ISTANBUL TECHNICAL UNIVERSITY ★ INSTITUTE OF SCIENCE AND TECHNOLOGY

BINDING DOMAIN ANALYSIS OF
PHAGE DISPLAY SELECTED PEPTIDES

M.Sc. Thesis by
Anıl CEBECİ, B.Sc.

Department : Advanced Technologies

Programme : Molecular Biology Genetics and Biotechnology

FEBRUARY 2007

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521051221**

Date of submission :	**4 January 2007**
Date of defence examination:	**30 January 2007**

Supervisors (Chairman):	**Assist. Prof. Dr. Nevin Gül KARAGÜLER**
	Assoc. Prof. Dr. Candan Tamerler BEHAR
Members of the Examining Committee:	**Assist. Prof. Dr. Alper Tunga AKARSUBAŞI (I.T.U.)**
	Assist. Prof. Dr. Sevil DİNÇER (Y.T.U.)
	Prof. Dr. Dilek KAZAN (Marmara Uni.)

FEBRUARY 2007

İSTANBUL TEKNİK ÜNİVERSİTESİ ★ FEN BİLİMLERİ ENSTİTÜSÜ

FAJ GÖSTERİM YÖNTEMİ İLE SEÇİLMİŞ PEPTİDLERİN BAĞLANMA BÖLGELERİNİN ANALİZİ

YÜKSEK LİSANS TEZİ
Anıl CEBECİ
521051221

Tezin Enstitüye Verildiği Tarih :   4 Ocak 2007
Tezin Savunulduğu Tarih :   30 Ocak 2007

Tez Danışmanları: Yrd. Doç. Dr. Nevin Gül KARAGÜLER

Doç. Dr. Candan Tamerler BEHAR

Diğer Jüri Üyeleri: Yrd. Doç. Dr. Alper Tunga AKARSUBAŞI (İ.T.Ü.)

Yrd. Doç. Dr. Sevil DİNÇER (Y.T.Ü.)

Prof. Dr. Dilek KAZAN (Marmara Üni.)

ŞUBAT 2007

ACKNOWLEDGMENT

FEBRUARY 2007   ANIL CEBECİ

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ABBREVIATIONS

Ala (A)	: Alanine
bp	: Base Pair
BSA	: Bovine Serum Albumin
Cys (C)	: Cysteine
dH$_2$O	: Distilled Water
DMF	: Dimethylformamide
DNA	: Deoxyribonucleic Acid
dsDNA	: Double-Stranded DNA
EB	: Elution Buffer
EDTA	: Ethylenediaminetetraacetic Acid
ELISA	: Enzyme-Linked Immuno Sorbent Assay
FM	: Fluorescence Microscopy
Gly (G)	: Glycine
HRP	: Horse Raddish Peroxidase
IPTG	: Isopropyl-α-D- thiogalactopyranosi
kb	: Kilobase
LB broth	: Luria Bertani Broth
LacZ	: α -galactosidase
MLB	: M13 Lysis and Binding Buffer
MP	: M13 Precipated Buffer
mRNA	: Messenger Ribonucleic Acid
Na-Ac	: Sodium Acetate
OD	: Optical Density
Pro (P)	: Proline
PC	: Potassium Phosphate-Sodium Carbonate Buffer
PFU	: Phage Forming Unit
PCR	: Polymerase Chain Reaction
PEG-8000	: Polyethylene Glycol-8000
Pt	: Platinum
Pd	: Palladium
RF	: Replicative Form of M13 Genome
Ser (S)	: Serine
ssDNA	: Single-Stranded DNA
Thr (T)	: Threonine
TBE	: Tris-Borat -EDTA
TMP	: 3,3',5,5' Tetramethylbenzidine
Gln (Q)	: Glutamine
Tris base	: Hydroxymethyl Aminomethane
X-Gal	: 5-Bromo-4-Chloro-3-Indolyl-D-Galactoside

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SUMMARY

Genetically engineered peptides with specific affinity for inorganic surfaces have the potential to be used as linkers and major building blocks for self-assembly of materials with controlled organization and specific functions. Phage display technology is adapted for the selection of inorganic surface specific proteins. An understanding of mechanism of surface interactions is necessary to control peptide building.

Protein domains are indepently folding units that represent a small number of polypeptides that creates a specific, 3D structure that leads to a desired interaction. In genetic engineering, it is essential to know the specific amino acid residues within the domain that directly bind polypeptides to their substrates. By identifying the conserved positions of amino acids, those necessary for stabilization and function can be determined.

The main focus of this study is to vary the functional capacity of the peptide chain through site directed mutagenesis to determine a core set of amino acids that is responsible for binding to a chosen inorganic surface. This task is accomplished using phage display selected peptides for platinum and palladium via site directed mutagenesis protocols, once they are displayed on phage. In particular, the modification of serine and threonine residues is carried out with respect to their neutral counterparts to reveal a possible role in their binding sequences. Mutants were constructed by a PCR based site directed mutagenesis procedure. Binding characterization of the cloned phages are assessed by fluorescence microscopy (FM) and enzyme-linked immuno sorbent assay (ELISA) to understand the mechanism of noble metal binding. The consequences of this study can be used in understanding of the binding domain of the peptides in their specific interactions with the inorganic surfaces. Smart peptides with selectivity can also be constructed and practiced in bionanotechnological applications.

FAJ GÖSTERİMİYLE SEÇİLMİŞ PEPTİDLERİN BAĞLANMA BÖLGELERİNİN ANALİZİ

ÖZET

İnorganik yüzeylere seçici özgüllüğü olan genetik olarak değiştirilmiş peptidler, malzemelerin kendiliğinden oluşturulmasında, kontrollü organizasyon ve özel fonksiyonlar için bağlayıcı molekül ve yapı taşları olarak kullanılabilme potansiyeline sahiptirler. Faj gösterim teknolojisi inorganik yüzeylere özgül olarak bağlanabilen peptidlerin seçilebilmesi için adapte edildi. Yüzey etkileşimlerinin mekanizmasının anlaşılabilmesi, peptid yapılanmasının kontrolü için gereklidir.

Protein bölgeleri, bağımsız bir şekilde katlanabilen ve istenilen etkileşimi sağlayabilecek özgül 3 boyutlu yapının oluşmasını sağlayan küçük polipeptidleri ifade eder. Gen mühendisliği alanında, bölge içersinde bulunan ve polipeptidlerin substratlarına doğrudan bağlanmasından sorumlu özel amino asitleri bilmek gereklidir. Amino asitlerin korunmuş pozisyonlarını belirleyerek, stabilizasyon ve fonksiyon için gerekli amino asitler de belirlenebilir.

Bu çalışmanın asıl odak noktası site directed mutagenez yöntemleri kullanarak peptidin fonksiyonel kapasitesini değiştirmek ve bu şekilde de seçilmiş belirli bir inorganik yüzeye bağlanmaktan sorumlu amino asit gruplarını bulmaktır. Bu görev, faj gösterim metodu ile seçilmiş platin ve paladyuma özgül olarak bağlanabilen peptidlerin, site directed mutagenez yöntemi ile peptitler faj üzerinde ekspres edildiğinde gerçekleştirilmiştir. Özellikle, serin ve treonin amino asitlerini, bunlara karşılık olarak gelen nötr amino asitler ile değiştirerek, bu amino asitlerin bağlanma üzerindeki etkilerinin ortaya çıkarılması amaçlanmıştır. Değiştirilmiş mutantlar, PCR tabanlı site directed mutagenez prosedürü ile oluşturulmuştur. Klonlanan fajların bağlanma karakterizasyonları florasan mikroskobu (FM) ve enzim-bağlı immün essey (ELISA) yöntemleri ile tayin edilerek asal metallerin bağlanma mekanizmaları anlaşılmaya çalışılmıştır. Bu çalışmanın sonuçları, inorganik yüzeylere özgül olarak etkileşime geçen peptidlerin bağlanmadan sorumlu bölgelerinin anlaşılması için kullanılabilir. Biyonanoteknolojik uygulamalar için "seçiciliğe sahip akıllı peptitlerin" oluşturulması ve denenmesi de gerçekleştirilebilir.

1. INTRODUCTION

1.1. Proteins and Their Roles in Biological Systems and Materials

When we investigate a cell with physical or chemical analysis methods, we always face with the same macromolecules. In fact, they can be in anywhere and in any suitable role for showing their activity and ability in the cell. These macromolecules are called as proteins.

"Protein" word came from the Greek word, *protas*, as the meaning of "primary importance". But then, what is the secret of being so important in the cell? The answer lies under the structural properties of proteins.

Three-dimensional conformation of the proteins gives them their functional properties and capacity. An important feature for proteins is that they spontaneously fold into very well-determined, selective and elaborate three-dimensional structures, which are configured by the amino acid residues they consist of. This self-folding ability of proteins provides the passage from one-dimensional sequence information to three dimensional biological functions [1].

 "Building blocks of the organism" term is mostly referred to proteins. Actually, proteins constitute most of a cell dry mass. They are not only the building blocks from which cells are built, but also execute nearly all cell functions. Enzymes, catalizer protein molecules, serve as the proper molecular surface supplier and promoters for the chemical reactions to take place in the cells. Protein made channel pumps enclosed in the plasma membrane are important in the control of transfer into and out of the cell. Some of the proteins have significance in signal transduction mechanisms. Also, for immune response and protection cells from extreme environmental conditions or outer effects, proteins are used by the immunity system of the organism. In DNA level, the importance of the proteins is getting higher like their role in the stabilization of DNA. All these variable properties of proteins rely on the fact that they are highly specific and selective towards their ligands [1, 2]. Some important protein based molecules can be seen in Figure 1.1.
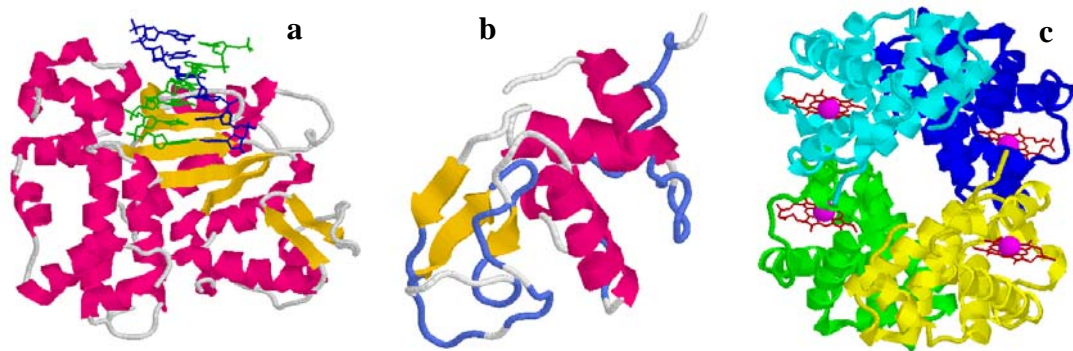
Figure 1.1. 3D structure of three important protein based molecules. *(a)* DNA polymerase beta (pol beta) (E.C.2.7.7.7) complexed with six base pairs of DNA [4]. *(b)* Lysozyme (E.C.3.2.1.17) [5]. *(c)* Hemoglobin (T-state, deoxygenated) [6].

The biological properties of protein molecules depend on the physical interactions with other molecules. All proteins bind to other molecules, however, this binding can be tight in some cases and weak and short-lived in others. In all of these cases, one thing is always the same: Great specificity. With this ability, protein will selectively bind its ligand while there are too many other molecules in the molecular environment. The ability of a protein to bind selectively and with high affinity to its ligand depends on the formation of both bonding and nonbonding interactions and their combinations. Not only the surface residues are important in binding of proteins to their ligands, but also the atoms buried in the interior of the protein which have no direct contact with the ligand, provide an essential scaffold giving the surface its chemical properties. Even small changes to the amino acids in the interior of a protein molecule can change its three-dimensional conformation enough to destroy a binding site on the surface [1-3].

Through this uniqueness and specificity, proteins, the building blocks of the cell, take place in tissue engineering, as well. Proteins take role in forming complex nano structures in soft (muscle, skin, tendon, membrane, silk of spider) and hard (bones, dental tissues, spicules, spines, shells, skeletal units of single-celled organisms or plants, bacterial thin film, nanoparticles) tissues [7, 8]. Soft tissues contain only proteinaceous phase whereas hard tissues contain both at least one proteinaceous and inorganic phase [8-10]. By genetic control, hard and soft tissues are synthesized in organisms when the organism needs them in necessary conditions and aqueous environment [7-10].

Figure 1.2 shows examples of organic-inorganic hybrid material formations in some organisms. By using protein – inorganic interactions, all these hybrid systems are

naturally produced in organisms which have naturally found the ways to use some of their proteins to produce and bind the inorganic materials and then use these magnificent systems for their need. The products of these systems can be used in such as forming protective layers, supportive tissues, transferring charge and ion, developing some optical and mechanical properties [8, 9, 11].
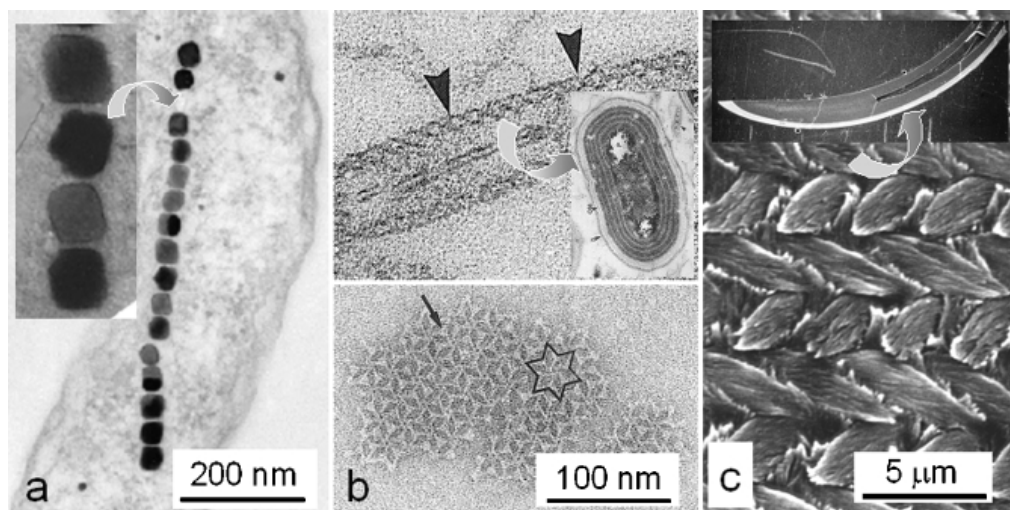
Figure 1.2. Examples of biologically synthesized organic-inorganic hybrid materials: *(a)* Single-crystalline, single-domained magnetic magnetite nanoparticles (Fe_3O_4) formed by a magnetotactic bacterium (*Aquaspirillum magnetotacticum*) (inset: higher magnification image of the magnetite nanoparticles revealing cubo-octahedral particle shape). *(b)* S-layer bacterium, *Synechococcus* strain GL24, has a nanostructurally ordered thin film calcite on its outer layer serving as a protective coating. *(c)* Hard, wear-resistant tooth enamel of mouse consists highly ordered micro/nano architecture of hydroxyapatite crystallites that assemble into a woven rod structure (SEM image). Each rod is composed of thousands of hydroxyapatite particles (inset: cross-sectional image of a mouse incisor; white region is enamel, backed by grayish dentine) [12].

1.2. Inorganic Binding Polypeptides

Inspiration from nature and using this with biology, we can now construct hybrid materials with molecular biology tools and nanotechnology. This combination is referred as "molecular biomimetics". Molecular biomimetics shortly searches for polypeptides which recognize and specifically bind to inorganics to form the hybrid systems. In this manner, formation and assembly of functional inorganic hybrid materials and systems can be controlled for the construction of biolinkers and molecular building blocks [12].

1.2.1. Obtaining inorganic binding polypeptides

There are some classical techniques that are used to create nano–technological systems, but these techniques have some drawbacks like requiring high temperature, pressure and pH adjustment and production of toxic byproducts [12]. Also, obtaining desired size nano particles can be difficult with these methods. However, when we look at the nature, these problems have been already overcome.

Up to now, only a few proteins have been characterized well to bind inorganic materials such as ice binding (antifreeze) proteins in some fish, plant and insect species [13]. Other than ice-binding protein, the organisms in Figure 1.2 have organic-inorganic hybrid systems where an inorganic- binding peptide relationship has occurred. Some of the proteins in these organisms are used to form inorganic material *in vivo* and then the organism uses these inorganics for its benefit.

