**ISTANBUL TECHNICAL UNIVERSITY ★ INSTITUTE OF SCIENCE AND TECHNOLOGY**

# SELECTION OF MICA BINDING POLYPEPTIDES BY USING PHAGE DISPLAY TECHNOLOGY

**M.Sc. Thesis by**

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**Programme: Molecular Biology – Genetics & Biotechnology**

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# MİKAYA ÖZGÜN OLARAK BAĞLANAN POLİPEPTİTLERİN FAJ GÖSTERİM TEKNİĞİ İLE TARANMASI

**YÜKSEK LİSANS TEZİ**

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**ABBREVIATIONS**

| | |
|---|---|
| **A** | : Alanine |
| **bp** | : Base pair |
| **BSA** | : Bovine serum albumin |
| **C** | : Cysteine |
| **cDNA** | : Complementary DNA |
| **CSD** | : Cell Surface Display |
| **D** | : Aspartic acid |
| **DMF** | : Dimethylformamide |
| **DNA** | : Deoxyribonucleic acid |
| **DTT** | : Dithiothreitol |
| **E** | : Glutamic acid |
| **EB** | : Elution Buffer |
| **EDTA** | : Ethylenediaminetetraacetic acid |
| **F** | : Phenylalanine |
| **FM** | : Fluorescence Microscopy |
| **G** | : Glycine |
| **GEPI** | : Genetically engineered polypeptides for inorganics |
| **H** | : Histidine |
| **I** | : Isoleucine |
| **IPTG** | : Isopropyl-β-D- thiogalactopyranoside |
| **K** | : Lysine |
| **L** | : Leucine |
| **LB- broth** | : Luria Bertani broth |
| ***lacZ*** | : β-galactosidase |
| **LM** | : Light Microscopy |
| **kD** | : Kilo Dalton |
| **M** | : Methionine |
| **ME** | : β-Mercaptoethanol |
| **MLB** | : M13 lysis and binding buffer |
| **MP** | : M13 Precipated Buffer |
| **mRNA** | : Messenger ribonucleic acid. |
| **Na-Ac** | : Sodium acetate |
| **OD** | : Optical density |
| **P** | : Proline |
| **PC** | : Potassium Phosphate-Sodium carbonate buffer |
| **PFU** | : Phage Forming Unit |
| **PCR** | : Polymerase chain reaction |
| **PD** | : Phage Display |
| **PEG-8000** | : Polyethylene Glycol-8000 |
| **R** | : Arginine |
| **S** | : Serine |
| **SDS** | : Sodium dodecyl sulfate |

| | |
|---|---|
| **SSB** | : Single stranded DNA binding protein |
| **ssDNA** | : Single stranded DNA |
| **T** | : Threonine |
| **TBE** | : Tris-borat -EDTA |
| **TCEP** | : Tris (2-carboxyethyl) phosphine |
| **Q** | : Glutamine |
| **V** | : Valine |
| **W** | : Trptophan |
| **Tris base** | : Hydroxymethyl aminomethane |
| **X-Gal** | : 5-Bromo-4-chloro-3-indolyl-D-galactoside |
| **Y** | : Tyrosine |

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## SELECTION OF MICA-BINDING POLYPEPTIDES BY USING PHAGE DISPLAY TECHNOLOGY


### SUMMARY

Many organisms form organic-inorganic hybrid systems as their hard tissues. Proteins have the major role in the formation of highly ordered micro and nano-structures *in vivo* in hard tissues. These hybrid materials have excellent functions such as forming protective layers, supportive tissues, transferring charge and ion, developing some optical and mechanical properties. These excellent functions by using any inorganic material and specific polypeptides are tried to be used in biotechnology and nano-biotechnology.

Phage Display as a combinatorial biology based molecular library method is one of the approaches for the selection of polypeptides that specifically bind to a given inorganic surface. There has been an increasing number of studies in the recent years that focused on the selection of genetically engineered polypeptides for inorganics (GEPIs) from noble metals, oxides and semiconductors using phage display technology. GEPIs offer a novel way of using biomolecular linkers in the synthesis and assembly of materials structures for use in nano- and bionanotechnology.

In this study, mica which is applicable both to biotechnological and nano-biotechnological areas, was chosen based on its interesting physical properties, such as being ionic crystal, having high dielectric strength, low thermal conductivity, high temperature resistance, and being chemically inert. During the selection of mica binding polypeptides, two screening experiments were performed by using Ph.D.-C7C phage library. Before applying phage display protocol on mica, characterization experiments of muscovite mica based on XRD, SEM and Zeta potential analyses were performed. During the first screen, 3 biopanning and during the second screen, 4 biopanning were performed and 14 sequences from the first and 64 sequences from the second screen were selected, so totally 78 mica-binders were obtained.
During selection of polypeptides, firstly chemical elution was applied to remove bound phage from mica surface, Different novel approaches, such as application of bound phage recovery and ultrasonication as a physical elution method were applied to overcome the inability of removing all specific binders from inorganic surface.
Following the screens identified polypeptides were analyzed on sequence and amino acid base to search for any trend among the sequences. As a next step, fluorescence microscopy experiments were performed to test the binding affinities of each sequence and grouping the sequences mainly as good, moderate or weak binders. Also cross-specificity experiments among the strong binders using titanium powder were performed.

# MİKAYA ÖZGÜN OLARAK BAĞLANAN POLİPEPTİTLERİN FAJ GÖSTERİM TEKNİĞİYLE TARANMASI

## ÖZET

Birçok organizma, sert dokularında organik-inorganik melez sistemler oluştururlar. Çok düzgün mikro ve nano yapıların sert dokulardaki oluşumunda proteinlerin önemli rolleri vardır. Bu melez sistemlerin, dokulara destekleyici olması, iyon ve yükleri transfer edebilmesi, optik ve mekanik özellikler geliştirebilmesi gibi mükemmel fonksiyonları vardır. Bu mükemmel fonksiyonlar herhangi bir inorganik malzeme ve malzemeye özgün peptitlerle biyoteknolojide ve nano biyoteknolojide kullanılmaya çalışılmaktadır.

Seçilen bir inorganik malzemeye özgün olarak bağlanan polipeptitlerin taranması için kullanılan yöntemlerden biri de kombinatoriyel moleküler kütüphane metoduna bağlı olarak geliştirilmiş olan faj gösterim tekniğidir. Soy metaller, oksitler ve yarı iletkenlere yapışan polipeptitlerin faj gösterim yöntemiyle taranmasına dair yapılan çalışmalar son yıllarda giderek artmaktadır Nano-malzemelerin sentezlenmesinde inorganik malzemelere özgün olarak bağlanan peptitlerin biyomoleküler birleştirici olarak kullanılması nano- ve nano biyoteknoloji alanlarına yeni yaklaşımlar sunmaktadır

Bu çalışmada, biyoteknoloji ve nano-biyoteknolojide uygulama alanları, iyonik kristal yapı oluşturabilme gibi önemli fiziksel özellikleri, elektriğe ve sıcaklığa karşı yüksek direnç gösterme, düşük ısı iletkenliği ve kimyasal tepkimelerde kararlılık gibi özellikleri olan mika seçilmiştir. Mikaya özgün polipeptitlerin seçilmesinde, Ph.D.-C7C faj kütüphanesi kullanılarak iki set deney yapılmıştır. Faj gösterim protokolünü mika üzerine uygulamadan önce, XRD, SEM ve Zeta potansiyel gibi karakterizasyon çalışmaları yapılmıştır. İlk set deney süresince, 3 döngü, ikinci set deney sırasında 4 döngü deney yapılmıştır. 14 sekans birinci ve 64 sekans ikinci set deneyden olmak üzere, toplam olarak 78 sekans elde edilmiştir. Polipeptitlerin seçilmesi sırasında, ilk olarak bağlanmış olan faj kimyasal yıkama ile yüzeyden uzaklaştırılır. Fiziksel bir yıkama olan ultrasonikasyon ve bağlanan fajın geri kazanılması gibi farklı yeni yaklaşımlar inorganik yüzeylerden özgün olarak bağlanan polipeptitlerin uzaklaştırılmasındaki kısıtları aşabilmek için uygulanmıştır.

Deney setini takiben, tanımlanan polipeptitlerin, amino asitlerine ve sekanslarına bağlı olarak aralarındaki benzerlikler analiz edilmiştir. Bundan sonraki aşama olarak, floresan mikroskobu çalışmasıyla her bir sekansın bağlanma ilgisi belirlenmiştir. Seçilen polipeptitler yüzeye olan ilgiye göre güçlü, orta ve zayıf bağlananlar olarak sınıflandırılmışlardır. Mika yüzeyine olan özgünlüğü analiz etmek üzere, floresan mikroskobu teknikleri, titanyum tozu kullanılarak sadece güçlü bağlananlar için uygulanmıştır.

# 1. INTRODUCTION

## 1.1. Significance of proteins in biological systems

The vast majority of the biochemical processes that take place in or outside the cell are performed by proteins. Proteins are the imperative part of the biological structure and systems. They are instrumental in the formation of cellular structures and functional activities in biological tissues as a consequence of their specific interaction with other macromolecules and materials. They control the tissue formation, biological function and physical performance of biomaterials as well as catalyzing biological reactions. Proteins mostly work in soft [1] and hard tissues, [2] which are highly organized in a manner that they exhibit complicated nano-structures and diverse functional characteristics.

The synthesis of hard and soft tissues, are genetically controlled via mainly proteins in aqueous environments, at ambient conditions. [3, 4, 5] As a matter of fact, proteins might be considered as the most valuable part of living tissues considering their functions as transporters and self- and co-assembly into short and long range ordered nuclei and substrates [6,7,8,9].

Soft tissues having only proteinaceous phase, like the ones belonging to muscle, membranes etc, are handled by soft tissue [3] engineering. On the other hand, hard tissues show an organic-inorganic hybrid structure, [2,4,8] in which inorganic materials like the ones found in bones, dental tissues, spicules, spines, shells, skeletal units of single celled organisms or plants, bacterial thin film and nanoparticles, are attached and organized by specific proteins [2,4,8]. These proteins, forming at least one proteinaceous phase, specify hard tissues [8]. Only the organisms that use the organic-inorganic hybrid systems have evolved to use a part of their proteins in order to produce and bind the inorganic materials *in vivo*. These organisms synthesize inorganic binding proteins that bind and organize inorganic materials to highly ordered structures to perform excellent functions such as forming protective layers, supportive tissues, transferring ions and developing some optical and mechanical

properties in favor of the organism. The inorganic material, on which the proteinaceous phase is bound, commonly include magnetite ($Fe_3O_4$) particles in magnetotactic bacteria or teeth of chiton [10]; silica ($SiO_2$) as skeletons of radiolarian [2] or tiny light-gathering lenses and optical wave guides in sponges [11]; hydroxyapatite ($Ca_2C(OH)_3$) in bones [12] and dental tissues of mammals [13] calcium carbonate ($CaCO_3$) in the shells of mollusks [14]. (Figure 1.1.) [8].

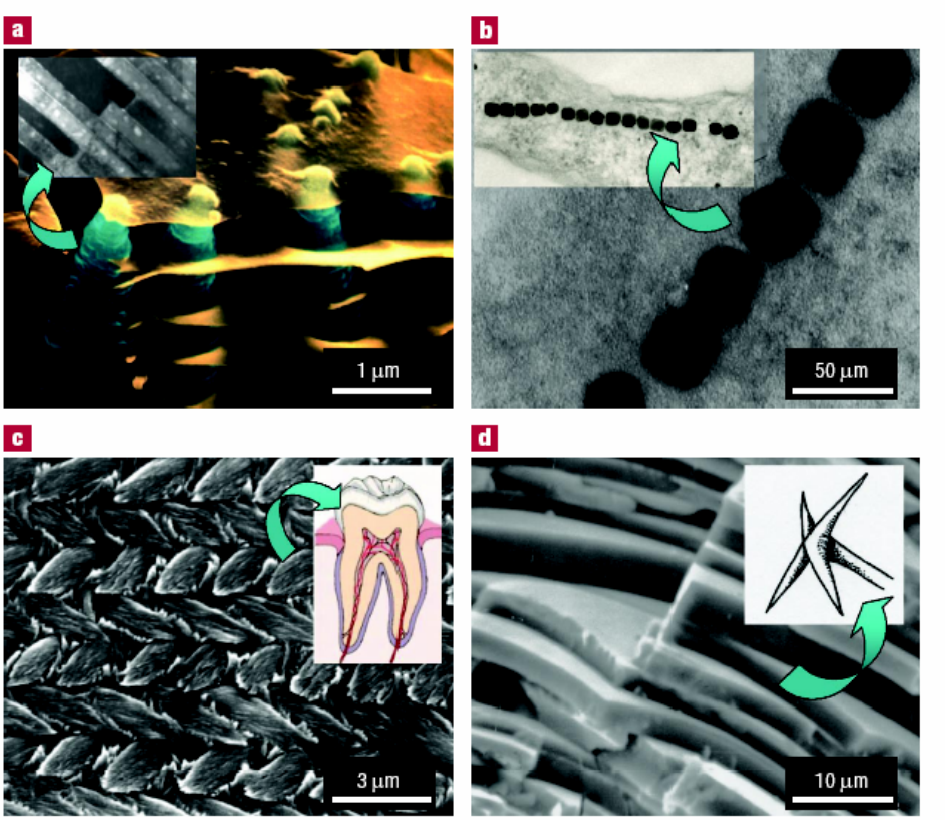



**Figure1.1** Examples of biologically synthesized complex materials **a)** Scanning electron microscope (SEM) image of a growth edge of abalone (*Haliotis rufescens*) displaying aragonite platelets (blue) separated by organic film (orange) that eventually becomes nacre (mother-of-pearl). This is a layered, tough, and high strength biocomposite (inset:transmission electron microscope (TEM). **b)** Magnetite nanoparticles formed by magnetotactic bacterium (*Aquaspirillum magnetotacticum*, inset:TEM image) are 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) [8].

Similar useful properties can be obtained from many inorganic materials, to be utilized in nano- and biotechnological applications. In nanometer scale, these materials differ from biological systems in the basis of their synthesis and control

patterns. This difference, together with their ordered assemblies in two and three dimensions are still hard to explain [5,8,14,15]. Working effectively with nano-scale structures systems using traditional chemistry is still handicapped. This obscurity is mainly caused by the difficulties envisaged in their synthesis and their assembly into fully potential functional structures and devices [8,15].

Following the path opened by basic biology and via examining proteins encountered in nature, new polypeptides can be manufactured genetically in order to make them select which inorganic compounds they will bind to create hybrid materials. The reason for choosing genetic synthesis methods is to give these materials useful functions as that observed in biological hard tissues. All the molecular tools developed for this aim are gathered under the name molecular biomimetics that offers efficient ways to overcome difficulties in the synthesis and assembly mentioned above through the combination of Mother Nature's way and synthetic nanosacle constructs [8,15].

Inorganic-binding biomolecules (proteins, polypeptides, oligonucleotides etc.) could potentially be used as biolinkers in the synthesis and assembly of functional nano-structures and they could also be the building blocks of self-assembled materials with controlled organization and specific functions [8,15].

### 1.2. How to Obtain Inorganic Binding Proteins?

There are many difficulties and limitations in the synthesis of nano-technological systems by using traditional "heat and beat" approach. This approach requires high temperature, pressure and pH. Another limitation for this traditional approach is the production of large amount of toxic byproducts [8,14]. Moreover, obtaining the desired size nanomaterial can be very difficult. However, hard tissues are biocomposites of structural macromolecules such as lipids, proteins, and carbohydrates. Synthesis of hard tissues in multicellular organisms is a well-coordinated event that takes place at mild physical conditions. There are some examples of characterized proteins that interact with the inorganics such as ice-binding (antifreeze) proteins. Ice-binding proteins are among the few identified polypeptides that specifically bind to ice and are synthesized in many fish species, plants, and insects [16]. These proteins, composed of repeating polypeptide units, control ice formation via binding and preventing the growth of ice crystals in the

internal fluids. Another example is *Cylindrotheca fusiformis*, [17] unicellular algae present in marine and fresh water habitats, which possesses proteins that interact with silica in its cell wall. In a similar study, *Tethya aurantia* was shown to produce proteins that interact with silica [18].

There are several possible ways of obtaining polypeptides or proteins specific to inorganics. One of the ways to obtain inorganic binding proteins is to extract biomineralizing proteins form the hard tissues [8,15]. In this case, extraction of proteins and cloning of their genes is complicated and time consuming. Besides, the number of known and well-characterized inorganic-binding polypeptides is also limited [19]. In addition to proteins, other macromolecules in multicellular and single celled organisms may affect biomineralization, thus making this process more complex [20,21]. Although the extraction is a laborious process, some isolated proteins have been used as enzymes in the synthesis of certain inorganics and growth modifiers [22,23]. Amelogenins used in mammalian enamel synthesis [20] and silicatein used in sponge spicula formation are among the best representatives of these functional proteins [23].

Inorganic binding peptides may be designed using a theoretical molecular approach similar to the one used for pharmaceutical drug research [24]. However this approach is currently impractical for design of inorganic binding polypeptides because it is a time consuming and expensive method [8,15]. Therefore, different and faster methods were chosen to apply in order to identify the inorganic binding proteins or polypeptides. One of the approaches is directed evolution based methodology where the peptides are selected from molecular libraries. Molecular libraries or combinatorial libraries are composed of completely random sequences of peptides or oligonucleotide. Common molecular libraries are generated based on combinatorial chemistry and biology methods. Combinatorial chemistry libraries of small organic molecules can be generated by a variety of synthetic methods. This method also has some possible limitations because of complicated peptide synthesis and requirement of large number of sequential chemical reactions [8,25,26].

Combinatorial biology libraries are large and random peptide libraries that are generated by the same number of amino acids, but with different sequence compositions [25,26]. Combinatorial biology libraries can be preferred for obtaining inorganic binding polypeptides [8,15]. “Phage display” and “Cell surface display”

are well known *in vivo* combinatorial biology techniques [27, 28, 29]. Phage and cell surface display technologies are proven to be very powerful tools for many biotechnological and biological applications. These include 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 otherwise altered binding characteristics for their ligands [30]. These display techniques also preferred to obtain the inorganic binding polypeptides [8, 15].

Up to date, phage display has been employed to identify peptides that are specific to gallium arsenide [31] silica [32] silver [33], zinc sulfide [34], calcite [35], cadmium sulfide [36], and noble metals such as platinum and palladium. [37]. Cell surface display also has been applied to identify the iron oxide [15,38], gold [15, 39], zinc oxide, zeolites and cuprous oxide [40] specific binders.

### 1.2.1. Phage Display Technology

Phage display, developed by G. Smith in 1985, is the most efficiently adapted molecular display technique [28, 29]. It is an extremely powerful tool for selecting peptides or proteins with specific binding properties from vast numbers of variants. Therefore, this technique can be used to study protein-ligand interactions, receptor and antibody binding sites, and to improve or modify the affinity of proteins for their binding partners [30].

Phage display involves the expression of proteins, including antibodies, or peptides on the surface of filamentous phage. DNA sequences of interest are inserted into a location in the genome of bacteriophage such that the encoded protein is expressed or "displayed" on the surface of filamentous phage as a fusion product to one of the phage coat proteins [25,28, 41].

A significant aspect of phage display lies in linking the phenotype of a bacteriophage-displayed peptide or protein with the genotype encoding that molecule, packaged within the same virion. This permits the selection and amplification of specific clones of phage, representing desired binding sequences from pools of billions of phage clones. In case of filamentous phage, amplification is simply accomplished by infecting male *E. coli.* The genotype-phenotype linkage also permits the rapid determination of the amino acid sequence of the specific binding

peptide or protein molecule by DNA sequencing of the specific insert in the phage genome [25, 28, 29, 41].

There various biological vehicles for phage display. These biological vehicles have been utilized as platforms for the presentation of random peptide, gene fragment, cDNA and antibody libraries on a genetic package. These genetic packages include λ, T4, T7 and M13 phage. T7-, T4- and λ-based display systems are not yet used on a routine basis. The filamentous bacteriophage M13 (Ff family) is the first and the best characterized library display vector so far [42].

### 1.2.1.1. M13 Filamentous Bacteriophage

M13 is an *Escherichia coli*-specific filamentous bacteriophage that has a length of about 1 μm but diameter of 6 nm. M13 contains a circular single-stranded DNA genome of 6407 nucleotides, surrounded by five phage-encoded proteins (Fig.1.2) and a proteinaceous coat. The M13 genome encodes a total of 10 genes as shown in Figure1.3. Five of these genes encode the coat proteins while the others encode proteins necessary for viral replication and assembly [41, 63].

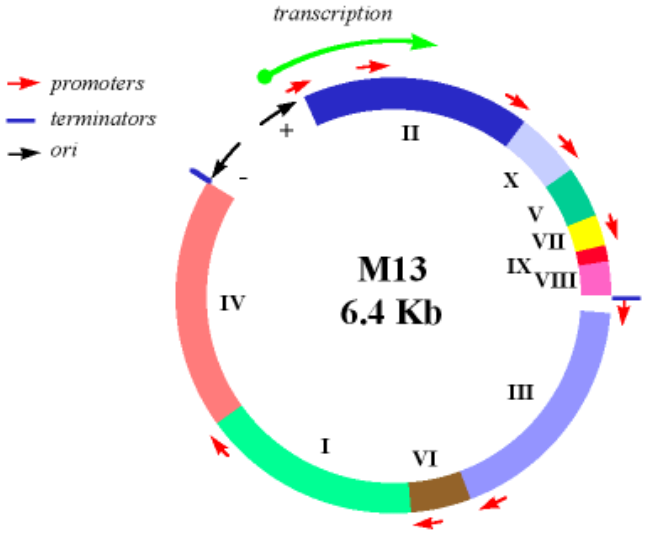



**Figure1.2** M13 genome [44]

M13' s 5 different coat proteins are named gp3, gp6, gp7, gp8, and gp9 (Figure1.3). The M13 genome is coated by a single layer constituting ~2700 subunits of protein gp8 (gpVIII) encoded by gene VIII, giving filamentous appearance to phage. Moreover, gp8 is also called major coat protein because of its ability to cover

particles. One end of the filament is bound by gp7 (gpVII) and gp9 (gpIX) proteins that are encoded by gpVII, gpIX respectively. To the other end are bound the gp3 (gpIII) and gp6 (gpVI) proteins that are encoded by gpIII and gpVI. The vast majority of the coat proteins are gp8. The other four coat proteins are represented at about 5 copies per particle; gp7 and gp9 cap at one end of the particle while gp3 and gp6 cap at the other end. [42]. These proteins are also called the minor coat proteins.

