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CONTROLLING BIOLOGICAL FUNCTIONALIZATION OF SURFACES BY ENGINEERED PEPTIDES

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ABSTRACT

Nature is a valuable source for inspiration in designing practical materials. Biological structures have diverse functions as a result of the intricate structures that are developed through evolution. With a growing understanding of the molecular processes involved, biological principles are revisited for solving the challenges in engineering design and applications. Biological systems are highly organized from molecular dimensions to macroscales often in a hierarchical manner. Molecular recognition and self-assembly are the key processes for many of the essential activities in the living cells that ultimately construct the functional tissues. In traditional biomimetics, biological micro and macrostructures have been mimicked using synthetic counterpart components with traditional fabrication approaches. In a new twist, recently developing approaches in molecular biomimetics follow the molecular scale principles of biology in developing novel materials and systems. Among the biomacromolecules, proteins and peptides are indispensable players of the biological systems. We, therefore, exploit designed and engineered peptides then utilize them in developing molecularly hybrid materials and systems for proof-of-principle practical applications. The procedures used in the identification of functional peptide sequences are based on directed evolution. Here, combinatorial peptide libraries are used in the selection of peptides with affinity to inorganic materials (e.g., gold, titanium, silica, and hydroxyapatite). Next, based on the selected peptide sequences, either experimental rational principles or *de novo* design is followed to develop engineered sequences. Finally, designed peptides are used as molecular building blocks as synthesizers, linkers and assemblers in the fabrication of functional hybrid materials. Coupled to a protein or another peptide, the inorganic-binding peptides can be a part of the resulting multifunctional molecules bridging biological function to a material surface. This review article summarizes part of the current work in which inorganic-binding peptides are developed in our collaborative group as biomolecular surface functionalization agents built upon their specific binding properties to variety of inorganic materials.

1. LESSONS FROM BIOLOGICAL SYSTEMS

In Nature, there are numerous examples on engineering structural and processing design criteria for the fabrication of practical materials with technological interest.[1-6] As we better understand of the biological systems, the biomimetic is increasingly integrated into biological materials design approaches to solve the engineering challenges. These include controlling biological and inorganic interfaces and surface forces that derive controlled self-organizations.[4-12]

Biological materials are structured hierarchicallyover multiple length scales starting from nanometer to macroscales.[11-16] These intricate structures derive their function from controlled size, morphology and self-organization into two- and three-dimensional constructions. Hierarchical structuring is one of the key features providing intricate architectures that ultimately provide multifunctionality to adapt the survival needs of an organism.[4-7] The diversity of the materials is the result of the natural evolution adapting to various conditions and environments.[8-14] Hard tissues such as bones, teeth, spicules, shells, beaks and bacterial nanoparticles are examples that contain a high percentage of mineral integrated with a mostly biopolymeric matrix.[11-15] This organic matrix contains

protein-based components that control structural formation and become an integral part of the biological composites.[8,13,15] Among these, examples include silaffins and silicateins in silica-based structures, amelogenin in enamel, calcite- or aragonite-forming proteins in mollusk shells and magnetite-forming proteins in magnetotactic bacteria.[14,16-18] All of these functional biological systems are simultaneously self-organized, self-repaired, dynamic, complex, and multifunctional, and have characteristics difficult to achieve in purely synthetic systems even with the recently developed bottom-up processes that use molecules and nanocomponents.

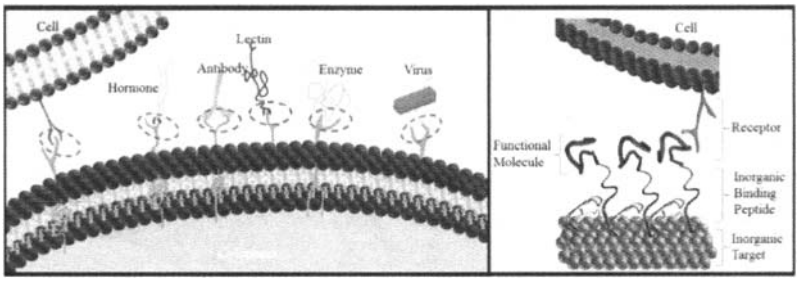

Figure 1: Schematics on molecular recognition in biological interactions (left panel) and peptide-enabled biofunctionalization of surfaces mimicking biological molecular recognition (right panel).

