Invited Review
Molecular Biomimetics: GEPI-Based Biological Routes to Technology

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ABSTRACT:

In nature, the viability of biological systems is sustained via specific interactions among the tens of thousands of proteins, the major building blocks of organisms from the simplest single-celled to the most complex multicellular species. Biomolecule-material interaction is accomplished with molecular specificity and efficiency leading to the formation of controlled structures and functions at all scales of dimensional hierarchy. Through evolution, Mother Nature developed molecular recognition by successive cycles of mutation and selection. Molecular specificity of probe-target interactions, e.g., ligand-receptor, antigen–antibody, is always based on specific peptide molecular recognition. Using biology as a guide, we can now understand, engineer, and control peptide-material interactions and exploit them as a new design tool for novel materials and systems. We adapted the protocols of combinatorially designed peptide libraries, via both cell surface or phage display methods; using these we select short peptides with specificity to a variety of practical materials. These genetically engineered peptides for inorganics (GEPI) are then studied experimentally to establish their binding kinetics and surface stability. The bound peptide structure and conformations are interrogated both experimentally and via modeling, and self-assembly characteristics are tested via atomic force microscopy. We further engineer the peptide binding and assembly characteristics using a computational biomimetics approach where bioinformatics based peptide-sequence similarity analysis is developed to design higher generation function-specific peptides. The molecular biomimetic approach opens up new avenues for the design and utilization of multifunctional molecular systems in a wide-range of applications from tissue engineering, disease diagnostics, and therapeutics to various areas of nanotechnology where integration is required among inorganic, organic and biological materials. Here, we describe lessons from biology with examples of protein-mediated functional biological materials, explain how novel peptides can be designed with specific affinity to inorganic solids using evolutionary engineering approaches, give examples of their potential utilizations in technology and medicine, and, finally, provide a summary of challenges and future prospects. © *2010 Wiley Periodicals, Inc. Biopolymers (Pept Sci) 94: 78–94, 2010.*

Keywords: *molecular biomimetics; materials binding peptides; nanotechnology; evolutionary engineering; materials synthesis; assembly*

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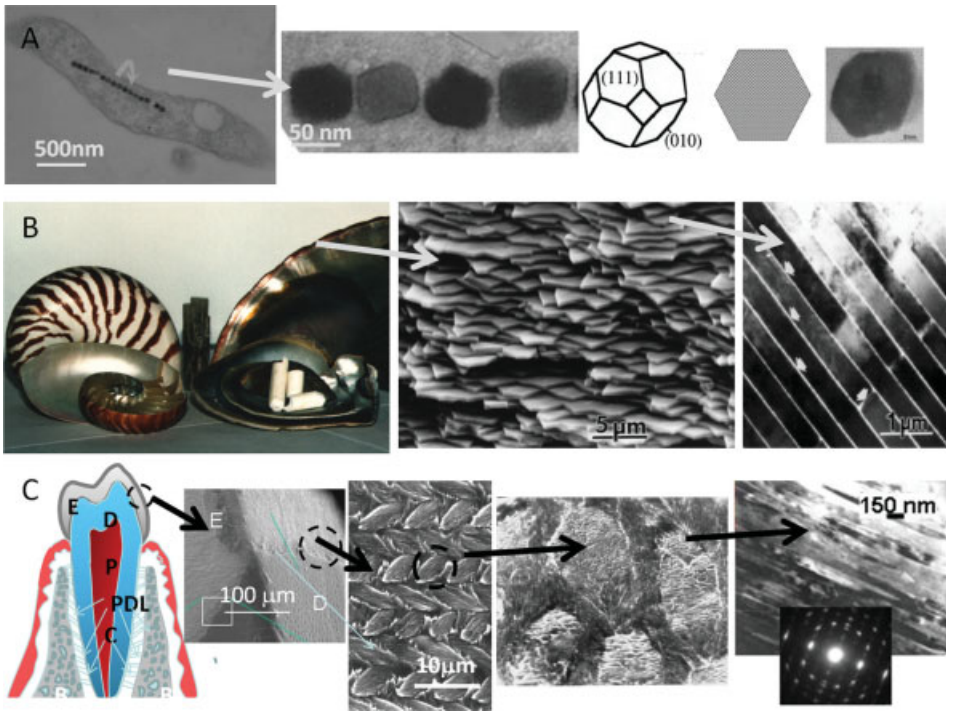

FIGURE 1 Examples of functional biological materials systems. (A) Magnetotactic bacteria, e.g., *Aquaspirillum magnetotacticum*, have aligned cubo-octahedral-shaped superparamagnetic particles.[8] (B) Nacre, mother-of-pearl is a segmented laminated composite of calcium carbonate and biomolecules having mechanical properties that provide an armor to the mollusks such as red abalone, pearl oyster, and nautilus.[8,10] (C) Mammalian tooth is a hierarchical multimaterial system composed of enamel (E), dentin (D), pulp (P), cementum (C) and periodontal ligaments (PDL). Enamel, such as the one shown here from mouse, is ~100% hydroxyapatite crystals that have a hierarchically ordered woven structure providing stability against complex mastication mechanical stresses.[11]

LESSONS FROM MOTHER NATURE AND MOLECULAR BIOMIMETICS

For the last three decades, biological hard tissues have been an inspiration for materials design and engineering due to their architectural organization from molecular to nano-, micro-, and macro-scales, often in a hierarchical manner with intricate nanoarchitecture that ultimately makes up a myriad of different tissues. They are simultaneously "smart," dynamic, complex, self-healing, and multifunctional, the characteristics difficult to achieve in purely synthetic systems. Based on their highly controlled nanostructures, biocomposites have properties of high-technological interest that surpass synthetic systems with similar phase compositions.[1–3] Biomimetics can be considered as a particularly promising path to realizing nano- and bionanotechnology. There is indeed a rich and long history of inspiration from Nature's biological structures to design practical materials and systems.[4,5] Traditionally, biomimeticists have focused on emulating or duplicating biosystems using mostly synthetic components and conventional approaches.[5,6] With a growing understanding of the processes involved, biological principles are now revisited as novel routes in materials assembly and fabrication for technological applications. By merging recent advances in molecular biology with state-of-the-art engineering and physical sciences, the new goal in the emerging field of "Molecular Biomimetics"[7] is to shift the biomimetic materials science paradigm from imitating Nature to engineering materials at the molecular level and up using molecular biological routes.

Nature provides abundant examples of materials and systems with a rich variety of interconnections of function and resource exchange. The biological design principles have been tested over time, improved, and refined through evolution and result in optimized and elegant properties for a given set of conditions. Based on our previous research, Figure 1 shows three examples from biology where proteins control formation of diverse functional materials systems.[8–11] The first one is a single-celled organism that incorporates a magnetic nanoparticle system (Figure 1A). One of the first organelles produced by an organism probably

is magnetosome, an organization of protein and lipid-based closed membrane incorporating a magnetic nanoparticle that forms within the bacteria In *Aquaspirillum magnetotacticum*, magnetosomes, numbering about 25, align to create a magnetic moment large enough to sense the Earth's magnetic field (0.5 Gauss), a biologically made nano-compass.[12] The particles are magnetite (Fe_3O_4) formed as perfect cubo-octahedral single crystals, about 50 nms. in diameter, each having a single superparamagnetic domain and stationary within the magnetosome membrane. The particles in this species are crystallographically and morphologically aligned with respect to each other as to maximize magnetic field sensing that allows the cell to have a directional motility via magnetotaxis. Both the nano-compass formation and sensing are accomplished via a network of proteins.[12]

Figure 1B shows a classic biomimetic example: mother-of-pearl of seashells, the natural armor of mollusks.[8–10] The shell, in the interior, is a layered and segmented hybrid composite of aragonite (orthorhombic $CaCO_3$) and biopolymer mixture, nano-structurally integrated proteins and polysaccharides (e.g., chitin), with a 95/5 inorganic/organic volume ratio. Both the architecture of the soft and hard component phases and their chemical and mechanical coupling result in a unique composite with the highest specific toughness and fracture strength among all the known ceramic-based materials.[10]

The third example is our mammalian tooth (Figure 1C), which is comprised of several tissues one of which is enamel, the crown of the tooth in mammalians.[11] Dental enamel is the hardest material in the body and provides the protective cover to the tooth. Underneath, the enamel is integrated to dentin, the softer and, therefore, tougher bone-like tissue that absorbs the stress during cutting and chewing. Hierarchical structure of the enamel is orchestrated by a plethora of proteins that control the formation of ordered woven-like fibers of 3 μm-diameter enamel rods, each consisting of thousands of 30 nm-diameter, mm-long, and crystallographically-aligned elongated hydroxyapatite (HA) crystallites. This unique architecture provides the resistance to stresses preventing premature fracture or failure. Understanding the roles of proteins during biofabrication of teeth would provide means to develop protocols for regeneration of enamel, as well as other hard tissues, curing of caries of various kinds, and restoration of periodontium.

