ISTANBUL TECHNICAL UNIVERSITY ★ INSTITUTE OF SCIENCE AND TECHNOLOGY

IDENTIFICATION OF CRYSTAL FACE SPECIFIC SAPPHIRE BINDING PEPTIDES BY PHAGE DISPLAY TECHNOLOGY

MASTER THESIS
Asiye Arzu ÖZMÜŞ
707011007

Date of Thesis Given to Institute : April 26, 2004
Date of Thesis Defence : May 18, 2004

Supervisors (chairmans)	**:**	**Assoc. Dr. Candan TAMERLER BEHAR**
		Prof. Dr. Mehmet SARIKAYA
Members of the examining committee:		**Prof. Dr. Mustafa ÜRGEN**
		Assist.Prof. Hakan BERMEK
		Assist.Prof. Nevin Gül KARAGÜLER

JUNE 2004

İSTANBUL TEKNİK ÜNİVERSİTESİ ★ FEN BİLİMLERİ ENSTİTÜSÜ

SAFİR KRİSTALOGRAFİK YÜZEYLERİNE SPESİFİK PEPTİDLERİN PHAGE DISPLAY YÖNTEMİ İLE BELİRLENMESİ

YÜKSEK LİSANS TEZİ

Asiye Arzu ÖZMÜŞ
707011007

Tezin Enstitüye Verildiği Tarih : 26 Nisan 2004
Tezin Savunulduğu Tarih : 18 Mayıs 2004

Tez Danışmanları : Yrd.Doç.Dr. Candan TAMERLER BEHAR

Prof. Dr. Mehmet SARIKAYA

Diğer Jüri Üyeleri : Prof.Dr. Mustafa ÜRGEN

Yrd.Doç.Dr. Hakan BERMEK

Yrd.Doç.Dr. Nevin Gül KARAGÜLER

HAZİRAN 2004

ACKNOWLEDGEMENT

I would like to express my deepest appreciation and thanks to grateful people who helped me to accomplish this work, especially to my advisors; Assoc. Prof. Candan TAMERLER BEHAR and Prof. Dr. Mehmet SARIKAYA.

I would also like to thank two very special people, Sevil DİNÇER and Dr. Hanson FONG for their great help, technical assistance and wonderful friendship. Also my other group members; Eswaranand Venkatasubramanian for his help and support, Dr. Ersin Emre ÖREN, Deniz ŞAHİN and Aydın TANKUT. Without their help, this work would not be this complete.

I am also grateful to my family and my 'Seattle family', friends who endured the long work hours and months I devoted to research, with extraordinary grace and provided constant encouragement; Nathan KOHLER, Marcela GONZALES, Mandana VEISEH, Mike BEERMAN, Jessica TORREY, Bradley ROBERTS, Dustin FRAME, Yuping BAO, Noriaki SUZUKI and Billy CLINTON for his technical help from UW-MSE. Also three special people Serkan KUTAN, Cüneyt HAVLİOĞLU and Surya VANKA. And Levent for keeping up with me along all this work....

A.Arzu Özmüş

June, 2004

CONTENTS

ABBREVIATIONS

Ala (A)	: Alanine
Val (V)	: Valine
Thr (T)	: Threonine
Lys (K)	: Lysine
Arg (R)	: Arginine
Trp (W)	: Tryptophane
Ser (S)	: Serine
Leu (L)	: Leucine
Ile (I)	: Isoleucine
His (H)	: Histidine
Gly (G)	: Glycine
Tyr (Y)	: Tyrosine
Asp (D)	: Aspartic acid
Glu (E)	: Glutamic acid
Gln (Q)	: Glutamine
Cys (C)	: Cysteine
Pro (P)	: Proline
Phe (F)	: Phenylalanine
Met (M)	: Methionine
Asn (N)	: Asparagine
HAP	**:** Hydroxyapatite
PD/ Ph.D.	: Phage display
CSD	: Cell surface display
RD	: Ribosome display
BSA	: Bovine serum albumine
QCM	: Quartz crystal microbalance
SPR	: Surface plasmon resonance

LIST OF TABLES **Page No**

LIST OF FIGURES

University : **İstanbul Teknik Üniversitesi**
Enstitüsü : **Fen Bilimleri**
Anabilim Dalı : **Moleküler Biyoloji ve Genetik**
Programı : **Moleküler Biyoloji, Genetik ve Biyoteknoloji**
Tez Danışmanları : **Doç. Dr. Candan TAMERLER BEHAR**
Prof. Dr. Mehmet SARIKAYA
Tez Türü ve Tarihi : **Yüksek Lisans – Haziran 2004**

ÖZET

PHAGE DISPLAY TEKNOLOJİSİ İLE KRİSTAL YÜZEY SPESİFİK PEPTİDLERİN TANIMLANMASI

Asiye Arzu ÖZMÜŞ

Büyük ve küçük proteinlerin, kemik, diş dokuları ve bakteri partikülleri gibi biyolojik sert dokuların, sentez ve oluşumu gibi biyomineralizasyonları boyunca, temel rolleri vardır. Tüm bu olaylar, minerallerin moleküler olarak tanınması, onların kataliz/sentez ve biyomineralizasyon boyunca geçici olarak alan içine dağılmış bir grup protein tarafından kendiliğinden düzenlenmesi (self-assembly), yoluyla gerçekleşir. Metal, metal oksit, karbonat, sülfit ve atomik kompozisyon, kristal yapı, nano- ve mikro-yapı ile fiziksel özelliklerde değişiklik gösteren yapılar, biyomineralize dokularin inorganik bileşenlerini olşturur.

Bu çalişmada, kristal yüzey spesifik peptidleri seçmek üzere, M13 Phage Display sistemini kullanarak, phage display teknolojisi benimsenmiştir. Seçim için, tek kristal safirin (α-alumina, Al_2O_3), [110] ve [001] olmak üzere iki farklı kristal yüzeyi kullanılmıştır. İnorganik yüzeye bağlanan peptidlerin seçimi için adapte ettiğimiz, New England BioLabs tarafından geliştirilmiş olan Ph.D.-C7C kütüphanesi ile, moleküler biyolojik protokol kullanılarak safir spesifik peptidler elde edilmiştir. Seçim süreci boyunca, yüzeye bağlanan herhangibir faj tercihli olarak ortamda tutulmuş, spesifik olmayan fiziksel olarak tutunan fajlar yıkanarak ortamdan uzaklaştırılmıştır. Proses, safire bağlanmasi istenen peptidleri ortaya koyan fajların zenginleştirilmesi için, 3-4 kez tekrarlanmiştır. İnorganik yüzeyden bağlanan fajlarin yetersiz bir şekilde elusyonu gibi, seçim protokolünün muhtemel dezavantajlarını gidermek amacıyla, yeni bir protokol uygulanmiştır. Seçilen sekansların karakterizesyonu için, pl ve yük gibi fizikokimyasal özellikleri ile hidrofilite değerlendirilmesi yapılmıştır. Bağlanma özellikleri izlenerek, immünoboyama temelli floresan mikroskopi çalışmaları ile, seçilmiş peptidler güçlü, orta ve zayıf bağlanma özellikli olarak üç gruba ayrılmıştır. Sekansların daha kapsamlı özelliklerinin belirlenmesi, atomik kuvvet mikroskopisi kullanılarak, gerçekleştirilmiştir.

Bu çalışmalar, seçilen peptidlerin kristalografik yüzey spesifik doğası ile ilgili bilgiler vermiştir.

Anahtar Kelimeler: Faj Gösterimi Teknolojisi, Tek Kristal Safir

University : **İstanbul Technical University**
Institute : **Institute of Science and Technology**
Science Programme : **Molecular Biology and Genetics**
Programme : **Molecular Biology, Genetics and Biotechnology**
Supervisor : **Assoc. Prof. Candan TAMERLER BEHAR**
Prof. Dr. Mehmet SARIKAYA
Degree Awarded and Date : **Master – June 2004**

ABSTRACT

IDENTIFICATION OF CRYSTAL FACE SPECIFIC SAPPHIRE BINDING PEPTIDES BY PHAGE DISPLAY

Asiye Arzu ÖZMÜŞ

Proteins, large and small, play major roles during biomineralization, i.e., in the synthesis and formation of biological hard tissues such as bone, dental tissues and, bacterial particles. This is accomplished via molecular recognition of minerals, their catalysis/sythesis and, self-assembly by a collection of proteins that are temporally and spatially distributed during the biomineralization processes. Inorganic components of the biomineralized tissues include metals, metal oxides, carbonates, sulfides and, semiconductors that vary in atomic composition, crystal structure, nano- and micro-structure and, physical properties.

In this research, in an attempt to select crystal–surface specific peptides, we adopted phage display technology using M13 Phage Display system. Single crystals of sapphire (α-alumina, Al_2O_3) in two different crystal orientations [110] and [001] were used as the substrates for selection. Sapphire specific peptides were recovered using a molecular biological protocol with Ph.D.-C7C library provided by New England BioLabs, that we adapted for the selection of inorganic-binding peptides. During the stages of selection, any phage that binds to the surface is preferentially retained and, non-specific physisorbed phages are washed out. The process is repeated 3-4 rounds to enrich for the phages displaying the desired sapphire-binding peptides. A new protocol was introduced to overcome possible disadvantages of the selection procedure like inefficient elution of the bound phages from the inorganic surface. Characterization of the selected sequences was performed both by evaluating their physicochemical properties such as pI, charge and hydrophilicity. By following their binding properties, the selected peptides were classified in three groups as strong, moderate and weak binders as aresult of immunolabelling based fluorescence microscopy studies. Further verifications on the properties of the sequences, were performed by use of atomic force microscopy experiments.

These studies provided insights into the nature of crystallographic surface specific binding of selected peptides.

Keywords: Phage Display Technology, Single Crystal Sapphire

IDENTIFICATION OF CRYSTAL FACE SPECIFIC

SAPPHIRE BINDING PEPTIDES BY PHAGE DISPLAY

SUMMARY

Proteins, large and small, play major roles during biomineralization, i.e., in the synthesis and formation of biological hard tissues such as bone, dental tissues and, bacterial particles. This is accomplished via molecular recognition of minerals, their catalysis/sythesis and, self-assembly by a collection of proteins that are temporally and spatially distributed during the biomineralization processes. Inorganic components of the biomineralized tissues include metals, metal oxides, carbonates, sulfides and, semiconductors that vary in atomic composition, crystal structure, nano- and micro-structure and, physical properties.

In this research, in an attempt to select crystal–surface specific peptides, we adopted phage display technology using M13 Phage Display system. Single crystals of sapphire (α-alumina, Al_2O_3) in two different crystal orientations [110] and [001] were used as the substrates for selection. Sapphire specific peptides were recovered using a molecular biological protocol with Ph.D.-C7C library provided by New England BioLabs, that we adapted for the selection of inorganic-binding peptides. During the stages of selection, any phage that binds to the surface is preferentially retained and, non-specific physisorbed phages are washed out. The process is repeated 3-4 rounds to enrich for the phages displaying the desired sapphire-binding peptides. A new protocol was introduced to overcome possible disadvantages of the selection procedure like inefficient elution of the bound phages from the inorganic surface. Characterization of the selected sequences was performed both by evaluating their physicochemical properties such as pI, charge and hydrophilicity. By following their binding properties, the selected peptides were classified in three groups as strong, moderate and weak binders as aresult of immunolabelling based fluorescence microscopy studies. Further verifications on the properties of the sequences, were performed by use of atomic force microscopy experiments.

These studies provided insights into the nature of crystallographic surface specific binding of selected peptides.

PHAGE DISPLAY TEKNOLOJİSİ İLE KRİSTAL YÜZEY SPESİFİK PEPTİDLERİN TANIMLANMASI

ÖZET

Büyük ve küçük proteinlerin, kemik, diş dokuları ve bakteri partikülleri gibi biyolojik sert dokuların, sentez ve oluşumu gibi biyomineralizasyonları boyunca, temel rolleri vardır. Tüm bu olaylar, minerallerin moleküler olarak tanınması, onların kataliz/sentez ve biyomineralizasyon boyunca geçici olarak alan içine dağılmış bir grup protein tarafından kendiliğinden düzenlenmesi (self-assembly), yoluyla gerçekleşir. Metal, metal oksit, karbonat, sülfit ve atomik kompozisyon, kristal yapı, nano- ve mikro-yapı ile fiziksel özelliklerde değişiklik gösteren yapılar, biyomineralize dokularin inorganik bileşenlerini olşturur.

Bu çalişmada, kristal yüzey spesifik peptidleri seçmek üzere, M13 Phage Display sistemini kullanarak, phage display teknolojisi benimsenmiştir. Seçim için, tek kristal safirin (α-alumina, Al_2O_3), [110] ve [001] olmak üzere iki farklı kristal yüzeyi kullanılmıştır. İnorganik yüzeye bağlanan peptidlerin seçimi için adapte ettiğimiz, New England BioLabs tarafından geliştirilmiş olan Ph.D.-C7C kütüphanesi ile, moleküler biyolojik protokol kullanılarak safir spesifik peptidler elde edilmiştir. Seçim süreci boyunca, yüzeye bağlanan herhangibir faj tercihli olarak ortamda tutulmuş, spesifik olmayan fiziksel olarak tutunan fajlar yıkanarak ortamdan uzaklaştırılmıştır. Proses, safire bağlanmasi istenen peptidleri ortaya koyan fajların zenginleştirilmesi için, 3-4 kez tekrarlanmiştır. İnorganik yüzeyden bağlanan fajlarin yetersiz bir şekilde elusyonu gibi, seçim protokolünün muhtemel dezavantajlarını gidermek amacıyla, yeni bir protokol uygulanmiştır. Seçilen sekansların karakterizesyonu için, pI ve yük gibi fizikokimyasal özellikleri ile hidrofilite değerlendirilmesi yapılmıştır. Bağlanma özellikleri izlenerek, immünoboyama temelli floresan mikroskopi çalışmaları ile, seçilmiş peptidler güçlü, orta ve zayıf bağlanma özellikli olarak üç gruba ayrılmıştır. Sekansların daha kapsamlı özelliklerinin belirlenmesi, atomik kuvvet mikroskopisi kullanılarak, gerçekleştirilmiştir.

Bu çalışmalar, seçilen peptidlerin kristalografik yüzey spesifik doğası ile ilgili bilgiler vermiştir.

1. INTRODUCTION

1.1. Importance of proteins in biological systems

The living systems have properties reflecting highly organized state like; being able to exchange matter and energy with their surroundings; respond to changes in those surroundings; transform energy and matter into different forms according to their needs; to grow and, reproduce . All this organization would begin with water and small organic molecules, in the smallest structure called cell which shows all the attributes of life. And, proteins (meaning “first/foremost” in Greek proteios/prōtos), are without a doubt, the most important and ubiquitous macromolecules in cells and organisms. They are the largest group of biological molecules among carbohydrates, nucleic acids and lipids. They are the biological polymers of small molecules called amino acids, joined in a genetically determined sequence. They play both structural and, functional roles. Bone and connective tissue proteins (for support) performing structural; enzymes catalyzing specific metabolic reactions, hormones carrying chemical messages to coordinate the organisms activities (signaling and regulating metabolism) and all antibodies providing essential environment for the organisms (defense), performing functional roles are only couple among many examples. [1,2]

Proteins are the most structurally sophisticated molecules known. Consistent with their diverse functions, they vary extensively in structure and conformation constructed from the same set of 20 amino acids. They are synthesized as polypeptide chains on ribosomes under genetic direction of a molecule of a messenger RNA that is in turn derived by transcription of the genetic information stored in the DNA. The amino acid analysis of a vast number of proteins indicates that they have considerable variation with respect to their amino acid compositions. Leu, Ala, Gly, Ser, Val and Glu are the most common amino acid residues (>6% abundance), whereas His, Met, Cys and Trp occur least frequently (>3% abundance). The ratio of polar to nonpolar residues is generally >1 for globular proteins and tends to decrease with increasing protein size. Because globular proteins have a hydrophobic core and, hydrophylic exterior. Nonpolar residues

predominate in membrane bound proteins, however, because these proteins, being immersed in a nonpolar environment must also have a hydrophobic exterior [2-4].

The structure of a protein is usually described in terms of four hierarchical levels of organization. Primary structure referring to the amino acid sequence (covalent peptide bonds); the secondary structure involving local interactions (α-helix, β-sheet conformations, hydrogen bonds); tertiary structure resulting from long-distance interactions of between stretches of amino acids from different parts of the molecule (hydrogen, disulfide bonds, electrostatic interactions, hydrophobic effect); and quaternary structure concerning the interaction of two or more individual polypeptides forming a single multimeric protein (having the same bond and interctions involved for tertiary structure) [2].

Proteins play important roles in hard tissues. Large and small, they play major roles during biomineralization like in the synthesis and formation of biological hard tissues such as bone, mollusk shells, dental tissues and bacterial particles. This is accomplished via molecular recognition of minerals, their catalysis/sythesis and, self-assembly by a collection of proteins that are temporally and spatially distributed during the biomineralization processes. All those biological tissues are synthesized in aqueous environments under mild physiological conditions using primarily proteins, biomacromolecules.In addition to enzymatic character in the synthesis of inorganic phases, proteins collect and transport raw materials and coassemble subunits into short- and long-range ordered nuclei and substrates. Inorganic components of the biomineralized tissues include metals, metal oxides, carbonates, sulfides and, semiconductors that vary in atomic composition, crystal structure, nano- and micro-structure and, physical properties [5, 6].

1.2. Biomimetic approach

The development of effective strategies for the reproducible control of crystal structure, size and morphology is gaining an important consideration as for importance of the applications in many areas of material fabrication to determine the exploitable (optical, magnetic, electrokinetic) properties of inorganic solids. Most traditional approaches to synthesize nanoscale materials are inefficient, requiring stringent conditions and often producing toxic byproducts. They also require considerable external manipulation that would limit the large-scale synthesis of complex architectures. At this point, the controlled formation of single domain inorganic materials under reasonable conditions like low temperature, is of considerable technological interest especially since crystallochemical specificity is

important determinant of functional efficiency. There is much demand for tailored inorganic materials which have defined catalytic, magnetic, optical or electrokinetic properties. In biological systems there are many examples of advanced “crystal engineering” in which organic solids are deposited in a highly controlled manner to produce mineral phases that are unique with respect to their structure and habit, and uniformity of size. It is evident that biology has already overcome many of the practical problems. Biomaterials, including natural hybrid materials are, highly organized from molecular level to macroscale; giving rise to a hierarchical system among itself which includes the different functional units. They are smart or self directed in their organization and formation, dynamic in their interaction with their surroundings, complex in their structures and functions, self-healişng in damage control, multifunctional in their physical and chemical properties, and have characteristics difficult to achieve in pure synthetic systems. Thus, it is hoped that new routes to the controlled synthesis and fabrication of technologically and medically important materials will be forthcoming from the detailed analysis of such systems [5, 7].

At this point, molecular mimicry or molecular biomimetics becomes very important in developing novel technological products. Molecular biomimetics can be defined as mimicking function, synthesis, or structure of materials and systems at the molecular scale using biological pathways [5]. This novel approach when applied for characterization of peptide-inorganic surface interctions, it would include polypeptide selections through display protocols, modification using molecular biology techniques, and application as molecular building blocks in controlled assembly and formation of functional inorganic and hybrid materials and systems in nano- and, nanobio-technology. These selected polypetides would generally have a short length [8-11] and, obtained via combinatorial biology using, cell surface or phage display libraries. Once selected, the inorganic binding poylpeptides could be further engineered using genetic engineering tools, like site directed mutagenesis, to tailor their properties for specific applications [12].

The potential of using engineered polypeptides is enormous due to the premise offered by molecular biolog, due to chemical and physical molecular recognition characteristics of inorganics, their self- and, co-assembly in higher order and, predictable structures and, the ability to manipulate their molecular composition and structure and, therefore properties.

For their utility as molecular erectors in the conjugation and hybridization with biomacromolecules and functional synthetic molecules (photonic-block co-polymers or conducting C60), their long-range ordered assembly as monomolecular films and, finally their uses as molecular substrates for practical engineering applications can be considered to be critical future applications [12].

1.3. How to obtain inorganic surface specific proteins?

There are couple ways of obtaining the inorganic surface specific proteins changing from traditional approaches to novel sophiticated tools using hybrid technologies. They could be available off the shell which makes their availability very limited. They could be extracted from hard tissues or designed theoritically by using computational tools together with biological ones. And finally they could be selected by use of the molecular library techniques based on combinatorial chemistry or biology based systems. Some of the main approaches are discussed below in detail.

1.3.1.Extraction from hard tissue

Many multicellular organisms produce hard tissues including bones, teeth, shells, skeleltal units and spicules. These hard tissues are biocomposites of structural macromolecules (lipids, proteins, polysaccharides) and, different kind of minerals like hydroxyapatite (HAP), calcium carbonate and silica. Other than multicellular organisms, many single cell organisms produce inorganic materials intracellularly or extracellularly. Magnetotactic bacteria synthesizing magnetite [13], crysophytes [14], diatoms that synthesize siliceous materials [15], and S layer bacteria that has gypsum and calcium carbonate surface layers [16], are two among many examples.

