

Molecular Biomimetics: Linking Polypeptides to Inorganic Structures

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Candan Tamerler and Mehmet Sarikaya

Abstract

In developing novel materials, Mother Nature gave us enormous inspiration with its already existing highly organized structures varying from macro to nano- and molecular scales. Biological hard tissues are the examples of composite hybrid materials having both inorganic and organic phases that exhibit excellent physical properties, all based on their evolved architectural design. Biocomposites incorporate both structural macromolecules, such as proteins, lipids and polysaccharides and minerals, such as hydroxyapatite, silica, magnetite, and calcite. Among these, proteins are the most instrumental components for use in materials fabrication because of their molecular recognition, binding and self-assembly characteristics. Consequently, based on this premise, inorganic surface specific polypeptides could be a key in the molecular engineering of biomimetic materials. Peptides can now be selected by directed evolution, adapted from molecular biology, by using combinatorial peptide libraries, analogous to natural selection. Adapting genetic approaches further allow to redesign, modify or engineer the selected first generation peptides for their ultimate utilization in bionanotechnological applications as molecular erectors, couplers, growth modifiers and bracers.

Introduction

Mother Nature has provided a high degree of sophistication in materials and systems at the nanometer scale. Naturally occurring materials have remarkable functional properties derived from their highly organized structures from the molecular to the nano-, micro-, and macroscales, with intricate architectures (Fig. 8.1). They are self-directed in their organization and formation, operate in water environment, dynamic in their interaction with the surroundings, complex in their structures and functions self-healing in damage control. Yet, they are not achievable in purely synthetic systems under the same efficient energy conserving, no waste delivering manner (Lowenstam, 1989; Sarikaya, 1999; Ball, 2001; Sanchez *et al.*, 2005). With the integration of recent developments in molecular and nanoscale engineering in physical sciences, and the advances in molecular biology, materials fabrication through biology, biomimetics, is now entering the molecular scale (Sarikaya *et al.*, 1995; 2003). Utilizing closely controlled molecular, nano- and micro-structures through molecular recognition, templating and self assembling properties of Nature, molecular biomimetics is evolving from the true marriage of physical and biological sciences (Niemeyer, 2001; Sarikaya *et al.*, 2004).

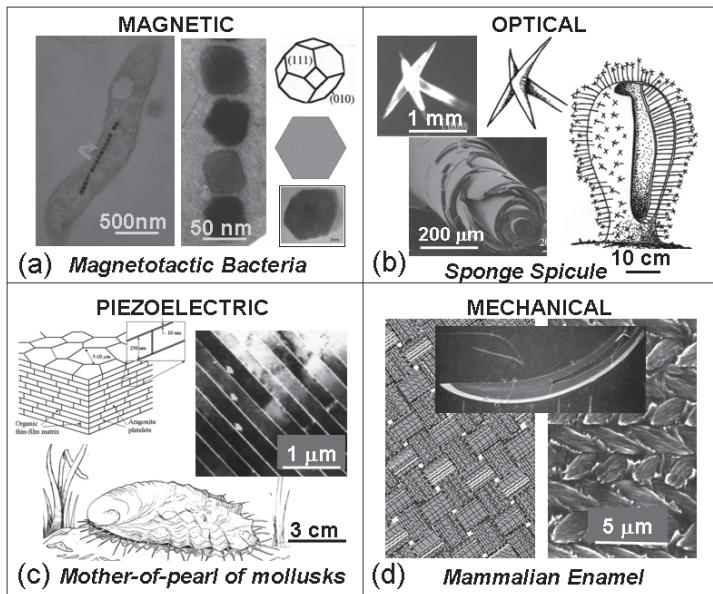


Figure 8.1 Examples of biologically fabricated complex nanomaterials. (A) Layered nanocomposite: growth edge of nacre (pearl) of abalone (*Haliotis rufescens*): Aragonite platelets separated by a thin-film of organic matrix. (B) Nanomagnetics: magnetite (Fe_3O_4) particles in magnetotactic bacteria: *Aquaspirillum magnetotacticum*. (C) Hierarchical structure: 3D woven enamel rods of hydroxyapatite crystallites of mouse teeth. (D) Biofiber-optics: a layered siliceous spicular optical fiber of a sponge (Rosella) and its apex (inset), novel design of a lens, a light collector.

Biological hard tissues are the examples of composite hybrid materials having both inorganic and organic phases and exhibiting excellent physical properties thereby creating ecological intakes for the host organisms (Mann 1996; Mann *et al.*, 1998; Ball, 2001). Biocomposites have incorporated both structural macromolecules such as proteins, lipids and polysaccharides and minerals, such as hydroxyapatite, silica, magnetite, and calcite (Berman *et al.*, 1988; Ratner *et al.*, 1996; Cha *et al.*, 1999; Mayer *et al.*, 2002). Among these, proteins are the most promising molecules because of their recognition, binding and self assembly characteristics. The advantage of a molecular biomimetic approach to nanotechnology, therefore, is that inorganic surface-specific proteins could be used as couplers, growth initiators and

modifiers, brazers and molecular erector sets, for self assembly of materials with controlled organization and desired functions. The realization of heterofunctional nanostructure materials and systems could be at three levels, all occurring simultaneously feed back each other as the Mother Nature produces her materials and components. The *first* is that the inorganic specific peptides are identified and peptide/protein templates are designed at the molecular level through directed evolution using the tools of molecular biology. This ensures the molecular-scale up processing for nanostructural control at the lowest dimensional scale possible. The *second* is that these peptide building blocks can be further engineered to tailor their recognition and assembly properties similar to the Nature's way of successive cycles of

mutation and generation can lead to progeny with improved features eventually for their utilization as couplers or *molecular erector sets* to join synthetic entities, including nanoparticles, functional polymers, or other nanostructures onto molecular templates (molecular and nanoscale recognition). Finally, the *third* is that the biological molecules self- and coassemble into ordered nanostructures. This ensures an energy efficient robust assembly process for achieving complex nano-, and possibly hierarchical-structures, similar to those found in Nature (self-assembly) (Sarikaya *et al.*, 2004).

There are different ways to obtain the inorganic surface specific proteins such as extraction from hard tissue, designing them via theoretical approaches or utilizing the limited number of already existing ones (Carlolou *et al.*, 1988; Paine *et al.*, 1996; Schneider *et al.*, 1998; Kroger *et al.*, 1999; Cha *et al.*, 1999; Liou *et al.*, 2000). Each of these approaches has its own major limitations and may not be practical enough to serve in all nanoscale-engineering applications. Inorganic surface specific peptides could be the key in the molecular engineering of bioinspired materials. However, there are only a few polypeptides have been identified that specifically bind to the inorganics. With the recent developments in recombinant DNA technology, these inorganic surface specific proteins can now be designed, modified or engineered for the production of nanostructured materials. During the last decades, combinatorial biology based molecular library systems have been developed for selecting substrate-specific peptide units, mostly for medical applications but only recently they are applied for selecting short peptides for inorganic surfaces (Brown, 1997; Whaley *et al.*, 2000; Gaskin *et al.*, 2000; Naik *et al.*, 2002; Sarikaya *et al.*, 2004). In these library sys-

tems, polypeptides are the major displayed molecules, which can be screened for the specific properties.

In the following sections, we provide an overview of molecular biomimetics approaches to achieve the premises of nanotechnology and summarize its potentials and limitations. Then, we look into the ways finding polypeptides that recognize inorganics, and describe the protocols of combinatorial biology for identifying, characterizing and engineering peptides to utilize them as molecular buildings blocks of future bimimetic materials and systems. Here we emphasize on the cell surface and phage display technologies that are well adapted for the identification of inorganic surface specific peptides, and to further tailor the characterized peptides using post-selection engineering. We then discuss the possible mechanisms through which a given protein might selectively bind to an inorganic based on their thoroughly binding characterization. We present examples of current achievements in utilizing engineered polypeptides are given to demonstrate their potential use and, finally, we present future prospects of molecular biomimetics in bio- to nanotechnologies.

Potentials and limitations of nanotechnology

The fundamental premise in the field of nanotechnology has been that the length scales, which characterize materials structure and organization, predominantly determine their physical properties (Drexler, 1992; Schmid, 1994; Ferry *et al.*, 1997; Katz *et al.*, 2004). Mechanical properties of nanocomposites, light harvesting properties of nanocrystals, stain defender properties of nanoparticles, magnetic properties of single-domain particles, barrier properties of nanoclays to extend the shelf lifes of bottles, and solution properties of col-

loidal suspensions are all examples to show that nanotechnology is not a futuristic technology, it is already establishing its place in our daily life (Jackson *et al.*, 2002; Shipway *et al.*, 2001; Hoenlein *et al.*, 2003; Thayer *et al.*, 2004). All of the given examples correlate directly to the nanometer-scale structures that characterize these systems. In building the nanometer scale structures, the approach is to design molecule by molecule with a purpose such as developing an advanced nanoscale machine or assembler or fabricator. Once, the materials are at the nanoscale then they present unique characteristics based on physical phenomena, and therefore, the physical and chemical rules governing the macroscale materials might not reflect their displayed properties (Ferry *et al.*, 1997; Muller, 2001). Recent experimental research in the field of nanometer-scale electronics and photonics has confirmed theoretical predictions in molecular and nanometer-scale structures, e.g. organized quantum dots, and electrical transport in nanotubes and wires. In addition, colloidal particles of metals, functional ceramics, and semiconductors have potentially useful electronic, optoelectronic and magnetic properties that derive from their small size. These properties may lead to their application as chemical, biological, and optical sensors, spectroscopic enhancers, nanoelectronics, and quantum structures, among others (Harris, 1999; Gittins *et al.*, 2000; Bachtold *et al.*, 2001; Huang *et al.*, 2001; McDonald *et al.*, 2005).

