İSTANBUL TECHNICAL UNIVERSITY ★ INSTITUTE OF SCIENCE AND TECHNOLOGY

STRUCTURE AND MULTIMERIZATION EFFECTS OF INORGANIC BINDING PEPTIDES: PHAGE DISPLAY SELECTED PT BINDERS AS A CASE STUDY

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İNORGANİKLERE BAĞLANAN PEPTİDLERE YAPISAL VE ÇOKLU TEKRARLARIN ETKİSİ: ÖRNEK ÇALIŞMA OLARAK FAJ GÖSTERİM YOLUYLA SEÇİLMİŞ PLATİNYUMA BAĞLANAN PEPTİT

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ABBREVIATIONS

A : Alanine
bp : Base pair
BSA : Bovine serum albumin
C : Cysteine
dH_2O : Distilled water
DMF : Dimethylformamide
DNA : Deoxyribonucleic acid
dsDNA : Double stranded DNA
EB : Elution Buffer
EDTA : Ethylenediaminetetraacetic acid
FM : Fluorescence Microscopy
G : Glycine
HRP : Horse Raddish Peroxidase
IPTG : Isopropyl-â-D- thiogalactopyranosi
kb : Kilobase
LB- broth : Luria Bertani broth
LacZ : â-galactosidase
MLB : M13 lysis and binding buffer
MP : M13 Precipated Buffer
mRNA : Messenger ribonucleic acid.
Na-Ac : Sodium acetate
OD : Optical density
P : Proline
PBS : Phosphate Buffer Saline
PC : Potassium Phosphate-Sodium carbonate buffer
PFU : Phage Forming Unit
PCR : Polymerase chain reaction
PEG-8000 : Polyethylene Glycol-8000
Pt : Platinum
RF : Replicative form of M13 genome
S : Serine
SDS : Sodium dodecyl sulfate
ssDNA : Single stranded DNA
T : Threonine
TBE : Tris-borat -EDTA
TMP : 3,3',5,5' tetramethylbenzidine
Q : Glutamine
Tris base : Hydroxymethyl aminomethane
X-Gal : 5-Bromo-4-chloro-3-indolyl-D-galactoside

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SUMMARY

Many organisms contain organic-inorganic hybrid systems consist of protein inorganic interactions. These hybrid systems have excellent functions such as forming protective layers, supportive tissues, transferring charge and ion, developing some optical and mechanical properties. These excellent as well as diversified functions give an enormous inspiration for novel design of genetically engineered materials and systems for applications in biotechnology and nano-biotechnology.

In order to control the formation and assembly of functional inorganic hybrid materials and systems, inorganic-binding polypeptides have a great potential to be used as biolinkers and molecular building blocks for controlled assembly processes. Polypeptides that selectively bind to a given inorganic can be identified by using combinatorial biology based molecular libraries such as phage display peptide libraries. Selected inorganic binding peptides can further be engineered by utilizing protein engineering tools to design second generation peptides with controllable and predictable affinities.

Protein engineering tools are composed of using both genetic and chemical techniques to change the structure and function of a protein; therefore they provide unique opportunity to further tailor the inorganic binding functionalities of the selected peptides. Here, the main design parameters are targeting the amino acids which may be playing structural or functional roles, deleting or replacing them with alternative ones or testing the impact of molecular structure as constrained or nonrestricted, hydrophobic forces, electrostatics and charge, and the placement of hydrogen bonds, salt bridges, disulfide bonds, water or metals.

Here, we developed engineering protocols for inorganic binding peptides displayed on phage using protein engineering approach. As a case study, we have studied the platinum binding peptide, which was identified and characterized by our group in a previous study. In our engineering approach, we have developed multimerization protocols for displayed peptides on phage through investigation of the effect of constrained to nonrestricted molecular structural on the second generation peptides. We also adapted characterization techniques as immuno-fluorescent labeling and ELISA analysis to investigate the binding affinities of second generation peptides. Our experiments demonstrated that binding affinity could be tuned up via genetic engineering approaches on the host organism. These protocols will be of great utility for optimizing length or the molecular conformation of the inorganic specific peptides for controllable and predictable affinities.

ANORGANİKLERE BAĞLANAN PEPTİTLERE YAPISAL VE ÇOKLU TEKRARLARIN ETKİSİ: ÖRNEK ÇALIŞMA OLARAK FAJ GÖSTERİM YOLUYLA SEÇİLMİŞ PLATİNYUMA BAĞLANAN PEPTİT

ÖZET

Bir çok organizma protein inorganik etkileşimlerinden meydana gelen organik-inorganik hibrid sistemler içerir. Bu hibrid sistemlerin koruyucu tabakalar ve destekleyici dokular oluşturmak, yük ve iyon transferi yapmak, bazı optik ve mekanik özellikler geliştirmek gibi mükemmel fonksiyonları mevcuttur. Çeşitlendirilmiş mükemmel fonksiyonlara sahip bu sistemler, biyoteknoloji ve nano-biyoteknoloji uygulamaları olan, genetik olarak değiştirilmiş materyallerin ve sistemlerin yeni dizaynına olanak tanır.

İnorganiklere bağlanan polipeptitler, kontrollü birleşim oluşumlarında biyolinker ve moleküler yapı taşları olarak kullanılabildiklerinden dolayı, fonksiyonel inorganik hibrid malzeme ve sistemlerin oluşum ve düzenlenmelerinin kontrol edilmesinde önemli potansiyele sahiptirler. Belirli bir inorganiğe özgün olarak bağlanan polipeptitler, faj gösterim kütüphaneleri gibi kombinatoriyel moleküler kütüphaneler kullanılarak seçilebilirler. Spesifik inorganiklere bağlanabilen seçilen peptitler, tanıma mekanizmalarının ve optimum yapılarının araştırılması amacıyla protein mühendisliği teknikleri kullanılarak tekrar dizayn edilebilirler.

Bir proteinin yapısını ve fonksiyonunu değiştirmek için genetik ve kimyasal tekniklerin yararlanıldığı protein mühendisliği yöntemleri; seçilen peptitlerin inorganiklere bağlanma fonksiyonlarını biçimlendirmede kullanılan eşsiz bir metoddur.. Yapısal ve fonksiyonel işlevi olan amino asitleri belirlemede kullanılan en önemli dizayn parametreleri, bu aminoasitlerin yapıdan çıkarılması veya alternatifleriyle değiştirilmesi ya da moleküler yapının sınırlandırılması ve serbest bırakılmasındaki önemini test etmek, hidrofobik, elektrostatik ve yük etkileşimlerinin, hidrojen bağları, tuz köprüleri, disülfit bağları, su ve metallerin değiştirilmesinin etkilerini incelemek seklinde sıralanabilir.

Bu çalışmada, protein mühendisliği yöntemlerini kullanarak faj gösterim yolu ile elde edilen inorganiklere bağlanan peptidlerin, yapısal ve fonksiyonel işlevlerinin tanınması ve geliştirilmesine yönelik protokoller geliştirdik. Örnek çalışma olarak, grubumuzda daha önce tanımlanmış ve karakterize edilmiş olan platinyuma bağlanan peptiti çalıstık. Kullandığımız yöntem ile, faj üzerinde gösterilen peptidlerin moleküler yapının sınırlandırılmış ve serbest bırakılmış durumundaki etkilerini de inceleyebilmek amacı ile çoklu tekrarlarını oluşturabileceğimiz ikinci jenerasyon peptid eldesine yönelik bir protokol geliştirdik. Elde edilen ikinci jenerasyon peptidlerin, bağlanma ilgilerini tanımlayabılmek icin immuno-florasan isaretleme ve ELİZA gibi karakterizasyon tekniklerini uyguladık. Deneylerimiz konak organizma üzerinde genetik mühendisliği yöntemleri ile bağlanma ilgilerinin kontrol edilebileceğini göstermistir. Bu protokoller, inorganiklere spesifik peptidlerin

uzunluk ve moleküler konformasyonlarının optimizasyonu ile bağlanma ilgilerinin kontrolu icin büyük fayda sağlayacaktır.

1. INTRODUCTION

1.1. Biological Materials and Systems

Proteins are abundant and imperative macromolecules of biological systems. Exhibiting enormous diversity of functions, they consequently perform most of the biological processes such as cellular structure formation, catalyzing biological reactions and controlling physical conditions of biomaterials. In fact, proteins constitute the most valuable part of tissues by serving as transporters and self- and co-assembly into short and long range ordered substrates [1-4].

Variable properties of proteins rely on the fact that they are highly specific and selective towards their ligands [5]. Proteins interact with other macromolecules and inorganics to control the structures and the functions of biological systems [3]. While macromolecular interactions are observed in soft tissues such as muscle, skin and etc. [6], macromolecular-inorganic hybrids form the basis of hard tissues [3, 7].

Hard tissues include bones, dental tissues, shells, spicules, spines, skeletal units of single cell organisms or plants, bacterial thin film and nanoparticles are composed of at least one proteinaceous phase together with inorganic materials. Specific inorganic binding proteins generate proteinaceous phase bind and organize inorganic materials to perform protective layer formation, ion transferring and developing some optimal and mechanical properties. The inorganic material, on which the proteinaceous phase is bound, commonly include magnetite (Fe_3O_4) particles in magnetotactic bacteria or teeth of chiton [8]; silica (SiO_2) as skeletons of radiolarian [9] or tiny light-gathering lenses and optical wave guides in sponges [10]; hydroxyapatite ($Ca_2C(OH)_3$) in bones [11] and dental tissues of mammals [12] and calcium carbonate ($CaCO_3$) in the shells of mollusks [13]. (Figure 1.1)

Similar properties of inorganic materials in nanometer-scale dimensions are utilized for nano - and biotechnological applications such as chemical, biological and optical sensors, spectroscopic enhancers, nanoelectronics and quantum structures. In contravention of great potential of nanotechnological systems, they are limited by the

reasons of difficulties in their synthesis and assembly into functional structures [3,14,15].

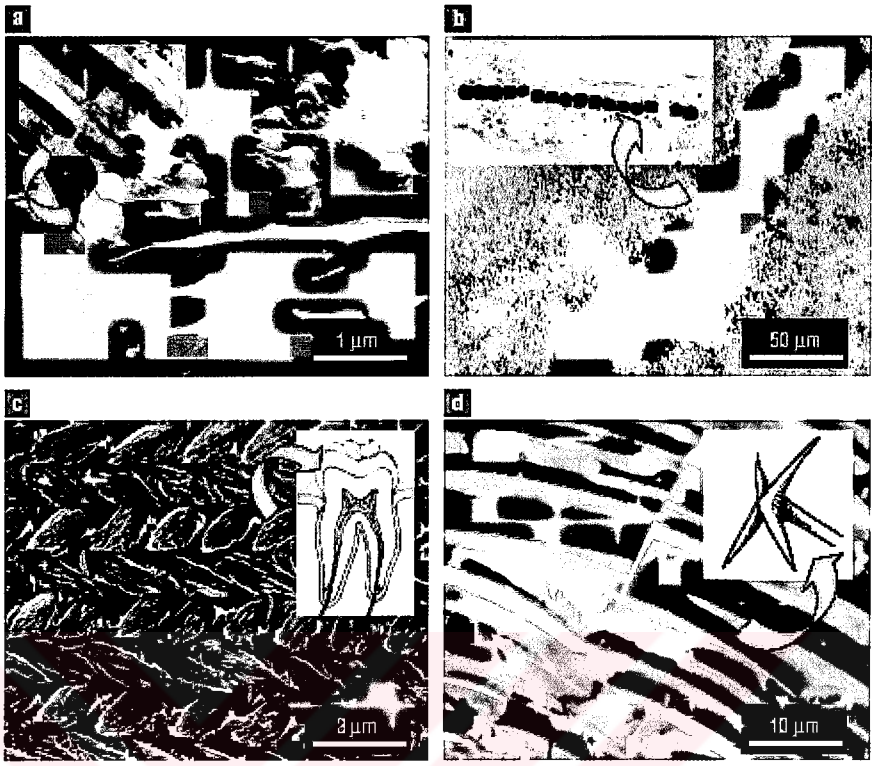


Figure1.1 Examples of biologically synthesized complex materials **a)** Scanning electron microscope (SEM) image of a growth edge of abalone (*Haliotis rufescens*) displaying aragonite platelets (blue) separated by organic film (orange) that eventually becomes nacre (mother-of-pearl) (inset: transmission electron microscope (TEM) image) **b)** Magnetite nanoparticles formed by magnetotactic bacterium (*Aquaspirillum magnetotacticum*, inset: TEM image) are singlecrystalline, single-domained, and crystallographically aligned. **c)** Mouse enamel (SEM image) is hard, wear resistant material with highly ordered micro/nano architecture consisting of hydroxyapatite crystallites that assemble into woven rod structure (inset: schematic cross-section of a human tooth) **d)** Sponge spicule (with a cross-shaped apex shown in inset) of Rosella has layered silica with excellent optical and mechanical properties, a biological optical fibre (SEM image). [4]

1.2. Inorganic binding proteins

Being inspired by nature, hybrid materials can be developed combining molecular biology tools with nanotechnology under the name molecular biomimetics to overcome difficulties faced up in nanotechnological applications. In order to control formation and assembly of functional inorganic hybrid materials and systems, inorganic-binding polypeptides are used as biolinkers and molecular building blocks [4,14].

1.2.1. How to Obtain Inorganic Binding Proteins

Till now a few proteins have been identified to bind inorganic materials. Most known example of these proteins is ice-binding protein found in many fish species, plants and insects [16]. Just unidentified inorganic-binding proteins could be obtained in several ways.

One of these ways is designing inorganic-binding proteins by using theoretical molecular approach [17]. However this is an impractical and expensive way to design inorganic-binding proteins.

Another way is to extract and purify proteins from hard tissues and cloning the genes coding them [18]. Adverse consequences of this approach are that hard tissues contain many proteins [19] and extracted protein could only be utilized for the regeneration of specific inorganics, could not be used for engineering of other nanostructures.

The use of combinatorial biology techniques is an emerging field in design of inorganic binding proteins [20]. In this approach, inorganic-binding proteins are selected against a specific inorganic material from combinatorial biology libraries [3,14]. These libraries composed of random peptides with the same number of amino acids but different in sequence compositions. "Phage Display" and "Cell Surface Display" are well-known *in vitro* combinatorial biology techniques [21, 22]. These display techniques mainly were carried out to perform the characterization of receptor and antibody binding sites, the study of protein-ligand interactions and the isolation and evolution of proteins or enzymes exhibiting improved or altered binding characteristics for their ligands [23] are also adapted for the selection of inorganic binding peptides [3,14].

Up to date, phage display has been employed to identify peptides that are specific to gallium arsenide [24], silica [25], silver [26], zinc sulfide [27], calcite [28], cadmium sulfide [29], and noble metals such as platinum and palladium [30]. Cell surface display also has been applied to identify the iron oxide [14, 31], gold [14, 32], zinc oxide, zeolites and cuprous oxide [33] specific binders.

