

**SELECTION AND SIMILARITY ANALYSIS OF
QUARTZ SPECIFIC DODECAPEPTIDES BY PHAGE
DISPLAY SELECTION PROTOCOL**

**M.Sc. Thesis by
Deniz ŞAHİN**

Department: Advanced Technologies in Engineering

Programme: Molecular Biology – Genetics & Biotechnology

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Deniz ŞAHİN, B.Sc.**

(707021009)

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Supervisor (Chairman) : Assoc. Prof. Candan TAMERLER

Prof. Mehmet SARIKAYA (UW)

Members of the Examining Committee Assoc. Prof. Pemra DORUKER (BÜ)

Asist. Prof. Hakan BERMEK (İTÜ)

Asist. Prof. Nevin G. KARAGÜLER (İTÜ)

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**FAJ GÖSTERİM SEÇİM TEKNİĞİ İLE KUARTZA
BAĞLANAN DODEKAPEPTİTLERİN SEÇİLMESİ VE
BENZERLİK ANALİZİ**

YÜKSEK LİSANS TEZİ
Deniz ŞAHİN
(707021009)

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Tez Danışmanı : **Doç. Dr. Candan TAMERLER**
 Prof. Dr. Mehmet SARIKAYA (UW)

Diğer Jüri Üyeleri **Doç. Dr. Pemra DORUKER (BÜ)**
 Yrd. Doç. Dr. Hakan BERMEK (İTÜ)
 Yrd. Doç. Dr. Nevin G. KARAGÜLER (İTÜ)

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TABLE OF CONTENTS

ABBREVIATIONS	vii
LIST OF TABLES	ix
LIST OF FIGURES	x
SUMMARY	xiv
ÖZET	xvi
1. INTRODUCTION AND BACKGROUND	1
1.1. Inorganic-binding Polypeptides and Their Importance in Organisms	1
1.2. Applications of Inorganic-binding Polypeptides in Nano and BioTechnology	3
1.3. Obtaining Inorganic-binding Polypeptides	4
1.4. Display Technologies	5
1.5. Adaptation of Phage Display for Selection of Inorganic-binding Polypeptides	8
1.6. Molecular Biomimetics	9
1.7. Selection of Inorganic Target Material for Biopanning Steps	10
1.7.1. Quartz as the Substrate	10
1.7.2. Silaffins	11
1.7.3. Silicateins	12
1.7.4. Phage Display Selected Silica Binders	13
1.8. Aim of the Study	15
2. MATERIALS AND METHODS	16
2.1. Materials	16
2.1.1. Bacterial strain- <i>E. coli</i> ER2738 host strain	16
2.1.2. Phage Display Peptide Library-Peptide 12-mer Phage Display Library (Ph.D.-12™)	16
2.1.3. Inorganic Targets: (100) and (001) Oriented Quartz Wafers	17
2.1.4. Bacterial Culture Media	18
2.1.4.1. Luria Bertani (LB) medium	18
2.1.4.2. LB Agar Medium	18
2.1.4.3. Top-Agar Medium	18
2.1.4.4. <i>E. coli</i> Overnight Culture	18
2.1.5. Stock Solutions	19
2.1.5.1. Tetracycline-HCl Stock	19
2.1.5.2. Xgal/IPTG Stock	19

2.1.5.3. Detergent Stock	19
2.1.5.4. Glycerol Stock Solution	19
2.1.5.5. MgCl ₂ Stock Solution	19
2.1.6. Buffer Solutions	19
2.1.6.1. PEG/NaCl	19
2.1.6.2. PC (Potassium Phosphate-Sodium Carbonate Buffer)	19
2.1.6.3. Elution Buffers	20
2.1.6.4. Tris Buffer	21
2.1.6.5. TBE (Tris / Borate / EDTA) Solution	21
2.1.7. Lab Equipments	21
2.2. Methods	22
2.2.1. Phage Display Protocol	22
2.2.2. Screening Procedure	23
2.2.3. Biopanning Steps	24
2.2.3.1. Cleaning of the Quartz	24
2.2.3.2. Washing Steps	25
2.2.3.3. Elution Steps	26
2.2.3.4. Purification Steps	28
2.2.4. Titters of the Selected Phages	29
2.2.4.1. Blue-white Screening Experiment	29
2.2.4.2. Preparation of Diluted Phage and Plating	29
2.2.4.3. Phage Titters for Each Round	31
2.2.4.4. Saving Clones for Sequencing	31
2.2.4.5. Preparation of Storage Plates	31
2.2.4.6. Preparation of Glycerol Stock of Phage	31
2.2.5. Single Stranded DNA Isolation	32
2.2.5.1. M13 Single Strand DNA Isolation	32
2.2.5.2. Measurement of DNA concentration by TECAN-Magellan	
SAFIRE Elisa Microplate Reader	34
2.2.6. Sequencing of DNA Sample	35
2.2.6.1. PCR Conditions for DNA Sequencing	35
2.2.6.2. PCR Product Purification	36
2.2.7. Immunofluorescence Microscopy Experiments	36
2.2.7.1. Cleaning of the Quartz Powder	36
2.2.7.2. Fluorescence Microscopy Experiment Procedure	37
2.2.8. Silica Formation Experiments	39
2.2.9. Similarity Analysis Tools-Dynamic Programming	40
2.2.10. AFM Imaging	40

2.2.11. TEM Imaging	41
2.2.12. SEM Imaging	41
3. RESULTS AND DISCUSSION	42
3.1. Identification of the Binder Polypeptides for (100) Oriented Quartz and Their Sequence Based Analysis	43
3.2. Statistical Analysis of the Selected Sequences	45
3.3 Physicochemical Properties of the Selected Binders	46
3.4 Affinity Characterization of the Selected Sequences	48
3.5. Analysis after Classifying Sequences into the Subgroups	50
3.6. Similarity Analysis for Verification of the Grouping of the Sequences	52
3.7. Understanding towards specificity: Cross-Specificity Experiments	53
3.8. Selection of Polypeptides for (001) Oriented Quartz	55
3.9 Silica Synthesis	58
4. CONCLUSION	61
REFERENCES	63
APPENDIX	67
CV	69

ABBREVIATIONS

AFM	: Atomic Force Microscope
bp	: Base pair
BSA	: Bovine Serum Albumine
CSD	: Cell Surface Display
CCW	: Counter clockwise
CW	: Clockwise
ddH₂O	: Double distilled water
DDT	: Dichloro-diphenyl-trichloroethane
DMF	: Dimethylformamide
DMSO	: Dimethylsulfoxide
DNA	: Deoxyribo Nucleic Acid
DNase	: Deoxyribonuclease
E. coli	: Eschericia coli
EDTA	: Ethylenediaminetetraacetic acid
EDS	: Electron Diffraction Spectrum
EtOH	: Ethanol
Fab	: Antibody Fragment
FM	: Fluorescence Microscopy
IPTG	: Isopropyl- β -D-1-thiogalactosidase
kb	: Kilobase
LB-broth	: Luria Bertani Broth
LMP agarose	: Low Melting Point Agarose
ME	: Mercaptoethanol
OD	: Optical Density
PC Buffer	: Potassium Phosphate-Sodium Carbonate Buffer
PCR	: Polymerase Chain Reaction
PD	: Phage Display
PEG	: Polyethylene glycol
RNA	: Ribonucleic acid
RT	: Room Temperature
SDS	: Sodium Dodecyl Sulfate
SDS-PAGE	: Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
ssDNA	: Single Stranded DNA
TBE	: Tris/Borate/EDTA
TBS	: Tris Buffered Saline
TCEP	: Tris(cyanoethoxy)propane
TE	: Tris/EDTA
TEM	: Transmission Electron Microscope
TEOS	: Tetraethylorthosilicate
TMOS	: Tetramethylorthosilicate
Tris	: Hydroxymethyl aminomethane
SEM	: Scanning Electron Microscope

Xgal	: 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside
XPS	: X-ray Photoelectron Spectroscopy
XRD	: X-ray Diffraction

LIST OF TABLES

	<u>Page No</u>
Table 2.1. Phage titers and amount of phage used for the next round after each round (D: Dilution number, A: Amount of phage solution used for the phage pool for the next round as microliter).....	31
Table 2.2. PCR conditions for DNA sequencing.....	35
Table 3.1. List of phage display selected quartz binders. (Color codes of amino acids: Red; Hydrophobic, Blue; Acidic, Purple; Basic, Light Green; Hydroxyl Containing, Dark Green; Amine+Basic) (*: The number after some sequences is the repetition number of that sequence).....	44
Table 3.2. List of phage display selected quartz binders with their charge, polarity and hydrophilicity-hydrophobicity values. (Color codes of amino acids: Red; Hydrophobic, Blue; Acidic, Purple; Basic, Light Green; Hydroxyl Containing, Dark Green; Amine+Basic) (*: The number after some sequences is the repetition number of that sequence)	47
Table 3.3. List of phage display selected quartz binders with their physicochemical properties and binding percentages. (Color codes of amino acids: Red; Hydrophobic, Blue; Acidic, Purple; Basic, Light Green; Hydroxyl Containing, Dark Green; Amine+Basic) (A: Classification with respect to % Binding calculated from FM pictures.*: The number after some sequences is the repetition number of that sequence)	51
Table 3.4. Phage display selected binders for (001) oriented quartz with their physicochemical properties.....	57

LIST OF FIGURES

	<u>Page No</u>
Figure 1.1 :Examples of biologically synthesized organic-inorganic hybrid materials with a variety of physical properties: (a) Single-crystalline, single-domained magnetic magnetite nanoparticles (Fe ₃ O ₄) formed by a magnetotactic bacterium (<i>Aquaspirillum magnetotacticum</i>) (inset: higher magnification image of the magnetite nanoparticles revealing cubo-octahedral particle shape). (b) S-layer bacterium, <i>Synechococcus</i> strain GL24, has a nanostructurally ordered thin film calcite on its outer layer serving as a protective coating. (c) Hard, wear-resistant tooth enamel of mouse consists highly ordered micro/nano architecture of hydroxyapatite crystallites that assemble into a woven rod structure (SEM image). Each rod is composed of thousands of hydroxyapatite particles (inset: cross-sectional image of a mouse incisor; white region is enamel, backed by grayish dentine) (Sarıkaya <i>et al.</i> , 2003).....	2
Figure 1.2 :Phage display and cell-surface display. Principles of the protocols used for selecting polypeptide sequences that have binding affinity to given inorganic substrates (Sarıkaya <i>et al.</i> , 2004).....	7
Figure 1.3 :Potential uses of inorganic-binding polypeptides. (a) linkers for nanoparticle immobilization, (b) functional molecules that assemble on specific substrates, and (c) heterofunctional linkers involving two (or more) binding proteins adjoining several nanoinorganic units. NSL, nonspecific linker (Sarıkaya <i>et al.</i> , 2004).....	9
Figure 1.4 : (A) Cell wall of <i>C. fusiformis</i> has rows of parallel silica strips running in a helical mode along the longitudinal axis of the cell. Bar: 2.5 mm. (B and C) Details of a <i>C. fusiformis</i> cell in cross section. The arrowheads indicate the position of the plasma membrane and point toward the extracellular space. Bars: 100 nm. (B) Lateral region. Each oval-shaped element represents a single silica strip of the cell wall in cross section. The arrow indicates a nascent silica strip within its SDV shortly before secretion. (C) Valve region. The ringlike structure and the two oval-shaped elements on either side are silicified cell wall elements (Kroger <i>et al.</i> , 1999).....	11

Figure 1.5	:Silica precipitation induced by silaffins (14). (A) Low molecular mass fraction of HF extract from <i>C. fusiformis</i> cell walls. The extract was subjected to Tricine–SDS–PAGE (13) and stained with Coomassie blue. (B) Correlation between silaffin concentration (27) and the amount of silica precipitated from a silicic acid solution. The dotted line represents the result obtained for the silaffin mixture; the solid line shows the result for pure silaffin-1A (15). (C and D) SEM micrographs of silica precipitated by silaffin-1A (C) and the mixture of silaffins (D). The diameter of silica particles is 500 to 700 nm (C) and 50 nm (D). The protein concentration was 5 mg/ml. Bar: 1 mm.(modified from Kroger <i>et al.</i> , 1999).....	12
Figure 1.6	:Primary structure of sil1p. The signal peptide sequence is shown in italics, the highly acidic peptide sequence (residues 20 to 107) is depicted in regular type, and the mature part of the polypeptide (residues 108 to 271) is shown in bold. The repeats within the mature part bearing silaffin sequences are labeled R1 to R7 on the left margin. Arginine and lysine residues within silaffin sequences are highlighted (modified from Kroger <i>et al.</i> , 1999).....	12
Figure 1.7	:Scanning electron micrographs of isolated silica spicules (x130) (A) and axial filaments (x 1,000) (B) from <i>Tethya aurantia</i> (modified from Weaver and Morse, 2003).....	13
Figure 1.8	:SEMs of silica products synthesized from tetraethylorthosilicate (TEOS) at 20°C, neutral pH, and atmospheric pressure. Silica nanoparticles grown in the presence of recombinantly derived silicatein β , the second-most abundant of the <i>Tethya aurantia</i> silicateins can be seen (modified from Weaver and Morse, 2003).....	13
Figure 1.9	:(A) Scanning electron micrograph of biosilica which was formed by the use of R5 peptide; scale bar, 1 μ m. (B) Multiple sequence alignment (with CLUSTALW) of selected silica-binders. Si3-4 means the clone was selected as the fourth clone from the third round. The shaded amino acids have the functional side chains with the ability to interact with the silica surface.Ge4-1 is a germanium-binding peptide and given as the control. R5 peptide is given for the comparison. The peptides with asterisk were isolated more than once (modified from Naik <i>et al.</i> , 2002).....	14

Figure 1.10	:(At left) Silica condensation of the phage-binding clones. Equal amounts of phage particles, 10^{11} from each, were incubated with hydrolyzed tetramethyl orthosilicate (TMOS) in Tris-buffered saline (pH 7.5). The silica precipitate was washed and dissolved in 1 M NaOH at 95 °C for 30 min. The amount of silica precipitated from silicic acid was measured with the spectrophotometric molybdate assay and it is proportional to the amount of Si ⁴⁺ -phage particles added (inset). (At right) SEM micrographs of silica precipitated by the phage peptide clones. The diameter of the silica nanoparticles is between 200 and 400 nm; scale bar, 500 nm. (modified from Naik <i>et al.</i> , 2002).....	14
Figure 2.1	:Image of a single <i>E. coli</i> ER2738 cell with 24,000X magnification and 2 s. exposure time. 2% Ammonium Molybdate solution was used for staining. Bar is 1µm.....	16
Figure 2.2	:(at left) AFM image of M13 phage on the quartz and (at right) TEM image of branches of phages. Bar is 1 µm in TEM image.....	17
Figure 2.3	:On the left, (100) plane quartz wafer as purchased from the company before cutting into the small pieces. In the middle, XRD result of the quartz wafer showing its (100) oriented structure. On the right, AFM image of the quartz wafer showing its surface roughness around 5 Å.....	17
Figure 2.4	:a) Structure of M13 phage and b) Phage display procedure, steps 1 through 9.....	23
Figure 2.5	:Making the serial dilutions on the Elisa plate.....	30
Figure 2.6	:Examples of blue-white screening experiment. The Petri dishes are shown presenting the results of different phage titers.....	30
Figure 2.7	:M13 ssDNA isolation procedure (QIAGEN, Catalog # 27704)....	33
Figure 2.8	:Labelling procedure for fluorescence microscopy (not scaled)....	38
Figure 3.1	:Relative abundances of amino acids from all sequences after 5 biopanning rounds.....	46
Figure 3.2	:Optical and fluorescence microscopy images of samples from 3 main groups, DS202 from strong, DS 150 from moderate and DS 72 from weak group.....	49
Figure 3.3	:Similarity scores of the strong-moderate and weak SiO ₂ binders. A) BLOSUM 62, and b) PAM 250 (The numbers on the parentheses, up: amino acid numbers of each sequence, bottom: total numbers of sequences used for the analysis).....	53
Figure 3.4	:Control experiments with Palladium.....	54
Figure 3.5	:(At left) Unit cell for single crystal quartz showing (100) and (001) planes. (At right) Orientation of the atoms at (100) and (001) planes. Blue dots; Silicon and Red dots; Oxygen Atoms...	56
Figure 3.6	:Relative abundance of the amino acids in all of the selected 12 amino acid peptide sequences for SiO ₂ (100) (gray bars) and SiO ₂ (001) (white bars) crystallographic surfaces.....	58
Figure 3.7	:Similarity scores for both (100) and (001) binders by using BLOSUM 62 (at left) and PAM 250 (at right) matrices (Oren <i>et al.</i> , 2004, in process).....	58

Figure 3.8	:SEM images from the ppt formed in the tube containing DS202 showing the formation of silica spheres.....	59
Figure 3.9	:SEM image of the gel formed after 24 hours in the absence of peptide.....	60
Figure A.1	:Amino acid distribution of the Ph.D.-12 library with expected and observed frequencies (Ph.D.-12 TM , Phage Display Library Kit Manual).....	67
Figure A.2	:N-terminal sequence of random 12-mer peptide-gIII fusion. The fusion is expressed with a leader sequence that is removed upon secretion at the position indicated by the arrow, resulting in the peptide positioned directly at the N-terminus of the mature protein. The hybridization positions of the –28 and –96 primers are indicated (Ph.D.-12 TM , Phage Display Library Kit Manual)..	68
Figure A.3	:Structure of M13KE display vector with cloning sites engineered for N-terminal pIII fusion. (Ph.D.-12 TM , Phage Display Library Kit Technical Manual).....	68

SELECTION AND SIMILARITY ANALYSIS OF QUARTZ SPECIFIC DODECAPEPTIDES BY PHAGE DISPLAY SELECTION PROTOCOL

SUMMARY

In nature, many organisms form organic-inorganic hybrid systems in their hard tissues by binding inorganic materials with polypeptides/proteins. These inorganic-binding proteins are highly effective in formation of highly ordered micro and nano-structures *in vivo* to have excellent functions such as forming protective layers, supportive tissues, transferring charge and ion, developing some optical and mechanical properties. Same kind of excellent functions by using any inorganic material we desire can be obtained for applications in nano- and biotechnology.

Phage Display as a combinatorial biology based molecular library method is one of the approaches for the selection of inorganic-binding polypeptides. The selected genetically engineered polypeptides for inorganics (GEPIs) offer a novel way of using biomolecular linkers in the synthesis and assembly of materials for use in nano- and bionanotechnology.

Here, (100) oriented single crystal quartz (SiO_2) was used as substrate for the selection of quartz-specific 12-aminoacid polypeptides from PhD-12 phage display library. As the second most abundant mineral of the earth's crust, silica is a highly valued element due to the wide range of industrially produced materials. Chemical synthesis of many useful silica-based materials like resins, molecular sieves, and catalysts requires extreme conditions of temperature, pressure and pH. All these limitations can be overcome by using phage display selected polypeptides at ambient conditions. The availability of phage display selected silica-binding proteins from the literature and also presence of the natural silica binding proteins called silicateins and silaffins, the newly selected quartz binding sequences was considered to provide a basis for biofabrication applications via molecular biomimetics approach.

5 biopanning rounds were carried out to obtain totally 50 sequences, 10 sequences from each round. Here, we show the necessity of detailed analysis of all sequences that were selected through the phage display rounds. Before doing binding experiments, first, all the sequences were analyzed on sequence and amino acid base to search for any trend along the sequences or any convergence to specific sequence or an amino acid group. As the first result, we show the sequences with hydrophobic amino acids were being selected through the rounds.

As the next step, Leica-fluorescence microscopy was used to test the binding affinities of each sequence and grouping the sequences mainly as good, moderate or weak binders. Although the measurement of the binding coverage of the sequences under the fluorescence microscopy was semi-quantitative, it seems to be the first and easiest experimental step to characterize the binding affinities of the clones.

To verify the grouping of the sequences as strong, moderate and weak binders, the similarity score analysis was made by using a dynamic programmatic method using BLOSUM 62 and PAM 250 scoring matrices. The results indicate that the self-

similarity scores of peptides for inorganics is relatively higher among the strong binders while it is very low between the weak binders verifying the distribution of the sequences into three groups; strong, moderate and weak binders.

Cross specificity experiments were carried out to test the specificities of each clone by using palladium. The results showed that only very few of the binders had some degree of affinity for Palladium indicating that the most of the sequences selected have high specificity for the quartz surface.

To understand the effect of surface orientation, (001) oriented single crystal quartz pieces were used for another phage display experiment. The selected sequences were compared with the sequences for (100) oriented quartz.

The best binder (DS202) for (100) oriented quartz was synthesized commercially and used for silica formation experiments. The results showed that in the presence of quartz binder, silica nanospheres were formed in silicic acid solution while in the absence of peptide there was no formation of silica.

