İSTANBUL TECHNICAL UNIVERSITY ★ INSTITUTE OF SCIENCE AND TECHNOLOGY

SELECTION OF TITANIUM SPECIFIC POLYPEPTIDES BY PHAGE DISPLAY TECHNIQUE

M.Sc. Thesis by

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ABBREVIATIONS

bp	: Base pair
BSA	: Bovine Serum Albumin
CSD	: Cell Surface Display
CCW	: Counter clockwise
CW	: Clockwise
dH_2O	: Distilled water
DDT	: Dichloro-diphenyl-trichloroethane
DMF	: Dimethylformamide
DNA	: Deoxyribo Nucleic Acid
DNase	: Deoxyribonuclease
E. coli	: Escherischia coli
EDTA	: Ethylenediaminetetraacetic acid
EtOH	: Ethanol
Fab	: Antibody Fragment
FM	: Fluorescence Microscopy
IPTG	: Isopropyl-β-D-1-thiogalactosidase
kb	: Kilobase
LB-broth	: Luria Bertani Broth
ME	: Mercaptoethanol
OD	: Optical Density
PC Buffer	: Potassium Phosphate-Sodium Carbonate Buffer
PCR	: Polymerase Chain Reaction
PD	: Phage Display
PEG	: Polyethylene glycol
RNA	: Ribonucleic acid
RT	: Room Temperature
SDS	: Sodium Dodecyl Sulfate
ssDNA	: Single Stranded DNA
TBE	: Tris/Borate/EDTA
TBS	: Tris Buffered Saline
TE	: Tris/EDTA
Tris	: Hydroxymethyl aminomethane
SEM	: Scanning Electron Microscope
Xgal	: 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside
XPS	: X-ray Photoelectron Spectroscopy

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SELECTION OF TITANIUM SPECIFIC POLYPEPTIDES BY PHAGE DISPLAY TECHNIQUE

SUMMARY

In nature, nearly all of the organisms utilize organic-inorganic hybrid systems. Either these systems play structural and/or defensive roles, or function in intercellular interactions between cells. Proteins are the major molecules in formation of this type of hybrid systems. Proteins are, in fact the major building blocks of all organisms. Polypeptides are used in biotechnologic and nano-biotechnologic areas due to their excellent functionality in complexes with inorganic materials.

Phage display technique is one of the combinatorial biology based selection techniques used in selection of inorganic binding peptides. Recent years, there is a great trend to study on selecting inorganic binding polypeptides by phage display technology. Some noble metals, metal oxides and semiconductors could be given as examples to these studies preformed recently. Inorganic specific polypeptides can be used in nanotechnological and bionanotechnological applications as molecular linkers and take role in inorganic biomineralization.

In the current work, titanium specific polypeptides were selected via using phage display technique. The rationale for selecting titanium as an inorganic subtrate may be explained by its biocompatibility and popularity in medical implants, especially the dental implants.

C7C phage library was used to select titanium specific polypeptides. Before screening, material characterization studies were carried out in order to get information about particle size, particle surface and morphology. After 3 rounds of screening 51 sequences were randomly selected to study the binding affinities and specificities.

At the end of the screening procedure amino acid distribution of selected sequences and observed amino acid frequencies in library were compared, after amino acids frequency analyses, fluorescence microscopy experiments were carried out in order to understand the binding affinity of selected polypeptides to titanium powder. After fluorescence microscopy studies, polypeptides were grouped as strong, moderate and weak binding polypeptides by their affinities. Selection of strong polypeptides was not considered sufficient to assure which polypeptide was “specific” to titanium therefore cross specificity experiments were performed with platinum, silver and silica by examination on fluorescence microscopy.

TİTANYUMA ÖZGÜN OLAN POLİPEPTİTLERİN FAJ GÖSTERİM YÖNTEMİYLE SEÇİMİ

ÖZET

Doğadaki hemen hemen tüm organizmalar organik-inorganik hibrid sistemlerini kullanırlar. Bu sistemler yapısal ve/veya koruyucu amaçlı olmakla beraber aynı zamanda hücreler arası etkileşimde de görev alırlar. Bu tip organik-inorganik hibritlerin oluşmasında başlıca rolü proteinler oynamaktadır. Proteinler organizmaların en önemli yapıtaşlarındandır. Bu mükemmel işlevsellikleri nedeniyle inorganik malzemelere özgün olan polipeptidler biyoteknolojide ve nanobiyoteknolojide kullanılmaya çalışılmaktadır.

Faj gösterim tekniği çeşitli inorganik malzeme yüzeylerine özgün olarak bağlanan polipeptitlerin seçiliminde kullanılan kombinatoryal biyoloji tabanlı bir yöntemdir. Son zamanlarda genetik mühendisliğinin büyük yol katetmesi sonucu inorganik yüzeylere bağlanan polipeptitler üzerine yapılan çalışmalar hızla artmaktadır. Bu malzemelere soy metaller, bazı metal oksitler ve yarı iletken malzemeler örnek verilebilir. İnorganiklere özgün olan polipeptitler moleküler bağlayıcı veya inorganik sentezi gibi nano ve bionanoteknolojik alanlarda kullanılabilir.

Bu çalışmada toz titanyuma özgün polipeptitler faj gösterim yöntemiyle seçilmiştir. Titanyumun inorganik malzeme olarak seçilmesinin sebebi biyouyumluluğu yüksek bir malzeme olması ve tıp alanında özellikle diş implantlarında sıklıkla kullanılmasıdır.

Titanyuma özgün polipeptitlerin seçimi C7C faj gösterim kütüphanesinden yapılmıştır. Faj gösterim protokolü uygulanmadan önce malzeme karakterizasyonu yapılıp, yüzey alanı, parçacık büyüklüğü ve yüzey morfolojisi hakkında bilgi edinilmiştir. Deney seti süresince 3 döngü yapılmıştır. Bu döngüler sonucunda rastgele 51 dizi seçilip titanyuma bağlanma eğilimi ve özgünlüğü incelenmiştir.

Deney setinin sonunda, tanımlanan polipeptit dizilerinin amino asit oranları hesaplanarak faj kütüphanesindeki amino asit oranlarıyla karşılaştırılmıştır. Daha sonra orantısal analizleri yapılan polipeptit dizilerinin titanyuma bağlanma eğilimlerini araştırmak için floresan mikroskobu çalışması yapılmıştır. Seçilen polipeptitlerin titanyumun yüzeyine bağlanma eğilimleri incelenip, güçlü, orta ve zayıf bağlanan olarak 3 kategoriye ayrılmıştır. Güçlü bağlanan polipeptitler titanyuma olan özgünlüğünü anlamak için platin, gümüş ve silika ile florasan mikroskobu çalışmaları yapılmıştır.

1. INTRODUCTION

1.1 Inorganic material binding polypeptides in biological organisms, and their significance.

Proteins are the major building blocks in biological organisms. Due to their unique and specific interactions with other macromolecules, they can build up cell structure and take major role in functional cell properties like tissue formation and catalyzing enzymatic reactions [1]. Proteins participate in forming complex nano structures in soft [2] and hard [3] tissues. Muscle, skin, tendon, membrane, silk of spider are the main soft tissues that contain only proteinaceous phase. In hard tissues such as bones, dental tissues (i.e., dentine and enamel), spicules, spines, shells, skeletal units of single-celled organisms (e.g., radiolarian) or plants, bacterial thin film, and nanoparticles [3,4] contain both at least one proteinaceous and inorganic phase. Hard and soft tissues are synthesized in organisms at ambient conditions and aqueous environment by genetic control [4,5,6]. Fig. 1.1 shows examples of organic-inorganic hybrid material formations in some organisms [7]. The inorganic material, on which the proteinaceous phase is bound, commonly includes magnetite (Fe_3O_4) particles in magnetotactic bacteria or teeth of chiton [8]; silica (SiO_2) as skeletons of radiolarian [3] or tiny light-gathering lenses and optical wave guides in sponges [9]; hydroxyapatite ($Ca_2C(OH)_3$) in bones [10] and dental tissues of mammals [11] calcium carbonate ($CaCO_3$) in the shells of mollusks [12]. All these hybrid systems are naturally produced in organisms by using protein – inorganic interactions. These systems play important roles in evolution such as supporting elements, defencing units, some optical and mechanical properties that help organism to adapt to its habitat. All these tissues are produced in organisms mainly by using proteins under genetic control. In addition to enzymatic reactions in the synthesis of inorganic phases, proteins collect and transport raw materials and consistently and uniformly self- and co-assemble subunits into short- and long-range ordered nuclei and substrates [9-11]. Whether controlling or participating in tissue formation and regeneration, or being an integral part of the tissue in its biological functions and

physical performance, proteins are an indispensable part of biological structures and systems. Therefore, proteins are the workhorses that control the fabrication of biological structures by orchestrating the assembly of materials in two and three dimensions [7].

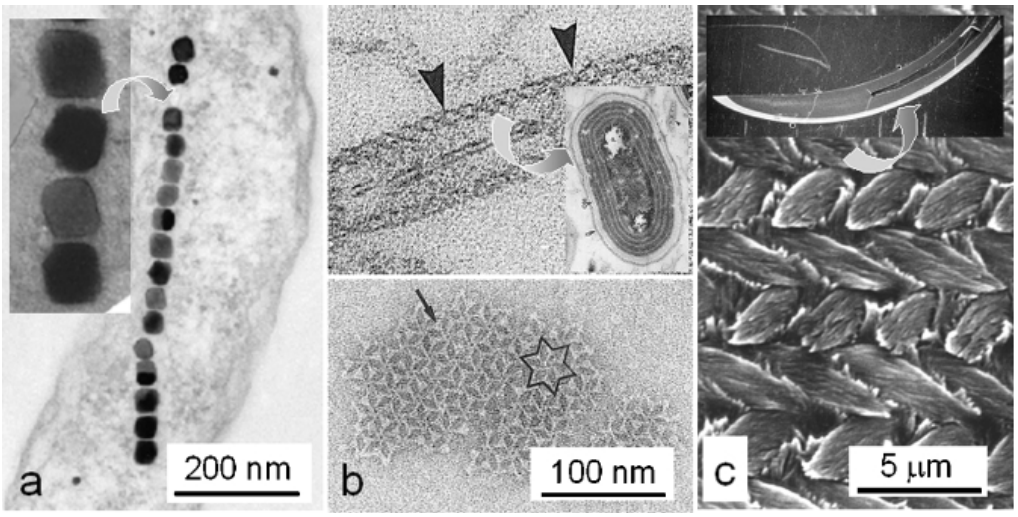


Fig. 1.1 Examples of biologically synthesized organic-inorganic hybrid materials: (*a*) Single-crystalline, single-domained magnetic magnetite nanoparticles (Fe_3O_4) formed by a magnetotactic bacterium (*Aquaspirillum magnetotacticum*) (inset: higher magnification image of the magnetite nanoparticles revealing cubo-octahedral particle shape). (*b*) S-layer bacterium, *Synechococcus* strain GL24, has a nanostructurally ordered thin film calcite on its outer layer serving as a protective coating. (*c*) Hard, wear-resistant tooth enamel of mouse consists highly ordered micro/nano architecture of hydroxyapatite crystallites that assemble into a woven rod structure (SEM image). Each rod is composed of thousands of hydroxyapatite particles (inset: cross-sectional image of a mouse incisor; white region is enamel, backed by grayish dentine) [7]

Inorganic-binding biomolecules (proteins, polypeptides, oligonucleotides etc.) could potentially be used as biolinkers in the synthesis and assembly of functional nanostructures and they could also be the building blocks of self-assembled materials with controlled organization and specific functions [1,7].

1.2 Obtaining Inorganic Binding Proteins

There are some traditional techniques which are used to create nano – technologic systems, melting and solidification processes, followed by thermo-mechanical treatments, or solution/vacuum deposition and growth processes can be exemplary for traditional techniques, but these techniques have some difficulties and limitations

like requiring high temperature, pressure and pH adjustment, and usually produce toxic byproducts [1-13]. On the other hand, obtaining desired size nano particles can be difficult with these methods. However, nature can overcome these limitations and produce hybrid materials in mild conditions. As an example ice binding (antifreeze) proteins are well characterized in some fish, plant and insect species. These proteins, composed of repeating polypeptide units, control ice formation via binding and preventing the growth of ice crystals in the internal fluids [13]. TiO_2 binding proteins were isolated from a Rhodococcus strain, which can adhere to TiO_2 layer with its nano - bridge shape proteins [14]. Another example is silica binding proteins in *Cylindrotheca fusiformis* (unicellular algae) [15] and *Tethya aurantia* [16].

There are several possible ways to isolate and determine the specific inorganic binding proteins. One of the known techniques is to extraction the proteins from tissues [6-12]. In this approach extraction of proteins and cloning of their genes are complicated and time consuming. Furthermore, the number of isolated and characterized proteins is limited [17]. In addition other biomolecules can affect the inorganic biomineralization so this technique becomes much more difficult and complex [18,19]. Although this technique is complex some proteins are isolated and used in inorganic substrate synthesis and growth [20,21]. In mammalian enamel synthesis amelogenin is the utilized protein [18] and in sponge spicula formation silicatein is used [17], these can be the best examples of these functional proteins. Inorganic binding proteins can be designed with [22] molecular approaches similar to those used in pharmaceutical drug research but these require the usage of time consuming and expensive methods such as difficult extraction procedures [12,6].

1.2.1 Application of Display Technologies in Selection of Inorganic Binding Polypeptides

Since traditional methods of isolation and identification of inorganic binding polypeptides have disadvantages, various and faster methods have been developed. In these approaches molecular libraries or combinatorial libraries which are composed of a random combination of a given number of amino acids display on biological systems. Combinatorial biology techniques also have limitations such as the requirement of complex polypeptide production and the need of having many chemical reactions [12,23,24]. Display technologies can improve these

disadvantages. In this approach phage display (PD) and cell surface display (CSD) *in vivo* techniques are used for selection of inorganic binding polypeptides [25,26]. A large, random library of peptides with the same number of amino acids, but of different sequence compositions, is screened to identify specific sequences that strongly bind to a target inorganic material surface [27,28,29]. These techniques can be applied in design of new drugs, enzymes, antibodies, DNA-binding proteins and diagnostic agents [26,30]. Nowadays these techniques are also adopted for selection of inorganic specific polypeptides. In this method there is no need to have prior information about the polypeptides sequences which are specific to the selected material.

Fig. 1.2 represents the procedure for *in vivo* display techniques [31],

Step 1→ Libraries are generated by inserting randomized oligonucleotides within certain genes encoded on phage genomes or on bacterial plasmid in PD and CSD.

Step 2→ A random polypeptide sequence is incorporated within a protein residing on the surface of the organism such as the coat protein of a phage or an outer membrane or flagellar protein of a cell.

Step 3→ Each phage or cell produces and displays a different, but random peptide.

Step 4→ The library which has a heterogeneous mixture of recombinant cells or phages is exposed on the inorganic substrate.

Step 5→ Non-binder cells or phages are eliminated by several washing cycles by disrupting weak interactions with the substrate and the cells or phages.

Step 6→ The next step is taking the binders out by eluting them from the surface.

Step 7→ In PD, the eluted phages are amplified by reinfecting the host;

Step 7-8→ Cells are allowed to grow in CSD which has only one host cell type.

This is the end of a round of biopanning which is generally repeated for 3-5 times to enrich for tight binders.

Step 9→ Individual clones are sequenced to get the related amino acid sequences of the polypeptides which bind to the target substrate material.

In CSD, outer membrane proteins, lipoproteins, fimbria and flagellar proteins have been used so far for heterologous surface display on bacteria. In PD, most of the

research has been performed using filamentous phages such as M13 or the closely related fd and f1. Random peptide libraries have been displayed on bacteriophages T7, T4 and λ, but these systems have not used on a routine basis yet.