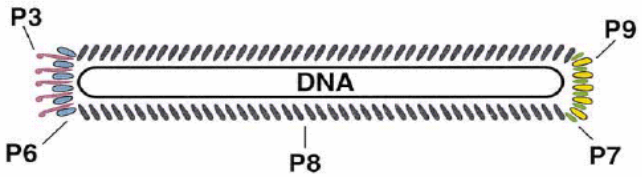



**Figure1.3** Structure of a filamentous bacteriophage. The major coat protein gp8 (P8), and also minor coat proteins also shown [41].

All five-coat proteins contribute to the structural stability of the phage particle, but gp3 is also necessary for host cell recognition and infection. Consequently, gp3 is the largest and most complex of the coat proteins and it contains three distinct domains. The N-terminal domain initiates translocation of the viral DNA into *E. coli* during infection while the second domain confers host cell recognition by binding to the F pilus on the *E. coli* surface. The C-terminal domain interacts with other phage coat proteins, and is thus responsible for the integration of gp3 into the phage [41]. Subsequently, gp7 and gp9 are required for the initiation of assembly and for the maintenance of virion stability [41, 42, 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 and infection is initiated by the binding of gpIII to the tip of the F pilus on the surface of an *E.coli* cell. Although the integration of the phage through the cell membrane is a complex and poorly understood mechanism, there are some possible suggestions for it. Considering these suggestions, gp3 may have a role in the mechanism of phage DNA entry into the cell. Moreover, it has the capability to fuse into cell membrane. It also forms the pores that are large enough for DNA to pass through cell membranes. By the way, viral single stranded DNA is extruded through this pore [41, 42, 43].

The M13 DNA that enters the cytoplasm is a circular single-stranded DNA molecule. The strand present in phage particle is known as the plus or + strand. (Figure 1.4 a and b) Following the entry of DNA into the cytoplasm, the + strand DNA is immediately coated with an *E. coli* single stranded DNA binding protein known as SSB. The SSB coating protects the DNA from degradation. The M13 plus (+) strand is converted to a double-stranded molecule immediately upon entry into *E. coli*. The complementary strand is synthesized entirely by *E. coli*'s DNA synthesis machinery. The complementary strand is called the minus or (-) strand. Only the minus strand is used as the template for mRNA synthesis and ultimately it is the template for the translation of the encoded M13 gene products. The SSB that coats the (+) plus strand upon entry of the DNA into the *E. coli* cytoplasm fails to bind to ~60 nucleotides of the molecule (Figure 1.4-c). These nucleotides form a hairpin loop that is protected from nuclease degradation [41, 42, 43].

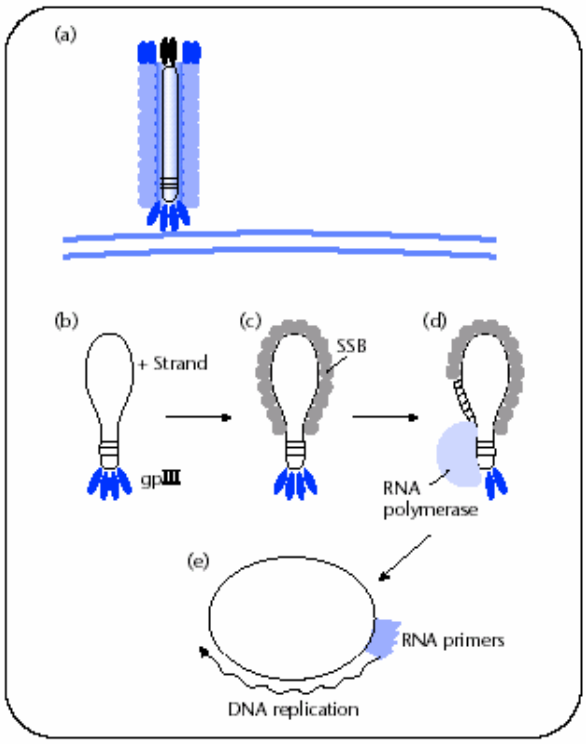



**Figure 1.4** The conversion of the M13 plus (+) strand to a double stranded DNA molecule. The plus strand enters the cell (a and b) with gpIII attached. It is immediately coated with host SSB (c). RNA polymerase synthesizes a short primer (d) and DNA polymerase synthesizes the minus strand. (e) [43].

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 (Figure 1.4-d). The

RNA primer is extended by *E. coli* DNA polymerase III to create the minus strand (Figure 1.4-e). The RNA primer is eventually removed by the exonuclease activities of *E. coli* DNA polymerase I. The gap is filled in by the 5' to 3' polymerizing activity of the same DNA polymerase. *E.coli* ligase forms the final phosphodiester bond resulting in a covalently closed double-stranded circular M13 DNA. The double-stranded form of M13 genome is called 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 as shown in figure 1.5 [43] While the M13 genome is being replicated, the genes encoding the coat proteins are being transcribed and translated. When M13 gp5 protein accumulates to sufficient levels, a switch from synthesizing RF DNA to synthesizing the plus strand occurs.

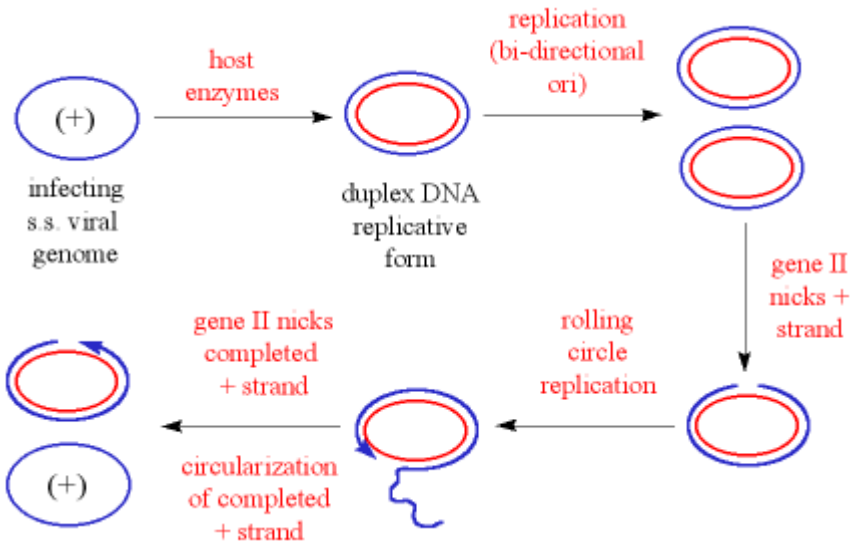



**Figure 1.5** DNA replication of M13 bacteriophage [45].

gp5 blocks the synthesis of the minus strand, presumably by displacing SSB on the plus strand and preventing the plus strand from being used as a template. The (+) plus strand is circularized [43].

M13 phage particles are assembled and released from *E. coli* cells through a process that does not disrupt the cell wall. The gp5 coated plus strand makes contact with the bacterial inner membrane. This interaction requires a specific packaging sequence on the DNA and gp7 and gp9. The protein-coated DNA traverses the membrane and gp5 is replaced by gp8 in the process.

Phage assembly is initiated by the incorporation of gp7 and gp9 to one end of the particle. The elongation process follows the initiation during which thousands of gp8 molecules are assembled along the length of the particle, and the process is terminated by the addition of gp6 and gp3 to the other end of the particle. The assembled phage particle is then released into the extracellular environment [43].

#### 1.2.1.2. Types of Phage Display Peptide Library

Phage display libraries are generated by inserting short sequences encoding randomized oligonucleotides within certain genes that are encoded on coat proteins of the phage.

There are different phage display peptide library types depending on the length of oligonucleotide insert and insertion site. Libraries can be constructed by inserting 5-38 amino acid long oligopeptides but increasing length of oligopeptides may affect the display efficiency and phage viability [27]. Commercialized libraries especially contain 7, 12 and 15 amino acid long insertions.

M13 phage display systems are generally based on N-terminal fusions to the coat proteins gp3 or gp8. gp6 coat protein is very rarely used to construct the library. gp7 and gp9 coat proteins are the small hydrophobic peptides that have not been employed for the phage display [27, 41, 43].

gp3 is present as 5 copies per virion and short polypeptides can be fused to all 5 copies of gp3 without interfering with the phage infectivity to generate library. The major coat protein gp8 is present at ~2700 copies per virion, short peptides can be fused only to 10 % of the gp8 copies. As a result, peptides expressed as gp3 fusions are present at low valency (1-5 copies per virion), while gp8 fusions are present at high valency (~200 copies per virion). The increased avidity effect of high valency gp8 display permits selection of very low affinity ligands, while low valency gp3 display limits selection to higher affinity ligands [27,43,46,56].

## 1.3. Adaptation of Phage Display Technology to Selection of Inorganic Polypeptides

Phage display technology, which is a combinatorial biology based system, is utilized for the selection of polypeptides with specific affinity to inorganic substrate.

However, phage display is especially used in order to study an organic-organic interaction. Therefore, phage display procedure should be adapted and optimized for protein-inorganic interaction and also for each different type of inorganics. During the adaptation of phage display procedure, material instability in the screening buffers could also be a problem. For instance, many materials rapidly develop an oxide layer on their surfaces, exposing different crystallographic faces to the solvent, and may become chemically or physically modified during the panning process [8,50]. To overcome this problem, spectroscopic and surface image analysis could be done to characterize inorganic surfaces, before and after the phage display procedure. Moreover, effect of all solutions used as washing and elution buffers in phage display procedure must also be analyzed. If any surface modification is detected, buffer conditions should be changed and optimized based on careful selection of buffer combination and inorganic materials. Besides, chemically inert materials might be preferred [4]. In this study, effects of buffers on mica surface were characterized by SEM [8, 15].

Following the characterization of materials, phage display screening procedure can be applied to the inorganic material. This procedure is based on an affinity selection, involving the following fundamental steps such as binding, washing, elution, amplification and purification step (Figure 1.6). All of these steps can be gathered under the name biopanning.

**Binding steps:** Heterogeneous mixture of phage libraries is exposed to the inorganic substrate to allow the phage and target interaction. Although libraries with very high diversities are available, some expressed sequences are incompatible with phage propagation, while others are highly susceptible to proteolysis during propagation. These factors impose constraints on effective diversity and it is therefore desirable to start with a library that is as diverse as possible. The possibility that some expressed sequences may be somewhat deleterious to phage propagation, can be militated up to some extent by adding a growth step to each panning round that creates less competitive growth conditions, for example by growing on solid media rather than exclusively in liquid culture. It is also important to empirically check the diversity of libraries before starting any screen because of the possibility that instabilities in libraries can lead to loss of inserts [8,15,43]. During this step, some possible factors may affect the target library interaction. Consequently, based on enthalpic or entropic

characteristics of binding interaction, stringency can be either increased or decreased via raising or diminishing the temperature of binding step, respectively. Different suggested binding temperatures, such as $4^0$C, room temperature, 37 $^0$C should be tried. Binding also can be performed at high temperatures like 55 $^0$C. However, high temperatures can cause the inactivation of phage [67,43,46].

Target concentration is another factor affecting screening. Lowering the target concentration can increase the stringency.

**Washing steps:** Following the library-inorganic surface interaction, non-binding phages are removed by several washing cycles that disrupt the weak interactions with the substrate. In the washing steps, nonspecific or weak interactions are reduced by the presence of detergent and blocking agent.

Lower detergent concentrations in early rounds will result in higher eluate titers and stringency can be gradually increased every round by raising the detergent concentration stepwise to a maximum of 0.5%. If washing step is too stringent, some of the binders will also be lost. Hence, the stringency of washing buffer should be balanced by adjusting washing times, and progressively increasing detergent concentrations [27,46].

**Elution steps:** Following the washing steps, phages that strongly bind to the target substrate are retained, while the non-binder ones are washed away. These bound phages then are recovered from the substrate, through the elution with a harsh wash that disrupts strong binding of phage to the substrate. Generally, increasing or lowering the pH and reducing agents that is used to disrupt disulfide linkages are often employed during the elution step. Although phage particles are resistant to relatively harsh conditions such as acid pH, high temperatures, using the harsh elution buffers may affect the integrity of the phage [8,15,27,46]. Therefore, amount of the reducing agents and pH of the buffers should be optimized. Another limiting factor is that a number of specifically binding polypeptides remains strongly bound on the substrate material even after an extended acid elution step during phage display. Stone *et al.* observed that these remaining strongly bound polypeptides were identified by polymerase chain reaction (PCR) method [47]. To overcome this limitation, some supplementary methods should be developed [8, 15].

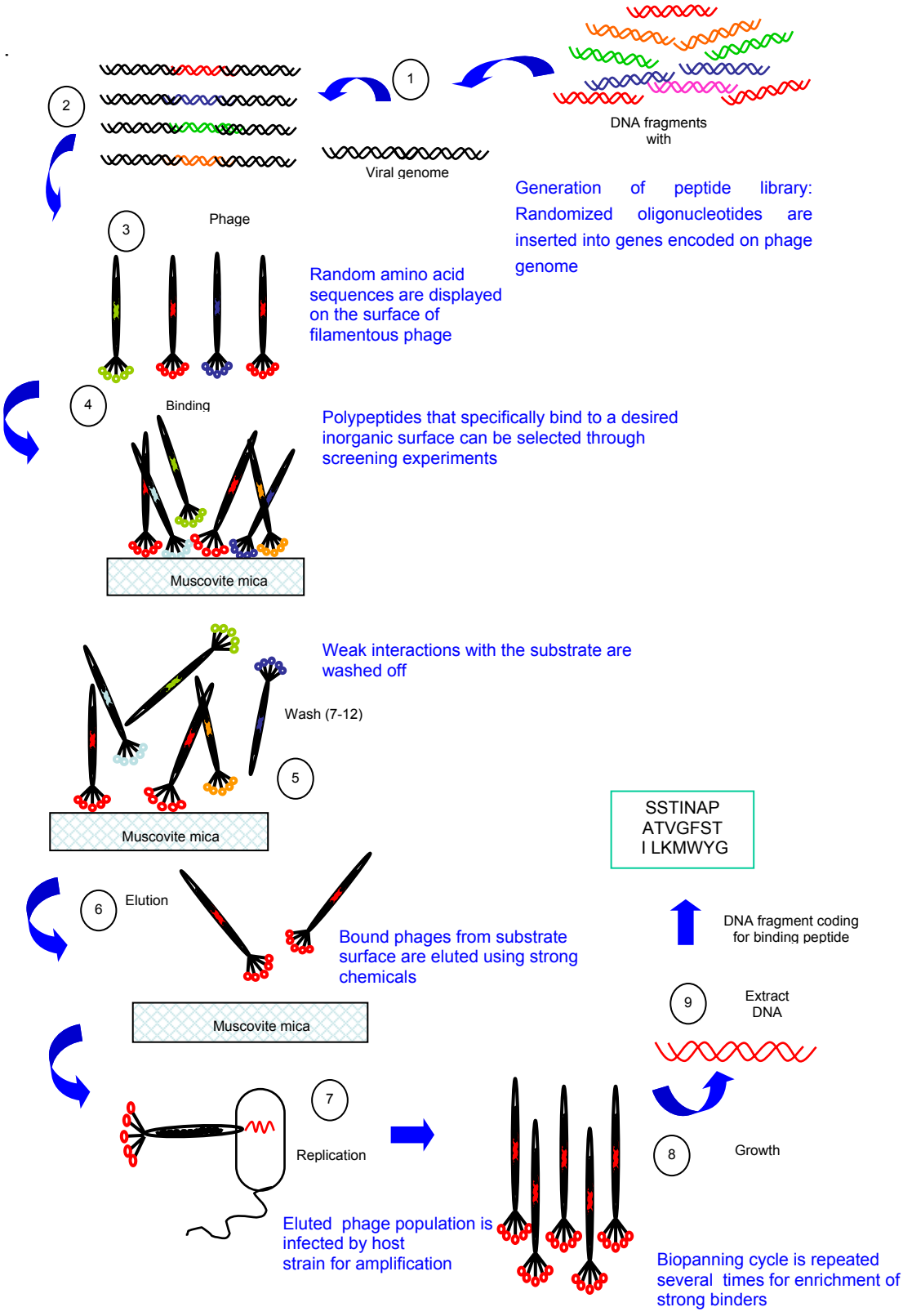



**Figure 1.6** Phage display procedure for selection of inorganic binding polypeptides [8, 15].

In this study, ultrasonication and bound phage recovery methods as a novel approach were used to obtain binders that are retained by inorganic surface and as a consequence, number of inorganic binders also increased.

**Amplification and purification step:** Following the elution of bound phage, it is essential to amplify the recovered phage population before starting to the next round of biopanning. During the amplification, phages that have better growth characteristics may be preferentially amplified. Some of good binders will be eliminated because of the weak growth characteristics of the phage it is contained in and also some of the weak binders that have the better growth characteristics will be enriched. Growth competition or poor expression of a peptide due to rare codons, misfolding, degradation or inefficient export, may also endanger the screen [8,15]. Thus, parallel use of multiple screening techniques may offer greater flexibility in working with unstable materials and maximizing the number of useful screens. This step completes one round of biopanning. Generally, three to five rounds of biopanning are needed for enrichment of tight binders. After the amplification period, to get the pure phage from the biopanning, a purification step is necessary. In this step M13 phage libraries have major advantages over the other phage libraries, such that M13 is non-lytic. Consequently, they do not lyse the host cell after amplification period; therefore, host cell can subsequently be removed from the phage-host cell culture, through centrifugation. In contrast, other phages that have been used for phage display (T4, T7, lambda) are all lytic, necessitating additional time-consuming purification steps. Because proteases, which are present in the cell can degrade phage, and cellular proteins, they cause a big problem during the purification of these phages. At the end of the biopanning, individual clones are sequenced to obtain the amino acid sequences of the polypeptides binding to the target substrate material [48].

### 1.3.1. Selection of Substrate Material

In this study, single crystal oriented in (001) muscovite mica sheet was the substrate. Selection of inorganic-binding polypeptides should ideally be performed using inorganic materials that have specific morphology, size, crystallography and surface stereochemistry. Furthermore, chemical inertness, surface stability and surface roughness should be taken into account while determining the substrate material for

selection [8, 15]. Muscovite mica, has well defined surface as an ionic crystal, with its chemically inert and very smooth surface is a proper inorganic material for the selection of specific binders. On the other hand, as silica containing mineral, mica has the advantage of being a preferable material in nanotechnology. Silicate-based nanotechnological structures are likely to be particularly valuable where an unreactive void-bearing framework is desirable, as in selective catalysis, ion exchange and adsorption, solid electrolytes. Besides, they could be used as arrays of semiconductor clusters for novel electronic and optical devices in nano scale [49].

As an excellent property, mica has an atomic layered structure and this has motivated its extensive use in scanning-force microscopy, namely in the characterization of nanostructured materials [49]. Because of its perfectly layered atomic structure, atomically flat surfaces of macroscopic (~$cm^2$) sizes can be achieved by simply cleaving the mineral. This perfect basal cleavage can only be obtained if mica has (001) crystallography [50]. Mica sheet also has applicability both in the biotechnological and nanobiotechnological areas because of its interesting physical properties, such as being ionic crystal, having high dielectric strength, low thermal conductivity, high temperature resistance.

Micas are an important group of minerals, representing the classic phyllosilicate mineral and are usually the first minerals to be thought of forming this subclass of the silicate class. Thin flakes of mica are generally flexible and brittle. However mica crystals are resistant and durable at high temperatures and at pressures in metamorphic regimes as well as against the erosional environments. There are over 30 different mica minerals in nature. They can be grouped into seven categories: Muscovite, paragonite, lepidolite, zinnwaldite, biotite, phlogopite and lepidomelane [50,51,52]. *Muscovite* is the pure potassium mica, whereas, *Paragonite* mica is a chromium rich variety of muscovite in which chromium cations substitute for some of the octahedral aluminum in the muscovite crystal structure. The chromium impurities in fuchsite are responsible for its emerald green color. *Lepidolite* mica is a lithian mica in which an occasional lithium cation substitutes for some of the octahedral and tetrahedral aluminum in the mica crystal structure. *Zinnwaldite* is a lithian ferrous mica, where lithium and iron cations take place of some of the octahedral and tetrahedral alumina. *Biotite* is one of the common types of mica, containing iron and/or magnesium impurities instead of octahedral aluminum.

