

ISTANBUL TECHNICAL UNIVERSITY ★ INSTITUTE OF SCIENCE AND TECHNOLOGY

**GENETIC DESIGN AND SYNTHESIS OF
BI-FUNCTIONAL PROTEIN FOR
BIONANOTECHNOLOGY**

**M.Sc. Thesis by
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**BİYONANOTEKNOLOJİ İÇİN ÇİFT İŞLEVİLİ PROTEİNİN
GENETİK TASARIMI VE SENTEZİ**

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ABBREVIATIONS

APS	: Amonium peroxo disulfate
AuBP	: Gold binding protein
bp	: Base pair
BLAST	: Basic Local Alignment Search Tool
BSA	: Bovine serum albumin
CBB	: Comassie Brilliant Blue
dH₂O	: Distilled water
ddH₂O	: Double distilled water
DMF	: Dimethylformamide
DNA	: Deoxyribonucleic acid
DTT	: Dithiothreitol
EDTA	: Ethylenediaminetetraacetic acid
EtBr	: Ethidium bromide
G, Gly	: Glycine
GEPI	: Genetically engineered polypeptides for inorganics
IPTG	: Isopropyl-2-D- thiogalactopyranoside
kb	: Kilobase
kDa	: Kilodalton
<i>lacZ</i>	: β-galactosidase
LB	: Luria Bertani
LMP agarose	: Low melting point agarose
MALDI	: Matrix-assisted laser desorption/ionization
MBP	: Maltose Binding Protein
Na-Ac	: Sodium acetate
OD	: Optical density
PAGE	: Polyacrylamide Gel Electrophoresis
PCR	: Polymerase chain reaction
P, Pro	: Proline
QBP	: Quartz binding peptide
RNase	: Ribonuclease
SAM	: Self-assembled monolayer
S, Ser	: Serine
SDS	: Sodium dodecyl sulfate
TBE	: Tris-borat-EDTA
Tris base	: Hydroxymethyl aminomethane

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GENETIC DESIGN AND SYNTHESIS OF BI-FUNCTIONAL PROTEIN FOR BIONANOTECHNOLOGY

SUMMARY

Nature provides the inspiration for fabrication of materials. Biological materials are highly organized from molecular to macro scales, and make different functional parts, such as hard and soft tissues. In hard tissues, bones, dental tissues, spicules, shells, bacterial nanoparticles are examples of hybrid materials with proteinaceous and inorganic layer formed together. Therefore, peptide and proteins can be utilized as versatile building block in fabricating materials. Traditionally, large proteins have been used in engineering, mostly to increase biocompatibility of implants, surface coating, and making molecular scaffolds for use in tissue engineering. Additionally, the integration of proteins with signal transducers, protein-based biosensors, has exceedingly been utilized in many technologies including medical diagnostics.

The current binding and assembly of biomolecules onto solid substrates for various sensing applications are predominantly accomplished using covalently bound chemical linkers, e.g. thiols and silane linkers. For chemo- and bio-sensing applications, nevertheless, more biology-friendly linkers are necessary that are both versatile and amenable to genetic manipulation. A novel alternative to current chemical coupling may be through the utility of combinatorial inorganic-binding peptides as specific molecular linkers. Phage and cell surface display have become the major *in vivo* techniques for the selection of material-specific peptides, called genetically engineered peptides for inorganics, GEPI. After display techniques selected inorganic binding peptides can be further modified, engineered through genetic engineering methods and synthesized to utilize as molecular tools with controlled functionalities. To provide new functionality to inorganic binding peptides, bi-functional proteins can be generated with functional proteins such as enzymes and GEPI through genetic engineering.

GEPI can be used alone or as a fusion partner in functional proteins. Therefore, the methods to produce them via recombinant DNA technology and to check their inorganic binding functionality once they are inserted into different protein domains, is crucial. We aim to develop a recombinant production method for GEPI as a fusion partner in maltose binding protein (MBP). Two main objectives of this study are genetic engineering and purification of MBP-GEPI bi-functional protein in order to demonstrate applicability of the proposed method. To demonstrate this, we selected two GEPIs to produce as fusion partners, namely gold binding peptide (AuBP1) and quartz binding peptide (QBP2). We also used two different peptide linkers as S(G)₃ and (PG)₃ between MBP and GEPI in the expression to test the effect of linkers in bi-functional activities of the fusion constructs. For the expression of the proteins, pMAL expression and purification system was used. The vectors, pMALc2X, were constructed, and bi-functional proteins were expressed in Luria Bertani medium and

purified by amylose affinity chromatography. Purification of the bi-functional protein has been then optimized and the mass of resulting protein has been confirmed by mass spectrometry. We believe these constructs will lead to new facile alternatives in the formation of protein-metal and protein-metal oxide biosensors and bioassays. Moreover, the genetically designed bi-functional proteins can be used to create different material architectures in biomedical fields and nano-electronics due to gold and quartz binding peptide features.

BİYONANOTEKNOLOJİ İÇİN ÇİFT İŞLEVİLİ PROTEİNİN GENETİK TASARIMI VE SENTEZİ

ÖZET

Doğa malzemelerin üretiminde ilham kaynağıdır. Biyolojik malzemeler, moleküler boyuttan makro boyuta yüksek derecede organizasyona sahiptirler ve yumuşak ve sert dokular gibi fonksiyonel kısımlardan oluşurlar. Sert dokulardaki, protein ve anorganik katmanın birlikte oluşturduğu melez malzemelere örnekler, kemikler, diş dokuları, spiküller, deniz kabukları ve bakteriyel nano parçacıklardır. Bu nedenle, peptid ve proteinler malzemelerin üretiminde çok yönlü yapı taşları olarak kullanılabilir. Buna ek olarak, proteinlerin sinyal dönüştürücüler ile tümleştirilmesiyle oluşturulan proteine dayalı biyo duyargalar tıbbi tanıları da kapsayan pek çok teknolojik alanda kullanılmaktadır.

Günümüzde çeşitli duyarga uygulamalarında kullanılmakta olan biyomoleküllerin katı bir tabakanın yüzeyine bağlanması ve montajı çoğunlukla kovalent bağ oluşturan tiyol ve silan bağlayıcılar gibi kimyasal bağlayıcılarla gerçekleşmektedir. Kimyasal ve biyolojik duyarga uygulamaları için, ne yazık ki, hem çok yönlü hem de genetik değişikliklere uygun olan daha çok biyolojik açıdan elverişli bağlayıcılara gereksinim vardır. Anorganiklere bağlanan peptidlerin spesifik moleküler bağlayıcı olarak kullanılması günümüzdeki kimyasal bağlamaya yeni bir alternatif olabilir. GEPI diye adlandırılan genetik olarak üretilmiş peptidlerin malzemeye spesifik seçilmesinde kullanılan faj ve hücre yüzey gösterimi *in vivo* tekniklerin başında gelmektedir. Gösterim teknikleriyle seçilmiş anorganik bağlayıcı peptidler, genetik mühendisliği yöntemleriyle daha da geliştirilip, değiştirilebilir ve kontrol edilebilir işlevsellik kazandırılarak moleküler araç olarak kullanılmak üzere üretilerler. Anorganik bağlayıcı peptidlerle genetik mühendisliği yöntemleri kullanılarak enzim gibi proteinlerle birleştirilip çift işlevli proteinler oluşturulabilir.

GEPI tek başına kullanılabileceği gibi fonksiyonel proteinlerin füzyon kısmı olarak da kullanılabilir. Bu sebeple, GEPI'yi rekombinant DNA teknolojisi ile üretmek ve farklı proteinlerle birleştirildiğinde anorganik bağlama işlevinin kontrolünü yapmak için gerekli olan yöntemler çok önemlidir. Bu çalışmada, GEPI'nin maltoz bağlayan proteinde (MBP) füzyon kısmı olarak davranacağı bir rekombinant üretim yöntemi geliştirmeyi amaçladık. Bu çalışmadaki iki ana hedef, ileri sürülen yöntemin uygulanabilirliğini göstermek için MBP-GEPI çift işlevli proteinin genetik mühendisliği ve saflaştırılmasıdır. Bunu göstermek için füzyon kısmı olarak kullanacağımız altına bağlanan peptid diye adlandırılan AuBP1 ve silikaya bağlanan peptid olarak bilinen QBP2 seçildi. Aynı zamanda füzyon yapıların çift işlevli aktivitelerinde bağlayıcıların etkinliğini kontrol etmek için MBP ve GEPI arasına S(G)₃ ve (PG) gibi iki farklı peptid bağlayıcı yerleştirildi. Proteinlerin üretilmesinde ise pMAL ekspresyon ve saflaştırma sisteminden faydalandık. pMALc2X vektörleri oluşturulup Luria Bertani besiyerinde ilgili proteinler üretilerek amiloz ilgi

kromatografisi ile saflařtırıldı. ift iřlevli proteinlerin saflařtırılması optimize edildikten sonra proteinlerin molekler ağırlıkları ktle spektrometresiyle doęrulandı. Bu yapıların, protein-metal ve protein-metal oksit biyoduyarga ve biyoanalizlerin oluřumunda yeni alternatiflere nc olacaklarına inanılmaktadır. Dahası genetik olarak tasarlanmış ift iřlevli proteinler biyomedikal alanda ve nanoelektronikte altına ve kuvarza baęlanma zelliklerinden dolayı farklı malzemelerin inřasında da kullanılabilir.

1. INTRODUCTION

The ability to synthesize and manipulate material at the nanometer length scales can have significant impact in different fields, including microelectronics, computation, therapeutics, and diagnostics [1,2]. A wide number of inorganic nanoparticles including carbon nanotubes, buckyball fullerenes and various semiconductor and metal nanoparticles have long been utilized potential applications at the nanoscale. In nature, there are many examples demonstrating nanoscale organization having specific proteins and peptides that serve various chemical, mechanical and structural functions. Thus, there is great interest in engineering peptide and protein based systems for potential applications in nanosystems and devices [1-12].

Peptides and proteins can be versatile building blocks for fabricating materials. In nature, there are many examples where the proteins are utilized as scaffolders such as collagen, keratin and have been instrumental in producing silica and calcite materials. Proteins have been used commonly in engineering, mostly to increase biocompatibility of implants, surface coating and making molecular scaffolds for use in tissue engineering [13-17]. Inorganic binding peptides can be used alone or as a fusion partner in functional proteins. Therefore, the methods to produce them via recombinant DNA technology and to check their inorganic binding functionality once they are inserted into different protein domains are crucial. The integration of proteins with signal transducers, protein-based biosensors, has mostly been utilized in many technologies including medical diagnostics [9, 11, 18-21].

More recently, hybrid materials composed of noble-metal nanostructures functionalized with protein receptor molecules (e.g. antibodies and enzymes) are of interest for developing novel chemo- and bio-sensors [22-27]. Ideally, the receptor coupling on the inorganic surface should be highly specific, highly ordered, reversible, and friendly to receptor molecules. Therefore, the multiplexed linking of proteins to a solid support in protein-based arrays is searched for the high-throughput study of proteins. Unfortunately, those devices are restricted via the challenges of

interfacing proteins with the solid support like loss of activity on immobilization owing to unfolding and the complex chemistries for coupling.

Currently, various approaches utilize covalently coupling such as thiolylation [28] and silanization [29] for direct conjugation to functionalized layers that is used as an intermediary between the protein and surface of substrate

Although current inorganic surface coupling approaches are based on creation of various self-assembled monolayer (SAM) linkages on metal inorganic surfaces, commonly using thiol or silane chemistry [4, 28-30] the SAM-surface interactions are nonspecific, e.g. thiols bind to any noble metal – gold, silver, or platinum – more or less equally likely [28]. An exciting alternative to chemical coupling may be the use of combinatorially selected inorganic-binding peptides that are genetically incorporated onto the protein and can direct it to solid support without the need for further chemistry on the surface or the protein. Besides, the combinatorially selected inorganic-binding peptides have the potential to control protein orientation in specific and provide reversibility.

1.1 The Purpose of the Research

Phage [31] and cell surface display [32, 33] have become the major *in vivo* techniques for the selection of material-specific peptides [8, 18, 19, 32, 34, 35], generally called genetically engineered peptides for inorganics, GEPI [1, 2]. Recently, peptide sequences specific for platinum, quartz, cuprous oxide and hydroxyapatite, as well as many other materials and minerals, have been identified [1,2, 5-10, 18-21, 34-36]. Those peptides have been also characterized in terms of binding kinetics, affinities, and molecular structure [7-9, 18, 20, 21, and 37].

GEPIs can be used alone or as a fusion partner in functional proteins. Therefore, the methods to produce them via recombinant DNA technology and to check their inorganic binding functionality once they are inserted into different protein domains, is crucial. We aim to develop a recombinant production method for GEPI as a fusion partner in maltose binding protein. Two main objectives of this study are genetic engineering and purification of bi-functional protein in order to demonstrate applicability of the proposed method. maltose binding protein was chosen in order to facilitate its folding and solubility. Purification of the bi-functional protein has been

then optimized and the mass of resulting protein has been confirmed so that the study of its structure and functional properties can be now approached.

We selected two GEPIs to produce as fusion partners, namely gold binding peptide (AuBP1, MW: 1454.7, WAGAKRLVLRRE) and quartz binding peptide (QBP2, MW: 1549, LPDWWPPPQLYH). We also used two different peptide linkers as S (G)₃ and (PG)₃ between MBP and GEPI in the expression to test the effect of linkers in bi-functional activities of the fusion constructs. For the expression of the proteins, pMAL expression and purification system was used. The vectors, pMALc2X, were constructed, and bi-functional proteins were expressed in Luria Bertani medium and purified by amylose affinity chromatography. Purification of the bi-functional protein has been then optimized and the mass of resulting protein has been confirmed by mass spectrometry. We believe these constructs will lead to new facile alternatives in the formation of protein-metal and protein-metal oxide biosensors and bioassays. Moreover, the genetically designed bi-functional proteins can be used to create different material architectures in biomedical fields and nano-electronics due to some unique peptide features: (1) inorganic-binding peptides are robust and can be genetically-engineered or chemically-modified for tailored functionalities, (2) bind selectively to the inorganic targets at ambient conditions providing biology-friendly environment desirable for most bionanotechnology applications, (3) the various specific inorganic-binding peptide linkages can modify simultaneously two or more inorganic entities with different functional molecules or nanostructures. Overall, those new biomaterials have implications in a wide range of potential practical applications including the controlled bottom-up assembly of hybrid nanostructures in bionanophotonic and biosensing devices.

2. THEORITICAL BACKGROUND

We summarize inorganic binding peptides and their potential use in nano and emerging technologies in the following sections. We also provide information on different fusion proteins and tagging systems and finally discuss maltose binding protein.

2.1 Inorganic Binding Peptides

In nature, proteins are reported to initiate, catalyze and mediate the fabrication of inorganic nano- and microstructures, which assemble into complex architectures. Therefore, a new emerging research field has been started in nanomaterials design termed molecular biomimetics and nanotechnology [1, 2]. The organisms using the organic-inorganic hybrid systems have evolved to use a part of their proteins in order to produce and bind the inorganic materials *in vivo*. These organisms synthesize inorganic binding proteins that bind and organize inorganic materials to highly ordered structures to perform excellent functions such as forming protective layers, supportive tissues, transferring ions and developing some optical and mechanical properties in favor of the organism. The inorganic material commonly include magnetite (Fe_3O_4) particles in magnetotactic bacteria or teeth of chiton [14]; silica (SiO_2) as skeletons of radiolarian [13] or tiny light-gathering lenses and optical wave guides in sponges [15]; hydroxyapatite ($\text{Ca}_2\text{C}(\text{OH})_3$) in bones [16] and dental tissues of mammals [17] calcium carbonate (CaCO_3) in the shells of mollusks [3].

In Figure 2.1, we demonstrate aragonite platelets separated by organic layer from abalone shell, magnetide particles produced in magnetotactic bacterium highly organized structures of hydroxyapatite crystallites in mouse enamel and layered silica structures in sponge spicule. a) Scanning electron microscope (SEM) image of a growth edge of abalone (*Haliotis rufescens*) displaying aragonite platelets (blue) separated by organic film (orange) that eventually becomes nacre (mother-of-pearl). This is a layered, tough, and high strength biocomposite (inset: transmission electron

microscope (TEM). b) Magnetite nanoparticles formed by magnetotactic bacterium (*Aquaspirillum magnetotacticum*, inset TEM image) are single crystalline, single-domained, and crystallographically aligned. c) Mouse enamel (SEM image) is hard, wear resistant material with highly ordered micro/nano architecture consisting of hydroxyapatite crystallites that assemble into woven rod structure (inset: schematic cross-section of a human tooth) d) Sponge spicule (with a cross-shaped apex shown in inset) of *Rosella* has layered silica with excellent optical and mechanical properties, a biological optical fiber (SEM image) [1].

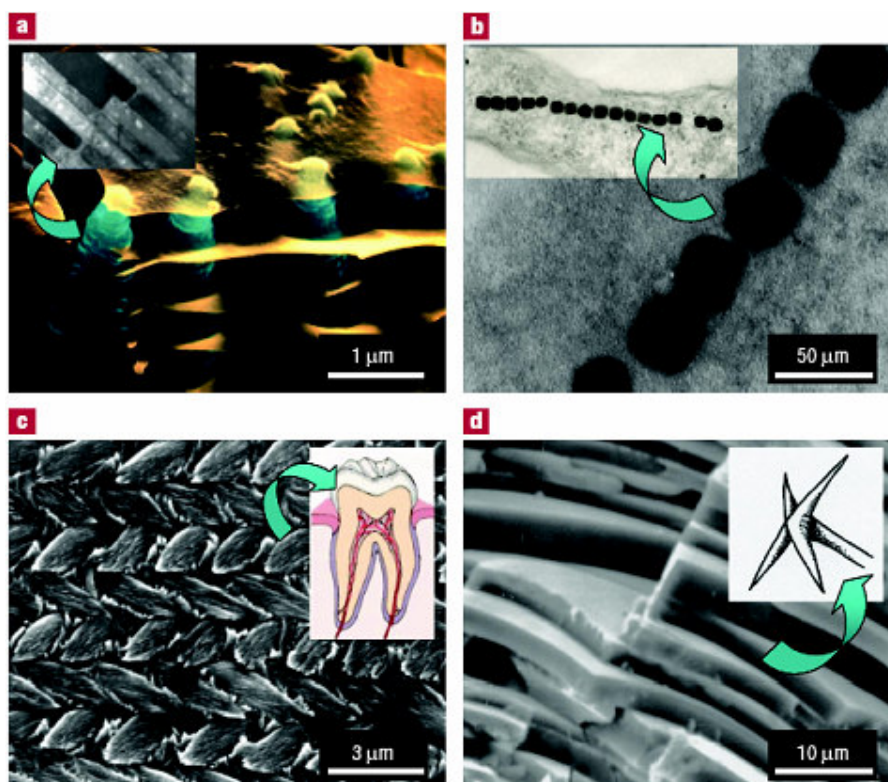


Figure 2.1: Examples of biologically synthesized complex materials

Because of the limited occurrence of naturally inorganic associated proteins, which could regenerate only inorganic material that they are associated with, some recent efforts have begun to instead identify small polypeptides that bind with high affinity to bulk materials using combinatorial biology approaches. The adapted phage display [31] and cell surface display [32, 33] technologies has emerged as a powerful strategy to select the inorganic-binding peptides. Nowadays, peptide sequences specific to metals [5, 18], metal oxides [8] and semiconductors [19, 35] and their potential use in material assembly and synthesis have been demonstrated. In addition

to the control of inorganic synthesis, self-assembly and recognition toward inorganic surfaces, combinatorial inorganic-binding peptides are robust and can be genetically-engineered or chemically-modified for tailored functionalities including binding, erecting and linking [1, 2]. Besides that, designed inorganic-binding peptides can control the hierarchical assembly of inorganic building blocks to form hybrid nanostructures with capable of being tuned properties for nanobiotechnology applications [9-12, 21, 37,].