1.2.2. Phage Display

Phage display is an *in vitro* combinatorial biology technique that enables peptides with desired binding affinities to be selected from a large library of variants. Phage display, first executed by G. Smith to follow antigen-antibody recognitions [34], is also be used to study protein-ligand interactions and to extract engineered proteins with altered affinities [23].

Phage display involves the expression of peptides on phage coat. The gene of interest is fused to the coat protein of phage, leading phage particle to display the encoded peptide and contain its gene. Thereby, a direct link is constituted between phenotype and genotype. This allows identifying selected colons by sequencing.

Various vectors are used as vehicles for phage display. The first developed vehicle, filamentous bacteriophage M13 (Ff family), is still widely used. This is due to ease of manipulating phage genome and obtaining high titers ($10^{11\text{-}12}$ particles per milliliter). Besides these advantages, use of filamentous phage vehicles has some drawbacks emanated from the entrance of coat proteins to the host cell membrane. Whereas these limitations such as limited peptide size to be displayed are interfere with the properties of desired protein to be expressed, phage λ, T7 or T4 can be preferred [35, 36]. However there are some studies on λ, T7 or T4 phage-based display systems, they are not yet used in a routine way.

1.2.2.1. Types of Phage Display Peptide Libraries

Phage display peptide libraries are constructed via inserting the randomized peptide coding sequences as a fusion into coat proteins of phage particle. With different phage vectors, different coat proteins can be utilized as peptide displaying agents. In the case of M13, all five coat proteins can be used to display peptides or proteins [37].

Different M13-based phage display peptide libraries can be generated according to the insertion site (Table 1.1.) and length of the insert. N-terminal display has been achieved with minor coat proteins g7p and g9p [38], while fusions into g6p have been performed into C-terminal [39]. Both C- and N-terminal display have been demonstrated with g3p and g8p [40, 41, 42].

Table 1.1. M13 coat proteins are given with their amino acid length, molecular weights and copy numbers exist on phage particle. N and C type display indicates the displayed peptide fusion site on coat protein. [43]

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

Among all five coat proteins, g3p and g8p are most widely used for generation of phage display libraries that are generally based on N-terminal fusions. Only short peptides (6-8 residues) can be displayed on every copy of major coat protein g8p. Larger peptides can be displayed only on 10% of the coat proteins because of the size restrictions of the channel through which phage pass during assembly. The minor coat protein g3p, present in 5 copies on phage coat, is more tolerant for large insertions and short peptides can be monovalent displayed without interfering with the phage infectivity to generate library [37]. As a result, peptides expressed as g3p fusions are present at low valency (1-5 copies per virion), while g8p fusions are present at high valency (~200 copies per virion). The increased avidity effect of high valency g8p display permits selection of very low affinity ligands, while low valency g3p display limits selection to higher affinity ligands.

Phage libraries can also be generated in constraint form by adding two cysteine residues on both ends of the insert peptide (Figure 1.2). In an oxidizing environment, such as the *E. coli.* periplasm, disulfide bond is formed between cysteine residues. The constraint libraries are useful for targets whose native ligands are in the context of a surface loop, such as antibodies with structural epitopes. Additionally, imposing structural constraint on the unbound ligand results in less unfavorable binding entropy, improving the overall free energy of binding compared to unstructured ligands. A major disadvantage of the constraint libraries is that the disulfide constraint may "freeze out" a conformation required for target binding. [44, 45]

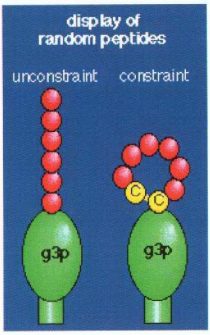


Figure 1.2. Diagram of 7 amino acid long, unconstraint (linear) and constraint phage displayed peptides. [46]

To sum up, libraries can be generated by inserting 5-38 amino acid long peptides but increasing length of peptides may affect the display efficiency and phage viability. Commercially available libraries are present with 7, 12 and 15 amino acid long insertions and both in linear and constraint form (C7C- disulfide constraint 7 amino acid long peptide displaying library).

1.2.2.2. M13 Filamentous Bacteriophage

Filamentous bacteriophages are bacterial viruses that infect many gram-negative bacteria. They have common characteristics that they contain a circular ssDNA (single stranded) genome covered with a flexible tube composed of thousands of a single major coat protein. "Ff" phages constitute one of filamentous phage families that infect *E. coli.* Phages f1, fd and M13 are commonly Ff phages and they are ideal vehicles for phage display technology [37, 43].

M13 is a filamentous phage with a diameter of 6 nm and a length of nearly 1μm. M13 phage genome consists of 6400 nucleotides that codes 10 genes as shown in Figure 1.3. Five of these genes encode phage coat proteins while the proteins encoded by the other genes take place in viral replication and assembly [37, 43].

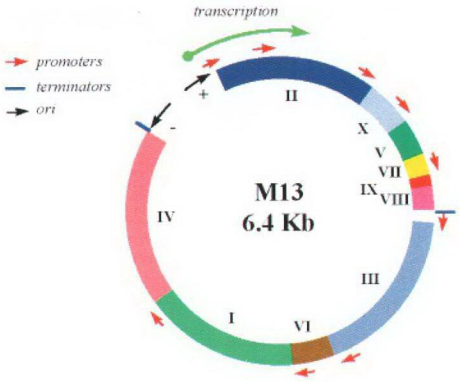


Figure 1.3. M13 phage genome [47]

Five coat proteins called g3p, g6p, g7p, g8p and g9p constitutes the structure of phage particle as shown in Figure 1.4. The hollow tube surrounding M13 genome is composed of ~2700 copies of g8p, major coat protein, held together by hydrophobic interactions. Positively charged residues of major coat protein located in the inner part of the particle interact with phosphates of viral ssDNA. The blunt end of phage particle is made up from 3-5 copies of minor coat proteins g7p and g9p. Phage assembly begins at the blunt end; therefore in the absence of either protein g7p or g9p, no phage particle is formed. The other coat minor coat proteins g3p and g6p are located in the pointed end of phage particle and are exist in five copies. While the function of g6p in phage particle is not known, g3p has an important role in host cell recognition and infection [37].

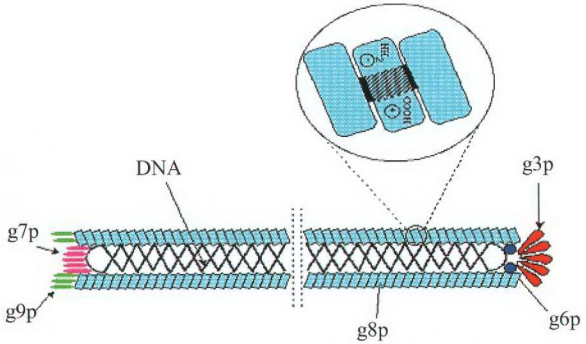


Figure 1.4. Structure of M13 bacteriophage [48]

The most is known about the structure and function of minor coat protein g3p. The largest and the complex coat protein, g3p consists of three domains. Two N-terminal domains namely N1 and N2 and one C-terminal domain called CT. These three domains are separated with two linker regions consist of a glycine-rich sequence. The structure of g3p is shown in Figure 1.5. [37].

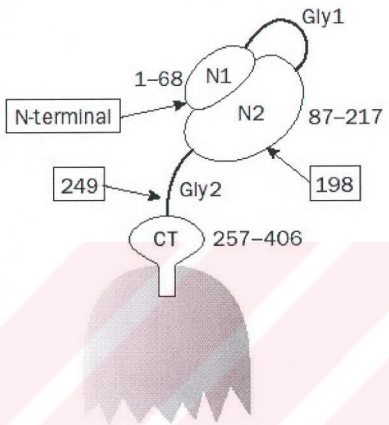


Figure 1.5. Domain structure of g3p and fusion points for display. Three domains are shown from N- to C- terminus and three positions at which display fusions are commonly created are indicated [37].

All filamentous phages require pili on host cell surface for infection. M13 phage bind to F pilus, thus it can only infect the *E. coli.* strains containing F type pilus. Infection begins when the N2 domain of g3p binds to the top of an F pilus. When N2 binds to pilus, N1 releases from its normal interaction with N2. Upon N2 binding to pilus, N1 domain interacts with the host cell membrane protein TolA and the pilus is retracted by an unknown mechanism. Pilus retraction brings phage particle closer to cell membrane and together with the other interactions between g3p and the bacterial membrane proteins TolQRA, phage DNA enters to host cell [49, 50]. The initial steps of infection mechanism can be seen in Figure 1.6.

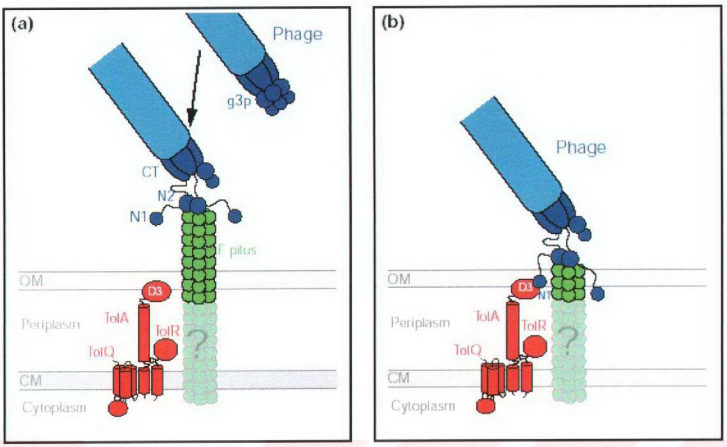


Figure 1.6. Initial steps of phage infection. **a)** phage binding on F pilus **b)** binding of N1 domain to D3 domain of TolA. [50]

Phage single stranded DNA that exists in cell cytoplasm just prior to infection is known as "+ strand" and has the same polarity as the mRNA [37]. Host RNA polymerase generates a primer that initiates the complementary strand synthesis from the noncoding region IG. Starting from this primer, DNA polymerases and topoisomares convert phage DNA to a double stranded form called Replicative Form (RF) and newly synthesized strand is named as "– strand". RF is used as template for phage gene expression and further replication. DNA replication is performed through a rolling circle mechanism in which phage protein g2p, specific-site nicking-closing enzyme encoded by phage gene II, acts an important role. g2p nicks the plus strand of RF at a specific site in noncoding region IG and DNA polymerase III synthesizes the new plus strand beginning from the 3' end of nick. When a round of replication is completed, displaced plus strand is circularized by the closing activity of g2p (Figure 1.7.). In the early stages of phage infection newly synthesized plus strands are converted to RF form because of the low concentration of ssDNA-binding protein, g5p. As its concentration increases, g5p binds to newly synthesized plus strands and avoid their conversion to RF. The interactions between g5p proteins on ssDNA perform the formation of a rod-like structured circular DNA. [37, 43, 47].

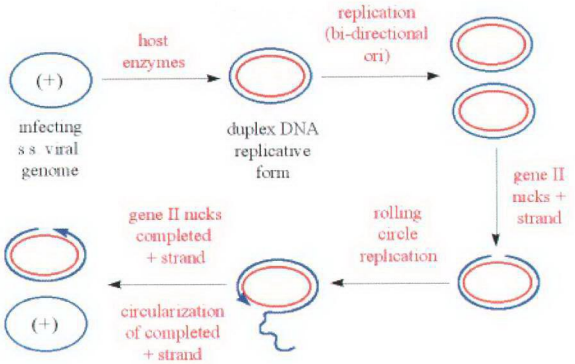


Figure 1.7. DNA replication of M13 phage [47]

The assembly and release of M13 phage particles occur in a secretory process that doesn't disturb the host cell. Phage particles are assemble in the cytoplasmic membrane and secreted from the cell as they assembled. In the early stage of phage assembly, coat proteins are imbedded into bacterial inner membrane and the other phage proteins generate a channel through the membrane. Assembly initiates when g7p and g9p interact with g5p coated DNA and form the one end of the particle (Figure 1.8.). In the elongation stage, g5p replaced by g8p until the phage DNA covered totally with major coat protein. The incorporation of g3p - g6p complex at the terminal end of particle, terminates the assembly. The assembled phage is then released to the extracellular environment. [37, 43, 47]

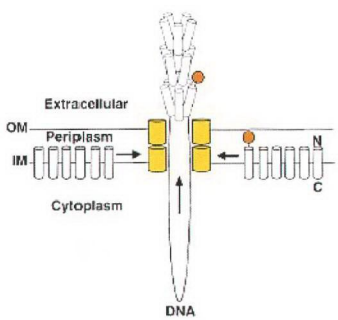


Figure 1.8. Diagram of M13 phage assembly. Coat proteins (white cylinders) are imbedded into inner membrane. Circular single-stranded DNA is extruded through the pore (yellow cylinders). [43]

1.3. Protein Engineering

Protein engineering can be defined as the use of genetic and chemical techniques to change the structure and function of a protein, thus producing a novel product with specific, desired properties. The act of protein engineering deals with catalysis, molecular recognition, folding and stability of proteins and protein-protein interactions. The approach for studying these 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 constraints, hydrophobic forces, electrostatics and charge, and the placement of hydrogen bonds, salt bridges, disulfide bonds, water or metals [51, 52].

There are two general strategies for protein engineering. The first is known as *rational design*, in which detailed knowledge of the structure and function of the protein is utilized to make desired changes. This has the advantage of being generally inexpensive and easy. However, there is a major drawback in that detailed structural knowledge of a protein is often unavailable.

The second strategy is known as *random approach* (directed evolution). This includes repeated rounds of random mutagenesis, followed by selection for the improved property of interest. This method mimics natural evolution and has advantage that require no prior structural knowledge of a protein. However, large amounts of recombinant DNA must be mutated and the products screened for desired qualities as a drawback

1.3.1. Rational Approaches

Rational protein design by site specific mutagenesis is a very efficient strategy to obtain proteins with improved features and reshape their structure and so specificities. These knowledge-based studies greatly benefit from the most recent computational analyses of protein structures and functions. The combination of rational and combinatorial methods opens up new vistas in the design of stable and efficient enzymes{references}.

Proteins can be engineering in rational way using “site-directed” and “cassette mutagenesis” techniques.

1.3.1.1. Site Directed Mutagenesis

Site directed mutagenesis allows one to change the gene sequence, and hence the amino acid sequence of an expressed protein. One important use of this method is to define the postulated role of specific amino acid residues because mutations can be placed precisely. To follow protein functions, amino acids in a specific site of protein can be substituted with another amino acid obtaining opposite properties. Comparing the binding affinity of each mutant to that of the wild type allows understanding the effect of mutated amino acid. [53, 54]

A number of strategies have been developed to construct site-directed mutants *in vitro*, all depend on the ability to chemically synthesize oligonucleotides [55, 56]. The oligonucleotide including the desired mutation is annealed with to one strand of the DNA of interest and serves as primer for initiation of DNA synthesis. By this way one of the strands of template includes the desired mutation. In the method developed by Michael Smith, the mutagenic strand is synthesized complementing the one of the strands of parental DNA resulting with one mutant strand. The heteroduplex is propagated by transformation in *E. coli*. After propagation, in theory, about 50% of the produced heteroduplexes will be mutants and the other 50% will be the "wild type" (no mutation). [57]

In order to increase the propagation of mutated DNA some selection and enrichment methods have been generated. By these methods unmutated strand can be eliminated by digestion or using the feasible characteristics of the host cell. Once parental DNA is methylated and used for mutagenesis reaction. This methylated DNA can be eliminated and seperated from unmethylated (mutated) DNA strand extended during PCR by digesting with *dnp*I enyme or by the cell. Thus, the cell could amplify only mutand strand.