### 1.3.1.1. Mica Lattice Structure

Being true phyllosilicates, all micas are thus composed of sheets of silicate tetrahedrons formed by interconnected six membered rings. These rings are responsible for the mica's typical six sided pseudohexagonal symmetry; in actuality they are only monoclinic or triclinic. Each tetrahedron in the rings shares three of their oxygens with three other tetrahedrons and all the tetrahedrons in a given sheet point their unshared oxygen in the same direction. The structure of micas is stacked like a building with several different layers. Two tetrahedral layers (***T***) with their tetrahedral points facing each other, sandwich small metal ions such as aluminum in an octahedral layer (***O***). This tetrahedral-octahedral-tetrahedral (***TOT***) sandwich is stacked in layers of large cations such as potassium or calcium. This cation layer is known as the interlayer (***i***) because it stands between the ***TOT*** sandwich layers. This interlayer is needed to balance the formula created for the substitution of the +3 charged aluminum for +4 charged silicons in the ***T*** layers. The whole structure can then be illustrated with the following sequence of layers: ***...iTOTiTOTiTOTiTOTi...*** (Figure 1.7) [51,52].

The general formula for the micas is $AB_{2-3}(X, Si)_4O_{10}(O, F, OH)_2$. The tetrahedral layers by themselves have a formula of $(X, Si)_2O_5$. In most micas the **A** ion is usually potassium but can also be sodium, calcium, barium, cesium and/or ammonium. These ions occupy positions in the interlayer ***i***, discussed above. The **B** ion can be either aluminum, lithium, iron, zinc, chromium, vanadium, titanium, manganese and/or magnesium. These ions occupy positions in the octahedral layers ***O***. The **X** ion is usually aluminum but can also be beryllium, boron and/or iron (+3) and they sit in the center of the tetrahedrons substituting for silicons by up to 50% [51,52].

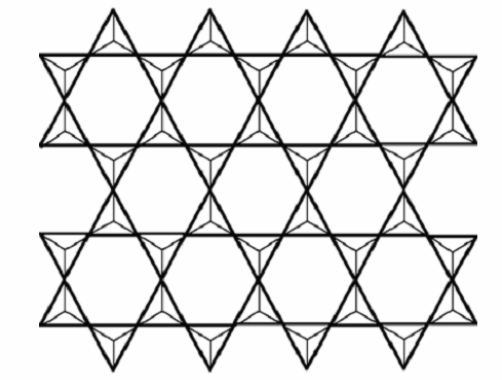


**Figure 1.7** Sheet structure of mica [51].

The most common mica is muscovite mica, known also as potassium mica (Figure 1.8). In the structure of muscovite mica, there are pyrophylitte layers with one aluminum atom replacing one of the silica atoms in each tetrahedral layer, linked together by potassium atoms in twelvefold coordination with oxygen [50,51,52].

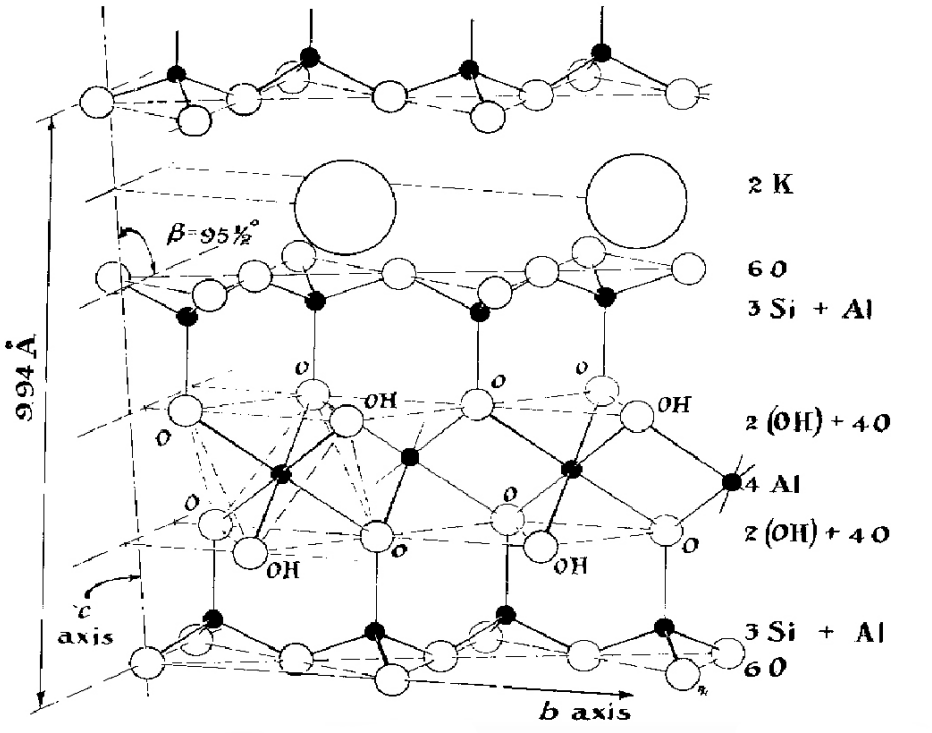



**Figure 1.8** Crystal structure of muscovite mica [50].

### 1.3.1.2. Mica as Engineering Material

As mica has interesting physical properties, the use of mica in industrial applications is a common desire. As mentioned before, mica has high dielectric constant, high temperature resistance and low thermal conductivity. Because of its high dielectric strength, mica is mostly used as an insulator. Electrical insulation in electronic equipment, in electric motors and generator armatures; field coil insulation, magnet and commentator core insulation, insulation of high-voltage conductors are some examples from the various mica applications. Mica with its good thermal properties such as high temperature resistance and low thermal conductivity is applied to the manufacture of heat-resistant bricks and tiles. One of the most important uses of mica powder is as filler in the plastics, paints and rubber industry. As filler in

rubber products, mica improves the hardness, tensile strength and tearing resistance of the product. Another common use of mica powder is the lubrication of mould in the manufacture of tyres. It prevents the mould from sticking to the tyre. Due to its pearly luster and glitter, mica is used in nail varnishes, lipsticks and eye shadows in cosmetic industry [50,51,52].

Based on the fact that mica is a strategic material in the scientific and industrial world, it is expected that mica could find vast applications as biolinkers in nano-scale engineering, especially in electronic industry [50,51,52].

### 1.3.2. Selection of phage library

The Ph.D.-C7C™ Phage Display Peptide libraries are based on a combinatorial library of random peptide 7-mers fused to a minor coat protein (pIII) of M13 phage. The randomized sequence is 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. Displayed peptides show conformational flexibility via cyclisation or disulfide loop formation. Cyclisation can be achieved by using an amide bond between the *N*-alpha group and the side chain of the last residue or by a disulphide bridge between cysteine residues positioned at the *N*- and *C*-termini. Disulfide-constrained peptide libraries have been proven to be useful in identification of structural epitopes, mirror-image ligands for D-amino acid targets, and they lead to peptide-based therapeutics [48]. Additionally, imposing structural constraint on the unbound ligands, results in less unfavorable binding entropy, improving the overall free energy of binding, in comparison to unstructured ligands. Unconstrained linear peptides can attend millions of different conformations, only very few of which may be able to bind to a target receptor. Constraining the conformational freedom and thus decreasing the entropy of unbound peptides in a library may result in isolation of higher affinity ligands for a receptor target. Moreover, constraining supplies some protection against proteolysis. A major disadvantage of the constraint library is that the disulfide constraint may "freeze out" a conformation required for target binding [48].

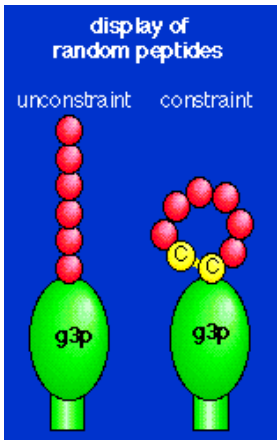



**Figure 1.9** Comparion of phage peptide C7C library and phage peptide 7 [45].

## 2. MATERIALS AND METHODS

### 2.1. Materials

#### 2.1.1. Bacterial Strains

***E. coli* ER2738 host strain**

F′ lacI$^{q}$ Δ(lacZ)M15 proA$^{+}$B$^{+}$ zzf::Tn10(Tet$^{R}$)/fhuA2 supE thi. (lac-proAB) Δ(hsdMS mcrB)5 ($r_k$ $^{-}$ $m_k$ $^{-}$ McrBC$^{-}$), not competent cell was purchased within Ph.D.-C7C™ Phage Display Peptide Library Kit, Catalog #E8120S [46].

#### 2.1.2. Phage Display Peptide Library

**Disulfide Constrained Heptapeptide Phage Display Library (Ph.D.-C7C™)**

In this study 100 µl (2 x $10^{13}$ pfu/ml) of Ph.D.-C7C™ Phage Display Peptide Library is used. This library purchased within Ph.D.-C7C™, Phage Display Peptide Library Kit, Catalog #E8120S contains ~ 1.2 x $10^{9}$ transformants supplied in TBS with 50% glycerol. These independent clones were fused to (gpIII) coat protein via a short spacer (Gly-Gly-Gly-Ser). The randomized sequence was flanked between 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 [46].

#### 2.1.3. Mica

Single crystal (001) muscovite mica sheets are used with the chemical formula of $KAl_2(AlSi_3O_{10})(OH)_2$.

#### 2.1.4. Bacterial Culture Media

##### 2.1.4.1. LB Medium

10 g tryptone (Acumedia), 5 g yeast extract (Acumedia), 5 g NaCl (Riedel-de-Haen) 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 for 15 min. under 1.5 atm at 121 ˚C.

#### 2.1.4.2. LB Agar Medium

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

#### 2.1.4.3. 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 for 15 minutes under 1.5 atm at 121 ˚C.

### 2.1.5. Stock Solutions

#### 2.1.5.1. Tetracyline Stock

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

#### 2.1.5.2. Xgal/ IPTG Stock

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

#### 2.1.5.3. 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.

#### 2.1.5.4. $MgCl_2$ Stock Solution

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

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.

### 2.1.6. Buffers

#### 2.1.6.1. 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.

#### 2.1.6.2. PC Buffers

PC buffer was prepared by dissolving 55 mM $KH_2PO_4$ (Merck), 45 mM $Na_2CO_3$ (Merck), 200 mM NaCl in distilled water and completing to 500 ml in a 1 liter flask. 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 (Riedel-de-Haen). PC buffers were sterilized by using 0.2μm single use sterile syringe filter.

#### 2.1.6.3. Elution Buffers

- **Elution Buffer I:** 0.2 M glycine (Merck) and 1mg /ml BSA (Sigma) were dissolved in distilled water up to 50 ml. and pH was adjusted to 2.2 with 10 M HCl and 0.1M HCl. The solution was sterilized by using 0.2 μm single use sterile syringe filter.

- **Elution Buffer II:** It is prepared by mixing equal amount of elution buffer A and B. The solution is sterilized by using 0.2 μm single use sterile syringe filter.

    - **Elution Buffer A:** 0.2 M glycine and 2 mg /ml BSA were dissolved in distilled water to 50 ml. and pH adjusted to 2.2 with 10 M HCl and 0.1M HCl .The solution was sterilized by using 0.2 μm single use sterile syringe filter.

    - **Elution Buffer B:** 1 M NaCl, 100 mM DDT (Sigma), 7mM TCEP (Sigma), 100 mM ME were dissolved in distilled water to 50 ml.

#### 2.1.6.4. Casein Blocking Agent

5 % casein, 10 mM Tris-base (Merck), 150 mM NaCI, %1 tween 20 was dissolved in 0.1M NaOH. pH was adjusted to 8.2 and added distilled water to 50 ml.

#### 2.1.6.5. Na-Ac Buffer

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

### 2.1.7. Agarose Gel Electrophoresis

Agarose gels (1%) were made by dissolving 1 g agarose (Sigma) 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.) 4 % (v/v) 0.5 M EDTA (Merck) pH 8.0 distilled water up to 1 l.

#### 2.1.7.1. DNA Molecular Weight Markers

**Lambda DNA/*EcoRI*+*HindIII* Marker 3:**

Lambda DNA was completely digested with *EcoRI* and *HindIII*, then phenol extraction and ethanol precipitation were performed by dissolving in storage buffer which consists of 10 mM Tris-HCl (pH 7.6) and 1 mM EDTA to form Lambda DNA/*EcoRI*+*HindIII* Marker 3, in a concentration of 0.5 mg DNA/ml (Figure 2.1.). This DNA Marker yields the following 13 discrete fragments (in base pairs): 21226, 5148, 4973, 4268, 3530, 2027, 1904, 1584, 1375, 947, 831, 564, 125. The cohesive ends of the 12 nt cos site of bacteriophage lambda from fragments 21226 bp and 3530 bp (indicated*) may anneal and form an additional band at 24756 bp [53].

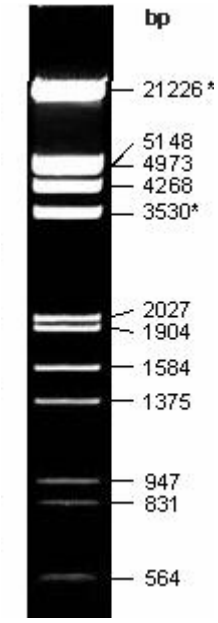



**Figure 2.1** λ DNA *EcoRI* and *HindIII* digested marker, 1.0% agarose, 0.5µg/lane, 8 cm length gel, 1X TAE, 17V/cm [53].

### 2.1.8. Lab Equipment

**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.

: 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.

: 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 procedure was carried out by incubating phage peptide library (Ph.D.C7C™) with target, washing away the unbound phage, and eluting the specifically bound phage (Figure 2.1). The eluted phage was then amplified by infecting the host strain ER2738. Following the amplification, phages were purified. These fundamental steps are called as biopanning or round to enrich the pool in favor of binding sequences. After 3-4 rounds, individual clones were characterized by DNA sequencing.

In this study, two screens were carried out. During the first screen, 3 rounds, during the second screen, 4 rounds were performed. In the first screen only ultrasonication, in the second screen both ultrasonication and bound phage recovery method were applied the mica surface after completion of chemical elution. At following subsections phage display protocol is discussed in detail and the other methods that are applied during the phage display procedure. The procedures of phage display described in subsections give the process flow of only one round.

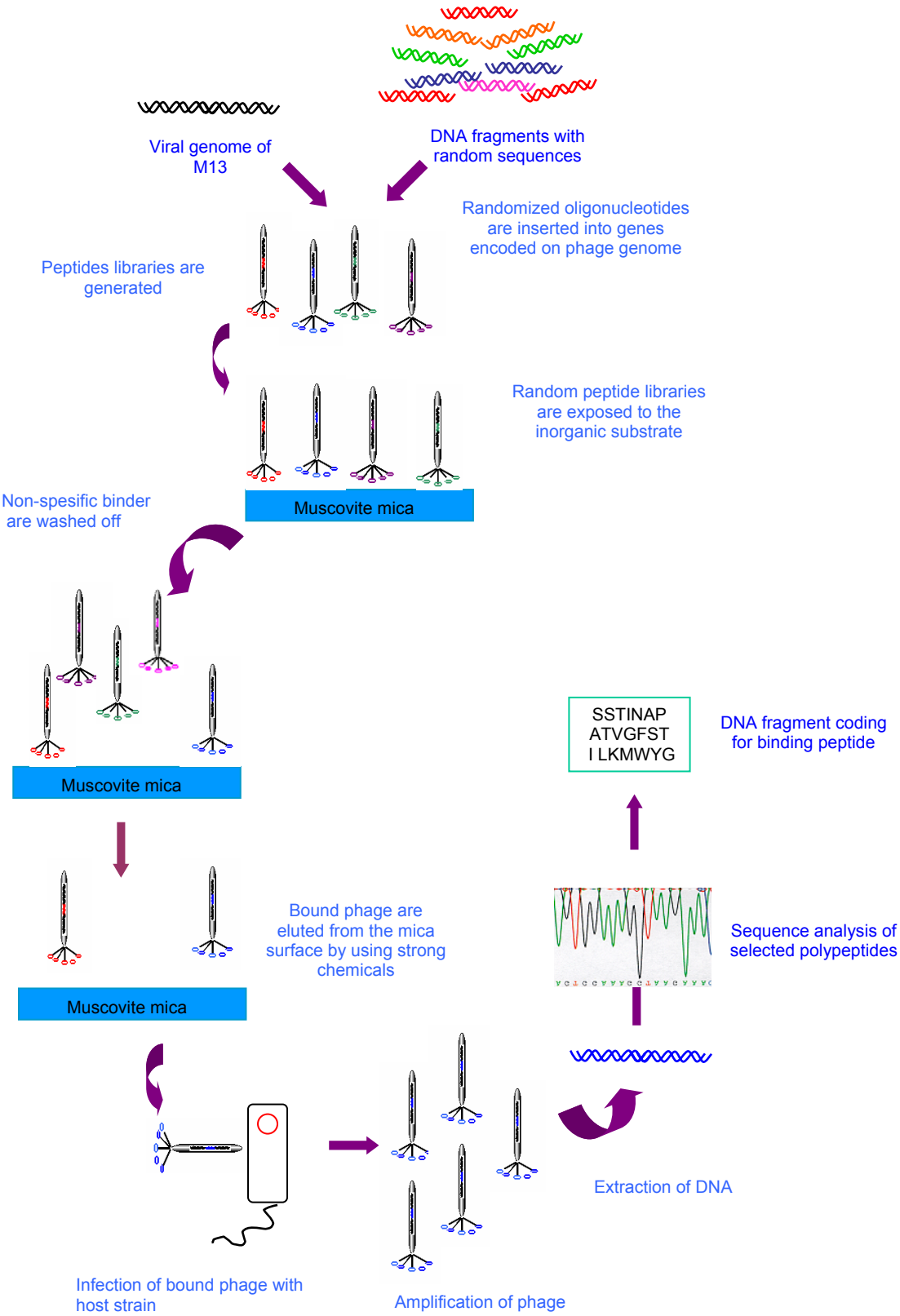



**Figure 2.2** Phage display procedure [8,15].

#### 2.2.1.1. Binding Step

In this step heterogeneous mixture of phage library was bound to the single crystal (001) muscovite mica for a specific period of time. Clean mica surface was obtained by cleaving a thin mica layer form the mica sheet. Ph.D.-C7C (New England Biolabs, MA) was exposed to the mica sheet in PC buffer containing 0.1% detergent for reducing phage-phage interactions on the surface. Mica-phage solution is rotated for 30 min in order to obtain better phage-mica interaction.

#### 2.2.1.2. Washing Step

After 30 min rotation, several washing cycles were performed in order to remove the non-specific phage from mica surface. Phages that strongly bind to the target substrate are retained, while the non-binder ones are washed away. These washing cycles were repeated for ten to thirteen times for each biopanning round. The detergent concentration was increased gradually up to 0.5%. Applied washing procedure was described below:

- Small mica sheet was divided into thin layers.
- One of the thin mica sheets was put into microfuge tube.
- 1 ml PC containing 0.1 % detergent was added into microfuge tube.
- 10µl of phage display peptide library (Ph.D.-C7C™) was added into microfuge tube.
- Microfuge tube containing mica and phage peptide library was put the on running cylinder 30 min for phage-mica interaction.
- The first supernatant was discarded and transferred into microfuge tube.
- Mica sheet was washed with 1 ml PC containing 0.1 % detergent.
- The washing steps were repeated 6 times.
- The supernatant was discarded at the end of the each washing steps and transferred into microfuge tubes.
- Microfuge tube containing mica and phage peptide library was put the on running cylinder 30 min for phage-mica interaction.
- The supernatant was discarded and transferred into microfuge tube. Mica sheet was washed with 1 ml PC containing 0.1 % detergent.
- Microfuge tube containing mica and phage peptide library was put on running cylinder 30 min for phage-mica interaction.

- The supernatant was discarded and transferred into microfuge tube.
- Mica sheet was washed with 1 ml PC containing 0.1 % detergent.
- The microfuge tube containing mica and phage peptide library was leaved on running cylinder overnight for phage-mica interaction.
- The supernatant was discarded and transferred into microfuge tube.
- Mica sheet was washed with 1 ml PC which contains 0.1% detergent.
- The microfuge tube containing mica and phage peptide library was put on running cylinder 30 min for phage-mica interaction.
- The supernatant was discarded and transferred into microfuge tube.
- Mica sheet was washed with 1 ml PC containing 0.1% detergent.
- The microfuge tube containing mica and phage peptide library was put on running cylinder 30 min for phage-mica interaction.
- The supernatant was discarded and transferred into microfuge tube.

#### 2.2.1.3. Elution Step

Specifically bound phages were recovered from mica surface through elution. With the use of elution buffers the strong interaction between phage and mica surface was disrupted. Applied elution procedure was described below:

- After the last washing step, 1 ml of elution buffer I was added onto the mica sheet in microfuge tube.
- Microfuge tube containing mica and elution buffer I was put the on running cylinder for 15 min to elute the phage from mica surface.
- The supernatant was transferred into microfuge tube XPYE1.
- 800µl supernatant XPYE1 was transferred into *E. coli* ER2738 culture ($OD_{600}$ ~ 0.5) and it was incubated 4.5 hours at 37ºC and 250 rpm.
- The remaining 200µl supernatant was neutralized by adding 40 µl of Tris, pH 9.1.
- 1 ml of Elution buffer II was put into microfuge tube XPYE2.
- Microfuge tube which contains mica and elution buffer II was put the on running cylinder for 15 min to elute the phage from mica surface.
- The supernatant was discarded and transferred into microfuge tube.
- 800µl supernatant into XPYE2 was transferred into *E. coli* ER2738 culture ($OD_{600}$ ~ 0.5) and it was incubated for 4.5 hours at 37ºC and 250 rpm.