2.1.1 Selection of Inorganic Binding Peptides

Phage [31] and cell surface display [32, 33] have become the major *in vivo* techniques for the selection of material-specific peptides [8, 18, 19, 32, 34, 35], generally called genetically engineered peptides for inorganics, GEPI [1]. Recently, peptide sequences specific for platinum, quartz, cuprous oxide and hydroxyapatite, as well as many other materials and minerals, have been identified and characterized with regards to binding kinetics, affinities, and molecular structure [7, 8, 20, 35]. Despite these most recent studies, there is still a need for detailed and quantitative data to address the molecular mechanisms that facilitate material-selective peptide binding on solid surfaces. The contribution of peptide primary and secondary structures most likely impact the recognition and binding to solid materials, and this information is essential for future peptide engineering, design, robust utility of these novel small biomolecules, either alone or as part of a larger protein unit, e.g., an enzyme [11, 38].

Recently, a FliTrx bacterial expression system [36] was used to select peptide sequences directed against cuprous and zinc oxide substrates [21] and as a scaffold for various metal nanoparticle self-assemblies [39] and formations. [40]. The FliTrx bacterial system uses the engineered extracellular flagellar protein (flagellin) to interact with external substrates. The main feature of FliTrx peptide libraries is that random peptides are inserted as fusions within the thioredoxin active-site loop [41], which is itself inserted into dispensable region of the flagellin gene, as opposed to the N-terminal location of fusions made to coat proteins in phage display techniques. Thus random peptides are displayed on the surface of bacteria within the structural context of the thioredoxin active-site loop, having both their N- and C- termini anchored by the rigid and stable tertiary structure of thioredoxin itself. in Figure 2.2

FliTrx Peptide Display

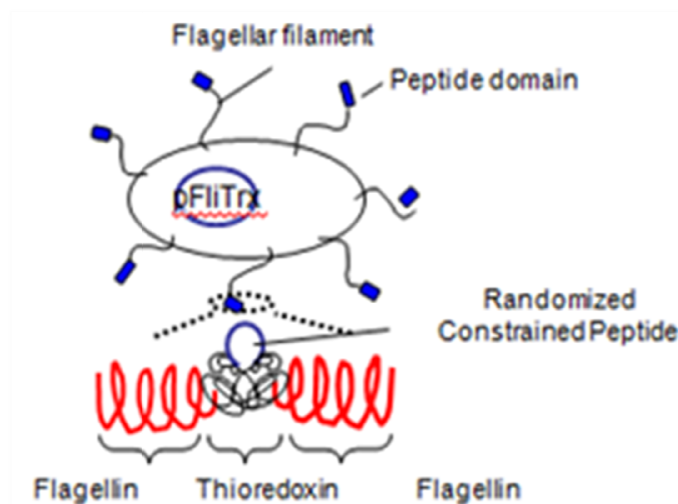
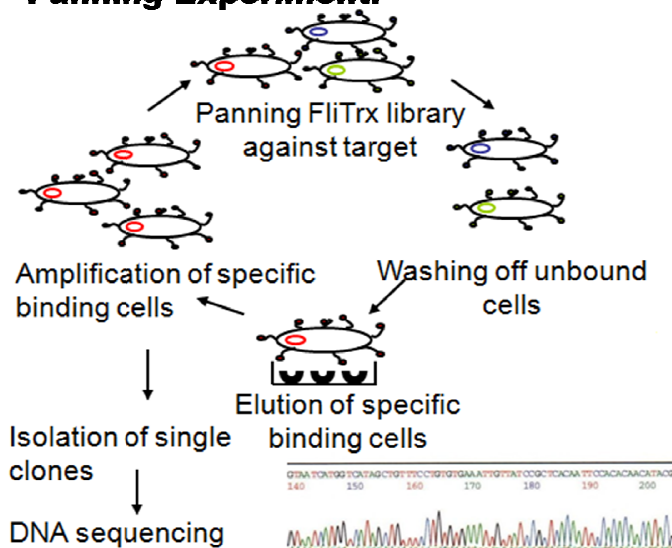


Figure 2.2 : Schematic of *E. Coli* used in FliTrx library

The novel gold-binding peptides (AuBPs) [37] were selected from FliTrx bacterial surface library [36]. After five rounds of biopanning selection to enrich the population for affinitive inorganic binders, several numbers of clones were selected and sequenced to determine amino acid composition of randomized insertions. The binding affinities of these 50 clones were analyzed using fluorescence microscopy technique [7, 37] (shown in Figure 2.3). The binding characterization study allowed us to sort selected clones into three groups: strong, moderate and weak binders depending on their gold-binding affinity [37].

Panning Experiment:



Characterization:

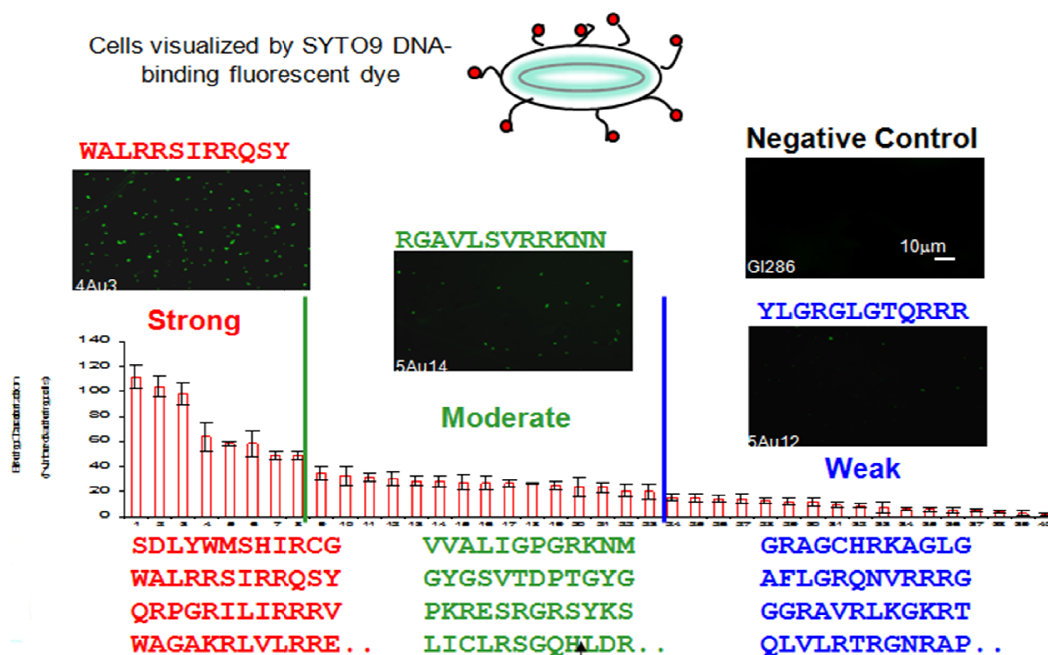


Figure 2.3 : After peptide selection panning and binding characterization experiments.

Prior to chemical peptide synthesis, the material specificity of strong binding clones were characterized to gold surfaces comparing to other materials (e. g. Ag, Pt, SiO_x) using the same technique. This preliminary analysis helps us to identify the peptide sequences showing both high affinity and selectivity to gold target when displayed on bacterial cells as FliTrx fusion surface proteins. The examples of selective gold-

adhered cells from strong binding group, weak binding group and control plasmid free GI826 cells are listed in (Figure 2.4).

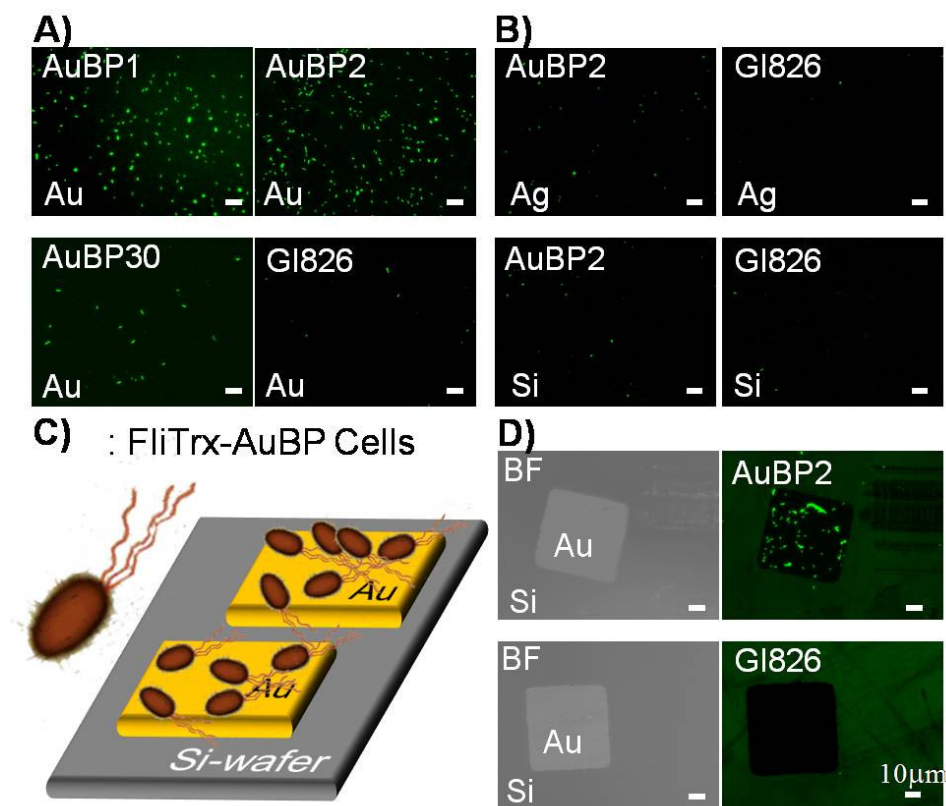


Figure 2.4 : Binding affinity and selectivity of selected novel gold-binding clones by fluorescence microscopy

As shown in Figure 1.4 examples of gold-adhered cells from strong binder group expressing strong gold-binding FliTrx protein (AuBP1, AuBP2) and from weak binder group expressing weak gold-binding FliTrx protein (AuBP30), in control experiment: plasmid free GI826 control cells used instead (A); examples of cross-specificity of strong gold-binding clone (AuBP2) on silver and silicon dioxide surface with respective plasmid free GI826 control cells (B); schematic of selected FliTrx gold-binding cell attachment to gold surface (C); examples of gold affinity and specificity of strong gold-binding clone (AuBP2) on gold-pattern silicon wafer with respective plasmid free GI826 control cells (D).

2.1.2 Proof of Demonstration Studies by Inorganic Binding Peptides

The utility of polypeptides in practical engineering is becoming common in recent years by the speeding up development of protocols and procedures using molecular

biology that allow selection, designing, and tailoring of these versatile biomolecules as molecular building blocks. Proteins control fabrication and assembly of inorganic structures in the form of hard tissues with myriad different functionalities, such as mechanical, magnetic, and optical [1, 2, 5, 19, 34]. Proteins have been used in engineering, mostly to increase biocompatibility of implants and surface coating. Recently, they are also used as engineering. More recently, hybrid materials composed of noble-metal nanostructures functionalized with protein receptor molecules (e.g. antibodies and enzymes) are of interest for developing novel chemo- and bio-sensors. [37, 38]. Ideally, the receptor coupling on the inorganic surface should be highly specific, highly ordered, reversible, and friendly to receptor molecules.

Current inorganic surface coupling approaches are based on creation of various self-assembled monolayer (SAM) linkages on metal inorganic surfaces, commonly using thiol or silane chemistry [4, 28-30]. However, the SAM-surface interactions are nonspecific, e.g. thiols bind to any noble metal – gold, silver, or platinum – more or less equally likely [28-30]. An exciting alternative to chemical coupling may be the use of combinatorially-selected inorganic-binding peptides as molecular linkers and assemblers [1, 2, 7, and 11]. In principal, in addition to the specific recognition of inorganic surfaces, combinatorially-selected inorganic-binding peptides are robust and can be genetically engineered or modified to tailor their functionalities such as synthesizing, binding, erecting and linking of inorganic nanostructures. [1, 2, 10, 11]

Peptides, synthesized using cell surface display and phage display, specific to inorganic substrates, can be used to pattern inorganic substrates with structures at the micro scale [1, 2, 5, and 37]. This functionality can be practically used in the medical field for biosensing and even serving as a scaffold for immobilized protein activity and cell growth. Proof of polydimethylsiloxane (PDMS) stamps are used in soft lithography in order to pattern inorganic surfaces with molecules, such as thiols [10, 11, 30, 28]. With the use of a PDMS stamp, one can apply the developed genetically engineered peptide specific to inorganic material (or GEPI) using as ink to the inorganic surface using physical force yielding high resolution patterning [11]. This has been shown with GEPI's on gold surfaces, but it is yet to be tested on quartz surfaces [1, 2, and 11]. GEPI has used for patterning should also have a means of

optical visualization; the preferred method in this manuscript is to use UV fluorescent quantum dots (QDs). The pattern of the PDMS stamp may also vary [11].

Genetically engineered peptides can be used as inks by PDMS stamping in creating functional bio-scaffolds. This can be applied to quartz surfaces, which are typically treated with silane chemistry for easy covalent bonding [10, 11, 30, and 28]. Current state of the art techniques using genetically engineered peptides are specifying peptide affinity to specific surfaces in order to use them as biosensors [1, 2, 5, 10, and 37].

2.2 Overview of Tag Protein Fusions

The use of recombinant proteins has gradually increased in recent years due to an increase demand towards more biobased approaches desired in many technologies. Recombinant proteins containing a fusion partner, called affinity tag, are widely used. Recently, several epitope peptides, different proteins and domains have been developed to over-produce recombinant proteins to facilitate the purification and detection of the target proteins is well-characterized. The advantages of these affinity-tag systems: (a) one-step adsorption purification; (b) a minimal effect on tertiary structure and biological activity; (c) easy and specific removal to produce the native protein; (d) simple and accurate assay of the recombinant protein during purification; (e) applicability to a number of different proteins. However, each affinity tag is purified under its specific buffer conditions, which could affect the functionality of the protein of interest [12, 43, and 44]. Therefore, it is difficult to choose the right purification system for a specific protein of interest. The most frequently used and interesting systems are His-tag, maltose-binding protein, glutathione S-transferase, FLAG-tag, calmodulin binding peptide and cellulose-binding domain. The tag systems and their conditions are summarized in Table 2-1.

Table 2.1: There are the most used affinity tags, their matrices and elution conditions

Affinity Tag	Matrix	Elution Condition
Maltose Binding Protein	Cross-linked amylose	10 mM maltose
Glutathione S-transferase	Glutathione	5-10 mM reduced glutathione
Poly-His	Ni- ²⁺ NTA, Co ²⁺ -CMA (Talon)	Imidazole 20-250 mM or low pH
HAT (natural histidine affinity tag)	Co ²⁺ -CMA (Talon)	150 mM imidazole or low pH
Poly-Arg	Cation exchange resin	NaCl linear gradient from 0 to 400 mM at alkaline pH > 8.0
FLAG	Anti-FLAG monoclonal antibody	pH 3.0 or 2-5mM EDTA
Strep-tag II	Strep-Tactin (modified strepavidin)	2.5 mM desthiobiotin
S	S-fragment of RNaseA	3 M guanidine thiocyanate, 0.2 M citrate pH 2.0, 3 M magnesium chloride
c-myc	Monoclonal antibody	Low pH
Calmodulin-binding protein	Calmodulin	EGTA or EGTA with 1 M NaCl
Cellulose –binding protein	Cellulose	Family I: guanidine HCl or urea > 4 M Family II/III: ethylene glycol
SBP	Strepavidin	2 mM Biotin
Chitin-binding domain	Chitin	Fused with intein: 30-50 mM dithiothreitol

Generally, it is hard to decide which fusion system is best for a specific protein of interest. The target protein features such as stability and hydrophobicity and application of the purified protein help us to choose suitable expression system. In Table 2.1, there are the most common tag-protein fusion systems.

Several different strategies have been developed to produce recombinant proteins on a large scale. One approach is to use a very small peptide tag that should not interfere with the fused protein. The most commonly used small peptide tags are poly-Arg-, FLAG-, poly-His-, c-myc-, S-, and Strep II-tag (Table 2.1). For some applications, small tags may not need to be removed. The tags are not as immunogenic as large tags and can often be used directly as an antigen in antibody production. The effect on tertiary structure and biological activity of fusion proteins with small tags depends on the location and on the amino acids composition of the tag [12, 26, and 49].

Another approach is to use large peptides or proteins as the fusion partner (Table 2.2). The use of a large partner can increase the solubility of the target protein. The disadvantage is that the tag must be removed for several applications e.g. crystallization or antibody production.