Using site-directed mutagenesis, at least one base change can be incorporated but also, mutagenic primers can be designed to generate multiple substitutions, deletions or insertions (Figure 1.9) [58].

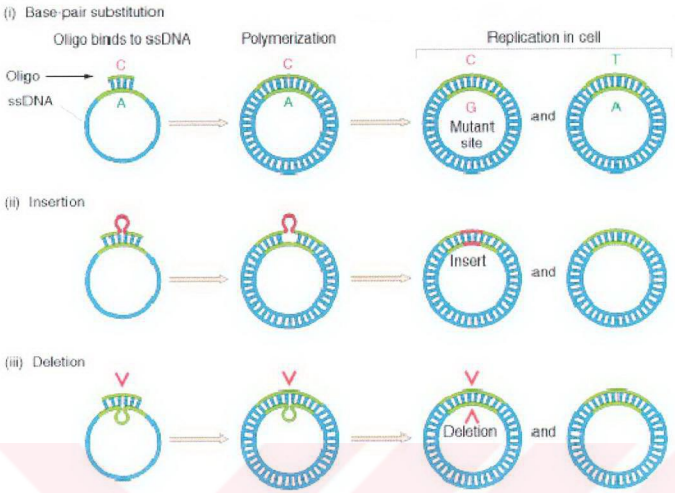


Figure 1.9. Site-directed mutagenesis; substitutions, insertions, deletions. [58]

1.3.1.2. Cassette Mutagenesis

Another approach to genetic alteration is through cassette mutagenesis. Cassette mutagenesis is the removal of a large section "cassettes" of DNA. The original cassette is replaced by a second cassette that contains synthetic DNA in which one or more of the base pairs have been altered (Figure 1.10) [58].

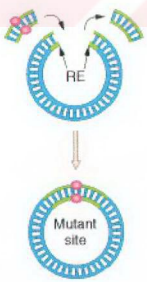


Figure 1.10. Cassette replacement [58]

It is also possible to replace the original cassette with a cassette for antibiotic resistance. This type of cassette mutagenesis is known as *gene disruption*. The

resulting clone will be antibiotic resistant, but will have lost the gene function where the insertion took place.

In cassette mutagenesis approach, synthetic oligonucleotide is extended to obtain double stranded DNA and this duplex "cassette" is incorporated can be replaced with a specific region but also can be inserted without any replacement. This technique can be used to obtain fusion proteins or to fuse single codons [59] at specific sites on double stranded DNA.

1.3.2. Random Approaches

Random approach is a fast and inexpensive way of finding variants of existing proteins that work better than naturally occurring under specific conditions. It mimics natural evolution in that it depends upon the selection of fitter individuals from a diverse population. Directed evolution allows us to explore enzyme functions never required in the natural environment and for which the molecular basis is poorly understood [60]. This bottom-up design approach contrasts with the more conventional, top-down one in which proteins are tamed 'rationally' using computers and site specific mutagenesis.

The frequently applied strategy comprises repeated rounds of random mutagenesis, starting with a given parent gene of interest. After each round, the best mutant(s) is (are) selected and used as parent sequence(s) in the following round of random mutagenesis. Typically, rather low mutation frequencies are employed to suppress accumulation of neutral or even deleterious mutations.

Random mutagenesis can be performed in different ways such as "Error-prone PCR", "DNA shuffling" and "Random Cassette mutagenesis".

1.3.2.1. Error-Prone PCR

Random mutagenesis systems are commonly based on error-prone PCR strategy. Changing the buffer conditions of PCR reaction and using DNA polymerases without □rnold□ading activity yields mutations [61, 62].

Diversify PCR mutagenesis provides control over the level of random mutation by independently varying the amounts of manganese and dGTP in the PCR reaction. The mutagenesis rate can be raised by increasing the amount of manganese in the

reaction. Further increases in mutation rate can be obtained by increasing the level of one of the dNTPs in the reaction to promote misincorporation [61]. This method generally provides mutation rates from 2 to 8 mutations per 1 kb. Performing a second round of PCR using a diluted aliquot of the primary mutagenesis reaction can produce higher mutation rates.

The mutated PCR products are then cloned into an expression vector and the resulting mutant library can be screened for changes in protein activity. In other way, whole plasmid can be mutated and transformed into bacterial host to obtain the mutant library.

1.3.2.2. Shuffling

DNA shuffling, a method for *in vitro* recombination is developed as a technique to generate mutant genes that would encode proteins with improved or unique functionality [63, 64]. It consists of a three-step process that begins with the enzymatic digestion of genes, yielding smaller fragments of DNA. The small fragments are then allowed to randomly hybridize and are filled in to create longer fragments. Ultimately, any full-length, recombined genes that are recreated are amplified via the polymerase chain reaction. If a series of alleles or mutated genes is used as a starting point for DNA shuffling, the result is a library of recombined genes that can be translated into novel proteins, which can in turn be screened for novel functions.

DNA shuffling can be applied to a single gene or within the gene families encoded by different species. DNA shuffling within gene families is termed as family shuffling and illustrated is given in figure 1.11.

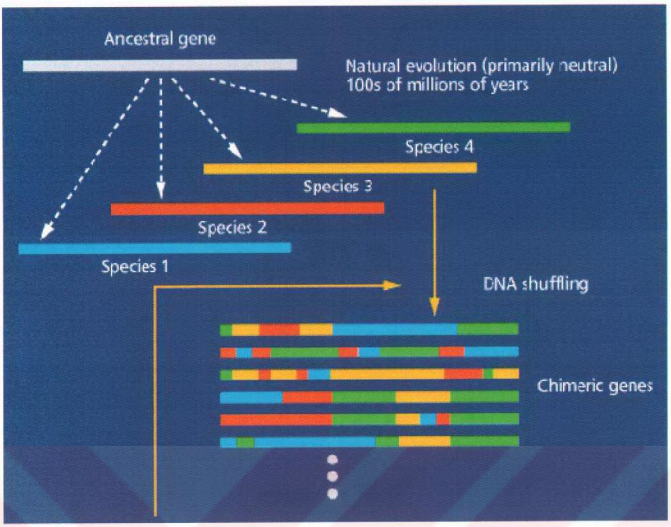


Figure 1.11. Schematic diagram of family shuffling [65]

1.3.2.3. Cassette Mutagenesis

Cassette mutagenesis is both considered in rational and random protein engineering approaches. All cassette mutagenesis techniques involve the synthesis of small double stranded DNA fragments to be ligated into a large vector. When a specific cassette with a known sequence is used for engineering, the approach is classified as rational. However, cassette mutagenesis can also be utilized for the generation of large mutant libraries with unknown sequences. In this manner oligonucleotides are synthesized with random codons that are encoding all 20 amino acids and converted to double stranded form to obtain the random cassette [66, 67]. Random library is screened to select the proteins demonstrating desired affinity.

1.4. Example to Inorganic Binding Peptides: Platinum Binding Peptide

The noble metal Pt (Platinum) has different properties including wavelength selective plasmon adsorption resonance characteristics, electrical conductivity and catalytic activity [68]. Thus, peptides selectively binding to Pt metal and the understanding of their binding properties would be very useful. Platinum and Pt-binding peptides could be used for development of therapeutic materials, such as in

anti-tumor drugs, catalysis and as binding agents [69], and for controlled nucleation, morphogenesis, and assembly through nanobiofabrication.

The peptides that exhibit binding properties against platinum have been selected in a previous study [30]. Phage display library with 7-amino acid random inserts in the g3p coat protein of M13 in polyvalent form was utilized to identify Pt-binders. The library used was constrained through disulfide linkages between two cysteine residues located at both ends of the peptide. Once the peptides were selected physicochemical properties of peptides have been calculated such as isoelectric points (pI), charges and hydrophilicities. Some of the selected Pt-binders and their physicochemical properties are given in Table 1.2.

Table 1.2. Examples of Pt binding peptides selected from a disulfide-constraint phage display peptide library. Color codes of amino acids are given in Appendix E.

Pt binders	Sequence	pI	Charge	Hydrophilicity
SD60	Q S V T S T K	8.75	+1	0.21
DH76	S V T Q N K Y	8.31	+1	-0.07
SD19	D R T S T W R	9.60	+1	0.73
SD152	P T S T G Q A	5.96	0	-0.11
SD125	I G S S L K P	8.75	+1	0.00
SD128	L G P S G P K	8.75	+1	0.21
SD51	S P V N P Y P	5.24	0	-0.47
SD144	T F L N P G T	5.19	0	-0.70
SD127	G T N M N A R	9.75	+1	0.17
SD37	T S P G Q K Q	8.41	+1	0.47
SD33	A Y P T Q L D	4.30	-1	0.26
SD124	E P Y N Q N M	4.00	-1	0.00
SD1	A P P L G Q A	5.57	0	0.37
SD6	L N D G H N Y	5.08	-1	-0.17
SD34	I M R D G P M	6.85	0	0.70
SD4	H K N V P Q A	8.76	+1	0.13

Amino acid distributions in selected peptides have been calculated and relative abundance for each amino acid has been obtained. However, this statistical analysis only shows the amino acid residues could be important in Pt selectivity but not affinity strength. Therefore each peptide have been characterized for their binding affinities to Pt powder through fluorescent microscopy analysis. Selected peptides have been classified as strong, moderate and weak according to characterization experiments. To follow the distributions of amino acids in strong and weak binders, statistical analyses applied to characterized peptides. Obtained relative abundances showed that strong and weak binders have represented different amino acid

distributions (Figure 1.12). A comparison of strong and weak binders seem to indicate that polar and basic residues may play an important role in the mechanism of polypeptide binding to metallic Pt [30, 70].

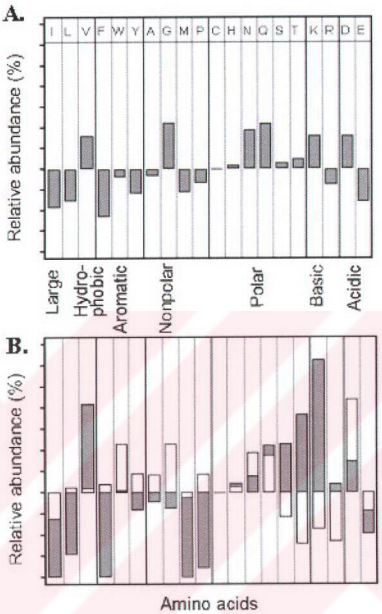


Figure 1.12. A. shows the relative abundances (%) of all selected Pt-binding peptides **B.** shows the relative abundance in strong (gray bars) and in weak (white bars) only.

1.5. Protein Engineering Approaches on Platinum Binding Peptide

In this study, we have recently applied multiple-repeat based strategy on phage display-selected platinum binding peptides. The genetic engineering tools were utilized for the generation of multiple repeats of binding sequences to incorporate the structural properties of the inorganic surfaces, e.g., optimizing the length of the sequence or shape of the sequence to achieve the desired effect. Multiple repeating form of the peptide was constructed both in linear and constraint form in order to investigate peptide conformation on binding affinity.

One of the strong Pt-binding peptides, SD152 was engineered to investigate the structural and multimerization effect of peptides on binding. SD152 is a disulphide constraint 7-mer peptide with an amino acid of PTSTGQA. In the first step linear peptide was constructed. In the next step 2 and 3 repeat peptides was obtained in different structures.

Binding affinity and specificity of multiple repeating peptides were compared with the original linear and constraint ones through immuno-fluorescent labeling and ELISA analysis.

2. MATERIALS AND METHODS

2.1. Materials

2.1.1. Bacterial Strains

2.1.1.1. *E. coli* ER2738 host strain

F′ lacI^{q}. (lacZ)M15 proA+B+ zzf::Tn10(Tet$_R$)/fhuA2 supE thi. (lac-proAB). (hsdMS mcrB)5 (r_{k-} m_{k-} McrBC–) which is supplied within Ph.D.-C7C™ Phage Display Peptide Library Kit (Catalog# E8120S, NEB Inc.), was used as a host for wild type M13 phage. Electrocompetent cells were prepared according to Sambrook J [71].

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

F- φ80*lac*ZΔM15 Δ(*lac*ZYA-*arg*F) U169 *deo*R *rec*A1 *end*A1 *hsd*R17 (rk-, mk+) *pho*A *sup*E44 λ- *thi*-1 *gyr*A96 *rel*A1 *ton*A, chemically competent cells were supplied with Gene-Tailor^{TM} Site-Directed Mutagenesis System (Catalog#12297-016, Invitrogen).

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

F- ö80*lac*Z Δ(*lac*ZYA-*arg*F)U169 *rec*A1 *end*A1 *hsd*R17(rk-, mk+) *pho*A *sup*E44 λ- *thi*-1 *gyr*A96 *rel*A1/F' *proAB*$^{+}$ *lac*I^{q}ZΔ15 Tn*10* (tet^{R}) was purchased from Invitrogen to be used as a host for mutated M13 phages (Catalog# 10643-013).

2.1.2. Cloning Vectors

2.1.2.1. M13KE Phage Vector

M13KE is a derivative M13 phage vector containing cloning sites at the 5' end of gene III for displaying of short peptides as N-terminal fusions. (See Appendix A. for vector map) and was purchased from New England Biolabs® (Catalog #E8101S, NEB Inc.).

2.1.2.2. pDrive Cloning Vector

pDrive cloning vector (given in Appendix-B) was purchased from QIAGEN (Catalog# 231122) to be used for cloning of PCR products containing the single A (Adenine) overhang at each end. Adenine nucleotide was added at 3' ends of blunt-ended PCR products using QIAGEN A-Addition Kit (Catalog# 231994, Qiagen).

2.1.4 Enzymes

2.1.4.1. Restriction Enzymes

***Eag* I** (C↓GGCCG, NEB Inc) and ***Acc65* I** (G↓GTACC, NEB Inc) restriction endonucleases and their reaction buffers were obtained from New England Biolab (Catalog # R0505L and Catalog # R0599L respectively).