Among many different biomacromolecules, proteins function as the leading enablers for specific interactions between various cell and tissue components as well as major components in the cellular communications.[7,19,20] Many essential biological interactions in living organisms are based on precise molecular recognition taking place among various biomolecules (Figure 1). The biomineral-associated proteins can be considered to operate on the same basic principles, i.e., sequence-related molecular structure that results in the specific affinity to the counterpart mineral and dictate its assembly function. Using specific affinity of a peptide to a given inorganic solid in the structural adaptability of biological composites provides new pathways for designing new materials and systems.[8,21,22] [11]

Recent interest in the molecular concepts is finding of inorganic-binding peptides that may be used in controlling surface interactions and, therefore, controlling assembly of nanoscale solid objects.[4,6,20,21] The peptide-based biomimetic systems follow a path similar to biological materials formation mechanisms and, therefore, can be realized in at least three steps:[23] (i) Inorganic-specific peptides are identified by initiating a fast evolution towards materials of interest using molecular biology tools; (ii) These peptide building blocks can be further engineered to tailor their recognition and assembly properties using rational design or computational biology approaches. This step is similar to natural evolution where successive cycles of mutation and generation potentially lead to improved progeny; finally (iii) Biological molecules self- or co-assemble into an ordered functional biomolecular layer controlling the bio/material interfaces.[23] Below, we provide a summary of the selection of inorganic binding peptides and their utilizations on biological surface functionalization.

2. ENGINEERED EVOLUTION OF INORGANIC-BINDING PEPTIDES

Inorganic-binding peptides are selected through affinity-based biocombinatorial protocols.[20] Over the past two decades various combinatorial selection techniques have been successfully applied to study a variety of biomolecular interactions, e.g., antibody-receptor, protein- or peptide-ligand interactions for a myriad of biotechnological and biomedical applications.[24-26] In combinatorial display

techniques random peptide or protein sequences that are encoded in either phage genome or plasmid bacterial DNA are displayed on the surface of the phage or cell, respectively. The link between phenotype and genotype of organisms is the common feature in all combinatorial display techniques. Randomized peptide sequences can be displayed on the phage or bacterial cell surfaces within the context of different surface proteins. Outer membrane proteins, lipoproteins, fimbria and flagellar proteins have been used to display the randomized peptide library on surface of bacteria.[27] Phage display utilizes the major or minor coat proteins of bacteriophage M13 to display the random peptides on virus surface.[24,28]

2.1. Selection of Inorganic Binding Peptides

We have selected peptides for a variety of materials including metals (e.g., Au, Ag, Pt and Pd), oxide and nitride semiconductors (e.g. Cu_2O, ITO, GaN, ZnO), minerals (e.g., mica, hydroxyapatite, calcite and aragonite) or biocompatible substrates (such as silica and titania) using either filamentous phage display or cell surface display [23,29-34] There are also a number sequences selected for various materials by other research groups.[35-37] Some of the peptides selected *via* cell surface display include for materials such as gold[25] and zinc oxide[38] whereas phage display selected ones are for gallium arsenide,[39] silica,[40,41] silver,[42] zinc sulphide,[43] calcite,[44] cadmium sulphide,[45] and titanium oxide.[46]

In inorganic binding peptide selection, typical biopanning steps consist of contacting the library with the solid material of interest, then washing out weak- or non-binder,s and repeating the process to enrich for tight binders to select a subset of the original library exhibiting the ability to tightly interact with the desired surface (Figure 2). During the biopanning procedure, a minimum of three to five cycles of enrichment are usually performed. Generally in early rounds, low affinity binders can be accessed if the selection is performed under mild conditions. In later rounds, as the conditions get harsher, tight binders are also recovered. Because the chimera is encoded within the phage genome or in a plasmid carried by the cell, the identity of the selected sequences (e.g., their amino acid sequences) can be deduced by DNA sequencing (Figure 2). To acquire initial information on the binding strength of the selected clones, we developed a simple binding assay using a fluorescence microscopy imaging technique.[21] The relative binding strength of selected individual phage or cell clones is estimated by enumerating either surface coverage of phage or adhered cells on the solid surface through immunolabeling by fluorescently labeled anti-M13 antibodies or DNA-binding fluorescence dyes.