Successful integration of nanoscale materials to technology requires creation of millions of these structures in parallel (Glotzer, 2004). The conditions for controlled structures at nanometer scale can be obtained by promoting the self-assembly nature of the molecules through balancing kinetic and thermodynamic forces, yet this does not provide a specific geom-

etry or functionality. One way to overcome this problem is to combine “self-assembly” with more conventional “bottom-up technology” to provide suitable functionalities with specific structures. However, there are a number of challenges to be overcome such as if the structures available from “self-assembly” technique can provide functionality comparable to that realized by “bottom-up” process, or if the architectures can be built by the choice of material functionality rather than the availability of materials which are applicable to the system (Seeman *et al.*, 2002; Sarikaya *et al.*, 2004) Self-assembled layers are often demonstrated using thiol-derived molecules on gold or silanes on oxides. This is because the sulfur or hydroxyl atoms chemisorb to the gold or silica surface, respectively. This is advantageous for the gold- or silica-based architectures. However, more practical approach to multifunctional materials is to use substrates other than gold or silica. Availability of new materials will extend current technology with biosorption in addition to traditional chemisorption.

The realization of the full potential of nanotechnological systems has so far been limited because of the difficulties in their controlled-synthesis and the subsequent assembly into useful functional structures and devices. Most traditional approaches to synthesis of nanoscale materials are energy inefficient, require stringent synthesis conditions, and often produce toxic byproducts. These techniques still use “top-down” approaches, and even the most advanced microtechnology and recently developed nanotechnology, such as self-assembly through chemistry, in-jet technology, dip-pen lithography, and microcontact printing, require considerable external manipulation that curtail the achievement of complex 3D architectures and robust scale-up, and, hence, limit the potential

of nanoscale related physical properties (Gooding *et al.*, 2003; Quist *et al.*, 2005). Furthermore, the quantities produced are small and the resultant material is often highly irreproducible because of uncontrolled agglomeration. Even in the case of carbon nanotubes, one of the most successful nanotechnological materials, there are still some practical limitations to their widespread use, including uniformity, and control of surface chemistry, and for two- and three-dimensional assembly (Harris, 1999; Hoenlein *et al.*, 2003). Despite all the promise of science and technology at the nanoscale, the control of nanostructures and ordered assemblies of materials in two- and three-dimensions remains not fully accomplished.

Inspiration from Nature for realization of nanotechnology

Mother Nature has been an inspiration in fully achieving the promises of nanotechnology. The key to nanotechnology is primarily to understand how nature works at the highest level of sophistication with efficient energy use without waste accumulating way (Sarikaya, 1999; Ball, 2000; Seeman *et al.*, 2002). Biomaterials are highly organized from the molecular to the nano-, micro-, and the macroscales, often in a hierarchical manner with intricate nanoarchitectures that ultimately make up a myriad of different functional units, soft and hard tissues. Hard tissues such as bones, dental tissues, spicules, shells, bacterial nanoparticles can be given as examples which all have one or more protein based components (Carilolou *et al.*, 1988; Berman *et al.*, 1988; Schultze *et al.*, 1992; Kaplan *et al.*, 1994; Paine *et al.*, 1996; Fallini *et al.*, 1996). The inorganic part could be the magnetite (Fe_3O_4) nanoparticules in the case of magnetotactic bacteria to

sense the direction of gravity with many different morphologies depending on the type of species, or it could be silica forming the sponge spicule to serve as light collector, or hydroxyapatite crystals in enamel providing 3D woven enamel rod structure or the aragonite platelets in abalone shell to present the microarchitecture (Fig. 8.1) (Fong *et al.*, 2000; Sarikaya *et al.*, 2001; 2003; 2004). They are simultaneously self-organized, dynamic, complex, self-healing, and multifunctional, and have characteristics difficult to achieve in purely synthetic systems even with the recently developed bottom up processes. Based on their closely controlled nanostructures achieved through molecular recognition, templating, and self-assembly, biological materials have properties of technological interest that surpass synthetic systems with similar phase compositions. Under genetic control of the organisms, biological tissues are synthesized in aqueous environments in mild physiological conditions using biomacromolecules, primarily proteins but also carbohydrates and lipids. Proteins both collect and transport raw materials, and consistently and uniformly self- and coassemble subunits into short- and long-range ordered nuclei and substrates. Whether in controlling tissue formation, participation in its formation, or being integral part of the tissue in its biological functions and physical performance, proteins are indispensable part of the biological structures and systems. A simple conclusion is that any future biomimetic system, whether for biotechnology or nanotechnology, should include protein(s) in its assembly and, perhaps, in its final structure.

Engineering materials, containing one of more phases, are synthesized via a combination of approaches using, for example, melting and solidification processes that are often followed by thermomechanical

treatments, or solution/vacuum deposition and growth processes and finally annealing. Chemical recognition and synthetic self-assembly processes are a step beyond these traditional approaches. Many examples have been shown in the last decade showing these processes can produce highly ordered and predictable structures, including, for example, mesoporous systems based on surfactant/ceramic precursor molecules; self-assembled monolayers, and hybrid macromolecules. In many cases, however, the final product is a result of a balance of interactions, dictated by the kinetics and thermodynamics of the system, that are often achieved through "heat-and-beat" approaches of traditional materials science and engineering (Sarikaya *et al.*, 1982; DeGarmo *et al.*, 1988). In biological systems, the same balance is achieved through evolutionary selection processes that result in the emergence of a specific molecular recognition. For example, in antigen/antibody interactions, lock-and-key is one of the main mechanisms by which two molecules specifically recognize each other. In the new field of molecular biomimetics, hybrid materials could be assembled from the molecular level using the recognition properties of the proteins that specifically bind to the inorganics. Using the peptide based molecular approaches, new generation of binding agents, couplers, or molecular erector sets could be designed for self assembly of materials with controlled organization and specific functions. The current state of protein folding prediction and surface binding chemistry do not provide sufficiently detailed information to perform rational design of these hierarchical structures. To circumvent this problem, massive libraries of randomly generated peptides can be screened for binding activity to inorganic surfaces via the use of phage and cell surface display techniques. In either case,

it may ultimately be possible to construct a "molecular erector set" in which different types of proteins, each designed to bind to a specific inorganic surface, could assemble into intricate, hybrid structures composed of inorganics and proteins. Below we demonstrate the general approaches with some examples.

Combinatorial biology approach in selecting inorganic-specific peptides

Combinatorial strategies in chemistry and biology have attracted great interest to search and generate active molecular compounds for various applications over the last decades. The discovery of the counter parting active molecule from a series or a large number of mixtures has been revolutionizing idea brought by the combinatorial methods. First, combinatorial chemistry-based methods started with large peptide libraries following the establishment of solid phase synthesis of peptides. Optimization and rapid development of parallel syntheses and automation resulted in screening large number of compounds for a particular pharmaceutically interesting property such as increasing selectivity, activity or lowering toxicity (Beck-Sickinger *et al.*, 2002). Later, the integration of combinatorial methods into biological selection strategies have brought advantages over the chemistry based ones since they utilize the production capacities of the living systems, phages and cells. Over the last 10 years, various methods using organisms have been established to produce large libraries of peptides, proteins or nucleic acids. These libraries were generated both *in vivo* environments where organisms such as cells and viruses have been utilized, and *in vitro* environments where biological molecules key to synthesis, have

been added into the reaction mixtures. The generated molecules are directed towards a certain target interaction where they are enriched and identified according to their desired property.

The idea of searching for its own active molecule is not new considering biological interactions, such as receptors on cell surfaces recognize their ligands among many different molecules or antibodies detect the certain fragments of bacterial or viral surface proteins. These high-throughput strategies are only bringing us closer to Mother Nature's ways in developing new molecular tools. Nature's building blocks indeed are based on simple elements; ribonucleic acid (RNA) or deoxyribonucleic acid (DNA) are formed through the polymerization of four different nucleotides and peptides or proteins are formed through the condensation reaction of 20 different natural amino acids. For a nucleic acid that is 300 base pair in length or composed of 100 codons, there would be a possibility of translating into 20^{100} different proteins. In combinatorial biology based library systems, polypeptides have been the major biological compounds because of their very efficient recognition properties at the nanometer scales. In this section, we will go over the basic principles of display technologies and discuss phage and cell surface display methods in detail, starting from their use as a tool for protein–protein interactions, then their adaptation for protein–inorganics interaction over viewing both advantages and drawbacks of the each display system.

Principle of display technologies

Display technologies, in general as a routine tool, refers to a collection of methods for creating libraries of biomolecules that can be screened for desired or novel properties, by optimizing the assembly of

building blocks with more diverse function. Since the invention of phage display nearly two decades ago, display technologies have proven to be an extraordinarily powerful tool for a various biotechnological and biological applications (Smith, 1985; Dani, 2001; Benhar, 2001; Ma *et al.*, 2001; Mrskich, 2002; Wernerus, 2004). Mainly protein–protein interactions were studied in a variety of contexts including characterization of receptor and antibody binding sites, ligand specificities, and the isolation and evolution of proteins or enzymes exhibiting improved or otherwise altered binding characteristics for their ligands.

