Nanoparticle assembly

Assembly of Gold Nanoparticles Using Genetically Engineered Polypeptides**

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Proteins initiate, catalyze, and mediate the fabrication of nano- and microstructures, which are then assembled into complex architectures necessary for specific biological functions.[1–5] Biomimetic systems could include proteins to control not only the synthesis but also the spatial distribution of inorganic materials into functional assemblies with useful electrical and optical properties.[6–11] There have been reports on the assembly of nanoparticles in solution and the directed immobilization of nanoparticles on substrates using protein-based recognition systems.[12–15] Over the last decade, the use of display technologies has emerged as a powerful strategy to screen peptides with recognition for the surface of an inorganic material.[16–24] In pioneering efforts, Brown et al. used *E. coli* cell-surface display (CSD) to develop repeating polypeptides that bind to metal oxides[16] and metals[17] while Belcher et al. used phage display (PD) to develop peptides that bind to semiconductors.[18] Utilizing both PD and CSD, we have developed genetically engineered polypeptides with surface recognition for metals[19,20] as well as technologically relevant metal oxides.[24]

Recently, a noteworthy advancement was reported by Levy et al., who employed a combinatorial approach in the rational design of peptide capping ligands to synthesize exceptionally stable gold nanoparticles with desirable chemical properties in aqueous media.[25] In principle, apart from synthesis, recombinant peptides can be instrumental in the hierarchical assembly of inorganic building blocks. Self-assembled monolayers (SAMs) with appropriate end-groups have been patterned to produce templates for the assembly of nanoparticles through covalent bonding[26a] or electrostatic interactions.[26b] The significant advantage of combinatorially selected peptides over SAMs is their ability to identify a specific atomic composition, crystallographic orientation, or morphology of an inorganic entity. Moreover, as molecular linkers, they can be covalently bonded with organic molecules to construct hybrid structures, which are potentially tunable in terms of physical conformation or chemical behavior.[27,28] However, to our knowledge, there have been no systematic studies on the fabrication of well-defined protein/organic hybrid structures on the surface or the direct patterning of genetically engineered polypeptides to assemble nanoparticles in a site-selective way. Here, in a proof-of-concept demonstration using gold-binding protein (GBP-1), we explored the patterning of genetically engineered polypeptides by microcontact printing (μCP). We investigated the spatial conformations of patterned GBP-1 by atomic force microscopy (AFM). We showed that patterns of hybrid structures, composed of polypeptides and organic molecules, can be generated on a substrate with submicrometer dimensions by combining μCP with surface chemical reactions. Finally, we demonstrate the utility of laterally structured hybrid assemblies in the controlled organization of gold nanoparticles on surfaces.

The amino acid sequence of gold-binding protein, GBP-1 (42 amino acids, $[MHGKTQATSGTIQS]_3$) can be seen in Figure 1. GBP-1 was originally developed by Brown et al. from a CSD library in which the randomized foreign gene product was displayed in the extracellular loops of outer-membrane protein, maltoprin, that was fused to the amino acid terminus of the alkaline phosphatase with retention of gold-binding activity.[17] Experimental and modeling studies indicated that strong binding with gold is achieved by three repeats of the sequence MHGKTQATSGTIQS.[19,20]