2.1.4.2. Platinum® *Taq* DNA Polymerase High Fidelity

Platinum® *Taq* DNA Polymerase High Fidelity is an enzyme mixture composed of recombinant *Taq* DNA polymerase, *Pyrococcus species* GB-D polymerase that has proofreading ability, and Platinum® *Taq* Antibody. Proofreading enzyme and *Taq* DNA polymerase mixture increases fidelity and allows amplification of long DNA templates up to 12 kb. not requiring any optimization. The *Taq* antibody provides a hot start for *Taq* Polymerase via inhibiting the polymerase activity that is restored after denaturation step, at 94°C. This automated Hot start in PCR increases sensitivity, specificity, and yield (Catalog # 11304-011, Invitrogen.).

2.1.4.3. Klenow Fragment (3' → 5' exo-)

Klenow Fragment (3′→5′ exo–)is a proteolytic product of DNA Polymerase I that retains polymerase activity but doesn't have 5′→3′ and 3′→5′exonuclease activities and was used for the second strand synthesis (Catalog# M0212S, NEB Inc.).

2.1.4.4. T4 DNA Ligase

T4 DNA ligase was purchased from Roche (Catalog # 10 481 220 001).

2.1.5. DNA Molecular Weight Markers

DNA molecular weight standard markers (given in Appendix C) were obtained from MBI Fermentas.

2.1.6. Oligonuclotides

Oligonucleotides given below were synthesised using an Applied Biosystems 308A DNA synthesizer by IONTEK.

3R-SD152	5' CATGTTTCGGCCGAACCTCCACCGCA**CGC**CTGACC AGTCGAAGTAGGCGCCTGACCAGTCGAAGTAGGCGC CTGACCAGTCGAAGTAGGACAAGCAGAGTGAGAATA GAAAGGTACCCGGG 3'
Extension Primer	5' CATGCCCGGGTACCTTTCTATTCTC 3'
GTSD152d1C-F	5' TTCTATTCTCACTCTGCTCCTACTTCGACTG 3'
GTSD152d1C-R	5' AGCAGAGTGAGAATAGAAAGGTACCACTAA 3'
GTSD152d2C-F	5' TACTTCGACTGGTCAGGCGGGTGGAGGTTC 3'
GTSD152trd2C-R	5' CGCCTGACCAGTCGAAGTAGGCGCCTGA 3'
GTSD152d1C-R	5' CGCCTGACCAGTCGAAGTAGGACAAGCA 3'
GTSD152i2C-F	5' GGTGGAGGTTCGGCCGAAACTGTTGAAAG 3'
GTSD152i2C-R	5' TTTCGGCCGAACCTCCACCGCACGCCTGACCA 3'
M13-40 Primer	5' GTTTTCCCAGTCACGAC 3'
M13-96 Primer	5′ CCCTCATAGTTAGCGTAACG 3′

2.1.7. Bacterial Culture Media

2.1.7.1. LB (Luria-Bertani) Media

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

2.1.7.2. LB Agar Medium

10 g tryptone, 5 g yeast extract, 5 g NaCl, 15 g bactoagar (Acumedia) were dissolved in distilled water up to 1 lt and the pH was adjusted to 7.0-7.5 with 10 M NaOH and sterilized by autoclaving.

2.1.7.3. Top Agar Medium

10 g tryptone, 5 g yeast extract, 5 g NaCl, 1 g $MgCl_2.6H_2O$ (Riedel-de-Haen), 8 g bactoagar were dissolved in distilled water up to 1 lt and sterilized by autoclaving.

2.1.7.4. SOC Medium

20 g tryptone, 5 g yeast extract, 0.5 g NaCl was dissolved in 950 ml deionized water. 10 ml of 250 mM KCl was added and pH was adjusted to 7.0 with NaOH. Water was added up to 1lt and sterilized by autoclaving. Just before use, 10mM of $MgCl_2$ and 20mM of glucose were added.

2.1.8. Stock Solutions

2.1.8.1. Tetracyline Stock

20 mg / ml of tetracycline (Sigma) was dissolved in 95 % ethanol and stored at -20°C in dark.

2.1.8.2. Amphicilin Stock

100 mg / ml ampicillin sodium salt was dissolved in deionized water, filter-sterilized and stored in dark at -20°C.

2.1.8.3. Xgal / IPTG Stock

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

2.1.8.4. Glycerol Stock Solution

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

2.1.8.5. $MgCl_2$ Stock Solution

5 mM $MgCl_2.6H_2O$ was dissolved in distilled water up to 100 ml and sterilized using 0.2 μm single use sterile syringe filter.

2.1.9. Buffers

2.1.9.1. PEG/NaCl

20 % (w/v) PEG-8000 (Sigma) and 2.5 M NaCl were dissolved in distilled water up to 100ml and sterilized for 15 min. under 1.5 atm at 121°C.

2.1.9.2. PC Buffers

500 ml of PC buffer was prepared by dissolving 55 mM KH_2PO4 (Merck), 45 mM Na_2CO3 (Merck), 200 mM NaCl in distilled water. Appropriate amount of detergent was added from the stock into PC buffer according to the desired detergent concentration (eg. 0.02%, 0.1%, 0.5%). Detergent stock was prepared by mixing 20 % (w/v) Tween 20 (Merck) and 20% (w/v) Tween 80 (Riedelde- Haen). PC buffers were sterilized by using 0.2µm sterile syringe filter.

2.1.9.3. PBS Buffer

8 g NaCl, 0.2 g KCl, 1.44 g Na_2HPO_4-$7H_20$ and 0.24 g KH_2PO_4 were dissolved in 1lt distilled water, pH 7.2 and sterilized for 15 min. under 1.5 atm at 121°C.

2.1.9.4. Na-Ac Buffer

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

2.1.9.5. 5 X TBE Buffer (1000ml)

54 g. of Tris base, 27.5 g. boric acid, and 20 ml 0.5 M EDTA at pH 8.0 were dissolved in 1 liter deionized water and sterilized for 15 min. under 1.5 atm at 121°C.

2.1.9.6. TE Buffer

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

2.1.10. Lab Equipment

Lab equipments are given in Appendix D.

2.2. Methods

2.2.1.Site-directed Mutagenesis

Cysteine deletion and insertion mutagenesis reactions were achieved via site-directed mutagenesis approach. GeneTailor Site-Directed Mutagenesis Kit was optimized for phage DNA manipulations (Catalog# 12397-014, Invitrogen). This mutagenesis system mainly involves three steps; DNA methylation, mutagenesis PCR and transformation. At following subsections protocol is discussed in detail.

2.2.1.1. DNA Methylation

DNA cytosine methylase encoded by the *dcm* gene of *E.coli.* that modifies the internal cytosine residue at the C^5-position on both strands within the sequence 5'...CC(A/T)GG-3' was used for methylation of template. Methylated DNA was splited with the Mcr and Mrr restriction systems of host strain DH5α-T1 when transferred into [72]. This system leads the separation of mutated PCR product from unmutated template during amplification period following transformation.

Methylation Reaction

dsDNA	1 µl (100 ng)
Methylation Buffer	1.6 µl
10X SAM	1.6 µl
DNA Methylase	1 µl (4U/µl)
dH_2O	10.8 µl
Total volume	16 µl

Reaction Conditions

Incubated at 37°C for 1 hour.

2.2.1.2. Mutagenesis PCR

Two overlapping primers, one of which containing desired mutation were designed for each cysteine deletion mutagenesis. Mutagenesis reactions for cysteine deletion were performed one by one using the replicative form of first mutated phage as template for the second reaction. 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_2O	34 µl
Total Volume	50 µl

Reaction conditions

94°C 2 min.

94°C 30 sec.
55°C 30 sec. } 20 cycles
68°C 7.5 min.

68°C 10 min.

Primers, methylated templates and annealing temperatures were different for all reactions.

Second cysteine deletion reaction for 3-repeat-SD152

Template constraint 3-repeat-SD152

Primers GTSD152trd2C-R, GTSD152d2C-F

Annealing Temprature 58°C

Second cysteine deletion reaction for 3-repeat-SD152

Template constraint 3-repeat-SD152

Primers GTSD152d2C-R, GTSD152d2C-F

Annealing Temprature 58°C

First cysteine deletion reaction for 3-repeat-SD152 and SD152

Template 3-repeat-SD152-d2C

Primers GTSD152d1C-R, GTSD152d1C-F

Annealing Temprature 55°C

Second cysteine insertion reaction for 2-repeat-SD152-d2C

Template 2-repeat-SD152-d2C

Primers GTSD152i2C-R, GTSD152i2C-F

Annealing Temprature 58°C

2.2.1.3.Transformation

1. 50 µl vial of One Shot MAX Efficiency DH5α-T1^R competent cells from –80 °C 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 heat shock, the tube was incubated in the 42°C water bath for exactly 30 seconds without mixing or shaking.
5. Vials were removed from the 42°C water bath and covered with ice for 1 minute.
6. 500 µl of pre-warmed SOC medium was added to the vial and than incubated at 37°C for exactly 30 minutes at 250 rpm in a shaking incubator.
7. 50-100-150 and 200 µl transformation reactions were aliquot and were put each of them into 5 ml top agar 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 has solidified, inverted and incubated at 37°C overnight.

The other day blue plaques were seen on the LB plate and storage plates were prepared with some plaques. These phages were amplified and ssDNA (single-

stranded) and dsDNA (double-stranded) of newly constructed phage were purified to use as a template for sequence analysis and next mutagenesis reaction respectively.

2.2.2. Phage Display Peptide Construction

An oligonucleotide is designed and annealed with extension primer purchased from NEB. Annealed duplex is extended. The gene product obtained is ligated into pDrive Cloning Vector and transformed into *E. coli* ER2738 competent cells and was sequenced in order to check if the desired gene was constructed. Plasmid DNA in pDrive Cloning Vector carrying the extended duplex is purified and digested to clone it into M13KE gIII vector with *Eag*I and *Acc65*I. It is ligated into M13KE gIII vector. The ligation product is transformed into ER2738 *E. coli* cells. The each step was discussed in detail below.

2.2.2.1. Synthesis of Double Stranded DNA from an Oligonucleotide

Synthesized oligonucleotide was annealed with an extension primer and extended by Klenow enzyme. Reaction conditions are described below;

- **Annealing Reaction**

Oligonucleotide	1.1 µl (5 ug)
Extension Primer	5.4 µl (5.3 ug.)
TE buffer	43,5 µl
Total volume	50 µl

Reaction conditions

Heated to 95°C and cooled slowly to 35°C in a water bath.

- **Extension Reaction**

Annealing reaction	50 µl
10X Klenow Buffer	20 µl
dNTP mix	40 µl
Klenow enzyme	3 µl (10U/ µl)
dH_2O	87 µl
Total volume	200 µl

Reaction conditions

The samples were incubated for 20 min. at 45°C and 15 min. at 65°C.

2.2.2.2. Cloning into a TA Vector

TA cloning vectors contain a U overhang at each ends providing easy and efficient ligaton of PCR products with an A overhang. Unpaired A residues are added to PCR products during PCR reactions generated by *Taq* or any other non-proofreading Polymerases but not Klenow enzyme. In this study pDrive Cloning Vector was used combined with an A-Addition procedure.

- **A-Addition Reaction**

PCR product*	6 µl (65 ng.)
5X A-Addition master mix**	2 µl
dH_2O	2 µl
Total volume	10 µl

* Here, PCR product indicates extended duplex coming from extension reaction.

** QIAGEN® A-Addition Kit from Qiagen. (Catalog # 231994, Qiagen)

Reaction conditions

Incubated for 30 min. at 37°C.

- **Ligation Reaction**

pDrive cloning vector***	1 µl (50 ng)
A-Addition reaction	2 µl (13 ng)
Ligation master mix***	5 µl
dH_2O	3 µl
Total volume	10 µl

*** PCR Cloning Kit from Qiagen. (Catalog # 231122, Qiagen)

Reaction conditions

The samples were incubated for 30 min. at 16°C and an additional incubation was performed at 70°C for 10 min to stop the reaction.

- **Preparation of Xgal/IPTG-amp plates**

50 µl Xgal/IPTG and 100 µl amphicillin were put into 50 ml liquid warm LB agar and it was poured onto plastic sterile petri dish. .

- **Transformation Procedure for Plasmids**

1. 10 µl ligation product was mixed with 20 µl *E. coli* ER2738 electrocompetent cells in a prechilled electroporation cuvette.
2. Cuvette was placed into electroporator and 1800 volts of voltage was applied.
3. 200µl SOC medium was added to the cuvette and whole mixture was transferred into a microfuge tube. Tube was incubated at 37°C, 200-250 rpm for 1 hour.
4. After incubation, transforming mixture was spreaded onto Xgal/IPTG-amp LB agar plates in serial dilutions such as 50, 75, 100 and 150 µl.
5. Plates were incubated overnight at 37°C.

2.2.2.3. Plasmid Purification

Following transformation, cell were incubated and then white colonies were selected and grown overnight in 5 ml. LB. 800 µl of this bacterial culture was mixed with 200 µl 80% glycerol and stored at –80°C. Plasmid DNA was purified from rests 4 ml for sequencing and further applications by using QIAGEN Plasmid Mini Kit. (Catalog # 12123, Qiagen). Procedure is described in detail below.

1. 4 ml. bacterial culture was divided into two 2 ml microfuge tubes and centrifuged in a standard bench-top microfuge at 5 000 rpm for 15 minutes.
2. The bacterial pellet in first tube was resuspended completely in 300 µl Buffer P1 and then transferred to second tube in order to combine the pellets.
3. 300 µl Buffer P2 was added. Suspension was mixed gently by inverting the tube 5 times and incubated at room temperature for 5 minutes.
4. 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.
5. After incubation on ice, mixture was centrifuged in a microcentrifuge for 10 minutes at 14 000 rpm. Supernatant was saved for further use.

6. QIAGEN-tip 20 colon was equilibrated by applying 1 ml of Buffer QBT and the column was allowed to empty by gravity flow.
7. The supernatant obtained and saved in the previous step was applied to the QIAGEN-tip 20 column and it was allowed to enter the resin by gravity flow.
8. QIAGEN-tip 20 was washed with 1 ml of Buffer QC for 4 times.
9. 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.
10. Eluted DNA was precipitated with 560µl room temperature isopropanol. It was then centrifuged at 10000 rpm for 30 minutes and the supernatant was decanted.
11. The DNA pellet was washed with 1 ml of room temperature 70% ethanol and centrifuged for 30 minutes at 10 000 rpm. Supernatant was decanted and the pellet was allowed to dry at room temperature.
12. DNA pellet was redissolved in 30 µl elution buffer EB and stored at –20°C

2.2.2.4. Plasmid DNA Sequencing

Big dye reaction mix*	2 µl
5X sequencing buffer*	1 µl
Template dsDNA	1 µl (200ng)
M13 –40 Primer	3.2 µl
dH_2O	2.8 µl
Total volume	10 µl

- Big dye® terminator v 3.1 cycle sequencing Kit from Applied Biosystems.