Biological libraries composed of peptides, antibodies, or proteins can be displayed by using either *in vivo* or *in vitro* display technologies. Regardless of the display technologies, there are three major components in the system: displayed molecule, its genetic code and a common linker to the displayed system. Following the interaction of the library with the counterpart molecule, high-throughput selection of the desired molecules with the possible specificity and affinity towards the counterpart molecule is carried out. All technologies are based on the common theme of cloned gene and its encoded protein is physically linked; therefore, the genetic information translating to the protein with the desired phenotypic character could be accessed easily. In *in vivo* display, stable genes are expressed either through transfection or introduction of foreign DNA into the cells, whereas *in vitro* systems cell free extracts will transcribe the cloned template (Hoess, 2001, Samuelson, 2002). Consequently, *in vitro* systems are not limited by the transformation efficiency of a cellular host (Dower *et al.*, 2002; Lipovsek *et al.*, 2004). In *in vivo* display technologies, biological host can be phage, such as very well established filamentous bacteriophage

M13, or alternative ones as λ , T4 or T7 phage, or cells, including prokaryotes and eukaryotes. In phage display, the coat protein genes are used to display the molecular library, whereas cell wall or periplasmic display systems are successfully applied to expression of molecular libraries. Outer membrane proteins, lipoproteins, fimbria, and flagellar proteins can be used for heterologous surface display on bacteria. With the data from human genome project, now determining the functions of the proteins is an important task especially for diagnosis and therapeutics. Mammalian cell surface expressions of receptor or transmembrane proteins have been attempted for being displayed on the current systems, however low efficiency of cloned gene delivery is a major problem. Many viral expression-cloning systems have been still continually searched for displaying mammalian proteins interactions in their *in vivo* environment. Among eukaryotic cells, yeast two hybrid systems have been very promising for expressing eukaryotic proteins (Ueda, 2004). However all of these systems still carry the limitations living cells such as rather restricted library size or suppression of certain mutant by the molecular machinery of the host or even sometimes they are not correctly folded or transported not contributing to the library diversity. Ribosome, mRNA and DNA display technologies are developed as cell free protein synthesis systems to overcome the limitations brought by the transformation efficiency of a cellular host, therefore they can be operated in the absence of a living cell (Fitzgerald, 2000; Amstatz *et al.*, 2001). Consequently, they can present high library size and also unique applicability to directed evolution of proteins, since selection with these systems can also be performed under the biologically in-

compatible environments (Takahashi, *et al.*, 2003).

Phage display

Phage display has been the most commonly practiced combinatorial peptide library display method since Smith (1985) first showed the linkage between phenotype and genotype in filamentous bacteriophage. In phage display, the majority of the research has been performed mainly by bacteriophages M13, fd and f1 which are closely related to each other, with 98% sequence identity. These phages require fertility plasmid (F-plasmid) in bacteria for infection and are called Ff type phages. Phage has two main components, genome and the protein coat composed of several different proteins. They are commonly used as vectors in recombinant DNA applications. Common feature of vectors is to accommodate the foreign DNA so that when they replicate within the host, foreign insert also replicates for the production of desired molecule. Some of the phage coat proteins tolerate additional domains coding for different biomolecules (e.g. antibodies, small peptides or proteins) at specific sequence positions (Kay *et al.*, 1996; Beck-Sickinger *et al.*, 2002). In general, DNA fragments coding for the random library population are ligated into phage genome such that the encoded biomolecule is expressed as a fusion product to the coat protein permitting the addition of these new segment. Once the recombinant phage DNA is introduced into *E. coli* cells, heterogeneous mixture of recombinant phages will be produced, each displaying a different member of the library on their surface (Benhar, 2001). Once a phage with the desired phenotype is selected, DNA sequence of the specific insert will give the amino acid sequence of the peptide.

The most widely used display system has been based on *E. coli* specific filamentous phage, M13, mostly because its life cycle is studied in detail and relatively easy to work with. It is in a flexible rod shape with helically arranged molecules about 1 μm in length but less than 10 nm in diameter (Fig. 8.2). A coat composed of five different proteins surrounds its single stranded DNA core. Four of the coat proteins are present at about five copies per phage, P7 and P9 cap one end of the virion while P3 and P6 cap the other end. Fifth one is present at \sim 2700 copies per phage and covers the length of the phage. Among all the coat proteins, P3 is the largest (42 kD) and the most complex one. It is responsible for host recognition and infection. P3 has three distinct domains connected by glycine rich linker regions. First domain is N-terminal

domain which initiates translocation of the viral DNA into *E. coli* during infection through binding to To1A receptor while the second domain confers host cell recognition by binding to the tip of F-pilus of the *E. coli* surfaces, finally C-terminal domain interacts with the other coat proteins for structural integrity, an important factor in viral morphogenesis (Riechmann *et al.*, 1997; Rodi *et al.*, 1999; Sidhu, 2001; Hoess, 2001). In display of proteins, P3 has been the primary scaffolds (Fig. 8.2), although P7 and P9 have been shown to tolerate fusion on their amino terminal and P6 has been shown to display fusions at its C terminus. M13 genome is single stranded however it is converted to double stranded plasmid like replicative form (RF) following the infection of its host to serve as template for the production of vi-

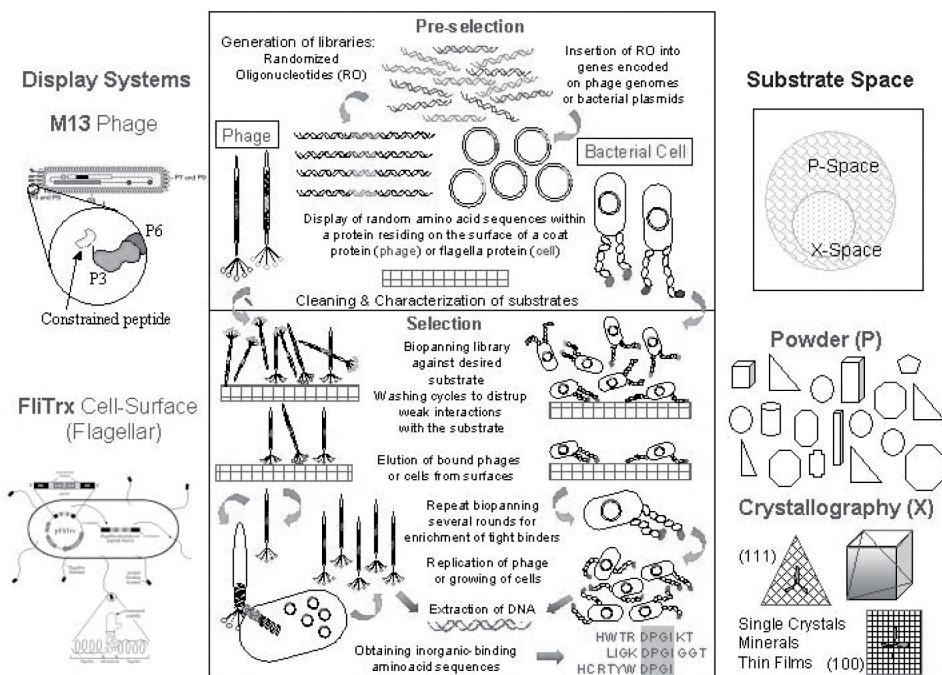


Figure 8.2 A schematic illustration of phage display and cell surface (flagellar) protocols adapted from molecular biology in this research for selecting polypeptide sequences with binding affinity to inorganic substrates and the substrate domains screened during the screen.

ral proteins and the single stranded DNA progeny. Phage progeny is assembled by encapsulating single stranded DNA with the viral coat proteins and extruded from the host cell into the medium. When the phages reproduce themselves gene fusion products pass along their properties to next generation (Benhar, 2001; Hoess, 2001; Sidhu, 2001). Phagemid vectors are also commonly applied in phage display technologies. Phagemids are hybrids of phage and plasmid vectors and they are designed to contain the origins of replications both for M13 and *E. coli*. In addition to gene III, they contain appropriate multiple cloning sites and an antibiotic resistance gene however, they lack all the other gene products required for generating a complete phage. Therefore they can be grown as plasmids in *E. coli* or alternatively coinfection with a helper phage results in the production of recombinant phage (Kay *et al.*, 1996).

In the construction of libraries, either all copies of the phage coat protein contain the insert (polyvalent display) or only a fraction of the coat protein contains the insert (monovalent display). In the first type of display, the foreign DNA is inserted into the phage chromosome eg. to encode a single type of P3 protein, therefore the insert is displayed in all of the five copies of P3 molecule. Whereas in the second type of display, one has to either include a second gene for the protein or use phagemid vectors to incorporate with the virus during morphogenesis. If the insert carrying gene and the native gene are both in phage genome, then the phage genome encodes for two different types of P3 molecule as being recombinant and wildtype. The insert carrying gene and the native gene are contained in a phagemid and in a helper phage, respectively. Phagemid carries both the plasmid and the filamentous phage

replication origin to replicate in host cell and when the host cell infected with the helper phage, phage replication protein acts both on the phagemid and the helper phage DNA (Smith *et al.*, 1997; Rodi *et al.*, 2002).

Cell surface display

Cell surface has been developed as an alternative to phage display only over a decade ago to display peptides and proteins on the surface of microbial cells. First examples of cell surface display of foreign proteins were reported in 1986 (Charbit *et al.*, 1986; Freudl *et al.*, 1986) and since then it has been utilized for various biotechnological and biological applications. The size of foreign protein to be displayed on the surface of the phage is rather limited; however, cell walls or periplasmic membranes can be utilized for fusion of larger proteins. Early examples of this approach were the display of gene fusion products on the outer surface of recombinant *E. coli* by insertion of short gene fragments into the outer membrane proteins LamB, OmpA and PhoE. (Charbit *et al.*, 1986; Freudl *et al.*, 1986, Agterberg, 1987, Stahl *et al.*, 1997). In addition to outer membrane proteins, fimbria and flagella proteins, lipoproteins have been applied for surface display on both Gram-positive and Gram-negative bacteria and yeast (Fig. 8.2) (Lu *et al.*, 1995; Stahl *et al.*, 1997; Scembri *et al.*, 1999; Wittrup, 2001). As a general principle, the displayed peptides or proteins (the target protein) are fused with anchoring motif (carried protein). Fusion mechanism such as N-terminal, C-terminal and the sandwich fusions, is one of the most important factors affecting the efficiency and the stability of the display (Beck-Sickinger *et al.*, 2002). First step is to identify the best available fusion site, which will allow the protein to be

displayed facing the surrounding environment, the target. Each anchoring motif will have a different characteristic, which can be utilized for specific applications such as membrane proteins can be useful for immunostimulation purposes. The next important parameter is the displayed protein, its amino acid sequence, transportation through different locations in the host cell and its folding structure. Next is the host cell as its easiness to work with, compatibility with the displayed figures. Among Gram-negative bacteria, *E. coli* is the common host system because of very well established genetic protocols, although its outer membrane is relatively fragile and it has high protease activities. Gram positive bacteria have more rigid cell wall and have only one membrane to achieve the secretion in contrast to two membranes existing in Gram negative ones. *Bacillus* has been the common host systems used in Gram-positive bacteria.

