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MATERIALS ASSEMBLY AND FORMATION USING ENGINEERED POLYPEPTIDES

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■ **Abstract** Molecular biomimetics can be defined as mimicking function, synthesis, or structure of materials and systems at the molecular scale using biological pathways. Here, inorganic-binding polypeptides are used as molecular building blocks to control assembly and formation of functional inorganic and hybrid materials and systems for nano- and nanobiotechnology applications. These polypeptides are selected via phage or cell surface display technologies and modified by molecular biology to tailor their binding and multifunctionality properties. The potential of this approach in creating new materials systems with useful physical and biological properties is enormous. This mostly stems from molecular recognition and self-assembly characteristics of the polypeptides plus the added advantage of genetic manipulation of their composition and structure. In this review, we highlight the basic premises of molecular biomimetics, describe the approaches in selecting and engineering inorganic-binding polypeptides, and present examples of their utility as molecular linkers in current and future applications.

INTRODUCTION

The Promise of Nanotechnology and Current Limitations

It is now commonly recognized that at nanometer-scale dimensions materials have unique functional properties that can lead to novel engineering systems with highly useful characteristics (1–4). For example, the mechanical properties of nanostructured single-particle systems or composites, the electronic properties of single molecules or low-dimensional semiconductors, the magnetic properties of single-domained particles, and the solution properties of colloidal suspensions are

attractive, and all directly correlate with their nanometer-scale dimension and organization. Recent research in electronics and photonics has confirmed theoretical predictions, such as the quantum properties of organized nanodots and electrical transport in nanotubes and nanowires (3–7). In addition, colloidal particles of metals, functional ceramics, and semiconductors have potentially useful properties that derive from their small size, morphology, or proximity effects (1–7). These properties may lead to a host of applications including chemical, biological, and optical sensors; spectroscopic enhancers and phase shifters; nanoelectronics; and quantum structures (1–7). The realization of the full potential of nanotechnological systems has so far been limited owing to the difficulties in their synthesis and subsequent assembly into useful functional structures and devices.

Most traditional approaches to synthesize nanoscale materials are inefficient, require stringent conditions, and often produce toxic byproducts (8, 9). These techniques still use "top-down" approaches, and even the most advanced microtechnology and recently developed nanotechnology require considerable external manipulation that limits the large-scale synthesis of complex architectures. This precludes the full exploitation of nanoscale-related physical properties and limits scale-up because the quantities produced are small and the resultant material is often irreproducible owing to nonspecific interactions and uncontrolled agglomeration. Even in the case of carbon nanotubes, one of the most successful nanotechnological systems, widespread use is hampered by nonuniformity, uncontrolled surface chemistry, and difficulties in multidimensional assembly (4). For the great promises of science and technology at the nanoscale to be realized, practical strategies are needed for the control and fabrication of large-scale nanostructures and ordered assemblies of materials in two and three dimensions.[1]

Natural Hybrid Materials

Biomaterials are highly organized from the molecular to the nano-, micro-, and macroscales, with intricate architectures often manifesting themselves in a hierarchical manner that ultimately makes up a myriad of different functional units (10–13). They are "smart," or self-directed, in their organization and formation,

[1]Abbreviations: AFM, atomic force microscopy; cDNA, complementary DNA; CSD, cell surface display; CTG, cytosine-thymine-guanine; CTA, cytosine-thymine-adenine; GBP, gold-binding protein; GEPI, genetically engineered polypeptide for inorganic; MBE, molecular beam epitaxy; mRNA, messenger RNA; M-sequence, morphological sequence space; NMR, nuclear magnetic resonance; PCR, polymerase chain reaction; PD, phase display; P-sequence, powder sequence space; QCM, quartz crystal microbalance; RD, ribosome display; RMSD, root mean square displacement; SEM, scanning electron microscopy; S-layer, surface-layer (bacteria); SPR, surface plasmon resonance; S-sequence, size sequence space; STM, scanning tunneling microscopy; STS, scanning tunneling spectroscopy; TOF-SIMS, time-of-flight—secondary ion mass spectroscopy; X-sequence, crystallographic sequence space; XPS, X-ray photoelectron spectroscopy.

dynamic in their interaction with their surroundings, complex in their structures and functions, self-healing in damage control, multifunctional in their physical and chemical properties, and have characteristics difficult to achieve in purely synthetic systems, even with recently developed molecular processes. Using closely controlled molecular, nano-, and microstructures through molecular recognition, templating, and self-assembly, biological materials have properties of technological and medical interest that surpass synthetic systems with similar phase compositions.

Examples of biomaterials include soft tissues, or all macromolecular materials and hard tissues, that is, macromolecule-inorganic hybrid systems (11–13). Muscle, membranes, skin, tendon, spiders' silks, and cuticles, to cite a few, belong to the former group and are of great interest in soft tissue engineering (14, 15). Hard tissues, the focus of this review, include bones, dental tissues (i.e., dentine and enamel), spicules, spines, shells, skeletal units of single-celled organisms (e.g., radiolarian) or plants, bacterial thin film, and nanoparticles (11). A common denominator in these hard tissues is that in addition to the presence of an inorganic component, there is at least one (and often several) proteinaceous phase (Figure 1).

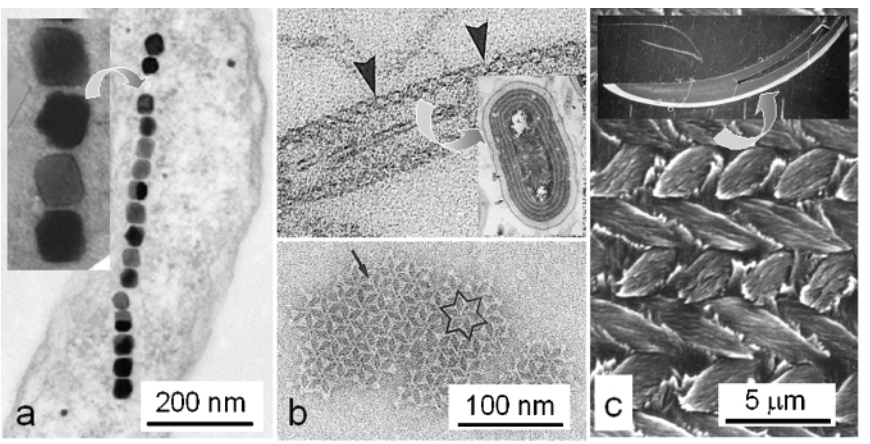

Figure 1 Examples of biologically synthesized hybrid materials with a variety of physical properties: (*a*) Magnetic nanoparticles formed by a magnetotactic bacterium (*Aquaspirillum magnetotacticum*) are single-crystalline, single-domained magnetite (Fe_3O_4) particles (inset: higher magnification image revealing cubo-octahedral particle shape). (*b*) Nanostructurally ordered thin film calcite on the outer layer of an S-layer bacterium, *Synechococcus* strain GL24, that serves as a protective coating (21). (*c*) Mouse tooth enamel is a hard, wear-resistant material with a highly ordered micro/nano architecture consisting of hydroxyapatite crystallites that assemble into a woven rod structure (SEM image). Each rod is composed of thousands of hydroxyapatite particles (inset: cross-sectional image of a mouse incisor; white region is enamel, backed by grayish dentine) (9, 19).

The inorganic phase could include magnetite (Fe_3O_4) particles in magnetotactic bacteria or teeth of chiton (16); silica (SiO_2) as skeletons of radiolarian (11) or tiny light-gathering lenses and optical wave guides in sponges (17); hydroxyapatite [$Ca_2C(OH)_3$] in bones (18) and dental tissues of mammals (19); calcium carbonate ($CaCO_3$) in the shells of mollusks as armor (20) or as thin protective films in some species of S-layer bacteria (21); and spines and tests of sea-urchins (11). These hard tissues could have a wide range of physical properties and are used as effective functional devices from single-celled organisms to higher organisms.

All these biological tissues are synthesized in aqueous environments under mild physiological conditions using biomacromolecules, primarily proteins, but also carbohydrates and lipids, under the genetic control of the organisms (22). In addition to enzymatic reactions in the synthesis of inorganic phases, proteins collect and transport raw materials and consistently and uniformly self- and coassemble subunits into short- and long-range ordered nuclei and substrates (11–13). Whether controlling or participating in tissue formation and regeneration, or being an integral part of the tissue in its biological functions and physical performance, proteins are an indispensable part of biological structures and systems. Therefore, proteins are the workhorses that control the fabrication of biological structures by orchestrating the assembly of materials in two and three dimensions.

Molecular-Level Biomimetics: Toward Realization of Nanotechnology

Future biomimetic systems, developed either for nanobiotechnology or nanotechnology, could include protein(s) in assembly, formation and, perhaps, in the final structure as an integral component leading to specific and controllable functions. In the new field of molecular biomimetics—a true marriage of traditional physical and biological fields—hybrid materials could potentially be assembled from the molecular level using the recognition properties of proteins that specifically bind to inorganics (9, 10). As schematically shown in Figure 2, molecular biomimetics offers three simultaneous solutions to the problem of control and fabrication of large-scale nanostructures and ordered assemblies of materials in two and three dimensions. The first is that inorganic-binding peptides and proteins are selected and designed at the molecular level and through genetics. This allows control at the lowest dimensional scale possible. The second solution is that such proteins can be used as linkers or "molecular erector sets" to join synthetic entities, including nanoparticles, functional polymers, or other nanostructures on molecular templates. Finally, the third solution is that biological molecules self- and coassemble into ordered nanostructures. This ensures a robust assembly process for the construction of complex nanostructures, and possibly hierarchical structures, similar to those found in nature.

Only a few polypeptides have been identified that specifically bind to inorganics. One example is ice-binding (antifreeze) proteins that are synthesized in many

fish species, plants, and insects (23). These proteins, which often have repeating polypeptide units, bind to ice in the internal fluids of organisms to control particle size, morphology, or distribution. Other inorganic-binding peptides could be designed using a theoretical molecular approach similar to those employed for the design and development of pharmaceutical drugs (24, 25). This route is currently impractical and too expensive for current materials research. An alternative possibility would be to extract biomineralizing proteins from hard tissues, followed by purification and the cloning of their genes.

Although this approach is difficult and time consuming, several proteins isolated in this fashion have been used as nucleators, growth modifiers, or enzymes in the synthesis of certain inorganics (26–31). Some examples include amelogenins in mammalian enamel synthesis (27); silicatein, in sponge spicular formation (31); and calcite- and aragonite-forming polypeptides in mollusk shells (26, 28, 29). The main drawback is that hard tissues usually contain multiple proteins that have different roles in biomineralization and are spatially and temporally distributed in complex ways. For instance, more than 20 known proteins have been implicated in the synthesis of human enamel (27), and over 10 polypeptides have been identified in mollusk shells (26). Furthermore, hard-tissue-extracted proteins may be used only for the regeneration of the inorganic that they are originally associated with and would be of limited practical use in the engineering of other nanostructures.

There is an emerging consensus that the preferred approach for obtaining inorganic-binding polypeptides is to use combinatorial biological techniques (9, 32–35). In this approach, a large, random library of peptides with the same number of amino acids, but varying compositions, is screened to identify specific sequences that strongly bind to an inorganic material of practical interest. In molecular biomimetics, the ultimate goal is to generate a molecular erector set in which different proteins, each engineered to bind to a specific surface, size, or morphology of an inorganic compound, promote the assembly of intricate, hybrid structures composed of inorganics, proteins, and even functional polymers (9). Achieving this would be a giant leap toward realizing nanoscale building blocks in which the protein and its binding characteristics are tailored using DNA technologies (36), whereas the inorganic component is synthesized for its specific functions (e.g., electronic, optical, or magnetic) (37) (Figure 3). We refer to these short polypeptides (or small proteins) as genetically engineered proteins for inorganics (GEPIs) (9).

