectroscopy (EDS) analysis was performed using EDAX Genesis software combined with the SEM microscope.

For TEM imaging, 300 mesh carbon-coated copper grids were used for sample preparation, where 10 μ l samples were deposited and incubated for 10 minutes at room temperature. The excess liquid was soaked up via a filter paper from the grid side. The samples were washed with ddH₂O two times, and 2% uranyl acetate was used to dye the samples for 20 seconds in order to enhance the contrast of the imaging. Then, the samples were left to air dry overnight in the box. The images were taken via TEM (FEI Tecnai) at 120 keV electron acceleration. A representative image was shown in the figures for all groups. EDS was also performed for the elemental analysis of the samples.

2.3.16 Preparation and Characterization of Bacterial Cell Systems via Magnetic Resonance Imaging

For magnetic resonance imaging (MRI) measurements, the five different iron concentrations were determined as 0.2, 0.4, 0.6, 0.8, and 1mM to prepare the bacterial and control samples. For extracellular magnetite accumulating bacteria (ECMAB) samples, *E. coli* BL21 (DE3) cells harboring Mms6-Ag43 fusion protein were induced by including an empty *E. coli* BL21 (DE3) cell group without a construct. The RTCP method was utilized for extracellular magnetite formation, and then 1 ml of samples were washed with 1 ml PBS (1×) and resuspended in 1 ml PBS (1×) again. An additional control group was also included for MRI measurements consisting of ferrous and ferric ions prepared in 1× PBS.

The intracellular magnetite accumulating bacteria (ICMAB) samples were induced, and empty *E. coli* BL21 (DE3) samples were included as a control group. For the MRI measurements, the samples were concentrated at OD₆₀₀ of 80 and diluted at 1:5 ratio in 1× M9 minimal media supplemented with 2 mM MgCl₂ and 0.1 mM CaCl₂. The control groups of 1mM ammonium ferrous sulfate hexahydrate in 1× M9 and 1× M9 media itself were prepared and used for the measurements.

The Magnetic Resonance Imaging (MRI) analysis was performed at Bilkent University's UMRAM (National Magnetic Resonance Research Center) by Associate Professor Emine Ülkü Sarıtaş Çukur. A 3T Siemens Magnetom Trio scanner using a 32-channel head coil was used for both transverse relaxation time (T₂) and transverse relaxivity (r₂) measurements of the samples prepared in 1.5 mL Eppendorf tubes. A multiple-echo spin-echo sequence with 12 different TE's between 10–1000 ms was used (TE = [10, 15, 25, 40, 60, 100, 150, 250, 400, 600, 800, 1000] ms), with a

repetition time (TR) = 2000 ms, 5 mm slice thickness, and $105 \times 140 \text{ mm}^2$ field-of-view (FOV) for the r_2 measurements of ECMAB samples and the control samples. A multiple-echo spin-echo sequence with 21 different echo times (TE) between 10–1000 ms was used (TE = [10, 15, 20, 25, 30, 35, 40, 50, 60, 100, 125, 150, 200, 250, 300, 350, 400, 500, 600, 750, 1000] ms), with TE = 3000 ms, 4 mm slice thickness, and $80 \times 80 \text{ mm}^2$ field-of-view (FOV) for T2 measurements of ICMAB samples and the control samples. MATLAB (Mathworks) was utilized via a custom script to analyze MRI images. For each tube in the MRI images, a circular region of interest (ROI) was selected. The pixel intensities as a function of TE were fitted to the standard monoexponential T2 relaxation curve. The inverses of T2 values as a function of concentration (C) were fitted to the linear relation for the determination of r_2 .

2.4 Results and Discussion

In this study, the iron-binding peptide Mms6 was utilized for synthesizing MNPs, [9], [10], [13]–[15], [17], [22], [108] and to create bacterial contrast agents. **(Figure 2.1)** An online modeling tool, I-TASSER, was used for the prediction of the 3D structure of the N-terminal hexahistidine tagged Mms6 peptide in order to be sure about the proper folding of the tagged protein and the availability of the iron-binding site. [109], [110] A model with the highest confidence score was selected among five predictions. Self-assembly of Mms6 protein provides an iron-binding surface for iron ions in the solution. The magnetite formation takes place at the negatively charged carboxylate-rich surface, which provides specific iron binding. [106] With these considerations, the bacterial cellular machineries can be engineered and designed to express recombinant iron-binding peptide and synthesize magnetites both inside the cell or on its surface under proper conditions. For this aim, in the research, the function of Mms6

protein was verified by using its purified form in IONPs' synthesis and their characterization. The magnetite accumulating bacterial cells, either extracellularly or intracellularly, were constructed by designing the responsible circuitries via molecular cloning techniques. The magnetites were characterized via electron microscopy imaging, elemental analysis, and MRI relaxivity measurements.

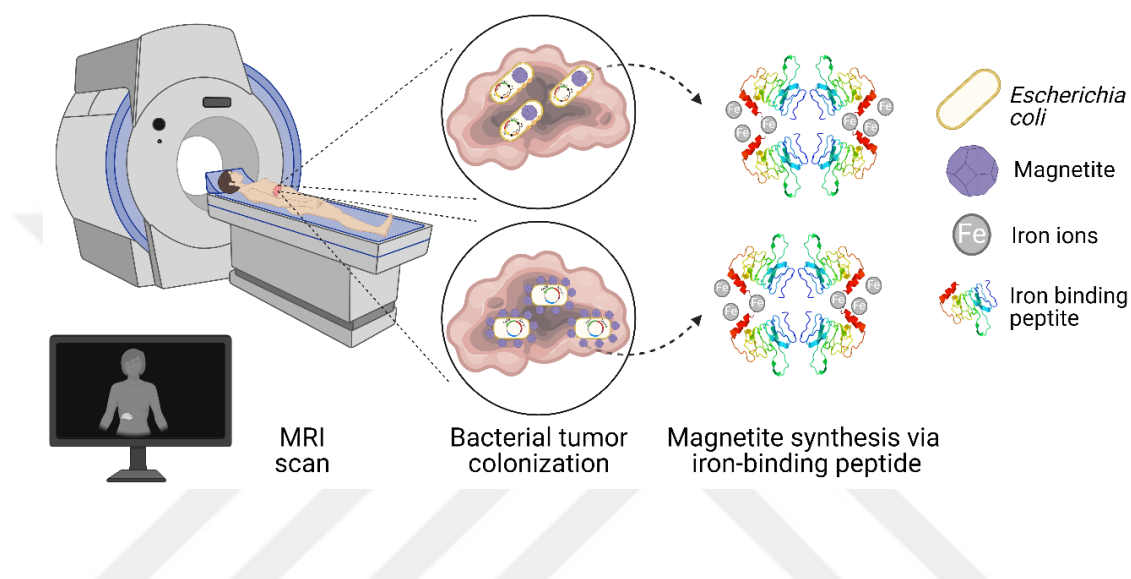


Figure 2.1 The magnetite accumulating engineered cells harboring related genetic circuits are designed for tumor diagnosis. Created with BioRender.com

2.4.1 Cloning, Expression, and Purification of Mms6

The chosen peptide, Mms6, comprises an iron-binding activity at the C-terminal which plays a role in templating iron-oxide crystal production and controlling the crystals' size and morphology. [9] This study aimed to construct MNP-producing *E. coli* cells by harboring the iron-binding peptide Mms6. First, the IPTG inducible Mms6 expression system was constructed in the pET22b backbone. After sequence verification, the *E. coli* BL21 (DE3) cells were harbored to express the Mms6 peptide. The purified version of the Mms6 protein was used in MNP synthesis to observe its functionality.

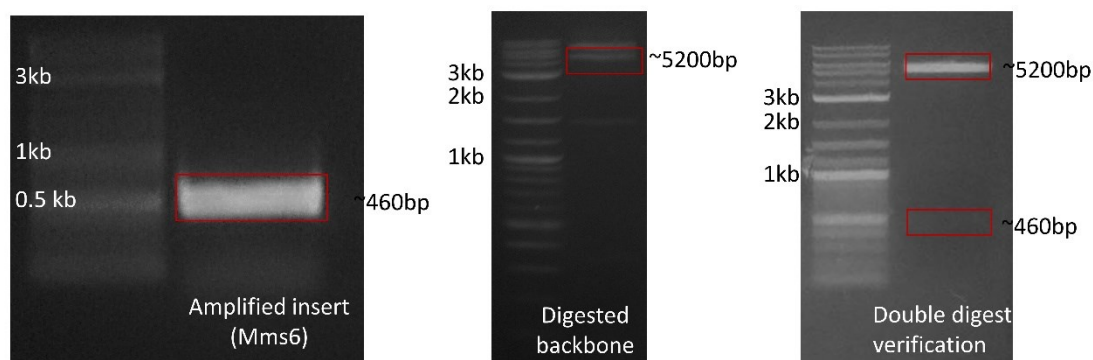


Figure 2.2 The construction of IPTG inducible pET22b 6H Mms6 cmr vector. All DNA bands were depicted as expected size. Double digest verification via XbaI and PspXI (NEB) restriction enzymes.

2.4.2 The Synthesis MNPs in the Presence of Purified Protein

The room temperature co-precipitation (RTCP) method was utilized for IONPs' synthesis due to its convenient methodology and the fact that it does not require any hazardous materials. [22] The Mms6 peptide has a tendency to agglomerate; thus, the purified protein was refolded in the refolding buffer and renatured via buffer exchange into 1× TBS. [22] The western blot procedure was performed for verification of the Mms6 peptide through a hexahistidine tag. (**Figure 2.3**) The online ExPASy tool was used to calculate the expected size of the peptide, which is 14 kDa. All the monomer, dimer, and trimer forms of Mms6 were observed in the analysis due to their tendency to oligomerize and form aggregates in solution. The N-terminal hydrophobic part of the peptide causes the aggregation, which forces the C-terminal to face out and facilitates iron-binding activity. [14]

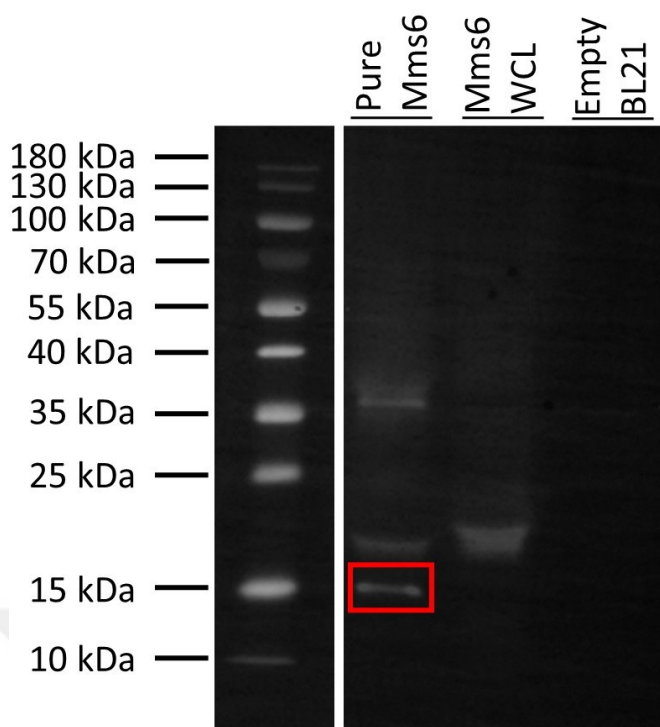


Figure 2.3 Protein expression analysis via Western Blot for pure Mms6 protein was depicted here.

Nitrogen purging was utilized throughout the process to provide the necessary environment for IONPs' synthesis. Also, the iron solutions were prepared in anoxic water in order to minimize the oxidation of iron ions before the synthesis. **(Figure 2.4)** The concentration of pure protein at around $20 \mu\text{g mL}^{-1}$ was efficient in the production of MNPs. The mixture of ferrous and ferric ions, as well as pure protein, was drop-by-drop supplemented with the NaOH solution to increase the pH value of the mixture. [108] The alkaline environment is convenient for the synthesis because it increases the affinity toward iron by making the protein aggregates highly charged. Thus, the base addition triggers the iron salts' precipitation and results in magnetite formation. [14], [108]

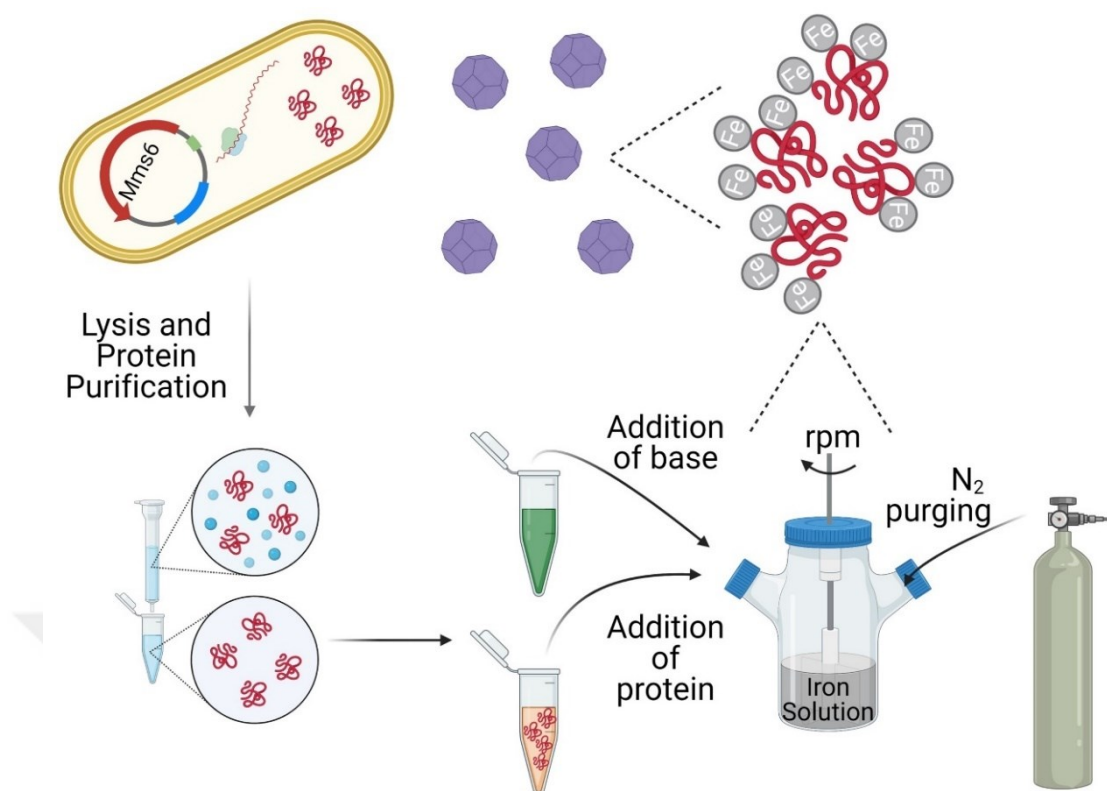


Figure 2.4 The purified iron-binding peptide functionality in MNP production is analyzed by ex vivo magnetite synthesis with pure Mms6 protein via the RTCP method. Created with BioRender.com

2.4.3 The Characterization of Protein-Based Nanomaterials

The characterization of the synthesized NPs in the presence and absence of pure Mms6 protein was done by scanning electron microscopy (SEM) and transmission electron microscopy (TEM) imaging (**Figure 2.5**) and analysis through X-ray spectroscopy to reveal elemental composition. (**Figure 2.6**) It was shown that the iron-oxide nanomaterials can be obtained both in the presence and absence of pure Mms6 protein according to SEM and TEM results. On the other hand, the nucleation function of the Mms6 protein in iron nanomaterial synthesis inspires the study to produce nanomaterials that accumulate bacterial cellular machineries through recombinant

protein expression. By interpreting the results, the Mms6 peptide contributes to the nanomaterials' size which show bigger dimensions and possess larger magnetic moment per particle as expectedly. [14], [111] Also, the protein creates a tendency to form a contact structure because of the probable aggregation inclination of the protein on the contrary to dispersed nanoparticles produced in the absence of the pure protein. In addition, the nanoparticles produced in the absence of Mms6 protein show smaller and inhomogeneous size distribution. However, the nanoparticles produced with and without Mms6 protein have a similar morphology to that of cuboidal.



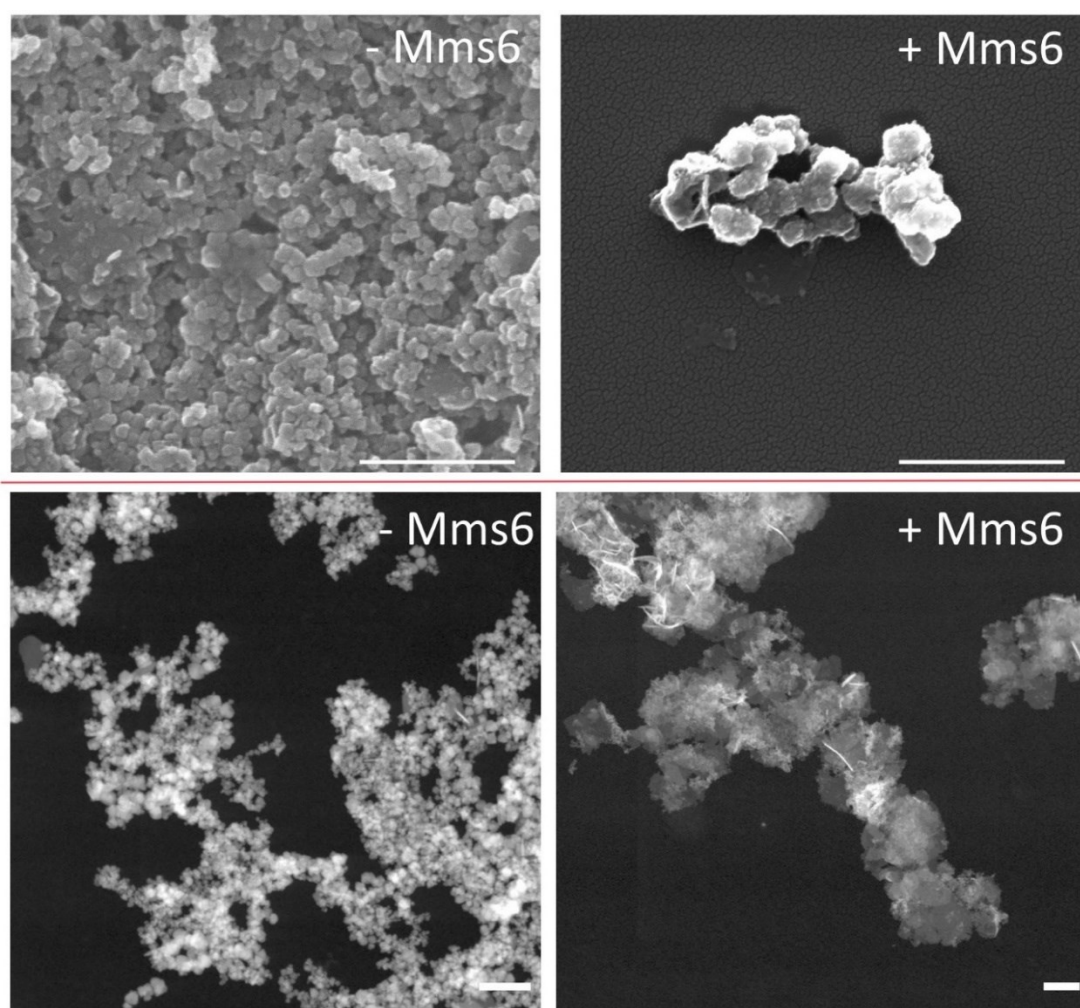


Figure 2.5 The characterization of MNP synthesized in the absence and presence of pure Mms6 protein via SEM images (upper panel) and TEM HAADF (lower panel). Scale bars are 500 and 100 nm, respectively. Samples are collected from replicates; representative images are presented here.

In the analysis of MNPs synthesized with pure protein via the energy-dispersive X-ray spectroscopy (EDS) module, carbon and oxygen peaks might be caused by the synthesized biohybrid material containing organic residues. The grid composition might cause to observe copper peaks as well as carbon and oxygen signals. The presence of iron ions in the selected reduced area was pointed out with red arrows indicating IONPs. (**Figure 2.6 upper panel**) Similarly, the EDS analysis of MNPs

produced in the absence of the pure protein revealed Cu, C, and O peaks, possibly due to the grid itself, and Fe peaks labeled with red arrows representing IONPs. (**Figure 2.6 lower panel**)

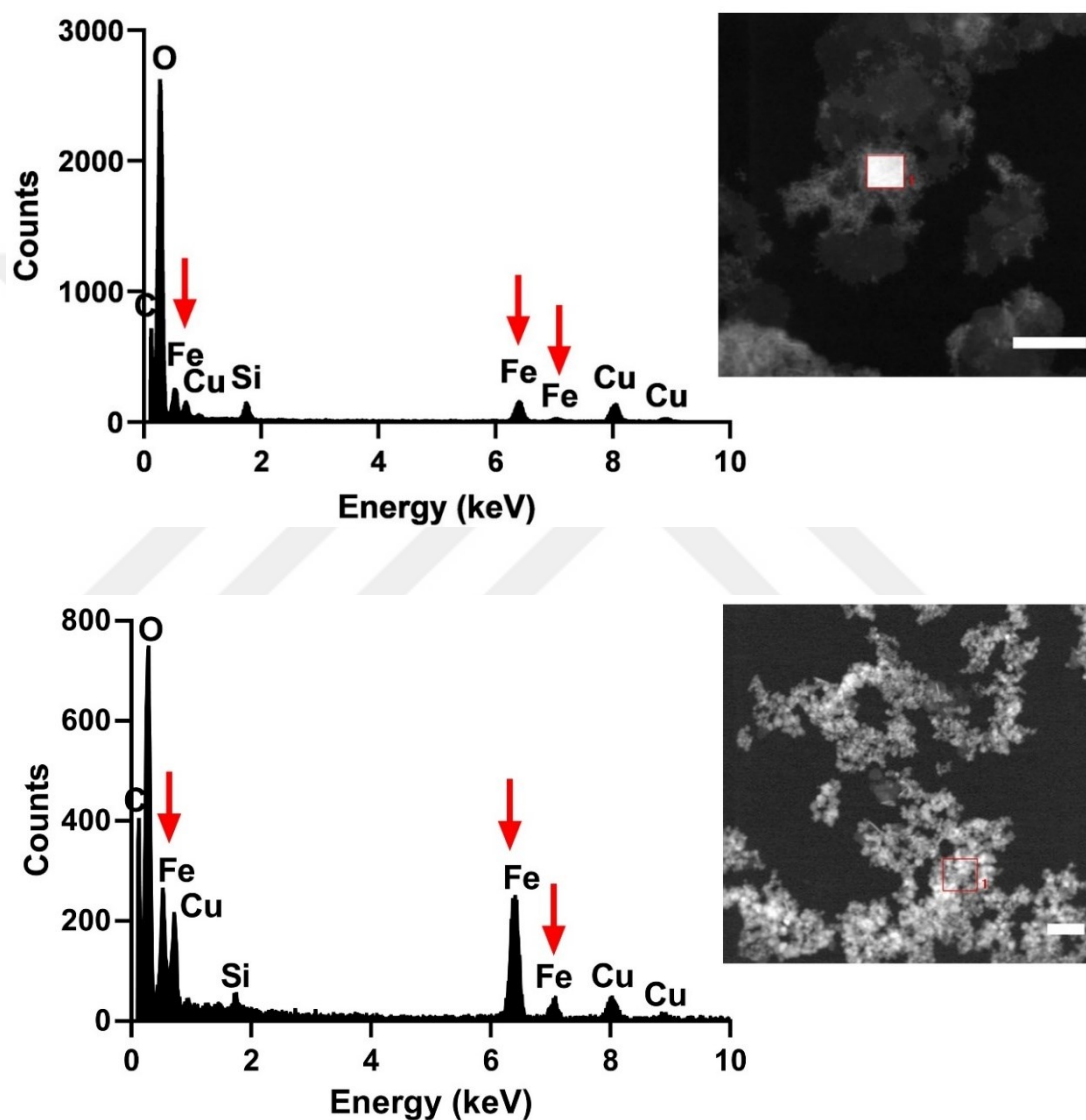


Figure 2.6 The EDS analysis of the selected area on a TEM specimen of MNPs produced with the pure Mms6 (upper panel) and without the pure protein (lower panel). Red arrows show the iron peaks. The white scale bar is 100 nm. Samples are collected from replicates; representative images are presented here.

In addition, X-ray diffraction (XRD) analysis of powder MNPs produced in the presence and absence of pure Mms6 protein (**Figure 2.7**) reveals that NPs in both conditions possess magnetite crystalline structures. [1], [9], [106] As an alternative to Mms6 peptide, it is reported that the proteins harboring iron-binding ability or not, like ferritin or bovine serum albumin (BSA), respectively, do not play a role in the size and morphology of the magnetic crystals. [14], [111] Furthermore, a poly-cationic peptide polymer attaches and affects the magnetites' size, morphology, and aggregation of crystals *in vitro*. [112] The results are consistent with the interference of the Mms6 peptide's nucleation mission in magnetite mineral species formation.

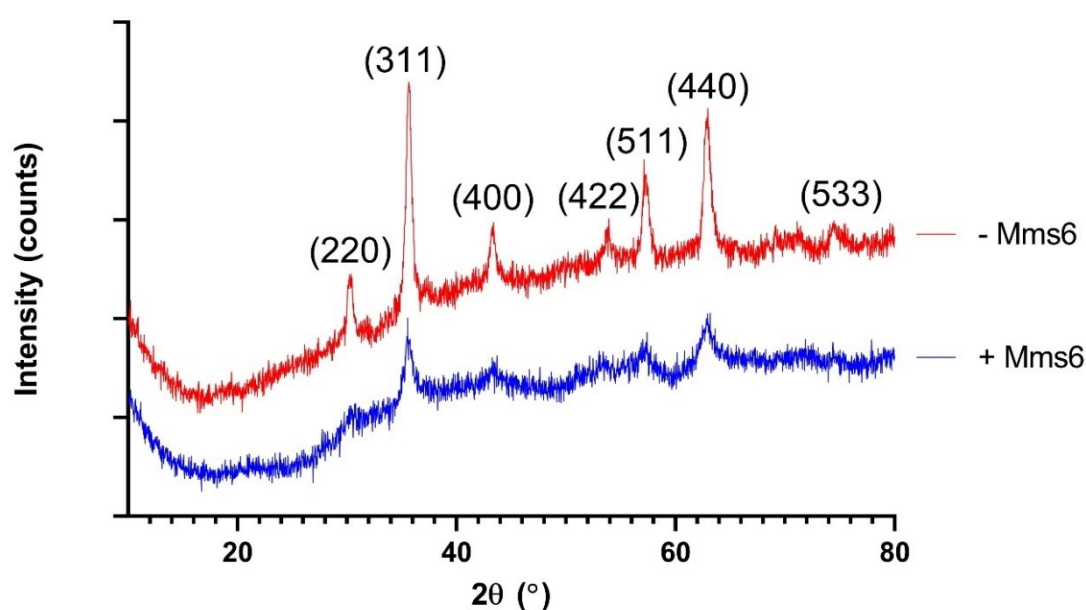


Figure 2.7 The XRD analysis of MNPs synthesized in the absence and presence of the pure Mms6 protein.

2.4.4 Construction of the Extracellularly Magnetite Accumulating Bacteria (ECMAB) system

For the construction of extracellular magnetite accumulating bacterial cell systems, Mms6 peptide was displayed on the cell surface via an autotransporter protein Ag43 which consists of an N-terminal signal sequence, α -passenger domain, and β -translocation domain. The first 160 amino acids of the α -passenger domain are responsible for self-recognition; thus, this region is used to display protein of interest (POI). [113] In this study, the genetic circuitries were designed as the iron-nucleating peptide located upstream of the truncated 160 N Ag43 sequence alongside a hexahistidine tag at N-terminal separated via a GS-linker (GGGGS) for preventing any possible misfolded peptide. (Figure 2.8)

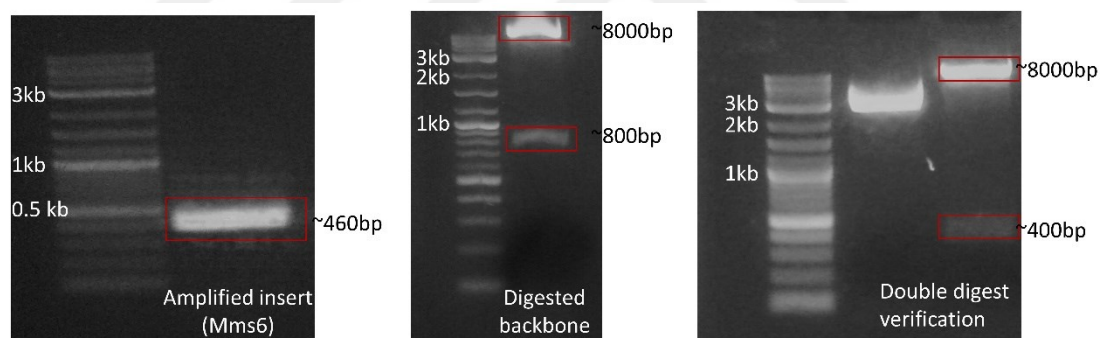


Figure 2.8 The construction of IPTG inducible pET22b PelB 6H Mms6 160N Ag43 ampR vector. All DNA bands were depicted as expected size. Double digest verification via BamH1 and Afl2 (NEB) restriction enzymes.

The bacterial cells displaying Mms6 peptide on the cell surface were used for MNPs production via the RTCP method like *ex vivo* synthesis condition because the peptide is easily accessible on the surface for responsible ions. The deposited ferrous and ferric ions can bind to the iron-binding site of displayed Mms6 peptide's C-terminus. The

NaOH solution was added drop by drop into the mixture as an oxidizing agent to initiate NP production. As an end product, the bacterial cells were expected to accumulate magnetites extracellularly. (**Figure 2.9**)

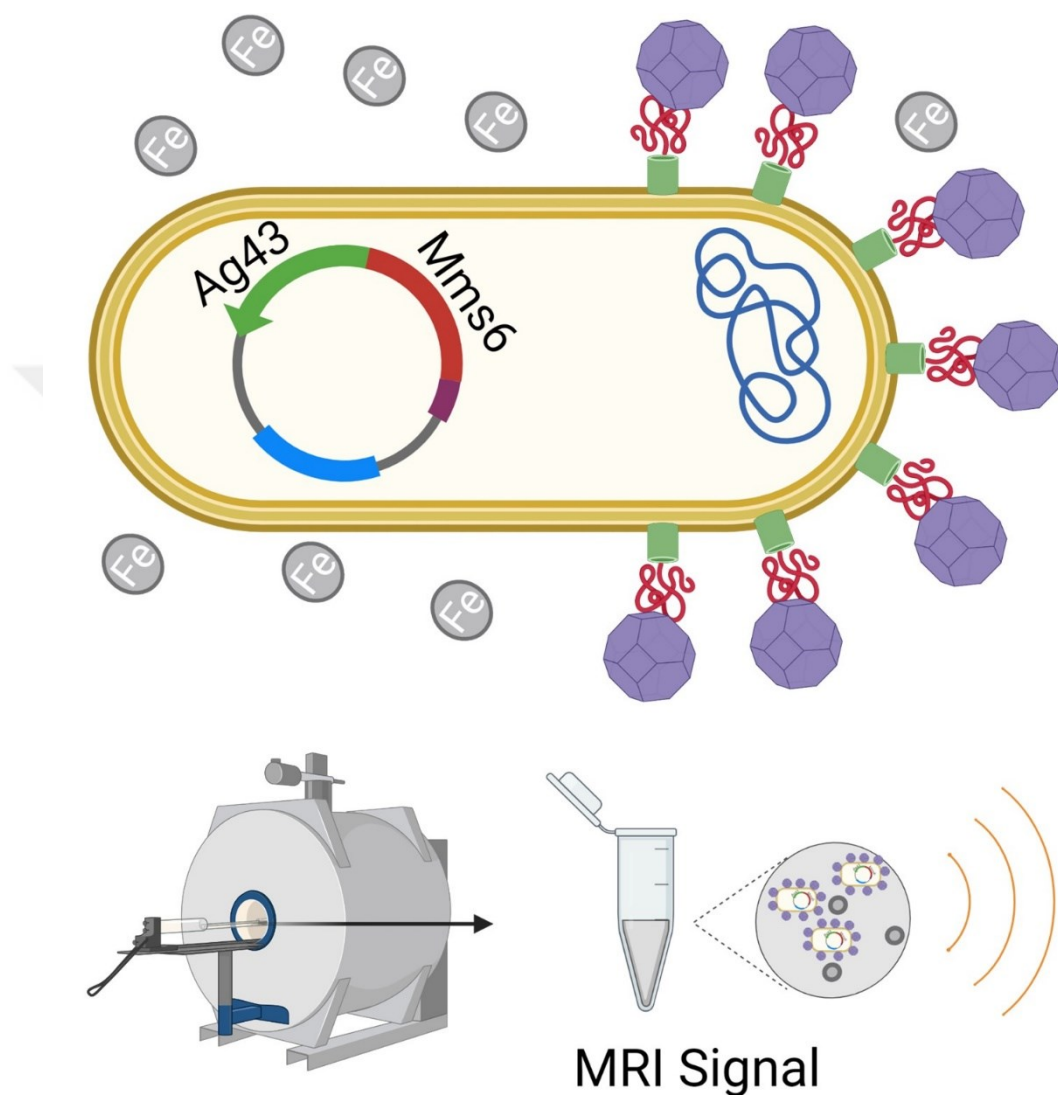


Figure 2.9 The design of the bacterial system accumulating magnetite on the cell surface. The representation of ECMAB by displaying the Mms6 protein on the cell surface. Created with BioRender.com.

Western blotting was performed to verify the expression of the Mms6-Ag43 fusion protein. (**Figure 2.10**) The Expsy online tool was used to calculate the theoretical

molecular weight of the recombinant proteins in which the Mms6-truncated α -subunit of Ag43 has around 48 kDa. On the other hand, the molecular weight of Mms6 with Ag43 protein is around 99 kDa that is responsible for the higher molecular weight band on the western blot.

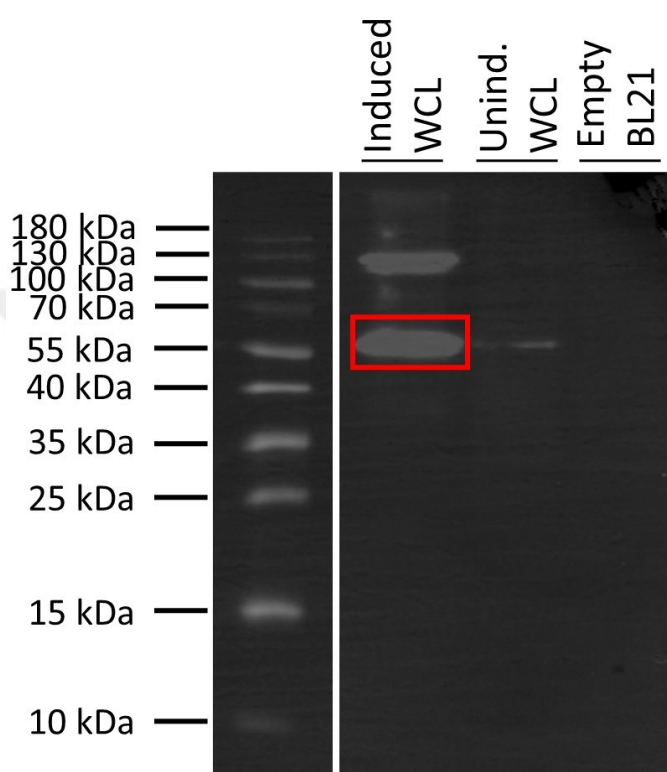


Figure 2.10 Protein expression analysis via Western Blot for ECMAB cells harboring Mms6-truncated alpha domain of Ag43 fusion protein was shown here.

The immunocytochemistry (ICC) assay was performed for ensuring the surface display of the Mms6 protein. The His-tagged Mms6 was labeled with His-tag-specific antibodies conjugated with DyLight550 in the absence of membrane solubilizing agents. The display of the Mms6 peptide was imaged by fluorescence microscopy. The red signals stand for the anti-his antibodies bound to the displayed Mms6 on the cell

surface because the cell membrane was not permeabilized for antibody passage.

(Figure 2.11)

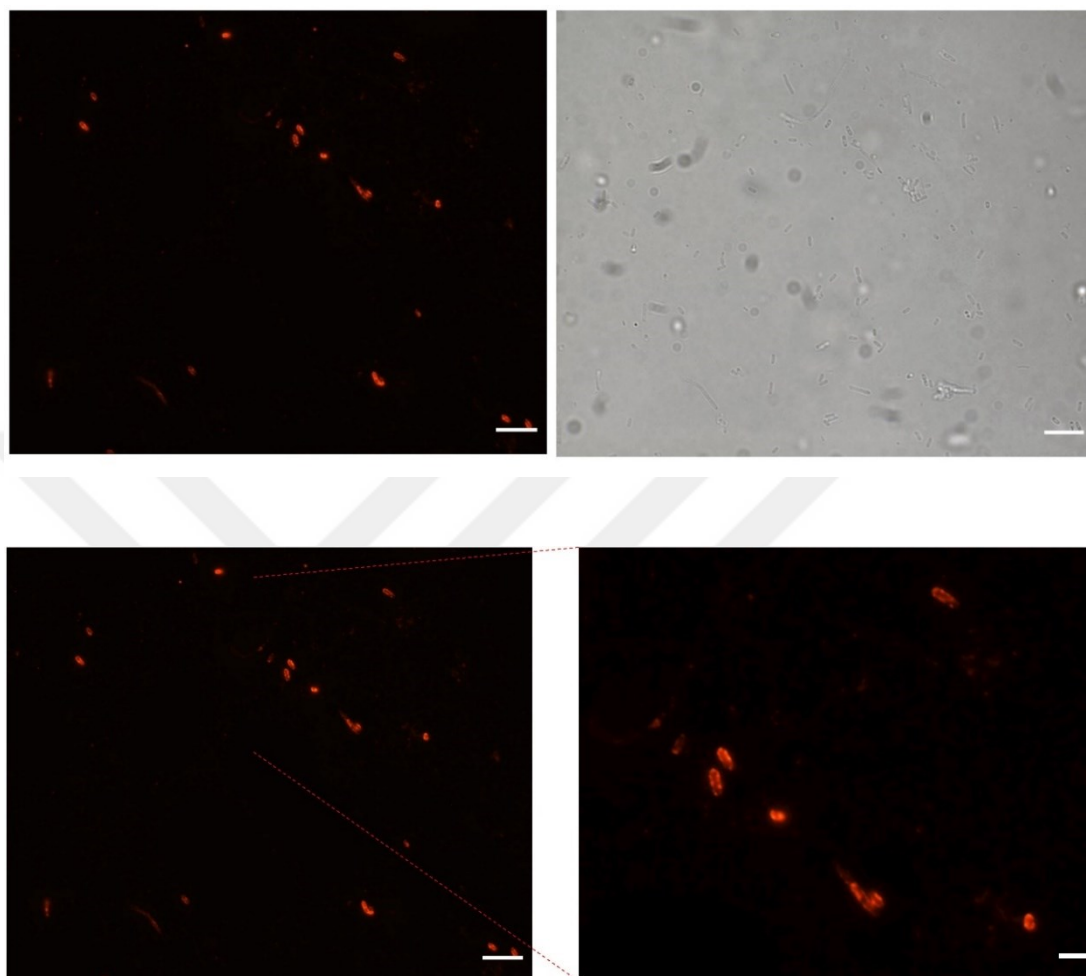


Figure 2.11 The immunocytochemistry assay showed expression of displayed His-tagged Mms6 on the cell surface stained with DyLight 550 antibody specific to His-tag and imaged under fluorescence microscopy. The left image is taken under the red filter, and the right one is a bright field image. (upper panel) Scale bars are 10 μm . The lower panel indicates the fluorescent microscope images (original at left and zoom-in at right) of the ICC assay for displayed His-tagged Mms6 protein on the cell surface. Scale bars are 10 μm and 2 μm , respectively.

2.4.5 Characterization of the Magnetites on the Bacterial Cell Surface

The analysis of MNPs synthesis on the cells was performed after utilizing the induced and empty cells in the NP synthesis procedure. The cell groups were treated with the mixture of iron ions and then the base addition. The induced cells expressing the Mms6-Ag43 fusion protein are expected to accumulate the magnetites on the cell surface, contrary to empty cells. However, in both cell groups, it is expected that there are free MNPs in the environment due to the synthesis procedure. The PBS wash step was performed after the synthesis procedure, before electron microscopy imaging. SEM imaging showed that induced cells performed a remarkable difference from the empty cells by means of accumulating magnetites on the cells' surface. (**Figure 2.12 upper panel**) Furthermore, the induced cells are surrounded by more MNPs in the environment. This might imply that the probable agglomeration of both nanoparticles and the Mms6 peptide displayed on the surface may cause nanoparticles to clump together around cells.

In order to obtain better contrast and high sensitivity, TEM imaging was also performed to differentiate IONPs clearly. (**Figure 2.12 lower panel**) TEM imaging provides the ability to visualize cuboidal magnetite crystals synthesized on the cell surface for induced cells. Mms6 peptide plays a role in nucleation and templating the iron-oxide nanomaterial synthesis; thus, the cells produced magnetites extracellularly with the help of displayed protein, as expectedly. In the empty cell group, there could be some nanoparticle attachment on cells, which might be caused by organic-inorganic attraction. [114] The outcomes were consistent with SEM results, and the EDX analysis of selected points on SEM images showed the elemental composition of the samples. (**Figure 2.12 middle panel**) For providing a conductive layer and minimizing

charging as well as thermal damage to organic and inorganic moieties, the samples were prepared on silica wafers and coated with gold-palladium alloy. As a result of these, the Si, Au, and Pd peaks present in both induced and empty cell specimens caused by sample preparation. The C and O signals in both cell groups' EDS analysis represent organic moieties, whereas iron peaks present in induced cells promote the conclusion of IONP production on the cell surface.