The common denominator in all of these examples of hard tissues, and countless soft tissues, is that, in addition to the inorganic materials, all contain biological molecules, mainly proteins. As shown earlier, in Nature, proteins are synthesizers directing nucleation, growth, and assembly of a variety of biological tissues with precise control of phase composition and architectures at the nano, meso-, and macroscales. In addition to their role in mineralization, proteins serve as enzymatic catalysts, are used as transport and storage molecules, mediate cell responses, are involved in immune protection and cell differentiation, and participate in virtually every process within cells.[13] Proteins, therefore, could be the key molecules in developing truly biomimetic, reliable hybrid materials systems for practical technological applications. Molecular biomimetics is an emerging field where assembly of hybrid materials can be achieved at the molecular scale using the specific recognition properties of proteins. The new field offers three unique advantages derived from Nature.[7] The first is that the peptides can be selected and engineered, using a set of design parameters, to molecularly recognize inorganic surfaces, each having a specific shape, crystallography, mineralogy, and chemistry. The second is that the peptide assembles on the specific surface or with the specific material in predictable manner leading to addressable molecules. Finally, the third one is that the applicability of genetic engineering approaches, i.e., the proteins and peptides can be produced using recombinant DNA technologies, where the genes of interest can be cloned and expressed in different organisms that synthesize them. This approach provides means to their mass production, creating fusion proteins with multiple functionalities by joining of two or more genes which originally coded for separate proteins. The process consequently leads to genetics based, elegant and precise control over engineering functions.[14] Current progress in the field points to a possible beginning of a new era where biological principles are not only used in designing materials which mimic the properties of biological counterparts, but also to take advantage of the functions and underlying principles of biomolecular processes to produce novel materials, systems and fabrication techniques that exhibit exquisite sensitivity, and accuracy, self healing, adaptability, control and, even, self replication. With this goal in mind, the following sections summarize the approaches in selecting solid binding peptides and applying evolutionary engineering tools to develop GEPIs, solid-binding peptides with material specificity, provide examples demonstrating their potential utilization in practical applications, and future perspectives.

GENETICALLY ENGINEERING PEPTIDES FOR INORGANICS, GEPI:

Biocombinatorial Methods for Selecting First Generation Inorganic Binding Peptides: The First Step in Directed Evolution of Materials

Development of combinatorial selection techniques more than two decades ago led to enormous progress in the

characterization of antibody-receptor, protein- or peptide-ligand interaction for a myriad of biotechnological and biological applications. Among them, phage[15] and cell surface displays[16–18] have been widely used. Both of the techniques rely on the link between phenotype and genotype of the organisms. Random peptide sequences that are encoded in either phage genome or plasmid bacterial DNA are displayed within the context of proteins localized on the surface of the phage particle or the cell, respectively. Outer membrane proteins, lipoproteins, fimbriae and flagellar proteins have been used to display the randomized peptide library on surface of bacteria,[5] while phage display utilizes the major or minor coat proteins of bacteriophage M13 to display the random peptides on the virus surface.[15–19]

Recently, we and others have adapted combinatorial selection methods and identified peptide sequences that are specific for various inorganic targets including noble metals (Au,[17,20,21] Ag[22,23] and Pt[24]), oxides (SiO_2,[25] ZnO,[26] Cu_2O,[26] TiO_2[27–29]), minerals (hydroxyapatite,[30] calcite,[31] graphite, sapphire[32]) and semiconductors (GaN,[33] ZnS and CdS[34]). Among biocombinatorial in vivo and in vitro methods, phage and cell surface displays became the predominant tools for material-specific peptide selection.[7,22,31,33,35] In the standard biopanning round, the library of phage or cell clones, displaying a vast population of randomized peptides on their surfaces, is exposed to inorganic targets. The un-bound clones are then washed away and the bound ones are eluted by physical or chemical methods and amplified for subsequent panning round. This biopanning cycle is then repeated, usually 3–5 times with increased stringency of washing steps, to enrich the population with clones having high-binding affinity to inorganic solid targets. In the final step, individual clones from the selected bound pool are isolated and binding peptide sequences are identified by DNA analysis of the phage genome (Figure 2). The advantage of the bacterial cell surface display system over phage display library is its greater efficiency in generating peptide sequences, simply because of the fact that the former does not need a second host organism to amplify and replicate genomic and plasmid sequences.

Much care must be taken when adapting and optimizing biopanning conditions for the selection of inorganic-binding peptides since the solid materials are quite different from proteinacous ligands for which the combinatorial selection techniques were originally developed.[7,36–38] Principally, any surface modification on an inorganic material, which might occur due to the solvent conditions used in biopanning, need to be prevented. In addition the form of the solid material used might limit the utility of particular display technology. For example, when the random peptide library is displayed

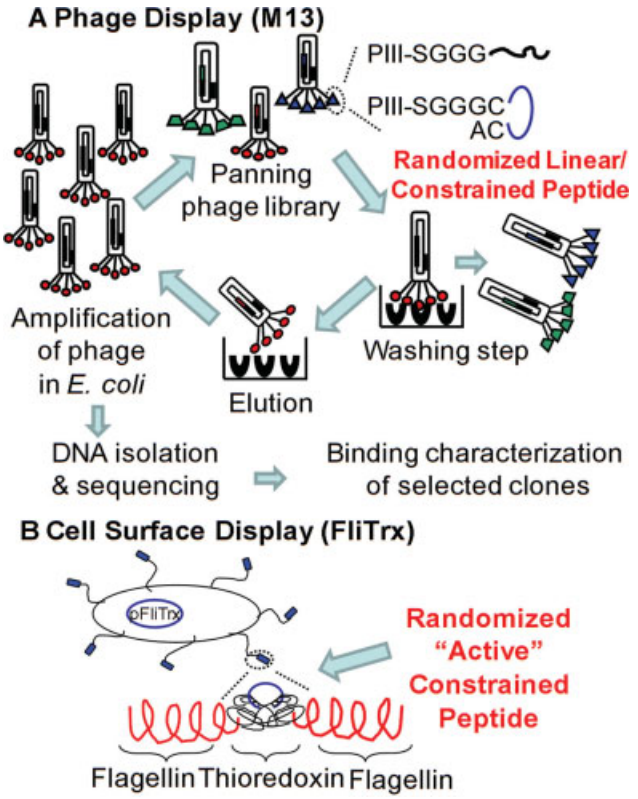

FIGURE 2 (A) Schematic summarizing the biopanning experiment using phage display system, M13, (B) Insertion site of the random peptide library in cell surface display system, FliTrx.

on flagellar proteins; any centrifugal force used in the biopanning step could disrupt and shear off the flagella from the cells and result in loss of tightly bound clones from the pool. Therefore, using inorganic powders or nanostructures is not feasible when flagellar proteins are used for display.[7,37] One may need to optimize the biopanning experiments for each material composition and morphology.

In general, a convergence towards a consensus binding motive is common in protein–protein interactions.[15,18,39] However the clear binding consensus might be difficult to identify in the selected inorganic-binding sequences based on our experience.[7,36] This probably reflects the heterogeneity of inorganic surfaces and various binding mechanisms implied in peptide-surface interactions. Consequently further binding analysis is essential in selecting truly material-specific peptides.[7,36] Following a successful biopanning experiment, we generally characterize around 50 clones, and each of the selected clones usually exhibits a different degree of binding strength to the solid of interest. In our group, we developed simple semi-quantitative binding assay via fluorescence microscopy (FM) to assess the affinity levels of individual