Among the microbial display methods, yeast display systems are promising and have many advantages over the bacteria mainly because yeast has a eukaryotic protein folding and secretion mechanisms. In yeast display, mammalian origin proteins can be displayed whereas it is rather hard to display them in phage or bacterial systems because of the lack of post-transcriptional modifications. Especially *Saccharomyces cerevisiae* strain is recently used increasingly for this purpose since it is safe and fermentation as well as genomic characters are very well known (Ueda, 2004). In general, microbial cell surface display has a wide range of biotechnological applications such as live bioadsorbents for bioremediation purposes, antibody production, whole cell biocatalysts, biosensor technology, live vaccine development, cell sorting etc (Samuelson *et al.*, 2002, Wernerus *et al.*, 2004, Feldhaus *et al.*, 2004).

Selection of peptides

Both phage and cell surface display rely on the use of chimaeric proteins that consist of a target sequence fused within (or to) a protein that naturally localizes on the surface of a bacteriophage (a bacterial virus) or a cell to achieve display. Using standard molecular biology techniques, the DNA sequence of the target region can be randomized to create a library of phages or cells, each of which will synthesize a different version of the chimera on its surface. Contacting the library with an immobilized ligand, washing out weak or non-binders and repeating the process to enrich for tight binders can select a subset of the original library exhibiting the ability to tightly interact with the desired ligand. This process is known as biopanning, which is an affinity, based method for a specific substrate. Generally in early rounds, low affinity binders can be caught if the selection is performed under mild conditions. In the later rounds, as the conditions gets harsher, tight binders gets recovered. Because the chimera is encoded within the phage genome or on a plasmid carried by the cell, the identity of the selected sequences (e.g. their amino acid compositions) can be deduced by DNA sequencing (Fig. 8.2) (Benhar, 2001).

In both of the selection processes, the stringency i.e. the degree of peptide's fitness, and the yield, i.e. the fraction of the surviving clones, are the two important parameters affecting the efficiency of the selection process. Ultimate aim is to have mild conditions in the early round to amplify the fittest clones, which may have a very low yield at the first round of selection. Whole optimization process during the rounds of selection will depend upon the relation between the stringency and the yield for the library population (Smith *et al.*, 1997; Hoess, 2001). Since the short peptide sequence might have difficulty to

fold, structural constraints can also be included in the encoded peptides. The simplest covalent constraint is the disulfide bond that becomes the nucleus of a microprotein. Bacterial disulfite bonds also form if the insert contains non-paired cysteine residues in random libraries because of dimerization through the formation of an intermolecular disulfide bond in solution.

Advantages and drawbacks of display technologies

Working with alternative display systems always helps to overcome the biases that might intrinsically exist in a particular display system, such as limited library size or expression problems. Therefore, it is necessary to integrate different display systems, which could be dictated by the character of the displaying molecule according to the desired application area. For example, yeast display has certain advantages for displaying proteins originating from mammalian cells; however this may also create false positives due to auto activating bait fusion proteins. In phage display, it is rather difficult to display mammalian or any other larger proteins; however this system can tolerate harsher conditions and a low copy number display can be easily achieved. Despite the easy applicability of phage display, phage requires bacterial cells for its amplification through infecting the cells. During the screening, some level of phage progeny might exhibit poor or no infectivity, which will result in their irretrievably lost from the library pool. Conversely, efficiently produced phages may yield a larger progeny and identical sequences may be selected at multiple occasions during subsequent rounds of biopanning (Hoess, 2001; Sarikaya *et al.*, 2004).

A critical parameter affecting the library size is the introduction of the library within a cell or a phage genome, since this

process can be limited by the transformation efficiency of the host. In general, *in vivo* display systems ($\sim 10^{10}$) contain smaller number of different library members than the *in vitro* display systems ($\sim 10^{15}$). However, a theoretical number of decapeptides that can be generated with the all possible permutations of the 20 amino acids is 20^{10} or about 10^{13} . This number or even higher size can be reached easily in *in vitro* display systems, but not in *in vivo* display systems. The greater numbers of individual members presented in the library will certainly increase the probability of identifying a high affinity and a specificity binder for the desired substrate. The functional expression of all the library members such as inefficient folding or expression biases associated with the living cells are among other factors affecting the library diversity (Sarikaya *et al.*, 2004). Major expression biases come from the fact that each amino acid is represented by different number of multiple codons, such that while leucine is specified by six codons, methionine is specified only by one codon. Consequently, there might be a great variation in the representation of each amino acid in the library, when the sequences are generated randomly. Another expression bias depends on the origin of the cells; the host system might synthesize different levels of transfer RNAs, which result in using certain codons preferentially. Transformation efficiency of the each codon might not be the same, then e.g. six codons specifying leucine might be represented by different percentage.

In spite of the above limitations, phage and cell surface display remain the techniques of choice in many applications because of their well established features, applicability, ease of use and commercial availability. One of the most extensively used system is the Ph.D. PD kit from New England Biolabs (Beverly, MA)

which relies on the exposure of a random hepta- or dodecapeptide on the surface of filamentous phage M13 by virtue of its fusion to the minor coat protein pIII (Fig. 8.2). Heptapeptide libraries are available in both linear and disulphite constrained forms. In the constrained library, a pair of cysteine residues forms a disulphite bond under oxidizing conditions and provides the display of the heptapeptide as a loop. The necessary items for construction of custom-made libraries are also available. The complexities of the libraries are at the level of two billion independent clones for a possible sequence space of 4×10^{15} (Parmley *et al.*, 1988; Smith *et al.*, 1993). In our group, we have selected the inorganic binding sequences via *in vivo* systems, includes M13 phage display system as well as cell surface display method utilizing both outer membrane and flagella protein based selection methods (Sarikaya *et al.*, 2004). In flagella display system, we have used FliTrx cell surface display system commercialized by Invitrogen Life Technologies (Carlsbad, CA). The library has random sequences of 12 amino acids as disulfide-constrained loops within Thioredoxin 1 (Trx), which is itself inserted into FliC, the major *E. coli* flagellar protein and it has an estimated diversity of about 1.8×10^8 (Lu *et al.*, 1995). The resulting fusion proteins are exported to the cell surface where they assemble into flagella, which are extended surface features used for cell motility. The display on flagella offers a unique feature as it provides the easy recovery of the bound cells following the physical breakage of the flagella from the substrate upon application shear stress such as vortexing (Fig. 8.2).

Application of phage and cell surface display methods to the selection of inorganic binding peptides

There has been a rapid accumulation of efforts on protein engineering towards im-

proved binding affinity and catalytic activity of existing proteins through directed evolution (Giver *et al.*, 1998; Petrouna *et al.*, 2000, Antikainen *et al.*, 2005). Nature uses unique characteristics that are desirable for material synthesis including high selectivity, nanoscale self-assembly, and precise structure control and follows the route of combining simple building blocks to form molecules with millions of different properties. Here, the molecular machinery is mostly based on the protein–material interactions and, therefore, proteins could be the biomolecules in the self assembly processes through their unique molecular recognition and binding capabilities. The premise is that, in addition to protein–protein interactions, polypeptides can recognize relatively simple and repetitive structure of inorganic surfaces and even short peptide sequences can discriminate between two closely related inorganic surfaces. These molecules, therefore, offer large variety of chemical and structural characteristics; with many advantages compared with the existing thiol and silane-based molecular systems that have been the hallmark of the self assembly in chemistry for the last two decades. With engineered polypeptides, there is now a possibility of producing millions of new potential linker molecules, each being specific to an inorganic surface and allowing fusion to create further tailoring for multifunctionality. With this in mind, molecular library systems have recently attracted great interest to generate molecular templates for diversified material synthesis and assembly. When we refer to combinatorial approaches, we mean the screening, synthesis, examination of millions of similar molecules, which are different in their compositions of amino acids and their arrangements. Here, the molecular templates can be generated for diversified material synthesis by altering the protein's properties. These systems allow

to form the self-assembly of nanostructure components with distinct functionalities especially in the areas of sensing, electronics and catalysis (Klaus *et al.*, 1999; Naik *et al.*, 2002, Seeman *et al.*, 2002, Koneracka *et al.*, 2002; Sarikaya *et al.*, 2003).

A genetically engineered polypeptide for inorganics (GEPI), selected through the display protocols as described in Fig. 8.2, normally defines a sequence of amino acids that specifically and selectively binds to an inorganic surface (Sarikaya *et al.*, 2004). The inorganic surface could be well defined, such as a single crystal or a nano-structure; it might be rough, or totally non-descriptive, such as a powder (Fig. 8.2). One of the first set of GEPIs selected were gold binding proteins that were isolated as extracellular loops of maltoporin which were subsequently fused to the amino terminus of the alkaline phosphatase with retention of gold-binding activity (Brown *et al.*, 2002). Most of our initial work focused on using the 14 amino-acid binding motif, MHGKTQATSGTIQS, called GBP-1. The motif does not contain cysteine that is known to form a covalent thiol linkage to gold, similar to thiolated molecules in self-assembled monolayers (SAM). To increase the binding activity, several repeats of the same sequence have been engineered and strong binding activity required at least three repeats.