Table 2.2: Residues, sequence and size of the most used affinity tags

Tag	Residues	Sequence	Size (kDa)
Maltose-binding protein	396	Protein	40.00
Glutathione S-transferase	211	Protein	26.00
Poly-His	2-10 (usually 6)	HHHHHH	0.84
HAT (natural histidine affinity tag)	19	KDHLIHNVHKEFHAAHANK	2.31
Poly-Arg	5-6 (usually 5)	RRRRR	0.80
FLAG	8	DYKDDDDK	1.01
Strep-tag II	8	WSHPQFEK	1.06
S	15	KETAAAKFERQHMS	1.75
c-myc	11	EQKLISEEDL	1.20
Calmodulin-binding protein	26	KRRWKKNFLAVSAANRFKKISSGAL	2.96
Cellulose –binding protein	27-189	Domains	3.00-20.00
SBP	38	MDEKTTGWRGGHVVEGLAGELEQLRAR LEHHPQGQREP	4.03
Chitin-binding domain	51	TNPGVSAWQVNTAYTAGQLVTYNGKTY KCLQPHTSLAGWEPSNPALWQLQ	5.59

A various number of expression systems designed for numerous applications and compatibilities are available. Approximately 80% of the proteins used to solve three-dimensional structures submitted to the protein data bank (PDB) in 2003 were prepared in an *E. coli* expression system. The T7 based pET expression system (commercialized by Novagen) is by far the most used in recombinant protein preparation (pET represents more than 90% of the 2003 PDB protein preparation systems). Systems using the λ PL promoter/cI repressor (e.g., Invitrogen pLEX), Trc promoter (e.g., Amersham Biosciences pTrc), Tac promoter (e.g., Amersham

Biosciences pGEX) and hybrid *lac*/T5 (e.g., Qiagen pQE) promoters are common. A radically different system is based on the *araBAD* promoter (e.g., Invitrogen pBAD).

One of the most popular tag proteins used in the fusion protein system is maltose-binding protein [27, 44, 45, 49, and 50]. MBP gained its popularity because it can achieve a high level of expression (up to 20–40% of total cellular proteins in *Escherichia coli* [46] and because it can increase the solubility of fusion proteins [42, 45].

2.2.1. Maltose Binding Protein Expression Systems

The 40 kDa maltose-binding protein (MBP) is encoded by the *malE* gene of *E. coli* K12 [46]. Vectors that facilitate the expression and purification of foreign peptides in *E. coli* by fusion to MBP were first described in 1988 [43]. Fused proteins can be purified by one-step affinity chromatography on cross-linked amylose. Bound fusion proteins can be eluted with 10 mM maltose in physiological buffer. Binding affinity is in the micro-molar range. Some fusion proteins do not bind efficiently in the presence of 0.2% Triton X- 100 or 0.25% Tween 20, while other fusions are unaffected. Buffer conditions are compatible from pH 7.0–8.5, and up to 1 M salt. Denaturing agents cannot be used. MBP can increase the solubility of overexpressed fusion proteins in bacteria, especially eukaryotic proteins [42, 49]. A spacer sequence coding for ten asparagines residues between the MBP and the protein of interest increases the chances that a particular fusion will bind tightly to the amylose resin. The MBP-tag can be easily detected using an immunoassay. It is necessary to cleave the tag with a site-specific protease. The MBP can be fused at the N- or C-terminus of the protein if the proteins are expressed in bacteria [46, 49]. N-terminal location can reduce the efficiency of translation. The MBP system is widely used in combination with a small affinity tag [49].

The maltose-binding protein (MBP) is one of the effective solubilizing agents when used as a fusion partner. In some cases, the fusion to MBP can promote proper folding of the attached protein into its biologically active conformation. Thus, MBP is likely to be capable of functioning as a general molecular chaperone in the context of a fusion protein [27, 42- 46, 49, 50]

2.2.2 Maltose Binding Protein Structure and Applications

The bacterial periplasmic-binding protein (PBP) superfamily members, in particular the maltose-binding protein, have been used extensively to prototype a variety of biosensing platforms. This protein superfamily has the same basic two domain structure, and upon binding a recognized ligand, almost all PBPs undergo a conformational change to a closed structure [43, 46]. Many direct detection or reagentless sensing modalities have been utilized with maltose-binding protein for both in vitro and in vivo detection of target compounds. Signal transduction modalities developed to date include direct fluorescence, electrochemical detection, fluorescence resonance energy transfer (FRET)- based detection, surface-tethered FRET sensing, hybrid quantum dot FRET sensing, and enzymatic detection, each of which have different benefits, potential applications and limitations [12, 26, 27].

A common functional conformational change is also found in PBP family, which involves switching from the 'open' ligand-free form to a 'closed' or ligand-bound form. This transition is accomplished through a bending and swiveling twist motion about the hinge region, which is analogous to a 'venus-fly-trap' closing, see Figure 2.5 (a, b) [26, 27]. A variety of analytical or signal transduction mechanisms have been used to monitor PBP binding and conformational changes, including direct and environmentally sensitive fluorescence, electrochemical and fluorescence resonance energy transfer (FRET), see Figure 2.5.

The interest in exploiting PBPs for biosensing arises both from their specific recognition of myriad analytes approaching nanomolar concentrations in some cases and from the ligand-induced conformational changes that they undergo [12, 50]. It is believed that the common functional mechanism and wide specificity of PBPs originated during evolution by positive selective pressure on their hosts as they mediated solute recognition and uptake in the periplasm and directed chemotaxis [46, 49]. Owing to the extensive understanding of its structure and function, maltose-binding protein (MBP) is the prototypical member of this superfamily. MBP specificity is well known, the crystal structure in both the open and ligand-bound form has been solved [12, 26, 27, 42, 44-46, 50], and a variety of mutants that have different maltose sensitivities (K_d s that range from nM to mM) have been created [27]. Moreover, MBP has been extensively utilized for recombinant protein purification over amylose resin [12, 26].

MBP, are an excellent platform not only for biosensing but also for prototyping a variety of diverse sensing modalities for eventual use in various environmental, clinical and security applications [26, 27, 42, 44-46, 50].

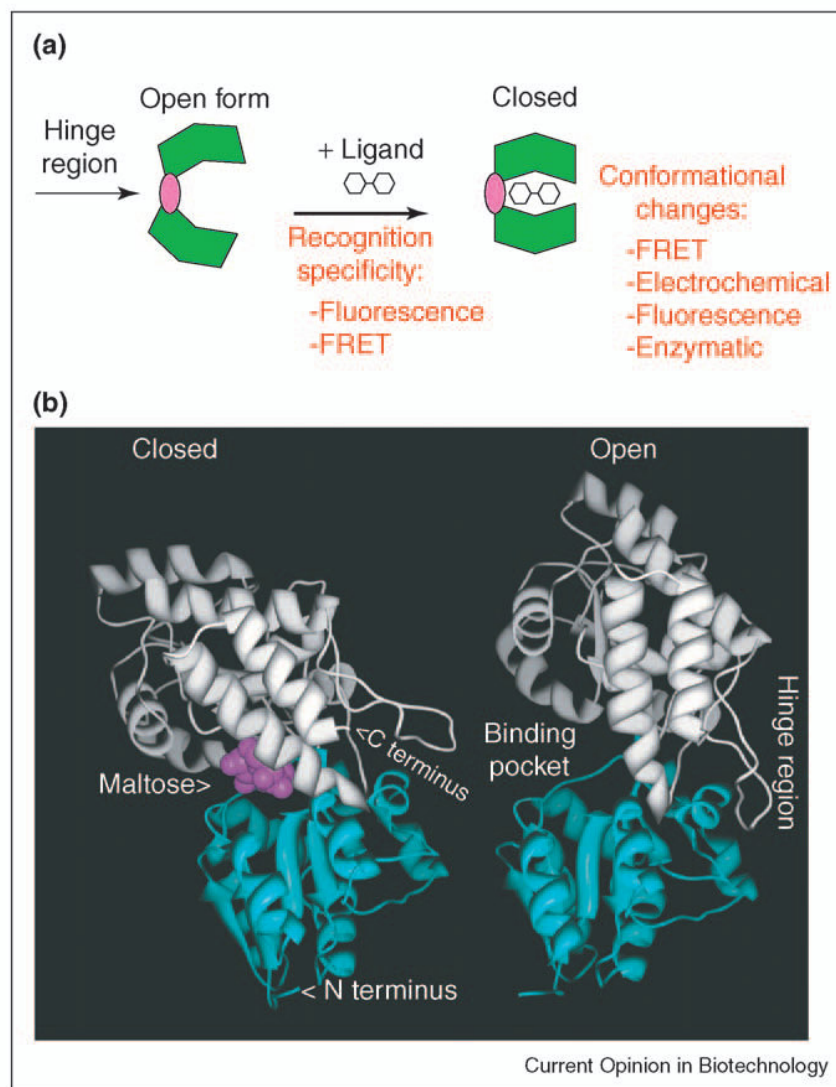


Figure 2.5 : MBP structure and function

In Figure 2.5 (a) A prototypic PBP structure is depicted; this has two lobes (green) that create the ligand-binding pocket and are joined by the hinge-binding region (pink). Upon binding the ligand, the PBP undergoes transition to the closed structure. PBPs present two intrinsic properties available for biosensor exploitation, either separately or when functioning in concert: recognition specificity and conformational change. Recognition specificity has been utilized for fluorescence- and FRET-based sensing. Conformational changes have been utilized for FRET-, electrochemical-,

fluorescent- and enzymatic-based sensing. (b) Ribbon and string rendering of MBP structure in the open form (right) and ligandbound closed form (left). MBP dimensions are $\sim 30 \times 40 \times 65 \text{ \AA}$. The two domains (lobes) are highlighted in green and grey. Upon binding maltose (purple), the lobes rotate $\sim 35^\circ$ and twist laterally $\sim 8^\circ$ relative to each other [47]. Overall the amino- and carboxy-termini move $\sim 7 \text{ \AA}$ closer to each other after binding. Note the change in conformation of the overall structure upon binding maltose [46, 47].

Proteins interact with other macromolecules and inorganics to control the structures and functions of all biological tissues in organisms [1,2]. Biological hard tissues such as bones, dentin, enamel, and spines contain proteins together with inorganic minerals [13-17]. Design and engineering of proteins with desired properties such as specific binding affinity to selected inorganics, would open avenues to the engineering of materials with novel properties and broaden the knowledge about protein physics. Thus, binding of polypeptides to metal surfaces is of great interest in the field of nanotechnology, biomaterials and biomimetics. In biomaterials, protein adsorption is critical for the integration of an implant with tissue [4]. In the nanotechnology aspect, protein-surface interactions constitute the basis for functional biological/electronic constructs such as sensors, activators, etc. [1, 2, and 30]. Biomimetics, i.e., gaining inspiration and guidance from nature have also gained considerable attention in recent years for the design concepts that introduce into technologies with novel properties [2]. In view of this approach, combinatorial and evolutionary techniques have been adopted for the creation and isolation of peptide sequences that bind specifically to solid surfaces, similar to the natural proteins that regulate crystal growth [2, 6, 19, 31-33, 47, 48].

2.3 Bioengineering New Bi-functional Proteins

Recent advantages in protein engineering have come from creating multifunctional proteins, nowadays more attention is being paid to protein than to the gene itself [44, 49, and 51]. High-throughput expression and purification strategies, such as affinity fusion tags, are necessary when conducting functional and structural studies of proteins. Using fusion proteins for protein purification is a routine practice in laboratories working on recombinant proteins. One of the most popular carrier proteins used in the fusion protein system is maltose-binding protein (MBP) [12, 43,

46, and 49]. MBP is one popular affinity tag because it can achieve a high level of expression (up to 20–40% of total cellular proteins in *Escherichia coli* [43, 46] and it can increase the solubility of fusion proteins. The MBP is a member of the family of the periplasmic binding proteins involved in active transport and chemotaxis [26, 49, 42, 46]. The protein expression is quietly high, especially; the expression level is higher in the cytoplasm than periplasm. It is shown that fusion with MBP can increase solubility of proteins expressed in *E.coli* [26, 49]. In addition to this, the system which maltose binding protein (MBP) is utilized for recombinant protein expression and purification provides the basis of general system for engineering families of reagentless biosensors [26].

The important challenge presented by the process of protein microarray fabrication is the requirement for high-throughput expression and purification of proteins, since thousands of purified proteins are required for the generation of high-density protein microarrays. Fortunately, the advent of recombinant DNA technology and Polymerase Chain Reaction has simplified protein expression, allowing not only the study of existing proteins but also the production of new proteins with enhanced features. Consequently, over the past decade several technologies have been developed where the target proteins are manipulated at the genetic level to increase productivity. The use of genetically-fused affinity tags has been particularly successful, and this technique has become ubiquitous in research [26, 49, 43, and 46].

2.3.1 Application Area of Bi-functional Protein

Nanostructures based on polypeptides have emerged as key hybrid systems for future nanobiotechnological applications so here peptides that have specific inorganic-binding are utilized as the most favorable building blocks for the design and synthesis of nanostructures. These simple building blocks that can be modified and decorated with functional elements, and they can be employed in different applications. The useful applications of these structures as novel components of highly sensitive biosensors and diagnosis tools, drug delivery vehicles, and scaffolds for tissue engineering and regeneration, were already known [10, 11, 47, and 48]. The premise in a molecular biomimetic approach to nanotechnology is that, genetically engineered polypeptides for inorganics (GEPs) could be used as linkers and building blocks for self-assembly of materials with controlled organization and

specific functions. The isolation methods and potential applications of genetically engineered peptides for inorganics (GEPIs) have been studying extensively in the recent years. Specific peptides can now be used for the nucleation and growth of inorganic nanomaterials and structure [10, 11, 34, 37, 48]. Peptides specific for inorganic binding can be combined (i.e., genetically fused) with proteins (i.e. enzymes) and other functional peptides that bind another moiety [38]. This approach can open up endless possibilities begin to emerge. Proteins have been used in engineering, mostly to increase biocompatibility of implants and surface coating, with their recent use in molecular scaffolds in tissue engineering, they can be extended towards new biobased approaches, utilized for novel technologies.

In relatively recent times, hybrid materials composed of noble-metal nanostructures functionalized with protein receptor molecules (e.g. antibodies and enzymes) are of interest for developing novel chemo- and bio-sensors [10, 11, 28-30, 48]. To assemble the recognition layer in these biosensor applications, different approaches have been used. The first approach is to immobilize the proteins by means of physisorption without adding any tags or chemical groups. In this approach, the main drawback is the un-oriented fashion of immobilization of the protein. These causes a loss in the activity of the protein adsorbed on the surface, because non-controlled and unoriented adsorption may triggers the adsorption of the active site of the proteins. Another approach is creating an active chemical layer on the surface of interest using thiol or silane based linkages [10, 11, 28-30]. Following surface preparation proteins were immobilized on these chemically active surfaces through the amine or carboxyl groups. However, the immobilization of protein on target surface is carried out in a random way. Therefore, the use inorganic binding peptides can be a unique utility on assembling biomolecules on any desired inorganic surface for many different applications including sensors and medical devices.

3. EXPERIMENTAL PART

3.1 Materials

3.1.1 Bacterial Strains

3.1.1.1 *Escherichia coli* K12 TB1

Escherichia coli strain K12 TB1 [F⁻ *ara* Δ(*lac-proAB*) [Φ80*dlac* Δ(*lacZ*)M15] *rpsL*(Str^R) *thi* *hsdR*] was purchased from New England Biolabs (Catalog # E4122S) and used for construction of expression vector.

3.1.1.2 *Escherichia coli* K12 ER2507

Escherichia coli strain K12 ER2507 [F⁻ *ara-14* *leuB6* *fhuA2* Δ(*argF-lac*)U169 *lacY1* *glnV44* *galK2* *rpsL20* *xyl-5* *mtl-5* Δ(*malB*) *zjc::Tn5*(Kan^R) Δ(*mcrC-mrr*)_{HB101}] was purchased from New England Biolabs (Catalog # E4121S) and used for construction of expression vector.

3.1.1.3 *Escherichia coli* Max Efficiency[®] DH5α[™]

Escherichia coli strain MAX Efficiency[®] DH5α[™] Competent Cells [F-φ80*lacZ*ΔM15 Δ(*lacZYA-argF*) U169 *recA1* *endA1* *hsdR17* (r_k⁻, m_k⁺) *phoA* *supE44* λ- *thi-1* *gyrA96* *relA1*] was purchased from Invitrogen (Catalog # K4520-01 20) and used for general cloning and blue/white screening without IPTG. Strain is resistant to T1 bacteriophage construction of cloning vector.

3.1.2 Expression Vectors

3.1.2.1 pMAL[™]-2 Vectors

The pMAL[™]-2 vectors (given in Appendix A) provide a method for expressing and purifying a protein produced from a cloned gene. The cloned gene is inserted downstream from the *malE* gene of *E. coli*, which encodes maltose-binding protein (MBP), resulting in the expression of an MBP fusion protein (1,2). The method uses the strong “tac” promoter and the *malE* translation initiation signals to give high-level expression of the cloned sequences (3,4), and a one-step purification of the

fusion protein using MBP's affinity for maltose (5). The vectors express the *malE* gene (with or without its signal sequence) fused to the *lacZα* gene. Restriction sites between *malE* and *lacZα* are available for inserting the coding sequence of interest. Insertion inactivates the β-galactosidase α-fragment activity of the *malE-lacZα* fusion, which results in a blue to white color change on Xgal plates when the construction is transformed into an α-complementing host such as TB1 or JM107 [52]. The vectors carry the *lacIq* gene, which codes for the *Lac* repressor. This keeps expression from Ptac low in the absence of IPTG induction. The pMAL-2 vectors also contain the sequence coding for the recognition site of a specific protease, located just 5' to the polylinker insertion sites. This allows MBP to be cleaved from the protein of interest after purification. The pMALc2X vector has a site for Factor Xa. Factor Xa cleaves after its four amino acid recognition sequence, so that few or no vector-derived residues are attached to the protein of interest, depending on the site used for cloning.

3.1.3 Enzymes

All the enzymes are recombinant and produced in *E. coli*. The restriction enzymes were purchased from New England Biolabs, the buffers used accordingly as provided and suggested by the supplier. All the restriction enzymes were supplied with the reaction buffer called 10X NEBuffer with each restriction endonuclease to ensure optimal (100%) activity. Most of the enzymes were supplied with one of four standard NEBuffers. Occasionally, an enzyme has specific buffer requirements not met by one of the four standards. Some restriction enzymes require BSA at a final concentration of 100 µg/ml for optimal activity.

3.1.3.1 Lysozyme (EC 3.2.1.17) from Chicken Egg white

50,000 units/mg lyophilized powder containing sodium acetate buffer salts and sodium chloride was purchased from Sigma (Catalog # L7001). It was dissolved in deionized water to get 10-20 mg/ml concentration. This enzyme breaks down the cell walls of bacteria so it was used for cell lysis.