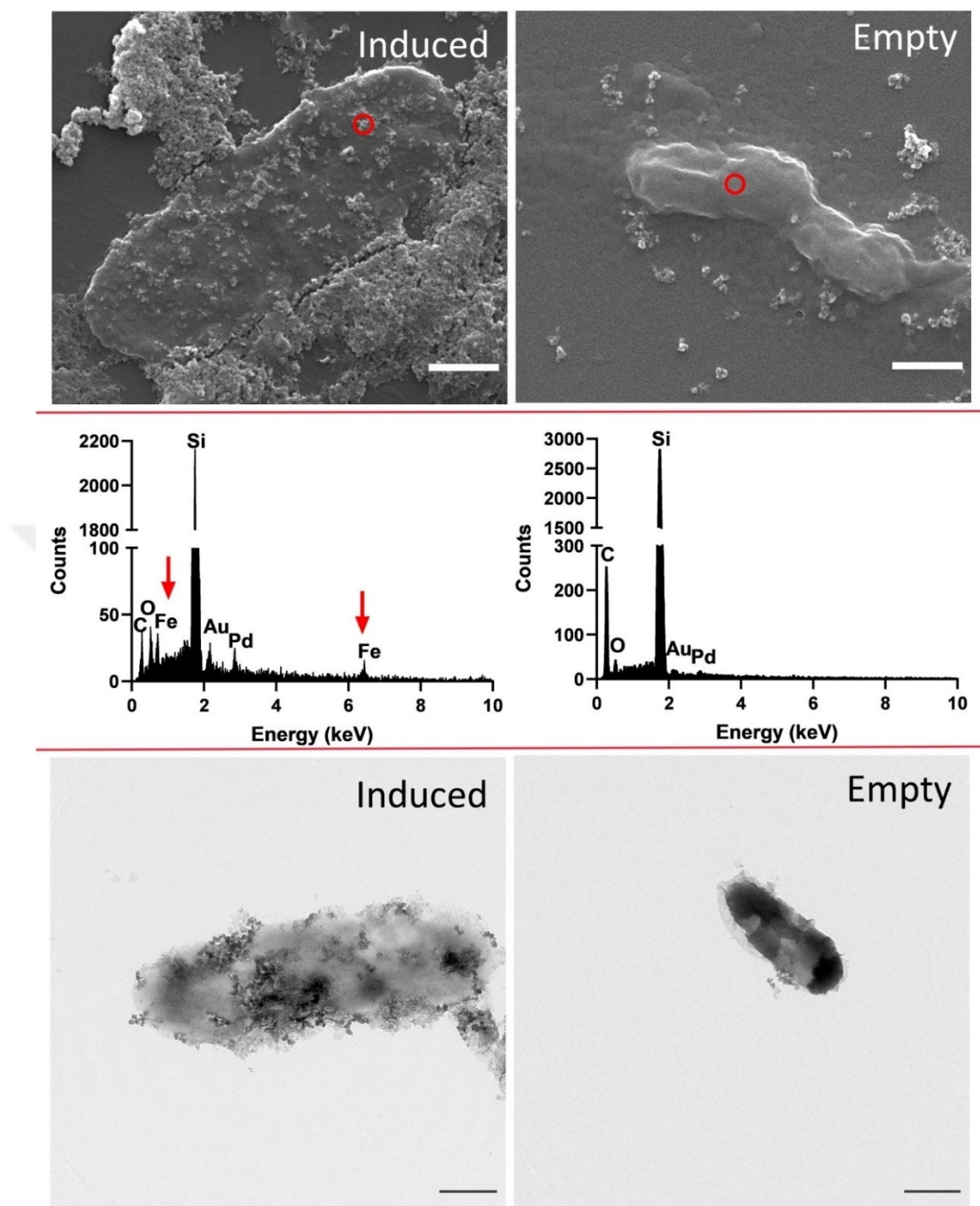


Figure 2.12 The characterization of magnetites synthesized on the cell surface via SEM (upper panel) and TEM imaging (lower panel). Scale bars are 500 nm. The middle panel gives the EDS analysis of the selected areas on SEM images. The given electron microscopy images are representative for the specified case where all images are obtained from minimum of two biological samples.

The construction of extracellular magnetite accumulating bacterial systems is a promising method for material synthesis because of its convenience in synthesis and easy manipulation of magnetic cells via external magnetic field application. The constructed system provides a convenient and fast preparation procedure and can be used easily according to need. The concentrations of iron were optimized for efficient material synthesis. Furthermore, it was shown that feeding the cells expressing recombinant fusion protein with only iron ions is not sufficient to synthesize the corresponding nanoparticles on the cell surface. However, there was nanoparticle coverage throughout the whole bacterial surface, similar to the situation observed with empty cells. (**Figure 2.13 middle panel**) Moreover, both the nanoparticles synthesized by the bacteria and those free in the environment do not possess a toxic effect on cells, which can grow after the synthesis procedure. (**Figure 2.13 upper panel**) In addition, it is concluded that 1mM ferrous and ferric ions supply more homogeneously dispersed nanoparticles throughout the cell surface by interpreting the SEM results of the bacterial cells harboring proteins for extracellular magnetite accumulation in changing concentrations of iron ions. (**Figure 2.13 lower panel**)

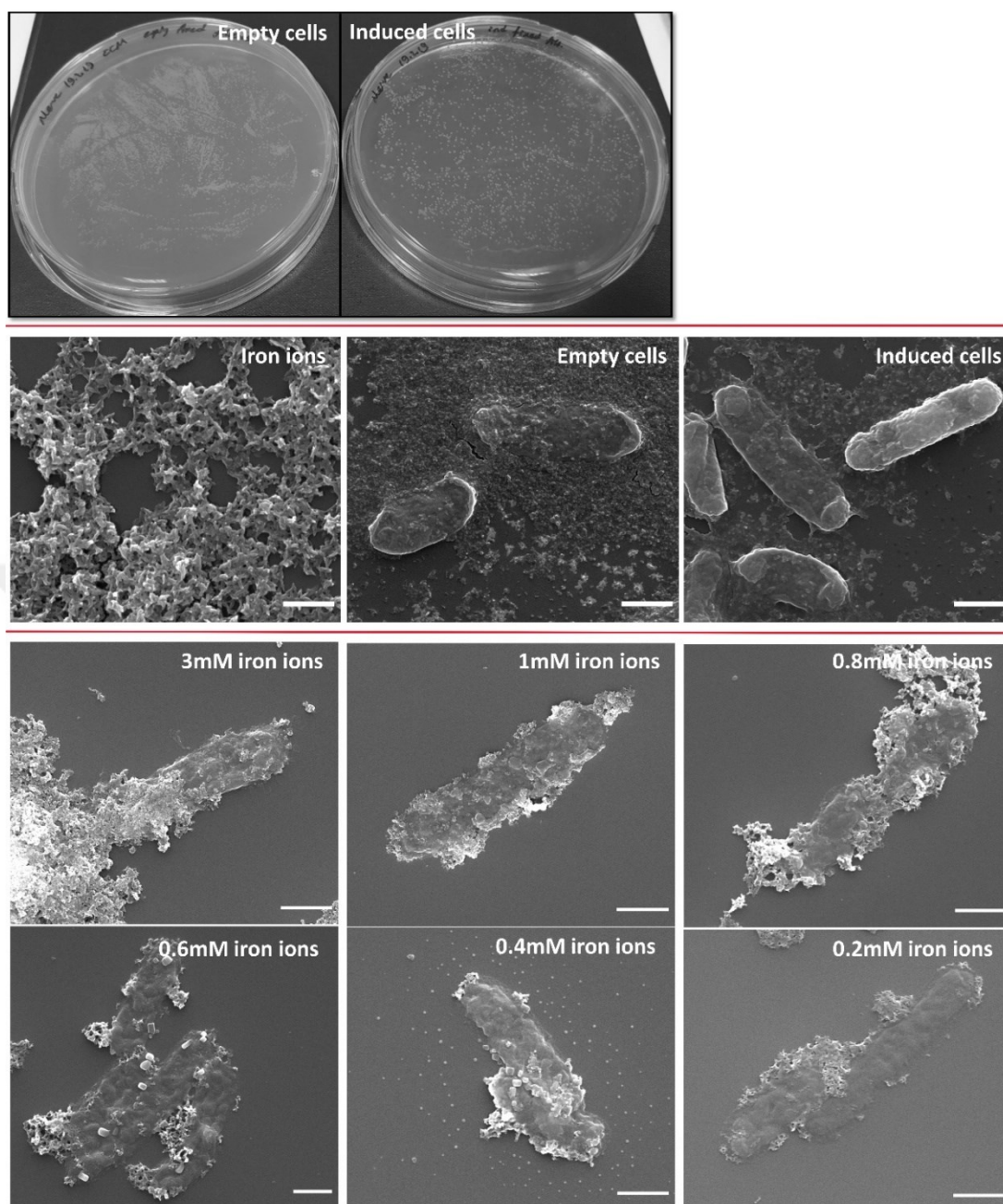


Figure 2.13 Growth of ECMAB cells after synthesis procedure. (upper panel) SEM images of ECMAB cells supplemented with only 1mM iron ions that are not enough to synthesize the corresponding nanoparticle on the cell surface. Scale bars are 1 μ m. (middle panel) SEM images for extracellular magnetite accumulation of induced cells with changing iron concentrations. Scale bars are 1 μ m. (lower panel)

2.4.6 Construction of the Intracellularly Magnetite Accumulating Bacteria (ICMAB) system

The intracellular magnetite accumulating bacteria (ICMAB) system was constructed for obtaining autonomous iron oxide nanomaterial synthesis in the cytoplasm, where the redox reactions take place by the bacterial cells. In the designed module, the iron-binding peptide was expressed as a fusion protein with ferroxidase enzyme at the N-terminus and with MBP at the C-terminus. Both the ferroxidase and MBP were linked to the Mms6 peptide via a GS linker in order to keep the correct folding. For avoiding any negative effect of possible misfolding on the C-terminus' iron-binding activity, the hexahistidine tag was kept upstream of Mms6, similar to other systems. The intracellular magnetite accumulating system was designed under an inducible promoter because the ferroxidase enzyme had shown a toxic effect on the cells if expressed under constitutively active promoters in both high-copy and low-copy vectors. **(Figure 2.14)**

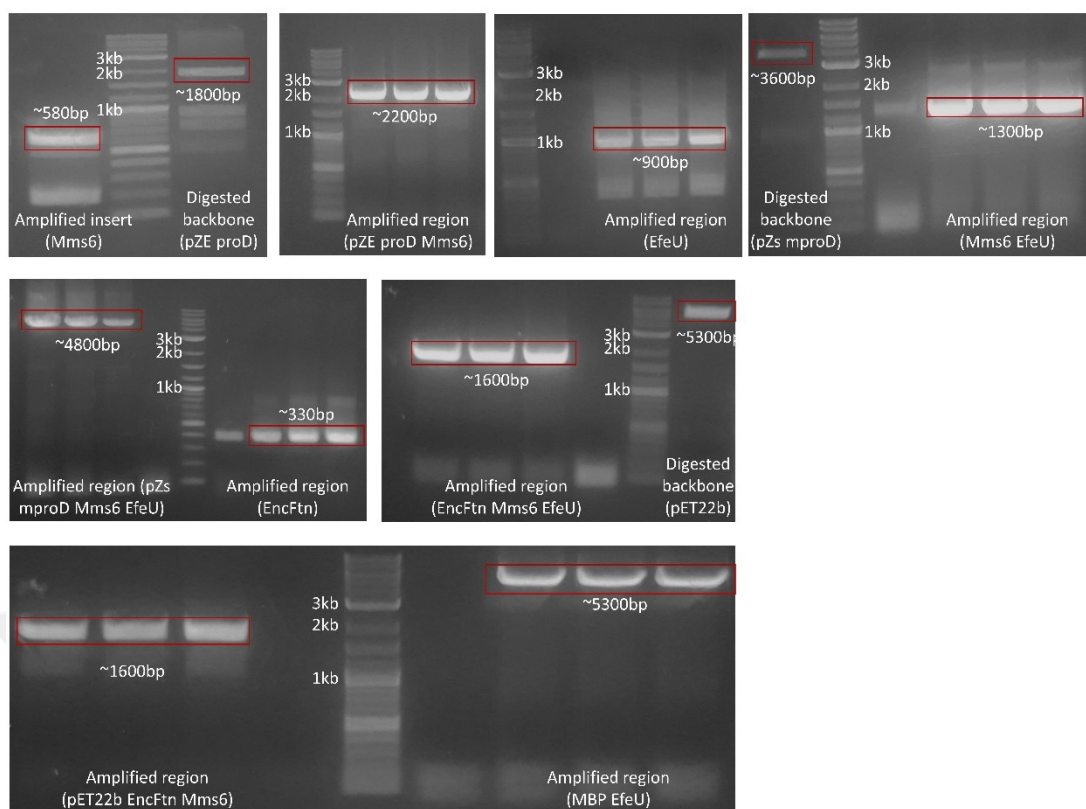


Figure 2.14 The construction of IPTG inducible pET22b EncFtn 6H Mms6 MBP6 EfeU cmr vector. All DNA bands were depicted as expected size.

The bacterial cells were engineered to express MBP for improving the cells' magnetic content. The selected MBP domain has an amino acid sequence found in metal-oxide binding proteins from *Thalassiosira pseudonana* [115], that plays a role in the nucleation and growth of the iron-oxide materials by increasing the inclination toward iron ions and gives a template for material growth. [101] Thus, the IONPs' growth was enhanced via the fusion of the MBP domain, which provides a template. After supplementing the cells' media with ferrous ions, the iron transporter protein enables the uptake of ferrous ions into the cells as a first step. When the ferroxidase oxidizes the ferrous ions into ferric ions, Mms6 binds iron ions and nucleates production, which is facilitated via MBP by increasing the tendency to iron ions and giving a template.

The given final design contains a ferroxidase enzyme, iron-binding peptide, MBP, and ferrous ion transporter for making the bacterial cells to produce iron-oxide nanomaterial in the cytoplasm. (**Figure 2.15**)

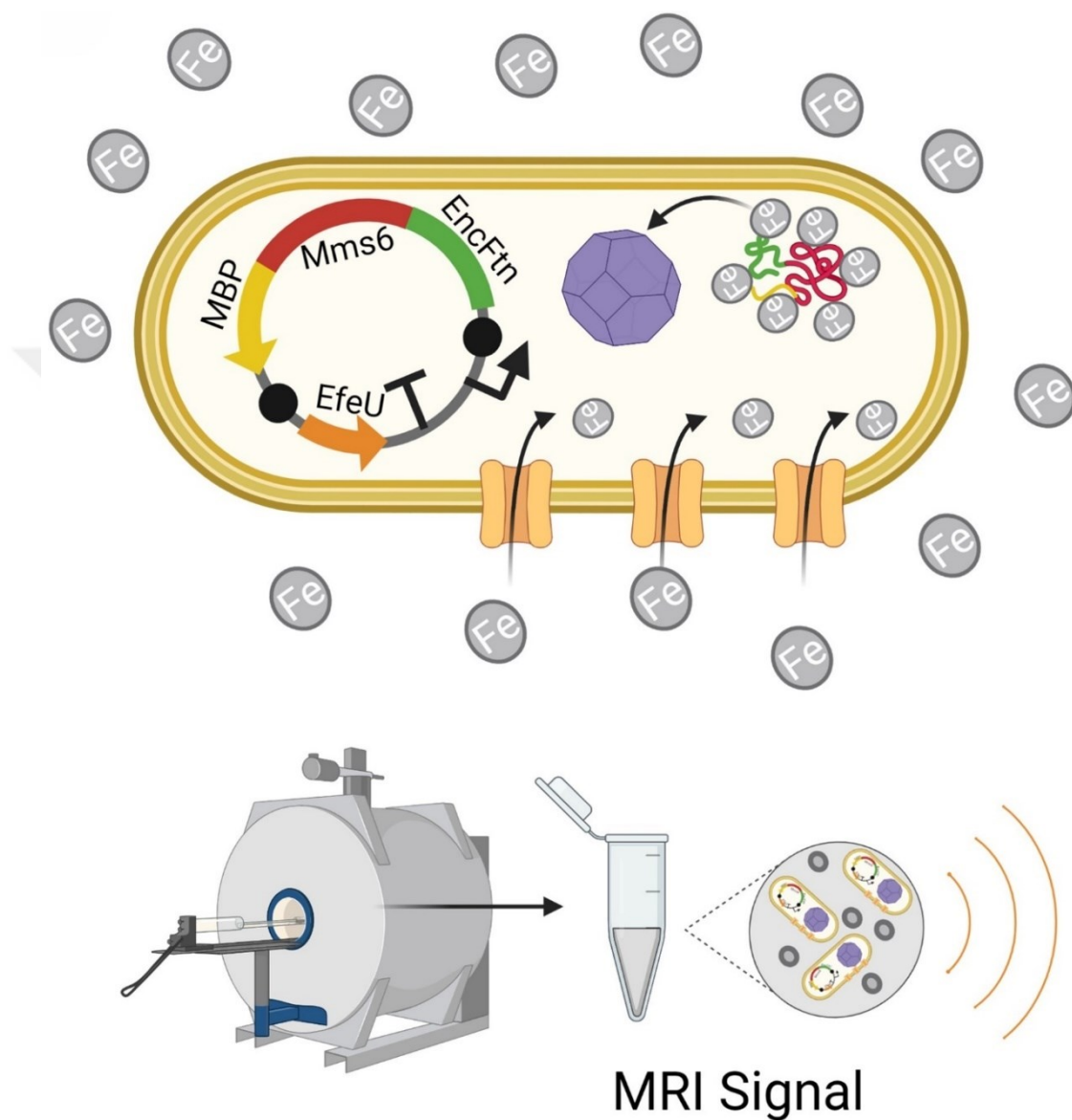


Figure 2.15 The design of the bacterial system accumulating magnetite inside the cell. The construction of ICMAB via ferroxidase, MBP, and iron transporter proteins besides the Mms6 peptide. Created with BioRender.com.

After the cells were induced and treated both in the presence and absence of ferrous iron ions, the protein expression was analyzed via western blotting. The calculated expected size of fusion protein composed of the ferroxidase enzyme (EncFtn), His-tagged iron-binding peptide (His₆Mms6), and MBP (MBP6) is 28 kDa in a monomer form. On the other hand, the ferroxidase protein stabilizes its structure via dimerization, [104]; the dimer form of the fusion protein at around 60 kDa molecular weight band was also observed in the western blot analysis. (**Figure 2.16**)

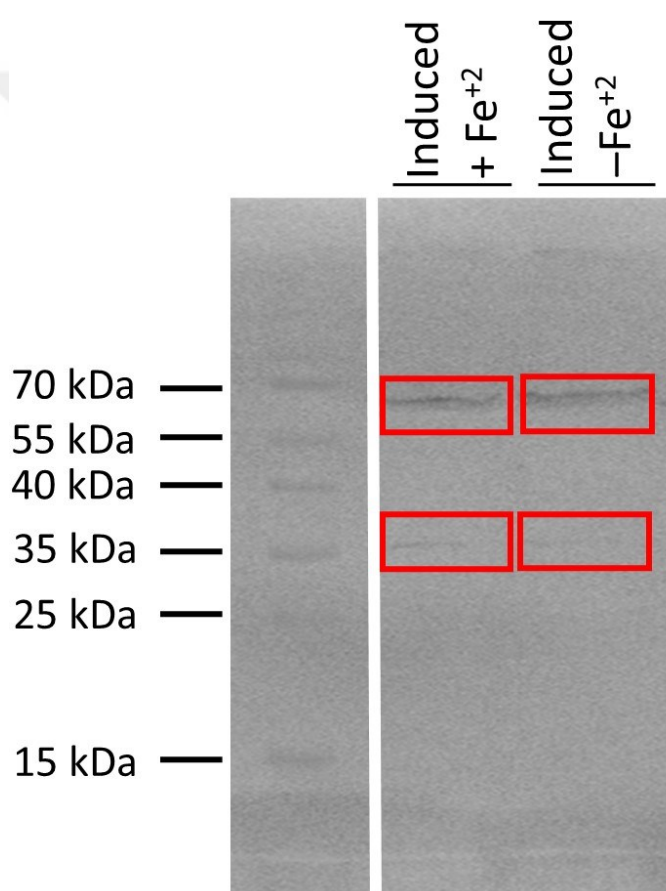


Figure 2.16 Protein expression analysis via Western Blot for ICMAB cells producing both monomer and dimer forms of the ferroxidase-Mms6-MBP fusion protein was provided here.

2.4.7 Characterization of the Magnetites inside the Bacterial Cell

There were three main groups constructed when analyzing the intracellular magnetite accumulating cells. In the first one, the bacterial cells express all the recombinant proteins, which are ferroxidase (EncFtn), iron-binding protein (Mms6), MBP, and iron transporter (EfeU). The purpose of MBP addition to the fusion protein is to improve the MNP content of the cells by increasing the affinity to bind and keep the iron nanomaterial. The second group was designed to deprive the cells from MBP expression in order to observe its active role in iron accumulation. The last group was selected as empty cells lack any recombinant proteins. According to these considerations, only the first two groups are expected to accumulate iron nanomaterial inside the bacterial cells when they are induced and supplemented with ferrous iron ions.

According to the electron microscopy imaging, the induced cell groups exhibited magnetite accumulation, whereas the empty cells didn't exhibit the same behavior as expected. (**Figure 2.17 upper and middle panel**) All groups possess free IONPs in the environment, which might be caused by the oxidation of ferrous iron ions during the cell growth conditions. The SEM analysis implies that the induced cells expressing MBP give more magnetite accumulation than the induced cells devoid of MBP expression. (**Figure 2.17 upper panel**) In addition, the induced cell groups and empty cell groups showed remarkable differences as means of magnetite accumulation. The nanoparticles surrounding the cells were observed because of possible organic-inorganic attraction, similar to the ECMAB case. The cells were also visualized via TEM in order to enhance the contrast and resolution of the images after growing the cells in the presence of chemical inducers and ferrous ions. (**Figure 2.17 middle**

panel) In TEM imaging, the results revealed that recombinant gene circuitries enable the cells to be capable of accumulating the iron nanoparticles intracellularly unlike the empty cells. The outcomes of SEM and TEM were consistent with each other, and the EDS module was utilized for the elemental analysis of the selected points on TEM images. **(Figure 2.17 lower panel)** In the given graphs, the grid composition caused to observe Cu peaks, and sample staining procedure led to obtain Mg and U peaks. The C and O signals were observed due to both biological moieties and the grid material. The peaks were labeled with red arrows, which indicated the presence of iron ions at selected points of TEM images.

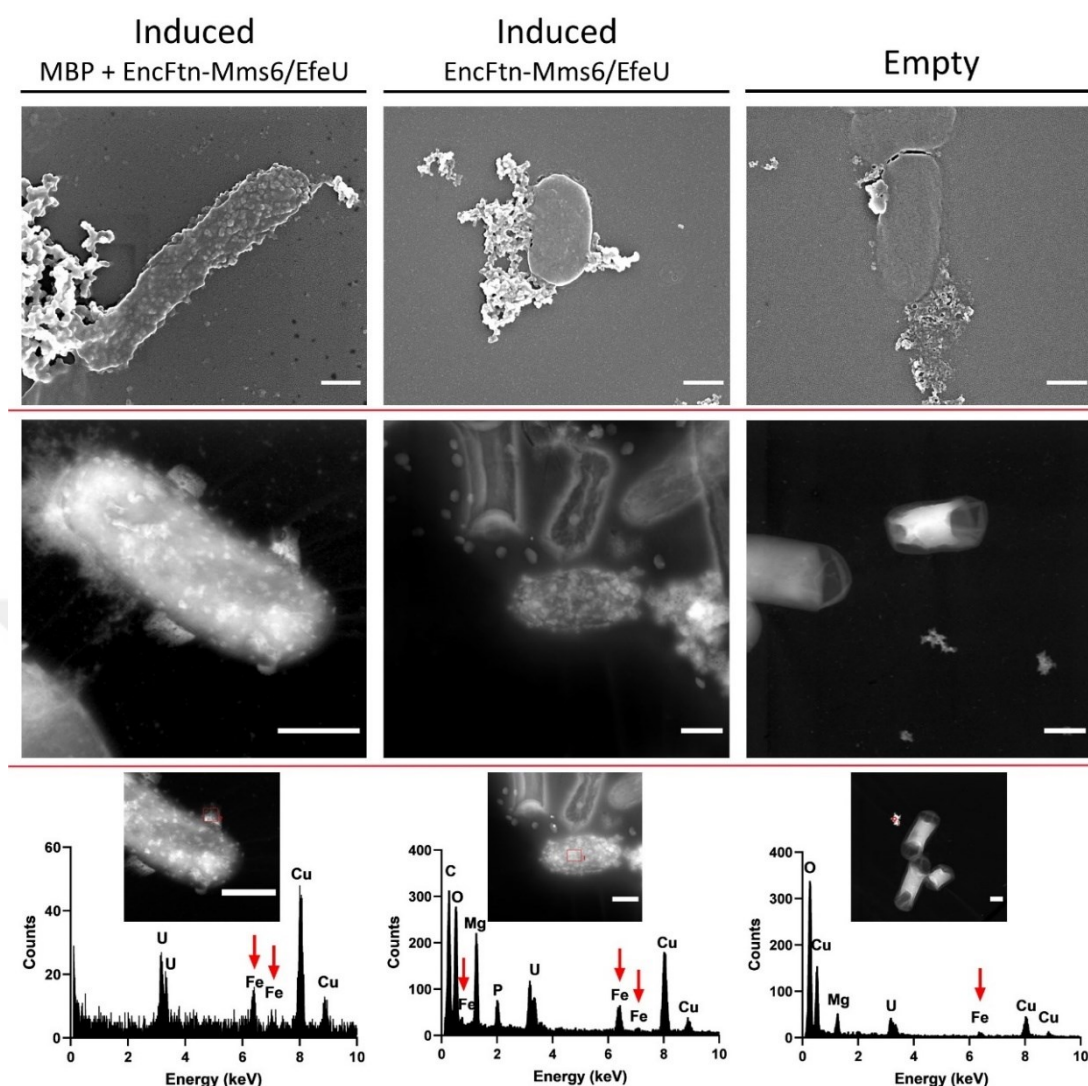


Figure 2.17 The characterization of produced magnetites inside the cell via SEM (upper panel) and TEM imaging (middle panel). Scale bars are 500 nm. EDS results of selected areas on the TEM images provide elemental analysis. (lower panel) Red arrows indicate iron signals. White scale bars are 500 nm. Samples are collected from replicates; representative images are presented here.

2.4.8 Quantification of the Iron Content of ICMAB Cells

The quantification of intracellular iron was performed via a colorimetric method called ferrozine assay (**Figure 2.18**), [105] in which the ferrozine gives a reaction with

ferrous iron ions to form a complex and results in a colorful end product. [116] It is important to determine the iron content per cell because cells' growth might show differences according to the contained synthetic gene circuit. Thus, the iron amount was calculated per colony-forming unit (CFU). The groups were compared with each other accordingly, where they differ in the expressed recombinant proteins' presence, type, and function the unpaired t-test (two-tailed) analysis was performed and gave a significant difference between MBP expressing *E. coli* BL21 strain (BL21 MBP+) and empty BL21 cell groups (P value = 0.0058). On the other hand, the difference between the BL21 MBP+ and BL21 MBP-, lacking MBP expression, was not significant (P value = 0.1028). As a result of these outcomes, it can be concluded that MBP improves the cells' iron content, though the difference was not remarkable.

In the EDS analysis, the free nanoparticles might lead to iron signal detection both in induced and empty cell groups, where the free nanoparticles might be synthesized due to the oxidation of iron ions in growth media. According to the ferrozine assay results, the empty cell group showed very little intracellular iron content. By considering these, it can be interpreted that the iron signal in empty cells might be mostly caused by free nanoparticles. In addition, the intracellular magnetite accumulating bacterial cell groups exhibited excess intracellular iron content in the ferrozine assay; thus, the iron signals in EDS were observed due to intracellular nanoparticles beside free nanoparticles for these cell groups.

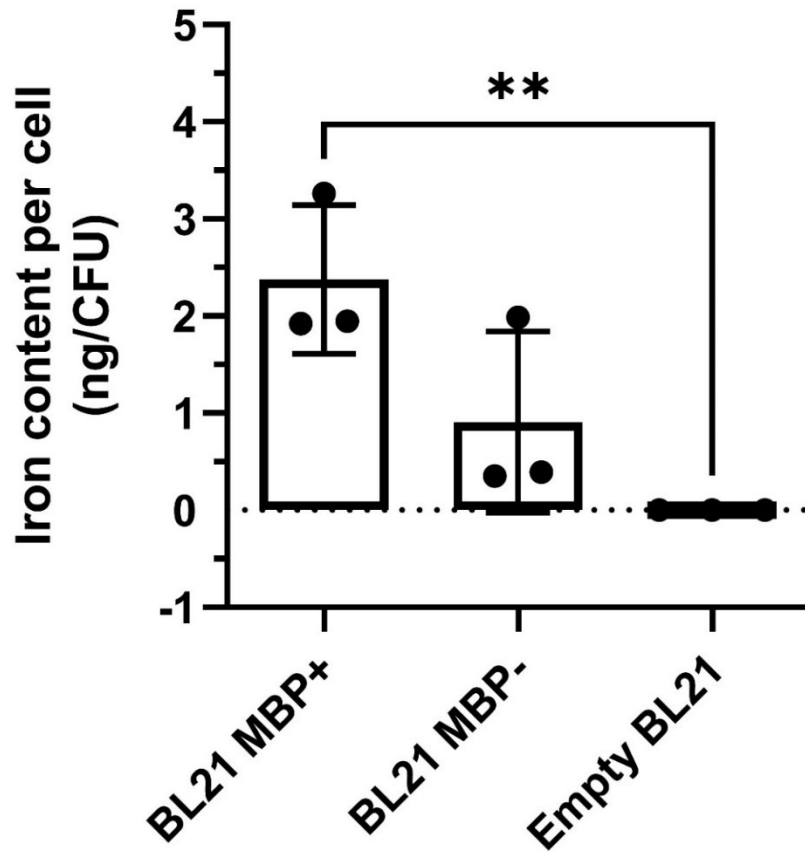


Figure 2.18 Quantification of intracellular iron via Ferrozine assay.

In order to observe the effect of ferrous iron ion supplementation on cell growth during induction, the growth curves were constructed. For this aim, the OD₆₀₀ values were measured at 0, 2, 4, 6, 12, and 24 h in triplicate (**Figure 2.19 A-C**), and it was seen that the overall cell growth was not negatively affected by iron ion addition. (**Figure 2.19 D**) Furthermore, the data sets were analyzed via grouped analysis – two-way ANOVA – and no significant difference was found between induced and uninduced cells except to some cases, that were seen mainly in the empty BL21 cell group, possibly caused by none use of iron.

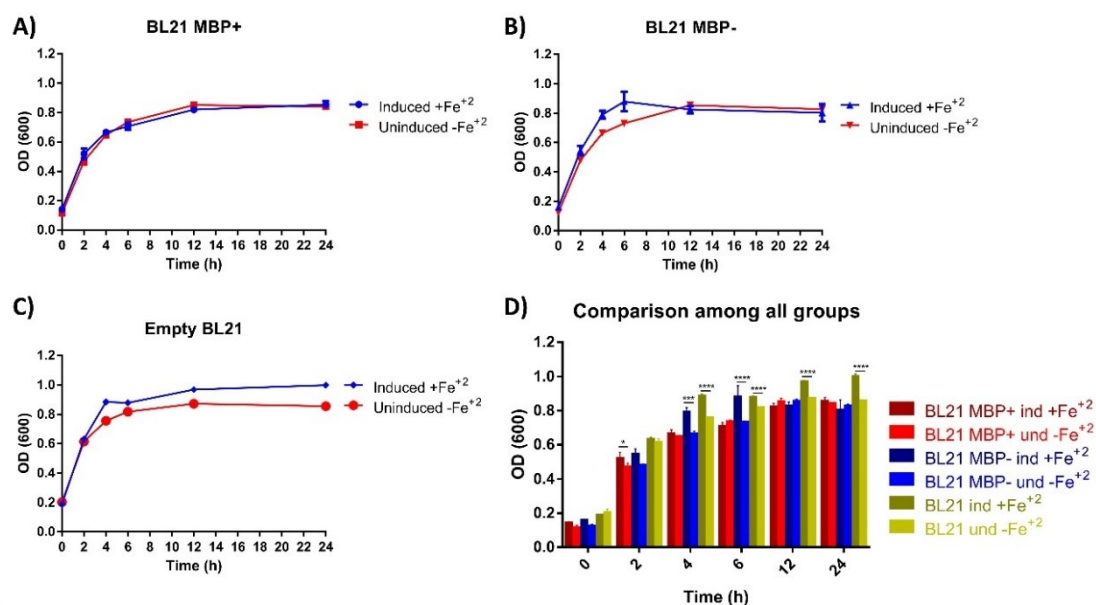


Figure 2.19 Growth curve of intracellular magnetite accumulating cells as well as control group at OD₆₀₀. All cell groups - (A) BL21 MBP+, (B) BL21 MBP-, and (C) empty BL21- were induced with IPTG and Fe²⁺ or left as uninduced, and growth under the same condition. (D) The comparison for induction and among cell groups was obtained via grouped analysis and two-way ANOVA.

In addition, a motility test was performed to control the effect of magnetite accumulation on bacterial locomotion. (**Figure 2.20**) According to the results, it was concluded that the overall motility was preserved when induced, and overnight cell growth was compared.

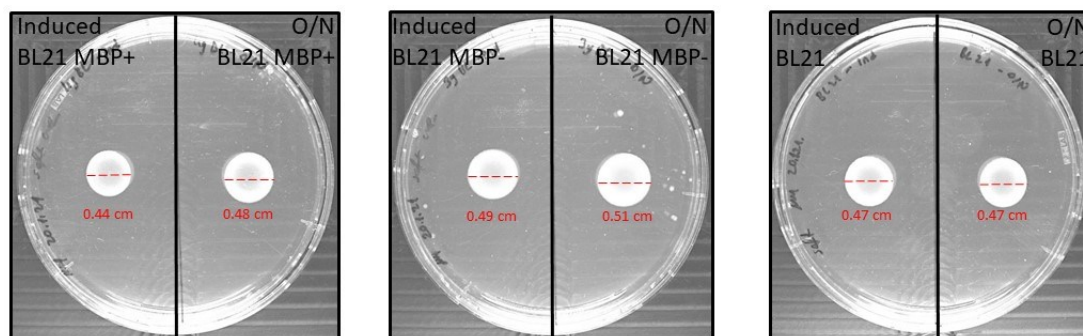


Figure 2.20 The motility test of intracellular magnetite accumulating cells as well as the control group plated on soft agar that incubated for 2 days.

2.4.9 Magnetic Resonance Imaging (MRI) of ECMAB and ICMAB Systems

For the ECMAB system, the r_2 transverse relaxivity estimations were provided in **Figure 2.21 A, B**. The negative contrast in MRI images is caused by the iron-based contrast agents' reducing the T_2 relaxation time in their environments. When the contrast agent induces a bigger reduction in T_2 for the same iron concentration, it means a higher r_2 value, which results in an improved MRI contrast agent performance. For the induced cells accumulating MNPs on the cell surface, the empty cells containing MNPs in the environment, and the control group consisting of only iron ions, the estimated r_2 values (mean $\pm$ std) were $34.1 \pm 1.6 \text{ mM}^{-1} \text{ s}^{-1}$, $27.6 \pm 2.4 \text{ mM}^{-1} \text{ s}^{-1}$, and $24.0 \pm 0.4 \text{ mM}^{-1} \text{ s}^{-1}$, respectively. Also, the r_2 value of the induced cells accumulating MNPs on the cell surface was significantly higher than the empty cells containing MNPs ($p < 0.05$, t -test) and the control group ($p < 0.05$). Thus, it can be concluded that the induced cells accumulating MNPs on the cell surface have significantly improved MRI contrast agent performance over both the empty cells containing MNPs in the environment and the control group according to the MRI measurements.

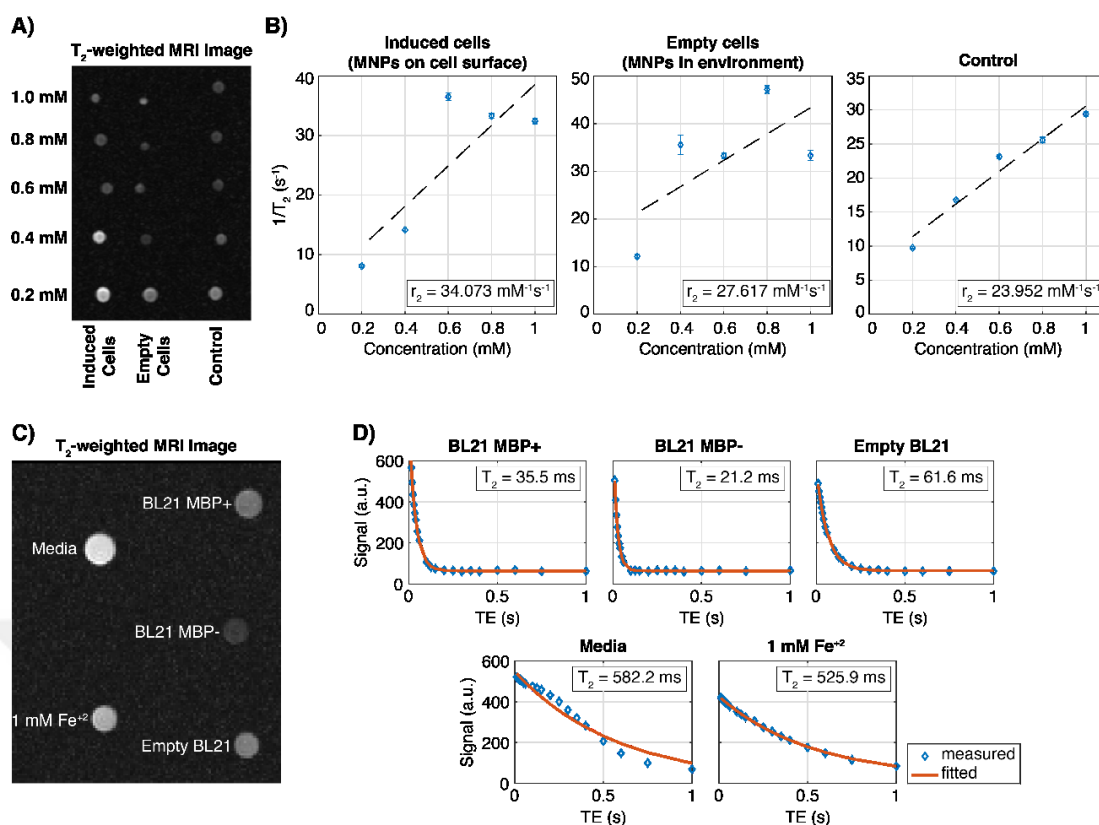


Figure 2.21 The MRI measurements for ECMAB and ICMAB systems. **A)** ECMAB r_2 relaxivity measurements. A representative T2-weighted MRI image at 3T with TR/TE = 2000/60 ms is shown. MNPs on the cell surface, MNPs in the environment, and the control group are prepared with iron concentrations of 0.2, 0.4, 0.6, 0.8, and 1 mM Fe. **B)** Inverse of T2 as a function of iron concentration (ranging between 0.2–1 mM Fe) for MNPs on the cell surface, MNPs in the environment, and the control group that only comprises ferrous and ferric ions. The T2 values are estimated using 12 different TE values between 10–1000 ms. The blue error bars denote the mean and the standard deviation for the ROI at each concentration, and the dashed lines represent linear fits. The fitted r_2 relaxivities are given inside the subfigures. **C)** ICMAB T2 relaxation time measurements. A representative T2-weighted MRI image at 3T with TR/TE = 3000/50 ms is shown. **D)** Pixel intensity as a function of TE for each sample at 21

different TE values between 10–1000 ms. The transverse relaxation time, T_2 , is estimated by fitting a monoexponential decay (red curves) to the data points (blue dots). The fitted T_2 values are given inside the subfigures.

For the ICMAB system, the T_2 relaxation time measurements were provided in **Figure 2.21 C, D**. For the BL21 MBP+, BL21 MBP-, and empty BL21 cell groups, the T_2 values (mean $\pm$ std across the corresponding ROI in the MRI images) were 35.5 ± 1.8 , 21.2 ± 0.9 , and 61.6 ± 2.4 ms, respectively. Additionally, the T_2 values of the media and 1 mM Fe^{+2} solution (control groups) were estimated as 582.2 ± 41.0 and 525.9 ± 76.2 ms, respectively. All the BL21 cell groups showed considerably shorter T_2 values when compared to both media ($p < 0.05$, rank sum test) and 1 mM Fe^{+2} solution ($p < 0.05$), pointing out significantly improved negative contrast capability in MRI, as can be seen in **Figure 2.21 C**. The shorter T_2 values represent higher iron concentrations for a fixed contrast agent and medium, allowing a comparison of the iron concentration of BL21 cell groups. Both the intracellularly magnetite accumulating bacterial cell groups, BL21 MBP+ and BL21 MBP-, show considerably shorter T_2 values than empty BL21 cells ($p < 0.05$), indicating higher iron concentrations and significantly improved negative contrast capability in MRI. Also, BL21 MBP- cells have slightly shorter T_2 values than BL21 MBP+ cells ($p < 0.05$). As can be deduced from the ferrozine assay, BL21 MBP- cells showed lower iron content per cell compared to BL21 MBP+ cells. On the other hand, BL21 MBP- cells also demonstrated denser colony formations, which might have counteracted the relatively low iron content per cell, resulting in an effectively higher iron concentration per unit volume and a shorter T_2 value. In other words, BL21 MBP- cell group showed more CFU than the BL21 MBP+ cells even if the cells' amount equalized before analysis which might be due to

lesser workload on BL21 MBP- cell group. Thus, the shorter T_2 value of BL21 MBP- cell group might be considered possibly due to the higher amount of the cells.

In this study, it was shown that the magnetite accumulating bacterial cell machineries, both extracellularly and intracellularly were constructed. Both systems represent better MRI contrast performance than the empty cells with the recombinantly expressed proteins; thus, the developed modules offer a promising diagnostic tool as a contrast agent. A probiotic *E. coli* strain, *E. coli* Nissle 1917 (EcN), can be engineered for cancer diagnosis. [73], [117] Danino et al. engineered EcN as a noninvasive diagnostic tool which defines the presence of hepatic tumors from urine signals. [73] Moreover, EcN can also be engineered to express acoustic genes that provide gas vesicle formation in bacterial cells, which enhances noninvasive ultrasound imaging with significant acoustic properties. [117] Here, the aim was to transfer our developed systems into EcN strain to get a probiotic magnetic contrast agent for cancer diagnostic applications. For constructing a contrast agent against a specific cancer type, the system can be improved by combining the agent with a targeted peptide that would play a role in the desired area of the body and minimize the toxicity of the imaging procedure. [89], [92] Thus, the engineered intracellularly magnetite accumulating bacterial system providing a preferred regime for *in vivo* trials that can be enhanced by incorporating a specified nanobody activity for creating a more reliable end product.

CHAPTER 3 : ENGINEERED BACTERIAL MAGNETS TARGETING BREAST CANCER

3.1 Objective of the Study

The demand for cancer targeting is still a need for minimizing the side effects of the therapeutic agents. The constructed intracellular magnetite accumulating bacteria (ICMAB) system, performing all required redox reaction in the cytoplasm, can be improved by specific targeting. The nanobody called 2Rs15d was integrated into ICMAB bacterial cells which binds to HER2 receptors over expressed on Jimt1 breast cancer cells. The dual-functionality of the bacterial cells leveraging from both magnetite accumulation and binding to specific tumor cell would serve as a promising platform in cancer diagnostics by eliminating the systemic toxicity.

3.2 Introduction

Nano-device development is an out-standing platform for constructing living therapeutic agents. The development of microbe-material platform is promising for creating biomaterials and next-generation nanomedicine featuring specific theranostic functionalities. [7] Utilizing the microbes attributes the capabilities for hypoxia tendency and oxygen production. The combinatorial effect of living machines with functional materials improves the efficacy of treatment via specified drug delivery, enhanced disease monitoring. The usage of microbial derivatives harbors various advantages for biomedical applications. For instance, bioimaging, anti-tumor, or disease detection modules are highly intended. [7]

Targeted therapy is a pivotal method to eliminate the traditional treatment's side effects as it leads to inevitable injury to the healthy tissues besides diseased cells. In order to get rid of the limitations of the therapeutic efficiency, the targeted therapy serves for a more effective cure and diminished systemic toxicity. Utilizing microbes stands out as a precise therapeutic delivery method for targeted biomedical applications. [7]

Microbe-material hybrids can be used for bioimaging in medical applications. For the diagnosis of cancer, magnetic resonance imaging (MRI) is preferable as a non-invasive, non-ionizing/ radiating imaging technique. [7] Synthetic biology enabled the cloning of genes from one cell to another, even from one organism to another. In this way, reprogramming cells to behave in a different manner became possible. In order to construct a biocompatible contrast agent for MRI, living microorganisms can serve as a cellular machinery to perform desired function. For instance, bacterial cells have the potential to be an actuator by an applied external magnetic field or light via covalently attached magnetic nanoparticles and light-responsive nanoliposomes for on-demand cargo delivery. [24] By taking inspiration from naturally magnetite crystal synthesizing bacteria, *E. coli* cells can also be genetically designed for magnetite accumulation. [74] The system for intracellular magnetite accumulation provides that all redox reactions take place in the cytoplasm of the cell and so the bacteria itself serve as a biohybrid microrobot. In order to achieve this, the combinatorial effect of helper proteins with the iron-binding peptide is taken into consideration. The ferroxidase enzyme provides the oxidation of ferrous iron ions into ferric ions, iron-binding peptide facilitates nucleation, material binding peptide increases the tendency for the iron ions and supplies a template, and iron transporter protein provides the uptake of ferrous iron ions into the bacterial cells. The development of bacterial cells

that are designed to do all the duties themselves for magnetite accumulation has significant potential for their use in cancer diagnosis and treatment. [74]

3.3 Materials and Methods

3.3.1 Cell Lines, Cell Growth and Maintenance, Chemical Competent Cell Preparation and Transformation

For cloning and protein expression experiments, *E. coli* DH5 α and *E. coli* BL21 (DE3) strains were used, respectively. The long-term storage condition for the bacterial cells was at -80°C in a freezing medium containing 25% glycerol in Lysogeny Broth (LB) media. *E. coli* BL21 (DE3) strain is lack of proteases and it was used for protein expression and purification. The strain encodes T7 polymerase gene under the control of Lac promoter. For bacterial cell growth, both *E. coli* strains were inoculated in LB medium and incubated on LB agar at 30 or 37°C. The liquid culture of bacterial cells was grown in a shaking incubator if not otherwise specified.