Similar to the protocols we followed in our initial collaboration with Brown (2002), our recent research focused on using materials that could be synthesized in aqueous environments at ambient conditions (biocompatible) and could have fairly stable surface structures and compositions in water. These include noble metals (such as Pt and Pd), oxide and nitride semiconductors (e.g. Cu₂O, ZnO, GaN, TiO₂, etc.), minerals (such as mica, calcite, sapphire) or biocompatible substrates

(such as silica and hydroxyapatite) that were selected by using either phage display (specifically, M13) or cell surface display (specifically, flagellar display, FliTrx) (Fig. 8.3) (Sarikaya *et al.*, 2004). There are also a number sequences selected for various materials by other groups either via cell surface display, including, iron oxide (Brown, 1992), zeolite (Nygaard *et al.*, 2002), gold (Brown, 1997) and zinc oxide (Kjærgaard *et al.*, 2000), and phage display, including gallium arsenide (Whaley *et al.*, 2000), silica (Naik *et al.*, 2002a), silver (Naik *et al.*, 2002b), zinc sulfide (Lee *et al.*, 2002), calcite (Li *et al.*, 2002), and cadmium sulfide (Mao *et al.*, 2003). The applications of the selected binders varied from the assembly of inorganic particles (Whaley *et al.*, 2000; Lee *et al.*, 2002; Mao *et al.*, 2003) to control the nucleation of the compounds they were selected for (Brown *et al.*, 2002; Li *et al.*, 2002; Naik *et al.*, 2002a; 2002b).

Inorganic materials are very different substrates than proteinaceous or general biomolecular ligands and, therefore, adapting display technologies developed with biology in mind to the realm of materials science would require a new set of conditions and protocols although this has not been widely discussed in the literature so far. Inorganic compounds come in a variety of forms, from polydisperse and morphologically uncharacterized powders of various particle sizes to single crystals with crystallographically defined flat surfaces. The chemical or physical nature of the inorganic substrate may disqualify a particular display technology. For instance, phage display is suitable for work with powders even if a gradient centrifugation step is used to harvest complexes between binding phages and particles. On the other hand the FliTrx CSD system would not be amenable to such an enrichment process since centrifugal forces would shear off the

GEP1 – Genetically-Engineered Polypeptides for Inorganics: A Molecular Toolbox			
Phage Display Selected GEP1:			
Pt-Binders:	TiO₂-Binders:	Al₂O₃-Binders:	SiO₂-Binders:
PtBP1: DRTSTWRL	SK12: PWSFQTP	(001) AO1: RTTHQAY	DS202: RLNPPSQMDPPF
PtBP2: TSPGQKQ	SK28: FEVGPLH	AO17: ELRPTVA	DS189: QTWPPLWFSTS
PtBP3: IGSSLKP	SK 31: QHGMTRQ	AO23: HLHEPWL	DS 30: LTPHQTTMAHFL
Pd-Binders:	HA-Binders:	(100)	(001)
PdBP1: SAGRLSA	HABP42: LDTPSPH	A07: SYQFSHH	DS250: APRDNFNTVNLL
PdBP2: TLPNHTV	HABP18: PPTRHLL	A06: ETQNRPMM	DS256: MPAKEWLPKHNN
PdBP3: HTSKLGI	HABP17: RTPDNTS	A04: QPYNKLTT	DS 262: SVSVGMKPSPRP
Mica-Binders:	GaN-Binders:		
MBP25: SAPTLRQ	GNBP132: TAAFERNMKPLL		
MBP89: IQSGHPQ	GNBP133: GALGAKMLHRPS		
MBP30: RVHEHPH	GNBP136: HSSPHFSRHGLL		
Cell-Surface Display Selected GEP1:			
ZnO Binders:	Cu₂O Binders:	Au Binders (identified by S. Brown):	
CN122: LGSWGELLWQRQ	CN225: RHTDGLRRIAAR	GBP1: MHGKTQATSGTIQS	
CN173: YRDLLLRSYRKRW	CN85: RTRRQGGDVSRD	GBP2: ALVPTAHLRDGNM	
CN177: HYANSIWALASQ	CN44: NTVWRLNSSCGM	GBP3: LGQS GASLGSEKLTNG	

Figure 8.3 Examples of inorganic binding sequences selected via phage and cell surface display methods that could be utilized as molecular toolbox for the next generation of peptide based materials and systems.

flagella from the cell. Similarly, while both phage and cell surface display are theoretically suitable for panning on single crystals, tightly bound cells or phages may be very difficult to elute out from the material, thereby leading to the loss of high affinity clones. In such cases, the use of the FliTrx system may be advantageous since all binders have an equal likelihood to be recovered following flagellar breakage. Another important factor affecting the efficiency of the display system, and consequently the selection of the right binder is to follow the stability of the inorganic nature (Sarikaya *et al.*, 2004). Many materials rapidly develop an oxide layer on their surface, expose different crystallographic faces to the solvent, and may become chemically or physically modified when incubated in the biological media used during the panning process. To avoid becoming a victim of the first law of directed evolution, “you get what you screen for” (Schmidt-Dannert *et al.*, 1999); it is therefore imperative to characterize

inorganic surfaces before and after panning using various characterization techniques such as XPS, Raman spectroscopy and AFM (Dai *et al.*, 2004, Sarikaya *et al.*, 2004). It may also be useful to monitor wash or elution buffers (e.g. using atomic adsorption spectroscopy to detect metals and metalloids). If any evidence of surface modification or deterioration is obtained, buffer conditions should be optimized to guarantee compatibility with the target inorganic. Another important parameter is that inorganic compounds come in a variety of forms, from polydisperse powders to single crystals (Fig. 8.2). Peptides may adjust themselves to recognize different substrates and, a new binding sequence could be obtained for a new morphology dictated by the application area.

In traditional biological applications of peptide libraries (e.g. antibody epitope characterization, mapping of protein–protein contacts and the identification of peptide mimics of non-peptide ligands), 3–4

biopanning cycles are usually performed in PD while 4–5 are carried out in CSD. After these cycles of enrichment, the selected sequences typically converge towards a consensus consisting of identical sequences. Such consensus sequences reflect precise interactions between the side chains of the protein under study and those of the selected polypeptides. However, current experience indicates that this rule does not hold for the case of inorganic binding sequences where similarities rather than a strict consensus are generally observed. This presumably reflects the heterogeneity of the inorganic substrate at the atomic, crystallographic and morphological levels and the fact that there are probably multiple solutions to the problem of inorganic binding. One could, for example, envision binding strategies relying on shape complementarities, electrostatic or stereochemical interactions, van der Waal's interactions or various combinations of these mechanisms. Clearly, a better understanding of the rules that govern the binding of polypeptides to inorganic compounds is needed to understand the nature of specificity, predict cross-specificity and affinity and, ultimately, for the rational use of inorganic binding peptides for the design of hybrid materials exhibiting controlled chemistry, structure and organization.

Post selection engineering of inorganic binding peptides

Up to now, we have discussed how the inorganic surface specific polypeptides can be screened and identified using selection techniques based on display technologies. Genetic engineering techniques can be further applied to the selected peptides not only to investigate the mechanism of binding through identification of core amino acids and but also to create second gen-

eration of libraries. This is very similar to the evolution process where recursive cycle of mutation and selection resulting in the progeny with the improved features. The information obtained from e.g. phage display libraries can be integrated into successive generations of phage display libraries. In the recent years, this type of approach led to 10–100 fold improvements in binding affinities in biological entities such as mapping of binding sites of proteins, eg antigenic epitopes, or searching for inhibitors for a specific function (Forrer *et al.*, 1999; Schmidt-Bannert *et al.*, 1999; Petrouna *et al.*, 2000; Hoess, 2001).

The ultimate aim of the protein engineering is to enhance the desired property of the proteins such as stability and catalytic activity via different methodologies. While in traditional biotechnology, available proteins and other macromolecules with known functionalities are used via top down approach (rational engineering), in genetic engineering, proteins and peptides are manipulated to tailor their structures and functions via the bottom up approach (directed evolution). Directed evolution and the rational engineering have been the two main methodologies utilized greatly for the improvement of enzymes. In directed evolution, the variant libraries are created through random mutagenesis, which could be directed over the entire gene or on a relatively small region of the gene. Mutations could be introduced by DNA shuffling which mimics natural recombination or by error-prone PCR at random without significant sequence bias. Here, the important requirements are to be able to make the libraries complex and large enough to contain a mutant having the desired property and also develop a rapid screen or selection protocol, as in the case of peptide libraries. These approaches have been very useful in biotechnological appli-

cations where more stable, active and better performing enzymes needed such as working in non-aqueous solvents or extreme pH or temperature or accepting different substrates. However, through randomization and including the right pressure for selection, completely novel property possessing proteins are obtained via directed evolution (Forrer *et al.*, 1999; Hoess, 2001; Joern *et al.*, 2002). Whereas rational engineering or the top-down approach requires a knowledge-based study in which proteins are designed rationally using site-directed mutagenesis and generally coupled with computer aided molecular modeling to specify the possible substrate interaction locations. Site-directed mutagenesis, where each amino acid is substituted by another, could be an invaluable tool in studying the effect of individual amino acids and in redesigning the proteins. This method could be perfectly well coupled with the combinatorial methods, which together would cover large and complex interactions to obtain the engineered sequences with improved controlled properties. Second generation libraries offers the right platform for the convergence of these two main protein-engineering approaches to obtain the improved progeny for the fully realization of directed evolution similar to natural evolution.