Reaction conditions

95°C	2’	
95°C	10’’	35 cycles
55°C	10’’	
60°C	4’	

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 added into each PCR product and samples were incubated on ice for 30 min.
3. After incubation, samples were centrifuged for 30 min. at 14000 rpm.
4. Supernatant was discarded and DNA pellet was washed with 250 µl cold ethanol.
5. Samples were centrifuged for 30 min. at 14000 rpm.
6. Ethanol was discarded; incubating at 95°C evaporated all residual ethanol.
7. DNA was dissolved in 20 µl di formamide.
8. Samples were denatured by putting first at 95°C and then at -20°C for 5 min.

ABI 3100 Avant (PE, Applied Biosystem, CA) automated sequencer was used for DNA sequencing.

2.2.2.5. Cloning into M13KE Phage Vector

pDrive plasmid vector was digested with restriction enzymes and desired DNA fragment was gel extracted. Cleaned and concentrated insert DNA was ligated into M13KE vector. Procedure is described in detail below.

- **Restriction Enzyme Digestion**

Digestion of M13KE

M13KE	2 µl
10X NEB buffer 3	2 µl
BSA	2 µl
EagI (10 U/µl)	1 µl
Acc65I (10 U/µl)	1 µl
dH_2O	12 µl
Total volume	20 µl

Reaction conditions

Incubated for 3 hrs. at 37oC and then, for 15 min. at 65oC for inactivation.

Digestion reaction was run on 1% agarose gel and DNA band belongs to digested vector DNA was extracted from gel.

Digestion of pDrive

pDrive	8 µl (18 µg.)
10X NEB buffer 3	4 µl
BSA	4 µl
EagI (10 U/µl)	3 µl
Acc65I (10 U/µl)	3 µl
dH_2O	18 µl
Total volume	40 µl

Reaction conditions

Incubated for 7 hrs. at 37°C and then for 15 min. at 65°C for inactivation.

Digestion reaction was run on 2.5% agarose gel and desired digestion product was extracted from gel.

- **Gel Extraction Procedure**

Using MinElute Gel Extraction Kit performed gel extraction reactions in this study (Catalog # 28604,Qiagen).

1. DNA fragment was excised from agarose gel with a clean scalpel. Gel slices were put into microfuge tubes and their weight was calculated.
2. A 3-gel volume of QC buffer was added to 1 volume of gel slice. (600 µl for 100 mg gel). When gels were excised from a %2 or more concentrated gel, a 6-gel volume of QC buffer was applied.
3. Sample was incubated at 50°C for 10 min. by vortexing every 2 min.
4. After the gel completely dissolved, the color of the mixture was checked. QC buffer contains a pH indicator and gives a yellow color at pH<7.5, the

optimum pH for DNA adsorption. If the color of the mixture was not yellow, 10 µl of 3 M sodium acetate, pH5.0 was added.

5. After the gel dissolved, 1 gel volume of room temperature isoproponal was added and mixed by inverting the tube several times.
6. Sample was applied to min-elute column and centrifuged for 1 min. at 13000 rpm.
7. Flow-trough was discarded from collection tube.
8. 500 µl QC buffer was applied to column and centrifuged for 1 min. at 13000 rpm.
9. Flow-trough was discarded from collection tube.
10. In order to wash, 750 µl PE buffer was applied to colon centrifuged for 1 min. at 13000 rpm.
11. Flow-trough was discarded from collection tube and centrifuged for an additional 1 min.
12. Spin colon placed in a microfuge and 10 µl EB buffer was applied to elute the DNA. After 1 min. incubation tube was centrifuged for 1 min.
13. Eluted DNA can be stored at –20°C for long term.

- **Ligation into M13KE**

Insert	11.5 µl (2 µg.)	mixed and incubated at 65°C for 5 min.
M13KE	0.5 µl (50 ng.)	and then cooled on ice.
T4 DNA Ligase	2 µl	
10X Ligase buffer	2 µl	
PEG	2 µl	
dH_2O	2 µl	
Total volume	20 µl	

Reaction conditions

Incubated for 16 hrs. at 16°C and then for 15 min. at 65°C for inactivation.

- **Transformation Procedure for M13KE**

Growing *E.coli* mid-log culture

12.5 µl *E.coli* (ER2738 *E.coli* strain was used) was inoculated from overnight culture into 5 ml LB containing 10 µl $MgCl_2$ and 5 µl tetracycline. The culture was incubated at 37°C, 225 rpm until mid-log phase (OD_{600} ~ 0.5).

1. Ligation product was mixed with 40 µl *E. coli* ER2738 electrocompetent cells in a prechilled electroporation cuvette.
2. Cuvette was placed into the electroporator and 1800 volts of voltage was applied.
3. 200 µl SOC medium was added to the cuvette and whole mixture was transferred into a microfuge tube. Tube was incubated at 37°C, 200-250 rpm for 30 minutes
4. After incubation, transforming mixture was separated in serial dilutions such as 50, 75, 100 and 150 µl. Meanwhile, 5ml. top agar were put in 15ml falcon tubes and kept warm in 50°C water bath. Each transforming dilution, 10 µl $MgCl_2$, 5 µl tetracycline and 180 µl mid-log culture were put in 5ml. warm top agar, mixed by vortexing and poured onto Xgal/IPTG-tet LB agar plates. Plates incubated at room temperature until top agar set and then incubated overnight at 37°C.

After incubation blue colonies were observed.

2.2.2.6. Saving Phage Clones

- **Preparation of storage stock of phages**

1. 170 µl 0.02% PC buffer was put in each well of a 96-well elisa plate.
2. Each phage plate observed on LB plates were picked up with a sterile pipette tip and put in different wells of elisa plate.
3. Elisa plate was incubated in a 60°C incubator for 45 minutes.

After incubation, plate was cooled in laminar flow and left at 4°C for overnight.

- **Preparation of glycerol stock of phages**

1. 60 µl, 80% glycerol was put in each well of a 96-well elisa plate.
2. 50 µl phage solution from storage culture was put in elisa plate wells filled with glycerol.
3. Plates covered with parafilm and stored in –80°C.

2.2.2.7. M13 single stranded DNA purification

5 µl phage from storage culture was amplified in 3 ml mid-log bacterial culture. Single stranded phage DNA was purified from amplified culture for sequence analysis. DNA isolation was performed using QIAprep ® Spin M13 kit (Catalog # 27704, Qiagen). The procedure is described below in detail.

- **Phage amplification**

Phage samples were infected into the host strain *E. coli* ER2738 and thereby amplified. Before the beginning of amplification period, *E. coli* host strain ER2738 was cultured to reach the OD600 ~ 0.5 and then phage samples transferred bacteria culture. OD600 ~ 0.5 is the best phage-host strain propagation period. The incubation period is approximately 4.5 hours at 37°C and 250 rpm. *E. coli* host strain ER2738 used in amplification is a robust F+ strain with a rapid growth rate and is particularly well-suited for M13 propagation. ER2738 is a recA+ strain and the F factor of ER2738 contains a mini-transposon, which confers tetracycline resistance.

- **Single stranded DNA purification procedure**

1. Amplified culture was centrifuged at 5000 rpm for 15 min. at room temperature to settle down bacterial cell. Supernatant containing M13 bacteriophage was transferred to a fresh reaction tube.
2. 1/100 volume of Buffer MP was added (i.e. 10µl per 1 ml of phage supernatant.) to the supernatant. It was mixed by vortexing and incubated at room temperature for at least 2 min. to precipitate phage particles.
3. A QIAprep spin column was placed in a 2 ml microcentrifuge tube and 700 µl of the sample applied to the QIAprep spin column.
4. Reaction tube was centrifuged for 15 sec. at 8000 rpm and flowthrough was discarded from collection tube.

5. The last two steps were repeated until all supernatant passed through QIAprep spin column.7
6. 700 µl MLB buffer was added for M13 lysis and binding, to the QIAprep spin column and centrifuged for 15 sec. at 8000 rpm.
7. Another 700 µl MLB buffer was added the QIAprep spin column and was incubated 1 min. at room temperature to lyse bacteriophage completely. QIAprep spin column was centrifuged for 15 sec. at 8000 rpm.
8. 700 µl Buffer PE was loaded to spin colon and centrifuged for 15 sec. at 8000 rpm for removal of residual salt.
9. Buffer PE was discarded from collection tube and QIAprep spin column was centrifuged for an additional 15 sec. at 8000 rpm to remove residual buffer.
10. QIAprep spin column was placed in a clean 1.5 ml microcentrifuge tube. 100µl Buffer EB (10 mM Tris.Cl, pH 8.5) was added to the center of the column membrane to elute the DNA. Elution buffer in the QIAprep spin column was incubated for 10 min. at room temperature to increases the recovery of single-stranded DNA molecules.

2.2.2.8. Single Stranded DNA Sequencing

Big dye reaction mix	2 µl
5X sequencing buffer	1 µl
Template dsDNA	1 µl (50 ng)
M13 –96 Primer	3.2 µl
dH_2O	2.8 µl
Total volume	10 µl

Reaction conditions

95°C	4’	
95°C	30’’	35 cycles
55°C	30’’	
60°C	4’	

PCR products were purified via Na-Ac Ethanol purification as previously described, before applying the samples into ABI 3100 Avant (PE, Applied Biosystem, CA) automated sequencer.

2.2.2.9. Phage Amplification And Purification

1. 50 µl phage from glycerol stock was amplified in 50 ml mid-log bacterial culture.
2. At the end of 4.5 hours of growth period, *E.coli*-phage culture was transferred to 250 ml sterile centrifuge tubes.
3. Samples were centrifuged at 8000 rpm for 10 min.
4. Supernatant was transferred to 250 ml sterile centrifuge tubes.
5. PEG/NaCl was added (1:6) into supernatant to precipitate phage and it was left overnight at 4°C.
6. Next day, samples were centrifuged at 8000 rpm for 20 min.
7. Supernatant was discarded and phage pellet was resuspended with 5 ml PC buffer (no detergent) by shaking.
8. Samples were centrifuged at 8000 rpm for 10 min.
9. Supernatant was transferred to 50 ml sterile centrifuge tubes.
10. PEG/NaCl was added (1:6) into solution and the solution was left 2 hours at 4°C.
11. Samples were centrifuged at 10000 rpm for 10 min.
12. Supernatant were discarded and phage pellet was resuspented with 1 ml PC buffer (no detergant).
13. Samples were centrifuged at 10000 rpm, for 10 min and supernatant were transferred to sterile microfuge tubes.
14. PEG/NaCl solution was added (1:6) into the microfuge tube, vortexed 5 sec, and left at room temperature for10 min.
15. Samples were centrifuged at 13500 rpm for 3 min to get the compact phage.

16. Supernatant was discarded and phage pellet was resuspended with 100 µl PC buffer (no detergent) by pipetting gently.

17. Samples were centrifuged at 13500 rpm for 1.5 min. and stored at –20°C.

2.2.2.10. Phage Titering

Blue-white screening was performed for tittering purified phages that will be used for characterization experiments.

1. 3 ml of LB was inoculated with 7.5 µl of ER2738 overnight culture and incubated at 37°C, 200-250 rpm until mid-log phase.
2. While cells were growing; Top Agar was melted and dispensed 3 ml into sterile falcon tubes, one per expected phage dilution. Tubes were left at 45°C until ready for use.
3. 10-fold serial dilutions of phage were prepared in PC buffer (without detergant); 190 µl PC buffer (without detergent) and 10 µl phage was put into 96-well plate well A1. 180 µl PC buffer (without detergent) was put into wells from A2 to A12. Ten fold dilutions were made from A1 to A12 by taking 20 µl samples from preceding wells.
4. When culture has reached to mid-log phase, 108 µl cultures were dispensed into microfuge tubes, 1 for each phage dilution.
5. 12 µl of each dilution was added to each tube, vortexed quickly, and incubated at room temperature for 1–5 minutes.
6. Each cell-phage mixture was added in one Top agar containing falcon together with 3µl tetracycline and 6 µl $MgCl_2$. Vortexed quickly and poured onto pre-warmed Xgal/IPTG-tet LB agar plates.
7. Plates were cooled 5 minutes, inverted and incubated overnight at 37°C.
8. Plaques counted on plates having ~10^2 plaques. Each number was multiplied by the dilution factor for that plate to get phage titer in plaque forming units (PFU) per 10 µl.

2.2.3. Characterization of Phage Display Peptides

Binding affinity and specificity of multiple repeating peptides were compared with the original linear and constraint ones through immunolabeling fluorescent microscopy and ELISA analysis. At the beginning of the characterization experiments, Platinum powder was cleaned following the steps described below.

Platinum Powder Cleaning Procedure

1. 100 mg powder was weighed and put into preweighed 1.5ml microfuge tube.
2. 100 µl dH_2O was added into the tube.
3. CH_3OH/acetone mixture (1:1) was added into the tube and powder pellet was dissolved gently by pipetting.
4. The sample was vortexed for 5-10 min. to prevent clump formation.
5. The powder was sonicated for 20 min. in ultrasonic bath to break the clumps.
6. Vortexed quickly for resuspention and centrifuged at 200 g for 3min.
7. The supernatant was removed and 1ml 50 % isopropanol was added onto the powder.
8. Vortexed for 5-10 min.
9. The powder was sonicated for 20 min. in ultrasonic bath.
10. Vortexed quickly for resuspention and centrifuged at 200 g for 3min.
11. The supernatant was removed and 1ml 0.5 % PC buffer was added.
12. The powder was sonicated for 60 min.
13. Vortexed quickly for resuspention and centrifuged at 200 g for 3min.
14. The supernatant was removed and 1ml 0.5 % PC buffer was added.
15. 100 µl of powder solution was transferred into each sterile microfuge tube. During the sample was aliqouted, each time sample was vortexed to block powder settle down.
16. Samples were centrifuged at 200 g for 3min.
17. Samples were washed twice with isopropanol (1:1).
18. Samples were washed twice with dH_2O and dried under vacuum.

2.2.3.1. Fluorescent Microscopy Analysis

Proper amount of cleaned Platinum powder suspension was incubated with 10µl phage (10^{11} PFU/10µl) in PC buffer containing 0.1 % detergent for overnight on a running cylinder to ensure binding. After attaching phage to the powder, phage sample was labeled according to immunolabelling procedure. Anti-M13 pIII monoclonal antibody (Amersham Biosciences) as a first antibody, 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 20 min. After this incubation period, 3 µl mixture was put in phage–platinum solution and incubated for 10 min. on running cylinder to increase phage-mixture interaction. Phage-powder-dye mixture was washed 2 times with PC buffer to eliminate non-specific interactions. Finally, these complexes were visualized by fluorescence microscopy (BX60, Olympus Corporation) at 20X magnification under WIB filter. Figure 2.1 shows the procedure.

For each phage, the same procedure was applied with Palladium powder as a control for cross-specificity.

