INORGANIC BINDING PEPTIDES DESIGNED BY PHAGE DISPLAY TECHNIQUES FOR BIOTECHNOLOGY APPLICATIONS

By

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To my family, friends, and lab members
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<td>Amino acids</td>
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<tr>
<td>ALP</td>
<td>Alkaline Phosphatase</td>
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<td>BG</td>
<td>β- D-galactosidase</td>
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<td>BioLBL</td>
<td>Biomimetic Layer-by Layer</td>
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<td>BLAST</td>
<td>Basic Local Alignment Search Tool</td>
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<td>BSA</td>
<td>Bovine Serum Albumin</td>
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<td>B/W Screen</td>
<td>Blue-White Screen</td>
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<td>CSD</td>
<td>Cell Surface Display</td>
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<td>DI</td>
<td>Deionized</td>
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<td>ELISA</td>
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<td>FITC</td>
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<td>GC</td>
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<td>HPLC</td>
<td>High Performance Liquid Chromatography</td>
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<tr>
<td>HRP</td>
<td>Horseradish Peroxidae</td>
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<tr>
<td>ICBR</td>
<td>The Interdisciplinary Center for Biotechnology Research</td>
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<tr>
<td>ICP-AES</td>
<td>Inductively Coupled Plasma-Atomic Emission Spectrometer</td>
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<tr>
<td>IF</td>
<td>Immunofluorescence</td>
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<tr>
<td>IgG</td>
<td>Immunoglobulins</td>
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<tr>
<td>IPTG</td>
<td>Isopropyl β-D-1-thiogalactopyranoside</td>
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<td>ITO</td>
<td>Indium Tin Oxide</td>
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<tr>
<td>MTB</td>
<td>Magnetotactic Bacterium</td>
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<tr>
<td>NCBI</td>
<td>National Center for Biotechnology Information</td>
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<tr>
<td>PD</td>
<td>Phage Display</td>
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<tr>
<td>PDMS</td>
<td>Polydimethyl Siloxane</td>
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<tr>
<td>PEG</td>
<td>Poly (ethylene glycol)</td>
</tr>
<tr>
<td>POM</td>
<td>Polarized Optical Microscopy</td>
</tr>
<tr>
<td>PZT</td>
<td>Piezoelectric Transducer</td>
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<td>RI</td>
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<td>SPR</td>
<td>Surface Plasmon Resonance</td>
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<tr>
<td>TIRF</td>
<td>Total Reflection Fluorescence</td>
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<tr>
<td>TMB</td>
<td>3, 3’, 5, 5’-tetramethylbenzidine</td>
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Biomacromolecules play an important role in the control of hard tissue structure and function via specific molecular recognition interactions between proteins of the matrix and inorganic species of the biomineral phase. During the construction of the tissue, biomacromolecules are usually folded into a certain conformation, analogous to a “lock” for fitting with other proteins or smaller molecules as a “key”. Currently, the rational design of molecular recognition in biomacromolecules is still hard to accomplish because the protein conformation is too complex to precisely predict based on the existing conformational information of proteins found in biological systems. In the past two decades, the combinatorial approach (e.g. phage display techniques) has been used to select short binding peptides with molecular recognition to an inorganic target material without a prior knowledge of the amino acid sequence required for the specific binding. The technique has been referred to as “biopanning” because bacteriophages are used to “screen” for peptides that exhibit strong binding to a target material of interest.

In this study, two diverse applications were chosen to demonstrate the utility of the biopanning approach. In one project, phage display techniques were used to pan for Indium Zinc Oxide (InZnO) binding peptides to serve as linkers between transducer devices and biosensing
elements for demonstration of the feasibility of reversibly electro-activated biosensors. The amorphous InZnO, with its homogeneous surface, led to three consensus peptide sequences, AGFPNSTHSSNL, SHAPDSTWFALF, and TNSSQFVVAIP. In addition, it was demonstrated that some selected phage clones of the InZnO binding peptides were able to be released from the InZnO surface after applying a voltage of 1400 mV on an electro-activated releasing device. In the second project, phage display techniques were used to select phage clones that bind specifically to francolite mineral in order to achieve separation of francolite particles from dolomitic particles within Florida phosphate ore. A phage clone with a 12-mer francolite binding peptide of WSITTYHDRAIV was able to concentrate the content of francolite from 25% to 42% in a bench-top flotation process of mixed minerals. The first system demonstrates an advanced technology application of the biopanning approach for the development of novel biosensors, while the second system demonstrates application of the biotechnology approach to a commodity industry.
CHAPTER 1
INTRODUCTION

1.1 Overview

In the past two decades, phage display techniques have brought a new dimension to the design of advanced materials. Beyond this field, however, there still exists abundant potential applications of organic-inorganic hybrid systems based on phage display techniques. This dissertation seeks to explore the diversity of these phage display techniques by focusing on two main projects, the first of which is centered on creating advanced materials for biosensor applications, whereas the second project seeks to evaluate the feasibility of these techniques for industrial production of commodities. In the advanced materials project, applications of phage display techniques were explored for the development of electroactive peptides which could be used for self-cleaning biosensors; the commodities project investigates the preferential separation of mineral particles based on surface modification of inorganic materials using inorganic binding peptides.

In the first application of phage display techniques, we considered that biosensing activities of biosensors can suffer because the receptors become clogged by analyte, whereby loss of detection usually causes underestimation in the measurement of concentration of analyte. Thus, self cleaning sensing components may be considered one of approach to provide biosensors with an effectively continuous detection. Thus, we proposed the concept of reversibly electro-activated peptide linkers to reversibly immobilize bioreceptors in close proximity to the surface of a transducer device for a self cleaning sensing component. With this idea in mind, we hypothesized that reversibly electro-activated peptides designed by phage display techniques could not only display specific binding affinity to an inorganic material of interest, but could also be released after applying a voltage to an inorganic surface. First, we utilized
immunofluorescence analysis and enzyme linked immunosorbent assay to evaluate the binding affinity and specificity of these peptides to inorganic materials. Then, we designed an electro-releasing device to test if these inorganic binding peptides could be released from the surface of the target material coated on the electro-releasing device.

With respect to the specific separation of mineral particles using inorganic binding peptides selected by phage display techniques, we considered that current commercial surfactants used in the phosphate mining industry are not able to remove dolomite from francolite in the froth flotation process. Recently, microorganisms have been found that are able to serve as flotation agents to float valuable minerals specifically, but low recovery rate of minerals were achieved at neutral pH, and a high dose compared to conventional chemical surfactants was required. Thus, we chose francolite pebbles as a target material for biopanning with M13 phage clones in order to find francolite binding peptides that could be used as flotation agents to achieve specific separation of francolite from phosphate ore containing dolomite contaminations. It was hypothesized that the phage display system could be based on a neutral working environment of phage display biopannings, and the small binding area of M13 phages on mineral particles might allow for a low concentration to be used. The feasibility of M13 phage amphiphiles as flotation agents in the commercial process of mineral recovery was evaluated, where the some representatives of phage amphiphiles were compared to standard commodity surfactants by way of recovery abilities. In this project, immunofluorescence analysis was used to choose clones with specific binding affinity to francolite relative to dolomite. We hypothesized that the phage body, which is relatively hydrophobic, could enhance the adhesion of francolite particles to air bubbles for floating francolite particles from phosphate ore containing dolomite particles. Therefore, we evaluated the hydrophobicity of phage coat proteins
using captive air bubble techniques to correlate with the effect of phage coat proteins on the recovery rate of minerals. Inductively coupled plasma-atomic emission spectroscopy (ICP-AES) was adopted to evaluate the purity of francolite in floated minerals after a mixture of francolite and dolomite was floated by a phage clone with an expressed francolite binding peptide.

1.2 Molecular Recognition of Biomacromolecules

In biological systems, biomacromolecules are usually involved in organization of molecules to hierarchical structures via their molecular specificity [1] to make up a myriad of different functions of soft tissues and hard tissues. For example, magnetite nanoparticles, cubo-octahedral single crystal of iron oxide, are aligned within *Aquaspirillum Magnetotacticum* [2], a kind of magnetotactic bacterium (MTB) (See Figure 1-1). Those magnetosomes are usually aligned in a chain within the MTB to form a permanent magnetic dipole. Thus, MTB swim along the magnetic field. For the formation mechanism of magnetosomes, the magnetosome specific proteins within magnetosome membrane (MM) are though to control the accumulation of iron, redox, and the nucleation of iron oxide [3].

Antarctic sponge, *Rosella racovitzea*, is another interesting example. The living environment of *Rosella racovitzea* is usually around 200 m under the ocean. However, green alga can survive in this environment’s lack of light by establishing symbiotic relations with *Rosella racovitzea* bodies [4]. The possible explanation is that silica-based spicules with star-shaped tips serve as light collectors. Those tips guide the light from optical silica-based fibers to the outer wall of the sponge. The structure of silica based optical fibers is a layer-by-layer silica shell surrounding the central core as shown in Figure 1-2 [2]. Some major protein components from the central core may behavior like enzymes that catalyze ions from seawater to form silica [5].
Furthermore, nacre, the structure of mother-of-pearl, is found at the interior of mollusk shells. Nacre is also a famous representative of the hybridization of organic and inorganic materials in nature. The apparent characteristic of nacre with high fracture toughness and strength is highly organized aragonite tablets separated by thin organic layers comprised of proteins and polysaccharides [6, 7] (See Figure 1-3 [2]). Those natural biological structures have inspired scientists and engineers to design human made materials for practical applications, utilizing the tools of molecular recognition that biological systems exemplify.

Basically, the concept of molecular recognition is defined as the relationship between lock-and-key host to guest molecules [8]. Then, guest molecule’s “key” must effectively fit into the host’s “lock”. In biochemistry, enzymes behave as the locks which fit with the desired substrates as the keys. For biomacromolecules, locks usually mean the crevices within the protein surface created by a certain conformation of the macromolecule, or the hallow sites within the molecular aggregates. However, keys are usually small molecules captured partially or completely by those crevice or hollow sites. In the other words, they are complementarily in molecular structure.

As to the flexibility of host/guest molecules, their conformations are not necessarily rigid. In most cases, their conformations are highly affected by the environment such as the change of pH, ion conductivity, and temperature. In addition, those conformations may continuously change to adjust the optimal steric arrangement during the occurrence of the host-guest binding event. The driving force of interaction between host and guest molecules is often entropically driven [9], as discussed below.

The chemical interaction between host and guest molecules consists of three components [10]: steric, polar, and hydrophobic fit. Steric fit means the interaction range between host and guest molecules must be within their van der Waals radii. Furthermore, their empty space
approaches a minimum. Electrostatic fit represents the maximum of polar interactions such as ionic bonding, hydrogen bonding, and aromatic ring-cation bonding. Finally, hydrophobic fit is the trend of association between non-polar groups in the biophase, an aqueous medium with dissolved ions and small molecules. This association tendency is defined by the density difference between host molecules and water. Thus, an association reaction becomes spontaneous because net dispersion attraction forces between host and guest atoms is larger that those between both of them separately and water, while there is an entropic gain from the release of hydration waters.

1.3 Challenges of Biopolymers in Biomimetics

Biological tissues are constructed with genetic control under mild physiological conditions and aqueous environments. During the construction of those tissues, biomacromolecules (proteins, polysaccharides, polynucleotides, or lipids) control those construction processes via their molecular recognition. In biomimetics, biomacromolecules are used to mimic the structure and biological function of proteins within the tissues. Those biomacromolecules are usually extracted from tissues followed by the process of purification. After the purification process, the composition of extraction may contain several biomacromolecules [11, 12]. In addition, the current knowledge leading to the prediction of protein structure and surface binding chemistry is not sufficient to perform the rational design of proteins [13].

1.4 Small Peptides Based on Combinatorial Techniques

At the start of this century, a combinatorial approach developed by the microbiology community was adopted, and that has brought a whole new dimension to the field of biomimetic engineering. The combinatorial biological techniques such as phage display (PD) [2, 14-54] and cell surface display (CSD) [55-58] had been adopted to select the peptide sequences that preferentially bind to inorganic or organic surfaces. Bacteriophages are virus particles that infect
bateria during their replication cycle. In combinatorial biological techniques, libraries need to be constructed to express random peptides on the phage or bacterial. For the construction of libraries, a set of random foreign oligonucleotides are inserted into the phage genomes or bacterial plasmids, which are responsible for expressing proteins on the surface of virus or bacterial cell. After those foreign oligonucleotides fuse with the phage genomes or bacterial plasmid respectively, they express random foreign peptides within those surface proteins (See Figure 1-4 [2]).

In this study, we focused on applications of phage display techniques. Using this approach, phage display libraries are used to “pan” for peptides that bind to the desired target materials. In the other word, those biopanning processes are basically an affinity selection technique which selects peptides that show binding affinity to a target. In the initial phage display techniques, antibodies were usually the target materials. In our studies, target materials were solid phase inorganic materials. Phage display techniques usually consist of four steps. First, phage libraries are constructed by inserting foreign desired gene segments into a region of the bacteriophage genome, the entirety of an organism's hereditary information. Thus, random peptides can display on the surface of a bacteriophage. Secondly, a phage library is incubted with a target material for capturing the phage on the surface of a target material. Subsequently, unbound phages are washed away. Then, bound phages are eluted. Expressed peptides from the DNA sequencing results of bound phages usually display strong and specific binding affinity to a target material. Some commercial phage display libraries such as Ph.D.-12™ (New England Biolab, Inc) consist of 2.7 x 10⁹ electroporated sequences.

The type of phages used in phage display includes Ff filamentous phages (M13, fd, and fl) and phages with the capsid shape (Lambda and T7). The amplification of Ff phage family is via a
non-lytic propagation mechanism. That means that all components of the phage coat are exported through the inner membrane of the bacteria prior to the assembly of mature phages. In this non-lytic mechanism, the density of coat proteins of filamentous phages is lower than phages with the capsid configuration because only proteins that can withstand export are displayed. However, the size of Ff phages is not related to their DNA. Thus, the insertion of foreign DNA within the genome does not affect the size of phages significantly. For Lambda and T7, their assembly is through a lytic mechanism in which the construction of the capsid occurs in the cytoplasm of cells. Thus, it is easy to display a high density of coat protein.

Most phage display libraries use filamentous phage strains due to their flexible and robust properties in display, even if the Ff family has the disadvantage of a low density of coat protein. Figure 1-5 shows the configuration of filamentous M13 phages which are flexible rods: 1μm in length and 6 nm in diameter [14]. Filamentous phages are composed of the major coat protein (pVIII) and minor coat proteins (pIII, pVI, pVII, and pIX). The phage body is composed of 2700 copies of pVIII protein (50 amino acids (AA) in length) with helical arrangement. Each end of a phage body is capped with two kinds of minor coat proteins: One end consists of pVII (5 copies, 33AA) and pIX (5 copies, 32AA). The other end includes pIII (5 copies, 406AA) and pVI (5 copies, 112AA).

Phage libraries can display random peptides within pVIII using insertion of foreign oligonucleotides into the gVIII genome. In general, the size of the expressed peptides is limited in this system. Furthermore, low and high affinity binders often can not be discriminated due to avidity effect, which means the combined strength of multiple bond interactions. However, this system is an ideal candidate if low affinity binders are mainly involved in studies. Currently, many phage libraries are generated to express random peptides at the amino terminus of pIII.
Although pIII proteins only has 5 copies to cause small avidity effect, the significant change of pIII proteins such as the fusion of heterologous proteins can prevent the viral assembly and block infectivity. In this research, the adopted phage library is New England Biolab-PhD.12™ which expresses random peptides in the terminus of pIII proteins. For the structure of inserted peptides in pIII proteins, there are two types currently available as commercial filamentous phage libraries: N-terminal constrained peptide insert and N-terminal linear peptide insert (See Figure 1-6 [27]). The phage libraries that we used express N-terminal linear peptide insert in protein pIII. In general, 12-mer peptides don’t display obvious conformations. However, 12-mer peptides expressed in pIII proteins composed of 406 amino acids may be folded into a periodic arrangement after binding on an inorganic surface.

In phage display selection (‘biopanning’), which is illustrated in the schematic in Figure 1-7, a large variant of phage mutants, 10⁹ random peptide sequences, are exposed to a desired target. Some phages with weak bonding to the target are removed with extensive washes. Then, more strongly bound phages can be eluted with a low pH solution. The eluted phages are amplified by infecting E. coli E2738 host bacterial strain, isolated, titered, and reexposed to fresh target to enrich the population of strong binders. The whole procedure is called a ‘biopanning’. Three to five biopannings are performed to evolve to the phages with strong binding affinity to targets (arrows in Figure 1-7). DNA of those selected phages is sequenced to determine the peptide binding sequences that provide molecular recognition to the target inorganic surface.

During the isolation of phage clones, a blue – white (B/W) screen is used for the purpose of phage isolation. The B/W screening method is a molecular biology technique for the detection of successful ligation in vector-based gene cloning which is based on the secretion of the enzyme β-galactosidase. In the PhD. 12™ M13 phage library, E. Coli ER2738 host strain does not
contain *lacZ* gene within its DNA. Thus, it cannot secrete β-galactosidase until *lacZ* within M13 phages fuse with its DNA to become bacteriophages (See Figure 1-8 (a)). The phage clones appear as blue plaques on the LB agar plate. An example is shown in Figure 1-8 (b).

The molecular mechanism of the B/W screen is related to the *lac* operon in *E. coli* strain as a host cell combined with a complementary subunit vector of the phage. Figure 1-9 [59] shows β-galactosidase can be secreted in the presence of inducer when the recombinant DNA of *E. coli* host strain with the target vector still keeps the existence of *lacZ* gene. Otherwise, the secretion of β-galactosidase will be blocked if *lacZ* gene is disrupted by foreign DNA, or repressors interact with operator.

β-galactosidase is the intracellular enzyme that can cleave the disaccharide lactose into glucose and galactose (See Figure 1-10 [59]). However, β-galactosidase is colorless. How do we detect the appearance of β-galactosidase? In general, the indicator, Xgal, is usually chosen to detect the existence of β-galactosidase. Xgal is a colorless modified galactose sugar that can be catalyzed by β-galactosidase to produce an insoluble blue product (5-bromo-4-chloroindole) (See Figure 1-11). At the same time, isopropyl β-D-1-thiogalactopyranoside (IPTG), which functions as the inducer of the *lac* operon, interacts with repressors to avoid them blocking the secretion of β-galactosidase.

### 1.5 Newly Designed Binding Peptides through Bioinformatics

After phage display selection, a consensus amino acid sequence of selected peptides which show strong binding affinity to the material of interest usually can be obtained. Figure 1-12 shows that platinum binding peptides on phage clones labeled with a fluorescein isothiocyanate (FITC) fluorescence probe display preferential binding affinity to the platinum area on a patterned substrate, rather than the quartz area [2]. Besides using phage display to pan for binding peptides from the biological and organic targets, such as antibodies, cells, and polymers,
inorganic binding peptides also have been selected for some practical applications. Table 1-1 includes some consensus amino acid sequences of inorganic binding peptides [18].

After post-selection of phage clones, a consensus amino acid sequence of a peptide insert just guarantees it exhibits strong binding affinity to a target of interest, rather than specific binding affinity. Thus, while some of the consensus peptide sequences display preferential binding affinity to the target of interest, other consensus peptide sequences can have binding affinity to several materials beside a target. Is there any strategy to pursue the peptides with specific binding abilities with using the biopanning approach?

Basically, a high degree of specificity is possible in biopanning through different approaches [24, 42, 46]. One approach named anti-selection is to take a set of clones that were determined to have affinity for the targeted surface, but then test the affinity of those clones with other surfaces that may be present in a given device (e.g. using immunofluorescence), and hope to find a clone that exhibits preferential affinity for the material of interest. Another interesting approach was demonstrated by Fang et al. [59], who used “subtractive” bacteriophage biopanning to identify 12-mer peptides that bind selectively, as well as induce the precipitation of titania, but not silica. Their approach consisted of two steps of biopanning: i) removal of phage particles containing silica-binding peptides from the phage library (the subtractive step), and then ii) isolation of phage particles bearing peptides that bind strongly to titania. Interestingly, the subtractive biopanning process yielded several acidic peptides enriched in hydroxyl-bearing residues, while prior reports of phage display biopanning with silica and titania targets led to the isolation of polycationic peptides enriched in basic residues. Thus, selectivity may need to be determined according to the demands of the system (or selectively may not even be required if a device only uses the one material that was targeted).
As a third option, the bioinformatics approach being developed by Sarikaya’s group also has the potential to design selective peptides de novo [60]. In this method (See Figure 1-13 [2]), a set of experimentally selected peptides are categorized for their binding affinities (such as quantifying % coverage of bound phage by immunofluorescence microscopy) and scoring matrices are defined. These include similarities within strong-binding sequences and the differences between the strong- and weak- binders. Experimentally, this entails categorizing a relatively large number of clones as strong, medium, and weak binders. They have demonstrated the capabilities of the approach by classifying experimentally characterized quartz-binding peptides and computationally designing new sequences with specific affinities, and found that binding of the computationally designed peptides correlated with their predictions with high accuracy [60], as described below.