Isolation and determination of the specific inorganic binding proteins consist of several possible ways. One of these ways is the extraction the proteins from related tissues [11, 14]. However, extraction of proteins and cloning of their genes is complicated and time consuming. Also, number of determined and characterized proteins is limited [15]. Inorganic binding proteins can be designed with [16] molecular approach which is similar used in pharmaceutical drug research but it is time consuming and expensive method like extraction methods [11, 14].

To overcome these problems, molecular libraries or combinatorial libraries which are generated completely random sequences of peptides or oligonucleotide is used. Combinatorial biology libraries are composed of same number of amino acids coding insertion but composition of different sequences. They have been used to screen and identify various biological activities, such as catalytic properties or altered affinity and specificity to target molecules in many applications including the design of new drugs, enzymes, antibodies, DNA-binding proteins and diagnostic agents [17-19]. Combinatorial biology techniques have also some limitations like requirement of complex polypeptide production and need to have many chemical reactions [14, 20, 21]. However, display technologies can improve these disadvantages. Although this limitations, phage display (PD) and cell surface display (CSD) techniques *in vivo* are adapted for selection of inorganic binding polypeptides [20, 22]. These techniques consist of a large, random library of peptides with the same number of amino acids,

but of different sequence compositions, are screened to identify specific sequences that strongly bind to a target inorganic material surface [17, 23, 24]. In this method there is no need to have some information about the polypeptides sequence which is specific to the selected material.

Till now, PD has been performed to isolate sequences binding to silica [24], silver [26], gallium arsenide [23], zinc sulfide [28], calcite [29], cadmium sulfide [30] and noble metals such as platinum and palladium [31] where CD has been used for investigation of peptides that recognize gold [12], zinc oxide [32], iron oxide [12,33], zeolites [32] and cuprous oxide [32].

1.2.2. Phage display technique

The display of a protein or peptide on the surface of a bacteriophage particle –phage display- is an efficient *in vitro* technique that involves insertion of the corresponding DNA into the gene of a structural protein and the expression of the foreign sequence as a fusion with the structural protein that is "displayed" on the surface of the phage particle [19]. This technique enables the selection of polypeptides with desired binding specificities and properties among a large collection due to the ability of certain phage allows additional peptide sequences to be incorporated into the coat proteins without disrupting their structure and function by linking the DNA sequence encoding the protein and the peptide itself. Thereby a direct link is constructed between phenotype and genotype [19, 35, 36].

There are many vector options for phage display. One of these alternatives, that is widely used, is bacteriophage M13 (Ff family). Bacteriophage M13 genome is easily manipulated and high titers can be obtained. However, the peptide size that is wanted to be displayed is limited. This disadvantage is tried to be overcome by using other vector types like phage λ, T7 or T4 [37, 38].

1.2.2.1. Phage display peptide libraries

Biological molecules that are active against other biological molecules and specifically interact with them can be distinguished in a vast population of randomly generated sequences. This principle can be used in discovery of ligands for certain proteins and peptides. This is mainly achieved through the insertion of random DNA fragments to the amino-terminal portions of structural coat proteins on viruses [39].

Peptide libraries generated in this way consist of a high number of peptides of a particular length, whose sequences are random and hence the amino acid residues are varied at each position. The peptides in some libraries also have fixed residues that provide formation of secondary structures such as disulfide bridged loops and alpha helices [40].

All five coat proteins can be used to display peptides or proteins for M13 bacteriophage case. Variety of the insertion site and length can be used as construction of different phage display libraries. g7p and g9p minor coat proteins are used in N-terminal display [41] and g6p is used for C-terminal display [42] whereas both C-and N-terminal display can be achieved with g3p and g8p [35, 43, 44].

Table 1.1. M13 coat proteins with their amino acid numbers, molecular weights, copies per phage and peptide fusion site [35].

Protein	Number of amino acids	Molecular weight	Copies per phage	Type of display
P3	406	42,500	~5	N or C
P6	112	12,300	~5	C
P7	33	3,600	~5	N
P8	50	5,200	~2,700	N or C
P9	32	3,600	~5	N

N-terminal based fusions mostly depend on g3p and g8p usage for the generation of phage display libraries. The limitation is that only short peptides (6-8 residues) can be displayed in every copy of major coat protein g8p. Due to the size restrictions of the channel through which phage ssDNA pass during assembly, larger peptides than 8 residues can be displayed only on 10 % of the coat proteins.

g3p, the minor coat protein, is more tolerant for large insertions and short peptides can be monovalent displayed without interfering with the phage infectivity to generate library [35, 43, 44].

Cyclic or "constrained" libraries encode the insert sequence between two cysteines. The idea behind the cyclic library is to constrain the peptide so that it assumes fewer conformations. Thus, if the library contains a good binder, it will be in the correct formation for binding more frequently than a floppy linear molecule. A cyclic peptide is easier to model using computational methods because there are fewer

degrees of freedom within a fixed loop than a floppy linear peptide. However, the disulfide constraint may "freeze out" a conformation required for target binding [43, 44].

As a summary, peptide libraries can be constructed by inserting 5-38 amino acid long peptides but increasing length of peptides may affect the display efficiency and phage viability.

1.2.2.2. M13 filamentous bacteriophage

Bacteriophage M13 is a filamentous virus belongs to the virus family of Inoviridae which encapsulate their circular single stranded DNA inside the proteinaceous coat. The length of the virus majorly depends on the size of the genetic material but it ranges between 0.7 and 2 μm and surprisingly with a diameter of 6-7 nm. The filamentous phage infects *Escherichia coli*. M13 phage assembly is a non-lytic and membrane associated process. Even at a slow rate, the phage particles continue to grow and released from *E.coli* without lysing the cell.

The genetic material of M13 bacteriophage is composed of single stranded circular DNA form (Figure 1.3). The M13 genome contains 6400 nucleotides and 11 genes, five of which are responsible for encoding phage coat proteins while others encode proteins used for replication and assembly of the virus [35].
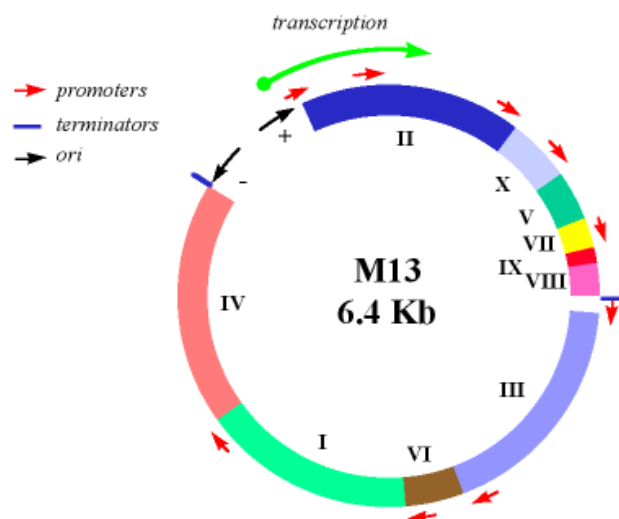
Figure 1.3. M13 genome [43].

The M13 filamentous coat is a symmetric array of several thousands of α-helical major coat P8 proteins which surrounding DNA core. Of minor coat proteins,

protein-7 and protein-9 cap one end while protein-3 and protein-9 cap the other end. There are about 5 copies of each minor protein per particle (Figure 1.4) [35, 46].
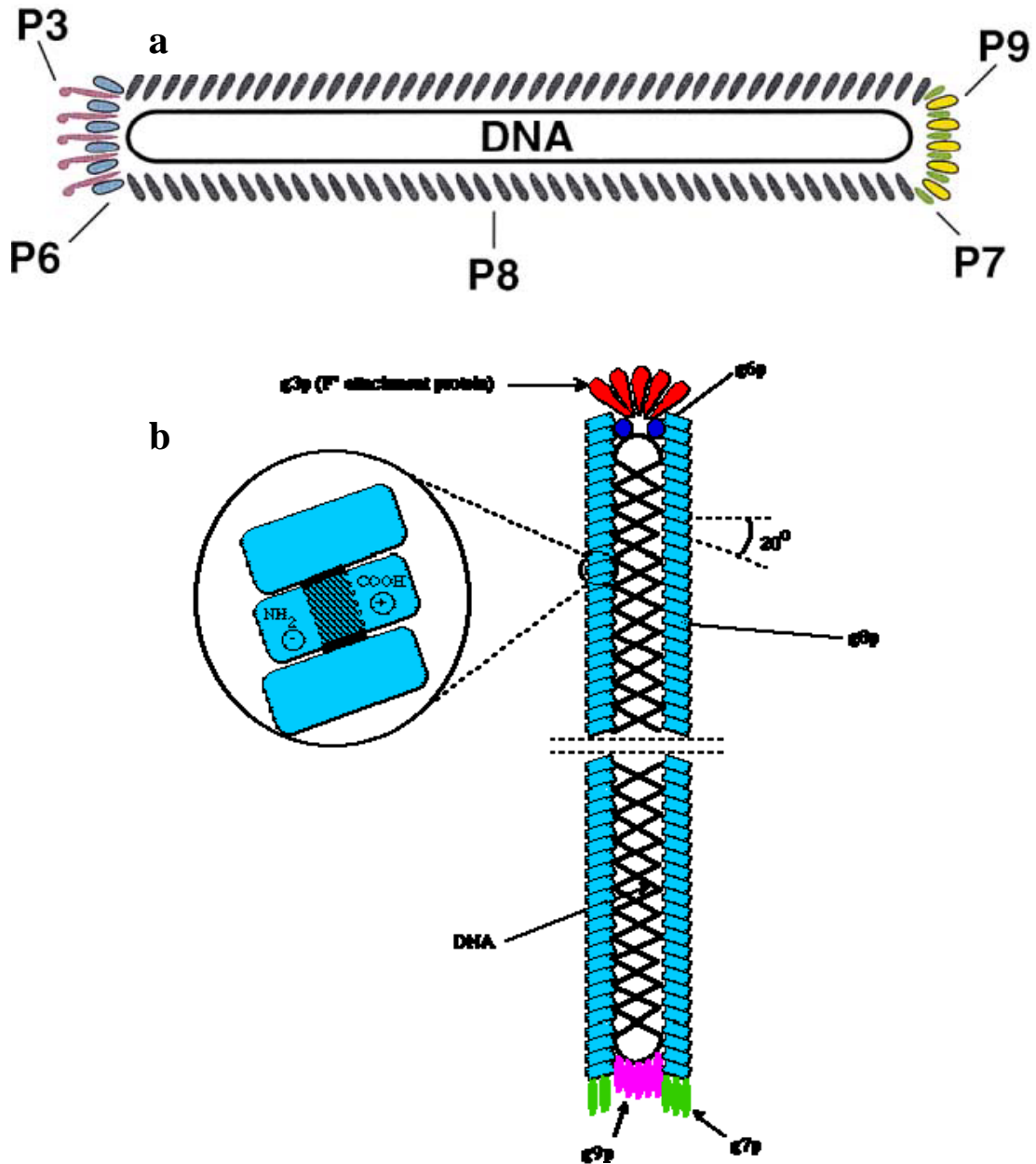
Figure 1.4. *(a)* A diagram of the bacteriophage particle shows the single-stranded DNA core surrounded by a proteinaceous coat [35]. *(b)* Structure of M13 bacteriophage and distribution of coat proteins [47].

All of these major and minor proteins have important roles in structural stability of the phage particle; pVI which works in the termination of the assembly process, pVII which is required for initiation of assembly, pIX which is involved in maintenance of virion stability. pIII is also a key factor for host cell recognition and infection.

Beyond this, pIII is the largest structurally most complex one among coat proteins with three different domains.

Several thousand copies of pVIII protein are available in each phage particle. The insertion of the sequences for phage display is done at the N-terminus, between signal sequence and protein coding sequence. The sequence accepts only short peptides (6-8) for display, for longer sequences, packaging of the phage particles is prevented. For most phage display fusions, the favorite protein pIII because of its tolerance for large insertions, compatibility with monovalent display and availability of vectors [36].

The N-terminal domain of the pIII protein starts the translocation of the phage DNA into the host cell while second domain is responsible for the recognition of the host cell and adsorption to the F pilus on *E.coli* cell surface. On the other hand, C-terminal domain of the pIII deals with other phage coat proteins, so that it is responsible for the integration of pIII into the phage coat. The structure of the first two domains have been solved by X-ray crystallography and NMR spectroscopy but the structure of the third domain and also other coat proteins have not been able to enlightened except for pVIII [35]. According to these studies, g3p consists of three domains. Two N-terminal domains namely N1 and N2, and one C-terminal domain called CT. These three domains are linked with two linker regions consist of a glycine-rich sequence [36] (Figure 1.5).
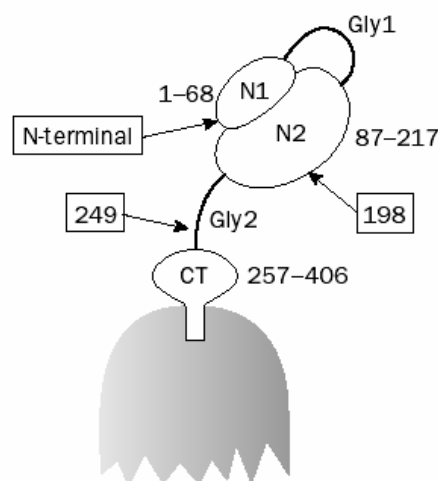
Figure 1.5. Domain structure of g3p [36].

M13 bacteriophage can only infect bacteria that have F pilus. This bacterial structure is an extracellular filament and is responsible for the communication between two bacteria. Also F pilus is the recognition sites for infectious bacteriophages. Only one or a few F-pili are present on the surface of a bacterial cell. Both pilus structural proteins and the proteins required for pili assembly are conjugative plasmid encoded [48].

Filamentous phage infection is a two step process: (i) recognition (which the bacteriophage binds to its primary bacterial cell surface receptor and (ii) translocation (which involves pilus retraction, capsid protein integration into the bacterial cell membrane, uncoating of viral DNA and its translocation into the host cell cytoplasm [49].

Infection of a male *E.coli* cell is initiated by binding of the N2 domain of pIII to the tip of the F-pilus. When N2 binds to pilus, N1 releases from its normal interaction with N2 and N1 domain interacts with the host cell membrane protein Tol A. The pilus is retracted by an unknown mechanism (Figure 1.6). This retraction provides the interaction between g3p and the cell membrane proteins TolQRA. Then viral DNA enters to the cell cytoplasm [50].
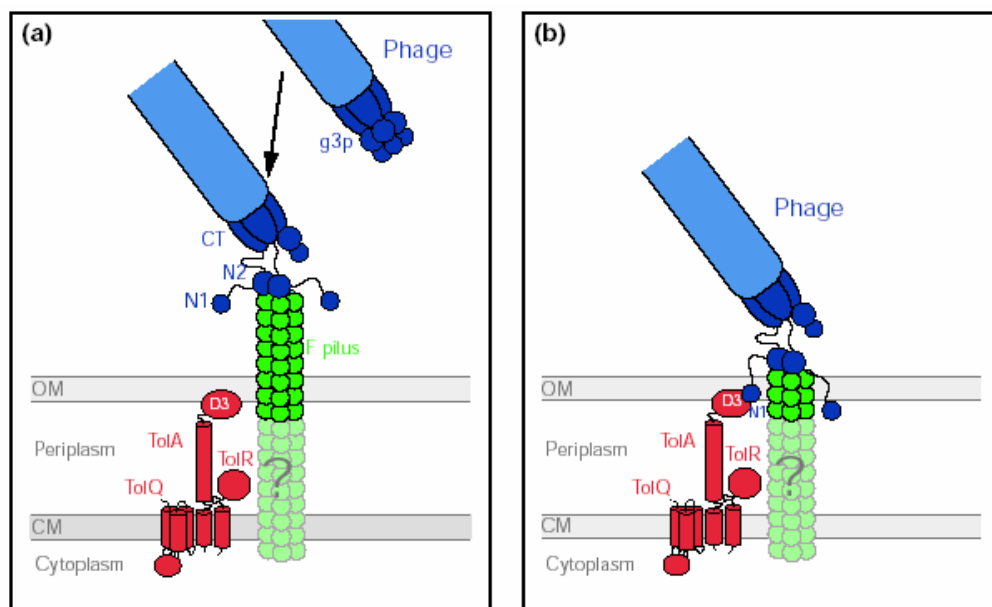
Figure 1.6. The Ff bacteriophage infection process. Pilus mediated separation of N1 from N2 at the amino terminus of pIII frees up N1 for interaction with the D3 domain of the coreceptor, TolA, thus mediating viral entry into the host cell [50].