- The remaining 200µl 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 contains mica and elution buffer II was put the on running cylinder for 15 min to elute the phage from mica surface.
- The supernatant was transferred into microfuge tube XPYE3.
- 800µl supernatant into XPYE3 was transferred into *E. coli* ER2738 culture ($OD_{600}$ ~ 0.5) and it was incubated for 4.5 hours at 37ºC and 250 rpm.
- The remaining 200µl 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 which contains mica and elution buffer II was put the on running cylinder for 15 min to elute the phage from mica surface.
- The supernatant was transferred into microfuge tube XPYE3.
- 800µl supernatant into XPYE3 was transferred into *E. coli* ER2738 culture ($OD_{600}$ ~ 0.5) and it was incubated for 4.5 hours at 37ºC and 250 rpm.
- The remaining 200µl supernatant was neutralized by adding 40µl of Tris, pH 9.1.

#### 2.2.1.4. Ultrasonication and Bound Phage Recovery Step

At the end of the chemical elution steps, ultrasonication was applied to the mica surface into PC (no detergent) with defined parameters such as Ultrasonic power: 150 W, duration: 30 sec, duty cycle: 0.5/sec. [65]. Following the ultrasonication, bound phage recovery method applied to mica surface and mica sheet was put into *E. coli* ER2738 culture host strain bacterial culture that is $OD_{600}$ ~ 0.5 to recover bound phage from the surface. By the way, phage that are remained on mica surface was infected *E.coli* ER2738 as host strain and amplified. At the end of the all different methods applied mica surface. Eluted phages were amplified as described in the next subsection.

#### 2.2.1.5. Amplification and Purification Steps

Eluted 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 $OD_{600}$ ~ 0.5 and then eluted phage samples transferred bacteria culture. $OD_{600}$ ~ 0.5 is the best phage-host strain propagation period. The incubation period is approximately 4.5 hours at 37ºC 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. After amplification eluted phages were purified from host cell according to the procedure below. Purification is the last step of biopanning cycle.

- At the end of 4.5 hours of growth period, *E.coli*-phage culture was transferred to 250 ml sterilized centrifuge tubes.
- Samples were centrifuged at 8000 rpm for 10 min.
- Supernatant was transferred to 250 ml sterilized centrifuge tubes.
- PEG/NaCl was added (1:6) into supernatant to precipitate phage and it was leaved overnight at 4 $^0$C.
- Samples were centrifuged at 8000 rpm for 20 min.
- Supernatant was discarded and phage pellet was resuspended with 5 ml PC buffer (no detergent) by shaking to remove any remaining *E.coli* ER2738.
- Samples were centrifuged at 8000 rpm for 10 min.
- Supernatant was transferred to 50 ml sterilized centrifuge tubes.
- PEG/NaCl was added (1:6) into the solution to precipitate phage and the solution was leaved 2 hours at 4 $^0$C.
- Samples were centrifuged at 10000 rpm for 10 min.
- Supernatant were discarded and phage pellet was resuspended by pipetting or shaking with 1 ml PC buffer (no detergent) to remove *E.coli* ER2738.
- Samples were centrifuged at 10000 rpm, for 10 min and supernatant were transferred to sterilized microfuge tubes.
- PEG/NaCl solution was added (1:6) into the microfuge tube to precipite phage, sample was vortex 5 sec, and leaved 10 min.
- Samples were centrifuged at 13500 rpm, 3 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 13500 rpm for 1.5 min.

- Supernatant was transferred to sterilized microfuge tubes and it was stored at -20$^0$C.

### 2.2.1.6. Blue-White Screening

This experiment was performed to estimate the phage titers at the end of each biopanning round. Blue-white screening experiment has three fundamental steps, namely preparation of Xgal/IPTG-tet plates, serial dilution of eluted phage samples, and estimation of phage titers for each round.

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

50 µl Xgal/IPTG, 250 µl 5mM $MgCI_2$ (1:200), 50 µl tetracyline (1:1000) were put 50 ml liquid warm LB agar in 100 ml glass medium flask and it was poured to plastic sterile petri dish. Plates were wrapped with parafilm and stored at 4$^0$C in the dark for a maximum of 1 month.

**Serial dilution of phage samples**

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 as shown in Figure 2.3.

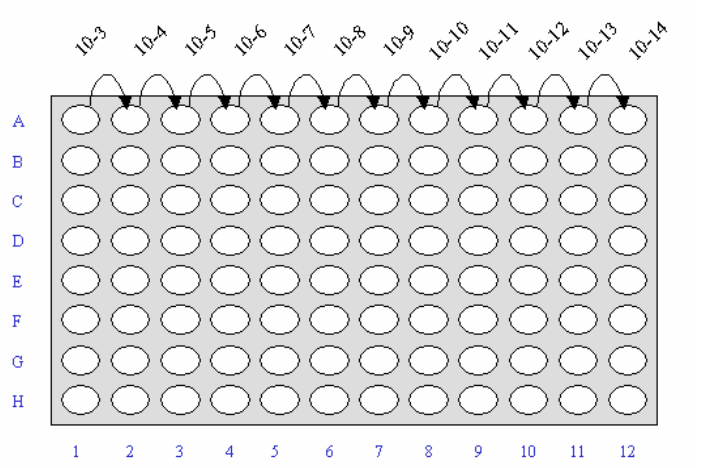



**Figure 2.3** Serial dilution of phage samples.

**Estimation of phage titers**

After serial dilutions Xgal/IPTG-tet plates that were prepared previously and stored at 4$^o$C, were put at room temperature. 3 ml melt top agar (for small petri dish) or 5

ml (for big petri dish) was aliqouted into 15 ml falcon tube and these tubes were put in 50°C water bath to prevent the solidification until other processes carried out.
6 µl 1M $MgCl_2$ (1:500), 3 µl tetracyline (1:1000) were put and 7.5 µl (1:250) *E. coli* ER2738 from overnight culture was inoculated into 3 ml LB in 50 ml falcon tube. The culture was incubated until mid- log phase ($OD_{600}$ ~ 0.5) at 37ºC and 250 rpm. After incubation period, 108 µl *E. coli* ER2738 culture and 12 µl diluted phage sample were put into 1.5 ml eppendorf tube for a small petri dish. 15 µl 5mM $MgCl_2$ (1:200), 3 µl tetracyline (1:1000) and phage - *E. coli* ER2738 mixture were added into 3 ml melt top agar and poured onto Xgal/IPTG-tet petri dishes. All petri dishes were inverted and incubated at 37°C for overnight. After incubation period, blue plaques were obtained as shown in Figure 2.4.

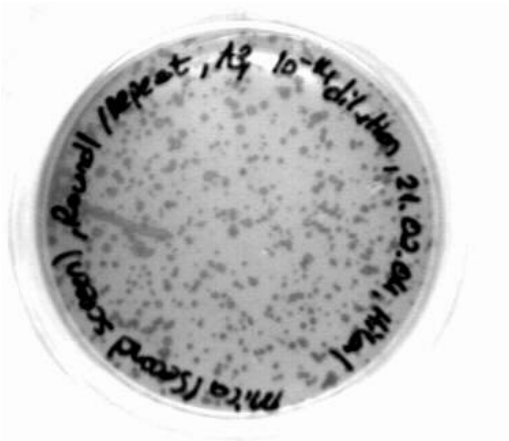


**Figure 2.4** Example of blue-white screening experiment

According to phage titers at each round, phage amount was determined to generate a phage pool for the next biopanning round. All phage titers and phage amount for each round were shown in Table 2.1.

**Table 2.1 Phage titers and amount of phage for each round**

| | First Round | | Second Round | | Third Round | |
|---|---|---|---|---|---|---|
| Elution | Dilution | Amount of phage | Dilution | Amount of Phage | Dilution | Amount of phage |
| A1 (first eluted phage) | $10^{-13}$ | 100µl | $10^{-14}$ | 100 µl | $10^{-19}$ | 50 µl |
| A2 (second eluted phage) | $10^{-16}$ | 50 µl | $10^{-15}$ | 50 µl | $10^{-12}$ | 75 µl |
| A3 (third eluted phage) | $10^{-14}$ | 75 µl | $10^{-15}$ | 75 µl | $10^{-11}$ | 75 µl |
| A4 (last eluted phage) | $10^{-15}$ | 50 µl | $10^{-15}$ | 75 µl | $10^{-9}$ | 100 µl |
| A5 (washing step before ultarsonication) | $10^{-15}$ | 50 µl | $10^{-16}$ | 50 µl | $10^{-14}$ | 50 µl |
| A6 (bound phage) | $10^{-15}$ | 50 µl | $10^{-16}$ | 50 µl | $10^{-15}$ | 50 µl |
| Total volume (phage pool) | | 375 µl | | 400 µl | | 400 µl |

### 2.2.1.7. Saving Clones for Sequencing

Blue-white screening experiments were performed on big petri dishes to pick up phage plaques easily. Preparation procedure of saving clones for sequencing was described in the following subsections.

**Preparation of Storage Plates with Different Phage Clones**

- 150 µl, 0.02 % PC buffer was put each well of 96-well elisa plate.
- Each phage plaque from big plates was picked and put into different well of elisa plate (Figure 2.5).
- 24 plaques were picked per each elution step. At the end of the fourth round 576 plaques were picked.
- 96-well plate containing phage clones was placed into the incubator at 60 $^0$C for 45 min. and 96-well plate was left at 4$^0$C for overnight.

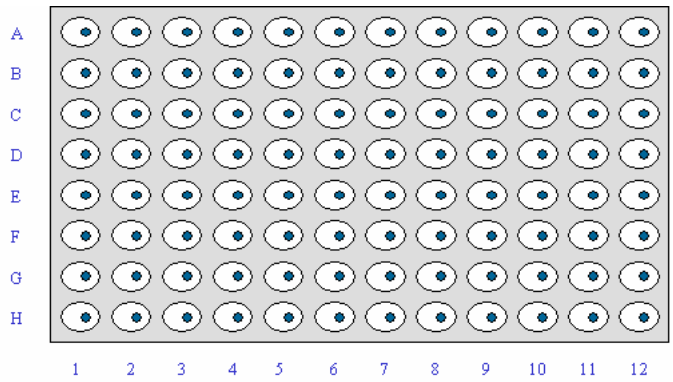



**Figure 2.5** Preparation of phage stock in glycerol

**Preparation of glycerol stock of phage**

- 60 µl sterilized 80% glycerol solution was put each well in two sets of 96-well plates (Overall glycerol concentration was kept as ~ 50 % in stocks).
- 50 µl clone was added from storage plate to glycerol containing plates.
- Plates were covered by parafilm and stored in –80$^{o}$C.

### 2.2.1.8. M13 Single Stranded DNA Isolation

After saving clones, phages DNA were isolated for DNA sequencing. DNA isolation was performed using QIAprep ® Spin M13 kit (QIAGEN, Catolog #27704) procedure shown in Figure 2.6. The procedure is described below in detail [54]:

1. 10µl sample, which belongs to only one phage plaque, was taken from the glycerol stock. And it was added into the 3ml *E. coli* ER2738 culture, which was incubated until mid-log phase ($OD_{600}$ ~ 0.5). After this step, culture was incubated 4.5 hours for phage –*E. coli* ER2738 infection.
2. Culture was centrifuged at 5000 rpm for 15 min. at room temperature supernatant containing M13 bacteriophage was transferred to a fresh reaction tube. During transferring the supernatant, bacterial pellet was not disturbed. Any carryover of bacterial cells will 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.) to the supernatant in 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 applied 0.7 ml of the sample to the QIAprep spin column.
5. Reaction tube was centrifuged for 15 sec. at 8000 rpm and discarded flow-through from collection tube. During this step, intact bacteriophage was retained on the QIAprep silica-gel membrane.
6. The last step was repeated until all supernatant passed through QIAprep spin column.

7. 0.7 ml MLB buffer was added for M13 lysis and binding, to the QIAprep spin column and centrifuged for 15 sec. at 8000 rpm. This step creates appropriate conditions for binding of the M13 DNA to the QIAprep silica-gel membrane. Bacteriophage lysis begins.
8. Another 0.7 ml MLB buffer was added 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. M13 single-stranded DNA is released from bacteriophage particles and adsorbed to the QIAprep to the silica gel membrane.
9. 0.7 ml Buffer PE was added and centrifuged for 15 sec. at 8000 rpm. In this step residual salt is removed.
10. Buffer PE was discarded from collection tube and centrifuged QIAprep spin column for 15 sec. at 8000 rpm to remove residual buffer PE. It is important to dry the QIAprep membrane with quick microcentrifugetion step. This prevents residual ethanol from being carried over into subsequent reactions.
11. QIAprep spin column was placed in a clean 1.5 ml microcentrifuge tube. 100µl EB buffer (10 mM Tris.Cl, pH 8.5) was added to 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. The DNA can also be eluted with water. When using water for elution, the pH of water should be in the range 7.0-8.5. Elution efficiency is dependent on pH and the maximum elution efficiency is achieved within this pH range [54].

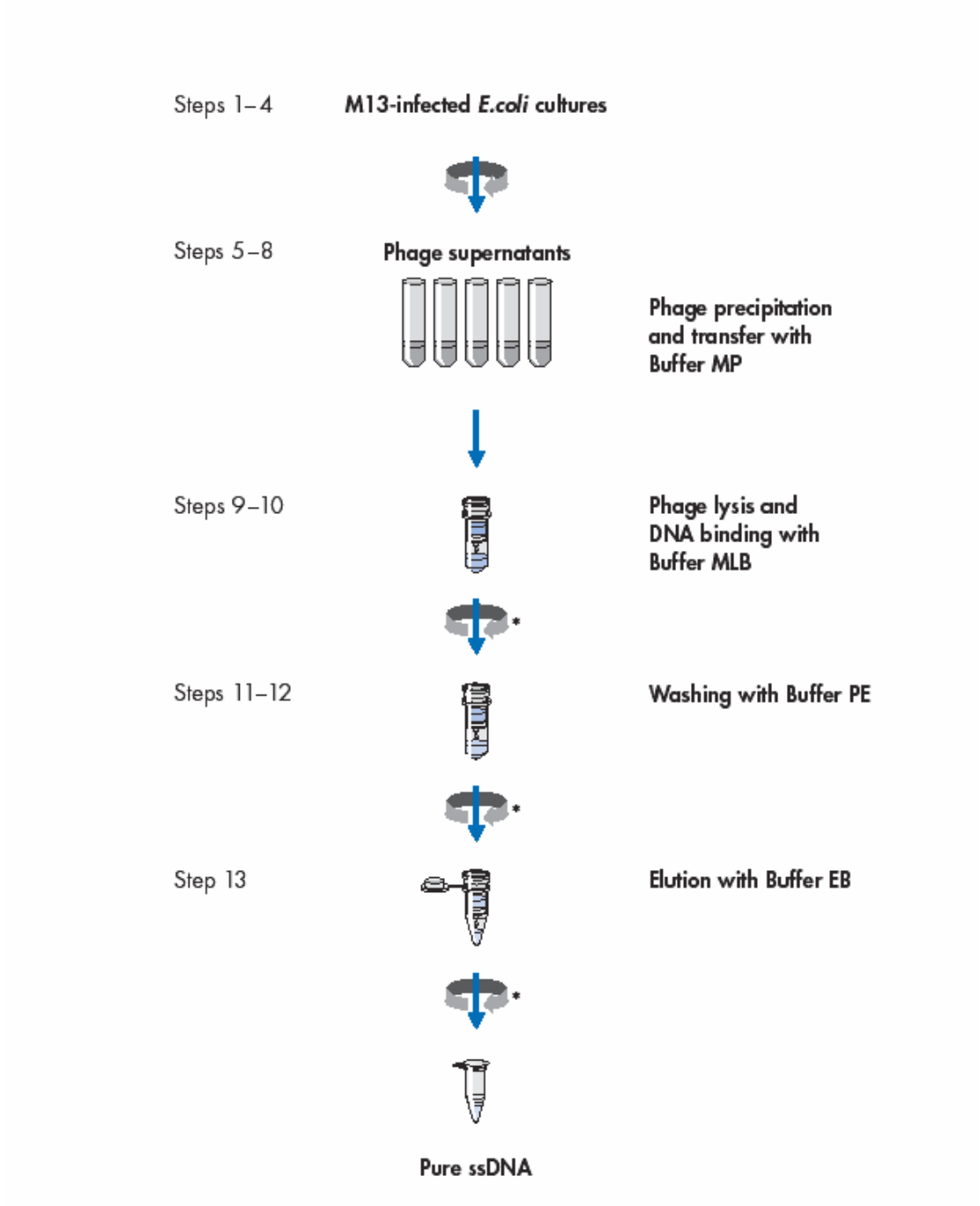



**Figure 2.6** M13 ss DNA isolation procedure [54].

### 2.2.1.9. Agarose Gel Electrophoresis

After DNA isolation and purification agarose gel electrophoresis was performed to control the amount of DNA. 2µl DNA of all samples was loaded into the each well of 1 %, agarose gel .1µl λ Marker was loaded into the last well. Figure 2.7 A and B shows the DNA in agarose gel.

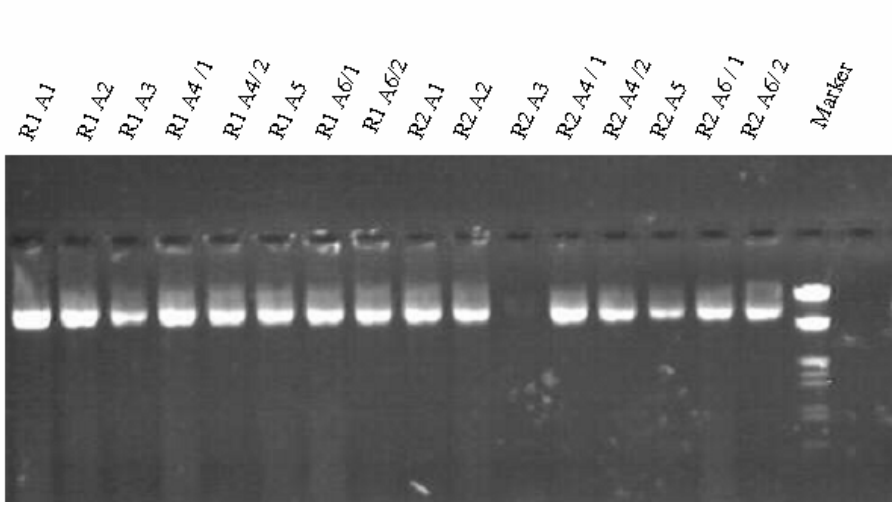



**Figure 2.7A** DNA samples that were isolated from selected phage colonies (2µl DNA+2µl loading dye +4µl $dH_2O$ were load into each well. λ Marker was used.)

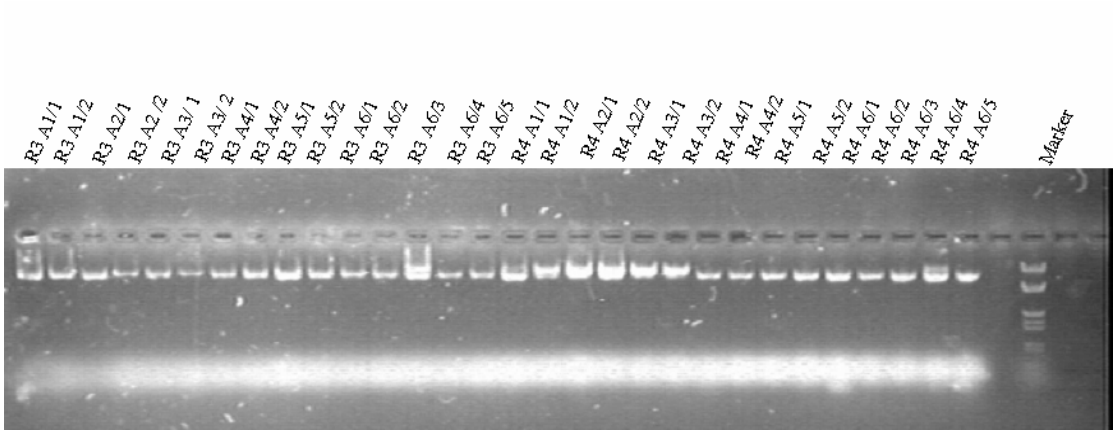



**Figure 2.7B** DNA samples that were isolated from selected phage colonies. (2µl DNA+2µl loading dye + 4µl $dH_2O$ were load into each well. λ Marker was used).

### 2.2.1.10. Measurement of DNA Concentration

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.

### 2.2.1.11. DNA Sequencing

**PCR conditions for DNA sequencing**

Reaction volume

| | |
|---|---|
| Big dye reaction mix* | 2µl |
| 5X sequencing buffer / $ddH_2O$ | 1µl |

| | |
|---|---|
| Template ssDNA | 1µl (75 ng) |
| Primer (-96 sequencing primer)** | 1.6 µl (3.2 pmol) |
| $dH_2O$ | 4.4 µl |
| Total volume | 10 µl |

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

** –96 gIII sequencing primer (5′- HOCCC 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 [46].

PCR conditions for cycle sequencing

| | | |
|---|---|---|
| 95 °C | 2’ | 35 cycle |
| 95 °C | 10’’ | |
| 55 °C | 10’’ | |
| 60 °C | 4’ | |

**PCR product purification**

- 2 µl, (3M pH 4.6 sodium acetate) and 50 µl, 95 % ethanol were mixed for each sample.
- 52 µl mixeture was put into each PCR product and all samples were put in 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°C and then -20°C for 5 min. to denaturate the samples.

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

### 2.2.2. Characterization of Sequences

This part contains detailed characterization of each obtained sequences and this characterization analyzed into three main subsections. The first part based on

determination of selected sequences physicochemical properties such as pI, MW and charge. The second part based on statically amino acid analyzes of each sequences. The last part based on analyzing the affinity level of each selected sequences by fluorescent microscopy experiment techniques.