The following example shows the approach of bioinformatics to design a new amino acid sequence of inorganic binding peptides. In this example, experimentally characterized quartz-binding peptides (Quartz I), BLOSUM 62, and PAM 250 were classified into strong, medium, and weak binding sets as the base respectively for developing the bioinformatic approach. Note-BLOSUM 62, and PAM 250 include quartz binding peptides sequences from natural proteins. Subsequently, the data base including 1,000,000 random peptide sequences were used to compare with the strong binder set in BLOSUM 62, PAM 250, and QUARTZ I to deduce newly designed quartz binding peptide sequences in the scoring matrix in Figure 1-14 [60]: The scoring matrix is the indication of similarity between two compared peptide sequences.

Finally, those predicted peptides must be confirmed by experimental validation, such as immunofluorescence (IF) analysis, to confirm their binding affinity matches with the experimental results. Basically, the quantity and quality of initial data determine the precision of the scoring
matrix. It is feasible to enhance the precision of the scoring matrix by expanding the quantity of experimental peptides. This is done by adding predicted peptides corresponding to the experimental validation of Quartz I to establish Quartz II for the second generation of scoring matrix, which can then design more peptides with specific binding affinity to targets.

1.6 Applications of Inorganic Binding Peptides

Inorganic binding peptides selected from phage display have been demonstrated for a variety of applications, such as morphology modifier, template nucleator, quantum dots, nanoparticle synthesis, and molecular linkers via their molecular recognition for two inorganic materials at opposite ends of dual peptide linkers. Additives can regulate the morphology of a crystal because the crystallographic faces with the adhesion of additives are stabilized by lowering surface energy. Thus, a distinctive application of inorganic binding peptides is as morphology modifiers. As in the example shown in Figure 1-15(a) [18, 61], gold can alter its crystal habit that is expressed when gold binding peptides interact with specific crystallographic faces. After gold binding peptides adhere onto the (111) faces of gold particles, the morphology of gold was changed into flat triangular or pseudo-hexagonal shapes. Under the equilibrium conditions without any additive, the shape of the gold particles is cubo-octahedral as shown in Figure 1-15(b) [61].

Furthermore, inorganic binding peptides also can be applied in the fabrication of nanoparticles. Naik et al. proposed a mechanism for silver nanoparticle formation using silver binding peptide to create a silver reduction layer as depicted in Figure 1-16 [23]. Silver binding peptides interact with the clusters of silver metal atoms in aqueous silver nitrate solution. Silver ions tend to undergo a reduction reaction to silver metal atoms, which deposit onto the silver clusters. This phenomenon is mainly as result of the reducing environment which is produced around the clusters via the interaction of silver binding peptides with silver clusters. Finally,
silver atoms from the reduction reaction aggregate around the clusters to form nanoparticles that precipitate from solution. The TEM images of silver nanoparticles created with the silver binding peptide base process from Naik et al. are shown in Figure 1-17 [23]. The size of the silver particles is 60-50 nm. The electron diffraction pattern indicates a face centered cubic lattice structure which matched with that of silver.

The silver binding peptides were further utilized by patterning the silver nanoparticles. In a micromoulding in capillaries (MIMIC) technique [62], the elastomer polydimethyl siloxane (PDMS) with patterned microfluidic channels was placed onto a glass substrate as a stamp (See Figure 1-18a [23]). A silver-binding peptides solution was guided to flow through those microfluidic channels as templates for silver deposition in a spatially ordered array. Next, silver binding peptides on the surface of microfluidic channels were incubated with silver nitrite solution to nucleate silver particles as shown in Figure 1-18b [23]. When the silver nanocrystal array was illuminated with a mercury lamp, fluorescence due to light scattering of silver nanoparticles was observed (See Figure 1-18c [23]).

In the microelectronics industry, top-down photolithography methods are used for the control of x-y positioning. However, a biomimetic layer-by layer (BioLBL) approach, which involves the usage of inorganic binding peptides, can enable one to control the stacking of molecules in the z-coordinate. By combining BioLBL with photolithography, it is possible to fabricate 3D nanoscale structures in an x-y-z controlled manner [63]. Furthermore, inorganic binding peptides also can be applied in the assembly and immobilization of inorganic nanoparticles in 2D and 3D geometries. In general, quantum dots are produced with vacuum techniques such as molecular beam epitaxy as shown in Figure 1-20a [18]. A desirable alternative would be not only to synthesize inorganic nanodots under mild conditions, but also to
immobilize/self-assemble the nanodots. A suitable method is to use inorganic binding peptides with specific recognition for inorganics for nanoparticle assembly. The advantage of this approach is inorganic binding peptides can genetically or synthetically be fused to other functional biomolecular units or ligands to form heterofunctional molecular entities. Figure 1-20b-c [18] shows the assembly of nanogold particles on a plate polystyrene surface coated with inorganic binding peptides, which resemble the distribution of quantum dots obtained by high vacuum deposition techniques.

Liquid crystalline materials have been the key component in optical and electronic devices that contain liquid crystal displays. The molecular structure of liquid crystalline materials often comprises two components: rod-like segment and flexible segment. Becher and coworkers have showed that rod shape viruses display a distinct liquid crystalline phase [64, 65]. Filamentous M13 phage also fit with the requirements of liquid crystals: The bodies of M13 phages have a rod-like structure. pIII minor proteins of M13 phages behave as flexible chains. Furthermore, peptide inserts in pIII of M13 phage can bind and nucleate desired inorganic materials at the nanometer scale. Thus, an ordered nanocrystalline thin film can be fabricated using the liquid crystalline phase displayed by the nanocrystal functionalized M13 virus [66, 67].

The dry Au-phage thin film was prepared in a diluted Au-phage solution (~ 6 mg ml⁻¹) as shown in Figure 1-21 (a) [66]. This viral nanocrystal hybrid thin film was transparent, corresponding to the optical property of nanocrystalline materials. Upon characterization by polarized optical microscopy (POM), the texture of the striped pattern in Figure 1-21 (b) [66] matches with the smectic phase of liquid crystals [67]. In scanning electron microscopy (SEM) analysis, the morphology of the Au-virus thin film also corresponded to a long range ordered smectic phase: the spacing of the zigzag periodic band (9.34±0.78μm) fit with the distinct feature
of a chiral smectic C structure [67]. Individual complexes of Au-M13 phage can be observed by TEM from particles extracted from a much diluted smectic suspension solution (See Figure 1-21 (d)) [66]. The TEM image in Figure 1-21 (d) shows that a 10 nm gold particle is bound to the pIII end of one phage. Thus, the recognition of inorganic binding peptides based on phage display techniques can achieve molecular construction for a variety of applications.
Table 1-1. Consensus peptide sequences that have binding affinity to the various inorganic materials. Table taken from Nat Mater 2003 Sep;2(9):577-585 By Sarikaya M, Tamerler C, Jen AKY, Schulten K, Baneyx F [18]. Permission was granted by Nature Publishing Group.

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*<sup>a</sup> Positive charges are calculated using Compute<sub>pI</sub>/MW<sub>gel</sub> method (http://service.expasy.org/compute_pI/mw_gel/).

<sup>b</sup> Molecular mass of peptides are calculated using Compute<sub>pI</sub>/MW<sub>gel</sub> method (http://service.expasy.org/compute_pI/mw_gel/).

<sup>c</sup> Charge was calculated by subtracting the number of basic residues (lysine and arginine) from the number acidic residues (aspartic acid and glutamic acid).

<sup>d</sup> Unpublished results by the authors.

<sup>e</sup> Most frequently observed sequences.
Figure 1-1. TEM images for nano magnetosomes particles within the bacterium *Aquaspirillum Magnetotacticum*. Image taken from Acta Biomater 2007 May;3(3):289-299 by Tamerler C, Sarikaya M. [2]. Permission was granted by Elsevier.

Figure 1-2. Images of the spicules with start shaped tips on the body of the spong *Rosella racovitzea*. Image taken from Acta Biomater 2007 May;3(3):289-299 by Tamerler C, Sarikaya M. [2]. Permission was granted by Elsevier.
Figure 1-3. Images for the nacre structure of mother-of-pearl mollusks. Image taken from Acta Biomater 2007 May;3(3):289-299 by Tamerler C, Sarikaya M. [2]. Permission was granted by Elsevier.

Figure 1-4. Schematic for the generation of random peptide libraries. Schematic taken from Acta Biomater 2007 May;3(3):289-299 by Tamerler C, Sarikaya M. [2]. Permission was granted by Elsevier.
pIII: 5 copies, 406aa  pVI: 5 copies, 112aa  pVIII: 2700 copies, 50aa
pVII: 5 copies, 33aa  pIX: 5 copies, 32aa

Figure 1-5. The configuration of a filamentous M13 phage. Image taken from Plant Mol Biol 2002 Dec;50(6):837-854 by Willats WGT. [14]. Permission was granted by Oxford University Press.

Figure 1-6. Schematic of inserted peptide structures within pIII proteins. (a) Minor proteins pIII (orange) and major proteins pVIII (green) of filamentous M13 phage are highlighted (b) The single strand DNA of M13 phages includes genome gIII and genome gVIII (c) Two types of structures of inserted peptides at the terminus of protein pIII. Image taken from J Mater Chem 2003;13(10):2414-2421 by Flynn CE, Mao CB, Hayhurst A, Williams JL, Georgiou G, Iverson B, et al. [27]. Permission was granted by the Royal Society of Chemistry.
Figure 1-7. Schematic of the phage display (PD) technique.

(a) Schematic of bacteriophage with lacZ gene. E. coli ER2738 host strain: F' lacP\(^{\Delta}(lacZ)M15\) proA+ \(\Delta\) (lac-proAB) \(\Delta(hsdS-mcrB)5\) (\(\gamma\) - \(\alpha\) - McrBC-). Recombinant of E. coli gene with M13 phage gene.

(b) β-galactosidase

Figure 1-8. Schematic of the blue-white screen. (a) Schematic of bacteriophage with lacZ gene. (b) An example of the B/W screen: the blue spots on LB agar plate are bacterial colonies that contain transfected M13 phage clones.
b. Inducer- Depression

**Figure 1-9.** Mechanism of the blue-white screen

**Figure 1-10.** Metabolism of lactose in the presence of β-Galactosidase
Figure 1-11. Metabolized reaction of Xgal under β-galactosidase

Figure 1-12. Image of a phage clone with the specific binding affinity to platinum. Image taken from Acta Biomater 2007 May;3(3):289-299 by Tamerler C, Sarikaya M. [2]. Permission was granted by Elsevier.
Figure 1-13. Schematic of the bioinformatics approach. Schematic taken from Acta Biomater 2007 May;3(3):289-299 by Tamerler C, Sarikaya M. [2]. Permission was granted by Elsevier.

Figure 1-14. The scoring matrix for quartz binding peptides. The amino acids are colored according to their chemical properties (hydrophobic, acidic, basic and polar). Image taken from Bioinformatics 2007 Nov;23(21):2816-2822 by Oren EE, Tamerler C, Sahin D, Hnilova M, Seker UOS, Sarikaya M, et al. [60]. Permission was granted by Oxford University Press.
Figure 1-15. The morphology of gold nanocrystals. (a) gold binding peptides on (111) faces; (b) no additive. Image taken from J Mol Biol 2000 Jun;299(3):725-735 by Brown S, Sarikaya M, Johnson E. [61]. Permission was granted by Elsevier.

Figure 1-17. TEM analysis of silver nanoparticles. (a) A TEM image of silver nanoparticles created using silver binding peptides: The size of the particles is around 60-150 nm. (b) High magnification of one silver nanoparticle. Insert: The array of crystal spots in the electron diffraction pattern indicates the structure of the nanoparticles corresponds to a face centered cubic arrangement of silver metal. Images taken from Nat Mater 2002 Nov;1(3):169-172 by Naik RR, Stringer SJ, Agarwal G, Jones SE, Stone MO. [23]. Permission was granted by Elsevier.

Figure 1-18. Schematic for the spatial control of an array of silver nanocrystals using silver binding peptides. (a) A patterned elastomer (polydimethyl siloxane, PDMS) mould was used to create microfluidic channels that serve to guide the AG4 silver binding peptide solution on the glass substrate by capillary action. The peptides adsorb on the glass surface in a pattern defined by the network. (b) Optical bright-field image showing the linear arrays of silver obtained after incubation of the AG4 patterned glass substrate with 0.1 mM silver nitrate for 48 h at room temperature. (c) Autofluorescence of the biomimetically synthesized silver particles when excited with a mercury lamp. Images taken from Nat Mater 2002 Nov;1(3):169-172 by Naik RR, Stringer SJ, Agarwal G, Jones SE, Stone MO. [23]. Permission was granted by Elsevier.
Figure 1-19. Images of gold quantum dots. (a) The atomic force microscope image shows quantum dots (GaInAs) assembled on GaAs substrate through vacuum techniques (b) Assembly of gold nanoparticles through inorganic binding peptides (c) Schematic illustration of the process used to create nanoparticle array in b, showing dual peptide linker that can bind to both the glutaradehyde and gold. PS: polystyrene substrate, GA: glutaradehyde crosslinking agent, GEP-1: inorganic binding peptides with specificity to gold. Image taken from Nat Mater 2003 Sep;2(9):577-585 by Sarikaya M, Tamerler C, Jen AKY, Schulten K, Baneyx F. [18]. Permission was granted by Nature Publishing Group.

Figure 1-20. Images of smectic ordered self-supporting Au-virus films. (a) Photography of a dry Au-phage thin film (b) Polarized Optical Microscopy image of the Au-phage film (Scale bar: 20μm) (c) SEM image of Au-phage film (Scale bar: 5μm) (d) TEM image of an individual complex of Au-phage. (Insert: A fast fourier transform image and lattice fringe image of a gold nanoparticle at the pIII end of the phage.). Images taken from Adv Mater 2003 May;15(9):689-692 by Lee SW, Lee SK, Belcher AM. [66]. Permission was granted by Wiley VCH.
CHAPTER 2
INORGANIC BINDING PEPTIDES BASED ON PHAGE DISPLAY FOR BIOSENSOR APPLICATIONS

2.1 Motivation

In this study, we applied the biopanning process to search for peptides that do not necessarily have a strong binding affinity for a surface, but instead have a reversible binding affinity. If the reversibility could be precisely regulated, then one could design “smart” surfaces for a whole host of applications. One possibility which is explored here is to pan for electroactivated peptides which can be electrically triggered to either adsorb or desorb (or both) from an electronic material’s surface as an electric field is applied.

An exciting application that results from electroactivated peptides is the possibility of self-cleaning device surfaces by electrically-triggered release of a coating. Biofouling contributes to a significant number of infections created with implanted biomaterials and devices, and is a significant problem with biosensor arrays as well. One could conceivably design a microsystem that could reduce or eliminate a biofilm. This could also help to overcome the hurdle in continuous sensing devices, because a system could be designed that replenishes spent receptors that have become clogged upon binding to the analyte or impurities (Figure 2-1). In this design, the surface of a transducer device could be patterned for multi-components as well. One could envision a system where the device is electronically stimulated to release the spent receptors, which is followed by a flow through system that contains a new batch of fresh receptors, which then bind and assemble on the surface once it is returned to the de-activated (or activated binding) configuration. This could then provide for long-term continuous biosensing if such as replenishment is done on an automated basis. This would be particularly valuable for fieldwork applications, ranging from biosensors for soldiers at the front, to point-of-care diagnostics in personal healthcare, to testing of animal/plant food products in the agricultural arena.
2.2 Background and Significance

Healthcare is an important issue worldwide. The death rate of diseases in the countries with few medical sources is higher than developed nations. The clinics in those countries have a need for drugs to treat illness. However, the problem is that diagnostic equipments are not widespread due to unaffordable prices for them. Diseases often are not identified, misdiagnosed or therapy is delayed. Thus, it is necessary to develop easy, economic, immediate diagnostic tools for the patients’ welfare.

Biosensors can be strong candidates for overcoming unbalanced distribution of medical sources between cities and countries based on several reasons as follow: Inexpensive cost of production is affordable for general clinics. All components of biosensors can be integrated in a small chip. In addition, biosensors have low energy consumption. This makes it possible to carry biosensors to poor areas. Furthermore, fast detection time also reduces the waiting time to allow doctors to initiate the effective therapy for patients immediately. In the past 40 years, biosensors have been developed as analytical tools for the detection of biological and chemical agents in diagnostic, environmental field monitoring, agriculture production, the pharmaceutical manufacturing and food processing.

The worldwide market of biosensors in 2003 was about $7.3 billion (Figure 2-2) [68]. In 2007, the market grew to 10.8 billion with 10.4% growth rate even under a weak global economy. The trend of increasing biosensor development is mainly in the health care industry. For example, 6% of the populations in Europe and America are diabetic. Thus, availability of glucose biosensors with simple, rapid, accurate detection is in urgent demand for the diagnosis of diabetes [69]. The research areas in biosensor development are wide and interdisciplinary including biological, biochemistry, physical chemistry, electrochemistry, electrical engineering, and software engineering.
2.2.1 Definition of Biosensors

What are biosensors? Biosensors are defined as analytical devices which can specifically, rapidly, and continuously convert biological/chemical response into an electric signal. The term “biosensor” is usually referred to as the sensor device which quantifies the concentration of biological substances when a biological system can not be used directly. In general, a biosensor device consists of a biosensing component chemically or physically immobilized onto the transducer element which converts a biological, chemical, or biochemical signal to a quantifiable and processable electrical signal [70]. As shown in Figure 2-3, biosensing probes could be enzymes [71-74], antibodies [75], organelles, nucleic acids [76-81], cells, tissues, microbes [75, 82-88], and more. The transducer in a biosensor device may be electrochemical (such as amperometric [89-91], potentiometric [92, 93], conductive [94], impedance [94], voltammetric [94], and etc.), optical (including optical fiber, surface plasmon resonance [95, 96], absorption, chemiluminescence, bioluminescence [97], fluorescence, and etc.), piezoelectric (quartz crystal microbalance [30, 63, 75, 98, 99], and surface acoustic wave [100-102], magnetic [103], calorimetric, and others [104, 105]).

When the detectable signal from an analyte-biosensing probe binding event is transferred to a transducer, this type of biosensing material can be directly linked to the transducer as a label free biosensor. Otherwise, labeling is required to achieve the delivering of a signal through the transducer. Basically, the function of labels is to amplify biological signals. Labels could be fluorescence, chemiluminescence, bioluminescence, enzymes, metal particles, and nanoparticles. Finally, a computer system is required for data processing, network connection, wireless communication, and the usage of a data base.

What characteristics are necessary for an effective biosensor? A successful biosensor must possess at least some of the following beneficial features:
• **Specificity and stability:** The biosensing probe must be highly specific to analyses, be stable under normal storage conditions and show good stability over a large number of assays.

• **Less sampling:** It should allow the minimum pre-treatment of samples for loading onto the biosensor device.

• **Binding event with less interference:** When biosensing materials interact with analytes, the binding event must not be altered by pH, temperature, and other physical parameters. In addition, if the binding event will involve co-zymes. The best way is that cozymes are co-immobilized with the enzyme.

• **Effective signal:** Electrical noise should not interfere with the real signal from a biosensor. The signal must be precise, accurate, reproducible and linear over the effective measurement range.

• **Biocompatibility:** The probe must be tiny and biocompatible, with no toxin release, if the biosensor is to be used for monitoring situations in biological systems. In addition, biosensors must have antifouling properties because biosensing materials such as enzymes may decompose under an autoclave environment.

• **Cost-effectiveness and easy operation:** The ideal biosensor should be cheap, small, portable and easy to operate.

### 2.2.2 Immobilization Methods of Biosensing Materials

One critical aspect in the fabrication of biosensors is how to link the biological components to the transducer device, and in a way that allows transduction of the signal, such as the occurrence of a receptor-binding event. Thus, it is necessary to immobilize the biosening molecules close enough to the transducer surface to provide sensitivity, operation stability, and response at a satisfactory level during the detection of analytes.

The immobilization of biosensing components on transducer elements can be divided into chemical and physical pathways [84, 85]. In chemical immobilization methods, the interaction between biosensing materials and transducer surfaces involve the formation of covalent binding or crosslinking. For covalent immobilization, biosening materials such as enzymes, antibodies, oligonucleotides, and carbohydrates can be bound onto the transducer surface by chemical bonding through amino, carboxyl, sulphydryl, or aromatic side groups of biosening molecules. In
addition, viable cells also can be covalently immobilized onto the transducer surface by the chemical reaction between functional groups on the cell walls of microorganisms and the transducer surface. During the formation of covalent bonding, microorganisms inevitably contact harsh chemicals. This may damage the cell membranes and biological activities.

Another chemical immobilization method is crosslinking, which involves the formation of a network structure via the linkages between functional groups on biosensing molecules and multifunctional agents such as glutaraldehyde, hexamethylene diisocyanate, and 1,5-dinitro-2,4-difluorobenzene [84]. Crosslinking is widely adopted in the immobilization of microorganisms onto transducer surfaces if the detection of analytes just relies on intracellular enzymes, rather than cell viability [84, 85]. The advantage of this method is that it can reduce the wear of biosensing probes. However, high mechanical strength of a transducer surface can not be obtained via crosslinking immobilization.