FAJ GÖSTERİM SEÇİM TEKNİĞİ İLE QUARTZA BAĞLANAN DODEKAPEPTİTLERİN SEÇİLMESİ VE BENZERLİK ANALİZİ

ÖZET

Doğada birçok organizmanın sert dokularında, polipeptit ve proteinlerin inorganik materyallere bağlanması ile organik-inorganik hibrid sistemleri oluşturulur. İnorganiklere bağlanan bu proteinler, canlı organizma içerisinde düzenli mikro ve nano-yapıların oluşturulmasında oldukça etkilidirler. Oluşturulan bu yapılarla organizma, koruyucu tabakalar kurma, destek dokuları oluşturma, yük ve iyon transferi sağlama, bazı optik ve mekanik özellikler geliştirme gibi kendisi için son derece yararlı fonksiyonlar elde eder. Benzer fonksiyonlar, arzu ettiğimiz herhangi bir inorganik materyal kullanılarak nano ve biyoteknolojide kullanılmak üzere elde edilebilir.

Faj gösterim seçim tekniği, inorganiklere bağlanan polipeptitlerin seçilmesi için kullanılan kombinatoryal biyoloji tabanlı bir moleküler kütüphane yöntemidir. Seçilen genetik olarak değiştirilmiş polipeptitler (GEPI), nano ve biyonanoteknoloji uygulamalarında materyallerin sentezlenmesi amacıyla biyomoleküler bağlayıcı olarak kullanılabilirler.

Quartza bağlanan 12 amino asitlik polipeptitlerin PhD-12 faj gösterim kütüphanesinden seçilmesi için, (100) oryantasyonlu basit kristal yapıdaki quartz (SiO_2) inorganik materyal olarak kullanıldı. Dünya yüzeyinde en çok bulunan minerallerden biri olarak silika, geniş endüstriyel kullanım alanlarından dolayı çok değerli bir materyaldir. Resin, moleküler elek ve katalizör gibi silika kaynaklı birçok yararlı materyalin kimyasal yolla üretilmesi yüksek sıcaklık, basınç ve pH gibi aşırı ortamlar gerektirir. Bütün bu sınırlamalar, uygun çevresel ortamlarda kullanılan faj gösterim seçim tekniği ile aşılabılır. Literatürde daha önceden faj gösterim seçim tekniği ile seçilmiş silikaya bağlanan proteinlerin bulunması ve aynı zamanda silicatein ve silaffin diye adlandırılan doğal olarak bazı organizmaların yapısında bulunan silikaya bağlanan proteinlerin varlığı, belli bir kristal yüzeyinde quartz kullanılarak seçilecek peptitlerin biyofabrikasyon çalışmalarına uyarlanmasına yol gösterecektir.

Herbir roundtan 10 tane olmak üzere, toplamda 50 tane peptit dizisi, 5 round sonunda seçildi. Burada biz, faj gösterim seçim tekniği ile seçilen peptitlerin detaylı analizlerinin gerekliliğini gösteriyoruz. İlk iş olarak seçilen bütün peptid dizileri, dizi ve amino asit bazında analiz edilerek herhangi bir bağlanma bölgesini oluşturmaya yönelik bir eğilim olup olmadığına ya da tümünden bir diziye doğru yönelim olup olmadığına bakıldı. Yapılan analizler sonucu, hidrofobik amino asitlere sahip peptitlerin döngü sayısı arttıkça daha fazla seçildiği gözlemlendi.

Bir sonraki adım olarak, herbir dizinin quartza bağlanma dereceleri, Leica-florasan mikroskop kullanılarak yapılan bağlanma deneyleri ile test edildi. Deneyler sonunda bütün diziler, iyi, orta ve zayıf bağlananlar olmak üzere 3 ana gruba ayrıldılar. Florasan mikroskobu ile alınan görüntülerden yola çıkılarak bağlanma yüzdelerinin

hesaplanması ve bu yüzdelere göre dizilerin gruplara ayrılması, yarı kantitatif olmasına rağmen, bu yöntem en kolay deneysel teknik olarak karşımıza çıkıyor.

Yapılan gruplamanın doğrulanması için, BLOSUM 62 ve PAM 250 matrisleri kullanılarak elde edilen dinamik programlama teknikleri kullanıldı ve diziler arasında benzerlik puanları hesaplandı. Sonuç olarak iyi bağlananlar arasındaki benzerlik skorlarının, zayıf bağlananlar arasındaki benzerlik puanlarına göre daha yüksek olduğu ortaya çıktı. Bu da, yapılan gruplamanın doğruluğunu ve iyi bağlananların birbirleri ile daha benzer diziler olduğunu gösterdi.

Paladyum kullanılarak yapılan bağlanma deneyleri ile herbir dizinin seçildikleri malzemeye özgünlüğü test edildi. Quartz bağlanan dizilerden sadece çok az bir kısmı aynı zamanda paladyuma da bağlanma göstererek, seçilen dizilerin büyük çoğunluğunun yüksek seçiciliğe sahip olduğu gösterildi.

Kullanılan inorganic materyalin yüzey oryantasyonunun etkisini anlamak için, (001) oryantasyonlu yüzeye sahip quartz kullanılarak yeni peptit dizileri seçildi ve seçilen diziler (100) oryantasyonlu kuartz için seçilen dizilerle karşılaştırıldı.

Son olarak, seçilen diziler arasında eniyi bağlanan dizi olarak ortaya çıkan DS202 dizisi, ticari olarak ürettirildi ve silika oluşturma deneylerinde kullanıldı. Silisik asit çözeltisine katılan peptit, nano boyutlu kristal silika oluşmasını sağlarken, peptitin eklenmediği tüpte hiçbir silika oluşumu gözlenmedi.

1. INTRODUCTION AND BACKGROUND

1.1 Inorganic-binding Polypeptides and Their Importance in Organisms

As the main building material of the cell, proteins do most of a cell's work. Through their unique and specific interactions with other macromolecules and inorganics, they control the formation of cellular structures and functional activities in the biological tissues. Soft (Kaplan *et al.*, 1994) and hard (Lowenstam and Weiner, 1989) tissues, as the main biological tissues in which the proteins do most of the work, have very organized structure from molecular to the nanoscale, often in a hierarchical manner, with very complex nano-architectures and infinite number of functional diversity. Soft tissues are the biomaterials containing only organic part as in the cases of muscle, membranes, skin, tendon, spiders' silks, and cuticles and they are of great interest in soft tissue engineering (Kaplan *et al.*, 1994, Sakiyama and Hubbell, 2001). On the other hand, hard tissues contain organic-inorganic hybrid systems (Lowenstam and Weiner, 1989, Mann, 1996) such as bones, dental tissues (i.e., dentine and enamel), spicules, spines, shells, skeletal units of single-celled organisms (e.g., radiolarian) or plants, bacterial thin film, and nanoparticles (Lowenstam and Weiner, 1989, Mann, 1996). In all these hard tissues, in addition to inorganic material, a proteinaceous phase is a common structural property. "Figure 1.1" shows examples of organic-inorganic hybrid material formations in some species. All these examples of hybrid systems are coming from the organisms which have naturally found the ways to use some of their proteins to produce and bind the inorganic materials *in vivo*, and then use these excellent systems for their benefit. In this evolutionary process, the inorganics produced *in vivo* has been used effectively to form highly ordered micro and nano-structures performing excellent functions such as forming protective layers, supportive tissues, transferring charge and ion, developing some optical and mechanical properties.

All these biological tissues are synthesized under genetic control of the organism in aqueous environments under mild physiological conditions by using main biomacromolecules, primarily proteins. By both collecting and transporting the raw materials, and forming self- and co-assembly subunits into short and long range ordered nuclei and substrates, proteins are indispensable part of all biological tissues (Sarıkaya and Aksay, 1995, Mann, 1996, Lowenstam and Weiner, 1989).

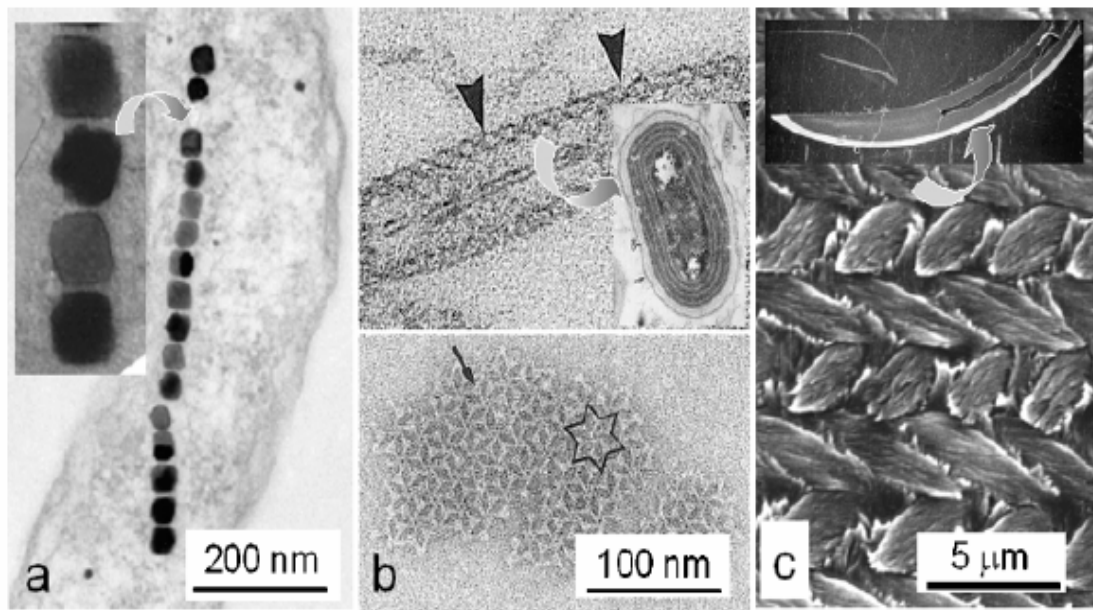


Figure 1.1 Examples of biologically synthesized organic-inorganic hybrid materials with a variety of physical properties: (a) Single-crystalline, single-domained magnetic magnetite nanoparticles (Fe_3O_4) formed by a magnetotactic bacterium (*Aquaspirillum magnetotacticum*) (inset: higher magnification image of the magnetite nanoparticles revealing cubo-octahedral particle shape). (b) S-layer bacterium, *Synechococcus* strain GL24, has a nanostructurally ordered thin film calcite on its outer layer serving as a protective coating. (c) Hard, wear-resistant tooth enamel of mouse consists highly ordered micro/nano architecture of hydroxyapatite crystallites that assemble into a woven rod structure (SEM image). Each rod is composed of thousands of hydroxyapatite particles (inset: cross-sectional image of a mouse incisor; white region is enamel, backed by grayish dentine) (Sarıkaya *et al.*, 2003).

By taking lessons from biology and observing the functions of the proteins in nature, now we can genetically engineer polypeptides to specifically bind to selected inorganic compounds to form hybrid materials. The aim here is to reach the same kind of excellent functions using any inorganic material we desire as it happens in hard tissues naturally, for applications in nano- and biotechnology.

1.2 Applications of Inorganic-binding Polypeptides in Nano and BioTechnology

For decades, traditional approaches try to overcome the limitations of nano-technological systems. The difficulty in the synthesis of nano-technological systems and their assembly into useful functional structures and devices prevents us to benefit from the full potential of nanotechnology in sciences and technology. Synthesis of nanoscale materials by traditional approaches such as melting and solidification processes, followed by thermo-mechanical treatments, or solution/vacuum deposition and growth processes (DeGarmo *et al.*, 1988), is inefficient, requiring stringent conditions such as high heat and pressure, and often producing toxic byproducts (Niemeyer, 2001, Sarıkaya *et al.*, 2003). Even most advanced micro and nanotechnology processes have the limitations of large scale synthesis of complex nano-scale architectures. The problem of scaling-up in nanotechnology is still standing against traditional approaches. The product is so small in quantity and not reproducible because of nonspecific interactions and uncontrolled agglomeration. Even today's one of the most successful nano-technological systems, carbon nano-tubes, have the same kind of limitations such as uncontrolled surface chemistry, and difficulties in multidimensional assembly for the widespread use (Harris, 1999).

For the control and production of large scale nano-structures, practical strategies are needed (Schmid, 1994). Observing and analyzing the examples from the nature can show the way to find those strategies. As exemplified in Figure 1.1, the nature itself produces ordered and complex nanostructures where inorganic materials are synthesized in aqueous environment under mild conditions such as ambient temperature, pressure and neutral pH. Magnetite (Fe_3O_4) particles in magnetotactic bacteria or teeth of chiton (Frankel and Blakemore, 1991); silica (SiO_2) as skeletons of radiolarian (Lowenstam and Weiner, 1989) or tiny light-gathering lenses and optical wave guides in sponges (Fong *et al.*, 2000); hydroxyapatite ($\text{Ca}_2\text{C}(\text{OH})_3$) in bones (Glimcher and Nimni, 1992) and dental tissues of mammals (Paine *et al.*, 2000); calcium carbonate (CaCO_3) in the shells of mollusks as armor (Mayer and Sarıkaya, 2002) or as thin protective films in some species of S-layer bacteria (Schultze *et al.*, 1992); and spines and tests of sea-urchins (Lowenstam and Weiner, 1989) are all examples of natural occurring

nanostructures and hard tissues where a proteinaceous phase is active in the synthesis (Sarikaya, 1999). In all these examples, proteins are the workhorses that control the fabrication of biological structures by orchestrating the assembly of materials in two and three dimensions.

1.3 Obtaining Inorganic-binding Polypeptides

There are several possible ways to obtain the proteins or polypeptides, specific to inorganic substances. A number of proteins may bind to inorganics, although they are rarely tested for this purpose. One naturally occurring example is ice-binding (antifreeze) proteins. These proteins are synthesized in many fish species, plants, and insects (Liou *et al.*, 2000). By binding to ice in the internal fluids of the organisms, these proteins control particle size, morphology, or disruption. Other than ice-binding protein, the organisms in Figure 1.1 have organic-inorganic hybrid systems where an inorganic-binding peptide is present. The proteins in these organisms are used to form inorganic material *in vivo* and then the organism uses these inorganics for its benefit.

First way of having inorganic-binding polypeptides is to extract biomineralizing proteins from hard tissues, followed by purification and the cloning of their genes. Several proteins isolated in this fashion have been used as nucleators, growth modifiers, or enzymes in the synthesis of certain inorganics (Cariolou and Morse, 1988, Paine and Snead, 1996, Berman *et al.*, 1988, Weizbicki, 1994, Kroger *et al.*, 1999, Cha *et al.*, 1999). Amelogenins in mammalian enamel synthesis (Paine and Snead, 1996); silicatein, in sponge spicular formation (Cha *et al.*, 1999); and calcite- and aragonite-forming polypeptides in mollusk shells (Cariolou and Morse, 1988, Berman *et al.*, 1988, Weizbicki *et al.*, 1994) are the examples for that case. Extraction and further analysis have the limitations which makes necessary to find other ways for obtaining inorganic-binding polypeptides. Firstly, extracting proteins is difficult and time consuming. Secondly, hard tissues usually contain multiple proteins that have different roles in biomineralization and are spatially and temporally distributed in complex ways. For instance, more than 20 known proteins have been implicated in the synthesis of human enamel (Paine and Snead, 1996), and over 10 polypeptides have been identified in mollusk shells (Paine and Snead, 1996). After extraction of the proteins, they must be

analyzed and the proteins responsible from the binding to inorganic material must be characterized. Thirdly, 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. Limited number of known inorganic-binding polypeptide is one of the main drawbacks of this methodology.

Other than naturally occurring proteins, inorganic-binding peptides could also be designed using a theoretical molecular approach similar to those employed for the design and development of pharmaceutical drugs (Schneider and Wrede, 1998, Schonbrun *et al.*, 2002). But that is currently impractical and too expensive for current materials research.

1.4 Display Technologies

There is an emerging consensus that the preferred approach for obtaining inorganic-binding polypeptides is to use combinatorial biological techniques (Sarikaya *et al.*, 2003, Brown, 1997, Whaley *et al.*, 2000, Gaskin *et al.*, 2000, Naik *et al.*, 2002). In this approach, a large, random library of peptides with the same number of amino acids, but of different sequence compositions, is screened to identify specific sequences that strongly bind to a chosen inorganic material surface (Naik *et al.*, 2002, Schembri *et al.*, 1999, Brown *et al.*, 2000, Whaley *et al.*, 2000, Naik *et al.*, 2002). There is no need for a priori knowledge of the desired specific polypeptide or single amino acid. The specific polypeptide sequence is simply selected and enriched. Phage display (PD) (Smith, 1985, Hoess, 2001) and cell-surface display (CSD) (Wittrup, 2001) are well-established *in vivo* display techniques for the isolation of polypeptides capable of binding inorganic materials with high affinity. They have been used to screen and identify various biological activities, such as catalytic properties or altered affinity and specificity to target molecules in many applications including the design of new drugs, enzymes, antibodies, DNA-binding proteins and diagnostic agents (Rosander *et al.*, 2002, Petrouna *et al.*, 2000, Smith and Petrenko, 1997, Benhar, 2001). Ribosomal and messenger RNA display technologies are the main *in vitro* methods (Amstutz *et al.*, 2001) which have been developed for increased library size (10^{15}) compared to those of *in vivo* systems (10^7 – 10^{10}).

Figure 1.2 simply presents the procedure for *in vivo* display techniques, PD and CSD, in which libraries are generated by inserting randomized oligonucleotides within certain genes encoded on phage genomes (Whaley *et al.*, 2000, Naik *et al.*, 2002) or on bacterial plasmids (Brown, 1997, Schembri *et al.*, 1999, Brown *et al.*, 2000) (step 1 in Figure 1.2). As a result, a random polypeptide sequence is incorporated within a protein residing on the surface of the organism such as the coat protein of a phage or an outer membrane or flagellar protein of a cell; (step 2). Each phage or cell produces and displays a different, but random peptide (step 3). The library which has a heterogeneous mixture of recombinant cells or phages is exposed on the inorganic substrate (step 4). Non-binder cells or phages are eliminated by several washing cycles by disrupting weak interactions with the substrate and the cells or phages (step 5). The next step is taking the binders out by eluting them from the surface (step 6). In PD, the eluted phages are amplified by reinfecting the host (step 7); on the other hand, in CSD which has only one host cell type, cells are allowed to grow (steps 7, 8). This is the end of a round of biopanning which is generally repeated 3-5 times to enrich for tight binders. Finally, individual clones are sequenced (step 9) to obtain the amino acid sequence of the polypeptides which bind to the target substrate material. In CSD, outer membrane proteins, lipoproteins, fimbria and flagellar proteins have been used so far for heterologous surface display on bacteria. In PD, most of the research has been performed using filamentous phages such as M13 or the closely related fd and f1. Random peptide libraries have been displayed on bacteriophages T7, T4 and λ , but these systems are not yet used on a routine basis. PD and CSD which have been used so far mainly for identifying the protein-protein interactions need to be investigated and optimized in the selection of inorganic-surface-binding polypeptides (Smith, 1985, Hoess, 2001, Wittrup, 2001, Rosander *et al.*, 2002, Petrouna *et al.*, 2000, Smith and Petrenko, 1997, Benhar, 2001).