3.1.3.2 *MscI* Restriction Endonuclease

The source of the restriction enzyme is an *E. coli* strain that carries the *MscI* gene from *Micrococcus* species. It was supplied with NEBuffer 4 (10X) from New

England Biolabs (Catalog # R0534S). Its recognition and cutting site is as follows, 5'-TGG[^]CCA-3'. It was used for construction of expression vector.

3.1.3.3 *Pst*I Restriction Endonuclease

The source of the restriction enzyme is an *E. coli* strain that carries the *Pst*I gene from *Providencia stuartii* 164. It was supplied with NEBuffer 3 (10X) and BSA (100X) from New England Biolabs (Catalog # R0140T). Its recognition and cutting site is as follows, 5'-CTGCA[^]G-3'. It was used for construction expression vector.

3.1.3.4 *Nla*IV Restriction Endonuclease

The source of the restriction enzyme is an *E. coli* strain that carries the *Nla*IV gene from *Neisseria lactamica*. It was supplied with NEBuffer 4 (10X) and BSA (100X) from New England Biolabs (Catalog # R0126S). Its recognition and cutting site is as follows, 5'-GGN[^]NCC-3'. It was used for construction expression vector.

3.1.3.5 *Xmn*I Restriction Endonuclease

The source of the restriction enzyme is an *E.coli* strain that carries the *Xmn*I gene from *Xanthomonas manihotis* 7AS1. It was supplied with NEBuffer 4 (10X) and BSA (100X) from New England Biolabs (Catalog # R0194S). Its recognition and cutting site is as follows, 5'-GAANN[^]NNTTC-3' It was used for digestion of expression vector.

3.1.3.6 *Taq* DNA Polymerase

It was 5 U/μl in its storage buffer with its discrete reaction buffer called 10X PCR Buffer (100 mM Tris-HCl (pH 8.3), 500 mM KCl, 1.5 mM MgCl₂) and was purchased from New England Biolabs (Catalog # M0273X).

3.1.3.7 Quick T4 Ligase

It provides ligation of cohesive end or blunt end DNA fragments in 5 minutes at room temperature (25°C). It was supplied with Quick Ligation™ Kit New England Biolabs (Catalog # M2200S). It was used for cloning into vectors.

3.1.3.8 Factor Xa Protease

Factor Xa cleaves after the arginine residue in its preferred cleavage site Ile-(Glu or Asp)-Gly-Arg. It will sometimes cleave at other basic residues, depending on the conformation of the protein substrate. The most common secondary site, among

those that have been sequenced, is Gly-Arg. There seems to be a correlation between proteins that are unstable in *E.coli* and those that are cleaved by Factor Xa at secondary sites; this may indicate that these proteins are in a partially unfolded state [46]. Factor Xa will not cleave a site followed by proline or arginine. It was from England Biolabs (Catalog # P8010S).

3.1.4 Protein Molecular Weight Markers

The Protein Molecular Weight Markers are Unstained Protein Molecular Weight Marker from Fermentas (Catalog # SM0431) and Protein Marker, Broad Range (2-212 kDa) from New England Biolabs (Catalog #P7702S) (shown in Appendix B)

3.1.5 DNA Molecular Weight Markers

The used DNA markers are Lambda DNA/*EcoRI*+*HindIII* Marker 3, purchased from Fermentas (Catalog #SM0193) and Lambda DNA/*Eco130I* (*StyI*) Marker 16 from Fermentas (Catalog # SM0161) and *PhiX174* DNA/*BsuRI* (*HaeIII*) Marker 9 from Fermentas (Catalog #SM0252) (see in Appendix C).

3.1.6 Oligonucleotides and Primers

The oligonucleotides and primers were purchased from Invitrogen as lyophilized form.

(PG)₃-AuBP1 5'- TAT ATA TTT TGG CCA GGC CCG GGC CCG
GGC TGG GCG GGC GCG AAA CGT CTG GTG
CTG CGT CGT GAA TAA TAA CTG CAG CGC
CCG CGC -3'

SGGG-AuBP1 5'- TAT ATA TTT GGA TCC GGC GGC GGC TGG
GCG GGC GCG AAA CGT CTG GTG CTG CGT
CGT GAA TAA TAA CTG CAG CGC CCG CGC -3'

(PG)₃-QBP2 5'- TAT ATA TTT TGG CCA GGC CCG GGC CCG
GGC CTG CCG GAT TGG TGG CCG CCG CCG
CAG CTG TAT CAT TAA TAA CTG CAG CGC
CCG CGC -3'

SGGG-QBP2 5'- TAT ATA TTT GGA TCC GGC GGC GGC CTG
CCG GAT TGGTGG CCG CCG CCG CAG CTG TAT
CAT TAA TAA CTG CAG CGC CCG CGC -3'

Forward primer (SGGG-GEPI)	5'-TAT ATA TTT GGA TCC GGC GGC GGC -3'
Reverse primer (SGGG-GEPI)	5'-GCG CGG GCG CTG CAG TTA TTA TTC-3'
Forward primer ((PG) ₃ -GEPI)	5'-TAT ATA TTT TGG CCA GGC CCG GGC -3'
Reverse primer ((PG) ₃ -GEPI)	5'-GCG CGG GCG CTG CAG TTA TTA TTC-3'

Sequencing *malE* Forward Primer (24-mer)

Sequence: 5'GGTCGTCAGACTGTCGATGAAGCC 3'

Sequencing Reverse Primer (21-mer)

Sequence: 5' GAAAGGGGGATGTGCTGCAAG 3'

3.1.7 Bacterial Culture Media

3.1.7.1 LB Medium

20 g LB Broth including 10 g tryptone, 5 g NaCl and 5 g yeast extract (Sigma) was dissolved in distilled water up to 1L. in addition, the pH was adjusted to 7.0-7.5 with 10 M NaOH and then sterilized for 15 minutes less than 1.5 Atm at 121°C.

3.1.7.2 LB Agar Medium

35 g LB Agar including 10 g tryptone, 5 g NaCl, 5 g yeast extract and 15 g agar (Sigma) was dissolved in distilled water up to 1 L. and the pH was adjusted to 7.0-7.5 with 10 M NaOH and then sterilized for 15 minutes under 1.5 Atm at 121°C.

3.1.7.3 LB Medium (containing 2% glucose)

LB Broth containing 10 g tryptone, 5 g NaCl , and 5 g yeast extract and 2 g glucose (Sigma) was dissolved in distilled water up to 1L. in addition, the pH was adjusted to 7.0-7.5 with 10 M NaOH and then sterilized for 15 minutes less than 1.5 Atm at 121°C.

3.1.7.4 SOC Medium

SOC medium including 2% tryptone 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM MgCl₂, 10 mM MgSO₄ and 20 mM glucose was supplied with pCR[®]2.1-TOPO[®] TA Cloning Kit (Catalog # K4500-01, Invitrogen)

3.1.8 Stock Solutions

3.1.8.1 1000x Ampicillin (Sodium Salt)

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

3.1.8.2 X-gal/IPTG Stock Solution

1.25 g IPTG (Sigma) and 1 g X-gal (Fermentas) were dissolved in 25 ml DMF (Riedel-de-Haen). The solution was stored at -20°C in the dark.

3.1.8.3 1 M IPTG Stock Solution

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

3.1.8.4 Glycerol Stock Solution

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

3.1.9 Buffers

3.1.9.1 Na-Ac Buffer

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

3.1.9.2 10X TBE Buffer (1000ml)

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

3.1.9.3 Column Buffer (1000ml)

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

β -mercaptoethanol were added into this solution and final volume was adjusted to 1 liter with distilled water.

3.1.9.4 Elution Buffer (1000ml)

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

3.1.10 Chemicals

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

Tris, NaOH, bromophenol blue, SDS, HCl, CBB-R250, CBB-G250, glacial acetic acid, glucose, MgCl_2 , TEMED, NaH_2PO_4 and EDTA were purchased from Merck., NaCl, NH_4Cl , CaCl_2 , isopropanol, glycerol, absolute ethanol, absolute methanol and acetic acid (glacial) were purchased from Fluka.

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

3.1.11 Computer Software

Gel Documentation System: UVIPhotoMW Version 99.05 for Windows 95 & 98, UVIttec Limited.

Homology search: Basic Local Alignment Search Tool, BLAST. Standard protein BLAST (blastp) (Altschul *et al.* 1990; Nakamura *et al.*, 2000). Available at <http://www.ncbi.nlm.nih.gov/BLAST/>

Nucleotide alignments: Multiple sequence alignment tools, ClustalW (Higgins *et al.*, 1994). Available at <http://www.ebi.ac.uk/clustalw/index.html>

Translator tool: JustBio Translator tool, (Rodriguez P.). Available at www.justbio.com.

3.2 Instruments

Instruments are given in Appendix D.

3.3 Methods

3.3.1 Construction of Bi-functional Protein Vector

The desired vector construction was accomplished by firstly design of oligonucleotides and primers for PCR, secondly PCR amplification of oligonucleotides, then restriction endonuclease digestion of DNA fragments and vector, and lastly cloning the DNA fragments into expression vector.

3.3.1.1 Oligonucleotides and Primer Design

In order to construct the bi-functional protein vector the required oligonucleotides and primers were designed according to amino acid sequence of GEPI with linker and cloning strategy of expression vector and synthesized as lyophilized form by Invitrogen (Figure 4.2, Figure 4.3, Figure 4.4, and Figure 4.5). The polylinker site of vector has a number of regions, which are recognized by a specific endonuclease. Therefore, the base sequence of GEPI with linker should contain the appropriate recognition sites for restriction endonuclease to be used in cloning strategy.

3.3.1.2 Amplification of Double Stranded DNA from Oligonucleotides by PCR

Designed oligonucleotides of SGGG-AuBP1, (PG)₃-AuBP1, SGGG-QBP2 and (PG)₃-QBP2 coding sequence (Figure 4.2, Figure 4.3, Figure 4.4, Figure 4.5) which have two restriction endonuclease recognition sites and two stop codons at the end of the specific peptide sequence were amplified. Forward and reverse primers, which are complementary to the DNA region targeted, were used for amplification. *Taq* DNA polymerase was used for the polymerase chain reaction. During optimization of PCR reaction, the following PCR reaction was set up for each template DNA and different annealing temperatures were tried to adjust the PCR reaction. After optimization, the proper PCR conditions were determined for each template (Table 3.1-3.4)

Table 3.1: Polymerase Chain Reaction (PCR)

Reagent	Volume (μl)
Sterile d H ₂ O	39,3
10X Taq Buffer	5,0
10 mM dNTP (2.5mM each)	2,0
25 mM MgCl ₂	3,0
Forward Primer (10 pmole)	1,0
Reverse Primer (10 pmole)	1,0
Template DNA (1 μg)	0,5
Taq DNA polymerase	0,2
Total volume	50

Table 3.2: PCR conditions for SGGG-AuBP1 and (PG)₃- AuBP1

Temperature(°C)	Time	Cycles
95	2 min	1
94	30 sec	30
58	30 sec	
72	1 min	
72	20 min	1

Table 3.3: PCR conditions for SGGG-QBP2

Temperature(°C)	Time	Cycles
95	2 min	1
94	30 sec	30
50	30 sec	
72	1 min	
72	20 min	1

Table 3.4: PCR conditions for (PG)₃-QBP2

Temperature(°C)	Time	Cycles
95	2 min	1
94	30 sec	30
60	30 sec	
72	1 min	
72	20 min	1

3.3.1.3 Agarose Gel Electrophoresis of PCR Products

Agarose gel electrophoresis was carried out for separating and analyzing of DNA fragments. The agarose gel was 2,5% because the gel of 2,5 % shows good resolution for small fragments (0.05–1kb). Our fragments are 87 mer or 81 mer. Lambda DNA/Eco130I (StyI) Marker 16 from Fermentas (Catalog # SM0161) in Appendix C was used as a marker.

3.3.1.4 Purification of PCR Products via Gel Extraction Procedure

PCR products were extracted from LMP agarose gel using MinElute Extraction Kit from Qiagen (Catalog # 28604)

1. DNA fragment is excised from agarose gel with a clean scalpel. Gel slices are put into microfuge tubes and their weight is calculated.
2. A 3-gel volume of QG buffer is added to 1 volume of gel slice. (600 µl for 100 mg gel). When gels are excised from a %2 or more concentrated gel, a 6-gel volume of QG buffer is applied.
3. Sample is incubated at 50°C for 10 min. by vortexing every 2 min.
4. After the gel completely dissolved, the color of the mixture is checked. QG buffer contains a pH indicator and gives a yellow color at pH < 7.5, the optimum pH for DNA adsorption. If the color of the mixture is not yellow, 10 µl of 3 M sodium acetate, pH5.0 is added.
5. After the gel is dissolved, 1 gel volume of room temperature isopropanol is added and mixed by inverting the tube several times.
6. Sample is applied to min-elute column and centrifuged for 1 min. at 13000 rpm.

7. Flow-through is discarded from collection tube.
8. 500 µl QG buffer is applied to column and centrifuged for 1 min. at 13000 rpm.
9. Flow-through is discarded from collection tube and centrifuged for additional 1 min.
10. Spin column placed in a 1, 5 ml. microfuge tube and 10 µl Elution Buffer is applied to elude the DNA. After 1 min. incubation tube is centrifuged for 1 min.
11. Eluted DNA is stored at -20°C for further use.

3.3.1.5 Quantization of Nucleic Acids

Determination of the concentration for unknown DNA fragments in microplates was carried out by measuring absorbance at 260 nm using followed by correction for light pathlength. The subsequent pathlength corrected absorbance was then transformed using the appropriate conversion factor indicated in Table 3.5.

Table 3.5: Commonly accepted absorbance to concentration conversion for nucleic acids. Indicated values are accepted average values for absorbance measurements at 260 nm with a 1 cm pathlength

Nucleic Acid Species	Concentration for 1 A ₂₆₀ Unit (µg/ml)
dsDNA	50
ssDNA (oligonucleotides)	33
ssRNA	40

3.3.1.6 Restriction Enzyme Digestions of DNA Encoding for Linker-GEPI

Amplified four kinds of DNA fragments which are capable of coding for each GEPI with linker sequence, in turn, SGGG-AuBP1, (PG)₃-AuBP1, SGGG-QBP2 and (PG)₃-QBP2 were digested with the specific restriction endonucleases for cloning them produced by PCR into pMAL vector. In order to obtain 5' - blunt end from each DNA different restriction enzymes were used, but the same restriction enzyme, PstI, was used to get 3'- sticky end from each DNA. The restriction enzyme digestion reactions are in Table 3.6, Table 3.7, Table 3.8, Table 3.9,. Reactions were incubated at 37°C for 3 h. and then at 80°C for 20 minutes for enzyme inactivation. Digestion reaction was run on a 2.5 % agarose gel.

Table 3.6: Digestion Reaction of SGGG-AuBP1

Reagent	Volume (μl)
DNA (631 ng/μl)	25
10X NEBuffer 4	5,0
NIaIV (1 U/μl)	6,0
PstI (20 U/μl)	0,5
Sterile distilled water	13,5
Total volume	50

Table 3.7: Digestion Reaction of (PG)₃-AuBP1

Reagent	Volume (μl)
DNA (514 ng/μl)	25
10X NEBuffer 1	5,0
MscI (3 U/μl)	2,0
PstI (20 U/μl)	2,0
Sterile distilled water	16
Total volume	50

Table 3.8: Digestion Reaction of SGGG-QBP2

Reagent	Volume (μl)
DNA (410 ng/μl)	25
10X NEBuffer 4	5,0
NIaIV (1 U/μl)	6,0
PstI (20 U/μl)	0,5
Sterile distilled water	13,5
Total volume	50

Table 3.9: Digestion Reaction of (PG)₃-QBP2

Reagent	Volume (μl)
DNA (474 ng/μl)	25
10X NEBuffer 1	5,0
MscI (3 U/μl)	2,0
PstI (20 U/μl)	2,0
Sterile distilled water	16
Total volume	50

3.3.1.7 Agarose Gel Electrophoresis of PCR Products

Agarose gel electrophoresis was applied for analyzing and separating of DNA fragments. The agarose gel was 2,5% because the gel of 2,5 % shows good resolution for small fragments (0.05–1kb). Our fragments are 54 mer and 48 mer. Lambda DNA/Eco130I (StyI) Marker 16 from Fermentas (Catalog # SM0161) in Appendix C was used as a marker.

1.8 Purification of PCR Products via Gel Extraction Procedure

Following digestion reaction, PCR products were extracted from LMP agarose gel using MinElute Extraction Kit from Qiagen (Catalog # 28604).

1. DNA fragment is excised from agarose gel with a clean scalpel. Gel slices are put into microfuge tubes and their weight is calculated.
2. A 3-gel volume of QG buffer is added to 1 volume of gel slice. When gels are excised from a %2 or more concentrated gel, a 6-gel volume of QG buffer is applied.
3. Sample is incubated at 50°C for 10 min. by vortexing every 2 min.
4. After the gel completely dissolved, the color of the mixture is checked. QG buffer contains a pH indicator and gives a yellow color at pH < 7.5, the optimum pH for DNA adsorption. If the color of the mixture is not yellow, 10 μl of 3 M sodium acetate, pH5.0 is added.

5. After the gel is dissolved, 1 gel volume of room temperature isopropanol is added and mixed by inverting the tube several times.
6. Sample is applied to min-elute column and centrifuged for 1 min. at 13000 rpm.
7. Flow-through is discarded from collection tube.
8. 500 µl QG buffer is applied to column and centrifuged for 1 min. at 13000 rpm.
9. Flow-through is discarded from collection tube and centrifuged for additional 1 min.
10. Spin column placed in a 1, 5 ml. microfuge tube and 10 µl Elution Buffer is applied to elude the DNA. After 1 min. incubation tube is centrifuged for 1 min.
11. Eluted DNA is stored at -20°C for further use.

3.3.2 Competent Cell Preparation

Competent cells have more easily altered cell walls that DNA can be passed through easily. These cells readily incorporate foreign DNA. If the cells are not already competent, it can be made more competent through calcium chloride and heat shock. This is especially easy with cells undergoing very rapid growth. Rubidium Chloride Method was used for *E. coli* K12 ER2507 and TB1 strains. This procedure works with most strains and should routinely give $> 10^7$ cfu/ug of pBR322 with reasonably healthy K-12 derivatives [52]. Frozen cells last at least a year. The following protocol was used to generate *E. coli* that is chemically competent for transformation of ligation reactions or plasmids.