In the following section we provide an overview of the display technologies that can be used to select polypeptides recognizing inorganic compounds and highlight unique aspects of using these systems. We next discuss the mechanisms involved in protein-inorganic binding and the techniques that may be used for their characterization. A separate section showcases achievements involving the use of inorganic-binding polypeptides. Finally, we present future prospects in bio- and nanotechnologies.

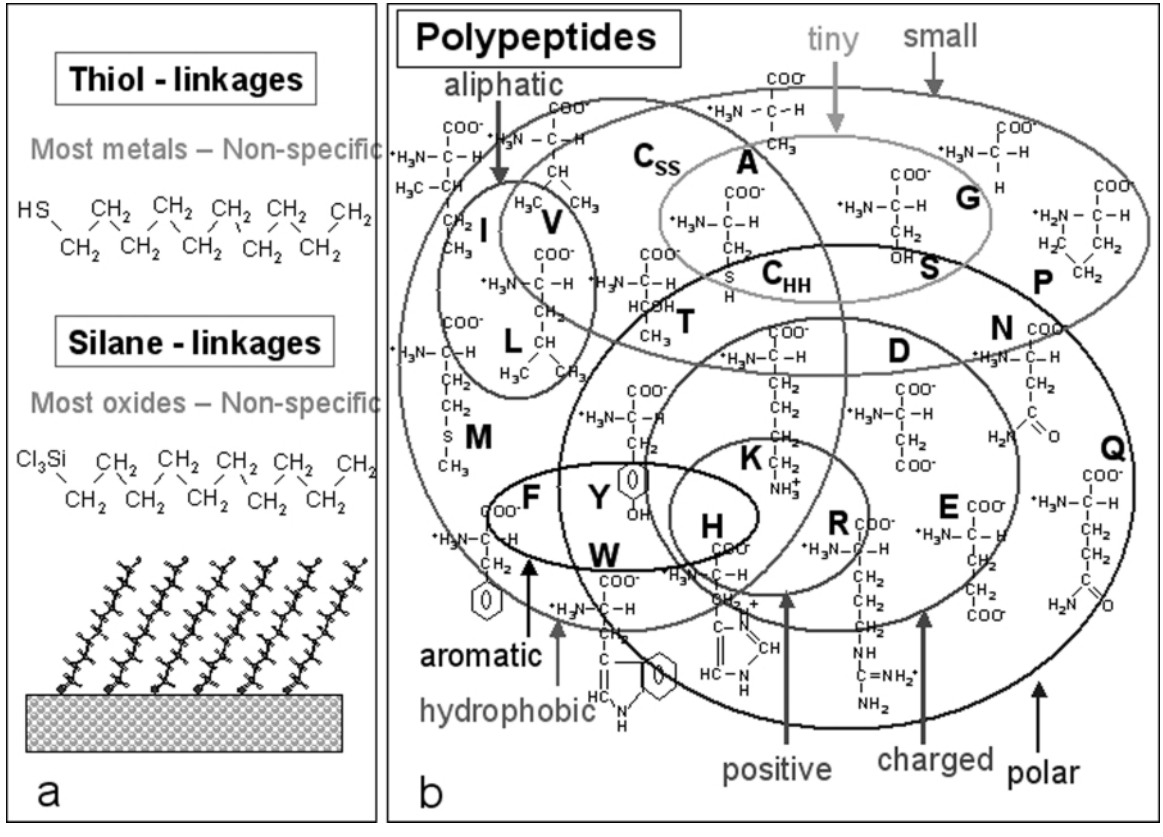

Figure 3 (*a*) Both *n*-alkane thiol ($n = 10$) and alkyltricholosilane form self-assembled monolayers nonspecifically on either metals or oxides, based on thiol (HS) and silane (Si-OH) linkers, respectively. (*b*) A large number of proteins, with a wide range of chemical and structural characteristics, may be potential linkers binding specifically to a variety of inorganic materials.

SELECTION OF INORGANIC-BINDING PEPTIDES

Combinatorial Biology

Since the invention of phage display nearly two decades ago (38), display technologies have proven an extraordinarily powerful tool for a myriad of biotechnological and biological applications. These include the characterization of receptor- and antibody-binding sites, the study of protein-ligand interactions, and the isolation and evolution of proteins or enzymes exhibiting improved or otherwise altered binding characteristics for their ligands. The three most common approaches, phage display (PD), cell surface display (CSD), and ribosome display (RD) have recently been reviewed (39–44). All technologies are based on the common theme of linking phenotype and genotype.

Both PD and CSD rely on the use of chimeric proteins that consist of a target sequence fused within (or to) a protein that naturally localizes on the surface of a bacteriophage (a bacterial virus) or a cell (bacterium) to achieve display (Figure 4,). Using standard molecular biology techniques (39), the DNA sequence of the target region (for instance the active site of an enzyme or the complete sequence of a small polypeptide) 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. By contacting the library with an immobilized ligand, washing out weak or nonbinders, and repeating the process to enrich for tight binders, a subset can be selected from the original library exhibiting the ability to tightly interact with the desired ligand. This process is known as biopanning. 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 (Figure 4).

In RD (also called polysome display), a synthetic DNA library, consisting of the mutated gene of interest fused to a C-terminal spacer region, which allows the polypeptide to fold, and lacking stop codons to prevent release from the ribosome, is first transcribed in vitro. The resulting collection of mRNAs is translated in vitro, and the encoded polypeptide folds as the ribosome travels along the mRNA. Owing to the lack of stop codons, a ternary complex is formed that consists of a particular mRNA, its translation product, and the ribosome as a coupler. These products can be panned on an immobilized ligand, as discussed above. Following dissociation of the ternary complexes that exhibit high affinity for the ligand, the mRNA is converted to a cDNA by reverse transcription, amplified by PCR, and sequenced to deduce the amino acid composition of the binding polypeptide (39, 41). Related strategies include the ribosome-inactivation display system, in which an alternative approach is used to stabilize the RD ternary complex (45), and mRNA display, in which the use of RNAs containing a 3′ puromycin moiety leads to the formation of a covalent bond between the polypeptide and its cognate RNA, thereby circumventing the requirement for ribosomes as a coupler.

Advantages and Drawbacks of Display Technologies

We and others have adapted CSD and PD technologies for the selection of inorganic-binding polypeptide sequences (Table 1). Each technology has distinct advantages and disadvantages, and one is preferred over the other depending on a number of factors. One of the most significant disadvantage of in vivo display technologies such as CSD and PD is that they rely on the introduction of the library within a cell or phage genome, a process limited by the transformation efficiency of the host (the number of cells that uptake naked DNA). As a result, the largest CSD or PD libraries contain at best 10^{10} different members. Although this number may seem high, a simple comparison with the possible sequence space reveals otherwise. For instance, the theoretical number of decapeptides that can be generated with all possible permutations of the natural 20 amino acids is 20^{10}, or about 10^{13}. Therefore, a library consisting of 10^{10} members will sample only 0.1% of the possible sequence space. This restriction does not apply to RD and other in vitro technologies where libraries as large as 10^{14} to 10^{15} members can be constructed. In addition, in vitro display is less sensitive to biases associated with in vivo expression, is more amenable to serial rounds of mutagenesis, and allows the possibility of using nonnatural amino acids. On the negative side, in vitro techniques demand a significant level of expertise, involve labile intermediates (single-stranded nucleic acids), and off the shelf systems are not commercially available.

In vivo display techniques also have common caveats. These include biases in the original (naïve) library introduced during the construction process, as well expression biases associated with the use of living cells. Because the genetic code is degenerate, certain amino acids are represented by multiple (synonymous) codons, and this number varies depending on the identity of the residue. For instance, leucine is specified by six codons, threonine by four, tyrosine by two, and methionine and tryptophan by one. As a result, the probability of randomly generating a codon specifying tryptophan will be lower than that for one encoding, for instance, leucine. A second level of bias is associated with cells from different origins synthesizing different levels of transfer RNAs and therefore preferentially using certain codons. For example, *Escherichia coli* genes use CTG (cytosine-thymine-guanine) to specify leucine 49% of the time, whereas the synonymous codon CTA (cytosine-thymine-adenine) is employed only $\sim$3% of the time. Thus a random sequence containing a CTA codon will not be as efficiently translated as one containing CTG, which may reduce the number of copies displayed at the surface of a cell or the number of progeny phages.

Additional issues that can eliminate library members include the introduction of stop codons (which will lead to the synthesis of a prematurely terminated protein), the inefficient folding of the chimeric protein (which may result in its aggregation or degradation), and its inefficient export from the cytoplasm. In addition, because PD requires infection of bacterial cells to amplify selected phages after each round of biopanning, subsets of clones exhibiting poor infectivity or otherwise unable to infect a bacterial cell may become irretrievably lost. Conversely, efficiently

TABLE 1 A list of inorganic-binding polypeptides selected by phage display and cell surface display

Metals and metal oxides	Sequence origin and reference	Sequences	Size	pI[a]	MW[a]	Charge[b]
Au	CSD outer membrane	MHGKTQATSGTIQS	14	8.52	1446.60	+1
	(32, 56)	SKTSLGCQKPLYMGREMRML	21	9.78	2430.94	+3
		QATSEKLVRGMEGASLHPAKT	21	8.60	2211.52	+1
Pt	PD: constrained 7aa	DRTSTWR	7	9.60	920.98	+1
	(S. Dincer, C. Tamerler &	QSVTSTK	7	8.75	749.82	+1
	M. Sarikaya, unpublished)	SSSHLNK	7	8.49	771.83	+1
Pd	PD: constrained 7aa	SVTQNKY	7	8.31	838.92	+1
	(S. Dincer, C. Tamerler	SPHPGPY	7	6.46	753.81	0
	& M. Sarikaya, unpublished)	HAPTPML	7	6.74	765.93	0
Ag	PD: 12 aa unconstrained (52)	AYSSGAPPMPPF	12	5.57	1221.39	0
		NPSSLFRYLPSD	12	6.09	1395.53	0
		SLATQPPRTPPV	12	9.47	1263.46	+1
SiO_2	PD:12 aa unconstrained (35)	MSPHPHPRHHHT	12	9.59	1470.63	+1
		RGRRRRLSCRLL	12	12.30	1541.89	+6
		KPSHHHHHTGAN	12	8.78	1359.43	+1
	PD:12 aa unconstrained	YSDQPTQSSQRP	12	5.83	1393.43	0
	(D. Sahin, C. Tamerler &	TYHSSQLQRPPL	12	8.44	1426.59	+1
	M. Sarikaya, unpublished)	SPLSIAASSPWP	12	5.24	1212.37	0
	Silaffins (30)	SSKKSGSYSGYSTKKSGSRRIL	22	10.66	2364.64	+6
		SSKKSGSYSGSKGSKRRIL	19	11.22	2013.28	+6
		SSKKSGSYSGSKGSKRRNL	19	11.22	2014.23	+6
	Silicatein α (87, 88)	. . .. .SSRCSSSS. . .. . .[c]	218	4.88	23,382.74	−5
		SSRCSSSS	8	7.96	799.81	+1

(*Continued*)

TABLE 1 (*Continued*)

Metals and metal oxides	Sequence origin and reference	Sequences	Size	pI[a]	MW[a]	Charge[b]
ZnO	CSD: flagella (51a)	RIGHGRQIRKPL	12	12.3	1430.72	+4
		VRTRDDARTHRK	12	11.5	1510.68	+3
	CSD: fimbria (50)	PASRVEKNGVRR	12	11.7	1368.56	+3
		NTRMTARQHRSANHKSTQRA	20	12.48	2351.59	+4
		YDSRSMRPH	10	8.75	1148.26	+1
Cu_2O	CSD: flagella (51a)	RHTDGLRRIAAR	12	12.0	1421.63	+3
		RTRRQGGDVSRD	12	11.5	1402.49	+2
		RPRRSAARGSEG	12	12.0	1299.42	+3
Zeolites	CSD: outer membrane (51)	VKTQATSREEPPRLPSKHRPG	21	10.90	2371.68	+3
		MDHGKYRQKQATPG	14	9.70	1616.82	+2
$CaCO_3$	PD: 12 aa unconstrained (34)	HTQNMRMYEPWFG	13	6.75	1696.92	0
		DVFSSFNLKHMRG	13	8.75	1537.76	0
Cr_2O_3	CSD: fimbria (34)	VVRPKAATN	9	11.00	955.13	+2
		RIRHRLVGQ	9	12.30	1134.35	+3
Fe_2O_3	CSD: outer membrane (49)	RRTVKHHVN	9	12.01	1146.32	+3
GaAs	PD: 12 aa unconstrained (33)	AQNPSDNNTHT	12	5.97	1335.31	0
		RLELAIPLQGSG	12	6.00	1253.46	0
		TPPRPIQYNHTS	12	8.44	1410.55	+1
ZnS	PD: 7aa constrained (53)	NNPMHQN	7	6.74	853.91	0

[a]Isoelectric points and molecular masses of peptides are calculated using Compute pI/Mw tool (http://us.expasy.org/tools/pi_tool.html).