If a viable cell is required during the detection event, the formation of covalent bonds between microorganisms and transducers should be avoided. The suitable strategy to keep the viability of cells is physical immobilization including adsorption and entrapment. Physical adsorption is a simple method with less disruption to biosensing materials. However, the interaction between biosensing materials and the transducer surface is weak causing a short lifetime (several days) in this kind of immobilization method. Biosensing materials, including enzymes, antibodies, and microbes, can be adhered onto a transducer matrixe via ionic, hydrophilic-hydrophilic, hydrophobic-hydrophobic, and hydrogen bond interactions. Although biosensing materials can survive in those mild environments, poor long term stability is the drawback in adsorptive immobilization.
With respect to the entrapment immobilization of biosensing materials, biosensing materials are confined to the proximity of the transducer surface by dialysis membranes. A dialysis membrane provides several functions such as structural framework, selective ion permeability, and mediation of the electron transfer process. Another way is to mix biochemical/polymer gels such as agaros, alginate, or polyurethane with microorganisms, and then coat those mixtures onto the transducer surface. The main disadvantages in entrapment methods are low sensitivity and detection limit due to the diffusion problem of entrapment materials.

2.2.3 A Challenge in Biosensor Development

Biological components immobilized onto a transducer device through physical interaction, including adsorption and entrapment, usually have the diffusion problems which cause low stability of the biosensors. Thus, functionalizing the inorganic interface with a covalent linker is usually adopted to achieve high stability of these biosensors. However, one of the major problems in the biosensors with covalent linkers is the loss of activity of molecular probes after surface immobilization because the molecular probes are gradually clogged by analyte.

Thus, one challenge with a biosensor device is the ability to have continuous detection, which means that the biosensing component needs to release its analyte and be restored back to its original active state. One way to achieve this might be to use a compound with relatively weak binding characteristics so that sufficient flow will pull off the compound after the detection event. An alternative approach is proposed in this study, in which inorganic binding peptides designed by phage display techniques could be tailored as linkers between the organic-inorganic interface, such as for the attachment of biosensing components (e.g. DNA aptomers, antibodies and peptide epitopes) to inorganic transducer elements, and be released by an electrical trigger from the transduction element in a biosensor device. An additional advantage of this system is
that peptides designed by phage display techniques are short (e.g. random expressed peptides in phage libraries kit from New England Biolab, Inc. are usually 7-mers or 12-mers), which bring the analyte closer to the transducer surface for improved sensitivity as compared to avidin-biotin linkages, which are macromolecular proteins.

There are some literature reports that support the premise of using an electric field to release a bound organic, where ‘electrodesorption’ has been of interest for anti-biofouling surfaces. Tang et al. [106] found that electrical stimulation could remove a triblock copolymer of \text{co(propylene sulfide-block-ethylene glycol)} from indium tin oxide (ITO) surfaces. These copolymers have been studied as protein resistant coatings that originally were found to chemisorb onto gold surfaces. This paper shows they also adsorb onto the transparent conducting surface of ITO, which was postulated to occur through direct sulfide-indium or tin interactions. They applied an ascending anodic electrical stimulus to the surface of the modified samples, and found that copolymer was steadily removed, with complete loss of a polymeric monolayer at a potential of 2000 mV.

Yeh et al. [107] have also demonstrated electric field desorption, and in this case, the protein bovine serum albumin (BSA) from a lead zirconate titanate substrate (a kind of piezoelectric transducer (PZT)) coated with either fired silver or titanium. They compared DC versus sinusoidal AC signals, and found the vibration mode of the piezoelectric aided in the removal, where 58% protein could be removed from the silver coated PZT, while 39% could be removed from the titanium coated PZT. Through modeling, they believe that the applied electric potential was the major contributor in reducing the adhesive force between protein and surface, where the desorbed protein was then taken away by acoustic streaming shear stress. They describe a mechanism which considers application of the voltage to lead to an accumulation of
charges on the surface due to the capacitance character of PZT, where at a certain voltage, the charge polarity will be the same as that of the adsorbed proteins, leading to repulsive forces between surface and proteins. In addition, because the acoustic streaming velocity is dependent on vibration amplitude, and the shear stress is linear to the streaming velocity, they proposed that the PZT plates with larger vibration amplitude would have greater BSA desorption, which was observed.

These reports demonstrate that the electodesorption principle is feasible, and given the large size of the synthetic copolymer or protein, both of which may have many binding sites, it seems reasonable to speculate that smaller peptides should be desorbed more quickly and completely, and at lower potential if the proper binding chemistry is selected. Therefore, my goal was to use phage display techniques to pan for short inorganic binding peptides that could be desorbed by an electric field for achieving reversibly electroactivated biosensors.

2.3 Materials and Methods

2.3.1 Materials

2.3.1.1 Dodecapeptide phage display peptide library (Ph.D.-12)

*New England Biolabs* Ph.D.-12 phage display kit is based on M13 phage vector modified for pentavalent display of peptides as N-terminal fusions to the minor coat protein pIII. The phage display Ph. D.-12 library contains 100μl 2 x 10^{13} pfu/ml (1.29 x 10^9 12-mers peptide sequences supplied in TBS with 50% glycerol). There is a short linker sequence between the displayed peptide and pIII: Gly-Gly-Gly-Ser.

2.3.1.2 *E. coli. ER2738* host strain

F’ proA+B+ lacIq Δ(lacZ)M15 zsf::Tn10 (TetR)/fhuA2 glnV thi Δ(lac-proAB) Δ(hsdMS-mcrB)5 (rk’ mk’ McrBC’). Host strain supplied as 50% glycerol culture was included in *New England Biolabs* Ph.D.-12 phage library kit.
2.3.1.3 Bacterial culture medium

**Lauria-Bertani (LB) Lennox medium:** Twenty gram LB Lennox (Fisher Brand) (trypton: yeast extraction: NaCl = 2:1:1) were dissolved in 1 liter distilled water and adjusted pH to 7.0-7.5. The LB medium was sterilized for 15 minutes at 1.5 atm (121°C) in an autoclave (NAPCO, model 800-DSE).

**LB agar medium:** Forty gram LB agar (Fisher brand) was dissolved in 1 liter distilled water until the solution become transparent by heating. The pH was adjusted to 7.0-7.5 and the solution was sterilized for 15 minutes at 1.5 atm (121°C) in an autoclave.

**Top agar medium:** Twenty gram LB (Fisher brand) and 15g agar were dissolved 1 liter distilled water by heating until the solution became transparent. The top agar medium was sterilized for 15 minutes at 1.5 atm (121°C) in an autoclave.

**10x Minimal salts (MS):** Three gram Na₂HPO₄-7H₂O (Fisher brand), 1.5g KH₂PO₄ (Fisher brand), 0.25g NaCl (Fisher brand), and 0.5g NH₄Cl (Fisher brand) were dissolved in 50 ml distilled water.

**M9 Solid medium plate:** Ten ml MS and 1.5g agar (Fisher brand) were dissolved in 89 ml distilled water by heating until the solution became transparent and sterilized for 15 minutes at 1.5 atm (121°C) in an autoclave. Then, sterilize 1M MgSO₄·6 H₂O (Sigma-Aldrich), 1M CaCl₂ (Fisher brand), and 40(w/v) % glucose (Fisher brand) were filtered by using 0.2μm single use sterile syringe filter. Then, 0.2 ml sterilized 1M MgSO₄·6 H₂O, 0.01 ml sterilized 1M CaCl₂, and 0.5 ml sterilized 40 % (w/v) glucose were added into the agar/MS mixture. Finally, pour liquid M9 medium was poured onto 90 mm petri dish until solidification.
2.3.1.4 **Stock solution**

**Tetracycline stock:** Twenty mg/ml tetracycline hydrochloride (Sigma-Aldrich) was dissolved in 70% ethanol and stored at -20°C.

**Xgal/IPTG stock:** Five gram IPTG (ultrapure grade, dioxane free, Molecul A) and 4g Xgal (Molecul A) were dissolved in 100 ml DMF (Sigma-Aldrich) and stored at -20°C.

**Glycerol stock solution:** Eighty ml glycerol (Sigma-Aldrich) and 20ml distilled water were mixed to make 80% (w/v) and sterilized for 15 minutes at 1.5 atm (121°C) in an autoclave.

2.3.1.5 **Buffer solutions**

**PEG-NaCl:** Twenty gram Poly (ethylene glycol) (PEG) -8000 (Sigma) and 14.61g NaCl (Fisher) were dissolved in distilled water up to 100ml and sterilized for 15 minutes under 1.5 atm at 121°C.

**Detergent stock solution:** Two ml (w/v) Tween 20 (Enzyme grade, Fisher) and 2 ml (w/v) Tween 80 (for molecular biology, Sigma-Aldrich) were added into 6 ml distilled water to get 20% (w/v) Tween 20/ 20% (w/v) Tween 80.

**PC buffer:** Three gram KH₂PO₄ (Fisher), 1.90 g Na₂CO₃ (Fisher), and 3.50 g NaCl (Fisher) were dissolved in 400ml distilled water to get 55 mM KH₂PO₄, 45 mM Na₂CO₃, and 200 mM NaCl. An appropriate amount of detergent was added from the detergent stock into the PC buffer according to the desired detergent concentration (eg. 0.02%, 0.1%, 0.5%). PC buffers were sterilized by using a 0.2μm single use sterile syringe filter.

2.3.1.6 **Elution buffers**

**Low pH elution buffer solution:** Three gram glycine (Sigma-Aldrich) and 400 mg BSA (Sigma-Aldrich) were dissolved in distilled water up to 200 ml to get 0.2 M glycine and 1 mg/ml
BSA, and the pH was adjusted to 2.2 with a combination of 10 M HCl and 1M HCl. The solution was sterilized by using a 0.2 μm single use sterile syringe filter.

**High salt elution buffer solution:** Five M MgCl$_2$.6H$_2$O was prepared and sterilized using a 0.2 μm single use sterile syringe filter.

### 2.3.2 Methods

#### 2.3.2.1 Fabrication of Indium Zinc Oxide (IZO) as a target material

In this study, Indium Zinc Oxide (IZO) was chosen for the initial substrate as a target material for panning with a M13 phage library to screen for IZO-binding peptides. Amorphous IZO thin film, n-type semiconductors with high electron mobility (~ 10cm$^2$V$^{-1}$S$^{-1}$), can be deposited onto suitable substrates such as Sapphire, SiO$_2$, or Si, using sputtering techniques near room temperature. IZO has the useful properties of chemical stability and thermal stability. Thus, IZO is a good candidate for the transducer element in a biosensor. Furthermore, consensus amino acid sequences that mean some amino acid residues frequently occur in some positions of a peptide sequence are dependent of chemical composition, pH of the environment, crystal structure, morphology, surface roughness, and the size of grains or particles. Amorphous IZO is anticipated to provide a more homogeneous surface than a crystalline one because it should not have grain boundaries and crystal defects, where consensus sequence is not always achieved. This property can have a high possibility of leading to a well defined consensus sequence in peptides. IZO thin films and devices for the phage releasing tests were provided by Dr. Norton’s group in the Department of Materials and Engineering. The detailed fabrication procedure of IZO thin films and devices is as follow:

**Deposition of IZO thin film:** Indium zinc oxide (IZO) thin films were deposited on 5mm square sapphire wafers in argon plasma with using rf-magnetron sputtering in Nanoscale Research Facilities (NRF), University of Florida. Before deposition, the sputter chamber was
pumped down to less than $5 \times 10^{-6}$Torr. During deposition of the IZO thin films, sapphire substrates were washed with trichloroethylene, acetone, and methanol in ultrasonic bath for 5 minutes successively for removing contamination from the sapphire surface, and then were dried by a nitrogen gas stream. In addition, a commercial 3-inch diameter IZO pellet was used as a target for producing IZO vapor by applying 200 W power under 5 mTorr working pressure (Figure 2-4).

**Devices coated with IZO for electro-releasing experiments:** In the fabrication of the IZO devices, SiO$_2$ / Si wafers with 5 mm x 10 mm size were used as substrates. Substrates were cleaned in an ultrasonic bath for 5 minutes each in trichloroethylene, acetone, and methanol, and were blown dry by nitrogen gas to remove organic or inorganic contamination. Substrates were heated in an oven at 105°C for 10 minutes to remove moisture on the substrate surface. S1813 positive photoresist (PR) (Shipley) was coated onto the substrate by a spinner (Headway) at 5000 rpm for 50 seconds. Subsequently, PR coated substrates were baked at 100°C for 90 seconds on a hot plate. Then, PR-covered substrates were exposed to light with wavelength in 365 nm for 15 seconds by Karl Suss MA-6 Contact aligner system with hard contact mode under exposure intensity in 8 m W/cm$^2$. To develop photoresist after exposure, MF-300 developer was used. We developed for 50 seconds and washed for 2 minutes in deionized (DI) water. After the developing process, we used the sputtering system to make metal electrodes. Ti and Au thin films were deposited by KJL CMS-18 Multi-source sputtering system. Ti was deposited with 10 nm and Au was deposited with 80 nm thickness. Final metal layers were Ti / Au / Ti. After metal deposition, a circular pattern was made with the PR (S1813) to connect gold wires later, which would then be removed after the InZnO (IZO) film deposition (Figure 2-5).
2.3.2.2 Phage display protocol

Phage display was performed by incubating the phage display library (Ph.D.-12, *New England Biolabs*) with the desired target material, washing away unbound phage, and then eluting the strongly bound phages. The eluted phages were amplified by infecting the host bacterial strain ER2738, and purified. These steps are called biopanning rounds, which enrich the pool in favor of binding sequences. This sequence of steps was repeated to enrich the phage clones with the binding affinity to the target material until consensus peptide sequences are obtained. However, not every material leads to consensus peptide sequences. In this case, the cycle of biopanning is stop until peptide sequences with preferentially strong binding affinity to a specific target material. After each round, single colonies were selected for DNA sequencing, and then characterized to evaluate the specific binding affinity for the target with Enzyme-Linked Immunosorbent Assay (ELISA) or Immuno-Fluorescence Microscopy (IF), for qualitative comparison. All chemicals and labware were autoclaved, and the reactions performed in an Airclean® 600 PCR workstation laminar flow hood. Phage display protocol and the other methods applied during the phage display procedure are discussed in detail as follows.

**Binding step:** In this step, 10 μl phage library Ph.D.-12 (New England Biolabs, MA) was exposed to an amorphous Indium Zinc Oxide (IZO) thin film coated on the top and bottom of a sapphire (0001) plate in PC buffer. The IZO-phage solution was rotated by an agitator (Labqueake®, Barnestend Thermolyne) for 30 min in order to obtain sufficient time for phage-IZO interaction.

**Washing step:** After a 30 min rotation, several washing cycles were performed in order to remove the non-specific phage from IZO surface. Phages that strongly bind to the target substrate are retained, while the non-binder ones are washed away. These washing cycles were repeated for ten to thirteen times for each biopanning round. The detergent concentration was
increased gradually up to 0.5%. Applied washing procedure is described below. First, one IZO sheet was put into a 1.5 ml microfuge tube, and one ml PC was added into a microfuge tube. Then, ten μl of phage display peptide library (Ph.D.-12™) was added into a 1.5ml microfuge tube (Fisher brand), and the microfuge tube containing IZO and phage peptide library was put the on an agitator 30 min for phage-IZO interaction. After 30 min agitation, the first supernatant was put into another microfuge tube, and the IZO sheet was washed with 1 ml PC containing 0.1 % detergent. The washing steps were repeated 6 times. The supernatant was put in a fresh microfuge tube at the end of the each washing step. Next, a microfuge tube containing the IZO sheet with bound phages in 1ml PC containing 0.1% detergent was put the on an agitator 30 min for removing unbound phage again. The supernatant was transferred into a fresh microfuge tube. 1ml PC containing 0.1 % detergent was added into the microfuge tube including the IZO sheet, and the microfuge tube containing the IZO sheetwas put on an agitator 30 min for removing unbound phages further. The supernatant was transferred into a fresh microfuge tube, and the IZO sheet was washed with 1 ml PC containing 0.1 % detergent. The microfuge tube containing IZO with bound phages was left on an agitator overnight. The supernatant was discarded and transferred into microfuge tube. Subsequently, the IZO sheet with bound phages was washed with 1 ml PC which contains 0.1% detergent. The microfuge tube containing IZO with bound phages was put on an agitator 30 min. The supernatant was discarded and transferred into microfuge tube. IZO was washed with 1 ml PC containing 0.1% detergent. The microfuge tube containing the IZO sheet with bound phages was put on an agitator 30 min. The supernatant was put into microfuge tube.

**Elution step:** Strongly bound phages were recovered from the IZO surface through elution. The strong interaction between phage and IZO surface was disrupted using a low pH
buffer solution. The elution procedure is described as follows. After the last washing step, 1 ml of low pH elution buffer solution was added into the IZO sheet with bound phages in a microfuge tube. The microfuge tube containing the IZO sheet with bound phages and low pH elution buffer was put the on an agitator for 8 minutes to elute the bound phages from IZO surface. The supernatant including eluted bound phages was transferred into a fresh microfuge tube. Then, one hundred µl supernatant was transferred into *E. coli* ER2738 culture (LB Lennox medium: *E. coli* ER2738 overnight culture = 100:1), and it was incubated 4.5 hours at 37ºC and 250 rpm. The remaining 900µl supernatant was neutralized by adding 40 µl of Tris, pH 9.1. One ml of low pH elution buffer solution was put into microfuge tube. The Microfuge tube which contains IZO and low pH elution buffer was put the on an agitator for 8 minutes to elute the phage from IZO surface again. Subsequently, the supernatant was transferred into a microfuge tube. One hundred µl supernatant into was transferred into *E. coli* ER2738 culture (LB medium: *E. coli* ER2738 overnight culture = 100:1) and it was incubated for 4.5 hours at 37ºC and 250 rpm. The remaining 900µl supernatant was neutralized by adding 40 µl of Tris, pH 9.1. Then, one ml of low pH elution buffer solution was put for the third time into microfuge tube. The Microfuge tube which contains IZO and low pH elution buffer solution was put the on an agitator for 8 min to elute the phage from IZO surface. The supernatant was transferred into microfuge tube, and 100µl supernatant was transferred into *E. coli* ER2738 culture (LB Lennox medium: *E. coli* ER2738 overnight culture = 100:1) and it was incubated for 4.5 hours at 37ºC and 250 rpm. The remaining 900µl supernatant was neutralized by adding 40µl of Tris, pH 9.1.Next, one ml of low pH buffer solution was put for the forth time into microfuge tube. The Microfuge tube which contains IZO and low pH buffer solution was put the on an agitator for 8 min to elute the phage from IZO surface. The supernatant was transferred into microfuge tube, and 100µl supernatant
was transferred into *E. coli* ER2738 culture (LB Lennox medium: *E. coli* ER2738 overnight culture = 100:1) and it was incubated for 4.5 hours at 37°C and shaked at 250 rpm. Finally, the remaining 900μl supernatant was neutralized by adding 40μl of Tris, pH 9.1.

**Amplification and purification steps:** Eluted phage samples were infected into the host strain *E. coli* ER2738 and amplified. Before the beginning of amplification period, *E. coli* host strain ER2738 was cultured to reach the OD$_{600}$ ~ 0.5 (the best phage-host strain propagation period) and then eluted phage solutions were transferred to bacteria culture. The incubation period was approximately 4.5 hours at 37°C and phage-*E. coli* host strain solution was shaken at 250 rounds per minute (rpm) on a shaker (Max 2000, Barnstead Lab-Line). *E. coli* host strain ER2738 used in amplification is a robust F+ strain with a rapid growth rate and is particularly well-suited for M13 propagation. ER2738 is a recA+ strain and the F factor of ER2738 contains a mini-transposon, which confers tetracycline resistance. After amplification, eluted phages were purified from host cell according to the procedure below. First, *E.coli*-phage culture was transferred to 50 ml sterilized centrifuge tubes after 4.5 hours of the growth period. Then, samples were centrifuged at 8000 rpm for 10 min, and the supernatant was transferred to 50 ml sterilized centrifuge tubes. PEG/NaCl was added (1:6) into supernatant to precipitate phage and it was left overnight at 4°C. Samples were centrifuged at 10000 rpm for 20 min. Then, the supernatant was discarded, and a phage pellet was resuspended with 5 ml PC buffer (no detergent) by pipetting to remove any remaining *E.coli* ER2738. Next, samples were centrifuged at 10000 rpm for 10 min. After that, the supernatant was transferred to 50 ml sterilized centrifuge tubes. PEG/NaCl was added (1:6) into the solution to precipitate phage and the solution was left for 2 hours at 4°C. Samples were centrifuged at 10000 rpm for 10 min. The supernatant were discarded and phage pellet was resuspended by pipetting with 1 ml PC buffer (no detergent) to
remove *E.coli* ER2738. Samples were centrifuged at 10000 rpm, for 10 min and supernatant were transferred to sterilized microfuge tubes. PEG/NaCl solution was added (1:6) into the microfuge tube to precipitate phage, sample was vortexed 5 sec, and leaved this solution in the air 10 min. Samples were centrifuged at 13200 rpm, 3 min to compact the phages. The supernatant was discarded and phage pellet was resuspended with 0.2 ml PC buffer (no detergent) by pipetting gently. Samples were centrifuged at 13200 rpm for 3 min. The supernatant was transferred to sterilized microfuge tubes and it was stored at -20°C.

### 2.3.2.3 Blue-white screening

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

**Preparation of LB-Xgal/IPTG plates:** 187 µl Xgal/IPTG was added 150 ml liquid warm LB agar in 150 ml glass medium flask and it was poured to 60 mm plastic sterile petri dish. Plates were wrapped with parafilm and Aluminum foil and stored at 4°C in the dark for a maximum of 1 month.

