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Review

Molecular biomimetics: nanotechnology and bionanotechnology using genetically engineered peptides

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Nature provides inspiration for designing materials and systems, which derive their functions from highly organized structures. Biological hard tissues are hybrid materials having both inorganics within a complex organic matrix, the molecular scaffold controlling inorganic structures. Biocomposites incorporate both biomacromolecules such as proteins, lipids and polysaccharides, and inorganic materials, such as hydroxyapatite, silica, magnetite and calcite. The ordered organization of hierarchical structures in organisms begins via the molecular recognition of inorganics by proteins that control interactions and followed by the highly efficient self-assembly across scales. Following the molecular biological principle, proteins could also be used in controlling materials formation in practical engineering via self-assembled, hybrid, functional materials structures. In molecular biomimetics, material-specific peptides could be the key in the molecular engineering of biology-inspired materials. With the recent developments of nanoscale engineering in physical sciences and the advances in molecular biology, we now combine genetic tools with synthetic nanoscale constructs to create a novel methodology. We first genetically select and/or design peptides with specific binding to functional solids, tailor their binding and assembly characteristics, develop bifunctional peptide/protein genetic constructs with both material binding and biological activity, and use these as molecular synthesizers, erectors and assemblers. Here, we give an overview of solid-binding peptides as novel molecular agents coupling bio- and nanotechnology.

Keywords: bioinspiration; material-specific peptides; molecular recognition; biological materials evolution; binding and assembly; bionanotechnology

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1. Introduction

(a) Inspiration and lessons from biology

Nature provides inspiration for engineering structural and processing design criteria for the fabrication of practical materials to perform life's functions (Sarikaya *et al.* 1990; Sarikaya 1994; Mann & Calvert 1998). During the last two decades, the realization that nanoscale inorganic materials have interesting physical characteristics based on their nanometre-scale size (1–100 nm)-driven promises and expectations from nanotechnology with potential applications in both engineering and medical systems. Although there have been significant advances in the applications of nanotechnology, there have also been serious limitations mostly based on the problems associated with the assembly of nanoscale objects. These stem from the limitations in nanotechnological systems in controlling surface forces, inability to synthesize homologous sizes or shapes, and limitations in their higher scale, controlled organizations. In biological systems, on the other hand, inorganic materials are always in the form of nanometre-scale objects, which are self-assembled into ordered structures for full benefits of their function, that derive from their controlled size, morphology and organization into two- and three-dimensional constructions. Recently, this realization, therefore, brought biomimetics back into the forefront for renewed inspiration for solving nanotechnological problems (Sarikaya 1999; Ball 2001; Seeman & Belcher 2002). Biological materials are highly organized from the molecular to the nano-, micro- and the macroscales, often in a hierarchical manner with intricate nanoarchitectures that ultimately make up a myriad of different functional elements, soft and hard tissues (Alberts *et al.* 2008). Hard tissues such as bones, dental tissues, spicules, shells, bacterial nanoparticles are examples that all have one or more protein-based organic components that control structural formation as well as become an integral part of the biological composites (Lowenstam & Weiner 1989; Sarikaya & Aksay 1995). These include slaffins and silicateins in silica-based structures, amelogenin in enamel and bone morphogenesis proteins or collagen in mammalian bone-, calcite- or aragonite-forming proteins in mollusc shells and magnetite-forming proteins in magneto-tactic bacteria (Berman *et al.* 1988; Cariolou & Morse 1988; Schultze *et al.* 1992; Paine & Snead 1996). The inorganic component could be of various types of materials (traditionally called 'minerals') with highly regular morphologies and three-dimensional organizations. These include piezoelectric aragonite platelets in nacre (figure 1*a*), precipitation-hardened single-crystal calcite with a complex architecture in sea urchin spines (figure 1*b*), optically transparent silica layers in sponge spicules (figure 1*c*) and superparamagnetic nanoparticles in magnetotactic bacteria (figure 1*d*).

The types of inorganics chosen by the organism have precursors or raw ingredients that are either in the soil, water or air that can relatively easily be accessed to (Lowenstam & Weiner 1989; Sarikaya & Aksay 1995; Mann 1996). In addition to the intrinsic physical properties, the overall function and performance of the biological material, therefore, is derived by the high degree of control that the organisms have over the formation of the structure of the material produced. The traditionally used term, 'biomineralization', therefore, is misnomer, as the inorganics produced are not minerals but are materials with

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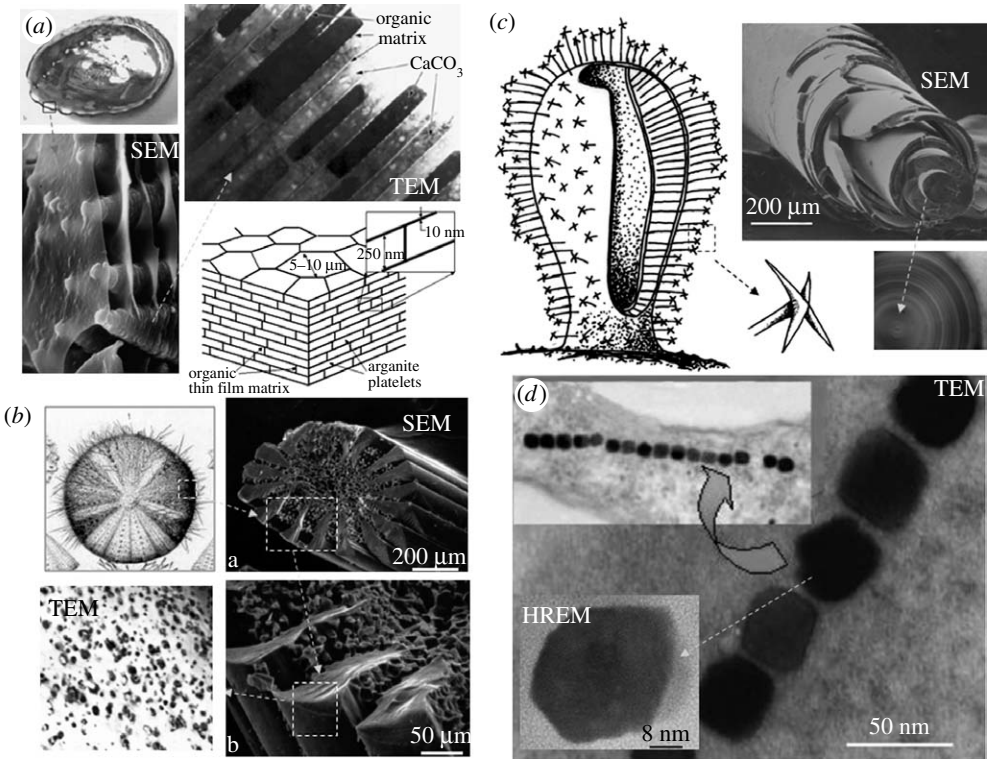


Figure 1. Examples of biologically fabricated, hierarchically structured (protein+inorganic solid) hybrid, functional nanomaterials. (*a*) Layered nanocomposite: growth edge of nacre (mother-of-pearl) of abalone (*Haliotis rufescens*). Nacre is made of aragonite platelets separated by a thin film of organic matrix. (*b*) Sea urchin spine is a single-crystal calcite with complex architecture containing internal nanometre-scale $MgCO_3$ precipitates. (*c*) Sponge spicule (*Rosella*) is an optical fibre made of layered amorphous silica with the central proteinaceous core. The apex of the spicule is a star-shaped lens, a light collector. (*d*) Magnetotactic bacteria (*Aquaspirillum magnetotacticum*) contain superparamagnetic magnetite (Fe_3O_4) particles aligned to form a nano-compass that senses the Earth's magnetic field.