[b]Calculated by subtracting of the number of basic residues (R and K) from the number of acidic residues (D and E).

[c]First row shows the physicochemical analysis of the silicatein α; second row shows the analysis only for the serine-rich hydroxyl cluster unit.

PD, phage display; CSD, cell surface display.

produced phages may yield a larger progeny, and identical sequences may be selected repeatedly during subsequent rounds of biopanning.

Despite the above limitations, PD and CSD remain the techniques of choice in many applications because of their ruggedness and ease of use and because premade libraries are commercially available. One of the most extensively used PD systems is The New England Biolabs PD system, 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 (also called g3p) (Figure 4). Heptapeptide libraries are available in both linear and constrained forms. The latter was constructed by flanking the random sequence by two cysteine residues that form a disulfide bond under oxidizing conditions and lead to the display of the heptapeptide as a loop. The diversity of the PD heptapeptide libraries is approximately 3×10^9, which should adequately cover the possible sequence space. The dodecapeptide library (which is available only in an unconstrained form) contains $\sim 2 \times 10^9$ independent clones for a possible sequence space of 4×10^{15} (46, 47). In our studies, we have used both constrained and linear forms of the libraries displaying hepta or dodecapeptides on the phage surface.

For CSD, we use the FliTrx CSD system (48), which positions random sequences of 12 amino acids as disulfide-constrained loops within Thioredoxin 1 (TrxA), which is itself inserted into FliC, the major *E. coli* flagellar protein (Figure 4). The resulting fusion proteins are exported to the cell surface, where they assemble into partially functional flagella (an extended surface feature used for cell motility that is 20 nm in diameter and several micrometers in length in its authentic form). The FliTrx library has an estimated diversity of $\sim 1.8 \times 10^8$ and does not contain any predefined structural motif. A unique feature of the FliTrx system is that elution of cells binding to the target ligand is accomplished by imposition of shear stress (typically by vortexing), which leads to breakage of the flagella.

Adaptation of PD and CSD to the Selection of Inorganic-Binding Peptides

The growing interest in hybrid materials incorporating both inorganic components and peptides or proteins for nanotechnology or nanobiotechnology applications has made PD and CSD highly appealing for the isolation of polypeptides capable of binding inorganic materials with high affinity. To date, CSD has been used to identify peptides that recognize iron oxide (49), gold (32), zinc oxide (50), zeolites (51), and cuprous oxide (51a), whereas PD has been employed to isolate sequences binding to gallium arsenide (33), silica (35), silver (52), zinc sulfide (53), calcite (54), cadmium sulfide (55), and noble metals such as platinum and palladium (S. Dincer,C. Tamerler & M. Sarikaya, unpublished data). We have also used both technologies to select peptides that bind to metals, semiconductors, and dielectrics (Figure 4). Some of these peptides have been used to assemble inorganic particles (33, 53, 55), and others have been used for the formation (i.e.,

biofabrication, synthesizing, and controlling the nucleation and growth) of the selected compounds (35, 52, 54, 56).

As subtrates, inorganic materials differ from those of proteinaceous ligands, but surprisingly little attention has been paid to assessing these differences in the realm of materials and biological sciences together. For instance, many materials rapidly develop an oxide layer on their surfaces, 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") (57), it is therefore imperative to use spectroscopic and imaging techniques to characterize inorganic surfaces before and after panning (58). It may also be useful to monitor wash or elution buffers (e.g., using atomic adsorption spectroscopy to detect metals and metalloids). If evidence of surface modification or deterioration is obtained, buffer conditions should be optimized to guarantee compatibility with the target inorganic.

Inorganic compounds come in various forms, from polydisperse and morphologically uncharacterized powders to single crystals (Figure 5). The nature of the inorganic substrate may disqualify a particular display technology. For instance, PD is suitable for work with powders even if a gradient centrifugation step is used to harvest complexes between binding phages and particles. However, the FliTrx CSD system would not be amenable to such an enrichment process because centrifugal forces would shear off the flagella from the cell. Similarly, although both PD and CSD are theoretically suitable for panning on single crystals, tightly bound cells or phages may be very difficult to elute from the material, thereby leading to the loss of high-affinity clones. In such cases, the use of the FliTrx system may be advantageous because all binders have an equal likelihood of being recovered following flagellar breakage.

In traditional biological applications of display technologies (e.g., antibody epitope characterization, mapping of protein-protein contacts, and identification of peptide mimics of nonpeptide ligands), three to four biopanning cycles are usually performed in PD, whereas four to five are carried out in CSD. After these cycles of enrichment, the selected sequences typically converge toward a consensus consisting of identical or conservatively replaced residues (e.g., an isoleucine for a leucine). Such consensus sequences reflect precise interactions between the side chains of the protein under study and those of the selected polypeptides. However, all available evidence indicates that this rule does not hold true in 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 and crystallographic levels and the fact that there are multiple solutions to the problem of inorganic binding. One could, for example, envision binding strategies relying on shape complementarities, electrostatic 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 will be needed for the design of hybrid materials that

exhibit controlled topology and composition. In this regard, some key questions need to be asked: What are the physical and chemical bases for recognition of inorganic surfaces by selected polypeptides? Are there differences between metals and nonmetals? How do the surface characteristics influence binding? What are the strength, kinetics, and specificity of binding? Do inorganic peptides exhibit long-range assembly characteristics? Can we use inorganic-binding peptides as molecular linkers, thereby opening new avenues in self-assembled molecular systems? In the following sections we provide preliminary answers to some of these questions, give examples of applications, and offer our perspective on the future potential of this approach.

THE NATURE OF POLYPEPTIDE BINDING TO INORGANICS

Significance of Protein-Inorganic Interactions

The selective binding of proteins to their ligands often invokes complementarity in molecular architectures and surface chemistries. For example, in one of strongest receptor-ligand interactions found in nature, four biotin molecules bind to each of the pockets of the tetrameric protein streptavidin via a "lock and key" mechanism (59, 60). By analogy, inorganic-binding polypeptides could ideally be selected using inorganic materials exhibiting specific morphology, crystallography, or surface stereochemistry (Figure 5). In the design and assembly of functional inorganics, it is crucial to understand the nature of polypeptide-inorganic recognition and binding to optimize and tailor these events. Although protein-surface interactions have been the object of considerable research, how proteins recognize inorganics remains unclear (61–63). The specificity of a protein for a surface may originate from both chemical (63–65) (e.g., H-bonding, polarity, and charge effects) and structural (66) (size and morphology) recognition mechanisms. An additional problem is that inorganic surface properties must be well characterized to provide an understanding of the nature of binding.

The surface could be as well defined as, for example, a single crystal or a nanostructure. It might be rough or totally nondescriptive, for example, a powder, where the sequence space is the largest (Figure 5). When powders of various sizes (ranging from a few nanometers to sub-micrometer) and morphologies (sharp corners, rods, spherical particles, etc.) are used during the selection process, it is expected that the diversity of sequences would be the largest because numerous morphological, crystallographic, and size features (encompassing the M-, X-, and S-sequence spaces in Figure 5) would be available for peptide binding. However, peptides selected on materials of carefully controlled size, morphology, crystallography, or stereochemistry may show higher sequence homology; for example, a genetically engineered protein for inorganics (GEPI) selected using a specific crystallographic (hkl) surface may or may not bind to another crystallographic plane of the same material. Finally, a GEPI that strongly binds to a crystal of composition A may

exhibit reduced affinity for a structurally related material B of different composition (e.g., $BaTiO_3$ versus $SrTiO_3$, both having perovskite structure).

Thus if one seeks to identify binders that are highly specific for certain surfaces, a detailed knowledge of the physical and chemical characteristics of the material must be available. However, once binders are selected from a large combinatorial sequence space (10^6 to 10^9) using powders, one could, in a second step, use a specific morphology, size, or surface to select a subset of peptides that are more likely to exhibit sequence similarity. Understanding how polypeptides recognize and bind inorganic materials, combined with genetic manipulation, should ultimately facilitate their rational design and allow for the successful integration of materials (e.g., quantum dots, rods, or polygons) at the molecular level (8–10, 15, 22, 67–69).

Fluorescence microscopy has proven very useful to quantitatively rank a population of polypeptides selected by PD or CSD in terms of affinity and specificity for a particular inorganic surface (33, 35, 49, 52, 58). Similar to the study of nanoscale structures of thiol- and silane-based self-assembled monolayers (70–73), scanning probe microscopy (SPM) [atomic force microscopy (AFM) and scanning tunneling microscopy (STM)] may also be used to investigate polypeptide assembly and binding onto inorganic surfaces (H. Zareie & M. Sarikaya, unpublished observation). The imaging results with SPM studies could be closely correlated with data from quartz crystal microbalance (QCM) (74) and surface plasmon resonance (SPR) (75) spectroscopy to quantitatively characterize protein adsorption kinetics under various solution conditions such as protein concentration, pH, and salinity on a variety of inorganic surfaces. Traditional spectroscopy techniques such as X-ray photoelectron spectroscopy and time-of-flight secondary ion mass spectroscopy (TOF-SIMS) techniques have recently proven useful in providing the fingerprint of specific peptide adsorbed onto solid surfaces (76, 77). For the evaluation of the molecular structure and conformation on solid surfaces, liquid state and solid-state nuclear magnetic resonance (NMR) spectroscopy has provided essential data toward understanding molecular recognition and binding mechanisms (66). As part of the theoretical tools, predictive molecular dynamics and statistical physics protocols could also provide valuable insights into the three-dimensional structure of inorganic-binding peptides on atomically flat surfaces (63, 78). It is clear that parallel studies on the same polypeptide-inorganic system using modeling and experimental techniques (e.g., molecular dynamics and NMR in addition to SPR) would be the most powerful approach to understanding the nature of binding. Some of these techniques are briefly discussed here through part of our ongoing studies (9, 78; M. Sarikaya, unpublished observation).

