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Biomimetic multifunctional molecular coatings using engineered proteins

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Abstract

A molecular biomimetics approach is presented in developing polypeptide-based coatings for inorganic surfaces. In general, inorganic surface-binding polypeptides are genetically engineered using cell surface and phage display technologies. These peptides contain short amino acid sequences, known to bind specifically to selected inorganics. Based on the sequences of the polypeptides that were recently selected by this (e.g. Au, Pt and Pd) and other groups, one may find certain specificity, e.g. hydrophobic and hydroxyl amino acids, common among noble metal-binders. We show that an engineered gold-binding protein self-assembles onto gold surface forming monomolecular and highly structured crystallographic domains. The protein-based molecular films could provide robust templates for potential utility in practical nanotechnological and bionanotechnological applications.

Keywords: Molecular biomimetics; Surface binding polypeptides; Display technologies

1. Introduction: why biomimetics pathways to coatings?

There is a rich and long history of gaining inspiration from nature. Biological systems carry a great wealth of engineering principles for the design, synthesis, and manufacturing of materials for practical uses [1–3]. It is well known that biomaterials are highly organized from the molecular to the nano-, micro-, and the macro-scales, often in a hierarchical manner, with intricate nanoarchitectures that ultimately make up a myriad different functional units, soft and hard tissues [1–5]. Hard tissues, such as bone, dental tissues, mollusk shells, and sponge spicules, are structures that are made of two components: one being a ceramic phase and the other an organic phase (Fig. 1). As such, biological hard tissues are composites forming truly hybrid materials (organic/inorganic) with excellent physical properties including mechanical, optical, piezoelectric, and magnetic. In many biocomposites, the micro- and nanostructures are organized in layered architectures leading to the control of shape and function especially in terms of wear and impact resistance, hardness, damage tolerance, and long-term durability [6,7]. For example, in mammalian bone [8] and enamel [9], the inorganic phase is in the form of small particles and the organic phase (collagen and amelogenin, respectively) is fibrous (at the molecular scale). In nacre section of the mollusk shells and spicules of many sponge species, the inorganic (crystalline $CaCO_3$ in the former and amorphous silica in the latter) is layered and separated by an organic matrix composed of proteinaceous macromolecules [7,8] (Fig. 1). Extraordinary properties such as toughness ($K_{IC} > 10\,MPa\,m^{1/2}$), high flexibility (despite high elastic modulus, $E > 50\,GPa$), and strength ($\sigma_F > 100\,MPa$) are all thought to be a direct result of this layering in addition to the presence of strong interface between the two dissimilar materials. Another desirable aspect of biological materials is that they are all synthesized under ambient conditions of room temperature, atmospheric pressure, and aqueous environments, resulting in materials that are environmentally friendly [2–5]. It is, therefore, desirable to use proteins as the soft and binding component that would act as the glue to produce organic/inorganic damage tolerant but highly functional composites and coatings.

Traditionally, biomimeticists, inspired by the biological structures and their functions, focused on emulating or duplicating biosystems using mostly synthetic components and by following traditional approaches [2,3]. For example, in an attempt to produce hybrid materials, researchers tried extracting proteins from hard tissues (e.g. from mollusk shells and spicules of sponges), isolated, purified, and cloned them, albeit this being a labor intensive approach [10–12]. Although some success has been achieved, especially in the use of some of the isolated proteins as catalyzers [12], this

