Nanopatterning Peptides as Bifunctional Inks for Templated Assembly**

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The ability to bind, assemble, synthesize, and grow technologically relevant materials on a solid surface under mild aqueous conditions is important for many applications including diagnostic biosensors,[1] nanophotonics,[2] and possibly even rapid prototyping.[3] One of the challenges in this regard is the selection and optimization of linking chemistry to bind functional structures to a variety of different technological surfaces such as metals, oxides, and semiconductors. A number of small molecules are routinely used to functionalize surfaces: thiols on gold, silanes on silica or other oxides, and phosphonic acids on metal oxides. Recently, peptides have emerged as a promising class of materials for surface functionalization. By using rapid combinatorial screening techniques, the molecular biomimetic community has already identified a library of peptide sequences with specific affinities for a variety of metals, semiconductors, oxides, and even organic materials like conducting polymers.[4–12]

Using the tools of molecular biology, these inorganic-binding peptide sequences can be incorporated into proteins,[13] linked with other peptides,[14] conjugated to functional small molecules such as organic dyes, or attached to quantum dots.[15–17] The resulting structures can potentially be utilized as molecular building blocks to direct assembly or in situ synthesis of functional inorganic nanostructures.[18–21] For example, one can imagine using heterobifunctional peptide inks in rapid-prototyping applications on many different surfaces: one end of a functional peptide would bind to a solid surface, while the other end would catalyze growth of another desired inorganic material, all in a site-specific template under the mild conditions typical of biological catalysis.[13,14,18,20,22–25]

Solid-binding peptides are typically identified by cell-surface or phage-display methods.[26] Although the fundamental mechanism of solid-binding is not fully understood,[27,28] NMR spectroscopy,[29,30] computational modeling,[31,32] and experiments with geometrically constrained peptides/proteins[13] have suggested that the secondary structures of these engineered peptides in solution can play an important role in their solid-binding and selectivity.

These factors have the potential to affect the success of peptides in substrate-templated assembly applications. It is known that biomolecules delivered by various printing techniques can denature or adopt other conformations on solid surfaces that differ from their native form in solution.[33–35] Biomolecules have been patterned by a wide range of techniques[36,37] including pin printing, inkjet printing, nanoimprint lithography, electron beam (e-beam) lithography, focused ion beam lithography, soft lithography, photolithography, scanning probe microscopy, and dip-pen nanolithography (DPN).[1,38–45] However, most of these demonstrations did not use biomolecules that were specifically engineered to bind to selected surfaces. Nonspecific binding of biomolecules often results in loss of biological function, which is especially true in the case of enzyme immobilization on solids. Given the possible role of solution-phase secondary structure in the affinity/selectivity of inorganic binding peptides for different surfaces, it is imperative to show that these engineered polypeptides can serve as viable inks for use in a variety of immobilization strategies.[46]

In this work, we demonstrate the utility of three different peptide sequences as inks for DPN with sub-100-nm resolution and for microcontact printing. These peptides were selected using combinatorial methods[5] followed by in silico design[47] to bind to silica or gold surfaces. Their binding affinities for inorganic substrates have been quantified using surface plasmon resonance (SPR) spectroscopy or a quartz-crystal microbalance (QCM) in earlier studies.[47,48] By using peptides functionalized with biotin at the NH_2-end to bind with fluorescently labeled streptavidin, we show that patterns made from these solid-binding peptides are robust enough to survive solution processing steps following deposition. We also

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compare the relative stability of the patterned peptides on the specific surfaces (i.e., gold and silica) to which they were selected to interact with their stability via nonspecific interactions on other substrates.

DPN is a direct-write, scanning probe-based lithography tool used in many applications such as generating etch resist masks on metals,[49,50] assembling nanowires,[51] growing peptides on a solid surface,[52] and controlling phase separation in polymer thin films.[53–55] DPN has been used to deposit biomolecules including DNA[1] and peptides.[45] However, to our knowledge, DPN has not been studied for use with engineered solid-binding peptides.