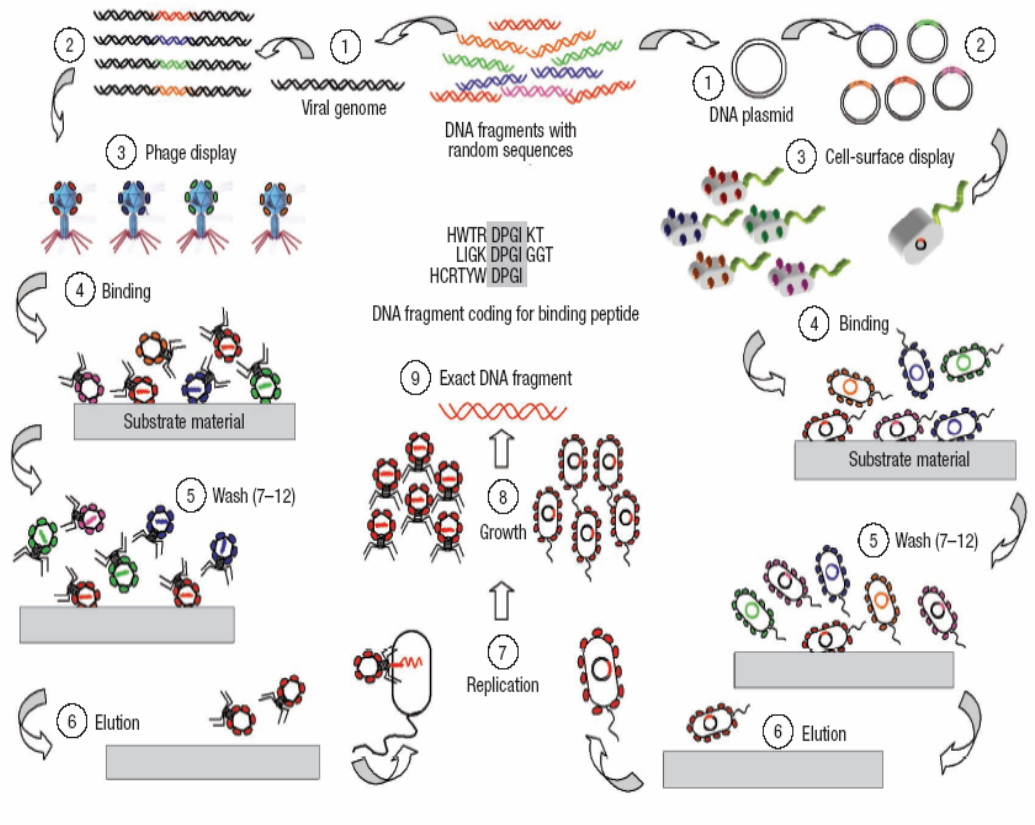


Fig. 1.2 PD and CSD. Principles of the protocols used for selecting polypeptide sequences that have binding affinity to given inorganic substrates [31].

1.2.1.1 Phage Display Technique

Phage display, developed by G. Smith in 1985 [26], is the most efficiently adapted molecular display technique [26,32]. Phage display proved to be an effective tool for various biological and biotechnological research. Indeed, this technology has been used to determine the target sequences for monoclonal antibodies and to identify ligands for antibodies in complex polyclonal serums [33]. It relies on the ability of certain phage to allow additional peptide sequences to be incorporated into the coat proteins without disrupting their structure and function by linking the DNA sequence encoding the protein and the peptide itself [34]. Since researches have started on inorganic binding polypeptides with combinatorial biology methods gallium arsenide [28], silica [35], silver [36], zinc sulfide [37], calcite [38], cadmium sulfide [39], and noble metals such as platinum and palladium [40] binding specific polypeptides have been identified by phage display technique.

1.2.1.2 M13 filamentous bacteriophage

M13 is a filamentous bacteriophage uses *E. coli* as its host. It is approximately 1μm in length and 10 nm in diameter [41]. The genetic material of M13 bacteriophage is composed of single-stranded circular DNA form (Fig.1.3) [42]. The particle is surrounded by a proteinaceous coat. M13 DNA encodes totally 10 proteins, 5 of them are related with coat, and the others are related with phage replication and assembly [43].

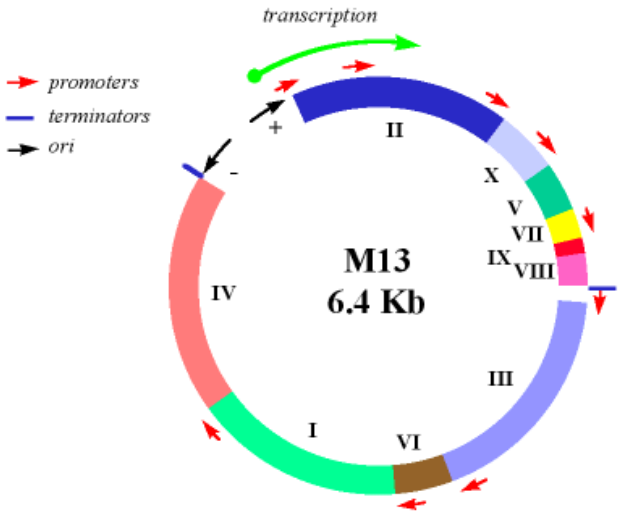


Fig. 1.3 M13 genome [42].

The coat consists of five different proteins, but the vast majority consists of several copies of the major coat protein P8 (~ 2700 subunits) which covers the length of the phage. Other four minor coat proteins are represented with 5 copies approximately. P3 and P6 proteins form the one end of particle while P7 and P9 proteins form the cap of other end [43]. In Fig. 1.4 structure and coat proteins of M13 bacteriophage are shown [44].

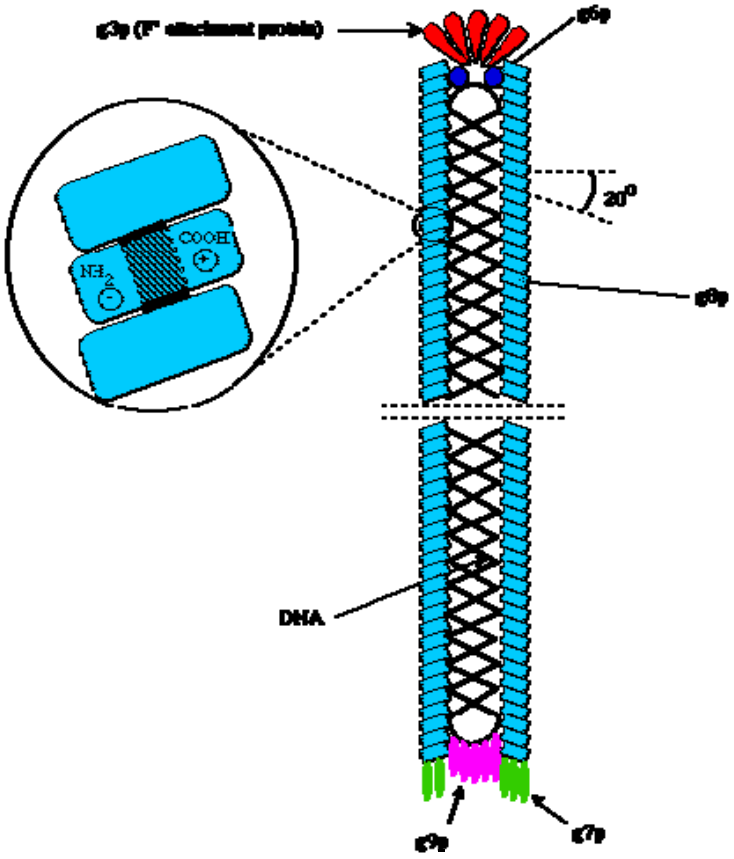


Fig. 1.4 Structure of M13 bacteriophage and distribution of coat proteins [44].

Both major and minor coat proteins contribute to the structural stability of the phage, but P3 is needed for host cell recognition and infection also. Besides, P3 is the most complicated coat protein and it has three distinct domains. The N-terminal domain initiates translocation of the phage DNA into *E. coli* during infection, while second domain recognizes the host cell by binding to the F pilus on the E.coli surface. The C-terminal domain attaches with other coat proteins to stabilize P3 protein [43].

M13 can only infect bacteria that carry an F or F-like conjugative plasmid that encodes the proteins that make up the F pilus. F pili are extracellular filaments found on F+ strains of Escherichia coli, where they are involved in the early stage of conjugation when cell-cell contacts are formed. The F pili are also the recognition sites for infectious bacteriophages, like fd and M13, where the initial recognition, mediated by the minor coat protein P3 of the bactariophage, is followed by phage DNA translocation into the bacterium. [45].

The DNA strand present in phage particle is named as the plus or + strand. After entry into the cytoplasm of the host cell, the (+) strand DNA is instantly coated with an *E.coli* single stranded DNA binding protein known as SSB. The SSB coating protects DNA from degradation [46].

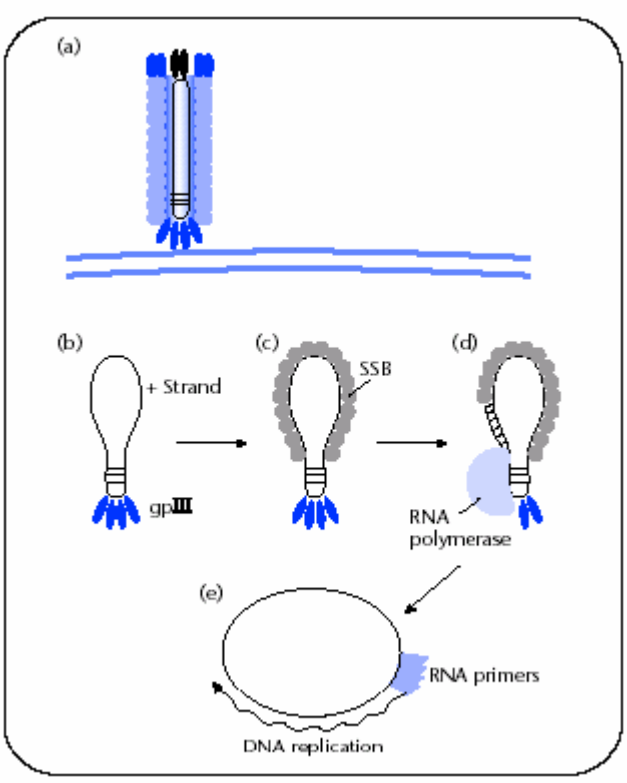


Fig. 1.5 The conversion of the M13 plus strand to a doublestranded DNA molecule. The plus strand enters the cell (a and b) with gpIII attached. It is immediately coated with host SSB (c). RNA polymerase synthesizes a short primer (d) and DNA polymerase synthesizes the minus strand [47].

The M13 plus strand is converted to a double-stranded molecule immediately after entry into *E. coli*. Synthesis of the complementary strand is performed by *E. coli*'s DNA synthesis machinery. The complementary strand is called as the minus or (—) strand. Minus strand is used as the template for mRNA synthesis and it is the template for the translation of the encoded M13 gene products. The SSB proteins fail to bind to ~60 nucleotides of the molecule. These nucleotides form a hairpin loop to prevent nuclease degradation. M13 gpIII is found associated with the hairpin loop. The hairpin loop is recognized by *E. coli* RNA polymerase as a DNA replication origin and is used to initiate transcription of a short RNA primer, in order to create the minus strand the RNA primer is extended by *E. coli* DNA polymerase III. The RNA primer is removed by the exonuclease activity of *E. coli* DNA polymerase I.

The gap is filled in by polymerizing activity of the same DNA polymerase. The final phosphodiester bond resulting in a covalently closed double-stranded circular M13 DNA is formed by *E. coli* ligase. The double-stranded form of M13 DNA is called as the replicative form (RF) DNA. The M13 gene II encoded protein is an endonuclease that nicks the plus strand of the RF DNA at a specific place to initiate the replication process for M13 RF DNA. Approximately 100 copies of M13 RF DNA are made. While the M13 DNA is being replicated, the coat proteins are being synthesized. When M13 gpV protein accumulates to sufficient levels, gpV blocks the synthesis of the minus strand, probably by displacing SSB on the plus strand and preventing the plus strand from being used as a template [48]. The plus strand is circularized.

1.3 Types of Phage Display Libraries

Phage display system described in this study is based on simple M13 phage vector for display of peptides as N-terminal fusions to the minor coat protein pIII [49]. In contrast to libraries of peptides fused to the major coat protein pVIII, which typically have on the order of 100 displayed peptides per virion, pIII libraries have a maximum valency of only five peptides per virion. As a result of this greatly reduced valency, pIII libraries are the best suited to the discovery of higher-affinity ligands. If the displayed peptide is sufficiently short (<100 residues), the infectivity function of pIII is not adversely affected, and all five copies can carry displayed peptides without appreciable attenuation of phage infectivity [50]. Commercial phage display libraries mostly contain 7, 12, 15 amino acids insertion.

1.4 The Substrate: Titanium Powder

Titanium has been selected as a substrate for this study. The biocompatible metallic materials used for manufacturing cement-less arthroprostheses are characterized by high fatigue stress and corrosion resistance; their surface finishing is extremely important for the bone integration of the implant [51]. It is well known that titanium and titanium based alloys exhibit high biocompatibility [52]. However, titanium and titanium alloys are still not sufficient for prolonged clinical use because the biocompatibility of these materials must be improved [53]. The development of surface modification is a real necessity for the biomedical community. Titanium with

a biocompatible coating such as hydroxyapatite has been successfully studied in recent years [54].

1.5 Adapting Phage Display Technology for Selecting Inorganic Binding Polypeptides

Although phage display is a usefull tool for identification of the peptides that take roles in protein protein interactions, it can be applied in selecting inorganic binding polypeptides. However, some morphological and specificity analyses must be performed on inorganic substrate. Density, surface area, average particle size and surface morphology are analysed in order to get a detailed information about substrate. Following the characterization of material, phage display screening procedure is applied to the inorganic material. This procedure based on affinity selection with 5 basic steps: Binding, washing, elution, amplification and purification. These steps may be called together as biopanning (Fig. 1.6) [55].

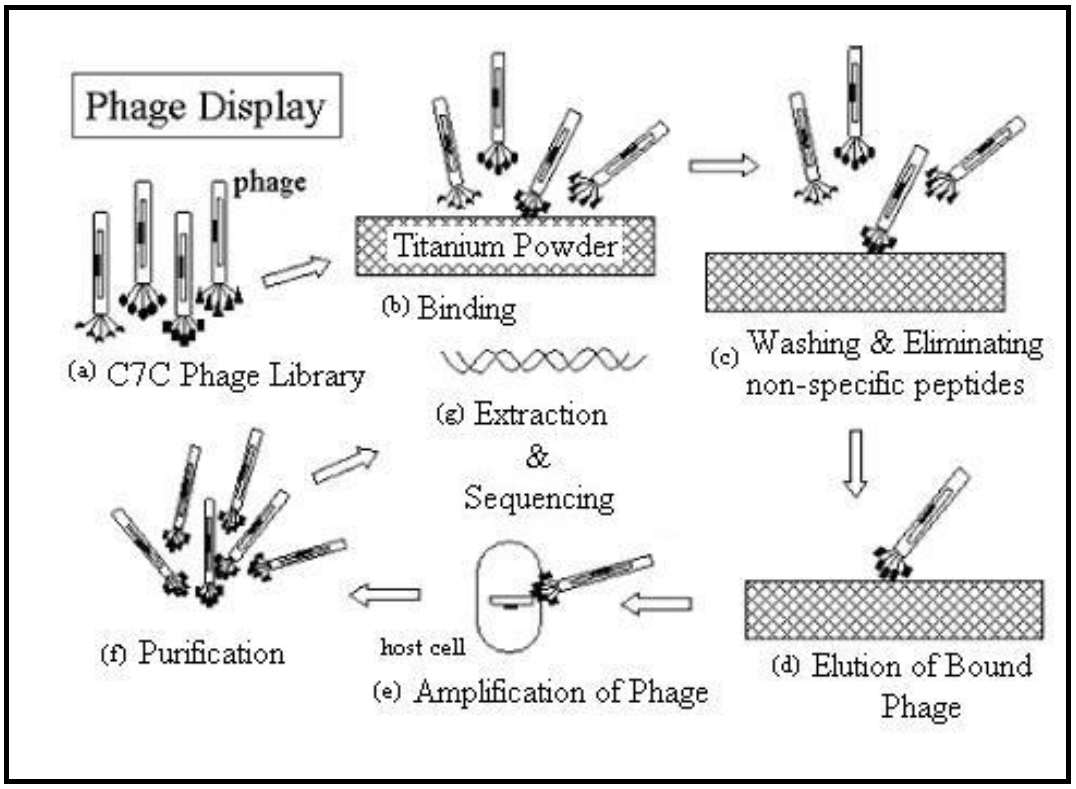


Fig. 1.6. Basic steps of biopanning. a) phage library, random insert in gpIII, b) binding of phage to the substrate, c) washing step to get rid of non specific phage, d) elution of specific phage, e) amplification of eluted phage, f) purification of amplified phage, g) Extraction of phage and DNA sequencing [55].