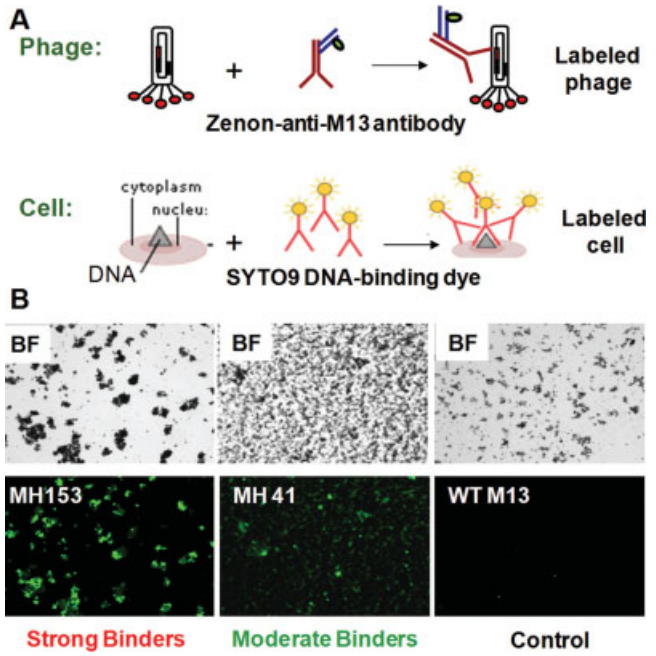

FIGURE 3 Initial binding characterization of biocombinatorially selected peptides using fluorescence microscopy (FM). (A) Schematics showing how the FM labeling is carried out on both phage and cell, (B) FM analysis of strong, moderate and weak binders texted on hydroxyapatite powder. Bright field and fluorescence images are provided on the first and the second row, respectively.

clones. Although qualitative, this approach proved to be highly efficient, in particular, in the design of second generation peptides (see below). The affinities of selected individual phage or cell clones are estimated by enumerating either surface coverage of either bound phage or cells via immunolabeling using fluorescently labeled anti-M13 antibodies or DNA-binding fluorescence dyes, respectively (Figure 3). The FM technique also enables characterization of binding specificities of the selected clones which are tested on various different inorganic solid surfaces. In our approach, a set of peptide sequences (e.g., 30 or 50) of experimentally selected clones from an initial random library is referred to as the first generation peptides. These first generation peptides could be used directly or can be further engineered by computational biomimetics approaches, incorporating bioinformatics tools and/or experimental mutagenesis approaches that mimic natural evolution, to produce second and higher generation, more functionally-specific peptides.

Computational Biomimetics: Designing Functionally Specific Solid Binding Peptides by Combining Experimental Knowledge with Bioinformatics

The understanding of protein recognition and binding to minerals and inorganic substrates with various affinities and specificities has been an important issue for some time for the purposes of understanding the correlation between solids and biomacromolecules, realizing hard tissue regeneration and, more recently, making practical materials using engineered peptides as molecular building blocks in synthesis, nanostructural organization, and directed assembly of functional materials. Experimental as well as modeling studies towards this understanding are in their infancy and accurate force field parameters required to model the protein/substrate interaction are still under development.[25,40–42]

Gaining insight from evolutionary biology, we developed a new way to address the design of inorganic-binding peptides with improved specific properties. In nature, proteins that perform functions similar to each other usually have similar sequences due to biochemical, biophysical and evolutionary constraints.[43] Founded on this observation, we proposed and have shown that the inorganic binding peptides, generated by in vivo selection, recognize the same material have similar sequences, much as evolutionarily related proteins do.[25]

Protein sequence alignment is one of the basic tools of modern biology and used for various analyzes, from detecting key functional residues to inferring the evolutionary history of protein families. In general, sequence pairs are aligned using an optimization procedure (e.g., dynamic programming)[44,45] which finds the best possible relative arrangement of the amino acids maximizing the overall similarity score. A scoring matrix is used to obtain the score for aligning two amino acids (match or mismatch) in an alignment of two protein sequences, and the overall score can be considered as a measure of the similarity between sequences. Among many, BLOSUM[46] and PAM[47] are two widely used scoring matrices derived from naturally occurring sequences. These matrices have been extensively evaluated for nucleotide and protein sequence comparisons with the primary goal of inferring homology or evolutionary relationships found in nature.

Here, we combine sequence alignment techniques[44,45] and produce unique, material-specific scoring matrices;[25,48] using these we then developed a bioinformatics method that allows design of new peptides that exhibit enhanced affinities and specificities to inorganic materials. As illustrated in Figure 4, the method starts with the sequence selection followed by binding characterization of inorganic binding peptides for specific materials. By doing so, the peptides selected are grouped into three sequence clusters, i.e., strong, moderate and weak, according to their material-binding affinities. Then, using sequence alignment methods[44,45] and the standard scoring matrices,[46,47] we generate a novel material-specific sequence scoring matrix[25] that would account for the

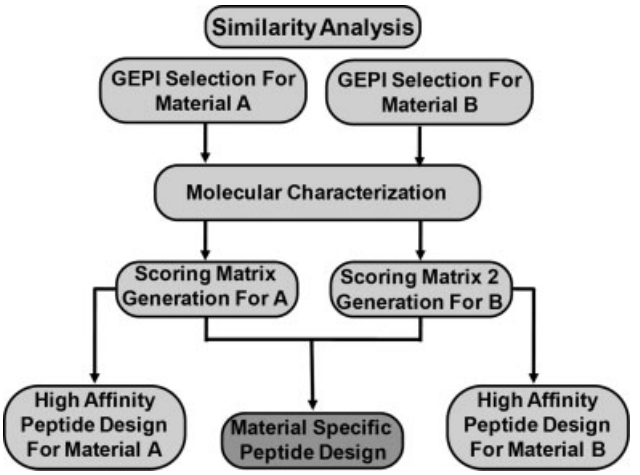

FIGURE 4 Knowledge based approach in generating next generation of peptides with superior binding properties.

specific sequence patterns responsible for binding (Figure 4) as opposed to sequence patterns found in weak binding group. Thus, to design novel high affinity binding peptides, one may generate random sequences and then calculate their sequence similarities to the experimentally known strong binding sequences. The sequences with the highest and lowest similarity scores are considered to represent the strongest and weakest binders, respectively. This technique has been prospectively validated in designing high affinity quartz binding peptides and thus our bioinformatics approach facilitated the *de novo* design of inorganic-binding peptides.[25]

The efficacy of the bioinformatics approach could be extended to design novel peptides with any arbitrary functional property (in addition to binding) as a utility in a wide range of applications in materials sciences, nano-technology, biology, and medicine. For example, using different material-specific matrices, one can design peptides that have multiple inorganic material recognition and binding functionalities (Figure 4).[48] Providing the initial sequence and characterization, this procedure could be applied to any given inorganic material. Alternatively, one can utilize the bioinformatics protocol to explore and better understand the very model system that inspired the original development of GEPIs: i.e., biomineralization proteins. In this case, GEPI sequences that are specific for hydroxyapatite could be used as a database for identifying mineral-binding regions within the sequences of poorly understood biomineralization proteins, such as those involved in tooth and bone formation. Such information could then be used to understand protein function in these medically important hard tissues and to eventually develop hard tissue-regeneration or practical engineering applications.

Understanding the Mechanism of Molecular Recognition: Sequence Versus Conformation

During the last decade, combinatorially-selected inorganic-binding peptides rapidly became important tools for bio- and nano-technological applications.[7,27,37,49] However, despite extensive recent reports about these peptides and their bio-nanotechnological utility, there is still only a limited understanding of the mechanisms that govern molecular binding to solids, assembly and organization.

The selected peptide sequence, when chemically synthesized outside the context of the chimera proteins, may exhibit decreased binding affinities than when still displayed from the surface of the phage or bacterium cells. One of the obvious explanations is the loss of multivalent peptide display. Moreover unlike natural proteins or protein domains, the short peptides do not generally fold into well defined three-dimensional structures and can often adopt multiple structural conformations in solution.[15,18,35] This may result in different structural conformations of the identical chemically synthesized peptide sequence from its displayed "active" conformation. Such effects on peptide binding may especially be observed when unconstrained "linear" peptides are selected from combinatorial libraries.[15,18] To overcome this disadvantage the random peptide sequences are displayed as cyclic peptides constrained by a Cys-Cys disulfide bond or displayed in the context of a protein scaffold, mainly to increase the probability of retaining active peptide conformations upon their chemical synthesis.[15] For example, in widely used M13 peptide display libraries (New England Biolab), 7 or 12 amino acid long peptides are displayed at the N-terminus of pIII coat protein in both either linear or Cys-Cys constrained loop forms. The randomized peptide sequences are separated from the phage coat protein by short flexible SGGG linker. In contrast, the FliTrx bacterial system (Invitrogen) uses the engineered extracellular flagellar protein (flagellin) to interact with external substrates. The main feature of FliTrx peptide libraries is that random peptides are inserted as fusions within the thioredoxin active site loop, which is itself inserted into a dispensable region of the flagellin gene. Thus random peptides are displayed on the surface of bacteria within the structural context of the thioredoxin active-site Cys-Cys loop, having both their N- and C-termini anchored by the rigid and stable tertiary structure of thioredoxin itself (Figure 2).[18]