Second generation of phage and/or cell surface display libraries, where the semirandom proposed alignments of peptides are designed for specific application purposes, could be also integrated in search for improved GEPIs. These libraries could be used to characterize the structural basis of binding by modifying the fragment expected to be responsible of binding. The main benefit of *second generation display libraries*; is the possibility of improved affinity and binding specificities of the already selected peptides. This has already been applied in

inorganic specific peptides by using cell surface display. Here, gold binding affinity of peptides was improved by constructing semirandom second generation peptide libraries expressed on the outer surface of *E. coli* as part of the maltodextrin porin, LamB. From the results of the earlier findings of two defined sequences contributing to gold binding were combined in the second generation libraries with repeating polypeptide populations to search for altered rate of gold appearance which in turn altered the morphology of the gold crystals. Genetic engineering tools can be further utilized for the generation of multiple repeats of the already engineered sequences to incorporate the structural properties of the inorganic surfaces, e.g. optimizing the length of the sequence or shape of the sequence to achieve the desired effect (Brown *et al.*, 2002). We have recently applied multiple-repeat based strategy on phage display-selected peptides, specifically, platinum binders. Based on the strong binding affinity of Pt-binders, examined via immunofluorescence microscopy, we genetically designed different number of repeats in both constrained and linear forms. Not surprisingly, depending on the number of repeats, or the molecular architecture of the peptides, each GEPI presented different affinity and selectivity for their substrates even if the basic amino acid sequence was the same in all (N. Gul-Karaguler *et al.*, unpublished results).

Understanding the nature of inorganic binding

In the design and assembly of functional inorganics, it is crucial to understand the nature of polypeptide recognition and binding onto inorganic materials for engineered utility of the GEPI. Although considerable research has been directed in the literature on how proteins recognize each

other or other biomacromolecules, it is not completely readily possible to design a peptide for desired functionality. Therefore, the studies on the understanding of the nature of peptides recognition of inorganic surfaces are in its infancy. The specificity of a protein for a surface may originate from both chemical (e.g. H-bonding, polarity and charge effects) and structural (stereochemistry, size and morphology) recognition mechanisms (Izrailev et al., 1997; Dai et al., 2000; Evans, 2003). These two key mechanisms may both be important and work collectively to create a complementarity and compatibility in molecular recognition followed by self assembly. Weak, non-covalent, chemical bonds can result in a stable structures, when many bonds act collectively. In addition, inorganic properties also affect binding as their surface could be well defined, such as a single crystal or a nanostructure or they might be rough and, or totally non-descriptive such as a powder. In the latter case, the sequence space is the largest as powders represent many possible topologies, sizes, and chemistries. Because of the availability and non-specificity of non-descriptive surfaces on powders, selected polypeptides, often have little or no consensus sequences. Even so, these selected (first generation) sequences may contain binding domains with similar functionalities. Binders selected for a given size, morphology, crystallography or stereochemistry may share a higher degree of homology. For example, a GEPI binding to a material of a certain size may also bind to a smaller particle of the same material, but less strongly. Similarly, a GEPI binding strongly to a specific crystallographic surface may bind with an altered affinity to another surface of the same material, e.g. (111) versus (100) of α -alumina (unpublished). Finally, a GEPI strongly binding to a material of composition A may bind less

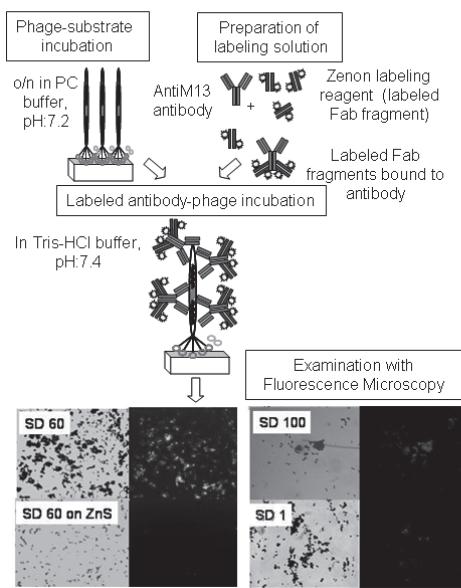
strongly to the material B with a different composition having similar structure (e.g. two different perovskite structures). Therefore, if one seeks highly specific binders, the physical and chemical characteristics of the material must be known. Binders selected for a specific size, morphology, crystallography or stereochemistry would constitute smaller number of binders.

Many sequences specific to different inorganics have been identified up to now via application of display technologies using molecular peptide libraries. In the next step, characterization and understanding of binding by examining both its chemical and physical basis providing the information for the evaluation of polypeptide-inorganic surface affinities and selectivities, essential for the realization of protein/inorganic hybrid materials (Niemeyer, 2001; Ball, 2001; Sakiyama et al., 2001; Sarikaya et al., 2003) As discussed below, surface assessment of inorganics could be carried out by suing both experimental and theoretical peptide/inorganic interactions characterization tools.

Experimental approaches to study the protein adsorption and binding

Among the experimental approaches to monitor protein adsorption on and binding to inorganics, fluorescence microscopy is a routine and practical tool as the first step in the semiquantitative evaluation of these sequences with respect to their affinity and selectivity (Fig. 8.4). Although the FM experiments are an essential part of the screening protocols, they do not provide quantitative adsorption and binding mechanism. In FM, immunofluorescence labeling detection using monoclonal antibody conjugated with secondary antibody fragments results in a powerful binding detection system (Brown, 1992; Whaley

Semi-quantitative approach of initial evaluation via FM



Quantitative approach on binding via QCM & SPR

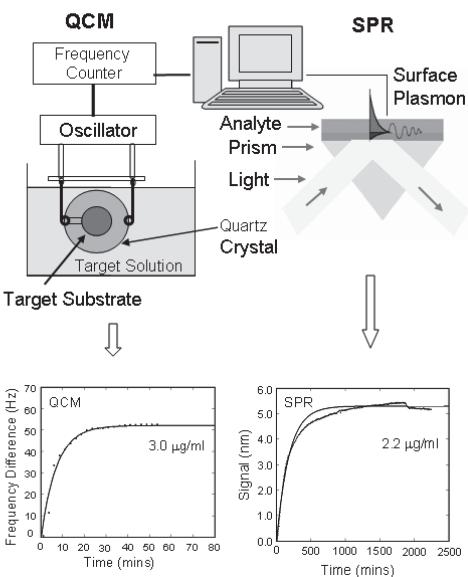


Figure 8.4 Examples of characterization tools for studying peptide binding. (A) Initial semiquantitative evaluation of binding affinity and specificity via immunolabelling fluorescent microscopy analysis. (B) The analysis of kinetics of binding via quartz crystal microbalance and surface plasmon resonance spectroscopy.

et al., 2000; Naik et al., 2002; Dai et al., 2004; Sarikaya et al., 2004). Scanning probe microscopy (SPM) protocols have been also integrated for inorganic sample surface preparation and peptide binding/self-assembly. Here the tip-design and engineering, observation conditions, data analysis and interpretations are all part of these new protocols to study specific polypeptide binding onto inorganic surfaces (Whitesides et al., 1991). While the SPM, specifically atomic force microscopy (AFM) and scanning tunnel microscopy (STM) techniques have been utilized to obtain structural information of inorganic binding, quartz crystal microbalance (QCM) and surface plasmon resonance (SPR) spectroscopy are used to quantitatively analyze protein adsorption kinetics and thermodynamics of binding and stability under various protein concentrations, solution properties such as pH, sa-

linity and surface conditions (Czenderna et al., 1984; Homola et al., 1999; Sarikaya et al., 2004). Conventional spectroscopy techniques, such as X-ray photoelectron spectroscopy (XPS) and time-of-flight-secondary ion mass spectroscopy (TOF-SIMS) techniques also recently proven to be viable techniques in providing the fingerprint of peptide adsorption onto the surfaces (Coen et al., 2001). Although, difficult and time-consuming, solid and liquid state nuclear magnetic resonance (NMR) spectroscopy supplies the essential experimental evidence of peptide molecular structures and provides clues towards understanding of mechanism of polypeptide binding on inorganics (Long et al., 2001; Evans et al., 2004). All of these techniques are being utilized in our on-going research, and some will be briefly discussed below as examples.

The QCM has been an established mechanical measurement tool in studying the adsorption of proteins on the metal surfaces (Murray *et al.*, 2000; Bailey *et al.*, 2002). Here we use it to evaluate the kinetics of binding of GBP on gold surface. In QCM, quartz crystal disk mounted with e.g. gold electrode, sense the resonance vibration behavior through the change of deposited molecular mass on the surface. When worked in air, QCM can accurately provide measurements even thick films although the viscosity and the thickness of the film could become important parameters in the case of liquids measurement.

In assessing quantitatively the nature of binding of GEPIs on inorganic surfaces, we have examined many of the selected binders as many materials can be coated on a quartz crystal electrode (Fig. 8.4). Our first example was the GEPI-Au interaction and, for this, we used several of the gold-binding polypeptide (GBP) series. Here, we demonstrated the adsorption behavior of GBP1 on gold (coated on quartz) as well as platinum (coated on quartz) by using QCM (M. Duman *et al.*, unpublished). We showed both the kinetics of adsorption as well as strength of binding, and thereby, demonstrated the substrate specificity-based kinetic parameters. The GBP-1 was found to be stronger binder and more stable on the gold surface compared with the platinum surface. Kinetic analysis of the experimental data followed Langmuir monolayer adsorption. A 20-fold differences between the equilibrium constants and fourfold frequency differences were obtained when GBP-1 bound to Au and Pt surfaces, respectively. We also examined other selected metal (e.g. Pd) and metal oxide (e.g. Cu₂O, ZnO, SiO₂) binders by QCM system, and found out their binding affinity and selectivity differences. Not only selected peptides can be

analyzed via QCM, but second generation engineered peptides can be also compared with the first generation peptides to assess their improved binding.