**Serial dilution of phage samples:** 190 µl PC buffer (without detergent) and 10 µl phage was added into A1 well of 96-well plate. 180 µl PC buffer (without detergent) was put into wells from A2 to A12. Ten fold dilutions were made from A1 to A12 by taking 20 µl samples from preceding wells (Figure 2-6).

**Calculation of Phage Titers:** After serial dilutions, LB-Xgal/IPTG plates that were prepared previously and stored at 4°C, were put at room temperature. Three ml melt top agar (for 60mm petri dish) or 5 ml (for 90 mm petri dish) was alyqotted into 15 ml cell culture tube and
these tubes were put in 55℃ water bath to prevent the solidification until other processes carried out. *E. coli* ER2738 (1:250) from overnight culture were inoculated into 5 ml LB medium in 50 ml falcon tube. The culture was incubated until mid- log phase (OD$_{600}$ ~ 0.5) at 37℃ and 250 rpm. After incubation period, 200 µl *E. coli* ER2738 culture and 10 µl diluted phage sample were put into 1.5 ml eppendorf tube. Phage - *E. coli* ER2738 mixture was added into 3 ml melt top agar and poured onto LB-Xgal/IPTG petri dishes. All petri dishes were kept up-side down and incubated at 37℃ for overnight. After incubation period, blue plaques were obtained (Figure 1-8 (b)). The plate with 30~100 plaques from each eluted page solution (E1-E4) were chosen to calculate the amount of phage with the equation as follow:

\[
\text{The amount of phage (pfu)} = \frac{\text{The number of plaques}}{\text{The volume of diluted phage solution (ml)}} \times \text{Dilution factor}
\]

*pfu*: plaque forming unit

For example, 10µl diluted phage solution from A9 well was added into 3 ml top agar. After vortexing, the mixed solution was pour onto a LB-Xgal/IPTG plate. After the incubation overnight, 100 phage plaques were counted on this LB-Xgal/IPTG plate. The calculation of the amount of phage was as follows.

\[(100 \text{ phage plaques/0.01 ml diluted phage solution}) \times 10^9 = 1.00 \times 10^{13} \text{ pfu/ml}\]

According to phage titers at each round, phage amount was determined to generate a phage pool for the next biopanning round.

**Saving Phage Clones for Sequencing:** Preparation procedure of saving clones for sequencing is described in the following subsections. First, 150 µl, 0.02 % PC buffer was put each well of 96 well ELSA plate. Each phage plaque from big plates was picked and put into different well of ELSA plate. Twenty four plaques were picked per each elution step. At the end
of the fourth round, 576 plaques were picked. Next, 96 well plate containing phage clones was placed into the incubator at 60°C for 45 min. and 96 well plate was left at 4°C for overnight. After that, 60 μl sterilized 80% glycerol solution was put each well in two sets of 96 well plates (Overall glycerol concentration was kept as 50 % in stocks). Subsequently, 50 μl of each phage clone was added from storage plate to glycerol containing plates. Plates were covered by parafilm and stored in –80°C. After saving clones, phages DNA were isolated for DNA sequencing. DNA isolation was performed using the QIAprep ® Spin M13 kit (QIAGEN) procedure. The procedure is described below in detail. Ten μl sample was taken from the glycerol stock of a single phage clone, and was added into the 3ml E. coli ER2738 culture (LB medium: E. coli ER2738 overnight culture = 100:1), which was incubated until mid-log phase (OD₆₀₀ ~ 0.5). After this step, culture was incubated 4.5 hours for phage-E. coli ER2738 infection. Next, culture was centrifuged at 5000 rpm for 15 min. at room temperature supernatant containing M13 bacteriophage was transferred to a fresh reaction tube. During transferring the supernatant, bacterial pellet was not disturbed. Any carryover of bacterial cells will result in contamination of the M13 precipitation with bacterial chromosomal DNA or double-stranded bacteriophage RF DNA. Buffer MP was added 1/100 volume (i.e. 10μl per 1 ml of phage supernatant) to the supernatant in reaction tube. It was mixed by vortexing and incubated at room temperature for at least 2 min. During this step, bacteriophage particles were precipitated from the culture medium. A QIAprep spin column was placed in a 2 ml microcentrifuge tube and applied 0.7 ml of the sample to the QIAprep spin column. Reaction tube was centrifuged for 15 sec. at 8000 rpm and discarded flow-through from collection tube. During this step, intact bacteriophage was retained on the QIAprep silica-gel membrane. The last step was repeated until all supernatant passed through QIAprep spin column. 0.7 ml MLB buffer was added for M13
lysis and binding, to the QIAprep spin column and centrifuged for 15 sec. at 8000 rpm. This step creates appropriate conditions for binding of the M13 DNA to the QIAprep silica-gel membrane.

When bacteriophage lysis begins, another 0.7 ml MLB buffer was added the QIAprep spin column and was incubated 1 min. at room temperature to lyse bacteriophage completely. QIAprep spin column was centrifuged for 15 sec. at 8000 rpm. M13 singlestranded DNA is released from bacteriophage particles and adsorbed to the QIAprep to the silica gel membrane. Buffer PE 0.7 ml was added and centrifuged for 15 sec. at 8000 rpm. In this step residual salt is removed. Buffer PE was discarded from collection tube and centrifuged QIAprep spin column for 15 sec. at 8000 rpm to remove residual buffer PE. It is important to dry the QIAprep membrane with quick micro-centrifugation 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. 100µl EB buffer (10 mM Tris.Cl, pH 8.5) was added to the center of the column membrane to elute the DNA. Incubation of elution buffer in the QIAprep spin column significantly increases the recovery of single-stranded M13 DNA molecules, which adsorb tightly to the silica membrane. The DNA can also be eluted with water. When using water for elution, the pH of water should be in the range 7.0-8.5. Elution efficiency is dependent on pH and the maximum elution efficiency is achieved within this pH range.

**DNA sequencing:** The Interdisciplinary Center for Biotechnology Research (ICBR) in the University of Florida provided service in PCR and DNA sequencing for single strain DNA of M13 phage clones. –96 gIII sequencing primer (5´- HOCCC TCA TAG TTA GCG TAA CG – 3´, 100 pmol, 1 pmol/µl) purchased from New England Biolabs Ph.D.-C7C™ Phage Display Peptide Library Kit was used in PCR. DNA samples were sequenced by using Genome Sequencer 20™ System (Roche Applied Science).
2.3.2.4 The determination of expressed 12-mer peptides from M13 phage DNA

For the DNA of M13 Phage in Ph.D.-12 phage library, the DNA codes are the same except 12-mer peptide-gIII fusion for each phage (Figure 2-7). In order to find out the 12 mer peptide gIII fusion, two segments of DNA sequences in the immediate vicinity of gIII fusion domain were used and aligned with any DNA sequence of phage clones by using Nucleotide alignment in Basic Local Alignment Search Tool (BLAST) from National Center for Biotechnology Information (NCBI): http://blast.ncbi.nlm.nih.gov/. After obtaining the DNA sequence of gIII fusion, DNA codes of gIII fusion can be translated into amino acid sequences of 12-mer peptides with the tool for DNA to protein translation in the website as follows:

http://bio.lundberg.gu.se/edu/translat.html.

An example is shown below:

The DNA sequence for a M13 phage clone is aligned by two segments of DNA sequences in the immediate vicinity of the gIII fusion domain as follow:

1. TTATTCGCAATTCTTTAGTGTTACCTTTCTATTTCTCACTCT
2. GGTGGAGGTTCCGCAAAACTGTGGAAAGTTGTTTAGCAAAATCCCATACAGAA

After the alignment using Nucleotide BLAST, the DNA sequence of gIII fusion (green domain) will be confined between two sets of aligned DNA domains (yellow domain):

ACCCCGAGCCGCAGTCATAGAAAAAGAAAGAGGCG

Subsequently, this DNA sequence is translated into amino acid sequence:

1 CGTCTTTCCA GACGTTAGTA AATGAATT TTGCTGCTAAA
2 CAACTTTCAA CAGTTTCCGGCCGAACCTCCA CCACCCCGAGCGCAGTCAT
101 AGAAAAAGAGAGGCGTAGAGGAGTAAATA GAAAGGTACC ACTAAAGGAA
151 TTGCGAATAA TAATTTTTCTC ACGTTGAATA TCTCCAAAA AAAGGCTCCA
201 AAAAGGACCT TTAATTGTAT CGGTTTATCA GCTTGCTTTGCAGGTTGAATT
251 TCTTAAACAG ATAGTATACC CGAAGTTGCGC CGACAATGAC ACAAACCATT
301 GCACGCAATTCA ACACGATATA TCTGTTGCTGCTGAGGCTGCC GGGAGTTAA
351 GGCCGCTTTTGCGGAGATCGT CACCCCTAGGCAGCGAAGAGAC AGCAGTCCCA
401 CGAGGGTACGC AACCGCTACA CAGCCCGTACA GAGGCTTTGAGAACTAAAAGGAA
451 AGGAAATTTTCTAAACCGG GTAATTGCTGCTGACTGCA CTATGCAACTGCAAGGACCA
2.3.2.5 Immunofluorescence (IF) microscopy experiment

At the beginning of the fluorescence microscopy experiment, both negative and positive control experiments were carried out to decide on the right procedure during the fluorescence experiment. In the negative control experiment, IZO sheet was incubated with Anti-M13 pIII monoclonal antibody (Amersham Bioscience) (Anti-M13 monoclonal antibody: PC buffer = 1:500) as the primary antibody which is specific to the M13 pVIII protein in 1ml PC buffer for 20 minutes on an agitator. Then, discarded supernatant and use 1 ml PC with suitable concentration of detergent washed IZO sheet twice (0.1 %, 0.3 %, and 0.5 % for phage clones from 1st, 2nd, and 3rd biopanning respectively). Subsequently, anti mouse IgG-FITC as secondary (Sigma-Aldrich) antibody (anti mouse IgG-FITC : PC buffer = 1 : 100) was incubate with IZO in 1 ml PC buffer for 20 minutes on an agitator. After 20 minute incubation, added 1 ml PC buffer with suitable concentration of detergent to wash IZO three times. IZO was visualized at 20X magnification and 2 sec exposure time by the fluorescence microscope (Nikon-Eclipse E600) with WIB filter.

In the positive control experiment (Figure 2-8), IZO was incubated with 10μl phage single clone (10^{11} pfu/ml) in 1 ml PC buffer on an agitator for 1 hour. Subsequently, discarded supernatant and washed IZO with 1 ml PC with suitable concentration of detergent three times. Next, anti M13 pIII monoclonal antibody in 1 ml PC buffer (1:500) was incubated IZO for 20 minutes on an agitator. After incubation period, discarded supernatant and washed IZO with 1 ml PC with suitable concentration of detergent twice. Then, anti mouse IgG-FITC in 1 ml PC buffer
(1:100) was incubated with IZO for 20 minutes on an agitator. Subsequently, discarded supernatant and washed IZO with 1 ml PC with suitable concentration of detergent three times to remove the residue of unbound anti mouse IgG-FITC from IZO. Finally, these complexes were visualized at 20X magnification and 2 sec exposure time by fluorescence microscope with WIB filter. In order to evaluate the specificity of IZO phage binders to IZO, all obtained good binders were analyzed by using the sapphire (0001), silicon (100), and silicon oxide (amorphous) for cross-specificity at 20X magnification and 2 sec exposure time by fluorescence microscopy with WIB filter.

2.3.2.6 Enzyme-linked immunosorbent assay (ELISA)

A phage single clone (10⁷ pfu/ml) in 1 ml PC buffer was incubated with IZO, sapphire (0001), silicon (100), silicon oxide (amorphous) sheet for 1 hour on an agitator respectively. After incubation, discarded supernatant and washed substrates with 1 ml PC buffer with suitable concentration of detergent three times. Then, monoclonal anti M13:HRP (GE Health) which is the secondary antibody conjugated to horseradish peroxide (HRP) for M13 phage detection (anti M13:HRP : PC buffer = 1:2500) with inorganic sheets mentioned above for 20 minutes on an agitator. Subsequently, discarded supernatant and washed inorganic sheets with 1 ml PC with suitable concentration of detergent three times to remove unbound anti M13:HRP. Substrate for the development reaction for HRP-conjugated secondary enzymes was prepared by dissolving one capsule of phosphate citrate buffer with sodium perborate (Sigma-Aldrich) in 100 ml distilled waster (0.05 M phosphate citrate buffer pH5.0, 0.3% (w/v) sodium perborate). A 10 mg tablet of 3, 3’, 5, 5’-tetramethylbenzidine (TMB) substrate (Sigma-Aldrich) was added to 10 ml of the buffer to give a final concentration of 1 mg/ml. 1 ml substrate solution was added into microcentrifuge tube containing inorganic sheet respectively and was developed for 20 minutes.
(Figure 2-9). After that, supernatant from each tube was transferred into a 96 well plate. The plate was read in an ELx 800UV plate reader (Bio Tek) at 630 nm.

2.3.2.7 Calculation of surface coverage of a phage clone on substrate sheets

Image processing software, Image J, was used to measure the surface coverage of phage clones on substrate (Figure 2-10). A fluorescence image of phage clones on IZO sheet (Figure 2-10(a)) was converted to a 32 bit black and white image (Figure 2-10(b)). Make a threshold of Figure 2-10(b) to Figure 2-10(c). Finally, measure the ratio of black area to the whole picture area. This ratio measures surface coverage of phage clones on substrate sheets.

2.4 Results and Discussions

In this study, Indium Zinc Oxide (IZO) was as a target material to select phage clones with 12 mer expressed peptides showing binding affinity to IZO determined from the phage display process. After each biopanning, phage titers were necessary to calculate the concentration of eluted phages. Based on the concentration of eluted phages, the volume of each elution solution was determined based on making the same contribution of the phage amount to phage pool for the succeeding round. The phage titer result with using low pH elution buffer is shown in Table 2-1. Based on these results, the level of phage concentration was over $10^{11}$ pfu/ml. This indicates that amplification efficiency of phages within a host bacterial, E. coli. ER2738 strain, was promising.

After phage titers, some phage clones were picked for DNA sequencing to deduce the IZO binding peptides sequences. Figure 2-11 shows the expressed 12-mer peptide sequences translated from the DNA of phage clones selected from IZO. For amino acid sequences shown in Figure 2-11, most of the peptide sequences contained a block of hydrophobic amino acids, and one or two basic amino acids. In addition, approx. 4 to 5 polar amino acids were distributed within 12 mer expressed peptides separately.
It can be seen that the IZO substrate led to a consensus sequence; in fact two sequences were fully duplicated with 6 clones for one (TKNMLSLPVGPG), and two clones for the other (MNRPSPPLPLWV). Although inorganic surfaces don’t always evolve to a consensus sequence, the amorphous nature of the IZO may have provided a very uniform surface. This has been observed in Sano’s work with TiO₂ particles with of amorphous oxide surface layer, where 33 of the 43 clones had an identical sequence [35].

However, a phage clone with expressed consensus 12-mer peptide sequences does not mean it could be a good binder for a target material because a consensus peptide sequence just represents the expressed fusion peptide from the most enriched phage clones observed after three or more biopannings. Thus, immunofluorescence (IF) analysis and enzyme linked immunosorbent assay (ELISA) were used to evaluate the properties of phage clones in binding affinity and specificity to a target material.

In ELISA, the secondary antibody conjugated to horseradish peroxide (HRP), anti M13:HRP, was used to detect M13 phages absorbed onto the surface of inorganic substrates semi-quantitatively. When HRP catalyze the enzymatic reaction of 3, 3’, 5, 5’-tetramethylbenzidine substrate, the oxidized product of TMB has a deep blue color. A deep blue color indicates a higher density of phages on those substrates. In addition, the degree of blue color can be quantified by measuring UV-Visible absorbance at 630 nm.

The seven representative phage clones eluted in low pH buffer were first evaluated for their binding affinity and specificity to IZO by using ELISA (Figure 2-12 and Figure 2-13). The phage clone with the consensus amino acid sequence (TKNMLSLPVGPG) preferentially bound to IZO based on the UV absorbance, but also displayed a high binding affinity to sapphire. With respect to another phage clone with the other consensus amino acid sequence
(MNRPSPPPLPLWV), it mainly tended to bind onto sapphire, even if IZO was chosen as target material. The reason is that IZO was sputtered onto the top and bottom surfaces of a sapphire plate. Thus, some thin edges of sapphire were exposed to a phage library during biopanning. This caused a few of the clones to be selected for this region. The phage clone with expressed peptide (MNRPSPPPLPLWV) was probably selected from the sapphire edge due to its strong binding affinity to sapphire. In addition, other phage clones with different types of expressed 12 mer peptides were also explored for their binding affinity to IZO in ELISA results as shown in Figure 2-12 and Figure 2-13. For example, ASQITHFPRPPW contained less polar amino acid residues than TKNMLSLPVGPG, but they have similar properties of peptide sequences. TEAHRQSMTLTW was comprised of an acidic amino acid residue which is not common in IZO binding peptides. Furthermore, ASQITHFPRPPW mostly consisted of polar amino acid residues.

In order to visualize the phage binding on inorganic substrates, immunofluorescence analysis (IF) was used by utilizing a fluorescence tag (FITC) conjugated with anti-mouse IgG to indicate the location of M13 phage on the inorganic substrate surface under fluorescence microscopy. However, Anti-mouse IgG-FITC may have a binding affinity to some inorganic substrates directly. Thus, it was necessary to test anti-mouse IgG-FITC to see if it has the ability to bind onto IZO, sapphire, Si, and SiO₂ in the absence of M13 phage and anti-M13 primary antibody. Figure 2-14 shows that anti-mouse IgG-FITC did not tend to bind onto those inorganic substrates because no green spots were observed without loading M13 phage and anti M13 primary antibody under fluorescence light.

The phage clone-TKNMLSLPVGPG exhibited preferential binding to IZO in ELISA (Figure 2-13a). In IF analysis, this phage clone had high binding affinity to IZO, but showed
minor binding affinity to the other materials (Figure 2-15). For the phage clone: MNRPSPPPLPLWV, it showed preferential binding affinity to sapphire, rather than IZO (Figure 2-16). This also proved this clone was selected from sapphire edge. The results in IF analysis corresponded to the ones in ELISA (Figure 2-13). In addition, IZO was etched using elution buffer with low pH (Figure 2-17). Thus, some phages with strong binding for IZO were released. This is not a benefit for the development of electro-active peptide linker. Thus, high pH or high salt elution buffer may be considered better to use in the elution step.

In high salt elution, high salt elution buffer solution, 5M MgCl₂·6H₂O, did not cause the etching of the IZO thin film on the sapphire substrate. In addition, high salt elution buffer also elute similar phage amount compared to low pH elution buffer solution (Table 2-2). This indicated high salt elution buffer also could provide enough diversity of eluted phage clone for the subsequent biopanning. Figure 2-18 shows that some expressed 12-mer peptide sequences eluted from high salt elution. High salt elution solution evolved into three consensus peptide sequence: AGFPWSTHSSWL, SHAPDSTWFALF, and TNSSSQFVVAIP. In most of peptides sequences from high salt elution, they were basically comprised of a block of hydrophobic amino acids (2~5 residues) which also appeared in peptides sequences from low pH elution. In addition, a block of polar amino acids (3~5 residues) were usually followed by a block of hydrophobic amino acids. In addition, Figure 2-11 and Figure 2-18 showed that the amino residue, histidine, frequently appeared in most IZO binding peptide sequences. Some metalloproteins have been proved to coordinate to zinc cations through basic amino acid residue such as histidine [108, 109]. Thus, histidine in IZO binding peptides may function as binding sites to interact with zinc components in IZO through coordination binding.
The evaluation of representative phage binding affinity through ELISA is shown in Figure 2-19 and Figure 2-20. Most of the representative 12-mer expressed peptide sequences, including three consensus peptide sequences, display significantly higher preferential binding to IZO, except one of consensus peptide sequence, AGFPWSTHSSWL. Figure 2-21 shows that the phage clone: SHAPDSTWFALF displayed high preferential binding affinity to IZO. In addition, it also displayed lower surface coverage on sapphire than phage clones from low pH elution. On the whole, the binding affinity phage clones eluted from high salt elution was more selective to IZO than low pH elution. This indicated high salt elution indeed avoided the selection of phage clones with strong binding affinity to different inorganic substrates. However, the surface coverage of phage clones from high salt elution is obviously lower than low pH elution.

After evaluating the binding of phage clones to IZO, the phage clones with good surface coverage on IZO surface were chosen to test electro-releasing ability using IZO devices. The phage clones from high salt elution showed insufficient surface coverage on IZO surface even if they have better selectivity to IZO. Thus, it is difficult to observe the difference of fluorescence intensity between phage binding and phage releasing on IZO device surface. Thus, we chose phage clones from low pH elution because of their high surface coverage on IZO surface. Although they still show obvious binding affinity to sapphire, sapphire is not a candidate for a transducer surface material due to its low conductivity.