#### 2.2.2.1. Physicochemical Analysis

In this section**,** isoelectric points (pI) and molecular weight (MW) of peptides were calculated by using compute pI/Mw tool http://us.expasy.org/tools/pi_tool.htm. Isoelectric point is the pH at which a protein carries no net charge. Below the isoelectric point proteins carry a net positive charge, above it a net negative charge. Molecular weight is the relative atomic masses of constituent atoms of a molecule. Charge of the polypeptides was calculated by subtracting the number of basic residues (R and K) from the number of acidic residues (D and E).

#### 2.2.2.2. Statistical Analysis

This section focused on comparison of obtained amino acid frequencies sequences with the observed and expected frequencies of the Ph.D.-C7C ™ library. In the library manual, expected and observed frequencies were shown and calculated .The detail data of the library observed and expected frequencies also shown in Table2.2

In the library manual, expected frequency was calculated by defined formula. The formula is expected frequency = number of clones for that amino acids\ 32*100. In this formula the codon number (32) shows the use of reduced genetic code in library construction thus expected frequencies give us more mathematical data.

Observed frequency was calculated with different way in the library manual. The number of all obtained amino acids and the overall amino acids distribution were calculated. As shown in Table 2.2, a total of 83 clones were sequenced from this library. All of the sequences should contain 7 amino acids flanked by cysteine residues. Total number of amino acids is 581 as shown in Table 2.2. Then % observed frequency was calculated.

In this study, during the first screen 14, during the second screen 64 clones were sequenced. The number of all obtained amino acids, overall amino acid distribution % observed frequency were calculated depends on the instruction manual.

By this data, we can easily analyze the expression level of the all observed amino acids by the comparison of the observed frequencies in the library thus relative

abundance graph shows us low expressed and over expressed for each amino acid type that were obtained in the screen [46].

**Table2.2** Observed and expected frequencies of Ph.D.-C7C ™ that was used in screening experiment [46].

| Amino acid type | Codon type | Expected frequency | Observed frequency |
|---|---|---|---|
| R | CGK.AGG | 9.4 % | 4.3% (25\581) |
| L | CTK.TTG | 9.4 % | 9.6 % (56\581) |
| S | TCK. AGT | 9.4 % | 8.6% (50\581) |
| A | GCK | 6.2 % | 6.5 % (38\581) |
| G | GGK | 6.2 % | 2.2 % (13\581) |
| P | CCK | 6.2 % | 10.7% (62\581) |
| T | ACK | 6.2 % | 13.1% (76\581) |
| O | CAG.TAG | 6.2 % | 7.1 % (41\581) |
| V | GTK | 6.2 % | 1.9 % (11\581) |
| N | AAT | 3.1 % | 6.4% (37\581) |
| D | GAT | 3.1 % | 4.1% (24\581) |
| C | TGT | 3.1 % | 0 % (0\581) |
| E | GAG | 3.1 % | 3.1 % (18\581) |
| H | CAT | 3.1 % | 6.9 % (40\581) |
| I | ATT | 3.1 % | 2.1 % (12\581) |
| K | AAG | 3.1% | 3.8 % (22\581) |
| M | ATG | 3.1 % | 3.3 % (19\581) |
| F | TTT | 3.1% | 2.1 % (12\581) |
| W | TGG | 3.1% | 1.9 % (11\581) |
| Y | TAT | 3.1% | 2.4 % (14\581) |

### 2.2.2. Immunofluorescence Microscopy Experiment

At the beginning of the fluorescence microscopy experiment, mica sheet was grinded to obtain mica powder. The powder was cleaned as described below.

#### 2.2.2.1. Cleaning Procedure for Mica Powder

- 100mg powder was weighed and put into preweighed 1.5ml microfuge tube.
- 100µl $dH_2O$ was added into the tube.

- $CH_3OH$/acetone mixture (1:1) was added into the tube. Powder pellet was dissolved gently by pipetting.
- The sample was vortexed for 5-10 min. to observe the forming clumbs.
- The powder was sonicated for 20 min. in ultrasonic bath to break the clumbs.
- The powder was vortexed quickly to resuspend.
- The powder was centrifuged at 200 g for 3min.
- The supernatant was removed and 1ml 50 % isopropanol was added onto the powder.
- Powder was vortexed for 5-10 min.
- The powder was sonicated for 20 min. in ultrasonic bath.
- The powder was vortexed quickly to resuspend.
- The powder was centrifuged at 200 g for 3min.
- The supernatant was removed and 1ml 0.5 % PC buffer was added.
- The powder was sonicated for 60 min.
- The powder was vortexed quickly to resuspend.
- The powder was centrifuged at 200 g for 3min.
- The supernatant was removed and 1ml 0.5 % PC buffer was added.
- 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.
- Samples were centrifuged at 200 g for 3min.
- Samples were washed twice with isopropanol (1:1).
- Samples were washed twice with $dH_2O$.
- Samples were dried under the vacuum.
- All samples were weighed to determine the amount of powder into the each tube.

#### 2.2.2.2. Fluorescence Microscopy Procedure

At the beginning of the fluorescence microscopy experiment, both negative and positive control experiments were carried out to decide on the right procedure during the fluorescence experiment.

In the negative control experiment, proper amount of powder suspension of washed and cleaned mica was incubated with 10µl phage (PFU/ml $10^{11}$) in PC buffer containing 0.1 % detergent for binding and left overnight. This mixture was incubated only 2.5µl secondary antibody for 10 min. on running cylinder. Following the centrifuge step, the sample was visualized under fluorescence microscopy (FM).

In the positive control experiment was performed by using different blocking agents such as gelatin (1 %), casein (5 %). In this experiment, two of the proper amount of powder suspension of mica sample was incubated with 10µl phage (PFU/ml $10^{11}$) in PC buffer containing 0.1% detergent for binding and left overnight. Following this step, one of the samples was incubated with 1ml casein blocking. Another sample was incubated with 1ml gelatin blocking agent for 15 min. Following the incubation period, mica samples were centrifuged and casein blocking and gelatin blocking agent were removed from the each sample. Only the primary antigen and secondary antibody dye mixture was added as described ratio in below and incubated for 10 min. Following the sample preparation, they were analyzed under FM.

After the determining the right procedure, each obtained sequences were analyzed by using the described procedure as below. Proper amount of powder suspension of washed and cleaned mica incubated with 10µl phage (PFU/ml $10^{11}$) in PC buffer containing 0.1 % detergent for binding and left overnight. After attaching phage to the powder, this conjugate was incubated with Tris buffer containing casein as a blocking agent for 10 min. in order to coat the powder and occupy any binding sites that the antibody might attach to. Each phage sample with a determined DNA sequence is labeled according to immunolabelling procedure (Figure 2.8). Anti-M13 pIII monoclonal antibody (Amersham Biosciences) as a first antibody, which is specific to M13 gp8 protein and Alexa-Fluor [55] 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, 3µl mixture was put in phage–mica solution and incubated for 10 min. on running cylinder to increase phage-mixture interaction. Finally, these complexes were visualized by fluorescence microscopy (BX60, Olympus Corporation) at 20X magnification under WIB filter.

Based on the FM experiment procedure, all obtained good binders were analyzed by using the titanium powders, which were used as a material for cross-specificity under fluorescence microscopy.

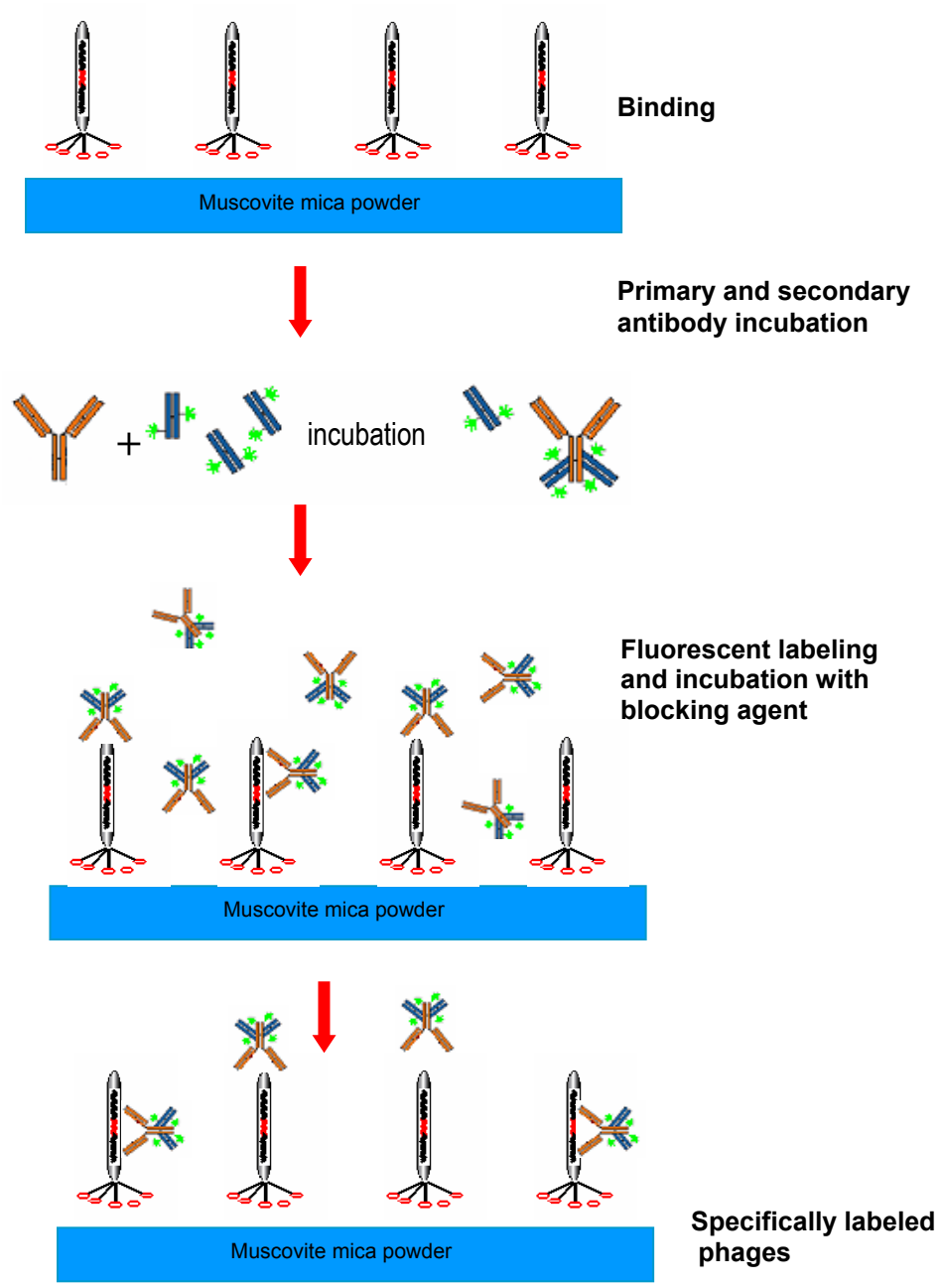



**Figure 2.8** Labeling procedure for fluorescence microscopy

## 3. RESULTS AND DISCUSSION

### 3.1. Identification of Mica Binders

This study has focused on selection of disulfide-constrained heptapeptides exhibiting affinity to single crystal (001) muscovite mica surface using phage display technique. Choosing mica has some advantages depending on its layered atomic structure. Since the mica naturally has very smooth and clean surfaces; it provides a good substrate for phage display experiments when splitted into thin layers [50]. Mica has applications both in the biotechnological and nanobiotechnological areas because of its interesting physical properties, such as being an ionic crystal, having high dielectric strength, low thermal conductivity, high temperature resistance, and being chemically inert [50].

During the selection of mica binders by applying the phage display technique, different buffer solutions containing strong chemicals with different pH ranges were used. These buffers can affect material surface morphology and cause formation of oxide layer on the surface [8,15]. In other words, when the material is treated with these buffers, surface conditions could be changed. Consequently, material stability in these buffers should be analyzed at the beginning of the screening procedure. This problem may also be overcome by a careful choice of buffer combination. Inertness of the material towards these chemicals is certainly important as well. In this study, material stability was analyzed by SEM. Besides SEM studies; detailed characterization experiments of the material were also carried out. For instance, XRD experiments were performed to determine crystallinity of mica. Zeta potential analyses were done to determine the surface charge of the mica substrate.

Following material characterization and selecting right buffer combinations, two screenings were carried out to select the mica binding polypeptides. During the first screening, 3 biopanning experiments, during the second screening, 4 biopannings were carried out. In the biopanning steps, strength of the washing buffer was increased gradually depending on the detergent concentration as the number of the rounds increased one by one to find better binders after each round. During the

washing steps, non-specific binders were removed from the mica surface. Following the washing steps, several cycles of chemical elution were used for the removal of bound and presumably, specific phage to the material. An often-limiting factor of biopanning procedure is the inability of removing all specific binders from inorganic surface. In this study, novel approaches, such as application of bound phage recovery and ultrasonication as a physical elution method were also tried to overcome the limitations of biopanning procedure. During the first screening, only at the last round, following the chemical elution, ultrasonication was applied to remove tightly bound phage from the mica surfaces. Ultrasonication parameters had already been chosen according to the results of a separate study that is related to effects of ultrasound energy on phage viability in our research group. These parameters were, Ultrasonic power: 150 W, duration: 30 sec. , duty cycle: 0.5/sec. [50]. During the second screening, following the chemical elution at each round, ultrasonication and bound phage recovery methods were applied to the mica surfaces. Ultrasonication parameters were kept constant for comparison at both screens. In bound phage recovery method, after applying the ultrasonication on mica surface, mica sheets were added into *E. coli* ER2738 host strain bacterial cultures. Thus, phages that remained on mica surfaces were used to infect *E. coli* ER2738 as host strain. Application of these approaches increased the number of mica binders.

Phage colonies were selected to isolate the ssDNA from chemical elution, physical elution and bound phage recovery methods. All the ssDNA were sequenced and 14 sequences from the first and 64 sequences from the second screen were selected, a total of 78 mica-binders were obtained.

Characterization of sequences was then performed. The characterization studies were grouped into three different sections. In the first section, physiochemical properties of the selected sequences (pI, MW and charge) were analyzed and compared. The second step was based on statistical amino acid analyses of each sequence. The overall amino acid distributions in each peptide were calculated. The overall distributions of amino acids obtained in our selected sequences were compared to expected and observed frequency of amino acids in the commercial phage library we used. Thus, the expression level of the all observed amino acids were easily analyzed by comparing the observed frequencies in the library, and the relative abundance graph demonstrated that low expression or over expression of

each different amino acids of selected sequences that were obtained in the screening. The last step was based on the analysis of the affinity level of each selected sequence by fluorescence microscopy visualization. At the beginning of the fluorescence microscopy, mica sheets were grinded to obtain wide surface area.

Individual phage with determined DNA sequences were phage with determined DNA sequences was exposed to grinded mica powder. Then the phages were labeled with anti-M13 gp8 monoclonal antibody and Alexa-Fluor conjugated secondary antibody fragments. This approach seems to be the best and easiest way of starting to categorize the sequence results relative to each other according to their affinities for a specific substrate. The way we used to measure the binding capability of each sequence was semi-quantitative. Depending on fluorescence microscopy results; binders were grouped as strong, moderate and weak binders.

The cross-specificity experiments among the strong binders with titanium powder were also performed. Identification of the high affinity binders to mica surface is not enough to assess the specificity of the selected sequences; therefore cross-specificity checks using other substrates should be performed to analyze the specificity of sequences for mica.

### 3.2. Characterization of Muscovite Mica

#### 3.2.1. Phase Analysis with Grazing Angle X-Ray Diffractometer

The crystallinity of the mica used in screening experiments was examined by grazing angle X-Ray diffraction method. Obtained diffraction data was compared with JCPDS (Joint Committee of Powder Diffraction Standarts) patterns. According to JCPDS patterns our mica had the chemical formula of $KAl_2(AlSi_3O_{10})(OH)_2$, namely muscovite mica, known also as potassium mica. The first peak in the graph (Figure 4.13, x=8˚) is dominant compared to other small peaks (Figure 4.13, x=13, 17, 27˚). Hence, it characterizes the single crystallinity of the structure with the [001] orientation.

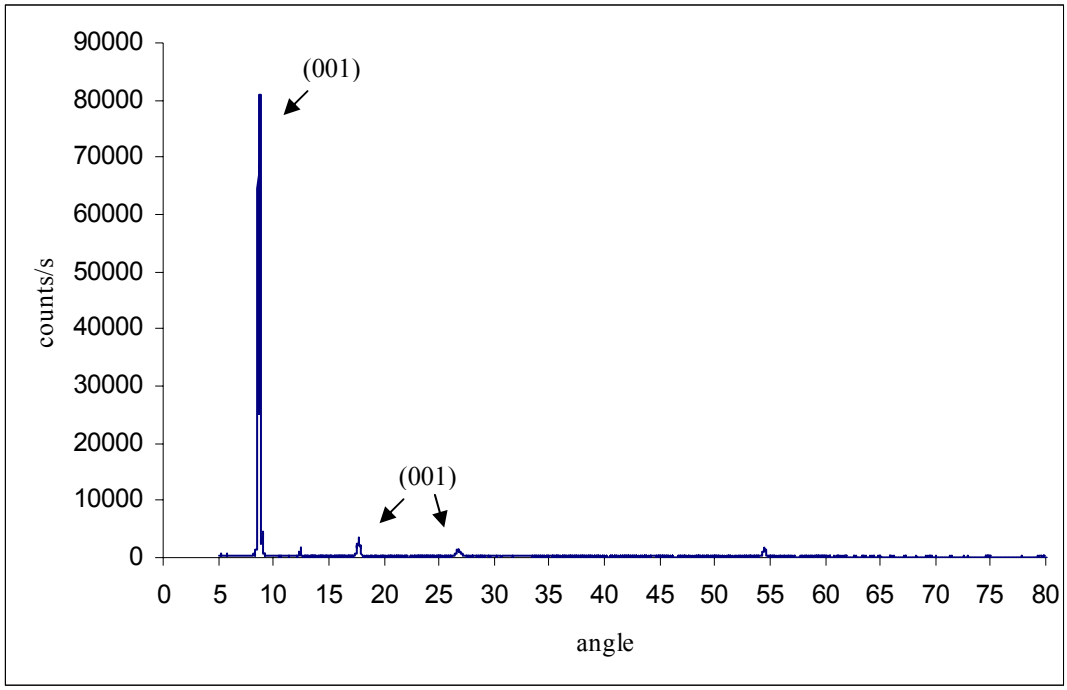



**Figure 3.1** X-Ray diffraction pattern of muscovite mica [50]

### 3.2.2. Surface Analysis with Scanning Electron Microscopy

Freshly cleaved mica surfaces were examined by scanning electron microscopy (SEM) to investigate the structural morphology (Figure 3.2). As expected, a flat surface was observed which is convenient for screening experiments [50].

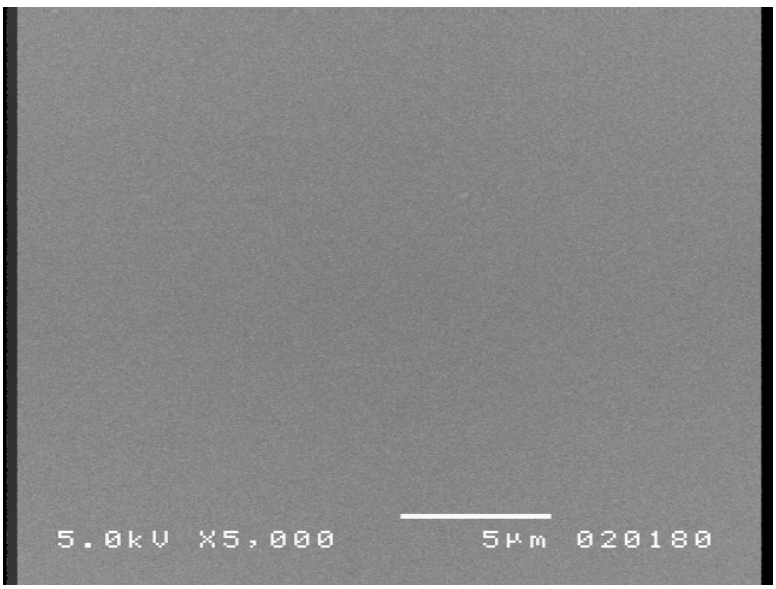
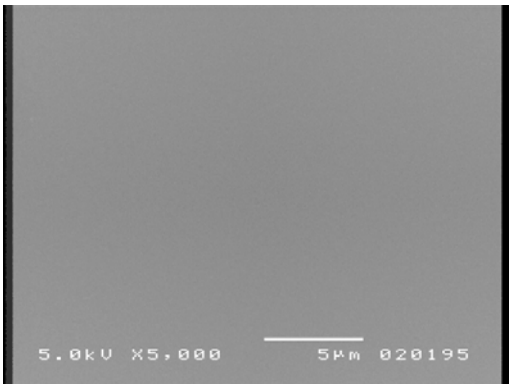
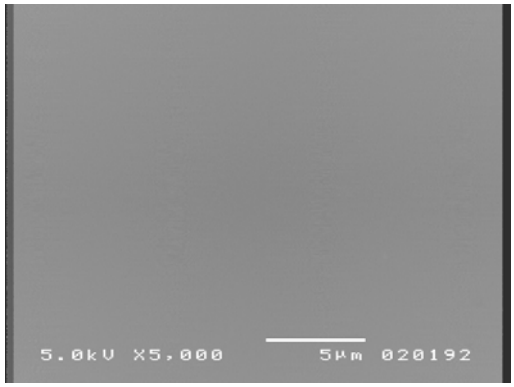



**Figure 3.2** SEM image of muscovite mica (5 kv voltage, 5000X magnification)

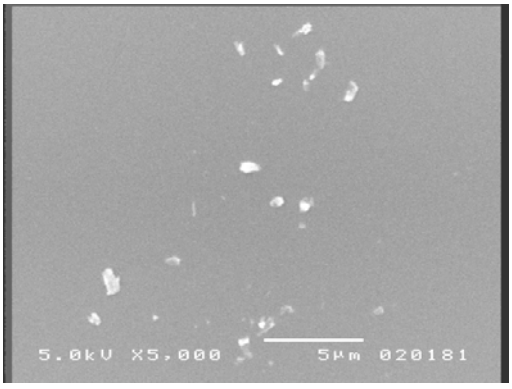
The influence of buffers and ultrasonic power on mica surface was also investigated through Scanning Electron Microscopy in order to analyze the surface conditions. Cleaved mica sheets were incubated in potassium carbonate (PC) and elution buffers according to the screening procedure. As it can be seen from Figure 3.3, PC buffer with detergent percentage (0.1%, 0.2%) did not damage mica surface, whereas elution buffers caused some damages on the muscovite surface (Figure 3.4.). However, the background of the image did not change significantly [50].