Chemically Competent Preparation Protocol:

1. Single line of colony of *E. coli* strain is inoculated to 5 ml Luria Bertani (LB) liquid medium and incubated at 37°C and 250 rpm overnight.
2. 0.5 ml of overnight culture is inoculated into 50 ml of LB liquid medium and incubated at 37° C, 250 rpm until optical density at 600 nm (OD₆₀₀) reached 0.2-0.3.
3. The culture is centrifuged for 10 minutes at 4000x g, 4° C in Beckman Coulter J109 Avanti centrifuge with JA30.50i rotor.
4. The supernatant is removed and the pellet dried on a tissue paper.

5. The pellet is gently resuspended in ice-cold 9 ml RF1 solution (100mM RbCl₂, 50 mM MnCl₂, 30mM KAc (pH: 7.5), 10mM CaCl₂, Glycerol 15 % (w/v); pH is adjusted to 5.8 with 0.2 M HCl, solution is sterilized with 0.22µm filter) and centrifuged for 10 minutes at 4000 x g, 4° C in Beckman Coulter Avanti J-30I centrifuge with JA-30.50i rotor.
6. The supernatant is removed and the pellet is gently resuspended in ice cold 1 ml RF2 solution (10mM MOPS (0.5 M stock, pH: 6.9), 10mM RbCl₂, 75 mM CaCl₂, Glycerol 15 % (w/v); adjust pH to 6.8 with NaOH, sterilize with 0.22 µm filter) and kept on ice 1-2 hours.
7. 50 µl of competent cell solution is sampled to pre-chilled microfuge tubes and stored at -80° C for transformation.

All tubes, rotors, solutions should be kept on ice or at -80°C before use. When resuspending pellet cells, motorized pipette was used instead of a vortex to avoid lysis.

3.3.3 Chemical Transformation of pMAL c2X into *E.coli* K12 TB1

The commercial expression vector was transformed into competent cell *E. coli* K12 TB1 to prepare glycerol stocks and express the wild type MBP. The used protocol was as follows:

1. The vial of TB1 chemically competent cells in microfuge tube is thawed on ice.
2. 0.1-0.5 µl of vector is added into the vial of competent cells, mixed gently and incubated on ice for 30 minutes.
3. The cells are heat shocked at 42° C for exactly 60 seconds without shaking.
4. The tubes are immediately transferred to ice and are incubated on ice for 10 minutes.
5. 0.2 ml of room temperature SOC medium and 0.8 ml LB medium are added to the mixture of vector and competent cells in the microfuge tube.
6. The tubes are tightly capped and shaken horizontally at 200 rpm at 37° C for 15 minutes.
7. 4 dilutions (25, 50, 75 and 150 µl) are spread from the transformation reaction onto pre-warmed LB agar plates containing Ampicillin by using a spreader.

8. The plates are incubated at 37° C overnight.

3.3.3.1 Glycerol Stock Preparation

After overnight incubation of plates, the colonies were picked with a sterile toothpick and stabbed onto a master LB agar plate with Ampicillin of 100µg/ml. At the same time, the toothpick carrying one colony was put in 3 ml liquid LB containing 100µg/ml of Ampicillin and incubated at 37° C overnight. In order to prepare glycerol stock for a long storage, 850 µl of this bacterial culture was mixed with 150 µl 80 % glycerol and stored -80° C at least one year.

3.3.3.2 Plasmid DNA Isolation (Mini Prep)

Following transformation, the colonies received the plasmid were chosen from LB agar containing 100 µg/ml Ampicillin plates and grown in 3 ml liquid LB containing 100 µg/ml overnight. Plasmid DNA was isolated for further applications by using High Pure Plasmid Isolation Kit from Roche (Catalog # 1 754 785). Isolation procedure is described below:

1. 3 ml of the culture is centrifuged 60 seconds at 6000 x g.
2. The supernatant is discarded.
3. The pellet is resuspended in 250 µl Suspension Buffer + RNase.
4. 250 µl Lysis Buffer is added, mixed gently by inverting the tube 3 to 6 times and incubated at 15-25 °C for 5 minutes.
5. 350 µl chilled Binding Buffer is added mixed gently and incubated on ice for 5 minutes.
6. The samples are centrifuged at 13 000 x g for 10 minutes.
7. High Pure Filter Tubes are inserted into Collection Tubes.
8. Supernatant is transferred to High Pure Filter Tube and centrifuged at 13 000 x g for 1 minute.
9. The flow-through is discarded.
10. 500 µl Wash Buffer I is added and centrifuged at 13 000 x g for 1 minute.
11. The flow-through is discarded.
12. 700 µl Wash Buffer II is added centrifuged at 13 000 x g for 1 minute.
13. The flow-through is discarded and an additional 1 minute centrifuged at 13 000 x g is performed.

14. The filter tube is placed in 1.5 ml microcentrifuge tube and 100 µl Elution Buffer is added.
15. The isolated plasmid is checked by running on an agarose gel.

3.3.3.3 Agarose Gel Electrophoresis of Purified pMALc2X Vector

Agarose gel electrophoresis was applied for analyzing of pMALc2X.. The agarose gel was 0.7 % because the plasmid size is over 6kb. Lambda DNA/*EcoRI*+*HindIII* Marker 3, purchased from Fermentas (Catalog #SM0193) in Appendix C was used as a marker

3.3.3.4 Restriction Enzyme Digestion of pMALc2X Vector

For cloning a fragment produced by PCR into a pMAL vector. The PCR fragment begins with a blunt end, and that a stop codon followed by a PstI site has been incorporated into the 3' end. Therefore pMALc2X vector was digested with 20 units of XmnI and 30 Units of PstI. For complete digestion of the pMALc2X digest was checked by running 4 µl on an agarose gel. At the same time, PCR fragments digested were run to estimate their concentrations electrophoretically. The digestion reaction reagents for pMALc2X vector are shown in Table 3.10.

Table 3.10: Digestion Reaction of pMALc2X vector

Reagent	Volume (µl)
DNA (180 ng/µl)	25
10X NEBuffer 1	5,0
XmnI (20 U/µl)	1,0
PstI (20 U/µl)	1,5
Sterile distilled water	17,5
Total volume	50

3.3.3.5 Agarose Gel Electrophoresis of Digested pMALc2X Vector

Agarose gel electrophoresis was carried out for analyzing f the vector is cut and separating from the gel. The agarose gel was 0.7 % because the plasmid size is over 6kb. Lambda DNA/*EcoRI*+*HindIII* Marker 3, purchased from Fermentas (Catalog #SM0193) in Appendix C was used as a marker

3.3.3.6 Purification of Digest pMALc2X via Gel Extraction Procedure

Following digestion reaction, vector was extracted from LMP agarose gel using Qiaquick Gel Extraction Kit from Qiagen (Catalog # 28604).

1. DNA fragment is extracted from agarose gel with a clean scalpel. Gel slices are put into microfuge tubes and their weight is calculated.
2. A 3-gel volume of QG buffer is added to 1 volume of gel slice. (600 µl for 100 mg gel). When gels are excised from a %2 or more concentrated gel, a 6-gel volume of QG buffer is applied.
3. Sample is incubated at 50°C for 10 min. by vortexing every 2 min.
4. After the gel completely dissolved, the color of the mixture is checked. QG buffer contains a pH indicator and gives a yellow color at pH<7.5, the optimum pH for DNA adsorption. If the color of the mixture is not yellow, 10 µl of 3 M sodium acetate, pH5.0 is added.
5. After the gel is dissolved, 1 gel volume of room temperature isopropanol is added and mixed by inverting the tube several times.
6. Sample is applied to min-elute column and centrifuged for 1 min. at 13000 rpm.
7. Flow-through is discarded from collection tube.
8. 500 µl QG buffer is applied to column and centrifuged for 1 min. at 13000 rpm.
9. Flow-through is discarded from collection tube and centrifuged for additional 1 min.
10. Spin column placed in a 1,5 ml. microfuge tube and 10 µl Elution Buffer is applied to elude the DNA. After 1 min. incubation tube is centrifuged for 1 min.
11. Eluted DNA is stored at -20°C for further use.

3.3.4 Cloning of GEPI Gene with Linker into pMALc2X Expression Vector

This section provides subcloning a gene of interest into a pMALc2X vector. This procedure provides that a fragment containing the gene of interest with a blunt end at the 5' end of the gene and an overhang at the 3' end to be inserted into the pMALc2X vector, using the restriction site adjacent to and downstream of the sequence encoding the protease site (the primary site), and a site further down in the polylinker with a compatible overhang. The DNA fragments were made by PCR After that, the

DNA fragments were digested with the suitable restricted endonucleases for construction a 5'-blunt 3'-sticky-ended fragment containing the gene of interest. The used restriction enzymes were shown in Figure 4.2, Figure 4.3, Figure 4.4, and Figure 4.5. The restriction site for creating the sticky end is one of the enzymes, PstI, which cuts in the pMALc2X polylinker downstream of the primary site. Later, the digest DNA inserts and digest vector were extracted from the gel. Cleaned and concentrated inserts were ligated with pMALc2X vector.

3.3.4.1 Ligation

The inserts digest and vector digest run onto agarose gel were isolated from LMP agarose gel and set up the following ligation reactions in Tablo-3.11, Tablo-3.12, Tablo-3.13 and Table 3.14. Using the equation (3-1), the volume of the vector component required in the ligation is calculated first, then this is subtracted from the total DNA component of the reaction (T) to give the required insert volume. The value T represents the combined volume of vector and inserts DNA solutions added to the ligation reaction. The lengths and concentrations of the DNA fragments must be inserted along with the desired insert-to-vector ratio.

$$V_v = \frac{T}{\left(\frac{V_c \cdot I_i \cdot I_r}{I_c \cdot V_i}\right) + 1} \quad I_v = T - V_v \quad (3-1)$$

Equation (3-1) described determination of the volumes required in a ligation reaction. Insert and vector parameters must both be in the same units (e.g. kilobases for length and micrograms per microlitre for concentration). I_i Insert length, V_i vector length, I_c insert concentration, V_c vector concentration, I_r required insert-to-vector ratio, T volume of total DNA solution component, V_v vector volume, I_v insert volume. The I_r should be inserted as insert/vector (e.g. 2 for a two-fold insert excess, 0.5 for a two-fold vector excess).

Table 3.11: Ligation reaction for SGGG-AuBP1 according to insert/vector ratio which is 10 for a two-fold insert excess

Component	Volume
pMALc2X vector digest (6611 bp, 143 ng/μl)	7,9 μl
Insert digest (48 bp, 85 ng/μl)	2,1 μl
2X Quick Ligation Buffer*	10 μl
Quick T4 DNA Ligase	1,0 μl
Total volume	21 μl

*Quick Ligation™ Buffer is supplied with Quick Ligation™ Kit from New England Biolabs (Catalog # M2200S)

Reaction condition

Ligation reaction was incubated at room temperature (25 ° C) for 1h and chilled on ice.

Table 3.12: Ligation reaction for (PG)₃-AuBP1 according to insert/vector ratio which is 10 for a two-fold insert excess

Component	Volume
pMALc2X vector digest (6611 bp, 143 ng/μl)	6,9 μl
Insert digest (54 bp, 58 ng/μl)	3,1 μl
2X Quick Ligation Buffer*	10 μl
Quick T4 DNA Ligase	1,0 μl
Total volume	21 μl

*Quick Ligation™ Buffer is supplied with Quick Ligation™ Kit from New England Biolabs (Catalog # M2200S)

Reaction condition

Ligation reaction was incubated at room temperature (25 ° C) for 1h and chilled on ice.

Table 3.13: Ligation reaction for SGGG-QBP2 according to insert/vector ratio which is 10 for a two-fold insert excess

Component	Volume (μl)
pMALc2X vector digest (6611 bp, 143 ng/μl)	3,5
Insert digest (48 bp, 6 ng/μl)	6,5
2X Quick Ligation Buffer*	10
Quick T4 DNA Ligase	1,0
Total volume	21

*Quick Ligation™ Buffer is supplied with Quick Ligation™ Kit from New England Biolabs (Catalog # M2200S)

Reaction condition

Ligation reaction was incubated at room temperature (25 °C) for 1h and chilled on ice.

Table 3. 14: Ligation reaction for (PG)₃-QBP2 according to insert/vector ratio which is 10 for a ten-fold insert excess

Component	Volume (μl)
pMALc2X vector digest (6611 bp, 150 ng/μl)	8,6
Insert digest (54 bp, 73 ng/μl)	1,4
2X Quick Ligation Buffer*	10
Quick T4 DNA Ligase	1,0
Total volume	21

3.3.4.2 Preparation of X-Gal/IPTG-Amp Plates

50 μl Ampicillin solution and 200 μl X-Gal/IPTG solution made up of 20 mg/ml X-Gal and 25mM IPTG were put into 50 ml liquid LB agar at 50 °C and was poured onto plastic sterile petri dishes, quickly. The final concentrations of X-Gal and IPTG on each plate should be 80 μg/ml X-Gal and 0.1 mM IPTG.

3.3.4.3 Chemical Transformation of Ligation Mixture into *E.coli* K12 TB1

The ligation reaction was transformed into competent cell *E. coli* K12 TB1. The used protocol was as follows:

1. The vial of TB1 chemically competent cells in microfuge tube is thawed on ice.
2. 5 μ l of ligation reaction is added into the vial of competent cells, mixed gently and incubated on ice for 40-60 minutes.
3. The cells are heat shocked at 42° C for exactly 1-2 minutes without shaking.
4. The tubes are immediately transferred to ice and are incubated on ice for 1 minute
5. 0.2 ml of room temperature SOC medium and 0.8 ml LB medium are added to the mixture of the ligation reaction and competent cells.
6. The tubes are tightly capped and shaken horizontally at 200 rpm at 37° C for 4 hours.
7. 4 dilutions (75, 150, 200 and 250 μ l) are spread from the transformation reaction onto pre-warmed LB agar plates with 100 μ g/ml Ampicillin and LB agar plate containing 100 μ g/ml Ampicillin, 80 μ g/ml X-Gal and 0.1 mM IPTG by using a spreader.
8. The plates are incubated at 37° C overnight.

3.3.4.4 Colony Screening

To select the recombinant colonies from LB Agar+ampicillin plates alpha complementation has been carried out. In α -complementation pMALc2X expression vector include partial *lacZ* gene. After ligation, the resulting DNA was used to transform *E. coli* K12 TB1 competent cells in which partial *lacZ* gene missing in the vector is present. In cells that contain a vector without an insert, the amino-terminal β -galactosidase subunit would interact with the carboxyl-terminal β -galactosidase subunit to form an active β -galactosidase enzyme, which catalyzes formation of a blue colored product from colorless X-Gal in the presence of inducer IPTG making the non-recombinant cells blue. However, clones containing an insert, recombinants, would not produce a functional β -galactosidase, and would consequently appear white in color [1-3]. Therefore, α -complementation was helped to impress if getting successful cloning and transformation. The colonies were picked with a sterile

toothpick and stabbed onto master LB amp plate and an LB plate containing 80 µg/ml Xgal and 0.1 mM IPTG. The plates were incubated at 37°C for 8 to 16 hours. The Lac phenotype on the X-Gal plate determined and recovered the “white” clones from the corresponding patch on the master plate. In addition, there was a control transformation with about 1 ng of the uncut pMAL vector. Since most inserts prevent expression of any lacZα fragment, it is possible to get some α-fragment activity in clones with inserts. In this case, a difference in the shade of blue can usually be seen by comparing to transformants containing the vector alone. At the same time, an alternative way was used for the blue-white screen. The replica plate on LB amp and LB amp Xgal IPTG were prepared. The ligation mixture was divided into two aliquots, and one of them was spread on 80 µg/ml X-Gal, 0.1 mM IPTG to observe the percentage of clones with inserts. It was acceptably high; therefore the transformants were screened on the LB amp plate directly by preparing plasmid DNA. Because of the strength of the Ptac promoter, transformants taken from a plate containing IPTG can contain mutant plasmids that have either 1) lost part or the entire fusion gene, or 2) no longer express it at high levels [1-3].

3.3.4.5 Plasmid Isolation from Inoculated Transformant Cell

Following transformation, the white colonies received the vector with inserts were chosen from master plates LB agar containing 100 µg/ml Ampicillin and grown in 3 ml liquid LB containing 100 µg/ml overnight. Plasmid DNA was isolated for DNA sequence analysis and further applications by using High Pure Plasmid Isolation Kit from Roche (Catalog # 1 754 785). Isolation procedure is described below:

1. 3 ml of the culture is centrifuged 60 seconds at 6000 g.
2. The supernatant is discarded.
3. The pellet is resuspended in 250 µl Suspension Buffer + RNase.
4. 250 µl Lysis Buffer is added, mixed gently by inverting the tube 3 to 6 times and incubated at 15-25 °C for 5 minutes.
5. 350 µl chilled Binding Buffer is added mixed gently and incubated on ice for 5 minutes.
6. The samples are centrifuged at 13.000 g. for 10 minutes.
7. High Pure Filter Tubes are inserted into Collection Tubes.
8. Supernatant is transferred to High Pure Filter Tube and centrifuged at 13.000 g. for 1 minute.

9. The flow-through is discarded.
10. 500 µl Wash Buffer I is added and centrifuged at 13.000 g for 1 minute.
11. The flow-through is discarded.
12. 700 µl Wash Buffer II is added centrifuged at 13.000 g for 1 minute.

The flow-through is discarded and an additional 1 minute centrifuged at 13.000 g. is done.

13. The filter tube is placed in 1.5 ml microcentrifuge tube and 100 µl Elution Buffer is added.
14. The isolated plasmid is checked by running on an agarose gel.

3.3.4.6 Agarose Gel Electrophoresis of purified pMALc2X Constructs

Agarose gel electrophoresis was applied for analyzing of plasmid. The agarose gel was 0.7 % because the plasmid size is over 6kb. Lambda DNA/*EcoRI*+*HindIII* Marker 3, purchased from Fermentas (Catalog #SM0193) in Appendix C was used as a marker.

3.3.4.7 Analyzing Transformants

The plasmids were analyzed by restriction analysis to confirm the presence and correct orientation of the insert. Firstly, the restriction enzymes and a combination of enzymes that cut in the vector and the insert were tested both by computer program named pDRAW32 to predict the size of the fragments and inserts After that, the result from agarose gel electrophoresis experiment was compared with that of computer program.