'unique' architectures with detailed micro- and nanostructures, including the defect structures such as dislocations and mechanical or crystallographic twins, all specific to the organism that is producing them (Sarikaya 1994). For example, even in the case of mother-of-pearl, each of the organisms, e.g. pinctada, nautilus or abalone, producing it has different single-crystal aragonite platelets that are different from each other and each different from that of geological aragonite single crystal, both in term of the crystal itself, morphology and, more significantly, intrinsic physical property, such as elastic modulus. From this point of view, these materials fabrication processes could be called biomaterialization to give the true meaning to the biological processes. The biological processing or fabrication (different from bioprocessing or biomimetic processing) is accomplished at ambient conditions of (near) room temperature, pH approximately 7.0 and in aqueous environments (Lowenstam & Weiner 1989; Coelfen & Antonietti 2008).

As we see in figure 1*a*, nacre has a brick-and-mortar architecture that is a layered segmented aragonitic (orthorhombic $CaCO_3$) tiles separated by an organic matrix. The organics is in the form of a 10 nm or thinner film that contains both proteins and polysaccharides, such as chitin. Either within the layer or on the surface of the organic film or within the particles themselves, the proteins possibly nucleate the inorganic, aragonite, establish its crystallography and control the growth. The resultant architecture, mother-of-pearl, is one the most durable hybrid composites with excellent specific toughness/strength combinations (Mayer & Sarikaya 2002). In figure 1*b*, sea urchin spines are single crystals of calcites (rhombohedral $CaCO_3$) with complex architectures. The spicule has high toughness and elastic modulus, unusual for a mineral calcite. Despite its single crystallinity, excellent mechanical property combinations in the spicule is probably due to the presence of nanoscale $MgCO_3$ precipitates, of which each associated with a strain field, toughening the, otherwise, brittle calcite matrix through microcrack closure (H. Fong & M. Sarikaya 2008, unpublished data). Both the formation of the complex architecture of the calcite and the presence of precipitates must, again, be due to the control that proteins have over these essential structural formations. Another example (figure 1*c*), the spicules of the sponge species, *Rosella* are known to have excellent light collection (via the lens-shaped tip) and transmission (via the stem) properties with interesting layered structure made up of non-crystalline silica (Sarikaya *et al.* 2001), all controlled by the silica-binding proteins known as silicatein (Morse 1999; Muller 2001). Finally, in magnetotactic bacteria (figure 1*d*), superparamagnetic single particles of magnetite (Fe_3O_4) form a string of particles aligned to sense the Earth's magnetic field, aligning the bacteria and directing its motion via magnetotaxis (Frankel & Blakemore 1991). Each of the magnetite particles forms within a proteinacous magnetosome membrane, a component of which directs the magnetite formation (Sakaguchi *et al.* 1993).

In each of the examples above, through materialization, the resultant hybrid composite structures, incorporating inorganic and proteinaceous components, are organized at the nanometre and higher dimensions, resulting in viable mechanical, magnetic and optical devices and each offer unique design, not yet seen in man-made engineered systems. These functional biological systems are simultaneously self-organized, dynamic, complex, self-healing and multifunctional, and have characteristics difficult to achieve in purely synthetic systems even with the recently developed bottom-up processes that use molecules and nanocomponents. Under genetic control, biological tissues are synthesized in aqueous environments in mild physiological conditions using biomacromolecules, primarily proteins but also carbohydrates and lipids. Proteins both collect and transport raw materials, and consistently and uniformly self- and co-assemble subunits into short- and long-range ordered nuclei and substrates (Tamerler & Sarikaya 2007). Whether in controlling tissue formation or being an integral part of the tissue in its biological functions and physical performance, proteins are an indispensable part of the biological structures and systems. A simple conclusion is that any future biomimetic system, whether for biotechnology or nanotechnology, should include protein(s) in its assembly and, perhaps, in its final hybrid structure (Sarikaya *et al.* 2003).

In traditional materials systems, the final product is a result of a balance of interactions, dictated by the kinetics and thermodynamics of the system, that are often achieved through 'heat-and-beat' approaches of the traditional materials

science and engineering, which provide the energy for structural formations (Kingery 1976; Reed-Hill 1991). In biological systems, on the other hand, the same balance, and the energy, is achieved through evolutionary selection processes that result in the emergence of a specific molecular recognition using peptides and proteins (Pauling 1946). As we discussed below, and throughout the paper with examples, our approach is to engineer peptides with materials selectivity and use these as molecular building blocks in organizing functional materials systems in practical proof-of-principle demonstrations. Availability of new platforms will bring to the forefront new materials functionalities provided by the solid-binding peptides that will extend current technology via coupling nanoentities using the principles of biosorption beyond those provided by the traditional chemisorption or physisorption.

(*b*) *Molecular biomimetics pathways to nano- and bionanotechnology*

Molecular biomimetics is using biology's molecular ways in genetic selection or design of proteins and peptides that can control the synthesis of nanoscale objects and self-assembly of higher ordered multifunctional materials systems (Sarikaya *et al.* 2003). In the development of the molecular biomimetics protocols in nanotechnology, therefore, one uses solid-binding peptides and control the formation, assembly and organization of functional nanoentities towards building useful technologies. To accomplish the overarching task, we integrate recent developments in molecular- and nanoscale engineering in physical sciences (nanoparticle formation, nano- and micropatterning such as dip-pen nanolithography and microcontact printing, and self and directed assemblies), and the advances in molecular biology, genetics and bioinformatics towards materials fabrication all at the molecular and nanometre scales (Sarikaya 1999; Sarikaya *et al.* 2003). Using closely controlled molecular, nano- and microstructures through molecular recognition, templating and self-assembly properties in biology, this field is evolving from the true marriage of physical and biological sciences towards providing practical application platforms (Niemeyer 2001; Sarikaya *et al.* 2004). The advantage of the new approach for nanotechnology is that inorganic surface-specific proteins could be used as couplers, growth initiators and modifiers, bracers and molecular erector sets, i.e. simply as building blocks for the self-assembly of materials with controlled organization and desired functions from the bottom-up.