Various research groups, including our laboratory, have shown that improved selectivity can be achieved by integrating simple modifications into the biopanning protocol (e.g., counter selection step, material specificity testing, etc.) to isolate peptides that not only hve high affinity, but also have high material selectivity.[20,47,48] Since proteins can non-specifically interact with surfaces through their side chains with diverse physicochemical properties, material selectivity becomes an important parameter towards the integration of the desired molecular recognition properties into peptide-based materials and systems.

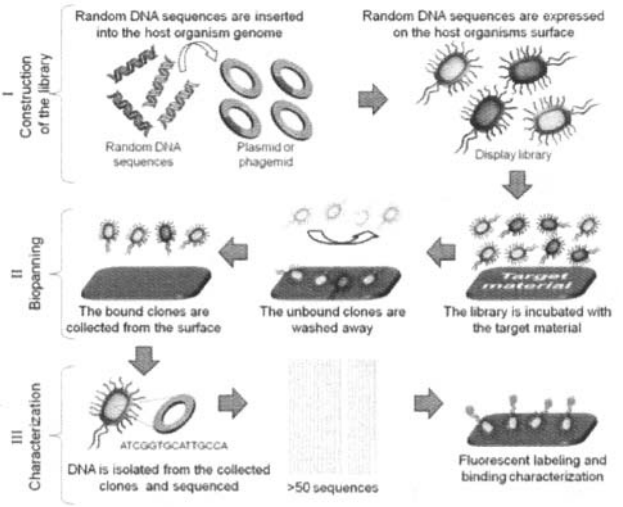

Figure 2: Schematics of combinatorial-biology based peptide selection using phage- and cell surface-display showing the library generation, biopanning procedure and binding characterization.

Adapting and continuously modifying the biopanning conditions are critical for the optimized selection of the inorganic-binding peptides depending on the chemical composition, surface properties, and physical characteristics of a given material. Material properties, e.g., charge, roughness, and reactivity in buffer environment are very different from proteinaceous ligands for which the combinatorial selection techniques were originally developed.[21,49-51] Furthermore the form of the inorganic solid material might limit the utility of particular display protocol. For example, when the random peptide library is displayed on flagellar proteins; the centrifugal force used in biopanning step could disrupt and shear off the flagella from cells and result in the loss of tightly bound clones from the pool.[21,49] Detailed procedures, therefore, have to be developed for a particular inorganic material in the powder, thin film or in single crystal forms as demonstrated in numerous publications.[24-45]

2.2. Molecular Interactions of Peptides on Inorganic Materials

When one is focusing on the inorganic-specific peptide interactions, finding a consensus sequence in the selected peptide pool might be desired for given particle size or a crystallographic surface. But this has so far been impossible to achieve, and consensus sequences, therefore, might often be misleading. This could be due to the high potential that a genetic bias in the selection by the organism may produce the same sequence without the diversity. The range of sequences may reflect the heterogeneity of a given inorganic substrate at the atomic, topographic, chemical and crystallographic levels. Furthermore, chemical diversity of the surfaces alone would contribute to produce a variety of sequences that the peptide library could entail. The shape and lattice complementarities, electrostatic interactions, van der Waals forces or various combinations of these mechanisms would collaboratively contribute the interactions between the peptides and the inorganic surfaces.[32,34,50,52-54]

Despite significant work carried out both experimentally and computationally, there is still a limited knowledge on the molecular binding and recognition mechanisms of combinatorialy selected inorganic-binding peptides.[55-57] Sometimes the selected peptide sequence, when chemically

synthesized without the context of the chimera proteins, may perform decreased binding affinity than when it is still displayed of the surface of the phage or bacterial cells. One obvious explanation may be the loss of multivalent peptide display. Therefore, producing the peptides containing the repetition binding motifs can limit the potential valency effect.[47,48,58] Another possible approach could be to bring the side chains within the context of the molecular structure and then tuning the material-specific peptide interactions. The methods available to molecular biology can be applied to the peptide sequences such as site-directed or random mutagenesis. The single residue or whole motif substitution and mutagenesis can be applied either at the genomic level to the peptide displayed on the phage or during the solid phase peptide synthesis.[33,55]