1.6 Selection of Phage Library

The Ph.D.-C7C^{TM} library consists of randomized 7-mer peptides, each flanked by a pair of cysteine residues. In the absence of reducing agents, these cysteines spontaneously form a disulfide bond, resulting in each peptide in the library being constrained in a disulfide loop. The Ph.D.-C7C^{TM} library contains 3.7 x 10^9 independent clones. Like the other libraries, the library is fused to pIII via the Gly-Gly-Gly-Ser spacer (Fig. 1.7). The Ph.D.-C7C library is 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 Ph.D.-C7C library is that the disulfide constraint may "freeze out" a conformation required for target binding [56].

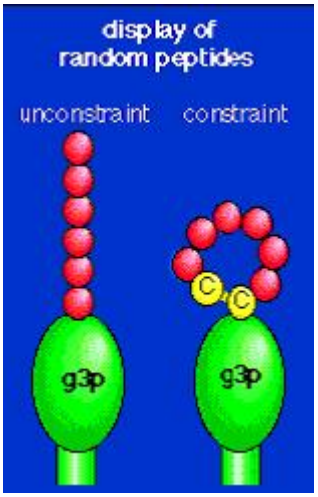


Fig. 1.7. Differences between constraint and unconstrainted library.

1.7 Synthetic Biology and Molecular Biomimetics

Synthetic Biology refers to the design and construction of new biological parts, devices and systems, and also re-design of existing, natural biological systems for useful purposes by inspiring from nature [1,57]. Molecular biomimetics, biological micro electro mechaniacl systems (bioMEMS), physics and bioinformatics can gather under the topic of synthetic biology. In this study phage display procedure is performed to design novel organic-inorganic hybrids. Molecular biomimetics is based on combination of traditional physics and biology applications. Molecular

biomimetics simultaneously offers three solutions to the development of heterofunctional nanostructures. The first is designing protein templates at the molecular level through genetics. This ensures complete control over the molecular structure of the protein template (DNA-based technology). The second is that surface specific proteins can be used as linkers and molecular errectors to bind synthetic entities, including nanoparticles, functional polymers, or other nanostructures onto molecular templates (molecular and nanoscale recognition) (Fig. 1.8).

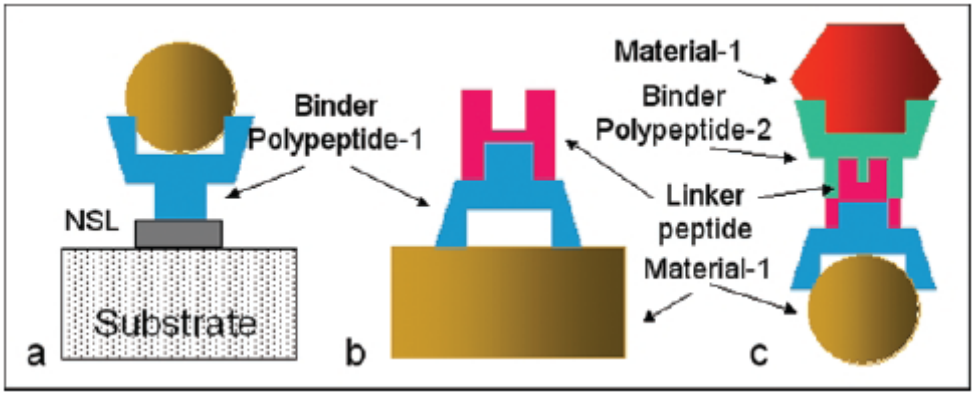


Fig. 1.8. Schematic illustration of the potential utility of inorganic-binding proteins as (*a*) linkers for nanoparticle immobilization, (*b*) functional molecules that assemble on specific substrates, and (*c*) heterofunctional linkers involving two (or more) binding proteins adjoining several nanoinorganic units. NSL, nonspecific linker [6].

Third, some complex nano, and possibly hierarchical structures, similar to those found in nature (self-assembly) can be designed by using the self- and co-assemble ability of biological molecules to into ordered nanostructures [1].

1.8 Aim of the Study

The aims of the study can be ordered as follow, selecting titanium specific C7C polypeptides and investigating their binding strength, identification of the polypeptide sequences from good binders which are suitable for biomimetic applications, determination of the cross specificity of strong binders with silica, silver and platinum, comparing the selected sequences with the sequence identified before. At the end, determined sequences may be utilized for nanobiotechnological studies.

2.MATERIALS AND METHODS

2.1. MATERIALS

2.1.1. Bacterial Strain- *E. coli* ER2738 Host Strain

E.coli ER2738 , F´ lacIq Δ(lacZ)M15 proA+B+ zzf::Tn10(TetR)/fhuA2 supE thi Δ(lac-proAB) Δ(hsdMS-mcrB) was used during the experiments. It is not a competent strain and it was purchased as 50 % glycerol culture within Ph.D.-C7C™ Phage Display Peptide Library Kit, (2003).

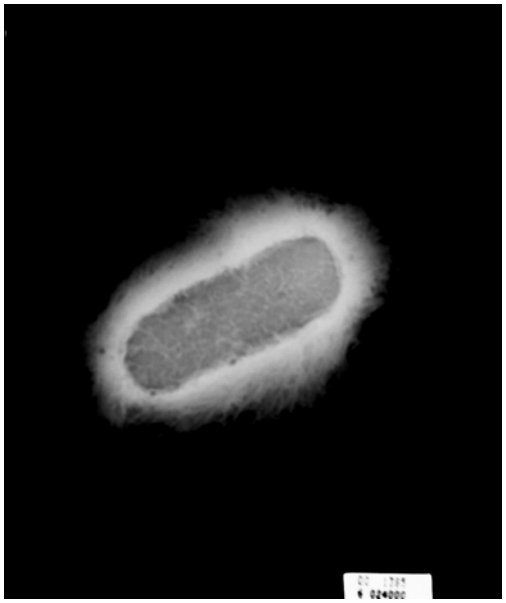


Figure 2.1. Image of a single *E.coli* ER2738 cell with 24,000X magnification and 2 s. exposure time. 2% Ammonium Molybdate solution was used for staining. Bar is 1μm.

2.1.2. Phage Display Peptide Library-Peptide C7C Phage Display Library (Ph.D.-C7C™)

Phage display peptide library is provided in a 100 μl, with 1.5 x 10^{13} pfu/ml. It is supplied in TBS with 50% glycerol. It has complexity of ~ 2.7 x 10^{9}. The library was purchased within Ph.D.-C7C™ Phage Display Peptide Library Kit, Catalog #E8120S.

2.1.3. Inorganic Targets: Titanium Powder

Titanium powder (Istanbul Technical University - Istanbul) was used as the substrate for the biopanning steps. Our SEM analyses of the powder show the size and morphology of the substrate.

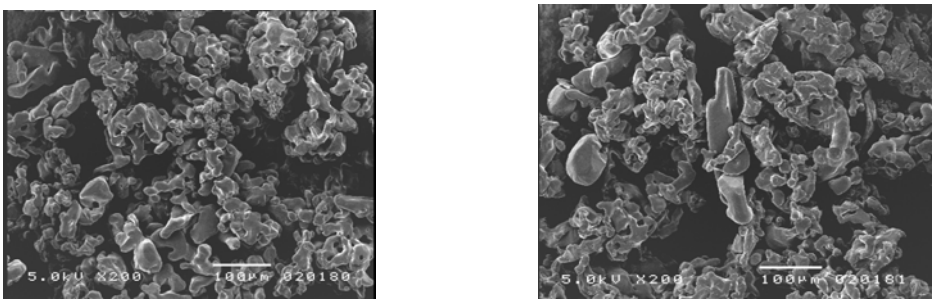


Fig. 2.2. Images of Titanium powder magnified at 5.0 kV x 200

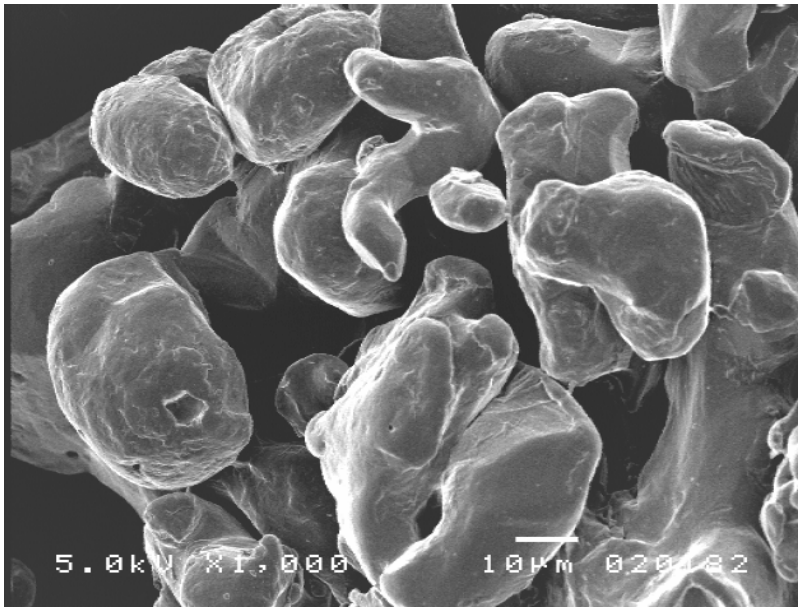


Fig. 2.3. Image of Titanium powder with detailed magnification at 5.0 kV x1000

2.1.4. Bacterial Culture Media

2.1.4.1. Luria Bertani (LB) Medium

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

2.1.4.2. LB Agar Medium

10 g tryptone (Acumedia Neogen Corporation USA/Canada), 5 g yeast extract (Acumedia Neogen Corporation USA/Canada), 5g NaCl (Riedel-de-Haen Seelze Germany) and 15 g bactoagar (Acumedia Neogen Corporation USA/Canada) were dissolved in distilled water and completed up to 1 lt and the pH was adjusted to 7.0-7.5 with 10 M NaOH and sterilized for 15 minutes under 1.5 atm at 121˚C. After autoclaving, tetracycline solution (Sigma Chemicals USA) (final concentration of 10 μg/ml) and X-gal/IPTG solution (final concentration of 40 μg/ml) (Fermentas/Sigma Chemicals USA) were added when the temperature of the medium was cooled down to 45-50 ºC. The medium was shaken properly and poured into the plates by avoiding any bubble formation (3.5 ml for small plates and 10 ml for big plates). After the medium was solidified in the plates, they were turned upside down and stored at 4 ˚C for later use.

2.1.4.3. Top-Agar Medium

10 g tryptone (Acumedia Neogen Corporation USA/Canada), 5 g yeast extract (Acumedia Neogen Corporation USA/Canada), 5 g NaCl (Riedel-de-Haen), 1 g $MgCl_2$ (Riedel-de-Haen Seelze Germany), 9 g LMP (Low Melting Point) agarose (Acumedia Neogen Corporation USA/Canada) were dissolved in distilled water and completed up to 1 lt and sterilized for 15 minutes under 1.5 atm at 121 ˚C. The medium was stored at room temperature and melted in microvawe as needed to pour onto the LB agar plates.

2.1.4.4. *E. coli* Overnight Culture

5 ml LB solution containing 1 mM $MgCl_2$ and tetracycline, was inoculated with *E. coli* ER-2738 stock (from -80°C). The culture was left in the shaker overnight at 37°C, 200 rpm.

2.1.5. Stock Solutions

2.1.5.1. Tetracycline-HCl Stock

20 mg/ml tetracycline-HCl (Sigma Chemicals USA) was dissolved in 95% ethanol. It was covered with aluminium foil to protect from sunlight and then stored at -20°C.

2.1.5.2. Xgal/ IPTG Stock

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

2.1.5.3. Detergent Stock

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

2.1.5.4. Glycerol Stock Solution

80 ml of 100% glycerol (Sigma Chemicals USA) was mixed with distilled water up to 100 ml total volume to have 80 % glycerol solution. It was sterilized for 15 minutes under 1.5 atm at 121˚C and then stored at room temperature.

2.1.5.5. $MgCl_2$ Stock Solution

1M $MgCl_2.6H_2O$ (Fisher Chemicals UK) was dissolved in distilled water and completed up to 100 ml. and sterilized with 0.2 μm single use syringe filter.

2.1.5.6. Gelatin Solution

10mM Tris-HCl pH 8.02, 150 mM NaCl, 0.05% Tween20 and 1% Gelatin dissolved in distilled water and completed up to 100 ml and sterilized with 0.2 μm single use syringe filter.

2.1.6 Buffer Solutions

2.1.6.1 PEG/NaCl

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

2.1.6.2. PC (Potassium Phosphate-Sodium Carbonate Buffer)

- **PC (no detergent):** 55 mM KH_2PO_4 (Fisher Chemicals UK), 45 mM Na_2CO_3 (Fisher Chemicals UK), 200 mM NaCl (Sigma Chemicals USA) were dissolved in

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

- **PC (containing 0.02% detergent):** 55 mM KH_2PO_4 (Fisher Chemicals UK), 45 mM Na_2CO_3 (Fisher Chemicals UK), 200 mM NaCl (Sigma Chemicals USA), 0.5 ml detergent stock solution were dissolved in distilled water and completed up to 500 ml and the solution was sterilized by using 0.2 μm single use syringe filter. The pH value was adjusted to 7.2-7.5.

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

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

Note: PC buffer can not be sterilized with autoclave because carbonate ions are converted to $C0_2$ due to high pressure. This causes an increase in pH up to 10. PC buffer is sterilized with 0.2 μm syringe filters.

2.1.6.3. Elution Buffers

- **Elution buffer I:** 0.2 M glycine (Merck Corporaiton USA) and 1mg /ml BSA (Sigma Chemicals USA) were dissolved in distilled water and completed up to 50 ml. and pH was adjusted to 2.2 with 10 M HCl and 0,1 M HCl. The solution was sterilized by using 0.2 μm single use syringe filter.

- **Elution buffer II:** Equal amount of elution buffer A and B are mixed and the solution is sterilized by using 0.2 μm single use sterile syringe filter.

- **Elution buffer A:** 0.2 M glycine (Merck Corporation USA) and 2 mg /ml BSA (Sigma Chemicals USA), 0.02% SDS were dissolved in distilled water and completed up to 50 ml. and the pH adjusted to 2.2 with 10 M HCl and 0.1 M HCl. The solution was sterilized by using 0.2 μm single use syringe filter.

- **Elution buffer B:** 1 M NaCl (Riedel-de-Haen Seelze Germany), 100 mM DDT (Sigma Chemicals USA), 7 mM TCEP (Sigma Chemicals USA), 100 mM ME were dissolved in distilled water and completed up to 50 ml.

2.1.6.4. Tris Buffer

5% casein (Sigma Chemicals USA), 10 mM Tris-base (Merck Corporation USA), 150 mM NaCl, 1% Tween 20 (Riedel-de-Haen Seelze Germany) were dissolved in 0.1 M NaOH. The pH was adjusted to 8.2 and distilled water was added up to 50 ml.

2.1.6.5. TBE (Tris / Borate / EDTA) Solution

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

2.1.7. Agarose Gel Electrophoresis

Agarose gels (1%) were made by dissolving 1 g agarose (Sigma Chemicals USA) in TBE and electrophoresed as described by Sambrook, J. [62]. 10X TBE buffer was prepared by dissolving 108 g tris-base, 55 g boric acid (Riedel-de-Haen Seelze Germany.) and 4 % (v/v) 0.5 M EDTA (Merck Corporation USA) at pH 8.0. Distilled water was added to complete up to 1 lt.

2.1.7.1. DNA Molecular Weight Markers

Lambda DNA/EcoRI+HindIII Marker 3 (MBI Fermentas, #SM0193) is used to determine the presence of single stranded 7222 base paired M13 DNA. Marker 3 can be used to discriminate 21226, 5148, 4973, 4268, 3530, 2027, 1904, 1584, 1375, 947, 831, 564, 125 base paired DNAs.

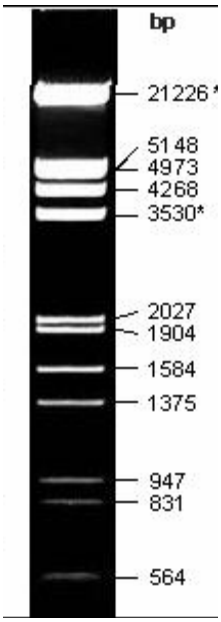


Fig. 2.4. Lambda DNA/EcoRI+HindIII Marker 3 (MBI Fermentas, #SM0193) 1.0% agarose, 0.5µg/lane, 8cm length gel, 1X TAE, 17V/cm.

2.1.7. Lab Equipments

Autoclaves **:** 2540 ML benchtop autoclave, Systec GmbH Labor-Systemtechnik.

: NuveOT 4060 vertical steam sterilizer, Nuve.

Balances **:** Precisa BJ 610C, order# 160-9423- 050, Precisa Instruments AG Dietikon.