The realization of heterofunctional nanostructure materials and systems could be at three levels (Sarikaya *et al.* 2004), all occurring simultaneously with a closely knit feedback similar to the biological materials formation mechanisms (Alberts *et al.* 2008). The *first* is that the inorganic-specific peptides are identified and peptide/protein templates are designed at the molecular level through directed evolution using the tools of molecular biology. This ensures the molecular scale and up processing for nanostructural control at the lowest practical dimensional scale possible. The *second* is that these peptide building blocks can be further engineered to tailor their recognition and assembly properties similar to the biology's way of successive cycles of mutation and generation can lead to progeny with improved features eventually for their usage as couplers or *molecular erector sets* to join synthetic entities, including nanoparticles, functional polymers or other nanoentities on to molecular

templates (molecular and nanoscale recognition). Finally, the *third* is that the biological molecules self- and co-assemble into ordered nanostructures. This ensures an energy-efficient robust assembly process for achieving complex nano, and possibly hierarchical structures, similar to those found in biology (self-assembly; Sarikaya *et al.* 2004).

Q14 In the following sections, we provide an overview of molecular biomimetics approaches to achieve the premises of bionanotechnology with specific applications, mostly, in medicine, and summarize their potentials and limitations. Here, we first summarize the protocols, adapted from molecular biology to materials science and engineering, for selecting polypeptides that recognize and bind to solids, and describe the protocols of combinatorial biology for identifying, characterizing and genetically engineering peptides for practical use. We emphasize cell surface and phage display approaches that are well adapted for the identification of solid material-specific peptides and to explain ways to further tailor peptides using post-selection engineering and bioinformatics pathways. The protocols, established over years in this group, are presented in the quantitative binding characterization of the peptides using various spectroscopic techniques. We also briefly discuss possible mechanisms through which a given peptide might selectively bind to a material. Finally, we present extensive practical examples of current achievements in the usage of the solid-binding polypeptides as building blocks to demonstrate their wide range of applications and, finally, discuss future prospects.

2. Genetic selection and directed evolution of solid-binding peptides

(*a*) *Biocombinatorial selection of peptides*

Genetically engineered peptide for inorganics (GEPI) is selected through affinity-based biopanning protocol (Sarikaya *et al.* 2003). Biopanning steps consist of contacting the library with the material of interest, then washing out weak or non-binders 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. During the biopanning step, a minimum of three to five cycles of enrichment is 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 on a plasmid carried by the cell, the identity of the selected sequences (e.g. their amino acid compositions) can be deduced by DNA sequencing (figure 2).

We selected peptides for a variety of materials including noble metals (such as Au, Pt and Pd), metals (Ag and Ti), oxide and nitride semiconductors (e.g. Cu_2O, ITO, GaN, ZnO), minerals (such as mica, hydroxyapatite, calcite, aragonite, sapphire and graphite) or biocompatible substrates (such as silica, titania and alumina) that were selected by using either phage display (specifically filamentous phage strain M13) or cell surface display (specifically flagellar display) (Sarikaya *et al.* 2004). There are also a number sequences selected for various materials by other groups. The ones selected via cell surface display includes gold (Brown 1997) and zinc oxide (Kjærgaard *et al.* 2000), whereas phage display selected ones are for their affinity towards gallium

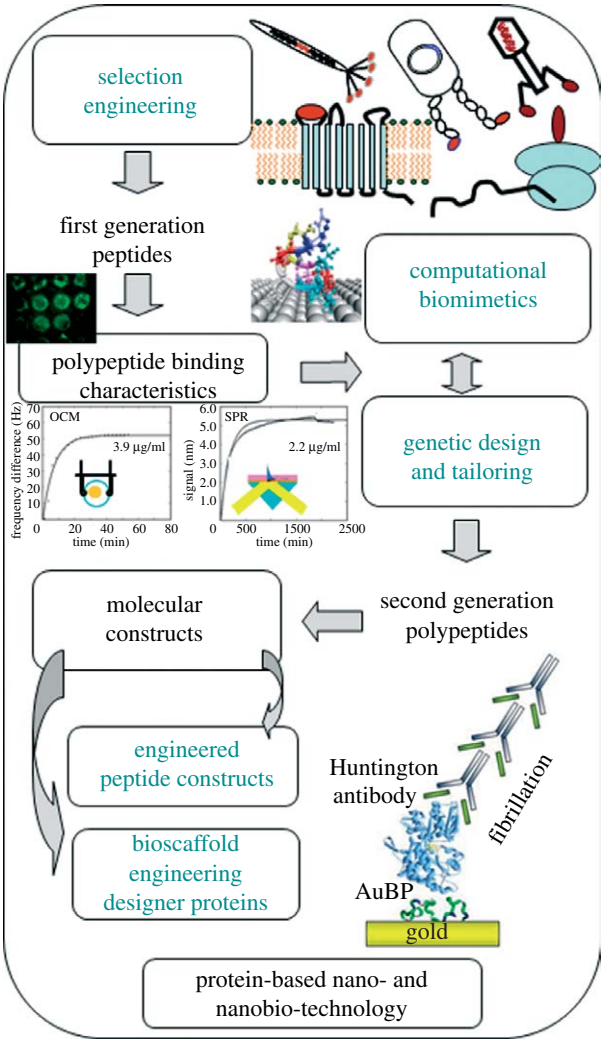


Figure 2. Standardized steps in the selection, binding characterization, designing/tailoring of solid-binding peptides and their usefulness as bifunctional molecular constructs.

arsenide (Whaley *et al.* 2000), silica (Naik *et al.* 2002*a*), silver (Naik *et al.* 2002*b*), zinc sulphide (Lee *et al.* 2002*a*,*b*), calcite (Li *et al.* 2002), cadmium sulphide (Mao *et al.* 2003) and titanium oxide (Sano *et al.* 2005). Some of biocombinatorially selected peptides have been used to assemble inorganic particles (Whaley *et al.* 2000; Lee *et al.* 2002*a*,*b*; Mao *et al.* 2003) or to control nucleation of the compounds that they were selected for (Li *et al.* 2002; Naik *et al.* 2002*a*,*b*).

When one is focusing on the material-specific peptide interactions, finding a consensus sequence might lead to a misleading result. 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. As it is well known, the health of genetic diversity leads to an assortment of sequences, which presumably reflects

the heterogeneity of the inorganic substrates at the atomic, topographic, chemical and crystallographic levels. Chemical diversity of the surfaces alone could produce a variety of sequences due to the different binding strategies that the peptide library could entail that are derived from the shape and lattice complementarities, electrostatic interactions, van der Waal's interactions or various combinations of these mechanisms (Kulp *et al.* 2004; Evans *et al.* 2008; Seker *et al.* in print). The ultimate robust usage of the inorganic-binding peptides for the fabrication and assembly of hybrid materials and systems requires fundamental studies towards better insights into peptide–solid molecular interactions and their incorporation into the design of desired material-specific peptides.