Scheme 1 illustrates our basic experimental approach. A DPN tip inked with the biotinylated engineered peptides is brought into contact with an inorganic surface to pattern the desired features. The patterned biotinylated peptides are then incubated with a solution containing the fluorescently labeled streptavidin. The specific attachment of the streptavidin to the patterned biotinylated peptides is confirmed with fluorescence microscopy.

We used DPN to pattern two silica-binding peptides (QBP1 and QBP3)[47] and a gold-binding peptide (3R-GBP1),[5] which have previously been identified for their strong selectivity and affinity to silica and gold surfaces, respectively (sequence information is provided in the Experimental Section).[5,47] Figure 1 shows the lateral force microscopy (LFM) images of the peptides patterned onto different surfaces: 1a) QBP1 patterned on SiO_x (hydrophilic, oxygen plasma-cleaned, thermally-oxidized silicon wafer), 1b) QBP3 patterned on SiO_x, and 1c) 3R-GBP1 patterned on a gold surface. The narrowest line we have obtained is ≈80 nm in width, and we routinely write ≈110-nm-wide lines. QBP1, QBP3, and 3R-GBP1 patterned well on the surfaces to which they were engineered to bind. While the DPN tips are generally inked from a peptide solution dissolved in phosphate carbonate (PC) buffer, we have also patterned with tips inked from peptides dissolved in deionized water without significant differences (Supporting Information, Figure S1).

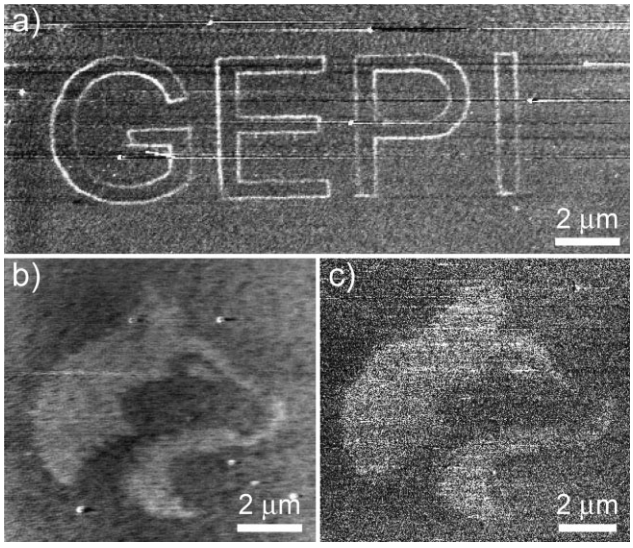
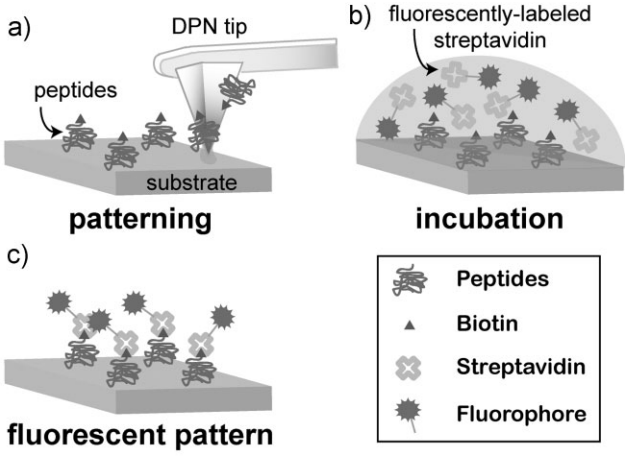


Figure 1. LFM image of DPN-patterned a) silica-binding peptide (QBP1) on SiO_x, b) another silica-binding peptide (QBP3) on SiO_x, and c) gold-binding peptide (3R-GBP1) on gold.



Scheme 1. Schematic illustration of the approach for using DPN-patterned peptides as bifunctional linkers for site-specific surface functionalization or assembly, in this case screened with a simple fluorescently labeled streptavidin. a) DPN delivers engineered polypeptides to an inorganic surface, b) reaction of pattern with fluorescently labeled streptavidin in solution, and c) DPN-generated patterns can be observed with fluorescence microscopy.