The chemical transformation method was utilized to uptake plasmids into bacterial cells. The bacterial cells were inoculated in LB medium containing proper antibiotic from -80°C cell stock overnight and diluted in fresh LB at 1:100 ratio on the next day morning, for preparation of chemically competent cells. Then, the bacterial cells were allowed to grow until OD₆₀₀ of 0.2-0.5 and cooled on ice for 10 min. The cells were collected via centrifugation at 3000 rpm for 10 minutes at +4°C. The TSS (Transformation and Storage Solution) buffer was used to resuspend the cell pellets in 1:10 culture volume of and aliquoted 100 μ l into chilled microcentrifuge tubes that were stored at -80°C.

For the transformation of a plasmid into chemically competent cells, the cell stock was thawed on ice for 20 minutes, and a plasmid or reaction mixture was added onto the cell. The heat shock was performed at 42°C for 45 seconds for plasmid uptake into the cells, after 20 minutes of incubation of the cells on ice. The cells were incubated on ice for 2 minutes and 250 µl LB was added onto the cells for growth at 37°C 200 rpm for 45 minutes. After incubation, the cells were centrifuged, and the excess supernatant was removed. The remaining supernatant was utilized to resuspend the cell pellet and all suspension was spread on LB-agar plate containing the appropriate antibiotic. The plate was incubated at 37°C stationary incubator for overnight growth.

3.3.2 Construction of Gene Circuits and Cloning

Nanobody expression was achieved via the construction of pZa ntetO 6H 2Rs15d 160N Ag43 AmpR vector. The cmr antibiotic resistance gene was replaced with AmpR. For cloning of AmpR, P22 and P23 primers were utilized for the amplification of pZa ntetO 6H 2Rs15d 160N Ag43 region, additionally, P24 and P25 primers were used for the amplification of AmpR gene. Then, the PCR products were assembled via the Gibson assembly protocol.

mCherry cloning was performed to integrate the red fluorescent protein into nanobody-expressing ICMAB cells. For constructing the pZs proD mCherry kan plasmid, the insert region as proD mCherry part was amplified with P26 and P27 primers, whereas the backbone region containing pZs ori kan parts was amplified with P28 and P29 primers. Then, the PCR products were assembled via the Gibson assembly protocol.

mCherry cloning was performed for integrating the red fluorescent protein into the pZa ntetO 6H 2Rs15d 160N Ag43 AmpR vector. For the construction of pZa proD

mCherry 6H 2Rs15d Ag43 ampR plasmid, the backbone was obtained by digest reaction of pZa ntetO 6H 2Rs15d ampR construct with Avr2 (NEB) and Mlu1 (NEB) enzymes. For the insert, proD mCherry rnnB T1 and T7 terminator regions were amplified with P30 and P31 primers. Then, the digest and PCR products were assembled via the Gibson assembly protocol.

3.3.3 Sequence Verification

An online tool, Benchling.com, was utilized to construct and design genetic circuitries. The plasmid maps were exported as .gb files. The sequencing process was obtained from Genewiz company as Sanger Sequencing or from InterGen company as Next Generation Sequencing (NGS). The Geneious® 9.05 software or Benchling alignment option was used to import both the vector sequence and sequence results as .ab1 files. The standard parameters were used for pairwise alignment of the sequence to the reference, and gaps or distinction reads were determined for the analysis.

3.3.4 Recombinant Proteins Expression in *E. Coli*

For the construction of nanobody integrated ICMAB system, pZa ntetO 6H 2RS15d 160N Ag43 AmpR vector was transformed into chemical competent cells harboring pET22b EncFtn 6H Mms6 MBP6 EfeU *E. coli* BL21 (DE3) strain. After transformation, single colonies from the plate were inoculated into LB media supplemented with proper antibiotics for overnight growth. The overnight grown cells were sub-cultured at 1:50 ratio into fresh 1×M9 minimal media that is supplemented with proper antibiotics as well as 0.4% (w/v) glucose, 0.2% (w/v) casamino acid, 2 mM MgCl₂ and 0.1 mM CaCl₂. For the optimization of protein expression from double transformant cells, the changing temperature conditions were performed while the

induction experiment. The induction of the cells was performed at around OD₆₀₀ of 0.1 with 0.1 mM IPTG, 1 mM ammonium ferrous sulfate hexahydrate, and 100 ng/ml or 250 ng/ml anhydrotetracycline (aTc) at for 24 hours at 30°C and 18°C, 200 rpm. In addition, heat release was performed for the analysis of surface-displayed nanobody-fused with truncated alpha passenger domain of Ag43. The aim was to release the surface-displayed protein in the extracellular medium. The heat treatment would disturb the non-covalent interaction between α - and β -domains. For that purpose, 5×10^8 cells were collected and resuspended in 200 μ l 1 $\times$ PBS. The samples were heated at 60°C for 5 minutes or left at room temperature as a control group. The cells were centrifuged at maximum speed for 5 minutes, and the supernatant was collected for following analysis.

The nanobody expressing ICMAB cells were also integrated with a red fluorescent protein, mCherry, as a reporter protein. The protein was express under a constitutively active promoter, so that there was a constant accumulation off reporter protein in the media.

3.3.5 Protein Expression Verification

For verification of recombinant protein expression, Western Blotting analysis was performed. The certain amount of induced cells (5×10^7) were collected, and the final concentration of 1 $\times$ SDS loading dye was added for sample preparation. After boiling at 95°C for 5 min for denaturation of cells, the samples were loaded on 15% SDS-polyacrylamide gel and ran at 120V for around 2 hours. For Western Blotting, the gel was transferred to a pre-activated PDVF membrane with methanol treatment via Trans-Blot Turbo (BioRad) utilizing the standard miniblott protocol. The incubation in 5% skim-milk dissolved in TBS-T buffer of the membrane was performed to block the

non-specific bindings at room temperature for 2 hours on the rotator. Subsequently, the membrane was transferred to the 1: 10000 primary anti-his mouse antibody (Abcam ab18184-100 μ g) in 5% skim milk in TBS-T solution at 4°C overnight on the rotator to enable binding of primary antibody to the His-tagged proteins on the membrane. The TBS-T washes were performed for the membrane three times with shaking in order to get rid of excess primary antibody. After washing, the membrane was incubated in 1: 10000 horseradish peroxidase (HRP) conjugated goat anti-mouse secondary antibody (Abcam ab205719-500 μ g) in 5% skim milk in TBS-T solution at room temperature for 1 hour. The TBS-T washing was done to remove excess secondary antibody solution for three times on the rotator at room temperature. ECL substrate (Biorad 170-5061) was used for HRP to give a reaction with His-tagged proteins on the membrane so that the protein bands can be visualized via the gel imaging system (Vilber Fusion Solo S).

3.3.6 Surface Display Verification of 2Rs15d Protein in ICMAB Cells

Immunocytochemistry (ICC) assay was utilized for labeling and ensuring the display of Mms6 peptide on the cell surface of the ECMAB system. After induction of the cells, a certain amount of cells (5×10^8) were collected and washed with $1 \times$ PBS. For blocking, the cells were resuspended in 1% BSA in $1 \times$ PBS solution for 2 hours at room temperature on a rocker. The cells' pellet was resuspended and incubated in 1: 250 anti-his primary antibody in 1% BSA in $1 \times$ PBS solution for overnight at +4°C on the rocker. The cells were washed with $1 \times$ PBS in order to remove the primary antibody that were later incubated in 1: 250 DyLight550 secondary antibody for 1.5 hours in dark at room temperature on the rocker. The cells were washed with $1 \times$ PBS and diluted at 1:10 ratio in $1 \times$ PBS. For imaging under a fluorescence microscope

(Scope A1, Zeiss), the cells were mounted on the slide via a drop of mounting media and a coverslip was sealed to the slide.

3.3.7 Mammalian Cell Culture

The breast cancer cell line, called Jimt1, was stably transfected with a green fluorescent protein, GFP. The stock was kindly provided by the Molecular Biology and Genetics Department at Bilkent University. The cell stocks were kept in nitrogen tank up until thawing. The cells were cultivated in low glucose Dulbecco's Modified Eagle Medium (low glucose DMEM) without L-glutamine, 10% heat-inactivated Fetal Bovine Serum (FBS), 1% Penicillin/ Streptomycin (Pen/Strep) antibiotic, and 2 mM L-glutamine. For thawing of a stock, the vial was kept in 37°C water bath for a few minutes and diluted in 10 ml of fresh media. After collecting the cells via centrifugation at 2500 rpm for 5 minutes, the pellet was resuspended in 1 ml of fresh media and the cells grown in T25 flask at 37°C in a 5% CO₂ humidified incubator. Around 80% confluency, the cells were harvested via trypsinization. After deactivation of the trypsin and collection of the cells via centrifugation, the cells were counted via a hemacytometer. The diluted cells at 1:10 ratio were stained with Trypan Blue and observed under inverted microscope (Zeiss Axio Observer A1). The new stocks were made from half or one million of the cells that were stored at 10% DMSO. The cell stock vial was placed in an isopropanol chamber and stored at -80°C overnight. The following day the vial was transferred into the nitrogen tank and stored there for further usage.

3.3.8 Binding Assay of Nanobody Integrated ICMAB Cells to Breast Cancer

Cell line

Infection of Jimt1 cells was performed by utilizing induced 2Rs15d ICMAB BL21 cells under proper conditions. When Jimt1 cells reached to 80% confluency, they were collected and co-cultured with the induced bacterial cell group at a 100:1 or 1000:1 multiplicity of infection ratio (bacteria: mammalian cells). The certain amount of Jimt1 cells (10^5 cells/ml) were incubated overnight (O/N) at 37°C, 5% CO₂ conditions. Then, the specified amount of the induced bacterial cells (10^7 or 10^8 cells/ml) were added onto mammalian cells and incubated for one hour. After washing the co-culture with $1 \times$ PBS for three times, microscopy images were obtained.

For 3D cell culture experiments, Jimt1 cells were used for formation of spheroids. Hanging drop method and the technique utilizing high purity agarose (HPA) were tried, on the other hand, for the easiness of cells collection and for maintaining the stability of spheroids, HPA method was utilized for further experiments. For that aim, a 96 well plate was coated with 1-2% HPA for creation of non-adhesive layer for mammalian cells. 3000 cells/well were incubated on HPA containing DMEM with L-glutamine for 3-5 days. The induced bacterial cells were added on the spheroidal cells at certain amount (3×10^6). After co-culturing bacteria and spheroids for 1 hour at 37°C, 5% CO₂ humidified incubator, they were carefully collected from the well plate and prepared for confocal imaging by fixing with formaldehyde and trapping the sample between a polished glass lam and coverslip

3.3.9 Characterization of the Binding Assay via Electron Microscopy Imaging

The scanning electron microscope (SEM) was utilized to characterize iron oxide nanoparticle formation. For that, silicon wafers were used that were cut into small pieces, then washed with ethanol and isopropanol prior to sample preparation. 10 μ l samples were dropped on the silica wafers and allowed to dry overnight. The wafers were coated with 8/10 nm Au-Pd via a precision etching coating system (PECS, Gatan 682) to obtain conductivity for the specimens. The visualization was performed via E-SEM microscope (FEI-Quanta 200F) at 10-15kV electron acceleration. A representative image was shown in the figures for all groups. Energy-dispersive X-ray spectroscopy (EDS) analysis was performed with EDAX Genesis software combined with the SEM microscope.

3.3.10 Visualization of the Binding Assay Confocal Imaging

Due to the difficulty in directly observing the binding of bacteria to the spheroids on the HPA substrate, the cells had to be collected and prepared on glass slides for confocal imaging. Consequently, the binding assay had to be performed multiple times to ensure reliable results. Confocal microscope imaging was employed to visualize the binding between the two cell types, and the experiments were performed a few times to demonstrate the repeatability of the results. The experiments were conducted using a 20X-oil objective lens for imaging. Three-dimensional (3D) maximum and volume imaging techniques were employed to capture detailed information. To ensure experimental consistency and reliability, technical replicas of the samples were prepared. This involved replicating the experimental procedure and treating each replica as an independent sample for analysis.

3.3.11 Preparation and Characterization of Bacterial Cell Systems via Magnetic Resonance Imaging

For magnetic resonance imaging (MRI) measurements, the induced bacterial cells were utilized for the infection of spheroidal mammalian cells. Non-engineered empty bacterial cell group, only spheroidal mammalian cell group and media were also included for interpretation of the results. After the co-culture of cells, they were carefully collected from well plate and transferred into an Eppendorf tube. The samples prepared for MRI measurements had to be handled gently to avoid disrupting the binding between the bacteria and the spheroids. Furthermore, it was crucial to perform MRI measurements immediately after the binding period to capture accurate data and minimize any potential changes or alterations in the binding state. This ensured that the MRI measurements accurately reflected the interaction between the bacteria and the spheroids at the specific time point of interest.

The Magnetic Resonance Imaging (MRI) analysis was performed at Bilkent University's UMRAM (National Magnetic Resonance Research Center) by Associate Professor Emine Ülkü Sarıtaş Çukur. A 3T Siemens Magnetom Trio scanner using a 32-channel head coil was used for transverse relaxation time (T₂) measurements of the samples prepared in 1.5 mL Eppendorf tubes. A multiple-echo spin-echo sequence with 16 different echo times (TE) between 8.5–1000 ms was used (TE = [8.5, 10, 15, 20, 30, 50, 80, 100, 150, 200, 250, 300, 400, 600, 800, 1000] ms), with a repetition time (TR) = 2000 ms, 4 mm slice thickness, and 120 × 120 mm² field-of-view (FOV) for T₂ measurements of bacteria spheroid co-culture samples and the control samples. MATLAB (Mathworks) was utilized via a custom script to analyze MRI images. For each tube in the MRI images, a circular region of interest (ROI) was selected. The

pixel intensities as a function of TE were fitted to the standard monoexponential T2 relaxation curve. The inverses of T2 values as a function of concentration (C) were fitted to the linear relation for the determination of r_2 .

3.4 Results and Discussion

Nanobodies are produced recombinantly to minimize size and target recognition module while possessing high stability and enhanced tissue penetration. They are fully functional antibodies containing only the heavy chains and lacking the light chains. Nanobodies can be tagged with peptides for labeling tumors and utilizing tag-specific T-cells. [118] Human epidermal growth factor receptor (HER2) is overexpressed in breast cancer in addition to bladder, pancreatic, ovarian, and stomach cancers. [119] An anti-HER2 nanobody called 2Rs15d can be used against the Jimt1 breast cancer cell line that harbors overexpression of HER2. The chosen nanobody displayed on *E. coli* bacterial cells via an autotransporter protein was constructed for specific targeting of breast cancer cells (**Figure 3.1**).

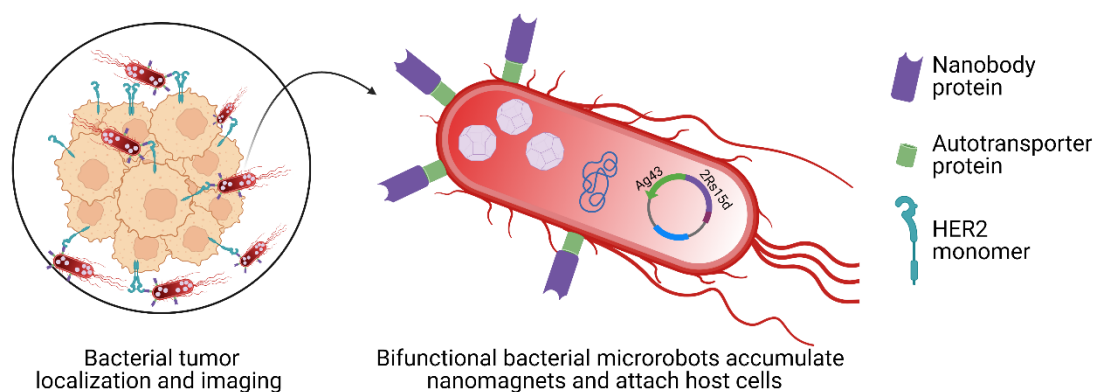


Figure 3.1 The intracellular magnetite accumulating bacterial cells were engineered for a nanobody display on the cell surface, enhancing on-target activity. Created with BioRender.com.

3.4.1 Cloning and Expression of 2Rs15d

In this chapter, it was purposed to construct nanobody-producing *E. coli* cells under an inducible circumstance. Also, it was aimed to display the nanobody on the surface of the bacteria that was achieved via the expression of 2Rs15d as a fusion protein with an autotransporter protein called Ag43. The transcriptional inducer anhydrotetracycline (aTc) was utilized for mediating the protein expression from the native TetO promoter, that is a bidirectional promoter. The genetic circuit was designed to express both the target protein, 2Rs15d-Ag43 fusion protein, and TetR repressor protein under the control of a native TetO promoter for the construction of an inducible system (**Figure 3.2**).

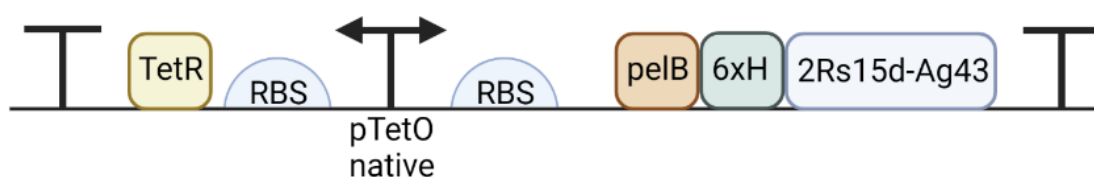


Figure 3.2 The genetic design for constructing a nanobody expression system under inducible conditions. Created with BioRender.com.

The system was constructed using a pZa mid-copy plasmid. The backbone pZa ntetO 6H 2Rs15d 160N Ag43 and the insert ampR were PCR amplified (**Figure 3.3 A**), and selected colonies were screened via double digestion with SpeI HF (NEB) and Aat2 (NEB) restriction enzymes (**Figure 3.3 B**).

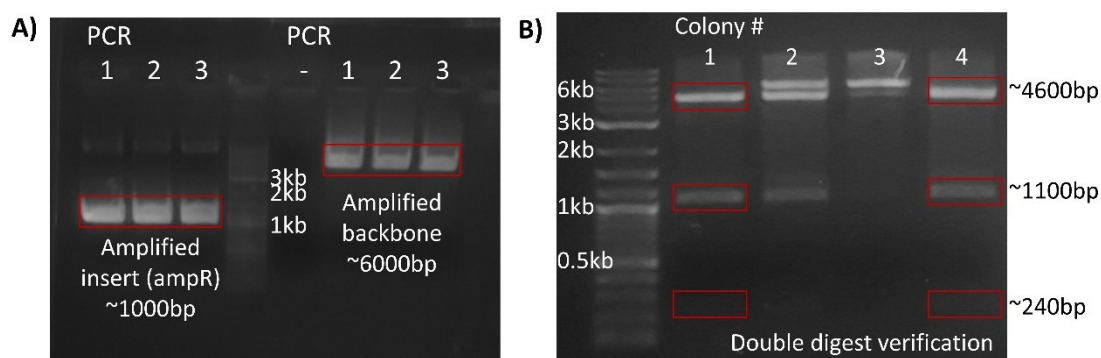


Figure 3.3 The construction of aTc inducible 2Rs15d expression system. A) The PCR products of ampR and pZa ntetO 6H 2Rs15d 160N Ag43 were shown. B) Selected colonies were verified via double digestion reactions.

3.4.2 Protein Expression Analysis for Nanobody Integrated ICMAB Systems

After sequence verification, the ICMAB *E. coli* BL21 (DE3) competent cells were transformed with pza nteto his 2Rs15d Ag43 ampR plasmid. In order to verify the protein expression, western blot analysis was performed for the induced cell groups. An online tool called Expasy was used to calculate the theoretical molecular weight of the recombinant proteins. In order to disturb the noncovalent bond between the α - and β -domains' of Ag43 protein, heat treatment was performed for the cells (**Figure 3.4**). Without a need for heat treatment, the dimer form of the ICMAB fusion protein, ferroxidase enzyme (EncFtn), His-tagged iron-binding peptide (His₆Mms6), and MBP (MBP6), was observed at around 60 kDa molecular weight band, the fusion protein of the hexahistidine tagged 2Rs15d and truncated α -domain of Ag43 was seen at around 48 kDa, and lastly the fusion form of 2Rs15d with α - and β -domains of Ag43 was visible at around 99 kDa molecular weight band on western blot.

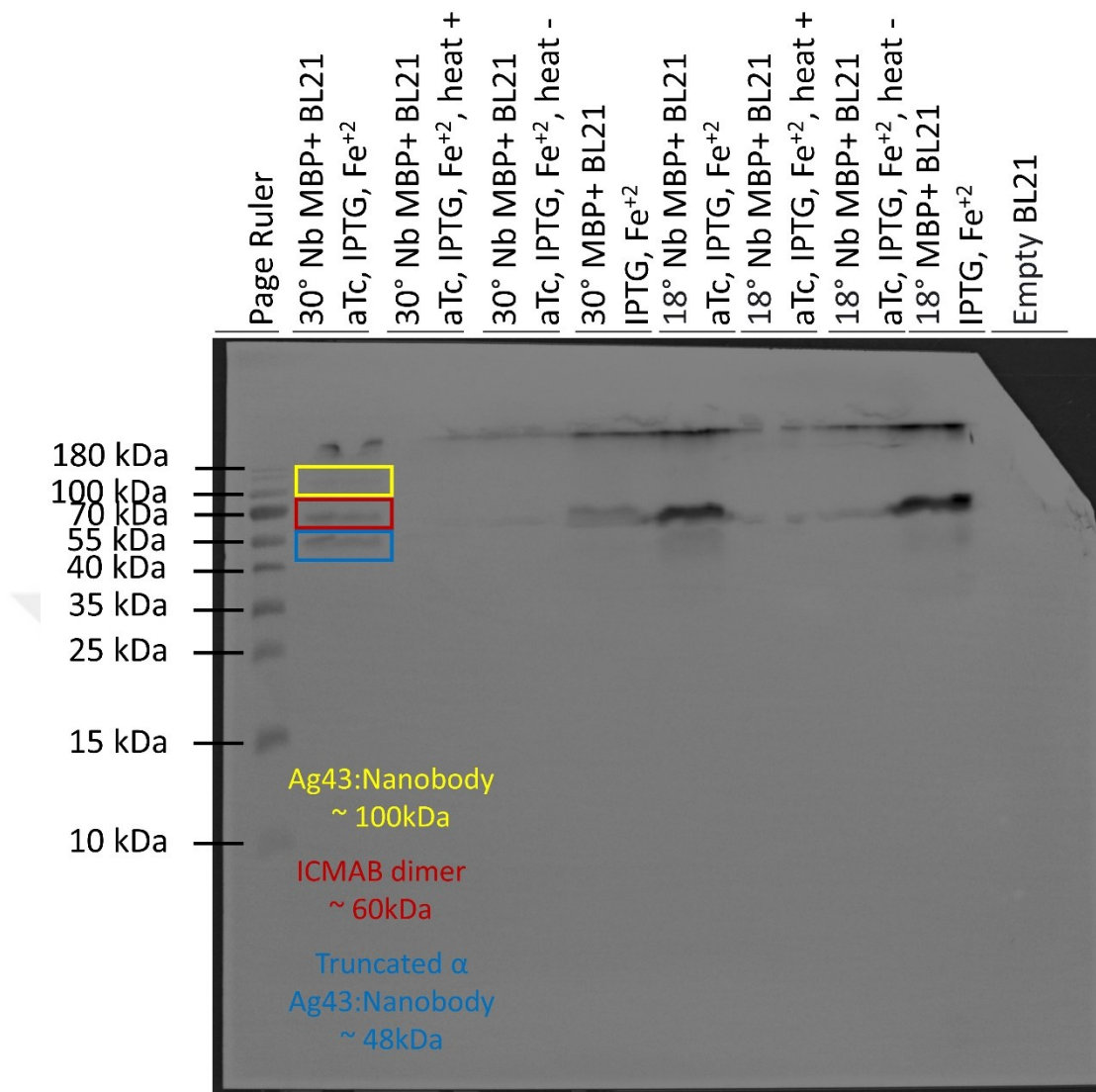


Figure 3.4 The protein expression analysis via Western Blot was shown for nanobody-integrated ICMAB BL21 bacterial cells. Changing temperature and heat treatment conditions were performed.

In order to optimize the inducer concentration for the nanobody expression, two different aTc concentrations were used for changing induction temperatures (**Figure 3.5**). The optimized condition for the best expression of involved proteins was determined as 100 ng/ml anhydrotetracycline (aTc) concentration for 24 hours at 30°C, 200 rpm.

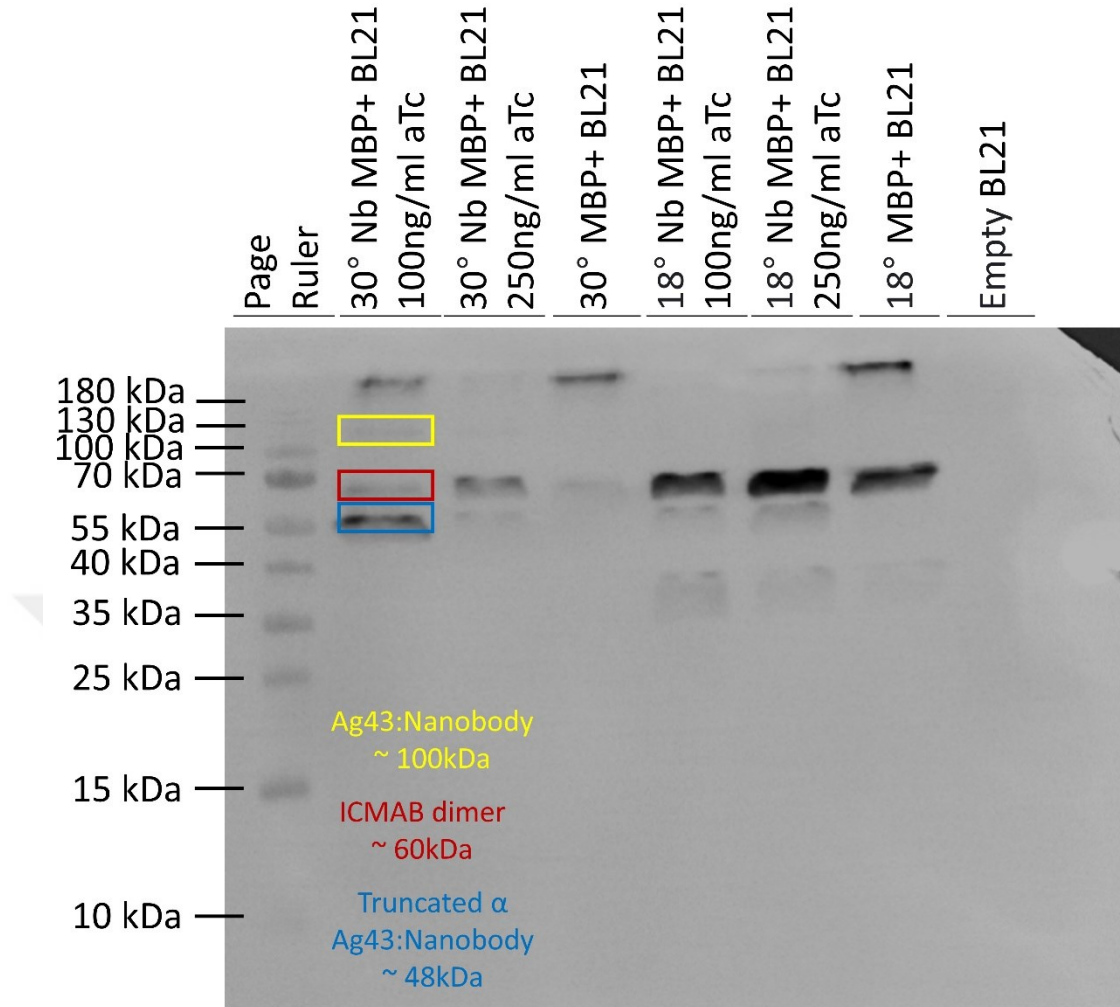


Figure 3.5 The protein expression analysis via Western Blot was shown for induced nanobody-integrated ICMAB BL21 bacterial cells. Changing inducer concentrations in different induction temperatures were indicated.

3.4.3 Verification of Surface Display of the Nanobody Protein on ICMAB Cells

The immunocytochemistry (ICC) assay was utilized for ensuring the surface display of 2Rs15d protein on ICMAB BL21 cells. The His-tagged 2Rs15d was labeled with His-tag-specific antibodies conjugated with DyLight550 in the absence of membrane solubilizing agents. Fluorescence microscopy imaging was performed for observing the display of 2Rs15d peptide. The red signals stand for the anti-his antibodies bound

to the displayed 2Rs15d on the cell surface because the cell membrane was not permeabilized for antibody passage (**Figure 3.6**).

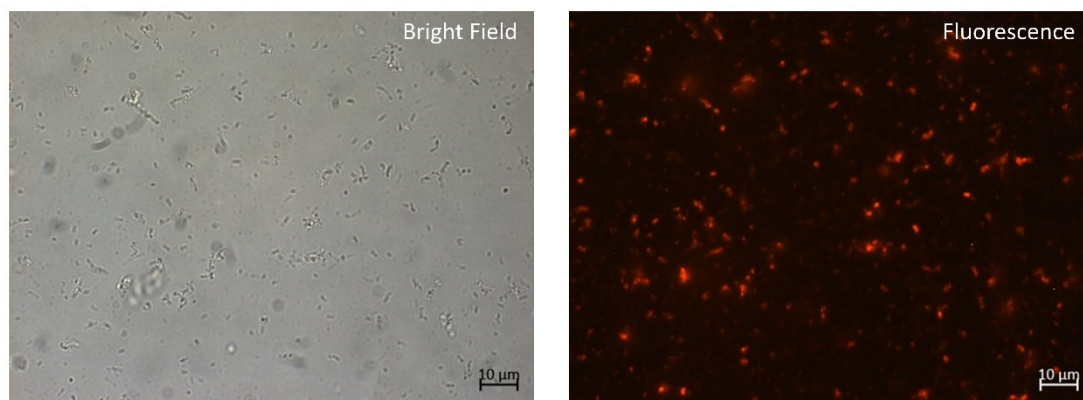


Figure 3.6 The immunocytochemistry (ICC) assay depicted the expression of displayed His-tagged 2Rs15d on the ICMAB BL21 cell-surface stained with DyLight 550 antibody specific to His-tag and imaged under fluorescence microscopy. The left image is a bright field image, and the right one is taken under the red filter at 100% intensity. Scale bars are 10 µm.

3.4.4 Infection of Mammalian Cells with Induced Nanobody Harboring ICMAB BL21 Cells

The binding assay was performed to observe the attachment performance of nanobody-integrated magnetite accumulating bacteria to the breast cancer cells. For that purpose, the induced bacterial cells under proper conditions were utilized to infect Jimt1 cells at 100:1 multiplicity of interest (MOI) ratio. After co-culturing bacteria and mammalian cells for binding to occur, samples were prepared for bright field and electron microscopy imaging. The first group was depicted as Jimt1 cells infected with induced 2Rs15d ICMAB BL21 cells, the second group was represented as the control group, including Jimt1 cells co-cultured with empty BL21 cells, and the third group

was Jimt1 itself (**Figure 3.7**). According to the results, Jimt1 cells were possibly surrounded by nanobody-integrated ICMAB cells, whereas the empty BL21 cells didn't show attachment to the mammalian cell surface. On the other hand, to increase the accuracy of outcomes, a reporter protein integration to nanobody expressing ICMAB cells was aimed.

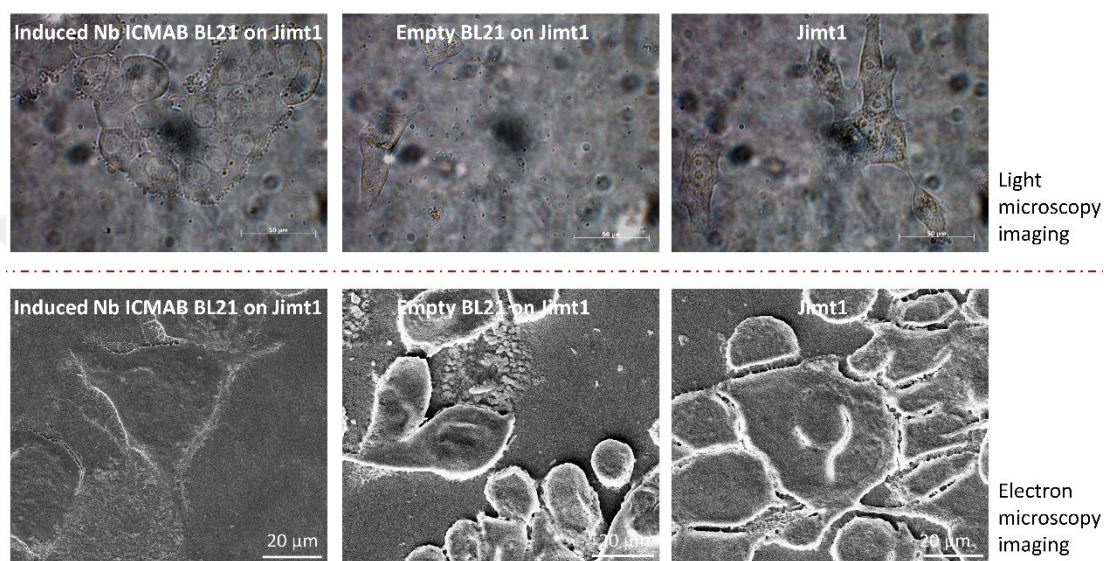


Figure 3.7 The binding assay for exploring the attachment function of 2Rs15 integrated ICMAB BL21 cells on Jimt1 cells was observed under light microscope (upper panel) and under scanning electron microscope (SEM) (lower panel). Scale bars are 50 μm for light microscope images, and white scale bars are 20 μm from SEM images.

3.4.5 Reporter Protein Combination to Nanobody Expressing ICMAB Cells

In order to be sure about the nanobody expressing ICMAB cells' attachment onto mammalian cells, the bacterial cells were integrated with a red fluorescent protein. For combining a fluorescent protein with nanobody expressing ICMAB cells, a red fluorescent protein called mCherry was utilized for bacterial cells as the GFP

transfected mammalian cells fluorescence green. For that purpose, constitutively active mCherry protein in a low-copy vector was constructed (**Figure 3.8 A**).

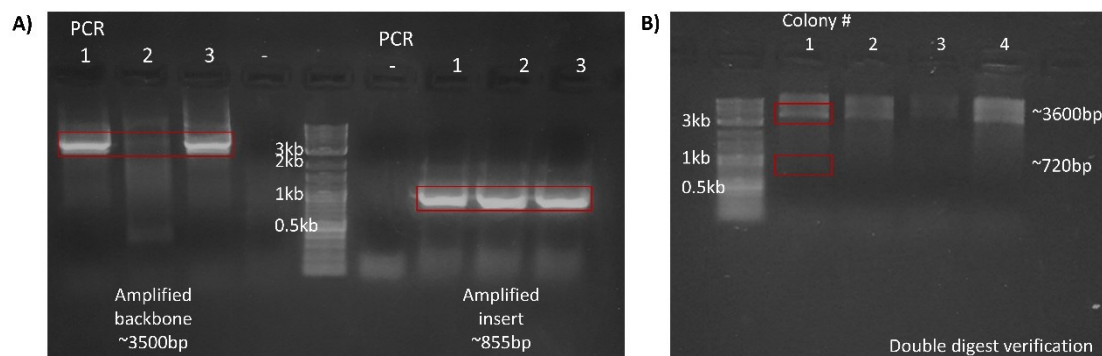


Figure 3.8 The construction of constitutively active mCherry protein in a low copy vector. A) The PCR products of the insert and backbone were shown. B) Selected colonies were verified via double digestion reactions.

For constructing the pZs proD mCherry kan plasmid, the insert mCherry and the pZs backbone were amplified and assembled. The selected colonies were screened via double digestion with BamH1 HF (NEB) and Kpn1 HF (NEB) restriction enzymes (**Figure 3.8 B**).

After verification of the cloning via NGS analysis, the plasmid was transformed into 2Rs15d ICMAB *E. coli* BL21 (DE3) cells. The end result was inducible for nanobody and paramagnetic proteins' expression, whereas constitutively active for reporter protein expression. After the induction of the cells, the fluorescent microscopy imaging revealed that the cells couldn't perform all the works and showed too little red fluorescence (**Figure 3.9**); the same situation was faced when the binding assay was performed with those cells. Therefore, the strategy was modified to clone the mCherry fluorescent gene in the nanobody coding plasmid.

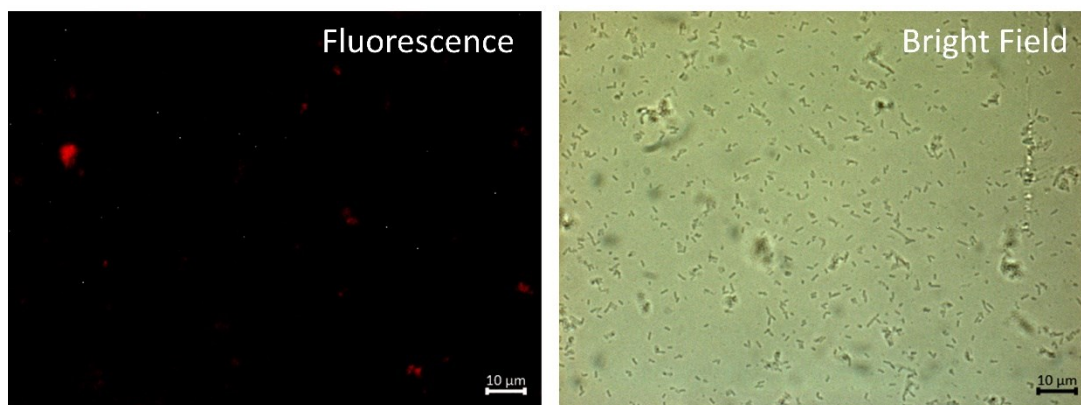


Figure 3.9 The fluorescent microscope imaging of induced mCherry nanobody ICMAB *E. coli* BL21 cells harboring three plasmids. Scale bars are 10 µm.

In order to overcome this issue, the red fluorescent protein was cloned into an already existing plasmid in the opposite direction used for nanobody expression. As a result of this, the pZa vector harbors aTc inducible 2Rs15d and constitutively active proD promoter for mCherry production (**Figure 3.10**).

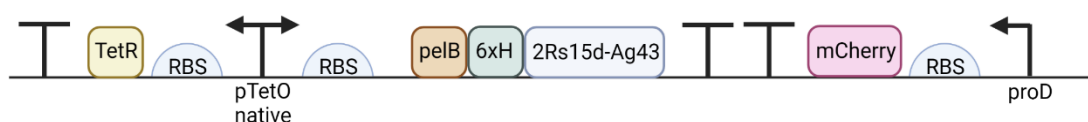


Figure 3.10 The genetic design for constructing constitutively active red fluorescence protein and inducible nanobody expression system. Created with BioRender.com.

For the construction of pZa proD mCherry ntetO 6H 2Rs15d Ag43 ampR plasmid, the digested backbone and amplified insert were assembled (**Figure 3.11 A**). The selected colonies were screened via double digestion with EcoR1 HF (NEB) and Sac1 (NEB) restriction enzymes (**Figure 3.11 B**).

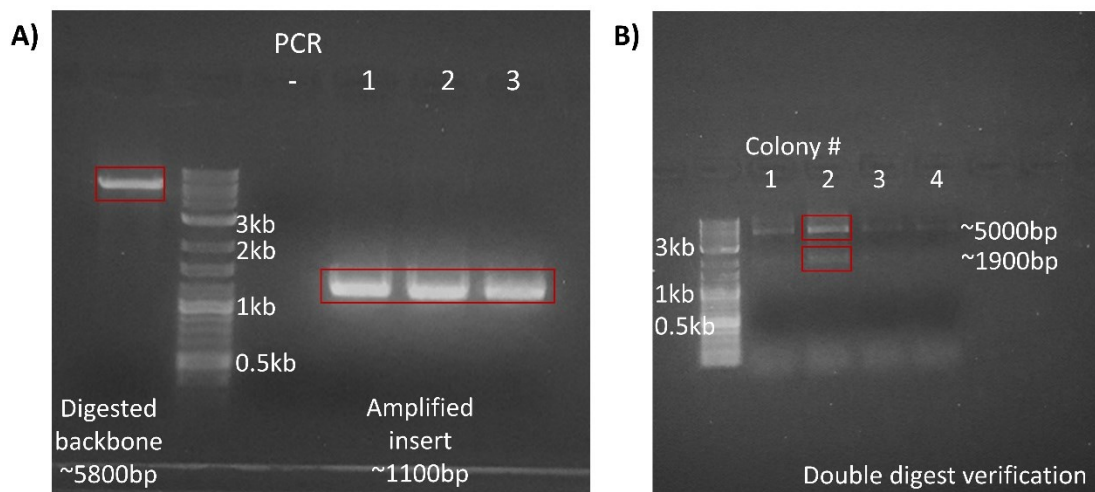


Figure 3.11 The construction of constitutively active mCherry protein expression and aTc inducible 2Rs15d production from the same plasmid. A) The digest and PCR products of the backbone and insert were shown, respectively. B) Selected colonies were verified via double digestion reactions.

After verification of the cloning via Sanger sequencing, pZa proD mCherry ntetO 6H 2Rs15d Ag43 ampR plasmid was transformed into freshly prepared ICMAB BL21 cells. Both the overnight grown and induced cells were imaged under fluorescent microscopy. According to the results, *E. coli* cells can express all the desired proteins, including the reporter protein, which can be seen from the fluorescence microscopy images (**Figure 3.12**) and western blot analysis (**Figure 3.13**).