(*b*) *Structural design concepts: mutation, multimerization, conformational constraints*

Both the amino acid content (chemistry) as well as the sequence of the amino acids (molecular conformation) in a given selected set of peptides could affect their binding characteristics. We have recently demonstrated that the molecular constraints can be used to tune the architectural features and, consequently, the binding properties of the first generation of selected peptides. Specifically, we used a high-affinity 7-amino acid Pt-binding sequence, PTSTGQA, to build two different constructs: one is a Cys–Cys constrained 'loop' sequence (CPTSTGQAC) that mimics the domain used in the pIII tail sequence of the phage library construction, and the second is the linear form, a septapeptide, without the loop (Seker *et al.* 2007). By incorporating surface plasmon resonance (SPR, measuring binding) and circular dichroism (CD, determining molecular architecture), one is able to analyse the consequence of the loop constraint on peptide adsorption and kinetics and the conformation of peptides. These studies are related to each other with a comparative approach (as determined in figure 2).

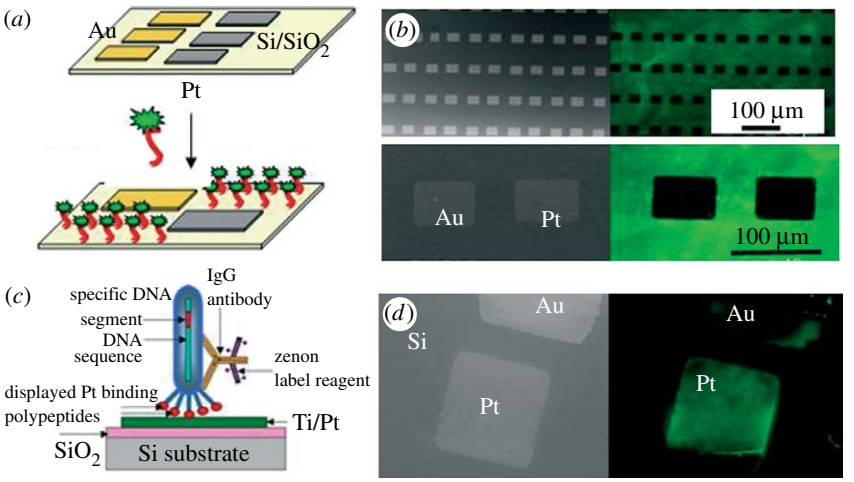
One may also modify the binding activity of a given selected peptide by simply increasing the number of repeats of the original sequence. This multimerization could be accomplished using the simple tandem repeat, i.e. sequential attachment of the original sequence. We applied multiple-repeat-based strategy on both phage display selected platinum and quartz binder (7 and 12 amino acid sequences each, respectively) and cell surface selected gold binders (14 amino acids each). One would expect that, as the number of repeats increased, there would be an increase in the binding activity of a given peptide. Surprisingly, however, not in all cases, the increase in the number of repeating peptide was reflected in the enhancement of binding activity. In addition, material selectivity behaviour of each of the single peptides also changed when they were used in multiple-repeat forms. These results indicate that, rather than the amino acid content in a given material-binding sequence, it is the molecular conformation (secondary structure) that is more relevant, which dictates the solid-binding function. These preliminary results, therefore, show that there is a correlation between conformational instability (or adaptability) and binding ability (Seker *et al.* in print). It is imperative that, in the next stage of multimerization studies, one could incorporate designed linkers between successive sequences to intentionally conform the overall multiple-repeat second-generation peptides for desired binding and other biological functions.

(*c*) *Binding and assembly of peptides on solids*

In the design and assembly of functional inorganic solids, it is essential to understand the nature of polypeptide recognition and binding on to solid materials. Although considerable research has been directed in the literature towards understanding peptide binding to solids, it is not yet clear how proteins recognize an inorganic surface and how it could be manipulated to enhance or reduce this binding activity. This problem is similar to protein–protein recognition in biology (Pauling 1946); in the current hybrid systems, the problem reduces to one of peptide–solid interface. Here, the peptide is relatively small, perhaps approximately 10 amino acids long (1 kD), and the inorganic solid is relatively flat but with atomic and molecular features with mostly crystallographic lattice organization. The specificity of a protein for a surface may originate from both chemical (e.g. H-bonding, polarity and charge effects) and physical (conformation, size and morphology) recognition mechanisms (Izrailev *et al.* 1997; Dai *et al.* 2000; Evans 2003; Evans *et al.* 2008). Recent studies have also demonstrated that the peptide overall molecular architecture (i.e. constraint versus linear) plays a key role in the solid recognition (Hnilova *et al.* 2008). For a given system, these mechanisms may be all significant, but with varying degrees depending on the peptide sequence, chemistry and topology of the solid surface, and the conditions of the solvent (water). Therefore, each, with a certain degree, would contribute towards a collective behaviour. Similar to the molecular recognition in biomacromolecular systems, the major contribution, however, comes from amino acid sequences that lead to a specific molecular conformation on the surface of the solid, and to a lesser extent on composition and overall amino acid content of the peptide, as demonstrated in the example below (see §2*e*).

(*d*) *Peptide binding to solids and kinetics*

Among the experimental approaches to rapidly monitor the protein adsorption and binding on inorganics is fluorescence microscopy (FM), which now become a routine tool as a first step in the qualitative evaluation of these sequences with respect to their affinity and selectivity (figure 3). The FM imaging is an essential part of the screening protocol in our laboratory. However, this type of characterization does not provide quantitative information of polypeptide adsorption or detailed binding kinetics or mechanism(s). Another frequently used technique in molecular biology binding assays is ELISA, an immuno-fluorescence labelling detection using monoclonal antibody conjugated with secondary antibody fragments (Brown 1992; Whaley *et al.* 2000; Naik *et al.* 2002; Dai *et al.* 2004; Sarikaya *et al.* 2004). Although time consuming and statistically less significant, scanning probe microscopy (SPM) protocols could also be used, which require the integration of sample preparation, self-assembly, tip design, observation conditions, data analysis and interpretations of specific polypeptides binding on to inorganic surfaces (Whitesides *et al.* 1991). Both atomic force microscopy (AFM) and scanning tunnel microscopy (STM) techniques have been used to acquire static information of peptide binding to solids. The quantitative data towards determining kinetic parameters of binding could, however, be obtained using more established techniques such as quartz crystal microbalance (QCM; Murray & Deshaires 2000; Bailey *et al.* 2002) and SPR spectroscopy (Czenderna & Lu 1984; Homola *et al.* 1999).