In multicellular organisms, bioinorganic materials are synthesized by a coordinated process in compertmentalized mediums like, bone synthesized at osteoblast [17] and dentin synthesized at dentin [18] which both are sythesized extracellularly . Also both resulting hard tissues are composites with structural proteins produced at mild conditions. Whereas the sythesis of nanoscale materials by most traditional methods require, high temperature, pressure and pH, often yielding very small amount of products with toxic byproducts [19].

In addition to its time consuming long procedure and difficulty, one of the main drawbacks of protein extraction from hard tissues is the inefficient application of the process. Amolegenin formation in human enamel [20] and lustrin in abalone nacre

sythesis [21] are the examples of application of proteins isolated from hard tissues and being used in materials sythesis. *In vitro* biomineralization process is investigated preliminarily. However, in biomineralization process, a large number of macromolecules are present in hard tissue and may effect the hard tissue formation in concert. Hard tissues usually contain multiple proteins that have different roles in biomineralizationand are distributed in complex ways. Furthermore, hard-tissue extracted proteins may be used only for the regeneration of the inorganic that they are originally associated with and, would be of limited practical use in the engineering of other nanostructures. At that point *in vivo* studies are essential, but complicated [5].

1.3.2. Selection of Proteins Through Molecular Library Based Systems

1.3.2.1. Combinatorial chemistry (based on peptide synthesis).

The classic technique of solid-phase method, developed in the 1963 by R.Bruce Merrifield of Rockefeller University. H. Mario Geysen, in the mid 1980s, then at Melbourne University in Australia, now at Glaxo Wellcome Company, adapted the technique to synthesize arrays of peptides on pin-shaped solid supports, first in parallel fashion and later as mixtures. Nearly at the same time, Richard Houghten of San Diego's Torrey Pines Institute for Molecular Studies developed a means of creating libraries of several thousand compounds by solid-phase parallel synthesis in tiny " tea bags" [22].

Solid phase synthesis, is a comprehensive reference of chemical reactions of substrates attached to solid supports (polystyrene, PEG, cellulose, controlled-pore glass) including methods for attachment and detachment from the supports [23].

The chemistry of peptide synthesis is also complicated by the poly-functional nature of the common α-amino acids. Traditionally, it has been slow, difficult and laborious, since large number of consecutive chemical reactions are usually required. With this method, all the synthetic operations take place on asolid but permeable particle, which can be asily separated from soluble reagents.

The method having a rapid way to create libraries of molecules with drug-like characteristics, quickly became the key to the production of entire proteins and enzymes during the past three decades. And, it has also become the basis fır explosion of solid phase organic synthesis, whereby molecular diversity can be introduced by producing a nearly infinite variety of heterocycles, steroids,

cardohydrates, and soon, organometallics, all while tethered by one reversible link to a suitable polymeric support.

By reacting a set of starting chemicals in every possible combination. Chem. Gives life scientists the ability to create molecules in huge numbers, and test them for sought-after properties. 6-7 years ago, a single chemist could make perhaps five compound per week. Now up to 100 new compounds, in the same period of time, can be created [22].

1.3.2.2. Combinatorial biology (selection through molecular libraries by display technologies)

Display technologies have proven an extraordinarily powerful tool for a wide range of applications of biology and biotechnology. This development enabled characterization of receptor- and antibody-binding sites, study of protein-ligand interactios and the isolation of proteins or enzymes exhibiting improved or altered binding characteristics for their ligands.

The three common approaches are; phage display (PD), cell surface display (CSD) and ribosome display (RD, also called as polysome display) [24-28].

Both PD and CSD technologies rely on the use of chimeric proteins that consist of a target sequence fused within or to a protein that naturally localizes on the surface of a bacteriophage (a bacterial virus) or a cell to achieve display. Using standard molecular biology techniques [24], the DNA sequence of the target region can be randomized to create a library of phages or cells, each of which will synthesize a different protein on its surface. By contacting the library with an immobilized ligand, washing out the weak or non-binders and, repeating the process to enrich for the tight (strong)-binders, a subset of interacting peptides can be selected from the original library and identified by sequencing. This process is known as biopanning.

In ribosome display (RD), a synthetic DNA library consisting of the mutated gene of interest is fused to C-terminal spacer region, which allows the polypeptide to fold, is transcribed *in vitro*. The stop codons are also eliminated to prevent release from the ribosome. Transcribed mRNAs are translated *in vitro,* as the encoded polypeptide folded as the ribosome travelled along the mRNA. Formed ternary complex consisting of mRNA, its translation product and, the ribosome as a coupler, is formed. These products can be panned also on an immobilized ligand. Following dissociation of the ternary complexes, the mRNA is converted to a cDNA by reverse transcription, amplified by PCR and, sequenced [5, 24-26].

Phage display, developed by George P. Smith in 1985 [29], is a selection technique in which a peptide or protein is expressed as a fusion with a coat protein of a bacteriophage (virus that only infects bacteria), resulting in display of the fused protein on the surface of the virion. He has shown that foreign DNA fragment could be inserted into the filamentous phage gene III which codes for phage coat protein pIII. From then, phage display has been used to create a physical linkage allowing rapid identification of peptide ligands for a variety of target molecules including antibodies, enzymes and cell-surface receptors by an *in vitro* selection process called biopanning [30,31].

Biopanning in original, is carried out by incubating a library of phage-displayed peptides (pool of phage) with a plate (or bead) coated with the target, washing away the unbound phage, and eluting the specifically-bound phage (Fig 1.1). The eluted phage is then amplified and taken through additional binding/amplification cycles to enrich the pool in favor of binding sequences. After 3-4 rounds, individual clones are characterized by DNA sequencing.

Random phage display protocol have been used in a number of applications [3,32] like; epitope mapping [32-34]; protein-protein contacts mapping [35]; and peptide mimics of non-peptide ligands identification [36-39]. Bioactive peptides have been identified either by panning against immobilized purified receptors [40], or against intact cells [41-43]. Protease substrates have been identified by attaching an affinity tag upstream from the randomized region, and seperating cleaved from uncleaved phage with the appropriate affinity matrix [44]. Conversely, larger proteins such as antibodies [45], hormones [46], protease inhibitors [47], enzymes [48], and DNA binding proteins [49], have been displayed on phage, and variants with altered affinity or specificıty have been isolated from libraries of random mutants.

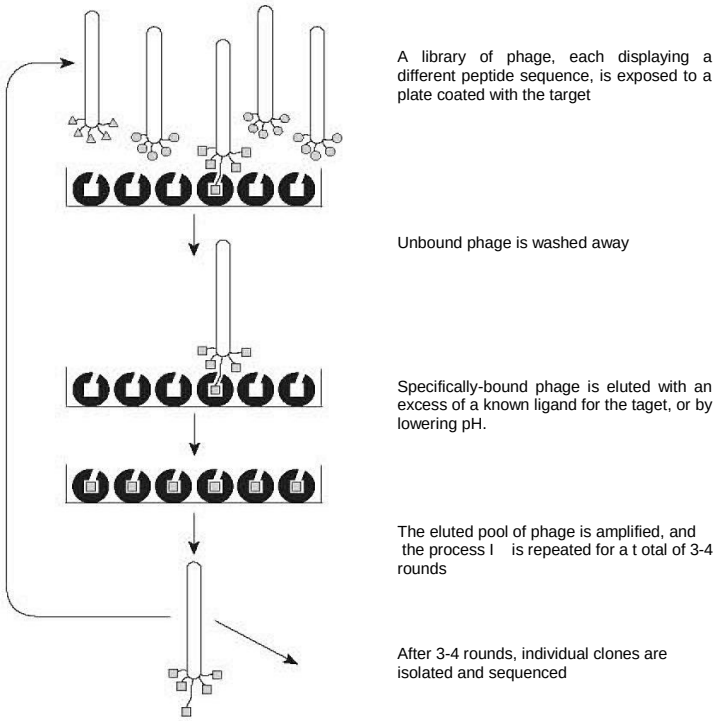


Figure 1.1. Panning with the PD peptide library. Adapted from the manual by the New England BioLabs [50].

The panning experiments with Ph.D.-C7C disulfide constrained peptide library is applied for streptavidin and monoclonal antibodies, demonstrating the library being in sufficiently high complexity for identical heptatpeptide sequences to be encoded by multiple DNA sequences is determined by the New England BioLabs.

K= G or T; M= A or C

Figure 1.2. N-terminal sequence of random disulfide-constrained heptapeptide-pIII fusion. The fusion is expressed with a leader sequence that is removed upon secretion at the position indicated by the arrow, resulting in the alanine preceding the first cysteine residue of the crosslink positioned directly at the N-terminus of the mature fusion protein. The hybridization positions of the –28 and –96 primers are indicated [50].

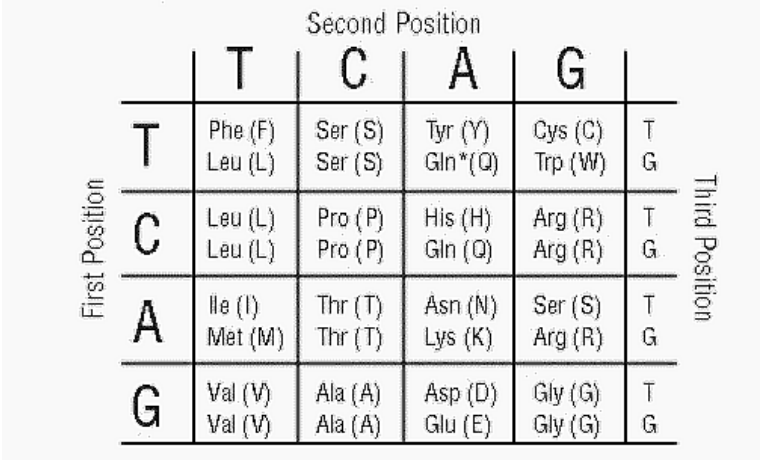


Figure 1.3. Reduced genetic code. The randomized region of the library encodes all 20 amino acids with only 32 codons. This increases the relative frequency of residues with a single codon, as well as removing 2 of the 3 stop codons. The amber stop codon TAG (*) is supressed by Gln in the strain used to construct the library [50].

There are several variables affecting the stringency of selection during panning which, should be considered for optimization of peptide binding. These are ;

a- Detergent: The presence of detergent (typically Tween-20) in buffers reduces nonspecific interactions between the phage and the target and/or blocking agent (Bovine Serum Albumin, BSA). Lower Tween concentrations in early rounds resulting higher eluate titers, can be gradually increased with each round by raising the Tween concentration stepwise to a maximum of 0.5% also increasing the stringency. In parallel experiments, when Tween concentrations were held constant at 0.5% or increased stepwise as 0.1, 0.3 and, 0.5% in 3 rounds of panning. however, identical consensus sequences are obtained. Still gradually increasing detergent concentration is still recommended since, the eluate titer (i.e. the number of bound sequences) in early round is expected to be very low.

b- Temperature: Depending on whether the binding interaction is enthalpically or entropically driven, stringency can be increased or decreased by raising the temperature of the binding step, respectively. Suggested binding temperatures to try is given by the New England BioLabs are : 4 ºC, room temperature and 37 ºC.

c- Binding and Elution time: Selection of peptides with rapid on rates (k_{on}) accompanied with shorter binding times, is favored. Conversely, with longer elution times, selection of peptides with off rates (k_{off}) is favored. Since the equilibrium association constant K_a for binding of the peptide to the target is equal to k_{on} / k_{off},

stringency can be increased by shortening the binding time and lengthening the elution time. When eluting with pH 2.2 glycine buffer, it is not recommended to elute for longer than 10 min. since phage infectivity would be reduced.

d- Target concentration: If panning against the target in solution, the stringency can be increased by lowering the concentration of target. An initial target concentration of 10nM is recommended; this can be lowered to 1 nM in later rounds for selection of ligands with nanomolar binding affinity.

e- Number of rounds: The pool of phage becomes enriched in favor of sequences that bind to the target, with each round of panning and amplification. Maintaining a constant input phage concentration in each round resulting a stepwise increase in the number of particles displaying a given sequence until a point is reached where most or all of the eluted particles display a consensus binding sequence. Depending on the interaction being studied and the applied stringecy, it is achieved after 2 or 3 rounds. If no clear consensus sequence emerges after 3 rounds, the 3rd round eluate should be amplified and a fourth round of panning carried out.

f- Choice of Library: The commercial phage display libraries are constructed as 7 mer constrained and unconstrained and, 12 mer unconstrained due to various applications. When, following 3 or 4 rounds of panning, clear consensus of ligand sequence is not observed (or all of the plaques are white), one possible may be that the library simply does not contain any clones that bind tightly to the target. One explanation for this is that the ideal ligand sequence is not statistically reperesented in the library. Or, any potential ligand sequence is not able to adopt a conformation necessary for target binding.

In the case of the Ph.D.-C7C library, where all of the displayed peptides are structurally constrained in a 7-residue disulfide loop, a ligand sequence where the imposed constraint allows a productive binding conformation will bind more tightly than the same sequence expressed in a linear peptide library (due to the improved entrophy of binding). If the imposed constraint does not allow a productive binding conformation, than that sequence will likely not bind to the target at all. In this case either of linear peptide libraries (ph.D.-7 or Ph.D.-12) may yield better results. Unfortunately, in the absence of detailed structural information about the target-ligand interaction, it's not possible to predict in advance which library will yield the most productive ligands [50].

One of the mostly used phage display system uses, filamentous phage (Ff) M13 for the process. It has the diameters of 930 nm x 6.5 nm. The random sequence of

amino acids are displayed at the N-terminus of the phage pIII protein. Filmentous bacteriophages are a group of viruses that contain a circular ssDNA genome in a long protein capsid. Many members of Ff class phages use bacterial pilus to facilitate infection. M13, uses the tip of F conjugative pilus as a receptor, so is specific for *E.coli* containing the F plasmid. It encodes 11 genes; genes II, V, X encodes proteins required for phage genome replication; genes I, IV, XI are involved in membrane associated assembly of bacteriophages and finally genes III, VI, VII, VIII, IX encode capsid proteins. pVIII being the major coat protein has nearly 2700 copies per virion which only, 10% of the copies can reliably fused to peptides giving rise to the selection of low affinity ligands. pIII being one of the minor coat proteins, is peresent 5 copies per virion which all can be fused to the peptides, resulting the selection of high affinity ligands. [51].

The schematic representation of constrained and unconstrained display of the peptide on pIII-coat protein of phage, is shown in the Figure 1.4.

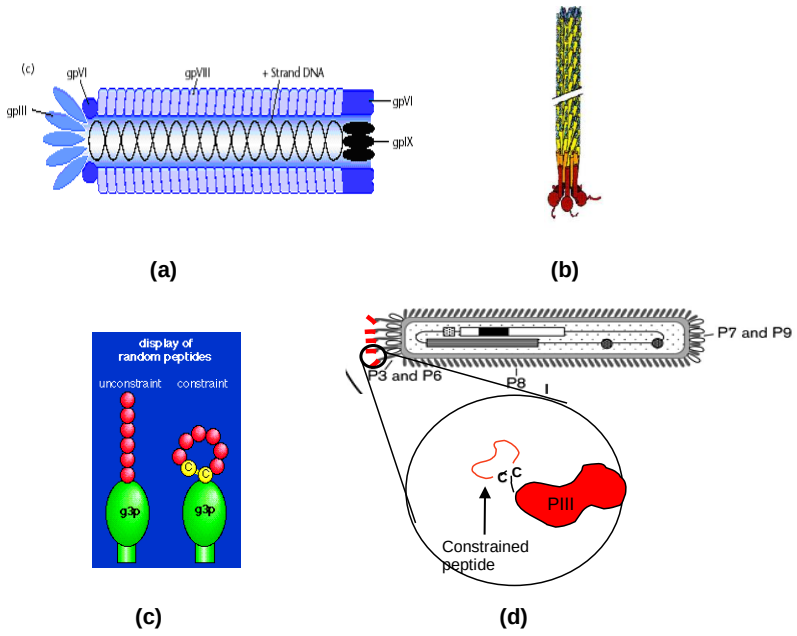


Fig. 1.4. Schematic representations of M13 bacteriophage **(a)** The filamentous Ff bacteriophage particle. Schematic representation of the phage particle showing the location of the capsid protein **(b)** Representation of orientation of the pVIII molecules with pVI and pIII molecules displaying peptides **(c)** Commercially available display of peptides on coat protein pIII of Ff phage [53] **(d)** Schematic presentation of the constrained peptide fused to PIII minor coat protein of the M13 bacteriophage.

All 5 coat proteins contribute to structural stability of the phage particle, PIII being necessary for host cell recognition and infection. Consequently PIII is the largest and most complex of the coat proteins. It contains three distinct domains. N-terminal domain, initiates translocation of the viral DNA into *E.coli* during infection while second domain confers host cell recognition by binding to the F pilus on the *E.coli* surface. C-teminal domain, interacts with other phage coat proteins, and is thus responsible for the integration of PIII into the phage coat [52]. One of the most important advantages of M13 over other phage systems is that, M13 is a non-lytic phage, meaning that it does not lyse the host during phage production. This simplifies the intermediate phage purification steps between rounds of panning, as a PEG precipitation step is sufficient to separate the phage from nearly all contaminating cellular proteins.

1.4. Phage display in identification of inorganic surface specific peptides

In biological hard tissues, proteins control synthesis , assembly, morphogenesis and, formation of inorganic materials through molecular recognition and surface-specific binding. Some of the natural proteins can be large and complex, hard to isolate, purify and, difficult to use in reconstruction of the hybrid structures. By using molecular biology techniques via the use of molecular libraries, inorganic material-binding poylpeptides can be obtained. Adapting phage display protocols, specific peptides can be selected for use in material assembly and, formation [54].

In adapting phage display technology to the selection of inorganic surface specific peptides, surface characteristics of the inorganics becomes very important. The binding mechanism of peptides to their ligands often require structural complementation and surface chemistries. One of the strongest interaction example in nature, would be biotin-streptavidin interaction. The binding of 4 biotin molecules to tetrameric protein sterptavidin occurs by a “lock and key” mechanism [55, 56]. For selection of inorganic specific peptides, similar kind of mechanism is considered in specific morphology, crystallography, or surface stereochemistry. In order to design and optimize the properties of functional inorganics, peptide-inorganic material interaction mechanism should be recognized which still remains unclear [51, 57, 58]. Chemical (hydrogen bonding, polarity, charge effects) [53, 56] and, structural (size, morphology) [60] recognition may be responsible for a peptide or a protein specificly interact with an inorganic surface. At this point, the accurate characterization of the inorganic surface properties plays a critical role. The surface

would be as well defined as a single crystal or, would be totally nondescriptive as a powder having the sequence space the largest.

In the peptide selection process for a powder, the sequence diversity is expected to be the largest, since a powder would have wide distribution of size, morphology (sharp corners, rods, spherical particles etc) and crystallography. On the other hand, when materials that have controlled morphology, crystallography or stereochemistry are used for those materials specific peptide selection, it may show higher sequence homology. For instance, GEPI (genetically engineered protein for inorganics) selected for [hkl] surface, may or may not bind to another crystallographic plane of the same material. Once binders are obtained for powders, then a specified material (specific morphology, size or surface) can be used to obtain a narrower peptide distribution showing probable sequence similarities. By getting some information on peptides recognizing inorganic materials, would aid designing and integration of materials at the molecular level [5].

1.5. Binding Characterization Tools

A better understanding of the rules that govern the binding of peptides to inorganic compounds would be needed for the design of hybrid materials that exhibit controlled topology and composition. In this regard, FM and AFM are heavily used as characterization tools, are routine tools used as a part of the inorganic surface specific polypeptide identification protocol. FM would also allow cross-specificity of a given polypeptide among a number of micro-patterned inorganics simultanously, while AFM, given the correct parameters, may provide three dimentional (3D) image of the interactions.

1.5.1. Qualitative Assesment of Binding by Fluorescent Microscopy (FM)

Fluorescence is a member of the ubiquitous **luminescence** family of processes in which susceptible molecules emit light from electronically excited states created by either a physical (absorption of light), mechanical (friction), or chemical mechanism. Generation of luminescence through excitation of a molecule by ultraviolet or visible light photons is a phenomenon termed **photoluminescence**, which is formally divided into two categories, **fluorescence** and **phosphorescence**, depending upon the electronic configuration of the excited state and the emission pathway.

Fluorescence is the property of some atoms and molecules to absorb light at a particular wavelength and to subsequently emit light of longer wavelength after a brief interval, termed the fluorescence lifetime.

Because of the tremendously sensitive emission profiles, spatial resolution, and high specificity of fluorescence investigations, the technique is an important tool in genetics and cell biology.

Fluorescence microscopy based on immunolabelling techniques has proven very quantitatively rank a population of polypeptides selected by phage or cell surface display in terms of affinity and specificity for a particular inorganic surface [61-65].

In general, amplified phages that each peptide sequence is fused to the pIII coat protein are contacted with inorganic surface. Bound phages are visualized by incubation with primary antibody that is specific for the phage coat protein and, with a secondary antibody conjugated with a fluorophore that recognizes the primary antibody. When the fluorophore is excited, the phages would reflect the light. Although, it is a qualitative technique, binding strength is estimated due to comparison with other materials.