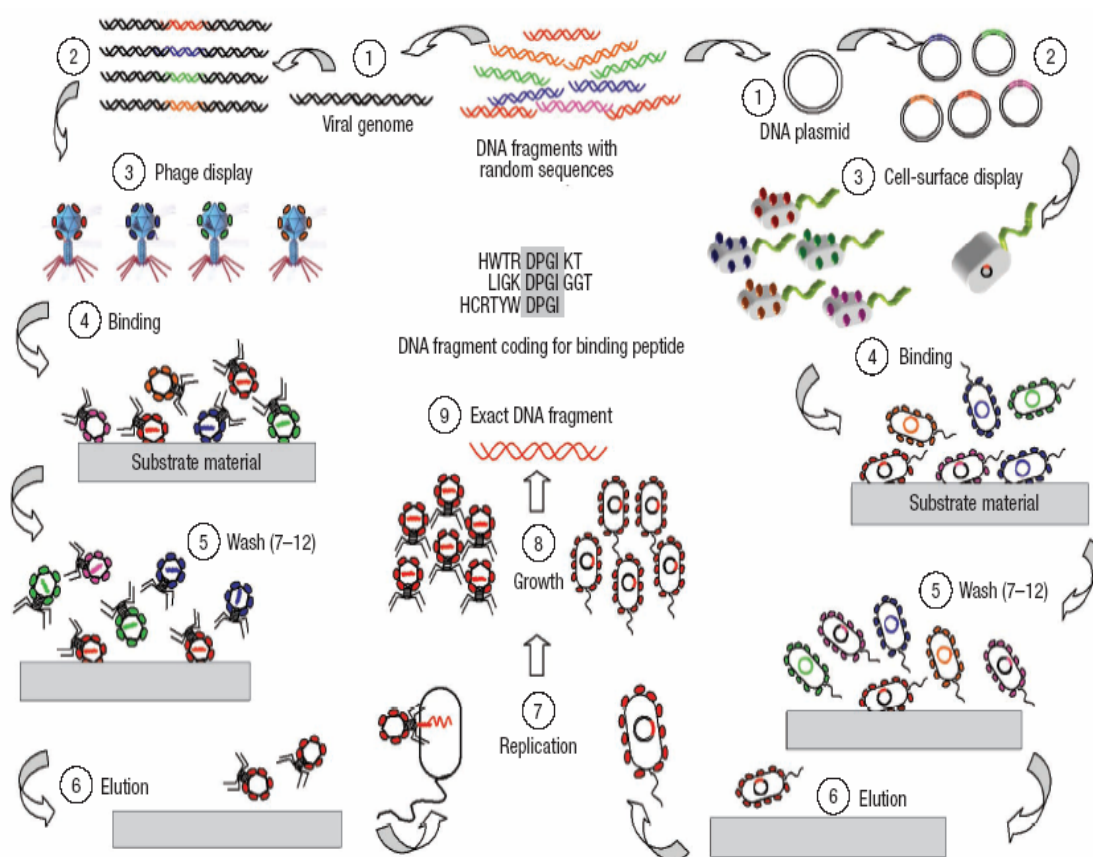


Figure 1.2 Phage display and cell-surface display. Principles of the protocols used for selecting polypeptide sequences that have binding affinity to given inorganic substrates (Sarıkaya *et al.*, 2004).

So far, CSD has been used to identify peptides that recognize iron oxide (Brown, 1992), gold (Naik *et al.*, 2002), zinc oxide (Kiargaard *et al.*, 2000), zeolites (Nygaard *et al.*, 2002), and cuprous oxide (Nygaard *et al.*, 2002), whereas PD has been employed to isolate sequences binding to gallium arsenide (Whaley *et al.*, 2000), silica (Naik *et al.*, 2002), silver (Naik *et al.*, 2002), zinc sulfide (Lee *et al.*, 2002), calcite (Li *et al.*, 2002), cadmium sulfide (Mao *et al.*, 2003), and noble metals such as platinum and palladium (S. Dincer, C. Tamerler & M. Sarıkaya, unpublished data). Even some of these selected peptides have been used to assemble the formation of inorganic particles (i.e., biofabrication, synthesizing, and controlling the nucleation and growth) (Whaley *et al.*, 2000, Naik *et al.*, 2002, Lee *et al.*, 2002, Mao *et al.*, 2003, Brown *et al.*, 2000).

1.5 Adaptation of Phage Display for Selection of Inorganic-binding Polypeptides

To use display techniques for selecting inorganic-binding polypeptides, we have to adapt the procedure which is recommended for the proteinaceous substrate-ligand interaction and optimize it for the organic-inorganic interaction. The problems such as the oxide layer which is formed on surfaces of many materials, chemically or physically changing the surface due to the buffers or biological media used for the biopanning rounds, need to be solved. When the library faces with different crystallographic surface other than the intended one due to the changes on the surface, not the true binders will be selected for that inorganic substance. It is therefore very crucial to use spectroscopic and imaging techniques to characterize inorganic surfaces before and after biopanning rounds to detect any change on the surface (Dai *et al.*, 2004). Effect of wash or elution buffers on the surfaces can possibly be checked by monitoring via using atomic adsorption spectroscopy and XPS to detect metals and metalloids. If evidence of surface modification or deterioration is obtained, buffer conditions should be optimized to guarantee compatibility with the target inorganic. The mechanism of interaction between peptide and the inorganic surface may rely on shape complementarities, electrostatic interactions, van der Waal's interactions or various combinations of these mechanisms. To understand these mechanisms, all parameters effective on the binding must be well studied. Affect of substrate size and shape, question marks on the library diversity, enrichment of some sequences during the biopanning rounds, changing levels of stringency and yield efficiencies, substrate specificities of sequences isolated from different rounds need to be carefully characterized and the procedure be optimized in that way. Various forms of inorganics, from polydisperse and morphologically uncharacterized powders to single crystals makes necessary to find optimum experimental procedures for each different material type. The effect of the conditions in the rounds on the biological materials, phages or bacteria, need also be optimized. PD is suitable for work with powders even if a gradient centrifugation step is used to harvest complexes between binding phages and particles in contrast to CSD using flagellar bacteria in which centrifugal forces would shear off the flagella from the cell.

1.6. Molecular Biomimetics

Combination of traditional physical and biological fields brings us a new field called molecular biomimetics. The ability of recognition and specifically binding to inorganics makes the proteins to form inorganic-organic hybrid materials which form the base for this new field (Sarıkaya *et al.*, 2003, Ball, 2001). Display technologies are used for the selection of inorganic-binder polypeptides specific for any desired substance. Three basic solutions are offered by molecular biomimetics for the problem of control and fabrication of large-scale nanostructures and ordered assemblies of materials in two and three dimensions as schematically. First solution is designing the protein templates through genetics at the molecular level. This is DNA-based technology and inorganic binding peptides and proteins are selected and designed. The designed protein template is the result of the DNA which codes for that protein. Secondly, synthetic entities, including nanoparticles, functional polymers, or other nanostructures can be bind onto molecular templates where the molecular and nanoscale recognition by the use of surface specific proteins are effective. In this step, the protein template is the linker or in other words “molecular erector sets” to join synthetic entities and the inorganic surface which the protein template is specific.

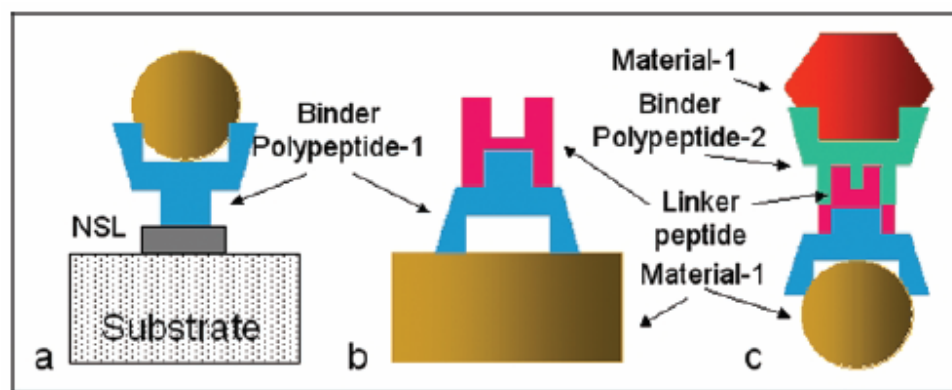


Figure 1.3 Potential uses of inorganic-binding polypeptides. (a) linkers for nanoparticle immobilization, (b) functional molecules that assemble on specific substrates, and (c) heterofunctional linkers involving two (or more) binding proteins adjoining several nanoinorganic units. NSL, nonspecific linker (Sarıkaya *et al.*, 2004).

Thirdly, complex nano-, and possibly hierarchical structures, similar to those found in nature (self-assembly) can be formed by using the ability of biological molecules to self-

and co-assemble into ordered nanostructures. The ultimate goal of molecular biomimetics is to generate a molecular erector set in which different proteins, each engineered to bind to a specific surface, size, or morphology of an inorganic compound, promote the assembly of intricate, hybrid structures composed of inorganics, proteins, and even functional polymers (Sarıkaya *et al.*, 2003). Some potential uses of inorganic-binding proteins are exemplified in Figure 1.3.

1.7 Selection of Inorganic Target Material for Biopanning Steps

1.7.1 Quartz as the Substrate

Quartz has been selected as the inorganic material for the biopanning steps. It has the same formula (SiO_2) as silica. The discriminating point between quartz and silica is the single crystal structure of quartz with ordered distribution of silicon and oxygen atoms in the structure, in contrast to amorphous silica in which the atoms distributed randomly showing no regular order. Silicon (Si), the second most abundant element of the earth's crust, is a highly valued element due to the wide range of industrially produced silica-based materials. Chemical synthesis of many useful silica-based materials like resins, molecular sieves, and catalysts requires extreme conditions of temperature, pressure and pH, as well as non-aqueous solvents, and highly ordered structures are still challenging to obtain. In literature, both combinatorily-selected silica binder polypeptides and binders those extracted from hard tissues have been reported recently. Silica-binding peptides, silaffins and silicateins, which have been isolated from diatom cell wall (Kroger *et al.*, 1999) and a marine sponge (Shimizu *et al.*, 1998, Weaver and Morse, 2003), respectively, are the examples of naturally occurring silica-binder polypeptides.

Widely use of silica-based materials in industry and technology, limitations of producing these materials and the presence of natural occurring and phage display selected silica-binders make quartz a good choice as an inorganic material to start searching peptide binders.

1.7.2 Silaffins

Kroger *et al.* (1999) extracted the silica-precipitating peptides, called silaffins, from the cell wall of a diatom, *Cylindrotheca fusiformis*, which are unicellular algae present in marine and fresh water habitats. In these organisms, biosilicification proceeds at ambient temperatures and pressures, producing an amazing diversity of nanostructured frameworks. In Figure 1.4, the structure of *C. fusiformis* with the silica spheres can be seen clearly.

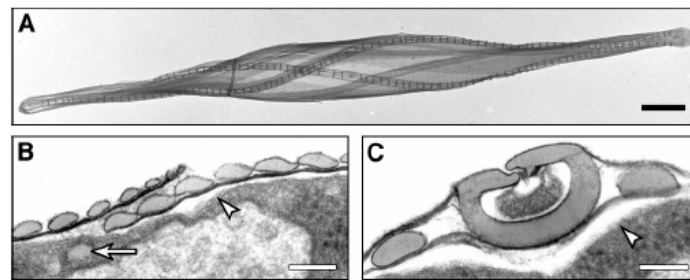


Figure 1.4 (A) Cell wall of *C. fusiformis* has rows of parallel silica strips running in a helical mode along the longitudinal axis of the cell. Bar: 2.5 μ m. (B and C) Details of a *C. fusiformis* cell in cross section. The arrowheads indicate the position of the plasma membrane and point toward the extracellular space. Bars: 100 nm. (B) Lateral region. Each oval-shaped element represents a single silica strip of the cell wall in cross section. The arrow indicates a nascent silica strip within its SDV shortly before secretion. (C) Valve region. The ringlike structure and the two oval-shaped elements on either side are silicified cell wall elements (Kroger *et al.*, 1999).

Silica is initially deposited in the form of nanoscale spheres, suggesting the presence of components within the diatom cell that control silica sphere formation. The amorphous silica in diatom cell walls is intimately associated with organic substances that have been hypothesized to act as regulating molecules in biosilicification.

Extraction of purified cell walls showed the presence of polypeptides which have affinity to silica, thus they are named as silaffins. Silaffins were fractionated into three components as silaffin-1A, silaffin-1B and silaffin-2. They have high degree of homology and each of them is able to form silica within seconds when added to a freshly prepared solution of metastable silicic acid as they act to form silica nano spheres *in vivo*, in the cell wall of diatom (Figure 1.5). A 265 amino acid long polypeptide from N-terminus of silaffin-1B, which is called sillp, was analyzed further (Figure 1.6). It is

composed of seven highly homologous repeating units (R1 to R7). It has been shown that sil1p has very high homology with some parts of N-terminus of silaffin-1A.

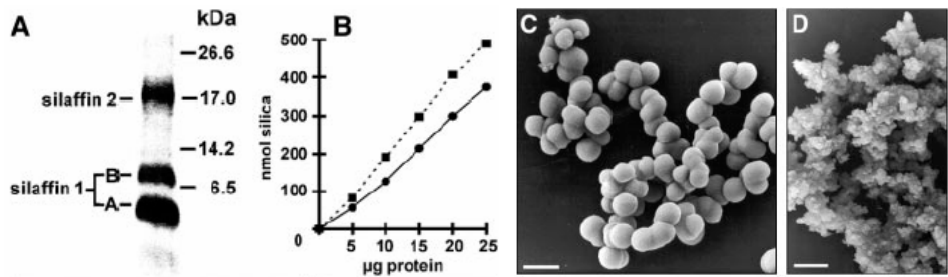


Figure 1.5 Silica precipitation induced by silaffins (14). (A) Low molecular mass fraction of HF extract from *C. fusiformis* cell walls. The extract was subjected to Tricine–SDS–PAGE (13) and stained with Coomassie blue. (B) Correlation between silaffin concentration (27) and the amount of silica precipitated from a silicic acid solution. The dotted line represents the result obtained for the silaffin mixture; the solid line shows the result for pure silaffin-1A (15). (C and D) SEM micrographs of silica precipitated by silaffin-1A (C) and the mixture of silaffins (D). The diameter of silica particles is 500 to 700 nm (C) and ,50 nm (D). The protein concentration was 5 mg/ml. Bar: 1 mm.(modified from Kroger *et al.*, 1999)

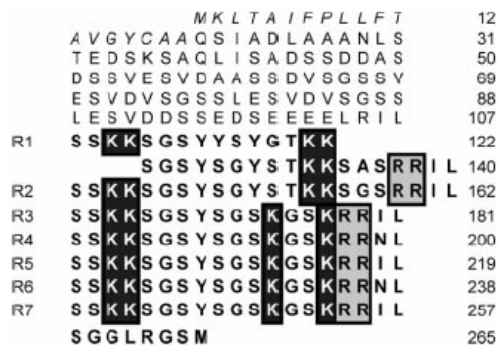


Figure 1.6 Primary structure of sil1p. The signal peptide sequence is shown in italics, the highly acidic peptide sequence (residues 20 to 107) is depicted in regular type, and the mature part of the polypeptide (residues 108 to 271) is shown in bold. The repeats within the mature part bearing silaffin sequences are labeled R1 to R7 on the left margin. Arginine and lysine residues within silaffin sequences are highlighted (modified from Kroger *et al.*, 1999).

1.7.3 Silicateins

In a similar study, Weaver and Morse (2003) extracted and characterized silica binding proteins, called silicateins, from needle like spicules of the sponge *Tethya aurantia*. Silica spicules constitute 75% of the dry weight of the sponge and support the organism and provide defense against predation (Figure 1.7). Three silicatein subunits, silicatein α , β , and γ present in the spicules show similar structure with regular repeating array

suggesting that they are members of a single protein family. Silicatein α is shown to belong to the cathepsin L class of the papain-like cysteine protease superfamily, supporting the suggestion that enzymatic activity may be involved in biosilicification in sponges. These three proteins promote the condensation of silica and organically modified siloxane polymers (silicones) from the corresponding silicon alkoxides.

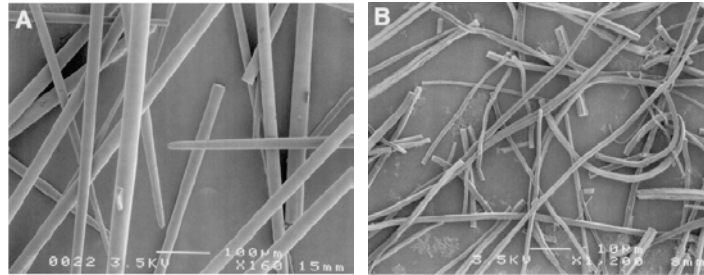


Figure 1.7. Scanning electron micrographs of isolated silica spicules (x130) (A) and axial filaments (x1,000) (B) from *Tethya aurantia*. (modified from Weaver and Morse, 2003)

Protein filaments occluded within these needles catalyze the synthesis of silica and polysiloxanes from the corresponding silicon alkoxides at neutral pH and 20 °C.

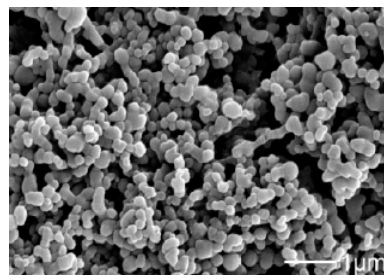


Figure 1.8 SEMs of silica products synthesized from tetraethylorthosilicate (TEOS) at 20°C, neutral pH, and atmospheric pressure. Silica nanoparticles grown in the presence of recombinantly derived silicatein β , the second-most abundant of the *Tethya aurantia* silicateins can be seen (modified from Weaver and Morse, 2003).

1.7.4 Phage Display Selected Silica Binders

On the other hand, silica-binding polypeptides have also been identified by phage display. In the study by Naik *et al.* (2002), phage display was used to select the silica-binding 12-amino acid peptides. They demonstrate that selected silica-binding peptides can be used in precipitating silica from a solution of silicic acid. They first synthesized the target, biogenic silica, using the silaffin-derived R5 peptide as described by Kroger *et al.* (1999). R5 peptide is an 18 amino acid long peptide unit of silaffin-1 precursor

polypeptide and it is able to precipitate silica when added to a freshly prepared solution of hydrolyzed silicic acid. The produced silica particles were washed and then incubated with a combinatorial library of random 12-amino acid peptides for the selection of silica-binding peptides. Figure 1.9 shows the R5 precipitated silica particles and the phage display selected silica-binder peptides.

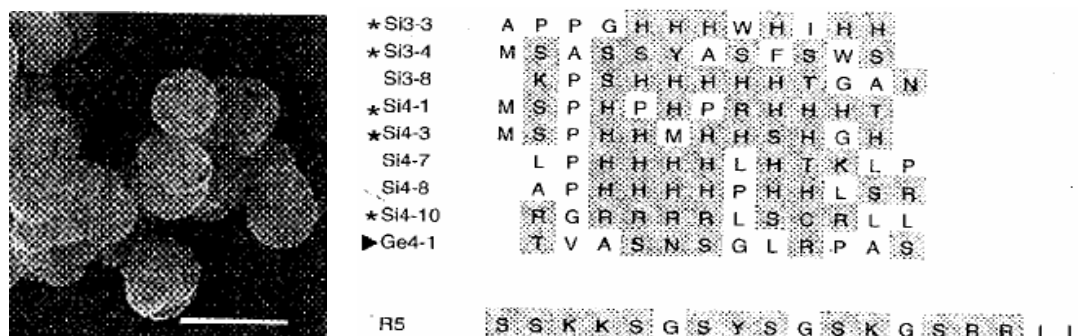


Figure 1.9 (at left) Scanning electron micrograph of biosilica which was formed by the use of R5 peptide; scale bar, 1 μm . (at right) Multiple sequence alignment (with CLUSTALW) of selected silica-binders. Si3-4 means the clone was selected as the fourth clone from the third round. The shaded amino acids have the functional side chains with the ability to interact with the silica surface. Ge4-1 is a germanium-binding peptide and given as the control. R5 peptide is given for the comparison. The peptides with asterisk were isolated more than once (modified from Naik *et al.*, 2002).

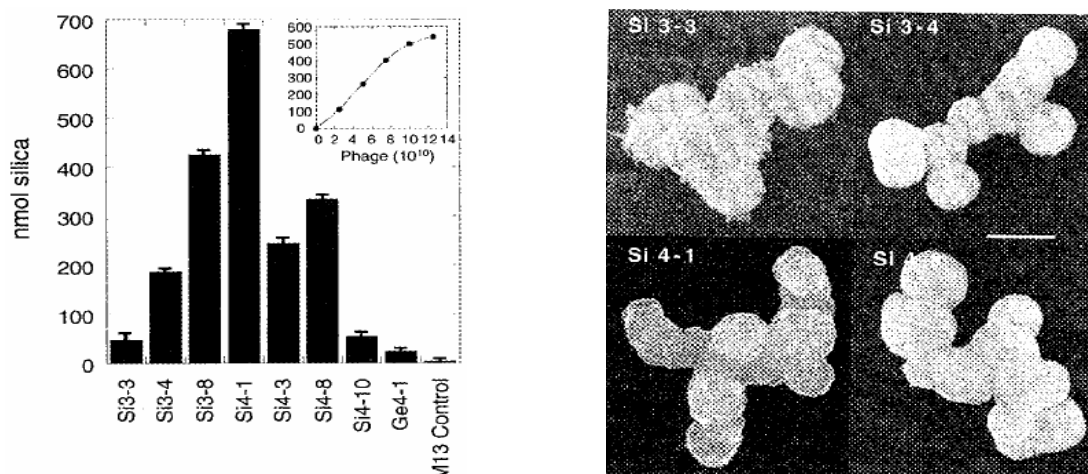


Figure 1.10 (At left) Silica condensation of the phage-binding clones. Equal amounts of phage particles, 10^{11} from each, were incubated with hydrolyzed tetramethyl orthosilicate (TMOS) in Tris-buffered saline (pH 7.5). The silica precipitate was washed and dissolved in 1 M NaOH at 95 $^{\circ}\text{C}$ for 30 min. The amount of silica precipitated from silicic acid was measured with the spectrophotometric molybdate assay and it is proportional to the amount of Si4-1 phage particles added (inset). (At right) SEM micrographs of silica precipitated by the phage peptide clones. The diameter of the silica nanoparticles is between 200 and 400 nm; scale bar, 500 nm. (modified from Naik *et al.*, 2002)

They examined the selected phage peptide clones for silica precipitation activity. Peptides rich in basic and hydroxy amino acids have been shown to exhibit silica-precipitating activity *in vitro*. Figure 1.10 shows the amounts of silica precipitated and the SEM micrographs of the precipitated silica particles.