Figure 2 shows fluorescence images of the patterned biotinylated silica-binding peptides (Figure 2a: QBP1 and Figure 2b: QBP3) on SiO_x surfaces after incubation in a solution containing Alexa Fluor 594-labeled (Figure 2a) or Qdot 605-labeled (Figure 2b) streptavidin. We used the biotin–streptavidin linkage to demonstrate two objectives: first, to confirm the presence of biologically active peptides after patterning using DPN, and second, to demonstrate the possibility of using peptides as heterobifunctional linkers to direct site-specific assembly (or, in the future, in situ synthesis of functional nanostructures) in subsequent steps. The fluorescent patterns are clearly visible, which confirmed the presence of the DPN-patterned peptides and demonstrated that their attachment to the surface is robust enough to be used for subsequent assembly steps. The ''spottiness'' of the fluorescent patterns is most likely due to attachment heterogeneity in the underlying peptide patterns, as can be seen in atomic force microscopy (AFM) topography images (Supporting Information, Figure S2). In control experiments, patterns were also made with DPN tips inked with peptide-free buffer and also completely non-inked tips (Supporting Information, Figure S3). While LFM patterns, were occasionally observed during these control experiments (probably from the deposition of buffer salts or water), no fluorescent patterns were ever observed from the control ''patterns'' generated with peptide-free buffer-inked or non-inked tips (Supporting Information, Figure S4). Due to the very small feature sizes generated by DPN and the quenching from the gold substrate,[56] we were only able to observe the fluorescence of the DPN-generated patterns on silica surfaces, although the DPN-generated patterns are clearly visible in the AFM images (topography and LFM) on both substrates.

In addition, microcontact printing of the peptides can also direct the site-specific assembly of fluorophores (Supporting

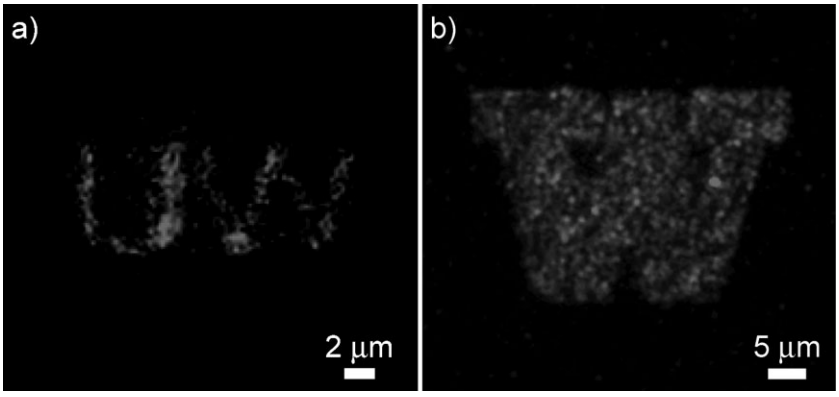


Figure 2. Fluorescence images indicate the presence of patterned peptides and demonstrate a proof-of-concept for site-specific assembly with engineered polypeptides. a) QBP1–biotin patterned on SiO_x labeled with Alexa Fluor 594 and b) QBP3–biotin patterned on SiO_x labeled with Qdot 605.

Information, Figure S5). Despite the reported specificity of the peptides for particular inorganic surfaces upon adsorption from solution,[57] we found that patterns of the silica-binding peptides on gold and the gold-binding peptide on silica could be generated by both DPN and microcontact printing (Supporting Information, Figures S5 and S6). While somewhat unexpected, this result is not surprising since a wide variety of peptides that can be patterned by both microcontact printing and DPN are thought to adhere primarily by nonspecific interactions with the surface.[58] Indeed, even peptides that have been selected by biocombinatorial approaches to exhibit affinities for specific inorganic surfaces show cross reactivity at very high concentrations. For instance, Tamerler et al. showed that although the equilibrium surface coverage of a gold-binding peptide is ten times larger on gold than on platinum at very low concentrations ($<0.1\,\mu M$), the differences in surface coverage decrease as the concentration increases, and eventually there is no significant difference at high concentrations ($>100\,\mu M$), likely due to supersaturation.[57] Since the local peptide concentration during microcontact printing and especially DPN can be very high (near saturation conditions), it is reasonable to expect that the specific inorganic binding peptides may be deposited with a high coverage on surfaces to which they were not selected to bind.