The QCM, being a mass detection system is a good tool for determination of kinetics of binding but it does not provide detailed information on the mechanism of physical adsorption. The SPR, the other hand, can provide more detailed adsorption data measuring very small amounts of molecular adsorption as this causes low level of refractive index changes due to the biological compounds bound on the sensor surface. Reflected light intensity from the interface between metal substrate and the analyte at a specific incident angle is measured as a result of the optical excitation of surface plasmon waves (Jung *et al.*, 1998). The shift in SPR wavelength is related to molecular adsorption on the metal substrate (which is either metallic Ag or Au). In our experiments, rapid assembly of GBP-1 onto sensor surface was observed followed by a sharp increase in SPR shift (Fig. 8.4). In the GBP-1 studies, we have observed two different regions as a result of the kinetic evaluation compared with QCM results. The SPR results suggest that following the binding of the GP-1 on the gold surface, reorganization of the molecules takes place towards a more stable and two-dimensional ordered molecular film formation. In fact, studying these self-assembled surfaces with AFM revealed ordered molecular domains formation of GBP-1 on atomically flat Au (111) surfaces. It is assumed that the monomolecular ordered GEPI formation on the inorganic substrate in this case is a result of a combination of forces involving intermolecular interaction as well as the lattice recognition of Au by GBP-1. To be able to study GEPI adsorption and as well as kinetic and thermodynamic parameters on selected surfac-

es other than Au and Au, we are presently developing a model for generating SPR signal from any surface (other metals, oxides, and semiconductors). This new approach will extend the applicability of SPR analysis of the peptide adsorption on any given interesting material and provide invaluable information not possible otherwise (L. Yee, unpublished).

Modeling tools on polypeptide-inorganic surface interactions

The experimentally determined binding information using the approaches discussed above combined with the simulation based structural results could give a coherent understanding of GEPI-inorganic surface interactions. These will include the characteristics of the side chains that are capable of interacting the surface atoms on various materials and crystallographies. Unfortunately, however, studies on molecular modeling of protein binding to inorganic surfaces have been very limited. Our first attempt to analyze this interaction was performed by gold binding protein in a collaborative work (Braun *et al.*, 2001). Different tandem repeats of gold binding peptide was compared with all known protein structures using FASTA searches and also traced if a particular secondary structure preference exists via Chou-Fasman and Holley/Karplus secondary structure prediction algorithms. Initial findings suggest that on Au{111} surface, GBP1 forms an anti-parallel β pleated sheet conformation. This constraint, places OH groups from serine and threonine residues into a regular lattice based on energy minimized in vacuo using X-PLOR. Again, based on these simulations, we also found that the binding of GBP-1 on {112} is not as it is on Au{111} surface because of the decoupling caused by the presence of water molecule that sweeps under the binding protein on

the substrate through atomic grooves intrinsically present on this {112} crystallographic surface.

We have also extended our preliminary computational approaches on understanding of molecular recognition of inorganic-binding polypeptides to phage display selected septapeptides with constrained molecular structure. Following their affinity characterization based on immunofluorescent microscopy to metallic platinum, we have investigated their crystallographic surface recognition by conformational analysis via HyperChem 7.5 molecular modeling system. We used CHARMM force field parameters and showed that the selected septapeptides conform into certain molecular architecture containing multiple protrusions that spatially match with the crystallographic metal surfaces (i.e. (111), (110), and (100)). While the physical recognition may originate from how well the molecular polypods spatially match a given crystallographic surface, the reactive groups on the protrusions through which the peptide binds to the inorganic surface dictates the strength of the binding. Our molecular modeling analysis is found to be consistent with the experimental binding characteristics of the platinum binders presenting different affinities (Oren *et al.*, 2005).

Conventional spectroscopic techniques along with molecular dynamics studies will shed more light into the chemical specificity of inorganic surface-specific polypeptides. Contribution of side chain of amino acids is appearing as one of the major interactions. Up until now among the studied noble metals, namely, gold, silver, platinum and palladium binding polypeptide sequences showed similarities in terms of conserved hydrophobicity and polarity. A similar trend was also observed in terms of conservation of small amino

acid molecules. These results might give us a perspective in terms of the existence of common binding domain for similar materials. The correlation between how functional groups are distributed within the polypeptide depending on the inorganic lattice structure or the topology of the substrate while conserving their binding domain would be a major contribution towards tailoring engineered linkers in many application areas.

Practical applications by utilizing GEPIs

Nanometer-sized particles and nanostructured inorganics are now fundamental building blocks for technological materials and devices both in nanotechnology and biotechnology as they are easy to prepare, store and manipulate. To make any nanoparticle significantly useful, in addition to its intrinsic properties (magnetic, photonic, semiconducting), it also needs to be chemically modified so that it can be assembled in an efficient and controlled manner and can be manipulated. This is possible if the physical and chemical characteristics of the particles can be controlled and assembly is directed. Therefore, numerous challenges must be addressed before nanoscience could be implemented successfully into working systems. Some of these challenges include synthesizing nanostructures (e.g. particles, rods, and tubes) with uniform size and shape, control their mineralogy, surface structures and chemistry, and organizing them in 2- and 3D with predictable spatial distribution. If biology is to be a guide, some of these challenges could be overcome utilizing the unique opportunities offered by the biomimetic approach at the molecular scale provided by the engineered inorganic binding polypeptides discussed herein.

We define molecular toolbox as a data bank containing fully characterized GEPIs that could be picked up and utilized for a wide range of applications. Just like what proteins can do in organisms, inorganic-binding polypeptides could also be used as molecular synthesizers, linkers, bracers, erectors, and assemblers in materials science and biotechnology. Furthermore, GEPI could also be useful when fused to another protein, macromolecule, or DNA-binding protein as a functional ligand. Alternatively, a GEPI could also be chemically conjugated, onto a synthetic polymer, to create multifunctional hybrid polymeric structures. When used in biological and synthetic macromolecules, GEPI's role is to create a heterofunctional polymer. The examples below give some areas of the diverse applications of inorganic binding peptides for materials engineering and biotechnology.

Morphogenesis of inorganics via engineered polypeptides

In biomineralization, a significant aspect of biological control over materials formation is via protein/inorganic interaction, such as in bone, dentin, mollusk shells, bacterial and algal particle formation. In traditional biomineralization, the studies in the search for the nature of proteins' affects on inorganic formation has traditionally focused on templating, nucleation, and enzymatic reactions. These studies were mostly carried out the protein(s) extracted, isolated, and purified from a given hard tissue and the effects were studied during the reconstruction of the specific inorganic(s) associated with it. Although instructive in pinpointing specific effects in biomineralization, i.e. mineral selection, habit modification, and enzymatic effects, these studies, besides being extremely slow in progress, have been

limited in their ability to study only a few inorganics that these hard tissues are associated with. Also, neither temporal nor spatial distributions of these proteins or their fragments have been quantitatively associated with the hierarchical architecture of the tissues.

With the emergence of combinatorial biology selected inorganic-proteins discussed herein, a natural first step is to examine how and which of these polypeptide sequences affect inorganic formation. Since done under controlled conditions, these studies could provide an opportunity to investigate various affects in mineralization, including nucleation, growth, morphogenesis, and enzymatic effects. These studies have necessarily been carried out under aqueous environments amenable for biological functions and show the potential use of inorganic binding polypeptides for biofabrication of a wide variety of material synthesis in biological environments. Here we demonstrate the first such study in which the morphology of gold particles were affected by GEPIs selected using the CSD route (Brown *et al.*, 2000) (similar studies were also carried out in Ag formation using GEPIs selected via the PhD route, Naik *et al.*, 2002). Nanogold (monosize, 120-Å diameter) particles can be formed at ambient conditions using the well-known Faraday's technique by reducing AuCl_3 by Na-citrate (or other reducing agents) (Turkovich, 1951). Reducing the gold concentration and temperature allow particle formation at a slower rate, giving the protein time to interact with surfaces during the growth and provides conditions to examine the effect of gold-binding during colloidal gold formation. In a collaborative study, one of us conducted a search for mutants that modulated the morphology of gold crystallites, i.e. the selection of mutants was based on the change of color of

the gold-colloid (from pale yellow to a red colloid) which was related to altered rate of crystallization. Fifty mutants were tested this way, and the sequence analysis showed that two separate mutants that accelerated the crystal growth also changed the particle morphology from cubo-octahedral (the usual shape of the gold particles under equilibrium growth conditions) to flat, triangular or pseudo-hexagonal, particles (Fig. 8.5a). This new observation is interesting from the point of enzymatic effect of protein in crystal growth rather than traditionally assumed templating effect. The polypeptides, in spite of being slightly basic, may have caused the formation of gold crystals similar to those formed in acidic condition. This suggests that the role of the polypeptides in gold crystallization is to act as an acid, a common mechanism in enzyme function.

Our second example focuses on silica, which exhibit diverse and extraordinarily designed shapes and structures in Nature from diatom skeletons to sponge spicules (Sarikaya, 1999). The proteins directing silica synthesis in biological systems have been studied extensively. For example, silaffins extracted from the cell wall of a diatoms *Cylindrotheca* (Kruger *et al.*, 2000) or silicatein extracted from the sponge spicules of *Terhyta aurantia* (Shimizu *et al.*, 1998) are very good examples of these proteins. Also, silica binding peptides also identified for the amorphous silica precipitate which was formed in the presence of the peptide unit of natural silicatein protein (Weaver *et al.*, 2003). In our research, we used the selected phage peptide clones and examined a silica precipitation assay. We found that the peptides that were rich in basic and hydroxyl amino acids exhibited more silica precipitating activity. We identified silica binding peptides via 12-amino acid phage display library on a single-crys-