: Precisa XB 220 A, order# 320-9204-001, Precisa Instruments AG, Dietikon.

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. 25

Deep freezes and refrigerators	**:** 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.
Glassware	**:** Technische Glaswerke Ilmenau GmbH.
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,
Laminar Flow Cabinet	**:** Ozge.
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.26

: UV-1601, Shimadzu Corporation.

Ultrasonic bath : Transsonic TP 690,elma®

Sterilizer : FN 500, Nuve.

Transilluminator : UV Transilluminator 2000, Catalog# 170-8110EDU, Bio- Rad.

Vacum Dryer : DNA Mini, JOUAN Nordic.

Vacuum Pump : Vacuum Station, Catalog# 165-5004, Bio-Rad.

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

2.2 METHODS

2.2.1. Phage Display Protocol

Phage display technique is based on incubating phage library (Ph.D.C7C™) with target substrate, and washing away the unbounded phage, and eluting the bound phage. Then the eluted phage was amplified by host strain ER2738. After amplification, phages were purified. Aim of these steps is to enrich the binding sequences pool. After 3 rounds, DNA sequencing was performed for the analysis of each individual clones.

2.2.2. Screening Procedure

As shown in the optimized screening procedure for selecting specific inorganic-binding polypeptides is given basically in (Figure 2.5.b). Biopanning is carried out by incubating a library of phage-displayed peptides with target, washing away the unbound phage, and eluting the specifically-bound phages. The eluted phage is then amplified and taken through additional binding/amplification cycles to enrich the pool in favor of binding sequences. After 3 rounds of biopanning, individual clones are selected and sequenced.

The screening protocol is given in the figure 2.5.b and follows as described here;

1) PhD-C7C library (New England Biolabs, MA) of M13 phage each displaying a different peptide sequence was used as the randomized peptide source.

2) Titanium powder was used as the substrate and cleaned prior to the biopanning rounds in the phage display screening method to select the titanium specific 7 amino acid polypeptides.

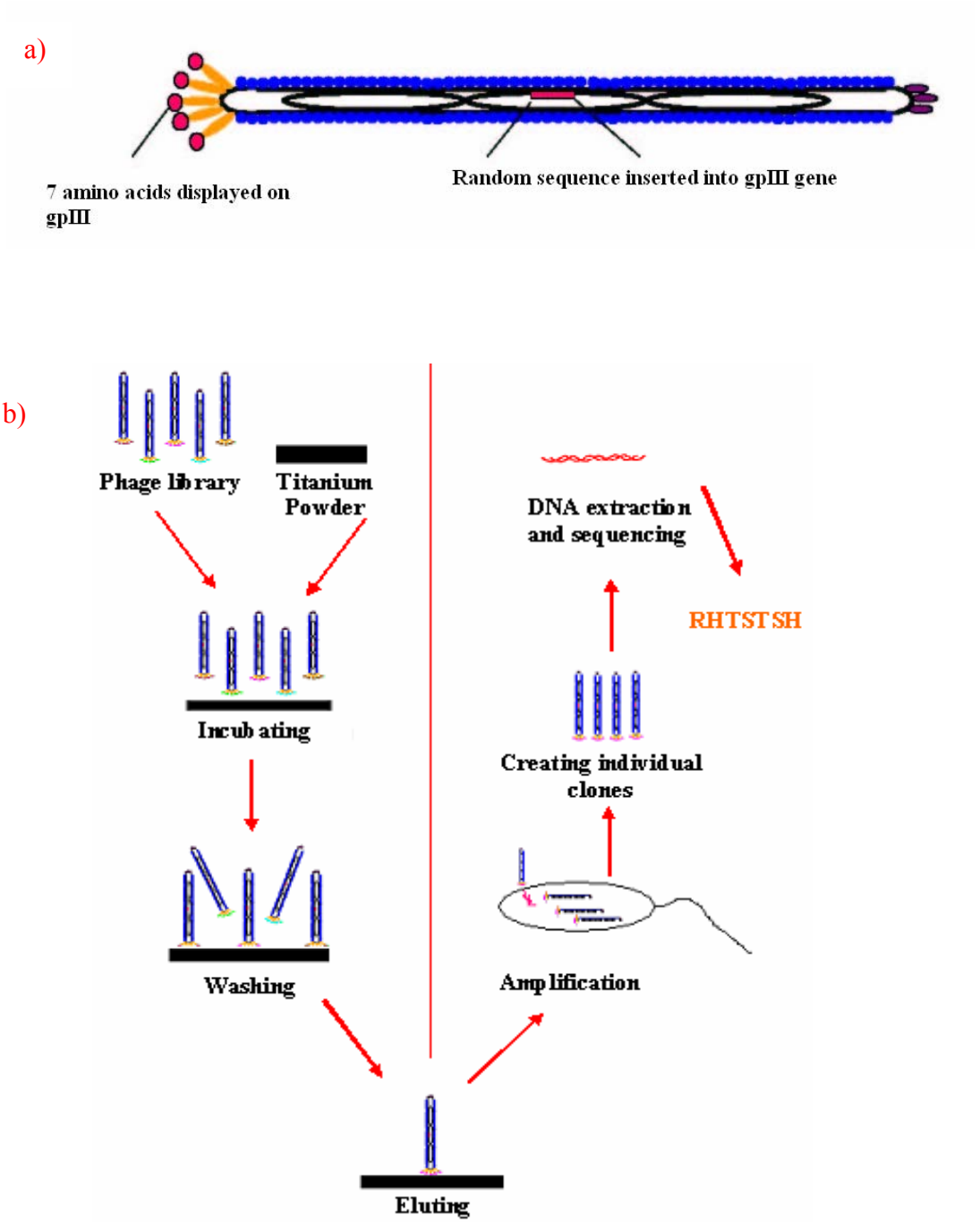


Figure 2.5. a) Structure of M13 phage and b) Phage display procedure

3) The phage library interacted with the surface of the substrate in potassium phosphate- sodium carbonate buffer (pH 7.5) containing 0.1% detergent solution

(20% Tween80 + 20% Tergitol). The detergent in the buffer was used to reduce the phage-phage interactions and by this way, the phages were expected to expose with the titanium surface individually.

4) Unbound phages were washed away by several washing steps using PC buffer (pH 7.5) containing 0.1% detergent solution. Washing cycles were repeated ten times, each one taking 30 min. The detergent concentration was increased gradually up to 0.5% as the number of the rounds is going up towards to 3.

5) Specifically bound phage was eluted from the surface with strong buffer solutions containing glycine-HCl (pH 2.2), 1 mg/ml bovine serum albumine (BSA), 0.02% sodium dodecyl sulphate (SDS), 1 M sodium chloride (NaCl), 100 mM dichloro-diphenyl-trichloroethane (DDT), 7 mM tris (chloroethyl) phosphate (TCEP) and 100 mM mercaptoethanol (ME). After 15 min treatment with the elution buffer, the eluted phages were transferred to a fresh tube and neutralized with tris-HCl (pH 9.1)

6) The eluted phage pool was amplified with *Eschericia coli* ER2738 host cell which is a robust F+ strain with a rapid growth rate and 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. The amplified phages were then plated on Luria Bertani (LB) plates containing 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (Xgal) and isopropyl-β-D-1-thiogalactosidase (IPTG).

7-9) Single plaques were picked and ssDNA isolated from these plates were sequenced. This is one round of biopanning. For the second round of panning, the eluted phage after the first round was used as the pool. After each round, the complexity of the pool decreases.

2.2.3. Biopanning Steps

2.2.3.1. Cleaning of the Titanium Powder

- In order to avoid the clump formation cleaning procedure was performed against titanium powder.
- ~ 3 gr titanium powder was sonicated for 15 min in absolute EtOH.
- The EtOH was discarded and distilled water was added into the substrate to sonicate the powder for another 15 min.

- After discarding distilled water from the eppendorf tube, PC buffer was added into the tube and the powder waited in the PC buffer containing 0.1% detergent overnight for the surface stabilization.
- After overnight, the titanium powder was taken into fresh PC buffer containing 0.1% detergent which was now ready to add library for biopanning rounds.

2.2.3.2. Washing Steps

- 20 mg of titanium powder was put into microfuge tube and 1 ml PC buffer containing 0.1 % detergent was added into the tube.
- 10 µl of phage display peptide library (Ph.D.-C7C™) was added into microfuge tube and placed on the running cylinder for 30 min rotation.
- After 30 min rotation, the supernatant was discarded and transferred into microfuge tube which was prelabelled as XTiRxS1*.
- Titanium powder was washed for 2 min with 1 ml PC containing 0.1 % detergent.
- The washing steps were repeated 6 times.
- The supernatants which were discarded at the end of the each washing steps were transferred into microfuge tubes which were prelabelled as XTiRxW1**, XTiRxW2, XTiRxW3, XTiRxW4, XTiRxW5, and XTiRxW6.
- After taking the supernatant out, microfuge tube containing titanium and phage peptide library was put on the running cylinder for 30 min rotation.
- The supernatant was discarded and transferred into microfuge tube XTiRxW7.
- 1 ml PC buffer containing 0.1 % detergent was added onto the tube and rotated for 30 min.
- The supernatant was discarded and transferred into microfuge tube XTiRxW8.
- Another 1 ml PC buffer containing 0.1 % detergent solution was added into the tube and left on rotation for overnight.
- Following overnight rotation, supernatant was discarded and transferred into microfuge tube XTiRxW9.

- 1 ml PC buffer containing % 0.1 detergent solution was added into the tube and rotated for 30 min on running cylinder.
- The supernatant was discarded and transferred into microfuge tube XTiRxW10.
- 1 ml PC buffer containing % 0.1 detergent solution was added into the tube and rotated for 30 min on running cylinder.
- The supernatant was discarded and transferred into microfuge tube XTiRxW11.

*: XTiRxS1: X→ Number of screening, Ti→ Titanium, Rx→Round number (here it is 1), S1→Supernatant Sample 1.

**: XTiRxW1: X→Number of screening, Ti→ Titanium, Rx→Round number (here it is 1), W1→Washing Sample 1.

The washing steps were performed to remove the non-specific phage to titanium surface. At the end of the all washing steps, phage which bound to titanium surface must be eluted from the surface. The elution steps come just after the washing steps and follow as written below. One round of biopanning contains washing, elution and purification steps. Basically all elution and purification steps are the same in each round but washing steps change as the number of the rounds increases. The detergent concentration in the washing buffers is increased gradually from 0.1% to 0.5% up to 3rd round.

2.2.3.3. Elution Steps

Following the washing steps, elution of the good binders from the surface of the titanium powder comes next. During the elution step, the eluted phages must be inoculated with the host bacteria for starting the amplification steps. Therefore, *E. coli* ER2738 liquid culture was needed for phage–host strain infection and amplification of phage prior to inoculating the phages with the host bacteria, we needed to have LB solution containing host bacteria of *E. coli* ER2738, growth of which reached OD_{600} value of 0.5. One night before starting the elution steps, 5 ml LB solution containing 10 µl 1M $MgCI_2$ (1:500), 5 µl tetracycline (1:1000) was incubated with a single colony which was withdrawn from the glycerol culture of the bacteria and inoculated LB waited overnight at 37ºC and 200 rpm. In the day of elution, 125 µl (1:400) *E. coli* ER2738 overnight culture was used to inoculate a fresh 50 ml LB solution containing 100 µl 1M $MgCI_2$ (1:500), 50 µl tetracycline

(1:1000) in 250 ml glass medium flask. 5 different 50 ml LB in 250 ml shake flasks were prepared for the elution steps. They were incubated until mid-log phase (OD_{600} ~ 0.5) at 37ºC and 200 rpm.

- After the last washing step, 1 ml of elution Buffer I was added onto the titanium powder in a clean microfuge tube and rotated for 15 min on running cylinder to elute the phage from titanium powder.
- The supernatant was transferred into microfuge tube XTiRxE1*.
- 800 µl supernatant was transferred into *E. coli* ER2738 culture (OD_{600} ~ 0.5) and incubated for 4.5 hours at 37ºC and 200 rpm.
- The remaining 200 µl supernatant was neutralized by adding 40 µl of tris, pH 9.1
- 1 ml of Elution buffer II was put into microfuge tube containing the titanium powder and put on the running cylinder for 15 min to elute the phage from titanium surface.
- The supernatant was discarded and transferred into microfuge tube XTiRxE2.
- 800 µl of the supernatant was transferred into *E. coli* ER2738 culture (OD_{600} ~ 0.5) and incubated for 4.5 hours at 37ºC and 200 rpm.
- The remaining 200 µl supernatant was neutralized by adding 40 µl of tris, pH 9.1
- 1 ml of Elution buffer II was put for the second time into microfuge tube.
- Microfuge tube which contained titanium powder and elution buffer II was put on the running cylinder for 15 min to elute the phage from titanium surface.
- The supernatant was transferred into microfuge tube XTiRxE3.
- 800 µl supernatant from XTiRxE3 was transferred into *E. coli* ER2738 culture (OD_{600} ~ 0.5) and incubated for 4.5 hours at 37ºC and 200 rpm.
- The remaining 200 µl supernatant was neutralized by adding 40µl of tris, pH 9.1
- 1 ml of Elution buffer II was put, for the third time, into microfuge tube.
- Microfuge tube containing titanium and elution buffer II was put on the running cylinder for 15 min to elute the phage from titanium surface
- The supernatant was transferred into microfuge tube XQRE3.

- 800 µl supernatant from XTiRxE3 was transferred into *E. coli* ER2738 culture (OD_{600} ~ 0.5) and incubated for 4.5 hours at 37ºC and 200 rpm
- The remaining 200 µl supernatant was neutralized by adding 40µl of tris, pH 9.1
- As a last step of the elution process, titanium powder itself was put into the LB solution containing *E. coli* ER2738 culture (OD_{600} ~ 0.5) and incubated for 4.5 hours at 37ºC and 200 rpm

*: XTiRxE1: X→ Number of screening, Ti→ Titanium, Rx→ Round number (here it is 1), E1→ Elution Step 1.

2.2.3.4. Purification Steps

After the elution steps, the eluted phages needs to be amplified and then the amplified phages must be isolated. The purification process of the amplified phages follows as written below.

- At the end of 4.5 hours of growth period, *E. coli* - phage culture (in 50 ml LB) was transferred into 250 ml sterilized centrifuge tubes.
- Samples were centrifuged at 8,000 rpm for 10 min.
- Supernatants were transferred into 250 ml sterilized centrifuge tubes.
- 8.33 ml (1:6) of PEG / NaCl solution was added into supernatant to precipitate phage and it was left for overnight at 4 ^{0}C.
- After overnight, samples were centrifuged clockwise (CW) and counter clockwise (CCW) at 8,000 rpm for 10 min.
- Supernatants were discarded and phage pellet was resuspended with 5 ml PC buffer (no detergent) by shaking to remove any remaining *E. coli*.
- Samples were centrifuged CW and CCW at 8,000 rpm for 10 min.
- Supernatant was transferred into 50 ml sterilized centrifuge tubes.
- 0.833 ml of PEG / NaCl (1:6) was added into the solution to precipitate phage and the solution was left for 2 hours at 4 ^{0}C.
- Samples were centrifuged CW and CCW at 10,000 rpm for 10 min.

- Supernatants were discarded and phage pellets were resuspended by pipetting or shaking with 1 ml PC buffer (no detergent) to remove *E-coli.*
- Samples were centrifuged CW and CCW at 10,000 rpm for 10 min and supernatants were transferred into sterilized microfuge tubes.
- 0.166 ml of PEG / NaCl solution (1:6) was added into the microfuge tube to precipitate phage, sample was vortexed for 5 sec, and left for 10 min at RT.
- Samples were centrifuged CW and CCW at 13,500 rpm for 1.5 min to get the compact phage.
- Supernatant was discarded and phage pellet was resuspended with 0.2 ml PC buffer (no detergent) by pipetting gently.
- Samples were centrifuged at 13,500 rpm for 1.5 min.
- Supernatant were transferred into sterilized microfuge tubes and stored at 4^0C.

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

2.2.4. Titers of the Selected Phages

2.2.4.1. Blue-white Screening Experiments

At the end of each biopanning round, blue-white screening experiment was carried out to determine the phage titers. Basically, amplified phages were grown in the plates which had Xgal, IPTG and tetracycline. 50 µl Xgal/IPTG from 20 mg/ml stock, 100 µl 1M $MgCl_2$ (1:500), 50 µl tetracycline (1:1000) were put into 50 ml liquid warm LB agar in 100 ml glass medium flask and it was poured into plastic sterile petri dishes. Plates were wrapped with parafilm and stored at 4^0C at dark for a maximum of 1 month. Purified phages needed to be serially diluted to estimate phage titers.