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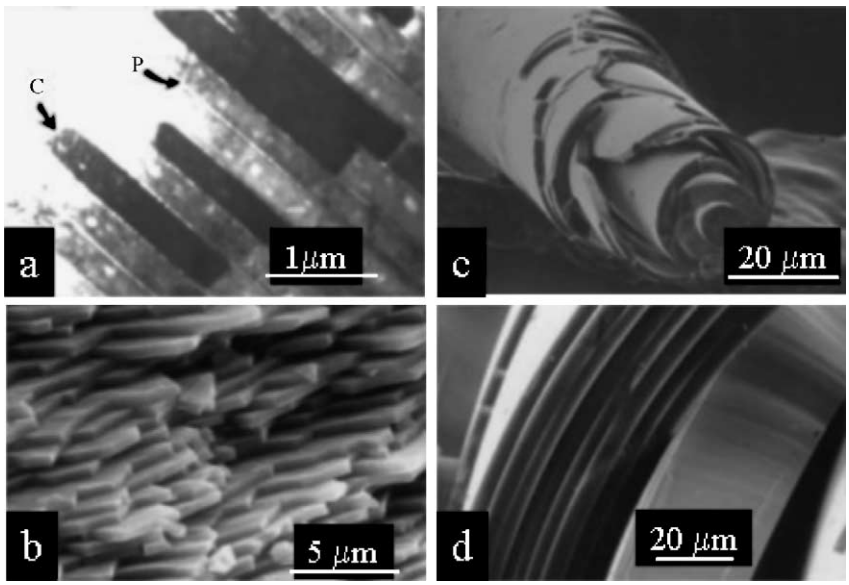

Fig. 1. Biological hard tissues: (a) TEM cross-section and (b) SEM fractured surface of nacre (mother-of-pearl) showing brick-mortar microarchitecture of the biocomposite, where the mortar is mostly composed of proteins. (c) and (d) SEM images of layered structure of sponge spicules in which layers are separated by an organic substance.

approach has been mostly futile. This is mainly because of the fact that the organic matrix of a biological hard tissue usually contains many protein components (>10 in nacre [10] and >30 in enamel [8]) and these are temporally and spatially distributed throughout the lifespan of the tissue. Even if this approach is successful, only the regeneration of the original hard tissue could be possible containing the inorganic component that may not have a practical utility.

With the recent developments of molecular and nanoscale engineering in physical sciences [13,14], and the advances in molecular biology [15], biomimetics is now entering the molecular scale [16]. The premise in a molecular biomimetic approach to nanotechnology is that, genetically engineered proteins specific to inorganic surfaces, could be used as linkers and building blocks for self-assembly of materials with controlled organization and specific functions (Fig. 2).

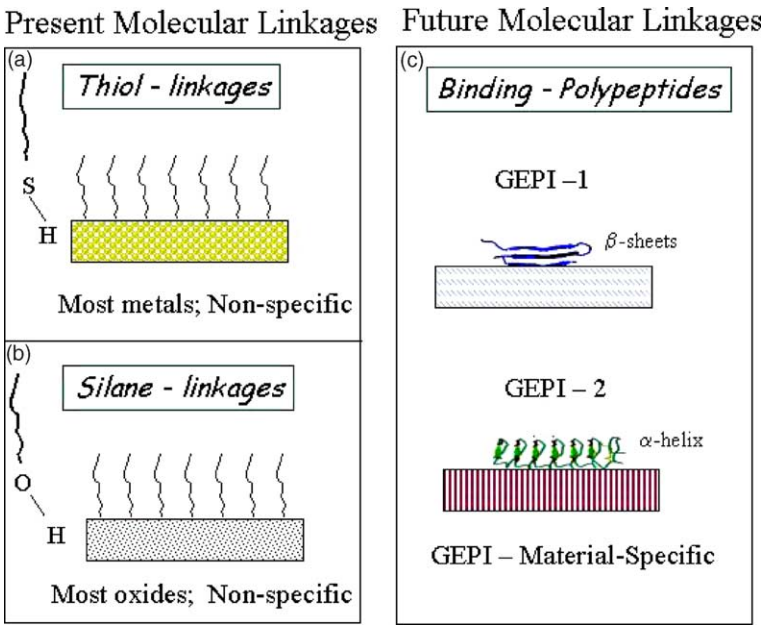

Fig. 2. Thiol- and silane-based self-assembled monolayers are produced based on chemical linking onto non-specific noble metals or oxides, respectively. Engineered proteins could bind to a given material specifically. While (a) thiol- and (b) silane-linkages are non-specific, (c) genetically engineered polypeptides could be material specific having a variety of conformational architectures.