Filamentous bacteriophages contain a single stranded DNA genome that replicates in three stages. In stage I, once the phage (+) strand ssDNA (SS) is translocated into the cytoplasm after infection, bacterial host enzymes synthesize the complementary (-) strand, producing a double-stranded covalently closed supercoiled DNA product called the parental or replicative form (RF) DNA (SS---RF). Stage II occurs when the RF DNA replicates to form a pool of approximately 100 RF DNA molecules per cell (RF---RF). In the final stage, the RF DNA molecules act as a template for the synthesis of progeny single- stranded DNA phage genomes (RF---SS) [36].

Transcription of the (-) strand of the RF DNA occurs in a clockwise direction as shown in Figure 1.3 and results with gene products produced in the order in which they are required for phage production [51]. The gene *II* protein is an endonuclease-topoisomerase that introduces a specific nick in the (+) strand of the RF DNA. The resulting 3' end serves as a primer for synthesis of new (+) strands via a "rolling circle" mode of replication carried out by host cell enzymes and terminated and circularized by pII (Figure 1.7). The resulting (+) strands can either be used as templates for the synthesis of more RF DNA or be coated by pV dimmers and prepared in the cytoplasm ready to be assembled [40,51].
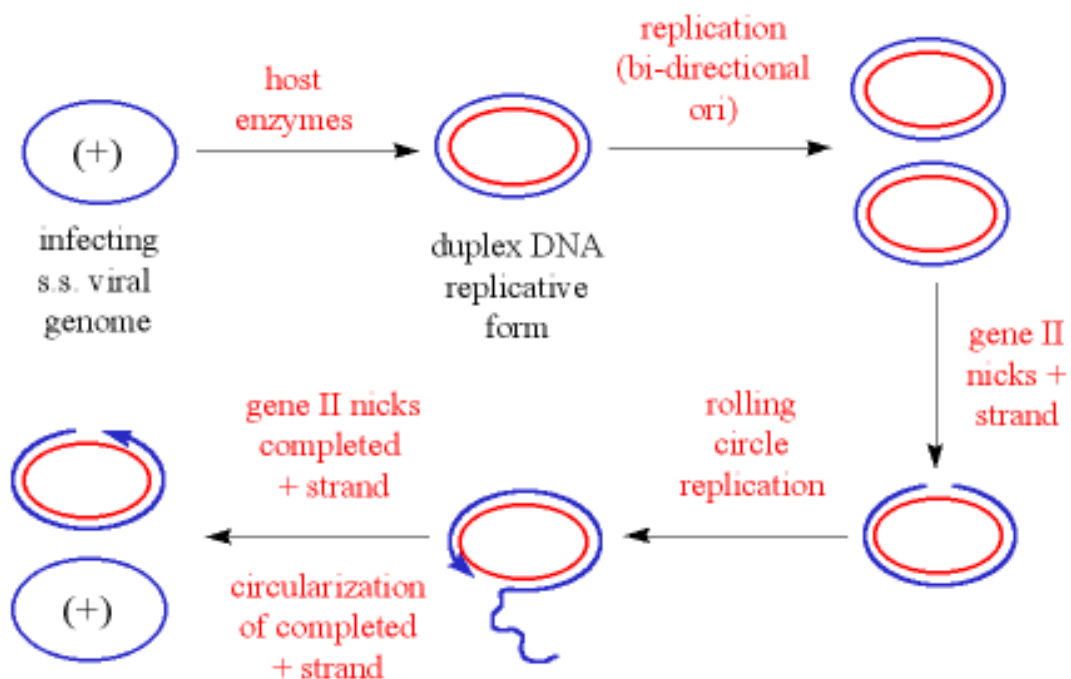
Figure 1.7. The "rolling circle" mode of M13 bacteriophage [47].

1.3. Protein Engineering

Protein engineering is the field of study involving the creation and modifications of proteins. Although the playground of protein engineering is very young, the applications of it range from creating structural motifs that test theories of protein folding and understanding of protein stability, to the production of first and second generation products for human therapeutics or industrial products.

The role of protein engineering deals with catalysis, molecular recognition, protein structure, protein folding, protein stability and protein-protein interactions. The aim for studying these addressed questions is to target the amino acids which may be playing structural or functional roles, deleting or replacing them with alternative ones to test the impact of the changed amino acids, hydrophobic forces, electrostatics and charge and the placement of hydrogen bonds, salt bridges, disulfide bonds, water or metals [52].

Up to now, two general approaches have been identified for protein engineering. One of them is known as *rational design*, in which one needs the detailed knowledge of the 3D structure and function of the protein to make desired changes. This approach has the advantage of being generally inexpensive and easy. However, the structural knowledge of a protein is often unavailable. Even the structure is known, it can not be predicted the effects of various mutations in the experiment.

The second approach is known as *random approach (directed evolution)*. Directed evolution involves repeated rounds of random mutagenesis. The key element in the process is the ability to screen large numbers of mutants. Once the desired mutant is identified, it can be isolated. However, because there is no control over the mutations made, large numbers of mutations must be analyzed to get practical indications. This also has the meaning of labor intensive and long work.

1.3.1. Site directed mutagenesis

Site-directed mutagenesis is a rational approach that has been widely used in studies of gene expression and protein structure-function relationships. This method is utilized for the modification of protein or enzyme properties by providing the applier to change the gene sequence and therefore, the amino acid sequence of an expressed protein. The importance of site directed mutagenesis is beyond structure-function

relationship, for the techniques allowing the generation of mutant proteins with very specific changes in certain amino acids. These kinds of mutations facilitate the study of catalysis mechanism, stability in case of harsh conditions and high specificity for the substrate [53].

There are many methods for site directed mutagenesis. Three of them are mostly used. These are cassette mutagenesis, primer extension and PCR-based mutagenesis. In cassette mutagenesis method, a synthetic DNA fragment including the wanted mutation on a certain sequence is replaced with the matching sequence in the non-mutated gene. This method is very simple with efficiency almost 100 %. The main drawback of the method is that the procedure requires the unique restriction sites flanking the region of interest [53].

Primer extension method, which is the simplest method of site-directed mutagenesis, involves priming *in vitro* DNA synthesis with a synthetic oligonucleotide carrying a base mismatch with its complementary strand. The requirement of the method is that the target DNA to be mutated should be in single-stranded form and M13-based vectors are the most suitable for these kinds of mutations [54]. Figure 1.8 shows basically how this process performs.
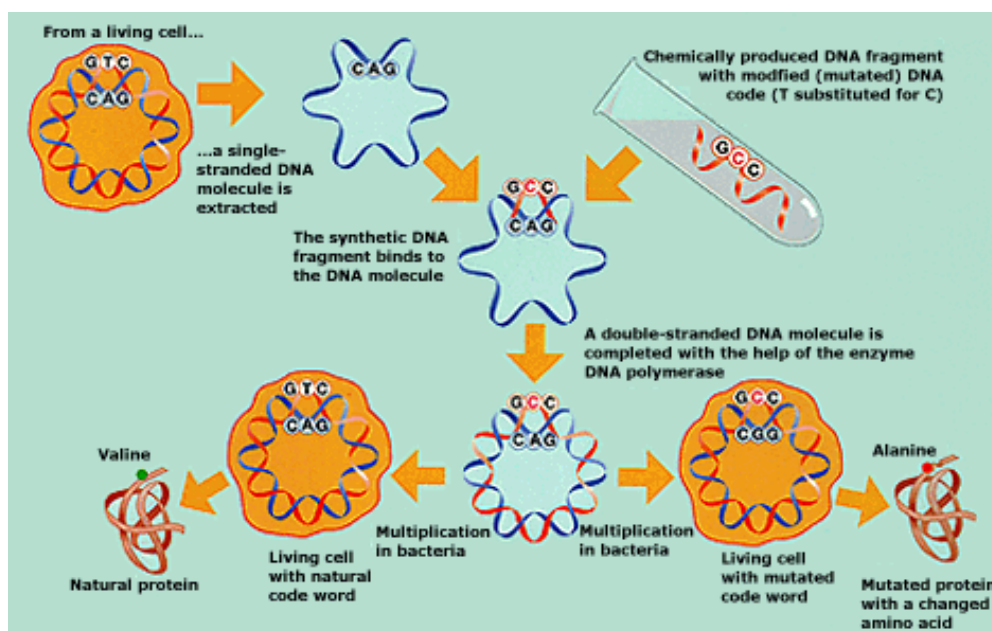
Figure 1.8. The flowchart of site directed mutagenesis process. The first applier of this method, Michael Smith, has won the Nobel Price in Chemistry 1993 [55].

PCR-based site-directed mutagenesis method has a high potential for mutagenesis reactions. This method allows the amplification of single stranded, double stranded,

circular or linearized mutant DNA. Desired mutations are incorporated into the oligonucleotide primers in the form of single base mismatch. Long fragments up to 6 kbs is possible to amplify and with the less undesired mutations by using thermostable DNA polymerases with 3'→5' proofreading activity. The desired mutation is obtained with 100% efficiency in PCR-based mutagenesis method. The main drawback of the method is that Taq polymerase copies DNA with low fidelity and this may cause undesired mutations on the template [54, 56]. But with the synthesis of high fidelity Taq polymerases, this drawback should be eliminated thus, high fidelity Taq polymerases have the ability of synthesis of long sequences without any nucleotide deficiency.

Widely usage of site directed mutagenesis forced researchers to find new ideas how site directed mutagenesis can be a "piece of cake". So, other methods for the selection and enrichment have been generated. The most common problem is the occurrence of the unmutated, template DNA in the mutagenesis reaction with the other mutant types after the reaction. This unmutated-mutated mixture makes the process more complicated and laborious for searching the mutated ones. To get rid of the template DNA, some methods are developed. In the beginning of the site directed mutagenesis reaction, parental DNA is methylated with DNA methylase. Thus, in the following steps after the mutagenesis reaction, the unmutated DNA can be digested by special endonuclease providing from host cell which transformation of reaction occurs in. Hence, the amplified DNA sequence will be only the desired ones [57].

1.3.1.1. Alanine scanning mutagenesis

Alanine scanning mutagenesis, systematic replacement of individual wild type residues with alanine, is a particularly useful technique for the identification of functional epitopes and consequently for uncovering biological insight [58]. This method has been applied to identify functional regions on human tissue type plasminogen activator, yeast cAMP-dependent protein kinase, human growth hormone receptor, human IL-8 and yeast cdc2 [57, 58].

The technique involves the substitution of each of the charged amino acids such as asparagine, glutamic acid, arginine, lysine and histidine in the peptide or protein with alanine. The resulting set of mutants can be observed for functional defects to identify the important regions.

The charged amino acids are selected for mutagenesis because they are more likely to be located on the surface of the protein [59, 60]. These residues are capable of forming ion pairs and hydrogen bounds. Therefore, they are likely to be important for enzyme catalysis and for the recognition of ligands. Alanine was chosen as the residue for all substitutions.

Alanine is the most common amino acid in proteins [61] (Figure 1.9). It is the smallest amino acid except for glycine, it is a hydrophobic molecule, it is located in α helices, β strands and turn regions and it is distributed amongst the buried and exposed residues in proteins [61, 62]. Substitution with alanine removes all side chain atoms past the β-carbon and does not change the hydrophobicity. Hence, it does not impose electrostatic and steric effects. Therefore, substitution with alanine allows the effects of removing the side chain to be evaluated while minimizing the chances of distrupting the secondary structures and tertiary conformation of the protein as do glycine and proline [63, 64].
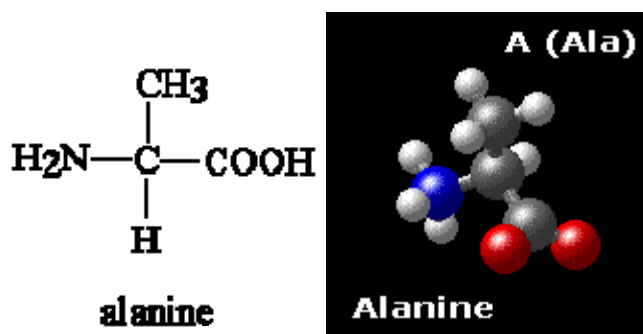
Figure 1.9. The molecular structure and 3D view of alanine amino acid [65].

1.4. Studies on Inorganic Binding Peptides

Peptides or proteins can bind to inorganic surfaces with specifically and selectively. The binding surface could be or a well-defined one such as a single crystal or a nanostructure, either a rough surface such as a powder. Biocompatible materials which are produced under physiological conditions in aqueous environment show stable surface structures and compositions. Noble metals such as Platinum (Pt) and palladium (Pd), and oxide semiconductors like Cu_2O and ZnO are examples to these surfaces which are used in experiments using both flagellar and phage display methods in our group [10, 66].

The specificity of a peptide for an inorganic surface may root from chemical recognition mechanisms such as hydrogen bonding, polarity or charge effects, and from structural recognition mechanisms such as size and morphology. By using the available set of some inorganic binding sequences, some outcomes may be obtained.

Phage display and flagellar display techniques are used to identify amino acid sequences that show high affinity to noble metals and semiconducting oxides. For instance, binders of metallic platinum and palladium powders are selected via phage display technology in our group [10]. In the display, a random peptide library formed by seven amino acids which is fused to bacteriophage M13 minor coat protein (pIII) is used. As the peptides were selected, they were categorized according to their physicochemical properties such as isoelectric points (pI), charges and molecular weights (Table 1.2).

Table 1.2. Examples of Pd and Pt binding peptides selected from a disulfide-constraint phage display peptide library. Color codes of amino acids are given in Appendix A [31].

Pd Binder Peptides	pI	Charge	M.W.
SPRLQGV	9.47	+1	755.87
TTLNPGT	5.19	0	702.76
VNSHPPL	6.71	0	762.86
TLPNHTF	6.40	0	828.92
SPHPGPY	6.46	-	753.80
Pt Binder Peptides			
QSVTSTK	8.75	+1	749.82
PTSTGQA	5.96	0	660.68
TSPGQKQ	8.41	+1	744.80
IGSSLKP	8.75	+1	700.83

Due to the fact that this categorization showed only the importance of the peptides for binding to Pt and Pd, but not showed the affinity strength, one of the widely used characterization method, fluorescent microscopy has been applied to elucidate the binding affinity.

As a result of some analysis, the selected sequences have shown conserved hydrophobicity and hydroxyl containing amino acids, threonine and serine (Table 1.2) [12, 66]. It has been concluded that hydroxyl groups in side chains of these amino acids seemed to play a key role in binding activity of the sequences to metal substrates, no matter which structure do the substrates have. The lack of cysteine residues in most of the isolated sequences and presence of histidine residues in only some sequences have surprising effects because these two residues are believed to

bind to metal ions. Further analysis of the presence and location of the certain amino acids may give a better angle of sight towards the presence of specific binding domains for inorganic materials [66, 67].

1.5. Aim of The Study

In this study, we have recently applied site directed mutagenesis i) to identify the core amino acids in phage display selected strong Pt binder SD152 with the amino acid sequence of PTSTGQA via alanine scanning approach applied once the peptides are on phage, ii) based on the results obtained from platinum binder, to make a reverse engineering comparing to the first one, changing glycine amino acid in the fifth position to a polar amino acid in phage display selected weak Pd binder SD188 with the amino acid sequence of SPHPGPY, again when the peptide is displayed on phage to observe any effect on enhancement for binding affinity.

Binding affinity and specificity of constructed mutants has been characterized via fluorescent microscopy (FM) and enzyme-linked immunosorbent assay (ELISA) analyses. The results have been compared with the unmutated, wild type SD152 and SD188.

2. MATERIALS AND METHODS

2.1. Materials

2.1.1. Bacterial strains

2.1.1.1. *E.coli* ER2738 host strain

F′ *lacI^q Δ(lacZ)M15 proA^+B^+ zzf::Tn10(Tet^R)/fhuA2 supE thi Δ(lac-proAB) Δ(hsdMS-mcrB)5 (r_k^- m_k^- McrBC^-)* was used as a host for wild type M13 phage. This strain was not a competent strain and it was purchased as 50 % glycerol culture within Ph.D.-C7C™ Phage Display Peptide Library Kit [68].

2.1.1.2. *E. coli* DH5α^TM-T1^R host strain

F^- Φ80*lacZΔM15* Δ(*lacZYA-arg*F) U169 *rec*A1 *end*A1 *hsd*R17 (r_k-, m_k+) *pho*A *sup*E44 *thi*-1 *gyr*A96 *rel*A1 *ton*A (confers resistance to phage T1), chemically competent cells were supplied with GeneTailor™ Site-Directed Mutagenesis System [69,70].