2.2.4.2. Preparation of Diluted Phage and Plating

190 µl PC buffer (no detergent) was put into the first well in Elisa plate. 180 µl PC buffer (no detergent) was put into other wells to dilute the sample as in Figure 2.6. 10 µl phage stock solution was put in the first well having 10^{-1} dilution. Phage and PC buffer were mixed by pipetting. 20 µl sample was taken from the first well and put into the second well to have totally 200 µl of solution mix having 10^{-2} dilution level

of the phages. The remaining serial dilutions were done the same way as it is labeled in Figure 2.5.

One night before plating, overnight culture was prepared to be used in the day of the plating. 5 ml LB solution containing 10 µl 1M $MgCI_2$ (1:500), 5 µl tetracycline (1:1000) was incubated with 12.5 µl *E. coli* ER2738 from overnight culture in 50 ml falcon tube. It was incubated until mid- log phase (OD_{600} ~ 0.5) at 37ºC and 200 rpm.

Xgal/IPTG-tet plates were taken out from 4°C refrigerator and put at RT and labeled. Previously melted top agar was placed in the water bath at ~ 50 °C to prevent solidification of the top agar till adding the diluted phage solutions.

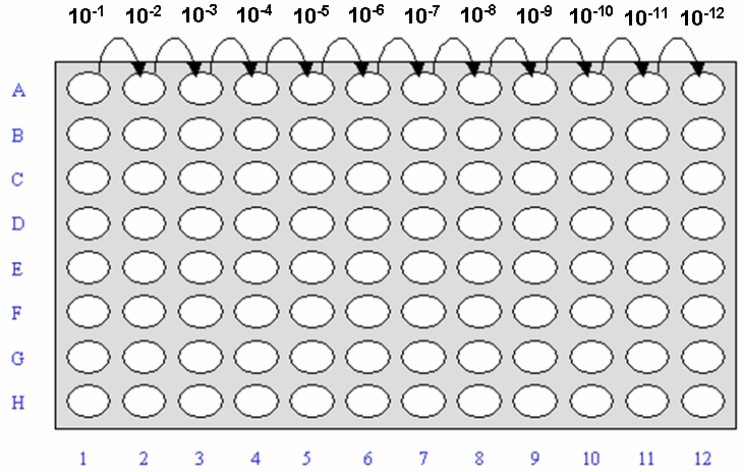


Fig. 2.6. Making the serial dilutions on the Elisa plate

20 µl diluted phage solution was mixed with 180 µl LB solution containing *E. coli* cells with OD_{600} ~ 0.5 resulting one more dilution of the phages. The resulting solution was mixed with warm LB agar (0.5 ml for small Petri dishes and 3.5 ml for big Petri dishes) in 15 ml falcon tubes and vortexed. The phage and bacteria mixture solution were poured onto the plates and waited for 5-10 min for the solidification of the top agar. All petri dishes were inverted and incubated at 37°C for 8-14 hours. After incubation period blue plaques were seen visually on the plate as in Figure 2.7.

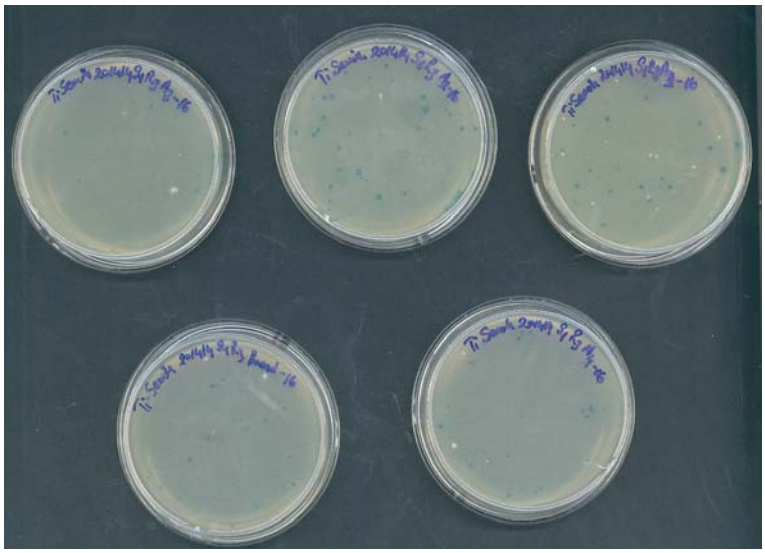


Fig. 2.7. Shows the individual colonies at the end of incubation in blue-white screening

2.2.4.3. Phage Titers for Each Round

At the end of the each round, phage titer was estimated by doing blue-white screening experiment. Amount of phage after each elution steps determines the amount of the phage which will be used from that elution step to form gene pool for the next round. Table 2.1 shows the titers after amplification of the phages from each elution steps, and the amount of the phage from that step to use forming the gene pool for the next round.

Table 2.1. Phage titers and amount of phage used for the next round after each round. (D: Dilution number, A: Amount of phage solution used for the phage pool for the next round as microliter)

	1st Round		2nd Round		3rd Round	
Elution	D	A	D	A	D	A
A1	10^{-12}	100	10^{-12}	100	10^{-13}	100
A2	10^{-12}	50	10^{-12}	50	10^{-15}	50
A3	10^{-14}	50	10^{-12}	50	10^{-9}	50
A4	10^{-13}	50	10^{-15}	50	10^{-11}	50
B	10^{-15}	50	10^{-17}	50	10^{-13}	50

2.2.4.4. Saving Clones for Sequencing

After estimation of titers by plating on small plates, eluted phages from each round were plated on the big plates to pick plaques to be saved for sequencing.

2.2.4.5. Preparation of Storage Plates

- 200 µl PC buffer containing 0.02 % detergent solution was put into each well of a sterile 96-well Elisa plate.
- At the end of the fifth round, 600 individual plaques have been picked.
- Elisa plate containing phage clones was placed into the incubator at 60 ^{0}C for 45 min.

2.2.4.6. Preparation of Glycerol Stock of Phage

- 60 µl sterilized 80% glycerol solution was put into each well of a fresh 96-well plate. Two sets of 96 well Elisa plate have been prepared, one for -20 ºC and one for -80 ºC stocks. (Overall glycerol concentration was kept as ~ % 50 in stocks.)
- 50 µl clone from the storage plate was added into the glycerol containing plates.
- Plates were covered by parafilm and placed into the freezers, -20 and–80 ^{o}C.

2.2.5. Single Stranded DNA Isolation

2.2.5.1. M13 Single Strand DNA Isolation

For the sequencing of the individual sequences, ssDNAs of the picked sequences needs to be isolated. QIAprep ® Spin M13 kit (QIAGEN, Catolog #27704) was used for the isolation of M13 phages which were previously stored at -20 ºC stock. The procedure is given schematically in Figure 2.8 and written below.

1) 50 µl from each individual sample which belongs to only one phage plaque was taken from the glycerol stock and added into 2 ml *E. coli* ER2738 culture which had been incubated until mid-log phase (OD_{600} ~0.5) in 2 ml microfuge tubes. Phage and bacteria mixture was incubated for 4.5 hours for *E. coli* ER2738 infection by phage and amplification.

2) Culture containing microfuge tubes were centrifuged at 5000 rpm for 15 min at room temperature. Supernatant containing M13 bacteriophage was transferred into a fresh reaction tube. During transferring the supernatant, bacterial pellet was not disturbed. Any carryover of bacterial cells result in contamination of the M13 precipitation with bacterial chromosomal DNA or double-stranded bacteriophage RF DNA.

3) Buffer MP was added 1/100 volume (i.e.10µl per 1 ml of phage supernatant.) onto the supernatant in the reaction tube. It was mixed by vortexing and incubated at room temperature for at least 2 min. During this step, bacteriophage particles were precipitated from the culture medium.

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

5) Reaction tube was centrifuged for 15 s. at 8,000 rpm and discarded flow-through from collection tube. Here, intact bacteriophage was retained on the QIA prep silica-gel membrane.

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

7) 0.7 ml MLB buffer was added for M13 lysis and binding, to the QIAprep spin column and centrifuged for 15 s at 8,000 rpm. This step creates appropriate conditions for binding of the M13 DNA to the QIAprep silica-gel membrane. Bacteriophage lysis begins.

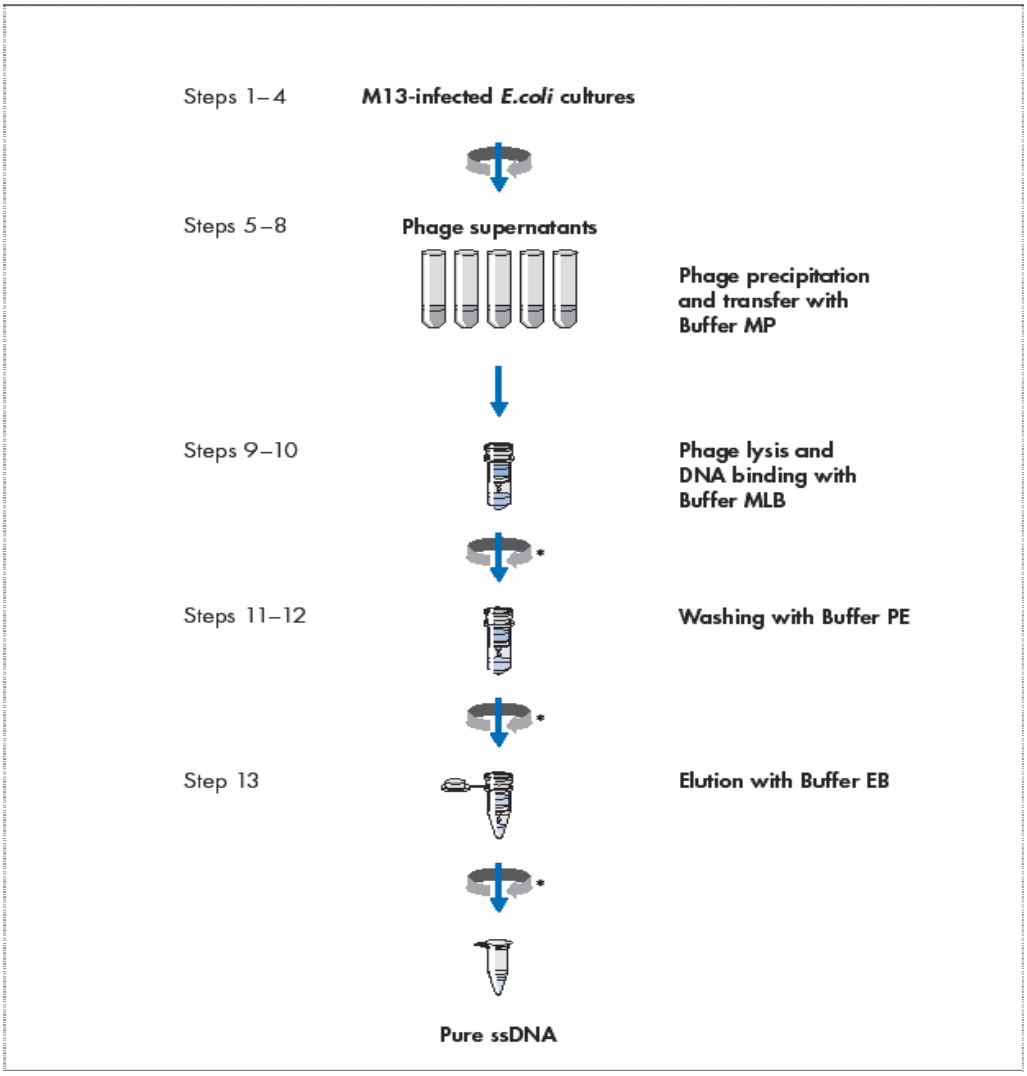


Figure 2.8. M13 ssDNA isolation procedure (QIAGEN, Catolog #27704).

8) Another 0.7 ml MLB buffer was added into the QIAprep spin column and incubated for 1 min at room temperature to lyse the bacteriophage completely. QIAprep spin column was centrifuged for 15 s at 8,000 rpm. M13 single-stranded DNA was released from bacteriophage particles and adsorbed to the QIAprep to the silica gel membrane.

9) 0.7 ml Buffer PE was added into the QIAprep column and centrifuged for 15 s at 8,000 rpm to remove the residual salt.

10) Buffer PE was discarded from collection tube and QIAprep spin column was centrifuged for 15 s at 8,000 rpm to remove residual buffer PE. It is important not to dry the QIAprep membrane quick microcentrifugation step. This prevents residual ethanol from being carried over into subsequent reactions. QIAprep spin column was placed in a clean 1.5 ml microcentrifuge tube. 100 µl EB buffer (10 mM tris.Cl, pH

8.5) was added onto the center of the column membrane to elute the DNA. Incubation of elution buffer in the QIAprep spin column significantly increases the recovery of single-stranded M13 DNA molecules, which adsorb tightly to the silica membrane.

11) After 10 min of waiting at RT, the tube is centrifuged for 30 sec at 8,000 rpm to collect the isolated ssDNA samples in the tube.

2.2.5.2. Measurement of DNA concentration by spectrophotometry

Each 2 or 4 µl DNA sample was diluted with 998 or 996 µl dH_2O (1:500, 1:250). The samples were put into the quartz cuvette. The absorbance of DNA was measured at 260 nm and at 280 nm to quantify the amount of DNA. The purity of DNA samples was determined by calculating the ratio of absorbent at 260/280 nm.

2.2.6. Sequencing of DNA Sample

2.2.6.1. PCR Conditions for DNA Sequencing

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

Table 2.2. PCR conditions for DNA sequencing.

PCR ingredients	Amount
Big dye reaction mix*	1µl
5X sequencing buffer / dH_2O	2 µl
Template ssDNA	2 µl (150 ng)
Primer (-96 sequencing primer) **	3.2 µl (3.2 pmol)
dH_2O	1.8 µl
Total volume	**10 µl**

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

** –96 gIII sequencing primer **(**5´- CCC TCA TAG TTA GCG TAA CG –3´, 100 pmol, 1 pmol/µl) was purchased from New England Biolabs Ph.D.-C7C™ Phage Display Peptide Library Kit

PCR conditions for cycle sequencing was;

95 ºC	2’	35 cycle
95 ºC	10’’	
55	10’’	
60	4’	

2.2.6.2. PCR Product Purification

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

• 52 µl mixture was put into each PCR product and all samples were put on dry ice for 30 min.

• All samples were centrifuged for 30 min. at 14000 rpm.

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

• All samples were centrifuged for 30 min. at 14000 rpm.

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

• Samples were put first 95^{o}C and then -20^{o}C for 5 min. to denature the samples.

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

2.2.7. Immunofluorescence Microscopy Experiments

2.2.7.1. Cleaning of the Titanium Powder

- Around 100 mg of titanium powder was weighted and put into pre-weighted 1.5 ml microfuge tube.
- 100 µl dH_2O and 900 µl CH_3OH/acetone (1:1) mixture were added into the tube. The powder was dissolved gently by pipetting.

- The sample was vortexed for 5-10 min to observe the forming clumps and then sonicated for 20 min in an ultrasonic bath to break the clumps.
- The powder was vortexed quickly to resuspend and centrifuged at 200 g for 1.5 min (CW and CCW).
- The supernatant was removed and 1 ml 50 % isopropanol was added onto the powder.
- Powder was vortexed for 5-10 min and then sonicated for 20 min in the ultrasonic bath.
- The powder was vortexed quickly to resuspend and spinned down at 200 g for 3 min (CW and CCW).
- The supernatant was removed and 1 ml 0.5 % PC buffer was added onto the powder.
- The powder was first vortexed for 5-10 min and then sonicated for 60 min.
- The powder was vortexed quickly to resuspend.
- The powder was centrifuged at 200 g for 1.5 min (CW and CCW).
- The supernatant was removed and 1ml 0.5 % PC buffer was added onto the titanium powder.
- 100 µl of powder solution was transferred into each sterile microfuge tube.
- Powder was spinned down at 200 g for 1.5 min.
- Powder was first washed twice with dH_2O and then with isopropanol.
- Samples were dried under the vacuum.
- All samples were weighted to determine the amount of powder into the each tube.