In our most recent reports, we studied the structural effect of originally selected Pt-, Au-, and Cu_2O-binding peptides on their binding to inorganic surfaces.[20,24,50] All tested GEPIs were originally selected from constrained peptide libraries (PhDC7C or FliTrx). We assessed the effect of molecular architecture on peptide binding onto solid surfaces. In all

cases, we chemically synthesized respective peptide sequences in two different forms, linear (without the original structural constrains) and cyclic (mimicking the original display structure). We found that when the respective peptide sequence retains its molecular conformation in both *c*– and *l*–forms, it also preserves similar adsorption behavior on noble metal surfaces. In contrast, when the molecular structure of the respective sequence in the linear forms differs from their cyclic versions (originally displayed – "active") we also observed a decrease in their solid-binding affinities.[20,24] Similarly, when Cu_2O-binding motif selected from FliTrx peptide library was fused to the DNA binding protein TraI in either the Cyc-Cys disulfide bonded loop region or without the loop constraint, the binding affinities were found to vary greatly.[50]

The quantitative data towards determining kinetic and thermodynamic parameters of binding can be obtained using established techniques such as quartz crystal microbalance (QCM)[51] and surface plasmon resonance (SPR) spectroscopy.[52–54], By incorporating SPR and QCM with circular dichroism (CD), we were able to analyze the consequence of the loop constraint on peptide adsorption kinetics and the conformation of peptides, and relate them to each other with a comparative approach.[20,24,54] 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 cases. Our results showed that there is a correlation between conformational instability (or adaptability) and binding affinity.[36] Molecular conformation could therefore be addressed as a parameter to further investigate and tune the adsorption kinetics of the inorganic binding peptides.

A detailed understanding of the peptide recognition and assembly processes, will inevitably lead to better insights into the design of novel peptides for tailored binding. A better knowledge of the mechanism(s) of the quantitative adsorption may become possible through high resolution surface microscopy (e.g., AFM and STM), molecular spectroscopy and surface diffraction studies (such as small angle x-ray diffraction). Many of these techniques, with their advantages and pitfalls, offer fundamental information towards understanding the chemistry of surface binding and the physics of diffusion and assembly of peptides on solids. As these approaches continue to be adapted and effectively utilized in correlating molecular structures and binding, peptide based materials and systems would be better realized for specific implementations in practice in a more controlled and robust manner. In our next section, we will provide examples of current utilizations of GEPIs followed by a summary of potential future prospects.

CURRENT AND POTENTIAL MULTIDISCIPLINARY APPLICATIONS OF ENGINEERED SOLID-BINDING PEPTIDES

Controlled binding and assembly of proteins onto inorganic substrates are at the core of nano-technology,[55] bio-nano-technology,[56] as well as biological materials science and engineering[57] with a wide-range of applications.[58] GEPI provides the molecular means to anchor, couple, brace, display, and assemble functional molecules, constructs nano-particles, and molecular structures.[14] The examples given below provide only a limited summary of potential utilizations of engineered peptides. Functional proteins and enzymes anchored onto specific substrates would provide platforms for controlling cell differentiation and tissue engineering.[59] Multicomponent nano-particle substrates and probes would be the basis of multidrug delivery.[14] Engineered peptides hybridized with functional synthetic molecules could be used as hetero-functional building blocks in nano-technology, and in particular in molecular electronics, magnetics, and photonics.[60,61] Controlled protein adsorption and genetically engineered macromolecular interactions at solid surfaces via GEPI would provide new platforms for high performance of implants and hard-tissue engineering.[14,55] These examples and many others provide a window to the potential of GEPI-based materials science and engineering.

Directed Self Assembly of Nanoparticles and Proteins Through GEPIs

Inorganic binding peptides are shown as assemblers and linkers in the literature.[32,59,62] Functional nano-entities, such as proteins, nano-particles as well as organic dyes, are immobilized onto inorganic surfaces using the peptides.[32,62–66] Here, we give example on the assembly of peptides and their use in the attachment of nano-components onto solid substrates which has various applications, e.g., protein immobilization, biosensing, microarray fabrication.

Recent developments in nano-technology have shown that nano-scaled materials have a great potential especially to build up devices which can be applicable in different areas, such as electronics, medicine, and biotechnology.[67–71] Nano-particles are one of the most central elements in nano-technology due to their structure, composition, size and surface-related optical, magnetic, and electronic properties.[72–77] For example, noble metal nano-particles, such as gold and silver, absorb and scatter light intensively, so that they can be detected using optical microscopy under dark-field conditions[71,78,79] and used as labeling agents in immunoassays,[80,81] and cellular imaging.[82,83] Since these metal nano-particles can convert minute changes in the local refractive index into

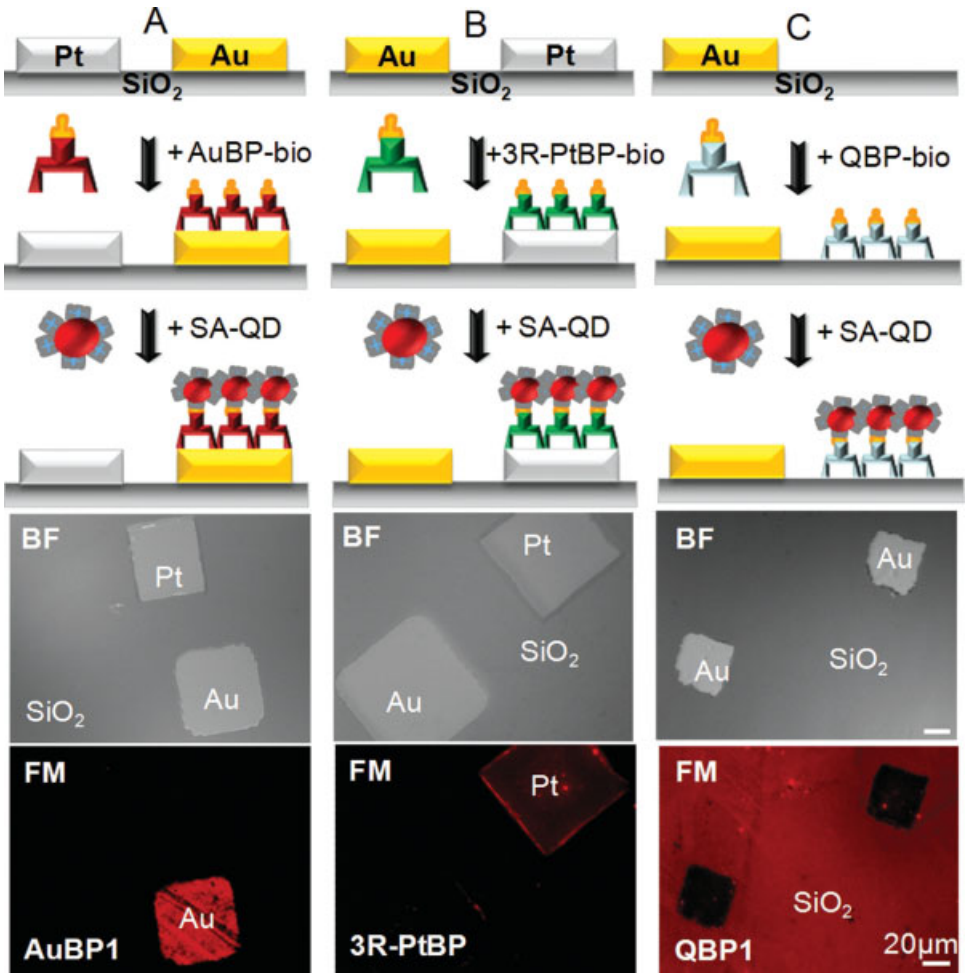

FIGURE 5 Preferential assembly of SA-QD through GEPI biotin conjugates on different inorganic surfaces. The substrates composed of either Pt and Au or only micropads on silica were incubated with either AuBP-bio (A), or 3R PtBP-bio (B) or QBP-bio (C). After the procedure schematically shown for each peptide was carried out the substrates were examined under FM.

spectral shifts in the scattering and extinction spectra, they can serve as nano-sensors to monitor binding of molecules onto the particle surface in real time with high sensitivity.[71,84,85]