1.5.2. Qualitative Assesment of Binding by Atomic Force Microscopy (AFM)

The atomic force microscope (AFM) is one of the scanned-proximity probe microscopes, providing very high resolution images of various sample properties. They work by measuring a local property - such as height, optical absorption, or magnetism - with a probe or "tip" placed very close to the sample.

Unlike traditional microscopes, scanned-probe systems do not use lenses, so the size of the probe generally limit their resolution, rather than diffraction effects [66].

The AFM measures topography of the sample with a force probe. As seen at figure 1.5, AFM operates by measuring attractive or repulsive forces between a tip and the sample [67]. Displacement of the tip is tracked with the laser.

Açıklamalı [p1]:

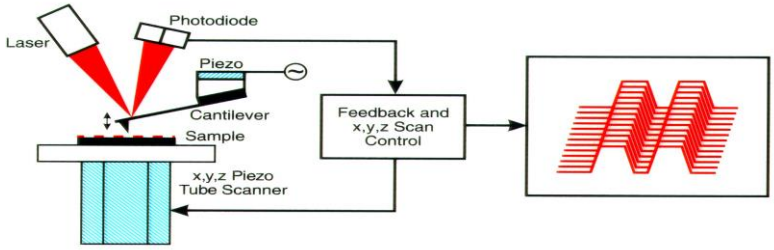


Figure 1.5. The concept of AFM, a cantilever touching a sample. Adopted from Park Scientific Instruments.

In its repulsive "contact" mode, the instrument lightly touches a tip at the end of a leaf spring or "cantilever" to the sample. As a raster-scan drags the tip over the sample, the vertical deflection of the cantilever is measured by some sort of detection apparatus , which indicates the local sample height. Thus, in contact mode the AFM measures hard-sphere repulsion forces between the tip and sample. In noncontact mode, the tip does not touch the sample and AFM derives topographic images from measurements of attractive forces [68]. AFMs can achieve a resolution of 10 pm, and unlike electron microscopes, can image samples in air and under liquids.

AFM incorporates a number of refinements that enables it to achieve atomic-scale resolution with sensitive detection, flexible cantilevers, sharp tips, high-resoltion tip-sample positioning and force feedback.

The AFM not only measures the force on the sample but also regulates it, allowing acquisition of images at very low forces. A high flexibility stylus exerts lower downward forces on the sample, resulting in less distortion and damage while scanning.

AFMs have two standard imaging modes. They can measure sample topography in two ways: by recording the feedback output ("Z") or the cantilever deflection. The sum of these two signals yield the actual topography.

Dull tips and tip-sample interaction forces prevent high-resolution imaging of biological structures. The ability of AFM to image at atomic resolution, combined with its ability to image a wide variety of samples under a wide variety of conditions, has created a great deal of interest in applying it to the study of biological structures. Images have appeared in the literature showing DNA, single proteins, structures such as gap junctions, and living cells [69].

Unfortunately, AFM cannot image all samples at atomic resolution. The end radii of available tips confines atomic resolution to flat, periodic samples such as graphite. In addition, because biological structures are soft, the tip-sample interaction tends to distort or destroy them.

When imaging in air, a layer of water condensation and other contamination covers both the tip and sample, forming a meniscus that pulls the two together [70].

1.6. Properties of single crystal sapphire

Since single crystal sapphire is the hardest natural substance next to diamond, it has a unique combination of physical, chemical and optical properties allowing it to withstand high temperatures, high pressure, the shock and, water or sand erosion. It is chemically inert, with alow friction coefficient, and excellent electrical, optical and, dielectric characteristics. Its radiation resistance makes it an excellent material for use in optical windows for space applications. It is much stronger than other optical materials. It has an extreme surface hardness. It is higly resistant to scratching and abrasion. It has a very wide optical transmission band from UV to near-IR. It has extremely high melting temperature as ~2053 ºC. Most properties are useful to (maximum) ~1800 ºC and has conductivity value as, 40 W/M ºK at 298 ºC.

It has high electrical resistance and high dielectric constant. It is chemically inert. It is totally unaffected by all chemicals except some very hot caustics. It has high thermal conductivity for a non-conductor, even better than copper at cryogenic temperatures.

Sapphire also has infinite number of **grades,** which is entirely arbitrary and, decided upon after inspection of each synthetic sapphire batch that has been grown. Synthetic sapphire is graded by what is important for a particular application, either optical or mechanical. A high grade of sapphire having little or no light scatter or lattice distortion can be used mainly for the most demanding optical applications. A lower grade of sapphire having extensive light scatter or lattice distortion, being used mainly for the mechanical and structural uses such as bearings, fixtures and, less demanding optical applications. An ultraviolet (UV) grade sapphire or non-browning sapphire would not solarize on exposure to UV light, giving rise to use in natural harsh UV conditions [71].

1.6.1.Optical and electronical properties of Sapphire

Single crystal saphire is a water clear material, having a trigonal (hexagonal) crystal system. It is non-porous, unlike alumina ceramics. It is extremely inert; unaffected by weathering and hydration. It is virtually unaffected by any solvents or acids at room temperature (some etching by hot phosphoric acid and strong caustics at temperatures exceeding 600 ºC – 800 ºC).

Because of its unique properties, sapphire is often machined into precision shapes for instruments and high temperature applications. Sapphire can be bonded to alumina, sapphire or other metal parts using glassy or non glassy fritz; or it can be metalized and brazed into metal holders.

Sapphire also has extremely wide applications on wide arrays of markets including, medical instruments, surgical devices, computer/electronics, nuclear power and vacuum systems, laser systems and applied laser systems, scientific instruments, imaging, military/aerospace, analytical measurement instruments, research and development at educational institutes, detectors, sensors and finally for semiconductor manufacuring equipments [71].

2. MATERIALS AND METHODS:

2.1. Materials

2.1.1. Sapphire substrate material

Sapphire (Al_2O_3) samples having two different surface characteristics as (110) and (001), were used. The digital images of the (110) and (001) sapphire samples are shown in Figure 2.1.

(a) **(b)**

Figure 2.1. Digital images of single crystal (110) and (001) sapphire. **(a)** Digital image of single crystal (110) sapphire. It has a light pink color since, it contains Ti in ppm amount. The diameters of each sample is around 0.5 X 0.6 cm having the thickness as 0. **(b)** Digital image of single crystal [001] sapphire. It is a clear crystal. The diameters are approximately 0.5 X 0.3-0.5 cm having the thickness of 0.1 mm.

Since sapphire is the second hardest material next to diamond, specialized instrument is required to perform substrate preparation. Sapphire samples are cut with the equipment shown in Figure 2.2.

Figure 2.2. The equipment used to cut the sapphire samples to perform substrate preparation.

2.1.2. Preparation of sapphire

i) Cleaning of single crystal sapphire

Single crystal sapphire (110) and (001) samples are cleaned by sonication, in order to remove any residual surface interactions. The procedure is; 15 minutes of sonication in eppendorph tube filled with 70% ethanol, 15 minutes of sonication with methanol-acetone (1:1) mixture, 15 minutes of sonication with isopropanol, 15 minutes washing by rotation with distilled water. Before using for screen, the sample is washed couple times with 0.02% PC buffer by pipetting.

ii) Cleaning of sapphire powder

The sapphire powder, alumina (Al_2O_3), that is used for fluorescent microscopy studies were prepared by grinding single crystal sapphire samples with, first steel grinder and then alumina pestle and mortar as shown Fig.2.2. Crushed Al_2O_3 is grounded in a small amount of ethanol in pestle and mortar to avoid powders flit. The details of cleaning procedure is given Appendix A.2.

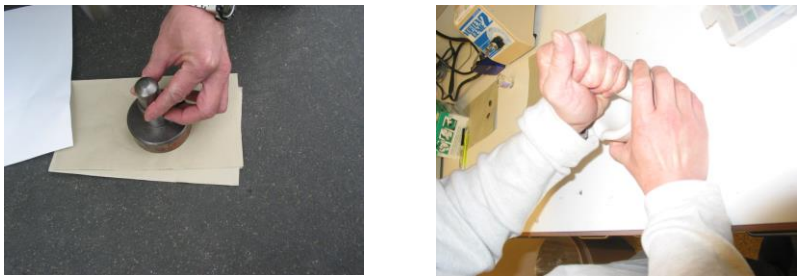


Fig. 2.3. Steel grinder (left) and alumina pestle and mortar (right), used for Al_2O_3 grinding. Sapphire crystal substrates are ground with applied force first with a steel grinder then, with alumina pestle and mortar.

2.2. Bacterial strain and phage specifications

2.2.1. *E. coli* ER2738 Host Strain

Host strain was purchased from New England BioLabs Inc. (*F' lacI^{q} Δ(lacZ)M15proA^{+}B^{+} zzf::Tn10(Tet^{R})/fhuA2 supE thiΔ (lac-proAB) Δ(hsdMS-mcrB) 5 ($r_k^-m_k^-$McrBC$^-$).* Host strain was supplied as 50 % glycerol culture, as non-competent. It is stored at -70°C. ER2738 is a robust F^+ strain with a rapid growth rate and it is well suited for, male specific M13 propagation. The F-factor of ER2738 contains a mini-transposon that confers tetracycline resistance, so cells harboring the F-factor is selected by plating and propagating on tetracycline-containing media.

2.2.2. Phage Display Library (Ph.D.-C7C ™)

Phage display library kit, also was purchased from New England BioLabs Inc. (E8120S). It is supplied in 100 µl TBS with 50 % glycerol. The concentration of the library was given as 2 x 10^{13} pfu/ml and the complexity of the library is given as 1,2 x 10^9 transformants. It is based on a combinatorial library of random peptide 7-mers fused to a minor coat protein (pIII) of M13 bacteriophage. The randomized 7 amino acids sequences are flanked by a pair of cysteine residues. Under nonreducing conditions the cysteins would spontaneously form disulfide cross-link, resulting in phage display of cyclized peptides. The disulfide-constrained heptatpeptides are expressed at the N-terminus of pIII, with the first cyteine preceded by an alanine residue, and the second cysteine followed by a short spacer (Gly-Gly-Gly-Ser) and the wild type pIII sequence (Figure100). The library consists of 1,2 x 10^9 electroporated sequences (compared to 20^7 = 1,28 x 10^9 possible 7-residue sequences), amplified once to yield ~ 200 copies of each sequence in 10 µl of the supplied phage.

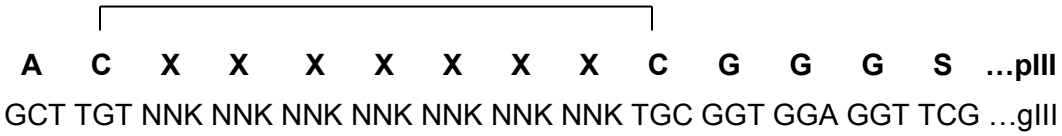


Fig.2.4. Constrained 7 amino acid insertion to pIII coat protein with two cysteine amino acids at both ends.

In order to avoid phage contamination, some precautions should be taken. The potential for contamination with environmental bacteriophage can be minimized by using aerosol-

resistant pipet tips for all protocols. Since the library phage is derived from the common cloning vector M13mp19, which carries the *lacZα* gene, phage plaques appear blue when plated on media containing X-Gal and IPTG. Environmental filamentous phage will typically yield white plaques when plated on the same media. It is recommended plating on LB / X-Gal / IPTG plates for all titering steps and, if white plaques are evident, picking only blue ones for sequencing.

2.3. Culture Media and Buffers:

***LB Media**:* It includes 10g. tryptone (USBiological, T8750) , 5g. yeast extract (USBiological, Y2010), 5 g NaCl (Sigma, S-3014) per 1L of dH_2O. pH of the solution is adjusted to 7.00, with concentrated NaOH. The solution is autoclaved and stored at room temperature.

LB Agar: It includes 10 g. tryptone, 5 g. yeast extract, 5 g NaCl, 15 g bactoagar (USBiological, A0930) per 1L of dH_2O. The solution is autoclaved.

LB/IPTG/X-Gal Plates: LB agar is prepared and autoclaved. After autoclaving, it is placed into a waterbath which is approximately 54°C (LAB-LINE, Aquabath) and let the solution cool down to <70°C. Then 5mM $MgCl_2$, (1:1000) tetracycline and, (1:1000) X-gal/IPTG is added. After shaking properly, LB agar is poured into plates by avoiding any bubble formation. For small plates 3.5 ml and for big plates 15 ml. of LB agar is poured. Plates are left at room temperature for 20 minutes for the agar to freeze and then, stored upside down in the freezer in the dark.

Top Agar: For 1 L of solution, it includes 10 g. tryptone, 5 g. yeast extract, 5 g. NaCl, 1 g. $MgCl_2.6H_2O$ and 9 g. LMP (ISC Bioexpress, GENEPure, E-3126-25) agar. The solution is autoclaved and stored at room temperature. It is melt in microwave as needed.

X-Gal/IPTG Stock Solution: 0.8 g. of X-Gal (*5-Bromo-4-chloro-3-indolyl-ß-D-galactoside*: USBiological, X1000, stored at -20°C) and 0.5 g. of IPTG (*isopropyl ß-D-thiogalactoside:*

USBiological, I8500, stored at -20˚C) is prepared in 10 ml. of DMF (dimethylformamide:). 1 ml/L of X-Gal/IPTG is used for plates. The solution can be stored at -20˚C in the dark.

Tetracyline solution: 10 mg tetracycline**.**HCl (USBiologicsal, T2965) is prepared in 1 ml. ddH_2O. It is vortexed and filter sterilized with filter having 200µm pore size (Cole-parmer, 47 mm membrane, 2916-34). Since tetracycline is a heat and light sensitive substance, interaction with light must be prevented. The tube is wrapped with an aluminium foil and stored in the freezer at +4 ºC . It should be vortexed each time used.

PEG/NaCl Solution: 20% (w/v) of polyethylene glycol-8000 (Sigma, P4463) containing 2.5M NaCl is prepared. The solution is autoclaved and stored at room temperature.

PC (Potassium phosphate-sodium carbonate) buffers: 55 mM KH_2PO_4 (Fisher, P285-500), 45 mM Na_2CO_3 (Sigma, S-2127) and 200 mM NaCl is prepared in water. The solution is filter sterilized by 0.2 µm filter paper. Appropriate amount of detergent is added. Stock detergent solution is 20% Tween® 20 (Sigma, P9416) and 20% Tergitol (Sigma, 15S12). For 500 ml PC containing 0.02%, 0.1% and 0.5% detergent, 0.5 ml, 2.5ml and 1.5 ml detergent from stock is added, respectively. The final pH of the solution should be between 7.2 and 7.5.

Elution Buffer I (Glycine-HCl buffer): 0.2 M glycine (Boehringer Mannheim Biochemicals,100 148) solution is prepared containing $1\mathrm{mg}/\mathrm{ml}$ BSA (Sigma, B-4287). pH is adjusted to 2.2 with concentrated HCl. The solution is sterilized by filter sterilization.

Elution Buffer II: Buffer A and B are mixed at the 1:1 ratio.

A: Glycine-HCl buffer containing 2mg/ml BSA and 0.02%SDS (Research Organics, 9602L). pH is adjusted to 2.2.

B: 7 mM TCEP (Molecular Probes, T-2556) buffer containing 100 mM DTT (Sigma, D-9779, stored at +4ºC) and 1 M NaCl (Sigma, S-3014).

Tris-HCl Neutralizing buffer: 80 ml. of 1M tris (hydroxymethyl) aminomethane solution is prepared. pH is adjusted to 9.1 with 1M HCl at room temperature. The solution is then completed to 100ml, .autoclaved and stored at room temperature.

E. coli overnight culture: Sterile LB solution, which contains 1mM $MgCl_2$ (Fisher Scientific, M33-500) and tetracycline (USBiological, T2965, stored at -20°C), is used. *E. coli* stock ER-2738 host strain *(*New England Biolabs, stored at –70°C), inoculated in 5 ml. LB media, is prepared daily and put for overnight incubation in a waterbath shaker at 200 rpm 37°C.

2.4. Phage Display Protocol

Phage display procedure consists of mainly two parts as; screening and sequencing.

2.4.1. Screening

Screening consists of three main processes as; biopanning rounds, purification and titer evaluations.

Prescreening preparation

***E. coli* culture preparation:** *E. coli* host strain ER2738 is supplied in 50 % glycerol solution as not competent. In order to use for screening, we selected the competent *E. coli* by, streaking out ER2738 from the glycerol culture onto an LB-Tet plate. It is inverted and incubated at 37 °C overnight and, frozen cultures were prepared (details of frozen culture preparation is given in Appendix A.3). ER2738 cultures for infection are grown in LB-Tet media.

Biopanning

For screening each round would consist of; biopanning experiment, amplification of the phages and elution of the amplified phages.

Biopanning experiments are applied as individual rounds for single crystal (110) and (001) sapphire. Schematic display of a round is demonstrated in Figure 2.4. and, detailed explanation about the protocol is available in Appendix A.7.

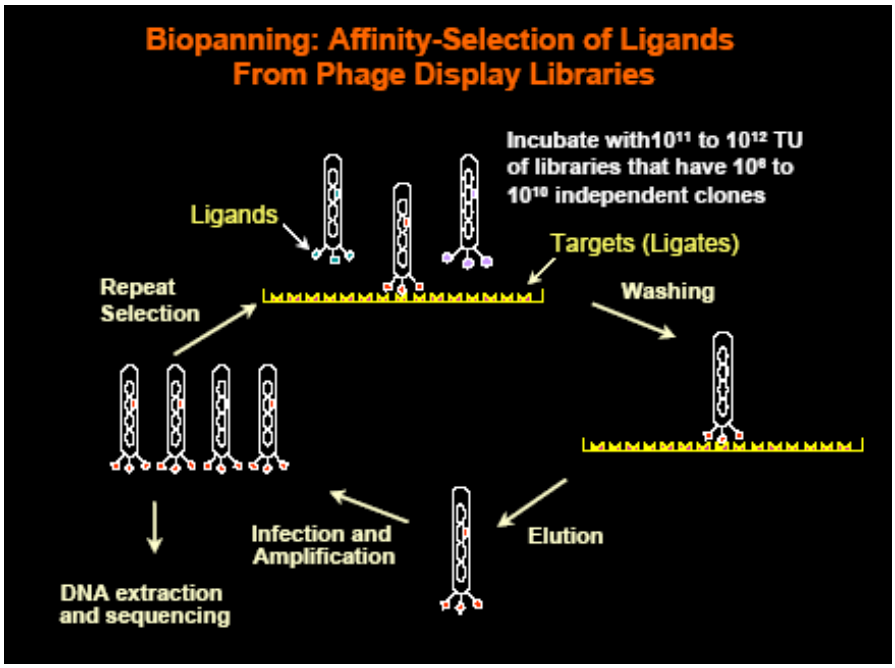


Figure 2.5. The biopanning procedure [72].

For each round, one sapphire crystal is washed with 0.01% PC buffer by pipetting and then, exposed to Ph.D.-C7C phage library in a sterilized eppendorf and rocked for 30 minutes. Supernatant is removed and put into prelabelled tube. Sapphire crystal is washed with 1 ml 0.1%PC buffer and the supernatants at the end of each washing is transferred into prelabelled tubes. Couple more washing steps were applied as 30 minutes of rotation with fresh buffer at each time and, non-specific phages are expected to be washed off from the sapphire surface. Elution is applied by using different pH buffers, change the medium conditions and, let the sapphire be exposed to harsh chemical environment in order to get strongly bound phages on the surface.

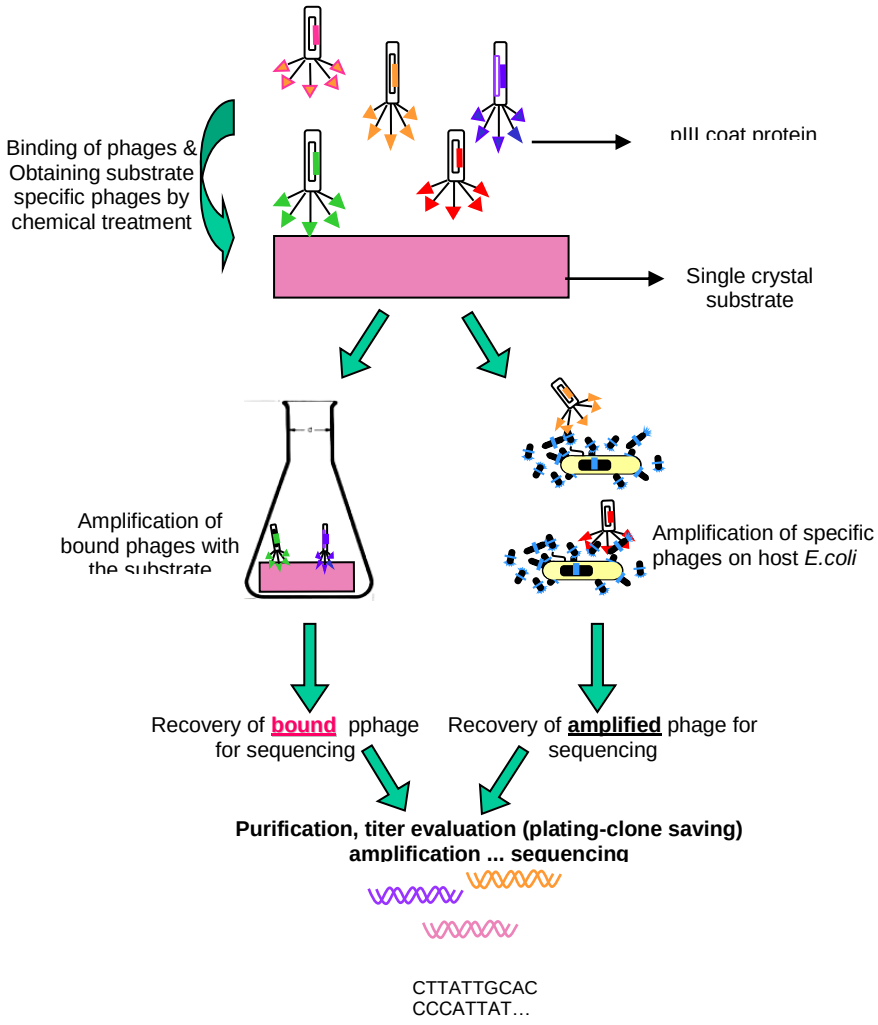


Figure 2.6. Modified biopanning procedure. Biopanning procedure (right part) is accompanied by, modified procedure of bound phage recovery (left part).