2.2.7.2. Fluorescence Microscopy Experiment Procedure

Following the sequencing results, each phage clone was checked for binding and specificity on titanium. The binding of the phage samples on the titanium suface can be observed visually under the fluorescence microscopy indirectly. Zenon complex (Zenon Alexa, Molecular Probes Inc) which has labeled Fab fragment bound on IgG

is used to label the phages for indirect observation. The procedure is basically as follows;

- A proper amount of previously cleaned titanium powder was incubated with 5 µl phage (PFU/ml 10^{13}) in PC buffer containing 0.1 % detergent and rotated overnight.
- After attaching phage to the powder, this conjugate was incubated with tris buffer containing gelatine blocking agent for 10 min, in order to coat the powder and occupy any binding sites that antibody might attach to.
- Each phage sample was labeled according to the immunolabelling procedure as in Figure 2.9. First, Anti-M13 pIII monoclonal antibody (Amersham Biosciences) which is specific to m13 gp8 protein and Alexa-Fluor conjugated secondary antibody fragments (Zenon Alexa, Molecular Probes Inc.) that contains flourescence dye were incubated (1: 5 ratio) for 30 min in a microfuge tube covered by aluminium foil.

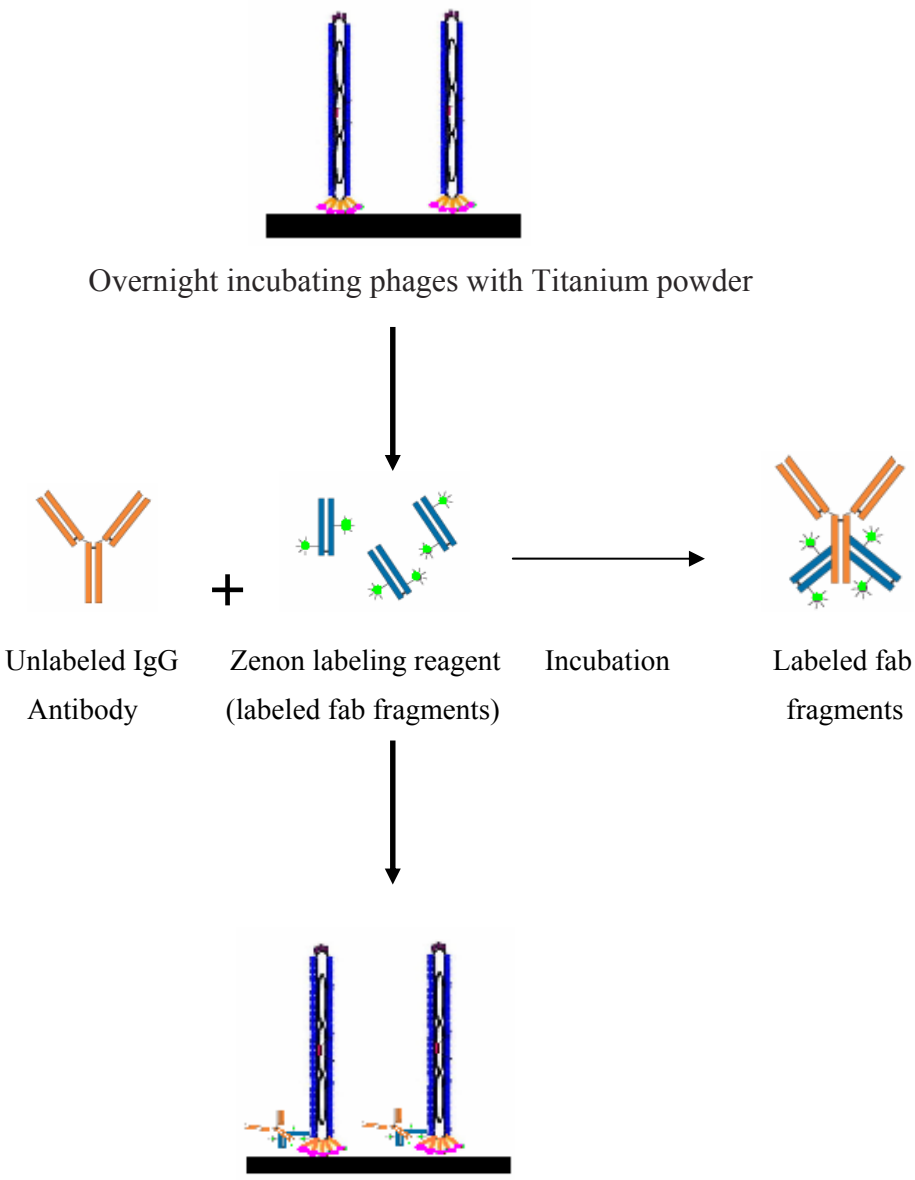


Figure 2.9. Labeling procedure for fluorescence microscopy (not scaled).

- While Zenon mixture was being prepared, phage-titanium powder solution was centrifuged at 1,000 rpm for 1.5 min, the supernatant was discarded and 0.1 % PC buffer was added on the sample for 15 min rotation on running cylinder to wash unbound phage out of the solution.

- The solution was centrifuged again at 1,000 rpm for 1.5 min and the supernatant was discarded.

- 3 µl Zenon dye complex and 497 µl PC buffer containing no detergent were put on the phage–titanium solution and incubated for 30 min on running cylinder to let phage- mixture interaction. The tube was covered with aluminium foil.

- The solution was centrifuged again at 1,000 rpm for 1.5 min and the supernatant was discarded.
- 0.5 ml 0.1 % PC buffer was added into the tube and the powder was washed 3 times by pipetting.
- The mixture solution was centrifuged again at 1,000 rpm for 1.5 min and the supernatant was discarded.
- Another 0.5 ml 0.1 % PC buffer was added into the tube and the powder was washed 3 times by pipetting.
- The mixture solution was centrifuged again at 1,000 rpm for 1.5 min and the supernatant was discarded.
- 30 µl 0 % PC buffer was added onto the powder solution and visualized by fluorescence microscopy (BX60 Olympus Corporation) at 20X magnification under WIB filter.

3. RESULTS AND DISCUSSION

3.1 Identification of the Binder Peptides for Titanium and Their Sequence Based Analysis

This study focuses on selection of disulfide-constrained heptapeptides exhibiting affinity towards titanium powder surface using phage display technique. Amorphous titanium powder was used as a substrate for the selection of titanium-specific 7 amino acid polypeptides from C7C phage display library. During the selection of titanium binders by phage display technique, different buffer solutions with different pH ranges were used. These buffers can cause oxide layer formation on the surface therefore material characterization studies were performed in order to obtain information about particle morphology and average particle size.

Following material characterization, screening was carried out to select titanium binding polypeptides. During screening 3 rounds of biopanning experiments were carried out. In biopanning steps, washing buffer strength gradually increased due to the round numbers to select better binders after each round. During the washing steps, non-specific binders were removed from the titanium powder surface. After the washing steps, chemical elution cycles were performed with low pH buffers to remove the specific phage bound onto the material. Bound phage recovery approach was also applied after elution steps to recover the phage remained on the material.

Phage colonies were selected to isolate the ssDNA from both chemical elution and recovered bound phage. After biopanning, 10, 21 and 20 sequences were studied from first, second and third rounds respectively. As a result of 3 rounds of biopanning, 51 sequences were identified as the titanium binding polypeptides.

In the next step sequence characterization was performed. Characterization studies could be divided into three different categories. Physicochemical properties of selected polypeptides were analyzed and compared with respect to their pI, MW, and charge in first characterization category. In the second categorization section statistical amino acid analyses of each selected sequences were carried out. Analyses

were based on calculation of overall amino acids distributions. The amino acid distributions of selected polypeptides were compared with the observed frequency of amino acids in the commercial phage library we used. Expression levels of observed amino acids were analyzed by comparing the observed sequences in the library and a relative abundance graph was prepared to observe the low or high expression levels of each amino acids. Last step of characterization employed semi-quantitative fluorescence microscopy studies. Sequences were analyzed by means of their affinities towards titanium. Individual phage colonies with determined DNA sequences were incubated with titanium powder, then the phage were labeled with monoclonal antibody IgG and Alexa Fluor secondary antibody fragment. This approach was the best and easiest way of categorize the sequences by their affinities to titanium. Depending on fluorescence microscopy results, binders were grouped as strong, moderate and weak binders. The sequences that bind to titanium with an affinity of 0%-10% were classified as “weak binders”, those that bind to titanium with an affinity of 10%-40% were classified as “moderate binders” and those that bind to titanium with an affinity of 40%-100% were called “strong binders”. Affinity percantages were calculated by software programme Image Pro Plus.

Selection of the titanium binding polypeptides did not grant us with the information about the specificity of these polypeptides for titanium metal and not for the others. In order to determine the level of specificity of these peptides for titanium, cross specificity fluorescence microscopy experiments were performed on platinum, silver and silica. Cross specificity experiments were perrformed with platinium, silver and silica. After cross specificity studies, strong and specific titanium binding polypeptides were determined.

3.1.2 Characterization of Titanium Powder

Characterization studies can be grouped into 4 types: Surface area studies, density measurements, particle size measurements, morphology observations.

3.1.2.1 Surface Area Calculation Results

Surface area was measured by Nova Station B (Quantachrome Instruments) as 13.78 m^2/g.

3.1.2.2 Density Measurement Results

3 runs were performed to measure density.

Table 3.1. Utilized volume of titanium and density for each rounds.

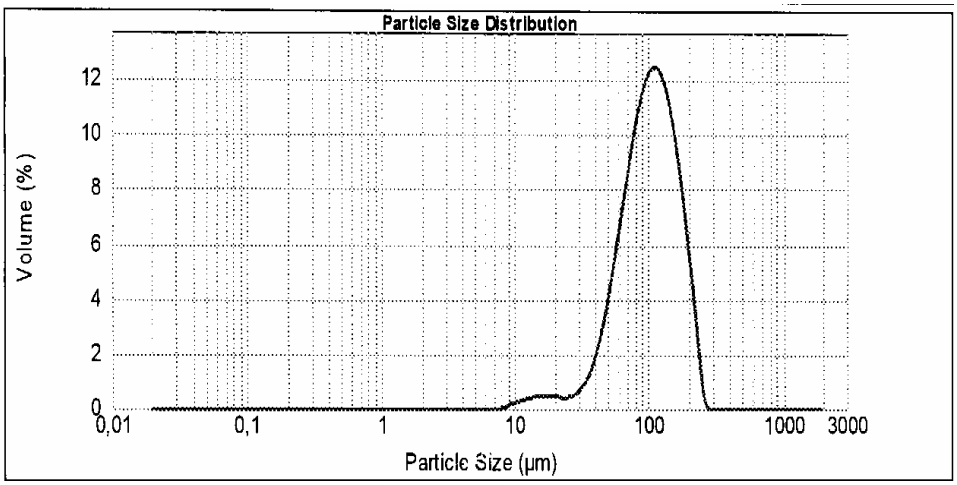
Run	Volume (cc)	Density (g/cc)
1	0,1849	4,3426
2	0,1869	4,2967
3	0,1931	4,1578

After performing 3 runs with titanium powder, average density measurement was found 4.2643 g/cc.

3.1.2.3 Particle Size Results

Particle size was measured by Hydro 2000 G(A) (Malvern Instruments, UK)

Table 3.2. Particle size distribution of titanium powder.



It was found that titanium powder contains different size of particles between 10µm-220µm. Average size of powder was calculated as 103µm.

3.1.2.4 Surface Morphology Analyses Results

Titanium Powder Morphology observation was performed at 2 magnification scale with JEOL JSM T-330 Scanning Electron Microscope (SEM) (JEOL, Japan).

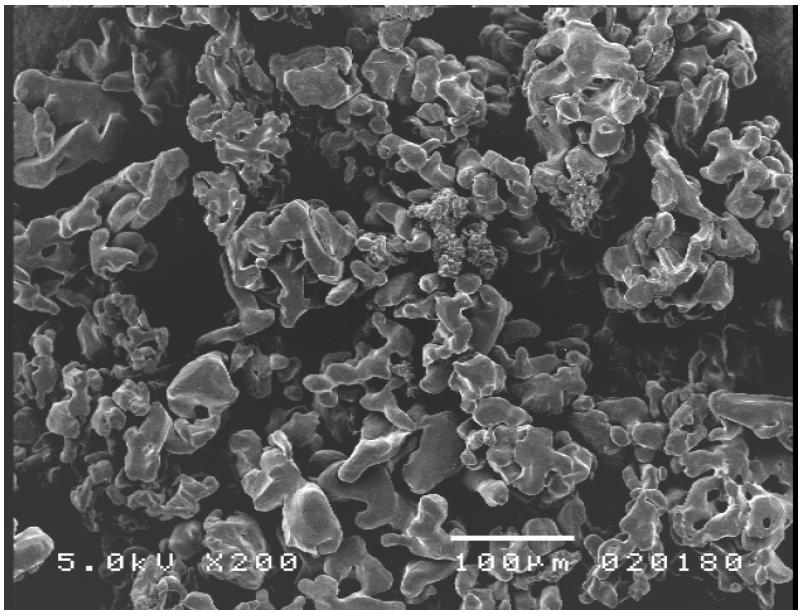


Fig 3.1. SEM image, magnification of titanium powder at 200X.

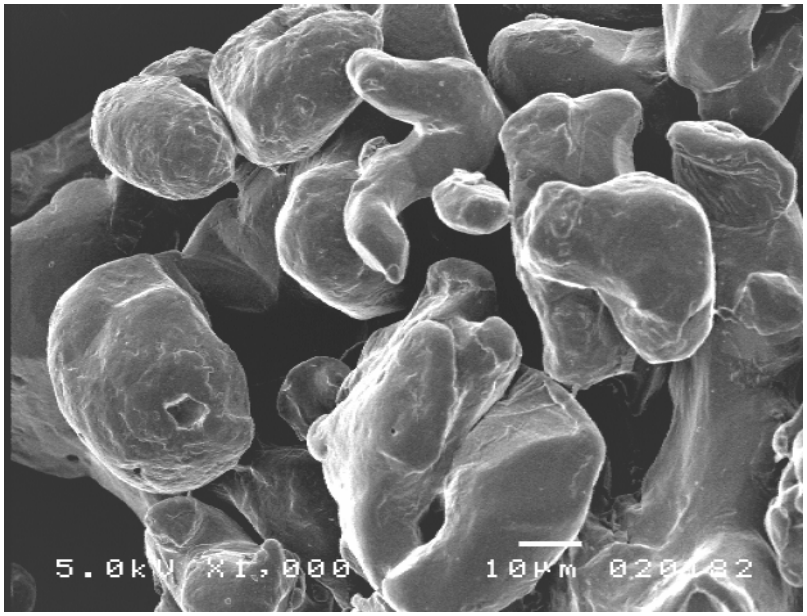


Fig 3.2. SEM image of titanium powder magnification at 1000 X.

3.2 Screening Results

3 rounds were performed to obtain titanium specific polypeptides in screening. Bound phage recovery was also studied at each round. Among a total of 51 sequences, 18 were from bound phage recovery. Physicochemical properties of each polypeptide were also calculated; isoelectric point (pI) and molecular weight (MW) were calculated by pI/MW computing tool [58]. The charges of the polypeptides

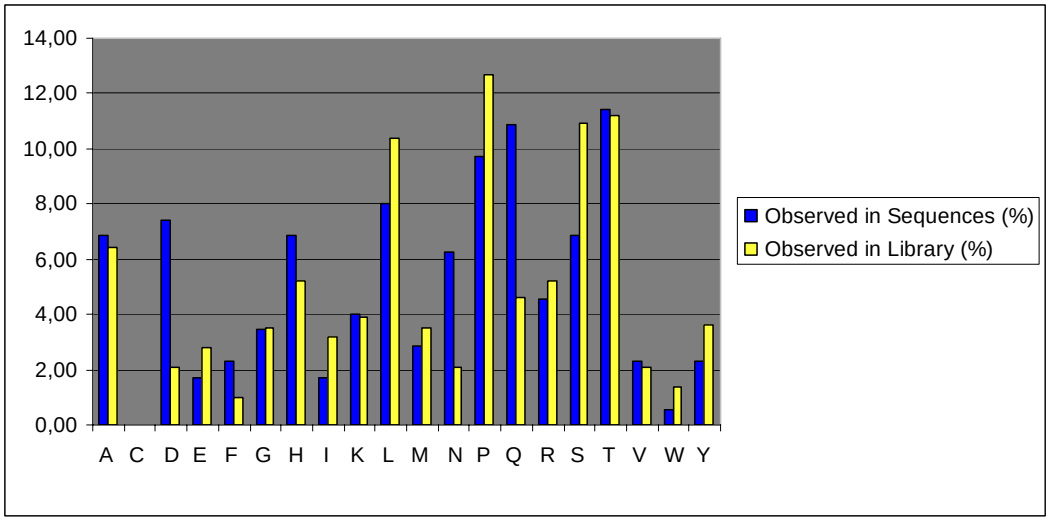
were calculated by subtracting the number of basic residues (**R** and **K**) from the number of acidic residues (**D** and **E**).