Morphogenesis: Flat gold particles

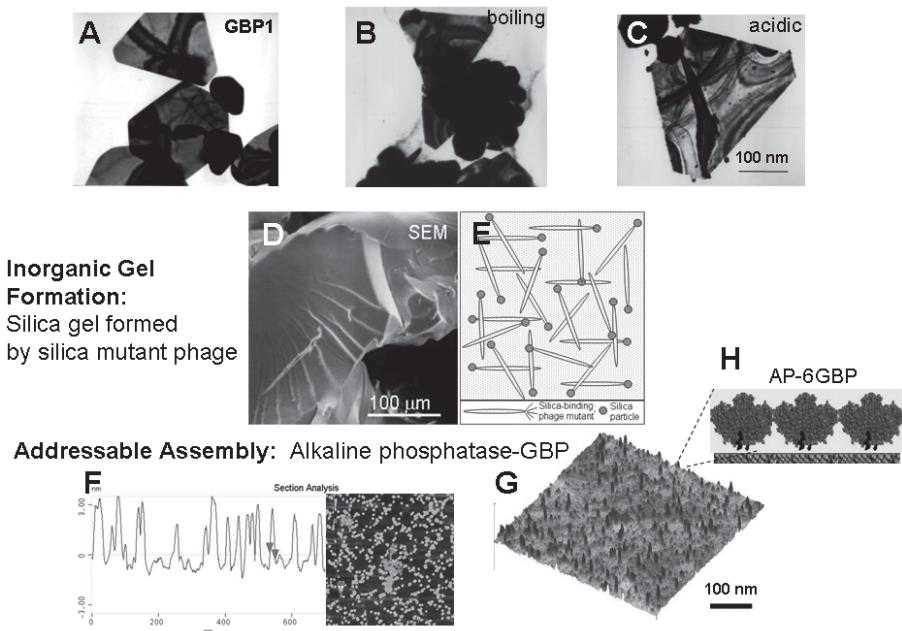


Figure 8.5 Examples of applications by combinatorial biology selected inorganic binding sequences. (A–C) Enzymatic effect of protein in crystal growth rather than traditionally-assumed templating effect, effect of gold binding polypeptides on the particle morphology. The effect of GBP (A), boiling (B), and acidic conditions (C). (D–E) Biosilica gel formation. An SEM image of the gel formed by silica-binding phage mutant and silica particles, a viable material (D). Schematics of the gel containing conjugates of phage mutant and silica particles in water (E). (F–H) Directed immobilization achieved via heterofunctionality introduced through genetic fusion of gold binding polypeptide. (F) Line height profile across a-a' shown in the AFM image. (G) 3D visualization of the AFM image. (H) Schematic illustration of AP-6GBP on gold.

tal silica (quartz) substrate and compared the differences between single crystal silica substrate and silicatein directed synthesized silica particles. We carried out five rounds of biopanning and 50 sequences were obtained presenting different affinities for silica. Binding characterization of the selected phage samples were examined by AFM, immunofluorescence microscopy experiments, and by SPR (D. Sahin *et al.*, unpublished). Some of the high silica-affinity presenting selected phage samples were then tested for their effect on silica synthesis.

For this, the samples containing 10^{13} phage forming units was incubated in

freshly prepared tetra methyl ortho silicate (TMOS) solution for various time intervals at room temperature. In the first process, silica gels were prepared using silicic acid precursors using alkoxides as a precursor in aqueous solutions using slightly acidic solutions. It was found that the time for gellation, in this case, depends on the pH of the precursor solution, in which this period being very long, sometimes days, depending on the pH value. In the presence of silica-binding phage particles, this gellation time is reduced to a less than an hour, even a few minutes depending on phage concentrations or the strength of the binding affinity of the inorganic-bind-

ing polypeptide. In addition, post-gellation processing, e.g. thin-film formation, can be controlled due to the opportunities provided for shaping and drying steps in the materialization procedures. The gellation of the TEOS alone takes about 30 h to fully gel while precursor/phage mixture can be readily shaped into thin film (tape casting, slip casting, screen printing) or cast into various architectural forms for specific and wide variety of applications (Fig. 8.5d). The solid material can be created through controlled drying and densification processes following the well-established, and simple, ceramic processing methodologies (D. Sahin et al, unpublished).

GEPI based directed immobilization

Gold binding polypeptides selected by cell surface display are one of the first examples of engineered polypeptides for inorganic surfaces. As discussed before, the cell surface displayed selected gold binding polypeptide sequences were screened via random peptide libraries expressed on the outer surface of *E. coli* as part of the malto-dextrin porin, LamB (Brown, 1997). Once they identified, GBPs were then fused to the amino terminus of the alkaline phosphatase (ALKP) with their retained gold binding activity. One of the identified sequences, GBP-1, has been studied in detail in our research group. Using the mutant strains of the bacteria supplied to us by Brown, we have incorporated further genetic engineering strategies for increasing binding affinity to gold surface via insertion of multiple repeats of GBP-1 into ALKP as 5 (5R-ALKP), 6 (6R-ALKP), 7 (7R-ALKP) and 9 (9R-ALKP). The idea here is to use the GBP-1 as molecular erector, and introduce mutifunctionality to the hybrid construct, i.e. enzymatic and inorganic binding simultaneously. ALKP con-

structs were produced by *E. coli* cultures by transforming the cells with the plasmid having the desired number of repeat units of gold binding polypeptide insert. By following the induction of the *E. coli* cultures, the constructs in the periplasmic fraction was isolated and concentrated fraction followed by purification using DEAE column chromatography. Subsequently, the eluted fractions showing ALKP activity was concentrated again and passed through gel filtration column. The enzymatic activity of ALKP was examined by monitoring the changes in the absorbance spectra of the solution containing the ALKP-GBP construct and p-nitrophenylphosphate as the substrate in an assay buffer in 500 mM Tris-Cl (pH:8,0), 1mM MgCl₂. To observe if enzyme is catalytically active in the presence of gold particles, we tested the ALKP-GBP constructs following an overnight incubation with gold particles at 37°C. The ALKP activity profile for both wildtype and recombinant ALKP with gold particles were shown no differences. ALKP-GBP constructs were also tested for stability of their fused gold binding polypeptide inserts in the main protein via SDS-PAGE gel electrophoresis. The gel analysis showed both the wildtype ALKP and the recombinant ALKP-GBP construct with an increased molecular weight corresponding to the number of repeat unit of GBP-1.

In the next step, we examined the possible use of gold binding polypeptide as a molecular linker when it is part of ALKP. Here, the protein solutions having either wildtype or ALKP-GBP were left for overnight binding in the presence of the gold substrate and then the substrate was taken out of solution and the remaining supernatant was checked for the ALKP catalytic activity. While the activity of the wildtype protein solution was retained fairly well, no

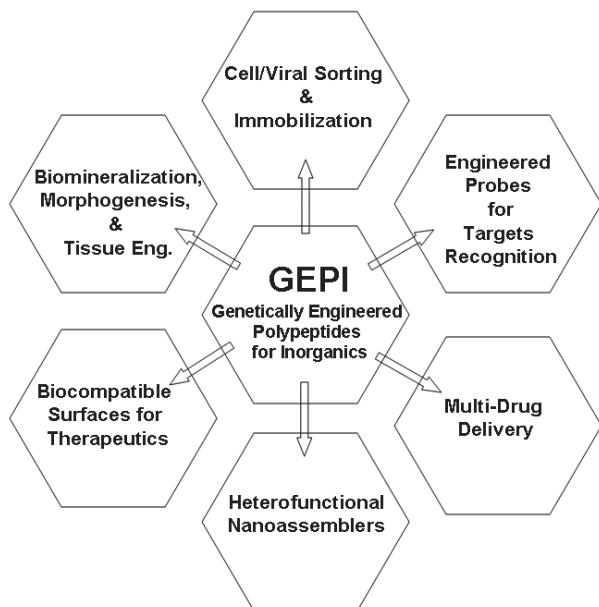


Figure 8.6 Potential Application areas of GEPI in the new field of molecular biomimetics.

activity was observed in the supernatant of the recombinant construct (Kacer *et al.*, unpublished observation). This means that most of the molecular construct was successfully immobilized on the gold substrate via the GBP. The result is significant from the point that that gold binding property of the recombinant alkaline phosphatase was retained simultaneously with the activity of ALKP, creating a portable sensor kit (Fig. 8.5f). Alkaline phosphatase is an essential enzyme for regulating (preventing or enhancing) biomineralization via the control of extracellular phosphate concentration by catalyzing the pyrophosphate degradation. For example, in the dental pulp, ALKP is responsible for dentin matrix formation. The ALKP-GBP bifunctional construct, therefore, could be promising probe especially in periodontal regeneration. In this application, the new construct provides excellent probing properties to follow the mechanism via the gold binding and enzymatic properties and, hence, may be utilized for providing a controlled

delivery of ALKP to the desired locations consequently promoting mineralization.

Our results showed that combinatorially selected polypeptides could be addressably immobilized and self-assembled on inorganic surfaces. Realizing the fact that chemical linkers, such as thiol and silane linkages are the other two major molecular linkers for noble metal and oxide (silica) surfaces, respectively, it is naturally expected that self assembled GEPI monolayers—SA(GEPI)M—as “molecular erector sets” will open up new avenues for designing and engineering new and novel functional surfaces for a wide variety of nano- and biotechnology applications, including chemical and biological sensors, nanobiotechnology, and proteomics.

Future prospects

For development of materials and systems architectures at molecular or nanoscale levels, proteins and polypeptides could be used as molecular erectors with controlled binding to and assembly on inorganics.

The GEPIs represent a class of genetically engineered polypeptides that can be tailored on technological demand through direction evolution for their affinity and specificity. The GEPIs could be utilized as part of hybrid molecular constructs (Fig. 8.6). The ordered assembly of GEPI on inorganic surfaces could have a significant impact on molecular biotechnology applications offering several novel immediate practical advantages. The attachment of biomolecules, in particular proteins, onto solid supports is fundamental in the development of advanced biosensors, bioreactors, affinity chromatographic separation materials, and many diagnostics such as those used in cancer therapeutics (Sarikaya *et al.*, 2004; Sanchez *et al.*, 2005). Protein adsorption and macromolecular interactions at solid surfaces play key roles in the performance of implants and hard-tissue engineering (Ranter *et al.*, 1996). Proteins adsorbed specifically onto probe substrates are used to build protein microarrays suitable for modern proteomics (Cutler, 2003; Chicurel *et al.*, 2002).

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