3.3.4.8 DNA Sequence Analysis

To confirm that the gene is cloned in the correct orientation the constructs were sequenced as well as restriction analysis Considering the results from restriction analysis the plasmids were sequenced by the help of ABI PRISM 3100-Avant Genetic Analyzer (PE, Applied Biosystem, CA) using Big Dye[®] Terminator V 3.1 cycle sequencing kit from Applied Biosystems with the procedure given in Table 3.15.

Table 3.15: Preparation of cycle sequencing reaction

Component	Volume
Template	2,5µl
M13 -40 primer (3.2 pmole)	1µl
5X Sequencing Buffer	1 µl
Big Dye Terminator	2 µl
Distilled water	3,5 µl
Total Volume	10 µl

The reaction tubes were mixed well and spin briefly. The sequence PCR was performed according to Table 3.16

Table 3.16: Sequence PCR Conditions

Temperature(°C)	Time	Cycles
95	5 min	1
95	1 min	30
50	30sec	
60	4 min	
4	∞	

3.3.4.9 NaAc-Ethanol Precipitation (Purification) of PCR Products

To obtain clean sequencing data, Ethanol/Sodium Acetate Precipitation method given in below was used after PCR. Then the samples were sequenced by ABI PRISM 3100-Avant Genetic Analyzer (PE, Applied Biosystem, CA).

1. 1 µl 3M pH 4.6 sodium acetate (cold) and 25 µl 95 % ethanol (cold) are mixed for each sample.
2. 26 µl mixture is added into each PCR product and samples are incubated on ice for 15 min.
3. After incubation, samples are centrifuged for 15 min. at 13000 rpm.

4. Supernatant is discarded and DNA pellet is washed with 250 µl cold 70% ethanol.
5. Samples are centrifuged for 15 min. at 13000 rpm.
6. Ethanol is discarded; the residual ethanol is evaporated by incubating at 95°C.
7. DNA pellet is dissolved in 20 µl formamide.
8. Samples are denatured by putting at 95°C for 3 minutes and then at -20°C for 5 min.

3.3.4.10 Sequence Alignment

EMBOSS Pairwise Alignment Algorithms tool was used to compare 2 sequences. The water local alignment method of Smith-Waterman was used for trying to find the best region of similarity between two sequences. The glycerol stocks of the cells received the correct genes were prepared.

3.3.4.11 Transformation of pMAL c2X with Insert into *E.coli* K12 ER2507

The plasmids confirmed sequence was transformed into competent cell *E. coli* K12 ER2507 for correct expression and their glycerol stocks were prepared as in 3.3.3.1. The expression available host strains used in this study is TB1 and ER2507 K12 *E. coli* strains. The most important property of these strains is their capability of MBP and MBP-fusion protein expression rare tRNAs that are found in *E. coli*. TB1 strain is a derivative of JM83 that has been made *hsdR* so it will not restrict unmodified DNA. It has *lacZ*ΔM15 allele that's why it is proper for α-complementation. ER2507 is a derivative of RR1. The *malE* gene, which encodes maltose-binding protein, is deleted as part of the *malB* deletion. The *malE* gene is included in the *malB* deletion, so this strain does not make any MBP from the chromosome. It does not have the *lacZ*ΔM15 allele, so it cannot be used for α-complementation (no blue-to-white screen on Xgal). This strain can be transformed with high efficiency, similar to RR1 and HB101.

The used protocol of chemical transformation was as follows:

1. The vial of ER2507 chemically competent cells in microfuge tube is thawed on ice.
2. 0.1-0.5 µl of vector with insert is added into the vial of competent cells, mixed gently and incubated on ice for 30 minutes.

3. The cells are heat shocked at 42° C for exactly 60 seconds without shaking.
4. The tubes are immediately transferred to ice and are incubated on ice for 10 minutes.
5. 0.2 ml of room temperature SOC medium and 0.8 ml LB medium are added to the mixture of vector and competent cells in the microfuge tube
6. The tubes are tightly capped and shaken horizontally at 200 rpm at 37° C for 15 minutes.
7. 4 dilutions (25, 50, 75 and 150 µl) are spread from the transformation reaction onto pre-warmed LB agar plates containing Ampicillin by using a spreader.
8. The plates are incubated at 37° C overnight.

3.3.5 Protein Expression Studies

3.3.5.1 Pilot Experiment

Following construction of the vector for the bi-functional protein, the pilot experiment, a small scale experiment, was carried out for determination the behavior of a particular MBP fusion protein and the optimization of the protein expression and purification. This protocol results in five pMALc2X vectors samples are uninduced and induced cells, a total cell crude extract, a suspension of the insoluble material from the crude extract, a fraction containing protein that binds to the amylose resin. In the expression step, a single colony which harbors only pMALc2X_SGGG_AuBP1, pMALc2X_(PG)₃_AuBP1, pMALc2X_SGGG_QBP2 and pMALc2X_(PG)₃_QBP2 from each LB agar plate of ER2507 strain was picked by a sterile toothpick and grown in 5 ml LB containing Ampicillin of 100 µg/ml overnight to analyze the target protein production. Then, the below protocol was followed.

The protocol is as follows:

1. 80 ml rich broth which is LB containing Ampicillin of 100 µg/ml, 2 % glucose with 0.8 ml of an overnight culture of cells containing the fusion plasmid is inoculated.
2. The bacterial culture is grown on shaker at 200 rpm at 37°C until optical density A₆₀₀ is ~0.5. A sample of 1 ml is taken and centrifuged for 2 minutes (sample 1:

uninduced cells). Supernatant is discarded and the cells are resuspended in 50 μ l 2X SDS-PAGE Sample Buffer by vortex and frozen at -20°C .

3. IPTG is added as to the remaining culture to a final concentration of 0.3 mM, e.g. 0.24 ml of a 0.1 M stock in H_2O . Incubation is continued at 37°C for 4 hours. A 0.5 ml sample is withdrawn and centrifuged for two minute (sample 2: induced cells). Supernatant is discarded and the cells were resuspended in 100 μ l 2X SDS-PAGE Sample Buffer by vortex and frozen at -20°C . Additional time points at 1 and 4 hours is helpful in trying to decide when to harvest the cells for large scale preparations.
4. The cells harvested by centrifugation at $4000 \times g$ for 10 minutes. The supernatant is discarded.
5. The pellet is frozen overnight at -20°C . The freeze one pellet is resuspended in 5 ml of Column Buffer and thawed in cold water.
6. The cells were placed on ice and sonicated in short pulses of 10 seconds. The release of protein is monitored using the Bradford assay Monitor (21), adding 10 μ l of sonicated sample to 1.5 ml Bradford reagent and mixing. Sonication is gone on until the released protein reaches a maximum (usually about 2 minutes).
7. The sonicated cells are centrifuged at $9,000 \times g$ at 4°C for 20 minutes. The supernatant (crude extract) is decanted and saved on ice. The pellet is resuspended in 5 ml Column Buffer. This is a suspension of the insoluble matter. 5 μ l of 2x SDS-PAGE Sample Buffer is added to 5 μ l of the crude extract and insoluble matter fractions (samples 3 and 4, respectively).
8. ~200 μ l of the amylose resin is placed in a microfuge tube and spun briefly in a microcentrifuge. The supernatant is removed by aspiration and discarded. The resin is resuspended in 1.5 ml Column Buffer, then centrifuged briefly and the supernatant is discarded. Again the resin is resuspended in 200 μ l of Column Buffer. 50 μ l of crude extract is mixed with 50 μ l of the amylose resin slurry. Mixture is incubated for 15 minutes on ice and centrifuged for 1 minute, then the supernatant is removed and discarded. The pellet is washed with 1 ml Column Buffer, centrifuged for 1 minute, and the resin is resuspended in 50 μ l SDS-PAGE Sample Buffer (sample 5: protein bound to amylose).

3.3.5.2 Cell Fraction Analysis

Following induction the samples 1,2,3,4 and 5 obtained from in step 2,3,7 and 8 in the above-mentioned protocol were analyzed via SDS PAGE and Bradford assay in 3.3.5.3. The procedure was explained in the SDS-PAGE methodology section 3.3.5.4.

3.3.5.2 Bradford Protein Determination Assay

Protein concentration was determined according to Bradford Assay to assess the sonication efficiency and to calculate the fusion protein amount in total cell protein. Bradford reagent was purchased from Sigma. (Catalog # B 6916) Bovine serum albumin (Sigma) was used as standard.

3.3.5.3 Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) was performed essentially as described by Laemmli et al., 1970) using vertical gel electrophoresis system. Proteins were resolved on 10 -12 % (w/v) acrylamide slab gels in the presence of 0.1 % SDS. Proteins were dyed with CBB-R250 or CBB-G25. Proteins were stained for 30 minutes in the stain solution at room temperature on rocker at 15.000 rpm. They were destained in destain solution for overnight on rocker at 15 rpm in room temperature. 30% acrylamide mix was prepared by dissolving acrylamide and N,N'-Methylenebisacrylamide in ddH₂O, the mixture was then filtered through 0,45 µm cellulose acetate membrane filter.

Solutions for preparing resolving gels for SDS-PAGE:

All the solutions in Table 3.17 were mixed to prepare %10 or %12 resolving gels for SDS-PAGE.

Table 3.17: Ingredients of separating gel for SDS-PAGE.

Components	Amount (ml)	
Concentration of acrylamide mix	10 %	12 %
dH ₂ O	4.79	4.29
40 % acrylamide mix	2.5	3.0
1.5M Tris (Hydroxymethyl) aminomethane (Tris) (pH 8.8)	2.5	2.5
20 % Sodium n-Dodecyl Sulfate (SDS)	0.10	0.10
10% AMPS	0.10	0.10
N,N,N',N'-Tetraethyl ethylenediamine (TEMED)	0.01	0.01
Total	10	10

Solutions for %5 stacking gels for SDS-PAGE:

All solutions given in Table 3.18 were mixed to prepare %5 Stacking Gels for SDS-PAGE.

Table 3.18: Ingredients of 5 % stacking gels for SDS-PAGE

Components	Amount (ml)
dH ₂ O	3.94
40% acrylamide mix (g/ml)	0.50
1M Tris pH 6.8	0.50
20% SDS	0.030
10% AMPS	0.030
TEMED	0.003
Total	4.0

CBB -R250 or CBB-G250 stain solution:

All ingredients in Table 3.19 were mixed and then filtered through chromatography paper.

Table 3.19: Ingredients of CBB -R250 or CBB-G250 stain solution.

Components	Amount (ml)
dH ₂ O	650
Isopropanol	250
Acetic acid glacial	100
CBB-R250	2.5 g
Total	1000

Destain solution:

All the ingredients in Table 3.20 were mixed to form the destain solution.

Table 3.20: Ingredients of destain solution.

Components	Amount (ml)
dH ₂ O	650
Methanol	100
Acetic acid glacial	50
Ethanol	200
Total	1000

3.3.5.4 Large Scale Protein Expression and Purification

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

1. 1 L rich broth containing 2% glucose and Ampicillin of 100 µg/ml with 10 ml of an overnight culture of cells containing the fusion plasmid is inoculated.

2. The cells are grown for 3 hours until to reach 0.5 of OD₆₀₀, 2 x 10⁸ cells/ml. IPTG is added to a final concentration of 0.3 mM, e.g., 85 mg or 3 ml of a 0.1 M stock in H₂O. The cells are incubated at 37°C for 3 h.
3. The cells are harvested by centrifugation at 4000 x g for 20 min and the supernatant is discarded. The cells are frozen overnight at -20 °C and thaw on ice.
4. The cells are resuspended with 50 ml column buffer containing 2-mercaptoethanol.
5. Sample is placed on ice sonicated in short pulses of 10 s and 10 s stop in 1.5 minutes.
6. Sonicated sample is centrifuged at 9,000 x g for 30 minutes and the supernatant called crude extract as well is saved. The crude extract is diluted 1:4 with column buffer.
7. Considering the total cell protein analysis the amylose resin column is packed. The column is washed with 8 column volumes of column buffer.
8. The diluted crude extract is loaded at a flow rate of 1 ml/min for a 2.5 cm column.
9. The loaded column is washed with 12 column volumes of column buffer
10. The fusion protein is eluted with elution buffer which consists of column buffer and 10 mM maltose. 10–20 fractions of 3 ml each is collected. The fusion protein usually starts to elute within the first 10 fractions, and the fractions are detected by UV absorbance at 280 nm and checked by SDS PAGE.
11. The protein containing fractions are pooled and concentrated in an Amicon[®] Ultra-15 Centrifugal Filter with 30,000 NMWL membrane (Milipore, Catalog # UFC9 030 24). The buffer of protein is changed with water and protein in water is lyophilized for long storage and further applications.

3.3.5.5 MALDI-TOF Mass Spectrometry

In order to confirm the purity and identity of the individually expressed proteins, Mass spectrometry (MS) was performed with the purified wt MBP, and the MBP-GEPI fusion proteins. Desalted samples were diluted in a matrix solution; 5mg/ml sinapinic acid (SA) (Sigma) in water: acetonitril solution (50%:50% vol:vol) and spotted onto the MALDI sample plate with 2µl/well to obtain strong signal. Droplets

were dried and then placed into mass spectrometer. The most intense peaks match expected m/z values for singly charged species for the individual proteins.

4 RESULTS AND DISCUSSIONS

4.1. Construction of Expression Construct pMALc2X-Linker-GEPI

In this study we used two inorganic-binding peptides: (1) combinatorially-selected gold-binding peptide and (2) *de novo* designed quartz-binding peptide. Each peptide with a flexible, SGGG-, and a rigid, PGPGPG-, linker was cloned as fusion partner to MBP through expression of pMALc2X harboring *malE* gene encoding for MBP.

The general strategy we followed to achieve the soluble fusion protein MBP-Linker-GEPI, was to clone the N-terminal of linker sequence conjugated with the GEPI in pMALc2X. DNAs of the GEPI with linker were obtained through PCR amplification of designed and synthesized oligonucleotides which were used as templates. The amplified fragment size was checked by agarose gel electrophoresis obtaining from a fragment between 100 and 70 bp in Figure 4.1

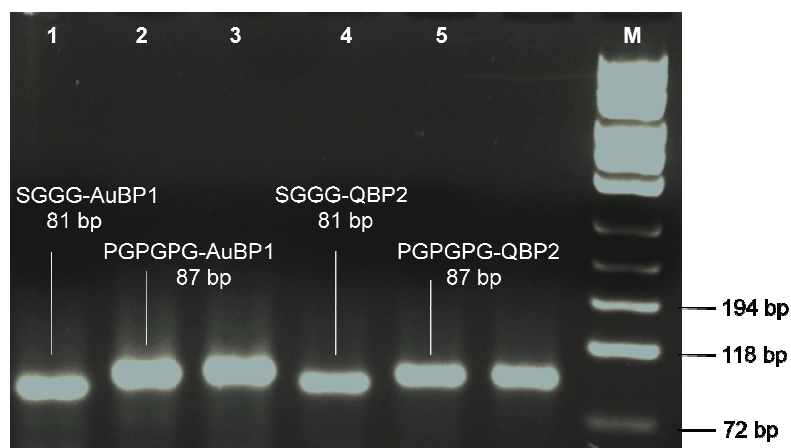


Figure 4.1: Electrophoretical mobility of the PCR amplification. Lane 1-5 are PCR products, Molecular weight marker is meant M.

The oligonucleotide belongs to SGGG-AuBP1 is 81 base in length and posses two restriction sites at each end, PstI at 3' and NlaIV at 5'. At the 3' there are two stop codons (TAATAA) before PstI recognition site. The sequence of the oligonucleotide with primer binding sites and the recognition sites yielding the gene to code for SSSG-AuBP1 are given in Figure 4.2. The construct of SGGG-QBP2

oligonucleotide (in Figure 4.3) has the same properties like that of SSSG-AuBP1. The major differences between the sequences of these oligonucleotides are the GEPI sequence in base and reverse primer binding site because of the different GEPI used at the 3' end.

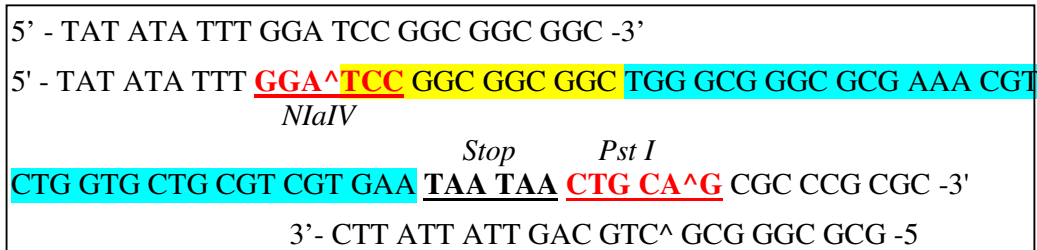


Figure 4.2: The sequence of SGGG-AuBP1 oligonucleotide and primer binding site

The oligonucleotide sequences of (PG)₃-AuBP1 and (PG)₃-QBP2 genes are shown in Figure 4.4 and Figure 4.5 respectively. The used restriction enzymes are MscI caused the blunt end at 5' end and PstI for sticky end at 3' in the sequence designed of 87 bp. At the 3' end, two stop codons (TAATAA) exist. GEPI sequence and reverse primer binding site are different in each oligonucleotide.

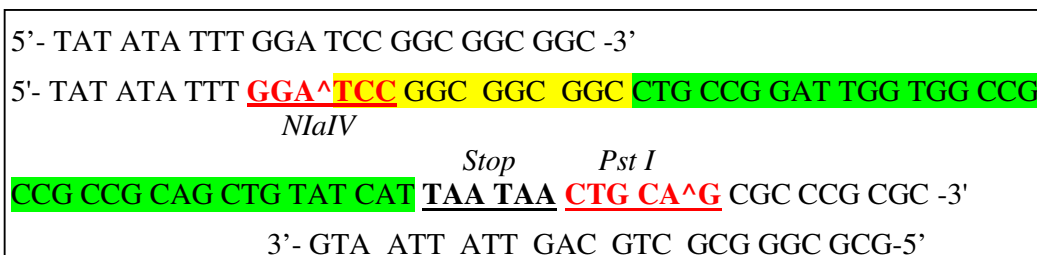


Figure 4.3: The sequence of SGGG-QBP2 oligonucleotide and primer binding site

The forward and reverse primers were used for the amplification of the sequence and at the end of the reaction a double stranded Linker-GEPI DNA with restriction sites was obtained from a single stranded oligonucleotide. The result of the PCR product is analyzed by agarose gel electrophoresis (Figure 4.1).