Table 3.3. 7 amino acids polypeptide sequences obtained from 3 rounds.

Amplification #	Sequence	Isoelectric point	MW	Charge
1st Round A1	S T T Q H S L	6,46	772	0
1st Round A2	D I S P P S H	5,08	752	-1
1st Round A2	N S S A L Q L	5,52	731	0
1st Round A3	N M A L S Q G	5,52	720	0
1st Round A3	P N V R T E A	6,43	785	-2
1st Round A3	N D K K D L L	5,96	844	0
1st Round A4	T P L L N T D	4,3	772	-1
1st Round Bound	H S L D S F N	5,08	818	-1
1st Round Bound	N T H A N H H	7,02	829	0
1st Round Bound	I D G D Q R S	4,21	789	-1
2nd Round A2	L H D T R S H	6,92	864	0
2nd Round A2	S T A Y P L N	5,24	764	0
2nd Round A2	A P A Q H K V	8,8	749	0
2nd Round A2	S V E E R G S	4,53	762	-1
2nd Round A2	N T A T L P S	5,52	702	0
2nd Round A3	I H Q D S H A	5,97	806	-1
2nd Round A3	P T A D S N M	3,8	735	-1
2nd Round A3	Q H G M T R Q	9,76	856	1
2nd Round A3	A P T A G L P	5,57	625	0
2nd Round A3	P L N D Q Y F	3,8	895	-1
2nd Round A4	P V T I H S S	7,17	739	0
2nd Round A4	A E Q L R P P	6,05	809	0
2nd Round A4	A Q V N T Y D	4,3	809	0
2nd Round A4	S T Q D P K P	5,55	771	0
2nd Round A4	R D P Q T F T	5,84	863	0
2nd Round Bound	T S R S E T M	5,66	810	0
2nd Round Bound	M P T S T L P	5,28	745	0
2nd Round Bound	P W S F Q T P	5,96	861	0

2nd Round Bound	P G T T N L N	5,96	715	0
2nd Round Bound	H D R H T P L	6,92	874	0
2nd Round Bound	R D Q T P A Y	5,84	849	0
3rd Round A1	H Q A H T S Q	6,92	807	0
3rd Round A1	L T R S S S H	9,76	787	1
3rd Round A1	H G Y S N S Q	6,74	791	0
3rd Round A2	M S Y V K P G	8,34	780	1
3rd Round A2	K A P L N S M	8,75	759	1
3rd Round A3	H S H N L H N	7	858	0
3rd Round A3	S G A P S A L	5,24	601	0
3rd Round A3	D L E N N R M	4,37	890	-1
3rd Round A4	H G N L H A T	6,92	749	0
3rd Round A4	K T T P Y A H	8,6	816	1
3rd Round A4	M G N K T P K	10	774	2
3rd Round Bound	L P H N N P Q	6,74	819	0
3rd Round Bound	P M S P H A L	7,17	752	0
3rd Round Bound	P G P Q Q M T	5,96	758	0
3rd Round Bound	N R Q D L P H	6,74	879	0
3rd Round Bound	F E V G P L H	5,24	797	-1
3rd Round Bound	A P L P N A S	5,57	668	0
3rd Round Bound	S T V S P N T	5,24	704	0
3rd Round Bound	S N S P V S S	5,24	676	0
3rd Round Bound	D L M T T H P	5,08	813	-1

Table 3.4. Shows the amino acid distribution. Blue bars show the amino acids percentage observed in sequences while yellow bars show amino acids percentage observed in library.



After selecting the sequences, D, H, N, Q were observed as overexpressed amino acids. D carries an acidic group on its side chain while H, N, Q carry amine and basic groups. In sequence base, there is no trend of convergence to a specific sequence or any accumulation in a specific round. Moreover, it is not possible to tell which sequence has high affinity or which one is more specific.

3.3 Immunofluorescence Microscopy Experiment Results

So far, all the sequences were evaluated with respect to sequence and statistical analyses. Lack of a consensus sequence formation makes necessary to have further analysis therefore each sequence was analyzed individually using fluorescence microscopy. At the beginning of the examination with fluorescence microscopy, control experiments were also carried out. Control experiments did not contain the phage but only contained the fluorescence dye mixture. Following the control experiment, fluorescence microscopy experiments were performed on all samples using the procedure described earlier.

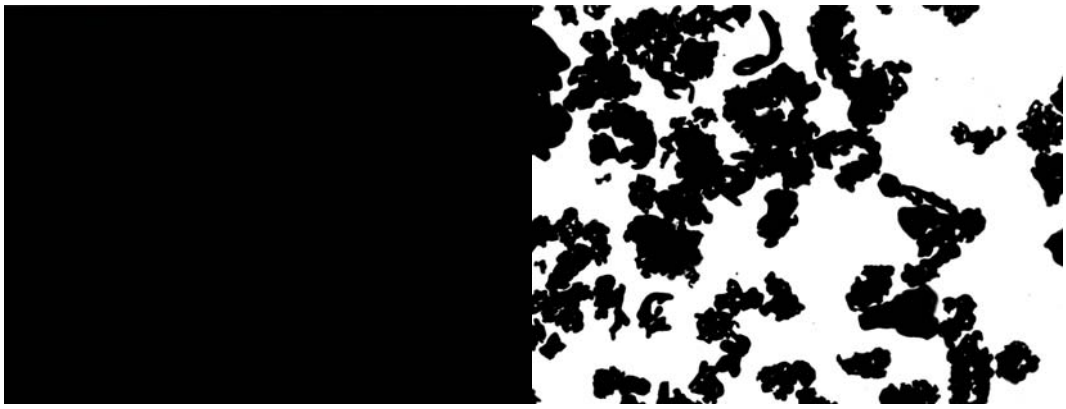


Fig 3.3. Shows control experiment images of fluorescence microscopy.

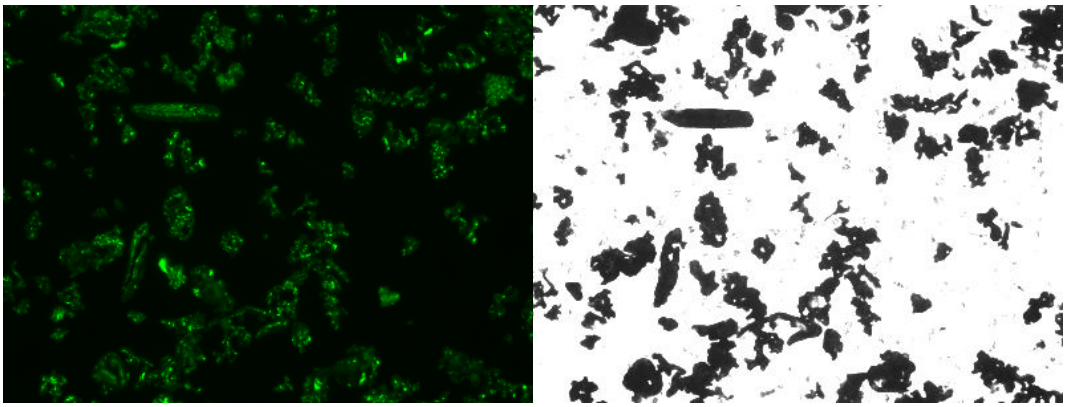


Fig 3.4. Shows **R D P Q T F T** sequence with binding affinity percentage 88% (a strong binder).

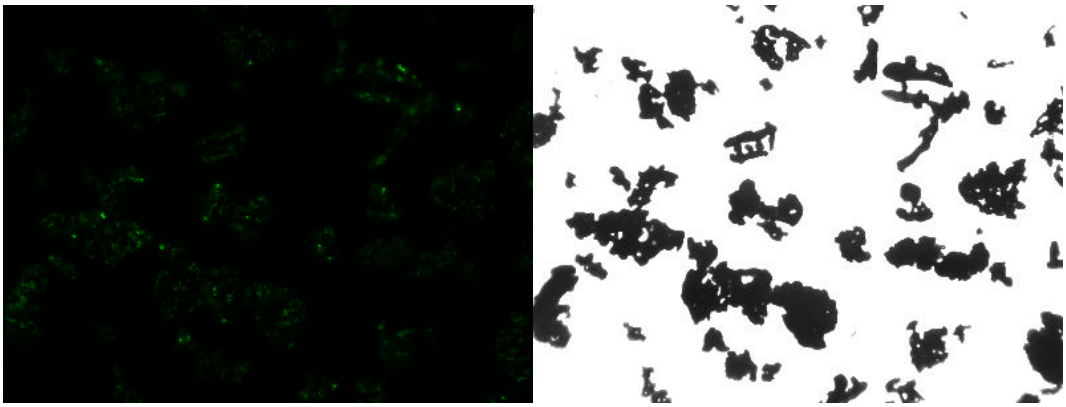


Fig 3.5. Shows **L H D T R S H** sequence with binding affinity percentage 28%(a moderate binder).

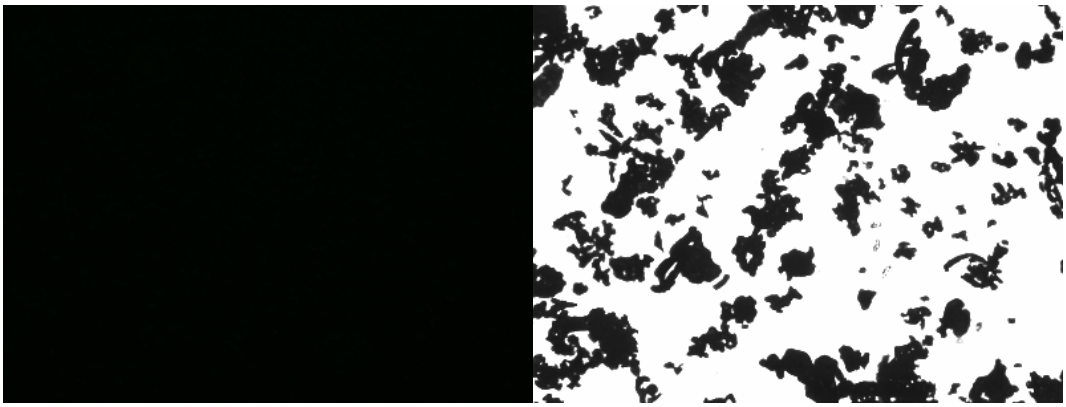


Fig 3.6. Shows A Q V N T Y D sequence with binding affinity percentage 0% (a weak binder).

Fluorescence and optical microscopy images of samples from 3 groups; strong**,** moderate and weak binders.

Table 3.5. Groups of selected polypeptides by their affinities to titanium after fluorescence microscopy studies; listed as strong, moderate and weak binders.

							Round	Name	MW (DA)	Charge	pI	%	Type
R	D	P	Q	T	F	T	2 / A4	SK1	863	0	5,84	88	S
R	D	Q	T	P	A	Y	2 / B	SK2	849	0	5,84	81	S
S	T	V	S	P	N	T	3 / B	SK3	704	0	5,24	74	S
S	T	T	Q	H	S	L	1 / A1	SK4	772	0	6,46	63	S
S	T	Q	D	P	K	P	2 / A4	SK5	771	0	5,55	58	S
L	P	H	N	N	P	Q	3 / B	SK6	819	0	6,74	52	S
P	Q	P	Q	Q	M	T	3 / B	SK7	758	0	5,96	51	S
H	S	H	N	L	H	N	3 / A3	SK8	858	0	7	46	S
S	N	S	P	V	S	S	3 / B	SK9	676	0	5,24	43	S
L	H	D	T	R	S	H	2 / A2	SK10	864	0	6,92	38	M
H	S	L	D	S	F	N	1 / B	SK11	818	-1	5,08	37	M
H	D	R	H	T	P	L	2 / B	SK12	874	0	6,92	37	M
H	G	N	L	H	A	T	3 / A4	SK13	749	0	6,92	36	M
K	T	T	P	Y	A	H	3 / A4	SK14	816	1	8,6	34	M
P	M	S	P	H	A	L	3 / B	SK15	752	0	7,17	31	M
P	V	T	I	H	S	S	2 / A4	SK16	739	0	7,17	29	M
M	P	T	S	T	L	P	2 / B	SK17	745	0	5,28	29	M
A	P	L	P	N	A	S	3 / B	SK18	668	0	5,57	29	M
P	G	T	T	N	L	N	2 / B	SK19	715	0	5,96	23	M
P	N	V	R	T	E	A	1 / A3	SK20	785	-2	6,43	20	M
S	T	A	Y	P	L	N	2 / A2	SK21	764	0	5,24	18	M
H	G	Y	S	N	S	Q	3 / A1	SK22	791	0	6,74	18	M
M	S	Y	V	K	P	Q	3 / A2	SK23	780	1	8,34	16	M
L	T	R	S	S	S	H	3 / A1	SK24	787	1	9,76	15	M
A	P	A	Q	H	K	V	2 / A2	SK25	749	0	8,8	15	M
Q	H	G	M	T	R	Q	2 / A3	SK26	856	1	9,76	13	M
P	W	S	F	Q	T	P	2 / B	SK27	861	0	5,96	12	M
D	I	S	P	P	S	H	1 / A2	SK28	752	-1	5,08	10	W
A	P	T	A	G	L	P	2 / A3	SK29	625	0	5,57	8	W

K	A	P	L	N	S	M	3 / A2	SK30	759	1	8,75	8	W
S	G	A	P	S	A	L	3 / A3	SK31	601	0	5,24	7	W
D	L	E	N	N	R	M	3 / A3	SK32	890	-1	4,37	7	W
N	T	H	A	N	H	H	1 / B	SK33	829	0	7,02	7	W
T	S	R	S	E	T	M	2 / B	SK34	810	0	5,66	7	W
H	Q	A	H	T	S	Q	3 / A1	SK35	807	0	6,92	7	W
F	E	V	G	P	L	H	3 / B	SK36	797	-1	5,24	5	W
D	L	M	T	T	H	P	3 / B	SK37	813	-1	5,08	5	W
P	T	A	D	S	N	M	2 / A3	SK38	735	-1	3,8	3	W
N	S	S	A	L	Q	L	1 / A2	SK39	731	0	5,52	3	W
N	M	A	L	S	Q	G	1 / A3	SK40	720	0	5,52	3	W
A	E	Q	L	R	P	P	2 / A4	SK41	809	0	6,05	0	W
A	Q	V	N	T	Y	D	2 / A4	SK42	809	-1	4,3	0	W
I	D	G	D	Q	R	S	1 / B	SK43	789	-1	4,21	0	W
S	V	E	E	R	G	S	2 / A2	SK44	762	-1	4,53	0	W
P	L	N	D	Q	Y	F	2 / A3	SK45	895	-1	3,8	0	W
N	D	K	K	D	L	L	1 / A3	SK46	785	0	6,43	0	W
T	P	L	L	N	T	D	1 / A4	SK47	772	-1	4,3	0	W
M	G	N	K	T	P	K	3 / A4	SK48	774	2	10	0	W
N	R	Q	D	L	P	H	3 / B	SK49	879	0	6,74	0	W
N	T	A	T	L	P	S	2 / A2	SK50	702	0	5,52	0	W
I	H	Q	D	S	H	A	2 / A3	SK51	806	-1	5,97	0	W

At the end of characterization experiments we observed strong binder polypeptides were not only from last round but also from early rounds but predominantly from last round. Although moderate and weak binders had positive or negative charges, strong binders had no charge; yet, all the strong binders had neutral charge. Moreover strong binding polypeptides carry some motifs like **RD** or **ST** and these motifs were thought to be important in binding to the substrate. For more statistical analyses, percent relative abundance of amino acids of strong binding polypeptides was compared against that of the full library.

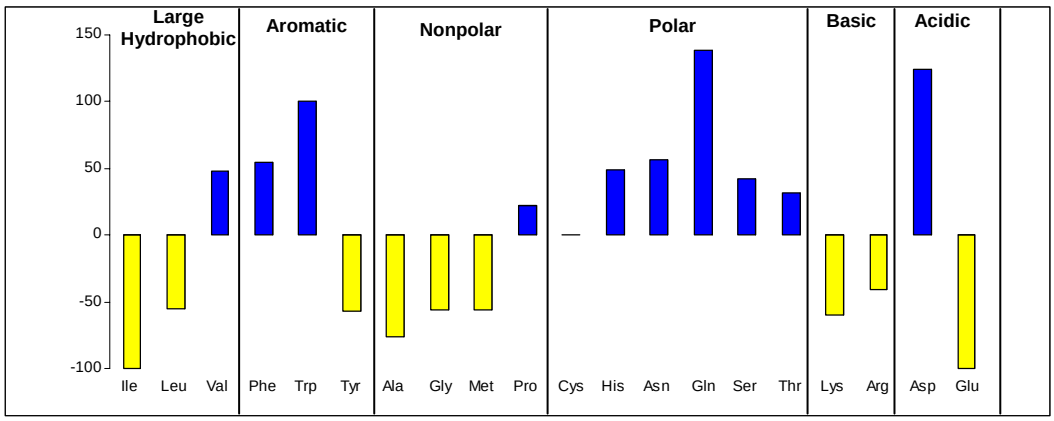


Figure 3.7. Shows relative abundance between amino acids at strong binding polypeptides and amino acids in the library.