2.1.1.3. *E. coli* DH5α^TM-FT host strain

F^- Φ80*lacZ* Δ(*lacZYA-arg*F) U169 *rec*A1 *end*A1 *hsd*R17(r_k-, m_k+) *pho*A *sup*E44 λ- *thi*-1 *gyr*A96 *rel*A1 / F' *proAB^+ lac*I^qZΔ15 *Tn*10(Tet^R), chemically competent cells were purchased to be used as a host for mutated M13 phages [71].

2.1.2. Enzymes

2.1.2.1. Platinum^® *Taq* DNA polymerase high fidelity

Platinum® *Taq* DNA Polymerase High Fidelity is an enzyme mixture including recombinant *Taq* DNA polymerase, *Pyrococcus species* GB-D thermostable polymerase that shows proofreading ability with its 3' to 5' exonuclease activity and Platinum® *Taq* Antibody. Presence of a proofreading enzyme in the enzyme mixture increases fidelity nearly six folds comparing to *Taq* DNA polymerase alone and allows amplification of complex and long DNA templates up to 20 kb with some optimization.

Besides, the Platinum® *Taq* Antibody provides an automatic "hot start" PCR condition by binding and inhibiting the polymerase activity that is restored after denaturation step in PCR cycling at 94 °C. This programmed "hot start" process provides more sensitivity, specificity and yield in PCR [72].

2.1.3. DNA molecular weight markers

2.1.3.1. Lambda DNA/*EcoRI*+*HindIII* marker, 3

Lambda DNA was completely digested with *EcoRI* and *HindIII*, then phenol extraction and ethanol precipitation were performed by dissolving in storage buffer which consists of 10 mM Tris-HCl (pH 7.6) and 1 mM EDTA to form Lambda DNA/*EcoRI*+*HindIII* Marker 3, in a concentration of 0.5 mg DNA/ml (Figure 2.1).

bp	ng/0.5µg
21226*	218.8
5148	53.1
4973	51.3
4268	44.0
3530*	36.4
2027	20.9
1904	19.6
1584	16.3
1375	14.2
947	9.8
831	8.6
564	5.8
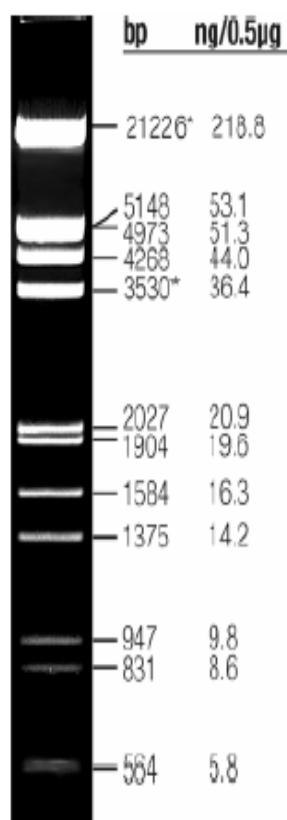
Figure 2.1. DNA *EcoRI* and *HindIII* digested marker-3, 1.0% agarose, 0.5µg/lane, 8 cm length gel, 1X TAE, 17V/cm [73].

This DNA Marker yields the following 13 discrete fragments (in base pairs): 21226, 5148, 4973, 4268, 3530, 2027, 1904, 1584, 1375, 947, 831, 564, 125. The cohesive ends of the 12 nt cos site of bacteriophage lambda from fragments 21226 bp and 3530 bp may anneal and form an additional band at 24756 bp [73].

2.1.4. Oligonucleotides

Synthetic oligonucleotides for desired mutants were designed as follow. Two overlapping primers namely; the forward (mutagenic) primer contains a two base substitution that introduces the desired amino acid and the reverse primer contains a 15- nucleotide sequence that is complementary to the forward primer and includes 12 additional nucleotides at its 3` end were synthesized using an Applied Biosystems 308A DNA synthesizer by IONTEK. These primers can be seen in Table 2.1.

Table 2.1. Synthesized synthetic oligonucleotides as used primers in mutagenesis reactions.

Primer Name	Nucleotide Sequence
GTSD152P1A-F	5' TCTCACTCTGCTTGTGCTACTTCGACTGG 3'
GTSD152P1A-R	5' ACAAGCAGAGTGAGAATAGAAAGGTACCAC 3'
GTSD152T2A-F	5' CACTCTGCTTGTCCTGCTTCGACTGGTCAG 3'
GTSD152T2A-R	5' AGGACAAGCAGAGTGAGAATAGAAAGGTAC 3'
GTSD152S3A-F	5' TCTGCTTGTCCTACTGCTACTGGTCAGGCGTGC 3'
GTSD152S3A-R	5'AGTAGGACAAGCAGAGTGAGAATAGAAAGGTAC 3'
GTSD152T4A-F	5' TGCTTGTCCTACTTCGGCTGGTCAGGCGTG 3'
GTSD152T4A-R	5' CGAAGTAGGACAAGCAGAGTGAGAATAGAA 3'
GTSD152G5A-F	5' TTGTCCTACTTCGACTGCTCAGGCGTGCGGTG 3'
GTSD152G5A-R	5' CAGTCGAAGTAGGACAAGCAGAGTGAGAATAG 3'
GTSD152Q6A-F	5' TTGTCCTACTTCGACTGGTGCTGCGTGCGGTG 3'
GTSD152Q6A-R	5' ACCAGTCGAAGTAGGACAAGCAGAGTGAGAA 3'
GTSD152S3AT2A-F	5' TCACTCTGCTTGTCCTGCTGCTACTGGTCAG 3'
GTSD152S3AT2A-R	5' AGGACAAGCAGAGTGAGAATAGAAAGGTACC 3'
GTSD188G5T-F	5' TTGTTCGCCGCATCCTACGCCGTATTGCGGTGGA 3'
GTSD188G5T-R	5' AGGATGCGGCGAACAAGCAGAGTGAGAATAGAA 3'
M13 -96 Primer	5' CCCTCATAGTTAGCGTAACG 3'

2.1.5. Bacterial culture media

2.1.5.1. Luria Bertani (LB) medium

10 g tryptone (Acumedia), 5 g yeast extract (Acumedia), 5 g NaCl (Riedel-de-Haen) were dissolved in distilled water and completed up to 1 lt and the pH was adjusted to 7.0-7.5 with 10 M NaOH and sterilized for 15 minutes under 1.5 atm at 121 ˚C. The medium was stored at room temperature.

2.1.5.2. LB agar medium

10 g tryptone (Acumedia), 5 g yeast extract (Acumedia), 5g NaCl (Riedel-de-Haen), 15 g bactoagar (Acumedia) were dissolved in distilled water and completed up to 1lt and the pH was adjusted to 7.0-7.5 with 10 M NaOH and sterilized for 15 minutes under 1.5 atm at 121 ˚C. Following autoclaving, tetracycline solution (Sigma) (final

concentration of 10 μg/ml) and X-gal/IPTG solution (final concentration of 40 μg/ml) (Fermentas/Sigma) were added when the temperature of the medium was cooled down to 45-50 ºC. The medium was shaken properly and poured into the plates by avoiding any bubble formation (3.5 ml for small plates and 15 ml for big plates). After the medium was solidified in the plates, they were turned upside down and stored at 4 ˚C for later use.

2.1.5.3. Top-agar medium

10 g tryptone (Acumedia), 5 g yeast extract (Acumedia), 5 g NaCl (Riedel-de-Haen), 1 g $MgCl_2.6H_2O$ (Riedel-de-Haen), 8 g bactoagar (Acumedia) were dissolved in distilled water and completed up to 1 lt and sterilized for 15 minutes under 1.5 atm at 121 ˚C. The medium was stored at room temperature and in the case of necessity, was melted with a heating source to pour onto the LB agar plates.

2.1.5.4. SOC medium

20 g tryptone (Acumedia), 5 g yeast extract (Acumedia), 0.5 g NaCl (Riedel-de-Haen) were dissolved in 950 ml deionized water. 10 ml of 250 mM KCl was added and pH was adjusted to 7.0 with NaOH. Mixture was completed to 1 lt with distilled water and sterilized for 15 minutes under 1.5 atm at 121 ˚C. The medium was stored at room temperature. Just before use, after melting and cooling, 10mM of $MgCl_2$ and 20mM of glucose were added.

2.1.5.5. *E. coli* overnight culture

5 ml LB solution containing 1 mM $MgCl_2$ and tetracycline, was inoculated with *E. coli* ER2738 glycerol stock. The culture was left in the shaker overnight at 37 °C, 200 rpm.

2.1.6. Stock solutions

2.1.6.1. Tetracycline stock

20 mg/ml tetracycline (Sigma) was dissolved in 95 % ethanol (Sigma). It was then stored at -20 °C at dark to protect from the light due to tetracycline is light-sensitive.

2.1.6.2. Xgal/ IPTG stock

1.25 g IPTG (isopropyl β-D-thiogalactoside) (Sigma) and 1 g Xgal (5-Bromo-4-chloro-3 indolyl-β-D-galactoside) (Fermentas) were dissolved in 25 ml DMF (Dimethyl formamide) (Riedel-de-Haen). Solution was stored at –20°C at dark to protect from the light.

2.1.6.3. Detergent stock

20 % (w/v) Tween 20 (Riedel-de-Haen) and 20 % (w/v) Tween 80 (Merck) were mixed and distilled water was added up to 20 ml.

2.1.6.4. Glycerol stock solution

80 ml of 100 % glycerol (Riedel-de-Haen) was mixed with distilled water up to 100 ml total volume to have 80 % (w/v) glycerol solution. It was sterilized for 15 minutes under 1.5 atm at 121˚C and then stored at room temperature.

2.1.6.5. MgCl$_2$ stock solution

5 mM MgCl$_2$.6H$_2$O (Riedel-de-Haen) was dissolved in distilled water up to 100 ml. and sterilized with 0.2 μm single use syringe filter.

2.1.7. Buffer solutions

2.1.7.1. PEG/NaCl

20 % (w/v) PEG 8000 (polyethylene glycol-8000) (Sigma), 2.5 M NaCl (Sigma) were dissolved in distilled water up to 100ml and sterilized for 15 minutes under 1.5 atm at 121 ˚C. The solution was stored at room temperature.

2.1.7.2. PC (Potassium phosphate-Sodium carbonate) suffers

- **PC (no detergent):** 55 mM KH$_2$PO$_4$ (Merck), 45 mM Na$_2$CO$_3$ (Merck), 200 mM NaCl (Sigma) were dissolved in distilled water up to 500 ml and the solution was sterilized by using 0.2 μm single use syringe filter. The pH value was adjusted to 7.2-7.5.

- **PC (containing 0.02% detergent):** 55 mM KH$_2$PO$_4$ (Merck), 45 mM Na$_2$CO$_3$ (Merck), 200 mM NaCl (Sigma), 0.5 ml detergent stock solution were

dissolved in distilled water up to 500 ml and the solution was sterilized by using 0.2 μm single use syringe filter. The pH value was adjusted to 7.2-7.5.

- **PC (containing 0.1% detergent):** 55 mM KH_2PO_4 (Merck), 45 mM Na_2CO_3 (Merck), 200 mM NaCl (Sigma), 2.5 ml detergent stock solution were dissolved in distilled water up to 500 ml. and the solution was sterilized by using 0.2 μm single use syringe filter. The pH value was adjusted to 7.2-7.5.

- **PC (containing 0.5% detergent):** 55 mM KH_2PO_4 (Merck), 45 mM Na_2CO_3 (Merck), 200 mM NaCl (Sigma), 12.5 ml detergent stock solution were dissolved in distilled water up to 500 ml. and the solution was sterilized by using 0.2 μm single use syringe filter. The pH value was adjusted to 7.2-7.5.

<u>**Note:**</u> PC buffers can not be sterilized by autoclaving because carbonate ions convert to CO_2 due to high pressure in the autoclave. This causes an increasing of pH up to 10.

2.1.7.3. Na-Ac buffer

3M Na-Ac (Riedel-de-Haen) was dissolved in 65 ml distilled water. pH was adjusted to 4.6 and distilled water added up to 100 ml.

2.1.7.4. TBE (Tris / Borate / EDTA) solution

10X TBE buffer was prepared by dissolving 108 g Tris-base (Merck), 55 g boric acid (Riedel-de-Haen.) and 4 % (v/v) 0.5M EDTA (Merck) pH 8.0. Distilled water was added to complete up to 1 l.

2.1.7.5. TE buffer

100 μl 1M Tris-HCl, 100 μl 100mM EDTA (Merck) and 1 ml 1M NaCl (Sigma) were mixed in 10 ml of distilled and sterilized for 15 minutes under 1.5 atm at 121 °C.

2.1.8. Lab equipment

Lab equipments are given in Appendix B.

2.2. Methods

2.2.1. M13 double-stranded DNA purification

M13 filamentous bacteriophage has an extraordinary morphology. The infecting single-stranded DNA is converted by host enzymes into a double-stranded circular form, called replicative form (RF) DNA after infection. Double stranded DNA (dsDNA) of newly constructed phages can be purified to use as a template for construction of the mutant phages. As RF DNA behaves like a plasmid in the host cell, plasmid purification methods can be used for obtaining RF DNA.

- **Phage amplification**

5 µl phage sample from glycerol stock were amplified in 5 ml mid-log E. *coli* ER2738 host cell (12.5 µl *E.coli* ER2738 host cell overnight culture was inoculated into 5 ml LB containing 10 µl MgCl$_2$ and 5 µl tetracycline. The culture was incubated at 37 ºC, 225 rpm until mid-log phase (OD$_{600}$ ~ 0.5) that is known as the best phage-host strain propagation period. The incubation period is nearly 4.5 hours. *E. coli* ER2738 host strain contains a mini-transposon, which confers tetracycline resistance. Besides, this strain has a rapid growth rate. Due to these acceptable properties, this strain is particularly well-suited for M13 propagation.). Amplified culture was centrifuged at 5000 rpm for 15 minutes at room temperature to settle down bacterial cell. Supernatant containing M13 bacteriophage was transferred to a fresh reaction tube.

- **Plasmid purification**

Plasmid DNA was purified from amplified culture by using QIAGEN Plasmid Mini Kit Procedure is described in detail below [74].

1. The bacterial pellet was resuspended completely in 300 µl Buffer P1.

2. 300 µl Buffer P2 was added. Suspension was mixed gently by inverting the tube 5 times and incubated at room temperature for 5 minutes.

3. 300 µl of chilled Buffer P3 was added. Mixture was mixed immediately but gently by inverting the tube 4-6 times and incubated on ice for 5 minutes.

4. After incubation on ice, mixture was centrifuged in a microcentrifuge for 10 minutes at 14000 rpm. Supernatant was saved for further use.

5. QIAGEN-tip 20 colon was equilibrated by applying 1 ml of Buffer QBT and the column was allowed to empty by gravity flow.

6. The supernatant obtained and saved in the step 5 was applied to the QIAGEN-tip 20 column and it was allowed to enter the resin by gravity flow.

7. QIAGEN-tip 20 was washed with 1 ml of Buffer QC for 4 times.

8. The DNA that was bound to the column matrix was then eluted with 800 µl of Buffer QF to a 1.5 ml microfuge tube by gravity flow.

9. Eluted DNA was precipitated with 560µl room temperature isopropanol. It was then centrifuged at 10.000 rpm for 30 minutes and the supernatant was decanted.

10. The DNA pellet was washed with 1 ml room temperature 70% ethanol and centrifuged for 30 minutes at 10000 rpm. Supernatant was decanted and the pellet was allowed to dry at room temperature.

11. DNA pellet was redissolved in 30 µl elution buffer EB and stored at –20 ºC.

2.2.1.1. Measurement of DNA concentration

Each 2 or 4 µl DNA sample was diluted with 998 or 996 µl dH$_2$O (1:500, 1:250). The samples were put into the quartz cuvette. The absorbance of DNA was measured at 260 nm and at 280 nm to quantify the amount of DNA. The purity of DNA samples was determined by calculating the ratio of absorbent at 260/280 nm.

2.2.2. Site directed mutagenesis

Alanine scanning and Glycine-Threonine substitution reactions were achieved via site-directed mutagenesis approach. GeneTailor Site-Directed Mutagenesis Kit was used for phage DNA manipulations [70]. In general, this mutagenesis system mainly includes three steps; DNA methylation, mutagenesis PCR and transformation. At following subsections protocol is discussed in detail.