Quantitative Protein Adsorption and Binding: QCM and SPR

Within the past few years, many amino acid sequences that promote binding to inorganics have been identified by PD and CSD techniques (9, 32–35). Upon

completion of a screen, a large number of sequences (usually from 10 to 50) is considered for further studies. Thus a rapid, first-pass technique is desirable for isolating the best (e.g., strongest or most specific) binders. Fluorescence microscopy is such a tool (32–35). In the case of PD, amplified phages encoding a given amino acid sequence fused to the pIII coat protein are contacted with an inorganic surface (33, 35). Bound phages are visualized by incubation with a primary antibody that is specific for a phage coat protein and with a secondary antibody conjugated with a fluorophore that recognizes the primary antibody (46). When the fluorophore is excited, the phages "light up" and the number of bound virions can be enumerated by counting. For CSD, we have developed a similar system that relies on the use of SYTO9, a fluorescent dye that penetrates metabolically active cells and binds to nucleic acids (51a). An example of a fluorescence study is given in Figure 6,

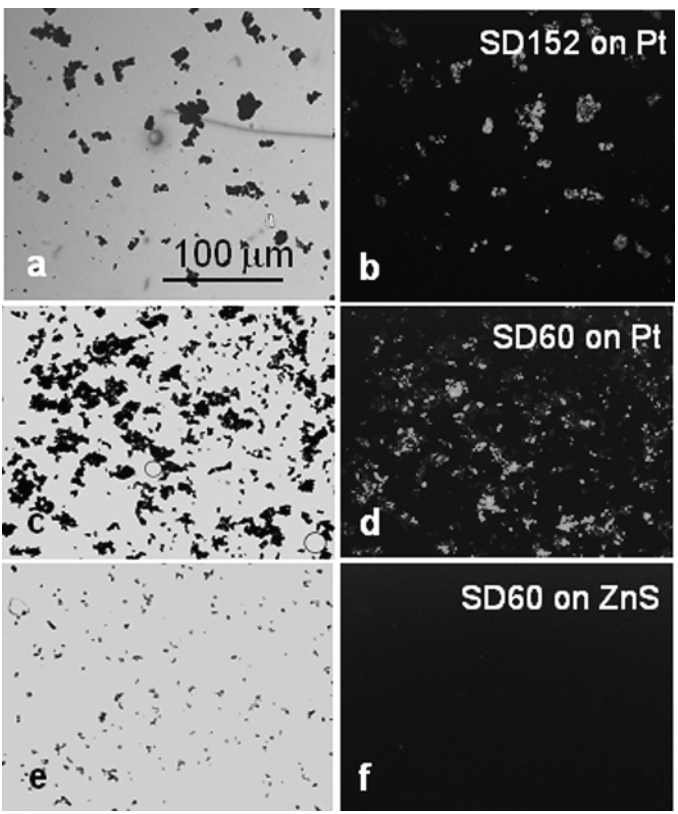

Figure 6 Evaluation of binding characteristics of Pt-selected polypeptides by fluorescence microscopy using immunolabeled phage carrying the preselected library inserts. Panels (*a*) and (*b*) show strong binders to Pt, whereas (*c*) shows no binding selectivity to ZnS using the same sequence as in (*b*) (see also Table 1) (from S. Dincer, unpublished observation).

where a strong Pt-metal binder produces a high-fluorescence signal on this metal while giving no signal when contacted with ZnS (S. Dincer, C. Tamerler & M. Sarikaya, unpublished observation). Although this technique is nonquantitative, it allows one to estimate binding strength (by assuming that the number of bound phages or cells is proportional to the affinity of the inorganic-binding sequence for its substrate) and specificity (by testing the phages on multiple materials, or better, by using substrates patterned with different inorganics).

Fluorescence microscopy, despite being well suited for qualitative binding studies, does not allow the determination of more fundamental binding parameters such as adsorption and desorption rate constants or equilibrium adsorption constants. Unfortunately, there is a scarcity of literature reporting quantitative adsorption properties of the inorganic-binding proteins. Nonetheless, there are a number of well-established tools for measuring molecular adsorption on inorganic surfaces, including both QCM and SPR measurements (79–82). The QCM is an established mechanical measurement tool to study the adsorption of proteins and synthetic molecules (such as self-assembling monolayers) on surfaces (79, 80). In QCM, a quartz crystal disk mounted with an electrode (typically gold), senses the resonance vibration behavior through the change of deposited molecular mass on the surface. When operating in air, QCM can accurately measure submonolayer films, but viscosity and thickness become important parameters in the case of liquids.

We have begun using QCM to evaluate the binding kinetics of gold-binding protein (GBP-1) on gold-coated quartz crystals (M. Duman, E. Venkatasubramanian, C. Tamerler, E.E. Oren & M. Sarikaya, unpublished data). These experiments were carried out in water under neutral pH conditions using buffers employed in the selection process and by varying polypeptide concentrations (0.1–4.0 ng/ml) at room temperature (Figure 7*a*). In these experiments, it was found that GBP-1

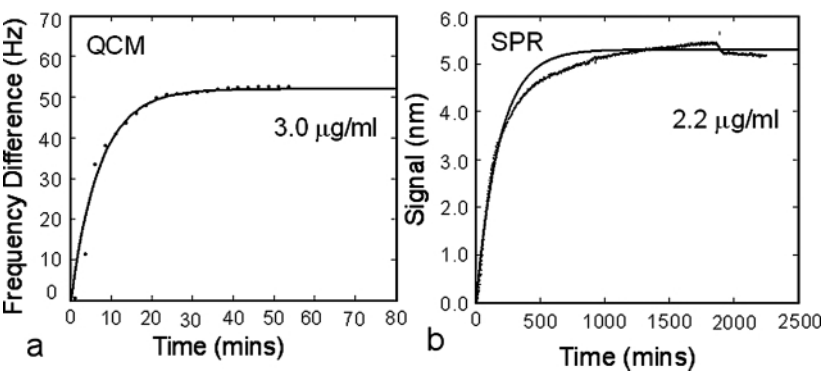

Figure 7 Experimental (*dotted line*) and model (*continuous line*) adsorption kinetics determined by (*a*) QCM and (*b*) SPR using a three-repeat gold-binding protein on flat gold substrates. The model is based on Langmuir monolayer adsorption (E. Venkatasubramanian, unpublished data).

binding to Au followed Langmuir kinetics, which provided adsorption rate constant (k_a) as well as free energy of binding. These values for GBP-1 on polycrystalline gold surface were 2.4×10^3 M^{-1} s^{-1} and -9.4 (kcal) $(mol)^{-1}$, respectively. Compared with alkanethiols [2×10^3 M^{-1} s^{-1} and -5.6 (kcal) $(mol)^{-1}$], these could be considered moderate values (83). However, the structural and assembly characteristics of the GBP-1 and the potential of its genetic manipulation make it a highly useful molecular linker. We have carried out binding experiments with Pt-coated quartz substrates to assess the cross-specificity of GBP-1 for Pt under the experimental conditions used for Au (M. Duman, E. Venkatasubramanian, C. Tamerler, E.E. Oren & M. Sarikaya unpublished data). Overall, the equilibrium constant (K_e) of GBP-1 for Pt was 20-fold lower than that for Au, suggesting that GBP-1 adsorbs on Au more efficiently and stably than on Pt.

SPR is complementary to QCM because it detects adsorption via changes in the refractive index of the interface, rather than net mass change (75, 81, 82). It is sensitive to the minute refractive index changes (0.0001) that occur when submonolayer amounts of protein bind to the sensor surface (82–84). Here, the reflected light intensity from the interface between metal and the glass substrate at a specific incident angle is measured as a result of the optical excitation of surface plasmon waves. The shift in position of the adsorption wavelength of the reflected light from the interface between glass prism and substrate is caused by the change in the evanescent wave in the metal film. This arises from the change in the refractive index as a result of the adsorption and coverage of the molecular species on the metal surface (75, 81). Figure 7*b* shows an example of SPR experiments carried out to evaluate GBP-1 binding on a polycrystalline gold film. Experimental conditions were similar to those used in QCM experiments except that the polypeptide solution was released in the SPR chamber via a fluid cell with a predetermined, optimum flow rate (E. Ventaka, unpublished data). Rapid adsorption of GBP-1 onto the sensor surface was observed as a sharp increase in SPR shift. The initial kinetics could be fitted with a single exponential term leading to adsorption parameters similar to those found in the QCM experiments (Figure 7*a* giving specifically $k_a = 6.5 \times 10^3$ M^{-1} s^{-1} and $\Delta G = -8.9$ (kcal)$(mol)^{-1}$].

Chemical Specificity of Inorganic Binding

With small molecules, one often assumes that low-to-moderate energy adsorption on surfaces is reversible and is not surface sensitive (physisorption). Moderate-to-high adsorption energy systems can undergo irreversible surface chemical bonding and possess specific surface selectivity (chemisorption) (85). Adsorption selectivity results from chemical bonding of functional groups such as thiol-terminated molecules for noble metal surfaces or silanes for metal oxide surfaces (Figure 3*a*) (70–75, 79–82). However, this simple picture of unselective physisorption versus highly selective chemisorption is not a sufficiently nuanced framework for understanding the reversible and selective interaction of a polypeptide or protein with its

target substrate. Instead, a single polypeptide or protein may interact with a surface in a complex manner that is dictated by the combination of amino acid sequence (i.e., spatial arrangement) and the nature of diverse amino acid side-chain interactions. These interactions arise from the widely varied chemical and physical traits of amino acids, as illustrated by the Venn diagram groupings shown in Figure 3*b*. In short, divining the right combinations of spatial configuration and choice among the 20 commonly occurring amino acids that impart both selectivity and strong binding is not possible. Nonetheless, a growing understanding of the features of known inorganic-binding polypeptides and proteins is providing some insights into the roles played by the surface, the amino acids, and water.

Gold-binding polypeptides are the early example of inorganic-binding polypeptides identified by CSD (32). More than 50 sequences were identified, and one, GBP-1, was studied in detail (Table 1). Most of these sequences did not contain cysteine, an amino acid that would be expected to give rise to binding through thiol linkage, as in the case of self-assembled monolayer formation. To increase binding activity, tandem repeats of the sequence (SEKL and GASL) were generated by genetic engineering, and it was found that, as the number of repeats increased, binding affinity also increased (56). In recent years, different display technologies have been used for the selection of polypeptides that bind to other inorganic compounds. Some of these sequences are listed in Table 1 together with those of polypeptides isolated from biological hard tissues.

The metal-binding sequences for silver (52), platinum, and palladium (S. Dincer, C. Tamerler & M. Sarikaya, unpublished data) were selected by the PD protocol. In silver binders, a positional conservation and enrichment in proline residues, as well as conservation of polar, hydrophobic, and hydroxyl-containing small amino acids, was observed (52). Although proline is not observed in the gold-binding polypeptide sequences (32), conserved hydrophobicity and polarity, along with the presence of hydroxyl-containing amino acids, are seen, reminiscent of silver-binding sequences (9). We used a disulfide-constrained heptapeptide library to isolate Pt and Pd binders. Although the selected binding sequences were much smaller (7 aa) than the ones identified for gold (14 aa) and silver (12 aa), Pt binders were also hydrophobic and contained aliphatic and hydroxyl side chain amino acids. Interestingly, most of these sequences did not contain cysteine (C), and only a few contained histidine (H), which have thiol and imidazole rings, respectively, among the noble metal binders These amino acids are traditionally expected to bind metals. However, both Pt and Pd binding sequences were enriched in serine (S), threonine (T), and proline (P) residues.