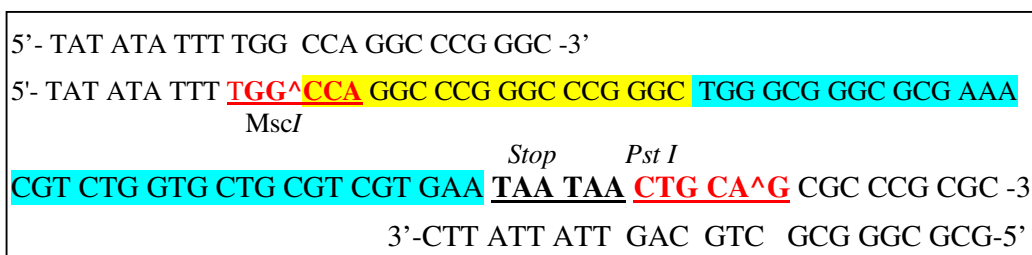


Figure 4.4: The sequence of (PG)₃-AuBP1 oligonucleotide and primer binding site

In the oligonucleotides, the sequences of the individual single gold binder region and quartz binder region were placed, following the linker region. Stop codons were placed to the end of the so- called binder regions. The restriction sites introduced to the sequence were selected as cloning strategy.

In designing the oligonucleotide of individual GEPI with linker, the sequence of the gold or quartz binder region are designed to have stop codons before the PstI restriction site which forms sticky end, and a few random bases for primer binding site to perform PCR successfully. In the beginning of sequence, there is a linker region which is either flexible, SGGG, or, rigid linker, PGPGPG. Following the linker region, a few random bases and a restriction site, which can be used for formation of blunt end, exist. The used restriction enzymes for blunt end formation are MscI at SGGG linker and NlaIV at PGPGPG linker.

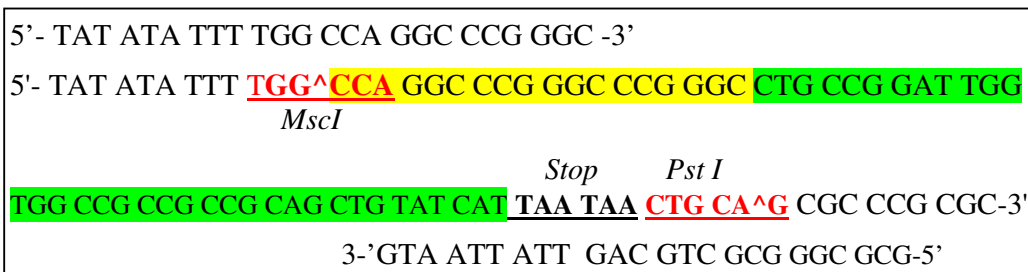


Figure 4.5: The sequence of (PG)₃-QBP2 oligonucleotide and primer binding site

The strategy for the construction of the bi functional protein was developed a method to ligate the desired domain. For this ligation, two different restriction sites placed in the multiple cloning site of the pMALc2x vector, were chosen (Figure 4.6). In addition, two other different restriction sites were preferred considering the linkers sequences. XmnI and PstI were selected for vector digestion. Here, the reason for choosing XmnI site was due to formation of the blunt end, and its protein cloning site. XmnI nucleotide recognition site in pMALc2X vector is G GAAGG[^]ATTTC which can be expressed as Glycine (Gly) and Arginine (Arg), respectively. The pMALc2X vector also contains the sequence coding for the recognition site of a specific protease, located just 5' to the polylinker insertion sites. This allows MBP to be cleaved from the protein of interest following the purification. The pMALc2X vector that is included in the system encode the site for Factor Xa. Factor Xa cleaves after its four amino acid recognition sequence, Isoleusine (Ile), Glutamine (Glu), Glycine (Gly) and Arginine (Arg), so that few or no vector-derived residues are

attached to the protein of interest, depending on the site used for cloning. *MscI* enzyme was selected since it can form blunt end following the digestion. It has TGG[^]CCA recognition site. The following the digestion remained CCA is expressed as Serine (Ser) that is the first amino acid of flexible linker, SGGG-. For the first amino acid of PGP GPG- peptide linker expression *NlaIV* restriction enzyme was used. Its recognition site is GGN[^]GCC and following the digestion GCC in linker region is expressed as Proline (Pro).

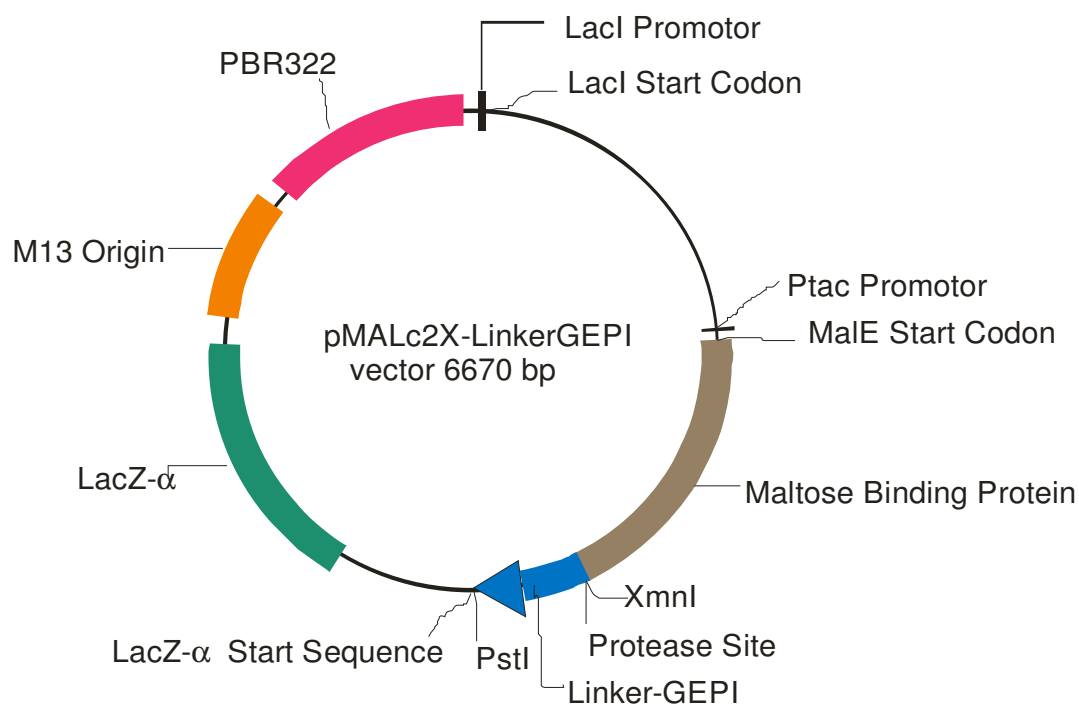


Figure 4.6: Schematic diagram of constructed recombinant plasmid pMALc2X-Linker-GEPI carrying maltose binding protein at its N-terminus for overexpression of recombinant MBP-GEPI bifunctional protein.

After ligation of the purified amplified fragment and linearized pMALc2X transformation of TB1 cells was carried out. Since the restriction sites between *malE* and *lacZα* are available for inserting the coding sequence of interest insertion inactivates the β -galactosidase α -fragment activity of the *malE-lacZα* fusion. This activity results in a blue to white color change on X-Gal plates when the construction is transformed into α -complementing host such as TB1 or JM107. Moreover, it has given the best results when considering plasmid stability, expression and purification. Individual colonies were screened for the insert by duplicate streaking on LB amp plates with and without IPTG and X-Gal since putative recombinant

clones do not form blue colonies on the LB amp broth with IPTG and X-gal. Recombinant vectors were electrophored for checking size (Figure 4.7).

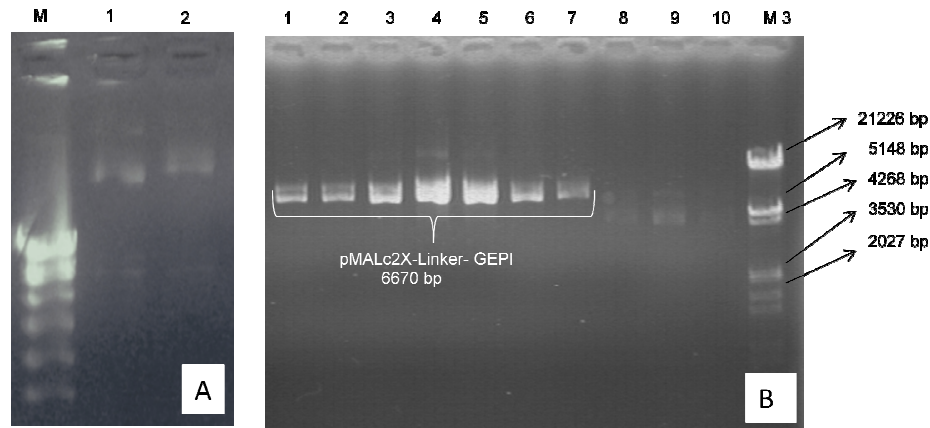


Figure 4.7: (A) The recombinant and non-recombinant plasmids. (B) The different recombinant plasmids are pMALc2X-S(G)₃-AuBP1, pMALc2X-S(G)₃-QBP2, pMALc2X-(PG)₃-AuBP1 and pMALc2X- (PG)₃-QBP2. Lane 1 is pMALc2X-Linker-GEPI and lane 2 is pMALc2X in (A)

We also used another strain in response to a particular problem which is expression. Therefore, transformation of ER2507 was carried out after the recombinant DNA sequence was assessed. Then the nucleotide sequence of recombinant clones was confirmed as the correct one so that the protein production was preceded. ER2507 was used since the *malE* gene is included in the *malB* deletion, so this strain does not make any MBP from the chromosome. It does not have the *lacZ*ΔM15 allele, so it cannot be used for α-complementation (no blue-to-white screen on Xgal). As a result of this, it is important to try different combinations of target protein and bacterial strain to avoid the complicated renaturation.

4.2. Expression Studies

We first verified the construction of the bi-functional protein via sequencing results. Next, we performed expression studies. To do this, first we carried out some pilot experiments to have the optimum parameters for expression and purification.

In order to optimize the production of the fusion protein, the concentration of the inductor (IPTG) was varied from 0.1 to 1 mM at 37 °C. A good expression was achieved with 0.3 mM IPTG. When the post-induction time of protein production was varied from 1 h to 4 h, the best result was achieved for 3 h as is shown in Figure 4.4

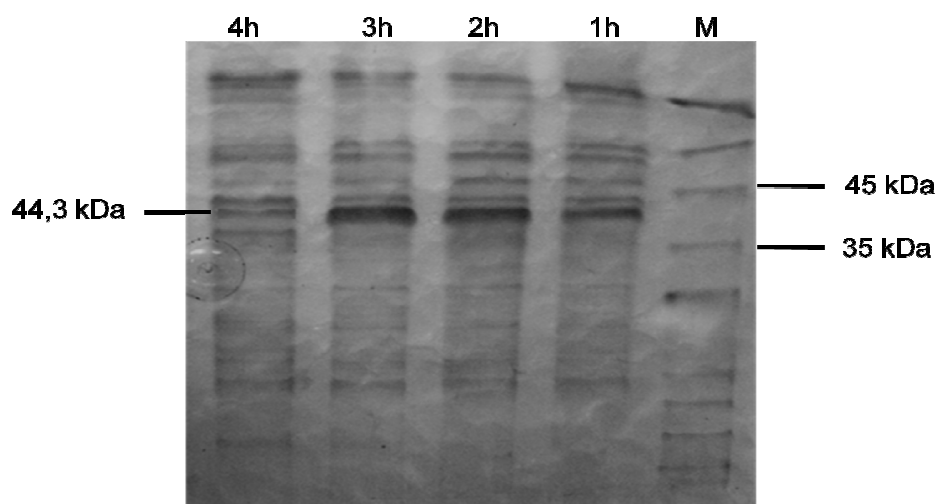


Figure 4.8: Effect of induction time on the expression of MBP-GEPI bi-functional protein. Aliquots of 1000 μ l were taken from cultures induced with 0.3 mM IPTG every hour up to 4 h. Samples were centrifuged at 13 000 rpm for 2 minutes and pellets were subjected to SDS-PAGE for the detection of fusion protein. M is marker.

To release the fusion protein from cells, the combination of three procedures were performed, including the addition of 50 mg lysozyme/ml, sonication and freezing. All methods included the addition to the column buffer of 10 mM 2-mercaptoethanol to prevent interchain disulfide bond formation and protease-inhibiting cocktail to prevent digestion of the fusion protein through proteolytic activity. The best results were obtained when the cells were frozen at -20°C , thawed, lysozyme addition and sonicated until protein release was constant. As the lysate was clarified by centrifugation, the soluble protein fraction was subjected to affinity chromatography.

4.3 Purification Analysis

In the pilot experiments, we first carried out optimization studies such as determination of the amount of amylose resin, size of column and the ingredients of column and elution buffer to obtain high yield purification. The optimization is necessary, since MBP fusion protein might not stick to the amylose column efficiently due to certain factors in the extracts that interfere with binding, or present a low intrinsic affinity. Factors in the crude extract that can interfere with binding and cellular components are released during alternative methods of lysis (lysozyme/sonication). In addition, cells grown in LB have substantial amounts of an

amylase that interferes with binding. By including glucose in the media, expression of this amylase is repressed and the problem is alleviated. A low intrinsic affinity could be caused by an interaction between the protein of interest and MBP that either blocks or distorts the maltose-binding site.

The most important variable in determining the useful life of the amylose resin is the amount of time that it is in contact with trace amounts of amylase present in the crude extract. Under normal conditions (crude extract from 1 liter of cells grown in LB + 0.2% glucose, 15 ml column), the column loses 1–3% of its initial binding capacity in each time it is used. If the yield of fusion protein under these conditions is 40 mg, this means that after 3 to 5 runs there would be a decrease in the yield.

The purification process of both wt-MBP and MBP-GEPI was performed using a modified New England Biolabs protocol. The first modification was addition of protease inhibitors to inhibit the undesired proteases. The second one was the use of β -mercaptoethanol of 1mM, which is 10-fold less than the standard procedure to prevent interchain disulfide bond formation upon lysis. Consequently, proteins were eluted from the cross-linked amylose column with a buffer containing 10mM maltose without reducing agent.

The elution profile of the fusion protein from an amylose affinity chromatography column is shown in Figure 4.9 and Figure 4.10. The pooled fractions containing protein were either subject to SDS-PAGE and stained with Coomassie reagent (Figure 4.10 and Figure 4.11). The apparent molecular mass of the fusion proteins is ~45 kDa which approaches the theoretical ones. The molecular mass of individual bi-functional proteins is given in Table 4.1. Moreover, wt MBP has an electrophoretic mobility of 42.5 kDa in Figure 4.10, being the expected mass 42.481 kDa.

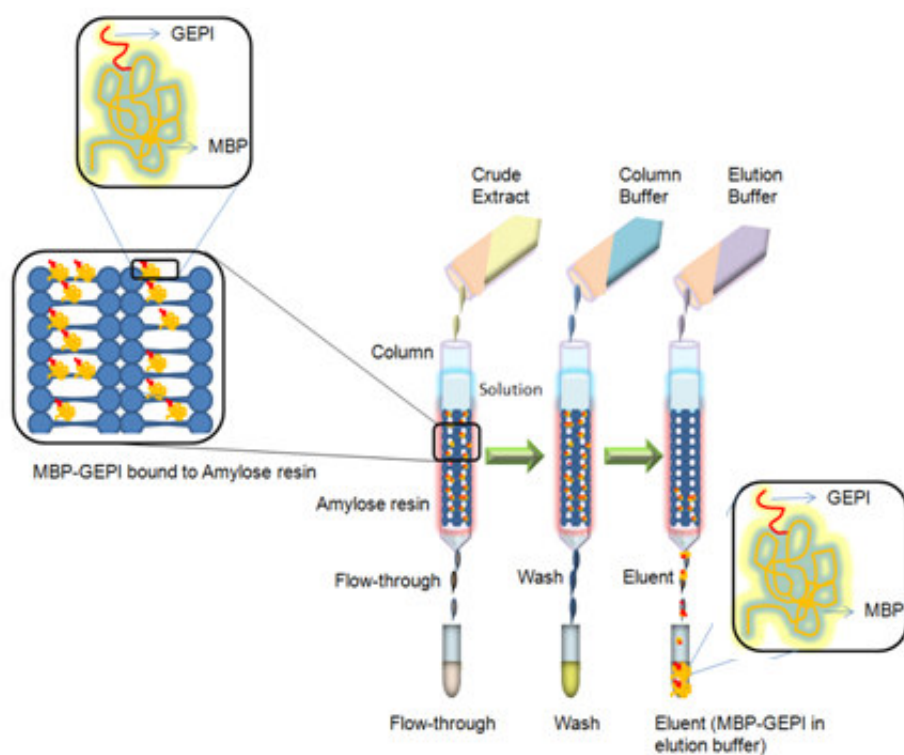


Figure 4.9: Schematic of Purification of bi-functional proteins

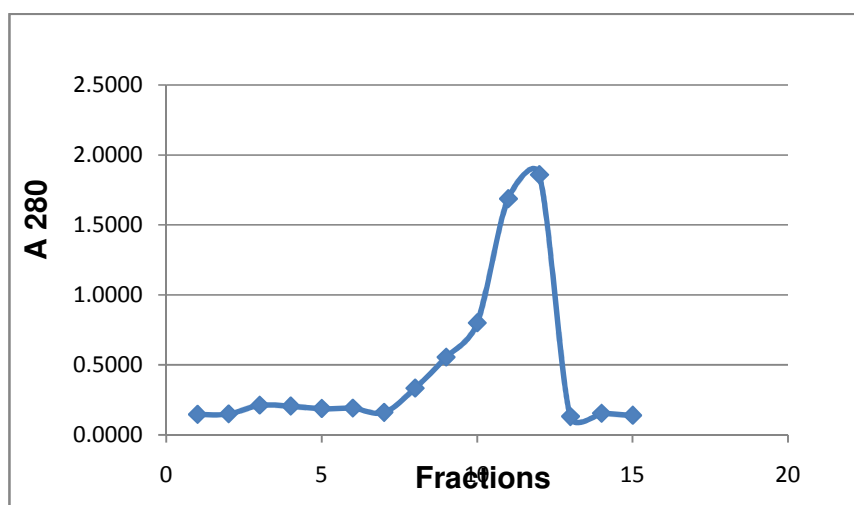


Figure 4.10: Elution profile from the amylose resin column. Purification of the MBP-GEPI bi-functional protein by affinity chromatography.