A new approach, recovery of the bound phage, is also introduced in order to obtain any possibly remaining binding peptide from the sapphire surface which was not recovered by chemical treatment. Schematic display of a modified round is demonstrated in Figure 2.5.

Elution

In order to begin elution, sterile 50 ml. LB medium in 250 ml. erlenmeyer flasks is required for each step.

To each erlenmeyer flask, 100µl of 1M $MgCl_2$, 50µl of tetracycline and 125 µl *E. coli* from overnight culture is added. After inoculation, the culture is incubated for 30 –40 min. (~2-3 doubling time) in orbital shaker at 37ºC, at 200 rpm. Cultures corresponding to elutions need to be inoculated in 30 minute intervals.

After elution step, 4.5 hours of incubation, amplified phages were purified.

Purification of the Amplified Phage Titers

Purified eluted and bound phage clones are saved for sequencing. Since the first round would not give specific phages to any given sample, the clones are saved from second, third and fourth round.

Plating

Plating is done for titer estimation for each round. For plating, dilutions are done in deep well ELISA plates. Top agar is melted in microwave oven and let it cool down to 40-50 ºC. Each well is filled with 180 µl of 0.02 % PC Buffer. 20 µl of phage is added to the first well and mixed on orbital shaker for 30 sec. For each dilution, 20 µl of diluted phage is taken from the previous well and added to the next one and mixed. 180 µl of *E. coli* overnight culture is put into the last well, 20 µl of previously diluted phage is added and mixed. For plating onto the small petri dishes, 500 µl of top agar is mixed with the *E. coli*-phage mixture by pipetting in the well and, poured onto the petri dish. After waiting for couple minutes for the mixture to be frozen, the petri dish is inverted and incubated at 37 ºC for overnight. For big petri dish platings, top agar amount at the last step is increased up to 1.5 ml.

Saving Clones for Sequencing

After estimation of titers by plating on small plates, all of the titers were plated on the big plates in order to be saved for sequencing. For each eluted phage plates, 24 individual clones were picked and stored in glycerol stocks.

Preparation of Storage Plates for Frozen Culture Stocks

The clones that are selected from petri dishes are stored in deep 96 well ELISA plates.

1) Each well on the ELISA plate is filled with 200 µl of 0.02 % PC Buffer.

2) Each blue clone (plaque) of phage on the petri dish is picked and put into individual wells on the plate.

3) Plate containing clones are placed into incubator at 60 ºC for 45 min. The plates can be stored at +4 ºC, until used for frozen cultures.

4) 80% of glycerol solution is prepared and autoclaved.

5) Two sets of new plates (one for –20 ºC and one for –80 ºC) are labeled.

6) In order to keep overall glycerol concentration as 50%, 60 µl of 80 % glycerol solution is put into the labelled plates.

7) 50 µl of clones from storage plate is added into the glycerol containing plates.

8) The plates are sealed by caps and placed into –20 ºC and –80 ºC freezers.

XMYE1* : Number of screening, material used, number of rounds, elution and step number.

2.4.2. Sequencing

In order to get sequences of selected sapphire (110) and (001) specific peptides, a series of applications are applied.

Growing up phage

2 ml of LB solution containing 4µl of 1M $MgCl_2$, 2µl of tetracycline, 5 µl of *E. coli* overnight culture is incubated for 30-40 min. 30-50µl of phage from frozen cultures are inoculated and incubated at orbital shaker at 200 rpm., 37 ºC for 4.5 hours.

Isolation of ssDNA of M13

For isolation of ssDNA of phage M13, QIAprep® Spin M13 Kit is purchased from QIAGEN (QIAGEN, 27704). QIAprep M13 kit utilizes a silica-gel-based membrane, under conditions which hade been optimized to promote the binding of ssDNA. The isolation procedure is applied as suggested by the company and demonstrated in Figure 2.6. Detailed explanation about the procedure is available in Appendix A.10.

QIAPrep M13 kit would allow the purification of 2 ml phage supernatant, giving the yield of 5µg of ss-M13 DNA with the concentration of 87 ng/µl. The yields from different M13 clones might vary due to differences in phage replication rates and titer.

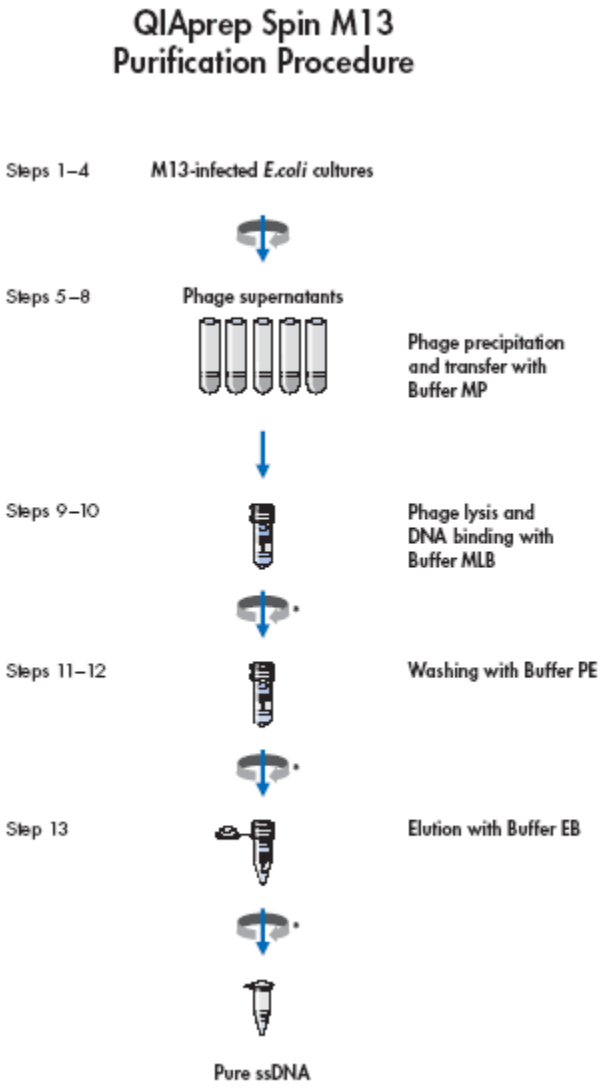


Figure 2.7. The QIAprep M13 Purification protocol by QIAGEN [73].

Determination of ssDNA Quantity by Fluorescence Spectrophotometer

DNA quantification of purified ss-M13 DNA is obtained by *SAFIRE* ELISA Microplate Reader. Quantitation is accomplished by incorporating a trace amount of a fluorogenic dye that exhibit fluorescence when bound to ssDNA.

For this purpose, *Oli*Green ssDNA Quantitation Reagent Kit is purchased (Molecular Probes, 011492). OliGreen is a fluorogenic dye that exhibits fluorescence when bound to ssDNA. Quantification as little as 100 pg/µl with a standard spectro fluorometer can be enhanced up to 1ng/µl with fluorescence microplate reader.

The kit includes;

- *Oli*Green ssDNA qantitation ragent, 1 ml. in anhydrous dimethyl-sulfoxide, DMSO (stored at –20 ºC, light sensitive). It is an ultrasensitive fluorescent nucleic acid stain for quantitating oligonucleotides and ssDNA in solution.
- 20XTE (Tris-EDTA) buffer 25 ml. of 200 mM Tris-HCl and 20 mM EDTA, pH 7.5 (stored at +4 to +24 ºC).
- Oligonucleotide standard, 1 ml. of 100 µg/ml solution in TE (stored at –20 ºC)

ssDNA quantitation procedure is as follows;

1. *Oli*Green reagent is thawed.

2. 1XTE Assay Buffer (working solution) is prepared by diluting the concentrated 20XTE with Dnase-free water. The assay buffer amount is calculated as 100 µl for standard and 200 µl for each sample.

3. Standard DNA solution is prepared by adding 4 µl oligonucleotide standard to 96 µl of 1XTE assay buffer. 100 µl of *Oli*Green reagent solution is added before reading fluorescence. The final concentration of standard DNA is 2 µg/ml.

4. *Oli*Green reagent solution is prepared immediately before the fluorescent reading, in a plastic tube wrapped with aluminum foil. The concentrated *Oli*Green reagent solution is diluted 300 folds with 1XTE assay buffer. Total volume of the *Oli*Green reagent solution is calculated as 200 µl for blank, 100 µl for standard and 198 µl for each sample.

5. On ELISA plate, 200 µl of *Oli*Green reagent solution is applied into the first well as blank.

6. 100 µl of *Oli*Green and 100 µl of standard solution is applied to the second well as standard.

7. For each sample 198 µl of *Oli*Green and 2 µl of ssDNA sample is applied to each well.

8. The plate is inserted into *SAFIRE* ELISA Microplate Reader and fluorescence intensity (FI) is measured.

9. TECAN *Magellan 3* is run.

10. For *Oli*Green reagent, excitation and emission wavelengths are given as 480 nm and 520 nm.

11. The plate is shaked for 15 sec. before fluorescence reading.

12. After obtaining raw FI data, blank is subtracted from standard and each sample. The concentration calculations are done proportional to standard.

Amplification of ssDNA by PCR:

For amplification of ssDNA, ABI Prism Big Dye® Terminator v1.1 Cycle Sequencing kit is purchased (Applied Biosystems, 4337450).

The primers are;

-28 gIII sequencing primer (recommended for manual dideoxy sequencing with ^{35}S or ^{33}P)

5' - ^{OH}GTA TGG GAT TTT GCT AAA CAA C – 3'

-96 gIII sequencing primer (recommended for automated sequencing with dye-labeled dideoxynucleotides)

5' -^{OH} CCC TCA TAG TTA GCG TAA CG – 3'

4 µl of Big Dye® Terminator reaction mix, 3.2 µl of primer, 2 µl of ssDNA and 0.8 µl of dH_2O is mixed. 25 sequencing cycles is run. Each cycle would consist of;

1. Hot start for 2 min. at 96 ºC
2. Rapid thermal ramp to 96 ºC (rapid thermal ramp: 1 ºC/sec)
3. 96 ºC for 30 sec.
4. Rapid thermal ramp to 50 ºC
5. 50 ºC for 15 sec.
6. Rapid thermal ramp to 60 ºC
7. 60 ºC for 4 min.
8. 25 cycles are run from step 2 to 7
9. Rapid thermal ramp to 4 ºC and hold for purification.

Purification of the PCR product

PCR product is purified by column cleaning, in order to remove unincorporated dye. For this purpose, Sephadex G-50 solution is prepared in the ratio of 1 g. of Sephadex G-50 for 15 ml. of dH_2O. Prepared G-50 should be hydrated at least for 4 hours.

For each sample 350 µl of hydrated G-50 solution is applied to 96 well plate and centrifugated at 2300rpm for 5 min., for the columns to be packed. Residual water at collection plate is removed. PCR products are applied onto the columns and, centrifugated

at 2300rpm for 5 min. Purified DNA samples were transferred to clean eppendorfs and, vacuum dried without heat.

Sequencing

The sequencing studies are applied at University of Washington, Biochemistry Department, with Applied Biosystems 3730 sequencer.

2.5. Data analysis

- Relative Abundance of each amino acid is calculated according to observed frequencies given by New England BioLabs (www.neb.com).
- Positional analysis of each sequence is obtained by EMBOSS *PEPINFO* (http://ebi.ac.uk/emboss/pepinfo) tool.
- pI, MW calculations and other physicochemical properties are determined by ExPASy Molecular Biology Server (http://au.expasy.org)
- Sequence alignment analysis are determined by ExPASy –clustalw alignment tool (http://au.expasy.org)

2.5.1. Fluorescence Microscopy (FM)

Fluorescence microscopy studies were performed in order to evaluate binding characteristics of peptides selected for single crystal sapphire (110) and (001). The selected phage samples with determined peptide sequence is immunolabeled with fluorescent dye Alexa Fluor 488. For FM studies, sapphire samples are ground in order to obtain sapphire powder which would increase the interaction between sapphire and each phage clone. The powder is cleaned as mentioned 2.1.2. *ii*. The immunolabelling procedure is summarized in Figure 2.8.

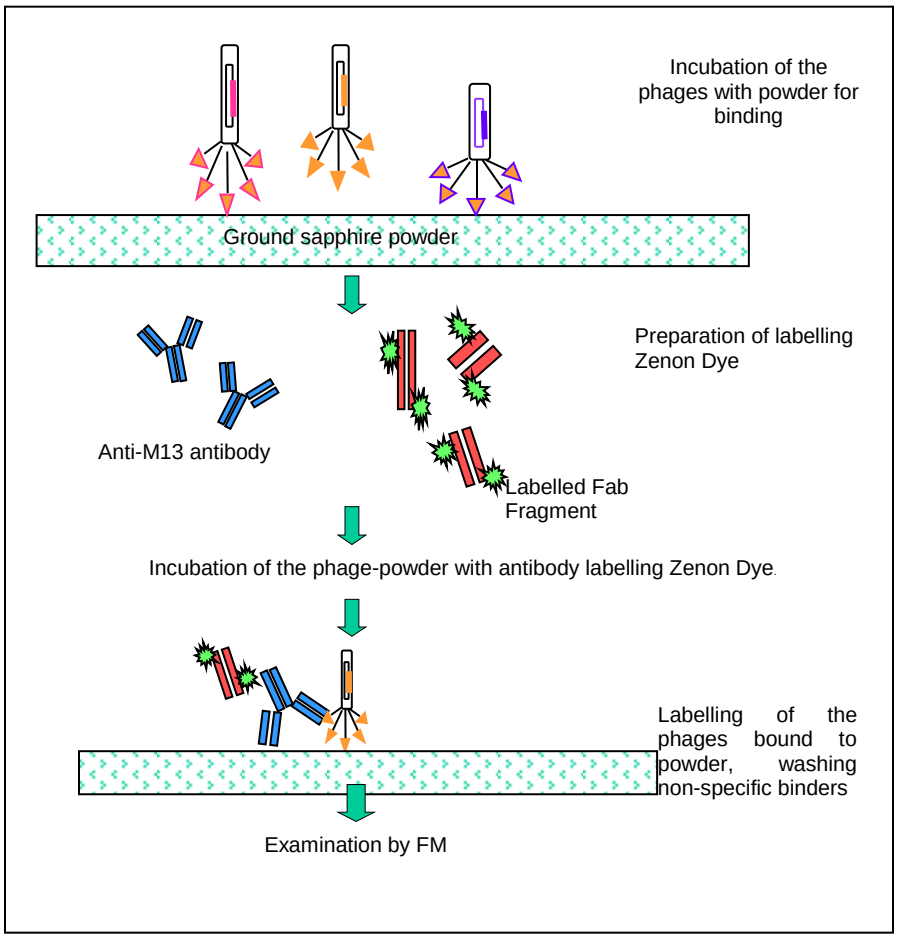


Fig. 2.8. Immunolabelling procedure of phage clones to be analysed by FM.

The immunolabelling procedure can be followed as;

1. 990 µl of 0.02 % PC Buffer is applied onto cleaned and vacuum dried powder and, 10 µl of phage clone is added.

2. The solution containing phage is incubated at rotation for 15 hours.

3. The sample is centrifuged for 1.5 min. at 500g and, supernatant is removed.

4. The remaining powder-phage precipitate is washed with 1.00 ml. of 0.1 % PC Buffer by rotating for 15 min.

5. At the same time, primary antibody, fluorescent dye Alexa Fluor 488 is conjugated with anti-mouse secondary antibody IgG at the ratio of 5:1 in an eppendorf, wrapped with aluminum foil for 30 - 45 minutes.

6. The solution is centrifuged for 1.5 min. at 500g and, supernatant is removed.

7. 997.5 µl of PC Buffer (no detergent) and 2.5 µl of fluorescent dye is applied, wrapped with aluminum foil and rotated for 30minutes.

8. The powder solution is centrifuged and supernatant is removed.

10 µl of PC Buffer is added on powder solution containing fluorescent labeled powder is put on a microscope slide, covered with 18 mm cover slip and studied under fluorescent microscope, Nikon Eclipse TE2000-U using Methamorph software.

2.5.2. Atomic Force Microscopy (AFM)

A.F.M. studies performed in order to, visualize binding of phages containing selected peptides for sapphire (110) and (001). The samples which were to be analyzed were cleaned with the same cleaning procedure, applied before screens. Ultrasonication with methanol-acetone, isopropanol, PC buffer and water is applied at least for 15 min. for each ultrasonication process. The samples are then incubated with appropriate amount of buffer and phage for 15 hours by rotation. After incubation, samples are washed with water added dropwise, then, air dried. Nanoscope III/Extended MMAFM (Digital Instrument Inc.) is used for the analysis.

2.6. Laboratory Equipment

Orbital shaker : New Brunswick Scientific – Benchtop Gyrotory® Water Bath Shaker *innova* 3100 (25-400 rpm; 5 – 100°C)

ddH_2O system : MilliQ Water

Rotator / Rocker : Barnstead International, Iowa – Labquake rotator

Shaker / Platform Mixer : Labline Instruments Inc., Illinois

Vacuum dryer: Brinkmann Instruments - Eppendorf®*vacufuge*® Concentrator 5301 (Heatings at 30°C, 45°C and 60°C)

Centrifuges : Kendro Laboratory Products – Sorvall® RC-5B Plus Superspeed Refrigerated Centrifuge (Fixed Angle, 500 - 21,000 rpm, -20°C – 40°C)

Brinkmann Instruments Inc. - Eppendorf® Micro Centrifuge 5415D (13,200 rpm ; 16,110 x g)

Freezers : (-80ºC) New Brunswick Scientific – Ultralow Temperature Freezer U535

Fluorescent Spectrophotometer : TECAN-*SAFIRE,* ELISA Microplate Reader operating *Magellan3*

Aromic Force Microscope (AFM) : Nanoscope III/Extended MMAFM by Digital Instrument Inc.

Fluorescent Microscope : Nikon Eclipse TE2000-U using software Methamorph.

Thermal Cycler : Brinkmann Instruments – Eppendorf Mastercycler ® gradient

Sterile cabin : ISC Bioexpress - Air Clean 600 Workstation

3. RESULTS AND DISCUSSION

Single crystal sapphire has a unique combination of physical, chemical and optical properties allowing it to withstand high temperatures, high pressure, the shock and, water or sand erosion, therefore it is an excellent material for wide array of applications. These applications may include chemical, biological, and optical sensors; spectroscopic enhancers and phase shifters; nanoelectronics; and quantum structures [13]. The goal of this work is, to select peptides specific to two different crystallographic structure of sapphire surface by use of phage libraries based on combinatorial biology based methods. Consequently utilize the most beneficial design features with the help of probable precursor peptides to improve the processing of synthetic sapphire and its properties.

3.1. Adaptation of Phage Display Protocols for selection of single crystal sapphire binders

3.1.1.Selection of (110) and (001) specific peptides

Sapphire has a hexagonal / rhombohedral structure and a number of properties are dependent on the orientation of the crystal such as C-plane sapphire is useful for infrared detector applications.

Single crystal sapphire (110) sample which has a polar surface, is incubated with 1000 µl of 0.01 % PC buffer containing 10 µl of phage library. From second round and on detergent concentration is gradually increased, increasing binding stringency and, bound phages are recovered by using elution buffer.

Single crystal sapphire [001] sample which has a non-polar surface, is incubated with 1000 µl of 0.01 % PC buffer containing 10 µl of phage library. From second round and on detergent concentration is gradually increased, increasing binding stringency and, bound phages are recovered by using elution buffer.

3.1.2. Novel approach: Bound phage recovery

Phage Display C7C Library is used in order to select and recover the single crystal sapphire (110) and (001) crystallographic surfaces specific peptides. During the biopanning procedure, there is a risk of leaving behind the bound phage on the surface of the inorganic material. Therefore, a novel approach was developed for the recovery of the phages that bound to sapphire surfaces and would not be recovered by chemical elution. Simple amplification protocol is developed by use of the inorganic surface to which the chemical elution is performed. Here, the single crystal sample is put into the LB medium containing *E.coli,* $MgCl_2$ and, incubated in erlenmeyer flask for amplification. The purification procedure is applied in the same way as for chemically eluted phages.