Building up micro- and nano-scaled devices may require assembly of the nano-components onto multimaterial solid substrates.[86,87] Conventional immobilization approaches are based on weak physical adsorption, e.g., electrostatic interactions,[88,89] hydrogen bonds, e.g., DNA base pairing[90,91] and chemical coupling between the nano-particle and surface of the substrate through self-assembled monolayers (SAMs) of synthetic molecules that act as linkers.[78,85] These linkers, such as aminoalkyl alkoxysilanes[92] for silica and carboxyl-terminated alkanethiols[93] for gold, are mostly toxic, nonspecific and need complex chemical steps for efficient immobilization.[85,94,95] Moreover, SAMs of thiols have been shown to be susceptible to oxidation and disassociation.[66,96,97] The oligonucleotides may also require functionalization with thiol groups to maintain the chemical affinity for metallic surfaces, e.g. gold and silver.[78,81]

Alternatively, GEPIs, genetically engineered peptides for inorganics, the theme in this work, have been used as linkers for the attachment of nano-entities onto different materials due to their high affinity and material specificity.[7,35,98] Our first example demonstrates both assembly and material specificity of gold binding peptide (AuBP), three repeats of a platinum binding peptide (3R-PtBP) and a quartz binding peptide (QBP) (Figure 5), all isolated and well characterized by our associated groups.[20,24,25] Here, the peptides are used in immobilization of streptavidin-coated quantum dots (SA-QD) that emit red light under the fluorescence microscope (FM). The experimental procedure is shown for each peptide

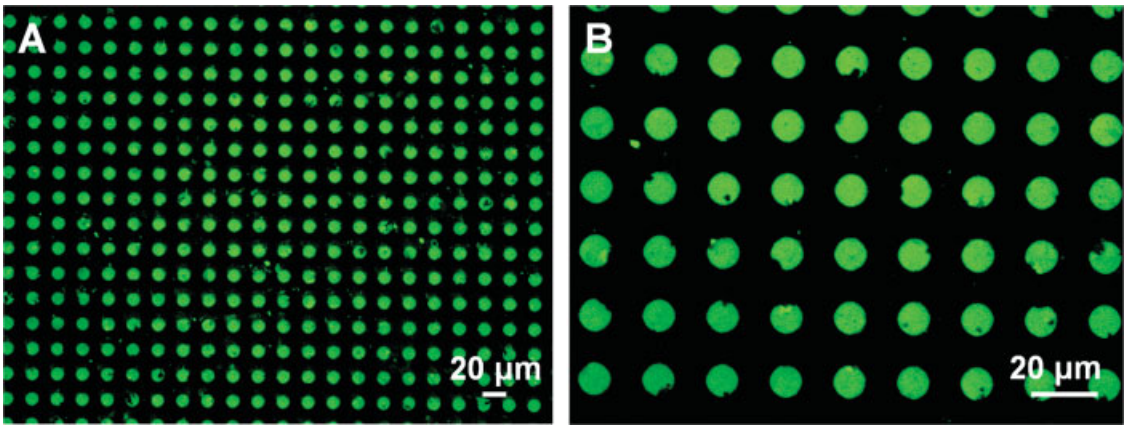

FIGURE 6 FM images of patterns produced by microcontact printing of QBP-F (A–B). After printing the peptide-fluorophore conjugate, each substrate was throughly rinsed with water and dried before FM characterization.

schematically in Figure 5. Basically, it includes sequential assembly steps for biotinylated GEPI (GEPI-bio) and SA-QD (strepavidin coated quantum dot). Following washing and drying steps, the substrates are examined using FM. Assembly of SA-QD is achieved on an array of two different microscale metal pads, i.e. gold and platinum, on silica using an appropriate GEPI-bio (In Figures 5A and 5B). For the substrate incubated with biotinylated AuBP (AuBP-bio), red emission is observed only on the gold pads (Figure 5A), whereas in the case of 3R-PtBP-bio treatment, red emission is only observed on the platinum pads (Figure 5B). Similarly, the same experimental procedure is repeated with QBP-bio to attach the SA-QD on silica regions of a separate substrate containing microscaled gold pads on it (Figure 5C). Here, we monitor the red emission emanating from the assembled SA-QD on the silica substrate, and not the gold pads. Overall results show that it is possible to directly assemble SA-QD on a micropatterned substrate composed of multimaterial regions, e.g. gold, platinum, silica, using the material-specific binding capabilities of GEPIs.

In biotechnology, in another example, site-specific protein immobilization is required for the fabrication of efficient tools such as enzymatic assays, protein chips, biosensors and microarrays.[99–104] Mainly, the conventional methods, e.g., physical and chemical immobilizations that have been used for the nano-particle attachment onto solid substrates are also applicable for protein immobilization[93,102] while the drawbacks mentioned earlier remain a concern. Moreover, both approaches may cause a decrease in protein activity due to the uncontrolled assembly (e.g., random orientation) following interaction or reaction between functional groups of the protein and the activated support surface.[101,102] Furthermore, physical interactions are weak and can cause protein desorption.[102]

As a recent alternative method, inorganic binding peptides have been called for their utilization in protein immobilization. Using inorganic binding peptides, we have demonstrated the immobilization of functional proteins, such as maltose binding protein (MBP) and alkaline phosphatase (AP), on various inorganic surfaces, such as sapphire, gold, and zeolite.[32,59,66,105–107] Genetic insertion of the desired peptide sequence into the protein genome can be used to produce hetero-functional constructs with an additional function, i.e., inorganic binding affinity. For this purpose, the insertion can be located at either one of the termini, i.e. C-terminus[32] and N-terminus,[59] or a permissive site identified on the protein.[108] In a particular example, AP has been assembled onto a gold surface using the gold binding peptide GBP1. The protein showed enhanced activity when bound to the surface.[59]

In protein and peptide microarray technology, there are several ways to fabricate micro/nano-scaled platforms, e.g., photolithography,[109] soft lithography,[110] dip-pen lithography[111] and others. With combination of these methods, advances in GEPI-driven assembly allow the preparation of biofunctional platforms under ambient conditions. Recent studies show that not only GEPI-based constructs can be immobilized onto targeted inorganic regions through self-assembly[98] but also they can be used as ink and printed onto the solid surfaces specifically using different lithography techniques, e.g., soft lithography[62,64] and dip-pen lithography.[112] We demonstrated that the quartz binding peptide QBP can be used as both ink and linker for microcontact printing (μCP) and self-assembly, for an organic molecule, fluorescein (FITC). As shown in Figure 6, QBP-F could be successfully stamped on quartz producing large scale micropatterns.

Apart from the peptide itself, GEPI-protein fusion products can also serve as ink in the μCP procedure, forming a

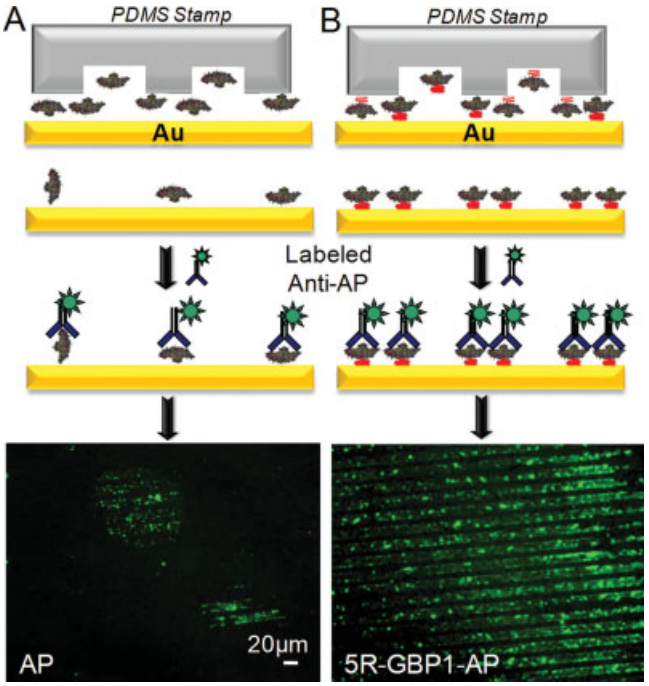

FIGURE 7 Assembly of Anti-AP on either AP (A) or 5R-GBP1-AP (B) printed on gold by μCP. AP alone was used as control. The experimental protocol for functionalizing the surface is schematically represented for each protein. The substrates were characterized by FM.

probe for subsequent antibody coupling on an inorganic surface. In Figure 7, assembly of AP-antibody through a coupling with microcontact printed 5R-GBP1-AP on gold surface is demonstrated. The polydimethylsiloxane (PDMS) stamp pretreated with either AP without GBP insert (Figure 7A) or 5R-GBP1-AP (Figure 7B) which was brought to contact with gold surface is shown. After washing and drying the substrates, FITC-labeled Anti-AP incubation was carried out on the protein-patterned surface. The substrates were then characterized by FM following final rinsing and drying the gold surfaces. The control experiment shows that the assembly of Anti-AP was inefficient (Figure 7A) whereas the GBP linkage maintains the immobilization of the protein, 5R-GBP1-AP (probe), leading to a successful Anti-AP (target) assembly.