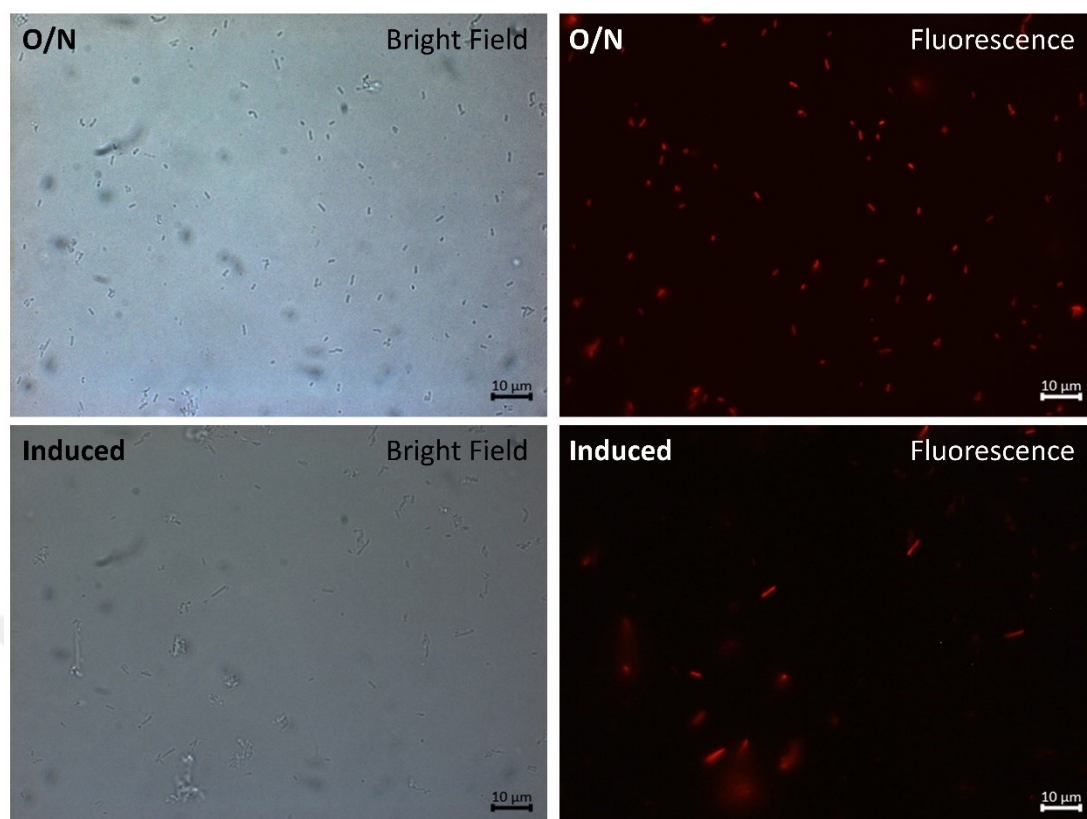


Figure 3.12 Fluorescence microscopy images were provided for BL21 cells harboring the following plasmids: pZa proD mCherry ntetO 6H 2Rs15d Ag43 ampR and pet22b EncFtn 6H Mms6 MBP EfeU cmr. Scale bars are 10 µm.

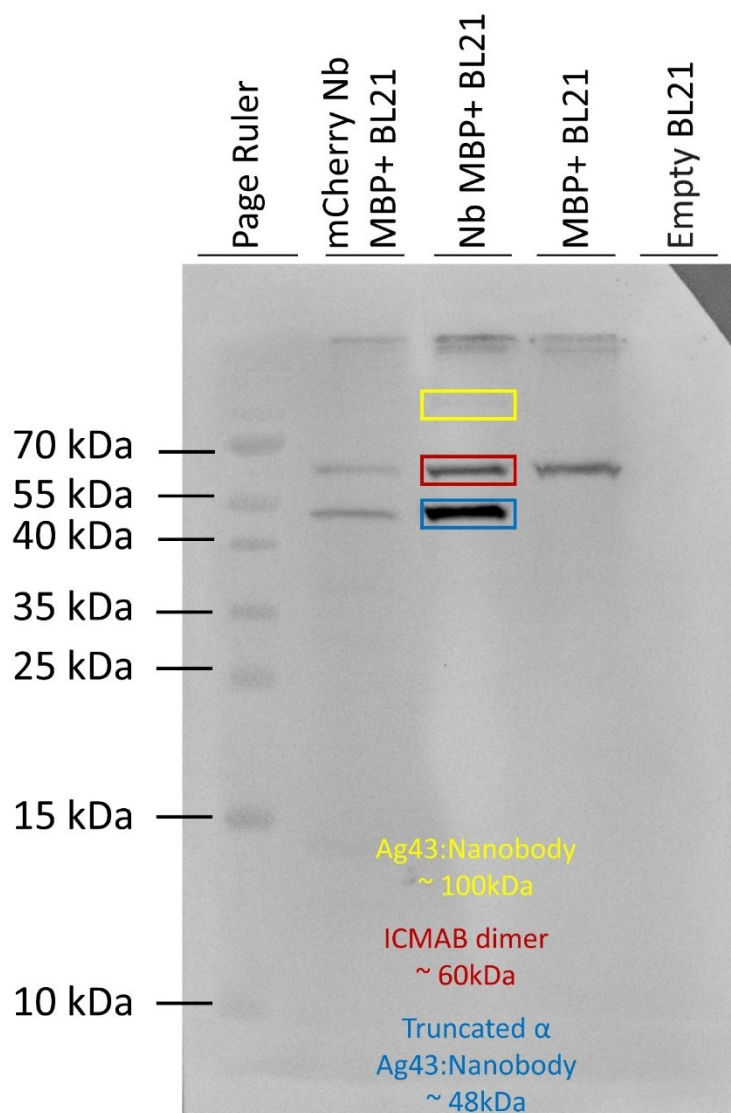


Figure 3.13 Western Blot analysis of induced BL21 cells harboring following plasmids: pZa proD mCherry ntetO 6H 2Rs15d Ag43 ampR and pet22b EncFtn 6H Mms6 MBP EfeU cmr.

3.4.6 Characterization of Monolayer Jimt1 Mammalian Cells Infection with Induced mCherry 2Rs15d ICMAB BL21 Bacterial Cells via Confocal and Electron Microscopy Imaging

The bacterial cells successfully expressing mCherry, 2Rs15d, and paramagnetic proteins were used for infection of the mammalian cell line, Jimt1, for binding assay.

The binding was observed via confocal microscopy imaging through a red fluorescent protein integration into the bacterial and the mammalian cells transfected with a green fluorescent protein. The Jimt1 mammalian cells were infected with induced bacteria at a multiplicity of infection (MOI) 1000:1 (bac: mammalian) on a glass surface. In order to get rid of autofluorescence or background fluorescence, poly-D-lysine (PDL) coating was performed onto the glass surface, and the binding assay was performed. The samples were washed with 1× PBS and imaged under the confocal microscope. According to the results, the Jimt1 cells were surrounded by mcherry and Nb-integrated ICMAB cells (**Figure 3.14**).

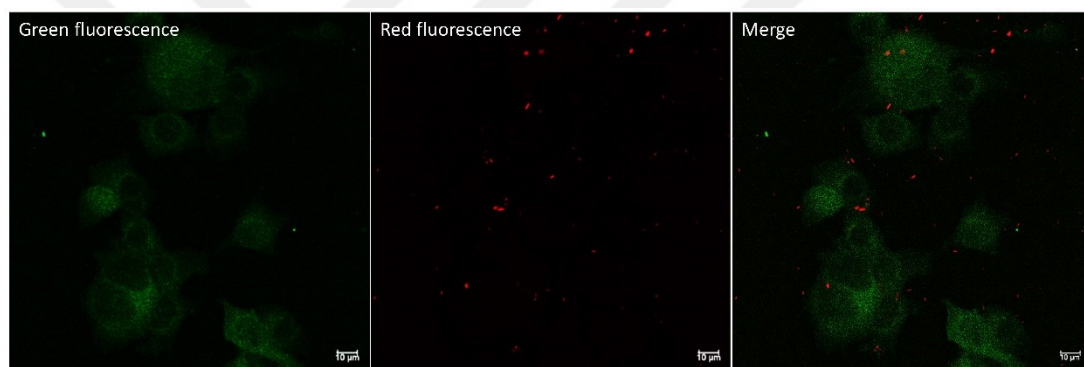


Figure 3.14 Confocal microscopy imaging of the infected Jimt1 mammalian cells with mCherry Nb ICMAB BL21 bacterial cells on PDL-coated glass surface. Scale bars are 10 μ m.

Additionally, electron microscope imaging and energy dispersive spectroscopy analyses were conducted to confirm that the bacterial cells produced nanoparticles that adhered to the mammalian cells.

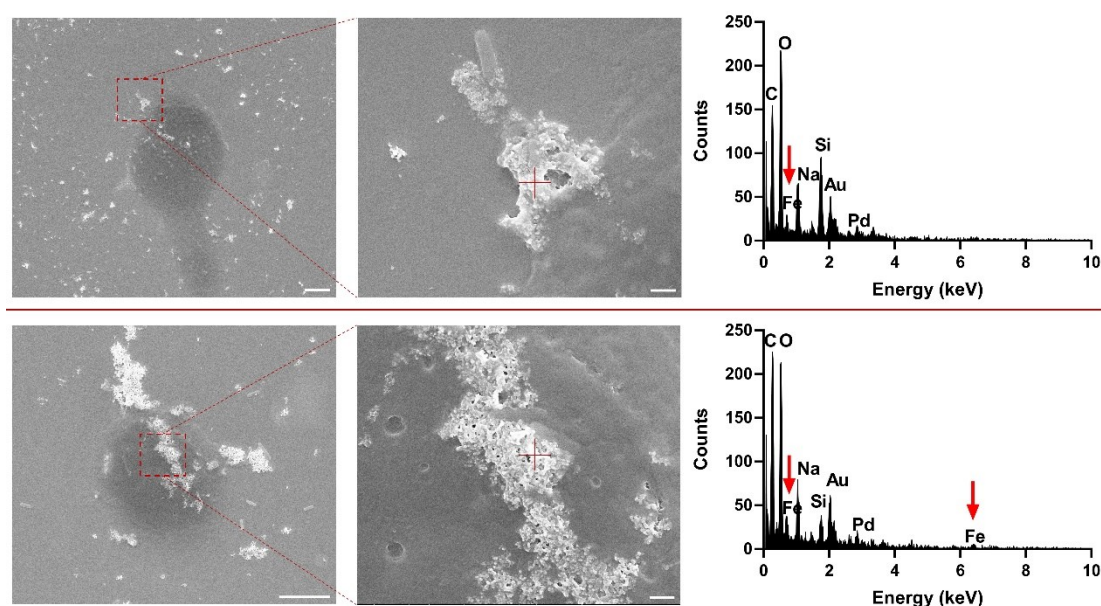


Figure 3.15 The characterization for the infection of monolayer Jimt1 cells with induced mCherry 2Rs15d ICMAB BL21 cells via environmental scanning electron microscopy (E-SEM) imaging and energy dispersive X-ray spectroscopic analysis (EDX). The top panel and the lower panel images stand for different areas from biological replicas. White scale bars are 10 μm (left panel), whereas the zoomed-in images' scale bars are 1 μm (right panel). Samples are collected from replicates; representative images are presented here.

The presence of iron peaks in the area where the bacterial cells were located on the mammalian cells, as observed through imaging and elemental analysis, indicates that the bacterial cells were capable of accumulating iron oxide nanoparticles and attaching themselves to the breast cancer cells (**Figure 3.15**). On the other hand, the control group, which consisted of empty bacterial cells and mammalian cells alone in the infection experiments, did not exhibit the expected iron peak (**Figure 3.16**).

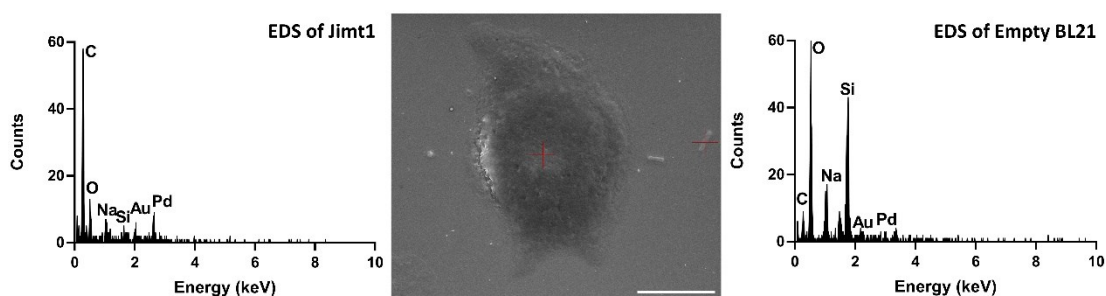


Figure 3.16 The characterization for the infection of monolayer Jimt1 cells with empty BL21 cells via environmental scanning electron microscopy (E-SEM) imaging and energy dispersive X-ray spectroscopic analysis (EDX). White scale bars are 10 μm . Samples are collected from replicates; representative images are presented here.

These results further support the notion that the presence of specific bacterial cells is necessary for the accumulation of iron oxide nanoparticles and their attachment to breast cancer cells (**Figure 3.17**).

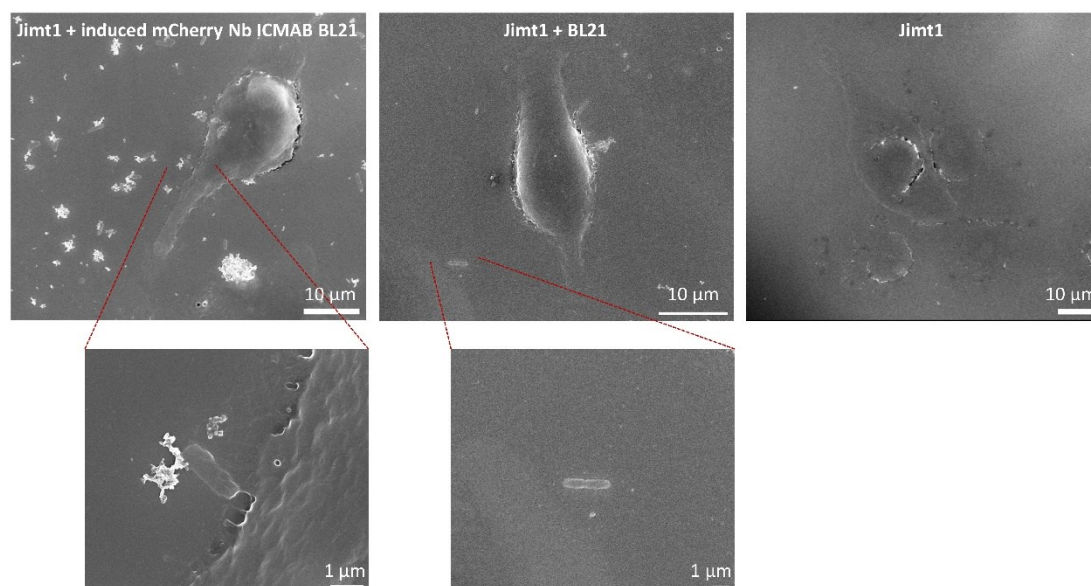


Figure 3.17 The comparison for the infection of Jimt1 mammalian cells with induced mCherry Nb ICMAB BL21 and empty BL21 cells at MOI of 1000:1 on

PDL-coated glass surfaces were visualized via SEM imaging. White scale bars are 10 μm (upper panel), whereas the zoomed-in images' scale bars are 1 μm (lower panel). Samples are collected from replicates; representative images are presented here.

3.4.7 Characterization of bacteria spheroid co-culture (BSCC) binding assay via confocal imaging

The binding assay aimed to be performed with three-dimensional cell cultures to represent human tissue outside the body better. Jimt 1 cells were utilized for spheroid formation in order to mimic the physicochemical environment *in vivo*, and then, the binding assay was performed to ensure the bacterial cell's adherence to the 3D cell colonies. The attachment was observed under a fluorescent/ confocal microscope.

The 3D cell culture for Jimt1 mammalian cells were performed via the high-purity agarose (HPA) method. For this purpose, the mammalian cells were seeded on 1.5-2% HPA to be 3000cells/well, and after spheroid formation for around three days, and they were infected with induced bacteria at a multiplicity of infection (MOI) 1000:1 (bac: mammalian) on the agarose. They were collected and imaged under a confocal microscope (**Figure 3.18**). According to the results, the Jimt1 spheroid cells were surrounded by mCherry Nb integrated ICMAB cells.

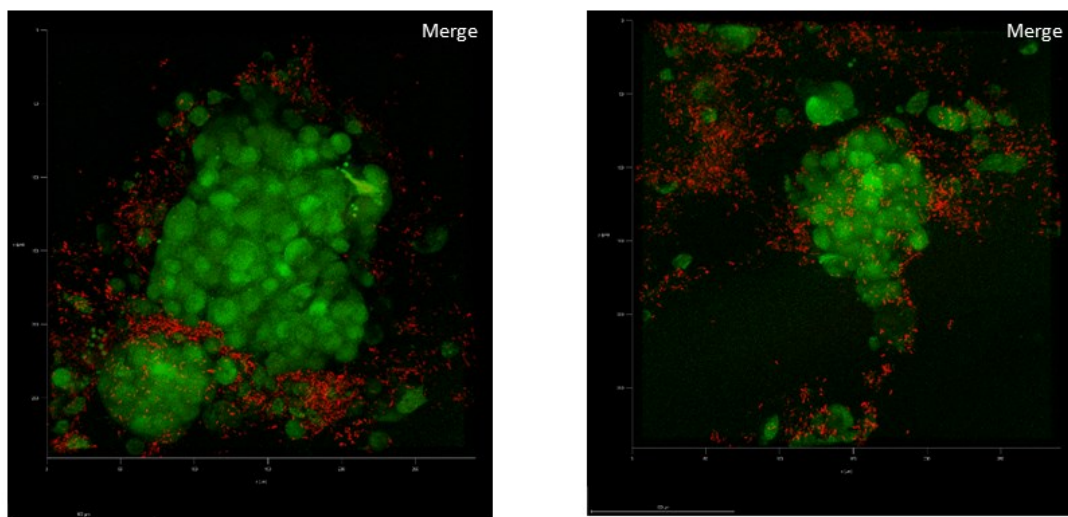


Figure 3.18 Confocal microscopy imaging of binding assay in which Jimt1 spheroid mammalian cells infected with induced mCherry 2Rs15d ICMAB BL21 bacterial cells at 1000:1 multiplicity of infection (MOI). The images were taken from different areas in the substrate. Scale bars are 100 μm .

3.4.8 MRI measurements for bacteria-spheroid co-culture (BSCC)

The multi-functional ICMAB cells', both accumulate iron-oxide nanoparticles and bind to mammalian cells, were characterized via electron microscopy imaging and spectroscopic elemental analysis. Also, the binding behavior of the bacterial cells onto the spheroid Jimt1 cells was shown via confocal microscopy imaging. Furthermore, confocal microscopy imaging was repeated to ensure the observation and characterization of the bacterial cells' adhesion behavior to Jimt1 cells, which possess a spherical morphology. Once the adhesion behavior of the bacterial cells to Jimt1 cells was confirmed, the co-culture samples were prepared for MRI measurements in order to see the contrast capability of the bacterial cells attached to mammalian cells (Figure 3.19).

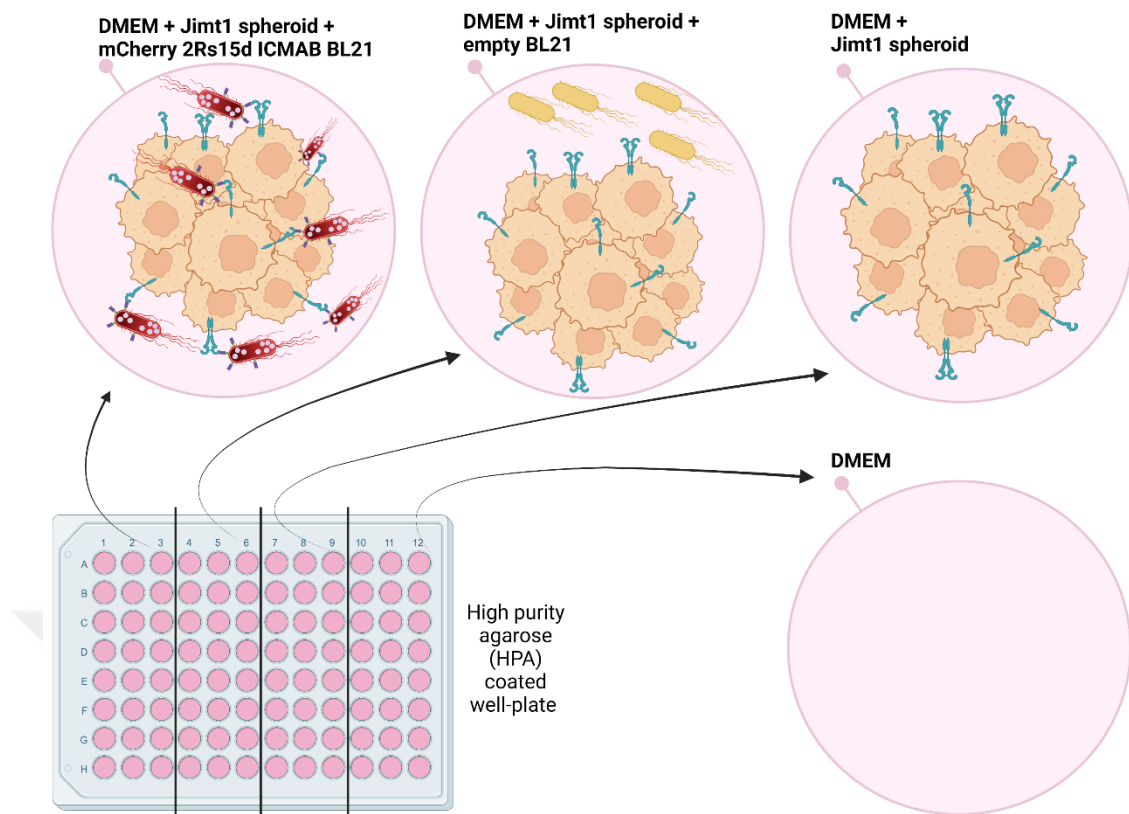
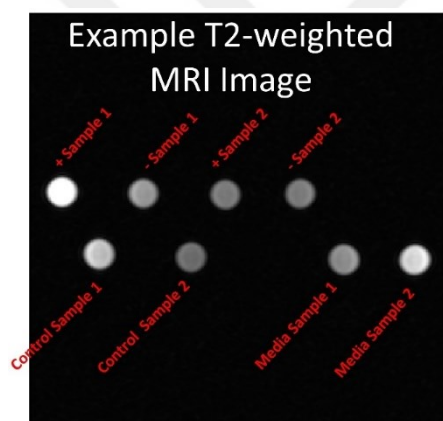


Figure 3.19 MRI sample preparation set-up is depicted for bacteria-spheroid co-culture to see the contrast performance. Created with BioRender.com.

As a first step for preparing samples for MRI analysis in a bacteria-spheroid co-culture experiment, spheroids were formed from Jimt1 mammalian cell on high purity agarose (HPA) in a suitable well-plate. The cells were allowed to grow and form 3D spheroids over a period of around five to six days. Then, the spheroids were co-cultured with bacterial cells at a 1000:1 MOI ratio for 1h to allow interaction between the bacterial cells and the mammalian spheroids. After incubation, the infected mammalian cells were carefully collected from the well-plate. The samples containing the cultures were gently inverted (upside-down) to assure about the equal distribution of the samples within the tube without disrupting the cell binding. The MRI measurements were performed for examining the infection dynamics and gathering imaging data.

For the BSCC sample, the T_2 relaxation time measurements were provided in **Figure 3.20**. For the Jimt1 spheroid + mCherry Nb ICMAB BL21, Jimt1 spheroid + empty BL21, Jimt1 spheroid, and media groups, the T_2 values (mean $\pm$ std across the corresponding ROI in the MRI images) were 741.3, 966.6, 880.2 and 819.3 ms, respectively. The Jimt1 spheroid + mCherry Nb ICMAB BL21 cell groups showed shorter T_2 values when compared to Jimt1 spheroid + empty BL21 cell group in addition to Jimt1 spheroid, and media groups, pointing out significantly improved negative contrast capability in MRI. The shorter T_2 values represent higher iron concentrations for a fixed contrast agent and medium.



T2 Changes		
Samples	T2 Values (ms)	Description
1) + Sample	741.3	Magnate + Bacteria + Tumor
2) - Sample	966.6	Bacteria + Tumor
3) Control Sample	880.2	Tumor
4) Media Sample	819.3	Medium

Figure 3.20 MRI T_2 relaxation time measurements at 3T were performed for bacteria-spheroid co-culture samples. A representative T2-weighted MRI image at 3T is shown (upper panel). Pixel intensity as a function of TE for each sample at 16 different TE values between 8.5–1000 ms. The transverse relaxation time,

T2, is estimated by fitting a monoexponential decay to the data points. The fitted T2 values are given in the table (lower panel).

MRI measurements were conducted on freshly prepared samples. The obtained results align with the initial expectations, indicating that the magnetite-accumulating bacteria co-cultured with mammalian spheroids exhibited the lowest T2 value (**Figure 3.20**). However, unexpected results were observed after storing the samples at +4°C. To ensure experimental consistency, duplicate samples were used. The presence of bacteria in the samples may be influencing the T2 values in some way. It was expected that control groups, which lack magnetite, exhibited values similar to those of the media samples. A simple turn-upside-down procedure was performed before the experiment to facilitate mixing. However, it was found that this procedure alone may not be sufficient for properly homogenizing the tube. Therefore, the replicas were used for ensuring about the results. The slice thickness used for the experiment was 4 mm.

In conclusion, the intracellular magnetite accumulating bacteria (ICMAB) system is engineered to construct more reliable agent possess promising usage in cancer diagnostics with minimized toxicity to healthy tissues. For this purpose, the bacterial cells were integrated with a nanobody protein in order to specifically target the breast cancer cells. Both the adherence and magnetite accumulation characteristics were characterized and ensured via electron microscopy imaging, spectroscopic analysis and confocal microscopy imaging. In addition, the engineered bacterial cell infecting breast cancer mammalian cells showed improved negative contrast capability when compared to the infection by non-engineered empty bacterial cells. The bacterial cells can be further developed to release a drug molecule upon stimulation to obtain efficient diagnostic and therapeutic agents in cancer targeting applications.

CHAPTER 4 : BIOELECTRONICALLY ACTIVE BACTERIAL SYSTEMS FOR WIRELESS BIOMONITORING APPLICATIONS

4.1 Objective of the Study

Designing the bacterial cellular machineries possesses promise for creating living electronic sentinel cells for biomedical applications. The cells would serve as an electron transfer module to the extracellular environment, and this strategy intends to engineer the well-studied *Escherichia coli* strain to take the role in extracellular electron transfer (EET) pathways. The system would be used to detect a disease molecule and act as a sensor. Upon the detection of the molecule, the signal would enable communication between the in-body and on-body antenna setup. Synthetic biology serves to enhance the control of electrochemical reactions. The developments in microbial electrochemical technologies open new avenues for high throughput electrochemical screening enabled by bioelectronically active microbes.

4.2 Introduction

The electron transfer process is one of the key mechanisms of molecular biology, and it is also utilized by electronically active bacteria to harness ionic metals from various sources. The main mechanism of electron transfer from cytoplasmic electron donors to the metallic final electron acceptors is characterized in the literature by using *Shewanella oneidensis* MR-1. [46], [81], [127], [82], [120]–[126] Extracellular electron transfer (EET) is the process where electroactive microorganisms produce electrons to extracellular acceptors or consume current from extracellular donors. [46] The redox-

active proteins and biomolecules take place in this process. Electroactive bacteria utilize these pathways for cellular respiration and homeostasis. Genetic engineering serves for creating new avenues to create novel bioelectronic sensors by enhancing the electric current applicable to both native and non-native host strains' functionality that have to be compatible with pathways' conditions like temperature, oxygen concentration, pH. Synthetic biology provides the integration of EET modules into *E. coli* cells. [46]

The organism *S. oneidensis* reduces iron or manganese via conductive proteins and redox-active molecules under anaerobic conditions. The mechanism of action is performed firstly as electrons are transferred from reduced quinol to periplasmic cytochromes, and the cytochrome-c proteins deliver the electrons from the cytoplasmic membrane to the outer membrane, and lastly, protein complexes deliver electrons to the extracellular acceptor. [46] This strain uses the Mtr pathway for both current production and current consumption by controlling the intracellular redox reactions to an electrode. [122] The outer membrane mtrCAB complex plays a role in current production with helper cytochrome c proteins when NADH is in intracellular oxidation. However, when there is a need for an electron inside the cell, the current consumption from an extracellular cathode is utilized for the intracellular reduction of fumarate. [122] Synthetic biology provides to study interactions between bacteria and physical/electrical components to overcome limitations for wireless in-body sensing applications. In this study, we utilized and engineered an electron transfer pathway from *S. oneidensis* to control transmitted signals from a metallic resonator via inducer-triggered or constitutive electron release. By using biologically safe probiotic bacterial strains such as chassis, *E. coli* Nissle 1917 (EcN) [128]–[131], we aim to obtain controlled degradation of designed and produced metal implants. Probiotic *E. coli* cells

were constructed in a way to express the combination of constitutively ccm-AH proteins for the cytochrome pathway and inducible mtrA/ mtrCAB proteins for e-shuttle. The electron release capability is aimed at being tested via a two-electrode test for permittivity and conductivity measurements. The iron metal was coated on glass surfaces via a physical vapor deposition (PVD) method, either e-beam evaporation or thermal evaporation, technique. The final thickness of the coating (50-100nm) was measured via an optical profilometer or mechanical surface profiler. Our overall design holds a promise to be used in the future as a new generation real-time biomonitoring system for tracking disease markers, cellular toxicity, and pathogenic infections in the human body.

Engineering bacterial cells via synthetic biology tools enable us to create electronic communication systems between living and nonliving modules, providing application areas in biosensing or bioenergy fields. The transfer of electrons outside of the bacterial membrane is a crucial step in preserving the bacteria's viability and reducing metal oxides at the same time. Synthetic biology assures the introduction and expression of variable essential protein combinations in *E. coli* cells to form a path for electrons to travel from the cell interior to the inorganic material outside of the cell. Solid metal oxides will be reduced via bacterial strain harboring mtrCAB proteins (mtrA and C are redox-active). For example, ferric (+3) irons can be reduced to ferrous (+2) iron via e- acceptance provided by bacteria. Ajo Franklin et al. constructed the probiotic *E. coli* strain in a way that ccmAH as atc inducible and mtrA/CAB as IPTG inducible. The mtrA can't release the electrons, so the reduction occurs in periplasmic space. In contrast, mtrCAB releases the electrons to the extracellular space, so the reduction occurs at the outside of the cell. [121]

An implant printed with a biodegradable material can be modified by a bacterial cell to send a signal to the outside of the human body. The living cells would transform the signals from the nanoscale to electronic devices at the human scale. The communication between in-body and on-body antennae is aimed at being actuated by engineered sentinel bacterial cells in order to develop a diagnostic tool for diseases.

4.3 Materials and Methods

4.3.1 Cell Maintenance and Growth

For cloning and protein expression experiments, *E. coli* DH5 α and *E. coli* BL21 (DE3) or *E. coli* Nissle 1917 strains were used, respectively. The long-term storage condition for the bacterial cells was at -80°C in a freezing medium containing 25% glycerol in Lysogeny Broth (LB) media. *E. coli* BL21 (DE3) strain is lack of proteases and it was used for protein expression and purification. The strain encodes T7 polymerase gene under the control of Lac promoter. For bacterial cell growth, both *E. coli* strains were inoculated in LB medium and incubated on LB agar at 30 or 37°C. The liquid culture of bacterial cells was grown in a shaking incubator if not otherwise specified.

The chemical transformation method was utilized for the uptake of plasmids into bacterial cells. The bacterial cells were inoculated in LB medium containing proper antibiotic from -80°C cell stock overnight and diluted in fresh LB at 1:100 ratio on the next day morning, for preparation of chemically competent cells. Then, the bacterial cells were allowed to grow until OD₆₀₀ of 0.2-0.5 and cooled on ice for 10 min. The cells were collected via centrifugation at 3000 rpm for 10 minutes at +4°C. The TSS (Transformation and Storage Solution) buffer was used to resuspend the cell pellets in

1:10 culture volume of and aliquoted 100 μ l into chilled microcentrifuge tubes that were stored at -80°C .

For the transformation of a plasmid into chemically competent cells, the cell stock was thawed on ice for 20 minutes, and plasmid or reaction mixture was added onto cell. The heat shock was performed at 42°C for 45 seconds for plasmid uptake into the cells, after 20 minutes incubation of the cells on ice. The cells incubated on ice for 2 minutes and 250 μ l LB was added onto the cells for growth at 37°C 200 rpm for 45 minutes. After incubation, the cells were centrifuged and the excess supernatant was removed. The remaining supernatant was utilized to resuspend the cell pellet and all suspension was spread on an LB-agar plate containing the appropriate antibiotic. The plate was incubated at 37°C stationary incubator for overnight growth.

4.3.2 Construction of Gene Circuits

For the construction of pBAD BBaJ23116 mtrA cmr plasmid, the mtrA insert region was PCR amplified with P32 and P33 primers and pBAD vector was amplified with P34 and P35 primers. Then, the PCR products were assembled via the Gibson assembly protocol.

4.3.3 Sequence Verification

An online tool, Benchling.com, was utilized to construct and design genetic circuitries. The plasmid maps were exported as .gb files. The sequencing process was obtained from Genewiz company as Sanger Sequencing. Benchling alignment option was used to import both the vector sequence and sequence results as .ab1 files. The standard parameters were used for pairwise alignment of the sequence to the reference, and gaps or distinction reads were determined for the analysis.

4.3.4 Protein Expression

The bacterial cell clones were grown overnight and diluted into a fresh medium the following day and induced with 0.2% (w/v) final concentration of L-arabinose when they reached the early logarithmic growth phase. The cell media was supplemented with 0.03% w/v final concentration of sodium sulfite (Na_2SO_3) and final 0.4% w/v concentration of sodium lactate. After 16 hours of incubation, the cells were precipitated and dissolved in M9 or MOPS minimal liquid medium.

4.3.5 Ferrozine Assay to Observe Electron Release Function of *E. coli* Cells

Triplicate bacterial cells were induced overnight and resuspended in M9 minimal media. The medium was supplemented with 5 mM ferric citrate as an electron acceptor. [132] Samples were collected at the beginning of citrate addition as T0 and at given time points. At each time points, both the OD_{600} measurements were done in addition to ferrozine readings. For ferrozine measurements, 1:10 dilution of sample was performed that 50 μl of sample was added into 450 μl of 0.5 M HCl solution. Then, 50 μl from the diluted sample was added in 450 μl of 4 mM ferrozine buffer. Therefore, the sample was diluted 10^{-2} in total. After incubation in dark for 10 minutes, absorbance measurements were performed at 562nm wavelength. A blank solution was prepared as M9 media containing 5 mM ferric citrate. The standards were prepared with ferrous ammonium sulfate hexahydrate at the concentration range from 0.1 mg/ml to 2 mg/ml. The standard solutions were also treated the same as the samples. Therefore, after 10^{-2} dilution, the standards had concentrations from 1 $\mu\text{g/ml}$ to 20 $\mu\text{g/ml}$. The spectrophotometry measurements were done at 562 nm via SpectraMax Microplate Reader. The constructed standard curve was used to determine the intracellular iron concentration of bacterial cells. All reactions were performed in

triplicate for statistical analysis. The OD₆₀₀ measurement results were used to calculate the cell amount in 1 ml to obtain the iron content of the cells. For the calculation of iron content per cell, the iron concentration was divided by the calculated cells/ml value.

4.3.6 Statistical Analysis

GraphPad Prism 6 software was utilized for the statistical analyses. p-value ≤ 0.05 was considered as significant for the represented data. Two-way ANOVA was used with multiple comparisons to compare the differences between the groups. All data were represented as mean $\pm$ standard error mean (SEM). At least three independent repeats were performed for each *in vitro* experiment.

4.3.7 SEM Imaging and EDS Analysis for Coated Surfaces

The freshly magnesium-coated glass surface and the ones used in the dissolution experiment for different cell groups were imaged by attaching the glass to SEM stubs with carbon tape. The visualization was performed via E-SEM microscope (FEI-Quanta 200F) at 10-15kV electron acceleration. A representative image was shown in the figures for all groups. Energy-dispersive X-ray spectroscopy (EDS) analysis was performed using EDAX Genesis software combined with the SEM microscope.

4.3.8 Photolithography Production and Characterization of Biodegradable Substrate

For obtaining the desired pattern structure on silk coated glass wafer, AZ4562 photoresist was rotated on a glass wafer at 3000rpm speed and 1000rpm acceleration for one minute, and 7 μm coating was aimed in this way. The coated sample was dried at 110 degrees and exposed to UV light with a mask of the desired model in hard

contact mode, at a separation distance of 100 μm , at a dose of 500 mJ/cm^2 in the lithography device. For the development of the model, it was kept in AZ400K diluted with 1:5 water for 250 seconds and cleaned with water and nitrogen. After the sample was coated with 200nm – 1 μm Mg by the sputter deposition method, it was kept in acetone for 30 seconds, and the lift-off was carried out and then cleaned with water-nitrogen gas sequentially.

4.3.9 Physical Vapor Deposition for Iron or Magnesium Metal Coating

The iron metal was coated on glass surfaces via a physical vapor deposition (PVD) method, either e-beam evaporation or thermal evaporation, technique. The final thickness of the coating (50-100nm) was measured via an optical profilometer or mechanical surface profiler (Veeco Dektak). The magnesium metal coating (200nm-1 μm) was performed via sputtering on direct current (DC) magnetron, 20 sccm Argon flow, 50% Turbo Pump at low speed and 100-watt power parameters.

4.4 Results and Discussion

4.4.1 Iron Dissolution by Probiotic *E. coli* Cells Expressing Cytochrome c Maturation Proteins Constitutively and Electron Conduit Proteins Conditionally

For the preparation of bacterial clones, cells grown overnight were diluted into a fresh medium the following day and induced with arabinose when they reached the early logarithmic growth phase. After 16 hours of incubation, the cells were precipitated and dissolved in M9 minimal liquid medium and kept on iron-coated glass surfaces for the specified lengths of time. Sample groups were arranged as following: EcN pzs-mprod-ccmah kan (maturation) and pbad-mtrCAB cmr (conduit), EcN pzs-mprod-

ccmah kan (maturation) and pbad-mtrA cmr (conduit), EcN harboring no synthetic gene circuit and M9 media (resuspension solution) in 2 replicates for each group. The time points were in minutes as following: T0, T30, T60, T120, T180, T240, T300, T360, T420, T480, T540, T600, T660, T720, T810. Sodium sulfite was used for anoxicity because it takes the role as oxygen scavengers in fluids. In other words, sodium sulfite is a reducing agent and is reported to scavenge more oxygen than sodium thiosulfate, thus making the oxygen unavailable to damage metals. Sodium lactate was used to increase e- release, but it is an antimicrobial and toxic to *E. coli* (glucose provides lower release). The cells were induced via L-arabinose.

Iron-coated glass surfaces were created using physical vapor deposition (PVD) methods, using electron beam or thermal evaporation paths, and thickness values were verified with surface topography analysis. After the induction, iron-coated glass surfaces were incubated with respective cell groups and glass surfaces were visualized via the VILBER imaging system at specified time points (**Figure 4.1 upper panel**). The extent of iron biodegradation on the glass surface was calculated by analyzing the photos on ImageJ by counting black and white pixels (**Figure 4.1 lower panel**).

The bio-electronically active probiotic bacterial cells were used for iron dissolution experiments after the induction procedure and the cells expressing mtr CAB protein showed a higher dissolution rate than the cells expressing mtr A protein, as expected (**Figure 4.1 left-hand side**). Also, arabinose-induced mtrA and mtrCAB cell groups were observed to dissolve significantly faster than the control group, the empty cell. For the bio-electronically active probiotic bacterial cells, the iron dissolution experiment was repeated to ensure all the cell groups and their expected activities were met. Then, the results were analyzed via image J software pixel analysis. However,

only the induced mtrA cell group in the biological replica performed was found to differ significantly from the medium control group (**Figure 4.1 right-hand side**).

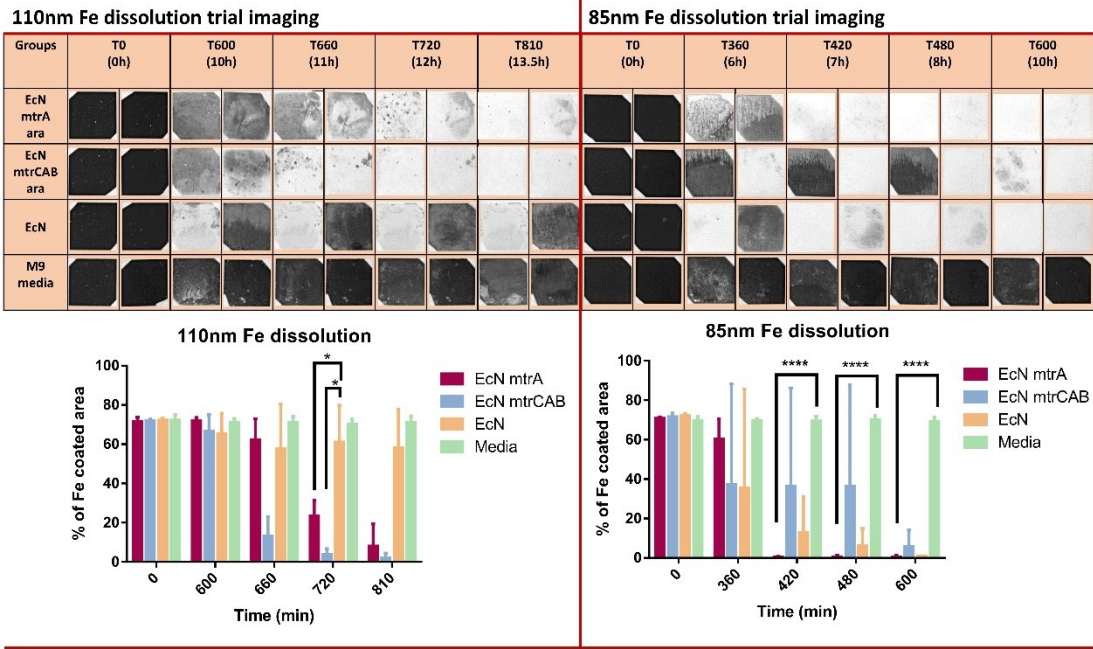


Figure 4.1 Electron-releasing (EcN ccmA-H mtrA, EcN ccmA-H mtrCAB) in the presence of arabinose inducer and non-releasing (EcN) *E. coli* groups were precipitated after incubation, then dissolved in minimal medium, and incubated on Fe-coated glass surfaces to observe metal dissolution. Analysis of iron dissolution via two-way ANOVA was provided after black-and-white pixel analysis on image J. The left-hand side stands for 110nm Fe-coated surfaces, and the right-hand side for 85nm Fe-coated surfaces.

4.4.2 Quantification of Iron Reduction Performance of Probiotic *E. coli* Cells Expressing Cytochrome c Maturation Proteins Constitutively and Electron Conduit Proteins Conditionally

Ferrozine assay was carried out to test the cells' electron-releasing behavior due to the differences observed in the biological replicas of the melting experiments. In other words, ferrozine assay might be used to test iron reduction capacities of the cells expressing mtrA or mtrCAB proteins. For this purpose, bacterial cells grown overnight were diluted into a fresh medium the following day and induced with arabinose when they reached the early logarithmic growth phase. At the time of induction, cells were grown by supplementing with sodium sulfite, which also acts as an oxygen scavenger in fluids, and sodium lactate to improve electron release. After 16 hours of incubation, the cells were precipitated and dissolved in M9 minimal broth supplemented with sulfite and lactate. Cell groups were divided into induced cell groups expressing one of the proteins required for electron release and control cell groups expressing any of them. It was aimed to analyze the reduced iron ions with the electrons released from the cells by adding 5mM ferric citrate to the cell media. Ferrozine makes a complex with ferrous iron ions, and this complex can be detected at a specific wavelength in light. OD₆₀₀ and 562nm absorbance measurements were performed by collecting samples at the time of ferric citrate addition and at the specified time points. Iron content per cell was calculated by finding the reduced iron concentrations of the cell groups according to the standard curve created (**Figure 4.2**).