Understanding the structure-function relationship in selected inorganic binding peptides is also another critical area in the peptide-based materials and systems. In biology, the molecular architecture of proteins and peptides affects their instrictic activity and biological functions. Peptides, unlike natural proteins or protein domains, do not generally fold into well defined structures and in solution they may often adapt multiple structural conformations.[28,59] This might pose limitation when unconstrained "linear" libraries are applied in biopanning selections since the molecular structures of chemically synthesized peptides outside the surface protein context might be significantly different from their original "active" displayed conformations. This may result in decreased peptide binding affinities.[28,59] Constraining the peptide structures when displayed in structural context of a protein scaffold in the original library by Cys-Cys disulphide bond increases the probability of retaining "active" peptide conformations upon their chemical synthesis.[28] In certain cases, however, the intrinsically disordered behavior, which is observed in naturally occurring proteins interacting with biominerals, might be the key feature in tuning the interactions on the surfaces.[50,60]

The continuing studies in the detailed understanding on the peptide binding, recognition and assembly processes will inevitably lead to better insights into the design of peptides with tailored inorganic surface interactions. The quantitative data towards determining kinetic and thermodynamic parameters of peptide binding can also be obtained using either established techniques such as quartz crystal microbalance (QCM)[61] and surface plasmon resonance (SPR) spectroscopy or by other techniques such as calorimeter.[48,62,63] By incorporating SPR and QCM with circular dichroism (CD) folding data, one may be able to analyze the effect of the peptide conformation on its adsorption kinetics.[32,34,48] A better knowledge of the mechanism(s) of the quantitative adsorption and surface diffusion is possible through the high resolution surface microscopy (e.g., AFM and STM), molecular spectroscopy and surface diffraction studies when solid surfaces are atomically flat.

3. BIOLOGICAL FUNCTIONALIZATION OF SURFACES

In the absence of total undertanding of the molecular recognition mechanisms, an immediate practical application of inorganic-binding peptides that have specific material affinity is to use them in functionalizing solid surfaces or as molecular linkers in displaying or immobilizing functional molecular or nanoentities *via* targeted or directed assembly approaches. Below, we provide two examples to demonstrate their utilization in oriented protein immobilization and designing peptide based implant coatings using both of the assembly approaches.

3.1.Oriented Protein/Enzyme Immobilization

Once a set of fully characterized inorganic-binding peptides is developed, then they could be used as specific surface-binding ligands assembling on solid interfaces and forming an self-immobilized biofunctionalization layer. These peptides bind to their respective materials with high affinity, having dissociation constant (K_D) values in the μM to nM range, while also exhibiting desired material

selectivity.[32,34,48,56,64] Several inorganic-binding peptides have already been shown to form densely packed monolayers on atomically flat solid surfaces, which is an advantage in surface engineering applications.[55,56] Another unique feature of these peptides is their ease to conjugate with complex proteins *via* site-directed genetic recombination. By taking advantage of molecular binding characteristics of inorganic-binding peptides our group and others have pioneered the mutli-functional proteins having these peptides as fusion partners.[29,65-69] Here, the genetic insertion can be located at either side of the protein's termini, i.e., C- terminus[70] and N-terminus[68], or on a permissive site identified on the protein.[29] Using various inorganic-binding peptides as specific tag partners, we have demonstrated the immobilization of functional fusion proteins, such as maltose binding protein, green fluorescent protein and alkaline phosphatase, on various inorganic surfaces, such as gold, silver, and silica.[68,70-74]