1.8. Aim of the Study

The aims of the study are as follow; selecting quartz-binding polypeptides by phage display selection method from a Ph.D.-12 Phage Library, detailed analysis of the selected sequences in terms of their binding strength and specificity which would lead to obtain the best binders capable of biomimetic applications. Similarity analysis was applied as a novel approach to verify the binding properties of the selected sequences. Finally, our main emphasis is to be able to use them for technological outputs, here nanosphere silica formation was performed under biological conditions as a proof of principle experiment for bionanotechnological applications by use of the selected quartz binding peptides.

2.MATERIALS AND METHODS

2.1. Materials

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

E.coli ER2738 , F' *lacI^f* $\Delta(lacZ)M15$ *proA⁺B⁺* *zzf::Tn10(Tet^R)*/*fhuA2 supE thi* $\Delta(lac-proAB)$ $\Delta(hsdMS-mcrB)5$ (*r_k⁻ m_k⁻ McrBC⁻*) was used during the studies. It is not competent strain and it was purchased as 50 % glycerol culture within Ph.D.-12™ Phage Display Peptide Library Kit, (2003).



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

2.1.2. Phage Display Peptide Library-Peptide 12-mer Phage Display Library (Ph.D.-12™)

Phage display peptide library is provided in a 100 μ l, with 1.5×10^{13} pfu/ml. It is supplied in TBS with 50% glycerol. It has complexity of $\sim 2.7 \times 10^9$. The library was purchased within Ph.D.-12™ Phage Display Peptide Library Kit, Catalog #E8110S.

Figure 2.2 shows our AFM and TEM images of the phage samples taken from the phage display library with ~ 900 nm in length and ~ 6 nm in diameter.

N-terminal sequence of random 12-mer peptide-gIII fusion and the structure of M13KE display vector with cloning sites can be found in Figure A.2 and Figure A.3 in Appendix.

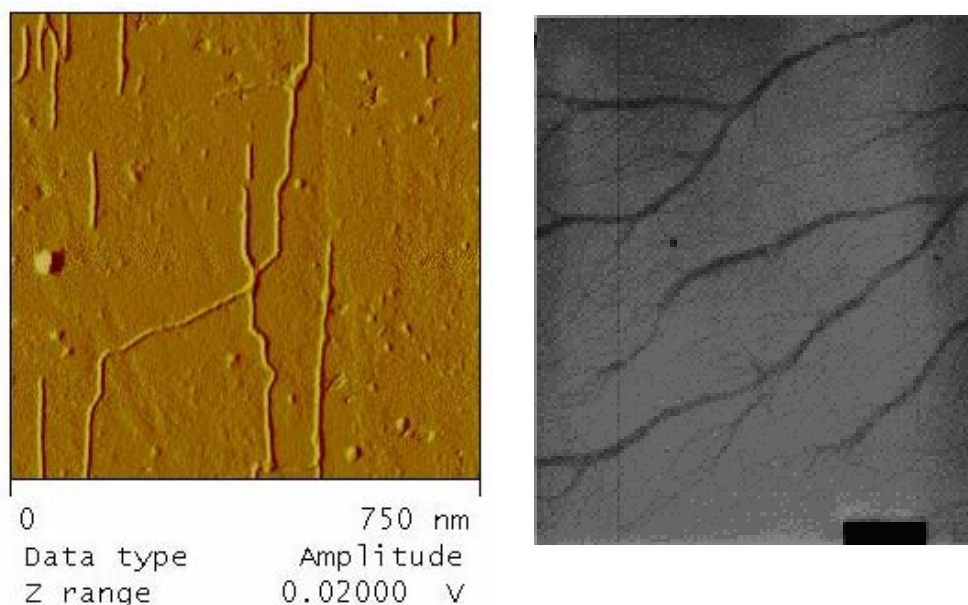


Figure 2.2 (at left) AFM image of M13 phage on the quartz and (at right) TEM image of branches of phages. Bar is 1 μm in TEM image.

2.1.3. Inorganic Targets: (100) and (001) Oriented Quartz Wafers

Single-crystal quartz wafers (University Wafer, Boston) with (100) and (001) atomic planes and chemical formula of SiO_2 were used as the substrate for the biopanning steps. Our X-Ray Diffraction and atomic force microscopy analyses of the substrate wafer showing the purity and the roughness level (5 Angstrom surface roughness) are given in the figure.

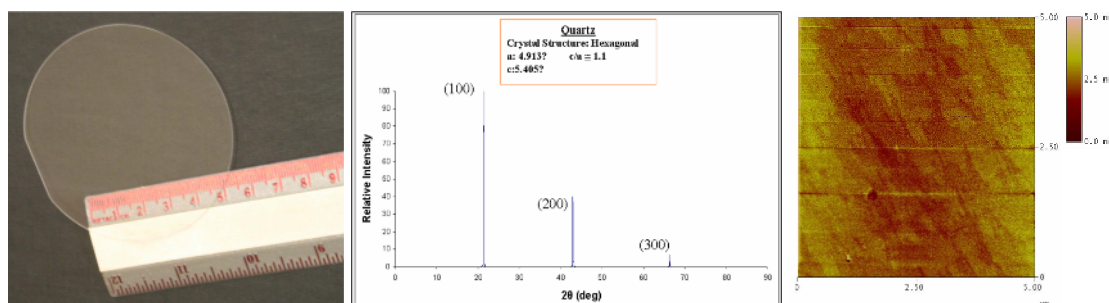


Figure 2.3 On the left, (100) plane quartz wafer as purchased from the company before cutting into the small pieces. In the middle, XRD result of the quartz wafer showing its (100) oriented structure. On the right, AFM image of the quartz wafer showing its surface roughness around 5 $\text{\AA}$.

2.1.4. Bacterial Culture Media

2.1.4.1. Luria Bertani (LB) Medium

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

2.1.4.2. LB Agar Medium

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

2.1.4.3. Top-Agar Medium

10 g tryptone (Acumedia), 5 g yeast extract (Acumedia), 5 g NaCl (Riedel-de-Haen), 1 g MgCl₂ (Riedel-de-Haen), 9 g LMP (Low Melting Point) agarose (Acumedia) were dissolved in distilled water and completed up to 1 lt and sterilized for 15 minutes under 1.5 atm at 121 °C. The medium was stored at room temperature and melted in microvawe as needed to pour onto the LB agar plates.

2.1.4.4. *E. coli* Overnight Culture

5 ml LB solution containing 1 mM MgCl₂ and tetracycline, was inoculated with *E. coli* ER-2738 stock (from -80°C). The culture was left in the shaker overnight at 37°C, 200 rpm.

2.1.5. Stock Solutions

2.1.5.1. Tetracycline-HCl Stock

20 mg/ml tetracycline-HCl (Sigma) was dissolved in distilled water. It was then stored at -20°C at dark to protect from the light. Tetracycline is light-sensitive.

2.1.5.2. Xgal/ IPTG Stock

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

2.1.5.3. Detergent Stock

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

2.1.5.4. Glycerol Stock Solution

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

2.1.5.5. MgCl₂ Stock Solution

1M MgCl₂.6H₂O (Fisher) was dissolved in distilled water up to 100 ml. and sterilized with 0.2 μ m single use syringe filter.

2.1.6 Buffer Solutions

2.1.6.1 PEG/NaCl

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

2.1.6.2. PC (Potassium Phosphate-Sodium Carbonate Buffer)

- **PC (no detergent):** 55 mM KH₂PO₄ (Fisher), 45 mM Na₂CO₃ (Fisher), 200 mM NaCl (Sigma) were dissolved in distilled water up to 500 ml and the solution was

sterilized by using 0.2 μ m single use syringe filter. The pH value was adjusted to 7.2-7.5.

- **PC (containing 0.02% detergent):** 55 mM KH_2PO_4 (Fisher), 45 mM Na_2CO_3 (Fisher), 200 mM NaCl (Sigma), 0.5 ml detergent stock solution were dissolved in distilled water up to 500 ml and the solution was sterilized by using 0.2 μ m single use syringe filter. The pH value was adjusted to 7.2-7.5.
- **PC (containing 0.1% detergent):** 55 mM KH_2PO_4 (Fisher), 45 mM Na_2CO_3 (Fisher), 200 mM NaCl (Sigma), 2.5 ml detergent stock solution were dissolved in distilled water up to 500 ml. and the solution was sterilized by using 0.2 μ m single use syringe filter. The pH value was adjusted to 7.2-7.5.
- **PC (containing 0.5% detergent):** 55 mM KH_2PO_4 (Fisher), 45 mM Na_2CO_3 (Fisher), 200 mM NaCl (Sigma), 12.5 ml detergent stock solution were dissolved in distilled water up to 500 ml. and the solution was sterilized by using 0.2 μ m single use syringe filter. The pH value was adjusted to 7.2-7.5.

Note: PC buffer can not be sterilized because carbonate ions convert to CO_2 due to high pressure in the autoclave. This causes an increasing of pH up to 10.

2.1.6.3. Elution Buffers

- **Elution buffer I:** 0.2 M glycine (Merck) and 1mg /ml BSA (Sigma) were dissolved in distilled water up to 50 ml. and pH was adjusted to 2.2 with 10 M HCl and 0,1M HCl. The solution was sterilized by using 0.2 μ m single use syringe filter.
- **Elution buffer II:** Equal amount of elution buffer A and B are mixed and the solution is sterilized by using 0.2 μ m single use sterile syringe filter.
- **Elution buffer A:** 0.2 M glycine (Merck) and 2 mg /ml BSA (Sigma), 0.02% SDS were dissolved in distilled water up to 50 ml. and pH adjusted to 2.2 with 10 M HCl and 0.1 M HCl. The solution was sterilized by using 0.2 μ m single use syringe filter.
- **Elution buffer B:** 1 M NaCl (Riedel-de-Haen), 100 mM DDT (Sigma), 7 mM TCEP (Sigma), 100 mM ME were dissolved in distilled water up to 50 ml.

2.1.6.4. Tris Buffer

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

2.1.6.5. TBE (Tris / Borate / EDTA) Solution

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

2.1.7. Lab Equipments

AFM: Extended MMAFM coupled with Nanoscope III, Veeco Instruments

Autoclave: Yamato sterilizer SE 510.

Balances: Denver Toledo AB 54

Centrifuge: Sorvall RC 5B Plus Kendro Laboratory Products Eppendorf Centrifuge 5415D

Centrifuge rotors: SA-600, SLA-1500, SH-3000, PN-11779.

Deep freezes and refrigerators: Heto Polar Bear 4410 ultra freezer, JOUAN Nordic A/S, catalog# 003431.

Deionized water: Millipore Milli Q Synthesis A 10

Fluorescence Microscope: Nikon Eclipse TE 2000-U

Glassware: Technische Glaswerke Ilmenau GmbH.

Ice Machine: Cornelius Ice Systems

Incubators: Quincy Lab. Inc. Model 10-14 Incubator VWR 1310

Laminar Flow Cabinet: Airclean 600 PCR Workstation ISC Bioexpress

Orbital shaker: Innova 3100 Water Bath Shaker New Brunswick Scientific

Magnetic stirrer: AGE 10.0164, VELP Scientifica srl.: ARE 10.0162, VELP Scientifica srl.

Pipettes: Pipetteman 1-20-200-1000-5000 ml

pH meter: MP 220, Mettler Toledo International Inc.: Inolab pH level 1, order# 1A10-1113, Wissenschaftlich-Technische Werkstätten GmbH & Co KG.

PCR cycler: Eppendorf Master Cycler Gradient

Power supply: EC 250-90, E-C Apparatus

Sequencer: Applied Biosystems 3730XL

Spectrophotometer: Tecan Safire

Sterilizer: FN 500, Nuve.

TEM: Philips 420 EM

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

Ultrasonic bath: Branson 1200

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

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

X-ray diffraction: PW1820 Diffractometer coupled with PW1830 Generator, Philips

2.2 METHODS

2.2.1. Phage Display Protocol

Phage display is a selection method in which a peptide or a protein is expressed as a fusion within a coat protein of a bacteriophage, resulting in display of the fused peptide on the surface of the virion, while the DNA encoding the fusion resides within the virion. Here, in this study, we used Ph.D.-12 Phage Display Library Kit which is based on a combinatorial library of random peptide 12-mers fused to a minor protein (pIII) of M13 phage. Figure 2.4.a schematically presents the structure of M13 phage with 12-mers peptide which is expressed at the N-terminus of pIII protein. The first residue of the mature protein is the first randomized position. A short spacer (Gly-Gly-Gly-Ser) resides between the randomized protein and the wild type pIII protein and binds them together. The randomized library consists of $\sim 2.7 \times 10^9$ electroporated sequences, amplified once to yield ~ 55 copies of each sequence in 100 μ l of the supplied phage.

2.2.2. Screening Procedure

Adapted and optimized screening procedure for selecting specific inorganic-binding polypeptides is given basically in Figure 2.4.b. In its simplest form, panning is carried out by incubating a library of phage-displayed peptides with target, washing away the unbound phage, and eluting the specifically-bound phages. The eluted phage is then amplified and taken through additional binding/amplification cycles to enrich the pool in favor of binding sequences. After 5 rounds of biopanning, individual clones are selected and sequenced.

The screening protocol is given in the figure 2.4.b and follows as described here;

- 1) PhD-12 library (New England Biolabs, MA) of M13 phage each displaying a different peptide sequence was used as the randomized peptide source.
- 2) (100) oriented single crystal quartz wafer was used as the substrate and cleaned prior to the biopanning rounds in the phage display screening method to select the quartz-specific 12 amino acid polypeptides.

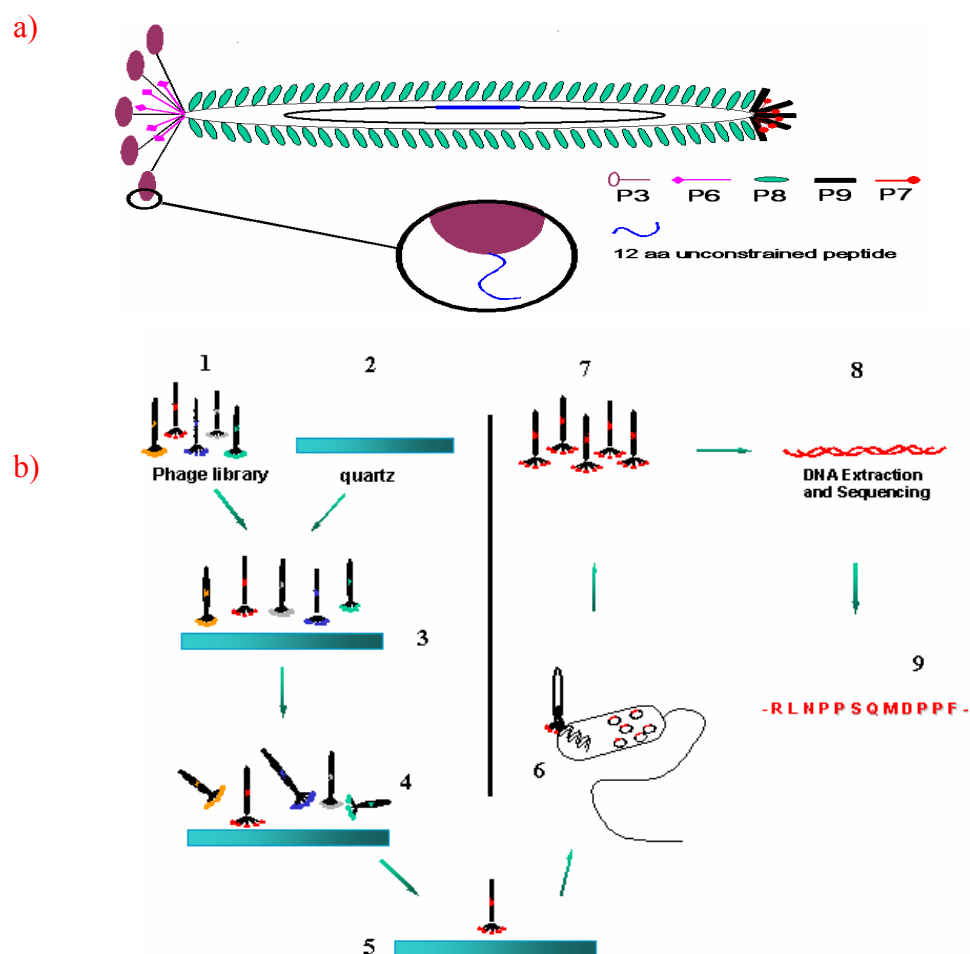


Figure 2.4. a) Structure of M13 phage and b) Phage display procedure, steps 1 through 9

3) The library is exposed to the surface of the substrate in potassium phosphate-sodium carbonate buffer (pH 7.5) containing 0.1% detergent solution (20% Tween80 + 20% Tergitol). The detergent in the buffer is used to reduce the phage-phage interactions and by this way, the phages are expected to interact with the quartz surface individually.

4) Unbound phages are washed away by several washing steps using PC buffer (pH 7.5) containing 0.1% detergent solution. Washing cycles were repeated ten times, each one taking 30 min. The detergent concentration was increased gradually up to 0.5% as the number of the rounds is going up towards to 5.

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

6) The eluted phage pool is amplified with *Eschericia coli* ER2738 host cell which is a robust F⁺ strain with a rapid growth rate and particularly well-suited for M13 propagation. ER2738 is a recA⁺ strain and the F-factor of ER2738 contains a mini-transposon which confers tetracycline resistance. The amplified phages are then plated on Luria Bertani (LB) plates containing 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside (Xgal) and isopropyl- β -D-1-thiogalactosidase (IPTG).

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

2.2.3. Biopanning Steps

2.2.3.1. Cleaning of the Quartz

- A quartz piece with the dimensions of $\sim 0.5 \times 0.5 \times 0.05$ cm is sonicated for 15 min in absolute EtOH.
- The EtOH is discarded and distilled water is added onto the quartz to sonicate the wafer for another 15 min.

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

2.2.3.2. Washing Steps

- One clean piece of quartz was put into microfuge tube and 1 ml PC buffer containing 0.1 % detergent was added into the tube.
- 10 µl of phage display peptide library (Ph.D.-12™) was added into microfuge tube and placed on to running cylinder for 30 min rotation.
- After 30 min rotation, the supernatant was discarded and transferred into microfuge tube which was prelabelled as XQRS1*.
- Quartz wafer was washed for 2 min with 1 ml PC containing 0.1 % detergent.
- The washing steps were repeated 6 times.
- The supernatants which were discarded at the end of the each washing steps were transferred into microfuge tubes which were prelabelled as XQRW1**, XQRW2, XQRW3, XQRW4, XQRW5, and XQRW6.
- After taking the supernatant out, microfuge tube containing quartz and phage peptide library was put on the running cylinder for 30 min rotation.
- The supernatant was discarded and transferred into microfuge tube XQRW7.
- 1 ml PC buffer containing 0.1 % detergent was added onto the quartz wafer and rotated for 30 min.
- The supernatant was discarded and transferred into microfuge tube XQRW8.
- Another 1 ml PC buffer containing 0.1 % detergent solution was added onto the quartz wafer and left on rotation for overnight.
- Following overnight rotation, supernatant was discarded and transferred into microfuge tube XQRW9.
- 1 ml PC buffer containing % 0.1 detergent solution was added onto the quartz wafer and rotated for 30 min on running cylinder.
- The supernatant was discarded and transferred into microfuge tube XQRW10.

- 1 ml PC buffer containing % 0.1 detergent solution was added onto the quartz wafer and rotated for 30 min on running cylinder.

- The supernatant was discarded and transferred into microfuge tube XQRW11.

*: XQRS1: Number of screening, Quartz, Round number (here it is 1), Supernatant Sample 1.

** : XQRW1: Number of screening, Quartz, Round number (here it is 1), Washing Sample 1.

The washing steps were performed to remove the non-specific phage to quartz surface. At the end of the all washing steps, phage which binds to quartz surface must be eluted from the surface. The elution steps come just after the washing steps and follow as written below. One round of biopanning contains washing, elution and purification steps. Basically all elution and purification steps are the same in each round but washing steps are changing as the number of the rounds increases. The detergent concentration in the washing buffers are increased gradually from 0.1% to 0.5% as the round number comes to 5.