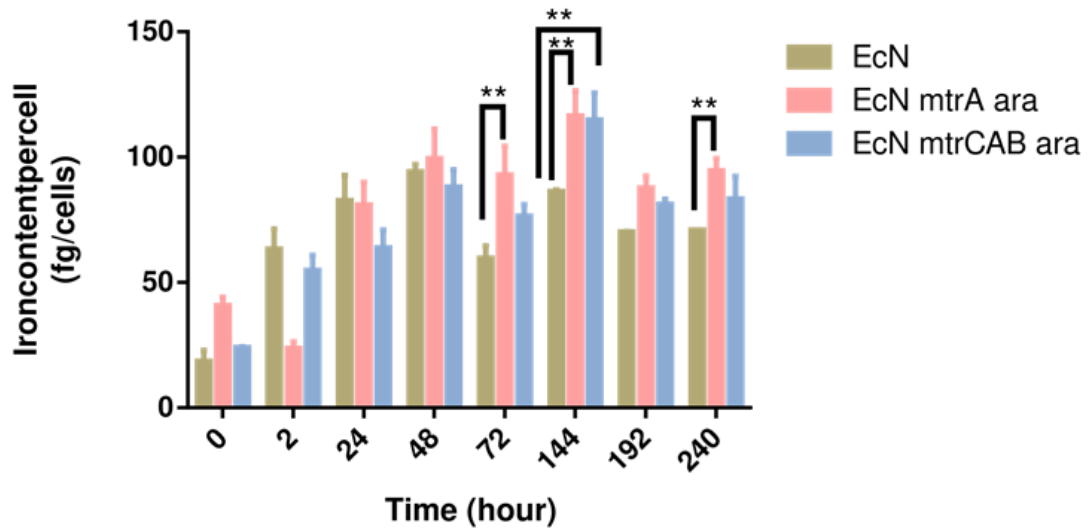


Figure 4.2 Iron content per cell was quantified via ferrozine assay for induced (EcN ccmA-H mtrA ara or EcN ccmA-H mtrCAB ara) cell groups expressing one of the proteins required for electron release and control groups (EcN) expressing none.

As a result, it has been shown that the produced bacterial systems reduce the iron +3 ions to iron +2 ions with the release of electrons significantly higher than the control group. EcN mtrA cell group showed a remarkable difference than the empty EcN cell group on third day. On the other hand, a significant difference for EcN mtrCAB cell group was seen on the sixth day. This might be as a result of that bacterial cells were loaded with more work to express also mtrC and mtrB in addition to mtrA. The ten-day-long experiment presented some discrepancies among the days possible due to difficulties in keeping the cells and the conditions in an ideal state. The cells can be engineered more to obtain a significantly higher electron release in shorter periods.

4.4.3 Magnesium Dissolution by *E. coli* BL21 Cells Expressing Cytochrome c Maturation Proteins Constitutively and Electron Conduit Proteins Conditionally

The focus of the biodegradation aspect of the project involved the production of bacterial cells capable of conditionally releasing electrons, and it was aimed to investigate whether these specific bacterial clones exhibit faster activity in metal, iron, or magnesium dissolution experiments compared to cells grown without induction in the absence of arabinose, *E. coli* ccmA-H, and non-engineered cell groups. The cell groups express cytochrome pathway genes (ccmA-H) continuously and conditionally producing proteins required for electron release (mtrCAB/mtrA) were subjected to experiments involving the dissolving of an alternative metal, magnesium (**Figure 4.3.**). It is important to note that the control cell type does not possess any synthetic genetic circuitry.

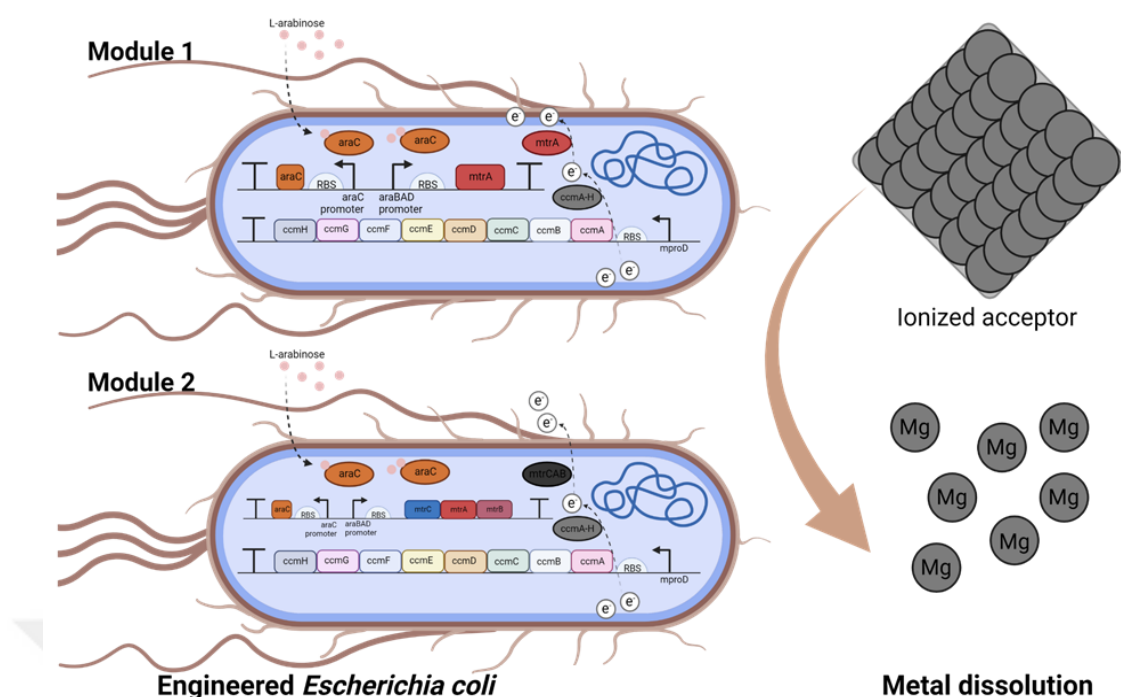


Figure 4.3 Metal dissolving bacterial concept was represented where there are two modules expressing either *mtrA* or *mtrCAB* proteins. Created with BioRender.com.

In the literature, it is stated that hemes are responsible for the red color of the pigment. Ajo Franklin et al. showed that the addition of as little as 10 μ M IPTG to *mtrA* and *mtrCAB* cultures, compared to cultures without IPTG, resulted in less distinct *mtrA* and *mtrC* bands in the 3,3',5,5'-tetramethylbenzidine (TMBZ) peroxidase stain to identify cytochromes c from whole cell extracts. Additionally, the cell pellets exhibited a less vibrant red hue. Interestingly, when the *mtrA* and *mtrCAB* plasmids were expressed at basal levels, they produced cytochrome c with better folding compared to when they were stimulated. [121] The used strains, EcN and *E. coli* BL21 (DE3), in the experiment were designed to be inducible that would exhibit higher basal protein expression even without induction. Moreover, it is important to note that the control cell type exclusively produces cytochrome pathway genes (*ccmA-H*). These genes

were included to maintain comparable metabolic workloads across all cell groups. Different media types were employed in the study. LB rich medium was used for growth; however, it led to rapid dissolution, which may not be desirable for the experimental setup. MOPS minimal medium was also used for growth, but it resulted in a faint pellet color. Similarly, M9 medium was utilized for growth, but it also resulted in a faint pellet color. MOPS and M9 media are a minimal type of growth media; therefore, the cell pellet colors were faint, but the dissolution occurs in an observable time duration. In the bioelectrically active *E. coli* BL21 bacterial cells aspect, magnesium dissolution experiments were conducted; however, the results did not demonstrate the anticipated bacterial activity. To address the challenges faced in the magnesium dissolution experiments, a new direction will be taken. The experiments will be repeated using EcN cells, a specific strain chosen for their potential capabilities. The aim is to investigate whether EcN cells exhibit improved performance in magnesium dissolution compared to previous experiments.

Drop-case incubation of the bacterial cells was performed on magnesium-coated glass surfaces. In other words, the 700 nm magnesium-coated glass surfaces were treated with a more concentrated bacterial solution to enhance the dissolution performance. For that purpose, the triplicates of bacterial BL21 cells were grown in LB media overnight and resuspended in MOPS minimal media supplemented with glucose and lactate in order to enhance the electron release activity (**Figure 4.4**). According to the results, BL21 ccmA-H showed faster dissolution than BL21 ccmA-H mtrA and BL21 ccmA-H mtrCAB cell groups visible at T60 and T90. On the other hand, the magnesium for the empty BL21 cell group was also almost completely dissolved at T90 which might be caused by coating defects.

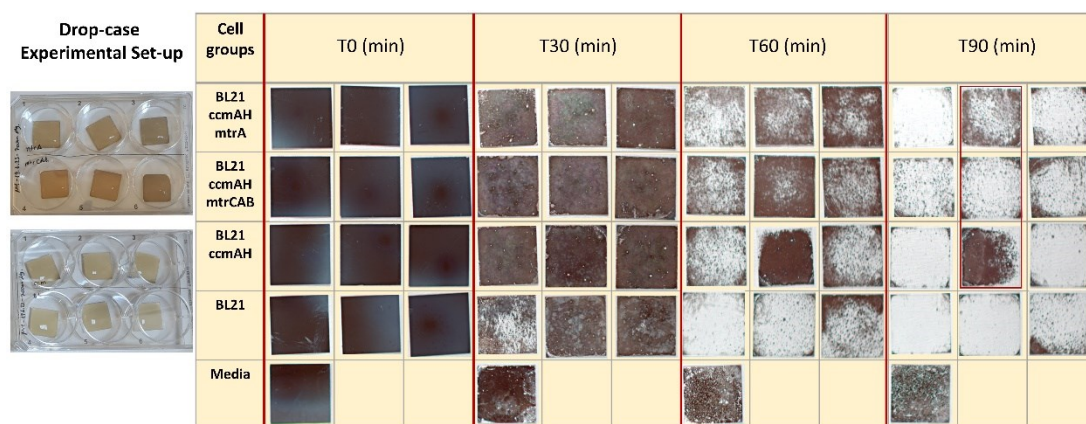


Figure 4.4 The 700 nm magnesium coated glass surfaces were incubated with BL21 ccmA-H mtrA, BL21 ccmA-H mtrCAB, BL21 ccmA-H, and non-engineered BL21 cell groups in a drop-case experimental set-up.

Due to inconsistency between observed and anticipated results in magnesium dissolution experiments, the magnesium-coated surfaces were examined to identify potential contamination. According to the results, it was confirmed that there were no unforeseen issues and that the problem did not stem from the coating (**Figure 4.5**). The Si peak might be due to the borosilicate glass surface, and Al could be from the SEM stub itself. To further ensure accuracy, the BL21 strain grown on 350nm coated glass surfaces in LB broth with lactate was suspended in MOPS minimal broth containing glucose and lactate the following day and allowed to interact, yet no difference in melting was observed compared to the control group.

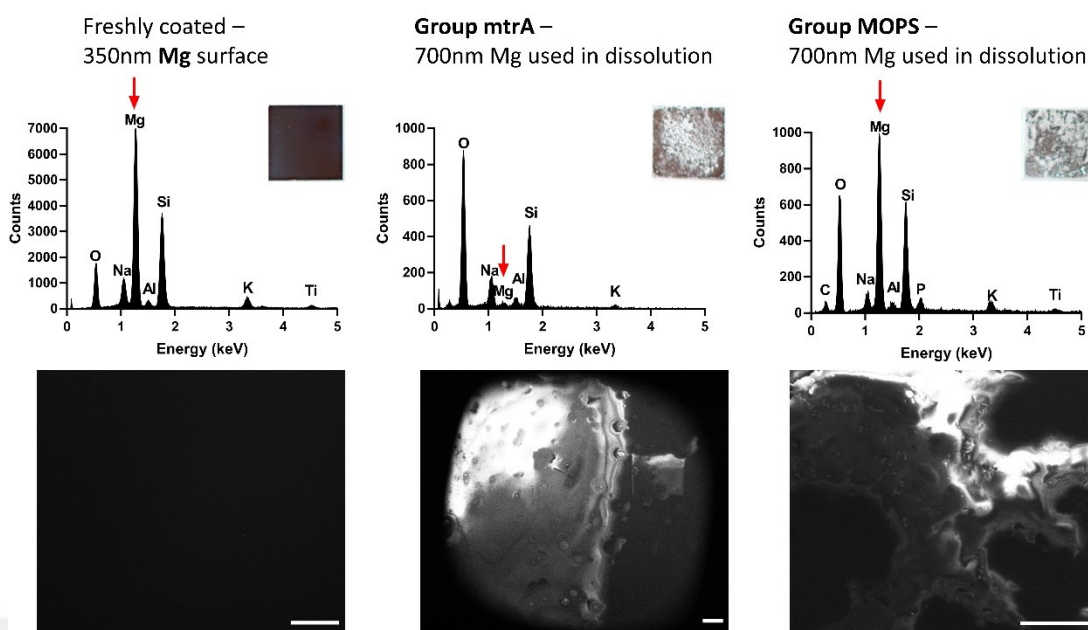


Figure 4.5 The Mg-coated glass surface and the residual Mg after dissolution underwent SEM-EDAX analysis, revealing no contamination in the coating. White scale bars are 500 μm .

4.4.4 Quantification of Iron Reduction Performance of *E. coli* BL21 Cells Expressing Cytochrome c Maturation Proteins Constitutively and Electron Conduit Proteins Conditionally

In response to the observed inconsistencies in dissolution experiments, a ferrozine assay was conducted on BL21 cells to assess their electron release behavior. Cell groups were segregated into those expressing one of the essential proteins for electron release and a control group expressing none, except for ccmAH proteins. To generate duplicate bacterial clones, cells cultured overnight in LB medium were resuspended in fresh M9 minimal medium the following morning upon reaching near OD_{600} values. The goal was to examine the iron ions reduced by the electrons released from the cells by introducing 5mM ferric citrate into the cell media. Samples were collected upon the

addition of ferric citrate and at specified time intervals, with subsequent measurements of OD₆₀₀ and 562nm absorbance. Iron content per cell was calculated by determining the iron concentrations reduced by the cell groups based on the standard curve established. The engineered bacterial systems' performance was not significantly different in reducing iron +3 ions to iron +2 ions through electron release, in comparison to the control groups, in eight days (**Figure 4.6**).

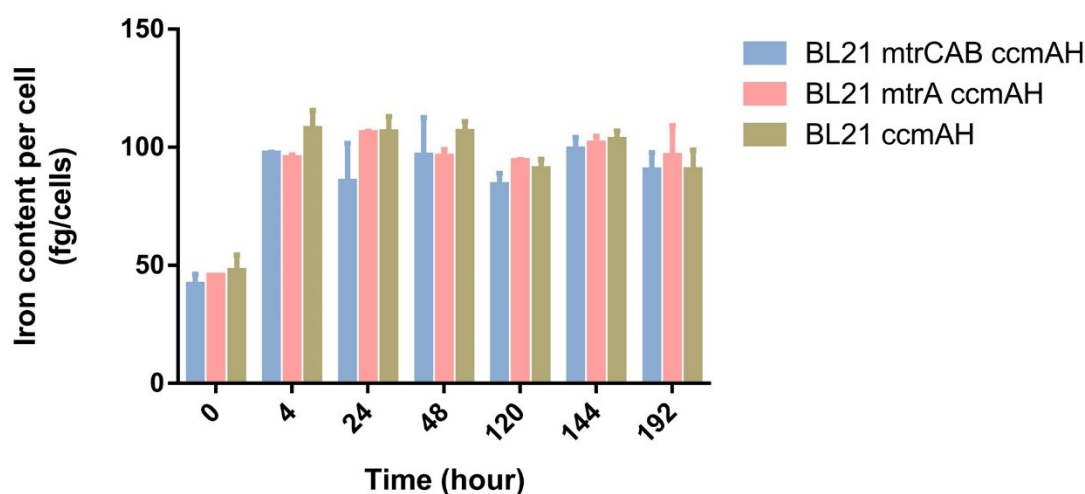


Figure 4.6 The ferrozine assay was utilized to quantify iron content per cell for experimental groups expressing one of the essential proteins for electron release (BL21 mtrA ccmAH or BL21 mtrCAB ccmAH) and control groups (BL21 ccmAH), exclusively expressing cytochrome pathway proteins (ccmA-H).

4.4.5 Construction of Probiotic *E. coli* Cells Constitutively Expressing mtrA Protein

An alternative approach was pursued to address the challenges encountered in magnesium dissolution experiments. The probiotic strain of *E. coli* was utilized to observe any improvements in the performance of magnesium dissolution compared to previous experiments. To achieve this goal, genetic circuits were established for the

constitutive production of *mtrA* protein, alongside *ccmAH* proteins (**Figure 4.7**). In order not to cause a metabolic burden in bacterial cells, the promoter was chosen as a minimal constitutive promoter; also, only the *mtrA* gene was utilized as electron releasing protein, not *mtrCAB* possessing large size and utilizing more amount of protein expression.

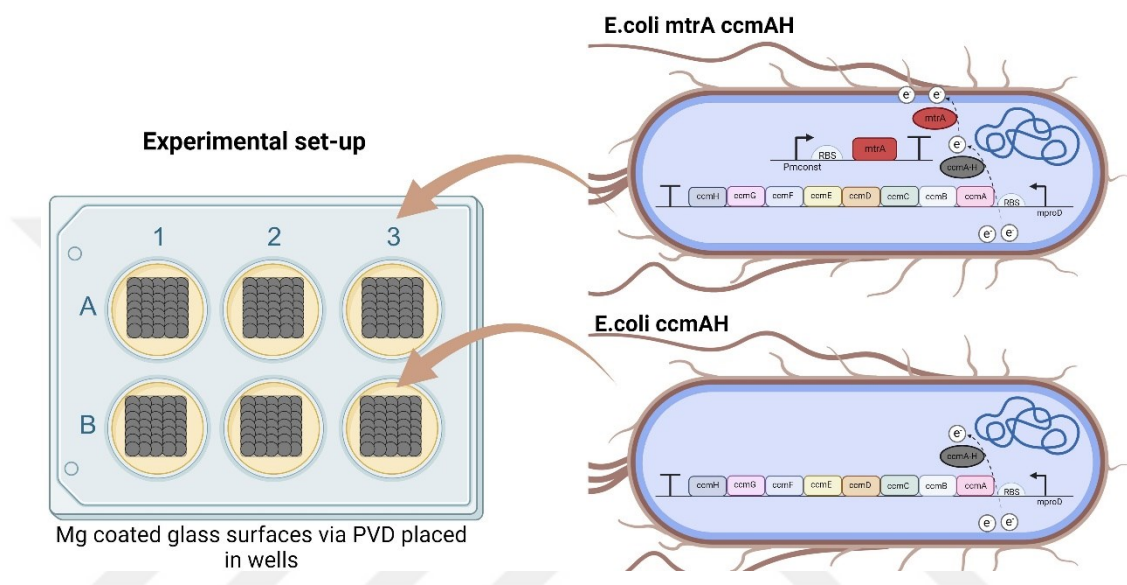


Figure 4.7 The genetic circuit design of EcN constitutively producing either *mtrA* *ccmA-H* or only *ccmA-H* as a control group and the experimental set-up were depicted here. Created with BioRender.com.

In cloning experiments, gene regions were amplified by PCR, and verifications were conducted for *mtrA* cloning (**Figure 4.8**). The cloning of pBAD BBaJ23116 *mtrA* construct was verified by restriction enzymes (REs) digestion with Avr2 (NEB) and Xho1 (NEB).

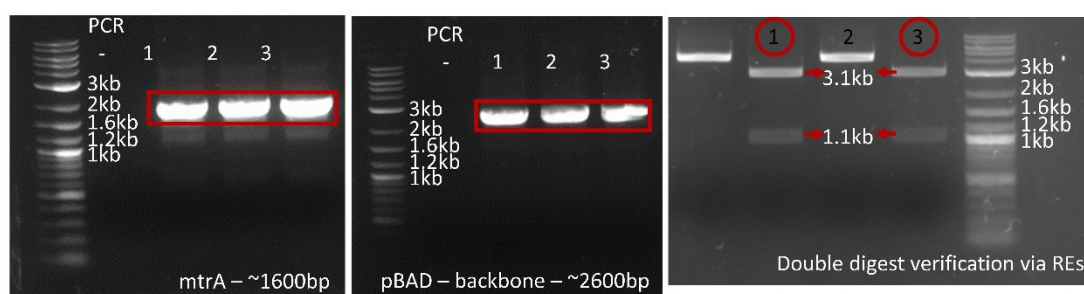
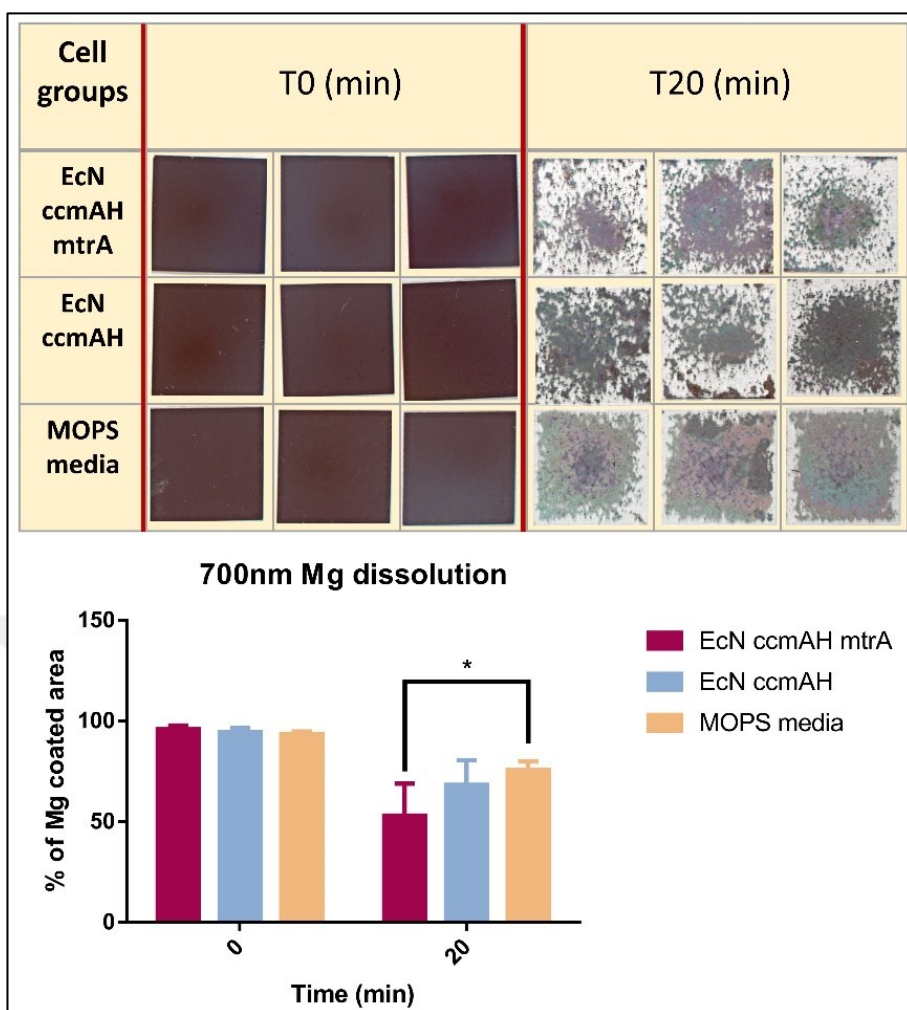


Figure 4.8 The cloning of *mtrA* under minimal constitutive promoter was done, and the PCR amplifications and double digest verification were depicted here.

4.4.6 Magnesium Dissolution by *E. coli* Cells Expressing Both *mtrA* and *ccmAH* Proteins Constitutively

Probiotic bacterial cells were transformed with constitutively expressed *mtrA* and *ccmAH* gene circuits, and then they were utilized in magnesium dissolution experiments. The triplicates of bacterial cells were prepared. For this purpose, cells were cultured overnight in LB medium and resuspended in MOPS minimal medium containing fresh glucose and lactate in a way to obtain five times concentrated cells, the next morning. These cells were then placed on magnesium-coated glass surfaces for a certain duration. The images were acquired via the VILBER imaging system at designated time points. The content of magnesium biodegradation on the glass surface was calculated by analyzing the images using ImageJ via black-and-white pixel analysis. According to the initial dissolution experiment results, there were significant differences between the EcN *ccmAH mtrA* cell group when compared to the MOPS media (**Figure 4.9-upper panel**). However, in the biological replica of the experiment conducted (**Figure 4.9-lower panel**), but the control group of EcN *ccmAH* cells showed faster dissolution than the EcN *ccmAH mtrA* cell group. A similar case was also observed for the BL21 strain.



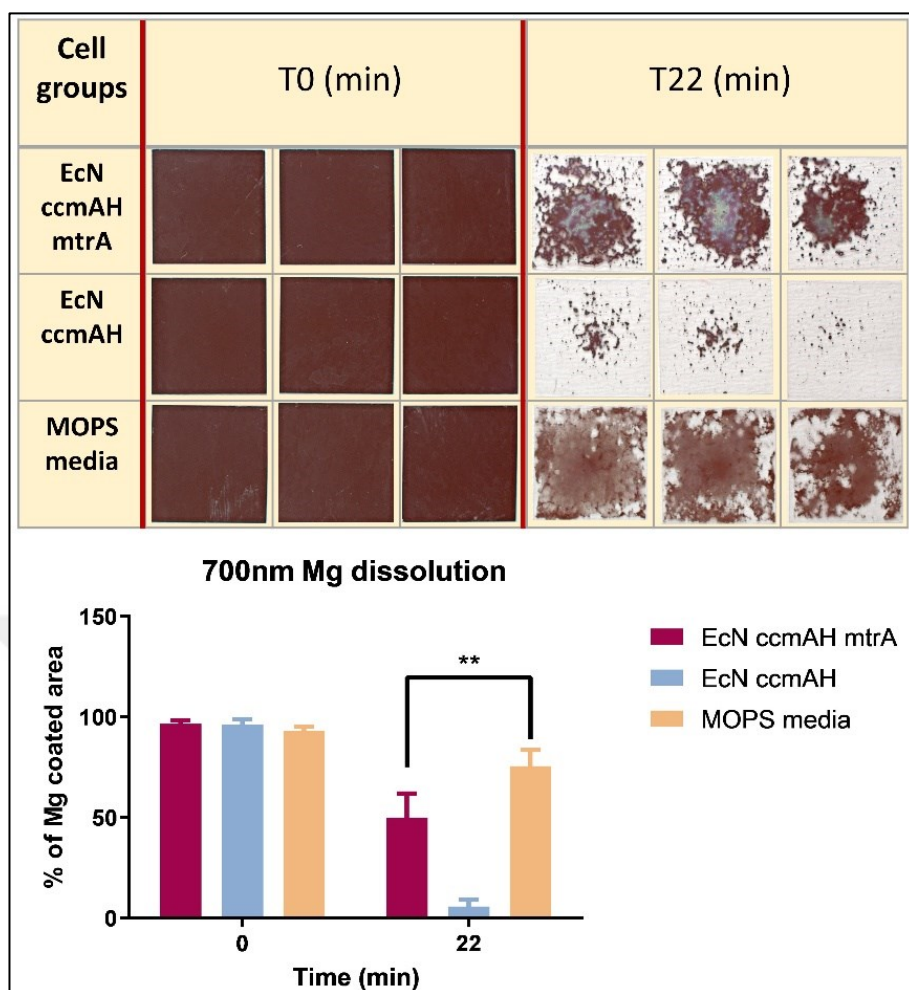


Figure 4.9 *E. coli* Nissle cells, expressing constitutively active gene circuits for *mtrA* and *ccmA-H*, used in the Mg (700nm) dissolution experiment, were cultured in LB broth overnight. They were then resuspended in MOPS medium, in a way to concentrate the cells five times, supplemented with glucose and lactate to enhance electron release before being applied onto metal-coated glass surfaces. The results were represented for different biological replicates.

The *mtrA* and *mtrCAB* proteins might cause not properly functioning the bacterial cells when expressed together with *ccmA-H* proteins. Therefore, an experimental set-up was planned to compare the activity of *ccmA-H* expressing cells with non-engineered empty cells. All the cell groups were concentrated five times by dissolving

in MOPS accordingly, and the OD₆₀₀ values were kept the same for all the groups for introducing the same amount of cells to the 700nm magnesium-coated surfaces. The images of the glass surfaces were captured after the water wash step when the incubation time with cells was reached. The magnesium dissolution experiment was conducted with constitutively ccmA-H expressing *E. coli* Nissle and BL21 strains (**Figure 4.10**). According to the results, EcN strain might not be suitable for expressing these heavy proteins because it already has two cryptic plasmids [128], so might cause not work expectedly. On the other hand, the BL21 ccm A-H cell group dissolves the metal faster than the empty BL21 cell group.

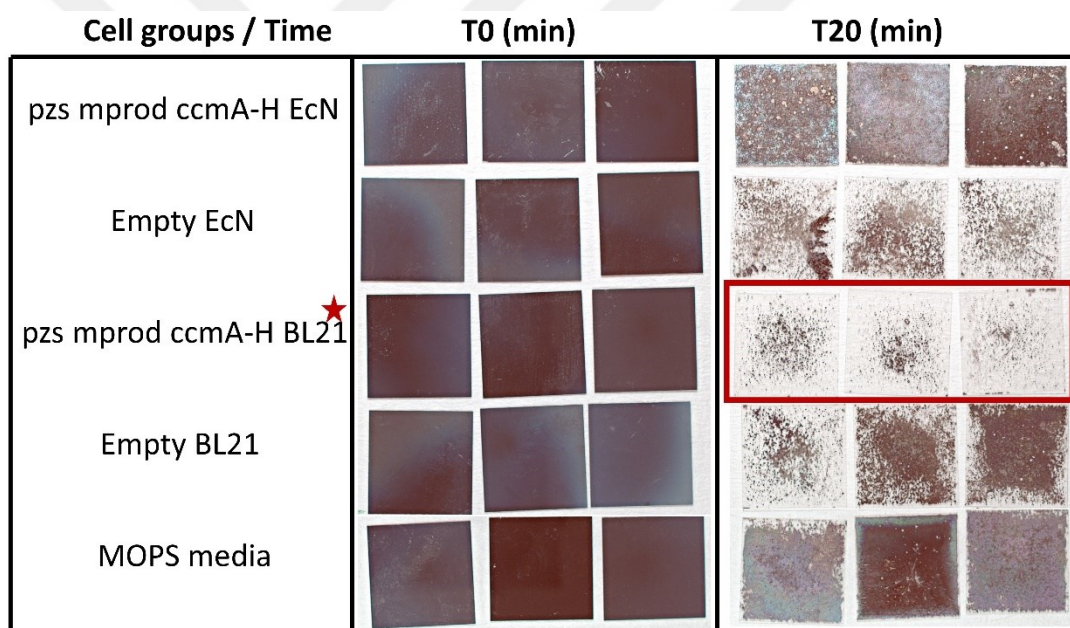


Figure 4.10 *E. coli* Nissle cells and BL21 strain expressing ccmAH proteins were used for 700nm Mg dissolution experiment. As compared to empty BL21 cells, ccmAH expressing BL21 cell group showed faster metal dissolution.

In conclusion, the proteins that play a role in electron release may overload synthetic protein production and lead to bacterial cells failing to perform the expected behavior.

Therefore, it was deduced that the ccmAH expressing BL21 cell group can be used for faster metal-dissolving machineries. Alternatively, to ensure the functionality of mtrA protein, the cells will be engineered to display the mtrA protein on the cell surface via an autotransporter protein. As a result, it aims to provide an environment where the protein can show its activity more freely and interact with the metal surface more easily.

4.4.7 Biodegradable Substrate Production and Characterization

The material where the bacteria aimed to be tested was designed to be produced. The mask design and production procedures were carried out at Boğaziçi University Electrical and Electronics Engineering Department by Associate Professor Sema Dumanlı Oktar. The production of Mg ring structures on silk altstone was aimed due to its biodegradable property. In this context, magnesium material was used for the production of ring structures. The smallest length of the ring structures designed in the simulation program is 115 μ m, aiming to produce the targeted structures with high quality. In this direction, studies have been carried out to produce rings by photolithography and physical vapor deposition (PVD) methods in a clean room environment. In order to observe the dissolution rate in a controlled manner, two different thicknesses of magnesium layers must be produced. For this, a two-stage photolithography process must be performed. For this purpose, the suitable masks were designed and produced for lithography stages were shown in **Figure 4.11** and **Figure 4.12**.

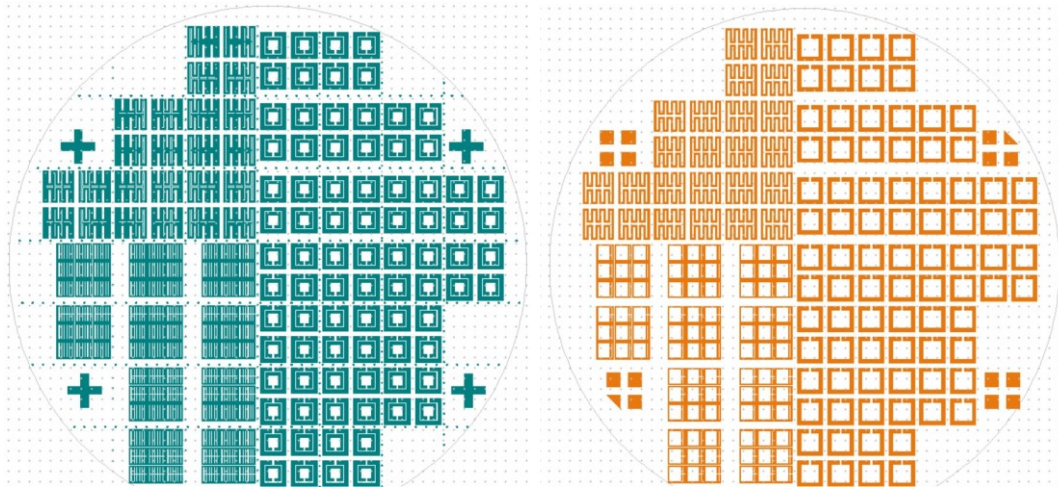


Figure 4.11 Masks designed for the photolithography stage. Left-1. mask, right-2. mask.

The designed masks were produced at Koç University. The images of the produced masks are shown in **Figure 4.4**.

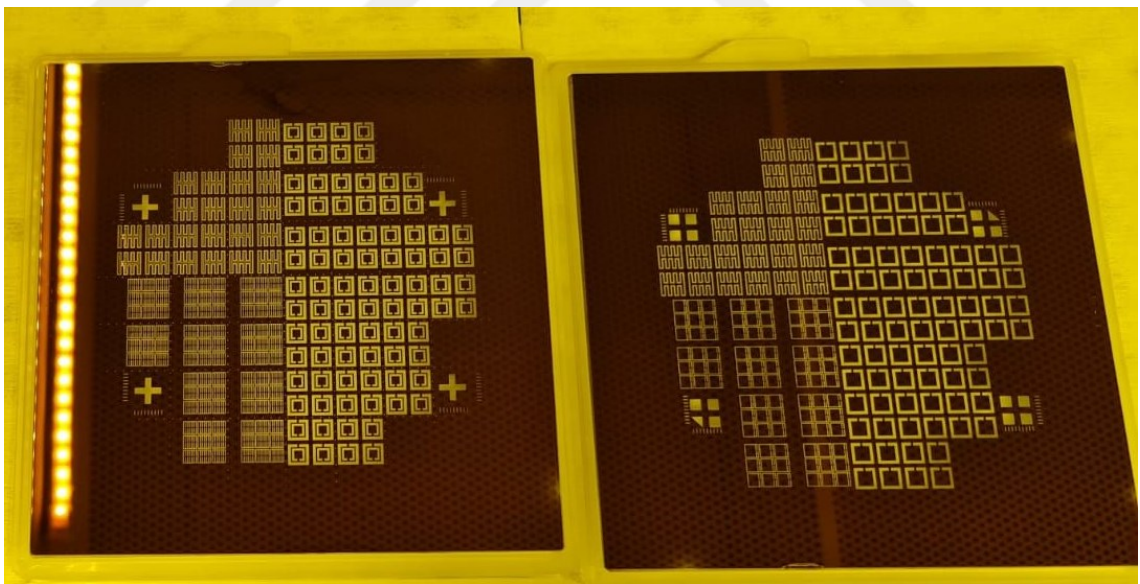


Figure 4.12 Masks produced for the photolithography stage. Left-1. mask, right-2. mask.

At this stage of the project, the glass wafer covered with silk was washed with water and dried with nitrogen gas, and then covered with the 7 μm AZ4562 photoresist. After UV exposure in the lithography device and the development steps, the sample was coated with 1 μm Mg by the sputter deposition method, and the lift-off was carried out and then cleaned with water-nitrogen gas sequentially. According to the results, the inability to obtain the desired pattern may be caused due to the diffusion of the photoresist by the increased temperature during Mg deposition. The possibility that the coated Mg thickness is too much results in coating with cracks on the photoresist, and in this case, the removal step was resulted in an undesirable pattern. It is thought that acetone attacks the photoresist from the cracked parts, preventing the resonator's definition with the desired clarity. Since the magnesium metal could not be formed in the desired pattern, the sample was kept in acetone in a sonic bath to clean the entire surface. It was observed that some of the magnesium residues and the silk coating were removed from the surface. As a result, it was found that the inability to obtain the targeted model used may be due to the fact that the photoresist is not resistant to the increased temperature during metal sputtering and, therefore, causes problems during the removal process. In order to solve this problem, it was planned to reduce the thickness of the metal coating, or use a different photoresist for material production.

At this point, cover glass lamel slides were used as a substrate to produce and optimize the material production. The Mg deposition thickness was reduced to 200nm after AZ4562 coating and lithography. However, cracked structures were observed. The metal used for deposition shifted aluminum (Al) to see if there was a problem with the metal, but there were again cracked structures on the model. The use of toluene for better adherence or a promoter (HMDS) for hydrophilicity of the surface were not a solution to the problem we faced. Therefore, a different positive photoresist, AZ4533,

was used on a titanium (Ti) coated borosilicate wafer, and after lithography, the model was coated with 200nm Mg via sputtering. The process was resulted without any problem, and the patterns were formed after the lift-off step (**Figure 4.13**). After optimization steps, it is concluded that the material production might be performed with AZ4533 photoresist on a glass surface first treated with HMDS and then a glass wafer coated with silk.

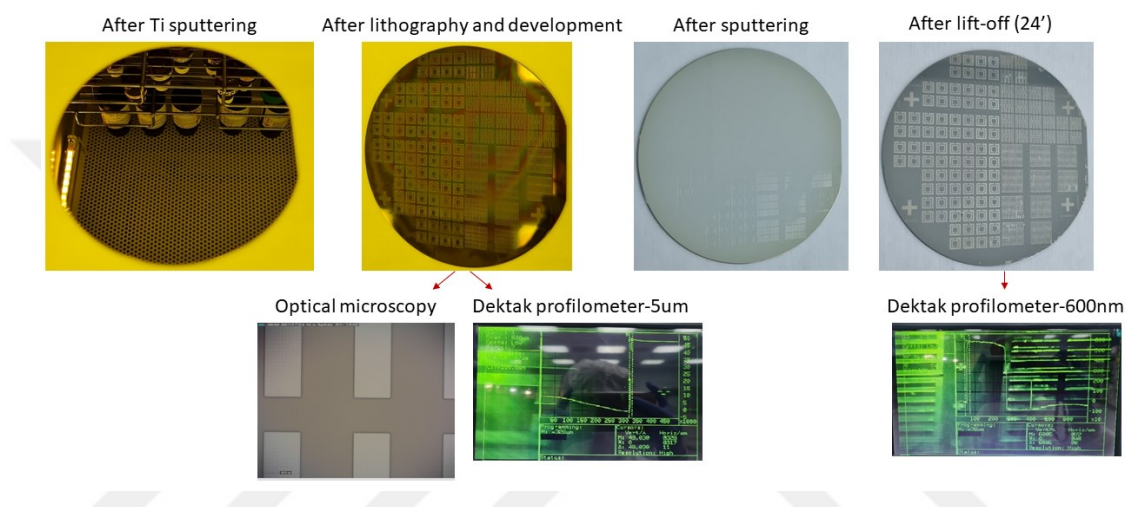


Figure 4.13 The material production process on titanium-coated borosilicate wafer via lithography and deposition.

In the bio-electronically active bacteria and magnesium interaction phase of the project, the aim was to utilize Mg ring structures on a silk substrate due to their biodegradable feature. To ensure controlled observation of the dissolution shell, two different thicknesses of material coating processes are required. For this purpose, two photolithography processes were planned. The reason for the failure to obtain the intended features on silk was the resistance of the photoresist to the increased temperature during metal sputtering, resulting in damage during the removal process. At this stage, glass coverslip slides were utilized as substrates for material fabrication

and optimizations. The AZ4562 coating was applied at a speed of 3000rpm with a 1000rpm acceleration. The coated slides were then dried at 110 degrees Celsius. Subsequently, they were exposed to UV light using a mask of the desired model in hard contact mode, with a separation distance of 100 μm and a dose of 500 mJ/cm^2 in the lithography device. After development in AZ400K diluted with water at a 1:5 ratio, the thickness of the Mg deposition was reduced to 200nm. The resulting structures were visualized under an electron microscope, revealing the presence of cracked structures. In this phase, the processes were tested on a 4-inch borosilicate wafer to generate all the models. Following the lithography and development stages, the sample coated with HMDS promoter and AZ 4533 photoresist was cut into 12x14mm scales. However, due to the cutting process occurring in an aqueous environment, applying the cutting process to the Mg-coated wafer sample resulted in the melting of the models. Consequently, the cut sample was recoated with a 200nm layer of Mg metal and subsequently removed (**Figure 4.14**).

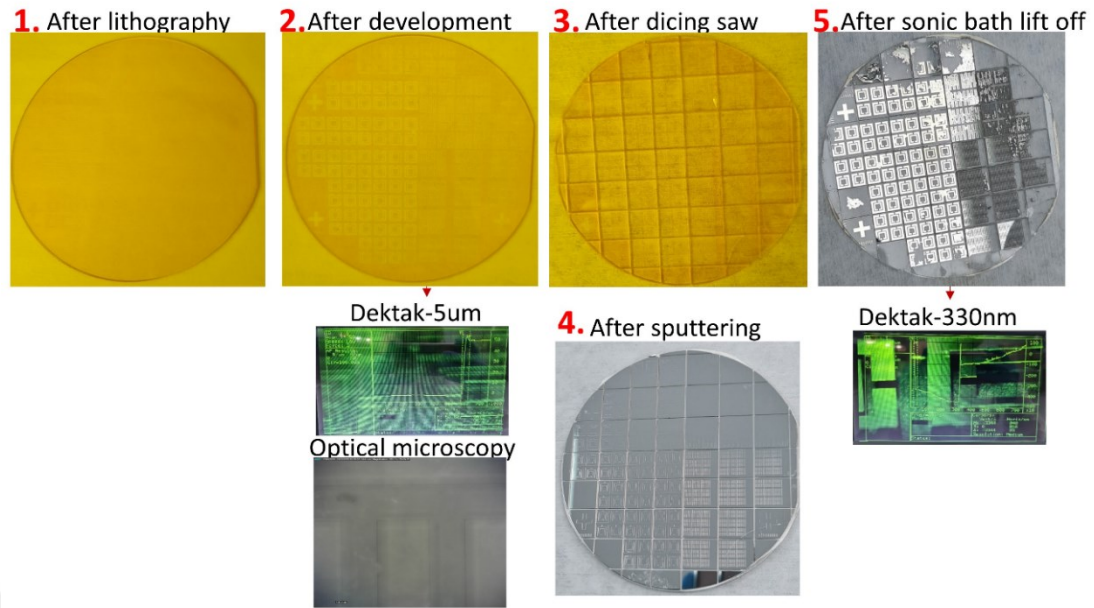


Figure 4.14 The material production process involving lithography, cutting, and deposition. The process begins with the coating of HMDS and AZ4533 on a borosilicate wafer.