In Table 1 some of the known inorganic-binding polypeptides are listed together with their molecular mass, isoelectric points, and charge distribution. Among metal oxide-binding sequences, both basic amino acids (arginine and lysine) and hydroxyl-containing amino acids are common regardless of the selection method (PD or CSD) (50, 51a, 58). In general, the sequences binding to metal oxides and ionic crystals exhibit strong basic characteristics and a net positive charge compared with metal-binding peptides.

As new inorganic-binding sequences appear in the literature, it will become possible to carry out more detailed and statistically significant analyses of the characteristics of inorganic-binding polypeptides. Even now one can assess similarities between combinatorially selected polypeptides and those extracted from hard tissues. Silica-binding sequences are a good example of the latter category (30, 31, 86–88). For example, the silaffins, polycationic peptides, isolated from diatom cell walls, were shown to exhibit silica-precipitating activity in silicic acid solutions (30). Silaffins have clusters of lysine (K) and arginine (R) pairs in addition to the hydroxyl-containing amino acids, serine (S), tyrosine (Y), and to a lesser extent, threonine (T). Another interesting example of a silica-binding protein, silicatein, comes from the axial protein extracted from a marine sponge (87, 88). These proteins are composed of three subunits, α, β, and γ, where α appears to play a role in biosilicification. Similar to silaffin, silicatein also contains serine, tyrosine, and threonine clusters. Site-directed mutagenesis studies proved the importance of serine and histidine residues at the expected domains of the protein for biological function (87, 88). It is also interesting to note that in silicateins, arginine and lysine are observed as individual pairs rather than in clusters. Certain silica-binding sequences selected by PD, especially those rich in histidine and arginine residues, were effective in silica precipitation (35). Although the mechanism of biosilicification remains unknow, it is known that the production of biosilicas in certain plants is associated with the presence of hydroxyl-rich polysaccharides, suggesting that hydroxyls in biomacromolecules provide conditions for thermodynamic stability of the material (88).

We have isolated quartz (crystalline silica)-binding sequences and found that they too contain hydroxyl-containing amino acids. In this case, in addition to the serine, tyrosine, and threonine residues observed in noncrystalline silica binders, there is also a significant number of arginine residues. As shown in Table 1, the quartz-binding sequences are similar to silica binders extracted from biological hard tissues and, therefore, their physicochemical properties (charge and isoelectric points) are also similar. In addition, there is a great deal of dissimilarity between noncrystalline and crystalline silica-binding sequences, which contain either histidine and arginine or hydroxyl-containing residues, respectively. Although both sets were selected using the same PD protocol, sequence differences may originate from the physical, as well as chemical, specificities where inorganic surface topography and crystallography may play crucial roles.

Current data suggest that amino acid side chains play an important role in inorganic binding. For instance, polypeptides that bind to noble metals (gold, silver, platinum, and palladium) contain small residues with hydroxyl side chains and exhibit similar hydrophobicity and polarity. This analysis has identified possible amino acid residues, their frequency of occurrence, and similarity as possible sources of binding in both noble metals and metal oxides. Understanding how these functional groups are distributed on the substrate and interact with the underlying structure would have a significant impact for practical applications. Additionally, the three-dimensional architecture of the polypeptides in solution and adsorbed

on the solid surface should be critical in dictating which amino acid side chains mediate substrate interactions. Therefore, a detailed knowledge of molecular architecture is essential to understand the specificity of a polypeptide on a solid surface. Normally X-ray crystallography, although tedious to perform, could provide this information. However, this technique is not an option for small polypeptides bound to a surface. Nuclear magnetic resonance (NMR) spectroscopy, on the other hand, can provide structural information under both liquid- and solid-state conditions. In the best of both worlds, conventional spectroscopic techniques (QCM, SPR, X-ray photoelectron spectroscopy) together with NMR and modeling studies (e.g., molecular dynamics) will likely prove valuable in furthering our understanding of the rules that dictate the interaction between proteins and inorganics (25, 59, 66, 78).

Physical Specificity and Molecular Modeling

The specificity of a polypeptide for an inorganic surface is likely to be rooted in its molecular structure. Structural information is, therefore, essential not only to elucidate the fundamentals of the recognition process, but also for practical applications. Such knowledge would allow genetic or chemical modifications to create additional functionalities (e.g., attachment of conducting or light-emitting polymers to create hybrid, heterofunctional molecules; ability to bind DNA or proteins), thereby yielding a molecular "tinker-toy." Ideally, using molecular dynamics and simulated annealing protocols and solution or solid-state NMR constraints, one could obtain an averaged lowest energy structure for as many GEPI as is feasible and utilize these structures, along with a simulation program, in modeling the orientation and binding energetics at specific interfaces. These data could then be used to rank peptides by interfacial interaction energies, allowing the identification of important side chains and preferred chain alignments for each polypeptide with specific interfaces. Experimental findings, together with structural information from simulations, should add up to give a coherent understanding of GEPI-inorganic surface interactions.

To date, only limited NMR studies have been performed to understand the molecular recognition principles of inorganic-binding polypeptides. These include the adsorption of salivary proteins on hydroxyapatite (89), experiments on $CaCO_3$-bound polypeptides from mollusk shells (90), and recently conducted NMR analysis of CSD-selected single-repeat polypeptide binding to gold (91). Molecular modeling of GEPI binding to inorganics has also been limited. In a collaborative work, we recently performed computer structure modeling studies to predict the shape of a GEPI (GBP-1) in solution and present a summary here as an illustration (Figure 8*a*) (78).

This preliminary work was carried out in the hope that any matching between the amino acid residues of the peptide and the spacing of the inorganic atomic lattice would shed light on how a GEPI binds to an inorganic surface. Raw amino acid sequence data for three repeats of the 14 amino acids in GBP-1 were compared with all known protein structures using searches and various first order

secondary-structure prediction algorithms (Chou-Fasman and Holley/Karplus) (78). Figure 8*a* shows the three-repeat GBP-1 above a {111} Au atomic lattice that highlights the correspondence of hydroxyl groups to gold atom positions. These initial results suggest that binding repeats of GBP-1 form an antiparallel β-pleated sheet conformation, which places hydroxyl groups from serine and threonine residues into a regular lattice based on energy minimized in vacuo (Figure 8*b*) (78). We also showed in these preliminary studies that GBP-1 does not bind to the Au{112} surface as tightly (Figure 8*a*) because of the migration of water molecules through the atomic grooves on this crystallographic surface, which decouples the protein from the surface.

Post-Selection Design and Engineering of Inorganic-Binding Polypeptides via Genetic Engineering

Up to now, we have discussed how inorganic-binding polypeptides can be screened and identified using display technologies and tools to understand the nature of binding. Genetic engineering techniques can be further used, not only to investigate the participation of specific residues in binding events but also to design second generation libraries to tailor binder properties (56, 57, 92, 93). This is very similar to the evolution process where successive cycles of mutation and selection are used to select a progeny with improved features (56, 94). The information obtained from PD libraries, for example, can be integrated into subsequent generations of PD libraries. In recent years, this type of approach has led to 10–100-fold improvements in binding affinities between antibodies and antigens and between proteins and their inhibitors (47, 93–95). It has also proven valuable for the isolation of more stable and active enzymes and for adapting their activities to nonnative substrates, nonaqueous solvents, and extremes of pH or temperature.

In one approach, termed directed evolution, libraries of variants are created by random mutagenesis of an entire gene or of a specific region (94, 96). Mutations are typically introduced by error-prone PCR at random locations, and diversity can be further increased by DNA shuffling, a technique that mimics natural recombination (96). An important requirement is to make the libraries complex and large enough to permit the isolation of mutants exhibiting the desired property. It is also essential to develop rapid screens or selection protocols, as in the case of peptide libraries.

On the other end of the spectrum are knowledge-based approaches where proteins are modified rationally using site-directed mutagenesis and information from crystallographic data or computer-aided molecular modeling. Site-directed mutagenesis in which any amino acid can be substituted by any of the other 19 should prove an invaluable tool to study the contribution of individual amino acids in binding and to redesign or optimize inorganic-binding sequences. Site-directed mutagenesis can also be combined with the irrational directed-evolution methods to obtain engineered sequences with desirable properties. For instance, second generation phage and cell surface display libraries, where randomness is introduced only in the residues that do not appear to be involved in binding (as judged

by sequence alignments or site-directed mutagenesis results), could be used in the search for improved GEPIs as well as for characterizing the structural basis of binding. This strategy has already been applied to GBP peptides (56). Here, binding affinity was improved by constructing semi-random peptide libraries and expressing them on the outer surface of *E. coli* as part of the maltodextrin porin LamB. Specifically, two defined sequences contributing to gold binding were combined in second generation libraries, and variants were selected for altered rates of gold particle formation, which in turn affected the morphology of the gold crystals (56; see below).

APPLICATIONS OF ENGINEERED POLYPEPTIDES AS MOLECULAR ERECTOR SETS

Controlled binding and assembly of proteins onto inorganic substrates is at the core of biological materials science and engineering, with wide ranging applications (Figure 9). Protein adsorption and macromolecular interactions at solid surfaces play key roles in the performance of implants and hard-tissue engineering (15, 97). DNA and proteins adsorbed specifically onto probe substrates are used to build microarrays suitable for modern genomics (69), pharmogenetics (99), and proteomics (96, 98). Engineered polypeptides hybridized with functional synthetic molecules could be used as heterofunctional building blocks in molecular electronics and photonics (8, 9). The unique advantages of engineered polypeptides, created through molecular biomimetics discussed here, include highly specific molecular surface recognition of inorganics, self-assembly into ordered structures, and tailoring of molecular structures and functions through molecular biology and genetics protocols. Using inorganic-binding polypeptides, one can create molecular erector

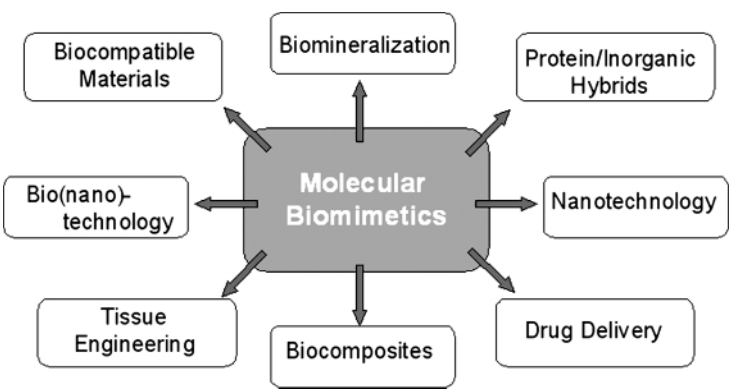

Figure 9 Potential applications of molecular biomimetics in nano- and nanobiotechnology using combinatorially selected and genetically engineered inorganic-binding polypeptides.

sets for potential nano- and nanobiotechnological applications (Figure 9). The examples given below represent a typical variety of these applications.

Self-Assembly of Inorganic-Binding Polypeptides as Monolayers

One of the central questions related to the genetically engineered proteins is whether they can assemble with a long-range order on a given crystallographic surface of a material in addition to chemically recognizing it. Although this aspect of molecular biomimetics is in its early stages, the AFM image of Figure 10 shows that it is possible to assemble a one-monolayer-thick gold-binding protein layer on the Au{111} surface (H.M. Zareie & M. Sarikaya, unpublished results). In fact, close inspection of this and other images reveals that GBP-1 assembles into domains with clear and straight boundaries that make 60° and 120° angles, suggesting that the polypeptide recognizes the sixfold lattice symmetry on the Au{111} surface. What is also significant in these results is that the assembly process progresses until the whole surface is completely covered. Instead of using the traditional thiol or silane linkers in self-assembled monolayers (70–73), this result indicates that self-assembled GEPI monolayers may be used as functional linkages, a central premise in this research (see demonstration of this concept below).