Overall, GEPIs have been demonstrated to be successful molecular linkers and assemblers to immobilize nano-particles and organic molecules as well as proteins on different inorganic platforms. The inorganic binding peptides can be chemically linked to other linker molecules, e.g., biotin (Figure 5) and organic molecules, e.g. FITC (Figure 6). Also, genetic insertion of the peptide sequence into a desired protein gene produces hetero-functional fusion biomolecules (Figure 7). GEPIs through their specific molecular recognition, self assembly properties and suitability to genetic modifications have increasingly proven to have enormous potential in a variety of applications, such as sensing, cellular imaging, and immunoassays.

GEPIs as Molecular Linkers in Biomaterials Applications

Molecular immobilization on inorganic surfaces has become an engineering consideration in a wide range of fields as the attention of the scientific community is drawn to nano- and subnano-technology. As discussed previously, it is relevant to the efforts in enzymatic and nano-catalysis,[113,114] sensor and detector applications,[115,116] lab-on-a-chip development,[117,118] as well as tissue and biomaterials engineering.[119–121] Particular requirements in each application vary significantly and include such variables as density of immobilization, chemical and biological stability, toxicity of the assembly processes and products, directionality, and material-specificity.

The most notable techniques for molecular immobilization include SAMs, Langmuir-Blodgett (LB) deposited films, direct chemical conjugation, especially on polymers, plasma deposition and physical adsorption.[120,122] The common thiol and silane SAMs bind to noble metals and oxides, respectively, and form very dense films due to the lateral van der Waal's interactions of their hydrophobic tails.[123,124] The major advantages of the SAMs are their binding strength, density, certain level of stability and in some cases, simplicity of application.[125,126] As discussed above, a number of molecules, however, require complex reaction conditions, as in the case of silanes, while very few can be assembled in biocompatible environments. Additionally, the toxicity of many of these constructs remains in question, making it harder to employ them for in vivo applications.[127] Finally, most of the head group chemistries are only applicable to a narrow range of solids, and are incapable of differentiating within that range of materials. By contrast, LB films can be assembled without any chemistry and can have the advantage of mimicking a cell membrane. They usually consist of amphiphiles that can be used to form an oligo-layer and functionalized further. Although these layers can be chemically cross-linked to increase their stability, many are relatively unstable as compared to other available techniques.[128] The LB deposition method allows for great control over the thickness, density and the displayed chemistry of the film. It is, however, a rather difficult approach to optimize, making it hard to transition between materials, and, therefore, their use has been significantly limited recently. Polymers can be easily modified chemically through functional groups. This type of surface modification is both robust and specific, but requires the

polymer to contain particular chemical arrangements, meaning that either the bulk or the surface has to be engineered with further modification in mind.[120,129] The plasma deposition technique has been used to modify a range of materials. This technique is best used to change the chemical properties of the surface, rather than to display functional species because the thickness of the adsorbed layer cannot be precisely controlled, and because more complex species are damaged in the plasma.[130] Another notable, recent, immobilization technique uses a mussel adhesive-protein derived amino acid, L-3-4-dihydroxyphenylalanine known as DOPA, which adsorbs strongly to many materials.[131,132] It has already been used to create both bioactive and bioinert surfaces via the RGD integrin binding sequence[133] and poly(ethylene glycol) antifouling polymer (PEG) respectively.[131] The modification can be carried out in biological conditions; however some questions have been raised in regard to possible toxicity of the linker, since it is used as a Parkinson's disease medication. DOPA also completely lacks material specificity necessary for more complex nano- and bionano-technological platforms.

GEPIs combine many of the advantages of the molecular immobilization techniques discussed above while being uniquely material-specific. Most solid-binding peptides bind quite strongly to their respective materials, with K_d values in the single μM range, while some display K_d's in the hundreds of nM and even 10s of nM range.[25,134] Several of them have already been shown to form densely packed monolayers, which is useful in surface engineering and modification applications. One of the more significant advantages of GEPIs is their ability to assemble in entirely aqueous solutions under ambient conditions (e.g., pH $\sim$7.0), making biologically safe modifications more straightforward. This capability also means that simultaneous application to multiple materials from a single solution is possible. While GEPIs have not yet been tested for toxicity in vivo, none of them so far have displayed any cytotoxicity in vitro. Additionally, absence of any chemical groups and elements normally associated with toxicity makes it reasonable to expect that none will be discovered. Although likely to be unstable in some chemically harsh environments, the peptides can remain bound and chemically stable in biological conditions. Although the GEPIs' effects on the immune system and susceptibility to peptidases remain to be examined, the wide range of possibilities in the peptide sequences will allow certain measures to be taken to prevent adverse effects by, for example, constraining the vulnerable binding sequence with cysteines. Another difficulty in using GEPIs as molecular erectors has been the preservation of the binding properties after conjugation with the active species. We showed that some gold-binding sequences lose their affinity when conjugated with PEG by amine-aldehyde chemistry. The loss has been attributed to the disruption of the secondary structure of the peptides by the excessive conjugation with the bulky polymer. This problem is currently being addressed through the development of connective sequences, which would allow both parts of the construct to maintain their function without interference from the other. Finally, GEPIs are unique in that they can be designed through in silico methods to bind specifically to one type of material over another (Computational Biomimetics: Designing Functionally Specific Solid Binding Peptides by Combining Experimental Knowledge with Bioinformatics section). The capability to *de novo* design GEPIs with multiple material-specificity enables self-assembly of various active species on complex multimaterial substrates specifically, thus enabling hitherto difficult implementations. In combination with the ability of solid binding under aqueous solution conditions across all currently identified GEPIs, single-step assembly and preparation of such system will be feasible in the future.

From the discussion above regarding the properties of GEPIs, it is evident that they can be useful in a variety of immobilization applications, but are also uniquely suited for modification of biomaterial surfaces. As such, several articles have already been published demonstrating bioactive and bioinert surfaces created via GEPI linkers on a number of materials.[135–137] Here we present the work that we carried out for four different materials surfaces (gold, platinum, silica glass, and titanium) as proof-of-concept substrates because they are relevant to the implant industry. The high chemical stability of gold and platinum make them ideal for bioinert applications in implants that are in direct contact with blood. Therefore, we use two different linkers (3R-GPB1 and PtBP1) to functionalize these surfaces with PEG anti-fouling polymer. For PEG immobilization we employ the two-step targeted assembly process (Figure 8B), in which the peptides are first immobilized on the surface and are then chemically conjugated with the activated polymer by Schiff-base chemistry. After optimization we obtain surfaces that are able to resist cell adhesion as well as those formed by oligo(ethylene glycol) thiol SAM on gold (Figure 9A). In the same study, we immobilize the RGD integrin-binding sequence on silica glass using QBP1 peptide because that type of material is often used for bone grafts and other tissue-integrated applications. This modification is accomplished through the single step directed assembly process (Figure 8A) by synthesizing the QBP1 sequence in tandem with the RGD through a GGG connector, and then placing it on the surface. The resulting samples promote both the adhesion and spreading of cells, exhibiting an increase only when both parts of the construct are present (Figure 9B). More recently we reproduced the RGD immobilization results on

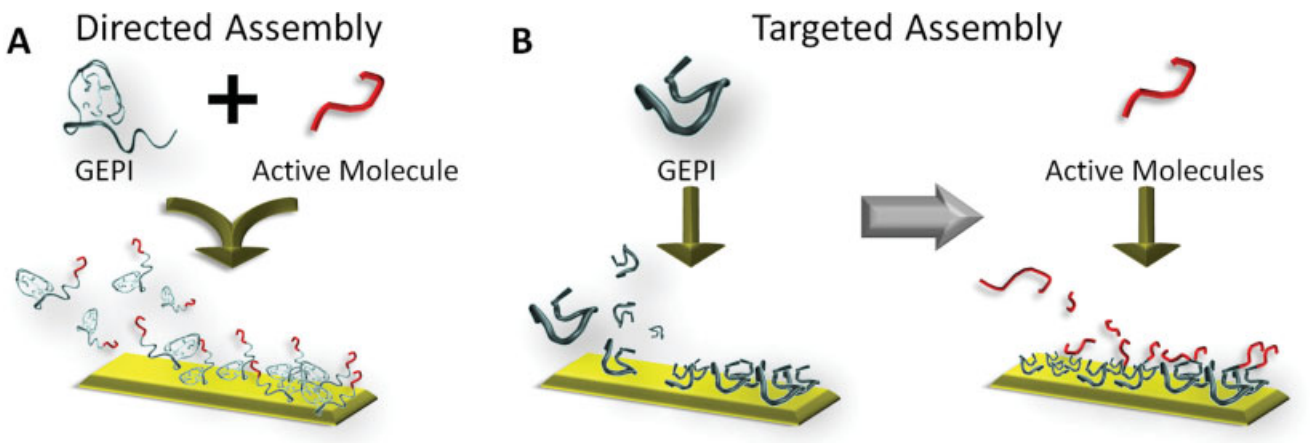

FIGURE 8 (A) Directed assembly—GEPI is conjugated with the active molecule before immobilization on the substrate. (B) Targeted assembly—GEPI is immobilized on the substrate alone, then conjugated with the active molecule by secondary means, such as Schiff base chemistry.

titanium, another tissue-integrating material. We used titanium-binding peptide, TiBP, synthesized with RGD to modify the surface in a single step. When the peptides are immobilized on the glass surface, the resulting layer displays an increase in both cell adhesion and spreading when both portions of the construct are present (Figure 9C).