The optimization steps required to achieve the desired model have been successfully completed. However, in previous material fabrication experiments, the choice of photoresist posed challenges due to its high viscosity. As a result, the material produced after lithography and metal deposition led to cracked structures, and the intended pattern could not be obtained. Various metals were tested for coating, and it was determined that the problem did not stem from the choice of metal. A different photoresist was employed to achieve the desired thickness measurement. Additionally, a promoter was utilized during the formation of the models on the borosilicate wafer to achieve a hydrophilic surface and successfully produce the device. The optimization and production of magnesium ring structures on borosilicate surfaces were carried out. The aim of this optimization process was to improve the quality and functionality of

the magnesium rings, which can potentially serve as key components in bioelectronic applications.

The activity of the bacterial cells was aimed to be tested on a biodegradable substrate coated with the desired metal. The steps of biodegradable substrate production and testing its suitability for lithography were explained below. During soft lithography experiments performed on silk fibroin substrate, it was observed that silk fibroin interacted highly with acetone used to remove the developer from the medium. Since acetone disrupted the structure of the silk fibroin film and damaged the surface before the physical vapor deposition (PVD) process, it was decided to continue the experiments with polyvinyl alcohol (PVA) as an alternative to silk. PVA was selected as an alternative biodegradable substrate because it is more resistant to aggressive solvents. A biodegradable substrate was cast on a borosilicate glass plate with a thickness of 1 mm and a diameter of 4 inches, and the effect of solvents such as developer, water, and acetone used in the lithography process in the production of in-body devices on the substrate was investigated. Due to the thinness of the substrate, it loses its surface homogeneity as soon as it comes into contact with the liquid. Another reason is that the substrate does not fully adhere to the glass surface and separates from the surface when it comes into contact with the liquid. As a result, the substrate is not suitable for lithography in its current form. The suitability of newly produced substrates for the lithography process will be tested, and if they are found to be suitable, in-body devices will be produced using lithography and DC sputtering methods.

The developed in-body device will be placed inside the serum. A physical phantom, a replicator medium, and an on-body antenna will be placed on the container containing the serum, respectively. Two of these devices will be prepared. The frequency

response of the on-body antenna and the biodegradation rate will be monitored for two separate situations with and without a trigger molecule. For bioelectrically active bacteria, the stable biodegradable substrate will be sputtered with magnesium, and the cellular machineries will be tested on them. Alternative substrates, like PVA, were considered alternative substrates to silk fibroin to address the challenges faced in biodegradable substrate usage in lithography. After successful magnesium sputtering on the stable biodegradable substrate, ccmAH expressing *E. coli* BL21 (DE3) cells is expected to dissolve the metal compared to the empty *E. coli* BL21 (DE3) cell group.



CHAPTER 5 : CONCLUSION AND FUTURE PERSPECTIVES

The work comprises the various strategies to construct genetically designed microbes hybridized with materials for theranostic applications. In Chapter 2, *E. coli* bacterial cells were engineered to synthesize protein-based nanomaterials inspired by iron-oxide biomineralization. The IONPs possess various advantages like magnetic characteristics and ease of manipulation when an external magnetic field applied. Therefore, IONPs are in demand as an MRI contrast agent. Synthetic biology enables the production of magnetic cell factories by eliminating harsh chemical synthesis conditions. Also, magnetic bacterial systems can be easily separated from other biological moieties, providing a promising contrast agent. The probable pathogenicity in the human body has to be get rid of by engineering the bacterial cells to express an iron-oxide nucleating protein to synthesize MNPs. In this work, two systems, called ECMAB and ICMAB, were developed as nanomaterial-producing factories that served as contrast agents with enhanced MRI performance. For that aim, Mms6 peptide was used as a primary protein to produce magnetite on the cell surface or inside the cell. For the constructed ECMAB system, the iron-binding peptide was fused with truncated autotransporter protein via molecular cloning in which the Mms6 display on the cell surface provides easiness in magnetite synthesis via its iron-binding activity at the C-terminus. The synthesis takes place at the cell surface, where an iron-binding site is available for ferrous and ferric ions, followed by dropwise base addition for crystalline formation. Electron microscopy imaging and spectroscopic analysis were utilized to characterize the ECMAB system. According to the results, the induced cell group showed remarkable enhancement in terms of magnetite accumulation with the

help of recombinant proteins' expression compared to the empty cell group, which lacks any synthetic gene circuit. On the other hand, it can be seen that there might be some attachment of nanomaterials to the cells' surface via the organic-inorganic attraction [114] because free MNPs are present in the environment. Additionally, characterizing the cells via taking the MRI relaxivity measurements revealed that the engineered cells demonstrated better MRI contrast performance than the non-engineered empty cells. As a second system, the intracellularly nanomaterial synthesizing cells were constructed by taking into consideration of the combinatorial effects of Mms6 with ferroxidase, MBP, and iron transporter proteins. The iron transporter protein eases the ferrous iron uptake into the cells upon ferrous iron ion addition to media, ferroxidase enzyme enables the effective oxidation of ferrous into ferric ions, [105] and the affinity toward the synthesized IONPs is enhanced via MBP. [101] When the intracellular iron content of the cells was quantified via ferrozine assay, the intracellular magnetite accumulating cells indicated higher iron content. Moreover, higher iron nanomaterial accumulation was observed in engineered cells compared to non-engineered one, according to the electron microscopy analysis and MRI measurements. This proof-of-concept study aimed to implement a synthetic biology route for mimicking natural iron biomineralization in *E. coli* cells that might serve magnetic imaging in biomedical applications. An alternative way can be implemented to the constructed system to minimize the side effects on healthy cells and improve *in vivo* cancer targeting via MRI diagnosis, hyperthermia treatment, or drug delivery strategies as transferred into a non-pathogenic tumor-targeting strain. To sum up, the designed systems have potential to be coupled with additional genetic circuitries in a probiotic bacterium for constructing a multi-functional agent in biomedical applications.

In Chapter 3, *E. coli* bacterial cells were modified to display a nanobody protein against the HER2 receptor. For this purpose, the bacterial cells were engineered to express the nanobody protein called 2Rs15d fused with an autotransporter protein named Ag43. The autotransporter protein serves to display the protein of interest (POI) provided by expressing it upstream of the α -domain of Ag43. This strategy was considered to be integrated into ICMAB cells that possess the magnetite accumulation by performing all redox reactions by themselves. After ensuring the display of nanobody on ICMAB cells via immunocytochemistry (ICC), they were utilized to infect the breast cancer cell line called Jimt1. The binding assay was visualized under a bright field microscope containing the control cell group; however, a reporter protein was needed in ICMAB cells for assuring that the Jimt1 cells were surrounded by 2Rs15d ICMAB cells. For this aim, a red fluorescent protein called mCherry protein was introduced to the cells. On the other hand, because an extra plasmid possibly increased the workload and caused a metabolic burden on the bacterial cells depicted via not giving fluorescence efficiently, mCherry expression was achieved by integration of it in the genetic circuit for nanobody display plasmid. The binding of bacterial cells fluorescence as red to the mammalian cells transfected with green fluorescence was verified via confocal imaging. In addition, the MRI measurements revealed that the ICMAB cells effectively attach to mammalian cells and provide contrast capability compared to non-engineered empty cells. According to the results, it was observed that the magnetite accumulating bacteria, which bound to the spheroids, exhibited the smallest T_2 relaxation time value, which indicates that these bacterial binding provide an enhanced T_2 contrast as MRI performance capability.

In chapter 4, it was aimed to construct electron-releasing bacterial machineries in order to be used for diagnostic tools. For this aim, *E. coli* bacterial cells were engineered to

express both ccmA-H proteins and mtrA/CAB complex. On the other hand, the workload of all these protein's proper expression and functionality possibly caused a metabolic burden in bacterial cells. This was depicted by considering the unexpected metal dissolution performance of the bacterial cell groups, in addition to ferrozine assays where the iron reduction performance of cells was quantified. Different strategies were performed to investigate the bacterial cells' activity. The dissolution was done for iron and magnesium metals. The magnesium surfaces were analyzed via EDX, but no contamination was found. The cells were designed to express mtrA protein under constitutive conditions, but the expected function couldn't be observed. Therefore, it was decided to continue with the BL21 cells expressing ccmA-H proteins that show faster dissolution compared to non-engineered empty BL21 cells. The bacteria-material interaction was aimed at being performed on a biodegradable substrate. For this purpose, magnesium ring structures were designed to be patterned on silk altstone. The patterns were obtained via photolithography and magnesium sputtering. At the end, the sentinel bacterial cells would be used as a sensor for wireless communication via in-body to on-body antenna signaling that would advance real-time monitoring of disease in the human body.

In this thesis, the synthetic biology approach was utilized for the construction of bacterial systems for bioimaging and biosensing applications. The efficacy of theranostic agents is crucial for obtaining on-target activity to minimize side effects on healthy tissues and to maximize the efficiency of therapeutic effects on diseased tissue. The magnetite-accumulating bacterial systems can be engineered to express and release an anticancer agent upon stimulation. The drug release at the desired area would decrease the off-target effect of the agent. Constructing the systems in a non-pathogenic bacterial strain, *E. coli* Nissle 1917, would further enhance the systems as

their specific localization on tumor cells. The hypoxic core of the tumor cells can be targeted by constructing the system under hypoxia-responsive promoter elements. This way, the penetration ability would be enhanced to target necrotic tumor centers. In order to ensure the functionality of the engineered bacterial cells, a microfluidic device is utilized to mimic the complex 3D architecture of tumors. Animal experiments pose ethical concerns and protocols in addition to time-consuming processes and inefficiency in replicating human tumor microenvironment. Conducting animal experiments compromises various hurdles to overcome, like obtaining a specific mouse strain to develop a xenograft breast cancer model following ethical guidelines and protocols. The potential of microfluidic devices to automate a diverse range of chemical and biological processes improves the usage in diagnostic and therapeutic applications more efficiently and productively. [133] These devices offer a controlled and scalable platform to mimic the complex 3D architecture of tumors. The precise manipulation of fluids at the microscale enables the creation of highly reproducible and physiologically relevant conditions for optimization and testing. The microfluidic platform would be used for first tumor spheroid formation, and later, the engineered bacterial systems would be integrated to observe the interaction between the bacteria and tumor cells. The expected scenario is that the bacterial cells infect the HER-2-positive mammalian spheroids when co-cultured. The therapeutic efficacy and mechanism of action are aimed to be observed in a more human-relevant context. The fidelity of preclinical models and the development of targeted cancer therapies would be improved with this strategy while minimizing the requirement of animal experiments. Therefore, both the ethical and scientific challenges would be diminished. These experiments aim to assess the bacterial cells' in vivo behavior and efficacy as MRI contrast agents in a relevant biological context. Introducing the

magnetite accumulating bacterial cells into animal models with breast cancer will evaluate their ability to generate MRI contrast and provide accurate imaging information. Furthermore, the electronically active bacterial cells possess a potency as sentinel cells that are activated to synthesize the electron conduit peptides upon detecting a disease biomarker molecule. The wireless communication between in-body and on-body antennas would operate real-time human body dynamics monitoring.



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APPENDIX A

DNA sequences of genes and gene fragments utilized in this thesis

Table A. Gene sequences used in the study (from 5' to 3')

Name of the gene	Sequence
Mms6	GGCGAAATGGAGCGCGAAGGGGCGGCTGCTAAGGCAGG CGCCGCTAAAACAGGAGCTGCCAAGACCGGAACTGTAGC GAAAACCTGGAATTGCGGCCAAGACCGGCGTTGCGACCGC GGTGGCAGCACCGGCCCGCACCAAGCTAACGTGGCTGCGGC TCAGGGTGCTGGCACTAAGGTAGCCTTAGGTGCAGGAAA GGCCGCTGCTGGCGCCAAAGTAGTTGGAGGGACCATCTG GACAGGTAAAGGGTTAGGACTGGGTCTTGGCTTAGGCCT TGGTGCGTGGGGACCGATTATTTGGGTGTCGTAGGGGCT GGAGCGGTTTACGCCTACATGAAGAGTCGTGATATTGAA TCTGCACAGTCAGACGAGGAAGTCGAGCTTCGTGACGCA CTTGCA
Ag43	CGCACAACCATCAATAAAAAACGGTCGCCAGATTGTGAGA GCTGAAGGAACGGCAAATACCACTGTGGTTTATGCCGGC GGCGACCAGACTGTACATGGTCACGCACTGGATAACACG CTGAATGGGGGATAACAGTATGTGCACAACGGCGGTACA GCGTCTGACACTGTTGTGAACAGTGACGGCTGGCAGATT GTCAAAAACGGGGGTGTGGCCGGGAATACCACCGTTAAT CAGAAGGGCAGACTGCAGGTGGACGCCGGTGGTACAGCC ACGAATGTCACCCTGAAGCAGGGCGGCGCACTGGTTACC AGTACGGCTGCAACCGTTACCGGCATAAACCGCCTGGGA GCATTCTCTGTTGTGGAGGGTAAAGCTGATAATGTCGTAC TGGAAAATGGCGGACGCCTGGATGTGCTGACCGGACACA CAGCCACTAATAACCCGCGTGGATGATGGCGGAACGCTGG ATGTCCGCAACGGTGGCACCGCCACCACCGTATCCATGG GAAATGGCGGTGTACTGCTGGCCGATTCCGGTGCCGCTGT CAGTGGTACCCGGAGCGACGGAAAGGCATTTCAGTATCGG

	AGGCGGTCAGGCGGATGCCCTGATGCTGGAAAAAGGCAG TTCATTACGCTGAACGCCGGTGATACGGCCACGGATACC ACGGTAAATGGCGGACTGTTACCGCCAGGGGCGGCACA CTGGCGGGCACCACCACGCTGAATAACGGCGCCATACTT ACCCTTTCCGGGAAGACGGTGAACAACGATACCCTGACC ATCCGTGAAGGCGATGCACTCCTGCAGGGAGGCTCTCTC ACCGGTAACGGCAGCGTGAAAAATCAGGAAGTGGCACA CTCACTGTCAGCAACACCACACTCACCCAGAAAGCCGTC AACCTGAATGAAGGCACGCTGACGCTGAACGACAGTACC GTCACCACGGATGTCATTGCTCAGCGCGGTACAGCCCTGA AGCTGACCGGCAGCACTGTGCTGAACGGTGCCATTGACC CCACGAATGTCACTCTCGCCTCCGGTGCCACCTGGAATAT CCCCGATAACGCCACGGTGCAGTCGGTGGTGGATGACCT CAGCCATGCCGGACAGATTTCATTTACCTCCACCCGCACA GGGAAGTTCGTACCGGCAACCCTGAAAGTGAAAAACCTG AACGGACAGAATGGCACCATCAGCCTGCGTGTACGCCC GATATGGCACAGAACAAATGCTGACAGACTGGTCATTGAC GGCGGCAGGGCAACCGGAAAAACCATCCTGAACCTGGTG AACGCCGGCAACAGTGCGTCGGGGCTGGCGACCAGCGGT AAGGGTATTTCAGGTGGTGGAAAGCCATTACGGTGCCACC ACGGAGGAAGGGGCTTTGTCCAGGGGAACAGGCTGCAG GCCGGTGCCTTTAACTACTCCCTCAACCGGGACAGTGATG AGAGCTGGTATCTGCGCAGTGAAAATGCTTATCGTGCAG AAGTCCCCCTGTATGCCTCCATGCTGACACAGGCAATGGA CTATGACCGGATTGTGGCAGGCTCCCGCAGCCATCAGAC CGGTGTAAATGGTGAAAACAACAGCGTCCGTCTCAGCAT TCAGGGCGGTCATCTCGGTACGATAACAATGGCGGTATT GCCCCGTGGGGCCACGCCGAAAGCAGCGGCAGCTATGGA TTCGTCCGTCTGGAGGGTGACCTGATGAGAACAGAGGTT GCCGGTATGTCTGTGACCGCGGGGGTATATGGTGCTGCTG GCCATTCTTCCGTTGATGTAAAGGATGATGACGGCTCCCG TGCCGGCACGGTCCGGGATGATGCCGGCAGCCTGGGCGG ATACCTGAATCTGGTACACACGTCCCTCCGGCCTGTGGGCT GACATTGTGGCACAGGGAACCCGCCACAGCATGAAAGCG TCATCGGACAATAACGACTTCCGCGCCCCGGGGCTGGGGC TGGCTGGGCTCACTGGAAACCGGTCTGCCCTTCAGTATCA CTGACAACCTGATGCTGGAGCCACAACCTGCAGTATACCT GGCAGGGACTTTCCCTGGATGACGGTAAGGACAACGCCG GTTATGTGAAGTTCGGGCATGGCAGTGCAACATGTGC GTGCCGGTTTCCGTCTGGGCAGCCACAACGATATGACCTT TGGCGAAGGCACCTCATCCCGTGCCCCCTGCGTGACAGT
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EncFtn	ATGGCGCAGTCCAGCAATAGCACCCATGAACCACTGGAA GTTCTGAAAGAAGAACTGTCAACCGTCATCGTGCAATT GTTAGCGTGATGGAAGAACTGGAAGCAGTGGACTGGTAT GACCAGCGCGTAGACGCATCCACCGATCCGGAACCTGACC GCAATTCTGGCCCACAACCGTGATGAGGAAAAAGAACAC GCAGCGATGACCCTGGAATGGCTGCGTCGTAATGATGCA AAATGGGCAGAACACCTGCGTACCTACCTGTTACCCGAG GGCCCGATTACGGCTGCTAACTCCTCTAGCGTCGATAAAC TGGCTGCGGCGCTGGAA
MBP6	CTGAGCACCGTGCAGACCATTAGCCCGAGCAACCAT
EfeU	ATGTTTGTTCCGTTTCTCATTATGTTGCGCGAAGGACTTG AAGCCGCGCTGATTGTCAGTTTGATTGCCAGCTATCTTAA GCGTACCCAGCGAGGCCGATGGATTGGTGTGATGTGGAT TGGCGTGTGCTTGCCGCTGCGTTGTGCCTGGGCTTGGGT ATCTTCATTAACGAAACCACCGGCGAATTTCCGCAAAAA GAACAGGAACTGTTTGAAGGTATCGTGGCGGTGATCGCC GTGGTGATCCTTACCTGGATGGTTTTCTGGATGCGCAAAG TGTCGCGCAACGTCAAAGTGCAACTGGAACAGGCAGTCG ATAGCGCATTGCAGCGTGGAATCATCATGGCTGGGCGC TGGTGATGATGGTCTTTTTTGCCGTTGCAAGGGAAGGGCT GGAGTCGGTCTTTTTCTGCTGGCGGCATTTCAACAAGAT GTCGGGATCTGGCCGCCGCTGGGTGCAATGCTCGGTCTTG CTACTGCCGTGGTGCTAGGCTTCCTGCTCTACTGGGGCGG TATTCGCCTCAATCTTGGTGCATTTTTTAAATGGACCAGC CTGTTTATTCTCTTCGTGCGCCGAGGGCTGGCAGCTGGTG CCATTCGCGCATTTTCATGAAGCCGATTGTGGAACCACTT TCAGGAAATCGCCTTCGATATGAGTGCGGTGCTCTCAACT CACTCGCTGTTTGGCACGCTGATGGAAGGGATTTTGGCT ATCAGGAAGCGCCGAGCGTCAGCGAAGTCGCCGTCTGGT

	TTATTTATCTCATCCCGGCGCTGGTGGCATTGCTCTGCCA CCACGCGCAGGGGCGACAGCGTCTCGCTCCGCG
mCherry	atggtttccaagggcgaggaggataacatggctatcattaagagttcatgcgcttcaaagtca catggaggggttctgtaacgggtcacgagttcagatcgaaggcgaaggcgagggccgtccgt atgaaggcaccagaccgccaactgaaagtgactaaaggcgcccgctgcctttgcgtgg gacatcctgagcccgcaatttatgtacggttctaagcgtatgttaaacaccagcgatatccc ggactatctgaagctgtctttccggaaggttcaagtgggaacgcgtaataatttgaagatgg tggtgtcgtgaccgtcactcaggactcctcctgcaggatggcgagttcatctataaagttaaact gcgtgggtactaattttccatctgatggcccggtgatgcagaaaaagacgatgggtgggagggcg tctagcgaacgcgatgtatccggaagatgggtgcgctgaaaggcgaaataaacagcgctgaaa ctgaaagatggcgccattatgacgctgaagtgaaccacgtacaaagccaagaaacctgtg cagctgcctggcgcgatcaatgtgaatattaaactggacatcacctctcataatgaagattatag atcgtagagcaatatgagcgcgcggaggggtcgtcattctaccggtggcatggatgagctgtac aaataa
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	TAAGGACCCAGCATCATGAATGATGCCAGAAATTGGTAA CGGACTGCTGTGCCTGGCGCTGGGAATTGCGCTGCTGCTG TCCGTGTATCCGCTATGGGGCGTGGCGCGCGGAGATGCG CGCATGATGGCGTCTTCCCGCTTGTTTGCCTGGCTGCTGTT TATGTCTGTGGCTGGCGCATTTCTGGTGCTGGTCAATGCT TTCGTGGTCAACGACTTCACCGTCACCTATGTTGCCAGCA ACTCCAATACCCAGCTTCCGGTGTGGTATCGCGTGGCGGC TACCTGGGGCGCGCATGAAGGCTCGCTACTGCTGTGGGT GCTGCTGATGAGCGGCTGGACCTTTGCGGTGGCGATTTTT AGTCAGCGTATTCCGCTGGATATTGTGGCCCGTGTACTGG CGATAATGGGGATGGTCAGTGTGGCTTTTTGCTGTTTCAT TCTCTTTACCTCTAACCCGTTCTCTCGCACGTTGCCGAAC TCCCGATTGAAGGTCGCGATCTTAACCCGCTATTGCAGGA TCCGGGGCTGATCTTCCATCCGCCTCTGCTTTATATGGGG TACGTGGGTTTCTCGGTGGCGTTTGCTTTTGCCATTGCTTC TTTGCTGAGCGGGCGTCTGGACAGCACTTATGCGCGTTTT ACTCGTCCGTGGACGCTGGCAGCGTGGATCTTCCTGACGC TCGGCATCGTGCTCGGTTCCGCATGGGCCTATTACGAACT CGGCTGGGGTGGCTGGTGGTTCTGGGATCCGGTAGAAAA CGCCTCGTTTATGCCGTGGCTGGTGGGGACTGCGCTGATG CACTCACTGGCGGTCACTGAACAACGCGCCAGCTTCAAA GCGTGGACATTACTGCTGGCAATCAGTGCCTTCTCGTTGT GTCTGTTGGGGACTTTCCTCGTGCGTTCCGGCGTGCTGGT ATCGGTACACGCGTTTGCGTCTGATCCGGCGCGCGGTATG TTTATCCTCGCCTTTATGGTGCTGGTGATTGGCGGTTTCGCT GCTGCTGTTTGCCGCGCGTGGACACAAAGTTTCGCTCACGC GTAAACAATGCGCTGTGGTCGCGGGAATCTTTGCTGTTAG CGAACAATGTTTTGCTGGTCGCTGCGATGCTGGTGGTGTT GCTGGGGACGCTGCTGCCGTTGGTGCATAAGCAACTGGG ACTGGGCAGTATTTTCGATTGGCGAACCCTTCAACACC ATGTTTACCTGGCTGATGGTGCCGTTTGCGCTACTGCTTG GTGTCGGTCCTCTGGTGCGCTGGGGGCGGGATCGCCCGC GTAAGATCCGCAATTTATTGATTATCGCCTTCATCTCTAC GCTGGTGCTGTCGCTGCTGTTGCCGTGGCTGTTTCGAAAGC AAAGTTGTGGCGATGACGGTGCTCGGCCTGGCAATGGCC TGCTGGATTGCGGTGCTGGCAATTGCGGAAGCTGCGCTAC GTATTTACGCGGCACGAAAACCACTTCAGTTATTGGGG GATGGTGGCGGCTCACCTTGGGCTGGCAGTGACAATTGTT GGCATTGCCTTTAGCCAGAACTATAGCGTTGAGCGTGATG TGCGCATGAAGTCCGGCGATAGCGTCGATATTCATGAAT ATCGCTTCACCTTCCGTGATGTCAAAGAGGTGACTGGCCC
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	GAAC TGGCGTGGCGGTGTGGCGACTATCGGCGTAACGCG CGATGGCAAGCCGGAAACGGTGCTGTATGCGGAAAAACG TTATTACAACACTGCCGGGTCGATGATGACCGAAGCGGC AATTGACGGCGGCATCACGCGTGACCTGTACGCGGCCCT CGGTGAAGAGCTGGAAAACGGCGCGTGGGCCGTGCGTCT TTACTACAAACCATTTGTTCGCTGGATTGTTGGGCGGGCGGG CTGATGATGGCGTTGGGCGGACTGCTGTGTCTGTTTGATC CTCGCTATCGTAAGCGCGTGAGTCCGCAAAAAACTGCGC CGGAGGCCGTATGAATGAAGCGCAAAGTATTGTTAATTC CGTTGATTATCTTCCTGGCGATTGCCGCGGCGCTGCTGTG GCAGCTGGCGCGTAATGCCGAAGGGGATGATCCGACCAA TCTGGAATCGGCGCTCATTGGCAAGCCTGTGCCGAAGTTT CGTCTCGAATCACTGGACAATCCGGGGCAGTTTTATCAGG CGGATGTGCTGACTCAGGGCAAACCAGTACTGCTTAACG TCTGGGCGACCTGGTGTCCGACCTGCCGTGCGGAACATCA ATATCTGAATCAGCTTTCTGCGCAGGGCATCCGCGTGGTC GGCATGAACTATAAAGACGATCGCCAGAAGGCAATCAGC TGGCTGAAAGAGCTGGGCAATCCTTACGCGCTAAGCCTG TTTGATGGCGATGGCATGTTAGGGCTGGATCTCGGTGTCT ATGGCGCGCCAGAAACGTTTCTTATTGACGGCAACGGCA TCATTGCTATCGCCATGCGGGCGATCTGAATCCTCGCGT CTGGGAAGAAGAGATCAAGCCGCTGTGGGAGAAATACAG TAAGGAGGCCGCACAATGAATGAGGTTTTTATTGGGCGT GCTGATGCTGATGATCTCCGGCTCAGCGCTGGCGACCATC GATGTGTTGCAGTTTAAAGATGAAGCACAGGAACAACAG TTCCGTCAGCTCACTGAAGAACTGCGCTGCCCGAAATGCC AGAACAACAGCATTGCCGATTCCAACCTCGATGATTGCCA CCGACCTGCGTCAGAAAGTGTATGAACTGATGCAGGAAG GTAAAAGTAAAAAAGAGATTGTCGATTATATGGTGGCGC GTTACGGCAACTTCGTCACCTACGATCCGCCGTTAACGCC GCTGACCGTGCTGCTGTGGGTGCTGCCAGTAGTGGCTATT GGCATTGGCGGTTGGGTCAATACGCCCGCTCGCGGCGTC GGGTACGCGTGGTGCCGGAAGCGTTTCCTGAACAAAGCG TGCCGGAAGGTAAGCGTGCCGGATATGTTGTTTATCTGCC GGGTATTGTGGTGGCGTTAATTGTGGCTGGCGTCAGCTAC TACCAGACTGGCAATTATCAGCAGGTGAAAATCTGGCAG CAGGCCACGGCACAGGCTCCGGCGTTGCTGGACAGGGCG CTGGATCCGAAAGCCGATCCGCTCAACGAAGAAGAGATG TCGCGTCTTGCGCTGGGGATGCGTACTCAACTGCAAAAA AATCCGGGAGATATAGAAGGCTGGATTATGTTGGGCCGC GTTGGCATGGCGCTGGGTAAACGCCAGTATCGCCACCGAT
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	GCATACGCCACTGCGTATCGCCTCGATCCGAAAAACAGT GATGCTGCACTGGGATACGCTGAAGCGTTGACTCGTTCAT CTGATCCCAACGACAACCGCCTCGGCGGTGAACTGCTAC GTCAGCTGGTGAGAACGGACCATAGCAATATCCGTGTGC TAAGCATGTATGCGTTTAATGCCTTTGAGCAGCAGCGATT TGGCGAAGCCGTTGCCGCGTGGGAGATGATGTTGAAACT CTTACCTGCCAACGATACTCGCCGTGCGGTGATTGAACGT AGTATCGCGCAGGCGATGCAACATTTGTCGCCGCAGGAG AGTAAATAA
Regulatory DNA part	Sequence
T7-LacO promoter	TAATACGACTCACTATAGGGGAATTGTGAGCGGATAACA ATTCC
Ribosome binding site (RBS)	ATTAAAGAGGAGAAA
6xHis-tag coding sequence	CACCACCACCACCACCAC
PelB signal sequence	ATGAAATACCTGCTGCCGACCGCTGCTGCTGGTCTGCTGC TCCTCG CTGCCCAGCCGGCGATGGCC
T7 Terminator	CTAGCATAACCCCTTGGGGCCTCTAAACGGGT CTTGAGGGGTTTTTTG
Cat promoter	TGATCGGCACGTAAGAGGTTCCAACCTTTCACCATAATGAA ATAAGATCACTACCGGGCGTATTTTTTTGAGTTATCGAGAT TTTCAGGAGCTAAGGAAGCTAAA
Chloramphenicol resistance gene	ATGGAGAAAAAAATCACTGGATATACCACCGTTGATATA TCCCAATGGCATCGTAAAGAACATTTTGAGGCATTTCAGT CAGTTGCTCAATGTACCTATAACCAGACCGTTCAGCTGGA TATTACGGCCTTTTTAAAGACCGTAAAGAAAAATAAGCA CAAGTTTTATCCGGCCTTTATTCACATTCTTGCCCGCCTGA TGAATGCTCATCCGGAATTCCGTATGGCAATGAAAGACG

	GTGAGCTGGTGATATGGGATAGTGTTACCCCTTGTTACAC CGTTTTCCATGAGCAAACGTAAACGTTTTTCATCGCTCTGG AGTGAATACCACGACGATTTCCGGCAGTTTCTACACATAT ATTCGCAAGATGTGGCGTGTTACGGTGAAAACCTGGCCT ATTTCCCTAAAGGGTTTATTGAGAATATGTTTTTCGTCTCA GCCAATCCCTGGGTGAGTTTCACCAGTTTTGATTAAACG TGGCCAATATGGACAACCTTCTTCGCCCCCGTTTTACCAT GGGCAAATATTATACGCAAGGCGACAAGGTGCTGATGCC GCTGGCGATTTCAGGTTTCATCATGCCGTCTGTGATGGCTTC CATGTCGGCAGAATGCTTAATGAATTACAACAGTACTGC GATGAGTGGCAGGGCGGGGCGTAA
AmpR promoter	CGCGGAACCCCTATTTGTTTATTTTTCTAAATACATTCAA ATATGTATCCGCTCATGAGACAATAACCCTGATAAATGCT TCAATAATATTGAAAAAGGAAGAGT
Ampicillin resistance gene	ATGAGTATTCAACATTTCCGTGTCGCCCTTATTCCCTTTTT TGCGGCATTTTGCTTCTCTGTTTTTGCTCACCCAGAAACG CTGGTGAAAGTAAAAGATGCTGAAGATCAGTTGGGTGCA CGAGTGGGTACATCGAACTGGATCTCAACAGCGGTAAG ATCCTTGAGAGTTTTTCGCCCCGAAGAACGTTTTCCAATGA TGAGCACTTTTAAAGTTCTGCTATGTGGCGCGGTATTATC CCGTATTGACGCCGGGCAAGAGCAACTCGGTCGCCGCAT ACACTATTCTCAGAATGACTTGGTTGAGTACTCACCAGTC ACAGAAAAGCATCTTACGGATGGCATGACAGTAAGAGAA TTATGCAGTGCTGCCATAACCATGAGTGATAAACTGCGG CCAACCTACTTCTGACAACGATCGGAGGACCGAAGGAGC TAACCGCTTTTTTGACACAACATGGGGGATCATGTAACCTCG CCTTGATCGTTGGGAACCGGAGCTGAATGAAGCCATACC AAACGACGAGCGTGACACCACGATGCCTGCAGCAATGGC AACAAACGTTGCGCAAACCTATTAACCTGGCGAACTACTTACT CTAGCTTCCCGGCAACAATTAATAGACTGGATGGAGGCG GATAAAGTTGCAGGACCACTTCTGCGCTCGGCCCTTCCGG CTGGCTGGTTTATTGCTGATAAATCTGGAGCCGGTGAGCG TGGGTCTCGCGGTATCATTGCAGCACTGGGGCCAGATGGT AAGCCCTCCCGTATCGTAGTTATCTACACGACGGGGAGTC AGGCAACTATGGATGAACGAAATAGACAGATCGCTGAGA TAGGTGCCTCACTGATTAAGCATTGGTAA
LacI promoter	GACACCATCGAATGGCGCAAAACCTTTTCGCGGTATGGCA TGATAGCGCCCGGAAGAGAGTCAATTCAGGGTGGTGAAT

LacI gene sequence	GTGAAACCAGTAACGTTATACGATGTCGCAGAGTATGCC GGTGTCTCTTATCAGACCGTTTCCCGCGTGGTGAACCAGG CCAGCCACGTTTCTGCGAAAACGCGGGAAAAAGTGGAAG CGGCGATGGCGGAGCTGAATTACATTCCCAACCGCGTGG CACAACAACCTGGCGGGCAAACAGTCGTTGCTGATTGGCG TTGCCACCTCCAGTCTGGCCCTGCACGCGCCGTCGCAAAT TGTCGCGGCGATTAAATCTCGCGCCGATCAACTGGGTGCC AGCGTGGTGGTGTTCGATGGTAGAACGAAGCGGCGTCGAA GCCTGTAAAGCGGCGGTGCACAATCTTCTCGCGCAACGC GTCAGTGGGCTGATCATTAATACTATCCGCTGGATGACCAGG ATGCCATTGCTGTGGAAGCTGCCTGCACTAATGTTCCGGC GTTATTTCTTGATGTCTCTGACCAGACACCCATCAACAGT ATTATTTTCTCCCATGAAGACGGTACGCGACTGGGCGTGG AGCATCTGGTCGCATTGGGTCAACCAGCAAATCGCGCTGTT AGCGGGCCCATTAAGTTCTGTCTCGGCGCGTCTGCGTCTG GCTGGCTGGCATAAATATCTCACTCGCAATCAAATTCAGC CGATAGCGGAACGGGAAGGCGACTGGAGTGCCATGTCCG GTTTTCAACAAACCATGCAAATGCTGAATGAGGGCATCG TTCCCACTGCGATGCTGGTTGCCAACGATCAGATGGCGCT GGGCGCAATGCGCGCCATTACCGAGTCCGGGCTGCGCGT TGGTGCGGATATCTCGGTAGTGGGATACGACGATACCGA AGACAGCTCATGTTATATCCCGCCGTTAACCAACCATCAA CAGGATTTTCGCCTGCTGGGGCAAACCAGCGTGGACCGC TTGCTGCAACTCTCTCAGGGCCAGGCGGTGAAGGGCAAT CAGCTGTTGCCCCGTCTCACTGGTGAAAAGAAAAACCACC CTGGCGCCCAATACGCAAACCGCCTCTCCCCGCGCGTTGG CCGATTCATTAATGCAGCTGGCACGACAGGTTTCCCGACT GGAAAGCGGGCAGTGA
ColE1 origin of replication	tcatgacaaaaatcccttaacgtgagttttcgtccactgagcgtcagaccccgtagaaaagatca aaggatcttcttgagatccctttttctgcgcgtaactctgctgcttgcaaacaaaaaaaccaccgct accagcgggtggtttggttgcggatcaagagctaccaactcttttccgaaggttaactggcttcag cagagcgcagataccaaatactgtccttctagttagccgtagtaggcccaccacttcaagaact ctgtagcaccgcctacatacctcgtctgctaactctgttaccagtggctgctgccagtggcgat aagtcgtgtcttaccgggttgactcaagacgatagttaccggataagggcgagcggctcgggc tgaacgggggggttcgtgcacacagcccagcttgagcgaacgacctacaccgaactgagata cctacagcgtgagctatgagaaagcgcacgcttcccgaaggagaaaggcggacaggtat ccggtgaagcggcagggtcggaacaggagagcgcacgagggagcttccaggggaaacgc ctggtatctttatagtcctgtcgggttcgccacctctgacttgagcgtcgatttttgtgatgctcgtc agggggggcgagcctatggaaaaacgccagcaacgcggcc

ROP coding sequence	GTGACCAAACAGGAAAAAACCGCCCTTAACATGGCCCCGCTTTATCAGAAGCCAGACATTAACGCTTCTGGAGAAACTCAACGAGCTGGACGCGGATGAACAGGCAGACATCTGTGAATCGCTTCACGACCACGCTGATGAGCTTTACCGCAGCTGCCTCGCGCGTTTCGGTGATGACGGTGAAAACCTCTGA
F1 origin of replication	ACGCGCCCTGTAGCGGCGCATTAAAGCGCGGCGGGTGTGGTGGTTACGCGCAGCGTGACCGCTACACTTGCCAGCGCCCTAGCGCCCGCTCCTTTTCGCTTTCTTCCCTTCCCTTTCTCGCCACGTTTCGCCGGCTTTCCCCGTCAAGCTCTAAATCGGGGGCTCCCTTTAGGGTTCCGATTTAGTGCTTTACGGCACCTCGACCCCAAAAACTTGATTAGGGTGATGGTTCACGTAGTGGGCCATCGCCCTGATAGACGGTTTTTTCGCCCTTTGACGTTGGAGTCCACGTTCTTTAATAGTGGACTCTTGTTCCAAACTGGAACAACACTCAACCCTATCTCGGTCTATTCTTTTGATTTATAAGGGATTTTGCCGATTTTCGGCCTATTGGTTAAAAAATGAGCTGATTTAACAAAAATTTAACGCGAATTTTAACAAAATAATAACGTTTACAATTT
proD promoter	CACAGCTAACACCACGTCGTCCCTATCTGCTGCCCTAGGTCTATGAGTGGTTGCTGGATAACTTTACGGGCATGCATAAGGCTCGTATAATATATTCAGGGAGACCACAACGGTTTCCCTCTACAAATAATTTTGTTTAACTTT
Native tetO promoter	tccctatcagtgatagaga
TetR repressor	atgatgtctagattagataaaaagtaaagtgattaacagcgcatagagctgcttaatgaggtcggaatcgaaggtttaacaacccgtaaactcgccagaagctaggtgtagagcagcctacattgtattgcatgtaaaaaataagcgggctttgctcgagccttagccattgagatgtagataggcaccatactcacttttgcccttagaaggggaaagctggcaagatttttacgtaataacgctaaaagttagatgtgctttactaagtcacgcgatggagcaaaagtacatttaggtacacggcctacagaaaaacagtatgaaactctcgaaaatcaattagccttttatgccaacaagggttttactagagaatgcattatgcactcagcgctgtggggcattttactttagggtgcgtattggaagatcaagagcatcaagtcgctaaagaagaaagggaacacctactactgatagtatgccgcatattacgacaagctatcgaaattttgatcaccaaggtgcagagccagccttcttattcgccctgaattgatcatatgcggattagaaaaacaacttaaatgtgaaagtggtcttaa
Kan promoter	gctggggcgccctctgtaagggtgggaagccctgcaa

Kan resistance gene	attgaacaagatggattgcacgcaggttctccggccgcttgggtggagaggctattcggctatg actgggcacaacagacaatcggtgctctgatgccgccgtgtccggctgtcagcgcagggg cgcccggttcttttgtcaagaccgacctgtccggtgccctgaatgaactgcaggacgaggcag cgcggtatcgtggctggccacgacgggcgttccttgccgagctgtgctcgacgttgactga agcgggaagggactggctgctattgggcgaagtgccggggcaggatctcctgtcatctcacct tgctcctgccgagaaaagtatccatcatggctgatgcaatgcggcggtgcatacgttgatccg gtacctgcccattcgaccaccaagcgaacatcgcatcgagcgagcacgtactcggatggaa gccggtcttgctgatcaggatgatctggacgaagagcatcaggggctcgcgccagccgaact gttcgccaggctcaaggcgcgatgccgacggcgaggatctcgtcgtgacctatggcgatg cctgcttgccgaatatcatggtggaaaatggccgcttttctggattcatcgactgtggccggctgg gtgtggcggaccgctatcaggacatagcgttggtacctgtgatattgctgaagagcttgccgg cgaatgggctgaccgcttctcgtgctttacggtatcgccgctcccagattcgagcgcacgcct tctatcgcttcttgacgagttcttga
p15A origin	GGATATATTCCGCTTCCTCGCTCACTGACTCGCTACGCTC GGTCGTTTCGACTGCGGCGAGCGGAAATGGCTTACGAACG GGGCGGAGATTTCTGGAAGATGCCAGGAAGATACTTAA CAGGGAAGTGAGAGGGCCGCGGCAAAGCCGTTTTTCCAT AGGCTCCGCCCCCTGACAAGCATCACGAAATCTGACGC TCAAATCAGTGGTGGCGAAACCCGACAGGACTATAAAGA TACCAGGCGTTTCCCCCTGGCGGCTCCCTCGTGCGCTCTC CTGTTCCCTGCCTTTCGGTTTACCGGTGTCATTCCGCTGTTA TGGCCGCGTTTGTCTCATTCCACGCCTGACACTCAGTTCC GGGTAGGCAGTTCGCTCCAAGCTGGACTGTATGCACGAA CCCCCGTTTCAGTCCGACCGCTGCGCCTTATCCGGTAACT ATCGTCTTGAGTCCAACCCGGAAGACATGCAAAAGCAC CACTGGCAGCAGCCACTGGTAATTGATTTAGAGGAGTTA GTCTTGAAGTCATGCGCCGGTTAAGGCTAAACTGAAAGG ACAAGTTTTGGTGACTGCGCTCCTCCAAGCCAGTTACCTC GGTTCAAAGAGTTGGTAGCTCAGAGAACCTTCGAAAAAC CGCCCTGCAAGGCGGTTTTTTCGTTTTTCAGAGCAAGAGAT TACGCGCAGACCAAAACGATCTCAAGAAGATCATCTTAT TAATCAGATAAAATATTTCTAGATTTTCAGTGCAATTTATC TCTTCAAATGTAGCACCTGAAGTCAGCCCCATACGATATA AGTTGTTA
rrnB terminator	T1 GGCATCAAATAAAACGAAAGGCTCAGTCGAAAGACTGGG CCTTTCGTTTTATCTGTTGTTTGTGCGGTGAACGCTCTCCTG AGTAGGACAAATCCGCCGCCCTAGA

APPENDIX B

List of primers used in this thesis

Table B. Primers used to amplify the specified region for corresponding cloning (nucleotide sequences provided from 5' to 3')

Cloning of pET22b 6H Mms6 vector	P1	GGTACCATGCACCACCACCACCACGGCGGTAGCGG TGGCGAAATGGAGCGCGAA
	P2	CTCGAGTCATCATGCAAGTGCCTCACGAAG
Cloning of pET22b PelB 6H Mms6 160N Ag43 vector	P3	GGATCCGGCGAAATGGAGCGCGAAGGG
	P4	CTTAAGTGCAAGTGCCTCACGAAGCTC
Cloning of pZE PROD	P5	TCATTTTCGCCAGATATCGACGTCCTCGAGcaaaaaaccctca agaccg

6H Mms6 T7 Ter	P6	ataattttgtttaactttAAGGAGgggatccATGCACCACCACCACCAC
Cloning of pZE PROD 6H Mms6 EfeU T7 Ter	P7	aaaaaaAAGCTTTCATCATGCAAGTGCGTCACG
	P8	aaaaaactcgagGACGTCGATATCTGG
	P9	GAGCTTCGTGACGCACTTGCATGATGAaagcttaaggagggtacc atgtttgtcc
Cloning of pZs mPROD 6H Mms6 EfeU	P10	atcgatcgggccctgaggcctgcagggatccaagcttttacgcgagcgagacgctgt
	P11	TGCTAGCtactagagAAAGAGGAGAAAGGTACCATGCACCA CCACCACCAC
Cloning of pZs mPROD EncFtn 6H Mms6 EfeU	P12	GCTAGCtactagagAAAGAGGAGAAAGGTACCATGGCGCA GTCCAGCAATAGC
	P13	ACCGCCGTGGTGGTGGTGGTGGTGCATctcgagTTCCAGCG CCGCAGCCAGTTT
	P14	AAAAAAGGTACCTTTCTCCTCTTTctctagTAGCTAGC
	P15	AAAAAActcgagATGCACCACCACCACCAC