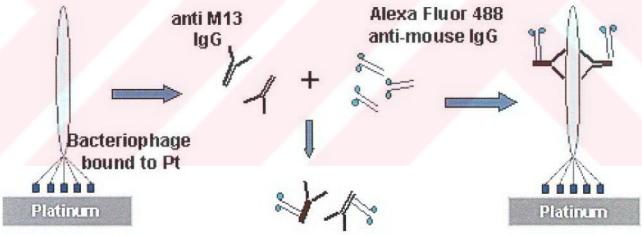


Figure 2.1. Immunolabeling fluorescent procedure [73]

2.2.3.2. ELISA Analysis

Proper amount of clean Platinum powder incubated with 10µl phage (10^{11} PFU/10µl) in 190µl PBS buffer containing 2% BSA for 2 hours on a running cylinder to ensure binding. After incubation period, phage-powder mix was washed 3 times with 200µl PBS buffer containing 0.05% detergent. 200µl PBS buffer containing 2%BSA was added on washed phage-powder mix and incubated with 0.4µl HRP-AntiM13 antibody for 30 min. by rotating. 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 PBS buffer containing 0.05% detergent. Enzymatic reaction was started when 100µl TMP (1-step TMP-ELISA, Catalog# 34022, Pierce) was added into solution. Reaction was ended when the color of the solution turned to blue with 100µl 1M H_2SO_4. Colorful supernatant was measured at 450nm.

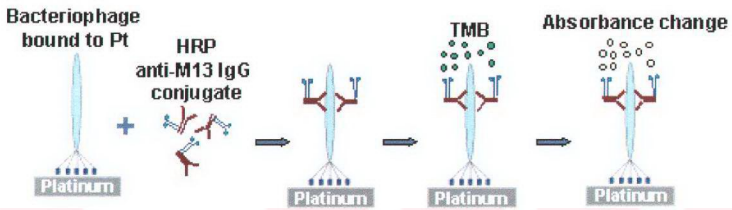


Figure 2.2. ELISA protocol [73]

3. RESULTS AND DISCUSSION

Linear peptide fragments are known to be flexible and exhibit numerous conformations in solution and even in the solid state. However, if one can restrict the conformational freedom of these linear peptides by introducing some constraints in the structure, it may render a biologically active and / or more specific peptide. Constraint form of peptides provides less unfavorable binding entropy, improving the overall free energy of binding compared to unstructured ligands. Disulphide constraint is one of the commonly structures in peptide libraries for the selection of the short peptides for desired targets. [44, 45]

Peptides displayed on phage libraries are generally composed of 7-12 amino acids. These kinds of short peptides can get multiple conformations however; this can result reducing the conformation of active forms. Multimerization of short peptides could change their properties, leading in a more rigid structure especially important in the controlled assembly processes. The increased number of binding sites in repeating peptides may provides an enhancement in binding strength or specificity. The optimal conformation for the surface recognition or desired characteristics of the peptide could be obtained by modifying the repeating forms, as a way of multimerization [32].

In this study, we have investigated the structural and the multimerazation effect on one of the strong Pt-binders, PTSTGQA. This peptide was previously identified from a random peptide phage display di-sulphite constrained library [30]. The second-generation peptides were genetically designed as different number of repeats (two and three) in both constrained and linear forms. Structural and multimerization effects on the Pt-binding property of the peptide have been investigated. The peptide was first linearized from a disulfide-constrained structure by deleting two of the cysteine residues, then multiple repeat studies were carried out. Binding properties of the each second generated peptides have been evaluated by FM and ELISA analysis, and they were compared with the phage display selected first set of binder.

3.1. Linearization of Pt-binding Peptide

Pt binding sequence SD152 was previously selected via 7 amino acid cyclic M13 phage display library. To overcome possible limitation of the cyclic nature of the peptide, constraint SD152 was linearized, Cysteine residues on both sides of the peptide (Figure 3.1) were deleted one by one to construct the linear form as explained in *Site directed mutagenesis studies* section.

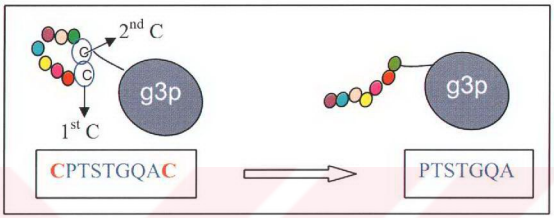


Figure 3.1. Constraint to linear conversion of SD152

Cysteine residues on SD152 are called as 1st and 2nd C according to their localizations in N- or C- terminus of the peptide respectively (Figure 3.1). Firstly, one of the C residues, 2nd C, was deleted by using Invitrogen GeneTailor Site-directed Mutagenesis Kit. Two overlapping primers namely; the forward (GTSD152d1C-F) primer contains the desired mutation which is the deletion of three bases coding cysteine amino acid and the reverse (GTSD152d1C-R) primer contains a 15- nucleotide sequence that is complementary to the forward primer and includes 12 additional nucleotides at its 3` end were designed. Replicative form (dsDNA) of phage genome containing SD152 as a fusion in g3p was used as a template for mutagenesis reactions. dsDNA of SD152 was methylated by DNA methylase and the second cysteine residue was deleted by site-directed mutagenesis using PCR. The performance of mutagenesis PCR reaction was analyzed by agarose gel electrophoresis (Figure 3.2).

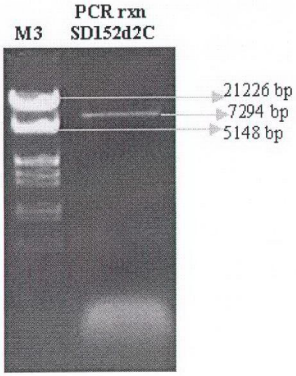


Figure 3.2. 2.5% agarose gel. **M3:** marker3, Lambda DNA / *Eco*RI+*Hind*III, **PCR rxn:** PCR product obtained from 2nd C mutagenesis reaction, SD152d2C: 2nd C deleted SD152

Following the observation of desired DNA fragment on agarose gel, the extracted PCR product was transformed into bacterial host. Two different *E. coli* strains namely DH5α-T1 and DH5α-FT were used for transformation process. PCR product was transformed into *E.coli* DH5α-T1 $CaCl_2$ competent cells. This strain provides the self-ligation of linear PCR product to form the circular phage DNA and digestion of methylated template leaving the mutated phage DNA generated during PCR. Mutated DNA obtained from PCR was seperated from unmutated template by DH5α-T1 cells, which have an endonuclase that digests methylated strands. However, *E.coli* DH5α-T1 doesn't contain F pilus, which is required for phage infection. Therefore, mid-log *E.coli* DH5α-FT containing F pilus was added to transformation mixture during plating onto LB plates. Thereby, assembled phage containing the desired mutation released from transformation host was amplified in *E.coli* DH5α-FT.

Blue-phage plaques observed on LB plate were amplified in *E.coli* DH5α-FT to obtain ssDNA for sequencing. However, it was impossible to obtain any ssDNA indicating that there was not any phage amplification, which designates to reduction or lost in phage viability. Phage infectivity can be affected with the unpaired C residue in the fused peptide. The unpaired C can constitute disulphide-bridge with any of the C residues in N1 or N2 domain of g3p resulting with unviable phage. In the case of SD152, unpaired C residue obtained after mutagenesis reaction affected

the viability of phage. In the light of this assumption, two cysteine residues in peptide were deleted on the same template leaving out the interval phage amplification.

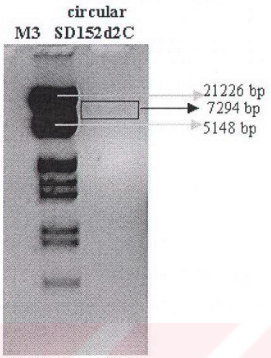


Figure 3.2. 2.5% agarose gel. **M3:** marker3, Lambda DNA / *Eco*RI+*Hind*III, **circular SD152d2C:** self-ligated 2nd C deleted SD152.

PCR product of 2nd C mutagenesis reaction was extracted from the gel and purified to self-ligate by using T4 DNA ligase (Figure 3.3). Self-ligated template was methylated before applying the second mutagenesis reaction. The 1st C residue was deleted from the methylated template by using PCR and the gel picture of linearized SD152 was shown in Figure 3.2.

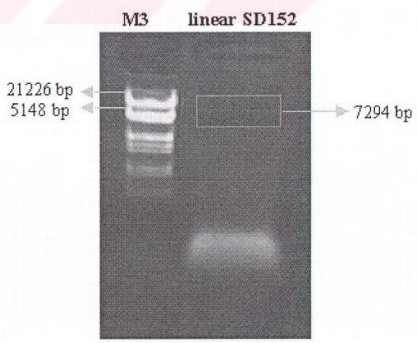


Figure 3.2. 2.5% agarose gel. **M3:** marker3, Lambda DNA / *Eco*RI+*Hind*III, **linear SD152:** PCR product of 1st C deletion performed on self-ligated 2nd C deleted SD152.

The PCR product was transformed into *E. coli* DH5α-T1 competent cells. Transformation mixture was plated onto LB plate with mid-log *E. coli* DH5α-FT cell culture required for phage amplification.

Each blue-phage plaque observed on LB plate was picked up with a pipette tip and put into PC buffer containing wells of an Elisa plate to obtain 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 phage DNA was purified from amplified phages and used for sequence analysis. Sequencing results confirmed that the "linear SD152" was constructed.

Glycerol stocks of newly constructed phage displaying linear SD152 were prepared and stored at –80°C.

3.2. Multimerization of Pt-binding Peptide

Multiple repeats of Pt-binding peptide SD152 were constructed both in constraint and linear form. Initially, constraint 3-repeat-SD152 was generated. Obtained constraint peptide was linearized by deleting cysteine residues via site-directed mutagenesis. 2-repeat-SD152 was constructed in a linear form containing one cysteine residue. Constraint and linear forms of 2-repeat-SD152 were generated via inserting and deleting the other cysteine residue respectively.

3.2.1. Construction of Multiple Repeating Peptide in Constraint Form

M13KE is an M13 derivative, which contains *Acc65*I and *Eag*I restriction sites located into 5' end of gene III. Thus, peptides cloned into, displayed on minor coat protein as N-terminal fusions. Because M13KE is a phage vector, the cloned peptide will be displayed on all 5 copies of g3p on the surface of the phage. This type of polyvalent display allows comparing binding affinities of different peptides. Since, peptides longer than generally 30 amino acids might have a deleterious effect on phage infectivity; display of short peptides is offered. Also the short peptides offer the flexibility to take part in as fusion product into many other functional proteins.

Here, we constructed three repeat constraint peptide displayed on M13KE minor coat protein. To achieve this, 3-repeat-SD152 oligonucleotide was synthesized in such a way that 3 repeat peptide contains two cysteines on both ends; a glycine rich linker in

C-terminus of the peptide and restriction enzyme recognition sites (Figure 3.3). Thereby, disulfide-constraint three repeat peptide would be inserted into N-terminus of g3p with a linker region between the peptide and g3p. Synthesized oligonucleotide was converted to double stranded form by using Klenow fragment and run on 2.5% agarose gel to confirm the fragment length (Figure 3.4).

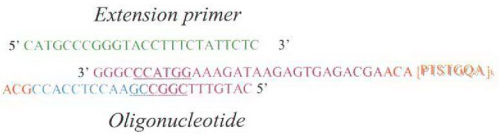


Figure 3.3. Annealing of three repeat SD152 oligonucleotide with the extension primer. Peptide sequence is written in orange, cysteine residues are in red, glycine rich linker sequence is in blue and restriction enzyme recognition sites are underlined.

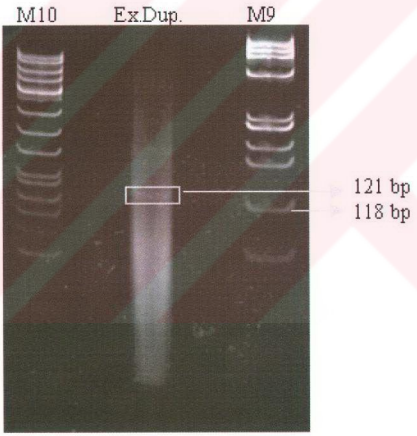


Figure 3.4. Extended duplex run on 12% PAGE (Polyacrylamide gel) with weight markers M9 and M10. Exact fragment length of 121 bp can be observed on gel.

In the first step extended duplex was cloned into a pDrive-cloning vector. Because of pDrive is a TA cloning vector, requires unpaired Adenine residues on 3' ends of insert DNA to be ligated. However, klenow fragment doesn't add adenine residues to 3' ends of DNA. Therefore, before cloning, unpaired Adenine bases were added to 3' ends of the extended duplex. By using this vector, we could easily control whether the three repeat sequence was constructed without any mutation and the positive

clones were easily checked through sequencing and then they were amplified to get the required amount of insert for ligation into phage vector.

pDrive vector with 3-repeat-SD152 insertion was amplified and digested with *Eag*I and *Acc*65I restriction enzymes. Three DNA fragments, which are 59 bp, 104 bp and 163 bp in length, were obtained after digestion. Digestion mixture was run on 2.5% agarose gel and desired 104 bp fragment which belongs to 3 repeat-SD152 was extracted (Figure 3.5.)

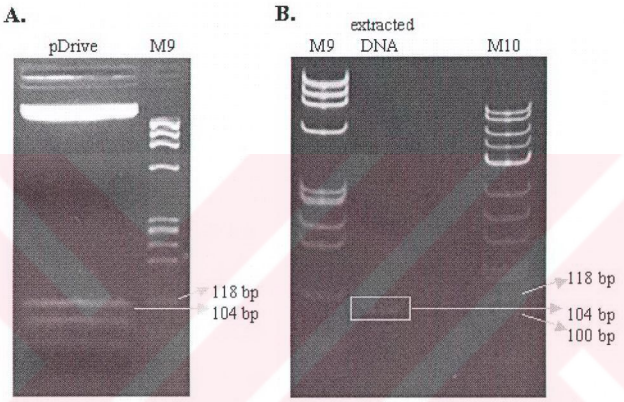


Figure 3.5. A. 2.5% agarose gel; DNA fragments obtained after *Eag*I and *Acc*65I digestion of pDrive containing 3-repeat-SD152, **B.** 12% PAGE (Polyacrylamide gel); 3-repeat-SD152 after gel extraction.

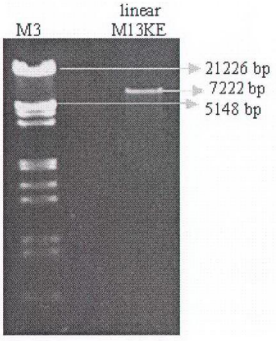


Figure 3.6. 1% agarose gel; linear M13KE phage vector obtained after *Eag*I and *Acc*65I restriction.

Cleaned and concentrated insert DNA was ligated into M13KE vector digested (Figure 3.6.) with the same restriction enzymes. Ligation product was transformed into *E. coli.* ER2738 electro-competent cell, which is the efficient host for phage amplification. Blue-phage plaques observed on LB plate were amplified and ssDNA of phage particles were purified.

Construction of phage-displayed "constraint 3 repeat-SD152" was confirmed via sequence analysis of single stranded phage DNA.