Q4 Figure 3. Material selectivity of inorganic-binding peptides (*a*) peptide alone, the case of quartz-binding peptide conjugated with a fluorescein molecule and (*b*) peptide displayed on the host organism, i.e. Pt-mutant phage, PtBP1, fluorescently labelled. The contrast reversal, as visualized using a fluorescence microscope in both cases, indicates the material specificity of QBP for silica against Au or Pt, and PtBP for Pt against Si and Au, respectively.

Both QCM and SPR (figure 2) have been successively used to quantitatively analyse peptide adsorption kinetics under various protein concentrations, solution properties, such as pH and salinity, and solid surface conditions (Sarikaya *et al.* 2004; Sano *et al.* 2005; Seker *et al.* 2007; Hnilova *et al.* 2008). Recently, conventional spectroscopy techniques, such as X-ray photoelectron spectroscopy and time-of-flight-secondary ion mass spectroscopy techniques, have also proven to provide the fingerprint of peptide adsorption on to the surfaces (Coen *et al.* 2001; Suzuki *et al.* 2007). Although difficult to carry out, the application of solid and liquid state NMR could provide quantitative information of molecular conformations of peptides, essential information towards the understanding of the mechanism of polypeptide binding on to solids (Evans 2003). Finally, molecular modelling that studies interface interactions between a peptide and a solid will lead to rapid evaluations of various types of hybrid interfaces. These studies, e.g. molecular dynamics, that make use of computational chemistry, biology and physics, are still in their infancy, but are expected to provide protocols in the near future through the implementation of model experimental systems coupled with theoretical approaches (Evans *et al.* 2008).

A detailed understanding of the peptide recognition and assembly processes will inevitably lead to better insights into the design of peptides for tailored binding. A better knowledge of the mechanisms of the quantitative adsorption may become possible through high-resolution surface microscopy (e.g. AFM and STM), molecular spectroscopy and surface diffraction studies as well (such as small angle X-ray diffraction). Many of these techniques, with their advantages and pitfalls, have been discussed extensively in the literature; in this review, we will discuss one technique, SPR, which provides the most practical information on binding kinetics and materials selectivity of peptides for solid and, therefore, frequently used in our research in the identification of the most promising peptides that are in frequent use today for practical implementations (§3).

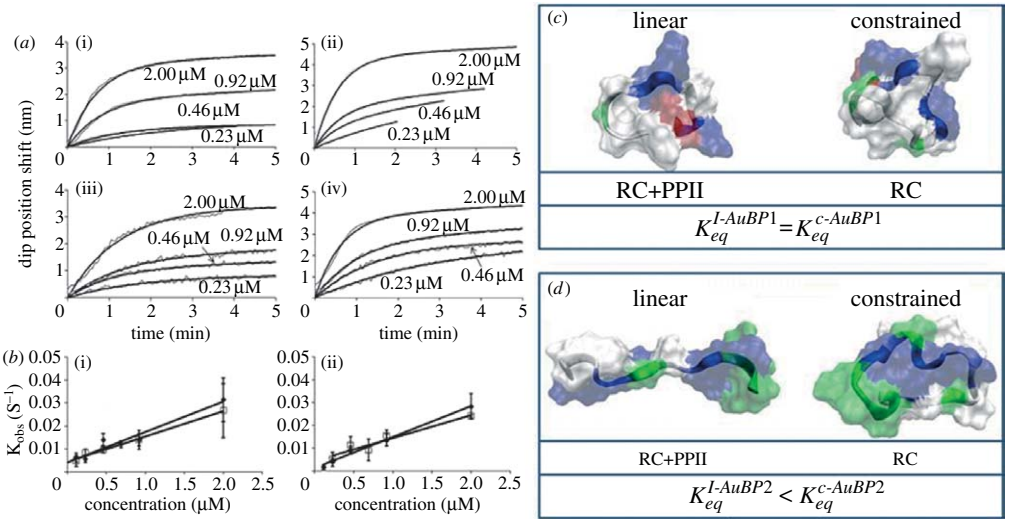


Figure 4. Effect of GEPI conformation on binding, the case study with gold-binding peptides. (*a*,*b*) SPR studies of the binding of AuBP1 and AuBP2, constraint and linear, respectively, are given ((*a*) (i) l-AuBP1, (ii) l-AuBP2, (iii) c-AuBP1, (iv) c-AuBP2; (*b*) (i) diamonds, l-AuBP1; squares, c-AuBP1; (ii) diamonds, l-AuBP2; squares, c-AuBP2). (*c*,*d*) Molecular architectures of the linear and constraint forms, respectively, are given ((*c*) AuBP1, (*d*) AuBP2). Note that the linear and constraint forms of AuBP2 have the same molecular conformation and, therefore, the same binding property while AuBP1 has two different conformations in two architectures and, therefore, the binding strengths are different.

(*e*) *Peptide adsorption via molecular architectural control*

Most studies on the adsorption behaviour of combinatorially selected inorganic-binding peptides on to solids have focused mainly on their amino acid compositions (Naik *et al.* 2002; Mao *et al.* 2003). Only recently some studies have addressed the peptide structural constraints on the adsorption behaviour and affinity to solids (Tamerler *et al.* 2006*a*,*b*; Makrodimitris *et al.* 2007; Seker *et al.* 2007; Gungormus *et al.* 2008; M. Gungormus, D. Khatayevich, C. So, C. Tamerler & M. Sarikaya 2008, unpublished data). It is well known in protein engineering that the protein molecular architecture affects its function (Alberts *et al.* 2008). In this example, we hypothesized that the structure–function relationship also persists in peptide binding to inorganic materials (figure 4). To assess the hypothesis, we used two gold-binding peptides that were originally selected in a cyclic form, i.e. constraint architecture, and compared their adsorption and conformational behaviours to those of their linear, free, forms using, respectively, SPR and CD spectroscopy and computational modelling. We used two gold-binding sequences that were originally selected using the FliTrx cell surface approach (Hnilova *et al.* 2008). These two peptides, AuBP1 (WAGAKRLVLRRE) and AuBP2 (WALRRSIRRQSY), were synthesized using solid-state technique in an open dodecapeptide version, called linear (*l*) as well as in constraint form, i.e. through an 18-aa Cys–Cys constrained loops, called cyclic (*c*), to mimic the original FliTrx displayed peptide conformations. We first carried out the CD spectroscopy to assess the molecular conformations and found that the cyclic versions of AuBPs have mainly random coil structures;

however, the linear versions of AuBPs also have some degree of polyproline type II (PPII) rigid structures in addition to the random coil structures (Hnilova *et al.* 2008). The percentage of PPII structure in *l*-AuBP2 is greater than that in *l*-AuBP1, and, thus, the structural differences between the *l*- and *c*-versions of AuBP2 are much bigger than the structural differences between the *l*- and *c*-versions of AuBP1.