Cloning of pET22b EncFtn 6H Mms6 EfeU	P16	cccctctagaATTAAAGAGGAGAAAGGatCCATGGCGCAGTC CAGCAATA
	P17	ggatctcagtggtggtggtggtggtgctGCagttacgcggagcgagacgc
Cloning of pET22b EncFtn 6H Mms6 MBP6 EfeU	P18	GACGTCGATATCTGGCGAAAATGAG
	P19	GGGCTAATGGTCTGCACGGTGCTCAGGCTGCCGCTGCC GCCTGCAAGTGCCTCACGAAG
	P20	TCCTTAGCTCCTGAAAATCTC
	P21	CTGAGCACCGTGCAGACCATTAGCCCGAGCAACCATTG ATGAaagcttaaggagggtacc
Cloning of pZa ntetO 6H 2RS15d 160N Ag43 AmpR	P22	ATATCGACGTCTCACACTGGCTC
	P23	TTTGATATCGAGCTCGCTTG
	P24	cccgaagtgagccagtgtaGACGTCGATATCGCGGAACCCCTAT TTGT
	P25	TCAACAGGAGTCCAAGCGAGCTCGATATCAAATTACCA ATGCTTAATCAGTGAGGC
Cloning of pZs	P26	cgcacgatcgggccctgaggcctgcagggatccaagctttattgtacagctcatccatgc

proD mCherry kan	P27	agggttcccaaccttaccagagggcgccccagctggcaattccgacgtcCACAGCT AACACCACGTC
	P28	aagcttggatccctgcag
	P29	gacgtcgggaattgccagctg
Cloning of pZa proD mCherry ntetO 6H 2Rs15d Ag43 ampR	P30	TGAGCGAGGAAGCGGAATATATCCCCTAGGCACAGCTA ACACCACGTCGT
	P31	GCCACACTGAATGTGACCTTCTGAACGCGTcaaaaaaccctca agacce
pBAD BBaJ231 16 mtrA cloning	P32	cagtcctagggactatgctagcaaagaggagaaaggtaccATGAAGAACTGCC TAAAAatg
	P33	CTCAAGACGATAGTTACCGG
	P34	tctttgctagcatagtcctaggactgagctagctgtcaactctgaatggcgggagta
	P35	CCGACAGGACTATAAAGATACC

APPENDIX C

Plasmid maps

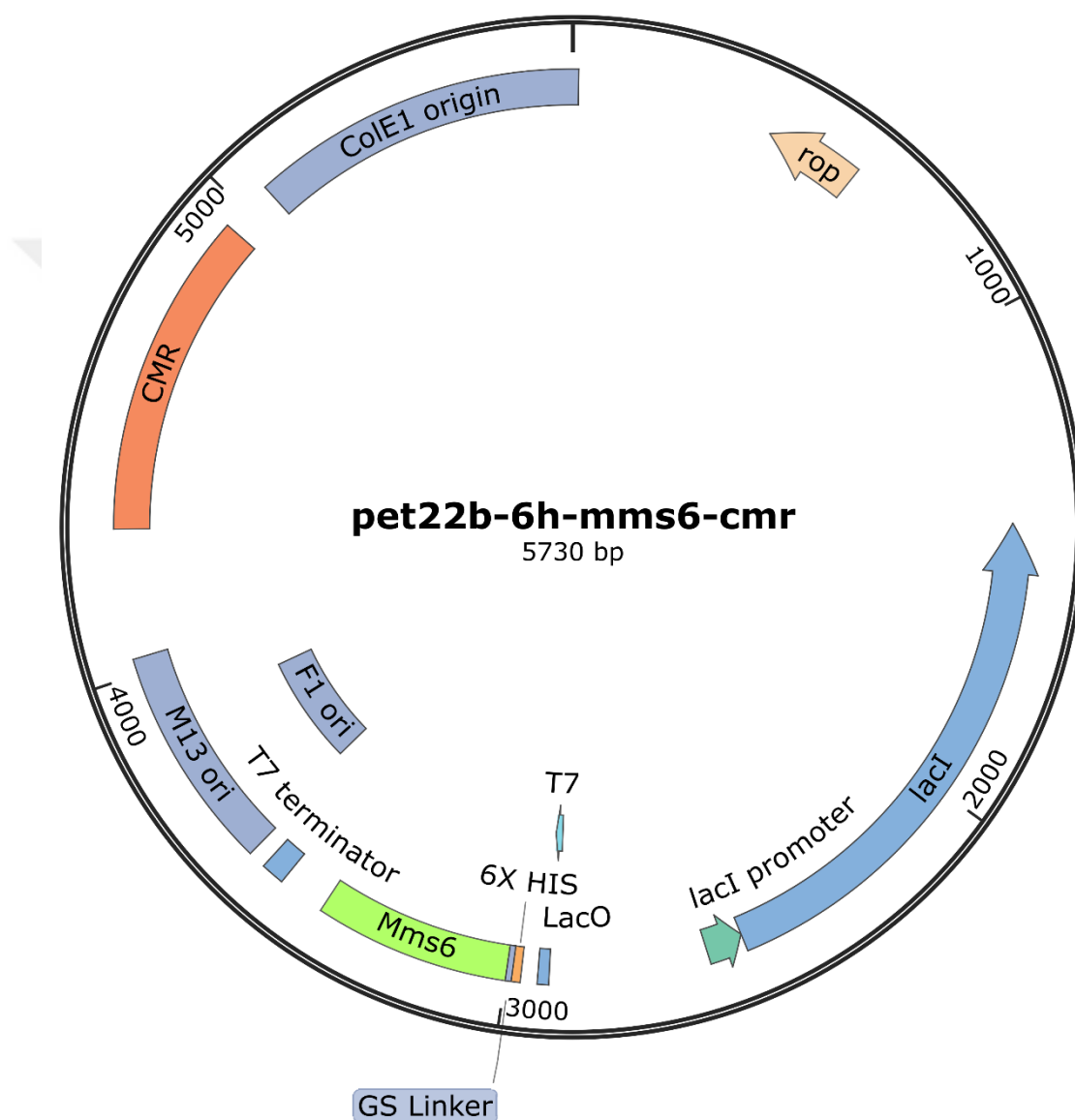


Figure C 1. Schematic representation of pET22b 6H Mms6 cmr

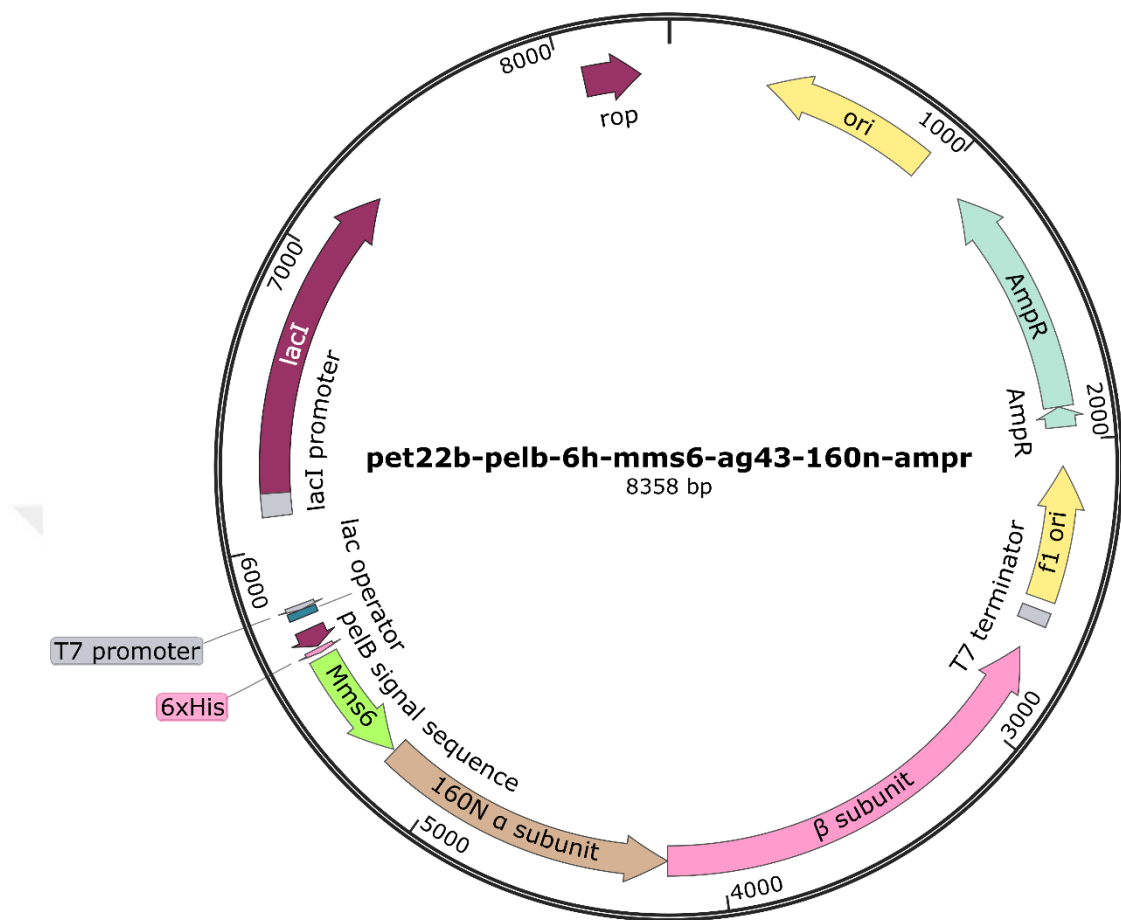


Figure C 2. Schematic representation of pET22b PelB 6H Mms6 160N Ag43

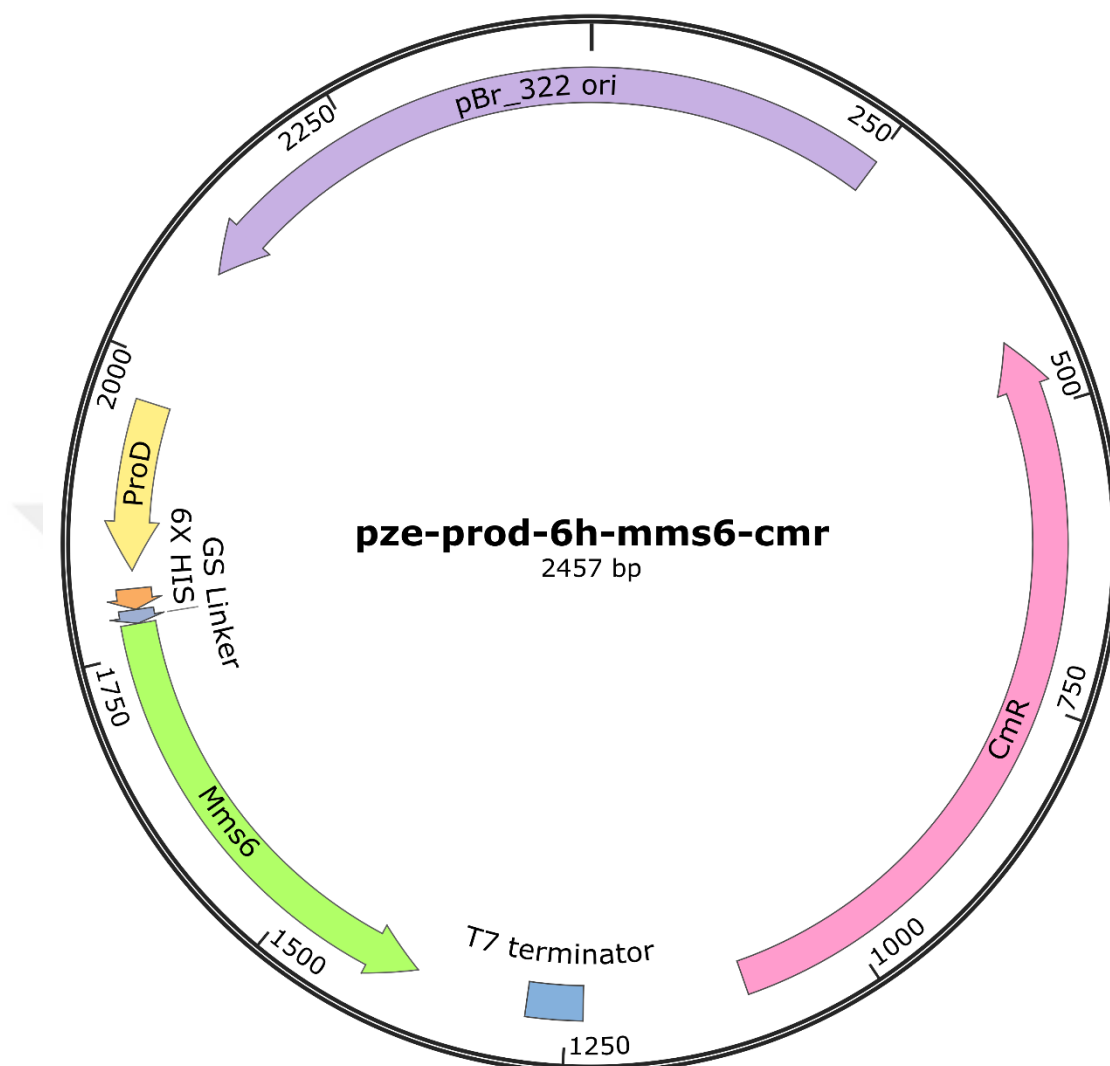


Figure C 3. Schematic representation of pZE PROD 6H Mms6 T7 Ter

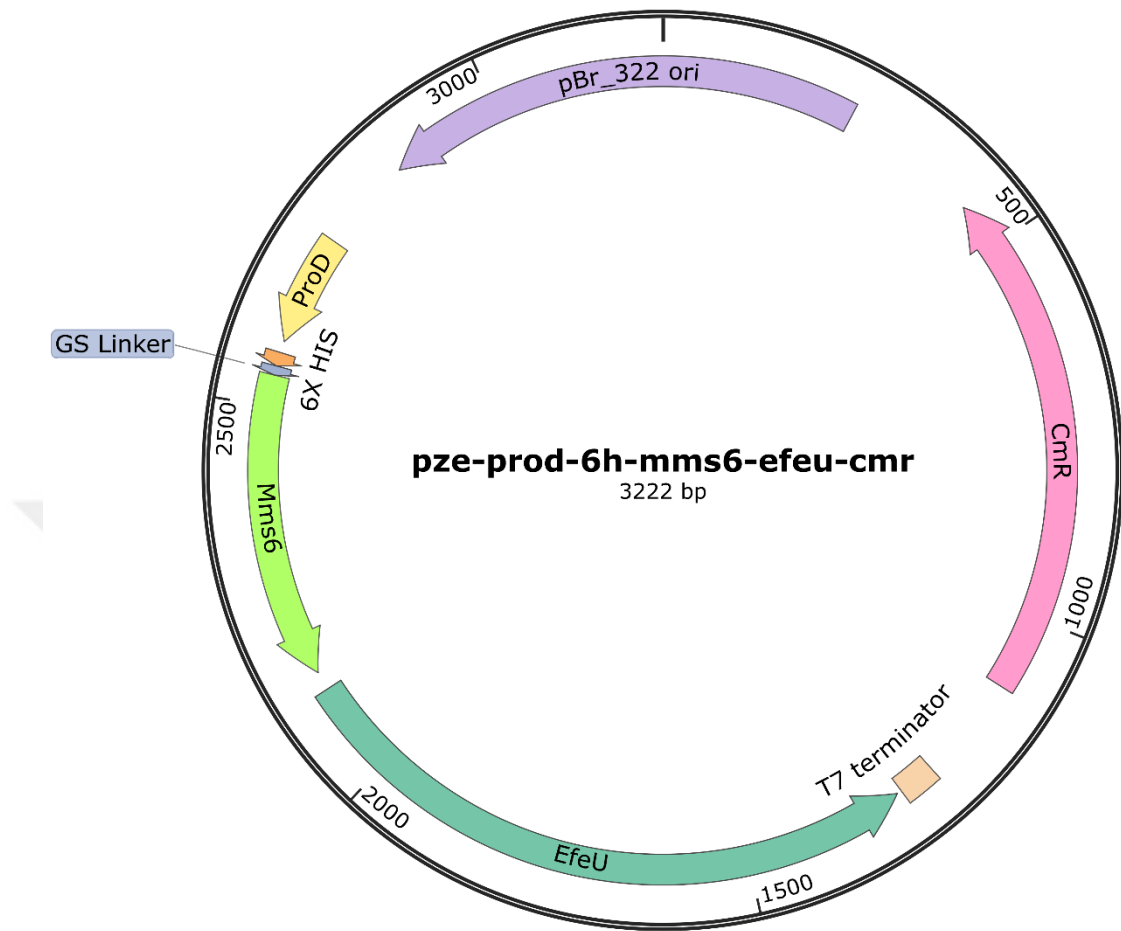


Figure C 4. Schematic representation of pZE PROD 6H Mms6 EfeU T7 Ter

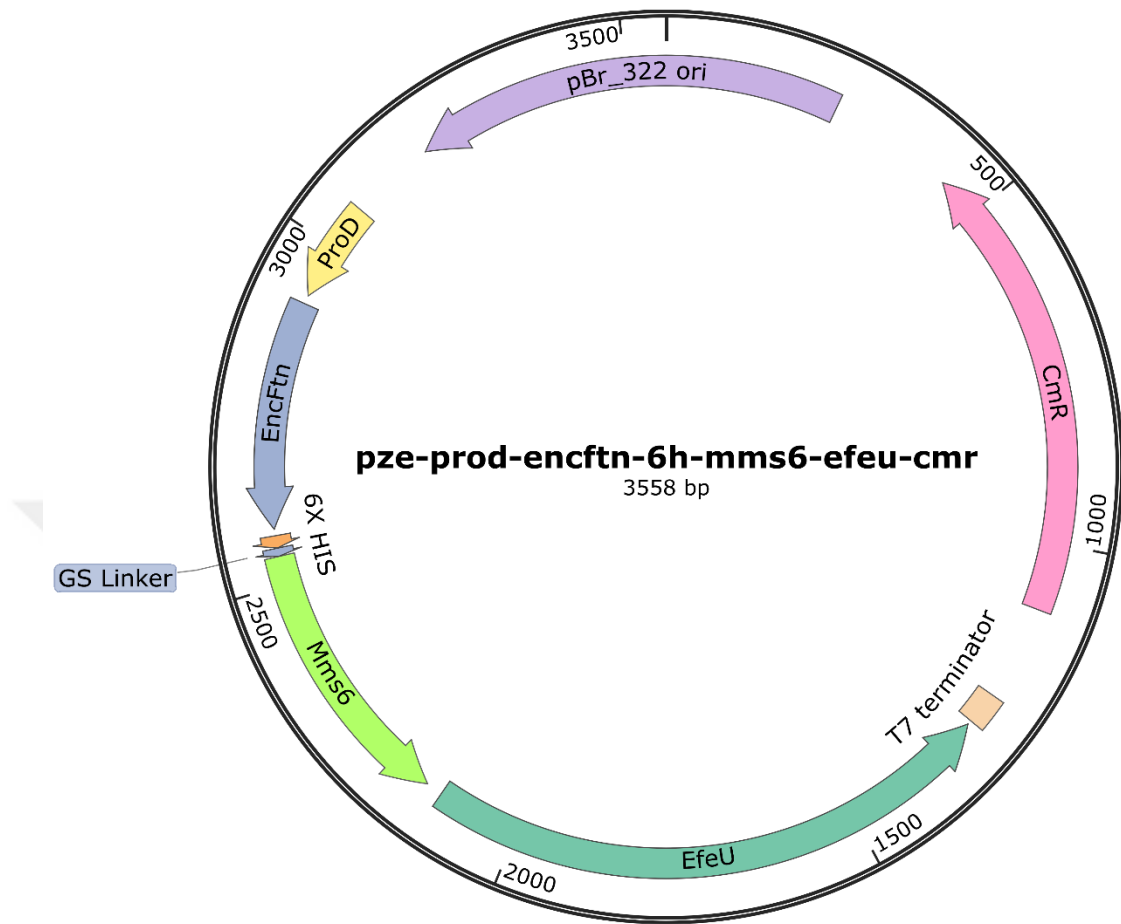


Figure C 5. Schematic representation of pZE PROD EncFtn 6H Mms6 EfeU T7 Ter

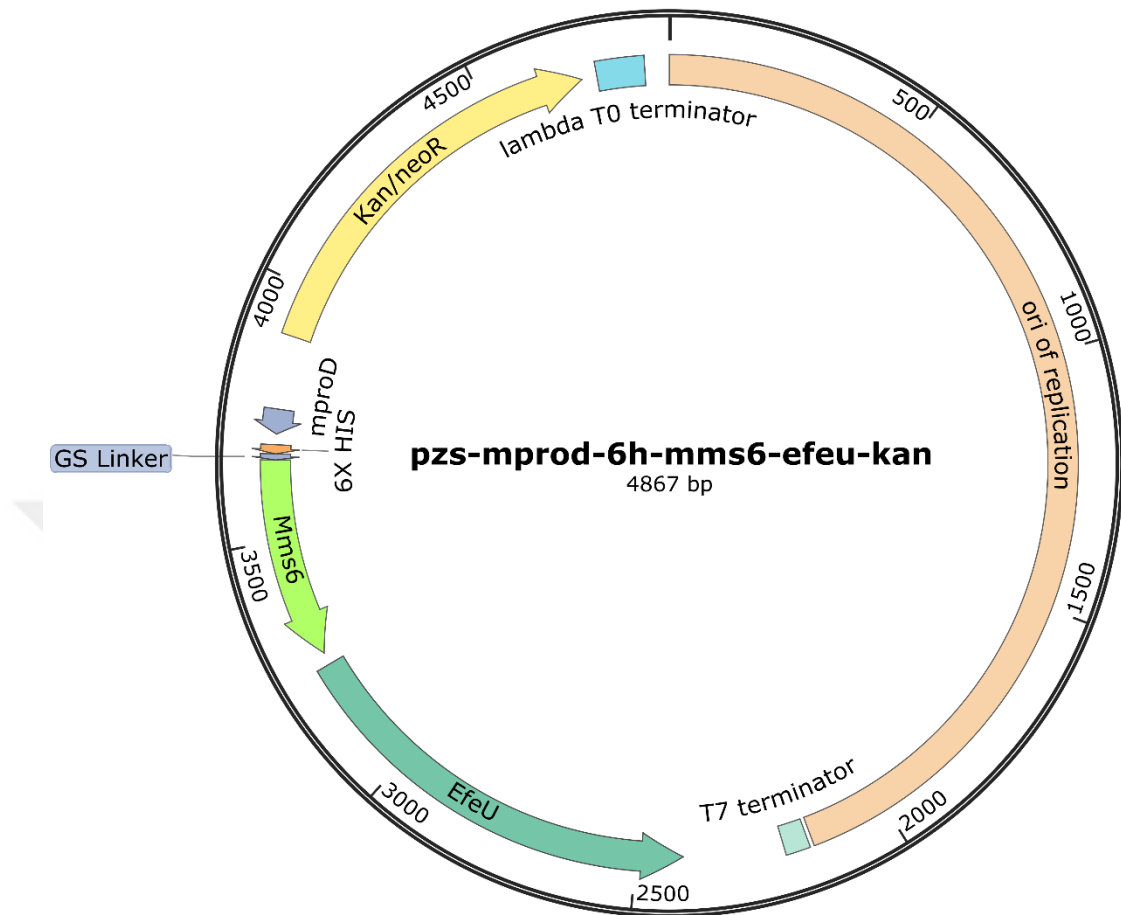


Figure C 6. Schematic representation of pZs mPROD 6H Mms6 EfeU

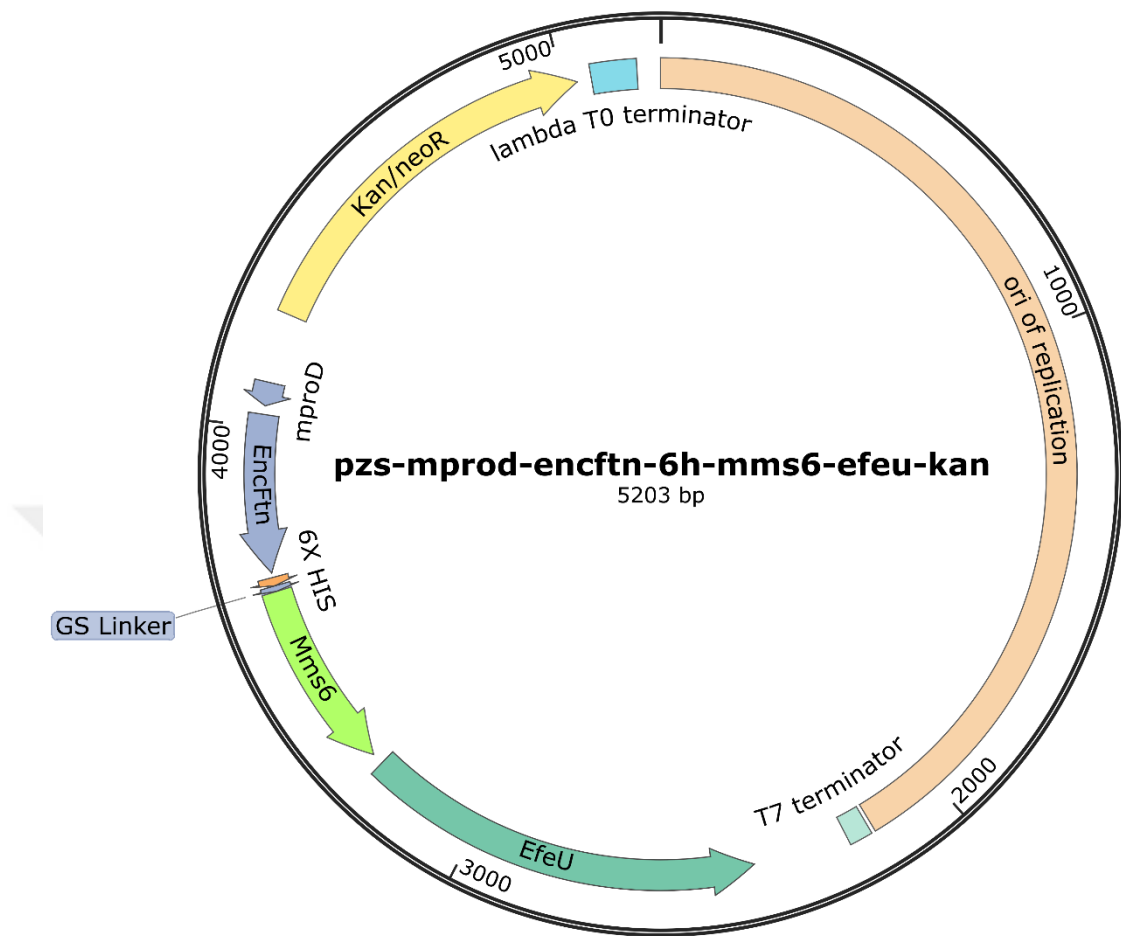


Figure C 7. Schematic representation of pZs mPROD EncFtn 6H Mms6 EfeU

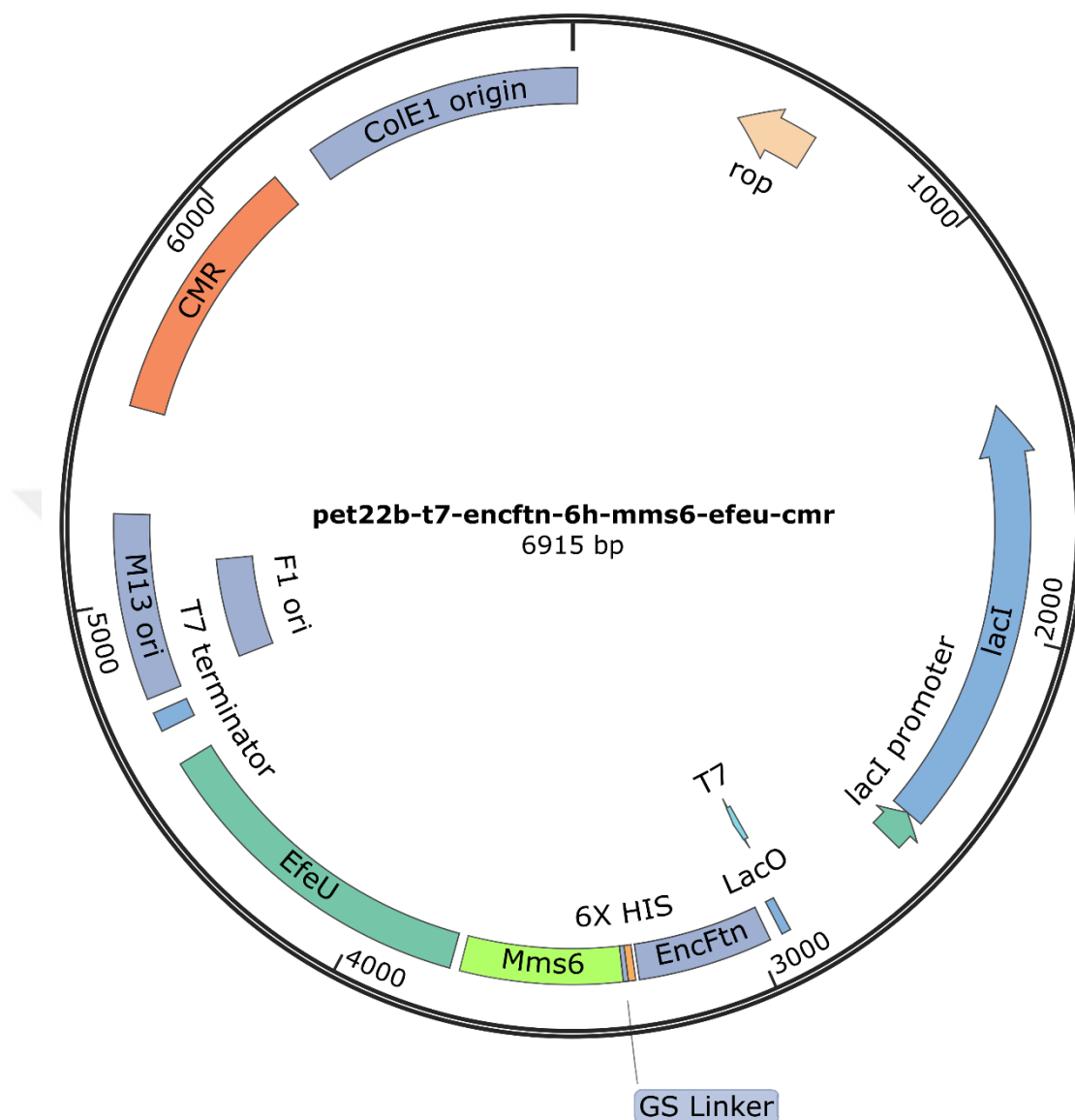


Figure C 8. Schematic representation of pET22b EncFtn 6H Mms6 EfeU

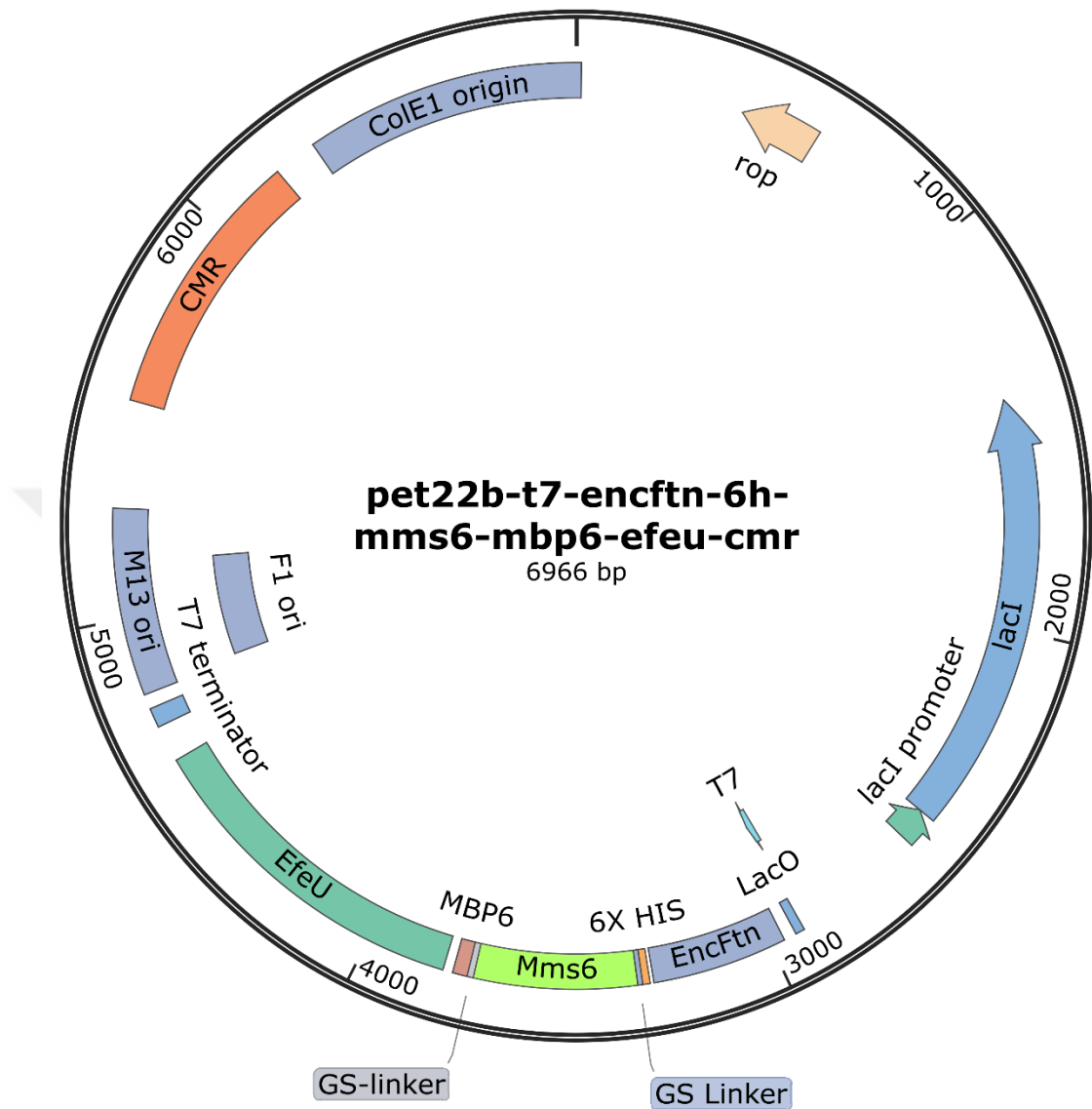


Figure C 9. Schematic representation of pET22b EncFtn 6H Mms6 MBP6 EfeU

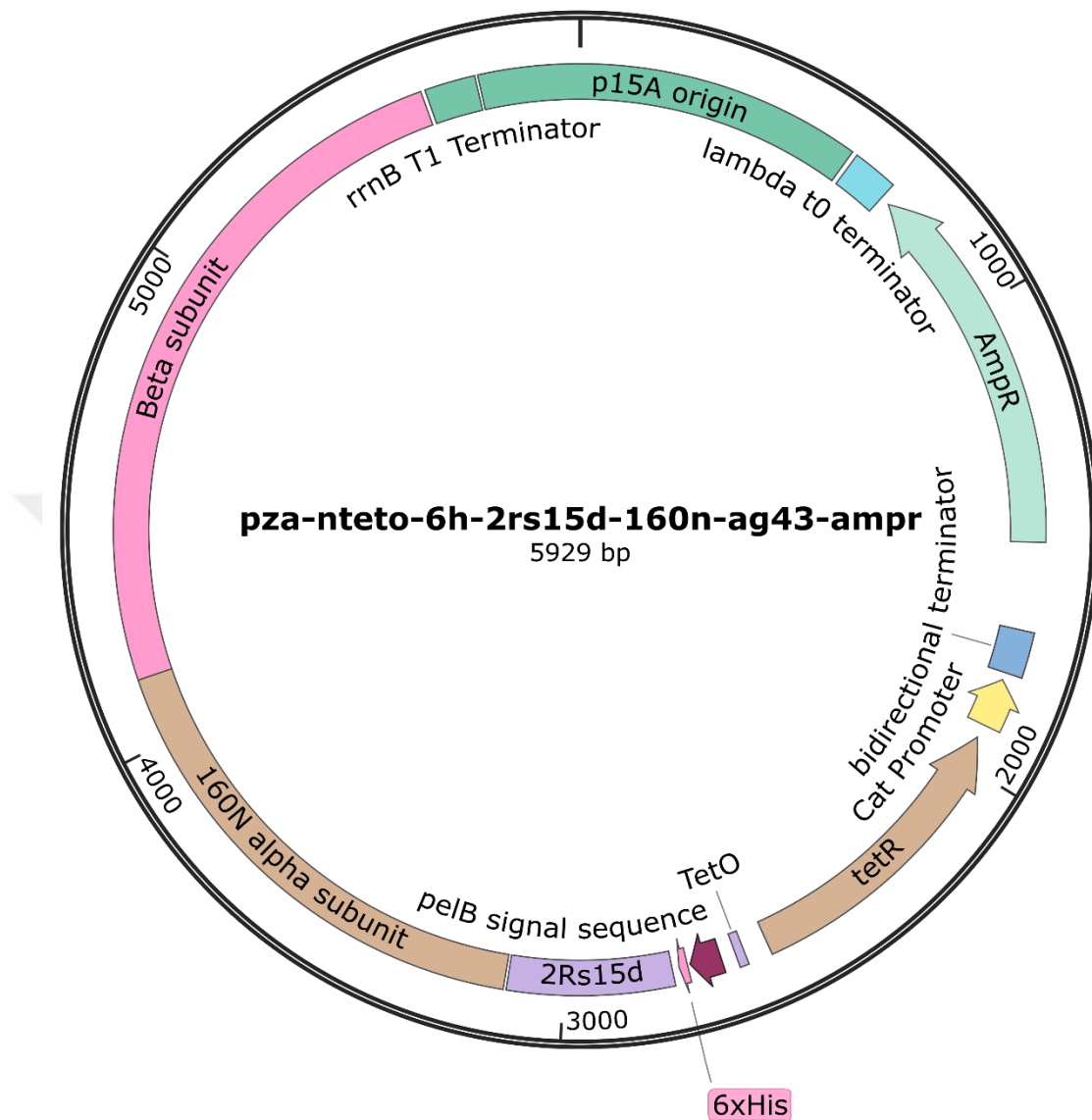


Figure C 10. Schematic representation of pZa ntetO 6H 2RS15d 160N Ag43 AmpR

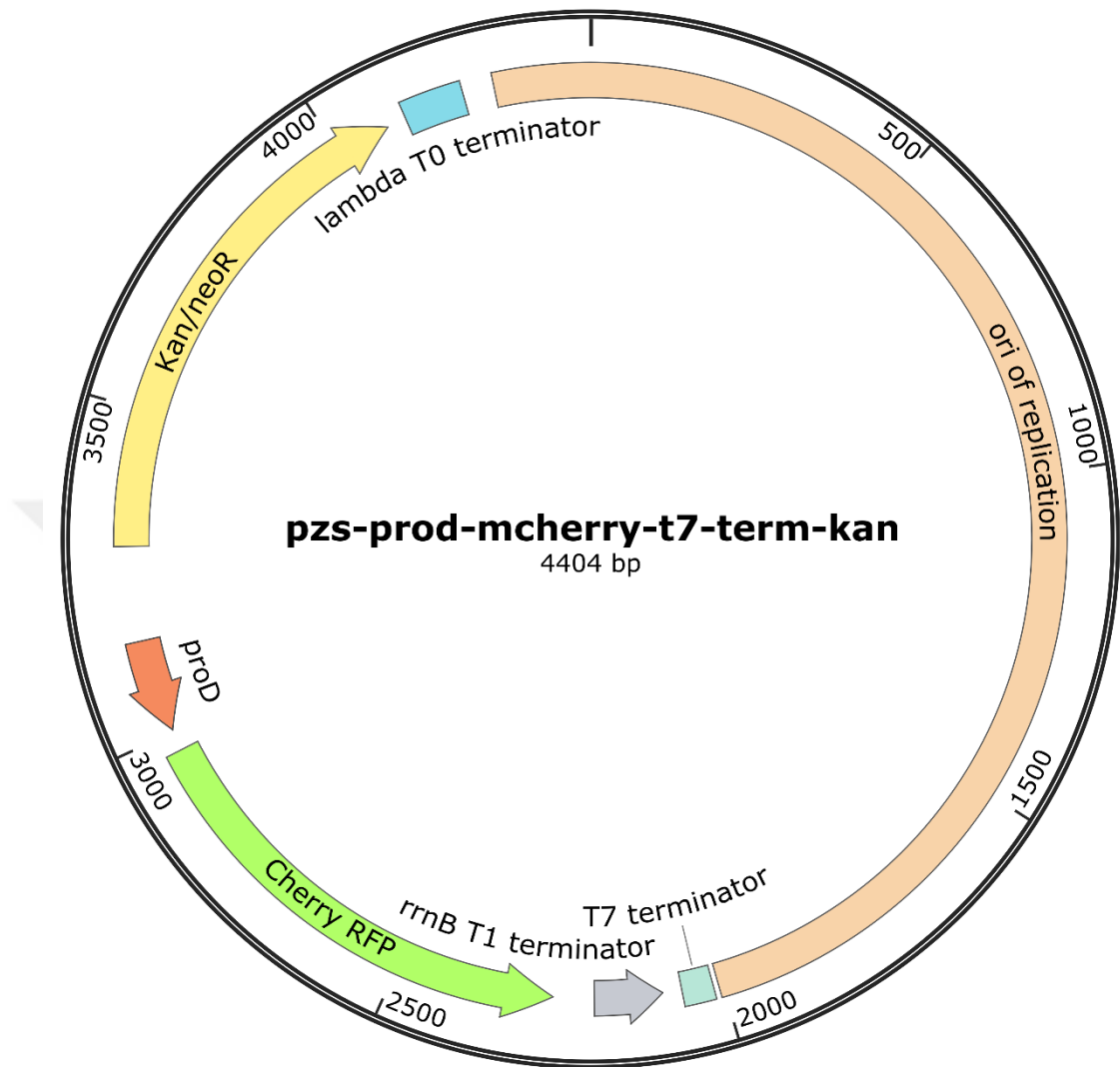


Figure C 11. Schematic representation of pZs PROD mCherry kan

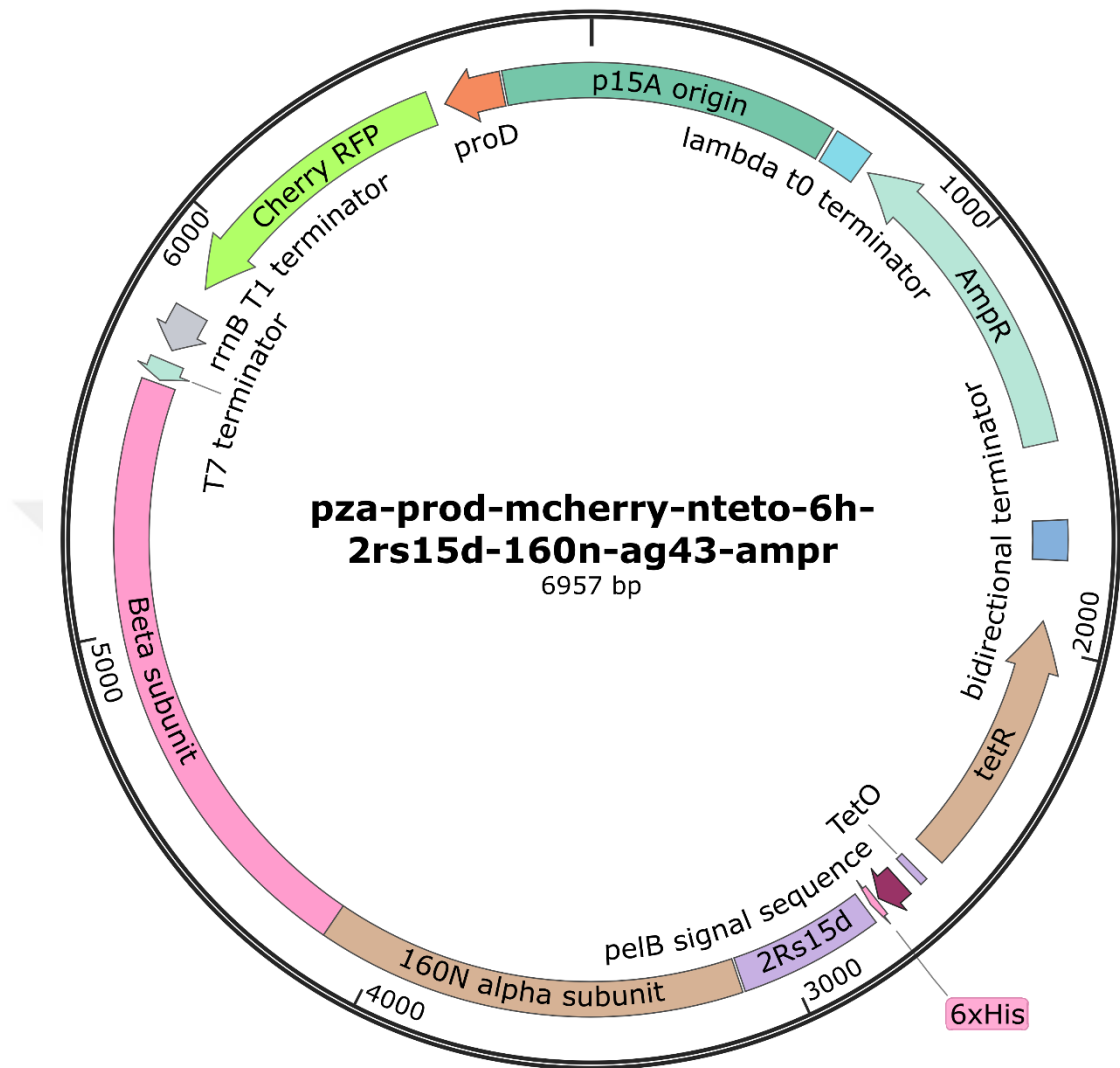


Figure C 12. Schematic representation of pZa PROD mCherry ntetO 6H 2RS15d 160N Ag43 AmpR