2.2.2.1. DNA methylation

For the methylation of the template DNA, DNA cytosine methylase encoded by the *dcm* gene of *E.coli* was used. This enzyme modifies the internal cytosine residue at the C^5- position on both strands within the sequence 5'...CC(A/T)GG-3'. As

methylated DNA was transferred into *E. coli* DH5α[TM]-T1[R] host strain, it was splited with the Mcr and Mrr restriction systems of the host. This system provides the separation of mutated PCR product from unmutated template during amplification period following transformation [69, 70].

Methylation reaction

dsDNA	1 µl (100 ng)
Methylation Buffer	1.6 µl
10X SAM	1.6 µl
DNA Methylase	1 µl (4U/µl)
dH$_2$O	10.8 µl
Total volume	16 µl

Reaction conditions

Incubated at 37 °C for 1 hour.

2.2.2.2. Mutagenesis PCR

For each mutagenesis reactions, two overlapping primers, one of which containing desired mutation were designed. For every mutation process, methylated replicative form of the template phage DNA was used. In one alanine mutagenesis reaction, template DNA was the mutated phage DNA to get a double alanine mutation. Mutagenesis reactions are described detail below.

Mutagenesis reactions

10X High Fidelity PCR Buffer	5 µl
10 mM dNTP	1.5 µl
50 mM MgSO$_4$	1 µl
Forward Primer (10 µM)	1.5 µl
Reverse Primer (10 µM)	1.5 µl
Methylated DNA	5 µl
Platinum® *Taq* DNA Polymerase High Fidelity	0.5 µl

dH$_2$O   34 µl

Total Volume   50 µl

Reaction conditions

94 ºC 2 minutes

94 ºC 30 seconds

55 ºC 30 seconds   } 20 cycles

68 ºC 7.5 minutes

68 ºC 10 minutes

2.2.2.3. Transformation

1. 50 µl vial of One Shot® MAX Efficiency® DH5α™-T1^R competent cells from − 80 ºC stock were taken and thaw on ice approximately 5-7 minutes.

2. 2-5 µl of mutagenesis reaction was added directly into vial of cell and mixed by tapping gently.

3. Tube was incubated on ice for 30 minutes.

4. To perform the heat shock, the tube was incubated in the 42 ºC water bath for exactly 30 seconds without mixing or shaking.

5. Vials were removed from the 42 ºC water bath and covered with ice for 1 minute.

6. 500 µl of pre-warmed SOC medium was added to the vial and then incubated at 37 ºC for exactly 30 minutes at 250 rpm in a shaking incubator.

7. 50-100-150 and 200 µl transformation reactions were put each of 5 ml top agar aliquots including 25 µl 5 mM MgCl$_2$ and 180 µl *E. coli* DH5α™-FT mid-log culture, mixed by vortexing and poured onto LB plates containing Xgal/IPTG-Tet according to the dilution rates (50-100-150-200 µl).

8. After the plates have solidified, they have been inverted and incubated at 37 ºC overnight.

Next day, blue plaques seen on the LB plate were put into the storage Elisa plates. These phages were amplified and ssDNA (single-stranded) and dsDNA (double-

stranded) of newly constructed phages were purified to use as a template for sequence analysis and following mutagenesis reaction respectively.

2.2.3. Saving phage clones

- **Preparation of storage stock of phages**

1. 200 µl PC buffer containing 0.02 % detergent solution was put into each well of a sterile 96-well Elisa plate.

2. Elisa plate containing phage clones was placed into the incubator at 60 ºC for 45 min. After incubation, plate was cooled in laminar flow and left at 4 ºC for overnight.

- **Preparation of glycerol stock of phage**

1. 60 µl sterilized 80% glycerol solution was put into each well of a fresh 96-well plate.

2. 50 µl clone from the storage plate was added into the glycerol containing plates.

3. Plates were covered by parafilm and placed into – 80 ºC.

2.2.4. M13 single-stranded DNA purification

For the sequencing of the individual plaques, ssDNAs of the picked plaques were needed to be isolated. QIAprep® Spin M13 Kit was used for the isolation of ssDNAs. The procedure is given schematically in Figure 2.2 and written below [75].

1. 50 µl from each individual sample which belongs to only one phage plaque was taken from the glycerol stock and added into 5 ml *E. coli* ER2738 host culture which had been incubated until mid-log phase (OD $_{600}$ ~0.5). Phage and bacteria mixture was incubated for 4.5 hours for *E. coli* ER2738 infection by phage and amplification.

2. Culture containing tubes were centrifuged at 5000 rpm for 15 minutes at room temperature. Supernatant containing M13 bacteriophage was transferred into a fresh reaction tube. During transferring the supernatant, bacterial pellet was not disturbed due to any carryover of bacterial cells will

result in contamination of the M13 precipitation with bacterial chromosomal DNA or double-stranded bacteriophage RF DNA.

M13-infected *E.coli* cultures

Phage supernatants

Phage precipitation and transfer with Buffer MP

Phage lysis and DNA binding with Buffer MLB

*

Washing with Buffer PE

*

Elution with Buffer EB

*

Pure ssDNA
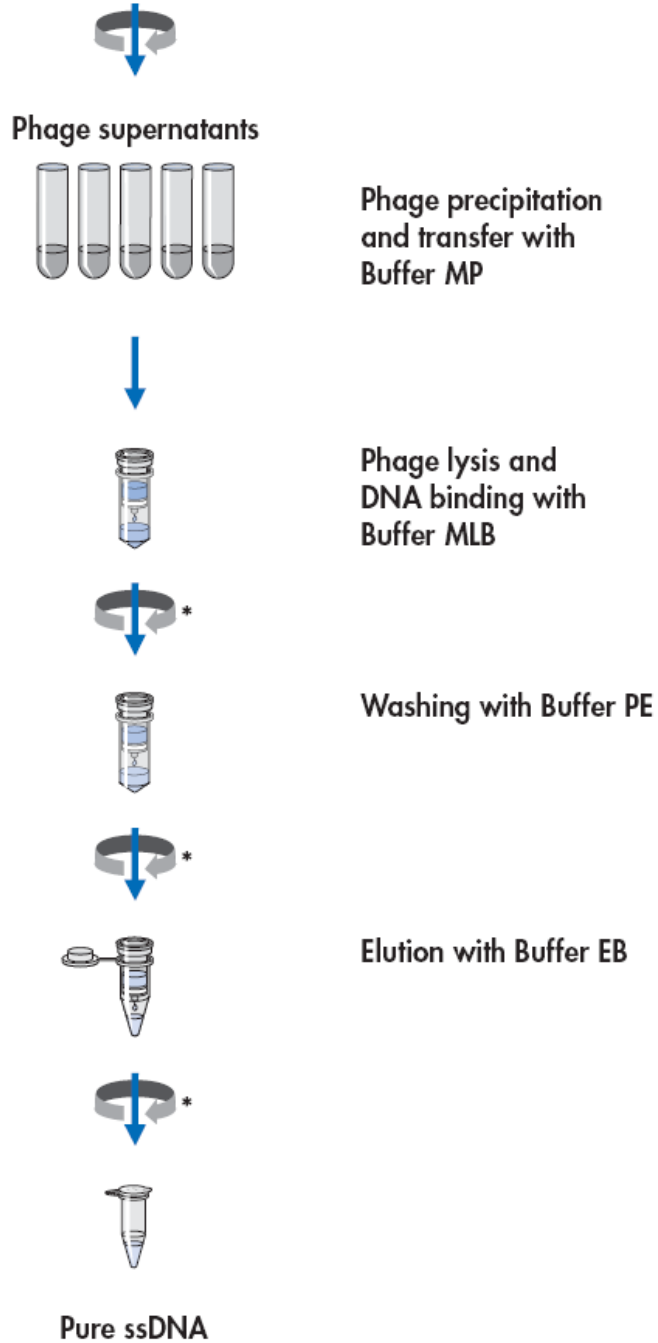
Figure 2.2. M13 ssDNA isolation procedure [75].

3. Buffer MP was added 1/100 volume (i.e.10µl per 1 ml of phage supernatant) onto the supernatant in the reaction tube. It was mixed by vortexing and

incubated at room temperature for at least 2 minutes. During this step, bacteriophage particles were precipitated from the culture medium.

4. A QIAprep spin column was placed in a 2 ml microcentrifuge tube and 0.7 ml of the sample was applied to the QIAprep spin column.

5. Reaction tube was centrifuged for 15 seconds at 8000 rpm and discarded flow-through from collection tube. Here, intact bacteriophage was retained on the QIAprep silica-gel membrane.

6. The last step was repeated until all supernatant passed through QIAprep spin column.

7. 0.7 ml MLB buffer was added for M13 lysis and binding, to the QIAprep spin column and centrifuged for 15 seconds at 8000 rpm. This step creates appropriate conditions for binding of the M13 DNA to the QIAprep silica-gel membrane. Bacteriophage lysis begins.

8. Another 0.7 ml MLB buffer was added into the QIAprep spin column and incubated for 1 minute at room temperature to lyse the bacteriophage completely. QIAprep spin column was centrifuged for 15 seconds at 8000 rpm. M13 single-stranded DNA is released from bacteriophage particles and adsorbed to the QIAprep to the silica gel membrane.

9. 0.7 ml Buffer PE was added into the QIAprep column and centrifuged for 15 seconds at 8000 rpm to remove the residual salt.

10. Buffer PE was discarded from collection tube and QIAprep spin column was centrifuged for 15 seconds at 8000 rpm to remove residual buffer PE. It is important not to dry the QIAprep membrane quick microcentrifugation step. This prevents residual ethanol from being carried over into subsequent reactions. QIAprep spin column was placed in a clean 1.5 ml microcentrifuge tube. 100 μl EB buffer (10 mM tris.Cl, pH 8.5) was added onto the center of the column membrane to elute the DNA. Incubation of elution buffer in the QIAprep spin column significantly increases the recovery of single-stranded M13 DNA molecules, which adsorb tightly to the silica membrane.

11. After 10 minutes of waiting at room temperature, the tube is centrifuged for 30 seconds at 8000 rpm to collect the isolated ssDNA samples in the QIAprep spin column.

2.2.5. Sequencing of DNA sample

2.2.5.1. PCR conditions for single-stranded DNA sequencing

PCR reactions were done for the total volume of 10 µl in the 0.2 ml PCR reaction tubes.

Big dye reaction mix*	2 µl
5X sequencing buffer	1 µl
Template ssDNA	1 µl (50 ng)
M13 –96 Primer	3.2 µl
dH$_2$O	2.8 µl
Total volume	10 µl

*Big dye ® Terminator v3.1 cycle sequencing Kit from Applied Biosystems for the amplification of ssDNAs.

Reaction conditions

95°C   4 minutes
95°C   30 seconds
55°C   30 seconds   } 35 cycles
60°C   4 minutes

2.2.5.2. Purification of PCR products

1. 2 µl (3M pH 4.6 sodium acetate) and 50 µl 95 % ethanol were mixed for each sample.

2. 52 µl mixture was put into each PCR product and all samples were put in dry ice for 30 minutes.

3. All samples were centrifuged for 30 minutes at 14000 rpm.

4. Supernatant was discarded from each sample and 250 µl cold ethanol was put into each sample.

5. All samples were centrifuged for 30 minutes at 14000 rpm.

6. Ethanol was discarded from each sample and 20 μl hi-di formamide was put into each sample.

7. Samples were put first 95 ºC and then – 20 ºC for 5 minutes to denaturate the samples.

DNA samples were sequenced by using an ABI 3100 Avant (PE, Applied Biosystem, CA) automated sequencer.

2.2.6. Phage amplification and purification

1. At the end of 4.5 hours of growth period, *E. coli* - phage culture (in 50 ml LB) was transferred into 250 ml sterilized centrifuge tubes.

2. Samples were centrifuged at 8000 rpm for 10 minutes.

3. Supernatants were transferred into 250 ml sterilized centrifuge tubes.

4. 8.33 ml (1:6) of PEG / NaCl solution was added into supernatant to precipitate phage and it was left for overnight at 4 ^{0}C.

5. After overnight, samples were centrifuged at 8000 rpm for 10 minutes.

6. Supernatants were discarded and phage pellet was resuspended with 5 ml PC buffer (no detergent) by shaking to remove any remaining *E. coli*.

7. Samples were centrifuged at 8000 rpm for 10 minutes.

8. Supernatant was transferred into 50 ml sterilized centrifuge tubes.

9. 0.833 ml of PEG / NaCl (1:6) was added into the solution to precipitate phage and the solution was left for 2 hours at 4 ^{0}C.

10. Samples were centrifuged at 10000 rpm for 10 minutes.

11. Supernatants were discarded and phage pellets were resuspended by pipetting or shaking with 1 ml PC buffer (no detergent) to remove *E-coli.*

12. Samples were centrifuged at 10000 rpm for 10 minutes and supernatants were transferred into sterilized microfuge tubes.

13. 0.166 ml of PEG / NaCl solution (1:6) was added into the microfuge tube to precipitate phage, sample was vortexed for 5 seconds, and left for 10 minutes at room temperature.

14. Samples were centrifuged at 13500 rpm for 1.5 minutes to get the compact phage.

15. Supernatant was discarded and phage pellet was resuspended with 0.2 ml PC buffer (no detergent) by pipetting gently.

16. Samples were centrifuged at 13500 rpm for 1.5 minutes.

17. Supernatant were transferred into sterilized microfuge tubes and stored at 4 ^{0}C.

At the end of the purification step, blue-white screening experiment was done.

2.2.7. Titers of the selected phages

2.2.7.1. Blue-white screening experiment

Blue-white screening experiment is carried out to determine the phage titers. Basically, amplified phages are grown in the plates which have Xgal / IPTG and tetracycline. 50 μl Xgal/IPTG from 20 mg/ml stock, 100 μl 1M $MgCl_2$ (1:500), 50 μl tetracycline (1:1000) were put into 50 ml liquid warm LB agar in 100 ml glass medium flask and it was poured into plastic sterile Petri dishes.

2.2.7.2. Preparation of diluted phage and plating

90 μl PC buffer (no detergent) was put into the first well in Elisa plate. 180 μl PC buffer (no detergent) was put into other wells to dilute the sample. 10 μl phage stock solution was put in the first well having 10^{-1} dilution. Phage and PC buffer were mixed by pipetting. 20 μl sample was taken from the first well and put into the second well to have totally 200 μl of solution mix having 10^{-2} dilution level of the phages. The remaining serial dilutions were done the same way as it is labeled in Figure 2.3.

One night before plating, overnight culture was prepared to be used in the day of the plating. 5 ml LB solution containing 10 μl 1M $MgCl_2$ (1:500), 5 μl tetracycline (1:1000) was incubated with 12.5 μl *E. coli* ER2738 from overnight culture in 50 ml

falcon tube. It was incubated until mid- log phase (OD$_{600}$ ~ 0.5) at 37 °C and 200 rpm.

Xgal/IPTG-tet plates are taken out from 4 °C refrigerator and put at room temperature and labeled. Previously melted top agar is placed in the water bath at ~ 50 °C to prevent solidification of the top agar till adding the diluted phage solutions.
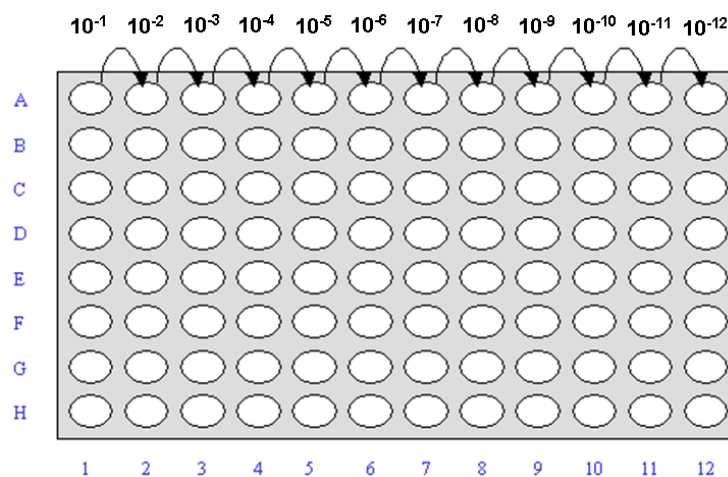
Figure 2.3. Making the serial dilutions on the Elisa plate.

20 μl diluted phage solution is mixed with 180 μl LB solution containing *E. coli* cells with OD$_{600}$ ~ 0.5 resulting one more dilution of the phages. The resulting solution was mixed with warm LB agar (0.5 ml for small Petri dishes and 3.5 ml for big Petri dishes) in 15 ml falcon tubes and vortexed. The phage and bacteria mixture solution was poured onto the plates and waited for 5-10 minutes for the solidification of the top agar. All Petri dishes were inverted and incubated at 37 °C for 8-14 hours.