2.2.3.3. Elution Steps

Following the washing steps, elution of the good binders from the surface of the quartz piece comes next. During the elution step, the eluted phages must be inoculated with the host bacteria for starting the amplification steps. So, *E. coli* ER2738 liquid cultures for phage–host strain infection and amplification of phage is needed. Just before inoculating the phages with the host bacteria, we need to have LB solution containing host bacteria of *E. coli* ER2738 with the OD₆₀₀ value of 0.5. One night before starting the elution steps, 5 ml LB solution containing 10 µl 1M MgCl₂ (1:500), 5 µl tetracycline (1:1000) is incubated with a single colony which was streak out from the glycerol culture of the bacteria and inoculated LB waits overnight at 37°C and 200 rpm. In the day of elution, 125 µl (1:400) *E. coli* ER2738 overnight culture is used to inoculate a fresh 50 ml LB solution containing 100 µl 1M MgCl₂ (1:500), 50 µl tetracycline (1:1000) in 250 ml glass medium flask. 5 different 50 ml LB in 250 ml shake flasks are prepared for the elution steps. They were incubated until mid-log phase (OD₆₀₀ ~ 0.5) at 37°C and 200 rpm.

- After the last washing step, 1 ml of elution Buffer I is added onto the quartz piece in a fresh microfuge tube and rotated for 15 min on running cylinder to elute the phage from quartz surface.
- The supernatant was transferred into microfuge tube XQRE1*.
- 800 μ l supernatant was transferred into *E. coli* ER2738 culture ($OD_{600} \sim 0.5$) and incubated for 4.5 hours at 37°C and 200 rpm.
- The remaining 200 μ l supernatant was neutralized by adding 40 μ l of tris, pH 9.1
- 1 ml of Elution buffer II was put into microfuge tube containing the quartz piece and put on the running cylinder for 15 min to elute the phage from quartz surface.
- The supernatant was discarded and transferred into microfuge tube XQRE2
- 800 μ l of the supernatant was transferred into *E. coli* ER2738 culture ($OD_{600} \sim 0.5$) and incubated for 4.5 hours at 37°C and 200 rpm.
- The remaining 200 μ l supernatant was neutralized by adding 40 μ l of tris, pH 9.1
- 1 ml of Elution buffer II was put for the second time into microfuge tube.
- Microfuge tube which contains quartz piece and elution buffer II was put on the running cylinder for 15 min to elute the phage from quartz surface
- The supernatant was transferred into microfuge tube XQRE3
- 800 μ l supernatant from XQRE3 was transferred into *E. coli* ER2738 culture ($OD_{600} \sim 0.5$) and incubated for 4.5 hours at 37°C and 200 rpm.
- The remaining 200 μ l supernatant was neutralized by adding 40 μ l of tris, pH 9.1
- 1 ml of Elution buffer II was put, for the third time, into microfuge tube.
- Microfuge tube containing quartz and elution buffer II was put on the running cylinder for 15 min to elute the phage from quartz surface
- The supernatant was transferred into microfuge tube XQRE3
- 800 μ l supernatant from XQRE3 was transferred into *E. coli* ER2738 culture ($OD_{600} \sim 0.5$) and incubated for 4.5 hours at 37°C and 200 rpm
- The remaining 200 μ l supernatant was neutralized by adding 40 μ l of tris, pH 9.1

- As a last step of the elution process, quartz piece itself was put into the LB solution containing *E. coli* ER2738 culture ($OD_{600} \sim 0.5$) and incubated for 4.5 hours at 37°C and 200 rpm

*: XQRE1: Number of screening, Quartz, Round number (here it is 1), Elution Step 1.

2.2.3.4. Purification Steps

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

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

- Samples were centrifuged CW and CCW at 13,500 rpm for 1.5 min to get the compact phage.
- Supernatant was discarded and phage pellet was resuspended with 0.2 ml PC buffer (no detergent) by pipetting gently.
- Samples were centrifuged at 13,500 rpm for 1.5 min.
- Supernatant were transferred into sterilized microfuge tubes and stored at 4°C.

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

2.2.4. Titers of the Selected Phages

2.2.4.1. Blue-white Screening Experiment

At the end of each biopanning round, blue-white screening experiment is carried out to determine the phage titers. Basically, amplified phages are grown in the plates which have Xgal, IPTG and tetracycline. 50 µl Xgal/IPTG from 20 mg/ml stock, 100 µl 1M MgCl₂ (1:500), 50 µl tetracycline (1:1000) were put into 50 ml liquid warm LB agar in 100 ml glass medium flask and it was poured into plastic sterile petri dishes. Plates were wrapped with parafilm and stored at 4°C at dark for a maximum of 1 month. Purified phages need to be serially diluted to estimate phage titers.

2.2.4.2. Preparation of Diluted Phage and Plating

90 µl PC buffer (no detergent) was put into the first well in Elisa plate. 180 µl PC buffer (no detergent) was put into other wells to dilute the sample as in Figure 2.5. 10 µl phage stock solution was put in the first well having 10⁻¹ dilution. Phage and PC buffer were mixed by pipetting. 20 µl sample was taken from the first well and put into the second well to have totally 200 µl of solution mix having 10⁻² dilution level of the phages. The remaining serial dilutions were done the same way as it is labeled in Figure 2.5.

One night before plating, overnight culture was prepared to be used in the day of the plating. 5 ml LB solution containing 10 µl 1M MgCl₂ (1:500), 5 µl tetracycline (1:1000) was incubated with 12.5 µl *E. coli* ER2738 from overnight culture in 50 ml falcon tube. It was incubated until mid- log phase (OD₆₀₀ ~ 0.5) at 37°C and 200 rpm.

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

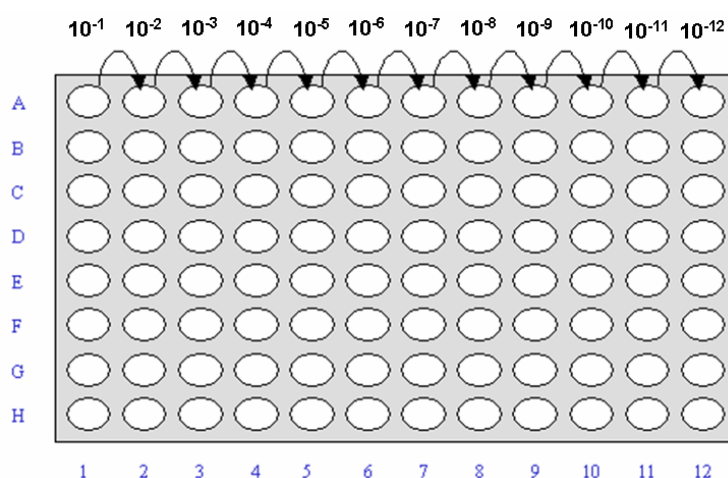


Figure 2.5 Making the serial dilutions on the Elisa plate

20 μ l diluted phage solution is mixed with 180 μ l LB solution containing *E. coli* cells with $OD_{600} \sim 0.5$ resulting one more dilution of the phages. The resulting solution was mixed with warm LB agar (0.5 ml for small Petri dishes and 3.5 ml for big Petri dishes) in 15 ml falcon tubes and vortexed. The phage and bacteria mixture solution was poured onto the plates and waited for 5-10 min for the solidification of the top agar. All petri dishes were inverted and incubated at 37°C for 8-14 hours. After incubation period blue plaques were seen visually on the plate as in figure 2.6.

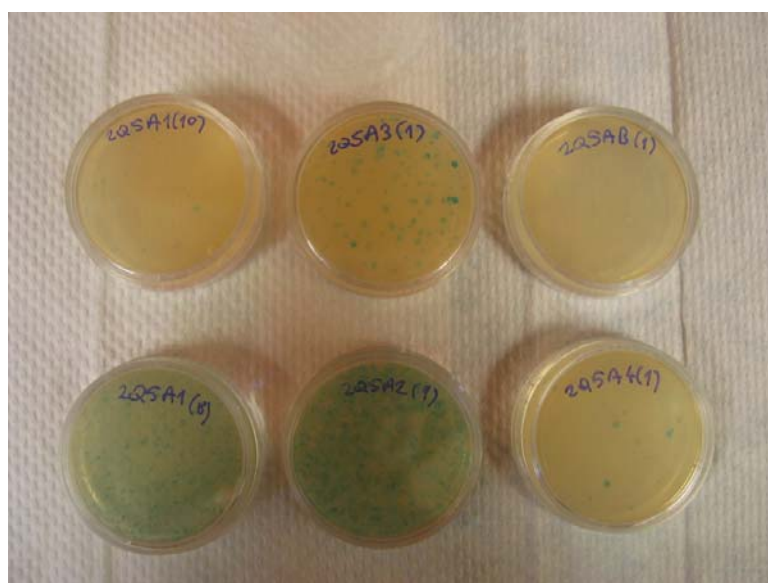


Figure 2.6. Examples of blue-white screening experiment. The Petri dishes are shown presenting the results of different phage titers.

2.2.4.3. Phage Titers for Each Round

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

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

	1 st Round		2 nd Round		3 rd Round		4 th Round		5 th Round	
Elution	D	A	D	A	D	A	D	A	D	A
A1	10 ¹²	100	10 ¹¹	100	10 ¹²	100	10 ¹¹	100	10 ¹²	-
A2	10 ¹	50	10 ¹	50	10 ¹	50	10 ¹	50	10 ¹	-
A3	10 ¹	50	10 ¹	50	10 ¹	50	10 ¹	50	10 ¹	-
A4	10 ¹	50	10 ¹	50	10 ¹	50	10 ¹	50	10 ¹	-
B	10 ¹	50	10 ¹	50	10 ¹	50	10 ¹	50	10 ¹	-

2.2.4.4. Saving Clones for Sequencing

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

2.2.4.5. Preparation of Storage Plates

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

2.2.4.6. Preparation of Glycerol Stock of Phage

- 60 µl sterilized 80% glycerol solution was put into each well of a fresh 96-well plate. Two sets of 96 well Elisa plate have been prepared, one for -20 °C and one for -80 °C stocks. (Overall glycerol concentration was kept as ~ % 50 in stocks.)
- 50 µl clone from the storage plate was added into the glycerol containing plates.

- Plates were covered by parafilm and placed into the freezers, -20 and -80 °C.

2.2.5. Single Stranded DNA Isolation

2.2.5.1. M13 Single Strand DNA Isolation

For the sequencing of the individual sequences, ssDNAs of the picked sequences needs to be isolated. QIAprep ® Spin M13 kit (QIAGEN, Catolog #27704) is used for the isolation of M13 phages which were previously stored at -20 °C stock. The procedure is given schematically in Figure 2.7 and written below.

1) 50 µl from each individual sample which belongs to only one phage plaque was taken from the glycerol stock and added into 2 ml *E. coli* ER2738 culture which had been incubated until mid-log phase (OD₆₀₀ ~0.5) in 2 ml microfuge tubes. Phage and bacteria mixture was incubated for 4.5 hours for *E. coli* ER2738 infection by phage and amplification.

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

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

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

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

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

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

for binding of the M13 DNA to the QIAprep silica-gel membrane. Bacteriophage lysis begins.

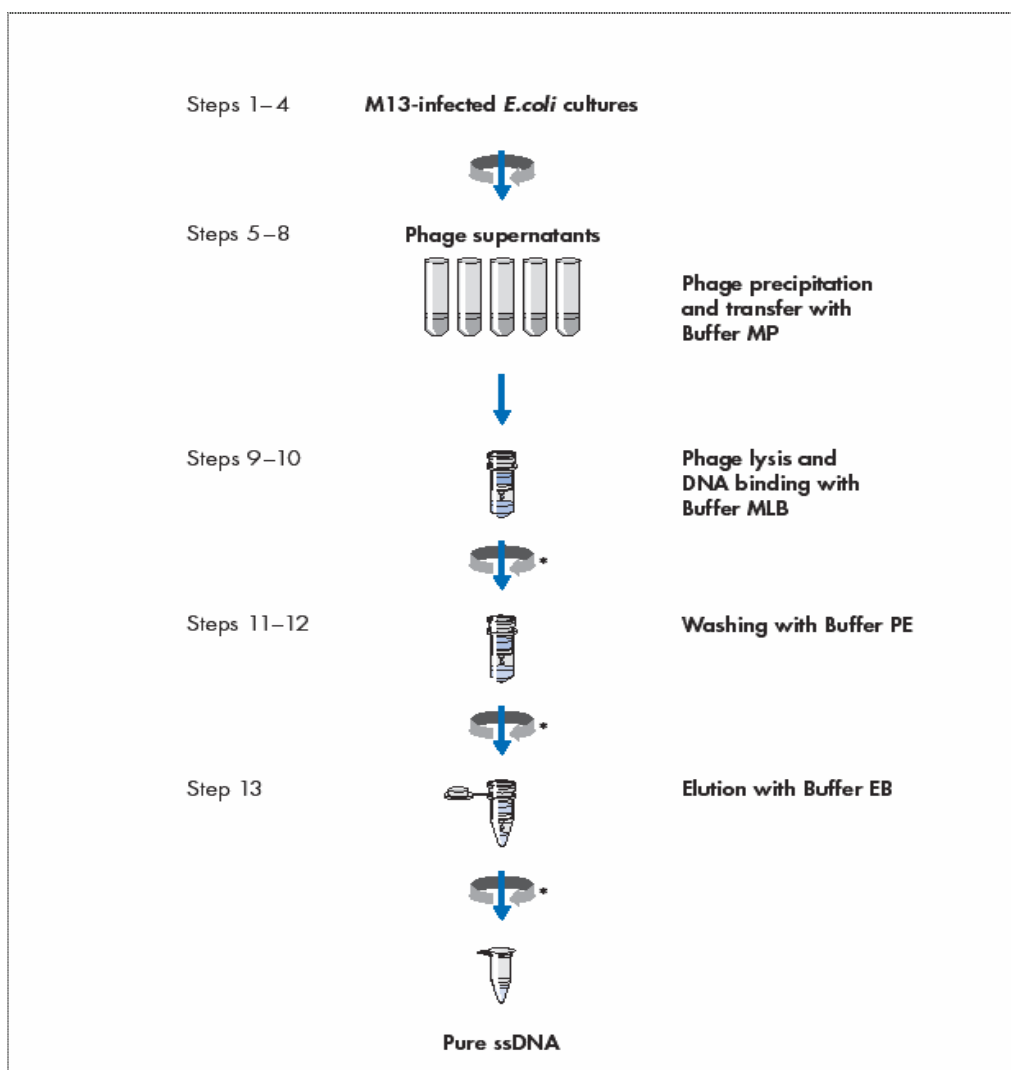


Figure 2.7 M13 ssDNA isolation procedure (QIAGEN, Catalog #27704).

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

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

10) Buffer PE was discarded from collection tube and QIAprep spin column was centrifuged for 15 s at 8,000 rpm to remove residual buffer PE. It is important not to dry the QIAprep membrane quick microcentrifugation step. This prevents residual ethanol from being carried over into subsequent reactions. QIAprep spin column was

placed in a clean 1.5 ml microcentrifuge tube. 100 µl EB buffer (10 mM tris.Cl, pH 8.5) was added onto the center of the column membrane to elute the DNA. Incubation of elution buffer in the QIAprep spin column significantly increases the recovery of single-stranded M13 DNA molecules, which adsorb tightly to the silica membrane.

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

2.2.5.2. Measurement of DNA concentration by TECAN-Magellan SAFIRE Elisa Microplate Reader

Before amplifying the ssDNA for sequencing, we need to determine the amount of DNA we have isolated. Oligreen ssDNA Quantitation reagent kit was used for this purpose. The kit includes:

- Oligreen ssDNA quantitation reagent (dye), 1 ml in DMSO,
- 20XTE, 25 ml of 200 mM Tris-HCl, 20 mM EDTA, pH: 7.5
- Oligonucleotide standard, 1ml of a 100 µg/ml solution in TE.

Determination of ssDNA quantity by fluorescence spectrophotometry- oligreen ssDNA Quantitation reagent kit was done as written below;

- 1XTE buffer was prepared by diluting the concentrated 20XTE buffer 20-fold with sterile, distilled, DNase-free water.
- Oligreen reagent solution was prepared by making 200-fold by using 1XTE buffer. It was made immediately before the experiment in a plastic tube and wrapped by aluminum foil to protect it from light. We need to have 200 µl for blank, 100 µl for standard and 198 µl for each sample.
- Standard DNA solution having 2000 ng/ml was prepared. concentration with 1XTE buffer by using proper amount of stock solution. Since our standard concentration is 100 ng/ml and working volume is 200 µl, we need to take 4 µl of standard in 96 µl 1XTE.

Measurement:

- 200 µl OliGreen reagent was put on polystyrene ELISA plate as blank
- 100 µl OliGreen solution and 100 µl of standard solution (96 µl 1XTE buffer + 4 µl standard ssDNA) was mixed in plate as the standard.

- 2 µl from each ssDNA sample and 198 µl OliGreen reagent solution was mixed in the plate.
- The plate was put in SAFIRE ELISA Microplate Reader and Fluorescence Intensity was measured.
- The excitation wavelength was taken as 480nm and emission wavelength as 520 nm. After running the samples (200 µl/well), we should subtract the blank from standard and each sample and calculate the concentration of ssDNA by standard concentration which is 2000 ng.

2.2.6. Sequencing of DNA Sample

2.2.6.1. PCR Conditions for DNA Sequencing

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

Table 2.2. PCR conditions for DNA sequencing

PCR ingredients	Amount
Big dye reaction mix*	1 µl
5X sequencing buffer / ddH ₂ O	3 µl
Template ssDNA	2 µl (150 ng)
Primer (-96 sequencing primer) **	3.2 µl (3.2 pmol)
dH ₂ O	0.8 µl
Total volume	10 µl

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

** -96 gIII sequencing primer (5'- HOCCC TCA TAG TTA GCG TAA CG -3', 100 pmol, 1 pmol/µl) was purchased from New England Biolabs Ph.D.-12™ Phage Display Peptide Library Kit

PCR conditions for cycle sequencing was;

95 °C	2'	} 35 cycle
95 °C	10''	
55	10''	
60	4'	

2.2.6.2. PCR Product Purification

- 1 g Sephadex G50 DNA Grade Fine was dissolved in 15 ml ddH₂O, in a plastic tube and hydrated for 3-4 hours.
- Hydrated Sephadex solution was vortexed just before use and loaded (0.3 ml for each well) into the wells of a sterile MultiScreen HV plate which has at bottom a 96-well microtiter plate as the holder for the samples after the centrifuge.
- The plate was spin down for 5 min, at 910 g (2,300 rpm) to remove the water and pack the mini-columns.
- After centrifuging, the 96-well microtiter plate at the bottom of the MultiScreen HV plate was replaced with a sterile one.
- Samples of PCR reactions were dropped to the center of the each column which has packed Sephadex as the column material (10 µl PCR sample for each column).
- The plates were centrifuged for 5 min at 910 g. Filtrates will consist of the sequencing product, free of the dye terminator.
- The purified DNA samples were transferred into fresh Eppendorf tubes and vacuumed for 30 min at RT in the Vacuum Pump.
- DNA was sequenced by using an ABI 3100 Avant (PE, Applied Biosystem, CA) automated sequencer.

2.2.7. Immunofluorescence Microscopy Experiments

2.2.7.1. Cleaning of the Quartz Powder

At the beginning of the fluorescence microscopy experiment, quartz piece was crashed and grinded to make the powder. The next step was the cleaning of the powder before interacting with phage clones. The cleaning procedure is basically as follows;

- Around 100 mg of grinded quartz powder was weighed and put into pre-weighed 1.5 ml microfuge tube.

- 100 µl dH₂O and 900 µl CH₃OH/acetone (1:1) mixture were added into the tube. The powder was dissolved gently by pipetting.
- The sample was vortexed for 5-10 min to observe the forming clumps and then sonicated for 20 min in an ultrasonic bath to break the clumps.
- The powder was vortexed quickly to resuspend and centrifuged at 200 g for 1.5 min (CW and CCW).
- The supernatant was removed and 1ml 50 % isopropanol was added onto the powder.
- Powder was vortexed for 5-10 min and then sonicated for 20 min in the ultrasonic bath.
- The powder was vortexed quickly to resuspend and spun down at 200 g for 3 min (CW and CCW).
- The supernatant was removed and 1 ml 0.5 % PC buffer was added onto the powder.
- The powder was first vortexed for 5-10 min and then sonicated for 60 min.
- The powder was vortexed quickly to resuspend.
- The powder was centrifuged at 200 g for 1.5 min (CW and CCW).
- The supernatant was removed and 1ml 0.5 % PC buffer was added onto the quartz powder.
- 100 µl of powder solution was transferred into each sterile microfuge tube.
- Powder was spun down at 200 g for 1.5 min.
- Powder was first washed twice with dH₂O and then with isopropanol.
- Samples were dried under the vacuum.
- All samples were weighed to determine the amount of powder into the each tube.