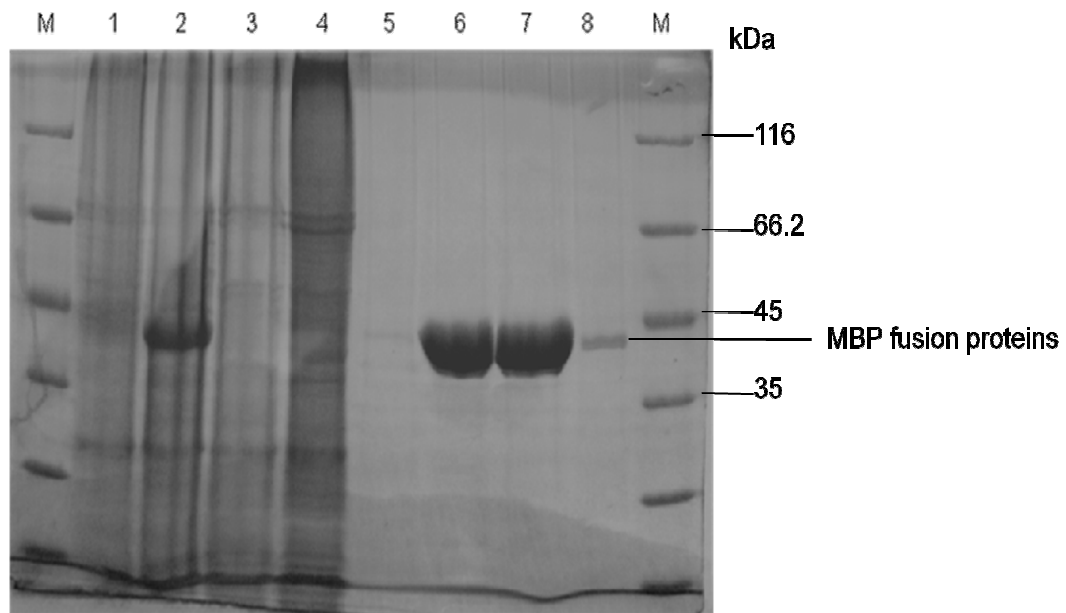


Figure 4.11: Electrophoretal mobility of the expressed and purified fusion proteins. Molecular weight marker is meant M. Lane 1 is uninduced cell, Lane 2 is crude extract, lane 3 is last flow through, lane 4 is first wash, lane 5 is last wash, lane 6, 7, 8 eluents which are MBP fusion proteins.

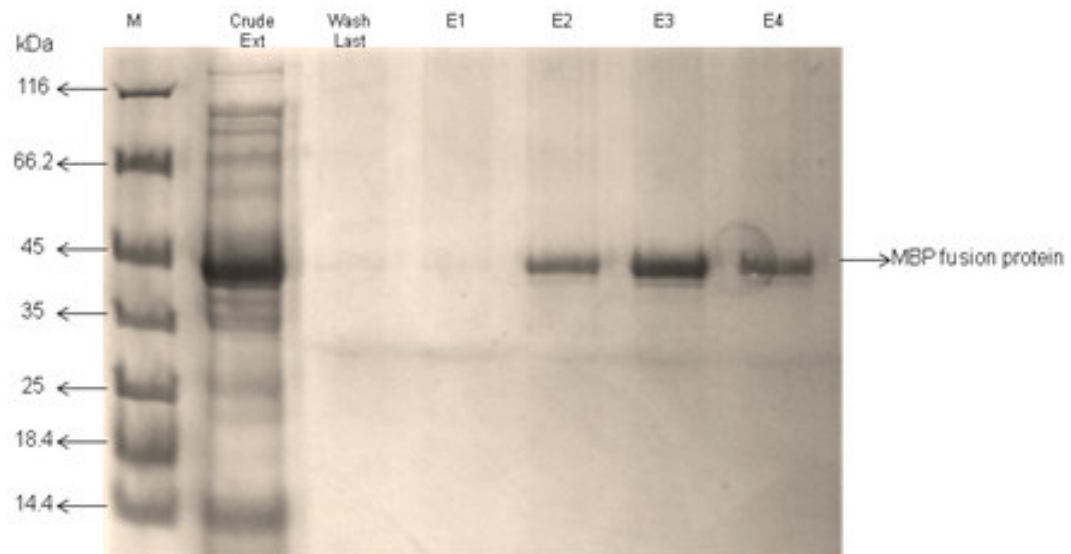


Figure 4.12: 5-110% SDS-PAGE result. Lane 1: Broad Range Marker, lane 2: crude extract, lane 3: fraction obtained from the last washing step, lane 4: fraction from the first elution step, lane 5: fraction from the second elution step, lane 6: fraction from the third elution step and lane 7: fraction from the fourth elution step

4.4. Molecular Characterization by Mass Spectrometry

In order to confirm the purity, identity and digestion reaction of the individually expressed proteins, we performed mass spectrometry (MS) with the purified wt MBP, and the MBP-GEPI bi-functional proteins. Desalted samples were diluted in a matrix solution, 5mg/ml sinapinic acid (SA) (Sigma) in water: acetonitril solution (50%:50% vol:vol) and spotted onto the MALDI sample plate with 2µl/well to obtain strong signal. Droplets were dried and then placed into mass spectrometer. The spectra for proteins MBP and MBP-GEPI are shown in Figure, respectively. As can be seen, the most intense peaks match expected m/z values for singly charged species for the individual proteins. The measured and predicted molecular masses are given in Table 4.1

Table 4.1: The apparent and measured molecular weight of bi-functional proteins

Constructs	Yield of Total Protein	Yield of Fusion Protein	Apparent Molecular Weight	Measured Molecular Weight
wt MBP	180 mg/L culture	40 mg/L culture	42.481 kDa	42.492 kDa
MBP-PGPGPG-AuBP1	150 mg/L culture	41mg/L culture	44.381 kDa	44.470 kDa
MBP-SGGG-AuBP1	85 mg/L culture	37 mg/l culture	44.176 kDa	44.200 kDa
MBP-PGPGPG-QBP2	90 mg/L culture	30 mg/L culture	44.475 kDa	44.600 kDa
MBP-SGGG-QBP2	150 mg/L culture	60 mg/L culture	44.270 kDa	44.247 kDa

The mass spectrums of the wt MBP and wt MBP cleaved by FactorXa and two main peaks were shown like 42 492 Da assigned to cleaved wt MBP (predicted molecular mass: 42 481 Da) and 43 301 Da assigned to wt MBP before cleavage. As mentioned wt MBP has a sequence of amino acids after protease site which is IEGR site. Therefore, purified wt MBP was digested by FactorXa to remove the residue the following protease site.

5. CONCLUSION

The major aim of this study, was to construct and purify maltose binding protein fused with a inorganic binding peptide thorough a linker. To demonstrate this, we have chosen two different inorganic binding peptides, a first generation cell surface displayed selected gold binding peptide, AuBP1(MW: 1454.7, WAGAKRLVLRRE) and a second generation designed *de novo* quartz binding peptide, QBP2 (MW: 1549, LPDWWPPPQLYH). Those two peptides were genetically fused into maltose binding protein through two different linkers, rigid (PG)₃ and a flexible S(G)₃. Each of the linkers were inserted to the beginning of the GEPI region. The expressed fusion protein with GEPI was designated as MBP-GEPI in the pMALc2x plasmid. The GEPI gene in the pMALc2x is connected with the peptide linker. The presence of a linker between MBP and GEPI was chosen to obtain sufficient flexibility to adopt optimal binding orientations. Therefore, the linker should be as rigid as possible in order to perform the bioactive conformation of the compound and to reduce the entropic cost of binding.

The four constructs have been generated by cloning the individual AuBP1 and QBP2 inorganic binding peptides in pMALc2X. The conditions to express these constructs in *E.coli* have been optimized. The desired bi-functional proteins have been then isolated by affinity chromatography. In order to obtain Wt MBP, cleaved extra amino acid residue has been separated by affinity chromatography. All steps along the process of production, cleavage and purification were checked by SDS-PAGE. The amount of soluble bi-functional proteins obtained from 1L culture are shown in Table 4.1. The purity and molecular weight of expressed and purified proteins were checked using mass spectrometry. The measured mass was shown to correspond to the expected molecular weight of each bi-functional proteins having MBP and corresponding inorganic binding peptide.

6. FUTURE WORK

Maltose binding protein is a membrane protein used in the transportation of the maltose from the outer membrane space into the cell. However, it is also used as a tag protein in protein purification, as it enhances the solubility of the proteins, and also used as a biosensor agent towards detection of some sugars as well as a photonic based detection system. Here, we designed bi-functional proteins using specific GEPIs cloned into MBP that would lead to the peptide-directed immobilization of various nanostructures or molecules onto different multimaterial patterned surfaces. We believe that a novel alternative to current chemical coupling may be the utility of combinatorially-selected inorganic-binding peptides as molecular linkers. Moreover using various specific inorganic-binding peptides as molecular coupling linkers would potentially enable the simultaneous immobilization of multiple functional molecules/nanostructures on one multimaterial array device. Therefore, in future studies, we will demonstrate the MBP-GEPI fusion proteins used as patterning substrates and demonstrate their sensing abilities via different methods. We also plan to characterize their binding kinetics and thermodynamics using Surface Plasmon Resonance (SPR) and Quartz Crystal Microbalance (QCM).

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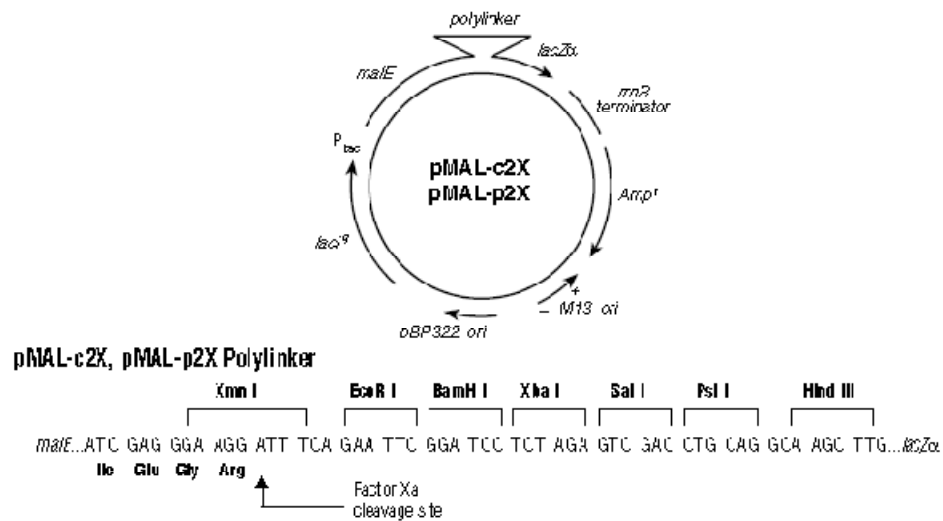
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APPENDICES

Appendix A

Vector Map of pMAL

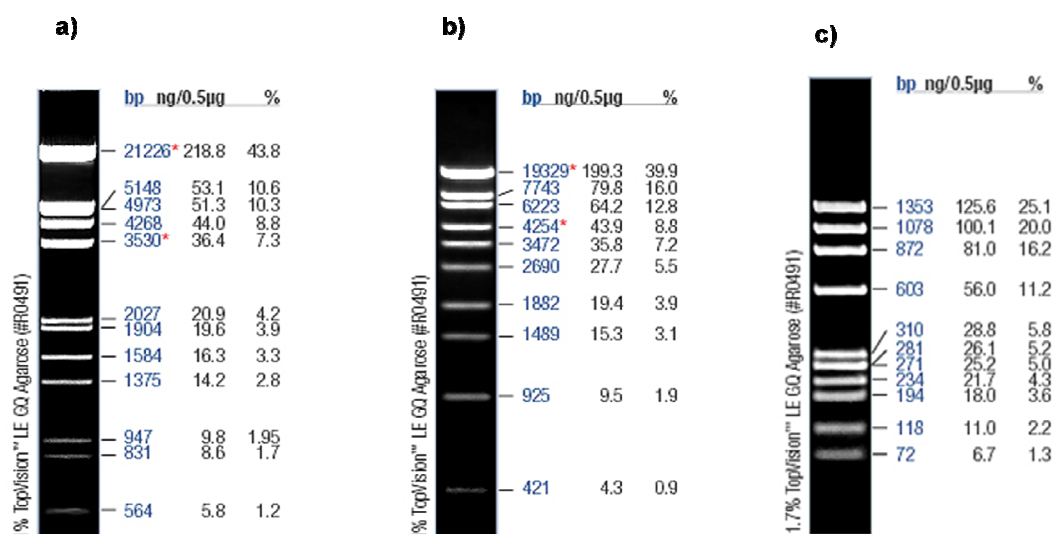


Features:

15-46	lacI promoter
1406-1433	Ptac promoter
1478	Begin <i>malE</i> DNA fragment
1528	<i>malE</i> start codon (ATG)
2629	Start polylinker, factor Xa site
2735	Start <i>lacZ</i> -alpha sequence (CA CTG GCC)
2914	First in-frame stop codon for <i>lacZ</i> -alpha
3327	Start pBR322 sequence
3418	beta-lactamase (Amp-r) start codon (ATG)
4276	beta-lactamase termination codon (TAA)
4320-4833	M13 origin (+ -)
4944-5532	pBR322 origin
6646	End

Appendix B

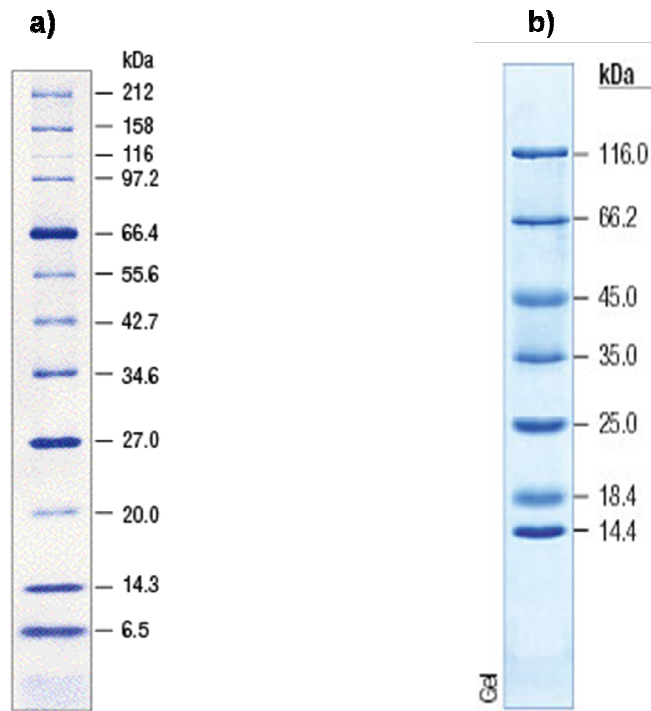
DNA Molecular Weight Markers



- a)** Lambda DNA/*EcoRI*+*HindIII* Marker 3, 1.0% agarose, 0.5 µg/lane, 8 cm length gel, 1X TAE, 17V/cm. (Catalog #SM0193, Fermentas)
- b)** Lambda DNA/*Eco130I* (StyI) Marker 16, 1.0% agarose, 0.5 µg/lane, 8 cm length gel, 1X TAE, 17V/cm. (Catalog # SM0161, Fermentas)
- c)** PhiX174 DNA/*BsuRI* (*HaeIII*) Marker 9, 1.7% agarose, 0.5 µg/lane, 8 cm length gel, 1X TBE, 17V/cm. (Catalog #SM0252, Fermentas)

Appendix C

Protein Molecular Weight Markers



- a) Protein Marker, Broad Range (2-212 kDa) is a mixture of purified proteins with known amino acid sequences. They are resolved to 13 sharp bands when analyzed by SDS-PAGE (Tris-Glycine) and stained with Coomassie Blue R-250. Two bands (BSA, MW 66.4 kDa and Triosephosphate isomerase, MW 26.6 kDa) are at double intensity to serve as reference points. (Catalog #P7702S, New England Biolabs)
- b) The Unstained Protein Molecular Weight Marker is designed for precise sizing of proteins by SDS-polyacrylamide gel electrophoresis. It is a mixture of 7 proteins (14.4-116 kDa) which appear as sharp bands after SDS-polyacrylamide gel electrophoresis (Catalog # SM0431, Fermentas)

Appendix D

Lab Equipments

Autoclave	: 2540 ML benchtop autoclave, Systec GmbH Labor- Systemtechnik : NuveOt 4060 vertical steam sterilizer, Nuve.
Centrifuges	: Avanti J-30I, Beckman Coulter. : Microfuge 18, Beckman Coulter.
Centrifuge rotors	: JA30.50.Ti, Beckman Coulter. : F421.5P, Beckman Coulter.
Deep freezes and refrigerators	: Heto Polar Bear 4410 ultra freezer, JOUAN Nordic A/S, catalog# 003431 : 2021 D deep freezer, Arcelik. : 1061 M refrigerator, Arcelik.
Electrophoresis equipments	:E-C Mini Cell Primo Ec320, E-C Apparatus. :Mini-POROTAN 3 Cell and Single-Row AnyGel Stand, Catalog# 165-3321, Bio-Rad. :Mini-V 8.10 Vertical Gel Electrophoresis System, Lifw Technologies GibcoBrl (now Invitrogen), Catalog# 21078.
Gel documentation system	: UVIpro GAS7000, UVItec Limited.
Ice Machine	: AF 10, Scotsman.
Incubators	: EN400, Nuve.
Magnetic stirrer	: AGE 10.0164, VELP Scientifica srl.

	:ARE 10.0162, VELP Scientifica srl.
Mass Spectrometry	: MALDI-TOF, Bruker
Orbital shaker	:Certomat S II, product# 886 252 4, B. Braun Biotech International GmbH.
Pipettes	: Pipetteman P10, Eppendorf.
pH meter	: MP 220

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Publications

Hnilova, M. , **Taktak, B.** , Carol, J. , Park. J. , So. C. , Ören. E. , Sarıkaya, M. , Tamerler, C. 2008. “ Targeted Immobilization of Nanostructures and Biomolecules via Peptide Based Biolinkers Towards Nanosensing Platforms” Molecular Biomimetics and Bionanotechnology-3, Friday Harbor WA, USA

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