2.2.8. Characterization of phage display peptides

Binding affinity and specificity of mutant phages were compared with the wild type phage through immunolabelling fluorescent microscopy and ELISA analysis. At the beginning of the characterization experiments, platinum or palladium powder was cleaned following the steps described below.

2.2.8.1. Cleaning of platinum and palladium powder

1. Around 100 mg of platinum or palladium powder was weighed and put into pre-weighed 1.5 ml microfuge tube.

2. 100 μl dH$_2$O and 900 μl CH$_3$OH/acetone (1:1) mixture were added into the tube. The powder was dissolved gently by pipetting.

3. The sample was vortexed for 5-10 minutes to observe the forming clumps and then sonicated for 20 minutes in an ultrasonic bath to break the clumps.

4. The powder was vortexed quickly to resuspend and centrifuged at 200 g for 1.5 minutes.

5. The supernatant was removed and 1 ml 50 % isopropanol was added onto the powder.

6. Powder was vortexed for 5-10 minutes and then sonicated for 20 minutes in the ultrasonic bath.

7. The powder was vortexed quickly to resuspend and spinned down at 200 g for 3 minutes.

8. The supernatant was removed and 1 ml 0.5 % PC buffer was added onto the powder.

9. The powder was first vortexed for 5-10 minutes and then sonicated for 60 minutes.

10. The powder was vortexed quickly to resuspend.

11. The powder was centrifuged at 200 g for 1.5 minutes.

12. The supernatant was removed and 1ml 0.5 % PC buffer was added onto the powder.

13. 100 μl of powder solution was transferred into each sterile microfuge tube.

14. Powder was spinned down at 200 g for 1.5 minutes.

15. Powder was first washed twice with dH_2O and then with isopropanol.

16. Samples were dried under the vacuum.

17. All samples were weighed to determine the amount of powder into the each tube.

2.2.8.2. Fluorescence microscopy experiment procedure

The binding of the phage samples on the platinum or palladium particles can be observed visually under the fluorescence microscopy indirectly. Zenon complex which has labeled Fab fragment bound on IgG is used to label the phages for indirect observation. The procedure is written below:

1. A proper amount of previously cleaned platinum or palladium powder is incubated with 5 µl phage (10^{11} PFU/10µl) in PC buffer containing 0.1 % detergent and rotated overnight on a running cyclinder to provide binding.

2. Each phage sample is labeled according to the immunolabelling procedure as in Figure 2.4. First, Anti-M13 pIII monoclonal antibody (Amersham Biosciences) which is specific to M13 gp8 protein and Alexa-Fluor conjugated secondary antibody fragments (Zenon Alexa, Molecular Probes Inc.) that contains fluorescence dye were incubated (1: 5 ratio) for 30 minutes in a microfuge tube covered by aluminum folio to protect from the light.
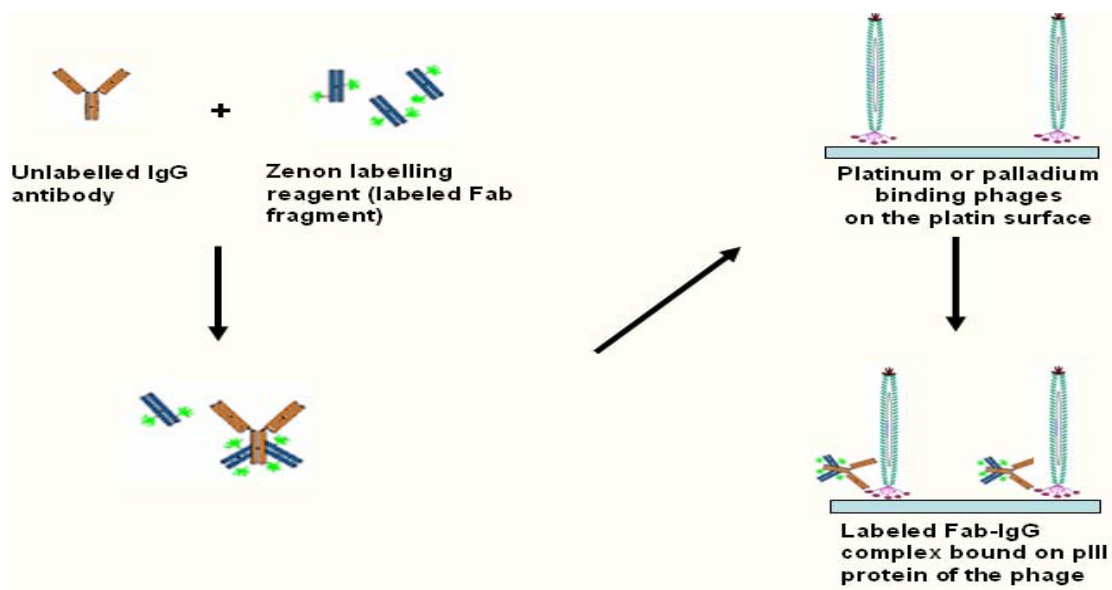
Figure 2.4. Immunolabelling procedure for fluorescence microscopy.

3. While Zenon mixture is being prepared, overnight phage-platinum or palladium powder solution is centrifuged at 1000 rpm for 1.5 minutes. The supernatant is discarded and 0.1 % PC buffer is added on the sample for 15 minutes rotation on running cylinder to wash unbound phage out of the solution.

4. The solution is centrifuged again at 1000 rpm for 1.5 minutes and the supernatant is discarded.

5. 3 µl Zenon dye complex and 497 µl PC buffer containing no detergent were put on the phage–platinum or palladium solution and incubated for 30 minutes on running cylinder to let phage- mixture interaction. The tube is protected from light by covering.

6. The solution is centrifuged again at 1000 rpm for 1.5 minutes and the supernatant is discarded.

7. 0.5 ml 0.1 % PC buffer is added into the tube and the powder is washed 3 times by pipetting.

8. The mixture solution is centrifuged again at 1000 rpm for 1.5 minutes and the supernatant is discarded.

9. Another 0.5 ml 0.1 % PC buffer is added into the tube and the powder is washed 3 times by pipetting.

10. The mixture solution is centrifuged again at 1000 rpm for 1.5 minutes and the supernatant is discarded.

11. 30 μl 0 % PC buffer is added onto the powder solution and visualized by fluorescence microscopy (BX 60, Olympus Corporation) at 20X magnification under WIB filter.

2.2.8.3. Enzyme-Linked Immuno Sorbent Assay (ELISA) analysis procedure

1. Proper amount of clean platinum or palladium powder incubated with 10μl phage (10^{11} PFU/10μl) in 190μl PC buffer containing 2 % BSA for 2 hours on a running cylinder to provide binding.

2. After incubation period, phage-powder mix was washed 3 times with 200μl buffer containing 0.05 % detergent.

3. 200μl PC buffer containing 2 % BSA was added on washed phage-powder mix and incubated with 0.4 μl HRP-AntiM13 antibody for 30 minutes by rotating as shown in Figure 2.5.
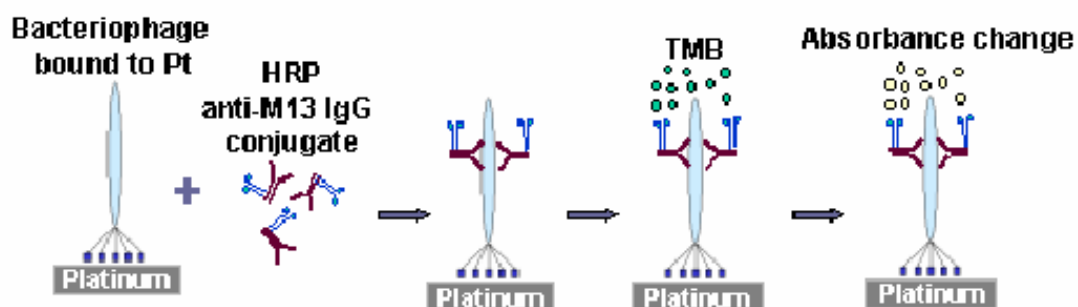
Figure 2.5. ELISA procedure [76].

4. After incubation period, 4 times washing step was performed to send away unbound antibody from solution and than phage-powder-antibody conjugate suspended in 200µl PC buffer containing 0.05 % detergent.

5. Enzymatic reaction was started when 100µl TMP (Pierce) was added into solution.

6. Reaction was ended when the color of the solution turned to blue with 100µl 1M H_2SO_4. Colorful supernatant was measured at 450nm.

3. RESULTS AND DISCUSSION

Binding events are complex and takes an important role in biomolecular recognition. Though the mechanism of binding of a polypeptide to the surface of an inorganic material has not been understood yet, identification of many short polypeptide sequences specific to metals like gold (Au), silver (Ag) and platinum (Pt) is growing field by many groups [12]. Already, these polypeptides are shown to have many effects to be potential building blocks for future engineering materials.

It can be clearly seen that the structure and function of a polypeptide is strongly linked with its amino acid sequence. Therefore, if the binding mechanism of a biomolecule - in this case polypeptide or protein – is desired to be lightened, investigation of amino acid sequence gains importance. When we look at the identified sequences for Pt via phage display, we face with much more smaller sequences (7 amino acids) than the ones defined for Au (14 amino acids) and Ag (12 amino acids) by cell surface display and phage display. Au and Ag binding peptides have similar amino acid properties: They have conserved hydrophobicity and polarity with hydroxyl containing amino acids [66].

This analogy also can be seen in the Pt and palladium (Pd) binding sequences. It was observed that most Pt binding sequences consist of serine (Ser) and threonine (Thr) repeating amino acids. These two amino acids have hydroxyl groups which make them more reactive. Therefore, it can be thought that hydroxyl groups in Ser and Thr seemed to play an important role for binding to the inorganic material, especially to platinum [31, 67].

In this study we have investigated the core amino acids that might be responsible for binding to Pt. To reach this aim, we have used one of the strong, 7- mer Pt binder, SD152, PTSTGQA. This peptide was previously identified from a random peptide phage display disulphide constrained library [12, 31, 66, 67]. We have applied site directed mutagenesis reactions to substitute the amino acid sequence one by one with alanine amino acid (Ala) thus Ala change is generally used for the investigation of side chains.

Next, based on the knowledge that we gained from platinum binder, we have used another phage clone displaying 7-mer peptide which was characterized as a weak binder for Pd, SD188, SPHPGPY. We have utilized site directed mutagenesis procedure this time to enhance the binding event by replacing glycine amino acid (Gly) to threonine (Thr).

Binding properties of these mutants have been assessed by Fluorescence Microscopy (FM) and Enzyme-Linked Immuno Sorbent Assay (ELISA) to compare with the unmutated, wild type SD152 and SD188.

3.1. Obtaining Double-Stranded DNA (dsDNA) of Pt and Pd Binding Sequences

Site directed mutagenesis procedure requires dsDNA of the binding sequences. To obtain these DNAs, M13 dsDNA purification has been applied. Firstly, SD152 and SD188 containing M13 phages were amplified. Thus, M13 dsDNA behaves like a plasmid (replicative form) when it is in the host cell, plasmid purification methods can be used for obtaining M13 dsDNA. So, QIAGEN Plasmid Mini Kit is used for obtaining dsDNAs. The performance of the dsDNA obtain was analyzed by agarose gel electrophoresis (Figure 3.1)
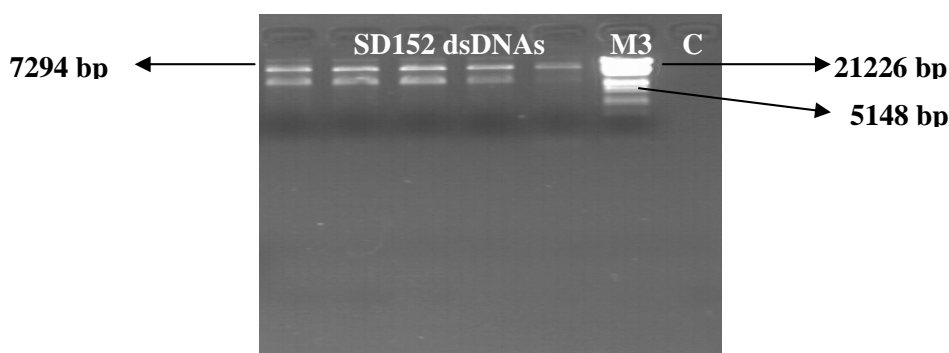
Figure 3.1. 1% agarose gel. M3: Lambda DNA/EcoRI+HindIII Marker, 3. C: Control reaction with no DNA. SD152 dsDNAs after plasmid purification method.

3.2. Site Directed Mutagenesis Reactions for Pt and Pd Binding Sequences

Site directed mutagenesis reactions mainly consist of three steps and all these steps were applied to both SD152 and SD188 peptides to obtain desirable mutants. These steps are:

    a) Methylation of the template dsDNA.

   b) Site directed mutagenesis with PCR and overlapping primers.

c) Transformation.

Replicative form (dsDNA) of phage genome containing SD152 and SD188 were methylated by DNA methylase. These methylated dsDNAs will be used as a template in PCR.

For SD152, two overlapping primers namely; the forward primer contains the desired Ala mutation and the reverse primer that is complementary to the forward primer were designed and used in site directed mutagenesis with PCR. The same concept was used for the design of SD188 primers. But this time, forward primer carries the Thr mutation. Table 3.1 shows these constructed oligonucleotides that were used as primers in PCR.

Table 3.1. Primers that were used in mutagenesis PCR.

Primer Name	Nucleotide Sequence
GTSD152P1A-F	5' TCTCACTCTGCTTGTGCTACTTCGACTGG 3'
GTSD152P1A-R	5' ACAAGCAGAGTGAGAATAGAAAGGTACCAC 3'
GTSD152T2A-F	5' CACTCTGCTTGTCCTGCTTCGACTGGTCAG 3'
GTSD152T2A-R	5' AGGACAAGCAGAGTGAGAATAGAAAGGTAC 3'
GTSD152S3A-F	5' TCTGCTTGTCCTACTGCTACTGGTCAGGCGTGC 3'
GTSD152S3A-R	5'AGTAGGACAAGCAGAGTGAGAATAGAAAGGTAC 3'
GTSD152T4A-F	5' TGCTTGTCCTACTTCGGCTGGTCAGGCGTG 3'
GTSD152T4A-R	5' CGAAGTAGGACAAGCAGAGTGAGAATAGAA 3'
GTSD152G5A-F	5' TTGTCCTACTTCGACTGCTCAGGCGTGCGGTG 3'
GTSD152G5A-R	5' CAGTCGAAGTAGGACAAGCAGAGTGAGAATAG 3'
GTSD152Q6A-F	5' TTGTCCTACTTCGACTGGTGCTGCGTGCGGTG 3'
GTSD152Q6A-R	5' ACCAGTCGAAGTAGGACAAGCAGAGTGAGAA 3'
GTSD152S3AT2A-F	5' TCACTCTGCTTGTCCTGCTGCTACTGGTCAG 3'
GTSD152S3AT2A-R	5' AGGACAAGCAGAGTGAGAATAGAAAGGTACC 3'
GTSD188G5T-F	5' TTGTTCGCCGCATCCTACGCCGTATTGCGGTGGA 3'
GTSD188G5T-R	5' AGGATGCGGCGAACAAGCAGAGTGAGAATAGAA 3'

After the PCR, reaction was observed on an agarose gel (Figure 3.2).
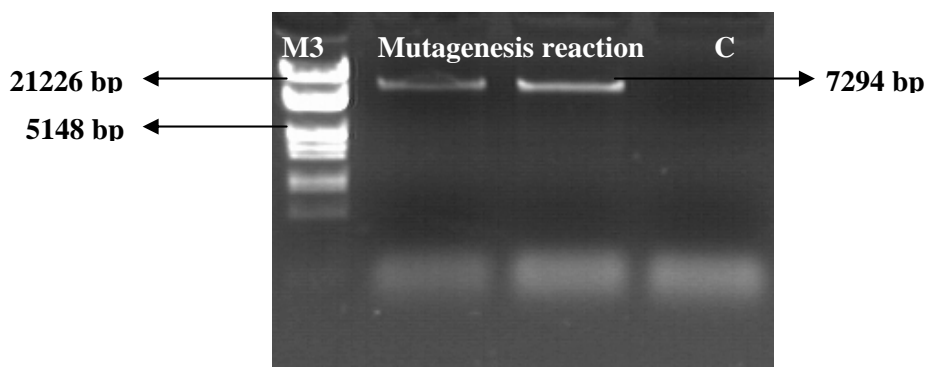
Figure 3.2. 1% agarose gel. M3: Lambda DNA/EcoRI+HindIII Marker, 3. C: Control reaction with no DNA. Mutagenesis reaction represents both SD152 and SD188 mutants.