Molecular biomimetics offers three solutions to the development of heterofunctional nanostructure problems simultaneously. The *first* is that the protein templates are designed at the molecular level and through genetics. This ensures the molecular scale-up processing for nanostructural control at the lowest dimensional scale possible (i.e. DNA-based, molecular synthesis). The *second* is the use of surface specific proteins, through their specific recognition, as *linkers* or *molecular erector sets* to bind synthetic entities, including nanoparticles, functional polymers, or other nanostructures onto molecular templates (molecular and nanoscale recognition). Finally, the *third* is that the biological molecules, with well-defined secondary structures, could self-assemble into ordered nanostructures. In this paper, we show a general approach of molecular biomimetics and describe genetic engineering protocols for the selection of inorganic binding polypeptides, discuss the nature of binding in known polypeptide sequences, show ordered self-assembly of one such polypeptide on a solid substrate, and discuss potential applications of these proteins as functional coatings.

2. Towards engineering proteins for use in nanotechnology—genetic display protocols

There has been increased activity in protein engineering specifically to improve their binding affinity and catalytic activity through directed evolution for specific applications [17–20]. Genetic engineering protocols have also been utilized for obtaining proteins or polypeptide sequences with specific affinity to inorganics for use as molecular erectors for the development of genetically engineered multifunctional nanostructural materials [16,21–25]. In this case, the combinatorial biology based library systems are utilized for the selection of proteins with specific affinity to inorganics through enrichment processes. Here, display technologies are used as routine tools for creating libraries of biomolecules that can be screened for desired and novel properties by optimizing the assembly of building blocks with more diverse function. Phage display (PD) [17] and cell surface display (CSD) [26] are well-established examples of *in vivo* display technologies. Outer membrane proteins, lipoproteins, fimbria and flagellar proteins can be used for heterologous surface display on bacteria. In PD, the majority of the research has been performed by using filamentous phage strains such as M13 [17] or the closely related fd and f1, although, recently T7, T4 and λ have also been used, but not yet on a routine basis [17–19,27].

Phages, i.e. viruses infecting bacterial hosts, are commonly used as vectors in recombinant DNA studies [18]. Common feature of a vector is to accommodate the foreign DNA so that when phages replicate in the host, foreign insert also replicates for the production of a desired molecule. PD has been the most commonly practiced combinatorial peptide library display method since the demonstration of the linkage between phenotype and genotype in filamentous bacteriophage [17]. The most widely used phage strain in PD is the filamentous, M13, infecting male *Escherichia coli*. This is partly because of the ease with which it can be used and a detailed knowledge of the phage life cycle. In display of proteins on M13, pIII has been the primary scaffold with five copies at one end of the virus.

The protocol for phage display involves generation of peptide libraries, displayed on the viral coat, by inserting randomized oligonucleotides into phage genomes (Fig. 3). The next step is to allow this heterogeneous mixture of phage clones to bind to the desired inorganic substrate for a specific period of time. The inorganic substrate could be in the form of powder, single crystal, or polycrystalline solid. The phages that strongly bind to the target substrate are retained, while the nonadherent ones are washed away. These bound phages can then be recovered from the substrate through