3.2.2. Linearization of Multiple Repeat SD152

Conversion of constraint 3 repeat-SD152 to linear form was achieved by deleting the C residues. Initially 2nd C residue was deleted. To perform the deletion, two primers were designed in such a way that both leading the construction of three repeat and two repeat of the peptides. When Reverse primer binds to the 3rd repeating unit (coloured blue in figure 3.7) linearized form of 3 repeat-SD152 is constructed. In the case of 2 repeat-SD152, Reverse primer binds to 2nd repeating unit (coloured yellow in figure 3.7) initiating the extension of 2 repeat-SD152 in linear form. Primer binding sites yielding three and two repeat SD152 are given in Figure 3.7.

Figure 3.7. Primer binding sites are shown in 2nd C deletion reaction. Multiple units of 3 repeat-SD152 are written in different colors, blue, yellow and green. Deleting cysteine is written as an upper symbol. **A.** Annealing of primers resulting with the extension of three repeat SD152 **B.** Annealing of primers resulting with the extension of two repeat SD152

Double stranded DNA of constrained 3-repeat-SD152 was used as a template to construct linearized form of three repeat and two repeat of SD152 in site directed mutagenesis reactions. Following the methylation of template DNA, mutagenesis reaction was applied as explained in site directed mutagenesis studies section. The obtained PCR product was visualized on an agarose gel electrophoresis as shown in Figure 3.8.

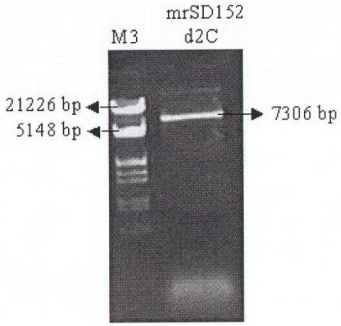


Figure 3.8. 1% agarose gel. **M3:** marker3, Lambda DNA / *Eco*RI+*Hind*III, **mrSD152 d2C:** PCR product of 2nd C deletion reaction performed on double stranded constraint 3 repeat-SD152. Obtained DNA fragment contains the double stranded multiple repeat (2 and 3) SD152 in which 2nd C was deleted.

Unpaired cysteine residues in the fusion protein can interfere with phage viability. To overcome this problem, the other C residue was also deleted before transforming the mutagenesis reaction into bacterial host.

The PCR product of 2nd C deletion was cleaned and self-ligated to obtain the template for the 1st C deletion reaction (this template was also used for 2-repeat-SD152 construction in constraint form- in section 3.2.3.). Self-ligated template was methylated and mutated using the reverse and forward primers of GTSD152-d1C (Figure 3.9).

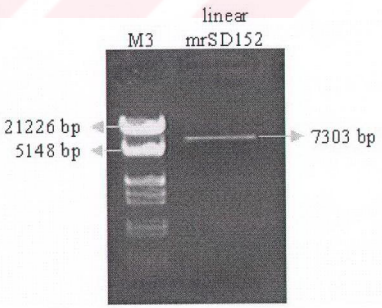


Figure 3.9. 1% agarose gel. **M3:** marker3, Lambda DNA / *Eco*RI+*Hind*III, **linear mrSD152:** PCR product of 1st C deletion reaction performed on the self-ligated template coming from mtSD152-d2C. Obtained DNA fragment contains the double stranded multiple repeat (2 and 3) SD152 in linear form.

Mutagenesis reaction that will be giving the linear multiple repeat peptides are transformed into *E. coli* DH5α-T1 competent cells. While plating the transformation mixture, *E. coli* DH5α-FT cells, which are in mid-log phase, were added into the cell culture tube. DH5α-FT cells are crucial to provide the F pilus, which is required for phage infection and thus phage amplification.

Each of all phage plaques observed on LB plate were picked up and transferred into the storage plates and then amplified in mid-log DH5α-FT culture in liquid media. ssDNA of phages were purified (Figure 3.10) to be sequenced. The results of sequencing confirmed that both 2 and 3 repeat of SD152 were obtained in linear form.

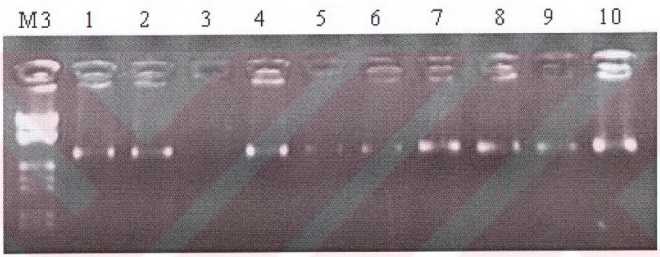


Figure 3.10. 1% agarose gel. **M3:** marker3, Lambda DNA / *Eco*RI+*Hind*III, **1-10:** DNA samples that were isolated from selected phage colonies

3.2.3. Construction of Two repeat SD152 in Constraint Form

There were two possible ways to obtain 2 –repeat-SD152 in constraint form. First one was to insert two of the C residues into the linear peptide that we have already constructed. However, in this situation we had to design two primers for each insertion mutagenesis. Thereby, we decided to apply the second way, which is to insert missing 2nd C into 2-repeat-SD152-d2C. Even though we don't have the construct of 2-repeat-SD152-d2C, dsDNA encoding this peptide already existed in mutagenesis reaction obtained from 2nd C deletion of 3-repeat-SD152 (in section 3.2.2.). On this account, PCR product of "2nd C deletion of 3-repeat-SD152" was cleaned and self-ligated.

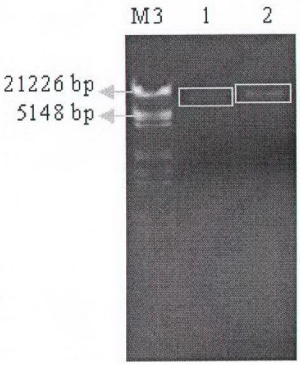


Figure 3.11. 1% agarose gel. **M3:** marker3, Lambda DNA / *Eco*RI+*Hind*III, **1:** Self-ligated mtSD152-d2C, **2:** PCR product obtained from 2nd C insertion mutagenesis

After methylation of self-ligated template, missing 2nd C was inserted using two primers namely GTSD152i2C-Forward and Reverse (Figure 3.11). Mutagenesis reaction was transformed into DH5α-T1 competent cells and phages were amplified in DH5α-FT cells. Sequence analysis of ssDNA purified from phage particles confirmed that 2-repeat-SD152 was constructed in linear form.

3.3. Characterization of Second Generation Peptides

Following site-directed mutagenesis of desired aminoacids, immunolabelling fluorescence microscopy (FM) and ELISA analysis's of mutants were performed to compare the binding strength of the mutants to the wild type (identified via first generation of phage display libraries). Binding affinities of second generation peptides were characterized through immunolabelling fluorescence microscopy (FM) and ELISA analysis to evaluate structure and multimerization effects of selected Pt binder SD152 peptide.

3.3.1. Fluorescent Microscopy Analysis

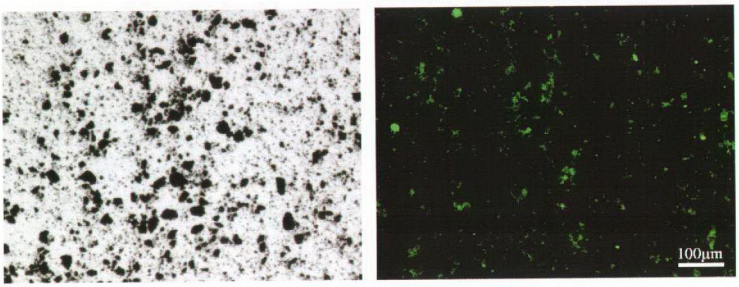
All the individual colonies were exposed to platinum powder. The mixture was rotated overnight and after washing the sample with PC buffer to remove unbound phage from the surface, anti-M13 g8p monoclonal antibody and Alexa-Fluor conjugated secondary antibody fragments were added to label the phage. The samples were visualized under fluorescence microscopy and the affinity of the

sequences was determined. Table 3.1 shows the second generation peptides characterized through fluorescent microscopy analysis and binding percentages of each peptide.

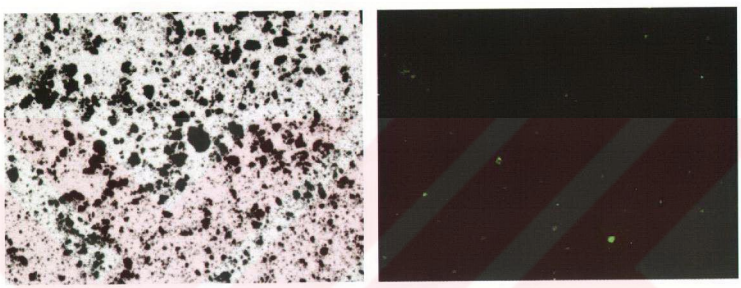
Table 3.1. Second-generation SD152 peptides, their amino acid sequences and binding percentages are given

Name	Sequence	Binding Percentage
Constraint SD152	CPTSTGQAC	% 10.45
Linear SD152	PTSTGQA	% 1
Constraint 2 repeat SD152	CPTSTGQA PTSTGQA PTSTGQAC	% 4
Linear 2 repeat SD152	PTSTGQA PTSTGQA PTSTGQA	% 1
Constraint 3 repeat SD152	CPTSTGQA PTSTGQAC	% 8
Linear 3 repeat SD152	PTSTGQA PTSTGQA	% 6

Binding percentages of each peptide were determined with fluorescent microscopy images obtained from individual peptide. Binding affinities of second-generation peptides were compared with each other to follow the effect of structure and multimerization of peptides.



Constraint SD152



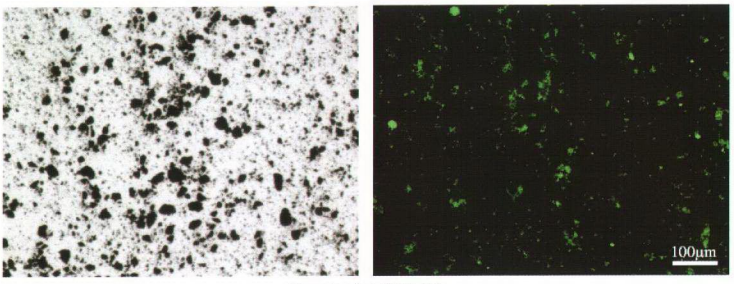
Linear SD152 linear

Figure 3.12. Fluorescent and optical microscopy images of constraint and linear SD125.

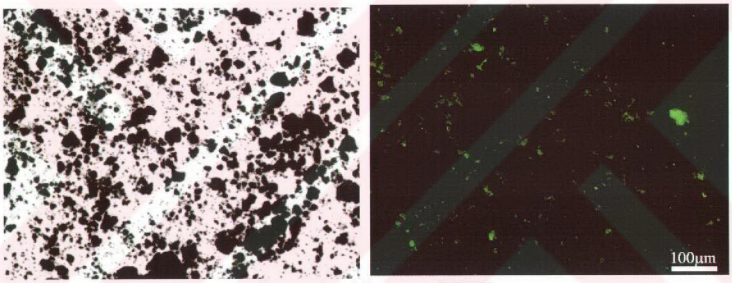
Figure 3.12 shows the fluorescent and optical microscopy images of constraint and linear SD152. Calculated binding percentages from these images indicated that while constraint SD152 has a binding affinity of nearly 10%, binding affinity of linear peptide is 1. This decrease in binding could be resulted from structure difference.

Linear peptides can get multiple conformations enabling them to obtain optimal one for intermolecular interactions. In the case of SD152, linearization of the peptide resulted in a binding affinity reduction. In this specific conformation, constrained form possibly provided a condition for the peptide to expose its active site responsible for binding allowing the peptide to use these sites for the intermolecular interactions with the substrate. While this specific conformation occurs in a high energy level in unconstraint form, constraint peptide may obtain this conformation in a lower energy level, resulted in higher affinity. Thus, the optimal conformation for binding could be gained when the peptide is constraint.

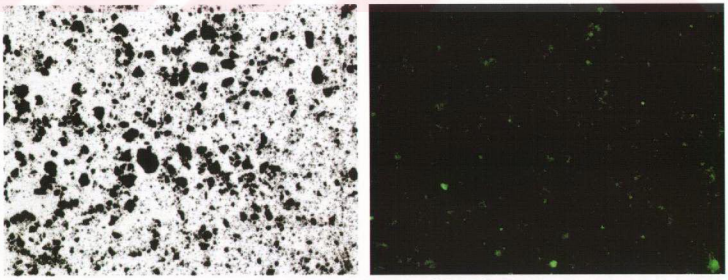
In the next step, we examined the effects of multiple repeat on the platinum binding property via our developed protocol. Multiple repeat peptides were constructed both in linear and constraint form. Thus, the effect of multimerization was followed with respect different structural conformations as constrained and nonrestricted.



Constraint SD152



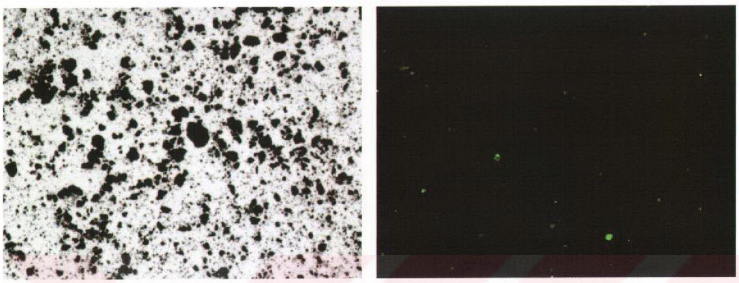
Constrained 3 repeat SD152



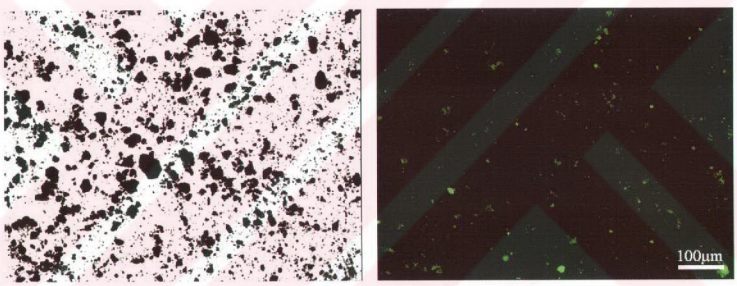
Constrained 2 repeat SD152

Figure 3.13. Fluorescent and optical microscopy images of multiple repeat SD152 peptides in constraint form.