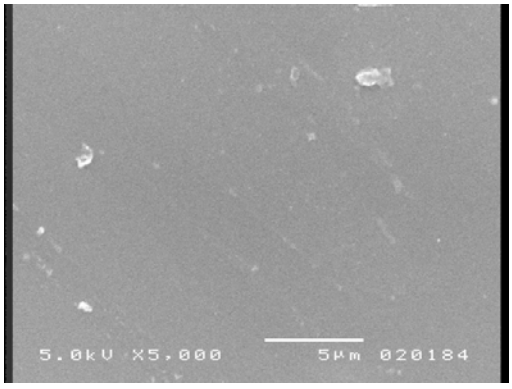



PC buffer containing 0.1 % detergent PC buffer containing 0.2 % detergent

**Figure 3.3** SEM image of muscovite mica hold in PC buffer with 0.1 % and 0.2 % detergent [50]



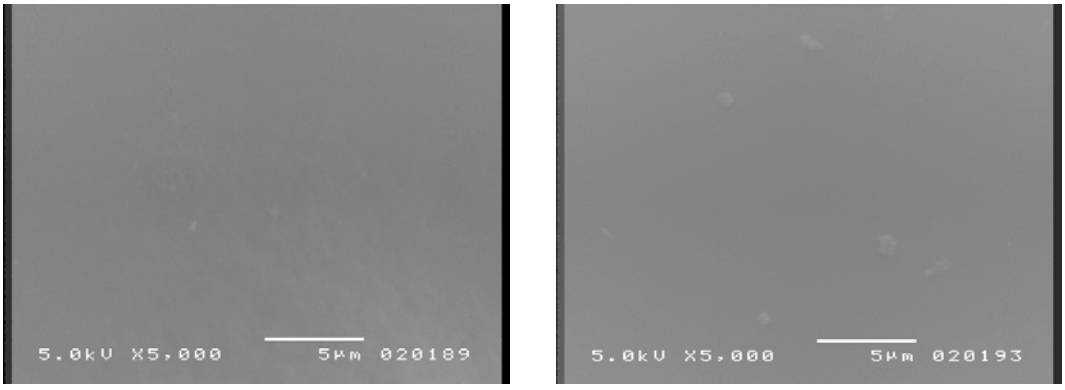




Elution Buffer I Elution Buffer II

**Figure 3.4** SEM image of muscovite mica hold in elution buffer I and II [50]

In sono-screening experiment, ultrasonic power was exposed to mica-surfaces that in PC buffer containing no detergent. To examine if the ultrasonic power introduced

any changes to the surface conditions, mica sheet was kept in PC buffer with no detergent for approximately 1 hour and then ultrasonication was applied using sono-screening experiment parameters. Results showed that there were no important changes in surface morphology. Besides, comparison of the SEM images of elution buffers and ultrasonication (Figure 3.4-3.5) indicated that ultrasonication could be a preferred method within the applied power because ultrasonication could cause less damage on surface than does elution buffers. It should also be added that these white spots on the SEM images could also be some dirt introduced during specimen preparation.




Before ultrasonication
(PC buffer without detergent)

After ultrasonication

**Figure 3.5** SEM images of muscovite mica before and after ultrasonication [50]

### 3.2.3. Surface charge analyses

Muscovite mica surface charges were determined. The zeta potential (surface charge) data was shown in Table 3.1. If the zeta potential data is below –25 or above +25, material is said to have high stability. In Table 3.1, Zeta potential data was below –25. This indicates that mica had high surface stability and chemically inertness. Zeta potential experiments also indicated that mica surface had negative charge. Surface charge experiments give us general information at macro level. At molecular level, different interactions may occur. However, this study does not give information about the molecular level interactions. Knowing the surface charge can provide an idea about the affinity of polypeptides to mica surface. The charge of amino acids and mica surface affect the binding affinity.

**Table 3.1** Surface charge of mica

| | KCps * | Mob** | Zeta*** |
|---|---|---|---|
| | 3264.7 | -3.961 | -49.8 |
| | 3475.8 | -3.976 | -50.0 |
| | 3322.9 | -4.005 | -50.04 |
| | 3174.6 | -4.109 | -51.7 |
| | 3741.7 | -4.136 | -52.0 |
| | 3321.2 | -4.256 | -53.5 |
| | 3287.4 | -4.385 | -55.2 |
| | 3157.4 | -4.374 | -55.0 |
| | 3226.8 | -4.531 | -57.0 |
| | 3303.1 | -4.610 | -58.0 |
| | 3064.7 | -4.569 | -57.5 |
| | 3326.6 | -4.551 | -57.3 |
| | 3293.9 | -4.714 | -59.3 |
| | 3055.1 | -4.573 | -57.5 |
| | 3166.6 | -4.722 | -59.4 |
| **Average** | 3278.8 | -4.365 | -54.9 |
| **+ / -** | **168.4** | **0.271** | **3.4** |

*** photon number **mobility *** Zeta potential**

### 3.3. Results of the 1st Screening

In the 1st screening, 3 biopanning rounds were carried out. Ultrasonication was applied at the end of the third round as a supplementary method to chemical elution. Here, a total of 14 sequences were selected after the last round of screening. 9 sequences were obtained by chemical elution (Table 3.2).

**Table 3.2** List of all sequences obtained by chemical elution in the 1st screening

| Sample number | Round | Occurance | Sequences |
|---|---|---|---|
| 1HY1 | 3 | - | S I T T N P S |
| 1HY2 | 3 | 7 | H P K P P R T |
| 1HY3 | 3 | - | Q M R T H S E |

The chemically eluted phage containing the sequence 1HY2 was repeated seven times and the obtained sequences were all different from each other. 5 sequences were obtained by ultrasonication (Table 3.3).

**Table 3.3** List of all sequences obtained by ultrasonication in the 1st screening

| Sample number | Round | Occurance | Sequences |
|---|---|---|---|
| 1ASD1 | 3 | - | I T P A S S T |
| 1ASD2 | 3 | - | G P W A S C E |
| 1ASD3 | 3 | - | Q V R T H P N |
| 1ASD4 | 3 | - | A K D S I P T |
| 1ASD5 | 3 | - | D G S T T R I |

No repeated sequences were observed among those obtained by ultrasonication. However, data from these results was not sufficient to find a logical relationship among the sequences. Since the data could not provide an idea about the affinity and specificity of the sequences to the substrate, detailed characterization of sequences were performed.

### 3.4. Characterization of Sequences Obtained from the 1st Screening

#### 3.4.1. Physicochemical Analysis

Isoelectric points (pI) and molecular weight of peptides were calculated by using compute pI/Mw tool (http://us.expasy.org/tools/pi_tool.htm). 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). The physicochemical properties of the polypeptides that were obtained by chemical elution are also shown in Table 3.4.

**Table 3.4** pI and molecular weight of mica binding sequences obtained by chemical elution in the 1st screening

| Sample number | Round | Occurance | Sequences | PI | MW | Ch |
|---|---|---|---|---|---|---|
| 1HY1 | 3 | - | S I T T N P S | 5.24 | 718.76 | 0 |
| 1HY2 | 3 | 7 | H P K P P R T | 11.0 | 831.97 | +2 |
| 1HY3 | 3 | - | Q M R T H S E | 6.79 | 887.97 | 0 |

pI and charge properties were found to be similar except for two selected phage samples one of which was seen seven times among the selected sequences. This sequence (1HY2) that had the highest occurrence among all chemically eluted sequences also had very high basic property and the only one with a charge of +2. During the phage display protocol, enrichment is one of the problems; so 1 HY2 can be an enriched sequence in this screening. The other sequences expect 1HY2, had neutral pI. The surface charge of the mica was negative; therefore peptides with positive charge would naturally have more affinity to the surface. 1HY2 was expected to demonstrate more affinity to the mica surface because of its positive charge, while 1HY1 and 1HY3 would be expected to have less affinity to mica surface compared to 1HY2 because of their neutral charge. No negative charged polypeptides were found. Following the analysis of charge of the amino acids and isoelectric points of each sequences were evaluated. Isoelectric point is the pH at which a protein carries no net charge. Below the isoelectric point proteins carry a net positive charge and above it they carry a net negative charge. For example, isoelectric point of 1HY1 was 5.24 as shown in table 3.4. Below this pH, 1HY1 had positive charge and at this pH, 1HY1 had more affinity to the negatively charged mica surface. However, above pH 5.24, 1HY1 had negative charge so at this pH, it had less affinity to mica surface.1HY1 could have more affinity to mica surface at basic pH.

Isoelectric point of 1HY3 is 6.79, very close to the pI of 1HY1. Therefore 1HY3 exhibited similar affinity with 1HY1 to mica surface at similar pH range. However, the isoelectric point of 1HY2 was high, 11.0 and above the pH 11.0, 1HY2 had a negative charge, while below this pH, 1HY2 had positive charge so around this pH, 1HY2 could have more affinity to mica. 1HY1 and 1HY3 have the negative charge

basic pH that is higher than 7. 1HY2 also had negative charge at pH higher than 11. All sequences may have a negative charge at basic pH but they present different pI values. Consequently, obtained sequences may have the different affinity to mica surface at different pH. So depends on the application area, different sequences can be used to get more efficient results. Molecular weight value of 1HY1, 1HY2 and 1HY3 are close to each other.

The physicochemical properties of the polypeptides that were obtained by ultrasonication are also shown in Table 3.5.

**Table 3.5** pI and molecular weight of mica binding sequences obtained by ultrasonication in the 1st screening

| Sample number | Round | Occurance | Sequences | PI | MW | Ch |
|---|---|---|---|---|---|---|
| 1ASD1 | 3 | - | I T P A S S T | 5.52 | 675.74 | 0 |
| 1ASD2 | 3 | - | G P W A S C E | 4.60 | 748.81 | -1 |
| 1ASD3 | 3 | - | Q V R T H P N | 9.76 | 850.93 | +1 |
| 1ASD4 | 3 | - | A K D S L P T | 5.88 | 730.82 | 0 |
| 1ASD5 | 3 | - | D G S T T R I | 5.84 | 748.79 | 0 |

The charges of 1ASD1, 1ASD4, 1 ASD5 were neutral. 1ASD2 had –1 and 1ASD3 had +1 charge. 1ASD3 may have more affinity because of its +1 charge. 1ASD2 may have the less affinity to mica surface than the other sequences because of its negative charge. pI values of 1ASD1, 1ASD2, 1ASD4 ,1ASD5 and molecular weights of 1ASD2, 1ASD4 and 1ASD5 were similar. However, pI and molecular weight values of 1ASD3 were higher than the others. 1ASD1, 1ASD2, 1ASD3 and 1ASD5 had negative charges at basic pH above the pH 6.0 and they may have the less affinity to mica surface. 1ASD3, which was the different one, had positive charge below pH 9.76. Although the sequences except for 1ASD3 had negative charge at pH levels between 6.0 and 9.76, 1ASD3 had positive charge around this pH. So 1ASD3 may have the more affinity to mica surface compared the other sequences around this pH. Statistical analyses were then performed and pI values and molecular weights of all sequences obtained via chemical elution or ultrasonication were similar with a few exceptions.

### 3.4.2. Statistical Analysis

This section was performed to analyze the number and distribution of the amino acids depending on its type. All obtained sequences were evaluated using the data in the instruction manual. All obtained sequences were compared with the observed amino acid frequencies in the library.

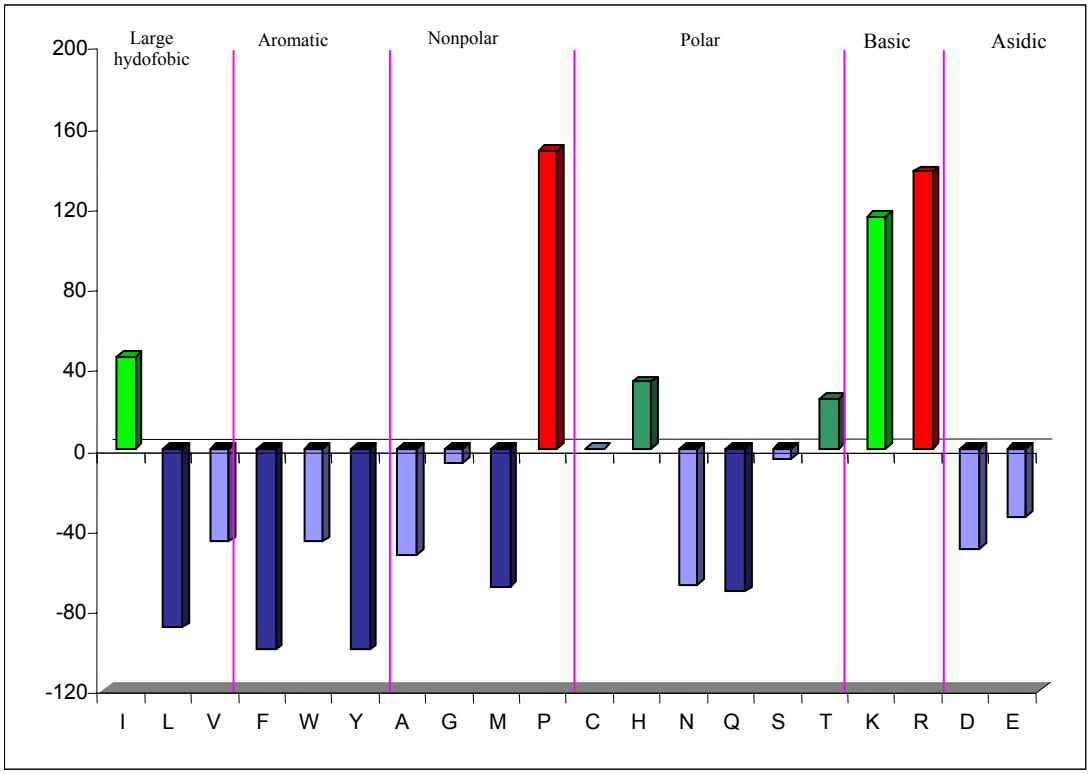



**Figure 3.6** Comparison of amino acid abundances of all selected sequences in the 1st screening.

Using this graph, the expression level of the all observed amino acids could easily be determined by the comparison of the observed frequencies in the library, thus, the relative abundance graph gave us information about the amino acids that are expressed low or over-expressed in the obtained sequences.

Amino acids like proline, lysine, arginine and isoleucine seemed to have high affinity to mica surface in comparison to other amino acids obtained in the selected sequences. Threonine and histidine were also over-expressed. But these amino acids are not expressed as proline, lysine and arginine as. Basic residues of amino acids

may have strong influence on binding compared to hydrophobic and hydroxyl residues

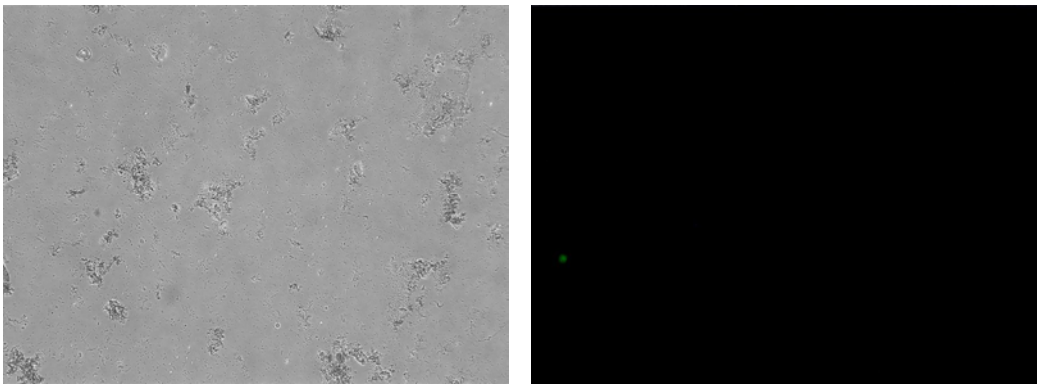
### 3.4.3. Immunofluorescence Microscopy Experiment of 1st Screening

All the sequences were investigated using various methods as described above to find a logical relationship among them. Sequence analysis and statistical analysis did not provide data showing a classification of sequences depending on affinity to mica surface, therefore immunofluorescence microscopy was utilized for a better understanding of binding affinities of each sequence. All the individual colonies were exposed to mica that had been grinded into powder. The mixture was rotated overnight and after washing the sample with a blocking agent to prevent nonspecific interaction between antibody and surfaces, anti-M13 gp8 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 3.6).

**Table 3.6** Affinity of mica binding sequences obtained by chemical elution in the 1st screening

| Sample number | Round | Occurance | Sequences | PI | MW | Ch | A |
|---|---|---|---|---|---|---|---|
| 1HY1 | 3 | - | S I T T N P S | 5.24 | 718.76 | 0 | S |
| 1HY2 | 3 | 7 | H P K P P R T | 11.0 | 831.97 | +2 | M |
| 1HY3 | 3 | - | Q M R T H S E | 6.79 | 887.97 | 0 | M |

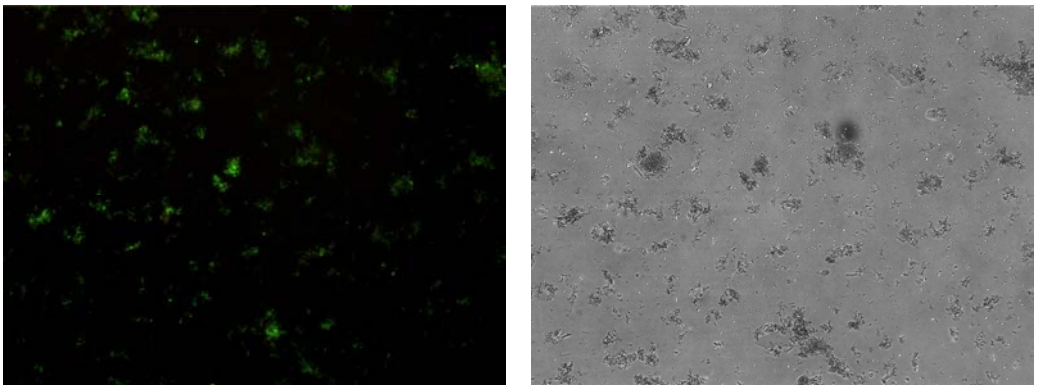
This table shows the classification of the sequences depending on the fluorescence images of the interaction of the mica and the phage with selected sequences. Before performing the fluorescence microscopy experiment, positive control experiments were also carried out in the absence of phage particles, using the fluorescence dye mixture consist of dye and the antibody solutions. And fluorescence image of control experiment was shown in Figure 3.7.



200X

**Figure 3.7** Fluorescence and optical microscopy images of the control experiment

In the first screening, the sequences to be examined in fluorescence microscopy (FM) experiments were selected among those observed most frequently. For example, 1HY2 was particularly selected among the chemically eluted sequences because it had the highest occurrence and therefore it could have been considered as a promising mica specific binder. Nevertheless, as it is seen in the fluorescence images in Figure 3.8, 1HY2 did not appear as a strong binder, but it was a moderate binder. As expected, 1HY2 had less specificity towards the mica surface. Although 1HY2 had positive charge (+2), it did not exhibit high affinity to negatively charged mica surface.



1HY2 **(M)** **H P K P P R T** 1HY2 **(M)** 200X

**Figure 3.8** FM and LM images for 1HY2

Enrichment is an important problem during chemical elution, and this was also exemplified in our studies; the most frequently observed sequence might not always be the most substrate-specific one. Other sequences, which were obtained via chemical elution, were shown in Figure 3.9.