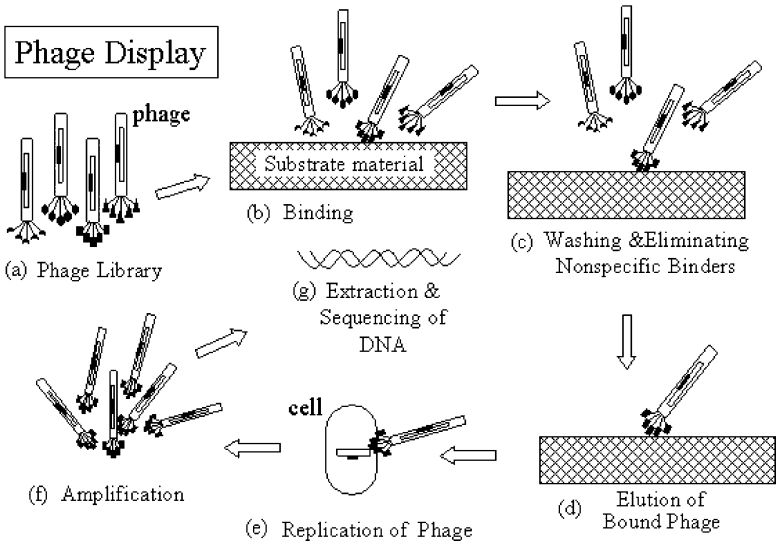

Fig. 3. A flowchart of the phage display protocol. In M13, the library was displayed on minor coat protein (pIII).

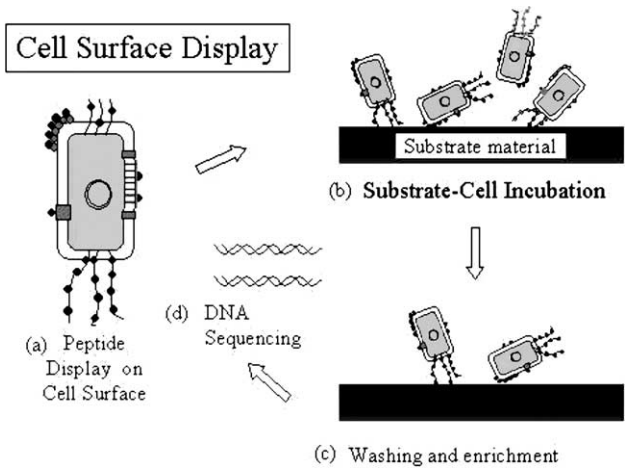

Fig. 4. A flowchart of the cell surface display protocol. The protein may be displayed on the cell surface, on a surface-bound protein, and anywhere on the flagella.

elution with a harsh wash that disrupts strong binding of the phages to the substrate. Once a phage has been recovered, it can be used to reinfect the cell for the amplification in the next step. This process may be repeated several times to progressively enrich the tight binders in the pool. The final step is to extract DNA from these tight binders for sequencing to identify the polypeptides that bind to the selected surface.

The CSD has also been utilized for various applications in microbiology, molecular biology, and biotechnology in addition to PD [21,22,26]. The main advantage of CSD is the improved strategies in terms of kinetics, equilibrium, and statistics of the screening process. Basically, following the growth of cells harboring the library, a hybrid protein is displayed on the surface of the cells (Fig. 4). Cells are then allowed to bind to the selected substrate for a specific period of time. Several cycles of washing eliminate non-binders while each cycle of biopanning provides the enrichment. Similar to PD, at the final step, individual clones are characterized by DNA sequencing to identify strongly binding polypeptides.

A genetically engineered polypeptide for inorganics (GEPI) [16], which may be selected through the display protocols described in Figs. 3 and 4, defines a sequence of amino acids that specifically and selectively binds to a specific inorganic surface. The inorganic surface could be well-defined, such as a single crystal or a nanostructure; it might be rough, or totally non-descriptive, such as a powder. Gold-binding proteins (GBPs) were the first examples of the proteins obtained using *E. coli* CSD technology from a population size of 5×10^6 clones prepared by using three different libraries [21,23]. The *E. coli* maltoporin membrane protein was used for isolation of gold-binding sequences and good binders were obtained even under extreme conditions. These conditions included high salt environment, which is known to have inhibitory effect in preventing binding of proteins to gold. Interestingly, none of the gold-binding polypeptide (Au°BP) sequences contained cysteine, which is known to form a covalent thiol linkage to gold. Multiple repeats of a gold-binding sequence, i.e. Au°BP1 (Fig. 5), have also been engineered and improved binding has been observed with the increased number of repeats [21,23]. Molecular dynamic studies carried out on the three repeats of this sequence (i.e. 42 residues) showed that the polypeptide has an antiparallel β-sheet conformation [28]. Following the success of this gold-binding protein, the search for specific peptide sequences that bind to various inorganic compounds has been carried out by us and others