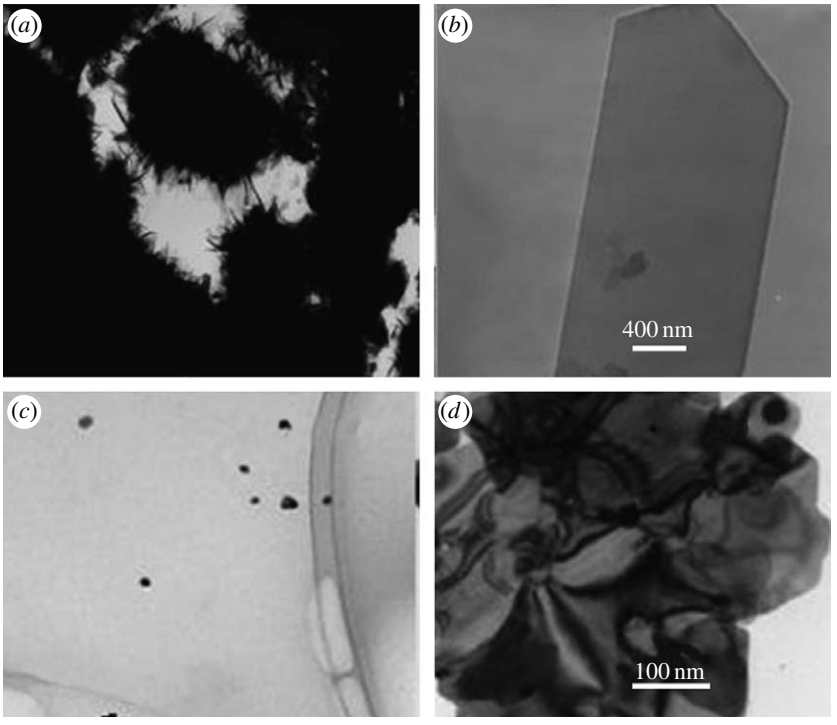
The SPR analysis showed that both the linear and cyclic forms of AuBPs have high affinities to gold (e.g. $\Delta G_{ads} = -8.7\,\mathrm{kcal\,mol^{-1}}$). We also found that both the linear and cyclic forms of AuBPs have random coil and PPII structures, which cooperatively promote unfolded, conformationally labile peptides that may enhance their adaptability to interfacial features that exist on gold surfaces. One would expect differences in the binding characteristics between the cyclic and linear forms as the structure may change. In fact, we found that AuBP2 has an order of magnitude higher affinity in the cyclic version than the linear one (figure 4). This difference is consistent with the observation of significant structural change in the molecular conformations of the cyclic and linear versions of AuBP2 in solution. On the other hand, the binding affinities of AuBP1 in the cyclic and linear forms are quite similar. In this case, the molecular structures of this peptide in the two architectures are similar, as we show both experimentally (CD) and via modelling. On the basis of all the evidence, we show that the sequence of the amino acids in a given peptide and its molecular conformation may be the key determinants that facilitate peptide-selective binding on solid materials (Hinlova *et al.* 2008).

3. Implementations of solid-binding peptides in bionanotechnology

Once a bank of fully characterized solid-binding peptides becomes available, then it could be used as a 'molecular toolbox' for a wide range of applications from solid synthesis to molecular and nanoscale assemblies. Here, the peptide is not only be useful in linking one nanomaterial to another, but a GEPI could also be used for genetically fusing it on to another functional protein and use the system as bifunctional molecular construct, where peptide would be the ligand. Alternatively, a GEPI could be fused, chemically, on to a synthetic polymer, to create multifunctional hybrid polymeric structures. Below, we will demonstrate a few uses of various GEPIs in generating new functional materials systems to understand their potential usage as molecular building blocks.

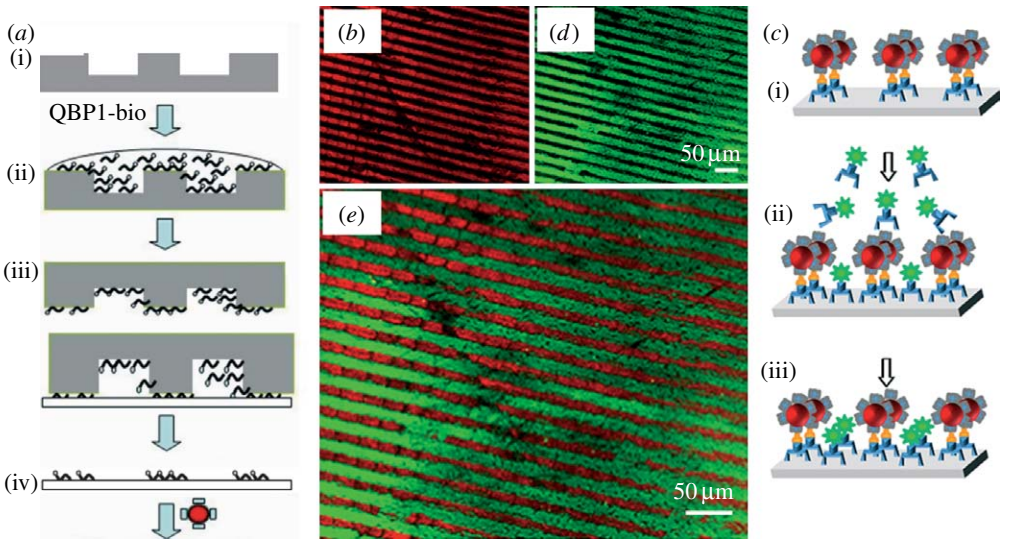
(*a*) *GEPI-assisted synthesis of nanoinorganics*

Given that these genetically engineered peptides recognize and bind to minerals, there may also be an inherent capability within the sequences to influence the morphology of these minerals as well, a prospect that has not yet been fully explored in great detail so far. Once this is achieved, peptide-based molecular scaffolds developed may have great potential for applications in tissue regeneration. An example from our recent work on biomineralization using hydroxyapatite (HA)-binding peptides (Gungormus *et al.* 2008; M. Gungormus, D. Khatayevich, C. So, C. Tamerler & M. Sarikaya 2008, unpublished data) is shown in figure 5*a*,*b*. We demonstrated that the biocombinatorially selected HA-binding peptides could offer a route for regulating calcium phosphate-based



Q6 Figure 5. Peptide-assisted biomaterialization using GEPIs. (*a*,*b*) Hydroxyapatite synthesis in the presence of phage display selected HABP1 with respect to a control containing no peptide. (*c*,*d*) Au nanoparticle synthesis in the presence of AuBP with respect to a control prepared by a non-specific peptide (*a*) control, (*b*) w/HABP1, (*c*) control, (*d*) w/AuBP1.