2.2.7.2. Fluorescence Microscopy Experiment Procedure

Following the sequencing results, each phage clone is checked for binding and specificity on the quartz. Grinded and cleaned quartz powder is used for the binding experiments. The binding of the phage samples on the quartz surface can be observed visually under the fluorescence microscopy indirectly. Zenon complex which has

labeled Fab fragment bound on IgG is used to label the phages for indirect observation. The procedure is basically as follows;

- A proper amount of previously cleaned quartz powder is incubated with 5 μ l phage (PFU/ml 10^{13}) in PC buffer containing 0.1 % detergent and rotated overnight.
- After attaching phage to the powder, this conjugate was incubated with tris buffer containing casein blocking agent for 10 min, in order to coat the powder and occupy any binding sites that antibody might attach to.
- Each phage sample is labeled according to the immunolabelling procedure as in Figure 2.8. First, Anti-M13 pIII monoclonal antibody (Amersham Biosciences) which is specific to m13 gp8 protein and Alexa-Fluor conjugated secondary antibody fragments (Zenon Alexa, Molecular Probes Inc.) that contains fluorescence dye were incubated (1: 5 ratio) for 30 min in a microfuge tube covered by aluminium foil to protect from the light.

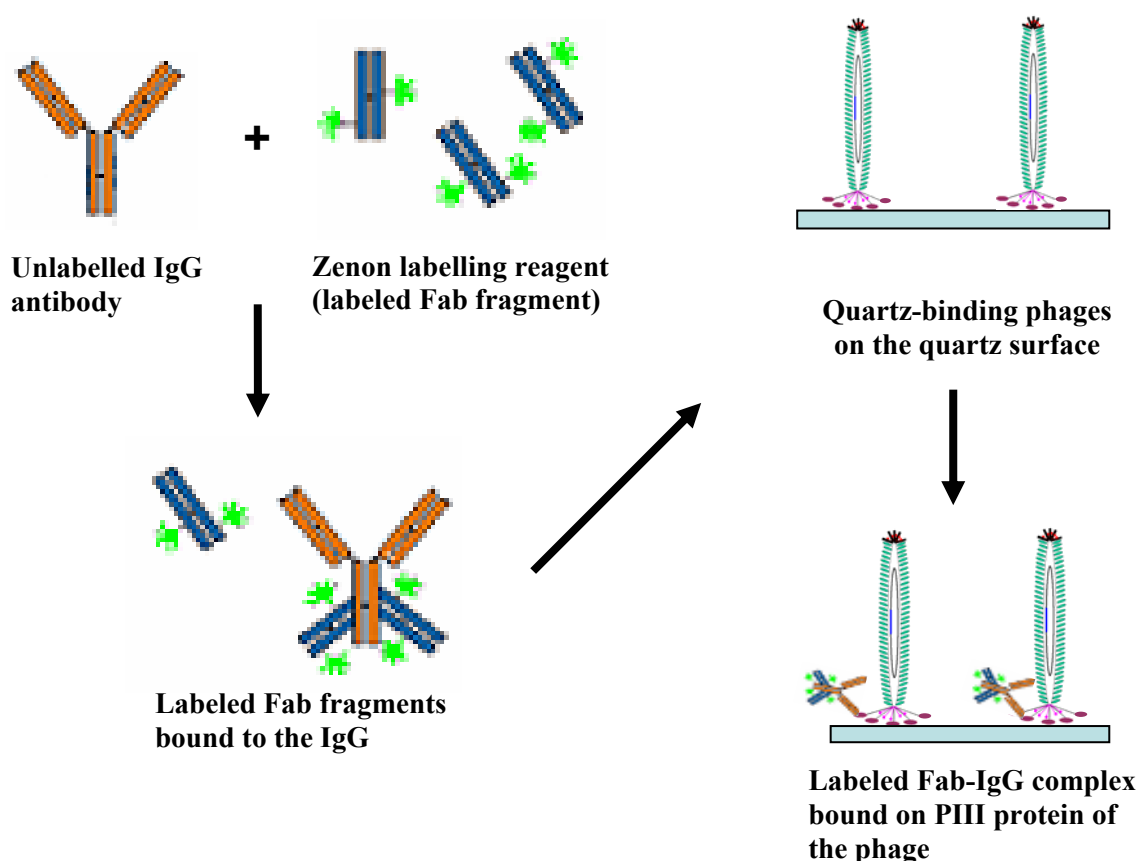


Figure 2.8. Labelling procedure for fluorescence microscopy (not scaled).

- While Zenon mixture is being prepared, overnight phage-quartz powder solution is centrifuged at 1,000 rpm for 1.5 min. The supernatant is discarded and 0.1 % PC

buffer is added on the sample for 15 min rotation on running cylinder to wash unbound phage out of the solution.

- The solution is centrifuged again at 1,000 rpm for 1.5 min and the supernatant is discarded.
- 3 μ l Zenon dye complex and 497 μ l PC buffer containing no detergent were put on the phage-quartz solution and incubated for 30 min on running cylinder to let phage-mixture interaction. The tube is covered with aluminium foil.
- The solution is centrifuged again at 1,000 rpm for 1.5 min and the supernatant is discarded.
- 0.5 ml 0.1 % PC buffer is added into the tube and the powder is washed 3 times by pipetting.
- The mixture solution is centrifuged again at 1,000 rpm for 1.5 min and the supernatant is discarded.
- Another 0.5 ml 0.1 % PC buffer is added into the tube and the powder is washed 3 times by pipetting.
- The mixture solution is centrifuged again at 1,000 rpm for 1.5 min and the supernatant is discarded.
- 30 μ l 0 % PC buffer is added onto the powder solution and visualized by fluorescence microscopy (Nikon Eclipse TE 2000-U) at 20X magnification under WIB filter.

2.2.8. Silica Formation Experiments

The best binder, DS202, was chosen first to analyze further. It was synthesized commercially as peptide powder and dissolved in distilled water. We followed the procedure for the silica formation in the presence of synthesized peptides as described in the paper by Stone et al. The procedure is as follows;

- 2 mg of synthesized peptide, DS202, was dissolved in 50 μ l TBS buffer containing no detergent, pH: 7.5. Stock peptide solution was 40 mg/ml in concentration.
- 11.8 μ l TMOS and 78.2 μ l HCl were mixed in 2 ml microfuge tube.
- 30 μ l from peptide stock solution was put into the tube forming the final peptide concentration in the tube as 10 mg/ml.

- The tube was rotated on running cylinder for 5 min and then centrifuged for 5 min at 13,200 rpm.
- The supernatant was discarded and the precipitate in the tube was observed under SEM to see any silica particle formation.

Control sample was run in the absence of peptide. Without addition of synthesized peptide, no precipitation formation was observed in the tube. The solution forms gel after 24 hours.

2.2.9. Similarity Analysis Tools-Dynamic Programming

Needleman-Wunsch (1970) dynamic programming method has been adapted and developed in our group for the sequence similarity/correlation analysis of inorganic-binding polypeptides with BLOSUM 62 and PAM 250 scoring matrices that give the score for aligning two amino acids (match or mismatch) in a pairwise alignment. The dynamic programming technique basically tries to find the optimal alignment by taking advantage of the optimal alignments already found for the prefixes of the sequence (For any letter sequence the segment of the sequence consisting of the letters from the beginning of the sequence up to the i^{th} letter in the sequence is called as prefix). Given a similarity or scoring matrix, e.g. PAM or BLOSUM which both calculate substitution frequencies between amino acids, and both are derived from known protein alignments, the overall similarity score of a pair wise alignment is defined by the sum of all similarity values of the aligned residue pairs minus a so-called gap penalty for every gap introduced into the alignment. (Oren et al. 2004, in process).

2.2.10. AFM Imaging

For AFM imaging, approximately 0.5cm by 0.5cm quartz wafer was incubated in the same condition as described in Section 2.4. Upon completion of incubation, quartz wafer was removed from buffer, rinsed in DI water, air dried until there was no visible moisture (approximately 2 hours) then further dried by storing in chamber drierite overnight until AFM imaging. AFM imaging was performed using a Extended MMAFM equipped with Nanoscoped III (Veeco Instruments), using tapping mode in air.

2.2.11. TEM Imaging

PC buffer containing bacteria or phages is dropped onto the carbon grid. After 20 min waiting, the liquid is gently wiped out by using tissue paper. To remove the dried salts from the grid, sample is washed with distilled water and dried. Philips 420 EM was used for imaging.

2.2.12. SEM Imaging

Before examination, sample is washed with distilled water and then taken into vacuum. After the sample is dry, it is sprinkled from eppendorf tube onto carbon tape which is taped to SEM stub. Sample is Sputter Au-Pd coated for 30 sec (roughly 5nm thick) with SPI Module Sputter Coater, SPI Supplies, Inc. Imaging is taken with SEM at 5 keV for imaging and 10 keV for EDS (SEM: JEOL 5200, JEOL, Inc.; EDS: QX2000, Link Analytical, Inc.).

3. RESULTS AND DISCUSSION

(100) oriented single crystal quartz (SiO_2) was used as substrate for the selection of quartz-specific 12-aminoacid polypeptides from PhD-12 phage display library. As a result of five rounds of biopanning experiments, 50 sequences were identified as the silica binders. The amino acids sequences were analyzed in terms of physicochemical properties and also statistical analysis were carried out on the selected sequences to search for any trend along the sequences for certain amino acids or any convergence to consensus sequence as silica binding domain. In the next stage, immunolabelling fluorescence microscopy technique was applied to characterize the binding affinities of each identified sequence. Here, fluorescence technique provided a semi-quantitative approach to group the sequences according to their relative binding strength for silica surface. The selected peptides were grouped into three groups as good, moderate or weak binders. In protein-protein interactions, high sequence similarity usually implies major functional or structural similarity. In our approach, we have also followed a novel Bioinformatics approach to find a correlation among the identified binding peptides and the silica substrate. Here, sequence alignment is an essential tool to compare the sequences and depending on the similarity among the compared ones, each one gets a score. In applying the same approach to the similarity analysis of inorganic binding peptides, we have followed a multiple alignment analysis. According to obtained the similarity scores, fluorescence microscopy based binding characteristics of the sequences were verified. We also carried out the cross specificity analysis of the selected clones to check if they will present any binding towards another material rather than the selected one. To understand the effect of surface orientation, another set of phage display experiment was applied on (001) oriented single crystal quartz pieces. Finally, the peptide presenting the best affinity and specificity for quartz surface was synthesized. Silica nanospheres were produced at room temperature under the aqueous environment in the presence of the synthesized peptide.

3.1 Identification of the Binder Peptides for (100) Oriented Quartz and Their Sequence Based Analysis

5 biopanning rounds were carried out as it was described in Section 2.2. (100) oriented quartz wafer was used as the target inorganic substrate. The strength of the washing buffer was increased gradually during the biopanning experiments to induce harsher conditions necessary for eluting the specific phages from the surface.

At the end of biopanning studies, 400 clones were saved for further necessary investigations. Among them, totally 50 phage colonies, 10 from each round, were selected for the binding characterization. Selected single phage colonies were grown and their ssDNAs were isolated and sequenced. The amino acid sequences were given in Table 3.1. Some of the sequences were observed more than once during the selection. Therefore, 39 different amino acid sequences were presented in the table and how many times the sequences were observed, were specified in the parentheses next to them. The first column gives the round numbers in which the sequences are selected and sequenced. The second column contains the names for each sequence given after the name of the author and the third column contains the amino acid sequences given in colors. At this point, it is rather hard to deduce any information from the sequences just by comparing the sequences in terms of their affinity or specificity for the quartz. One might consider the sequences being observed more than once as the high affinity presenters.

A consensus sequence could not be obtained even after the fifth round of bio panning in contrast to the traditional applications of phage display for the protein-protein interactions in which a consensus sequence is obtained generally after 2 or 3 rounds of bio panning. Once the protein-inorganic interactions are present, the binding mechanism can be expected to be more complicated. As a result, the further analyses were carried out on the sequences to elucidate the information for their binding properties. Next step, analysis consists of the statistical and physicochemical properties evaluation of the amino acid contents of the selected sequences.

Table 3.1. List of phage display selected quartz binders. (Color codes of amino acids: Red; Hydrophobic, Blue; Acidic, Purple; Basic, Light Green; Hydroxyl Containing, Dark Green; Amine+Basic) (*: The number after some sequences is the repetition number of that sequence)

Round #	Name	Sequence*
1	DS202	RLNPPSQMDPPF
1	DS189	QTWPPPLWFST
1	DS171	HFTFPQQQPPRP (3)
1	DS194	DAFTQMPWVWTH
1	DS193	GSTQAWMSPPLA (3)
1	DS190	LLADTTHHRPWT
1	DS165	HITLRMTDTER (3)
1	DS191	VAPRVQNLHFGA
1	DS201	MEGQYKSNLLFT
2	DS199	AMVLEGESTVWP
2	DS198	IPVPKFDHPWRG
2	DS197	SPLSIAASSPWP (2)
2	DS152	KTLNWLSYAQLA
2	DS150	YHSGLHPMPFP
2	DS153	QSPLLQLIVGTP
2	DS148	NVASYLSSVPDT
2	DS147	DSPSYKAIPGAS
2	DS146	ATGTMKITTHWF
2	DS200	SPQHMFLPTNSV
3	DS30	LTPHQTTMAHFL
3	DS142	APPGNWRNYLMP
3	DS143	MWPSTTHSSPYH
3	DS36	MIPNTWEMRLPF (3)
3	DS38	YVHNPHYHLPNP
3	DS145	DHQRMNDA MKVL
3	DS144	STPAHEPMPRCC
4	DS71	AIAETMSLFTKL
4	DS76	TLPAFGPRAHVL
4	DS80	SNFTTQMTFYTG
4	DS72	YESIRIGVAPSQ
4	DS69	TMGFTAPRFPHT
4	DS78	MLTPRYMALTVN (2)
4	DS75	EIQPRYPSTLTG
4	DS73	DNANSSIRSQTY
5	DS123	VPHMPSTLDVKR (2)
5	DS125	AILRPQLMPGSS
5	DS84	LIAHSMPPRTRI
5	DS91	QPFTTSLTPPAR
5	DS88	QSFTTLTGPDNR

3.2 Statistical Analysis of the Selected Sequences

Phage display libraries contain different number of members ranging from 10^8 to 10^{10} depending on the size of the amino acid sequence displayed on the coat proteins. They also contain some biases associated with the use of living cells since the genetic code is degenerate and certain amino acids are represented by multiple codons. For example, threonine is represented by four codons whereas tryphophan is specified by one codon. Therefore in creating libraries, both theoretical and experimentally obtained values for amino acid distribution are quiet important. Once these values are compared with the selected sequences, then the relative abundance of amino acids for quartz surface will be obtained.

We calculated the relative abundances of the amino acids in the selected sequences as described in Ph.D.-12 phage library catalog (New England Biolabs, MA) which is shown in Figure A.1. Figure 3.1 shows the relative abundances of the amino acids from all sequences after 5 rounds of biopanning. Zero level in the figure is the base level meaning that the amino acid is observed in the biopanning rounds after the 5 rounds same as it is observed in PhD-12 library. Positive values mean the over-expression of the amino acids and the negative values mean the under-expression. Overexpressed amino acids can be considered as the necessary amino acids for binding to the substrate. The first obvious data Figure 3.1 gives is that methionine (M) is mostly over expressed one which is ~130% more observed than it is observed in the Ph.D.-12 library. In contrast to sequence based analysis in which any information has not been found to understand the affinity levels of each binder, in amino acid based analysis, as an overall, we could see that mostly over expressed amino acids were non-polar and hydrophobic such as methionine (M), tryptophan (W) and phenylalanine (F) while mostly under expressed amino acids were generally polar and hydrophilic ones like cysteine (C), lysine (K) and glutamate (E). Indeed, the figure shows overall, a trend of an over expression of non-polar and hydrophobic ones in contrast to polar and hydrophilic ones which were generally under expressed.

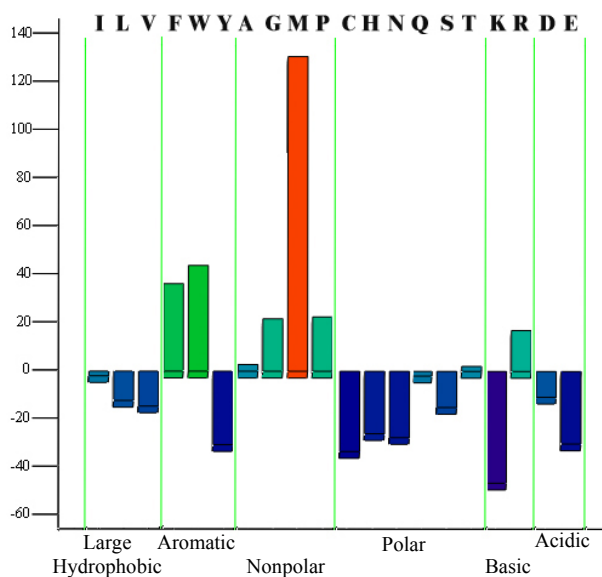


Figure 3.1 Relative abundances of amino acids from all sequences after 5 biopanning rounds.

3.3 Physicochemical Properties of the Selected Binders

The physicochemical properties of a protein are determined by the analogous properties of the amino acids forming its sequence. The side chains of the amino acids give the characteristics and the functional properties such as polarity, ionization and their tendency to interact with water. In the further analysis of the selected sequences, we have performed the physicochemical properties analysis of the sequences and they are shown in Table 3.2 Again, similar to Table 3.1, the first three columns are the round numbers, name of the binders and the amino acid sequences of the binders, respectively. Next three columns give the total charge, polarity and hydrophilicity-hydrophobicity values of each sequence. Total charges of all sequences are found to be generally neutral or very close to neutral. Similarly polarity and the hydrophilicity-hydrophobicity values seem to be distributed randomly not forming any specific trend.

In all these analysis, the selected sequences were considered as presenting the equal binding properties for the quartz substrate. Therefore, in our next step of characterization, we have performed experimental evaluation to deduce any information on the existence of different degree of affinity among the sequences.

Table 3.2. List of phage display selected quartz binders with their charge, polarity and hydrophilicity-hydrophobicity values. (Color codes of amino acids: Red; Hydrophobic, Blue; Acidic, Purple; Basic, Light Green; Hydroxyl Containing, Dark Green; Amine+Basic) (*: The number after some sequences is the repetition number of that sequence)

Round #	Name	Sequence*	Charge	P	H
1	DS202	RLNPPSQMDPPF	0	102.6	-3.20
1	DS189	QTWPPPLWFST	0	91.0	5.54
1	DS171	HFTFPQQQPPRP (3)	1	103.4	-0.99
1	DS194	DAFTQMPWVWTH	-1	94.8	2.42
1	DS193	GSTQAWMSPPLA (3)	0	94.7	2.89
1	DS190	LLADTTHHRPWT	0	101.4	-4.89
1	DS165	HITLRMTDTESR (3)	0	107.5	-14.90
1	DS191	VAPRVQNLHFGA	1	98.1	0.66
1	DS201	MEGQYKSNLLFT	0	99.4	-2.79
2	DS199	AMVLEGESTVWP	-2	95.3	0.83
2	DS198	IPVPKFDHPWRG	1	99.9	-0.68
2	DS197	SPLSIAASSPWP (2)	0	92.5	4.56
2	DS152	KTLNWLSYAQLA	1	93.7	1.62
2	DS150	YHSGLHPMPFP	0	93.0	7.96
2	DS153	QSPLLQLIVGTP	0	89.6	5.72
2	DS148	NVASYLSSVPDT	-1	99.8	-3.17
2	DS147	DSPSYKAIPGAS	0	104.5	-4.25
2	DS146	ATGTMKITTHWF	1	94.7	0.16
2	DS200	SPQHMFLLPTNSV	0	97.2	1.00
3	DS30	LTPHQTTHMAHFL	0	93.9	2.67
3	DS142	APPGNWRNYLMP	1	97.0	1.97
3	DS143	MWPTTTHSSPYH	0	98.3	-0.22
3	DS36	MIPNTWEMRLPF (3)	0	91.1	2.17
3	DS38	YVHNPHYHLPNPP	0	99.2	4.92
3	DS145	DHQRMDNDAMKVL	0	110.6	-12.76
3	DS144	STPAHEPMPRCC	0	99.8	-4.92
4	DS71	AIAETMSLFTKL	0	92.1	0.55
4	DS76	TLPAFGPRAHVL	1	91.6	4.38
4	DS80	SNFTTQMTFYTG	0	97.0	-1.48
4	DS72	YESIRIGVAPSQ	0	99.3	-2.85
4	DS69	TMGFTAPRFPHT	1	95.9	0.28
4	DS78	MLTPRYMALTVN (2)	1	88.7	1.85
4	DS75	EIQPRYPSTLTG	0	101.0	-4.31
4	DS73	DNANSSIRSQTY	0	112.9	-13.92
5	DS123	VPHMPSTLDVKR (2)	1	101.4	-6.05
5	DS125	AILRPQLMPGSS	1	93.2	1.06
5	DS84	LIAHSMPPRTRI	2	94.3	-1.99
5	DS91	QPFTTSLTPPAR	1	98.2	-2.04
5	DS88	QSFTTLTGPDNR	0	107.7	-10.13

3.4 Affinity Characterization of the Selected Sequences

Up to now, the characterization of the sequences was performed based on theoretical evaluation of their amino acid content. By doing this, all the binders were considered as having equal binding properties. However, they might present different degrees of binding affinity and the specificities for the substrate that they are selected for. Although the sequence-based analysis did not give any data showing a grouping or convergence of some sequences, amino acid based analysis show us the trend of selecting hydrophobic and non-polar amino acids during the biopanning rounds. The discriminating point of this study is to have a better understanding of binding affinities of each sequence by performing all binding experiments.