The ability to pattern proteins has a wide range of applications from the fundamental study of cell adhesion to the technological development of diagnostic platforms.[29,30] As depicted in Figure 1, we devised two schemes based on μCP to pattern GBP-1. μCP is a versatile soft-lithographic technique to deposit a variety of molecules (referred to as "inks") onto both flat and curved surfaces using elastomeric stamps—usually poly(dimethylsiloxane) (PDMS)— in a parallel fashion over large areas with down to submicrometer resolution.[31] We refer to the scheme shown in Figure 1B as "physical patterning", since GBP-1 was transferred via deposition onto a Au(111) substrate. However, it should be noted that there is a binding (i.e., surface recognition) between GBP-1 and the Au(111) surface. The scheme in Figure 1A, referred to as "chemical patterning", involved Schiff base formation (-HC=N-) between amine ($-NH_2$) groups on the GBP-1 and aldehyde (-CHO) end-groups of the SAMs of (10-mercaptomethyl-9-anthryl)(4-aldehydephenyl)acetylene (MMAPA) within the areas where the PDMS stamp is in contact. Each unit (i.e., three repeats) of the GBP-1 contains four $-NH_2$ groups—three from lysine residues and a more reactive N-terminus—for potential reaction with the surface -CHO groups. Localized surface chemical reaction on the reactive SAMs, as defined by a patterned PDMS stamp, enabled the generation of patterns as well as the formation of hybrid assemblies simultaneously. A key advantage of using reactive SAMs presenting terminal -CHO groups as molecular substrates is that the polypeptides can be patterned in a single step. Previous μCP of biological molecules has typically been carried out on SAMs with carboxylic (-COOH) functionality in a two-step procedure, which requires the conversion of -COOH end-

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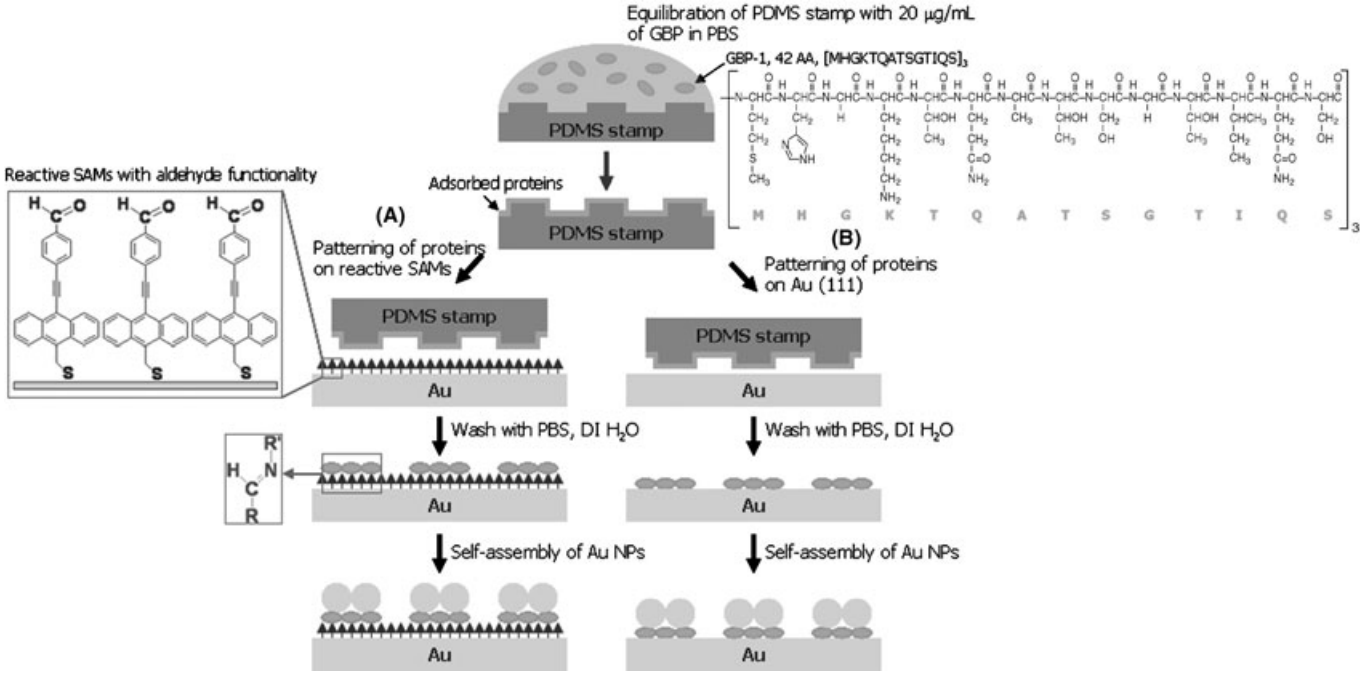