3.2. Selected (110) and (001) sapphire specific peptides

(110) and (001) crystallographic surface specific sapphire peptides are given Table 3.1 and 3.2, respectively. Some of the sequences were selected more than once. Interestingly some of these sequences were obtained both by elution and bound phage recovery method. Each color would indicate the group of which, each amino acid is included according to its chemical behaviour and characteristics. Red would indicate hydrophobic (**A, G, I, L, M, P, V, W, F**); pink would indicate basic (**K, R**); blue would indicate acidic (**D, E**); light green would indicate hydroxyl containing (**S, T, Y**) and, dark green would indicate amine containing, basic (**H, N, Q, C**) character of each individual amino acid.

Table 3.1. List of single crystal sapphire (110) specific peptides.

Code	Sequence	Repeat	Recovery Method	Binding Characteristics[a]
AO 13	T P L N P G T	1	Bound	S
AO 28	T E T K R L Q	1	Eluted	S
AO 351	Q P Y N K L T	1	Eluted	S
AO 362	H A P F P M L	2	Eluted & Bound	S
AO 370	S P T G I T S	1	Eluted	S
AO 4	E T Q N R P M	1	Eluted	S/M
AO 5	S Y Q F S H H	1	Eluted	S/M
AO 382	Q Y N H S A N	2	Bound	S/M
AO 385	E A K P R F H	6	Bound	M
AO 1	P N M R A I S	1	Eluted	M/W
AO 2	M N H I N S L	1	Eluted	W
AO 14	T L P N H T P	1	Bound	W
AO 26	V P T R L D P	1	Eluted	W
AO 34	M L M P W T G	21	Eluted & Bound	W
AO 38	Q T T P L R H	1	Bound	W
AO 352	S P H G L H F	1	Eluted	W
AO 364	D S K L D R I	7	Bound	W
AO 366	P P S P S L P	1	Bound	W
AO 381	L T S T P F F	2	Eluted	W
AO 350	L S N N S T N	3	Eluted & Bound	NB
AO 378	D R N T T L L	4	Eluted	NB

Table 3.2. List of single crystal sapphire (001) specific peptides.

Code	Sequence	Repeat	Recovery Method	Binding Characteristics[a]
AO 86	T P S T T R Q	2	Eluted	S
AO 333	E L R P T V A	7	Eluted & Bound	M/W
AO 343	R T T H Q A Y	2	Eluted & Bound	M/W
AO 346	V G A V P S K	12	Eluted & Bound	M/W
AO 117	H L H E P W L	1	Bound	W
AO 345	S V T Q N K Y	3	Eluted	W
AO 347	Q M S N A L V	3	Eluted & Bound	W

[a] Binding characteristics of each peptide clone is obtained according to FM studies. S would refer to strong; M would refer to moderate; W would refer to weak and NB would refer to non-binding characteristics of each individual clone

3.3.Analysis of selected sequences based on their amino acids

3.3.1.Relative abundance analysis of selected sequences

The relative abundance study, would give information on selectivity values for each amino acid at a particular sequencing cycle in the recovered peptides to that of each amino acid in the original peptide library mixture at the same position.

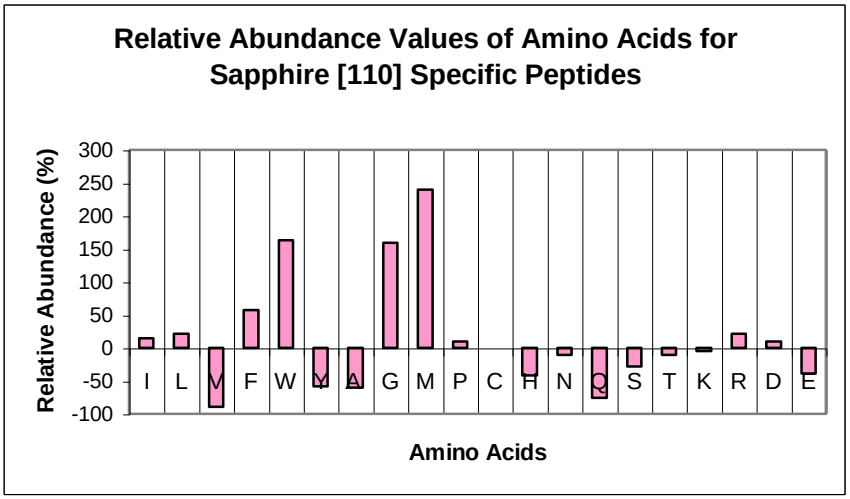


Fig.3.1. Relative abundance analysis of (110) surface specific peptides for single crystal sapphire

For relative abundance analysis of single crystal sapphire [110] surface specific peptides, methionine (M), glycine (G), tryptophan (W) and phenylalanine (F) are the overexpressed amino acids; valine (V), glutamine (Q), tyrosine (Y), alanine (A), histidine (H) and, glutamic acid (E) are the underexpressed amino acids.

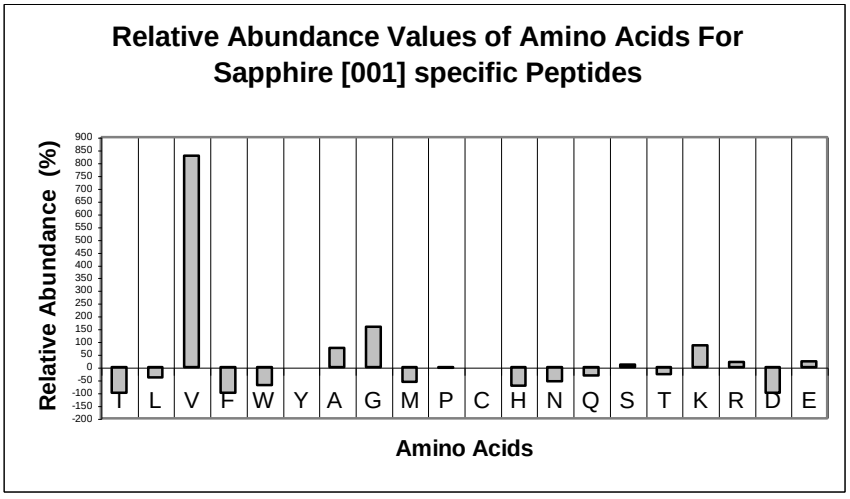


Fig.3.2. Relative abundance analysis of (001) surface specific peptides for single crystal sapphire

For relative abundance analysis of single crystal sapphire (001) surface specific peptides, valine (V), glycine (G), lysine (K) and alanine (A) are the overexpressed amino acids; isoleucine (I), phenylalanine (F), aspartic acid (D), tryptophane (W) and, histidine (H) are the underexpressed amino acids. The drastic overexpression can clearly be seen as valine, which is a hydrophobic and essential amino acid.

Overexpression of an amino acid could be a commented as a parameters referring to specificity to the selected inorganic surface. Interestingly a different relative abundance profiles were obtained for different sapphire crystallographic surfaces. Still without performing a detailed analysis on the binding specificity of the selected sequences, it is too early to draw a conclusion on the binding domain.

3.3.2. ClustalW Alignment Analysis of selected sequences

An important contribution of molecular biology studies was, the discovery of similar genes were conserved across widely divergent species, often performing identical or similar functions. Diversity might result from sometimes these genes being mutated and, their function altered according to the natural selection. At this point, multiple alignment analysis would provide an extreme help.

Multiple alignment, whether made of DNA or protein sequences can yield much more information than analysis of a single or even two sequence. When dealing with a new protein, with unknown function, the presence of several domains similar to domains in other “known” sequences, can imply a similar structure or function.
In proteins, maintaining their function generally requires a specific three dimensional (3D) structure. At this point, protein multiple alignments can give some information about 3D structure. The aligning procedure includes; pairwise alignment, database search and multiple alignment steps.
The multiple alignment program that is used for single crystal sapphire (110) and (001) was ClustalW produced by Julie D. Thompson and Toby Gibson from EMBL, Germany and Desmond Higgins from EBI, Cambridge, UK.

Clustalw is cited as improving the sensitivity of progressive multiple sequence alignment through sequence weighting, positions specific gap penalties and weight matrix choice [74]. It would create multiple alignments by manipulating existing alignments and create phylogenic trees.

ClustalW algorithm consists of 3 steps

1-Pairwise alignments are performed between all sequences in the compared group. Alignments scores are then used to build a distance matrix in calculating the distance matrix, the program takes into account the divergence of the sequences
2-A guide (phylogenetic) tree is created from the distance matrix using the neighbour-joining method. This guide tree has branches of different lenghts. Their length is proportional to the estimated divergence along each branch.

3-Progressive alignment of the sequences is done following the branch order of the guide tree. The sequences are aligned from the tips to the root. The alignment of the sequences is guided by the phylogenetic relationships indicated by the tree.
It would calculate the gaps in a novel way, designed to place them between conserved domains.

ClustalW calculates the genetic distances as:

$$D = \frac{\text{Number of mismatches in the alignment}}{\text{Number of matches in the alignment}}$$

The ClustalW analysis is run by EMBL-EBI, ClustalW tool. The alignment results are demonstrated at tables below.

In one of the sequences (DSKLDRI), the observed gap between D and S showed that an alignment could not be obtained for the whole sequence during the alignment process. The program itself divided the sequence into two section where the better alignment could have been obtained in terms of similar functional groups.

Table 3. 3. ClustalW analysis of amino acids for single crystal sapphire (110) specific peptides

					S	Y	Q	F	S	H	H	
		S	P	H	G	L	H	F				
		H	A	P	F	P	M	L				
			P	P	S	P	S	L	P			
	L	S	N	N	S	T	N					
	Q	Y	N	H	S	A	N					
		M	N	H	I	N	S	L				
T	L	P	N	H	T	P						
		D				S	K	L	D	R	I	
		D	R	N	T	T	L	L				
				V	P	T	R	L	D	P		
						T	P	L	N	P	G	T
				Q	T	T	P	L	R	H		
			P	N	M	R	A	I	S			
			Q	P	Y	N	K	L	T			
		L	T	S	T	P	F	F				
			E	A	K	P	R	F	H			
			T	E	T	K	R	L	Q			
		E	T	Q	N	R	P	M				
				S	P	T	G	I	T	S		
				M	L	M	P	W	T	G		

Table 3.4. ClustalW analysis of amino acids for single crystal sapphire (001) specific peptides

T	P	S	T	T	R	Q		
		R	T	T	H	Q	A	Y
		S	V	T	Q	N	K	Y
E	L	R	P	T	V	A		
	V	G	A	V	P	S	K	
Q	M	S	N	A	L	V		
H	L	H	E	P	W	L		

3.3.3. Amino Acid Analysis in the Selected Sequences

Different peptide sequences were obtained as a result of the phage display selection protocol. Among the 20 amino acids, the selected ones are profiled and cumulative amino acids shown in Figure 3.3 and 3.4 for both crystallographic surfaces.

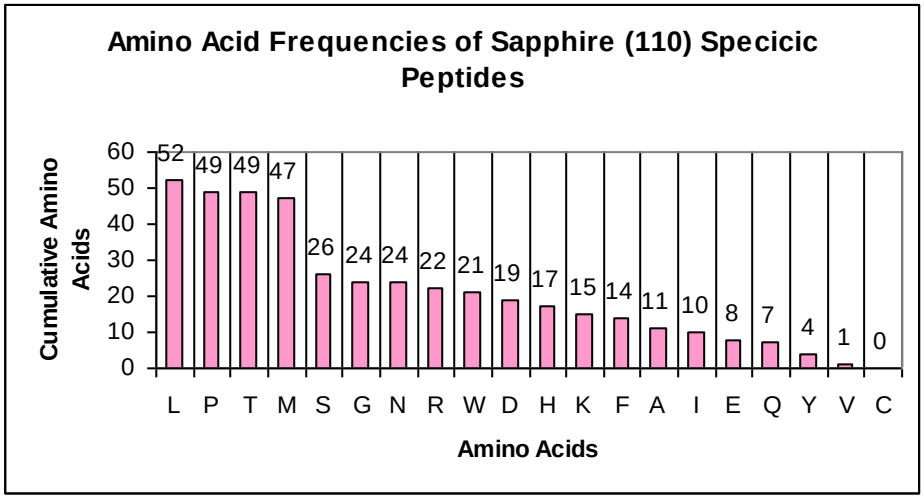


Figure 3.3. Amino acid frequency distribution of single crystal sapphire (110) specific peptides.

The amino acid distribution of single crystal sapphire (110) specific peptides are, leucine (L), proline (P), threonine (T) and methionine (M). Except threonine, 3 other amino acids are hydrophobic amino acids. Aliphatic threonine has a hydroxyl group. Methionine is a sulfur containing amino acid.

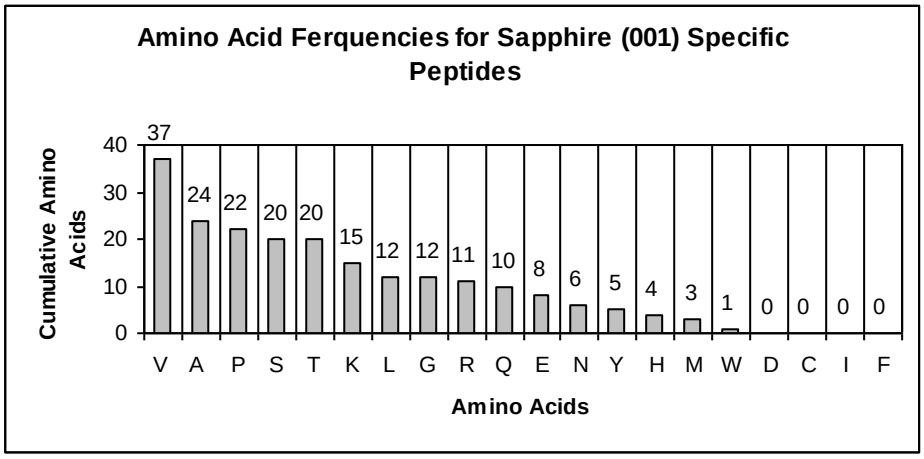


Figure 3.4. Amino acid frequency distribution of single crystal sapphire (001) specific peptides.

The amino acid distribution of single crystal sapphire (001) specific peptides are, valine (V), alanine (A), proline (P), serine (S) and threonine (T). Other than serine and, threonine, other 3 amino acids are hydrophobic amino acids. Serine and threonine has a hydroxyl group on their side chains.

3.3.4.Physicochemical analysis of the selected peptides

Physicochemical properties of the selected aminoacids are given in Table 3.5, and 3.6 for both crystallographic surfaces. In addition to their molecular weigths, pI, charge and hydrophilicities were presented. In terms of charge distribution, (110) surface specific peptides were found to be mostly having no charge, only a few of them carried +1 charge. Whereas in (001) surface specific peptides, charge carrying peptides were dominant, again mostly as +1 charge. Their pI values were mostly in the neutral range only a few of them showed basic behaviour.

Table 3.5. Physicochemical analysis of single crystal sapphire (110) specific peptides.

Code	Sequence	Repeat	MW[a]	pI[a]	Charge[b]	Hydrophilicity[c]
AO 385(B)	E A K P R F H	6	884.00	8,85	+1	5,5
AO 1(E)	P N M R A I S	1	787.90	10,18	+1	-0,1
AO 28(E)	T E T K R L Q	1	874.90	8,41	+1	6,6
AO 38(B)	Q T T P L R H	1	851.90	9,76	+1	0,1
AO 351(E)	Q P Y N K L T	1	862.90	8,59	+1	-1,1
AO 2(E)	M N H I N S L	1	827.90	6,49	0	-4,7
AO 4(E)	E T Q N R P M	1	874.90	6,10	0	5,5
AO 5(E)	S Y Q F S H H	1	904.90	6,66	0	-5,0
AO 13 (B)	T P L N P G T	1	698.70	5,19	0	-2,4
AO 14(B)	T L P N H T P	1	778.80	6,40	0	-2,9
AO 26(E)	V P T R L D P	1	796.90	5,81	0	2,3
AO 34 (E+B)	M L M P W T G	21	835.00	5,28	0	-8,2
AO 350(E+B)	L S N N S T N	3	748.70	5,52	0	-1,0
AO 352(E)	S P H G L H F	1	793.80	6,66	0	-5,0
AO 362(E+B)	H A P F P M L	2	812.00	6,74	0	-6,6
AO 364(B)	D S K L D R I	7	845.90	5,96	0	8,7
AO 366(B)	P P S P S L P	1	693.80	5,96	0	-1,2
AO 370(E)	S P T G I T S	1	661.70	5,24	0	-2,0
AO 378 (E)	D R N T T L L	4	831.90	5,84	0	1,8
AO 381(E)	L T S T P F F	2	811.90	5,52	0	-7,3
AO 382(B)	Q Y N H S A N	2	832.80	6,74	0	-2,4

Table 3.6. Physicochemical analysis of single crystal sapphire (001) specific peptides.

Code	Sequence	Repeat	MW[a]	pI[a]	Charge[b]	Hydrophilicity[c]
AO 346(E+B)	V G A V P S K	12	656,70	8,72	+1	-0,2
AO 345 (E)	S V T Q N K Y	3	838,90	8,31	+1	-0,5
AO 86 (E)	T P S T T R Q	2	789,80	9,41	+1	2,3
AO 343 (E+B)	R T T H Q A Y	2	875,90	8,75	+1	-0,9
AO 333 (E+B)	E L R P T V A	7	784,90	6,10	0	1,3
AO 347 (E+B)	Q M S N A L V	3	761,80	5,52	0	-4,4
AO 117 (B)	H L H E P W L	1	931,00	5,98	-1	-5

[a] MW of each individual peptide clone was obtained using the pI/MW calculator at ExPASy (Expert Protein Analysis System), proteomics server of the Swiss Institute of Bioinformatics (SIB) (http://us.expasy.org/)

[b] Charge of each individual peptide was calculated by subtracting the number of basic residues (R and K) from the number of acidic residues (D and E)

[c]Calculated using the hydrophilicity index of Hopp and Woops where a score of +1.5 corresponds to a very hydrophilic protein and a score of –1.5 to a very hydrophobic one.Ref: Hopp, T.P.; Wood, K.R., Proc. Natl. Acad. Sci. USA 1981, 78, 3824-3828

3.4. Characterization of selected peptides through FM analysis

Fluorescent microscopy experiments were performed on crashed sapphire surfaces to increase the surface area interaction between the selected peptide and the selected material. In fluorescent microscopy experiments, (110) and, (001) crystal

surface specific peptides were grouped under three catagories as strong, moderate and weak binders according to their binding affinity to Al_2O_3 (ground single crystal sapphire) powders. Binding experiments are performed as 15 hours of incubation of powder with selected specific peptides, washing non-specific binders and, labelling with fluorescent dye for 30 minutes.

Fluorescent microscopy images were observed by use of FITC filter. Excitation and emission wavelengths for fluorescent dye AlexaFluor 488.

3.4.1. FM images of (110) surface specific peptides

Fluorescent microscopy images for (110) specific peptides were grouped as strong, moderate and, poor binders and, shown as in figures 3.5, 3.7, 3.9, 3.11 and 3.13. In addition to binding experiments, cross specificity analysis were performed on the selected peptides by use of another inorganic surface. As an inorganic surface platinum powders were chosen and the selectivities of the sapphire binders towards the other surfaces were examined.

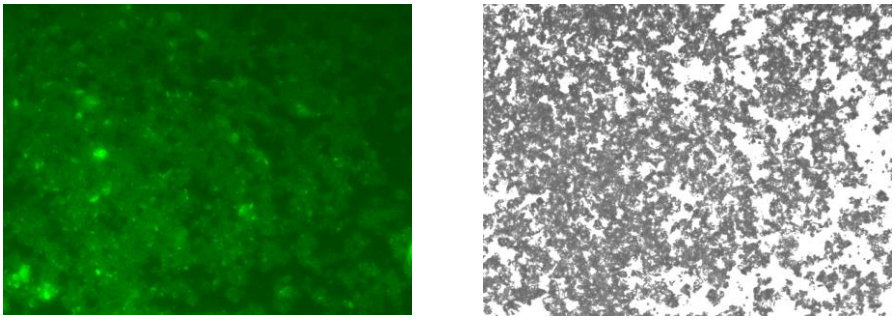


Fig.3.5. Strong binder for (110) surface, AO13-TPLNPGT

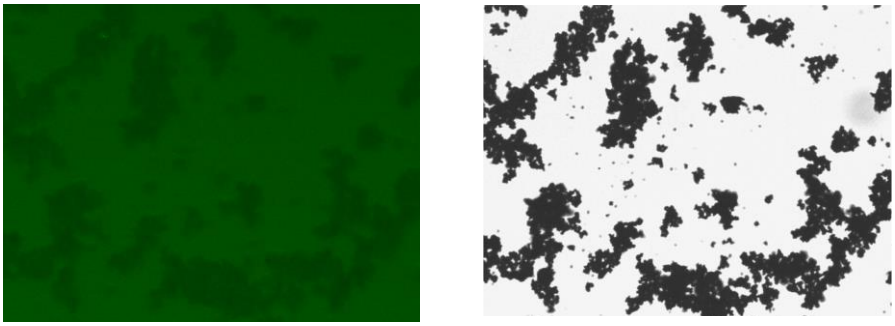


Fig.3.6. Pt control experiment for AO13-TPLNPGT

As can be seen from Figure 3.5., binding was obtained on the (110) sapphire surface by A013 -TPLNPGT. Moreover, no binding towards Pt surface as seen on

Figure 3.6 was obtained showing the specificity and the selectivity of the sequence towards Al_2O_3. Therefore this sequence was grouped in the strong binders.