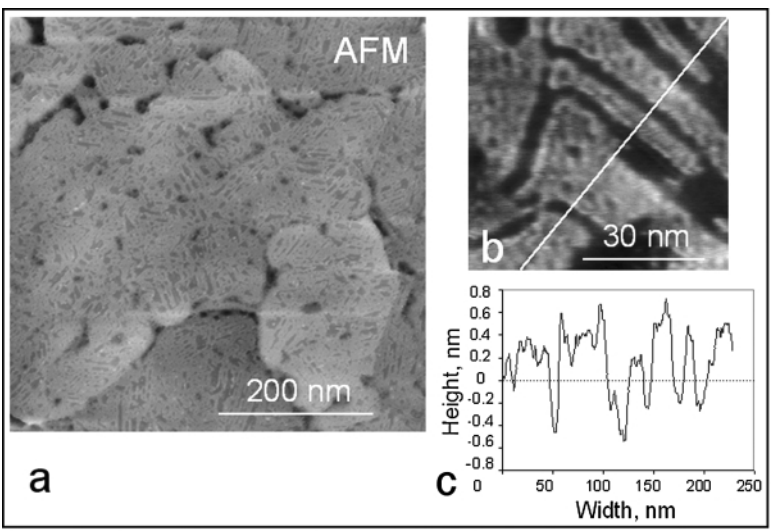

Figure 10 (*a*) Self-assembled three-repeat GBP-1 on Au(111) (AFM image) showing ordered-domained structure. The angle among the domain boundaries is either 120° or 60°, implying recognition of the symmetry of the top surface layer on Au(111). The line across several domains in the AFM image (*b*) produces a profile with 0.5-nm-high platforms revealing the monolayer thickness of the GEPI film (H.M. Zareie & M. Sarikaya, unpublished results).

GEPI-Assisted Cell-Sorting and Differentiation

Cell sorting and separation are critical in the study of cell differentiation, growth-factor interaction, apoptosis, and cell proliferation (100–102). Existing techniques, such as fluorescently activated cell sorting, sedimentation, and centrifugal elutriation, require batch processing, mostly rely on diffusion, and require large volumes (100). As a first step in cell separation and spatial organization, we have demonstrated a novel approach in which cells with genetically fused inorganic-binding polypeptides on their flagella are selectively attached on micropatterned substrates.

In our laboratory, we isolated *E. coli* variant CN48 on the basis of its binding activity to Cu_2O, using the flagellar display protocol described above (51a). As substrates, striped surfaces of Cu_2O and $Cu(OH)_2$ were prepared using an electrochemical process (58). The variant bacteria labeled with a fluorescent dye was contacted with the micropatterned substrate. The $Cu(OH)_2$ region in the micrograph (Figure 11) is easily distinguished because it adsorbs free dye and as a result exhibits a mild fluorescent background. However, many CN48 cells efficiently adhere to the Cu_2O stripes (bright rods of approximately 2 μm length, i.e., size of the cells), and only a few adsorb to $Cu(OH)_2$, possibly owing to nonspecific adsorption. These results indicate that the flagella-displayed GEPI (CN48) exhibits a strong affinity for Cu_2O but not for $Cu(OH)_2$. In these experiments, negative control *E. coli* cells did not adhere to either material. This protocol could be applied to cell sorting or differentiation in heterocellular, viral, and other macromolecular systems, for example, in the separation of DNA from RNA in genomics, nuclear antigens in cell cycles, and proteins in proteomics application (100–102).

Morphogenesis of Inorganic Nanoparticles Using Engineered Polypeptides

In biomineralization, a significant aspect of biological control over materials formation is via protein-inorganic interactions that control the growth morphology in tissue such as bone, dentin, mollusk shells, and bacterial and algal particle formation (16–22). Studies aiming at finding how proteins affect biomineralization have traditionally focused on templating (26–31), nucleation (103–106), and enzymatic reactions (30, 31, 35). With the emergence of combinatorially selected peptides that strongly bind inorganics, a natural step is to examine how these polypeptides affect inorganic formation and to investigate their effect in mineralization (including nucleation, growth, morphogenesis, and enzymatic effects). Such studies have been carried out under aqueous environments necessary for biological functions of selected GEPIs, most notably those binding to Au (56) and Ag (52). For example, the morphology of solution-grown gold particles was shown to be controlled by the presence of gold-binding GEPIs selected by CSD (56). More than 50 mutants were tested for their influence on the rate of crystallization of nanogold particles formed by the reduction of $AuCl_3$ with $Na_3C_6H_5O_7$. Two mutants accelerated the rate of

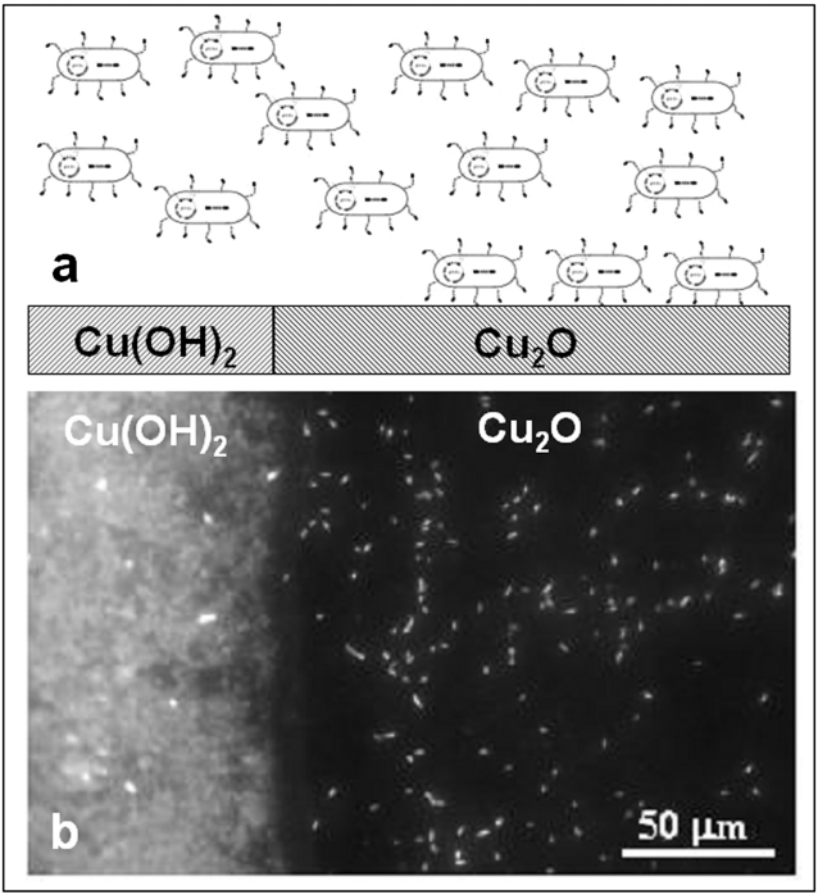

Figure 11 Selective adsorption of *E. coli* on the cuprous oxide, rather than on the cupric hydroxide, section of a striped substrate. The cell flagella express a dodecapeptide that binds to cuprous oxide (Cu_2O) (58).

crystal growth and changed the particle morphology from cubo-octahedral (the usual shape of gold particles grown under equilibrium conditions) to flat, triangular, or hexagonal, particles (Figure 12). Similar crystals usually form under acidic conditions in the absence of Au-binding polypeptides (107). It was proposed that slightly basic polypeptides acted as an acid in flat gold crystallization.

Assembly of Inorganic Nanoparticles via Engineered Polypeptides

Organization and immobilization of inorganic nanoparticles in two- or three-dimensional geometries are fundamental in the utilization of nanoscale effects (1–4, 108, 109). For example, quantum dots can be produced using vacuum

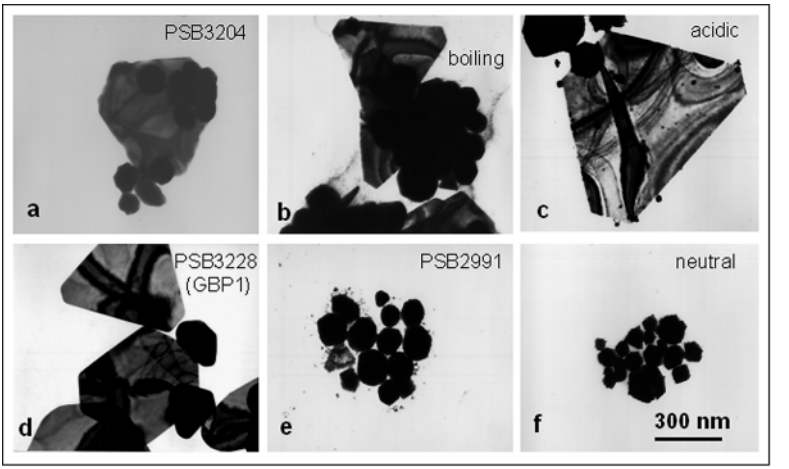

Figure 12 Effect of GEPI on nanocrystal morphology. Two mutants out of more than 50 from a library of gold-binding GEPIs were tested for their ability to form flat gold particles, (*a*) and (*d*), similar to those formed under acidic (*b*) or boiling (*c*) conditions. Particles formed in the presence of vector-encoded alkaline phosphatase (*e*) and neutral (*f*) conditions did not result in morphological change of gold particles (56).

techniques [e.g., molecular beam epitaxy (MBE)] (110), and such an organization is shown in Figure 13*a* in the GaInAs/GaAs system (T. Pearsall, unpublished data). However, this can be accomplished only under stringent conditions of high temperature, very low pressures, and a toxic environment. A desirable approach would be not only to synthesize the inorganics under less stringent conditions but also to assemble and immobilize them via self-assembly using functional molecules as coupling agents. Inorganic particles have been coupled and functionalized using synthetic molecules (e.g., thiols, lipids, and biological molecules, including amino acids, polypeptides, and ligand-functionalized DNA) and assembled to generate novel materials using the recognition properties of these molecules (8–10, 108, 109). Nanoparticles synthesized in wet-chemical conditions in the presence of these molecules (e.g., citrate, thiol, silane, lipid, and amino acids) not only cap the particle, which results in controlled growth, but also prevent uncontrolled aggregation (8, 111, 112).

Synthetic molecules used as coupling agents, however, are not specific for a given material. For example, thiols couple gold and silver nanoparticles in similar ways (8). Likewise, citrate ions cap noble metals indiscriminately (111–113). A desirable next step in molecular recognition and assembly via molecules would be to use polypeptide sequences that recognize inorganics specifically. In this context, GEPIs could potentially be used for nanoparticle assembly. In addition to inorganic surface recognition, a further advantage of a GEPI is that it can be genetically fused to other functional biomolecular units or ligands to produce heterobifunctional (or

multifunctional) molecular entities (114, 115). Figure 13*b* illustrates assembly of nanogold particles on GBP1-coated flat mica surfaces (116). When seven-repeat GBP1 was used as the linker, high-density gold particles formed on the surface, resembling a distribution similar to that seen in Figure 13*a*. Unlike the conditions of MBE, assembly was accomplished under ambient conditions of temperature and pressure and in an aqueous solution. The homogenous decoration of the surface with nanogold suggests that proteins may be useful in the production of tailored nanostructures as quantum dots. The recognition activity of the protein could provide the ability to control particle distribution, whereas particle preparation conditions may allow size control.