Figure 1. Schematic representation of the experimental procedure. GBP-1 was patterned by μCP using two schemes: A) GBP-1 was chemically patterned by covalent bonding between -NH_2 groups on the GBP-1 and -CHO end-groups of the SAMs of MMAPA through Schiff base formation; B) GBP-1 was physically patterned onto a Au(111) surface through deposition. In both cases, patterned GBP-1 functions as a template to direct the assembly of gold nanoparticles.

groups to activated esters prior to the patterning.[32] Consequently, unnecessary chemical reagents and, thus, additional contaminants may be introduced to the surface. Being a straightforward procedure involving a single reaction with water as a by-product, μCP on reactive SAMs presenting terminal -CHO groups is well-suited for creating patterned interfaces for studies involving proteins. Two patterning schemes allowed for a head-to-head comparison of spatial conformations of the GBP-1, and made possible the evaluation of structural effects on the assembly of gold nanoparticles. Cross-sectional profile analysis by AFM was carried out after patterning to optimize the conditions so that a monolayer of GBP-1 was formed on the surface. We also performed a control experiment for physisorption, in which GBP-1 was patterned onto SAMs of (4-mercaptophenyl)-anthrylacetylene (MPAA)—an analogous molecule to MMAPA, but without the -CHO groups.[33] Under identical conditions to chemical patterning, no well-registered pattern was generated on the SAMs of MPAA (see Supporting Information for details). Moreover, since the surface was not gold, GBP-1 did not show any binding to the surface and the deposited GBP-1 was washed away after rinsing in buffer solution and deionized water.

As shown in Figure 2, the surface properties of Au(111) and reactive SAMs induced GBP-1 to assume different spatial conformations. GBP-1 is bound tightly onto the surface of reactive SAMs due to covalent bonding. Cross-sectional profile analysis by AFM revealed that the chemically patterned GBP-1 ($\approx$1.2 nm) is more constrained than the physically patterned GBP-1 ($\approx$1.5 nm). Templated by the SAMs underneath, chemically grafted structures of GBP-1 are uniform, well-packed, and highly ordered (Figure 2 A,B). Study is underway to better understand the orientation of sterically demanding amino acid chains on the reactive SAMs and the nature of interactions between GBP-1/MMAPA hybrid structures. On the other hand, physically patterned GBP-1 displays a porous morphology, which is attributed to the forced immobilization of GBP-1 due to the mechanical nature of μCP (Figure 2 C,D). A certain degree of nonspecific binding is likely to exist between physically patterned GBP-1 and the Au(111) substrate. Open structures indicate the loss of recognition and self-assembly characteristics of GBP-1. Nonetheless, deposited GBP-1 structures showed strong binding to the Au(111) surface and were not removed from the surface even after repeated rinsing in buffer solution and deionized water.

The function of patterned GBP-1 as a template was investigated by the assembly of citrate-stabilized gold nanoparticles with an average diameter of 20 nm. In contrast to longer assembly times (from a few hours to over 24 h) reported in the literature, we found that 25 min was sufficient for gold nanoparticles dispersed in aqueous solution to assemble onto the hydrated GBP-1 templates. To reliably compare the difference in the binding ability of chemically and physically patterned GBP-1, the assembly of gold nanoparticles was carried out by pipetting the same volume of colloidal solution onto the GBP-1 templates of equal surface areas. Arrays of 700-nm-wide lines (Figure 3 A,B) were used to compare the abilities of physically patterned GBP-1 and chemically patterned GBP-1 in guiding the assembly of gold nanoparticles. It was found that only a couple of gold nanoparticles assembled onto the physically patterned GBP-1 (Figure 3 A). In comparison, gold nanoparticles almost fully occupied the regions of chemically patterned GBP-1