One of our examples given here includes the site-directed immobilization of alkaline phosphatase (AP) on gold surface using a genetically inserted gold-binding peptide (GBP1). Alkaline phosphatase is a common enzyme which is currently used in many diagnostics analysis in addition to its role in the biomineralization.[75] In our earlier studies, we studied a 14-amino acid long GBP1 binding and assembly on various gold surfaces. [47,48] On a planar gold surface, GBP1 has a binding energy of -8.4 ± 0.1 kcal/mol [47,48,52,76] Here, we genetically inserted five-repeat of GBP1 to AP to provide higher binding affinity and stability by the displayed content of the peptide. Depending on the concentration used, 5GBP1-AP reached to nearly 90% surface coverage with an equilibrium adsorption constant (K_{eq}) of 1.65×10^{8}. We next performed the enzymatic analysis of the AP-5GBP on the surface to analyze whether the GBP1 insertion has an effect on the AP activity. We found that, when immobilized onto bare gold surface, only 2% (±0.4) of wild-type AP activity was transferable, whereas in case of 5GBP1-AP, transferable enzymatic activity to the surface was 66% (±0.6). The AP-GBP1 self-immobilization on the gold surface resulted in an increased enzymatic activity on the surface. This could be contributed to the genetic fusion of the peptide to AP provided an orientation control which directed the self-immobilization of the enzyme onto the solid substrate. The enhanced enzymatic activity on flat gold surface is illustrated in AFM image in Figure 3 (b).[68]

Our next example involves a similar approach, where we demonstrated a bio-enabled technique for fabrication of multi-layered protein and nanometallic assemblies. Here, we used another gold-binding peptide (AuBP1, a dodecapeptide) as a fusion tag which has been recently selected due to shorter length and its stronger gold binding ability and selectivity as well as its synthesis capability; it has, therefore, been employed in our recent studies. The binding energy of GBP1, single repeat, is -8.4 ± 0.1 kcal/mol and the three repeat is -9.68 ± 0.28 kcal/mol compared to the Kd values of AuBP-family of peptides, around , which is -10.0 ± 2 kcal/mol.[32-65] The significantly lower binding energy of AuBPs compared to 1R-GBP1 indicates that they bind onto gold surface more tightly. In the present work modular AuBP1 peptide tag demonstrated as an enabler to immobilize multiple layers of nanostuctures and fusion proteins onto gold surface using a combination of soft-lithography and peptide-based directed-assembly techniques (Figure 3 (c-e)). Using this experimental strategy, we first produced a bifunctional molecule by genetic fusion of AuBP1 tag to the C'-terminus of maltose-binding protein (MBP). Next, we tested the effectiveness of proposed bio-enabled layer-by-layer assembly process on an Au nanoparticle-arrayed silica glass surface. To accomplish assembly on a glass surface, we first conjugated AuBP1 to a glass-binding peptide (QBP1), and patterned the surface with the resulting bifunctional peptide (AuBP1-QBP1) using soft-lithography technique (Figure 3 (c)). The Au nanoparticles assembled on the peptide patterns (Figure 3 (d-e)). Next a third layer of MBP-AuBP1 derivatives was self-immobilized selectively onto the gold nanoparticles decorating the glass surface. Finally, immobilized fusion protein localized on the assembled hybrid structures was detected with high precision with fluorescence microscopy using anti-MBP antibody labeled with a fluorophore (Figure 3 (d-f)).[65,66]