Although the steps already taken, in employing GEPIs as linkers in biomaterials applications, are significant, the path to clinical application has not been covered yet. Demonstrating the ability of GEPIs to function in vivo, as well as confirming their safety, is necessary for their extensive utilization in the biomaterials area. Understanding the mechanisms of binding in a number of peptide-solid systems may allow the design of new sequences that combine different functional domains that do not interfering with each other. Cell patterning is another area where GEPIs may play an important

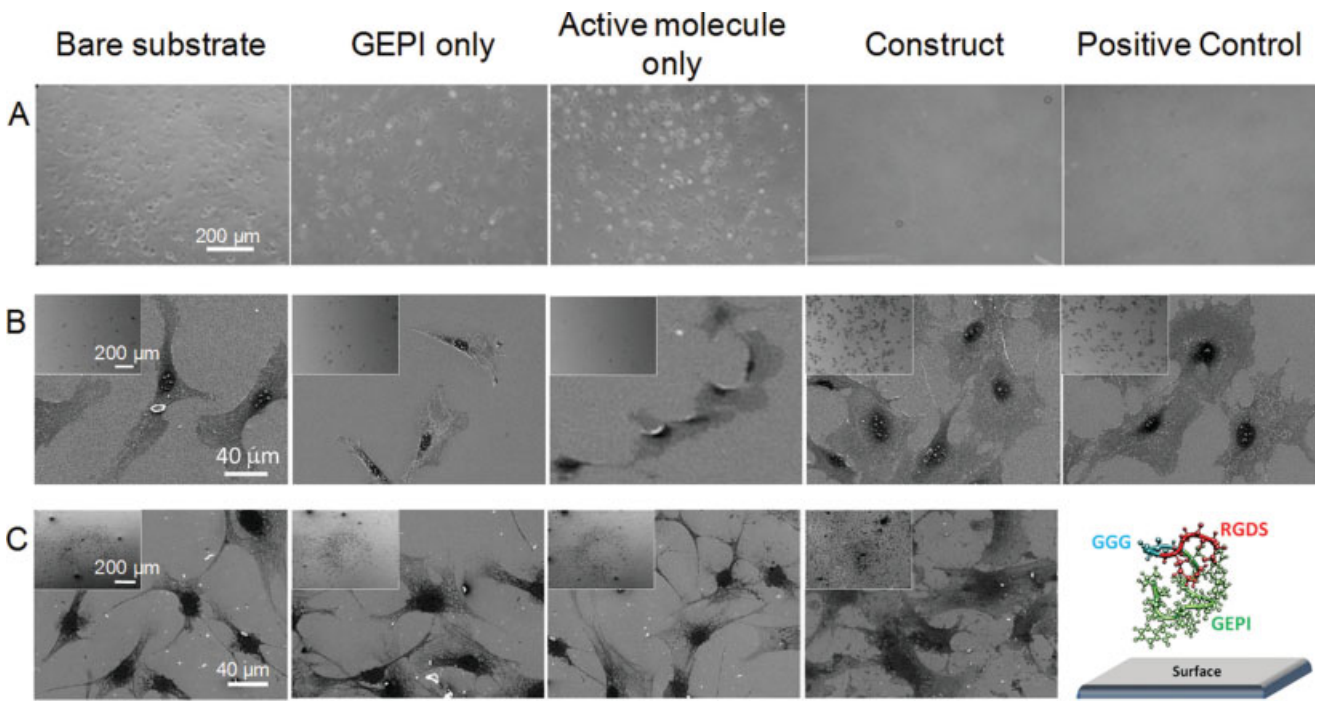

FIGURE 9 (A) Light optical microscopy images of NIH3T3 fibroblast cells (3×10^5 cells, 1.5 hrs, 200×) on gold substrate modified with GEPI-PEG conjugate; (B) Scanning electron microscopy (SEM) images of NIH3T3 (5×10^3 cells, 24 hrs) on glass substrate modified with GEPI-RGD conjugate; (C) SEM images of NIH3T3 (8×10^4 cells, 24 hrs) on titanium surface modified with GEPI-RGD conjugate.

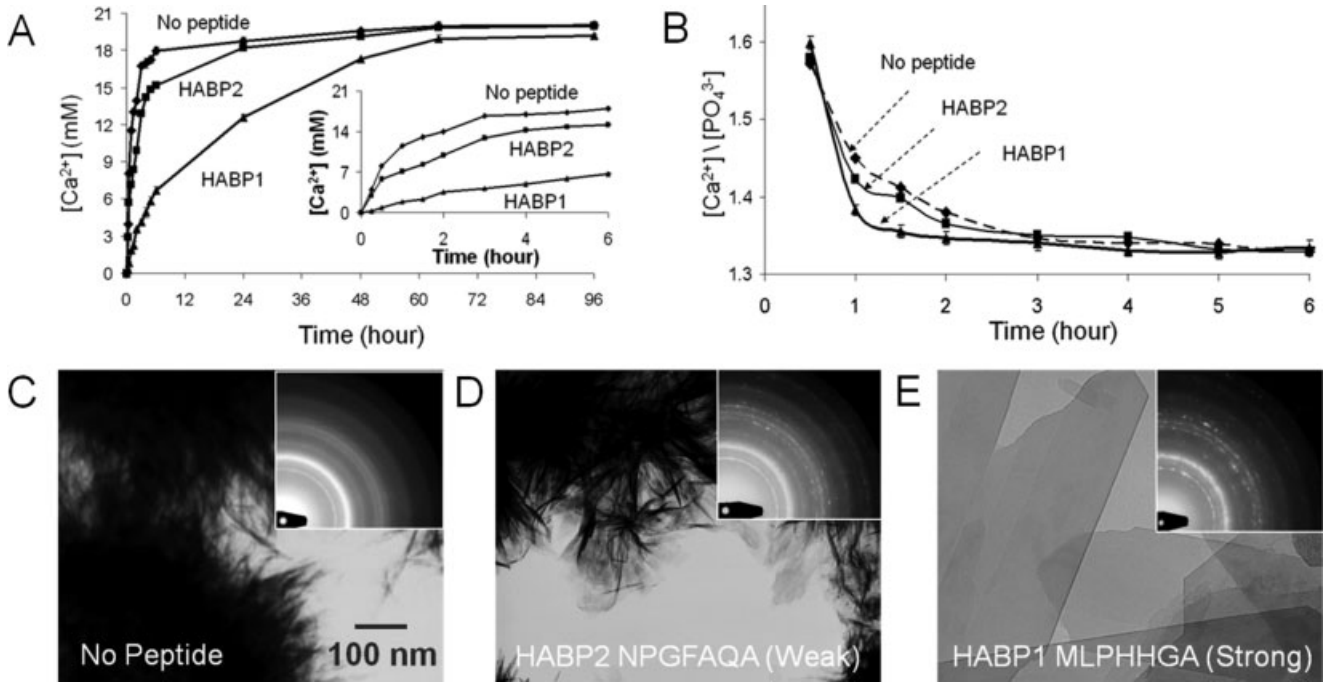

FIGURE 10 (A) Rate of Ca^{2+} consumption in the presence of peptides; inset: the consumption rate during the first 6 h. (B) Ca/P ratio of the mineral phase at different time points. The final morphology of the minerals after 96 hours of mineralization in the presence of (C) no peptide, (D) weak binding control peptide and (e) strong binding peptide, HABP1.

role by allowing modification of inorganic patterns produced by standard lithography techniques to display different functionalities on different materials on complex substrates. The usefulness of these versatile, easy-to-use and specific linkers will only increase as we and others continue to standardize the conjugation processes, hone the in silico selection procedures, expand the range of identified sequences, develop heterofunctional peptide-based linkers, and learn more about the inner workings of GEPIs.