Given that all three inorganic binding peptides can be patterned on both gold and silica surfaces, it is important to assess the advantage of using high-affinity peptides. To this end, we qualitatively compared the stability of the patterns after rinsing with buffer by monitoring the intensity of the fluorescence: as the peptides are desorbed and washed away, so are the fluorescent markers.

Figure 3 shows fluorescence images of the DPN-patterned biotinlyated silica-binding peptide (QBP1; Figure 3a and c) and gold-binding peptide (3R-GBP1; Figure 3b and d) on Si[59] (hydrophilic, oxygen-plasma-cleaned silicon wafers with a layer of native oxide and plasma-induced oxide)[60] before (Figure 3a and b) and after (Figure 3c and d) washing in phosphate buffered saline solution (PBS). Even though both silica-binding peptide and gold-binding peptide are patterned on Si surfaces using DPN, the gold-binding peptide was washed off the Si surface after ≈30 min or more of rinsing with PBS. However, the patterns of silica-binding peptide remained visible after washing for the same amount of time. In addition, the silica-binding peptide patterns on Si exhibited brighter fluorescence than the patterned gold-binding peptide on the same surface (Supporting Information, Figure S7), suggesting that the peptide selected to be a silica binder is indeed anchoring to this surface more effectively (the data in Figure 3a and c and 3b and d were taken with exposure times of 2.0 s and 8.6 s, respectively).

Likewise, Figure 4 compares the stability of the microcontact-printed gold-binding peptide (3R-GBP1; Figure 4a and c) and silica-binding peptide (QBP1; Figure 4b and d) on gold surfaces before (Figure 4a and b) and after (Figure 4c and d) 1 h of washing in PBS. The microcontact-printed silica-binding peptide is less stable than the gold-binding peptide on gold surfaces. Washing with deionized (DI) water also shows similar results (Supporting Information, Figure S8). The microcontact-printed gold-binding peptide patterns on gold exhibit brighter fluorescence than the silica-binding peptide on the same surface, and the differences become even more pronounced after the washing (Supporting Information, Figures S9 and S10). On the other hand, both of the microcontact-printed peptides of 3R-GBP1 and QBP1 on hydrophilic, oxygen-

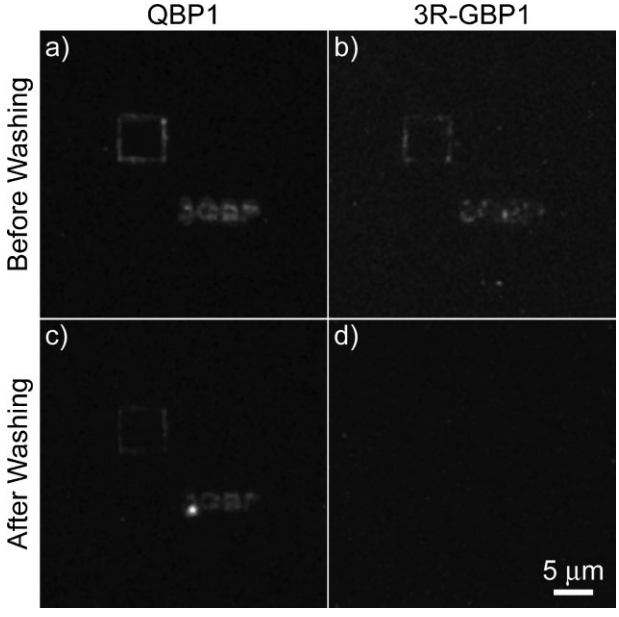


Figure 3. Stability of the DPN-patterned biotinylated engineered peptides on Si surfaces. The stability of the patterns is compared by the fluorescence of the patterns before (a and b) and after (c and d) washing in PBS for 30 min. The DPN-generated silica-binding peptide (QBP1; a and c) patterns still exhibit detectable fluorescence intensity while the DPN-generated gold-binding peptide (3R-GBP1; b and d) patterns are washed away. (Exposure time of QBP1: 2.0 s, 3R-GBP1: 8.6 s.)