In this study, we mixed three phage clones with good surface coverage on the IZO thin film from low pH elution to incubate with the IZO device (Figure 2-22(a)). Figure 2-22 (b) showed those phage clones also had good surface coverage on the IZO device surface. After applying 1400 mV for 5 minutes, the releasing area, the bending channels between electrodes, turned darker than the electrode area covered with IZO thin film (Figure 2-22(b)). The decrease
of fluorescence light is mainly due to the release of phage clones, rather than the degradation of fluoresce tags caused by applying a voltage. In order to prove that voltage is not the main factor to cause the decrease of fluorescence intensity, the phage clone-NMTMSFPTYPIA that had irreversible binding was used to examine the change of fluorescence intensity after applying a voltage. Figure 2-23 showed fluorescence intensity of the IZO device was not changed even if a voltage was applied onto the IZO device. These electro releasing results could be envisioned the potential of peptides designed by phage display technique to serve as linkers for the development of refurbishable biosensors.
Table 2-1. Phage titers in the IZO system with low pH elution buffer

<table>
<thead>
<tr>
<th>Elution</th>
<th>First round</th>
<th>Second round</th>
<th>Third round</th>
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<tr>
<td></td>
<td>Cum-p (pfu/ml)</td>
<td>Cam-p (pfu/ml)</td>
<td>Vtotal (μl)</td>
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<tr>
<td>E1 (1st elution)</td>
<td>2.7x10^5</td>
<td>3.3x10^14</td>
<td>75</td>
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<tr>
<td>E2 (2nd elution)</td>
<td>1.9x10^4</td>
<td>3.8x10^16</td>
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</tr>
<tr>
<td>E3 (3rd elution)</td>
<td>2.0x10^4</td>
<td>1.2x10^15</td>
<td>25</td>
</tr>
<tr>
<td>E4 (4th elution)</td>
<td>1.0x10^4</td>
<td>2.2x10^15</td>
<td>150</td>
</tr>
<tr>
<td>Total</td>
<td>252</td>
<td>476</td>
<td></td>
</tr>
</tbody>
</table>

Cum-p: The concentration of unamplified phages  
Cam-p: The concentration of amplified phages  
Vtotal: The total taken volume of eluted phages

Table 2-2. Phage titers in the IZO system with high salt elution solution

<table>
<thead>
<tr>
<th>Elution</th>
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<th>Second round</th>
<th>Third round</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cum-p (pfu/ml)</td>
<td>Cam-p (pfu/ml)</td>
<td>Vtotal (μl)</td>
</tr>
<tr>
<td>E1 (1st elution)</td>
<td>1.2x10^5</td>
<td>1.8x10^18</td>
<td>1</td>
</tr>
<tr>
<td>E2 (2nd elution)</td>
<td>2.3x10^4</td>
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<td>1</td>
</tr>
<tr>
<td>E3 (3rd elution)</td>
<td>4.0x10^4</td>
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<td>10</td>
</tr>
<tr>
<td>E4 (4th elution)</td>
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<td>1.4x10^15</td>
<td>100</td>
</tr>
<tr>
<td>Total</td>
<td>112</td>
<td>227</td>
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</table>

Cum-p: The concentration of unamplified phages  
Cam-p: The concentration of amplified phages  
Vtotal: The total taken volume of eluted phages
Figure 2-1. Schematic of the electro-releasing mechanism using electro-activated peptides. The ‘clogged’ receptors are depicted as brown spots. The electroactive peptide linkers are red curved lines.

Figure 2-2. Schematic of the market size and potential applications of biosensors in the worldwide market.
Figure 2-3. Schematic of the main components of biosensor devices

Figure 2-4. Schematic for the deposition of IZO on a sapphire sheet

Figure 2-5. Design of the device for releasing the phage clones
Figure 2-6. Serial dilution of phage samples

Figure 2-7. N-terminal sequence of random 12-mer peptide-gIII fusion for M13 phage DNA in Ph.D.-12 phage library
Figure 2-8. Schematic of immunofluorescence analysis. ImmunoFluorescence (IF) analysis uses an antibody raised against the phage, but in this case the antibody contains a docking domain for attachment of a secondary antibody that contains a fluorescent tag (FITC). Fluorescence analysis can be done qualitatively on a fluorescence microscope to compare binding to select particles, or quantified on an overall surface using a fluorimeter.

Figure 2-9. Schematic of enzyme linked immunosorbent assay. Enzyme Linked ImmunoSorbent Assay (ELISA) uses commercially available antibodies which have been raised to bind to this specific M13 phage. The antibodies have an attached enzyme (HRP) which can provide a quantitative measure of the amount of enzyme (and therefore phage) that are bound to a surface. The reaction product of the enzyme with a particular substrate (TMB) produces a blue color from which the absorbance can be measured at the wavelength of 630 nm.
Figure 2-10. Measurement of the surface coverage of a phage clone on the IZO surface. (a) The fluorescence image of phage clones on substrate sheet; (b) converted from color image to black and white image using image J; (c) The threshold from (b)

<table>
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<th>The second round</th>
<th>The third round</th>
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<tr>
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<td>F N G R H G T T D H P T</td>
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<td>7</td>
<td>T E A H R Q S M T L T W</td>
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</tbody>
</table>

Figure 2-11. 12-mer amino acid sequences of selected phages from InZnO with low pH elution

- **Hydrophilic, acidic**
- **Hydrophilic, basic**
- **Polar, uncharged**
- **Hydrophobic**
Figure 2-12. Images of ELISA plate containing phage clones eluted from IZO with low pH elution buffer. (a) An image of ELISA plate containing 4 phage clones from the third biopanning with low pH buffer elution after 10 minute development time: these phage clones with expressed 12 mer peptides (STTLNNTTWRLY, TFKYSHELESRG, TKNMLSLPVGPG, and MNRPSSPLPLWV) (b) An image of ELISA plate containing 3 phage clones from the second biopanning with low pH buffer elution after 10 minute development time: these phage clones with expressed 12 mer peptides (TEAHRQSMTLTW, GNHSTTNMHPPL, and ASQITHFPRPPW).
<table>
<thead>
<tr>
<th>STTLNNTWRLY</th>
<th>TFKYSHELESG</th>
<th>TKNMLSLPVPG</th>
<th>MNRPSPPLPLWV</th>
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<td>UV absorbance (a.u.)</td>
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<td></td>
<td></td>
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<tr>
<td>0.000</td>
<td>0.010</td>
<td>0.020</td>
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</table>

**12-mer expressed peptide sequences**

(a)

Figure 2-13. UV-Visible absorbance of the enzymatic substrate solution (a) from ELSA plate in Figure 2-12(a) (b) from ELISA plate in Figure 2-12(b)
Figure 2-14. Images of the immunoﬂuorescence analysis for the negative control experiment. Only monoclonal anti-M13 antibody and monoclonal anti mouse IgG-FITC were incubated with IZO, sapphire, Si, and SiO2 respectively. No ﬂuorescence light was observed from these inorganic substrates. Scale bar: 100μm

Figure 2-15. IF analysis for the phage clone-TKNMLSLPVGP. It showed binding afﬁnity to the both of IZO and sapphire. However, this phage clone preferentially bound to IZO because the intensity of ﬂuorescence from IZO is markedly stronger than sapphire. Scale bar: 250μm
Figure 2-16. IF analysis of the phage clone-MNRPSPPPLPLWV. It showed preferential binding affinity to sapphire, rather than IZO. This indicated this phage clone might be selected from sapphire edge without IZO coating. Scale bar: 100μm

Figure 2-17. Photography of the etching InZnO with using the low pH elution buffer
Figure 2-18. 12-mer amino acid sequences of selected phages from InZnO with high salt elution

![Figure 2-18] 12-mer amino acid sequences of selected phages from InZnO with high salt elution buffer after 10 minute development time

<table>
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<th>The second round</th>
<th>The third round</th>
</tr>
</thead>
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<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
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<td></td>
</tr>
<tr>
<td>3</td>
<td>S H A P DSTWFA LF</td>
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<td>S H A P DSTWFA LF</td>
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<td>8</td>
<td>ALDDLARRFPLP</td>
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</tbody>
</table>

**Legend**
- **Hydrophilic, acidic**: Red
- **Hydrophilic, basic**: Blue
- **Polar, uncharged**: Purple
- **Hydrophobic**: Orange

Figure 2-19. Image of ELISA plate containing 6 phage clones from the third biopanning with high salt elution buffer after 10 minute development time
Table 2-1. List of styles included in Version 9.0 of the MS Word Formatting

Figure 2-20. UV-Visible absorbance of the enzymatic substrate solution from ELISA plate in Figure 2-19

Figure 2-21. IF images for the phage clone-SHAPDSTWFALF from the low pH elution
Figure 2-22. Releasing test for the mixture of phage clones. (a) A picture of the electro-active device for releasing phage clones. (b) The electro-releasing test for phage mixture comprised of phage clones with expressed peptide sequences FNGRHGTDDHPT, TNPLSSWTFTPY, and ASQITHFPRPPW obtained from low pH elution.
Figure 2-23. Electro-releasing test for the phage clone-NMTMSFPTYPIA. It was not released from the IZO device by applying voltage. The fluorescence intensity on this phage clone also was not changed.
CHAPTER 3
PARTICLE SEPARATION USING INORGANIC BINDING PEPTIDES DESIGNED BY PHAGE DISPLAY TECHNIQUES

3.1 Motivation

Phosphate rock is the main raw material in the production of fertilizers. In the whole world, the United States has been the biggest phosphate producer. Florida phosphate contributes 80% of the phosphate production in the United States. Currently, the phosphate rock in Florida phosphate reserves is of low concentrate grade due to high dolomite contamination. The MgO content in dolomite affects the filtration of phosphoric acid, an important intermediate during the production of fertilizers. In general, the MgO content in a phosphate concentrate must be less than 1% for commercial applications.

Currently, some conventional flotation agents such as fatty acids can float francolite, which is a fluoroaptite (Ca₅(PO₄)₃ (F, OH)), at a high recovery rate. However, they also float dolomite. Thus, there are no commercial flotation agents that can differentiate francolite from dolomite (CaMg(CO₃)₂) very well due to their similar surface properties, such polarity. The goal of this study is to demonstrate the feasibility of using a biotechnology approach to particle-based applications that may benefit from a high degree of specificity achieved by molecular recognition between peptides and inorganic surfaces. More specifically, a biopanning approach based on phage display will be used to screen for peptides that have high binding affinity and selectivity for francolite particles. Phage display is a combinatory approach that utilizes a commercially available library of phage (virus) particles that have a set of foreign oligonucleotides encoding short random peptide inserts into the protein ‘tails’ (pIII) of the phage.

If a high degree of selectivity can be demonstrated in this study, inorganic binding peptides based on phage display techniques have the potential to lead to a design for a peptide amphiphile to accomplish particle separation by flotation. For this, the inorganic binding
peptides could be covalently linked to a hydrocarbon tail (e.g. a short hydrocarbon chain or a short hydrophobic peptide chain) using a terminal amino acid with either amine or cysteine functionality, creating a peptide amphiphile. It is anticipated that the hydrophobic tail of the peptide-surfactant would segregate to the air bubbles, such that selective flotation occurs with the specifically targeted particles attached to the froth (Figure 3-1).

3.2 Background and Significance

In nature, a particular mineral is usually found in association with one type of rock. For instance, cassiterite usually accompanies granitic rocks. With mechanical and chemical weathering, a mineral deposit may be converted to ore consisting of the extractable mineral of interest and gangue, extraneous rocky materials. Within an ore, the mineral of interest is found in a sufficient concentration for recovery. In the mining industry, a main issue is to extract a valuable mineral from gangue. In addition, mining processes called ore dressing, or milling, mainly reduce the volume of waste minerals (e.g. gangue minerals) to concentrate valuable minerals which reduces the shipping and handling costs.

3.2.1 Liberation

The mining process contains two parts: liberation of valuable minerals and separation of these valuables from gangue minerals. During liberation of valuable minerals from gangue minerals, comminution is necessarily involved for grinding or crushing lumps of ore into a mixture of relatively clean particles of valuable minerals and gangue minerals. The degree of liberation is the key toward the efficient recovery in the mining process. The degree of liberation is defined as the percentage of free mineral particles in total ore content. In general, valuable minerals usually strongly bind with gangue minerals. Thus, different constituents are contained in free mineral particles to cause much middling and a low degree of liberation when an ore is
ground. Wills et al.[110] developed a new approach in which breaking stresses were directed at mineral crystal boundaries to crush rock without breaking grain boundaries.

3.2.2 Concentration

After liberating mineral particles from ore minerals by crushing and grading, the following processing separates free valuable mineral particles from gangue mineral particles. This process is known as concentration. The efficiency of concentration can be quantified by measuring the ratio of concentration: the ratio of weight of freed to the weight of concentrate. The ratio of concentration is related to the grade of concentrate which is defined as the content of end valuable mineral products of interest in the materials. The ratio of concentration usually increases with the grade of concentrate. In addition, recovery is another measure in the efficiency of concentration: the percentage of the total valuable minerals in the ore that is recovered in the concentrate. There is the inverse relationship between recovery and the grade of concentrate (Figure 3-2).

After liberating mineral particles from gangue minerals, the ore is subsequently classified into two or more products in a concentration process. During the concentration process, separation of mineral particles of interest from gangue minerals is usually based on some difference in physical or chemical surface properties between valuable minerals and gangue minerals. In the mining industry, some physical separation methods have been widely adopted for concentrating ore [111] including photometric sorting [111], gravity concentration [111-113], magnetic concentration [111], electrical conductivity concentration [111], and froth flotation [111, 114-120].

For the separation of mineral particles, froth flotation is currently the most widely adopted method in the concentration of mineral particles of interest and the method of interest in this study. Froth flotation is based on the difference in physio-chemical surface properties between
valuable mineral particles and gangue mineral particles [114-117]. By adjusting the chemical environment within a flotation pulp, an aqueous ground ore suspension, it is possible to enable froth flotation to achieve a specific separation of valuable minerals from a complex ore. Originally, froth flotation was aimed at the separation of sulphides of copper, lead, and zinc. However, various chemical agents were designed to enhance the specific separation of minerals in froth flotation. Thus, froth flotation has been expanded to separate many kinds of minerals from an ore [111] (e.g. oxides: hematite and cassiterite; oxidized minerals: malachite and cerussite; non-metallic minerals: phosphates, fluorites, and fine coal). In the froth flotation process, an air stream is pumped into a pulp solution to create air bubbles. Gangue minerals within a flotation pulp tend to display aerophobic properties and are kept in the pulp solution. However, valuable minerals with areophilic properties attach to air bubbles and are lifted to the surface of the liquid phase, isolated from gangue minerals (Figure 3-3 [111]).

Large particles may not work well in froth flotation because their gravity force is larger than buoyant force. In this case, larger particles can not be lifted by air bubbles. Thus, froth flotation is mainly suitable for fine particles (5 - 500μm) [111]. In addition, most minerals show the tendency toward aerophobic properties in their natural state. Thus, the interaction between mineral particles and air bubbles is too weak to lift mineral particles in the froth phase. In order to enhance the efficiency of flotation concentration, a collector, a kind of surfactant, is added into the pulp to be absorbed onto the valuable mineral particle surface to render them areophilicity which can help mineral particles adhere onto air bubbles [118].

As an ideal collector, it needs to have complete wetting for the valuable mineral particles to adhere to the air bubbles. That means that there is the high work of adhesion at the interface between mineral particle and the air bubble: the work of adhesion indicates the required energy
that separates mineral particles from the air bubbles. The wetting activity of a collector on a particle can be quantified by measuring the contact angle of the mineral particle surface/bubble interface (Figure 3-4 [118]). The relationship between the work of adhesion and the contact angle is expressed as followed:

$$W_{s/a} = \gamma_{w/a} (1 - \cos \Theta)$$

Where $W_{s/a}$: the work of adhesion

$\gamma_{w/a}$: the surface energy between water and air

$\Theta$: the contact angle between mineral particle surface and the air bubble

Based on the equation above, mineral particles become more and more aerophilic (increase in $W_{s/a}$) with increasing contact angle. The interaction between the mineral particle and the air bubble also becomes strong to promote the flotation ability of mineral particles due to the enhancing surface tension. Collector molecules can be classified into two types: non-ionizing and ionizing compounds [111, 119, 120]. Non-ionizing collectors are insoluble in an aqueous medium and render mineral aerophilic abilities by forming a thin film on the mineral surface. Ionizing collectors are basically composed of polar head goups and hydrophobic tails. Collectors can adsorb to the particle surface through chemical, electrostatic, or physical interaction between polar groups and the mineral particle surface. Then, the hydrophobic tails are segregated into air bubbles. Ionizing collectors can be further divided into the anionic type and the cationic type based on the ionic properties of their polar head groups (Figure 3-5 [111]).

### 3.2.3 Challenges of Current Mining Processing Technologies

The objective of mining processing is to separate valuable minerals from gangue minerals regardless of the concentration methods applied. However, the current techniques of mineral particle separation are never perfect because they always isolate two or more mineral into concentrate. Thus, the big issue has been to improve the purity of the concentrate. Furthermore,
the concentration efficiency of current techniques is still low in the treatment of fine size particles. This often causes the loss of valuable minerals. For example, 30% of phosphate mined in Florida was lost due to the discard of fine particles [121]. With the development of froth flotation, it has become the most important and powerful concentration method widely used in the mining industry. Froth flotation can be used to treat various kinds of ores such as oxides and metal oxides. In addition, it also can selectively separate valuable mineral particles by changing the chemical environment of the flotation pulp in the large scale.

Even though froth flotation has been proven as a useful application in the concentration of mineral particles, it still has a limitation in the separation of two minerals with similar surface properties. For instance, dolomite is the main impurity in Florida phosphate ore which is an indispensable material during the production of fertilizers. However, dolomite severely affects the formation of phosphoric acid, an intermediate of fertilizers. Dolomite has not been effectively removed from phosphate ore during the conventional flotation process due to their similar surface properties.

Several flotation techniques were developed to reduce the content of dolomite in the phosphate ores [122-126]. However, there are some disadvantages that limit their commercial application in flotation processes. First, those flotation processes involve a secondary separation process in order to remove dolomite from the final phosphate concentrate. Secondly, many flotation reagents are not cost-effective due to high doses and high price. Finally, the most complex problem is the chemical environment that is changed from the alkaline to the acidic medium. This made the flotation operation very complex.

El-Midany et al. proposed reactive flotation to improve the separation efficiency of dolomite from phosphate ore in one step [127]: high dolomitic phosphate pebbles are dipped
with a surfactant composed of polyvinyl alcohol (PVA) to form a coating around an individual particle. Subsequently, the particles with PVA coating are immersed into an acidic medium. When dolomite particles with PVA coating are exposed to the acidic solution, CO₂ is produced through chemical reaction between carbonate and acid, and is confined between a particles surface and a PVA coating layer. When the average density of the dolomite particle with the CO₂ layer around it is smaller than the acidic medium, the dolomite particles will float. However, reactive flotation only works well for dolomite particles in the scale of mm. In addition, the composition of francolite also contains carbonate which has the ability to produce CO₂ in an acidic solution. This may reduce the specificity of the reactive flotation because the acidic solution reacts with both francolite and dolomite. Furthermore, the acidic solution also causes a harsh environment.

3.2.4 Role of Inorganic Binding Peptides in Separation of Minerals

In the past two decades, phage display techniques have proved the ability to screen for peptides with highly binding affinity for inorganic surfaces, including semiconductors (CdS, GaN) [2, 18, 24, 26, 46, 47], ceramics (SiO₂, TiO₂) [2, 18, 28, 30], metals (Au, Ag, Pt) [2, 18, 21, 23, 32, 33, 35, 39-42], and minerals (CaCO₃, Fe₂O₃, hydroxyapatite) [2, 18, 31, 53]. The whole procedure of screening for inorganic binding peptides is called biopanning (Fugre 1-7). After three biopanning rounds, peptide binding peptide sequences determined by the DNA of selected phage clones display not only strong binding affinity to a target material, but also the ability of the molecular recognition to it. In this study, francolite pebbles provided by Mosaic, Inc. are chosen as a target material to pan for 12-mer francolite binding peptide sequence. Those peptide sequences are expected to display strong and specific binding affinity to francolite, and are considered as the hydrophilic head group of a collector. If the francolite binding peptides were modified with hydrocarbon tails which could render francolite particles hydrophobicity, the
specific separation of francolite could be achieved from phosphate ore by using those peptides amphiphilicites in the flotation process.

### 3.3 Materials and Methods

#### 3.3.1 Materials

**Francolite particles** (Ca₅(PO₄)₃(F, OH)): Francolite pebbles with the size of mm were provided by *Mosaic, Inc.* from their plants at two different locations, Four Corners-Francolite and South Fort Meade-Francolite.

**Dolomite rock** (CaMg(CO₃)₂): Dolomite rock was purchased from *Wards’ Natural Science*.

**Ingredients for phage display screening**: All materials involved in phage display are the same as the one used in chapter 2.

#### 3.3.2 Methods

**Grinding**: Dolomite rock (Wards Natural Science) was first crushed into small pieces less than 5 mm in size using a hammer. Subsequently, planetary ball-milling which consists of 250 ml ZrO₂ bowl and 40 Zirconium balls (FRITSCH Pulverisette 5) was used to ground small dolomite pieces into powder under 150 rpm for 30 minutes. For francolite pebble (Mosaic, Inc.), planetary ball-milling was also applied to crush francolite pebbles into powder under the same operation conditions as dolomite. All grinding processes were operated by Dr. Wolfgang Sigmund’s group in the department of Materials Science and Engineering, University of Florida.