All individual colonies were interacted with quartz, which had been crashed and grinded into powder size. By doing this, their surface area was increased and that made FM analysis easier to follow. Next, the mixture containing the substrate and the phage population consisting of individual phage was rotated overnight. At the end of binding experiments, unbound phages were washed from the substrate. Anti-M13 pIII monoclonal antibody (Amersham Biosciences, Piscataway, NJ) and Alexa-Fluor conjugated secondary antibody fragments (Zenon One, Molecular Probes Inc., Eugene, OR) were added to immunolabel the phages. After washing the unbound dyes, the samples were observed under fluorescence microscopy and percentage coverage of bound phages on the powder was measured based on pixel counting.

The FM results are rather interesting, since the identified phages showed a different degree of affinity for the quartz substrate as the examples shown in Figure 3.2. In the figure, optical and fluorescent microscopy images of three binders each showing different binding levels were given. The scale bar is 100 micrometer. Control samples has no phage molecule, to observe if any unspecific interaction of the antibody or the dye molecule occurs with the substrate. The way we used to measure the binding percentages of each sequence was semi-quantitative and the percentage values may not exactly represent the affinities for the substrate but it still provides a basis for comparing each sequence relative to the other. This type of characterization seems to be the best and

easiest way of starting to group the phage display results relative to each other according to their affinities for a specific substrate.

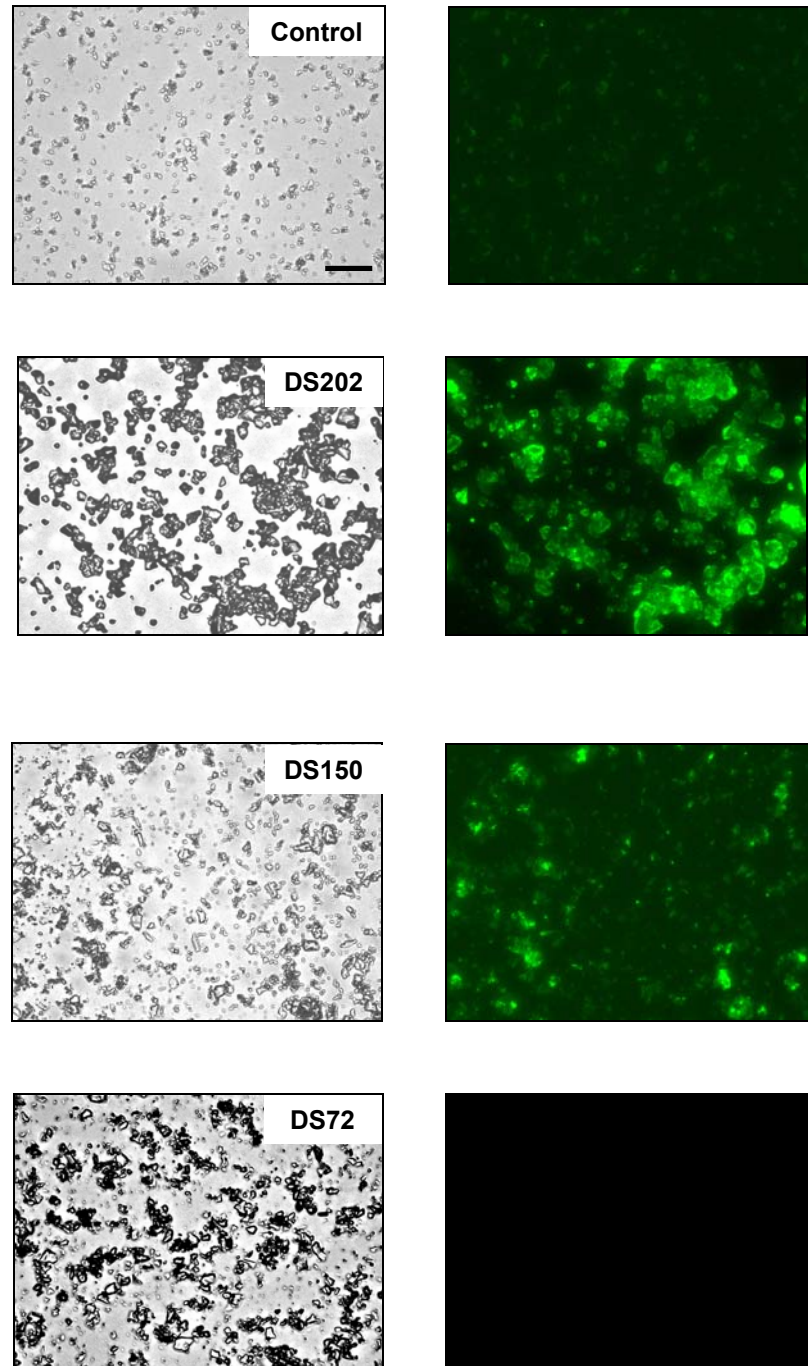


Figure 3.2 Optical (left) and fluorescence microscopy (right) images of samples from 3 main groups, DS202 from strong, DS 150 from moderate and DS 72 from weak group (Bar: 100 μm).

In our semi-quantitative approach, first, the ratio of powder area to the total area, (R1), was measured by using the fluorescence microscopy pixel counting software. Then, the ratio of binding area to the total area, (R2), was measured. The $(R2/R1) \times 100$ value gave us the percentage coverage (binding) for each clone which was presented in Table 3.3. The sequences in Table 3.3 were listed in the order varying from high to low coverage. They were divided into 3 main groups as strong, moderate and weak according to their percentage bindings. The order of sequences from high to low shows affinity of each sequence for quartz substrate. The sequences with high percent-coverage have high affinity for the quartz, and the sequences with low-percent coverage have less.

These results show the importance of performing fluorescence microscopy based binding experiments for all sequences obtained as a result of biopanning. As a result of binding experiments, we can align the sequences according to their binding affinities and group them into subgroups. We decided to name the sequences with 0-10 % binding as weak, 10-30 % binding as moderate and 30% and more as strong binders.

3.5 Analysis after Classifying Sequences into the Subgroups

After calculating the binding percentages and then grouping of the sequences, it is observed that all of the strong binders are from the first three rounds as it is given in Table 3.3. This is exactly opposite of the results expected in traditional phage display selecting organic binders for organic substrates. It is clear that as the number of the rounds increases, we are not going towards the best binders; one important finding was that all of the strong clones were coming from the first 3 rounds of biopanning. Indeed, after the 3rd round of biopanning, we observed mainly the repetitions of some of the sequences observed in the first 3 rounds and the clones from moderate and weak groups. This may be caused by some sort of enrichment which caused by the interaction of inorganic surface and protein. Following the grouping of the binders as shown in Table 3.3, it became also easier to deduce information from their physicochemical properties. As shown in Table 3.3, the strong binders are generally hydrophobic and the weak binders are hydrophilic. Overall, there is a selection towards the peptides having hydrophobic amino acids, and mostly they are selected as the strong ones.

Table 3.3. List of phage display selected quartz binders with their physicochemical properties and binding percentages. (Color codes of amino acids: Red; Hydrophobic, Blue; Acidic, Purple; Basic, Light Green; Hydroxyl Containing, Dark Green; Amine+Basic) (A: Classification with respect to % Binding calculated from FM pictures. *: The number after some sequences is the repetition number of that sequence)

Round #	Name	Sequence*	Charge	P	H	% Binding	A
1	DS202	RLNPPSQMDPPF	0	102.6	-3.20	94	S
1	DS189	QTWPPPLWFST	0	91.0	5.54	68	S
3	DS30	LTPHQTTMAHFL	0	93.9	2.67	54	S
3	DS142	APPGNWRNYLMP	1	97.0	1.97	44	S
1	DS171	HFTFPQQQPPRP (3)	1	103.4	-0.99	43	S
1	DS194	DAFTQMPWVWTH	-1	94.8	2.42	34	S
2	DS197	SPLSIAASSPWP (2)	0	92.5	4.56	33	S
3	DS143	MWPTTTHSSPYH	0	98.3	-0.22	33	S
2	DS152	KTLNWLSYAQLA	1	93.7	1.62	33	S
3	DS36	MIPNTWEMRLPF (3)	0	91.1	2.17	31	S
2	DS153	QSPLLQLIVGTP	0	89.6	5.72	26	M
5	DS123	VPHMPSTLDVKR (2)	1	101.4	-6.05	24	M
2	DS150	YHSGLHPMPFP	0	93.0	7.96	23	M
1	DS193	GSTQAWMSPPLA (3)	0	94.7	2.89	20	M
1	DS190	LLADTTHHRPWT	0	101.4	-4.89	20	M
3	DS38	YVHNPHYHLPNPP	0	99.2	4.92	20	M
2	DS148	NVASYLSSVPDT	-1	99.8	-3.17	15	M
5	DS125	AILRPQLMPGSS	1	93.2	1.06	14	M
4	DS69	TMGFTAPRFPHT	1	95.9	0.28	13	M
5	DS84	LIAHSMPPRTRI	2	94.3	-1.99	13	M
4	DS78	MLTPRYMALTVN (2)	1	88.7	1.85	12	M
5	DS91	QPFTTSLTPPAR	1	98.2	-2.04	12	M
1	DS165	HITLRMTDTER (3)	0	107.5	-14.90	12	M
2	DS147	DSPSYKAIPGAS	0	104.5	-4.25	11	M
4	DS75	EIQPRYPSTLTG	0	101.0	-4.31	9	W
1	DS191	VAPRVQNLHFGA	1	98.1	0.66	7	W
3	DS145	DHQRMNDAKVL	0	110.6	-12.76	7	W
4	DS73	DNANSSIRSQTY	0	112.9	-13.92	7	W
2	DS146	ATGTMKITTHWF	1	94.7	0.16	7	W
5	DS88	QSFTTTLTGPDNR	0	107.7	-10.13	6	W
1	DS201	MEGQYKSNLLFT	0	99.4	-2.79	5	W
2	DS200	SPQHMFLLPTNSV	0	97.2	1.00	4	W
3	DS144	STPAHEPMPRCC	0	99.8	-4.92	3	W
4	DS71	AIAETMSLFTKL	0	92.1	0.55	0	W
2	DS199	AMVLEGESTVWP	-2	95.3	0.83	0	W
2	DS198	IPVPKFDPHPWRG	1	99.9	-0.68	0	W
4	DS76	TLPAFGPRAHVL	1	91.6	4.38	0	W
4	DS80	SNFTTQMTFYTG	0	97.0	-1.48	0	W
4	DS72	YESIRIGVAPSQ	0	99.3	-2.85	0	W

3.6 Similarity Analysis for Verification of the Grouping of the Sequences

By grouping the sequences into the groups, we aim at least to separate the strong binders from the rest which will make any further analysis easy. The calculation of the binding percentages from the fluorescence microscopy images makes us to form the groups. For the verification of the grouping of sequences, dynamical programming algorithms were used for sequence analysis. In biomolecular sequences (DNA, RNA, or amino acids), high sequence similarity usually implies major functional or structural similarity. Sequences that are very similar probably have a similar structure and/or function such as binding to the specific inorganic surfaces. Dynamical programming algorithms were adapted and used for sequence analysis with BLOSUM 62 and PAM 250 scoring matrices that give the score for aligning two amino acids (match or mismatch) in a pairwise alignment. The results indicate that the self-similarity scores of peptides for inorganics is relatively higher, especially if only the strong binders are used, than the cross-similarity scores between the different peptide groups specific to different inorganic materials.

In figure 3.3 similarity scores for all binders together and for each subgroup are given. The numbers are relative to each other and as the numbers increase the correlation between the sequences increases. The negative values mean the similarity (correlation) between the sequences is very low. Similarity analysis using BLOSUM 62 matrix gives 1.071 for all binders together. When strong binders are correlated with strong binders, the similarity score is 3.507 meaning that there is high similarity between the strong binders than the similarity between all binders together. The similarity score for weak binders vs weak binders is -0.928 meaning that the correlation between the weak binders is very low. Similar results were obtained with PAM 250 matrix. When similarity score for all binders is -1.094, the similarity score for strong binders is 1.482 and for weak binders -2.962.

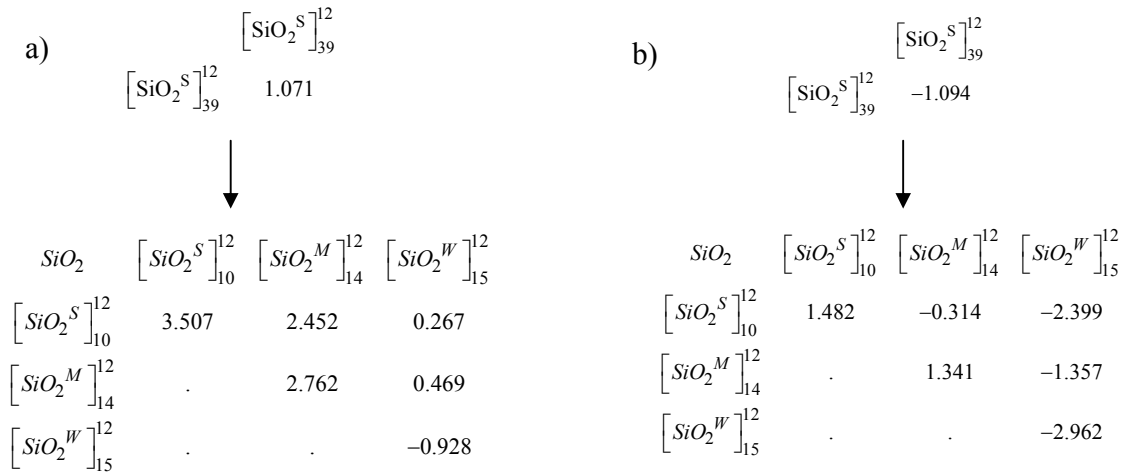


Figure 3.3 Similarity scores of the strong-moderate and weak SiO₂ binders. a) BLOSUM 62, and b) PAM 250. (The numbers on the parentheses, up: amino acid numbers of each sequence, bottom: total numbers of sequences used for the analysis)

The similarity score analysis was made to verify the grouping of the binders as strong, moderate and weak binders. The lack of any consensus sequence even after the fifth round makes necessary to separate the binders into subgroups. The way we used for grouping was the binding experiments by immunolabelling the binders and checking under fluorescence microscopy. The similarity analysis supports our way for grouping. After grouping, we can concentrate on the analysis of the strong binders especially when trying to use the binders for silica formation experiments which is mentioned in Section 3.9.

3.7 Understanding Specificity: Cross-Specificity Experiments

Up to now, we have obtained information with respect to the affinities of the sequences for quartz substrate. We do not have any information yet related to their specificity, meaning if they have any affinity for another substrate rather than the ones they are selected for. A sequence with high affinity for quartz does not necessarily need to be specific to quartz.

Another detailed set of binding experiment is needed with materials other than quartz. For example, both DS202 and DS171 have strong affinities for quartz but when another material such as palladium was used to test the specificity of the sequences, the results may affect the list of quartz binders.

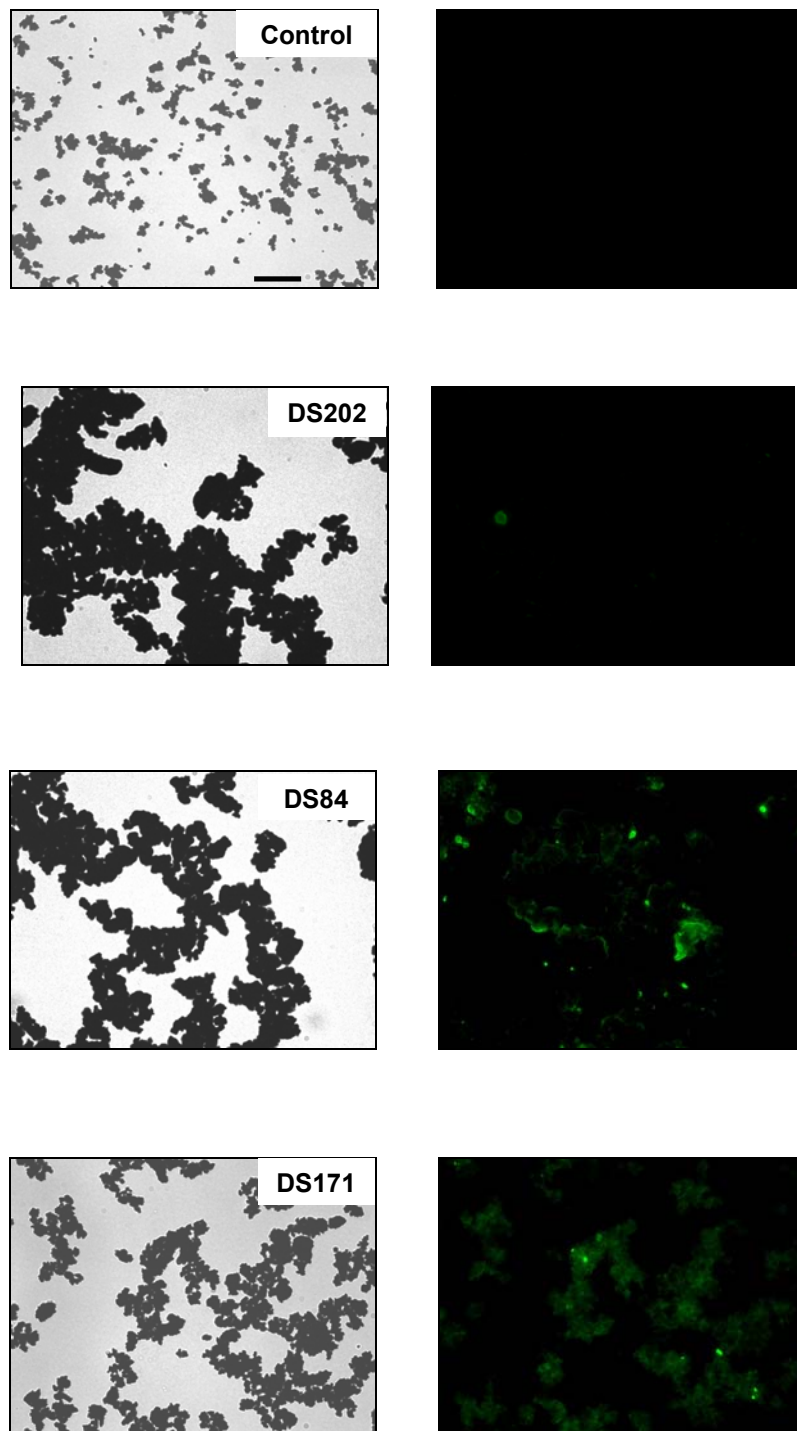


Figure 3.4 Control experiments with Palladium (Bar: 100 μm).

At this point, all selected sequences were tested against palladium powder sample. The experimental procedure is same with the procedure which was carried out for silica binding experiments. The results showed that just two of all sequences showed a slight binding on the Palladium sample as given in Figure 3.4, but others not. Figure 3.4 shows the two samples (DS 84 and DS171) with binding on the palladium and the images of control sample containing no phage.

Palladium was selected as control for two reasons. First of all, as a metal, it is very reactive and very possible to give binding with any material. We could start cross-specificity experiments with any material and then new materials can be used for further materials. Second reason is the availability of the sequences which were selected as palladium binders by phage display in our group in the study by Dincer et al. (unpublished data). If the quartz binders bind also on palladium, the sequences can be compared with the sequences of phage display selected palladium binders for further analysis. As similarity analysis or fluorescence microscopy experiments, cross-specificity experiments can also be used to narrow the list of quartz binders and find the most specific and strong binders.