Relative abundance graph points out that all polar amino acids and some aromatic amino acids were overexpressed when compared with library. Another important result was the overexpression of aspartic acid and underexpression of glutamic acid although both of them were acidic amino acids.

After comparison of strong binding polypeptides with library, strong binding polpeptides were compared with weak binding polypeptides to reveal the relative abundance of amino acids.

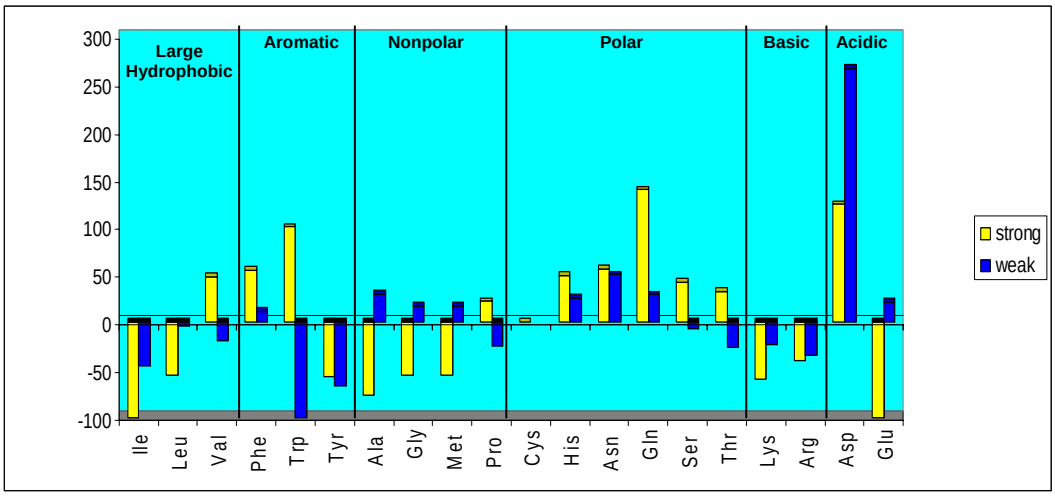


Figure 3.8. Shows relative abundance between amino acids at strong binding polypeptides and amino acids in weak binding polypeptides.

At relative abundance graph between strong binder polypeptides and weak binder polypeptides, nonpolar and acidic amino acids show wide range differences. In weak binders nonpolar amino acids were overexpressed while underexpressed in strong binder. Meanwhile aspartic acid was overexpressed in both strong and weak binders but glutamic acid was underexpressed in strong binders while overexpressed in weak binders.

The results shown in the figures above provides sequences of strong, moderate and weak binding polypeptides selected in biopanning experiments, yet the specificity of the selected polypeptide sequences is still unknown. In order to determine the unique titanium binding specificity of the selected strong binders we have to carry out cross specificity experiments by fluorescence microscopy with different materials.

3.4 Cross Specificity Experiments

Titanium binding peptides were selected and classified as strong, moderate and weak binders. Although binding affinities were known, there was no information about their actual specificity for the titanium substrate. In order to determine the specific binding peptides, cross specificity experiments were performed with different materials with strong titanium-binding polypeptides. In this step platinum, silver and silica were used to understand the binding capability of the titanium-binding polypeptides against other materials.

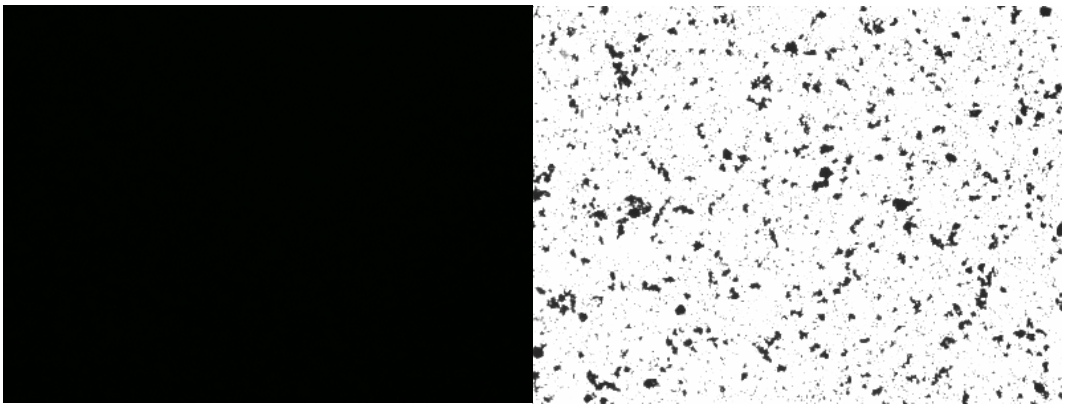


Fig 3.9. Cross specificity experiment image of **R D P Q T F T** (SK1) with platinum powder.

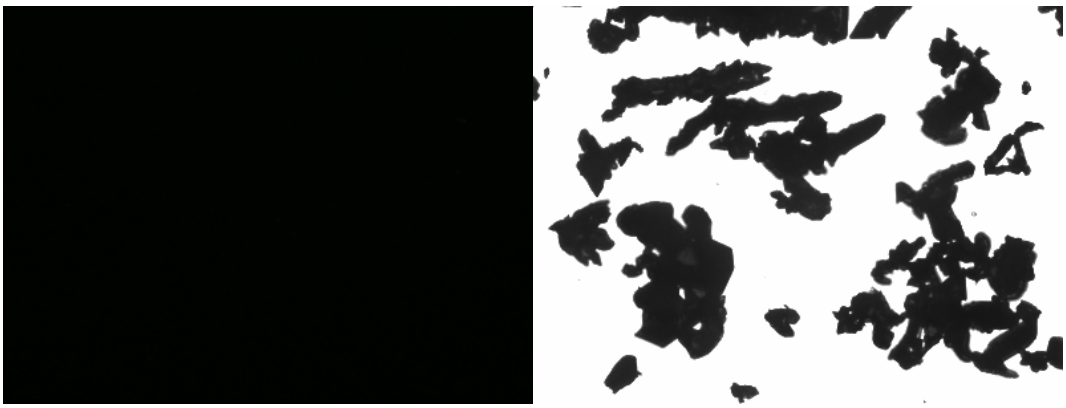


Fig 3.10. Cross specificity experiment image of **R D P Q T F T** (SK1) with silver.

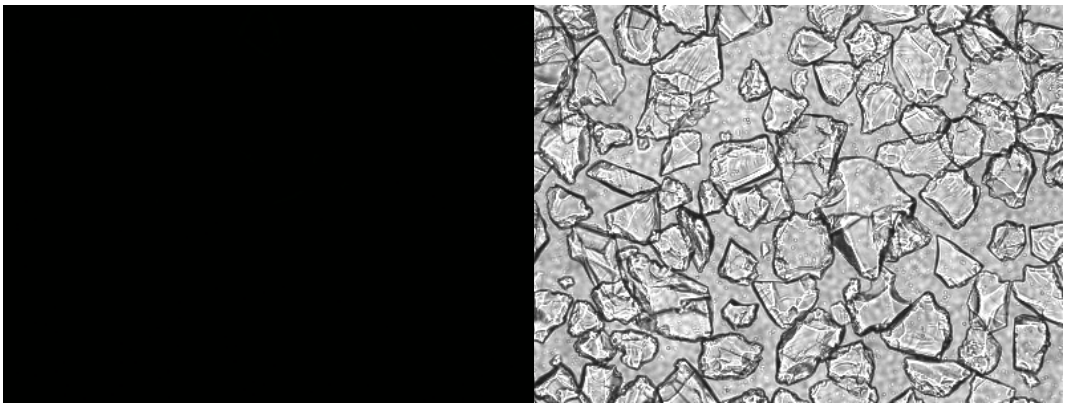


Fig 3.11. Cross specificity experiment image of **R D P Q T F T** (SK1) with silica.

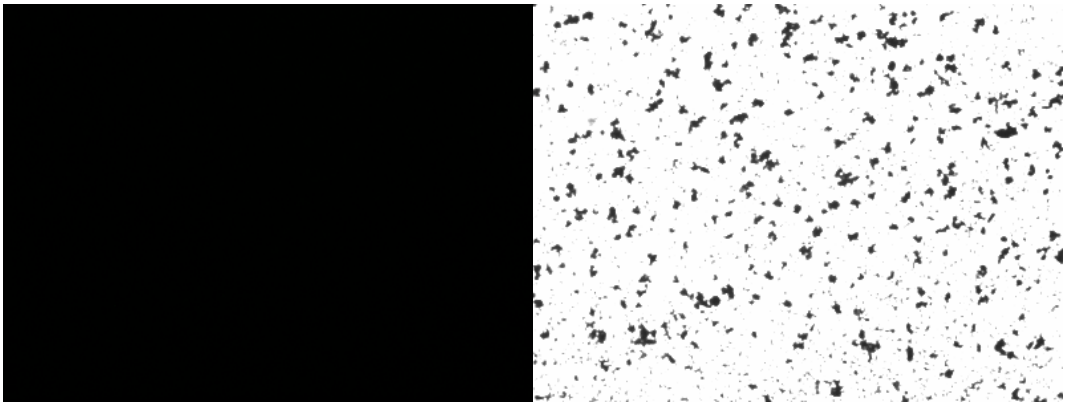


Fig 3.12. Cross specificity experiment image of **L P H N N P Q** (SK6) with platinum powder.

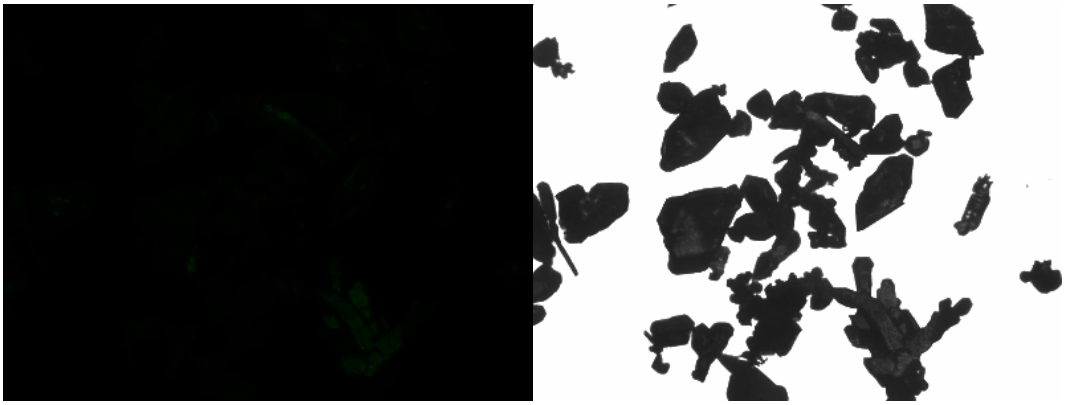


Fig 3.13. Cross specificity experiment image of **L P H N N P Q** (SK6) with silver.

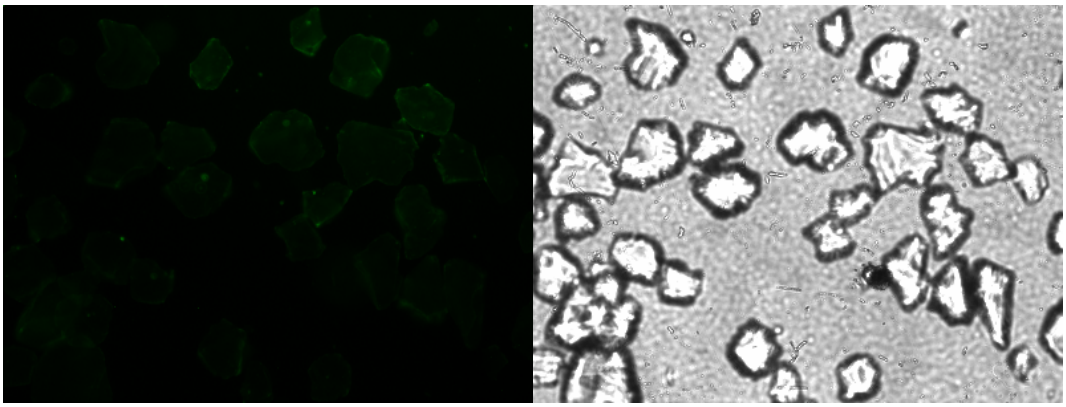


Fig 3.14. Cross specificity experiment image of **L P H N N P Q** (SK6) with silica.

3.5 Statistical Results of Specification Analyses

At the end of cross specification experiments, strong binding and titanium specific polypeptides were selected. **R D P Q T F T (SK1)** showed the highest affinity to titanium and showed no affinity to platinum, silver and silica while some other strong binders showed low affinities.

Strong binding polypeptides exhibited high level expression of polar and aromatic amino acids. Strong binding polypeptides carry mostly aspartic acid as an acidic amino acid, however low level expression of glutamic acid was observed. Non polar amino acids were observed as low expressed amino acids in strong binding polypeptides when compared with library frequencies.

Weak binding polypeptides sequences were mostly rich in non polar amino acids and glutamic acids in contrast with the strong binding polypeptides sequences.

4.CONCLUSION

Titanium binding polypeptides were selected by phage display technique. Before screening procedure, material characterization experiments were applied to get information about particle size, surface morphology and density. After screening experiments 9 strong binder polypeptides were obtained by observing their affinities with fluorescence microscopy. At the end of screening procedure, strong binders were obtained from all rounds but predominantly from last round, pI of all selected polypeptides were calculated and shown that all strong binders were neutral charged, RD and ST motifs were observed in strong binder polypeptides 2 and 3 times respectively, relative abundance of the amino acids of strong binding polypeptides against the frequency demonstrated that strong binders carry mostly polar amino acids and aspartic acid, non polar amino acids and glutamic acid were over expressed in weak binders, cross specification studies showed that most strong binders had no affinities for platinum, silver and silica.

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APPENDIX

Further informations about amino acids distribution observed in C7C Phage Display Library^{TM}. (Table A.1)

Amino Acid	Codons	Expected frequency*	Observed frequency
Arg	CGK, AGG	9.4%	5.2% (37/714)†
Leu	CTK, TTG	9.4%	10.4% (74/714)
Ser	TCK, AGT	9.4%	10.9% (78/714)
Ala	GCK	6.2%	6.4% (46/714)
Gly	GGK	6.2%	3.5% (25/714)
Pro	CCK	6.2%	12.7% (91/714)
Thr	ACK	6.2%	11.2% (80/714)
Gln	CAG, TAG‡	6.2%	4.6% (33/714)
Val	GTK	6.2%	2.1% (15/714)
Asn	AAT	3.1%	6.0% (43/714)
Asp	GAT	3.1%	2.1% (15/714)
Cys	TGT	3.1%	0.1% (1/714)†
Glu	GAG	3.1%	2.8% (20/714)
His	CAT	3.1%	5.2% (37/714)
Ile	ATT	3.1%	3.2% (23/714)
Lys	AAG	3.1%	3.9% (28/714)
Met	ATG	3.1%	3.5% (25/714)
Phe	TTT	3.1%	1.0% (7/714)
Trp	TGG	3.1%	1.4% (10/714)
Tyr	TAT	3.1%	3.6% (26/714)

*Expected frequency = # codons for that amino acid ÷ 32 codons x 100%. Note use of reduced genetic code NNK (32 codons) in library construction.

†Arginines and single cysteines in the random peptide sequence interfere with secretion of pIII and phage infectivity, respectively; consequently, clones with peptides containing Arg or Cys are selected against.

‡The stop codon TAG is suppressed by Gln in the strain used to propagate the library.

Table A.1 Shows the expected and observed amino acid distributions [56].

Color coding of amino acids were represented Table A.2

Basic properties		Some other properties	
Hydrophobic	AGVFPMILW	Small	AGPTSDN
Acidic	DE	Thiol	CM
Basic	RK	Amine	K
Hydroxyl	STY	Amide	NQ
Amine+Basic	HCNQ	Alkyl	AVLIPKM

Table A.2 Shows the amino acids categorization by their basic properties.

CV

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