Biocompatible Surfaces and Drug Delivery

Thiol and silane linkages, two major molecular linkers for noble metal and oxide surfaces, have dominated the field of self-assembled molecules for the past 20 years (71, 72). The self-assembled GEPI monolayers described above could open up new avenues for designing and engineering surfaces for a wide variety of nano- and biotechnology applications, in particular for studying fundamental chemistry and biological problems (8, 9, 15, 97, 117, 118). Whereas a GEPI has a binding affinity for an inorganic material, it can also be engineered to bind to another organic molecule (synthetic or biological) simultaneously. This multifunctionality could be used in drug delivery applications as demonstrated schematically in Figure 14. For instance, a GEPI could bind to an inorganic nanoparticle that exhibits interesting functionality such as magnetic (113) or photonic characteristics (119). Through a linker protein (attached genetically or via chemical synthesis), a drug of interest could be immobilized onto the bioreactive nanoparticle system, and the drug could be carried to the targeted tissue (for example a cancerous tumor in a brain, bone marrow, or an internal organ) while it is distribution and effectiveness were monitored via a magnetic or photonic signal. By taking advantage of the specificity of the polypeptides for selected materials, two (or more) types of GEPIs could be used for multidrug delivery systems as depicted in Figure 14*a*,*b*.

Similarly, using this multifunctionality, a GEPI recognizing and assembling on to the surface of a therapeutic device could be fused to a human protein to enhance biocompatibility. Some therapeutic device materials include gold, titanium, alumina, and stainless steel (15, 97, 120). We have already isolated polypeptides that bind to some of these materials (e.g., gold and alumina), and work is under way to select for others in this group. Complete coverage of the surface of a biomaterial by inorganic-binding polypeptide provides a promising platform exhibiting highly bioreactive properties. This is because such films could be genetically or chemically fused fairly easily, using a fusion protein or a short polypeptide, to a blood protein or a ligand, to make the device completely biocompatible (Figure 14*c*). These platforms could be durable for long-term applications including hip replacements, stents, bone-repair, and pace devices (15, 97).

Target Immobilization Using Engineered Polypeptides as Molecular Erector Films

Protein adsorption and immobilization on inorganic materials have been critical issues in biosensing and biochips used in genomics and proteomics (68, 69, 98, 99, 121). A common way of immobilizing a probe protein on an inorganic material relies on the use of self-assembled monolayers as linker molecules with, for example, thiol or silane linkages that preferentially attach to a noble metal (gold) or an oxide (silica), respectively (71–73). Both molecular types are then self-assembled as ordered domains composed of rigid, rod-like molecules. Typically the free ends of these molecules are chemically modified to a probe protein and create functional surfaces on to which a target is selected (60, 122). This approach has significant shortcomings, including the fact that thiolates are narrow, rigid, and one-dimensional, which restricts their versatility. In addition, they are not specific for a given material (metal or oxide), which restricts their use in some substrates; probe biomolecules can be linked only chemically, not genetically. More significantly, self-assembled monolayers are prepared in nonbiological environments, preventing their widespread use in in vivo applications.

We have recently demonstrated the usefulness of the self-assembled GBP-1 as a functional molecular erector set by immobilizing a probe protein onto it (H.M. Zareie & M. Sarikaya, unpublished data). Because of its high-binding affinity for biotin, streptavidin is often used as a molecular linker, for example, in biological assays, sensors, synthesis, and purification (60). For immobilization (Figure 15*a*) biotinylated GBP-1 (bio-GBP-1) was first assembled onto a gold surface, with the same ordered characteristics as unliganded GBP-1. Streptavidin (SA, the probe molecule) was then immobilized onto this ensemble with a high surface coverage. We next immobilized biotinylated ferritin (FE, a model target protein) onto this functional molecular substrate to confirm that the immobilized SA had retained specificity and biological activity. In addition to its significance in medicine, ferritin was chosen as a target protein because of its well-characterized structure, stability under heat and chemicals, ease of preparation (e.g., biotinylation), and imaging with AFM (123). Upon FE immobilization, a high surface coverage (determined from the SPR shift) was obtained corresponding to nearly 95% of the SA binding sites (Figure 15*b*). This is a highly efficient immobilization and compares well with the self-assembled biotin-terminated alkylthiols on gold surface that are traditionally used for biosensor applications for immobilizing probe proteins (e.g., SA) and for target protein (60, 122) or DNA selection (121).

FUTURE PROSPECTS AND POTENTIAL APPLICATIONS IN NANOTECHNOLOGY

Proteins hold great promise for the creation of architectures at the molecular or nanoscale levels (8–10). Genetically engineered proteins for inorganics represent a new class of biological molecules that are combinatorially selected to

bind to specific inorganic surfaces (9). The ordered assembly of GEPIs on inorganic surfaces could have a significant impact in molecular biotechnology applications. The results described above are the first demonstrations that combinatorially selected polypeptides can self-assemble specifically on a selected inorganic single-crystal surface and that a GEPI may molecularly recognize an inorganic surface. Realizing that thiol and silane linkages are the two other major molecular linkers for noble metal and oxide (silica) surfaces that have thus far constituted the field of self-assembly of molecules, it is naturally expected that self-assembled GEPI monolayers as molecular erector sets will open up avenues for designing and engineering new functional surfaces. We have already demonstrated that inorganic materials can be assembled at the nanoscale by proteins that have been genetically engineered to bind to selected materials surfaces. It is also the first time that engineered proteins were shown to affect crystal morphology (56).

The combinatorial genetic approach is general, one that could be applicable to numerous surfaces (32–35, 49–54, 56). The modularity of binding motifs should allow genetic fusion of peptide segments that recognize two different materials. The resulting heterobifunctional molecules could be used to attach different materials to each other and may permit assembly of complex nanocomposite and hybrid materials. This could lead to new avenues in nanotechnology, biomimetics, biotechnology, and crystal and tissue engineering such as in the formation, shape-modification, and assembly of materials and the development of surface-specific protein coatings.

One particular potential use of GEPIs is as a molecular linker in nanotechnology. Both nanostructured inorganics and functional molecules are becoming fundamental building blocks for future functional materials nanoelectronics, nanophotonics, and nanomagnetics applications (6–10, 124). Before nanoscience can be implemented in practical and working systems, however, there are numerous challenges that must be addressed. Some of these challenges include molecular and nanoscale ordering and scale-up into larger architectures. A nanotechnological system, for example, could require several components made up of materials of different physical and chemical characteristics. These different materials have to be connected and assembled without an external manipulation. As schematically shown in Figure 16, the components may include two or more inorganic nanoparticles, several functional molecules, and nanopatterened multimaterial substrates.

Using the example of Figure 16, four fundamental issues could be addressed concerning the utility of GEPIs as linkers. (*a*) Organization and display of several components (inorganic or organic) on a substrate require patterning at the nanometer scale. Nanoscale patterning has not yet been possible using the traditional approaches of micro- or nanolithography on a routine basis, especially with several different material components. It may be feasible, however, using designer proteins (such as chaperonins and S-layer proteins) (see for example, 114, 115) as molecular templates because they self-assemble into long-range, two-dimensional

structures with well-defined crystallography. (*b*) To utilize physical effects originating from ordered nanostructures, inorganic particles (or dots or wires) need to be assembled on these templates with specific positions and separations. Molecular linkers may serve as binding agents for assembly or immobilization of inorganic particles. As discussed above, synthetic molecular linkers, such as thiol or silanes, may not be useful for this purpose because these traditional linkers are not specific enough for binding similar materials (e.g., two noble metals, or two oxide semiconductors). Inorganic-binding short polypeptides and GEPIs could be of great utility for this purpose. (*c*) In addition, inorganic-binding polypeptides could also be genetically fused to a designer protein, thereby creating multifunctional linkers. (*d*) A synthetic molecule with a desired function could be designed to attach to an inorganic [designed and synthesized with yet another functionality (*c*)] directly or, better yet, hybridized with a GEPI.

One of the most attractive aspects of this approach is that all of the components can be separately synthesized or hybridized (chemically or biologically), released into an aqueous solution, and allowed to self-assemble, with all the organic and inorganic nanocomponents self-directed to the desired locations, to create a complex, multicomponent, multifunctional nanosystem that may not be possible by traditional approaches.

Although significant advances have been made in developing protocols for surface-binding polypeptides, many questions must be answered before their robust design and practical applications are affectively realized: What are the physical and chemical bases for recognition of inorganic surfaces by the genetically engineered polypeptides? What are the long-range assembly characteristics, kinetics, and stability of the binding? What are the molecular mechanisms of engineered-polypeptide binding onto (noble) metals compared with those on nonmetals? How do surface characteristics affect binding? Based on the insights achieved, can we develop a road map to use GEPIs as molecular linkers and open new avenues in self-assembled molecular systems in nanotechnology based on biology? Considering the rapid developments in the inorganic-binding polypeptide selection protocols and the increased variety of materials utilized as substrates, many of these questions are expected to be answered in the near future and have a significant impact on broad multidisciplinary fields, with potential wide-ranging applications.

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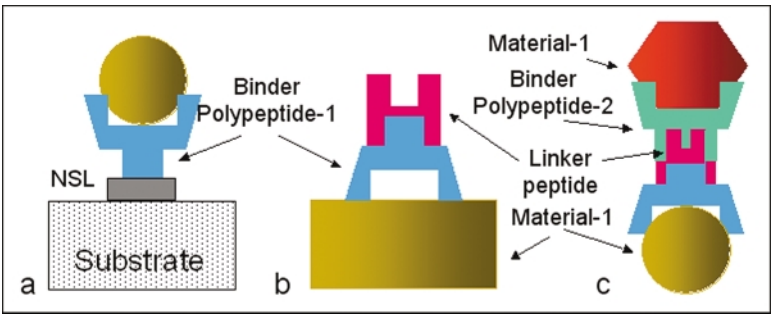

Figure 2 Schematic illustration of the potential utility of inorganic-binding proteins as (*a*) linkers for nanoparticle immobilization, (*b*) functional molecules that assemble on specific substrates, and (*c*) heterofunctional linkers involving two (or more) binding proteins adjoining several nanoinorganic units. NSL, nonspecific linker.

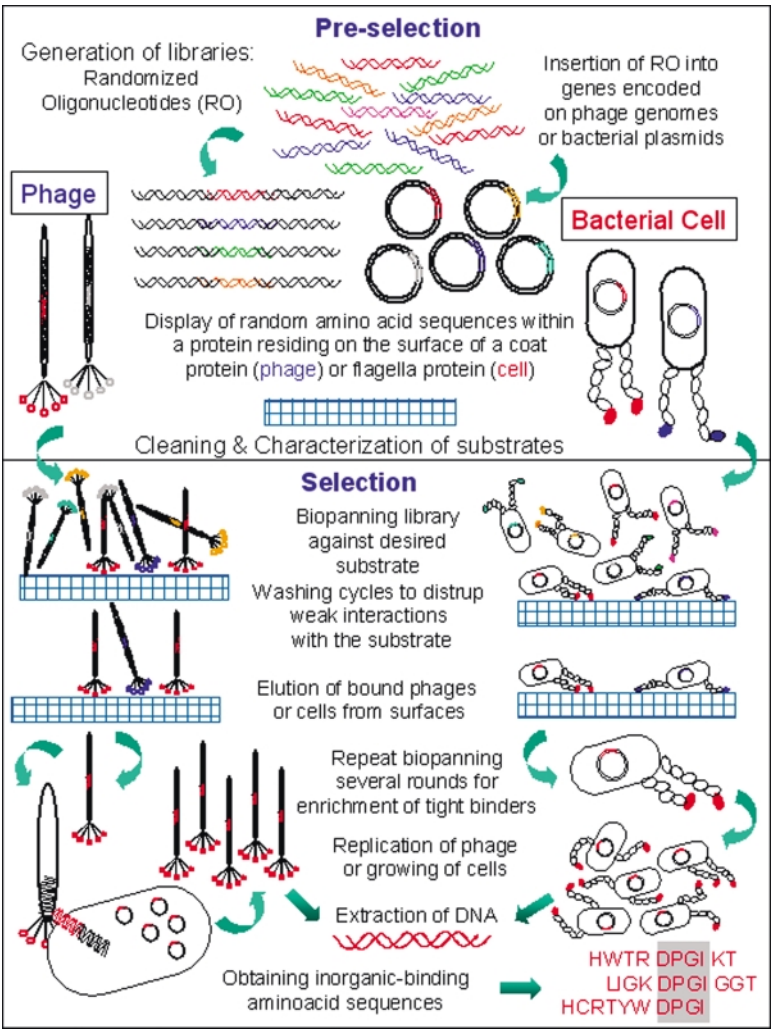

Figure 4 Principles of phage display (*left*) and cell surface display (*right*) protocols adapted for selecting polypeptide sequences with binding affinity to a given inorganic substrate.