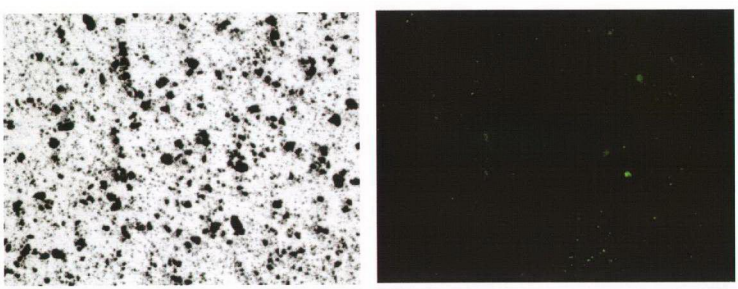
Fluorescent microscopy images and binding affinities of constraint multiple SD152 peptides are given in Figure 3.13. Fluorescent microscopy analysis showed that binding affinity was reduced in multiple repeating. However, 3–repeat-SD152 has a higher binding affinity compared to 2-repeat-SD152 indicating that increasing the repeating unit, binding affinity can be increased.



Linear SD152 linear



Linear 3 repeat SD152



Linear 2 repeat SD152

Figure 3.14. Fluorescent and optical microscopy images of multiple repeat SD152 peptides in linear form.

Figure 3.14 is showing the fluorescent microscopy images and binding affinities of linear multiple SD152 peptides. Multimerization of peptide resulted in an increased binding affinity. When the repeating unit was increased from two to three, binding affinity was observed to be higher than the linear peptide.

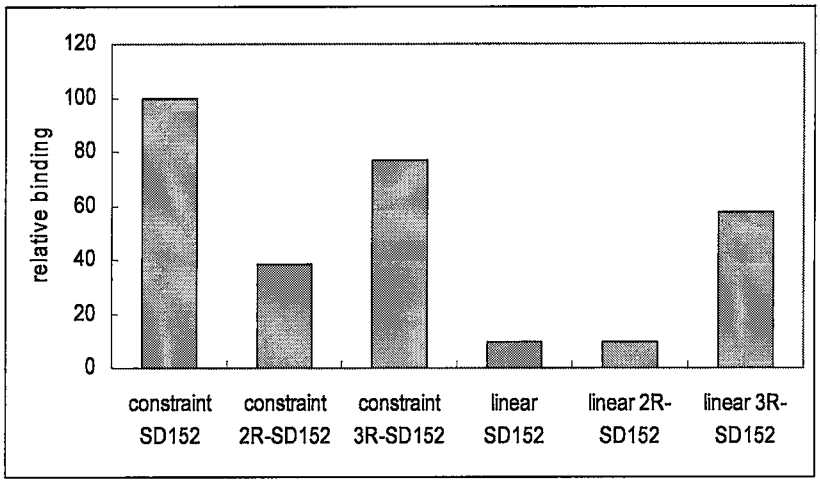


Figure 3.15. Relative binding affinities of second-generation peptides

Figure 3.15 shows the relative binding affinities of second-generation peptides to the phage library selected constraint SD152.

In the next step, ELISA analyses were performed as an alternative method to characterize the affinity property of the second-generation peptides and their results were compared with the fluorescent microscopy analysis.

3.3.2. ELISA Analysis

All the individual colonies were exposed to platinum powder. The mixture was rotated 2 hours and washed the sample with PBS buffer to remove unbound phage from the surface. HRP-AntiM13 antibody conjugate was added to the mixture and after the incubation period washed several times to remove the unbound antibody. By adding TMP (the substrate of HRP) an enzymatic reaction was occurred resulting with change in the color from blue to yellow. The change in the color was measured at 450nm.

ELISA analyses were performed for SD152 and 3-repeat-SD152 both in linear and constraint forms. Experiment was performed with different phage concentrations and in a triplet set for each concentration. Wild type M13KE was used as a blank in all

measurement. Figure 3.16 shows the results of ELISA experiment performed with 10^9/µl of phage particles.

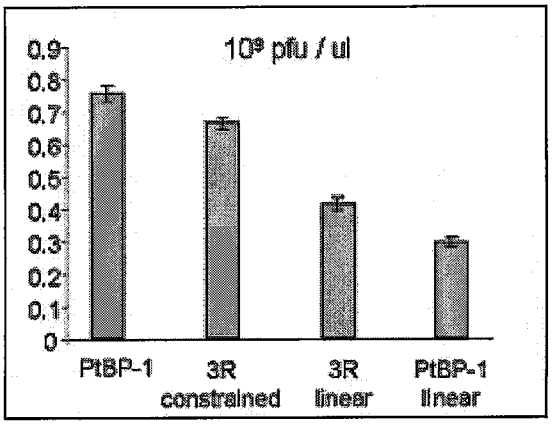


Figure 3.16. The graph shows the measured absorbance degrees during ELISA analysis of constraint SD152, linear SD152, constraint 3-repeat-SD152 and linear 3-repeat-SD152.

ELISA analysis verified the data obtained from fluorescent microscopy analysis. Peptides tend to loose their affinity for the surface as they get a linear structure rather than constrained. However, increasing the repeating unit results in increasing their binding affinity even if they are in linear structure. Constraint peptides both in single and multiple repeated forms resulted in a high binding affinity comparing with the linear forms of the peptide.

4. CONCLUSION

Second generation platinum binding peptides were genetically constructed on the filamentous M13 phage based on a phage display selected and well characterized one, SD152. This peptide is a 7-mer peptide in a disulphide constraint form with the amino sequence of PTSTGQA. Constraint SD152 has been characterized as a strong Pt binding peptide on the basis of varying characterization methods such as fluorescent microscopy analysis.

Peptides were engineered to evaluate the effect of structural conformations and multimerization on the platinum affinity levels. Multimerization effects were investigated for single to three repeats of phage display selected platinum binding peptide. Constraint SD152 is first linearized. Than, 2 and 3 repeat peptides were constructed in constraint form as well as in linear form.

In developing our engineering protocols for the second generation of peptides, we utilized different protein engineering tools such as site-directed and cassette mutagenesis. The mutagenesis techniques are widely used for the manipulation of plasmid vectors were adapted for engineering peptides displayed on filamentous phage in our study. Cassette mutagenesis techniques were used to construct multiple repeating peptides in constraint form. Constraint peptides were than linearized by deleting both cysteine residues one by one.

Characterization experiments showed that the change in the structural property of peptides from constraint to linear resulted in a loss of the platinum affinity. However, the platinum affinity was increased with three repeat of the peptide in linear form.

Our experiments indicated that binding affinity could be tuned up via genetic engineering approaches depending on the desired structure of the peptide for the controlled assembly processes. These novel engineering protocols will be of great utility for optimizing length or the molecular conformation of the inorganic specific peptides for controllable and predictable affinities.

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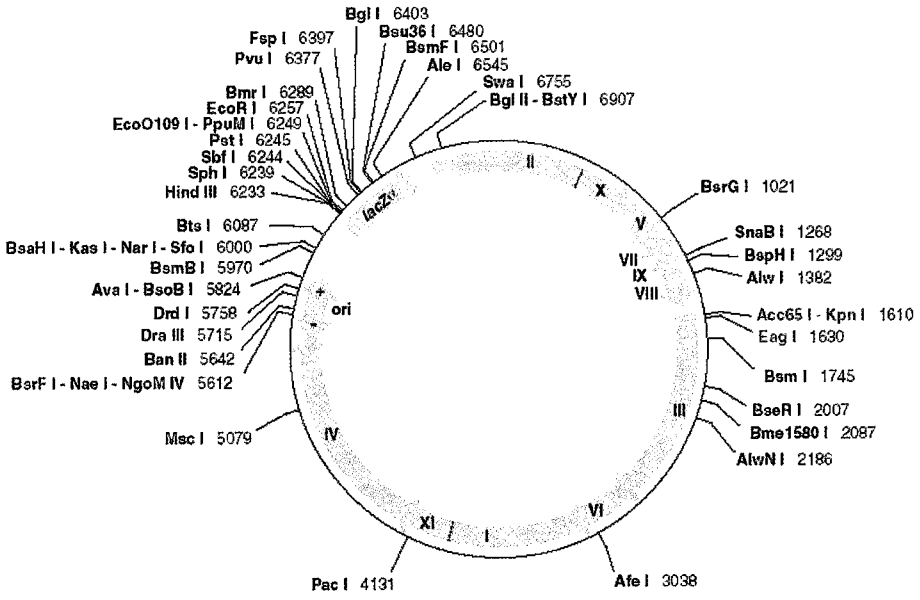
APPENDIX

Appendix A.

Vector Map of M13KE

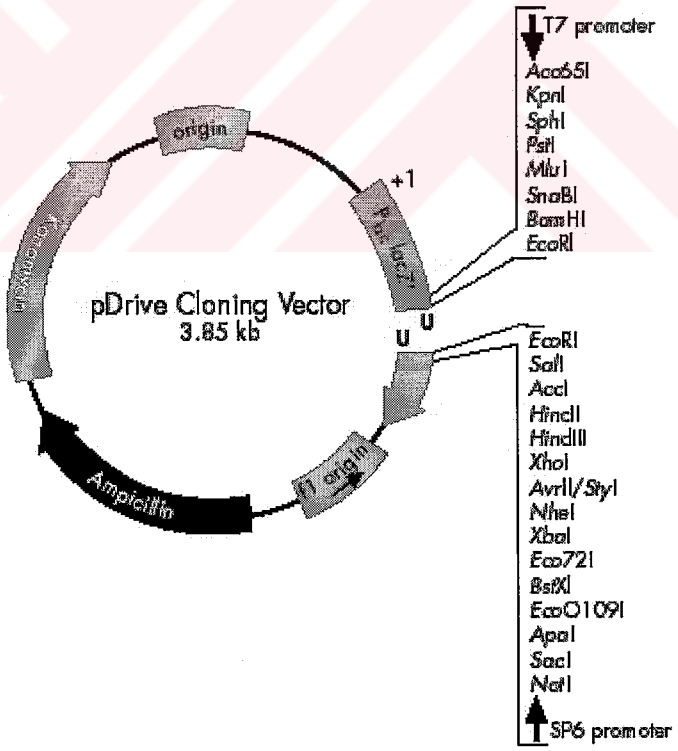
Position (bp)	Element
6821- 831	gene II CDS (start 6821)
496- 831	gene X CDS (start 496)
843-1106	gene V CDS (start 843)
1108-1209	gene VII CDS (start 1108)
1206-1304	gene IX CDS (start 1206)
1301-1522	gene VIII CDS (start 1301)
1578-2852	gene III CDS (start 1578)
2855-3193	gene VI CDS (start 2855)
3195-4241	gene I CDS (start 3195)
3915-4241	gene XI (gene I*) CDS (start 3915)
4219-5499	gene IV CDS (start 4219)
5487-5867	M13 origin of replication (- +)
6216-6695	lacZ alpha CDS (start 6216) [74]



Appendix B.

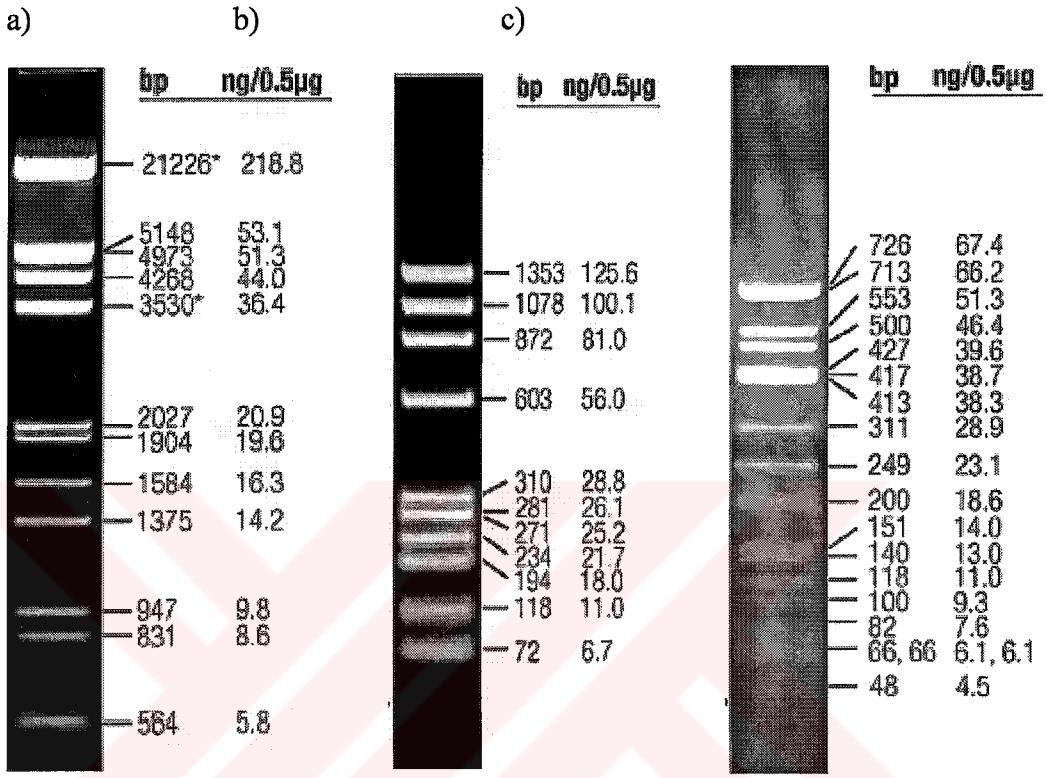
Vector Map of pDrive

Position (bp)	**Element**
266–393	Multiple cloning site
216–593	LacZ ⟨-peptide
239–258	T7 RNA polymerase promoter
start 256	T7 transcription
398–417	SP6 RNA polymerase promoter
start 400	SP6 transcription
1175–2032	Ampicillin resistance gene
2181–2993	Kanamycin resistance gene
3668	pUC origin
588–1043	Phage f1 origin



Appendix C.

Molecular Weight Markers



a) λ DNA *EcoRI* and *HindIII* digested marker-3, 1.0% agarose, 0.5µg/lane, 8 cm length gel, 1X TAE, 17V/cm. (Catalog # SM0191, NBI Fermentas).

b) ΦX174 DNA *Bsu*RI digested marker-9, 1.7% agarose, 0.5µg/lane, 8 cm length gel, 1X TBE, 5V/cm. (Catalog # SM0251, NBI Fermentas).

c) ΦX174 DNA *HinfI* digested marker-10, 2.5% agarose, 0.5µg/lane, 8 cm length gel, 1X TBE, 5V/cm. (Catalog # SM0261, NBI Fermentas).

Appendix D.

Lab Equipment

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.

: 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# 170-8110EDU, Bio- Rad.

Vortexing machine : Reax Top, product# 541-10000, Heidolph2.2.

Appendix E.

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**

RESUME

Sibel Çetinel was born in Kadıköy - İSTANBUL in 1980. Following her high school graduation from Ümraniye Anatolian High School, in 1998, she started to study in Istanbul University, Cerrahpaşa Medicine Faculty, Department of Medical Biology. She had her Bachelor degree from the same department in 2003 and consequently, she was accepted to Advanced Technologies in Molecular Biology, Genetics and Biotechnology's program in Department of Molecular Biology and Genetics. She is still pursuing her studies in the same department. Biotechnology, protein engineering and molecular genetics are among her professional interest topics.