Some Examples to Inorganic Binding Polypeptides

Nobel Metals

Au Binders:[21,23]
Au°BP1: MHGKTQATSGTIQS (14 AA)
Au°BP2:SKTSLGQSGASLQGSEKLTNG(21AA)
Au°BP3:QATSEKLVRGMEGASLHPAKT(21AA)

Ag-Binders:[29]
Ag°BP1: AYSSGAPPMPPF (12 AA)
Ag°BP2: NPSSLFRYLPSD (12 AA)
Ag°BP3: SLATQPPRTPPV (12 AA)

Pt-Binders: [31]
Pt°BP1: DRTSTWR (7 AA)
Pt°BP2: TSPGQKQ (7 AA)
Pt°BP3: IGSSLKP (7 AA)

Pd-Binders:[31]
Pd°BP1: SAGRLSA (7 AA)
Pd°BP2: TLPNHTV (7 AA)
Pd°BP3: HTSKLGI (7 AA)

Uncharged polar side chains: STNQYC
Charged polar side chains: KRHDE
Non-polar side chains: AVGLIMPFW

Metal Oxides

Silica-Binders:[25]
Si4-1 : MSPHPHPRHHHT (12 AA)
Si4-10: RGRRRRLSCRLL (12 AA)
Si3-8 : KPSHHHHHTGAN (12 AA)

ZnO Binders:[30]
pJKS9: RSNTRMTARQHRSANHKSTQRARS
pJKS15:RSYDSRSMRPHRS (9 AA)

Cr_2O_3 Binders:[22]
pKKJ62: RSVVRPKAATNRS (9 AA)
pKKJ66: RSRIRHRLVGQRS (9 AA)

CoO Binders: [22]
pKKJ75: RSGRMQRRVAHRS (9 AA)
pKKJ76: RSLGKDRPHFHRS (9 AA)

Hydrophobic: AGVPMILWF
Acidic: DE
Basic: RKH
Hydroxyl : STY

Fig. 5. Sequences of some known inorganic binding polypeptides from various sources.

using either PD or CSD [21–25,29–31]. Some of these known sequences are listed in Fig. 5.

The quest for inorganic binders focused on noble metals and those materials with semiconducting properties. Noble metals are used because of their stable surface structures in aqueous solution. For example, silver-specific peptides were obtained by using an unconstrained phage display library [29]. Positional conservation of some of the amino acids was reported in the silver-specific peptides. In particular, in these binders, enrichment of proline and conservation of polar, hydrophobic and hydroxyl containing amino acids were observed. Also, in the reported sequences, more than one third were composed of small amino acids. Another group identified ZnS binder by using constrained PD library [24]. Using a surface organelle, type 1 fimbriae, another group showed display of random peptide libraries on metal-oxide substrates, such as CoO [22], Cr_2O_3 [22] and ZnO [30].

We have recently used PD and flagellar display technologies to identify amino acid sequences that interact strongly with noble metals and semiconducting oxides [31]. For example, we used PD to select binders to metallic platinum and palladium powders. In our display, seven amino acid constrained random peptide library was used that was fused to a minor coat protein (pIII) of M13 phage. The number of amino acids in our library is considerably smaller than other metal binding domains, e.g. 14 amino acids in Au-binding sequences displayed on AP of *E. coli* [21] and 12 amino acids in Ag-binding, unconstrained, sequences displayed on M13 phage [25]. The question we specifically wanted to address was whether there was any similarity conserved among the metal binding domains even in the reduced size, but constraint, sequences. The cleaning of the metal powders was carried out by methanol/acetone/isopropanol, to ensure that the impurities were removed from the surface so that sequences were not recognizing a contaminant. As discussed above, the specific peptides were selected by a procedure including multiple rounds of target binding, elution and amplification of the specifically bound phage.