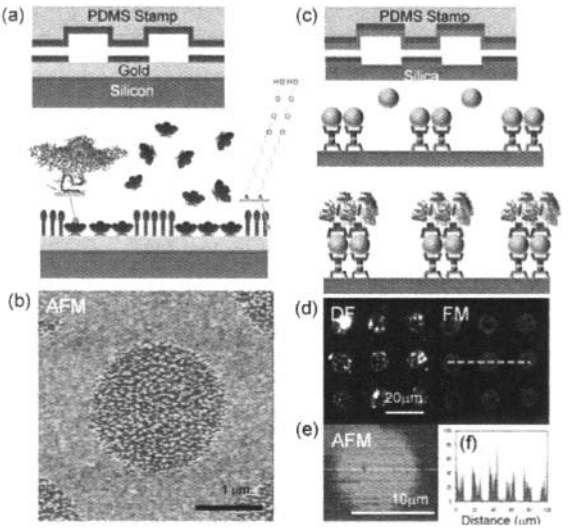

Figure 3: Oriented enzyme and layer-by-layer protein immobilization on planar and nanoparticle Au surfaces. (a) Schematics of the oriented alkaline phosphatase (AP) enzyme immobilization using -5-repeat gold-binding peptide tag (5GBP1). (b) An AFM scan of immobilized 5GBP1-AP fusion enzyme on planar gold surface. (c) Schematics of layer-by-layer maltose-binding protein (MBP) immobilization on AuNP-arrayed silica surface using peptide tags.(b) Representative fluorescence and dark field images of MBP-$(PG)_3$-AuBP1 proteins immobilized on AuNP arrays labeled with anti-MBP-Alexa-488 antibody. (e) AFM scan of AuNP patterns immobilized on glass surface through QBP-AuBP1 bifunctional peptide. (f) Corresponding line scan plot showing the light intensity of the fluorescence images. The respective positions on fluorescence images used in scan analysis are indicated by dashed lines.

Overall, combinatorial inorganic-binding peptides have been demonstrated as molecular biological surface functionalization agents to immobilize various proteins on different inorganic solid platforms.[22, 29,65-74] Furthermore, besides genetic fusion with protein units, the inorganic-binding peptides can be chemically conjugated to linker molecules, *e.g.*, other inorganic-binding peptide or biotin. Inorganic-binding peptides through their specific solid affinity and assembly properties, and suitability to genetic or chemical modifications are continuing to be employed in a variety of proof-of-principle applications, such as sensing, cellular imaging, and immunoassays.[22]

3.2. Biofunctionalization for Biomedical Applications

Controlling the biological-inorganic interfaces between the implant material and living tissues is still one of the major challenges in current biomaterials research.[77-82] Although a variety of implantable materials with desirable physical and mechanical properties are available, there are still limitations in controlling the biological response at the material interfaces. The implant materials should be carefully designed depending on the type of implantation and their intended use. For example, materials for

stents and pacemaker electrodes need to be bio-inert limiting interaction with environment; whereas orthopedic implant materials are generally required to be bio-active enhancing material's cyto-compatibility.[79,81,83,84] Modifying the material surface with covalently attached functional molecules according to intended use is the most common strategy in the literature.[85] Even though these methods offer solutions to a certain degree, many of these surface functionalization methods, however, are restricted to a limited range of materials and require the presence of specific functional groups at or complex chemical processes on material surfaces.[86,87]

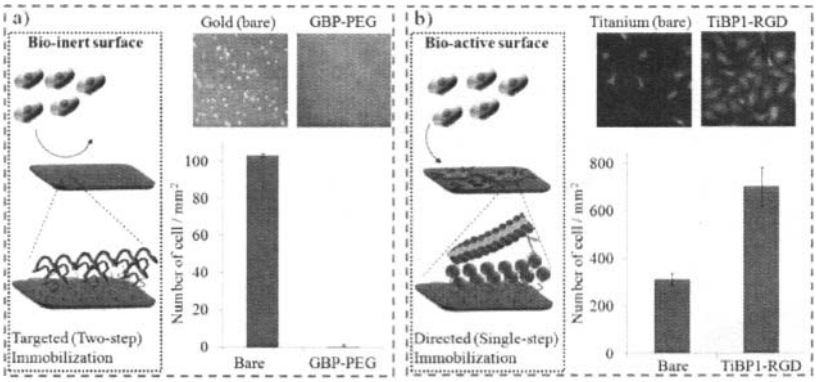

Figure 4: Biofunctionalization of Biomedical Surfaces. (a) Schematics of targeted immobilization (left panel). The corresponding NIH 3T3 cells enumerated per area showing the cells adhesion on gold surface using GBP-PEG functionalized- and bare gold-surfaces. (b) Schematics of directed immobilization (right panel).The fluorescence microscopy images of adhered NIH 3T3 cells on functionalized- and non-functionalized-titanium surfaces. The corresponding number of NIH 3T3 cells enumerated per area showing the enhanced cells adhesion and proliferation on titanium implant material using bi-functional TiBP-RGD peptide compared to control bare surfaces having no peptide