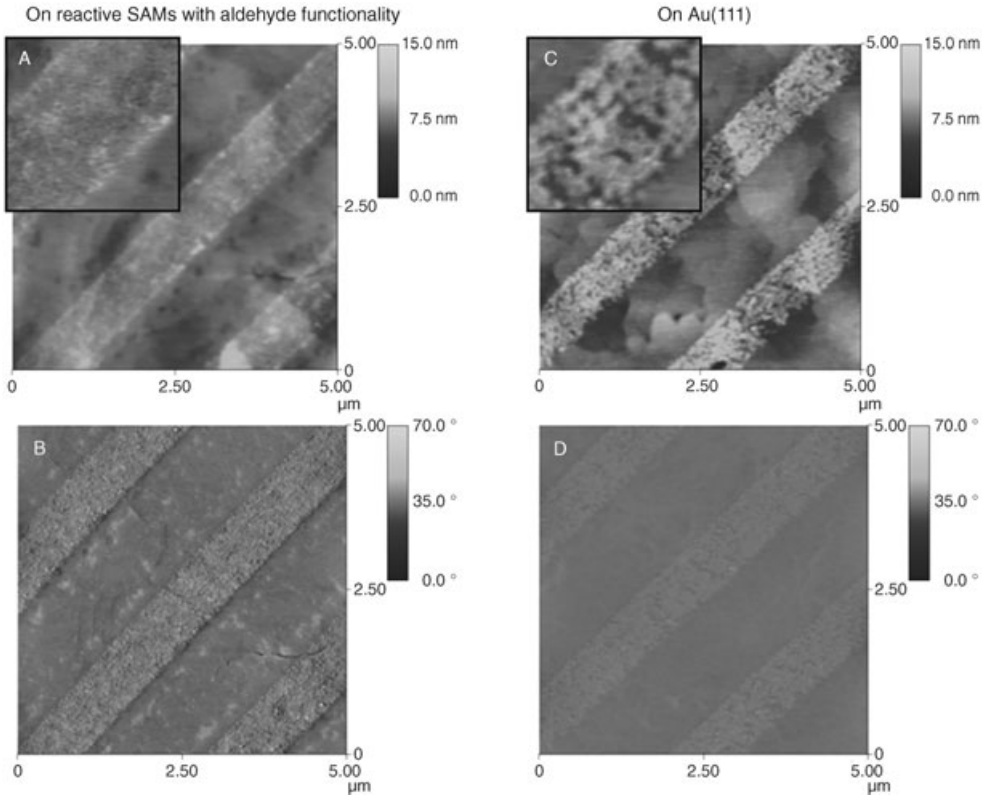

Figure 2. The influence of surface properties on the spatial conformation of patterned GBP-1. Height (A, C) and phase (B, D) images were acquired simultaneously with AFM in tapping mode. A, B) As a result of covalent bonding with the supporting SAMs, chemically patterned GBP-1 showed a compact morphology. GBP-1/MMAPA hybrid structures are uniform, well-packed, and highly ordered. C, D) Physically patterned GBP-1 showed a porous morphology. Deposited GBP-1 structures are not ordered as a result of forced immobilization.

(Figure 3B). The results suggest that the spatial conformation that maximizes interaction with gold nanoparticles may be stabilized by the covalent bonding between GBP-1 and reactive SAMs. Based on the porous morphology, the extended conformation would not be expected for physically patterned GBP-1, which implies a reduced availability of binding domains. After binding with the Au(111) substrate upon deposition, only a few binding domains were left for interactions with gold nanoparticles. In contrast, -CH=N- linkages at specific sites helped in regulating the organization of GBP-1/MMAPA hybrid structures. The binding domains are more exposed for interactions with gold nanoparticles in an extended, rather than folded, conformation. In addition, the flexibility of binding domains is critical for strong binding. Attachment along the side-chains promotes the extended conformation of GBP-1 on the reactive SAMs, whereas the attachment at the N-terminus allows movement and enhances flexibility of binding domains. Hence, interplay of both factors contributes to the assembly of gold nanoparticles in large numbers onto the chemically patterned GBP-1. Because of the limited ability of physically patterned GBP-1 as templates, our subsequent experiments utilized the chemical patterning of GBP-1 to make smaller templates. Figure 3C shows the assembled gold nanoparticles on the smallest template we were able to achieve using µCP. The average number of assembled gold nanoparticles on the arrays of 500-nm-wide squares was remarkably consistent ($\approx$15 particles per square), suggesting a homogeneous coating of GBP-1 within each pattern.