nanocrystal formation within a biomedical context. Specifically, a successful generation of cysteine-constrained M13 bacteriophage heptapeptide library were screened against HA powder. Using the library, we selected 49 sequences and two were identified for further investigation. One of these peptides exhibited the highest binding affinity (HABP1), and the other, a much lower binding affinity (HABP2) to HA, for subsequent calcium phosphate formation and biophysical characterization studies. Here, we were interested in learning whether HA-binding polypeptide sequences could also 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 both peptides affected calcium phosphate formation, with the former exhibiting a higher inhibitory activity over the latter, inducing a desired morphology on the formed Ca-phosphate mineral (figure 5*a*). The resulting nanoparticles are plate shaped, several 10s of nanometres in length and only a few nanometres in thickness. These particles resemble hydroxyapatite particles in dentine in human tooth (Fong *et al.* 2000). These results reveal a possibility of peptides in controlling particle morphology that is the major difference in differentiating the dental hard tissues (dentine, cementum and enamel) as well as the bone architectures. Peptide-controlled morphogenesis of Hap nanoparticles could be used in regulating materialization in hard-tissue regeneration or filler design for tissue restoration.



Q7 Figure 6. (*a–e*) Targeted co-assembly of molecular functional entity (fluorescein attached to QBP1-bio, silica-binding peptide) and nanoparticle (a QD) functionalized with streptavidin targeting biotinylated QBP on a microcontact-printed micropatterned Si substrate (containing native silicon oxide) (*a*) (*i*) incubation, (ii) washing and drying, (iii) stamping, (iv) incubation; (*c*) (i) SA-QD micropattern, (ii) self-assembly of QBP1-F, (iii) fluorescein and QD micropatterns on quartz.

Another example is in the morphology control of gold particles using gold-binding peptides (figure 5*c*,*d*). Gold nanoparticles with 12 nm diameter monosize can be formed at ambient conditions using the well-known Faraday's technique Q23 by reducing $AuCl_3$ by Na-citrate (or other reducing agents; Turkevich *et al.* 1951). In the presence of peptide, reducing the gold concentration and lowering temperature allow particle formation at a slower rate, giving the protein time to interact with surfaces during the growth and provides conditions to examine the effect of gold binding during colloidal gold formation. We conducted a search for mutants that modulated the architecture, i.e. particle versus thin film, of gold crystallites (Hnilova *et al.* 2008). The selection of mutants was based on the change of colour of the gold colloid (from pale yellow to a red colloid), which was related to altered rate of crystallization. Forty gold mutants were tested this way, and the sequence analysis showed that two separate mutants that accelerated the crystal growth also changed the particle shape from cubo-octahedral (the usual shape of the gold particles under equilibrium growth conditions) to flat, thin films (figure 6*c*,*d*). This new observation is interesting from the point of enzymatic effect of protein in crystal growth rather than traditionally assumed templating effect. The polypeptides, in spite of being slightly basic, may have caused the formation of gold crystals similar to those formed in acidic conditions. This suggests that the role of the polypeptides in gold crystallization is to act as an acid, a common mechanism in enzyme function, and the protocol could be used to regulate the shape of metal nanoparticles for photonic and electronic applications.

As demonstrated with the examples above, biocombinatorially selected peptides can have enzymatic effects in the synthesis, morphogenesis and fabrication of inorganic nanomaterials. Similar to biological systems, it may be

expected that the solid-binding peptides may have further potential for size, crystallography and mineral selectivity, with potential usage in a variety of practical applications, from filler material in papers to paints, as well as specialized coatings (Sarikaya *et al.* 2004).

(*b*) *Directed and mediated assembly of functional nanoentities*

Protein microarray technologies, used in proteomics and clinical assays, require efficient patterning of biomolecules on selected substrates (Gristina 1987; Blawas & Reichert 1998; Chicurel & Dalma-Weiszhausz 2002; Cutler 2003; Min & Mrksich 2004; Cretich *et al.* 2006), which is possible provided that the proteins are spatially immobilized on solid substrate via various lithography techniques, e.g. soft lithography (Xia & Whitesides 1998), dip-pen lithography (Lee *et al.* 2002*a*,*b*) and photolithography (Revzin *et al.* 2001). Recently, protein immobilization has become a key issue in bionanotechnology since immobilization provides physical support to the molecule, resulting in improved stability and activity and, furthermore, helps to separate proteins from solution, rendering them reusable (Castner & Ratner 2002; Bornscheuer 2003) The approaches for biomolecule immobilization on glass or metal (e.g. gold) substrates generally require surface functionalization by self-assembled monolayers (SAMs) of bifunctional molecules, such as amino-terminated aminoalkylalkoxysilanes for silica and carboxyl-terminated alkanethiols for gold substrates (Mrksich & Whitesides 1996; Ostuni *et al.* 1999). Despite their widespread usage, these traditionally available linkers have certain limitations, such as causing random orientation of the protein on solid surface and requiring multistep chemical reactions and, furthermore, the assembled monolayers can be unstable during immobilization (Fujiwara *et al.* 2006; Park *et al.* 2006). To overcome these limitations, it is preferable to have molecules as direct linkers to the solid substrate of interest, which not only have all the desired features of the conventional chemically prepared SAMs but also have specificity to a given solid substrate and assemble on to it efficiently. In addition, the molecule used as the linker could be amenable to genetic manipulation for selecting the best linker site to the displayed protein or nanoentity without causing any effect in reducing the binding activity. Solid-binding peptides can provide the multifunctionality as a preferred linker with high structural stability incorporating a target molecule aligned consistently to carry out a desired function (Sarikaya *et al.* 2003).

Here we demonstrate the solid-binding peptide as a molecular assembler for two different nanoentities, quantum dots (QDs) and fluorescent molecules, and sequentially assemble them on a micropatterned surface using the material specificity of the GEPI (Kacar *et al.* in press). In this case, directed immobilization of the QDs is followed by the GEPI-mediated assembly of the fluorescent molecule using the microcontact printing and self-assembly procedures schematically illustrated in figure 6*a*. The directed immobilization of SA-QD on a QBP1-biopatterned surface is shown in figure 6*b* as red stripes, imaged with a fluorescent microscope using a QD605 filter, revealing red fluorescent contrast. Here, the dark stripes represent the regions originally unoccupied, exposing the bare quartz surface (figure 6*a*(i)). Next, following the procedure in figure 6*a*, the assembly of the fluorescent molecule, i.e. fluorescein,

is mediated using the QBP1-F molecular conjugate. The assembled conjugate molecules are imaged in green, as shown in figure 6*c*, using a FITC filter. At this step, the QBP1-F molecular conjugate diffuses towards the regions of the substrate previously unoccupied, after the initial directed immobilization of QDs (figure 6*d*). Both images in figure 6*e*,*c* were recorded from the same area of the sample, showing regular alternating lines of red and green stripes, corresponding to the directed-assembled QDs and mediated-assembled fluorescein molecules, respectively. This result demonstrates that the QBP1 is active as an efficient molecular linker as well as a versatile PDMS ink. Furthermore, we demonstrate here the co-assembly of two diverse nanoentities without the involvement of complex surface modification, often involved in the silane-based traditional procedures (Fujiwara *et al.* 2006). The patterning protocol developed here would be useful as microscale platforms for wide range of applications from generating photonic lattices to co-assembling multi-enzyme or multi-protein assays.