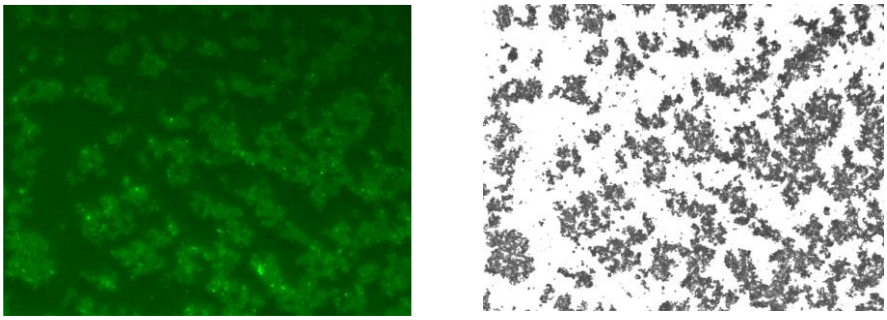


Fig 3.7. Moderate binder for (110) surface, AO4- ETQNPRM

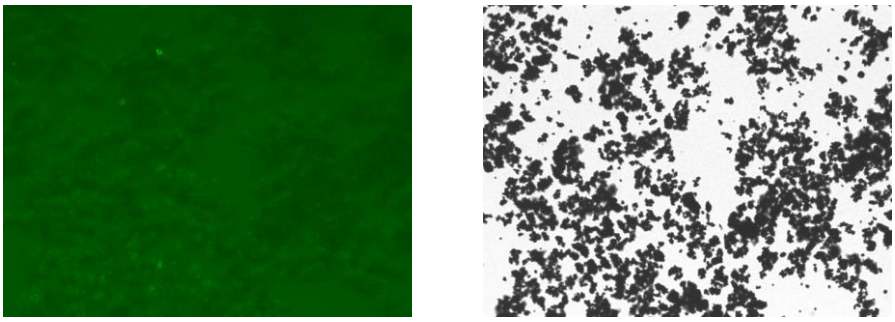


Fig 3.8. Pt control experiment for AO4- ETQNPRM

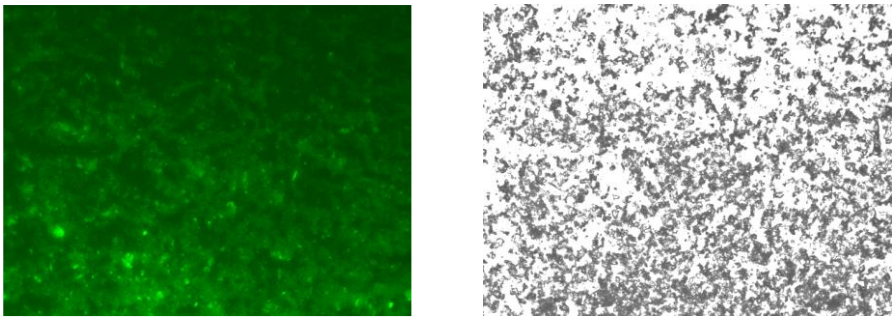


Fig. 3.9. Moderate binder for (110) surface, AO5- SYQFSHH

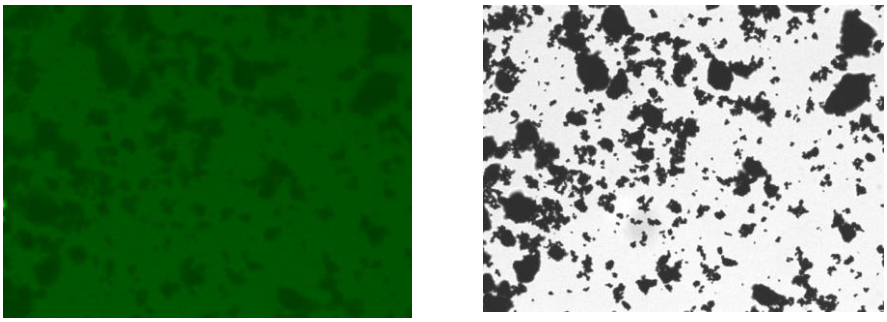


Fig. 3.10. Pt control experiment for AO5- SYQFSHH

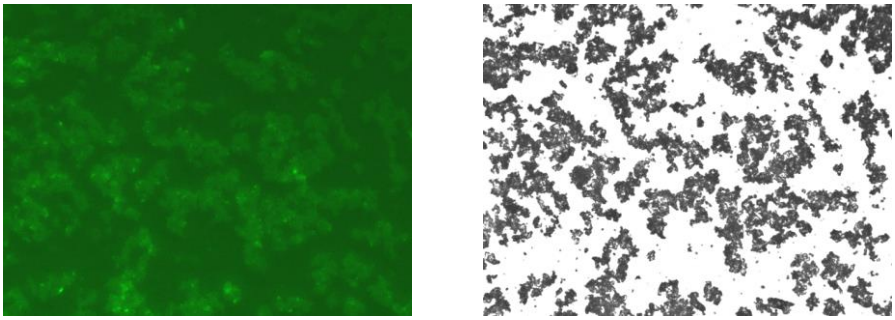


Fig. 3.11. Moderate binder for (110) surface, AO1-PNMRAIS

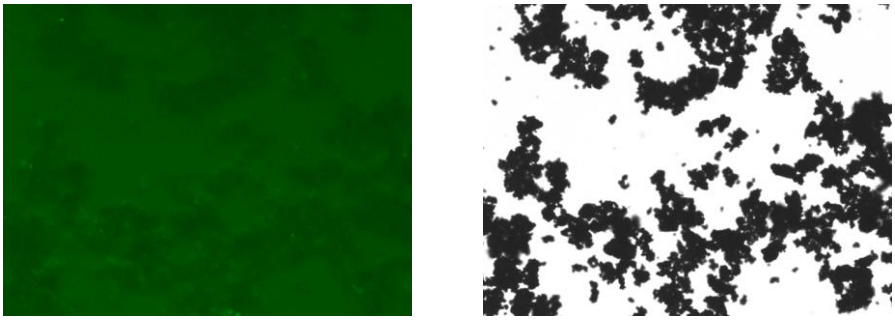


Fig. 3.12. Pt control experiment of AO1 PNMRAIS

As can be seen from Figures 3.7, 3.9, 3.11, relatively less amount of bindings were obtained on the (110) sapphire surface by sequences AO4, AO5., AO1. These sequences resulted in either no or minimum level of binding towards Pt surface (Figures 3.8, 3.10, 3.12). Therefore these sequences can be still considered as showing the specificity and the selectivity of the sequence towards Al_2O_3 but definetly having less affinity for sapphire surface. Therefore these sequences were grouped in the moderate binders. The differences in the affinities shows the importance of the necessity of characterization of selected sequences towards the inorganic surfaces. As expected, the results showed that there is a variation in their binding affinities.

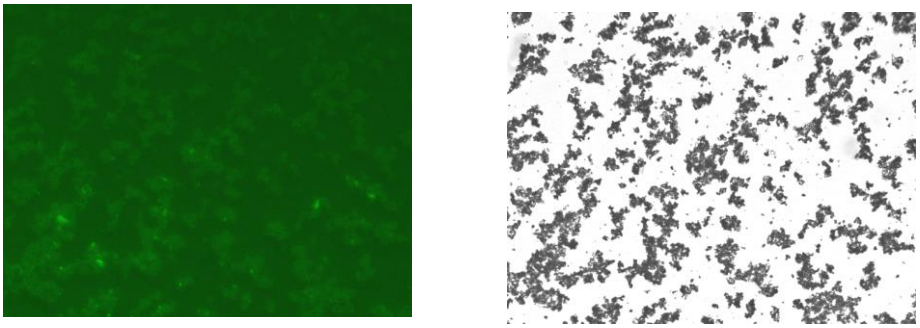


Fig 3.13 Weak binder for (110) surface, AO2-MNHINSL

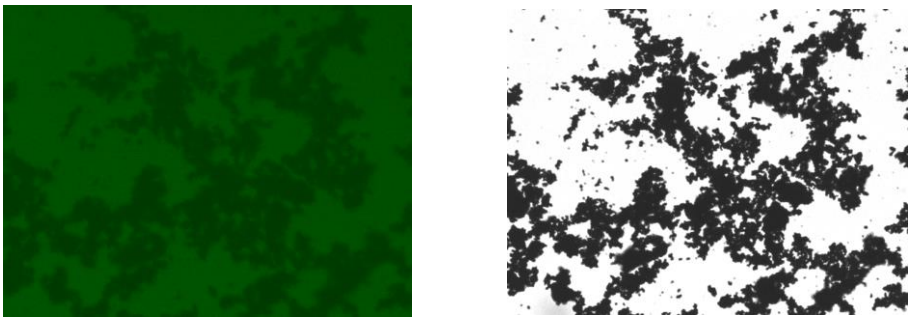


Fig 3.14. Pt control experiment for AO2 MNHINSL

As can be seen from Figure 3.13., this group showed the least affinity among the tested sequences but still no affinity was obtained towards the Pt surface in cross specificity experiments.

3.4.2. FM images of (001) surface specific peptides

Fluorescent microscopy images for (001) specific peptides were also grouped as strong, moderate and, poor binders and, shown as in figures 3.15, 3.16 and 3.17.

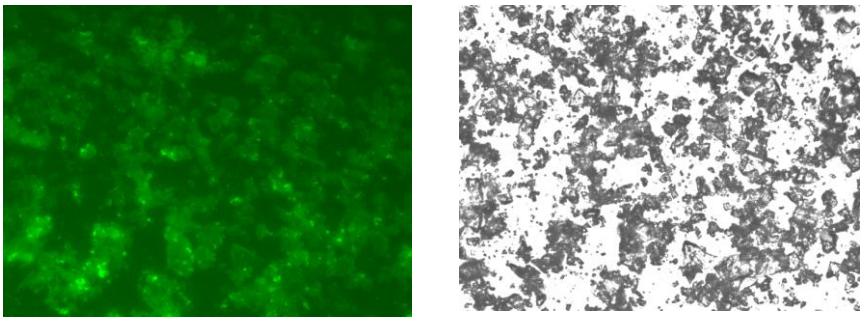


Fig.3.15. Strong binder for (001) surface, AO86- TPSTTRQ

As can be seen from Figure .3.15., a good amount of binding was obtained on the (001) sapphire surface by A086-TPSTTRQ.

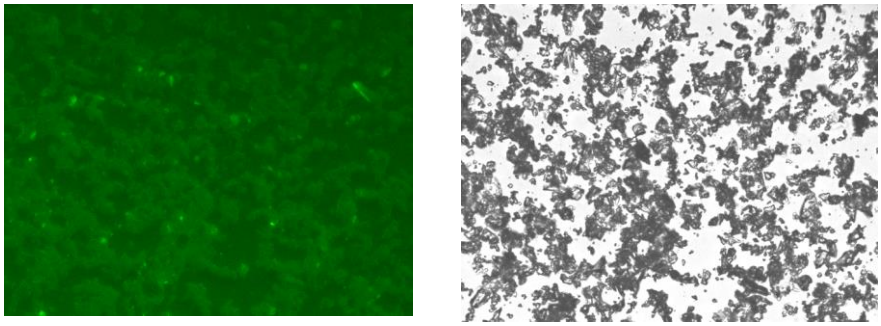


Fig.3.16. Moderate binder for (001) surface, AO346-VGAVPSK

As can be seen from Figure .3.16., relatively less amount of bindings were obtained on the (001) sapphire surface by sequence AO346.

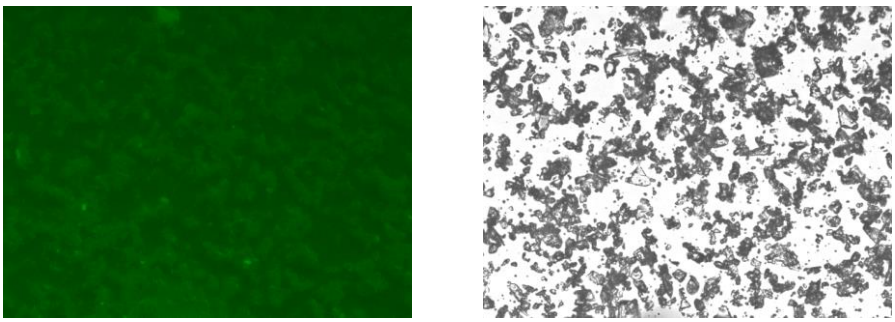


Fig 3.17. Weak binder for (001) surface, AO345- SVTQNKY

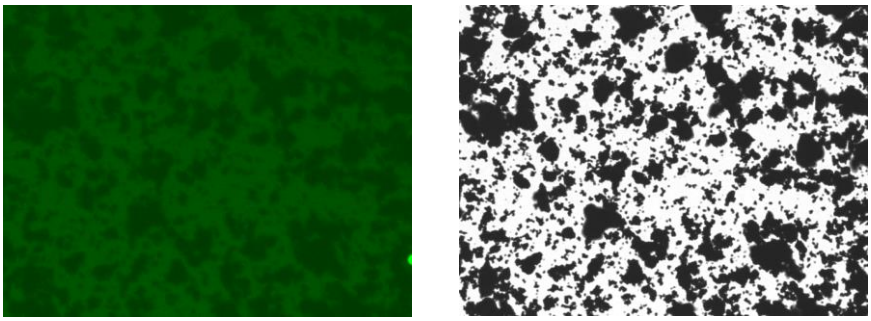


Fig 3.18. Pt control experiment for AO345- SVTQNKY

As can be seen from Figure .3.17., this group sequences showed the least affinity among the tested sequences but still no affinity was obtained towards the Pt surface in cross specificity experiments.

As a result of the F.M. analysis, phage display technology was shown to be very useful in selecting sapphire specific peptides with different degrees of affinity. Immunolabelling based techniques are shown to be very useful tools for the first stage characterization of the selected sequences. Through this method, it is possible to group the binders according their binding affinity.

3.4. AFM analysis of selected peptides

AFM images of selected peptides were analysed, in order to visualize the binding characteristics of selected peptides on single crystal sapphire and with real height information, get 3D images.

Single crystal sapphire samples were incubated with 10 µl of selected phages as individual clone for 15 hours, rinsed with water, air dried and analysed.

All AFM images are captured at tapping mode with cantilever's resonant vibration frequency in 100-350 mHz. The roughness value of the images are less than 2nm.

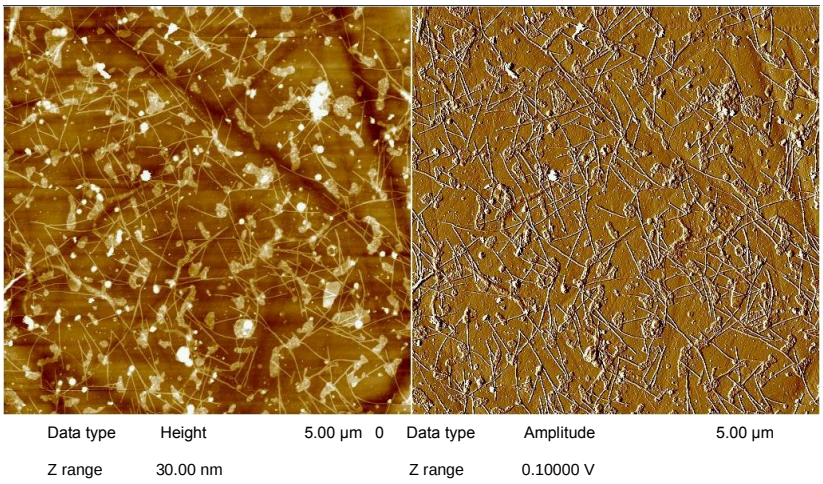


Fig.3.19. AFM analysis of strong binder AO351-QPYNKLT . Scan size is 5 microns.

AFM analysis given in Figure 3.19 for AO351-QPYNKLT. This sequence was grouped among the strong binders. Here, the AFM image of the sequence, shows a high coverage of the phage on the surface. AFM studies also allowed us approved the size of the phage molecules on the surface. Higher magnification of the same image was shown in Figure 3.20 , here phage molecules are easily seen.

One of the weak binders of (110) surface specific binders was also analysed by AFM. As can be seen in Figure 3.21, compared to AO351, surface coverage of the specific phage molecules is at very low level verifying the FM results.

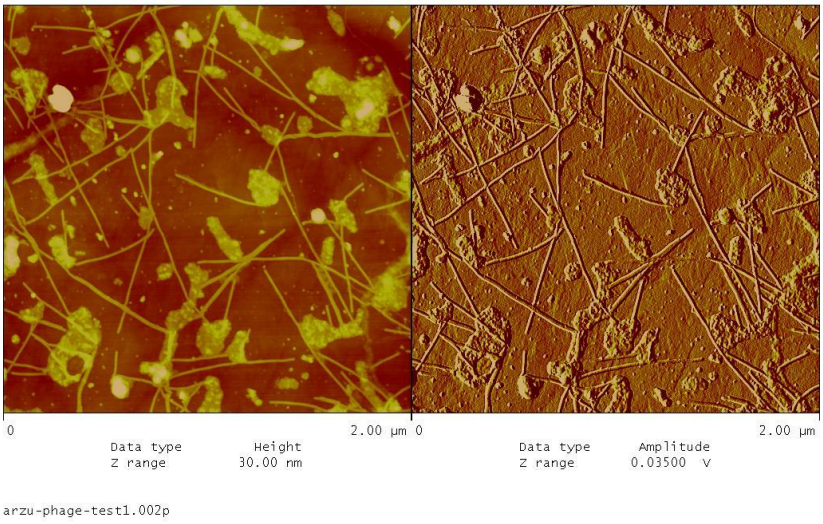


Fig.3.20. AFM image of strong binder AO351- QPYNKLT. Scan size is 2 microns.

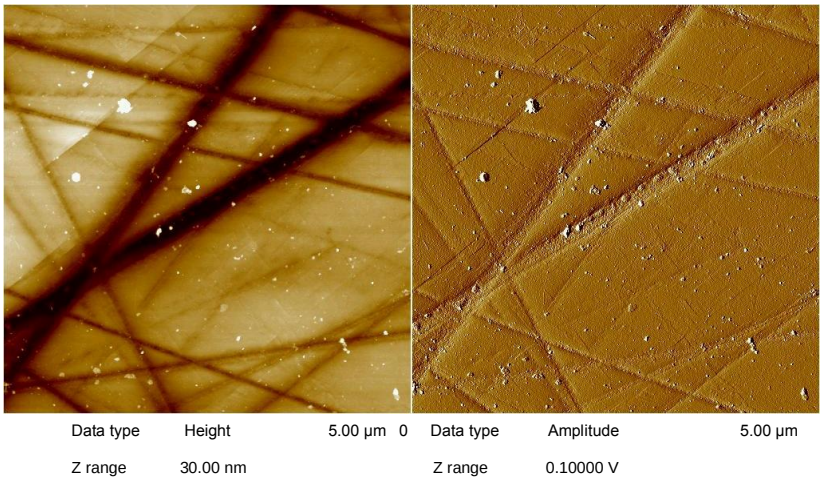


Fig.3.21. AFM image of weak binder, AO361-MLMPWTG. Scan size is 5 microns.

Both AFM and FM studies showed the importance of characterization of the selected sequences following the identification of inorganic specific peptides. By these tools, one can choose a peptide sequence for different purposes with the desired level of affinity and selectivity towards the screened surfaces.

3.5. Surface characteristics of single crystal sapphire (110) and (001)

Single crystal sapphire (Al_2O_3) has a and c values as, 4.759Å and 12.992Å. The two crystallographic orientations shown at Figures 3.22 and 3.24 are two selected sapphire samples for this study. Also XRD measurements were performed on the single crystalline sapphire surfaces.

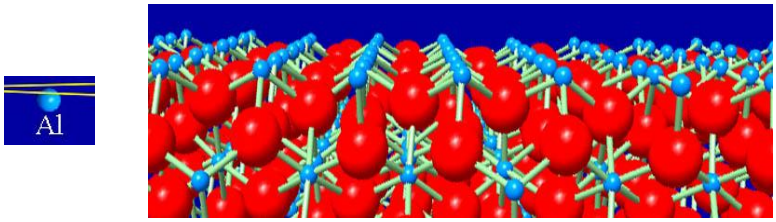


Fig.3.22. Crystallographic representation of single crystal sapphire (110) , showing atomic representations of Al and O atoms.