**Cleaning procedure of target particles**: One hundred mg powder was weighed and loaded into 1.5ml microfuge tube. 1 ml CH₃OH/acetone mixture (1:1) was added into the tube. The powder pellet was dispersed by pipetting, and then was vortexed for 5-10 minutes. Subsequently, the powder was sonicated for 20 minutes in ultrasonic bath to break the clumps. After sonication, the powder was vortexed quickly to re-disperse. The powder was centrifuged at
13200 rpm for 1 minute. The supernatant was removed and 1 ml 70% ethanol was added onto the powder for sterilization. The powder was vortexed for 5-10 minutes, and then was sonicated for 20 minutes in ultrasonic bath. After sonication, the powder was centrifuged at 13200 rpm for 1 minute. The supernatant was removed and 1 ml 0.5% PC buffer was added. The powder was sonicated for 30 minutes. The powder was vortexed quickly to resuspend. The powder was centrifuged at 13200 rpm for 1 min. The supernatant was removed and 1 ml 0.5% PC buffer was added. Then, 100 μl of powder solution was aliquoted into each sterile 1.5 ml microfuge tube. Samples were centrifuged at 13200 rpm for 1 minute. After centrifugation, supernatant was removed from each tube. Samples were washed twice with 1 ml sterilized DI water, and rinsed with 1 ml ethanol. Samples were centrifuged at 13200 rpm for 1 min., and then removed supernatant from each tube. Samples were dried under vacuum.

**Phage display protocol:** The experimental steps of the phage display protocol are the same as the description in Chapter 2 except for the target materials.

**Binding affinity assay:** Image processing software, Image J, was used to measure the surface coverage of phage clones on francolite powders. In this method, the ratio (R1) of particle surface area to picture area was first calculated in white light (after white light image was set as the threshold), and then the ratio (R2) of fluorescence area to picture area (after fluorescence image is threshold). Phage surface coverage on powders is defined as the ratio of R2 to R1. The following example shows how phage surface coverage is calculated.
R1 = 0.0327 (The ratio of black area to the whole picture under white light)

R2 = 0.0315 (The ratio of black area to the whole picture area under fluorescence light)

Surface coverage of phage on powder = R2/R1 = (0.0315/0.0327) x 100% = 96.9%

**Measurement of zeta potential:** The measurement of the zeta potential was mainly used to determine the net surface charge of particles. In this measurement, dolomite/francolite particles were dispersed respectively in PC buffer at pH 2.0, 4.0, 7.0, 8.0, and 10.0. Then, Zeta Reader Mark 21 (in Particle Engineering Research Center, University of Florida) was utilized to determine the net surface charge of particles.

### 3.4 Results and Discussions

In this chapter, we mainly explore the effect of M13 phage with expressed inorganic binding peptides on the separation of francolite particles from dolomite particles. For the conventional flotation strategies, it has been a tough issue to concentrate francolite particles from dolomitic phosphate ore in Florida due to the similar surface properties such as polarities between the francolite and dolomite particles. Phage display techniques have demonstrated the potential in the selection of inorganic binding peptides with specific binding affinity to a target material in the past two decades [2, 14-29, 31-36, 39-42, 44-49, 51, 53, 56, 57, 60-63, 128].

However, selected inorganic binding peptides based on phage display techniques may display cross-binding affinity to some inorganic materials in addition to the target material. Thus, we first panned for the dolomite binding peptides using phage display techniques for
understanding the properties of their peptide sequences in the beginning of this study. After panning for the francolite binding peptides, some of the francolite binding peptide sequences similar to dolomite binding peptide sequences could be excluded from the sequence database of francolite binding peptides by applying bioinformatics, the alignment tool of amino acid sequences.

Table 3-1 indicates that the concentration of eluted phage was in the scale of $10^5$ pfu/ml in each biopanning round. That means the diversity of phage clones is enough to amplify the phage pool for the subsequent biopanning. After selecting the eluted phage with binding affinity to dolomite powder, the chemical properties (such as polarity) of some dolomite binding peptides sequences are summarized in Figure 3-6. Basically, one notable feature is that acidic amino acids are common in dolomite binding peptides. In addition, the 3rd round dolomite binding peptide sequences are always accompanied by a block of hydrophilic amino acids, but do not seem to contain a very large hydrophobic block region compared to 2nd round dolomite binding peptides.

The binding affinity of dolomite binding peptides are evaluated as the surface coverage of phage clones with expressed 12 mer binding peptide sequences with IF analysis. Generally, images taken at a high magnification is more accurate than at a low magnification. However, the drawback using a high magnification is to cause a smaller view of an image. In order to compromise the accuracy and view of an image at different magnifications, images of phage clone-ADYFTARPGPIT selected from a dolomite system were taken at an objective magnification 20X and 40X respectively (Figure 3-7). At the objective magnification 20X, the surface coverage of this phage clone was $94.0\% \pm 3.0\%$ which is no significant different from $96.4\% \pm 3.6\%$ at the objective magnification 40X statistically. Thus, surface coverage of phage clones were taken IF images to calculate their surface coverage on mineral surfaces at the
objective magnification 20X. Figure 3-8 shows that the binding affinity of expressed 12-mer dolomite binding was classified into strong, medium, and weak binders. Those binders, especially strong binders, could be input data in bioinformatics for excluding the similar peptide sequences from francolite binding peptides. Figure 3-9 describes the difference in the surface coverage of phage clones with strong, medium, and weak binders respectively.

The binding behavior of binding peptides could be affected by the conformation of the peptides, the chemical properties of amino acid residues of peptides, and the interaction between inorganic substrates and peptide binders, especially electrostatic interaction. For expressed 12-mer inorganic peptides, their chain length is too short to display an obvious conformation. Thus, the electrostatic interaction may play an important role in the determination of peptide binding affinity to a target material. In the exploration of electrostatic interaction between inorganic materials and peptide binders, the charge character of the inorganic surface and peptides need to be confirmed first. For the charge character on inorganic surfaces, the zeta potential can be applied to determine this. Figure 3-10 indicated the zeta potential of dolomite/francolite particle surface as it varies with pH. The isoelectric point is at pH 4.3. After pH 4.3, the zeta potential on the dolomite particles surface became more and more negative with the increase of pH. Otherwise, the zeta potential on dolomite particles was more and more positive with the decrease of pH.

The charge character of the inorganic peptides is dependent on pKₐ of amino acid residues and pH of the aqueous medium according to Henderson- Hasselbalch as follow [30]:

\[ \text{pH} = \text{pK}_a + \log_{10} \left( \frac{[\text{base}]}{[\text{acid}]} \right) \]

The charge property of carboxylate group and amino group as side groups of amino acid residues with pH of aqueous medium can be described in the form of Henderson- Hasselbalch below [30]:

\[ \text{RCOOH} = \text{RCOO}^{-1} + \text{H}^+, \quad \text{pH} = \text{pK}_a + \log_{10} \left( \frac{[\text{RCOO}^{-1}]}{[\text{RCOOH}]} \right) \]
RNH$_3^+$ = RNH$_2$ + H$^+$, \( \text{pH} = \text{pK}_a + \log_{10}[^{[RNH_2]}/[RNH_3^+]] \)

For carboxylate groups, they tend to bear negative charge due to the abundance of RCOO$^-$ when the pH of the aqueous medium is larger than the pK$_a$. Furthermore, amino groups easily accumulate positive charge while the pH of the aqueous medium is smaller than the PK$_a$. For instance, the phage clone: QTLPLPLTIAHP showed higher surface coverage at pH 7.4 (71.7% ± 7.5%) than pH 4.0 (19.0% ± 4.5%) and pH 10.0 (3.6% ± 0.8%) (Figure 3-11). In the expressed 12-mer amino acid sequence, QTLPLPLTIAHP, the most of amino residues did not bear charge in the range of pH4.0-10.0 except histidine. Histine contains a tertiary amine side group with PK$_a$ 6.0. At pH 7.4, some amine groups of histidine were partially protonated to bear positive charge, and dolomite surface showed the negative zeta potential (Table 3-2). In this case, QTLPLPLTIAHP tended to adhere onto dolomite particle surfaces due to the electrostatic attraction force (Figure 3-11). At pH 10.0, no charge was produced on QTLPLPLTIAHP. Thus, the interaction between this peptide and dolomite surface was from the weak Van der Waals force, which caused low surface coverage (3.6% ± 0.8%) (Figure 3-11).

At pH 4.0, each histidine was fully protonted to bear a +1 charge. However, the dolomite surface tended to have the positive zeta potential to repell the peptides with the posive charges to cause lower surface coverage than the one at pH7.4. Although the dolomite surface contained the positive charges in high percentage, little negative charge still existed to attract peptide via electrostatic interaction. That is why the surface coverage of this peptide at pH4.0 is higher than pH 10.0 (Figure 3-11).

With repesct to the phage clone: GFASDPSSSPWT, aspartic acid (Asp, D) with PK$_a$ 3.9 dominated the adhesion of the peptides on dolomite surface. This peptide sequence displayed higher surface coverage (78.0% ± 6.7%) on the dolomite surface at pH 4.0 than pH 7.4 and pH
10.0 because the partial dissociated aspartic acid with little negative charge was adhered onto the dolomite surface with the highly positive zeta potential because of the electrostatic attraction force (Table 3-3 and Figure 3-12). At pH 7.4 and pH 10.0, aspartic acid was fully dissociated to bear a -1 charge. In addition, negative charge density is higher and higher on dolomite surface with increasing pH. Thus, the electrostatic repulsive force is stronger at pH 10.0 than pH 7.4. Thus, the surface coverage of expressed peptide GFASDPSSSPWT at pH 7.4 was higher than at pH10.0 (Figure 3-12).

In this study, our goal was to use biopanning techniques to achieve the separation of francolite particles from dolomite particles specifically. In order to select binding peptides with suitable binding affinity to dolomitic phosphate ore in Florida, we chose Four Corner (FC) and South Fort Meade (SFM) pure francolite particles provided by Mosaic, Inc to pan for francolite binding peptides. Table 3-4 and Table 3-5 showed the phage titers for FC and SFM francolite: the concentration of eluted phage was in the scale of $10^6$ pfu/ml. These results reflected that the diversity of phages with binding affinity to francolite particles was satisfied: an acceptable diversity of bound phages is usually above $10^4$ pfu/ml.

Although those two target materials are all francolites, their purity is different, and thus their overall morphology/structure may display some differences in surface characteristic. Thus, we anticipated that a consensus amino acid sequence of francolite binding peptides from those samples may be different, but also may have some similarities in chemical characteristics.

The francolite binding peptide sequences from FC and SFM samples are listed in Figure 3-13 and Figure 3-14 after three biopanning rounds, respectively. The peptide sequences of phage clones from FC and SFM were roughly comprised of a block of polar and a block of hydrophobic amino acids. In addition, one or two basic amino acids were also found in most of
the peptide sequences. Although some similarities can be seen between the chemical properties of the 12-mer peptide sequences from FC and SFM, they were not identical (as expected), and a complete consensus sequence was not found at 3 rounds of panning for either one. This is not unusual for inorganic materials, particularly powders, which contain many different surface characteristics and thus binding sites. The differences between the peptide sequences for FC and SFM samples are roughly quantified in Table 3-6 with respect to block lengths and basic amino acids. In addition, the 3rd round dolomite binding peptide sequences do not seem to contain a very large hydrophobic block region compared to francolite binding peptide sequence (Table 3-7). The examination of the binding affinity of francolite binding peptides was shown in Figure 3-15. Most of the francolite binding peptides displayed preferential binding affinity to francolite, even if there were similar surface properties between the francolite and dolomite particles, especially, the peptide WSITTYHDRAIV which displayed highly preferential binding affinity to francolite (Figure 3-16). Thus, francolite binding peptides have the potential to function as hydrophilic head groups linked with hydrophobic molecules to float francolite particles from phosphate ores specifically.
Table 3-1. Phage titers for the phages selected from dolomite particles

<table>
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<tr>
<td></td>
<td>$C_{um-p}$</td>
<td>$C_{am-p}$</td>
<td>$V_{total}$</td>
<td>$C_{um-p}$</td>
<td>$C_{am-p}$</td>
<td>$V_{total}$</td>
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<tr>
<td>E1 (1st elution)</td>
<td>$4.5 \times 10^5$</td>
<td>$2.7 \times 10^{13}$</td>
<td>50</td>
<td>$2.7 \times 10^5$</td>
<td>$1.4 \times 10^{13}$</td>
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</tr>
<tr>
<td>E2 (2nd elution)</td>
<td>$1.2 \times 10^4$</td>
<td>$5.0 \times 10^{11}$</td>
<td>75</td>
<td>$5.2 \times 10^4$</td>
<td>$4.6 \times 10^{12}$</td>
<td>75</td>
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<tr>
<td>E3 (3rd elution)</td>
<td>$1.3 \times 10^4$</td>
<td>$5.2 \times 10^{10}$</td>
<td>125</td>
<td>$3.2 \times 10^4$</td>
<td>$1.4 \times 10^{11}$</td>
<td>125</td>
</tr>
<tr>
<td>E4 (4th elution)</td>
<td>$3.3 \times 10^3$</td>
<td>$6.5 \times 10^8$</td>
<td>145</td>
<td>$4.8 \times 10^3$</td>
<td>$3.3 \times 10^{10}$</td>
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<td>Total</td>
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<td>375</td>
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<td>350</td>
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Table 3-2. Charge of Histidine in peptide sequence QTLPLPLTIAHP and zeta potential of dolomite surfaces in 3.3% PC buffer at pH 4.0, pH 7.4, and pH10.0

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<thead>
<tr>
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<th>pH 4.0</th>
<th>pH 7.4</th>
<th>pH10.0</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zeta potential on dolomite surfaces (mV)</td>
<td>+2.2</td>
<td>-7.6</td>
<td>-15.0</td>
</tr>
<tr>
<td>His, H</td>
<td>+1</td>
<td>$\delta^+$</td>
<td>0</td>
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</table>

Interaction force

$\delta^+$: Partial positive charge

Table 3-3. Charge of Aspartic acid in peptide sequence GFASDPSSSPWT and Zeta potential of dolomite surfaces in 3.3% PC buffer at pH 4.0, pH 7.4, and pH10.0

<table>
<thead>
<tr>
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<th>pH 4.0</th>
<th>pH 7.4</th>
<th>pH10.0</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zeta potential on dolomite surfaces (mV)</td>
<td>+2.2</td>
<td>-7.6</td>
<td>-15.0</td>
</tr>
<tr>
<td>Asp, D</td>
<td>$\delta^-$</td>
<td>-1</td>
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Interaction force

$\delta^-$: Partial negative charge
### Table 3-4. Phage titers for phage selected from Fortier Corner francolite pebbles

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<th>Third round</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>C\textsubscript{um-p} (pfu/ml)</td>
<td>C\textsubscript{am-p} (pfu/ml)</td>
<td>V\textsubscript{total} (μl)</td>
<td>C\textsubscript{um-p} (pfu/ml)</td>
<td>C\textsubscript{am-p} (pfu/ml)</td>
<td>V\textsubscript{total} (μl)</td>
</tr>
<tr>
<td>E1 (1\textsuperscript{st} elution)</td>
<td>2.0 x 10\textsuperscript{6}</td>
<td>1.9 x 10\textsuperscript{13}</td>
<td>27</td>
<td>3.9 x 10\textsuperscript{6}</td>
<td>8.2 x 10\textsuperscript{12}</td>
<td>100</td>
</tr>
<tr>
<td>E2 (2\textsuperscript{nd} elution)</td>
<td>1.4 x 10\textsuperscript{6}</td>
<td>2.1 x 10\textsuperscript{13}</td>
<td>25</td>
<td>1.7 x 10\textsuperscript{6}</td>
<td>9.7 x 10\textsuperscript{12}</td>
<td>85</td>
</tr>
<tr>
<td>E3 (3\textsuperscript{rd} elution)</td>
<td>1.7 x 10\textsuperscript{6}</td>
<td>6.3 x 10\textsuperscript{13}</td>
<td>83</td>
<td>1.8 x 10\textsuperscript{5}</td>
<td>1.0 x 10\textsuperscript{13}</td>
<td>82</td>
</tr>
<tr>
<td>E4 (4\textsuperscript{th} elution)</td>
<td>5.4 x 10\textsuperscript{5}</td>
<td>5.2 x 10\textsuperscript{12}</td>
<td>100</td>
<td>4.2 x 10\textsuperscript{5}</td>
<td>1.0 x 10\textsuperscript{13}</td>
<td>82</td>
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<tr>
<td>Total</td>
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<td></td>
<td></td>
<td>349</td>
<td></td>
</tr>
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</table>

\textit{Cum-p}: The concentration of unamplified phages  
\textit{Cam-p}: The concentration of amplified phages  
\textit{V\textsubscript{total}}: The total taken volume of eluted phages

### Table 3-5. Phage titers for phage selected from South Fort Meade francolite pebbles

<table>
<thead>
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<th>Elution</th>
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<th></th>
<th>Second round</th>
<th></th>
<th>Third round</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>C\textsubscript{um-p} (pfu/ml)</td>
<td>C\textsubscript{am-p} (pfu/ml)</td>
<td>V\textsubscript{total} (μl)</td>
<td>C\textsubscript{um-p} (pfu/ml)</td>
<td>C\textsubscript{am-p} (pfu/ml)</td>
<td>V\textsubscript{total} (μl)</td>
</tr>
<tr>
<td>E1 (1\textsuperscript{st} elution)</td>
<td>6.1 x 10\textsuperscript{5}</td>
<td>3.7 x 10\textsuperscript{12}</td>
<td>100</td>
<td>1.6 x 10\textsuperscript{7}</td>
<td>1.0 x 10\textsuperscript{13}</td>
<td>74</td>
</tr>
<tr>
<td>E2 (2\textsuperscript{nd} elution)</td>
<td>7.9 x 10\textsuperscript{5}</td>
<td>6.1 x 10\textsuperscript{12}</td>
<td>61</td>
<td>1.0 x 10\textsuperscript{7}</td>
<td>7.3 x 10\textsuperscript{12}</td>
<td>100</td>
</tr>
<tr>
<td>E3 (3\textsuperscript{rd} elution)</td>
<td>2.8 x 10\textsuperscript{5}</td>
<td>6.6 x 10\textsuperscript{12}</td>
<td>56</td>
<td>5.3 x 10\textsuperscript{6}</td>
<td>9.5 x 10\textsuperscript{12}</td>
<td>79</td>
</tr>
<tr>
<td>E4 (4\textsuperscript{th} elution)</td>
<td>6.1 x 10\textsuperscript{5}</td>
<td>7.5 x 10\textsuperscript{12}</td>
<td>50</td>
<td>2.9 x 10\textsuperscript{6}</td>
<td>8.7 x 10\textsuperscript{12}</td>
<td>84</td>
</tr>
<tr>
<td>Total</td>
<td>267</td>
<td></td>
<td></td>
<td></td>
<td>337</td>
<td></td>
</tr>
</tbody>
</table>

\textit{Cum-p}: The concentration of unamplified phages  
\textit{Cam-p}: The concentration of amplified phages  
\textit{V\textsubscript{total}}: The total taken volume of eluted phages
Table 3-6. Chemical properties of peptide sequences selected from FC and SFM francolite pebbles

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<th></th>
<th>Basic amino acid (amino acid# / peptide)</th>
<th>Block of Polar amino acid (amino acid# / block)</th>
<th>Block of Non-polar amino acid (amino acid# / block)</th>
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<tbody>
<tr>
<td>FC</td>
<td>1.7</td>
<td>Long (3.2)</td>
<td>Short (non-obvious, 2.1)</td>
</tr>
<tr>
<td>SFM</td>
<td>1.5</td>
<td>Short (2.3)</td>
<td>Long (4.1)</td>
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</tbody>
</table>

Table 3-7. The comparison of peptide sequences selected from francolite and dolomite powders

<table>
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<tr>
<th></th>
<th>Basic amino acid (amino acid# / peptide)</th>
<th>Acidic amino acid (amino acid# / peptide)</th>
<th>Block of Polar amino acid (amino acid# / block)</th>
<th>Block of Non-polar amino acid (amino acid# / block)</th>
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<tbody>
<tr>
<td>Francolite</td>
<td>1.6</td>
<td>Low (0.5)</td>
<td>Long (2.8)</td>
<td>3</td>
</tr>
<tr>
<td>Dolomite</td>
<td>1.5</td>
<td>High (1.0)</td>
<td>Short (1.6)</td>
<td>Non-obvious (1.7)</td>
</tr>
</tbody>
</table>

Figure 3-1. Schematic of a peptide amphiphile as a flotation agent. In a suspension containing the target particles of francolite, along with other undesired impurities, the peptide selected for francolite binds specifically to those particles, which are then collected at the interface of air bubbles due to the hydrocarbon tails, enabling flotation separation.
Figure 3-2. A curve representing the relationship between recovery and grade of concentrate

Figure 3-3. Schematic for the flotation process
Figure 3-4. Contact angle between bubble and particle in an aqueous medium

Figure 3-5. Types of collectors used in the flotation concentration
Clone #

<table>
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<tr>
<th>Clone #</th>
<th>The first round</th>
<th>The second round</th>
</tr>
</thead>
<tbody>
<tr>
<td>No consensus</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Clone 1 | A | D | Y | F | T | A | R | P | G | P | I | T |
Clone 2 | M | P | N | P | H | L | A | L | P | H | G | S |
Clone 3 | S | P | N | P | P | A | N | A | V | I | T | N |
Clone 4 | Q | T | L | P | L | P | T | I | A | H | P |
Clone 5 | A | N | D | G | L | A | T | R | P | R | D | L |
Clone 6 | N | I | Q | T | T | H | L | F | P | L | P | R |
Clone 7 | G | M | E | L | H | S | K | L | P | I | Y | R |

Clone #

<table>
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<tr>
<th>Clone #</th>
<th>The first round</th>
<th>The second round</th>
</tr>
</thead>
<tbody>
<tr>
<td>No consensus</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Clone 1 | Q | H | H | T | L | S | T | A | P | Y | L | Y |
Clone 2 | Q | Q | N | Y | L | T | Q | N | I | G | R | A |
Clone 3 | Q | L | T | V | D | N | N | H | Q | G | N | D |
Clone 4 | H | Y | T | E | A | S | F | D | I | R | T | R |
Clone 5 | H | T | E | P | A | N | W | Y | P | H | T | H |
Clone 6 | D | T | N | F | V | K | A | P | R | Q | P | N |
Clone 7 | S | T | D | M | S | P | S | P | M | S | H | S |
Clone 8 | T | S | E | N | N | Y | A | V | E | S | F | H |
Clone 9 | A | P | K | G | L | T | N | T | S | Q | L | M |

**Figure 3-6.** Summary of expressed 12-mer dolomite binding peptide sequences. The letters correspond to the commonly used one letter code for the 20 amino acids.