In conclusion, we have explored the patterning of genetically engineered polypeptides by microcontact printing using GBP-1 as a proof-of-concept demonstration. While it

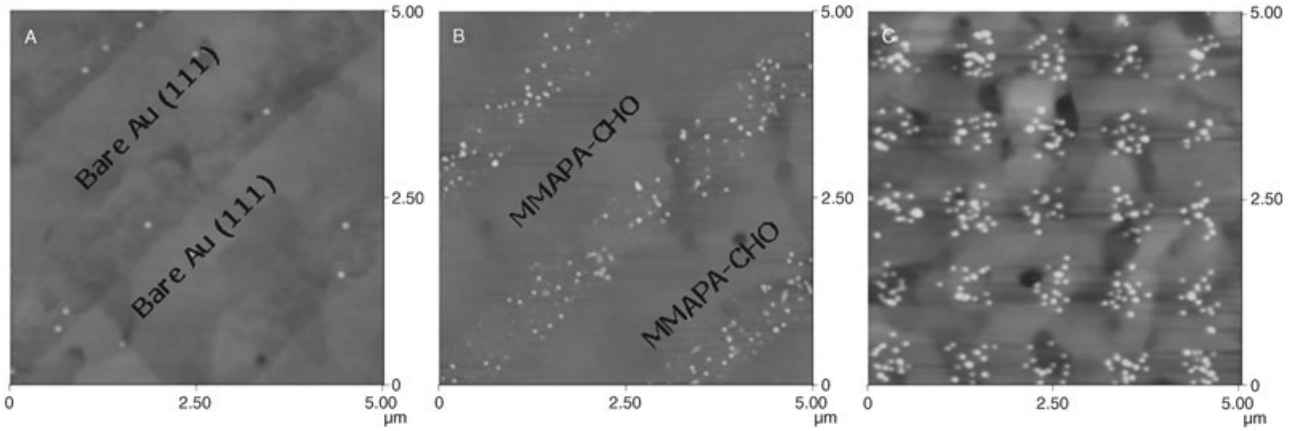

Figure 3. Physically patterned GBP-1 (A) and chemically patterned GBP-1 (B and C) as templates in directing the assembly of gold nanoparticles. A, B) For templates of a given surface area, gold nanoparticles assembled on the chemically patterned GBP-1 in much greater number than on physically patterned GBP-1. C) Assembled gold nanoparticles on arrays of 500-nm-wide squares—the smallest template made using µCP on reactive SAMs. During the assembly, no significant adsorption of gold nanoparticles in unpatterned regions was observed over a large area. The *z*-scale was 50 nm in all images.

is possible, forced immobilization of proteins on the surface may lead to the loss of recognition or self-assembly characteristics. As proven in the present study, the spatial conformation has a profound effect on the ability of GBP-1 in guiding the assembly of gold nanoparticles. Therefore, care should be taken to minimize or avoid forced immobilization in depositing peptides onto substrates with additive lithographic techniques. We have shown that the availability of $-NH_2$ groups on the GBP-1 can be exploited for covalent bonding with SAMs by presenting –CHO end-groups via a surface chemical reaction. Schiff base formation occurs in a localized manner within the areas where a patterned PDMS stamp is in contact with the SAMs of MMAPA. It requires no activation and involves only water as a by-product. AFM characterization confirms that the laterally structured GBP-1/MMAPA hybrid assemblies are uniform, well-packed, and highly ordered. Furthermore, we have demonstrated that chemically grafted structures of GBP-1 on the SAMs of MMAPA molecules are more functional than deposited structures of GBP-1 as binding agents in capturing gold nanoparticles. Since peptides contains -NH_2 groups on the amino acid side-chains and/or at the N-terminus, we believe that chemical patterning based on Schiff base formation could become a viable approach to directly pattern genetically engineered polypeptides.