4. Future prospects of solid-binding peptides as molecular building blocks in bionanotechnology

The joining of biology with materials requires an ability to design, engineer and control interfaces at the materials/bio intersections as these sites are significant in the implementation of nanotechnology, developments of new materials and protocols in molecular engineering, and realization of bionanotechnology (figure 7). Biology controls all interfaces between molecular materials, tissues and organs using peptides and proteins which are also the agents of molecular communication. In a sense, proteins are the workhorses in biology carrying out the chemical, physical and biological functions of the organisms. Similar to biology, in engineering and technological systems, we can genetically select peptides with an ability to bind to inorganic materials to create a new fundamental building block to couple bio and synthetic entities. As we describe here, genetically engineered polypeptides for inorganics (GEPI) have short amino acid sequences with material selective binding and self-assembling properties. Once selected using combinatorial mutagenesis, GEPIs can be further tailored to enhance/modify their binding ability and multifunctionality. The multifunctionality could be introduced either using two or more material-binding peptides to create novel ways of making dissimilar materials thermodynamically compatible, or by genetically fusing a functional protein, e.g. enzyme or an antibody, to develop heterofunctional molecular constructs.

Solid-binding peptides coupled with solid substrates form a new generation of novel hybrid materials systems (Sarikaya *et al.* 2003). Genetic control of the coupling and the resulting function of the hybrid material are new approaches with potential to overcome limitations encountered in the progress of wide range of applications in which traditionally synthetic linkers, such as either thiol or silane, have been used. The attachment of biomolecules, in particular proteins, on to 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 (Blawas & Reichert 1998; He *et al.* 2006; Behrens & Behrens 2008). Protein adsorption and macromolecular interactions at solid surfaces play key roles in the performance of implants and

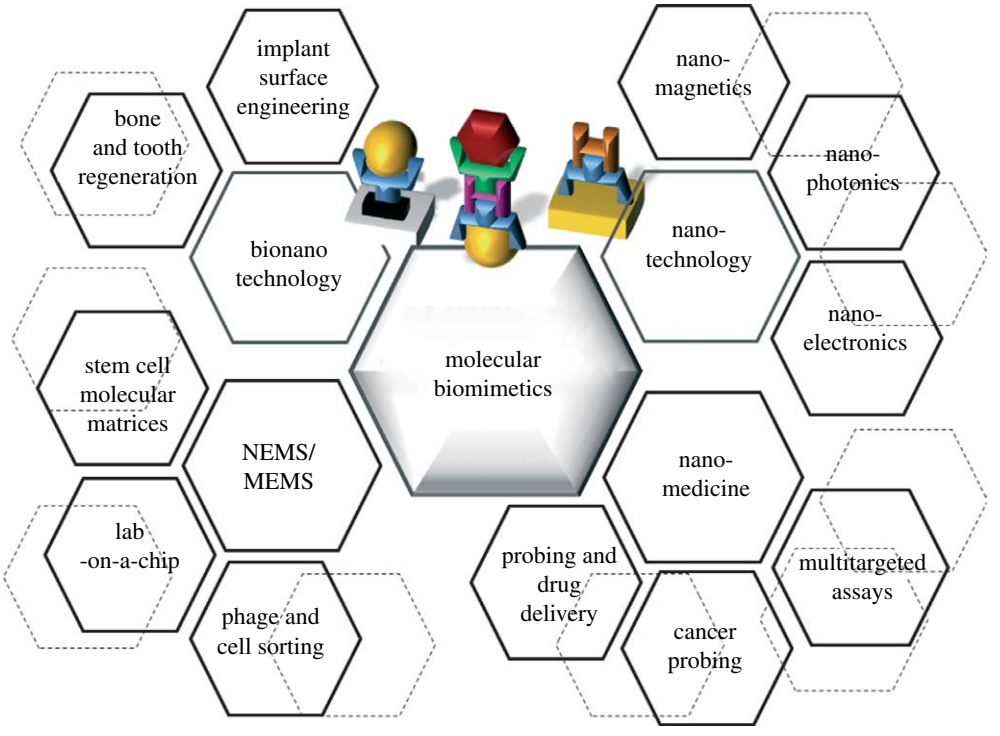


Figure 7. Potential application areas of GEPI in molecular biomimetics field, which include molecular probing, separation, nanotechnology and nanomedicine, with potential of growing in to new areas (dotted hexagons).

hard-tissue regeneration (Gottlieb *et al.* 2008; Ma 2008). Proteins adsorbed specifically on to probe substrates are used to build protein microarrays suitable for modern proteomics (Cuttler 2003; Cretich *et al.* 2006). Enzyme immobilization on substrates (e.g. nanoparticles in a colloid) will greatly enhance the usage of industrial enzymes (Kasemo 2002). Designing bifunctional peptides (e.g. attached to a probe) coupled to nanoparticles, e.g. QDs or fluorescent molecules will provide new avenues for multicomponent biosensor design (Li *et al.* 2007). The same (nanoparticle/GEPI-probe) platform, where the probe is an antibody and the nanoparticle is a therapeutic or imaging entity, will provide new molecular platform for cancer probing (Weissleder 2006; Tamerler & Sarikaya 2007). The examples given above illustrate only a part of achievable goals by these new classes of functional molecular linkers. All these and a wide variety of other applications form the core of biological materials science and engineering (Sarikaya *et al.* 2003) which can be designed and genetically engineered (figure 7). Based on its recognition and self-assembly characteristics, the role of GEPI 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 the functional (e.g. mechanical) durability. Owing to the intrinsic properties mimicked after natural proteins, in the coming years and decades, we are likely to see engineered inorganic-binding polypeptides to be used more and in wide range of applications

from particles synthesis and assembly with genetically controlled physical and chemical characteristics in materials science to probing for biological targets in biology and medicine (Eisledder 2006; Sengupta & Sasisekharan 2007; Tamerler & Sarikaya 2008).

5. Uncited references

Gaskin *et al.* (2000), Eteshola *et al.* (2005), Sanchez *et al.* (2005), Feldheim & Eaton (2007), Guo *et al.* (2007), Rusmini *et al.* (2007), Matthes *et al.* (2008), Shu *et al.* (2008), Tomzcak *et al.* (2008) and Tullman *et al.* (2008).

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