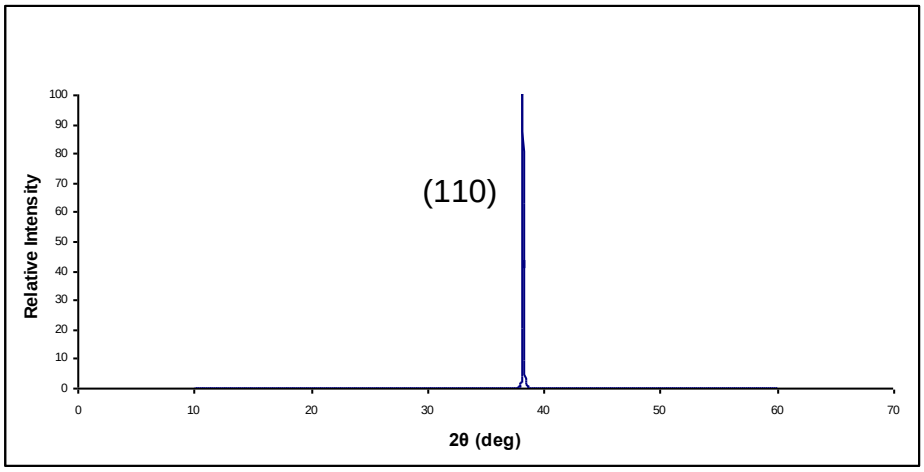


Fig.3.23. X-RD graph of sapphire (110).

In Figure 3.23, XRD graph of sapphire (001) is presented. The single peak shows the (110) single crystal sapphire surface. 2θ values of sapphire obtained from X-RD studies, are determined by comparing with 2θ values belonging to various crystals on JCPDS (Joint Committee on Powder Diffraction Standarts) cards.

On the other hand, single crystal (001) sapphire has the properties indicated as Figures 3.24, 3.25.

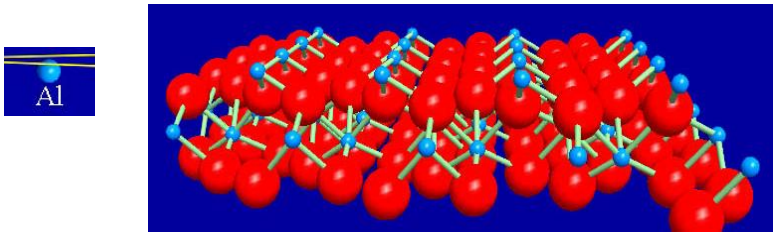


Fig. 3.24. Crystallographic representation of single crystal sapphire (001), showing atomic arrangements of Al and O atoms.

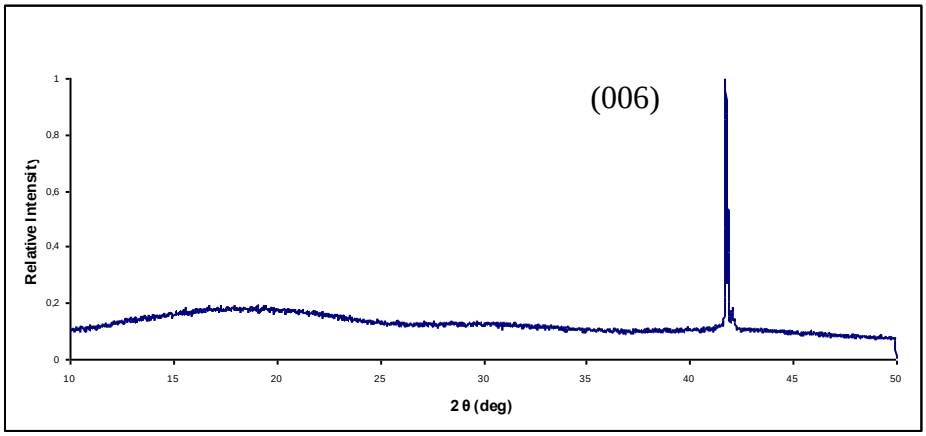


Fig.3.25. X-RD graph of sapphire (001).

In Figure 3.25, XRD graph of sapphire (001) is presented with a single crystal surface peak. 2θ values of sapphire obtained from X-RD studies, are determined by comparing with 2θ values belonging to various crystals on JCPDS (Joint Committee on Powder Diffraction Standarts) cards.

CONCLUSION

Concluding remarks are summarized as follows as a result of the study:

- Phage display technology was applied for the selection of different crystallographic surfaces of sapphire substrate.
- A novel approach during the biopanning procedure was developed as the recovery of bound phage. In the studies both eluted and bound phage inserts were identified.
- As a result of the F.M. analysis, phage display technology was shown to be very useful in selecting sapphire specific peptides with different degrees of affinity. Strong, moderate and weak binders were grouped and their cross specificities with control substrate, Pt, were performed to understand the existence of different degree of specificity. Immunolabelling based techniques are shown to be very useful tools for the first stage characterization of the selected sequences. Through this method, it is possible to group the binders according their binding affinity.
- In the different group of binders, both eluted and bound phage selected sequences were obtained. Studies showed not much difference between the elution method from the surface. Nevertheless, bound phage studies need to be carried out during the identification of inorganic surface specific binders.
- Both AFM and FM studies showed the importance of characterization of the selected sequences following the identification of inorganic specific peptides. By these tools, one can choose a peptide sequence for different purposes with the desired level of affinity and selectivity towards the screened surfaces.

APPENDIX

Appendix A.1.AMINO ACID DISTRIBUTION OF THE Ph.D.-C7C™ LIBRARY

Amino Acid	Codons	Expected frequency*	Observed ferquency
Arg	CGK, AGG	9,40%	4,3% (25/581)'
Leu	CTK, TTG	9,40%	9,6% (56/581)
Ser	TCK, AGT	9,40%	8,6% (50/581)
Ala	GCK	6,20%	6,5% (38/581)
Gly	GGK	6,20%	2,2% (13/581)
Pro	CCK	6,20%	10,7% (62/581)
Thr	ACK	6,20%	13,1% (76/581)
Gln	CAG, TAG''	6,20%	7,1% (41/581)
Val	GTK	6,20%	1,9% (11/581)
Asn	AAT	3,10%	6,4% (37/581)
Asp	GAT	3,10%	4,1% (24/581)
Cys	TGT	3,10%	0% (0/581)
Glu	GAG	3,10%	3,1% (18/581)
His	CAT	3,10%	6,9% (40/581)
Ile	ATT	3,10%	2,1% (12/581)
Lys	AAG	3,10%	3,8% (22/581)
Met	ATG	3,10%	3,3% (19/581)
Phe	TTT	3,10%	2,1% (12/581)
Trp	TGG	3,10%	1,9% (11/581)
Tyr	TAT	3,10%	2,4% (14/581)

Amino acid distribution of the Ph.D.-C7C™ library. A total of 83 clones were sequenced from the naïve library. All of the sequenced clones correctly contained a 7-residue peptide flanked by cysteine residues. The overall amino acid distribution from the 581 sequenced codons (83 clones x 7 randomized codons) are shown.

* Expected ferquency is the number of codons for that amino acid divided by 32 codons, multiplied by 100 %.

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

‘’ The stop codon TAG is supressed by Gln in the strain used to propagate the library.

Appendix A.2.THE CLEANING OF THE INORGANIC SUBSTRATE

1) 100 mg powder is weighed and, put into a clean 1.5 ml eppendorf tube.

2) 100 µl dH_2O and 900 µl CH_3OH/acetone mixture (1:1) is added. CH_3OH/acetone mixture should be added carefully since some powders might get flamed. Powder pellet is dissolved gently by pipetting.

3) Powder solution is vortexed for 3-5 minutes. The forming clumps are observed**.**

4) The powder soution is sonicated for 20 min. in a ultrasonic bath to break the clumps.

5) It is vortexed quickly to resuspend.

6) It is spin down at 200g for 1.5 min (clockwise and counterclockwise rotation).

7) Supernatant is removed and 1 ml isoprapanol is added.

8) The powder solution is vortexed for 3-5 minutes.

9) The powder solution is sonicated for 20 min.

10) It is vortexed quickly to resuspend.

11) It is spin down at 200g (0.2 rcf) for 1.5 min (CW and CCW rotation).

12) Supernatant is removed and add 1 ml. 0.5% PC buffer is added.

13) The powder solution is vortexed for 3-5 minutes.

14) The powder solution is sonicated for 60 min.

15) It is vortexed quickly to resuspend .

16) It is spin down at 200 g. for 1.5 min (CW and CCW rotation).

17) Supernatant is removed and 1ml of 0.5% PC buffer is added.

18) Here, in order to get relatively uniform size distribution, the heavy and light powder particles should be eliminated.

To eliminate heavy ones: The homogenized powder is transferred into a new tube leaving the easily settling powder particles.

To eliminate the light ones: The powder solution is spin down at 200g. for 1 min. It is then resuspended in 1 ml of 0.5% PC buffer. A quick punch by pipetting is given. After waiting for 10-20 seconds, the supernatant is discarded. This procedure is repeated for 5 times. The powder yield would be around 50-60%.

19) 1 ml of 0.5% PC buffer is added onto powder and pipetted gently.

20) 100 µl of powder solution is tansferred into 10 sterile preweighed eppendorf tubes. It is not let the powder settle down, a quick vortex is given each time.

21) For each eppendorf centrifugation at 200g is applied and supernatant is discarded.

22) The powders are washed twice with dH_2O and twice with isopropanol.

23) They are dried at vacuum drier, weighed and the data is recorded.

Appendix A.3. FROZEN CULTURE PREPARATION:

E.coli host strain ER2738 is plated by streaking, on M9 Minimal Medium (For 1 liter of water it includes, 200 ml. of 5XM9 salts, 2 ml. of 1M Mg_2SO_4, 20 ml. of 20% solution of carbon source (glucose) and, 0.1 ml. of $CaCl_2$. 5XM9 salt is made by dissolving 64 g.of $Na_2HPO_4.7H_2O$, 15 g. of KH_2PO_4, 2,5 g. of NaCl and 5.0 g. of NH_4Cl. The salt solution is divided into 200 ml. aliquots and sterilized. Mg_2SO_4 and $CaCl_2$ solutions should be autoclaved and added after diluting the 5XM9 salts to 1 liter with sterile deionized water. Glucose should be sterilized by filtration before it is added to the diluted M9 salts, plates using a sterile platinum wire. The plate is incubated for 16 hours at 37 ºC. Couple of well-isolated colonies are transferred into 5 ml. LB medium containing tetracycline and $MgCl_2$ for overnight incubation. Next day, overnight *E.coli* culture is inoculated into 50 ml. LB medium containing tetracycline and $MgCl_2$ and, incubated at 37 ºC for 2.5-3.0 hours until OD_{600} value is determined as 0.5. For each *E.coli* frozen culture, 700µl LB containing *E.coli* is added onto 300 µl of 80% glycerol in an eppendorf. Eppendorfs are swirled gently to mix and transferred into liquid nitrogen and stored at –80 ºC.

Appendix A.4. TITER CALCULATION FOR DILUTIONS

Titers are calculated according to dilution step numbers.

20/200 = 0.1 = $1x10^{-1}$ (dilution from the first step)

20/200 = 0.1 = $1x10^{-1}$ (dilution from the second step)

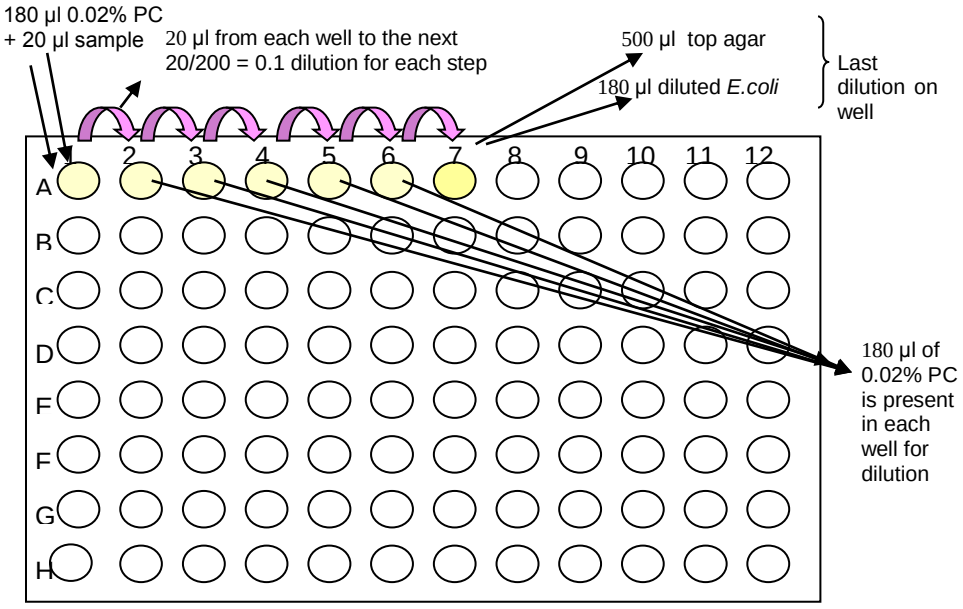
……

……

20/200 = 0.1 = $1x10^{-1}$ (dilution from the n^{th} step)

Total dilution = $1x10^{n}$

For titer evaluation, any number of colonies observed on petri dishes, are multiplied by total dilution.



Appendix A.5.NIKON ECLIPSE TE2000-U FLUORESCENCE MICROSCOPE

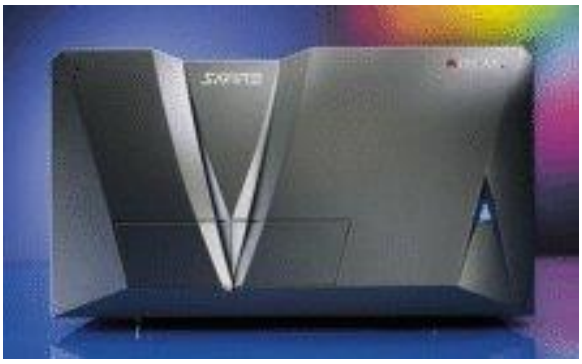
Inverted microscope used for research. Compatible for all advanced live cell applications and provides the highest level of optical imaging.

Major Features

- Has four ports, in order to integrate various pieces of imaging equipment (camera etc.) in desired combination and placement.
- Has extendible optical configuration. Distance between the objective and the tube lenses can be extended up to 80 mm., providing to add optional attachments (laser light source etc.)
- Motorized control adaptability
- Unprecedented signal to noise ratio by eliminating stray light, resulting high contrast (extending the detection level limit).
- CF160 optical system for crisp and clear images at any magnification
- Stability for greater precision. A new high-strength alloy material (M-45) on base for minor ambient temperature fluctuations not to adversely affect the image.
- The 25°-inclination eyepiece tube minimizes fatigue during long hours of observation
- Uses Xenon lamp

Fluorescence microscope is used to detect structures, molecules or proteins within the cell. Fluorescent molecules absorb light at one wavelength and emit at another. As fluorescent molecules absorb a specific absorption wavelength for an electron in a given orbital, the electron rises to a higher energy level (the excited) state. Unstable electrons return to the ground state, releasing energy in the form of light and heat. This emission of energy is **fluorescence.** In fluorescence microscopy, a cell is stained with a dye and the dye is illuminated with **filtered light** at the absorbing wavelength; the light emitted from the dye is viewed through a filter that allows only the emitted wavelength to be seen.

Appendix A.6. TECAN SAFIRE ELISA MICROPLATE READER



SAFIRE is the monochromator-based microplate reader for absorbance and fluorescence application needs. Major Features:

- Absorbance, fluorescence intensity or time-resolved fluorescence at various wavelengths. It can also record full range absorbance excitation and emission spectra.
- Top / bottom reading for optimum sensitivity for homogenous or cell based assays.
- Autofocus feature helps to optimize results by automatically adjusting the optical system to cope with different liquid levels in wells or variations in plate height.
- Selectable bandwidth allows performance optimization of the instrument with regard to resolution and sensitivity.
- Temperature uniformity
- Linear / orbital shaking
- Kinetic measurement for enzyme assays

SAFIRE microplate readers major application fields are pharmaceutical compound characterization, assay development, cell-based applications, fluorescent dye characterization.

Appendix A.7. BIOPANNING ROUNDS

Round 1

1. Single crystal sapphire is washed 4 times with 0.01% PC buffer by pipetting and then, buffer is removed.
2. 990 µl of 0.01% PC buffer and 10 µl of phage (Ph.D.-C7C) is added onto the material in the eppendorph and rotated for 30 minutes. Supernatant is removed and put into prelabelled tube (eppendorf) XM1S1* (1S1S1) Material is washed six times with 1 ml 0.1%PC buffer by pipetting, and the supernatants at the end of each washing is transferred into prelabelled tubes, 1S1W1, 1S1W2, 1S1W3, 1S1W4, 1S1W5 and 1S1W6.
3. After 6 washings, 1 ml 0.1% PC is added and rotated for 30 minutes.
4. Supernatant is transferred into tube 1S1W7
5. 1 ml 0.1 % PC is added and rotated for 30 minutes.
6. Supernatant is transferred into tube 1S1W8
7. 1 ml 0.1 % PC is added and left for rotation overnight Supernatant is transferred into tube 1S1W9 the next day.
8. 1 ml 0.1 % PC is added and rotated for 30 minutes.
9. Supernatant is transferred into tube 1S1W10.
10. 1 ml 0.1 % PC is added and rotated for 30 minutes.
11. Supernatant is transferred into tube 1S1W11.
12. Supernatants S1-W11 are used for plating out, in order to figure out the phage titers at each step
13. Elution is applied

*XM1S1: X; Number of screening, M; Material that is used, Round number (1), S; Supernatant (1) or W; Washing (1, 2, 3 etc.). In our case, M is `S` indicating Sapphire.

Round 2

1. Second single crystal sapphire is cleaned with the same procedure that applied to the first material, and washed 4 times with 0.01% PC buffer by pipetting and then buffer is removed.
2. The plaques are counted and the amplified phage titers are calculated. Panning should be carried with the volume corresponding to 10^9 – $2x10^{11}$ pfu phages.
3. 1 ml of phage solution is added to the second single crystal sapphire material. Phage solution is obtained through counter selection. The amounts of each eluents that are added to second round is calculated due to titers of first round. The phage solution is completed to 1 ml with PC buffer, obtaining a final PC concentration of 0.1% (~10^{11} phage titer) of the solution and rotated for 30 minutes.
4. Supernatant is removed and put into prelabelled tube 1S2S1
5. Material is washed six times with 1 ml 0.1%PC buffer by pipetting, and the supernatants at the end of each washing is transferred into prelabelled tubes, 1S2W1, 1S2W2, 1S2W3, 1S2W4, 1S2W5 and 1S2W6.
6. After 6 washings, 1 ml 0.1% PC is added and rotated for 30 minutes.
7. Supernatant is transferred into tube 1S2W7
8. 1 ml 0.1 % PC is added and rotated for 30 minutes.
9. Supernatant is transferred into tube 1S2W8
10. 1 ml 0.1 % PC is added and rotated for 30 minutes.
11. Supernatant is transferred into tube 1S2W9
12. 1 ml 0.1 % PC is added and left for rotation overnight.
13. Supernatant is transferred into tube 1S2W10 the next day.
14. 1 ml 0.2 % PC is added and rotated for 30 minutes. Supernatant is transferred into tube 1S2W11.
15. 1 ml 0.2 % PC is added and rotated for 30 minutes.
16. Supernatant is transferred into tube 1S2W12.
17. 1 ml 0.2 % PC is added and rotated for 30 minutes.
18. Supernatant is transferred into tube 1S2W13.

19. Supernatants S1-W11 are used for plate out, in order to figure out the phage titers at each step
20. Elution is applied.

Round 3

1. Third single crystal sapphire is cleaned with the same procedure that applied to the first material, and washed 4 times with 0.01% PC buffer by pipetting and then buffer is removed.
2. The plaques are counted and the amplified phage titers are calculated.
3. 1 ml of phage solution is added to the third single crystal sapphire material. Phage solution is obtained through counter selection. The amounts of each eluents that are added to third round is calculated due to titers of second round. The phage solution is completed to 1 ml with PC buffer, obtaining a final PC concentration of 0.1% (~10^{11} phage titer) of the solution and rotated for 30 minutes.
4. Supernatant is removed and put into prelabelled tube 1S3S1.
5. Material is washed six times with 1 ml 0.1%PC buffer by pipetting, and the supernatants at the end of each washing is transferred into prelabelled tubes, 1S3W1, 1S3W2, 1S3W3, 1S3W4, 1S3W5 and 1S3W6.
6. After 6 washings, 1 ml 0.1% PC is added and rotated for 30 minutes.
7. Supernatant is transferred into tube 1S3W71 ml 0.1 % PC is added and rotated for 30 minutes.
8. Supernatant is transferred into tube 1S3W8
9. 1 ml 0.1 % PC is added and rotated for 30 minutes.
10. Supernatant is transferred into tube 1S3W9
11. 1 ml 0.2 % PC is added and left for rotation overnight.
12. Supernatant is transferred into tube 1S3W10 the next day.
13. 1 ml 0.2 % PC is added and rotated for 30 minutes.
14. Supernatant is transferred into tube 1S3W11.
15. 1 ml 0.2 % PC is added and rotated for 30 minutes.
16. Supernatant is transferred into tube 1S3W12.

17. 1 ml 0.2 % PC is added and rotated for 30 minutes.

18. Supernatant is transferred into tube 1S3W13.

19. Supernatants S1-W11 are used for plating out, in order to figure out the phage titers at each step.

20. Elution is applied.

Round 4

1. Fourth single crystal sapphire is cleaned with the same procedure that applied to the first material, and washed 4 times with 0.01% PC buffer by pipetting and then buffer is removed.