**Figure 3-7.** Images of the phage clone-ADYFTARPGPIT on dolomite particles at the objective magnification 20X and 40X. (a) Image under white light at 20X; (b) image under fluorescence light at 20X; (c) image under white light at 40X; (d) image under fluorescence light at 40X (Surface coverage at 20X: 94.0% ± 3.0%; surface coverage at 40X: 96.4% ± 3.6%)
Figure 3-8. Classification of phages with expressed 12-mer peptide sequences into three categories based on the surface coverage on dolomite.

Red: Strong binders   Yellow: Medium binders   Green: Weak binders

Figure 3-9. The binding affinity of three representatives of phages on dolomite particles. The definition of binding affinity is the surface coverage of phages on dolomite powder surface (R2/R1); (a) The phage clone-SNITPQTSTPSL exhibits strong binding (94.3% + 3.5%); (b) The phage clone-SPNPPANAVTTN exhibits moderate binding affinity (71.7% + 7.5%); (c) The phage clone-THYTRGLSPFLS exhibits weak binding affinity (47.3% + 6.3%)
Figure 3-10. Zeta potential for francolite/dolomite powders in the PC buffer

Figure 3-11. Images of the phage clone-QTLPLPLTIAHP on dolomite surfaces. (a)~(c) Images under white light at pH 4.0, pH 7.4, and pH 10.0 respectively; (d)~(e) images under fluorescence light at pH 4.0, pH 7.4, and pH 10.0 respectively. The surface coverage of this phage clone on dolomite surfaces is 19.0% ± 4.5%, 71.7% ± 7.6%, and 3.6% ± 0.8% at pH 4.0, pH 7.4, and pH 10.0 respectively.
Figure 3-12. Images of phage clone-GFASDPSSSPWT on dolomite surfaces. (a)–(c) Images under white light at pH4.0, pH7.4, and pH10.0 respectively; (d)–(e) images under fluorescence light at pH4.0, pH7.4, and pH10.0 respectively. The surface coverage of this phage clone is 78.0% + 6.7%, 9.7% + 2.5%, and 2% + 0.7% at pH 4.0, pH7.4, and pH10.0 respectively.

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<td></td>
</tr>
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<td>3</td>
<td>T S P Q V A I P T L S G</td>
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<td></td>
</tr>
<tr>
<td>4</td>
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<td></td>
</tr>
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<td>E M W Q D N W M P W I T</td>
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</tr>
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<td>6</td>
<td>E T I S D O Y A W P Y A T</td>
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</tr>
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<td>3</td>
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<tr>
<td>4</td>
<td>S E M T H Q V A R V D T</td>
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<tr>
<td>5</td>
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<tr>
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<td>8</td>
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<td>10</td>
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<td>11</td>
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<td>12</td>
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<tr>
<td>13</td>
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Figure 3-13. Four Corner-francolite binding peptide sequences
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**Figure 3-14.** South Fort Meade-francolite binding peptide binding sequences

**Figure 3-15.** The surface coverage of phage clones with expressed francolite binding peptides on francolite and dolomite respectively
Figure 3-16. Images of the phage clone with expressed peptide-WSITTTYHDRAIV on francolite particles and dolomite particles. (a) A schematic for the glass slide containing francolite powder and dolomite powder; (b) francolite particles under white light; (c) francolite particles under fluorescence light; (d) the interface between francolite and dolomite under white light; (e) the interface between francolite and dolomite particles under fluorescence light; (f) dolomite particles under white light; (g) dolomite particles under fluorescence light.
CHAPTER 4
M13 PHAGE AMPHIPHILE AS COLLECTORS FOR THE SPECIFIC SEPARATION OF MINERAL PARTICLES

4.1 Motivation

In the Florida phosphate industry, froth flotation is the most effective method in separating phosphate particles from contamination by other minerals. However, it is difficult to remove dolomite contamination from Florida dolomitic phosphate ores because the characteristics of Florida dolomitic phosphate rock are CO₂ substitution, porous structure, and cryptocrystalline structure, in which the crystalline nature is vague and can only be characterized by a polarizing light microscope. In the work here, we hypothesized that the selected phage clones with expressed 12-mer francolite binding peptides would act as bio-amphiphilic flotation agents because some of the domains of coat proteins on M13 phage bodies are hydrophobic, having aerophilic ability to attract them to air bubbles (Figure 4-1). For example, in the froth flotation process (Figure 3-1), francolite binding peptides would display preferential binding affinity to francolite particles, and with the attached phage, these particles would be attracted to the air bubbles. Then, when air bubbles with attached phage-particles rise to the top of the flotation cell, the francolite particles will become separated from dolomite particles that remain on the bottom of the flotation cell.

4.2 Background and Significance

In the froth flotation process, the concentrate of valuable minerals is largely dependent of the collectors. Currently, the conventional collectors are usually organic surfactants such as fatty acids. Those chemical reagents are not environmentally friendly. In addition, the mining industry may also face the fluctuation of price due to the shortage of certain flotation agents. Aside from using organic surfactants as a collector, in 1993 Smith et al. first explored *Mycobacterium phlei*, a kind of bacterium, that could be applied as collectors to concentrate hematite in the flotation
process as collectors [129]. The main composition on the surface of *Mycobacterium phlei* is lipids which have hydrophobic tails [130]. In the aqueous medium at pH 2.5 (the isoelectric point of these lipids), the head regions of those lipids tend to bear a negative charge and easily adhere onto the surface of mineral particles bearing neutral, or positive charges. The hydrophobic domain on *Mycobacterium phlei* rendered the hematite particles hydrophobic to enhance the adhesion with air bubbles. In the froth flotation using *Mycobacterium phlei*, the specific separation of hematite particles could be achieved.

Florida contributes 80% of USA phosphate products. However, phosphate reserves in Florida usually contain high MgO contamination due to a high quantity of dolomite [131]. As mentioned before, one of the biggest challenges is to remove dolomitic carbonate from the phosphate ores using the froth flotation technology. In the usage of microorganisms as flotation agents, *Bacillus licheniformis* JF-2 was found to have the ability to interact with the metal cations through their anionic cell walls composed of teichuronic acid, teichoic acid, and peptidoglycan [132-134]. Beveridge *et al.* proved cationic metal ions were mainly captured in teichuronic acid and teichoic acid domains [132]. In addition, it was also confirmed that *Bacillus licheniformis* showed a stronger binding affinity to Mg$^{2+}$ than Ca$^{2+}$ [132, 135].

This selective binding of *Bacillus licheniformis* to Mg$^{2+}$ had been demonstrated in the separation of dolomite particles from phosphate ore by Misra *et al.* [136]. Thus, Misra i utilized *Bacillus licheniformis* as a collector for removing the dolomite particles from phosphate ores. At pH 10.0-12.0, *Bacillus licheniformis* displayed preferential binding affinity to Mg$^{2+}$. However, the fraction of floated dolomite is below 20% which is far lower than the 60% commercial floated fraction [136]. That means *Bacillus licheniformis* is not a good collector for floating dolomite powder because of the competition of OH$^-$ cations. Besides viable microorganisms,
freeze dried bacteria also can be used as flotation collectors [137]. Smith et al. used freeze dried *Staphylococcus carnosus* as a collector to float apatite, calcite, and quartz at pH 9.0 respectively. The recovery rate of apatite was 50% -70% with microbe dosage. However, *Staphylococcus carnosus* also can float calcite with the recovery rate of 25%-55%. Thus, *Staphylococcus carnosus* is a good collector, but lacks of high recognition capability in the differentiation between apatite and calcite.

As mentioned above, microorganisms have been considered as alternative flotation agents in mining processing due to the molecular recognition of their cell walls and non-toxicity compared to chemical flotation reagents. However, all of these bacteria only display preferential binding affinity to a certain metal cation, such as Mg$^{2+}$, in a low pH or high pH solution which is still harsh to the environment. Furthermore, the floated fraction of mineral particles of interest is usually too low to satisfy the requirement of commercialization in a neutral environment. Finally, the required quantity of microorganisms is usually 10-100 times greater than the conventional surfactants in order to reach a satisfied recovery rate of valuable minerals. Thus, it is desirable that the dose of microorganism is reduced to the level of the chemical agents as flotation agents for commercial applications.

In this study, phage display techniques [18-67] were adopted to select the phage clones with expressed inorganic binding peptides. The amino acid sequences of inorganic binding peptides can display strongly specific binding affinity to a certain inorganic substrate based on their chemical composition, surface morphology, particle size, pH, and crystal structure. Thus, phage display biopanning can be performed to select inorganic binding peptides with strong preferential binding affinity to minerals of interest at pH 7.0. In addition, some domains of the coat proteins on M13 phage bodies may be hydrophobic. Thus, the M13 phage clones acting as
bio-reagents could be used under mild environmental conditions, where they have the potential to be used as collectors for floating mineral particles of interest in one step within a neutral aqueous medium. In addition, the M13 phage is a kind of filamentous virus with the expressed inorganic binding peptides at one end of the phage body. If the adhesion of M13 phages on the mineral surface is through those inorganic binding peptides, the binding domain of selected M13 phage clones may be in the scale of nano-meters, far smaller than other microorganisms in the scale of micro-meters. In this case, the dose of M13 phages might be anticipated to be less, and possibly close to the dose used with chemical surfactants.

4.3 Materials and Methods

4.3.1 Materials

- Phage display ingredients: See Chapter 2
- M13 phage clones: Three selected phage clones with expressed 12-mer peptide sequences, WSITTYHDRAIV, TNSNWTPWEPLP, and SSMTHQHARVDT
- Fluorapatite: Ca$_5$(PO$_4$)$_3$ (F, OH): Crystalline fluorapatite with green to brown color was purchased from Ward’s Natural Science, Inc.
- Dolomite: CaMg(CO$_3$)$_2$: Crystallized dolomite with large and gray cleavages was purchased from Ward’s Natural Science, Inc.
- Florida francolite pebbles: Ca$_5$(PO$_4$)$_3$ (F, OH): Francolite pebbles were provided by Mosaic, Inc. after the flotation process.

4.3.2 Methods

**Immunofluorescence (IF) analysis:** See chapter 3-3.3.2 methods

**Flotation test:** In the bench-top flotation experiment, a flotation column (Figure 4-2(a)) was used to float mineral particles in the scale of 1 to 10 gram. A plastic tube served as the connection between the bottom of the flotation column and an air gas cylinder. An air stream adjusted by the gas valve on the bottom of the flotation column was pumped into the aqueous medium to create air bubbles in the flotation column. Before pumping the air stream into the
flotation column, 1 gram of mineral particle such as francolite (size: 50-250 $\mu$m), dolomite (size: 20-60 $\mu$m), or a mixture of francolite/dolomite was incubated with the desired phage clone (0.96 mg per gram mineral) in 5 ml PC buffer solution at PH 7.4 on an agitator for 1 hour. After that, the mixture of mineral particle/phage solution and a magnetic stir bar were added to fill into the flotation column, and then 145 ml distilled water (at pH 7.4) was filled into the flotation column. When the mineral particles were precipitated (Figure 4-2(b)), the air stream was pumped into the flotation column and the magnetic stir bar was allowed to agitate the mineral particles for 3 minutes. Floated particles on the top of the flotation column were collected by filtration and dried to weigh the floated mineral particles (Figure 4-2 (c)).

**Measurement of contact angles:** In the measurement of contact angles, a mineral rock was first cutted into mineral sheets with approximate dimensions of 2 cm x 2 cm x 2 mm by using a precision sectioning saw (Isomet 1000, Buehler, Inc.) with a 4” 15HC diamond waffering blade (Buehler, Inc.). The surface of each mineral sheet was polished using 600 and 1200 grit SiC sand papers (Alleid, Inc.). Subsequently, the mineral sheets were rubbed over a wet polishing cloth on which a 0.05 $\mu$m alumina suspensions was applied. Then, the mineral sheets were washed using distilled water for removing the residue of alumina suspension on their surfaces.

In this method, the captive bubble technique was adopted to determine the advancing and receding contact angles for dolomite and fluorapatite sheets using a Contact Angle Goniometer image system equipped with an auto pipetting system (Rame-Hart, Inc., USA). The polished mineral sheets were attached on the sample holder (Figure 4-3 (a)). Then, they were immersed into an optical acrylic cell containing distilled water or 3.3% PC buffer solution at pH7.4 (Figure 4-3 (b)). An air bubble created by the auto pipetting system was attached to the mineral surface
through a plastic needle (Figure 4-3 (c)). The sample holder was raised until the area between the air bubble and the mineral surface was in contact. In this situation, the inflection point could be observed.

In order to explore the effect of M13 phage clones on the contact angle of minerals, mineral sheets were immersed in 3 ml phage/PC solution (phage concentration: $10^{11}$ pfu/ml in 3ml PC buffer), and agitated for 50 minutes. Subsequently, mineral sheets were rinsed with the solution which is the same as the liquid in the acrylic cell. The measurement was made after 10 minutes after the mineral coated with M13 phage clones was immersed into the solution in the acrylic cell. At least five measurements were made for each condition, and the angle of each side of the air bubble was recorded at 23°C.

**Inductively coupled plasma-atomic emission spectroscopy (ICP-AES) analysis:** In this study, ICP-AES (Optima 3200KL, Perkin Elmer) was used to determine the content of Magnesium/Calcium in the minerals. In this analysis, 20 mg mineral sample was added into the 20 ml concentrated HCl solution, and was stirred for approximately 24 hours in order to prepare 1000 mg/L (ppm) mineral solution. Subsequently, 1000 mg/L (ppm) was diluted down to 100 mg/L (ppm) using distilled water. The emission of calcium was detected at 315.89 nm, 317.93 nm, and 393.37 nm. The emission of magnesium was detected at 279.77 nm and 285.23 nm. A calibration curve of Magnesium/Calcium was established from 100 ppm to 0.1 ppm before the measurement of the samples: the 1000 ppm standard Magnesium/Calcium solution (RICCA Chemical Company) was diluted into 100 ppm, 10 ppm, 1 ppm, and 0.1 ppm respectively. After that, the mineral solution with 100 mg/L (ppm) was loaded into ICP and the magnesium/calcium content was determined in the mineral sample. The concentration of Mg and Ca was converted into the content of MgO and CaO based on the calculation as follows:
The content of MgO (%) = \( \frac{C_{\text{Mg}}}{C_{\text{dissolved mineral}}} \times \frac{M_{\text{MgO}}}{M_{\text{Mg}}} \times 100\% \)

The content of CaO (%) = \( \frac{C_{\text{Ca}}}{C_{\text{dissolved mineral}}} \times \frac{M_{\text{CaO}}}{M_{\text{Ca}}} \times 100\% \)

Where:
- \( C_{\text{Mg}} \): The measured concentration of Mg (mg/L or ppm)
- \( C_{\text{Ca}} \): The measured concentration of Ca (mg/L or ppm)
- \( C_{\text{dissolved mineral}} \): The concentration of mineral dissolved in HCl
- \( M_{\text{MgO}} \): The molecular weight of MgO (g/mol)
- \( M_{\text{CaO}} \): The molecular weight of CaO (g/mol)
- \( M_{\text{Mg}} \): The atomic weight of Mg (g/mol)
- \( M_{\text{Ca}} \): The atomic weight of Ca (g/mol)

4.4 Results and Discussions

A successful flotation process is often dependent on the choice of a collector with the molecular configuration like a surfactant where the hydrophilic head selectively adsorbs on the valuable mineral and the hydrophobic tail renders valuable mineral hydrophobicity to reduce the work of adhesion to an air bubble. Phage display techniques have been proven as a potential in the selection of phage clones with the expressed inorganic binding peptides that have specific binding affinity to a target material. In addition, the hydrophobic domains of M13 coat proteins may have the potential to function as the hydrophobic tail of a collector. In this study, M13 phage clones with expressed francolite binding peptides were used to test the hypothesis that selective flotation could be achieved, whereby francolite particles would be concentrated from the mixed mineral containing the dolomite contamination.

In this study, there were three phage clones selected from the francolite pebbles after three biopannings for exploring the effect of expressed 12-mer francolite binding peptides on the performace of the selective flotation. Their expressed francolite binding peptide sequences are WSITTYHDRAIV, TNSNWTPFWPLP, and SSMTHQHAVDT respectively. In IF analysis
(Figure 4-4 - 4-5), the phage clone-WSITTYHDRAIV was found to preferentially adhere onto francolite particles, but not dolomite particles. However, phage clone-TNSNWTPFWPLP tended to bind onto dolomite particles, rather than francolite. With respect to phage clone-SSMTHQHARVDT, it did not seem to differentiate between francolite versus dolomite. The surface coverage of these phage clones is summarized in Table 4-1.

In the beginning of the flotation test, a fatty acid that is widely used as a conventional collector used widely for the concentration of francolite from phosphate ores in the mining industry, was first used to evaluate the recovery rate of pure minerals such as francolite and dolomite in the bench-top flotation process (Figure 4-2). Here, the recovery rate of pure minerals (a positive control) was defined as follows:

\[
\text{Recovery rate} (\%) = \left( \frac{W_{\text{floated minerals}}}{W_{\text{feed minerals}}} \right) \times 100\%
\]

Where \( W_{\text{floated minerals}} \): The weight of the floated minerals

\( W_{\text{feed minerals}} \): The weight of feed minerals

Figure 4-6 shows that the fatty acid didn’t differentiate francolite from dolomite due to their similar recovery rate. In the mining industry, the dose of fatty acid is usually 0.5mg per gram mineral to achieve a satisfactory recovery rate of francolite (approximately 100%). In the bench-top flotation test, the recovery rate of francolite was 52.5% in the distilled water solution with pH 7.4 using this suggested dose of fatty acid, but the recovery rate of dolomite was as high as 60%. When the selected phage clone-WSITTYHDRAIV was used as the collector in the flotation process, the recovery rate of francolite was clearly higher than dolomite (Figure 4-7). At 52.7% recovery rate of francolite, the dose of this phage clone is 0.96 mg per gram mineral which is twice as much as fatty acid (0.5 mg per gram mineral in 52.5% recovery rate of francolite).
dose of M13 phage clone is far lower than the microorganisms described early, in which the dose of the general microorganisms is 10-100 times higher than chemical agents.

The low required quantity of M13 phages could possibly be explained by the adhered domain on the mineral surface. As mentioned in chapter 1, the configuration of M13 phage is like a flexible filamentous rod (Figure 1-5). The strong interaction between M13 phage and a target material is usually through the expressed inorganic binding peptides at the end of the PIII proteins [30, 35]. The dimension of PIII protein is less than 10 nm. For other microorganisms used as collectors in the froth flotation techniques, their binding area is in the scale of micrometer on the mineral surface. That may be why the dose of M13 phage is in the same scale as chemical agents. Figure 4-8 shows another phage clone-TNSNWTPFWPLP that can still differentiate francolite from dolomite, but it tended to float dolomite even though this clone was selected from francolite pebbles. However, the phage clone-SSMTHQHARVDT behaved like a fatty acid, with limited specificity, but also with a low recovery rate of minerals (Figure 4-9). Figure 4-10 summarizes the recovery rate of francolite by comparing those three selected phage clones as collectors. These results support my hypothesis that a high surface coverage of phages can lead to a high recovery rate of francolite.

It was considered that the high recovery rate of mineral might be related to the high hydrophobicity on the mineral surface after being coated with the phage. The hydrophobicity can be evaluated with the measurement of contact angle (Table 4-2 to 4-3); the higher the contact angle, the more hydrophobic the mineral surface. The phage clone-WSITTYHDRAIV increased the contact angle of francolite from 12.5° to 50° in 3.3% PC buffer (Table 4-2 and Figure 4-11) due to 99% surface coverage of this phage clone on the francolite surface. In contrast, this phage clone displayed weak binding affinity to dolomite (11% surface coverage). As was expected, the
contact angle of dolomite modified with this phage clone just was increased only around 2°, as compared to bare dolomite. Thus, high surface coverage of phage clones can render the minerals to become more hydrophobic. If the solution medium was changed from 3.3% PC buffer to distilled water, the contact angle of the francolite surface modified with this phage clone was still maintained at 50° (Table 4-2 to 4-3). This can be explained by the hydrophobic coat protein of M13 phages, which shields the hydrophilic mineral surface from the influence of hydrophilic solution medium. Thus, I believe the hydrophobic domains of M13 coat protein rendered the francolite hydrophobic, and decreased the work of adhesion between the francolite surface and the air bubble. That may be why this phage clone tends to float francolite, rather than dolomite.