3. The nature of possible binding of the engineered polypeptides onto inorganics

One of the central issues in biomolecular recognition of inorganic surfaces is the nature of the binding. In the case of proteins, or engineered polypeptides, physical conformation depends on the sequence of the amino acids in addition to their overall composition. An inorganic may be recognized physically or chemically through its surface composition, structure, crystallography, or morphology. Based on the limited information available so far, we focus our current efforts on chemical ligand (amino acid) recognition of surfaces (Fig. 5). For example, both gold and silver-binding sequences, selected by different display routes, seem to have some similarities in terms of conserved hydrophobicity and polarity in addition to the preserving hydroxyl containing amino acids. Interestingly, although the overall length of the sequences were considerably shorter, i.e. 7 versus 14 or 12, the platinum and palladium binding sequences, selected through PD, also conserved hydrophobicity and hydroxyl containing amino acids. These amino acids, therefore, may constitute the metal binding domains. Another observation is that all binding sequences contained small amino acids in similar proportions. Furthermore, hydroxyl groups in serine and threonine seemed to play an important role in all four of the metal substrates employed regardless of the structure of the substrate. It is interesting to note that, even at this early stage in the quest for metal-binders, the sequences obtained so far through different display technologies or the use of different substrate structures did not make considerable differences in terms of these common features.

In the case of metal-oxide binders, the presence of basic amino acids, mainly amine derivative, i.e. arginine and histidine, were prevalent. Hydroxyl functional groups, although to a lesser extent, were also present in metal-oxide binders, perhaps showing a possible link to metal binding domains. A further analysis of the presence and position of certain amino acids might give us a better perspective towards the existence of specific binding domains for a given inorganic material or a group of specific materials. The knowledge of these specific sequences would lead to further research in using these peptides as heterofunctional molecular linkers with utility in self-assembly of nanostructure components with distinct functionalities. Even so, many more sequences for materials and their specific surfaces need to be isolated to quantitatively establish a possible link between binder sequences and the specifics of the associated surfaces, including stereochemistry, morphology, crystallography and size. Once inorganic binding properties are established, these specific sequences could then be introduced, through genetic manipulation, into large protein frameworks. These molecular platforms would potentially provide desirable structure and function with a new generation of nanostructured multifunctional materials that can be manufactured for nano- and nano-biotechnology applications [14,31–34].

4. Controlled assembly of engineered polypeptides on solid surfaces—a significant step towards developing engineered functional molecular coatings

Controlled binding and assembly of proteins onto inorganics is at the core of biological material science and engineering with applications ranging from biotechnologies and sensor-technologies to practical engineering such as coatings [14,30–33]. The attachment of biomolecules, in particular proteins, onto solid supports is fundamental in the development of advanced biosensors and bioreactors [32–34]. Protein adsorption and macromolecular interactions