Experimental Section

Materials: The 42-amino-acid-long polypeptides were synthesized and purified by United Biochemical Research, Inc. (Seattle, WA). Au(111) substrates were purchased from Molecular Imaging. They are high-purity gold epitaxially grown onto mica in high vacuum to obtain a thickness of 150 nm. Prior to use, the substrates were annealed in a hydrogen flame to produce contaminant-free, reconstructed flat Au(111) terraces. Gold nanoparticles (average diameter of 20 nm) were purchased from Aldrich. Both AFM and TEM characterizations showed a well-dispersed population of gold nanoparticles under neutral conditions (pH 7.0).

Preparation of self-assembled monolayers: Solutions of MMAPA (0.05 mM) were prepared in absolute ethanol. After Au substrates were immersed into the solution, deprotection of the thiol moieties by deacylation of thioacetyl groups using NH_4OH (28.0–30.0 % NH_3) permitted the formation of SAMs. After 48 h of self-assembly under N_2 gas, the samples were removed from solution, rinsed thoroughly with ethanol, and blown dry under N_2 gas. Freshly prepared SAMs of MMAPA were immediately subjected to μCP.

μCP: Stamps for μCP were prepared by casting a 10:1 (v/v) mixture of poly(dimethylsiloxane) and curing agent (Sylgard 184, Dow Corning) against a patterned master that had already been silanized to aid in release. Patterned regions, consisting of arrays of 500-nm-wide squares and 700-nm-wide lines, covered an area of $1.5 \times 0.5\ cm^2$; the distances between the lines and squares were 1.5 μm and 500 nm, respectively. Stamps were used as cast and the surface chemistry of the stamp was not modified. Proteins were deposited by covering the surface of the stamp with a solution of GBP (20 $\mu g\,mL^{-1}$) in phosphate buffer saline (PBS) for 2 h. The concentration of 20.0 $\mu g\,mL^{-1}$ was found to be optimal for achieving a full monolayer of polypeptides on the surface. During inking, the stamps were placed in a polystyrene Petri dish containing droplets of water to prevent the evaporation of GBP solution. After inking, the stamps were rinsed briefly with PBS and deionized water before being dried under a stream of N_2 gas to prevent the precipitation of residual proteins. The inked stamps were then immediately used for patterning. For μCP of GBP on a bare Au(111) surface, the conformal contact between the stamp and the substrate was maintained for 20 s. To chemically pattern the GBP on reactive SAMs, the duration of conformal contact between the stamp and the MMAPA/Au(111) surface was adjusted to 1.5 h for the Schiff base reaction to complete. After patterning, the GBP templates were placed in a PBS bath for 30 min, rinsed copiously with deionized water followed by PBS, and dried in a N_2 gas flow.

Assembly of gold nanoparticles: Solutions of gold nanoparticles were pipetted onto the GBP templates for the assembly to take place for 25 min. The samples were then rinsed thoroughly with deionized water, and blown dry under N_2 gas.

AFM characterizations: Tapping-mode measurements (both topographical and phase-contrast data) in air were made using a Nanoscope III AFM (Digitial Instruments). Unlike contact mode, tapping mode minimizes sample damage due to lateral shear force and is suitable for investigating soft biological samples. The scan rate for all images of all sizes was 1 Hz. Images included 512×512 data points and were flattened after recording using algorithms contained in the software; a low-pass filter was applied once in some images. Three areas were analyzed in each sample under the same conditions, and the images were reproducible.

Keywords:

biomimetics · microcontact printing · nanoparticles · polypeptides · self-assembled monolayers

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