The phage clone-TNSNWTPFWPLP that displayed strong binding affinity to dolomite was found to increase the contact angle of dolomite from 15.6° to 48.4° (in 3.3% PC buffer), but the contact angle of francolite was not changed very much. Thus, this phage clone would be anticipated to preferentially float dolomite, as was observed. With respect to the phage clone-SSMTHQHARVDT, it didn’t increase the contact of mineral of either mineral presumably due to its weak binding affinity to both of the two minerals (Table 4-2 to 4-3). Thus, this would explain why this phage clone did not have a good ability to float either of minerals. In addition, the contact angle of the bare minerals was lower in 3.3% PC solution than distilled water medium. Especially, the contact angle of the bare dolomite surface, which was reduced from 28.7° to 15.6° when distilled water was substituted with 3.3% PC buffer (Table 4-2 to 4-3). This result also reflected the decrease in the recovery rate of the bare dolomite (from 38.5% to 25.4%). Thus, PC buffer is able to function as a depressant to dolomite.

The most important requirement of a collector is to concentrate valuable mineral from ore particles. Although M13 phages had shown the ability of floating the pure minerals, it is
necessary to validate their effectiveness in the separation of francolite from phosphate ores containing the dolomite contaminations. In this study, we simulated the dolomitic Florida phosphate ore by mixing francolite with dolomite in the weight ratios of 1:1, 3:1, and 1:3. Subsequently, the selected M13 phage clones were applied to concentrate the francolite from the mixed minerals. The purity of francolite can be evaluated using ICP-AES in the floated minerals. In order to determine the content of francolite in the floated minerals, the content of magnesium and calcium are necessary to be measured in the pure francolite and dolomite respectively using ICP-AES. Table 4-4 showed the result of ICP-AES measurement for pure francolite and dolomite.

The content of magnesium mainly appeared in the pure dolomite. Thus, the result of the ICP-AES measurement shown in table 4-4 can be used to calculate the percentage of dolomite in the floated mixture of minerals after the content of magnesium is determined in the mixed floated minerals. If the concentration of dissolved minerals is the same, the content of magnesium is calculated as follows:

\[
11.91 \times X_{\text{Dolomite}} + 0.17 \times X_{\text{Francolite}} = 11.91 \times X_{\text{Dolomite}} + 0.17 \times (1-X_{\text{Dolomite}})
\]

\[
= C_{\text{Mg, mixed mineral}}
\]

\[\implies X_{\text{Dolomite}} = (C_{\text{Mg, mixed mineral}} - 0.17)/10.74\]

\[\implies X_{\text{Francolite}} = 1- X_{\text{Dolomite}}\]

Where \(X_{\text{Dolomite}}\): the percentage of dolomite in the floated mixed mineral

\(X_{\text{Francolite}}\): the percentage of francolite in the floated mixed mineral

\(C_{\text{Mg, mixed mineral}}\): the concentration of magnesium in the floated mixed mineral

Furthermore, the recovery rate of francolite and dolomite is also deduced in the mixed minerals based on \(X_{\text{Dolomite}}\) as follows:
Floated percentage of dolomite = \( \frac{X_{\text{Dolomite}} \cdot W_{\text{Floated mixed mineral}}}{W_{\text{Total dolomite}}} \)

Floated percentage of francolite = \( \frac{X_{\text{Francolite}} \cdot W_{\text{Floated mixed mineral}}}{W_{\text{Total Francolite}}} \)

\[ = \frac{((1 - X_{\text{Dolomite}}) \cdot W_{\text{Floated mixed mineral}})}{W_{\text{Total Francolite}}} \]

Where 
- \( W_{\text{Floated mixed mineral}} \): the weight of floated mixed mineral
- \( W_{\text{Total dolomite}} \): the weight of total dolomite in the mixed mineral before the flotation process
- \( W_{\text{Total Francolite}} \): the weight of total francolite in the mixed mineral before the flotation process

When the mixed mineral (francolite: dolomite = 1:1) was floated using fatty acid and selected M13 phage clones as collectors respectively, the ICP-AES analysis for the floated mixture is shown in Table 4-5. The floated mineral contained 10.5% MgO contamination using fatty acid as a collector. In this case, the content of dolomite occupied 52.3% of the floated minerals. This also indicates that the fatty acid didn’t enable differentiation francolite over dolomite. When the phage clone-WSITTYHDRAIV (phage #1) was chosen to float the mixed minerals, the content of MgO contamination was lowered from 10.5% to 6.7% in the floated minerals, and the content of CaO indicated the purity of francolite was increased from 35.2% to 36.8%. The percentage of dolomite was reduced from 52.3% to 39.3% in the floated mixture of minerals.

When phage #2 functioned as a collector, it behaved similarly to the fatty acid due to its similar binding affinity to francolite and dolomite. The only difference between phage #2 and fatty acid is that phage #2 caused lower recovery rate of mineral than fatty acid due to its weak binding affinity to minerals. With respect to phage #3, it tended to concentrate dolomite, rather than francolite. Thus, the content of MgO caused by phage #3 was 11.6% even higher than the MgO content of 10.5% obtained using fatty acid as a collector. In addition, the intrinsic recovery
rate of dolomite was 21.3\% in 3.3\% PC buffer in the absence of collectors (See Figure 4-10). For the mixed mineral (francolite: dolomite = 1:1), the minimum content of dolomite contamination is around 27.6\% due to the intrinsic recovery rate of dolomite (21.3\%) even if the selected phage clone did not display binding affinity to dolomite. The recovery rate of francolite/dolomite floated from mixed minerals (Table 4-5) matched with the results from the flotation of pure minerals (Figure4-6 to Figure 4-9). When the ratio of francolite to dolomite was changed from 1:1 to 1:3 or 3:1, the selected phage clones versus fatty acid followed basically the same trend of flotation proportional to the feed mixture of minerals composed of francolite and dolomite (Table 4-6 and Table 4-7, respectively). In particular, the ability of concentrating francolite was enhanced gradually using phage #1 versus the fatty acid as a collector with decreasing the ratio of francolite to dolomite in the feed mixture. Overall, the preferential binding affinity of M13 phages to francolite and the amphiphilic properties of M13 phages can envision that M13 phages has a great potential to achieve the concentration of francolite from dolomitic phosphate ores.
Table 4-1. Summary for the surface coverage of selected phage clones on francolite and dolomite

<table>
<thead>
<tr>
<th>The expressed 12-mer peptide sequences on selected phage clones</th>
<th>The surface coverage (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Francolite</td>
</tr>
<tr>
<td>WSITTYHDRAIV</td>
<td>94.1 ± 5.7</td>
</tr>
<tr>
<td>TNSNWTPFWPLP</td>
<td>61.7 ± 6.5</td>
</tr>
<tr>
<td>SSMTHQHARVDT</td>
<td>50.7 ± 3.1</td>
</tr>
</tbody>
</table>

Table 4-2. Contact angle on francolite and dolomite in 3.3% PC buffer solution at pH7.4

<table>
<thead>
<tr>
<th>Contact angle-Francolite (Degree)</th>
<th>Contact angle-Dolomite (Degree)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Advancing</td>
</tr>
<tr>
<td>No Phage</td>
<td></td>
</tr>
<tr>
<td></td>
<td>11.2±1.7</td>
</tr>
<tr>
<td>WSITTYHDRAIV</td>
<td>49.0±3.4</td>
</tr>
<tr>
<td>TNSNWTPFWPLP</td>
<td>14.2±2.3</td>
</tr>
<tr>
<td>SSMTHQHARVDT</td>
<td>12.0±2.3</td>
</tr>
</tbody>
</table>

Table 4-3. Contact angle on francolite and dolomite in distilled water at pH7.4

<table>
<thead>
<tr>
<th>Contact angle-Francolite (Degree)</th>
<th>Contact angle-Dolomite (Degree)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Advancing</td>
</tr>
<tr>
<td>No Phage</td>
<td></td>
</tr>
<tr>
<td></td>
<td>13.5±2.9</td>
</tr>
<tr>
<td>WSITTYHDRAIV</td>
<td>49.3±3.6</td>
</tr>
<tr>
<td>TNSNWTPFWPLP</td>
<td>15.6±3.5</td>
</tr>
<tr>
<td>SSMTHQHARVDT</td>
<td>12.5±4.2</td>
</tr>
</tbody>
</table>
Table 4-4. ICP-AES analysis for the pure minerals

<table>
<thead>
<tr>
<th>Mineral</th>
<th>Conc. of dissolved minerals (mg/L)</th>
<th>Mg (mg/L)</th>
<th>Ca (mg/L)</th>
<th>MgO (%)</th>
<th>CaO (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Francolite</td>
<td>100</td>
<td>0.17±0.01</td>
<td>33.40±0.33</td>
<td>0.28</td>
<td>46.81</td>
</tr>
<tr>
<td>Dolomite</td>
<td>100</td>
<td>11.91±0.02</td>
<td>19.96±0.11</td>
<td>19.85</td>
<td>27.94</td>
</tr>
</tbody>
</table>

Table 4-5. ICP-AES analysis of mixed minerals (Francolite:Dolomite=1:1) at pH7.4

<table>
<thead>
<tr>
<th>Collector</th>
<th>Percentage of floated mineral (%)</th>
<th>MgO (%)</th>
<th>CaO (%)</th>
<th>Distribution (%)</th>
<th>Recovery rate (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fatty acid</td>
<td>53.5</td>
<td>10.5</td>
<td>35.2</td>
<td>47.7</td>
<td>52.3</td>
</tr>
<tr>
<td>Phage #1</td>
<td>41.0</td>
<td>6.7</td>
<td>36.8</td>
<td>60.7</td>
<td>39.3</td>
</tr>
<tr>
<td>Phage #2</td>
<td>34.0</td>
<td>9.5</td>
<td>36.1</td>
<td>52.9</td>
<td>47.1</td>
</tr>
<tr>
<td>Phage #3</td>
<td>45.0</td>
<td>11.6</td>
<td>33.2</td>
<td>42.0</td>
<td>58.0</td>
</tr>
</tbody>
</table>

Phage #1: WSITTYHDRAIV (F: 99%, D: 11%)
Phage #2: SSMTHQHARVDT (F: 52%, D: 41%)
Phage #3: TNSNWTPFWPLP (F: 66%, D: 100%)

Table 4-6. ICP-AES analysis of mixed minerals (Francolite:Dolomite=3:1) at pH7.4

<table>
<thead>
<tr>
<th>Collector</th>
<th>Percentage of floated mineral (%)</th>
<th>MgO (%)</th>
<th>CaO (%)</th>
<th>Distribution (%)</th>
<th>Recovery rate (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fatty acid</td>
<td>52.4</td>
<td>5.7</td>
<td>40.7</td>
<td>72.2</td>
<td>27.8</td>
</tr>
<tr>
<td>Phage #1</td>
<td>43.8</td>
<td>3.7</td>
<td>44.4</td>
<td>83.0</td>
<td>17.0</td>
</tr>
<tr>
<td>Phage #2</td>
<td>33.4</td>
<td>5.9</td>
<td>39.2</td>
<td>71.4</td>
<td>28.6</td>
</tr>
<tr>
<td>Phage #3</td>
<td>34.0</td>
<td>7.1</td>
<td>37.5</td>
<td>65.8</td>
<td>48.3</td>
</tr>
</tbody>
</table>

Phage #1: WSITTYHDRAIV (F: 99%, D: 11%)
Phage #2: SSMTHQHARVDT (F: 52%, D: 41%)
Phage #3: TNSNWTPFWPLP (F: 66%, D: 100%)
Table 4-7. ICP-AES analysis of mixed minerals (Francolite:Dolomite=1:3) at pH7.4

<table>
<thead>
<tr>
<th>Collector</th>
<th>Percentage of floated mineral (%)</th>
<th>MgO (%)</th>
<th>CaO (%)</th>
<th>Distribution (%)</th>
<th>Recovery rate (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Francolite</td>
<td>Dolomite</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Francolite</td>
<td>Dolomite</td>
</tr>
<tr>
<td>Fatty acid</td>
<td>55.0</td>
<td>15.2</td>
<td>31.9</td>
<td>24.0</td>
<td>76.0</td>
</tr>
<tr>
<td>Phage #1</td>
<td>33.7</td>
<td>11.9</td>
<td>32.7</td>
<td>41.8</td>
<td>58.8</td>
</tr>
<tr>
<td>Phage #2</td>
<td>35.1</td>
<td>15.0</td>
<td>31.2</td>
<td>27.2</td>
<td>72.8</td>
</tr>
<tr>
<td>Phage #3</td>
<td>42.2</td>
<td>18.0</td>
<td>28.2</td>
<td>12.6</td>
<td>87.4</td>
</tr>
</tbody>
</table>

Phage #1: WSITTYHDRAIV (F: 99%, D: 11%)
Phage #2: SSMTHQHARVDT (F: 52%, D: 41%)
Phage #3: TNSNWTPFWPLP (F: 66%, D: 100%)

Figure 4-1. Schematic of froth flotation using M13 phage with expressed francolite binding peptides as collectors for separation of francolite particles from dolomite particles
Figure 4-2. The set-up for the bench-top flotation. (a) Design of a flotation tube (while empty); (b) the tube filled with 1.00 gram mineral/5 ml PC buffer in 145 ml distilled water; (c) an air stream is pumped into solution to create air bubbles for 3 minutes, and then floated minerals can be collected on the top of the tube and dried for characterization of the powder.

Figure 4-3. The method of the contact angle measurements. (a) The mineral sheet was attached on the sample holder; (b) the sample holder was placed side down, and was immersed into the solution in the acrylic cell; (c) an air bubble was attached to the mineral surface through a small plastic needle.
Figure 4-4. Images of francolite particles modified with phage clones selected from francolite. (a)(d) The phage Clone-WSITTYHDRAIV; (b)(e) the phage clone-TNSNWTPFWLP; (c)(f) the phage clone-SSMTHQHARVDT; under white light (a) to (c) and fluorescence light (d) to (f)

Figure 4-5. Images of dolomite particles modified with phage clones selected from dolomite. (a)(d) the phage Clone-WSITTYHDRAIV; (b)(e) the phage clone-TNSNWTPFWLP; (c)(f) the phage Clone-SSMTHQHARVDT; under white light (a) to (c) and fluorescence light (d) to (f)
Figure 4-6. The recovery rate of pure francolite and dolomite particles versus dose of fatty acid as a collector in distilled water at pH 7.4

Figure 4-7. The recovery of pure francolite and dolomite particles versus dose of phage clone-WSITYHDRAIV as a collector in 3.3% PC buffer at pH 7.4
Figure 4-8. The recovery of pure francolite and dolomite particles versus dose of phage clone-TNSNWTPFWPLP as a collector in 3.3%PC buffer at pH 7.4

Figure 4-9. The recovery of pure francolite and dolomite particles versus dose of phage clone-SSMTHQHARVDT as a collector in 3.3%PC buffer at pH 7.4
Figure 4-10. Comparison of recovery rate of francolite showing all three representative phage clones as collectors respectively at pH 7.4

Figure 4-11. The hydrophobicity of francolite through phage clone-WSITTYHDRAIV in 3.3%PC buffer solution at pH7.4 (a) Phage clone-WSITTYHDRAIV rendered francolite hydrophobicity as judged by the increased contact angle from 12.5° to 50°; (b) Images of an air bubble on francolite surface in 3.3%PC buffer solution (left) no phage coating (right) the addition of phage clone-WSITTYHDRAIV.
CHAPTER 5
CONCLUSIONS

In this dissertation, inorganic binding peptides selected by phage display techniques were used to demonstrate the feasibility of the approach for two applications, reversibly electroactive peptides and specific separation of minerals. In the first case, amorphous IZO was used as a target material to pan for IZO binding peptides as electro-activated peptide linkers for the development of refurbishable biosensors. Amorphous IZO led to some consensus binding peptide sequences, which I attribute to its homogeneous surface (i.e., lack of crystal facets that express various crystallographic planes). It was found that the IZO inorganic binding peptides showed preferential binding affinity to the amorphous IZO as compared to sapphire, Si, and SiO₂. Thus, although not studied here, the specificity of inorganic binding peptides may provide spatial control with multi-component systems. In the electro-releasing test, the releasing phenomenon of the selected phage clones with expressed IZO binding peptides was demonstrated that an electro-releasing device. These preliminary studies are promising, suggesting that this technique can contribute to the development of self-cleaning biosensors. In the future, an electro-biopanning protocol, in which an electric field substitutes the traditional low pH or high salt buffers, may aid in the selection of binding peptides more sensitive to an electric field.

In the second project of this dissertation, the separation of mineral particles, it was found that the phage clone-WSITYHDRAIV displayed preferential binding affinity to francolite. The data suggests that this occurs through electrostatic interaction between the expressed binding peptide and the francolite surface. After the adhesion of phages onto the francolite surface, the coat protein of M13 phages rendered the francolite particles hydrophobic. Furthermore, the body of the phage appeared to serve as an amphiphilic "tail" while the peptide ‘head’ was selective for the particles of interest, allowing for the separation of francolite particles from dolomite particles.
In addition, M13 phage amphiphiles can lead a satisfactory recovery rate of minerals in a neutral environment. Thus, it could be valuable to substitute organic chemical agents with M13 phage amphiphiles for reducing the toxicity to our environment. In a cost effectiveness consideration, the dose of the filamentous M13 amphiphile is in the same scale as chemical surfactants. In addition, M13 phages can be replicated by infecting host cells. Thus, there is no problem in the shortage of chemical agents. In the amplification process of M13 phages, the main expense is the cost of nutrient media such as the Luria-Bertani (LB) Lennox. The reason is that the quantity of M13 phage clone and host cell stock solution is negligible compared to the LB media, and M13 phages and host cells are reusable. For the longevity of M13 phages, their viability can be maintained over two years at -70°C in a refrigerator. These advantages suggest that M13 phages are more feasible as flotation agents than other microorganisms as collectors in an industrial froth flotation process.

Although most of the inorganic binding peptides selected with phage display techniques can show preferential binding affinity to a target material, they still displayed minor binding affinity to other inorganic materials. In the future, the bioinformatics approach could potentially improve the specificity of inorganic binding peptides. In this approach, a set of experimentally selected peptides are categorized for their binding affinities (such as quantifying the surface coverage of bound phages on an inorganic surface by immunofluorescence microscopy). Those experimental peptide sequences serve as the input data for defining of scoring matrices, which include similarities within strong-binding sequences and the differences between the strong- and weak- binders. New computationally designed peptide sequences have the potential to display enhanced specificity to a target material, depending on the accuracy of the computational prediction.
For the commercial 12-mer random peptide used in this dissertation, the diversity of peptide sequences, $10^9$ sequences, is only a small portion of all possible sequences based on probability theory, $20^{12}$ sequences. Thus, some possible binding peptide sequences may not be obtained from the commercial phage display library. Thus, we may consider extending the diversity of expressed peptide sequences by genetically modifying phage libraries for selecting more experimental binding peptide sequences. This could also provide more input data in bioinformatics, where the prediction accuracy of newly designed peptides can be enhanced further.

For understanding the binding mechanism of phage clones with expressed inorganic binding peptides on an inorganic surface, it is necessary to determine if the binding of phages to the inorganic surface is via the expressed inorganic binding peptides or the phage body. This can be done using quartz crystal microbalance-dissipation (QCM-D) with the aid of AFM image analysis. For the electroactive peptides project, releasing kinetics could also be explored for comparison among the different reversibly binding clones using QCM-D or surface plasmon resonance (SPR). In addition, it would be necessary to determine if the surrounding phage coat proteins and overall mass of phage particles impacts the releasing characteristics by comparing phage clones containing the genetically expressed targeted peptides with chemically synthesized peptides of corresponding amino acid sequences. In summary, the biotechnology approach of phage display can be applied to these types of advanced materials applications, or can even enhance the productivity of commodity industries, such as the mining industry demonstrated in this work.
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BIOGRAPHICAL SKETCH

Chih-Wei Liao was born in Yilan, Taiwan in 1977. His family is a standard nuclear family: father, mother, younger sisters, and he. His parents strongly emphasized the importance of education even if they just had the degree of elementary school. Thus, his parents always encourage him to pursue his own dream through a good education. He pursued his master’s degree in the Department of Applied Chemistry, National Chiao Tung University, Taiwan, from 2000 to 2002, his research mainly focus the synthesis of polyfluorene with blue emitting light and construct the devices for polymer light emitting diodes (PLED). After getting his master’s degree, he went to military for one and half a year for finishing his obligation as a Taiwan citizen. Then, he served as research assistant in the center for condensed mater science, National Taiwan University one year. In 2005, he decided to go to the United States for pursuing his Ph.D.

In the United States, his major was materials science and engineering at University of Florida. His specialty was to utilize the molecular recognition of peptides based on phage display techniques in the application of electro-activated reversible biosensors and the specific separation of mineral particles under Dr. Laurie Gower’ group. He believes that people have the right to live with the high quality of health. He expects himself to apply his specialties to develop the technologies for improving people’s life quality as his life goal.