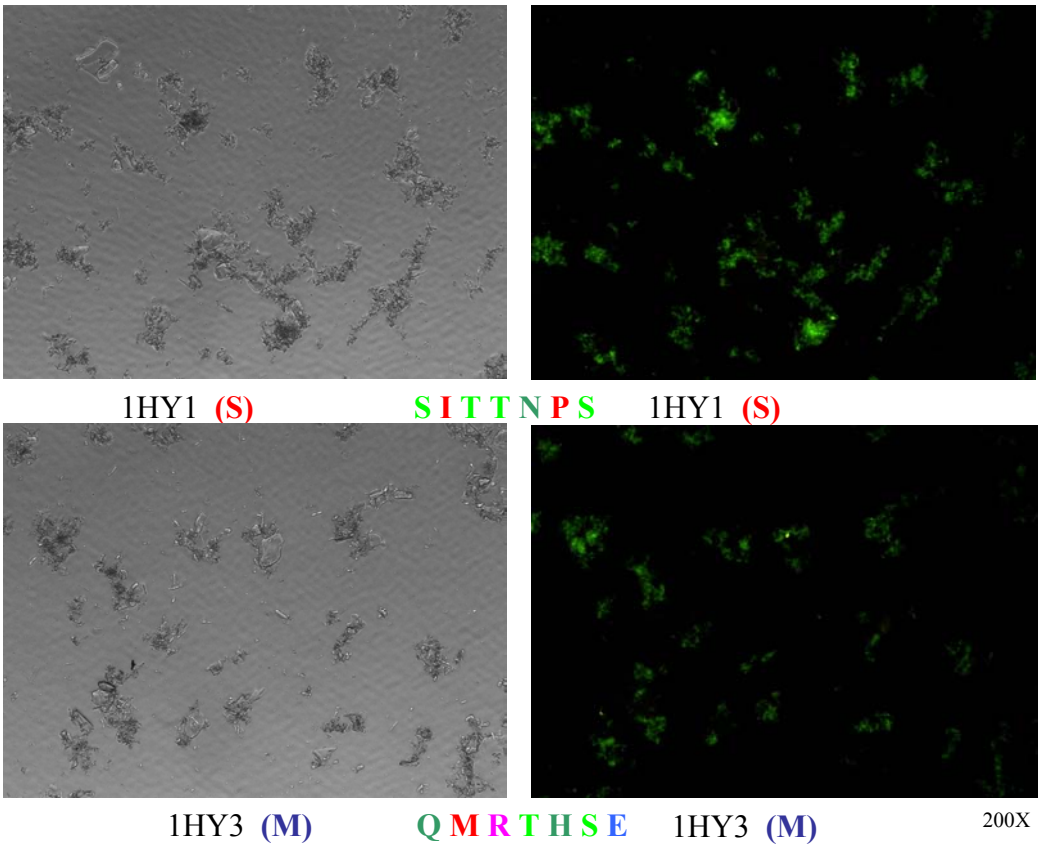
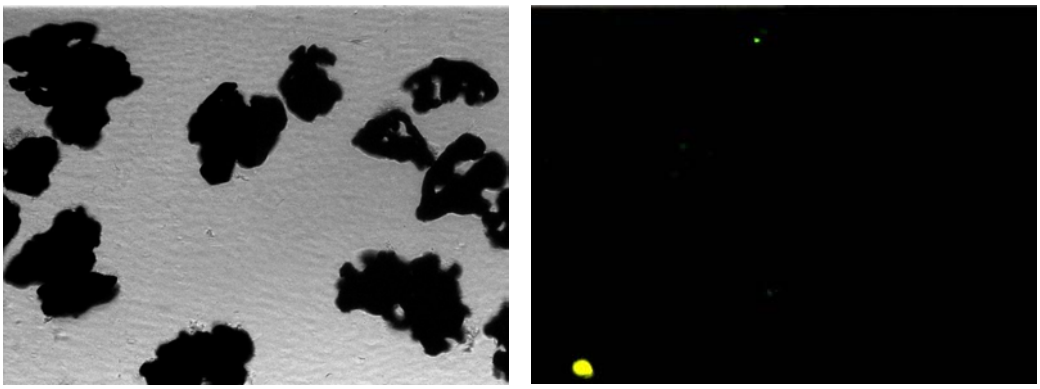



**Figure 3.9** FM and LM images for 1HY1 and 1HY3

1HY1 was one of the strong binders that were obtained by chemical elution during the first screening. 1HY3 was a moderate binder, and both binders had neutral charge. To obtain more data about the mica specific binders second screening was carried out.

### 3.4.4. Cross Specificity Experiment of 1st Screening

Until this step, all sequences were evaluated by using sequence and statistical analyses as well as fluorescence microscopy analysis. Using the fluorescence microscopy analysis, a visual knowledge about the binding affinity of the sequences was obtained. It must also be confirmed whether strong binders demonstrate any affinity to other materials or not. In other words a confirmation of we have to understand how specific is our specific sequences. Therefore we chose titanium powder as a substrate to perform the cross specificity experiments. Figure 3.10 shows the result of the cross specificity experiment of 1HY1.



200X

**Figure 3.10** Cross specificity experiment of 1HY1 with titantium

It is clearly seen that 1HY1 had no binding affinity to titanium powder. In the first screening, phage elution after ultrasonication of the mica surface indicates that there is still some phage particles remained on the mica surface and we also obtained different sequences by ultrasonication. Consequently, second screening was carried out to increase the number of mica binders and analyze the advantage of ulrasonication on phage display procedure.

### 3.5. Results of the 2nd Screening

In this screening, 4 rounds of biopanning were carried out. Following the chemical elution ultrasonication was applied at the end of the each round. Here, after each ultrasonication step bound phage recovery method was also performed to increase the number of mica specific binders. Ultrasonication parameters were kept constant for comparison at both screens. Among a total of 64 sequences, 40 were obtained by chemical elution and no repetition of the sequences were observed from those analysed except for one (Table 3.7). Besides, sequences obtained in the 1st screen were not observed in the 2nd screen. A consensus sequence could not be obtained even after the fourth round of bio panning in contrast to the traditional applications of phage display protocol for the protein-protein interactions where a consensus sequence is obtained generally after 2 or 3 rounds of biopanning.

**Table 3.7** List of all sequences obtained by chemical elution in the 2nd screen

| Sample number | Round | C7C Sequences |
|---|---|---|
| 2HY9 | 2 | I Q S G H P Q |
| 2HY12 | 2 | T N T I T H S |
| 2HY20 | 3 | N S R T F A S |
| 2HY29 | 4 | C H N T A R N |
| 2HY30 | 4 | T A N S T A L |
| 2HY1 | 1 | Q T T S L P E |
| 2HY2 | 1 | Q M A Q G L I |
| 2HY4 | 1 | S Q P S T A L |
| 2HY36 | 1 | F P P N S L F |
| 2HY15 | 3 | P K S A S H Y |
| 2HY17 | 3 | I T T E Y Q D |
| 2HY19 | 3 | Q L Y H A N A |
| 2HY22 | 3 | I A K H S T Y |
| 2HY23 | 3 | F N S P M H Q |
| 2HY24 | 3 | M F P S T P F |
| 2HY49 | 4 | Q K W A S P L |
| 2HY3 | 1 | L Q K P E P N |
| 2HY5 | 1 | I T R S D P Y |
| 2HY6 | 1 | N T M T P T S |
| 2HY34 | 1 | A H S F P L N |
| 2HY35 | 1 | A T V S G G S |
| 2HY37 | 1 | S R P T V H W |
| 2HY10 | 2 | K G D W T R N |
| 2HY11 | 2 | N S A T Q M A |
| 2HY39 | 2 | I S P E P G A |
| 2HY40 | 2 | K N I E T S R |
| 2HY41 | 2 | T P S G P R S |
| 2HY42 | 2 | V H P M P F I |
| 2HY43 | 2 | E P A N N G R |
| 2HY44 | 2 | A N C L P G S |
| 2HY16 | 3 | H P T L L T S |
| 2HY18 | 3 | N Q G H Y S T |
| 2HY21 | 3 | P L P N K Y E |
| 2HY28 | 4 | S G M T G A Y* |
| 2HY46 | 4 | L T A S P K N |
| 2HY47 | 4 | K H L C G G G |
| 2HY48 | 4 | P H D S P R A |
| 2HY50 | 4 | A L G I K N S |

*Repeated sequences

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

12 sequences were obtained by ultrasonication. Table 3.8 shows the list of all sequences obtained by ultrasonication.

**Table 3.8** List of all sequences obtained by ultrasonication in the 2nd screening.

| Sample number | Round | C7C Sequences |
|---|---|---|
| 2ASD4 | 2 | S A P T L R Q |
| 2ASD7 | 3 | R V H E H P H |
| 2ASD1 | 1 | L R Q H L N S |
| 2ASD2 | 1 | P L Q P L P T |
| 2ASD5 | 3 | S S T L P D H |
| 2ASD6 | 3 | P Q P N E D N |
| 2ASD9 | 4 | Q P A S S R Y |
| 2ASD10 | 4 | S K S D P R Q |
| 2ASD12 | 4 | P F T N A F G |
| 2ASD3 | 2 | N S L N G S A |
| 2ASD8 | 4 | S M S P A S L |
| 2ASD11 | 4 | L P A E R S Q |

In sequence base, a consensus or repeated sequence could not be obtained by ultrasonication either. The results in Table 3.8 show that, some of the bound phage could remain on mica even after the chemical elution at each round. This demonstrates that chemical elution is not sufficient to remove all bound phage from mica surface. Application of a 30 second-ultrasound after chemical elution has increased the number of mica-binding sequences to 35 as shown in Table 3.8. [50]. Therefore ultrasonication could be an alternative for better phage removal from inorganic surfaces. If the data in Table 3.7 and 3.8 are compared, it can be seen that the sequences obtained with ultrasonication and chemical elution were totally different from each other.

12 sequences obtained by bound phage recovery method were selected (Table 3.9).

**Table 3.9** List of all sequences obtained by bound phage recovery method in the 2nd screening

| Sample number | Round | C7C Sequences |
|---|---|---|
| 2HY7 | 1 | T D T R S Q T |
| 2HY8 | 1 | L S S D T P A |
| 2HY38 | 1 | H H V G P P Y |
| 2HY13 | 2 | T Q E S R L S |
| 2HY14 | 2 | S T K Q T S A |
| 2HY45 | 2 | T P H L M L S |
| 2HY25 | 3 | N P F L R T H |
| 2HY26 | 3 | T Q L I T Q M |
| 2HY27 | 3 | T V S S P E G |
| 2HY31 | 4 | S D R D S A F |
| 2HY32 | 4 | K S D Q T R T |
| 2HY33 | 4 | N G P S P F H |

It can be seen that the sequences obtained with bound phage recovery method, were different from each other like the other sequences obtained by different methods. By this method, number of mica binders also increased. Ultrasonication and bound phage recovery methods could be the alternatives for better phage removal from inorganic surfaces.

### 3.6. Characterization of sequences obtained from 2nd Screening

#### 3.6.1. Physicochemical Analysis

Isoelectric points (pI), molecular weight (MW) and molecular weight of peptides were calculated as explained in the first screening section. Charge of the peptides was calculated by subtracting the total number of basic residues (R and K) from the total number of acidic residues (D and E). The physicochemical properties of the peptides obtained by chemical elution in the second screening were also shown in the table below.

**Table 3.10** pI and molecular weight of mica binding sequences obtained by chemical elution in the 2nd screening

| Sample number | Round | C7C Sequences | PI | MW | Ch |
|---|---|---|---|---|---|
| 2HY9 | 2 | I Q S G H P Q | 6.74 | 765.82 | 0 |
| 2HY12 | 2 | T N T I T H S | 6.40 | 772.81 | 0 |
| 2HY20 | 3 | N S R T F A S | 9.75 | 781.82 | +1 |
| 2HY29 | 4 | C H N T A R N | 8.26 | 814.87 | +1 |
| 2HY30 | 4 | T A N S T A L | 5.19 | 676.72 | 0 |
| 2HY1 | 1 | Q T T S L P E | 4.60 | 774.83 | -1 |
| 2HY2 | 1 | Q M A Q G L I | 5.52 | 759.92 | 0 |
| 2HY4 | 1 | S Q P S T A L | 5.24 | 702.76 | 0 |
| 2HY36 | 1 | F P P N S L F | 5.52 | 820.94 | 0 |
| 2HY15 | 3 | P K S A S H Y | 9.01 | 788.86 | +1 |
| 2HY17 | 3 | I T T E Y Q D | 4.03 | 868.90 | -2 |
| 2HY19 | 3 | Q L Y H A N A | 6.74 | 815.88 | 0 |
| 2HY22 | 3 | I A K H S T Y | 8.60 | 818.93 | +1 |
| 2HY23 | 3 | F N S P M H Q | 6.74 | 859.95 | 0 |
| 2HY24 | 3 | M F P S T P F | 5.28 | 825.98 | 0 |
| 2HY49 | 4 | Q K W A S P L | 8.75 | 858.97 | +1 |
| 2HY3 | 1 | L Q K P E P N | 6.00 | 824.93 | 0 |
| 2HY5 | 1 | I T R S D P Y | 5.84 | 850.93 | 0 |
| 2HY6 | 1 | N T M T P T S | 5.52 | 750.82 | 0 |
| 2HY34 | 1 | A H S F P L N | 6.79 | 784.87 | 0 |
| 2HY35 | 1 | A T V S G G S | 5.57 | 577.59 | 0 |
| 2HY37 | 1 | S R P T V H W | 9.49 | 881.99 | +1 |
| 2HY10 | 2 | K G D W T R N | 8.75 | 875.94 | 0 |
| 2HY11 | 2 | N S A T Q M A | 5.52 | 721.78 | 0 |
| 2HY39 | 2 | I S P E P G A | 4.00 | 669.73 | -1 |
| 2HY40 | 2 | K N I E T S R | 8.75 | 846.94 | 0 |
| 2HY41 | 2 | T P S G P R S | 9.41 | 700.75 | +1 |
| 2HY42 | 2 | V H P M P F I | 6.71 | 840.05 | 0 |
| 2HY43 | 2 | E P A N N G R | 6.10 | 756.77 | 0 |
| 2HY44 | 2 | A N C L P G S | 5.56 | 660.74 | 0 |
| 2HY16 | 3 | H P T L L T S | 6.74 | 767.88 | 0 |
| 2HY18 | 3 | N Q G H Y S T | 6.74 | 805.80 | 0 |
| 2HY21 | 3 | P L P N K Y E | 6.63 | 859.98 | 0 |
| 2HY28 | 4 | S G M T G A Y* | 5.24 | 685.75 | 0 |
| 2HY46 | 4 | L T A S P K N | 8.75 | 729.83 | +1 |
| 2HY47 | 4 | K H L C G G G | 8.23 | 670.78 | +1 |
| 2HY48 | 4 | P H D S P R A | 7.17 | 778.82 | 0 |
| 2HY50 | 4 | A L G I K N S | 8.80 | 701.82 | +1 |

The charge of majority of the chemically eluted sequences found in this screening was either neutral or +1. It is logical to expect the sequences with negative charges would be attracted to the mica surface. However there were some exceptions such as 2HY17 (-2), 2HY1 (-1) and 2HY39 (-1) with negative charge. There was a similar correlation in the first screening. Most of the obtained sequences had positive or neutral charge. Generally, the distribution of pI values of sequences carried by the chemically eluted phage was between 9.75 and 4.00 for the second screening, 5.24 and 11.0 for the first screening. The distributions of pI values of the sequences obtained in first and second screening distribution of pI values were similar. The physicochemical properties of the polypeptides that were obtained by ultrasonication in the second screening were also shown in Table 3.11.

**Table 3.11** pI and molecular weight of mica binding sequences obtained by ultrasonication in the 2nd screening

| Sample number | Round | C7C Sequences | PI | MW | Ch |
|---|---|---|---|---|---|
| 2ASD4 | 2 | S A P T L R Q | 9.47 | 771.87 | +1 |
| 2ASD7 | 3 | R V H E H P H | 7.03 | 910.99 | 0 |
| 2ASD1 | 1 | L R Q H L N S | 9.76 | 866.98 | +1 |
| 2ASD2 | 1 | P L Q P L P T | 5.96 | 764.92 | 0 |
| 2ASD5 | 3 | S S T L P D H | 5.06 | 755.78 | -1 |
| 2ASD6 | 3 | P Q P N E D N | 3.67 | 812.79 | -2 |
| 2ASD9 | 4 | Q P A S S R Y | 8.75 | 807.86 | +1 |
| 2ASD10 | 4 | S K S D P R Q | 8.46 | 816.87 | +1 |
| 2ASD12 | 4 | P F T N A F G | 5.96 | 752.82 | 0 |
| 2ASD3 | 2 | N S L N G S A | 5.52 | 661.67 | 0 |
| 2ASD8 | 4 | S M S P A S L | 5.24 | 691.80 | 0 |
| 2ASD11 | 4 | L P A E R S Q | 6.00 | 799.88 | 0 |

As shown in Table 3.11, the charge of the all sequences obtained by ultrasonication are similar (neutral or +1), to chemically eluted sequences and also there were few exceptions such as 2ASD5 (-1), 2ASD6 (-2) that had negative charges. Distribution of pI values was widespread, between 9.76 and 3.67. This data also overlap other ones, which was accomplished in the first and second screening.

**Table 3.12** pI and molecular weight of mica binding sequences obtained by bound phage recovery method in the 2nd screening

| Sample number | Round | C7C Sequences | PI | MW | Ch |
|---|---|---|---|---|---|
| 2HY7 | 1 | T D T R S Q T | 5.50 | 807.82 | 0 |
| 2HY8 | 1 | L S S D T P A | 3.80 | 689.72 | -1 |
| 2HY38 | 1 | H H V G P P Y | 6.92 | 805.89 | 0 |
| 2HY13 | 2 | T Q E S R L S | 5.66 | 819.87 | 0 |
| 2HY14 | 2 | S T K Q T S A | 8.47 | 721.77 | +1 |
| 2HY45 | 2 | T P H L M L S | 6.40 | 797.97 | 0 |
| 2HY25 | 3 | N P F L R T H | 9.76 | 884.01 | +1 |
| 2HY26 | 3 | T Q L I T Q M | 5.19 | 834.00 | 0 |
| 2HY27 | 3 | T V S S P E G | 4.00 | 675.69 | -1 |
| 2HY31 | 4 | S D R D S A F | 4.21 | 796.79 | -1 |
| 2HY32 | 4 | K S D Q T R T | 8.75 | 834.88 | +1 |
| 2HY33 | 4 | N G P S P F H | 6.74 | 754.80 | 0 |

The physicochemical properties of the polypeptides obtained by bound phage recovery method in the second screening are also summarized (Table 3.12). Here, isoelectric points of the sequences varied between 4.00 and 8.75. The charge of the sequences varied between –1 and +1 without exception. Charge of the polypeptides indicates a constant trend in both screenings and elution methods. In the first and second screening, molecular weight distribution was between 577 and 881. Physicochemical properties of mica-binding sequences show a consistency in sequence results obtained by all the methods studied. The charges, molecular masses and isoelectric points of all mica-binding sequences vary in certain intervals, however no significant trend could be observed for a better understanding of mica binders.

### 3.6.2. Statistical Analysis

Following the sequence and physicochemical analysis, a statistical analysis was performed. Relative abundances of amino acids were measured in proportion to the observed frequency values of Ph.D.-C7C phage library as described in Ph.D.-C7C phage library catalog (New England Biolabs) and in the following section, relative abundancies of all obtained mica binders were documented (Figure 3.11).

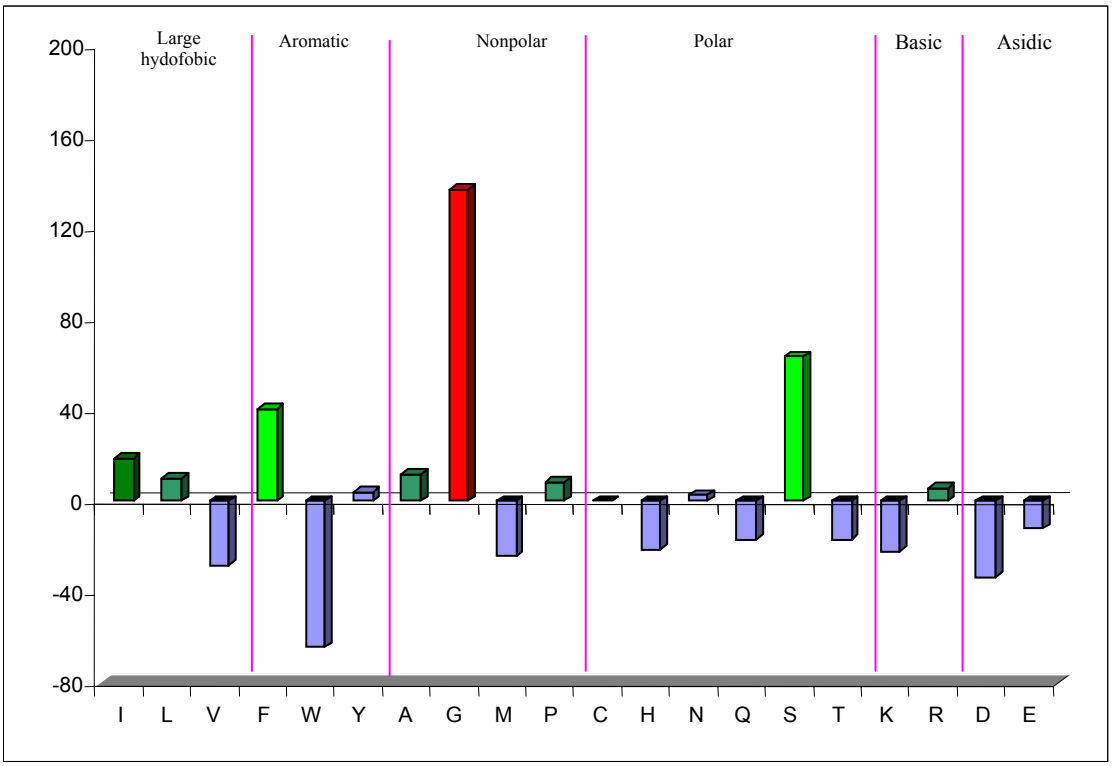



**Figure 3.11** Relative abundances of all obtained mica-binding sequences

Figure shows that glycine, serine and phenylalanine were over-expressed in all the sequences obtained by different methods. Isoleucine, leucin, arginine and prolin were also over-expressed but this expression was not high as that in the case of glycine, serine and phenylalanine expression. In the first screening ısoleucine was observed more than that in the second screening. Moreover, in the first screening nonpolar proline, and in the second screening another nonpolar amino acid glycine was found requently. Therefore, nonpolar amino acids could be postulated to have affinity to the mica surface. Although threonine and histidine appeared to be over-expressed amino acids in the first screening, in fact they are under-expressed based on the instruction manual. Comparing selected sequences in first and second screen, different amino acids contents were obtained

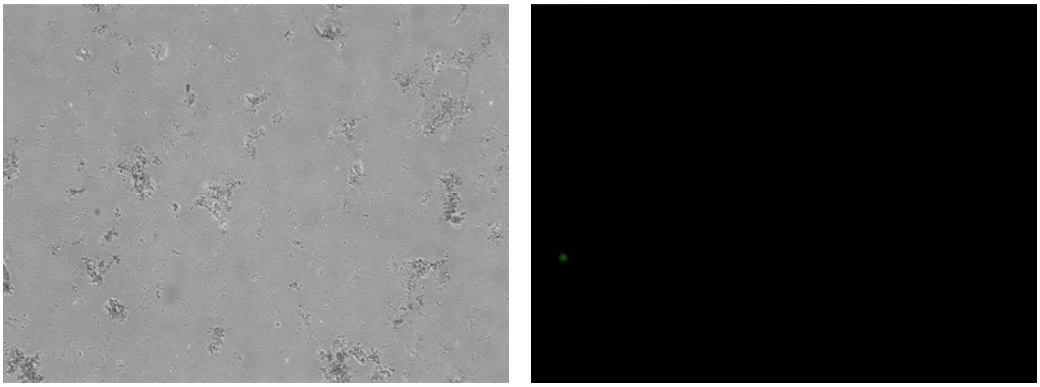
### 3.6.3. Immunofluorescence Microscopy Experiment of 2nd Screening

To this point all the sequences were evaluated with respect to sequence and statistical analyses. Lack of a consensus sequence formation makes necessary to have detailed analysis as a starting point so each sequence was analyzed individually using fluorescence microscopy.