2. The plaques are counted and the amplified phage titers are calculated.

3. 1 ml of phage solution is added to the fourth single crystal sapphire material. Phage solution is obtained through counter selection. The amounts of each eluents that are added to third round is calculated due to titers of second round. The phage solution is completed to 1 ml with PC buffer, obtaining a final PC concentration of 0.1% (~10^{11} phage titer) of the solution and rotated for 30 minutes.

4. Supernatant is removed and put into prelabelled tube 1S4S1.

5. Material is washed six times with 1 ml 0.1%PC buffer by pipetting, and the supernatants at the end of each washing is transferred into prelabelled tubes, 1S4W1, 1S4W2, 1S4W3, 1S4W4, 1S4W5 and 1S4W6.

6. After 6 washings, 1 ml 0.1% PC is added and rotated for 30 minutes.

7. Supernatant is transferred into tube 1S4W7

8. 1 ml 0.1 % PC is added and rotated for 30 minutes.

9. Supernatant is transferred into tube 1S4W8

10. 1 ml 0.1 % PC is added and rotated for 30 minutes.

11. Supernatant is transferred into tube 1S4W9

12. 1 ml 0.3 % PC is added and left for rotation overnight.

13. Supernatant is transferred into tube 1S4W10 the next day.

14. 1 ml 0.5 % PC is added and rotated for 30 minutes.

15. Supernatant is transferred into tube 1S4W11.

16. 1 ml 0.5 % PC is added and rotated for 30 minutes.

17. Supernatant is transferred into tube 1S4W12.

18. Supernatants S1-W11 are used for plate out, in order to figure out the phage titers at each step.

19. Elution is applied, If required, 5^{th} round can be applied as 4^{th} rounds' protocol.

Appendix A.8. ELUTION

In order to begin elution, sterile 50 ml. LB medium in 250 ml. erlenmeyer flasks is required for each step.

To each erlenmeyer flask, 100µl of 1M $MgCl_2$, 50µl of tetracycline and 125 µl *E. coli* from overnight culture is added. After inoculation, the culture is incubated for 30 –40 min. (~2-3 doubling time) in orbital shaker at 37ºC, at 200 rpm. Cultures corresponding to elutions need to be inoculated in 30 minute intervals.

1. Elution is started by adding 1 ml of Elution Buffer I to the sapphire sample and rotating for 15 minutes.
2. 800 µl of this solution is inoculated into the erlenmeyer flask XMYE1* (1S1E1) and incubated in orbital shaker waterbath for 4.5 hours at 37ºC, at 200rpm (LAB-Line Aqaubath). Since increased time of growth might yield to mutations, 4.5 hours of incubation is very critical.
3. Remaining 200 µl of recovered phage solution is transferred into the prelabelled eppendorf tube 1S1E1 and neutralized by adding 40 µl of pH 9.1 Tris-HCl neutralizing buffer.
4. 1 ml of Elution Buffer II is added to the sapphire sample and rotated for 15 minutes.
5. 800 µl of this solution is inoculated into the second erlenmeyer flask 1S1E2 and incubated in orbital shaker for 4.5 hours at 37ºC, at 200rpm.
6. Remaining 200 µl of recovered phage solution is transferred into the prelabelled eppendorf tube 1S1E2 and neutralized by adding 40 µl of pH 9.1 Tris-HCl neutralizing buffer.
7. 1 ml of Elution Buffer II is added to the sapphire sample and rotated for 15 minutes.
8. 800 µl of this solution is inoculated into the third erlenmeyer flask 1S1E3 and incubated in orbital shaker for 4.5 hours at 37ºC, at 200rpm.
9. Remaining 200 µl of recovered phage solution is transferred into the prelabelled eppendorf tube 1S1E3 and neutralized by adding 40 µl of pH 9.1 Tris-HCl neutralizing buffer.

10. 1 ml of Elution Buffer II is added to the sapphire sample and rotated for 15 minutes.

11. 800 µl of this solution is inoculated into the fourth erlenmeyer flask 1S1E4 and incubated in orbital shaker for 4.5 hours at 37ºC, at 200rpm.

12. Remaining 200 µl of recovered phage solution is transferred into the prelabelled eppendorf tube 1S1E3 and neutralized by adding 40 µl of pH 9.1 Tris-HCl neutralizing buffer.

13. Sapphire sample is put into the fourth erlenmeyer flask 1S1B, (B indicating bound phages). This step is added to main elution procedure, that is recommended by the company which phage display library kit was purchased. The aim of this step was to recover any possible binding phage that could not be obtained by Elution Buffers I and II.

Appendix A.9. PURIFICATION OF THE AMPLIFIED PHAGE TITERS

1. *E. coli* / phage cultures were transferred to sterilized 250 ml. centrifuge tubes at the end of 4.5 hours of growth period.
2. The culture is cetrifuged twice (CW & CCW)*, at 8000 rpm for 10 min. at +4 ºC (Sorvall SS-34, Beckman JA-17)and, supernatant is transferred to clean sterilized centrifuge tubes.
3. PEG/NaCl solution is added at the ratio of 1:6, to precipitate the phage and left overnight at 4ºC.
4. The solution is spin down twice CW & CCW to get the compact phage.
5. The supernatant is discarded and the precipitate is resuspended with 5 ml. PC buffer (no detergent) to remove any remaining *E. coli,* pellet is dissolved by shaking.
6. The solution is centrifuged at 8000 rpm for 10 min.
7. Supernatant is transferred to 50 ml. centrifuge tubes.
8. PEG/NaCl solution is added at the ratio of 1:6, to precipitate the phage and left for 2 hours at 4ºC.
9. The solution is centrifuged 10 min. at 10000 rpm. twice, CW & CCW, to get the precipitated phage.
10. Supernatant is discarded and resuspended with 1 ml. PC buffer (no detergent), to remove *E. coli,* pellet is dissolved by shaking or vortexing.
11. The solution is centrifuged at 10000 rpm twice, CW & CCW, for 10 min. and supernatant is transferred to eppendorf tubes.
12. PEG/NaCl solution is added at the ratio of 1:6, to precipitate phage and, the solution is vortexed for 5 sec. and left for 10 min.
13. The solution is centrifuged at 13200 rpm. twice, CW & CCW, for 1.5 min. to get the compact phage.
14. Supernatant is discarded and resuspended with 0.2 ml. PC buffer (no detergent), pellet is dissolved by pipetting gently.
15. The solution is centrifuged at 13200 rpm. for 1.5 min.
16. The supernatant is transferred to clean tubes, labelled as 1S1E1, 1S1E2, 1S1E3, 1S1E4 and 1S1B and, stored at 4ºC.

Appendix A.10. ISOLATION OF ssDNA OF M13

1) After 4.5 hours of incubation, 2 ml. of solution that is containing M13 bacteriophage is transferred to two sterile eppendorf tubes in 1 ml. of portions.

2) The bacterial cells are centrifuged at 5000 rpm. for 10 minutes at room temperature.

3) The supernatant containing M13 bacteriophage is transferred to fresh sterile eppendorf tubes.

4) 1/100 volume of Buffer MP is added for M13 precipitation, mixed by vortexing and incubated at room temperature for at least 2 min.

5) QIAprep spin column is placed in a 2 ml. microcentrifuge (collection) tube and, 0.7 ml. of the sample is applied into the QIAprep spin column.

6) The solution is centrifuged for 15 sec. at 8000 rpm and, the flow is discarded from the collection tubes.

7) The loading and centrifugation steps are repeated until all of the sample has been loaded onto the QIAprep spin column.

8) 0,7 ml. of Buffer MLB is added to the QIAprep spin column for M13 lysis and binding and, centrifuged for 15 sec. at 8000 rpm.

9) Another 0,7 ml. of Buffer MLB is added to the QIAprep spin column, incubated for 1 min. at room temperature to lyse bacteriophages completely and, centrifuged for 15 sec. at 8000 rpm.

10) 0,7 ml. of Buffer PE is added and centrifuged for 15 sec. at 8000 rpm.

11) Buffer PE is discarded from collection tube. To remove residual Buffer PE, QIAprep spin column is is centrifuged for 15 sec. at 8000 rpm.

12) QIAprep spin column is placed in clean 1.5 eppendorf tube. In order to elute DNA, 100 µl of Buffer EB (10 mM Tris.Cl, pH 8.5) is applied to the center of the column membrane, incubated for 10 min. and, centrifuged for 30 sec. at 8000 rpm.

REFERENCES

[1] **Ebbing, D.D.,** 1996. General Chemistry,5th Ed., Houghton Mifflin Company, Boston

[2] **Becker, W.M, Reece, J.B., Poenie, M.F.,** 1996, The World of the Cell, The Benjamin/Cummings Publishing Company, California.

[3] **Voet, D., Voet, J.G.,** 1995. Biochemistry, 2nd Ed. John Wiley and Sons, New York.

[4] **Campbell, N.A., Reece, J.B.,** 2002. Biology 6th Ed., Benjamin Cummings Publishing Company, San Francisco.

[5] **Sarikaya, M., Tamerler, C., Schwartz, D.T., Baneyx, F.,** 2004. Materials assembly and formation using engineered polypeptides, *Annual Reviews in Advance,* **34,** 373-408

[6] **Tamerler,C., Ventaka, E, Duman,M, Dinçer, S., Ören, E., Sarıkaya,M.,** unpublished work.

[7] **Heywood, B.R., Mann, S., Frankel, R.B.,** 1991. Structure, morphology and growth of biogenic greigite, *Mat. Res. Soc. Symp. Proc.,* **218,** 93-107.

[8] **Niemeyer, C.M.,** 2001. Nanoparticles, proteins and, nucleic acids: Biotechnology meets materials science. *Angew. Chem. Int. Ed.,* **40,** 67-69.

[9] **Sarıkaya, M., Tamerler, C., Jen, A.Y., Schulten, K., Baneyx, F.,** 2003. Molecular biomimetics: nanotacehnology through biology. *Nat. Mater.,* **2,** 577-585.

[10] **Ball, P.,** 2001, Life's lessons in design. *Nature,* **409,** 413-416

[11] **Sarıkaya, M., Aksay, İ.A., eds.,** 1995. Biomimetics: Design and Processing of Matreials. New York: Am. Inst. Phys.

[12] **Sarikaya, M., Tamerler, C., Traxler, B.,** Unpublished work.

[13] **Frankel, R.B., Blakemore, R.P. eds,** 1991. Iron Biominerals, Plenum, New York.

[14] **Kristiansen, J., Andersen, R.A. eds,** 1986. Chrrysophytes: Aspects and Problems, Cambridge University Press, New York.

[15] **Margulis, L., Schwartz K.V.,** 1998. Five Kingdoms, Freeman, New York.

[16] **Schultze-Lam, S., Harauz, G., Beveridge, T.H.,** 1992. *Journal of Bacteriology*, **174,** 7971-7981

[17] **Glimcher, J.,** 1981. In Chemistry and Biology of Mineralized Tissues. ed. Veis, A., Elsevier, Amsterdam.

[18] **Smith, C.E.,** 1998. *Crit. Rev. Oral Biol.Med.,* **9,** 128-161.

[19] **Sarikaya, M.,** 1999. Biomimetics: Matreials fagrication through biology, *PNAS*, **96,** 14183-14185.

[20] **Snead, M.L., Zeichner-David, M., Chandra, T., Robson, K.J., Woo, S.L., Slavkin, H.C.,** 1983. *Proc. Natl. Acad. Sci. USA,* **80,** 7254-7258.

[21] **Carioluo, M.A., Morse, D.E.,** 1987. *J.Comp. Physiol. B.,* **157,** 717-729.

[22] http://recruit.sciencemag.org/feature/cperspec/combi.shl/

[23] http://www.acelrys.com

[24] **Dani, M.,** 2001. Peptide display libraries: design and construction. *J. Of Recpt. Signal Transduct. Res.* **21:** 469-488

[25] **Wittrup, K.D.,** 2001. protein engineering by cell surface display. *Curr. Opin. Biotechnol.* **12:** 395-399

[26] **Azzazy, H.M.E., Highsmit W.E. Jr.** 2002. Phage display rechnology: clinical applications and recent innovations. *Clin. Biochem.* **35:**425-445

[27] **Dower, W.J., Mattheakis, L.C.,** 2002. In vitro selection as a powerful tool for the applied evolution of proteins and peptides. *Curr. Opin. Chem. Biol.* **6:**390-398

[28] **Samuelson, P., Gunneriusson, E., Nygren, P.A., Stahl, S.,** 2002. Display of proteins on bacteria. *J. Biotechnology,* **96:** 129-154.

[29] **Smith, G.P.,** 1985, Filamentous fusion phage: novel expression vectors that display cloned antigens on the virion surface. *Science,* **228:** 1315-1317.

[30] **Parmley, S.F., Smith, G.P.**, 1988. *Gene,* **73,** 305-318

[31] **Smith, G.P., Scott, J.K.,**1993. *Methods in Enzymology,* **217,** 228-257.

[32] **Felici, F., Castagnoli, L., Musacchio- A., Japelli, R., Ceasrini, G.** (1991) *J. Mol. Bio.,* **222,** 301-310.

[33] **Scott, J.K., Smith, G.P.,** 1990. *Science,* **249,** 386-390.

[34] **Cwirla, P.A., Peteres, E.A., Barrett, R.W., Dower, W.J.,** 1990. *Proc. Natl. Acad. Sci. USA*, **87,** 6378-6382.

[35] **Hong, S.S., Boulanger, P.**, 1995. EMBOJ. **14**, 4714-4727.

[36] **Devlin, J.J., Panganiban, L.C., Devlin, P.E.,** 1990. *Science,* 249, 404-406.

[37] **Oldenburg, K.R., Loganathan, D., Goldstein, I.J., Schultz, P.G., Gallop, M.A.,** 1992. *Proc. Natl. Acad. Sci. USA89*, 5393-5397.

[38] **Scott, J.K., Loganathan, D., Easley, R.B., Gong, X., Goldstein, I.J.**, 1992. *Proc. Natl. Acad. Sci. USA*, **89,** 5398-5402.

[39] **Hoess, R., Brinkmann, U., Handel T., Pastan, I.,** 1993. *Gene,* **128**, 43-49.

[40] **O'neil, K.T., Hoess, R.H., Jackson, S.A., Ramachandran N.S., Mousa, S.A., DeGrado, W.F.,**1992. *Proteins,* **12**, 509-515.

[41] **Doorbar, J., Winter, G.,** 1994. J. Mol. Bio., **244,** 361-369.

[42] **Goodson, R.J., Doyle, M.V., Kaufman, S.E., Rosenberg, S.,** 1994. *Proc. Natl. Acad. Sci. USA,* **91**, 7129-7131.

[43] **Barry, M.A., Dower, W.J., Johnston, S.A.,** 1996. *Nature Medicine,* **2,** 299-305.

[44] **Smith, M.M., Shi, L., Navre, M**., 1995. *Journal of Biol. Chem*., **270,** 6440-6449.

[45] **Barbas, S.M., Barbas, C.F.,** 1994. *Fibrinolysis 8,* **Suppl. 1**, 245-252.

[46] **Lowman, H.B., Bass, S.H., Simpson, N., wells, J.A.,** 1991. *Biochemistry,* **30,** 10832-10838.

[47] **Roberts, B.L. et al.**, 1992. *Proc. Natl. Acad. Sci*. **USA89**, 2429-2433.

[48] **Soumillion, P., Jespers, L., Bouchet, M., Marchand-Brynaert, J., Winter, G., Fastrez, J.,** 1994. *J. Mol. Bio*. 237, 415-422.

[49] **Choo, Y., Klug, A.,** 1995. *Current Opinion in Biotechnologhy,* **6,** 431-436.

[50] Ph.D.-C7C Phage Display Kit Manual, 2003, New England BioLabs, Cambrigde.

[51] **Dai, Y., Evans, J.C.,** 2000. An energy-based mapping method for identifying the inplane orientations of the polypeptides and other macromolecules at crystalline interfaces, *J. Chem. Phys.,* **112,** 144-157.

[52] **Weiss, G.A., Sidhu, S.S.,** 2000. Design and evolution of artificial M13 coat proteins. *J. Mol. Bio.* **300,** 213-219.

[53] http://www.biosci.ohio-state.edu/~mgonzalez/Micro521/Lambda/M13.html

[54] **Özmüş, A., Tamerler, C., Sarıkaya, M.,** Unpublished work.

[55] **Izrailev, S., Stepaniants, S., Balsera, M., Oono, Y., Schulten, K.,** 1997. Molecular dynamics study of unbinding of avidin-biotin complex. *Biophys. J.* **72,** 1568-1581.

[56] **Perez-Luna,H.V., O'Brien, M.J., Opperman, K., Hampton, P.D., Stayton, P.S. et al.**, 1999. molecular recognitio between genetically engineered streptavidin and surface-bound biotin. *J.Am.Chem. Soc*. **121,** 6469-6478.

[57] **Kruger, N., Deutzmann, R., Bergsdork, C., Sumper, M.**, 2000. Species-specific polyamines from diatoms control silicamorphology. *Proc. Natl. Acad. Sci. USA,* **97,** 14133-14138

[58] **Knight, C.A., Cheng, C.C., DeVries, A.L.,** 1991. Adsorption of alpha-heical antifreeze peptides on specific ice crystal surface planes. *Biophys. J.* **59,** 409-418.

[59] **Weissbuch, I., Addadi, L., Lahav, M., Leiserowitz, L.,** 1991. Molecular recognition at crystal interfaces. *Science,* **253,** 637-645.

[60] **Evans, J.S.,** 2003. ´Apples` and ´ oranges`: Comparing the structural aspects of biomineral- and ice-interaction proteins. *Curr. Opin. Colloid Interface Sci.,* **8,** 48-54.

[61] **Whaley, S.R., English, D.S., Hu, E, L., Barbara, P.F., Belcher, M.A.,** 2000. Selection of peptides with semiconducting binding specificity for directed nanocrystal assembly. *Nature,* **405,** 665-668.

[62] **Naik, R.R., Brott, L., Carlson, S.J., Stone, M.O.,** 2002. Silica precipitating peptides isolated from acombinatorial phage display libraries. *J.Nanosci. Nanotechnol.* **2,** 95-100.

[63] **Brown, S.,** 1992.Engineering iron oxide-adhesion mutants of *Escherichia coli* phage λ receptor. *Proc. Natl. Acad. Sci. USA,* **89,** 8651-8655.

[64] **Naik, R.R., Stringer, S.J., Agarwal, G., Jones, S.E., Stone, M.O.,** 2002. Biomimetic synthesis and paterning of silver nanoparticles. *Nat. Mater.* **1**, 169-172.

[65] **Dai, H. Nguyen, S., Sarıkaya, M., Baneyx, F., Schwartz, D.T.,** 2004. Through-mask anodic patterning of coppper surfaces and film stability in biological madia. *Langmuir*, In press

[66] **Baselt, D.** 1993. The tip-sample interaction in atomic force microscopy and its implications for biological applications, Ph.D. Thesis, California Institute of Technology.

[67] **Binnig, G., Quate, C.F., Gerber, Ch.,** 1986. Atomic force microscope*. Phys. Rev. Lett.* **56(9)**, 930-933.

[68] **Albrecht, T.R., Grütter, P., Horne, D., Rugar, D.,** 1991. Frequency modulation detection using high-Q cantilevers for enhanced force microscope sensitivity., *J. Appl. Phys.* **69(2)**, 668-673.

[69] **Hoh, J.H., Hansma, P.K.**, 1992. Atomic force microscopy for high-resolution imaging in cell biology., *Trends Cell Bio*. **2,** 208-213.

[70] **Weisenhorn, A.L., Hansma, P.K., Albrecht, T.R., Quate, C.F.,** 1989. Forces in atomic force microscopy in air and water. *Appl. Phys. Lett.,* **54(26),** 2651-2653.

[71] Guild optical associates, Inc**.(**http://guildoptics.com/properties.html)

[72] http://www.niddk.nih.gov/fund/other/genoproteo/wang.pdf

[73] QIAGEN QIAprep® M13 Handbook, March 2002 (http://www.qiagen.com).

[74] **Thompson, J.D., Higgins, D.G., Gibson, T.J.,** 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weightening, position specific gap penalties and weight matrix choice. *Nucleic Acid Research,* **22,** 4673-4680.

BIOGRAPHY

A.Arzu Özmüş was born in Malatya, 1977. She graduated from Fırat Elementary School and Malatya Anatolian High School. She also graduated from Greensburg Salem Senior High School in Greensburg, Pennsylvania, U.S.A. She enrolled to Ege University, Biochemistry Department in 1997. She got Bachelor of Science degree from the department, in 2001 with the Biotechnology option . She has enrolled to Molecular Biology-Genetics and Biotechnology graduate program as a part of Advanced Technologies in Engineering Center in İstanbul Technical University in 2001. As a part of her MS.c. studies, she has studied under supervision of Assoc. Prof. Candan Tamerler Behar from Molecular Biology, Genetics and Biotechnology program and, Prof. Dr. Mehmet Sarıkaya from University of Washington, in Materials Science and Engineering department. She enrolled to University of Washington in Seattle, Washington, U.S.A., Materials Science and Engineering Department to complete her MSc studies. She has been as a visiting graduate student in University of Washington between May 2003-March 2004. She is a research assistant in İTÜ, Molecular Biology and Genetics Department since 2001.