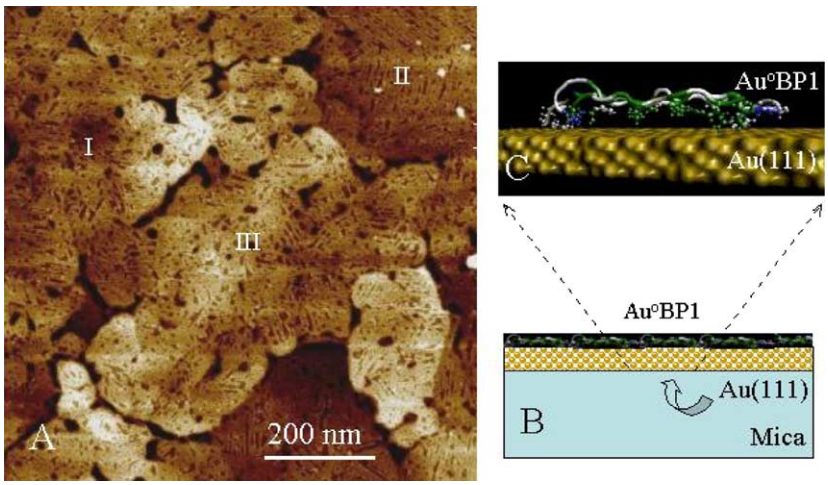

Fig. 6. Ordered monolayer-thick gold-binding protein on Au(1 1 1). (A) AFM image showing six different domains of the protein assembly on Au(1 1 1) surface. I, II, and III indicate different grains all textured about ⟨1 1 1⟩. (B) A scheme of the ordered molecular substrate; and (C) A molecular model of the Au°BP1on Au(1 1 1) lattice, a molecular dynamics study (adapted from ref. 28).

at solid surfaces play key roles in the performance of implants and hard tissue engineering [35]. Finally molecular adsorption on engineering materials is critical in the development of durable systems exposed to fluidic and gaseous environments in both civilian and military application [36]. In a new approach for creating functional molecular substrates, we show here the self-assembly of one of the genetically engineered proteins. For this, we used a gold-binding protein, specifically, GBP1, or Au°BP1, as shown in Fig. 5. In this case, the binding sequence was repeated three times to (proportionally) increase the binding activity as well as to improve the polypeptide stability.

For assembly, a gold-coated mica substrate was first prepared and then heat-treated to have (1 1 1) oriented growth of the grains that had an average size of about 0.5 μm^2. The gold substrates were then inserted into the aqueous solution at pH 7.0 containing the three-repeat gold binders at room temperature. The samples were then imaged using an atomic force microscope (AFM, Nanoscope-III, Digital Instruments, Santa Barbara) in humid air in non-contact mode. The example image shown in Fig. 6 displays regions of monomolecular thick film on several grains of Au, all oriented about ⟨1 1 1⟩. The thickness of the polypeptide film was ensured using a line profile across the AFM image of the film, which had an average thickness of about 0.5 nm, i.e. the height of the β-sheet conformation [28]. As it can be seen in the image, the molecular domains have straight edges and sharp corners. The coverage of more than 95% is possible within about 2 h of the assembly, which was monitored using a surface plasmon resonance spectrometer (SPR). Detailed measurements of the angles among the edges reveal either 60° or 120° values. Knowing that Au(1 1 1) has a six-fold symmetry in this projection, these angles may indicate that the protein had conformed onto the crystallography of the gold surface and formed specific variants of the molecular domains.

5. Potential applications and future prospects

The result that an engineered polypeptide assembles onto its associated inorganic surface forming a monomolecular film has significance in terms of providing practical utility in the molecular biomimetic approach. Firstly, the assembly process is carried out under mild biological conditions of aqueous solution at pH ∼ 7.0 and at room temperature which provide environmentally friendly conditions for thin film formation. Secondly, the formation of a monolayer thick film is a direct demonstration of self-assembly aspect of the engineered proteins. Furthermore, the presence of ordered domains conforming into six-fold symmetry is an indication that it may be commensurate with the Au(1 1 1) lattice, suggesting crystallographic recognition. These results, therefore, illustrate a new class of functional molecular substrates: these are self-assembled genetically engineered polypeptides. The conditions of their assembly suggest that they are biologically-friendly and may be used in more versatile functional applications, in particular, in the large scale manufacturing of practical, cheap, and robust molecular substrates and coatings.