**Table 3.13** Affinity of mica binding sequences obtained by chemical elution at the end of the 2nd screening

| Sample number | Round | C7C Sequences | PI | MW | Ch | A |
|---|---|---|---|---|---|---|
| 2HY9 | 2 | I Q S G H P Q | 6.74 | 765.82 | 0 | S |
| 2HY12 | 2 | T N T I T H S | 6.40 | 772.81 | 0 | S |
| 2HY20 | 3 | N S R T F A S | 9.75 | 781.82 | +1 | S |
| 2HY29 | 4 | C H N T A R N | 8.26 | 814.87 | +1 | S |
| 2HY30 | 4 | T A N S T A L | 5.19 | 676.72 | 0 | S |
| 2HY1 | 1 | Q T T S L P E | 4.60 | 774.83 | -1 | M |
| 2HY2 | 1 | Q M A Q G L I | 5.52 | 759.92 | 0 | M |
| 2HY4 | 1 | S Q P S T A L | 5.24 | 702.76 | 0 | M |
| 2HY36 | 1 | F P P N S L F | 5.52 | 820.94 | 0 | M |
| 2HY15 | 3 | P K S A S H Y | 9.01 | 788.86 | +1 | M |
| 2HY17 | 3 | I T T E Y Q D | 4.03 | 868.90 | -2 | M |
| 2HY19 | 3 | Q L Y H A N A | 6.74 | 815.88 | 0 | M |
| 2HY22 | 3 | I A K H S T Y | 8.60 | 818.93 | +1 | M |
| 2HY23 | 3 | F N S P M H Q | 6.74 | 859.95 | 0 | M |
| 2HY24 | 3 | M F P S T P F | 5.28 | 825.98 | 0 | M |
| 2HY49 | 4 | Q K W A S P L | 8.75 | 858.97 | +1 | M |
| 2HY3 | 1 | L Q K P E P N | 6.00 | 824.93 | 0 | W |
| 2HY5 | 1 | I T R S D P Y | 5.84 | 850.93 | 0 | W |
| 2HY6 | 1 | N T M T P T S | 5.52 | 750.82 | 0 | W |
| 2HY34 | 1 | A H S F P L N | 6.79 | 784.87 | 0 | W |
| 2HY35 | 1 | A T V S G G S | 5.57 | 577.59 | 0 | W |
| 2HY37 | 1 | S R P T V H W | 9.49 | 881.99 | +1 | W |
| 2HY10 | 2 | K G D W T R N | 8.75 | 875.94 | 0 | W |
| 2HY11 | 2 | N S A T Q M A | 5.52 | 721.78 | 0 | W |
| 2HY39 | 2 | I S P E P G A | 4.00 | 669.73 | -1 | W |
| 2HY40 | 2 | K N I E T S R | 8.75 | 846.94 | 0 | W |
| 2HY41 | 2 | T P S G P R S | 9.41 | 700.75 | +1 | W |
| 2HY42 | 2 | V H P M P F I | 6.71 | 840.05 | 0 | W |
| 2HY43 | 2 | E P A N N G R | 6.10 | 756.77 | 0 | W |
| 2HY44 | 2 | A N C L P G S | 5.56 | 660.74 | 0 | W |
| 2HY16 | 3 | H P T L L T S | 6.74 | 767.88 | 0 | W |
| 2HY18 | 3 | N Q G H Y S T | 6.74 | 805.80 | 0 | W |
| 2HY21 | 3 | P L P N K Y E | 6.63 | 859.98 | 0 | W |
| 2HY28 | 4 | S G M T G A Y | 5.24 | 685.75 | 0 | W |
| 2HY46 | 4 | L T A S P K N | 8.75 | 729.83 | +1 | W |
| 2HY47 | 4 | K H L C G G G | 8.23 | 670.78 | +1 | W |
| 2HY48 | 4 | P H D S P R A | 7.17 | 778.82 | 0 | W |
| 2HY50 | 4 | A L G I K N S | 8.80 | 701.82 | +1 | W |

Table 3.13 indicates that strong binders were not only from the late rounds but also in rounds as early as second round. It was clear that the increase in the number of the rounds did not help in finding the most specific binders. All the strong binders had either positive (+1) or neutral charge. Although moderate and weak binders also had positive or neutral charges, no binder with negative charge was found among the strong binders. At the beginning of examination with fluorescence microscopy, negative and positive control experiments were also carried out. Positive control experiments did not contain the phage but only contained the fluorescence dye mixture (Figure 3.10). Following the control experiment, fluorescence microscopy experiments were performed on all samples using the procedure described earlier. In Figure 3.11 and 3.12, some of the examples of strong, moderate or weak mica binders obtained by chemical elution were shown.



200X

**Figure 3.12** Fluorescence and optical microscopy images of control experiment

In the second screening, 2HY28 –similar to 1HY2 of the first screening- is the only binder that was observed more than once. Nevertheless, as it can be seen from the fluorescence images (Figure 3.13), 2HY28 appeared to be one of the weak binders. This demonstrates that, high occurrence of the sequences does not necessarily mean that they have more affinity to the substrate.

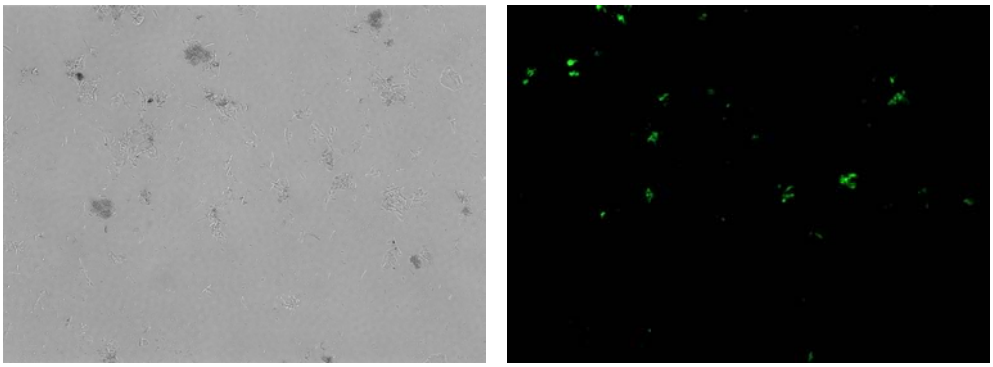



**Figure 3.13** Fluorescence and optical microscopy images of 2HY28

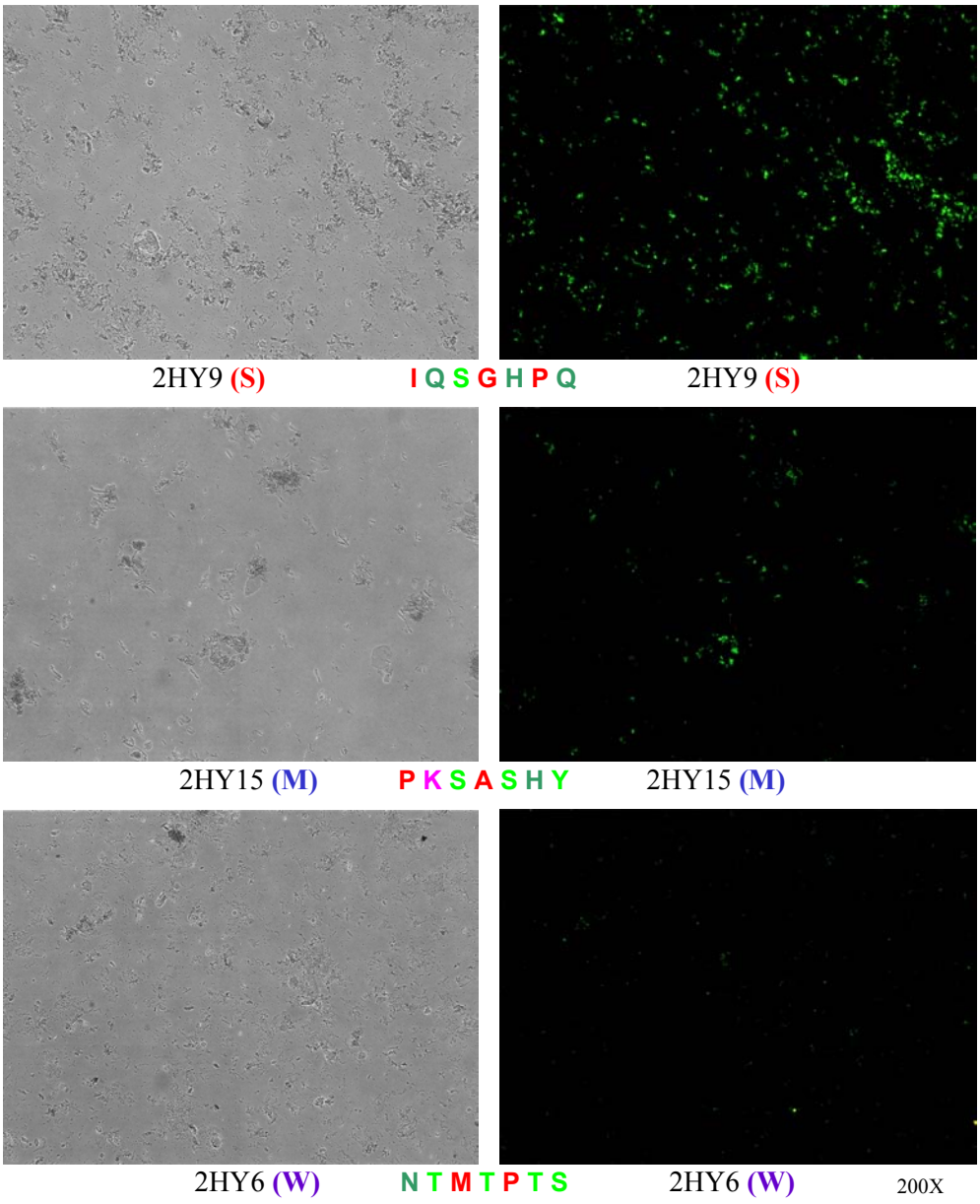



**Figure 3.14** Fluorescence and optical microscopy images of chemically eluted samples from 3 groups; strong (S), moderate (M) and weak (W) binders

**Table 3.14** Affinity of mica binding sequences obtained by ultrasonication in the 2nd screening

| Sample number | Round | C7C Sequences | PI | MW | Ch | A |
|---|---|---|---|---|---|---|
| 2ASD4 | 2 | S A P T L R Q | 9.47 | 771.87 | +1 | S |
| 2ASD7 | 3 | R V H E H P H | 7.03 | 910.99 | 0 | S |
| 2ASD1 | 1 | L R Q H L N S | 9.76 | 866.98 | +1 | M |
| 2ASD2 | 1 | P L Q P L P T | 5.96 | 764.92 | 0 | M |
| 2ASD5 | 3 | S S T L P D H | 5.06 | 755.78 | -1 | M |
| 2ASD6 | 3 | P Q P N E D N | 3.67 | 812.79 | -2 | M |
| 2ASD9 | 4 | Q P A S S R Y | 8.75 | 807.86 | +1 | M |
| 2ASD10 | 4 | S K S D P R Q | 8.46 | 816.87 | +1 | M |
| 2ASD12 | 4 | P F T N A F G | 5.96 | 752.82 | 0 | M |
| 2ASD3 | 2 | N S L N G S A | 5.52 | 661.67 | 0 | W |
| 2ASD8 | 4 | S M S P A S L | 5.24 | 691.80 | 0 | W |
| 2ASD11 | 4 | L P A E R S Q | 6.00 | 799.88 | 0 | W |

Table 3.14, indicates that, mica-binding sequences obtained ultrasonication also exhibited affinity of different levels and no strong binders were obtained in rounds 1 or 3. All the strong binders had positive or neutral charges and all negatively charged sequences were moderate binders. Negatively charged sequences could have less affinity to mica surface because of their charge.

### 3.6.4. Cross Specificity Experiment of 2nd Screening

Experiments to this point provided information about the relative affinities of the sequences to the substrate surface. However, relative affinity does not provide any information about the binding specificity, i.e. a sequence with high affinity for mica does not necessarily need to be specific to mica. Another detailed set of binding characterization experiments was needed to be performed using another inorganic material. For example, both 2HY29 and 2HY30 had strong affinities for mica but when another material was used to test the specificity of the sequences (Figure 3.15), we could be able to categorize them not only with respect to the binding affinity but also with respect to binding specificity which is a very significant aspect when these materials and sequences are considered in an application. A sequence with a strong

affinity for mica as well as other materials may not provide a suitable application. We have tested all the strong binders for cross specificity using titanium as a second substrate. As it can be clearly seen, none of the two strong mica binders exhibited affinity for titanium (Figure 3.15). This result demonstrated that these binders are specific for mica but not for titanium.

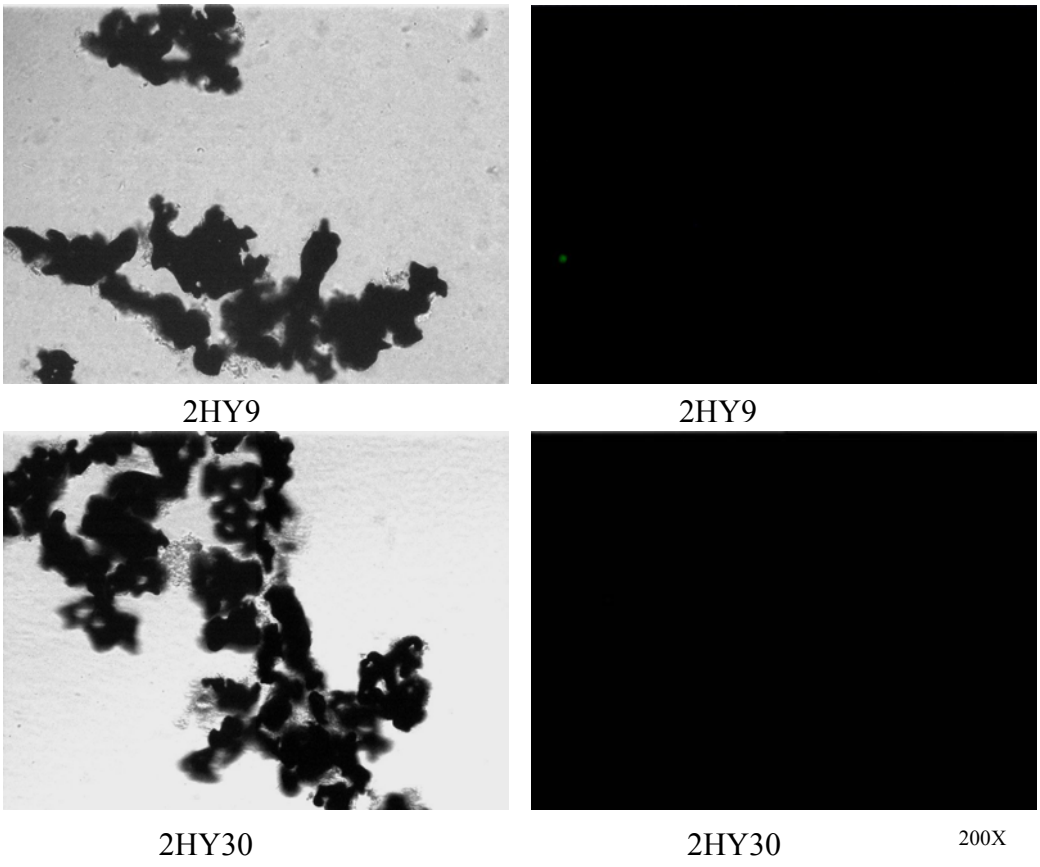



**Figure 3.15** Fluorescence and optical microscopy images of two strong binders for cross specificity experiment

### 3.6.5. Functional Groups Analysis of Classified Mica Binders

Following the binding characterization experiments (affinity and cross-specificity analyses), we have also searched for any existing similarity among the sequences in terms of functional groups of the amino acids. The analysis was based on evaluation of the functional groups of amino acids considering that these groups might have stronger interactions with the inorganic surfaces. All the sequences obtained through ultrasonication, chemical elution or bound phage recovery studies presented similar characteristics in terms of their amino acid functional groups. Amino acids with

amine ($NH_2$) + basic functional groups appeared to have an important role in binding (Figure 3.16).

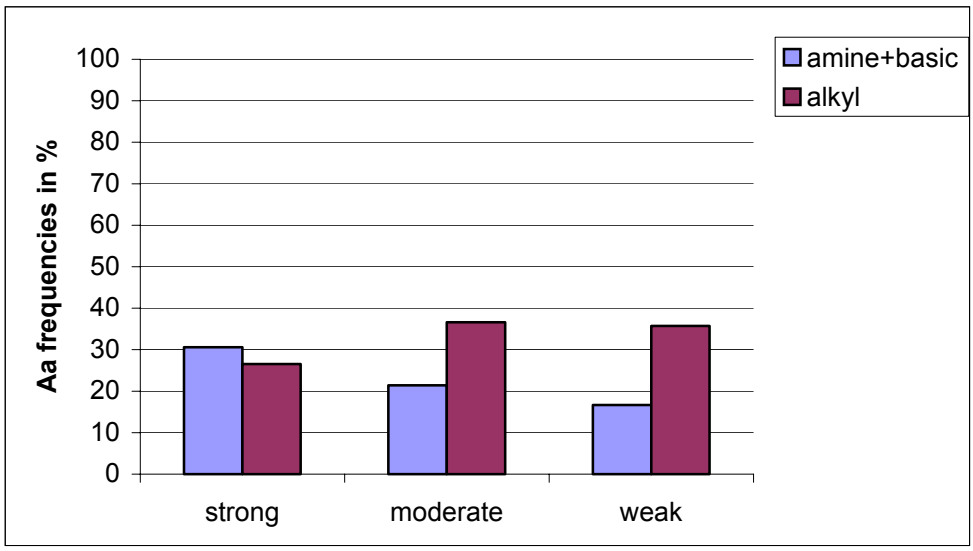



**Figure 3.16.** Frequencies of amino acids including amine+basic or alkyl functional groups encountered within all mica-binding sequences.

Strong mica-binders possessed more amine+basic groups than the weak ones whereas alkyl ($CH_3$) functional groups were rarely encountered in strong mica-binders. It could be reasoned that, positively charged amine groups may have more affinity to negatively charged mica surface.

## 4. CONCLUSION

- Mica binding polypeptides were obtained by using phage display and novel approaches.
- Different novel approaches, such as application of bound phage recovery and ultrasonication as a physical elution method are applied to overcome the inability of removing all specific binders from inorganic surface.
- Ultrasonication and bound phage recovery as combined method are suggested to make phage display protocol more efficient in the analysis of inorganic binders
- pI of all strong binders are mostly 0 and +1 charge, there is no negatively charged polypeptides to have much affinity to negatively charged mica surface**.**
- Strong binders are from not only last rounds but also in early rounds in contrast to the traditional applications of phage display where best binders are considered to be obtained as the round numbers increased
- As the general assumptions, enrich sequences are shown not be the most specific ones. Sometimes, they might be an end result of grow advantage which reflects as less specific binders.
- Our studies showed that the strong binders have mostly amine ($NH_2$)+basic functional groups suggesting that these functional groups may play important role in binding. Therefore they need to be further investigated.
- All strong binders obtained exhibited no affinity towards titanium powder.
- As mica is a strategic material in the scientific and industrial world, mica binding polypeptides could be applied in many different application areas to be used as biolinkers in nano-scale engineering, especially in electronic industry and also in the design of molecular assembly studies.

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## APPENDIX

### A.1. Color Coding of the Aminoacids

**Table A.1** Color Coding of the Aminoacids

| **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 |

**RESUME**

Hilal YAZICI was born in GEBZE in 1980. After getting her high school diploma from İzmit high school in 1997, she has continued her undergraduate degree in İstanbul University, Department of Biology in 1998. She had her Bachelor degree in 2002. She has continued to her Graduate studies in Advanced Technologies in Molecular Biology, Genetics and Biotechnology 's program. She has been also working as a research assistant in Department of Molecular Biology and Genetics. She is still pursuing her studies in the same department.