As discussed above, inorganic-binding peptides self-adhere onto inorganic surface under physiological conditions.[22,85] During the past decade, versatility of these short peptides as a multi-purpose molecular tool for biofunctionalization of various biomedical surfaces has been reported in numerous studies.[32-34,46,48,75,88,89] The inherent nature of these peptides allows the use of different strategies during the immobilization of functional molecules depending on their availability as a single or a multifunctional unit. We refer these approaches as two step targeted assembly versus single step directed-assembly approach. Both approaches can be used to immobilize small or large molecules to material surfaces depending on the desired surface conditions (Figure 4a and b). Here we provide two examples to explain these approaches. In the first example, we employed a two step targeted assembly where the gold-binding peptide (GBP) was first self-immobilized on the surface and then chemically conjugated with the activated aldehyde terminated poly(ethylene glycol) (PEGCHO) anti-fouling polymer by using Schiff-base chemistry (Figure 4a left panel). Biofunctionalized gold surfaces exhibited excellent cell resistant properties based on non-adhered NIH 3T3 cells on GBP-PEG functionalized gold surface compared to non-functionalized bare surface.

In our next example, we demonstrated the directed assembly approach where the inorganic surface was functionalized in a single step using a bi-functional peptide. To accomplish this goal, first the

single chimeric molecule with bifunctional domains was synthesized (RGD-inorganic binding peptide) and then this was applied onto material surface. Following the cell-culture studies, cell adhesion was evaluated using fluorescence microscopy imaging technique. Here a combinatorially selected peptide binding to implant-grade titanium surface was used, named asTiBP. The synthesized chimeric molecules composed of TiBP-RGD domains were directed immobilized onto the titanium surface in a single step process (Figure 4b). Fluorescence microscopy imaging show that TiBP1-RDG coated titanium surface has higher cell adhesion compared to the non-functionalized bare titanium surface. Also, the TiBP1-RGD coated titanium surface displays a significant enhancement of NIH 3T3 fibroblast cells proliferation.[88]

We note that this combination of techniques can easily be manipulated to provide high-throughput screening for a variety of biomaterial surfaces. The resulting peptides, for example, may be coupled to other biological domains towards enhancing osteointegration of bone implants or targeting specific cells for diagnostics.

4. FUTURE PERSPECTS AND POTENTIALS OF PEPTIDE-BASED MATERIALS FOR BIOMEDICAL APPLICATIONS

Joining biology with materials science at the molecular level requires the ability to design, engineer and control material/biology interfaces as these sites are central to the implementation of nanobiotechnology, development of new hybrid materials and novel protocols in molecular engineering. Biology controls interfaces among biomolecular materials, tissues and organs using peptides and proteins which are also the agents of cellular communication. Similar to biology, in engineering and technological systems, one can genetically select peptides with an ability to bind to inorganic solid materials to generate new fundamental building blocks to couple bio- and synthetic-entities. One can introduce multifunctionality either using two or more material-binding peptides to produce novel ways of making materials compatible across their interfaces, or by genetically fusing a functional protein, e.g., enzyme or antibody, to form heterofunctional molecular constructs. Solid-binding peptides coupled with solid substrates form a new generation of novel hybrid materials systems.[21] Genetic control of coupling and the resulting function of the hybrid material are key approaches with potential to overcome limitations encountered in a wide range of applications where traditional synthetic linkers, such as thiol or silane, have been used up until now with major limitations such as bioincompatibility, instability, and nonspecificity. The attachment of biomolecules, in particular proteins, onto solid supports is fundamental in the development of advanced biosensors, bioreactors, affinity chromatographic separation materials and many diagnostics such as those used in cancer therapeutics.[91-93] Precise control of bio/inorganic interfaces and protein adsorption at solid surfaces play key roles in the performance of implants and hard-tissue regeneration or restoration.[94,95] The examples given above illustrate only some of the achievable goals using these new classes of functional molecular linkers. Based on their specific affinity and assembly characteristics, the role of combinatorially selected inorganic-binding peptides in these hybrid structures is to be an integral component of the overall structure providing to it functional (e.g., mechanical) durability, in addition to providing the essential molecular linkage between the inorganic components. Owing to the intrinsic properties mimicked after natural proteins, in the coming years, we are likely to see engineered inorganic-binding polypeptides used more widely and in a broad range of applications from particle synthesis and assembly with genetically controlled physical and chemical characteristics in materials science to probing for biomolecular targets in biology and medicine.[51,96,97]

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