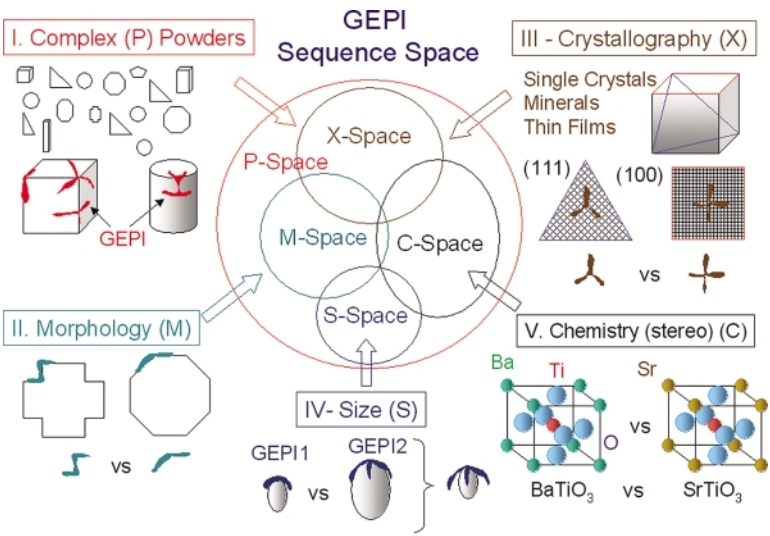

Figure 5 Engineered proteins may bind to inorganics depending on their physical shapes and surface chemistries. The largest number of different binding sequences (i.e., largest sequence space) could be obtained using powders (P- space). A smaller number of binders would belong to the sequence spaces of specific morphological shapes (M-space), sizes (S-space), single-crystal surfaces (X-space), and surface chemistries (C-space). Some polypeptides from the same material, but from different sequence spaces, could overlap, resulting in reduced specificity.

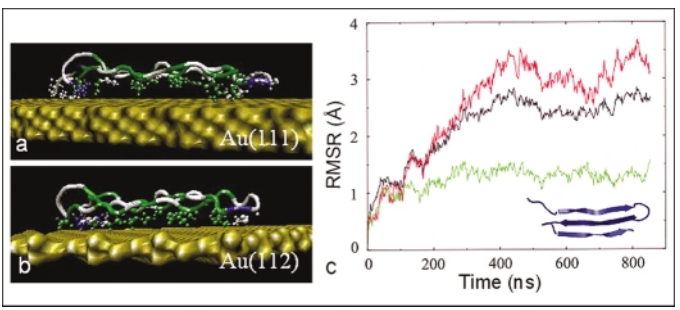

Figure 8 Panels (*a*) and (*b*) are molecular dynamic renderings of gold-binding protein (three-repeat GBP-1) on Au{111} and {112} surfaces, respectively, viewed edge-on. Coloring corresponds to residue type: polar residues are highlighted in green, charged in blue, and hydrophobic in white. (*c*) Root-mean-square displacements (RMSD) of Ca atoms on Au{111} were the result of an equilibration relative to the predicted starting structure (*black*). The protein is stable after 500 ps. Polar residues (*green*) exhibit a small RMSD compared with the fluctuations observed in the hydrophobic residues (*red*) (78).

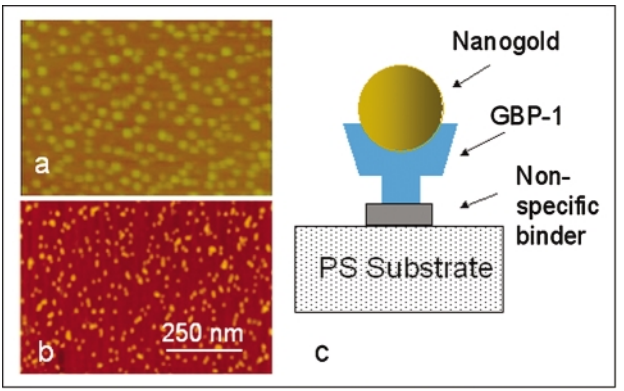

Figure 13 AFM images show quantum (GaInAs) dots assembled on GaAs substrate (*a*) via high-vacuum (MBE) strain-induced self-assembly (courtesy of T. Pearsall), and (*b*) via seven-repeat GBP-1. (*c*) Schematic illustration of (*b*), where PS is polystyrene substrate, and the nonspecific binder is glutaraldehyde.

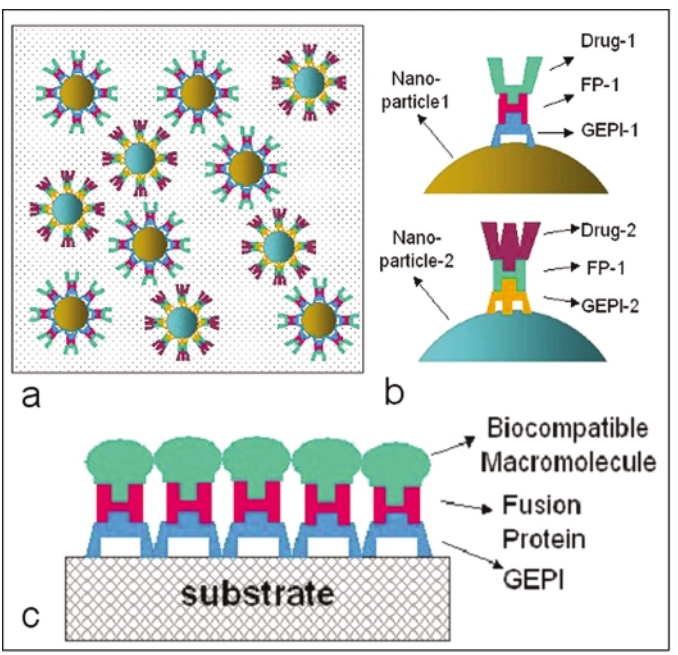

Figure 14 The schematics illustrate potential applications of GEPI. In drug delivery, two different GEPIs are used, each attached to a different inorganic particle carrying two separate drug molecules, (*a*) and (*b*). In creating a biocompatible surface in (*c*), a GEPI is first assembled to cover a therapeutic device and then completely covered with a biocompatible macromolecule.

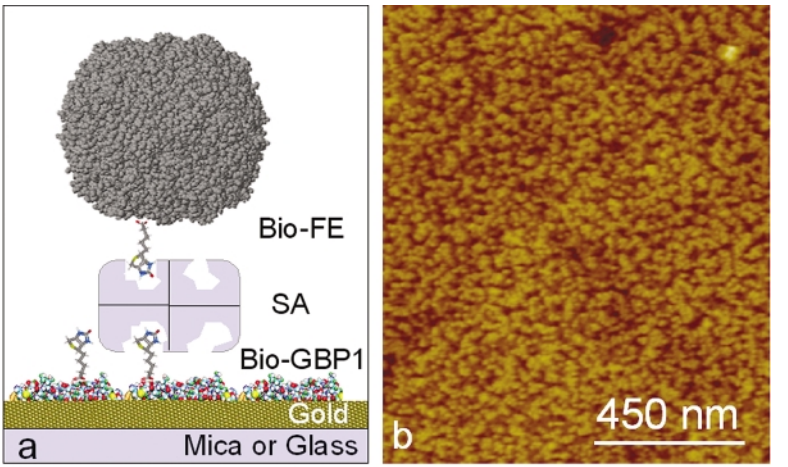

Figure 15 An experimental demonstration of GEPI as a possible molecular linker in nanobiotechnology. (*a*) A schematic illustration of targeted binding of biotinylated ferritin (bio-FE) on strep+avidin (SA, probe), immobilized on bioreactive, biotinylated GBP-1 monomolecular film assembled on Au(111). (*b*) AFM image of the assembled molecular structure (M.H. Zareie & M. Sarikaya, unpublished data).

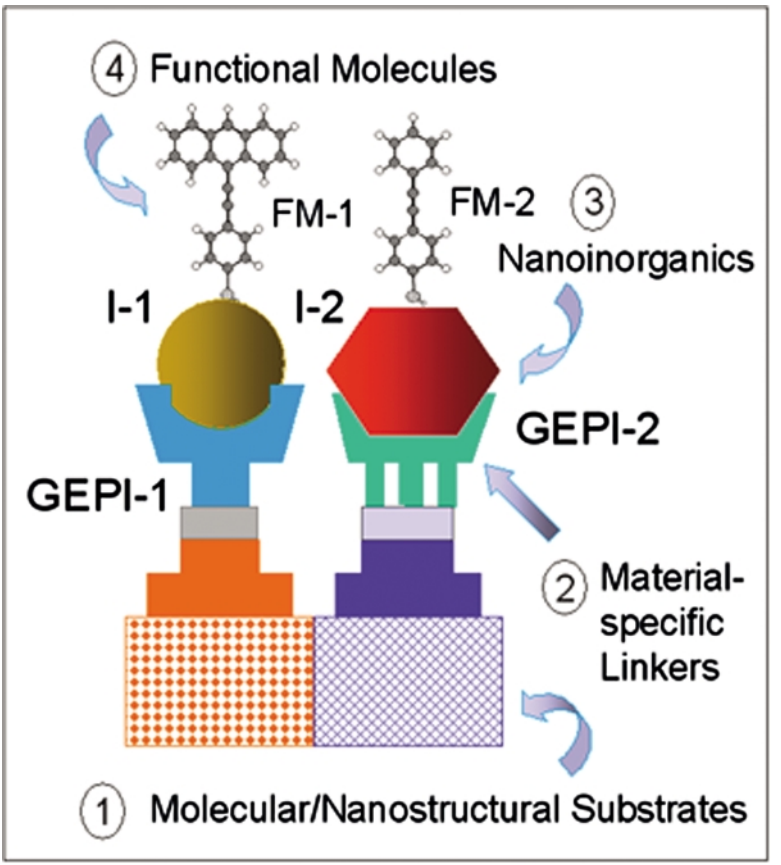

Figure 16 An illustration of the potential of GEPIs as molecular erectors in functional nanotechnology. Two GEPI proteins (GEPI-1 and GEPI-2) assemble on a patterned substrate. One could use either a designer protein, followed by genetic fusion of the respective GEPIs, or directed assembly GEPIs on a nanopatterned substrate. Two inorganic particles (I-1 and I-2) are immobilized selectively on GEPI-1 and GEPI-2, respectively. Synthetic molecules (i.e., conducting or photonic) via functionalized side groups or hybridized GEPIs are assembled on the nanoparticles.

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