3.8 Selection of Polypeptides for (001) Oriented Quartz

The wafer we used for the selection of polypeptides was (100) oriented single crystal quartz. It has the same formula with silica as SiO_2 . The basic difference between the quartz and the silica is that the atoms are ordered regularly in quartz making it single crystal structured while the atoms are randomly distributed in silica making it amorphous. To compare the sequences selected for (100) oriented quartz, we decided to do one more phage display experiment but this time with (001) oriented quartz. Both of (100) and (001) oriented quartz have the same formula, same surface roughness and same purity. The difference between them is the orientation of the atoms on the surface where the phages interact with the quartz. Figure 3.5 shows the unit cell for single crystal quartz where the upper plane is (100) plane and the plane at the left is (001) plane.

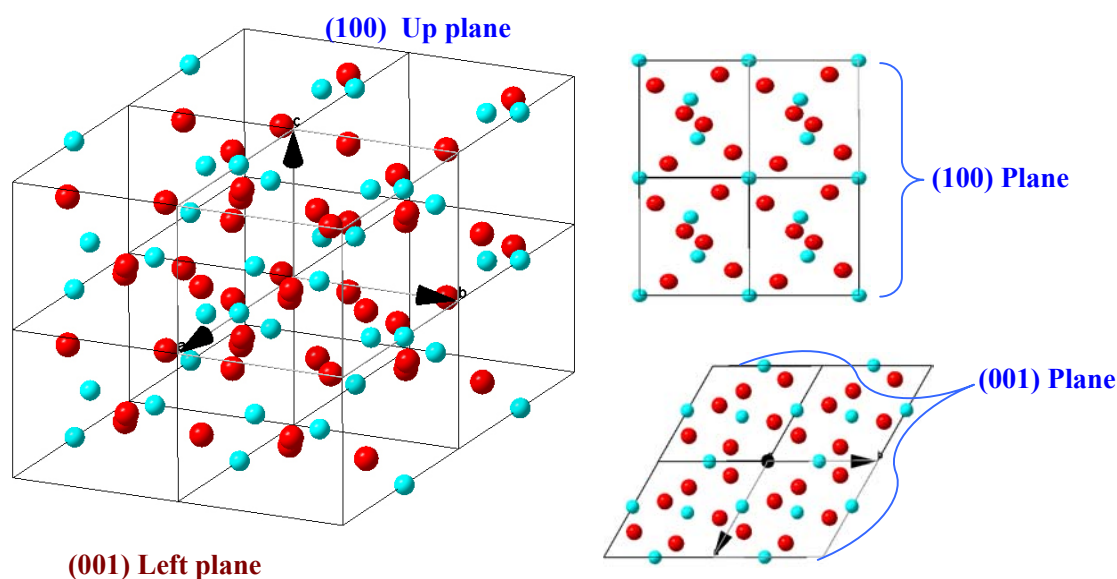


Figure 3.5 (At left) Unit cell for single crystal quartz showing (100) and (001) planes. (At right) Orientation of the atoms at (100) and (001) planes. Blue dots; Silicon and Red dots; Oxygen Atoms. (Lattice parameters for quartz: $a=4.89 \text{ \AA}$, $c=5.375$)

Inspection of $\{100\}$ family surfaces shows that these surfaces end with Si atoms with underlying O atoms. The net charge on the surface becomes neutral. (001) is different in terms of atomic distances. Five rounds of biopanning were carried out to select totally 25 clones. ssDNA of the selected clones were isolated and then sequenced. The sequences are given in Table 3.4.

Table 3.4 contains the (001) oriented quartz binders and some physicochemical properties of the sequences. The charge values are close to neutral as it is for (100) oriented quartz. Polarity and hydrophilicity values are close to the values for (100) oriented quartz. By comparing the sequences for both (100) and (001) oriented quartz substrates, we basically try to understand to see whether there is any effect of atomic orientation on the selection process or not. DS315 is a binder for (001) quartz and same with DS189 which is a binder for (100) oriented quartz. Presence of a common sequence and the similarity of physicochemical properties possibly means that the distribution of the atoms is not that much effective. Although binders for (001) oriented quartz substrate need more analysis such as fluorescence microscopy experiments, grouping and cross-specificity

experiments, we can still use them for the similarity analysis between binders for (100) oriented quartz.

Table 3.4 Phage display selected binders for (001) oriented quartz with their physicochemical properties.

Round #	Name	Sequence*	Charge	P	H
1	DS249	FPNLTVIPLSSR (3)	1	91.2	-6
1	DS250	APRDNFNTVNLL (2)	0	95.9	-2.1
1	DS254	QQGMEHRTTELW	-1	108.7	7.4
2	DS256	MPAKEWLPKHNN	1	108.6	3
2	DS257	HPMQPLSSPSAA	0	101.1	1.1
2	DS259	TDRSAHMTASPI	0	104.6	1.2
2	DS244	TNHRAAAHQTYV (2)	1	108.8	-0.9
2	DS236	QNLMPYGARHTP	1	103.3	-0.5
1	DS294	QNSHASAFMFSA	0	103.3	-4.2
3	DS296	KPGDTSPALWLI	0	96.4	-5.3
4	DS315	QTWPPPLWFSTS (3)	0	91	5.5
5	DS337	SVSVGMKPSRP (5)	2	96.9	-2.6

Figure 3.6 has the relative abundances of the amino acids for both (100) and (001) oriented quartz. The relative abundances are mostly matching for both groups. Generally, non-polar amino acids are over expressed while polar amino acids are under-expressed for the case of (001) quartz binders which were same for the case of (100) quartz binders.

Similarity analysis was done for (001) quartz binders and the similarity scores are given in Figure 3.7. The self-similarity scores for (100) binders is 1.071 and for (001) binders 0.902 with BLOSUM 62. Similarity score between (100) and (001) binders is 0.902 which is very close to the self-similarity scores. Similar results were obtained by using PAM 250 matrix.

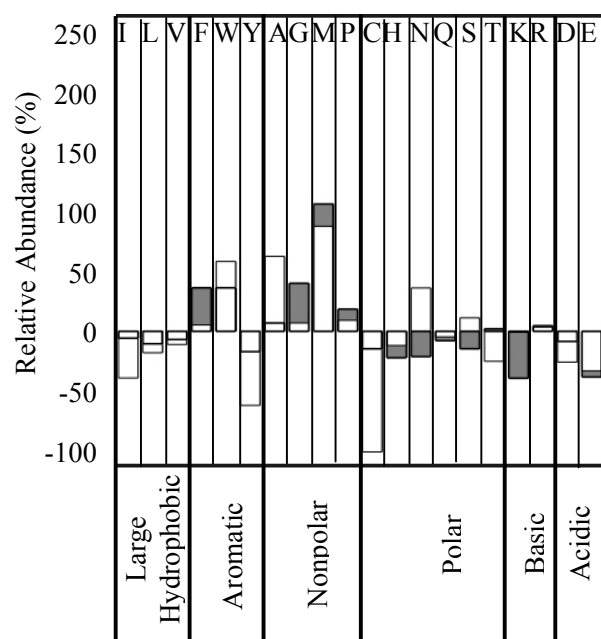


Figure 3.6 Relative abundance of the amino acids in all of the selected 12 amino acid peptide sequences for SiO₂ (100) (gray bars) and SiO₂ (001) (white bars) crystallographic surfaces.

SiO ₂	$\left[\text{SiO}_2^{(100)} \right]_{39}^{12}$	$\left[\text{SiO}_2^{(001)} \right]_{12}^{12}$	SiO ₂	$\left[\text{SiO}_2^{(100)} \right]_{39}^{12}$	$\left[\text{SiO}_2^{(001)} \right]_{12}^{12}$
$\left[\text{SiO}_2^{(100)} \right]_{39}^{12}$	1.071	1.089	$\left[\text{SiO}_2^{(100)} \right]_{39}^{12}$	-1.094	-1.119
$\left[\text{SiO}_2^{(001)} \right]_{12}^{12}$	.	0.902	$\left[\text{SiO}_2^{(001)} \right]_{12}^{12}$	.	-1.700

Figure 3.7. Similarity scores for both (100) and (001) binders by using BLOSUM 62 (at left) and PAM 250 (at right) matrices (Oren *et al.*, 2004, in process).

The physicochemical properties of the sequences, the relative abundances of the amino acids and the self similarity scores for both (100) and (001) quartz binders are very similar most possibly meaning that the selection process is free from the orientation of the atoms in the structure of quartz.

3.9 Silica Synthesis

As mentioned earlier, natural occurring silica binders, silicateins and silaffins, and phage display selected silica binders were used for the synthesis of silica *in vitro* when added to

a solution of silicic acid solution. It has been already known that natural silica binders can form silica nano particles *in vivo*. Silica synthesis is the first application for us to show that the quartz binders are really working. The best binder (DS202) for (100) plane quartz was synthesized commercially, to use in silica formation experiments. The procedure for the synthesis of silica is easy and fast which is carried out at ambient temperature and pressure. The conventional chemical synthesis of silica-based materials requires harsh conditions such as extreme temperature, pH and pressure, whereas biosilification occurs at RT, ambient pressure and neutral pH. The low cost biomimetic formation of silica nanostructures using polypeptides overcomes all of these limitations.

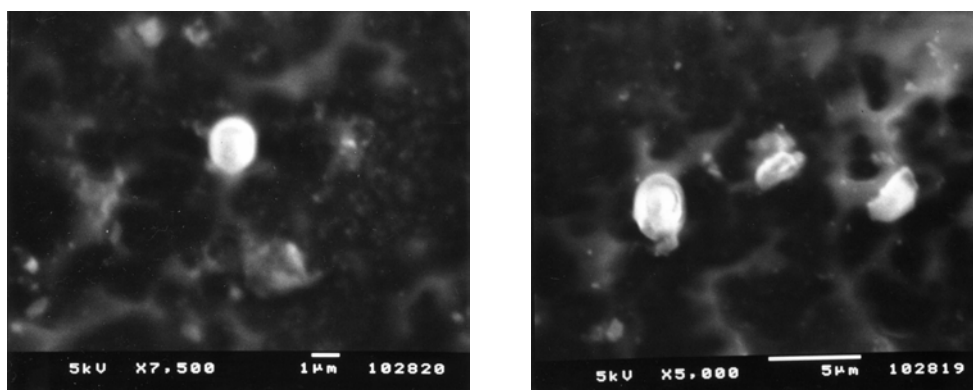


Figure 3.8 SEM images from the ppt formed in the tube containing DS202 showing the formation of silica spheres.

1 M tetramethylorthosilicate was hydrolyzed with addition of 1mM HCl. After synthesized peptide is added, the mixture is incubated for 5 min and then centrifuged. In the presence of the peptide, precipitation is formed in the tube. The precipitate is washed with water and then checked under SEM. Figure 3.8 shows the silica (SiO_2) particles formed in the presence of the peptide. EDS analysis shows that the precipitated particles are in the structure of SiO_2 . This is basically the same procedure when the silica particles are formed naturally in organisms or formed *in vitro* with the addition of silica binders.

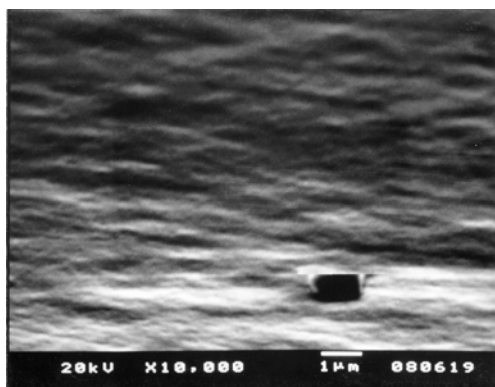


Figure 3.9 SEM image of the gel formed after 24 hours in the absence of peptide.

In the absence of peptide, the mixture of HCl and TMOS does not form any precipitate. The solution forms gel after 24 hours. Figure 3.9 shows SEM image of the gel formed after 24 hours without any silica nanosphere formation. The images in Figure 3.8 and Figure 3.9 clearly show that the selected quartz-specific binder is effective in the formation of silica.

4. CONCLUSION

(100) oriented single crystal quartz (SiO_2) was used as substrate for the selection of quartz-specific 12-aminoacid polypeptides from PhD-12 phage display library. 5 biopanning rounds were carried out to obtain totally 50 sequences, 10 sequences from each round. Table 3.1 gives the amino acid sequences of selected clones and their physicochemical properties. All the sequences were analyzed on amino acid base to search for any trend along the sequences or any convergence to specific sequence or an amino acid group as binding domain. Although charge and polarity values seem to be distributed randomly, hydrophobicity/hydrophilicity values show that the sequences with hydrophobic amino acids were being selected through the rounds. Indeed, when the relative abundances of each amino acid is calculated, it is clear that hydrophobic amino acids are over expressed while hydrophilic ones are under expressed as in Figure 3.1.

In the experimental binding characterization, Leica-fluorescence microscopy was used to test the binding affinities of each sequence and grouping the sequences mainly as good, moderate or weak binders. All of the sequences were immunolabelled and checked under microscopy to follow their binding affinities visually as shown in Figure 3.2. Semi quantitative approach was applied to derive relative binding strength of the selected sequences simply by calculating the binding percentages from the images simply by subtracting the background from the area containing labeled phages. The sequences were grouped into groups as strong, moderate and weak binders according to their binding percentages which basically show their affinities for the quartz substrate. Although the measurement of the binding coverage of the sequences under the fluorescence microscopy was semi-quantitative, it is the main and fast experimental step to characterize the binding affinities of the clones.

In our studies, we also followed a novel approach to verify the grouping of sequences as strong, moderate and weak binders obtained via immunolabelling techniques. The similarity score analysis was developed by using a dynamic programming method using BLOSUM 62 and PAM 250 scoring matrices. Self-similarity score for strong binders is relatively higher than the one for the weak binders which is verifying the distribution of the sequences into three groups; strong, moderate and weak binders. The strong binders has more correlation

between each others than the moderate or weak binders. Figure 3.2 shows the similarity scores calculated by using two matrices, BLOSUM 62 and PAM 250. Both of the analysis have the similar results verifying the grouping methodology as well as the necessity of grouping among the selected ones.

After testing the affinity levels of each clone, we carried out the specificity analysis of the selected clones. Palladium was used as the substrate for the cross specificity binding experiments. The results showed that very few of the binders (Figure 3.3.) had also affinity for palladium.

To understand the effect of surface orientation, (001) oriented single crystal quartz pieces were used for another phage display experiment (Table 3.2). Although surface distribution of Si and O atoms are basically similar to (100) oriented quartz, the distances of the Si atoms to the surface are different. The selected sequences were compared with the sequences for (100) oriented quartz. They were basically similar in physicochemical properties and even some of them were common in both phage display results.

The last step was the first application of the selected sequences. The best binder (DS202) for (100) oriented quartz was synthesized commercially and used for silica formation experiments *in vitro*. The results showed that in the presence of quartz binder, silica nanospheres were formed in silicic acid solution while in the absence of peptide there was no formation of silica. The silica nanospheres were imaged by using SEM and the images are given in Figure 3.7. The result clearly shows that the selected quartz-specific binder is effective in the formation of silica nanospheres.

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APPENDIX

The detailed information on amino acid distribution of the library used during the studies, N-terminal distribution of the 12 mer peptide –gIII fusion and structure of the M13 KE display vector are given in Figure A1, Figure A2 and Figure A3.

Amino Acid Distribution of the Ph.D.-12 Library

A total of 104 clones from the naive library were sequenced. Six clones (5.8%) did not contain a displayed peptide insert. The overall amino acid distribution from the remaining 1176 sequenced codons (98 clones x 12 random codons) is as follows:

<u>Amino Acid</u>	<u>Codons</u>	<u>Expected frequency*</u>	<u>Observed frequency</u>
Arg	CGK, AGG	9.4%	4.7% (55/1176) [†]
Leu	CTK, TTG	9.4%	9.3% (109/1176)
Ser	TCK, AGT	9.4%	10.0% (118/1176)
Ala	GCK	6.2%	6.0% (71/1176)
Gly	GGK	6.2%	2.6% (30/1176)
Pro	CCK	6.2%	12.2% (143/1176)
Thr	ACK	6.2%	11.1% (131/1176)
Gln	CAG, TAG [‡]	6.2%	5.1% (60/1176)
Val	GTK	6.2%	3.9% (46/1176)
Asn	AAT	3.1%	4.6% (54/1176)
Asp	GAT	3.1%	2.8% (33/1176)
Cys	TGT	3.1%	0.5% (6/1176) [†]
Glu	GAG	3.1%	3.1% (36/1176)
His	CAT	3.1%	6.3% (74/1176)
Ile	ATT	3.1%	3.4% (40/1176)
Lys	AAG	3.1%	2.8% (33/1176)
Met	ATG	3.1%	2.6% (30/1176)
Phe	TTT	3.1%	3.3% (39/1176)
Trp	TGG	3.1%	2.2% (26/1176)
Tyr	TAT	3.1%	3.6% (42/1176)

Figure A1. Amino acid distribution of the Ph.D.-12 library with expected and observed frequencies (Ph.D.-12TM, Phage Display Library Kit Manual).

*Expected frequency = # of codons for that amino acid ÷ 32 codons x 100%.

Note use of reduced genetic code NNK (32 codons) in library construction.

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

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

pIII leader sequence Kpn I

5' - ...TTA TTC GCA ATT CCT TTA GTG GTA CCT TTC TAT TCT CAC TCT
 3' - ...AAT AAG CGT TAA GGA AAT CAC CAT GGA AAG ATA AGA GTG AGA
 ...Leu Phe Ala Ile Pro Leu Val Val Pro Phe Tyr Ser His Ser

Start of mature 12-mer peptide-gIII fusion
 ↓
 NNK NNK NNK NNK NNK NNK NNK NNK NNK NNK NNK NNK GGT GGA GGT
 NNH NNH NNH NNH NNH NNH NNH NNH NNH NNH NNH NNH CCA CCT CCA
 Xxx Xxx Xxx Xxx Xxx Xxx Xxx Xxx Xxx Xxx Xxx Xxx Gly Gly Gly

Eag I

TGG GCC GAA ACT GTT GAA AGT TGT TTA GCA AAA TCC CAT ACA GAA
 AGC CGG CTT TGA CAA CTT TCA ACA AAT CGT TTT AGG GTA TGT CTT
 Ser Ala Glu Thr Val Glu Ser Cys Leu Ala Lys Ser His Thr Glu
 ← -28 sequencing primer

AAT TCA TTT ACT AAC GTC TGG AAA GAC GAC AAA ACT TTA GAT
 TTA AGT AAA TGA TTG CAG ACC TTT CTG CTG TTT TGA AAT CTA
 Asn Ser Phe Thr Asn Val Trp Lys Asp Asp Lys Thr Leu Asp

CGT TAC GCT AAC TAT GAG GGC...-3'
GCA ATG CGA TTG ATA CTC CCG...-5'
 Arg Tyr Ala Asn Tyr Glu Gly...
 ← -96 sequencing primer

K = G or T; M = A or C

Figure A2. N-terminal sequence of random 12-mer peptide-gIII fusion. The fusion is expressed with a leader sequence that is removed upon secretion at the position indicated by the arrow, resulting in the peptide positioned directly at the N-terminus of the mature protein. The hybridization positions of the -28 and -96 primers are indicated (Ph.D.-12TM, Phage Display Library Kit Manual).

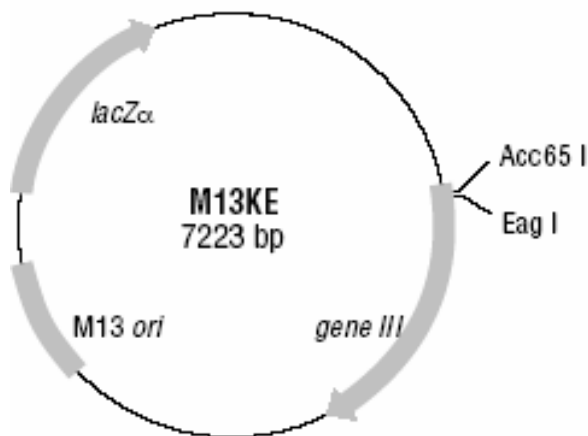


Figure A3. Structure of M13KE display vector with cloning sites engineered for N-terminal pIII fusion. (Ph.D.-12TM, Phage Display Library Kit Technical Manual).

CV

Deniz Şahin was born in 1978 in Manisa. He graduated from Gemlik High School in 1995. He had his Bachelors Degree in Molecular Biology and Genetics at Boğaziçi University in 2001. He worked as a visiting researcher in Westminster University/London in 2002. He has continued to his Masters Degree in Molecular Biology and Genetics-Biotechnology Program which is a part of Advanced Technologies in Engineering Graduate Program at İstanbul Technical University. He worked as a research assistant in the Department of Materials Sciences and Engineering at the University of Washington/Seattle from July 2003 to November 2004(funded by ARO-DURINT, PI: Prof. Dr. Mehmet Sarıkaya).