GEPIs as Synthesizers of Nanoinorganic Structures

Inorganic materials synthesized by biological organisms, by processes such as biomineralization, have astonished scientists for a long time because of their unique and optimal morphological, structural, and functional properties. The observation that proteins control nucleation, crystallography, polymorphism, and morphology of biogenic inorganics[4,138] has led to development of biomimetic approaches to utilize them in designing and engineering of functional materials. The traditional approach involves extracting and purifying proteins from organism of interest and utilizing them for in vitro material synthesis.[138–141] Although there are exciting examples, performing biomineralization using isolated proteins is limited and the results widely reported are inconclusive, mainly because of the difficulties involved in the extraction and purification of these complex proteins from the biological systems and detailed characterization of their individual functions. Another approach in using proteins in materials is *de novo* design via predictive methods.[142,143] Usually there may be a large number of proteins with various temporal and spatial distribution involved in a given biological mineralization process. It is impractical, for the time being, to expect all proteins to be extracted and purified or their sequences to be available in the existing protein databases. Therefore, practical biomineralization toward tissue regeneration using proteins extracted from hard tissues or those *de novo* designed remains elusive.

GEPIs offer unique and a more practical approach in biomineralization towards restorative and regenerative tissue engineering. Given that the GEPIs recognize and bind to solid materials, there may also be an inherent capability within the sequences to influence fabrication process of these inorganic solids as well. This premise has proven to be an effective approach in developing truly biomimetic materials by making templated conjugates, producing protein-encapsulated particles and controlled synthesis of nano-particles and minerals.[30,31,108,144] Besides those materials that are important for nanotechnological applications, these peptides can be utilized to control the formation of biologically

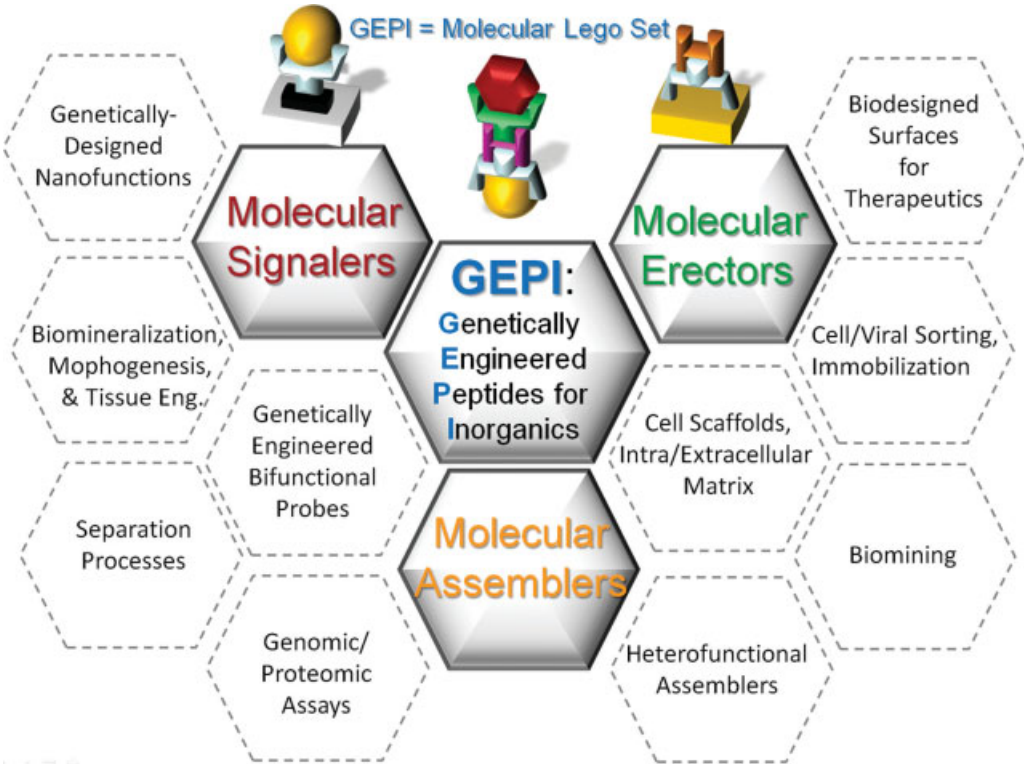

FIGURE 11 GEPI as molecular building block as nanoinorganic synthesizers, linkers for nanoentities, and assemblers of functional nanostructures.

important hard tissues, i.e., bone and teeth, an opportunity which has not yet been fully explored in sufficient detail so far.

To test this possibility we have selected hydroxyapatite, HA,-binding peptides that could offer a route for controlling calcium phosphate-based biomineral formation within a biomedical context. Specifically, this study involves a successful screening of a cysteine-constrained M13 bacteriophage heptapeptide library against HA powder. We were interested in exploring the possibility of HA-binding peptides to regulate calcium phosphate formation in vitro and, likewise, determine the contributions of primary sequence and secondary structural properties that are associated with HA affinity as well as calcium phosphate formation capability. We found that a strong-binding peptide (HABP1, NH_2—CMLPHHGAC—COOH) affects formation of calcium phosphate mineral in several aspects (Figure 10). We observed that the addition of HABP1 slowed the rate of initial mineralization, resulting in the formation of much larger plate-like particles compared to control samples (weak-binding peptide, HABP2: NH_2—CNPGFAQAC—COOH, and no-peptide containing solutions) and finally, increased the rate of transformation of the amorphous phase to the crystalline phase.[30] This conversion may involve interactions of HABP1 with the amorphous mineral surface, which in turn stabilizes the crystal structure by lowering the surface energy, therefore resulting in a growth-dominated mineralization pathway. Although the exact mechanism of HABP-mediated mineral formation is not known, our molecular structural studies using CD show that HABP1 and HABP2 exhibit important differences with regard to primary sequence, secondary structure, and conformational stabilities. More specifically, HABP1 adopts an equilibrium of random coil and PPII type structure while HABP2 adopts a relatively stable PPII type structure in the absence of the surface,[30] We believe that these molecular features have an impact on the observed differences in HA binding affinities and mineralization behaviors and could be further "tweaked" to select or synthesize crystalline minerals with desired properties.

FUTURE PROSPECTS AND POTENTIALS OF MOLECULAR BIOMIMETICS

There is an ever increasing multidisciplinary effort in shifting the biomimetic materials and technologies from imitating Nature to producing practical materials using biological routes from the molecular scale and up. Combining the

expertise, maturity, and opportunities provided by the biological fields such as molecular biology and genetics and the new developments of concepts and applications in molecular and traditional materials sciences and engineering could open novel pathways towards establishing molecular and nanoscale technologies both in engineering/nanotechnology and biotechnology/medicine. Based on the potentials of GEPI, genetically engineered peptides for inorganics, the emerging field of molecular biomimetics may serve as a platform for achieving the goal of efficient ways for the design and fabrication of complex materials with multiple functionalities. As we demonstrated here, GEPIs can act as fundamental molecular building blocks in nanoinorganic assembly as well as materialization processes through their unique and tailored functionalities. GEPIs coupled with solid substrates from nanometer to microscales, and from single to multimaterial substrates, are already increasingly helping to establish new protocols towards functional hybrid material systems. Genetic design and control of coupling for versatile multifunctional molecular probes offer an enormous potential in a wide range of applications from medicine to fundamental technologies such as electronics, photonics, and magnetic, with provisions for device fabrications. The multifunctionality of the hybrid system could be introduced either having two or more of the peptides combined to create novel ways of making dissimilar materials (e.g., gold and silica) kinetically compatible, or they can be genetically inserted into other functional biological molecules (such as enzymes, antibodies, and DNA) to create hybrid biomaterial constructs. In Figure 11, we summarize some of the potential application areas in the foreseeable future in which GEPIs can be one of the major molecular tools. Here, they may provide the ways for genetic control on biomineralization from tissue restoration to regeneration. Their use as hybrid molecular probes may find application in areas ranging from diagnostics to monitoring rare biological proteins, viruses, and cells. Their intrinsic solid binding and assembly properties combined with biofunctionality of a conjugated biomolecule through recombinant technologies could provide new generation of genetically engineered materials and systems (GEMS).

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