Realizing the fact that thiol and silane linkages are the other two major molecular linkers for noble metal and oxide (silica) surfaces that have constituted the field of self-assembled molecules until now [3,34] (Fig. 2). It is naturally expected that self-assembled engineered polypeptide monolayers could also be used as “molecular erector sets”. If so, this would open up new avenues for designing and engineering novel functional surfaces for a wide variety of nanotechnology and biotechnology applications, including chemical and biological sensors, nanobiotechnology, and engineered coatings [36]. In designing new coatings or layered material, both proteins and inorganics would be integral parts of the engineering material system (Fig. 7). In particular, the modularity of binding motifs should allow

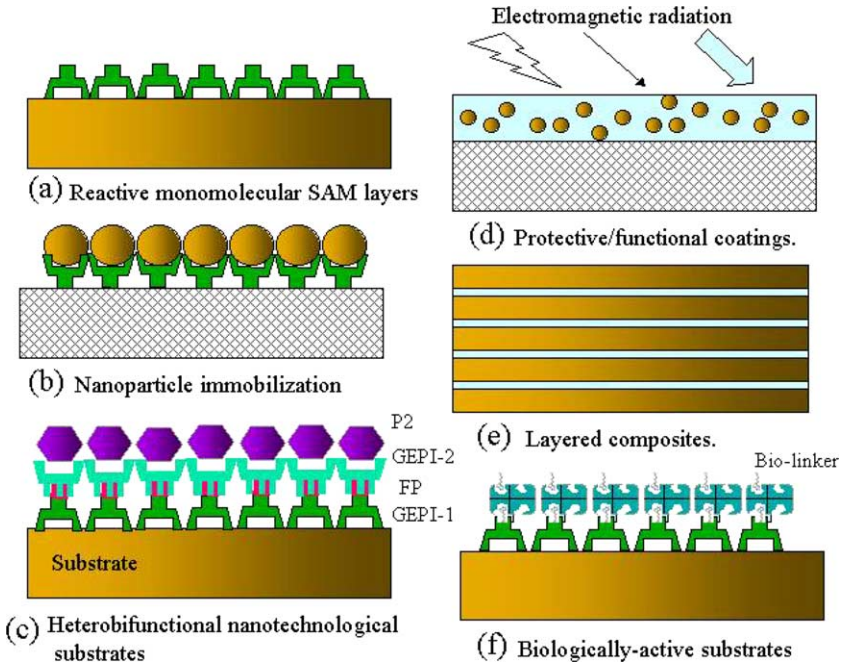

Fig. 7. Possible scenarios are shown for the use of GEPI as molecular substrates for various nano- and bionanotechnological applications. (a) A functional molecular substrate bound to an inorganic, ready for use in molecular immobilization. (b) Assembly of nanoinorganic particles immobilized via GEPI onto a solid substrate. (c) Immobilization of secondary nanoparticles (P2) via a heterobifunctional GEPI complex: GEPI-1 + fusion protein + GEPI-2 onto a primary inorganic substrate. (d) Nanoparticle embedded (GEPI assisted) composite coating against electromagnetic radiation or external mechanical stress. (e) Layered composite using GEPI as the binder between inorganic layers. (f) Biologically active surface using GEPI as the linker that binds to an inorganic.

genetic (or chemical) fusion of peptide segments recognizing two different materials, one being a nanoparticle and the other being a surface of an inorganic engineering material (Fig. 7a–c). Based on its recognition and self-assembly characteristics, the role of the engineered protein in these hybrid structures would be to provide the essential molecular linkage between the inorganic components, and at the same time, be an integral component of the overall structure providing to it functional (e.g. mechanical) durability. Amount, structural, and property characteristics of the nanoparticulate inorganic might be adjusted to produce highly controllable functionality to the coating applied to an existing bulk material (or a substrate) (Fig. 7d–f). The structural characteristics would include morphology, size, and mineralogy; controlling mechanical properties such as hardness, wear resistance, and impact resistance, and chemical stability. Possible functional characteristics would include optical, magnetic, and semiconducting properties, leading to the development of light-sensitive or magnetic field-sensitive materials, or coatings used in mirrors or shielding (e.g. electromagnetic or microwave) (Fig. 7d).

Acknowledgements

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