The enzyme mixture in site directed mutagenesis reactions is also important. This mixture includes a proofreading enzyme that increases fidelity comparing to *Taq* DNA polymerase alone and allows amplification of complex and long DNA templates up to 20 kb with some optimization. So, PCR for long DNA sequences can be done easily with no doubt.

In the procedure of SD152 double Ala mutation, the primer design idea was the again same. But this time, we used an Ala mutant as a template to get a double Ala mutation. The forward and reverse primers were again designed with the same limitations.

After observation of desired DNA fragments on agarose gel, transformation of these DNA fragments into the bacterial host step were accomplished. In this step, two bacterial strains, *E. coli* DH5α-T1^R and *E. coli* DH5α-FT, were used. Firstly, PCR product was transformed into *E. coli* DH5α-T1^R chemically competent cell which provides self-ligation of linear PCR product to form the circular phage DNA and destruction of methylated template DNA consequently, leaving only the mutated phage DNA. Destruction of methylated DNA was performed by an endonuclease which is active in *E. coli* DH5α-T1^R host cells. Though this strain helps to get rid of the template DNA, it does not contain F pilus. For phage infection, F pilus is a must. Then the second bacterial strain, *E. coli* DH5α-FT, takes role. Assembled phage containing the desired mutation was amplified in this second bacterial strain. At the end, the transformation reaction was plated onto LB agar plates with mid-log *E. coli* DH5α-FT cell culture to obtain the mutant phages with blue-white screening.

3.3. Obtaining Single-Stranded DNA (ssDNA) for Sequencing

After the last step of site directed mutagenesis procedure, transformation into the host cell of the mutant dsDNA, blue phage plaques observed on LB plates were picked up with a pipette tip and put into PC Buffer containing wells of an ELISA plate to get storage plates. Phage solutions in storage plates were inoculated into mid-log *E. coli* DH5α-FT cell culture in liquid media for amplification. Single stranded DNA (ssDNA) was purified from amplified phages by using QIAprep$^{®}$ Spin M13 Kit and observed on an agarose gel (Figure 3.3).
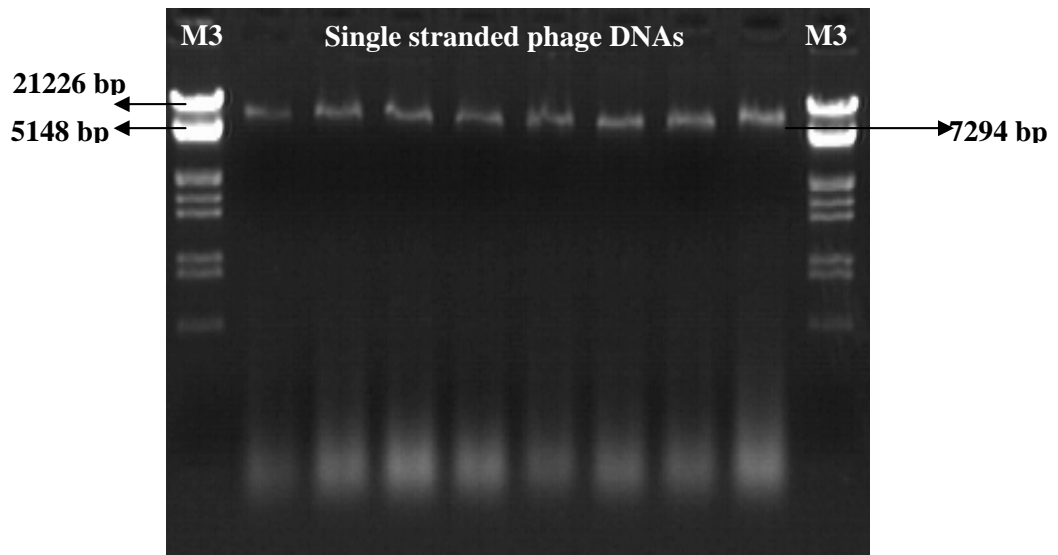
Figure 3.3. 1% agarose gel. M3: Lambda DNA/EcoRI+HindIII Marker, 3. ssDNA obtained from phage amplification and ssDNA purification.

These obtained ssDNAs then were prepared for the DNA sequencing if they were correct mutants to check if the desired mutants were obtained correctly.

After all of these steps, DNA sequencing analysis confirmed that we had constructed all the desired mutant phages. These mutants and the changes in their amino acid sequences can be seen on Table 3.2 and Table 3.3.

Table 3.2. The desired mutants for SD152 and the changes in amino acid sequences.

SD152 WT	PTSTGQA
SD152P1A	ATSTGQA
SD152T2A	PASTGQA
SD152S3A	PTATGQA
SD152T4A	PTSAGQA
SD152G5A	PTSTAQA
SD152Q6A	PTSTGAA
SD152T2AS3A	PAATGQA

Table 3.3. The desired mutant for SD188 and the change in amino acid sequence.

SD188 WT	SPHPGPY
SD188G5T	SPHP**T**PY

$$+H_3N \!-\! CH \!-\! \overset{\displaystyle O}{\overset{\|}{C}} \!-\! OH \quad \longrightarrow \quad +H_3N \!-\! CH \!-\! \overset{\displaystyle O}{\overset{\|}{C}} \!-\! OH$$

(with H below left structure; with CH–OH and CH₃ below right structure)

## 3.4. Characterization of Mutant Peptides

Following site directed mutagenesis of desired mutants, FM and ELISA analysis of mutant peptides were carried out to compare the binding efficiency of the mutants with the wild type peptides that were selected by phage display.

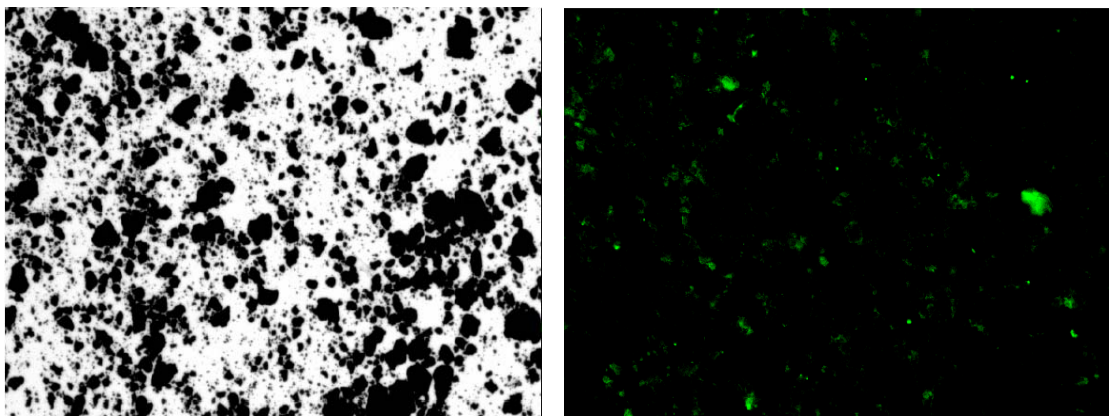### 3.4.1. Fluorescent microscopy analysis

All the mutant phages were purified and mixed with Pt or Pd powder. The mixture was rotated overnight and washed with PC buffer to remove any unbound phage from the organic surface. After then, anti-M13 g8p monoclonal antibody and Alexa-Fluor conjugated secondary antibody fragments were added to detect the phage as explained in Material and Methods section. The samples were observed under FM and the affinity of the mutant sequences was determined. Binding percentages of each peptide were determined with FM images obtained from each peptide.

The binding percentages of the wild type and mutant peptide binders were calculated as following logic: First, the ratio of powder area to the total area, (R1), was calculated by using the fluorescence microscopy pixel counting software. Then, the ratio of binding area to the total area, (R2), was calculated. The (R2/R1) x 100 value gave us the percentage coverage (binding) for each measured peptide.

Figure 3.4 shows the optic and fluorescent microscopy images of constraint SD152 and its two mutations, SD152P1A and SD152T2A. According to the calculated binding percentages from this image, SD152 has a nearly 11% binding affinity to Pt. These percentages also are different for mutant phages; for SD152P1A it is 8% and for SD152T2A the percentage is decreased to 0.15%.
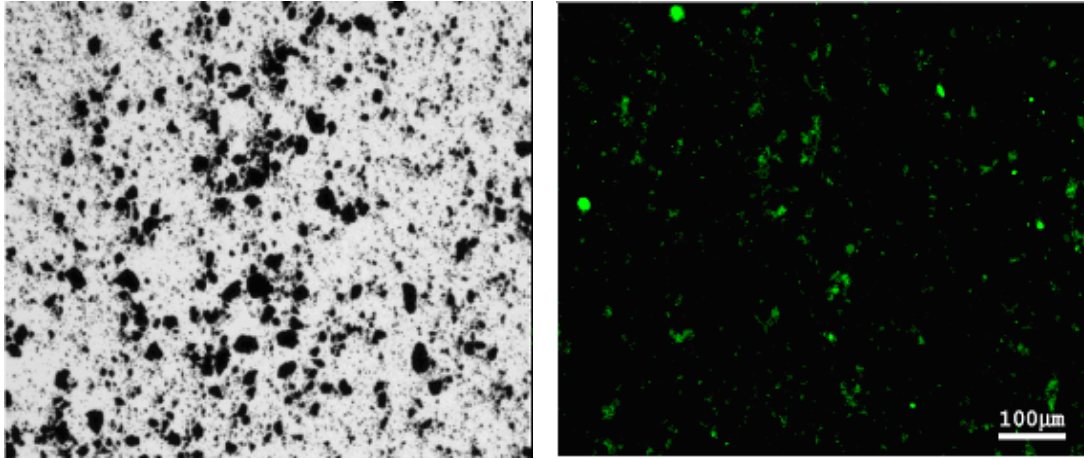
SD152 wild type (10.45% binding.)

SD152P1A mutation (8 % binding)
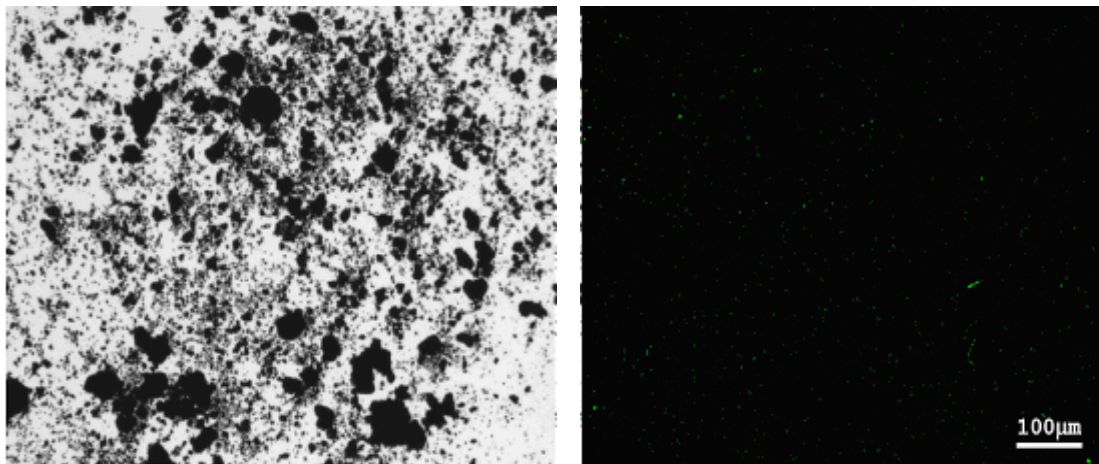
SD152T2A mutation (0.15 % binding)
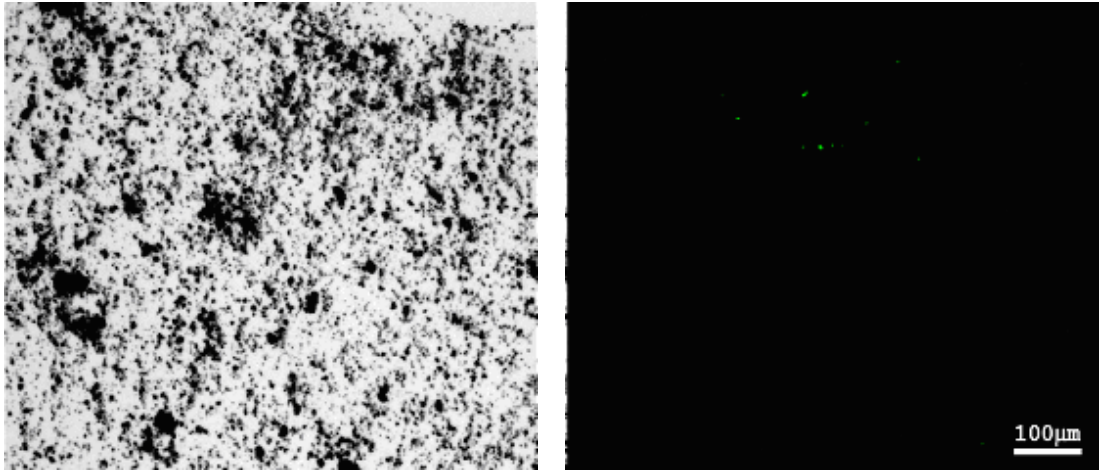
Figure 3.4. Optical and fluorescent microscopy images of SD152, SD152P1A and SD152T2A.

Figure 3.5 shows the optic and fluorescent microscopy images of mutations, SD152S3A and SD152T4A.

SD152S3A mutation (0.1% binding)

SD152T4A mutation (1.45% binding)

Figure 3.5. Optical and fluorescent microscopy images of SD152S3A and SD152T4A.

The decrease on binding percentage continues with these two mutant phages. But the decrease on SD152S3A is so certain that Ala mutation is said to be so powerful on that phage thus the binding affinity is nearly 0. With only this result, the importance of Ser can be seen on binding.
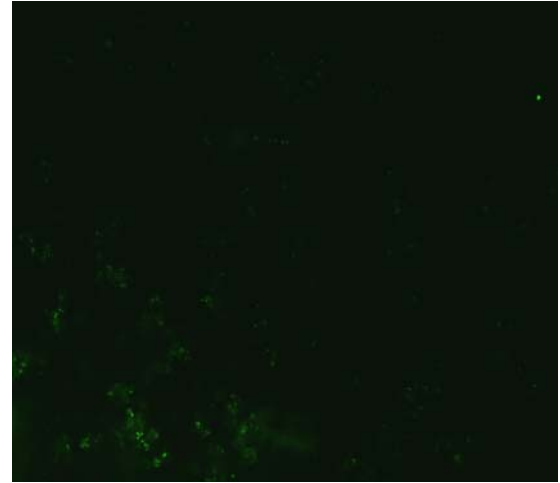
SD152G5A mutation (4% binding)
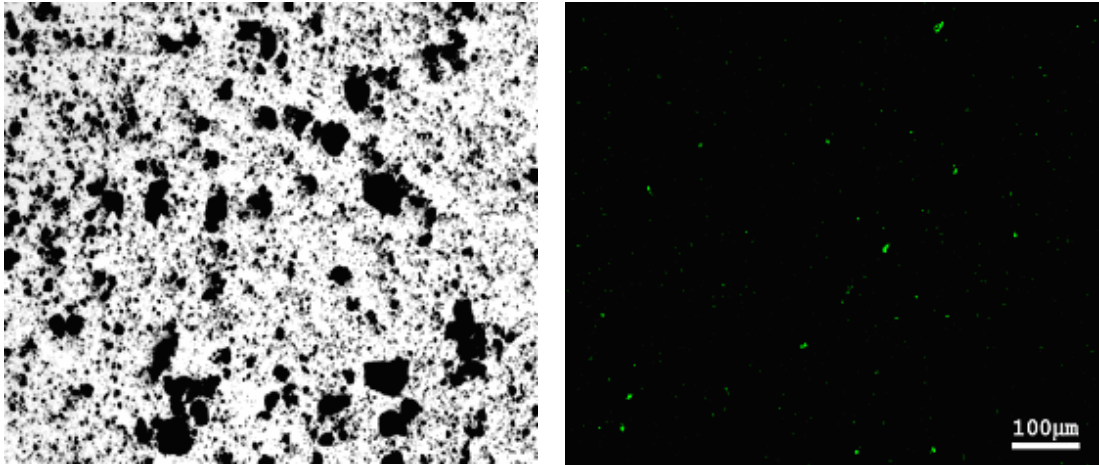