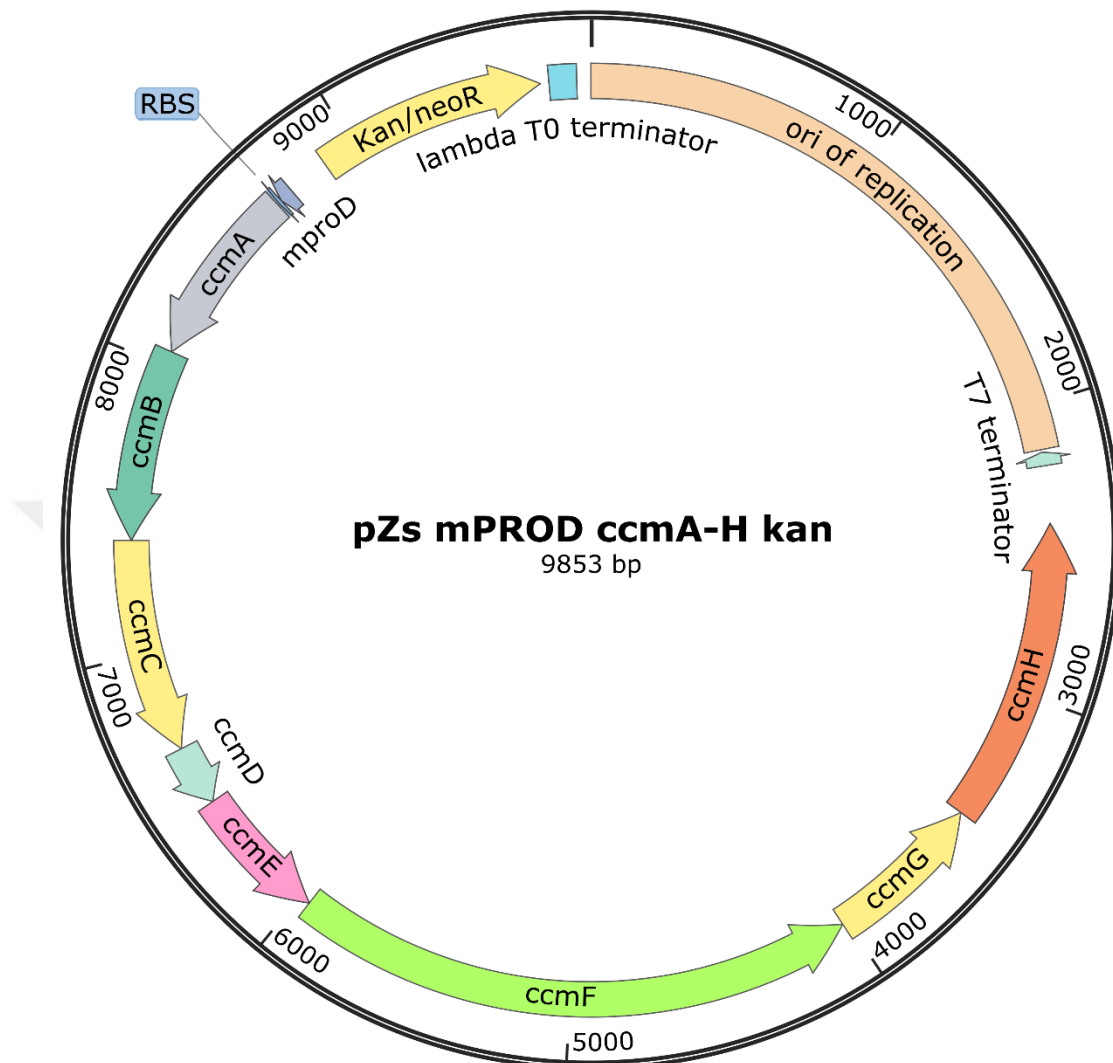


Figure C 13. Schematic representation of pZs mPROD ccmA-H kan cloning

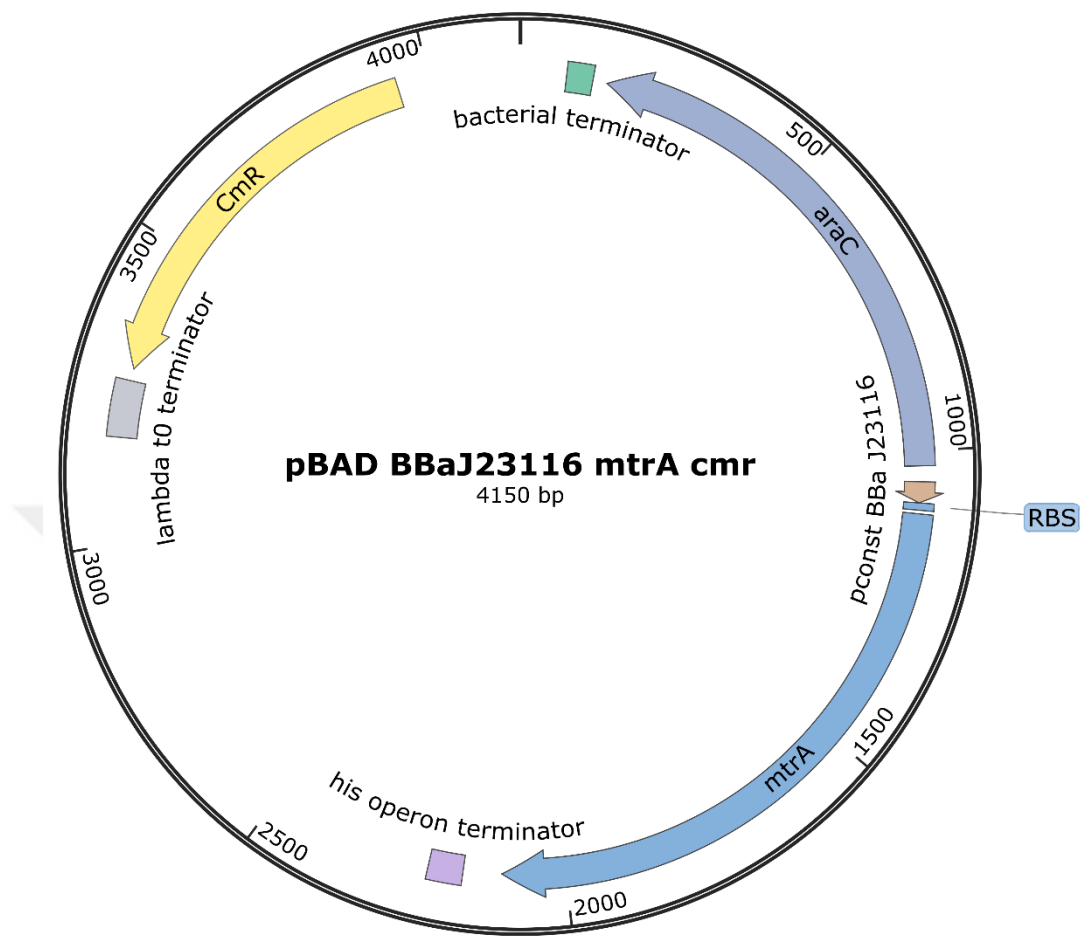


Figure C 14. Schematic representation of pBAD BBaJ23116 mtrA cmr

APPENDIX D

Sequencing results (Sanger or Next Generation Sequencing (NGS))

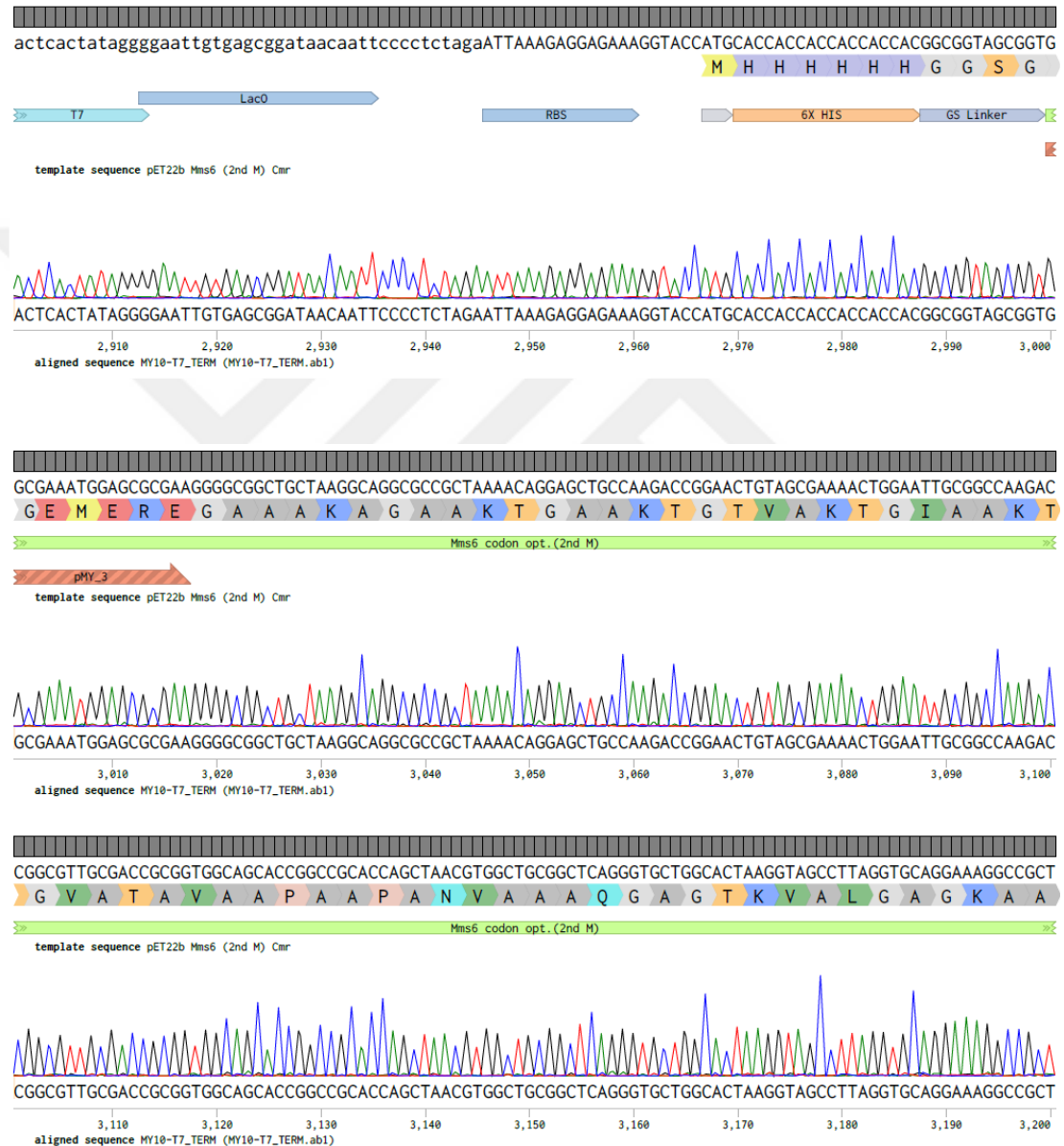
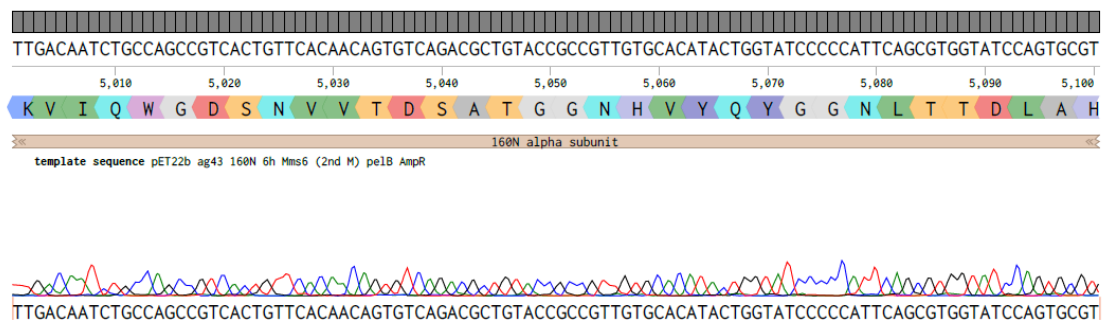
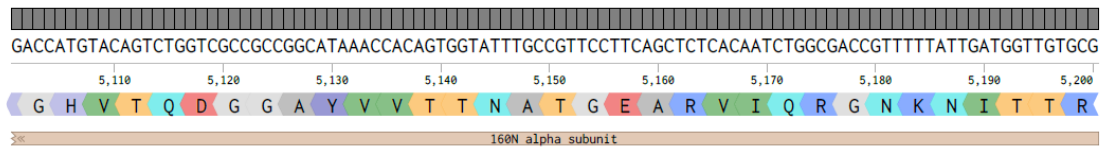


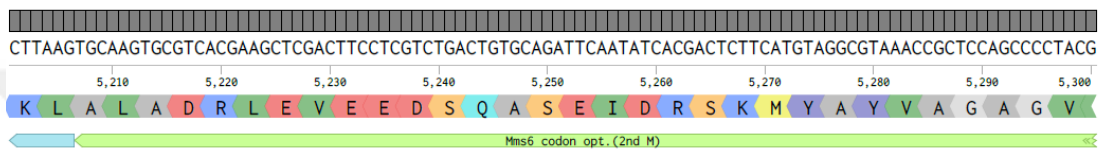
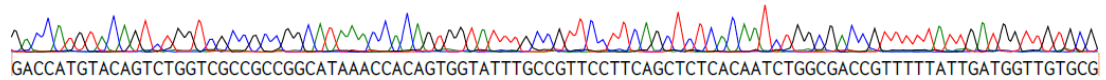


Figure D 1. Sanger DNA sequence verification results for pET22b 6H Mms6 cloning.

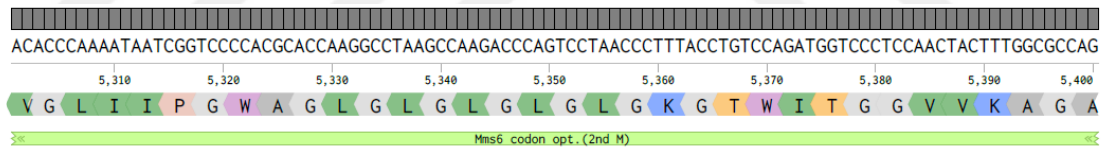
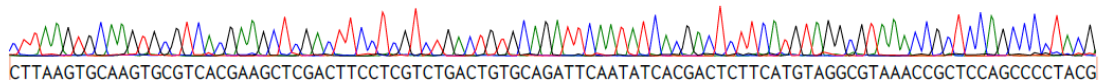




template sequence pET22b ag43 160N 6h Mms6 (2nd M) pe1B AmpR



template sequence pET22b ag43 160N 6h Mms6 (2nd M) pe1B AmpR



template sequence pET22b ag43 160N 6h Mms6 (2nd M) pe1B AmpR

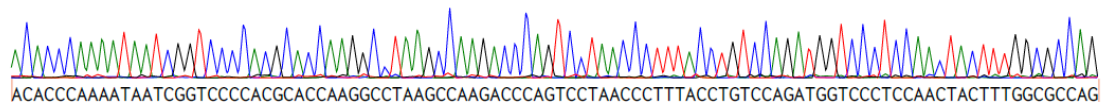
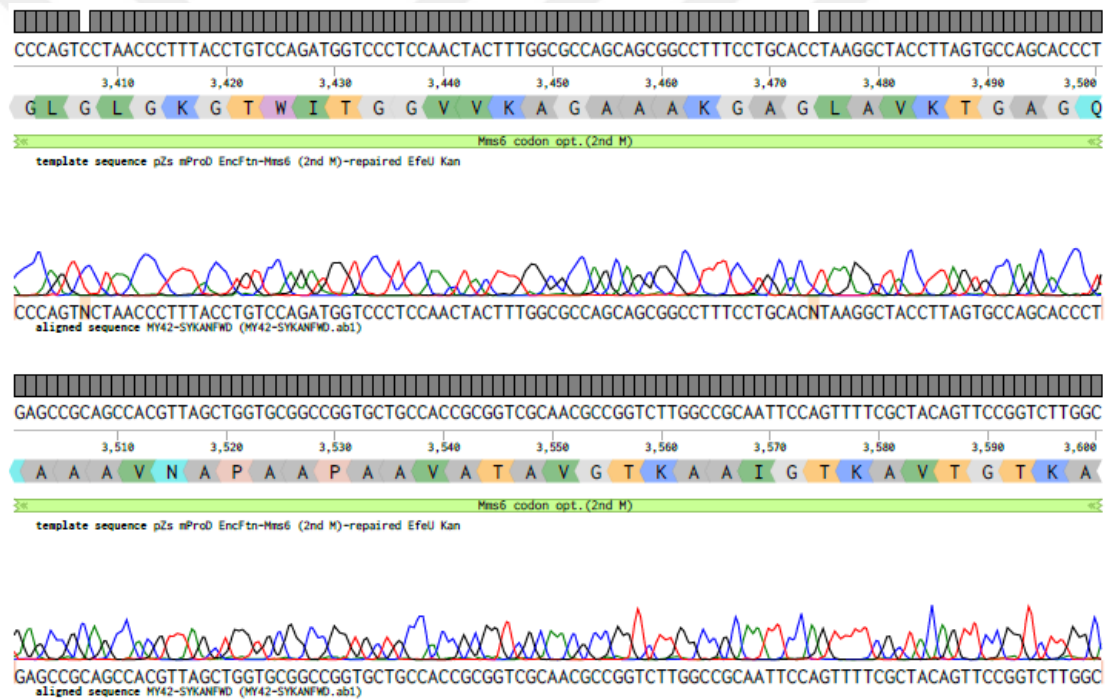


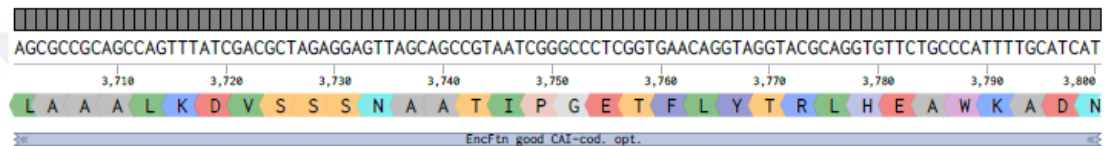
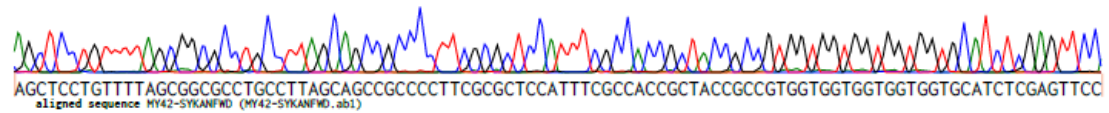
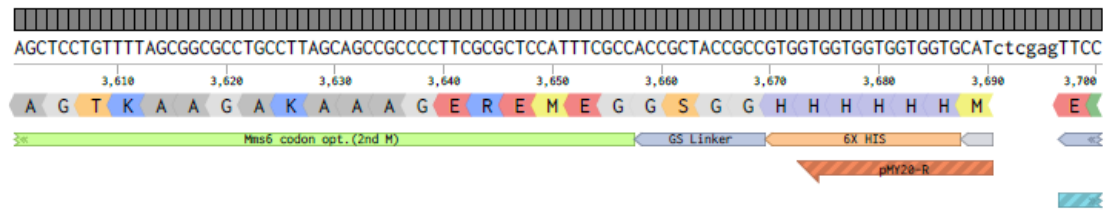


Figure D 2. Sanger DNA sequence verification results for pET22b PelB 6H Mms6 160N Ag43 cloning.



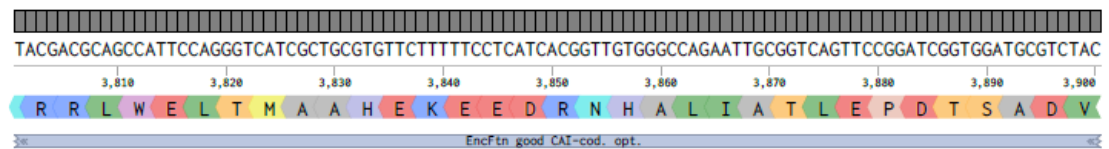
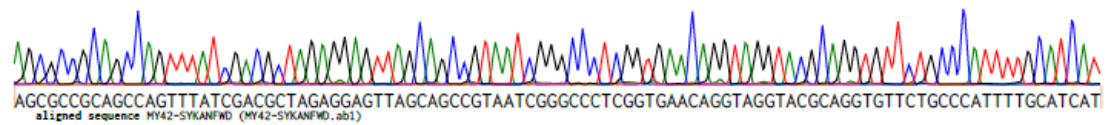
Figure D 3. Sanger DNA sequence results showing nucleotide deletion for pZE proD EncFtn 6H Mms6 EfeU cloning.





pMY18-F

template sequence pZs mProD EncFtn-Mms6 (2nd M)-repaired EfeU Kan



template sequence pZs mProD EncFtn-Mms6 (2nd M)-repaired EfeU Kan

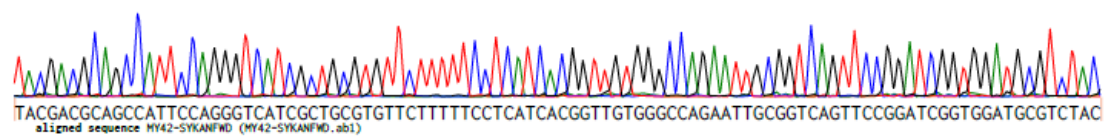




Figure D 4. Sanger DNA sequence verification results for pZs mPROD EncFtn 6H Mms6 EfeU cloning provided with close-up view showing the nucleotides and chromatogram in detail and zoomed-out image indicating nucleotide deletion circled in red.

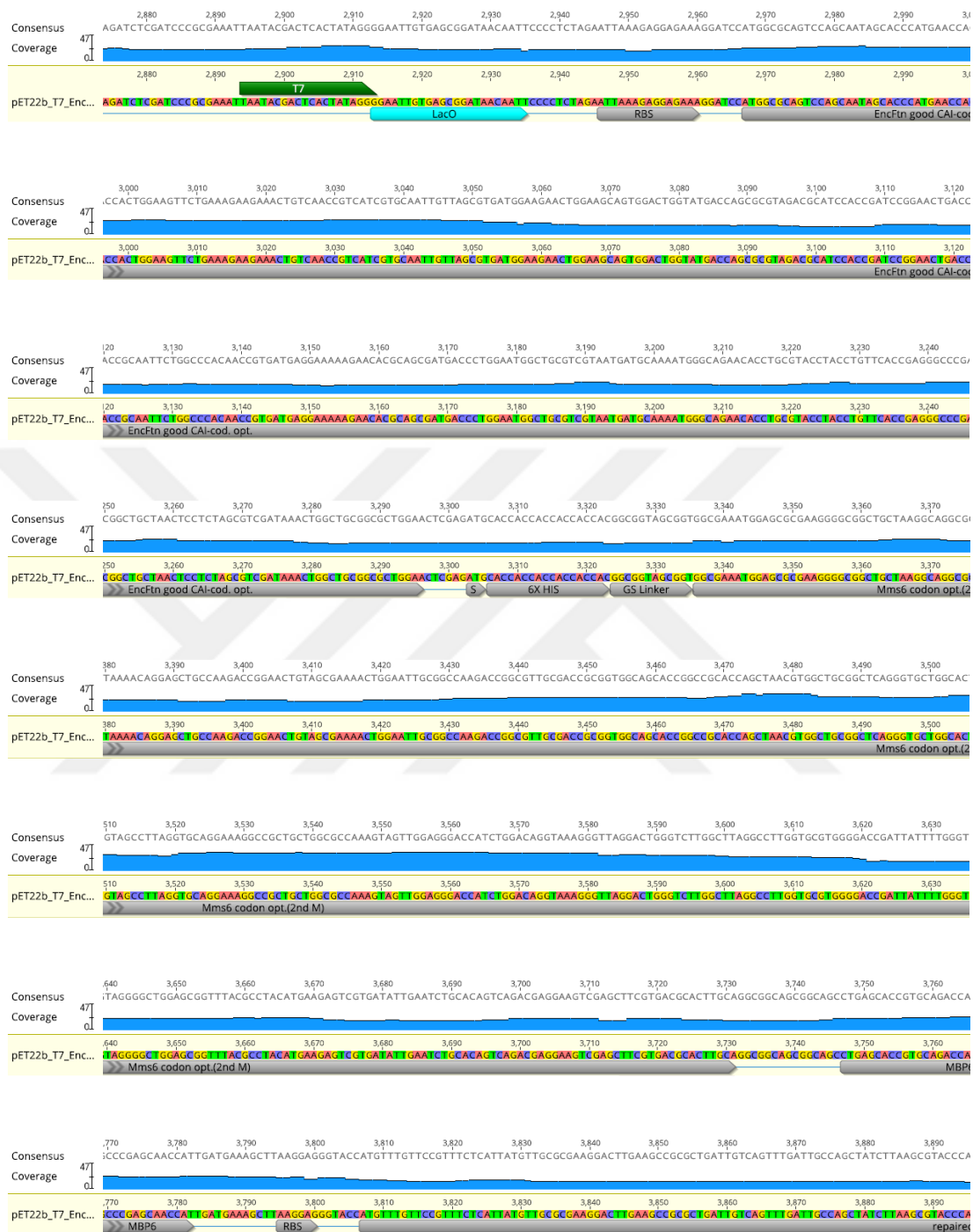




Figure D 6. Next generation sequencing (NGS) verification results for pza ntetO His 2Rs15d Ag43 ampR plasmid. Sequence result was aligned with reference construct.

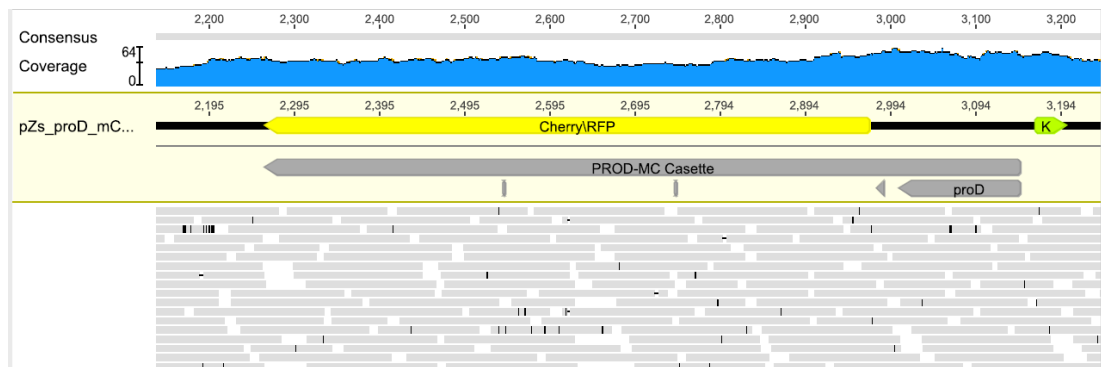
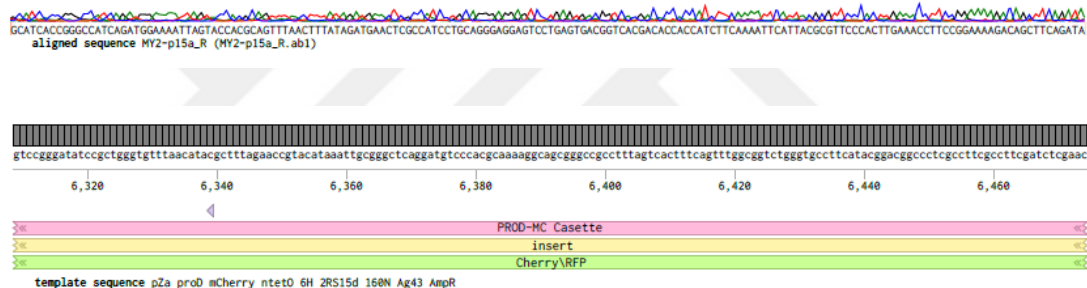
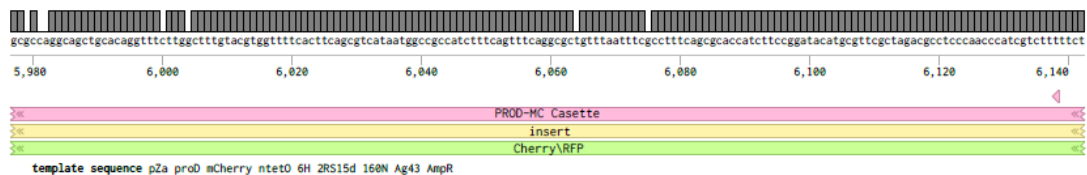


Figure D 7. Next generation sequencing (NGS) verification results for pZs proD mCherry kan plasmid. Sequence result was aligned with reference construct.



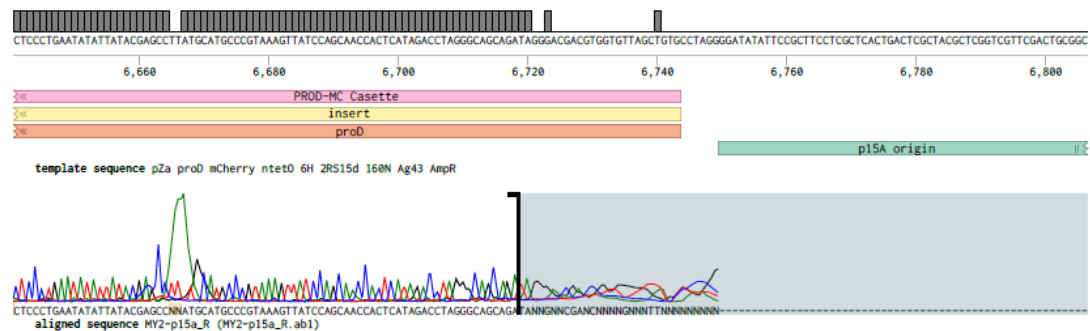


Figure D 8. Sanger DNA sequence verification results for pZa proD mCherry ntetO 6H 2Rs15d Ag43 ampR cloning.

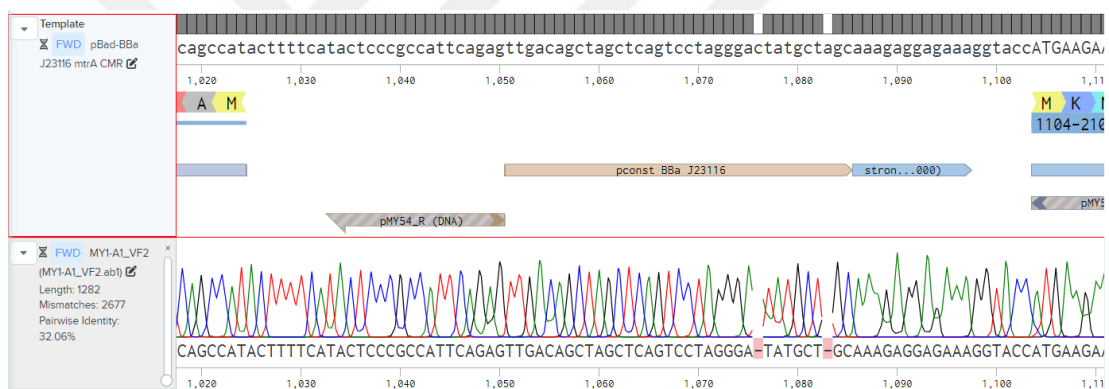


Figure D 9. Sanger DNA sequence verification results for pBAD BBaJ23116 mtrA cmr cloning.

APPENDIX E

Additional reaction recipes and methods

Table E 1. 5× isothermal buffer recipe

Components	Amount
PEG-8000	25%
Tris-HCl	500mM, pH 7.5
MgCl ₂	50mM
DTT	50mM
NAD	5mM
dNTPs	1mM each

Table E 2. Gibson Assembly mix recipe (1.33×)

Components	Amount
Taq ligase (40U/μl)	50μl
5× isothermal buffer	100μl
T5 exonuclease (1U/μl)	2μl
Q5 Hi-Fi DNA polymerase (2U/μl)	6.25μl
Nuclease-free water	216.75μl

Table E 3. Q5 polymerase PCR conditions

STEP	TEMPERATURE	TIME
Initial denaturation	98°C	30sec
Polymerization (25-35 cycles)	98°C 50–72°C 72°C	5–10 sec 10–30 sec 20–30 sec/kb
Final extension	72°C	2min
Hold	4–10°C	

Table E 4. Restriction digestion reaction

Components	Amount
CutSmart (10×)	2μl
Vector DNA	1000-2000ng
Enzymes (each)	0.4μl
Nuclease-free water	to 20μl

Table E 5. T4 ligation reaction recipe

Components	Amount
T4 DNA ligase	1μl
T4 DNA ligase buffer (10×)	2μl
Vector DNA	50ng
Insert DNA	equal molar to vector DNA
Nuclease-free water	to 20μl

Table E 6. 5× M9 media recipe

Components	Amount
Na ₂ HPO ₄	6 grams
KH ₂ PO ₄	3 grams
NH ₄ Cl	1 gram
NaCl	0.5 gram
Water	200 ml

Table E 7. 1× M9 media supplements

Components	Amount
Glucose	0.4 % (w/v)
Casamino acid	0.2 % (w/v)
MgCl ₂	2 mM
CaCl ₂	0.1 mM

APPENDIX F

Additional data

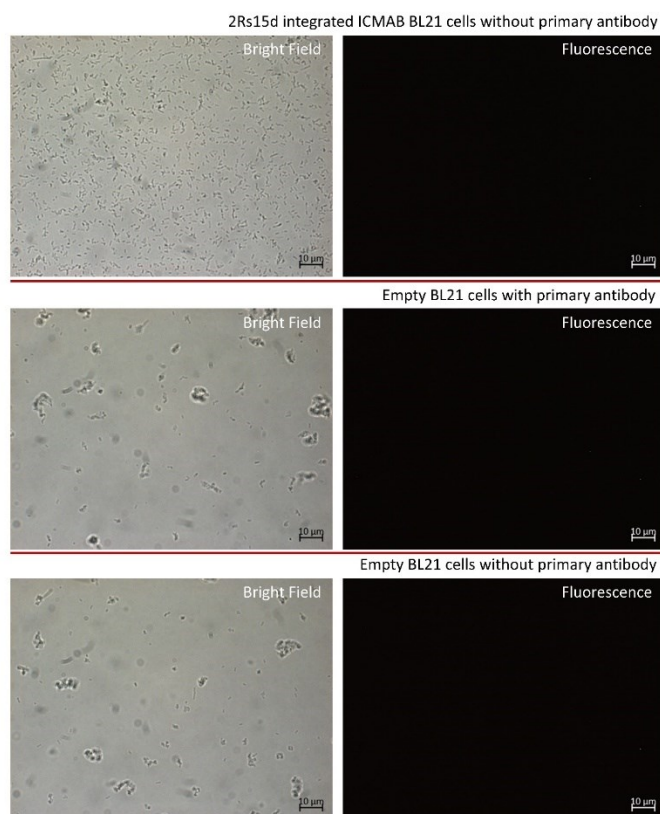


Figure F 1. The control groups were provided for ICC assay performed for expression of displayed His-tagged Mms6 on the cell surface stained with DyLight 550 antibody specific to His-tag and imaged under fluorescence microscopy. The left image was a bright field image, and the right one was taken under the red filter. Scale bars are 10 µm. Fluorescent images were obtained with 100% intensity.

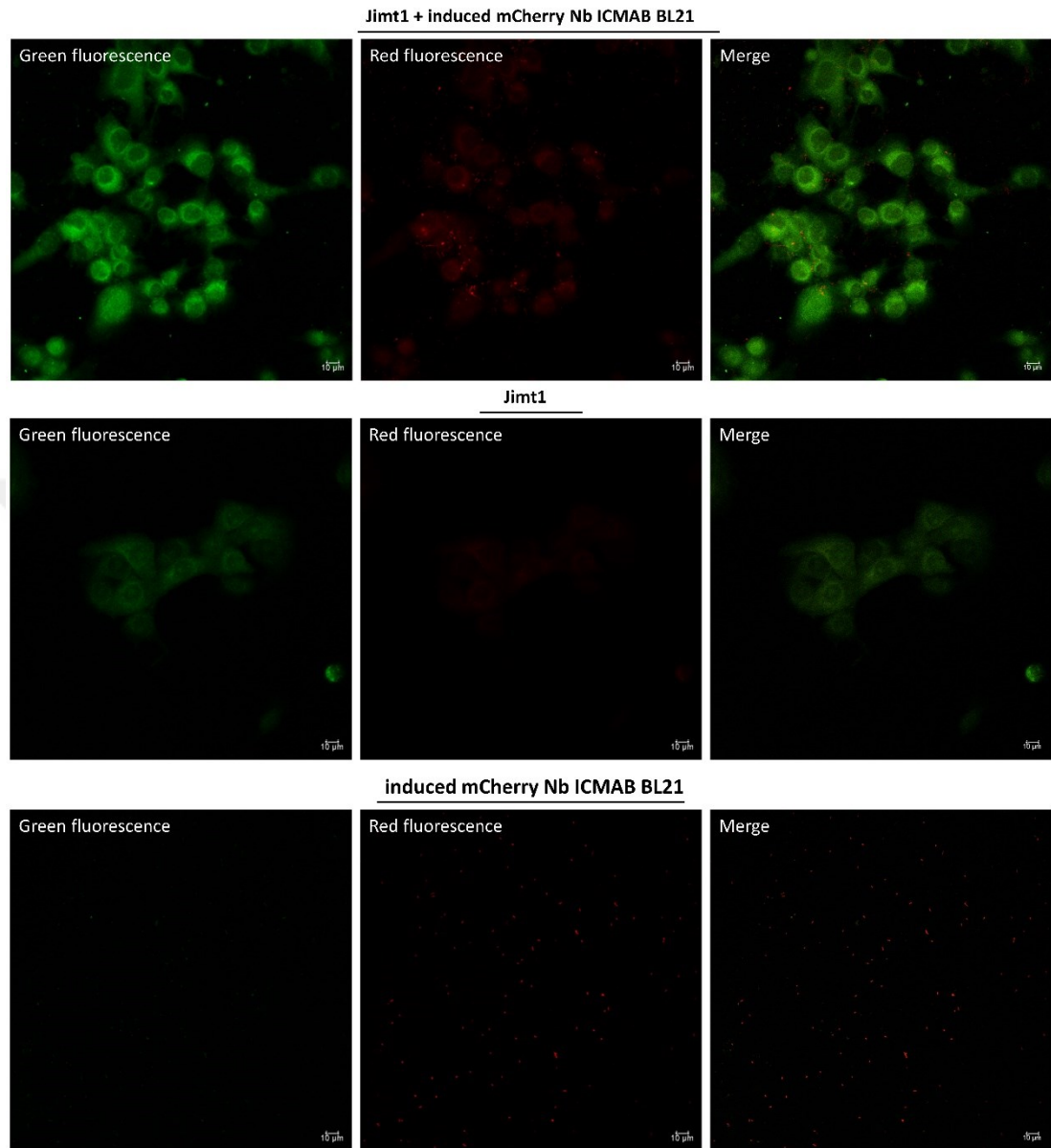


Figure F 2. Confocal microscopy imaging of the infected Jimt1 mammalian cells with induced mCherry Nb ICMAB BL21 bacterial cells on PDL coated glass surface. Same steps were performed and then visualized for only Jimt1 and only induced mCherry Nb ICMAB BL21 cells. Scale bars are 10 μm.

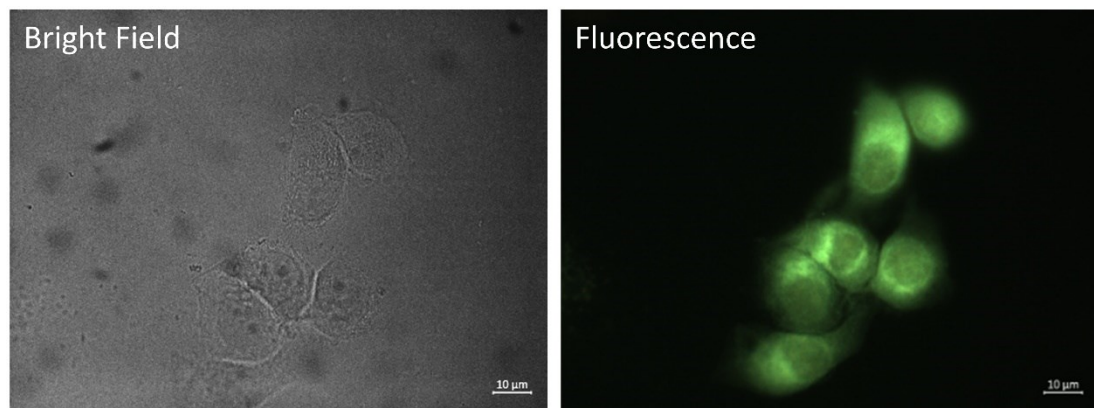


Figure F 3. Fluorescence microscopy imaging of the infected Jimt1 mammalian cells with empty BL21 bacterial cells on PDL coated glass surface. Scale bars are 10 µm.