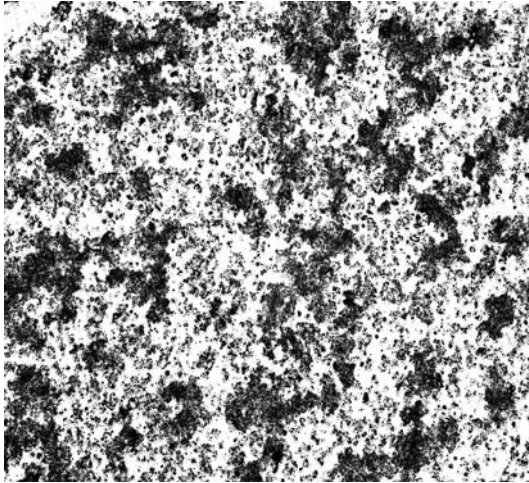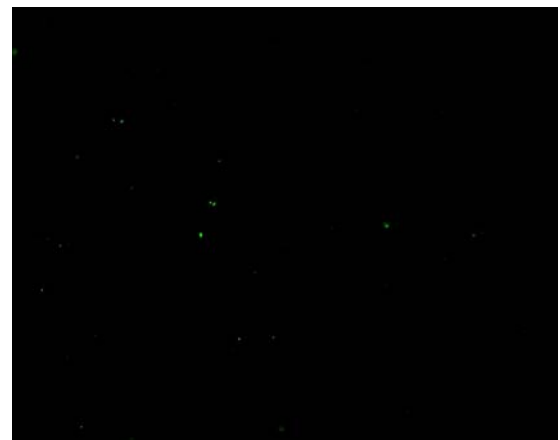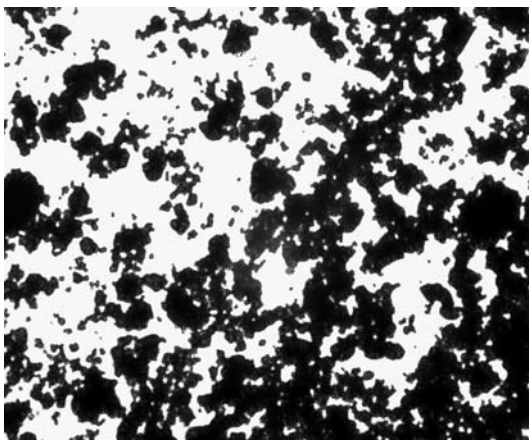SD152Q6A mutation (1.75% binding)

SD152T2AS3A mutation (0,15% binding)

Figure 3.6. Optical and fluorescent microscopy images of SD152G5A, SD152Q6A and SD152T2AS3A.

In Figure 3.6, the effect of Ala mutation can be clearly seen. Except SD152G5A mutant, the mutations force the binding affinity to nearly nonbinding level. We also see that the double Ala mutation (PAATGQA) has a drastic effect on SD152.

It is observed that the substitution of the target amino acids with Ala resulted with valuable indications. These indications show the importance of Thr and Ser on binding to Pt.

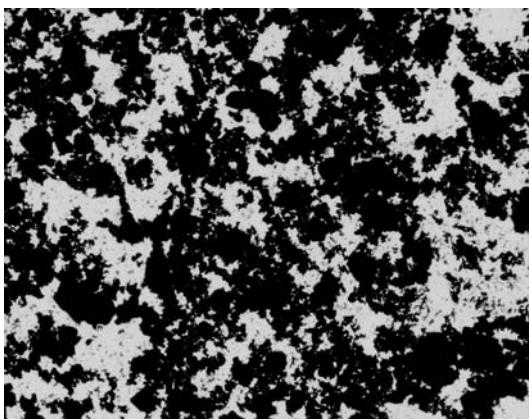The same FM analyses were carried out to characterize the binding affinity of SD188G5T (Figure 3.7).
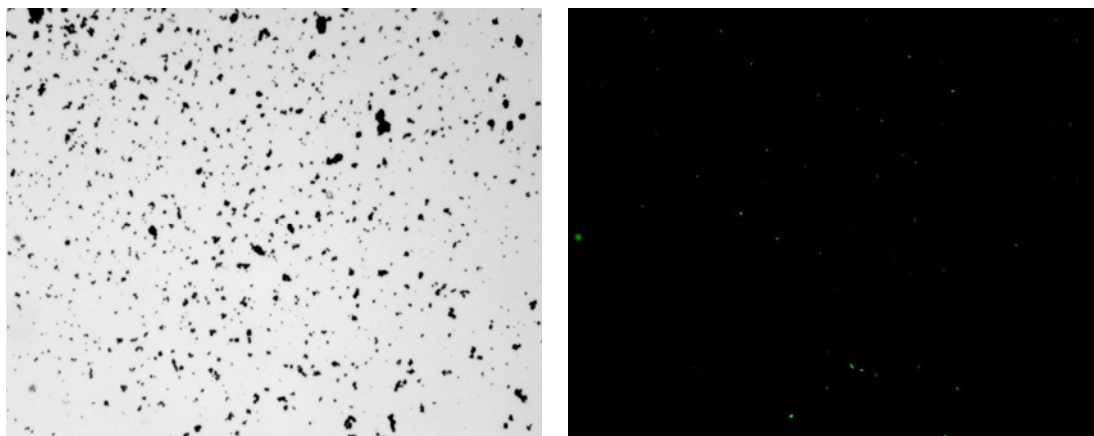
SD188 wild type (1.7% binding)
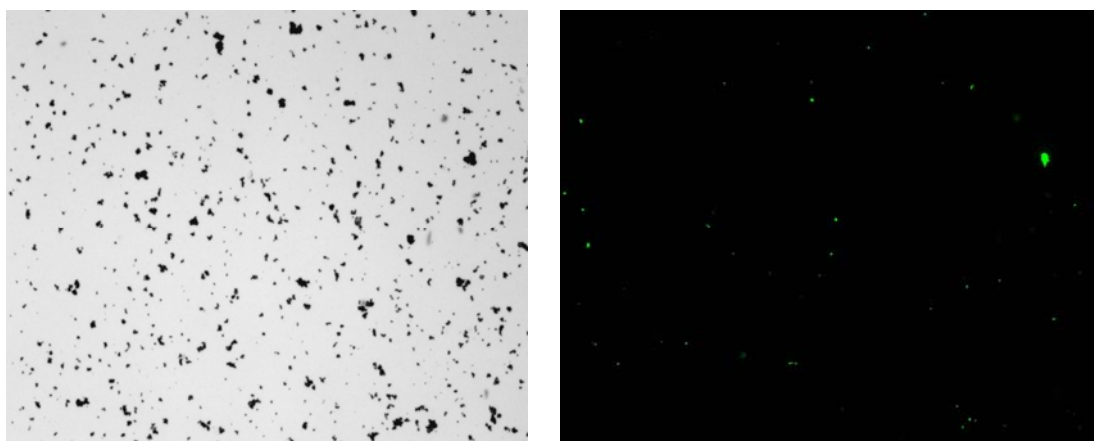
SD188G5T mutation (2.5% binding)

Figure 3.7. Optical and fluorescent microscopy images of SD188 wild type and its mutant, SD188G5T.

Repeating Ser and Thr consisted peptides are mostly observed in Pt binding sequences. These two amino acids have similar structures containing aliphatic hydroxyl groups which make them much more reactive than all other amino acids with inert side chains.

That is why Gly is changed to Thr in SD188. We see a meaningful increase on Pd binding in FM images (Figure 3.7). So we can assume that Thr may have the same boosting effect on binding to Pd.

In the next step, ELISA analyses were performed as an alternative method to characterize the affinity property of the constructed mutants and their results were compared with FM analysis to verify.

3.4.2. ELISA analysis

All the mutant phages were purified and mixed with Pt or Pd powder. The mixture was rotated for 2 hours and washed with 0.05 % detergent to remove any unbound phage from the organic surface. HRP-AntiM13 antibody conjugate was added to the mixture and left for incubation for 30 minutes. Then the mixture was washed several times to eliminate the unbound antibody. The substrate of HRP named TMP was added to this washed mixture and color change was observed due to the enzymatic reaction in the mixture. The color changed yellow to blue. This color change was measured at 450nm.

ELISA analyses were accomplished for SD152 and SD188 mutants. As a standard, the phage concentration is held on the same concentration for every mutation. For each mutation, a triple experiment set is formed. Wild type M13KE was used in all measurements as a control. The results of ELISA experiments performed with $10^9/\mu L$ of phage particles for Pt and Pd are given in Figure 3.8 and Figure 3.9.
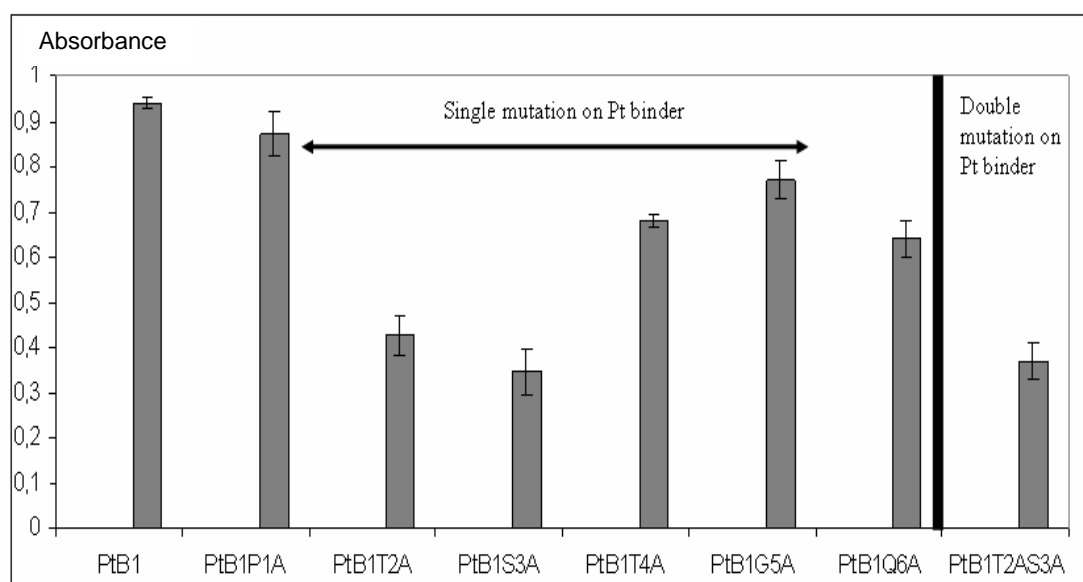
Figure 3.8. The graph shows the measured absorbance levels after ELISA analysis of PtBP1(SD152wt) and associated mutants.
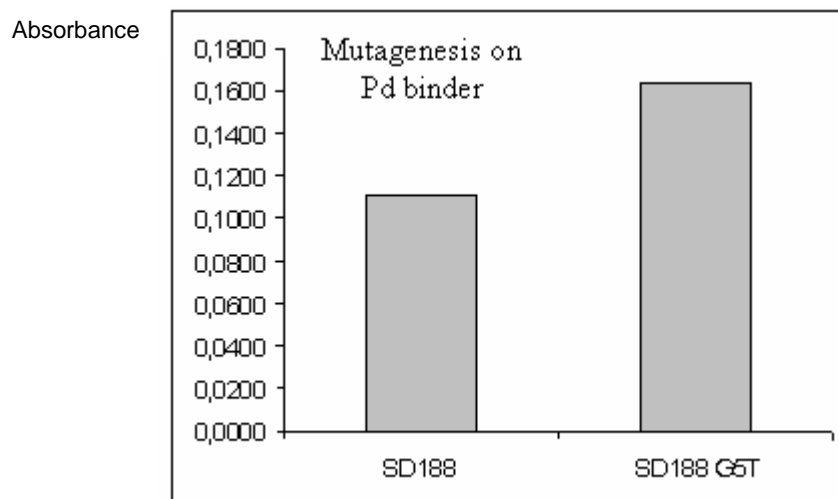
Absorbance

Figure 3.9. The graph shows the measured absorbance levels after ELISA analysis of SD188 and its mutant, SD188G5T.

Our results show that, ELISA analyses verified the results of FM analyses. Binding on platinum surface is related with amino acids which has hydroxyl side chains and Thr and Ser were the most effective ones. When these amino acids leave the peptide sequence with the change of Ala, there is a significant decrease in all mutations for Pt binding peptide, SD152. In addition, these amino acids can effect the binding for Pd. Experiments show that Thr also triggers the binding event on Pd surfaces.

4. CONCLUSION

Site directed mutagenesis reactions for two phage clones displaying selected and well characterized platinum and palladium binding peptides, SD152 and SD188, were accomplished.

SD152 and SD188 are 7-mer peptides with the amino acid sequences of PTSTGQA and SPHPGPY. SD152 has been characterized as a strong platinum (Pt) binding peptide and SD188 has been characterized as a weak palladium (Pd) binding peptide via characterization methods such as fluorescence microscopy (FM) when they were in disulphide constraint forms.

Site directed mutagenesis allows one to substitute the amino acids in a specific site of protein with another amino acid obtaining different properties. Through this ability, comparing the binding affinity of each mutant with originally selected peptide provides understanding the effect of mutated amino acid. With this knowledge, one can tune up the binding from strong to weak, or from weak to strong.

To reach this aim, amino acids that were thought to be responsible for binding in SD152 changed with alanine amino acid (Ala) one by one. Because substitution with Ala removes all side chain atoms past the β-carbon and the effects of single Ala substitution can be used to understand the roles of side chains.

Also a double Ala mutation was performed using a single Ala mutant as a template to test the effect of double Ala mutation in peptide function on binding.

Furthermore, site directed mutagenesis was utilized for weak Pd binder peptide, SD188, this time to increase the peptide affinity for the inorganic surface. For enhancement for binding, we performed polar group insertion as the result obtained from platinum binder showed that polar groups are effective in the binding process.

The binding affinity of palladium binder, SD188, with threonine substitution (Thr) to glycine (Gly) position was enhanced.

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APPENDIX

A. Color coding of amino acids

Table A.1. Color coding of amino acids.

Hydrophobic	A G V F P M I L W
Acidic	D E
Basic	R K
Hydroxyl	S T Y
Amine + Basic	H C N Q

B. Lab Equipments

Autoclaves	: 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	: JA30.50Ti, Beckman Coulter.
	: F241.5P, 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-PROTEAN 3 Cell and Single-Row AnyGel Stand, Catalog #165-3321, Bio-Rad.
	: Mini-V 8.10 Vertical Gel Electrophoresis System, Life Technologies GibcoBrl (now Invitrogen), Catalog #21078.
Gel documentation system	: UVIpro GAS7000, UVItec Limited.
Ice Machine	: AF 10, Scotsman.
Incubators	: EN400, Nuve
Orbital shaker	: Certomat S II, product# 886 252 4, B. Braun Biotech International GmbH.
Magnetic stirrer	: AGE 10.0164, VELP Scientifica srl.
	: ARE 10.0162, VELP Scientifica srl.
Microscope	: BX60, Olympus Corporation.
	: CH60, Olympus Corporation.
Microscope Camera	: RT Slider, Spot Diagnostic Instruments.
Pipettes	: Pipetteman P10, P 100, P1000, Eppendorf
pH meter	: MP 220, Mettler Toledo International Inc.

pH meter	: Inolab pH level 1, order #1A10-1113, Wissenschaftlich-Technische Werkstätten GmbH & Co KG.
Power supply	: EC 250-90, E-C Apparatus.
Pure water systems	: USF Elga UHQ-PS-MK3, Elga Labwater.
Spectrophotometer	: DU530 Life Science UV/ Vis, Beckman. : UV-1601, Shimadzu Corporation.
Sterilizer	: FN 500, Nuve.
Transilluminator	: UV Transilluminator 2000, Catalog #170-8110 EDU, Bio-Rad.
Vortexing machine	: Reax Top, product #541-10000, Heidolph2.2

RESUME

Anıl Cebeci was born in İzmit, Kocaeli in 1980. After getting his high school diploma from Kocaeli Seymen Science High School in 1998, he has continued his undergraduate degree in Istanbul University, Cerrahpaşa Medical Faculty, Department of Medical Biology in 1999. He had his Bachelor Degree from the same department in 2003. In the same year, he was accepted to Molecular Biology-Genetics and Biotechnology Program which is a branch of Advanced Technologies in Engineering Graduate Program at Istanbul Technical University to continue his Master Degree. He is still in the same program. His interest topics are about molecular genetics, protein engineering and biotechnology.