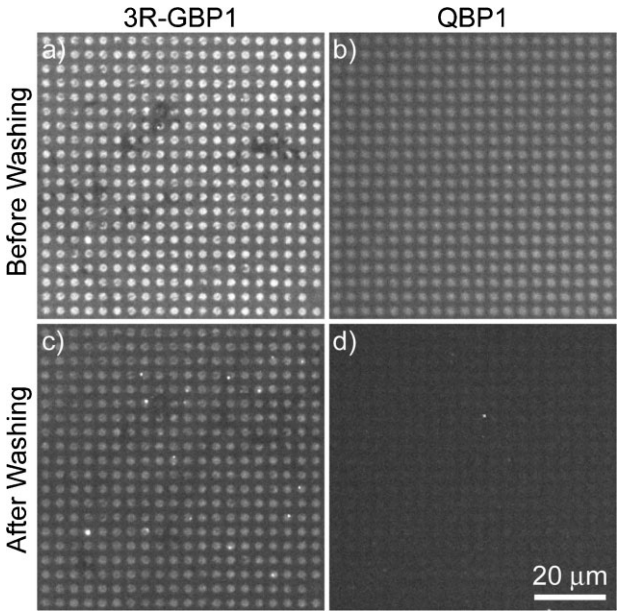


Figure 4. Stability of the microcontact-printed engineered peptides on gold surfaces. Fluorescence images indicate that the patterned 3R-GBP1 (a and c) are robust enough to undergo washing in PBS for 1 h. However, the QBP1 patterns (b and d) are almost completely washed away after the 1 h rinsing step.

plasma-cleaned Si surfaces behaved similarly (within experimental error) after washing in either PBS buffer or DI water. Nevertheless, at all stages of washing the silica-binding peptide patterns were brighter than the gold-binding peptide patterns on the same oxygen-plasma-cleaned Si surface (Supporting Information, Figure S11), indicative of higher attachment densities for the silica binder.

We have demonstrated that DPN can deliver engineered solid-binding peptides to both metal and semiconductor surfaces with high spatial resolution. Using streptavidin–biotin for fluorescent labeling, we have confirmed the presence of patterned peptides on the solid surface and the ability to use these peptides as biomolecular anchors to attach other materials to the solid substrates. We qualitatively compared the stability of the DPN-patterned and microcontact-printed silica-binding peptide and gold-binding peptide by comparing the relative fluorescence intensity when rinsing with buffer solution. While both peptides can be patterned on both surfaces by nonspecific interactions, our results indicate that 3R-GBP1 on gold (patterned via microcontact printing) and QBP1 on silica (patterned via DPN) have brighter overall fluorescence and/or are more stable against washing than QBP1 on gold and 3R-GBP1 on silica, respectively.

Furthermore, patterns of surface-binding peptides, when delivered by DPN, are robust enough to consider applications such as high-spatial-resolution surface bifunctionalization, followed by targeted immobilization, and potential in situ biosynthesis of inorganic materials by using nanometer- and micrometer-sized peptide patterns in future experiments. In the future, it should be interesting to study the effect of peptide length and sequence repeat number on the stability and/or selectivity of the DPN-generated peptide patterns, since other work has shown that the peptide length can improve surface binding when depositing from a solution.[5]

Experimental Section

The gold-binding peptide is a three-repeat tandem sequence of MHGKTQATSGTIQS (3R-GBP1).[5,57] The silica-binding peptides are single-repeat forms, that is, PPPWLPYMPPWS (QBP1) and SPPRLLPWLRMP (QBP3).[47] Engineered peptides were synthesized by Bio Synthesis, Inc. (Lewisville, TX) or United Biochemical Research Inc. (Seattle, WA).

DPN tips for writing solid-binding polypeptides were prepared by first plasma-cleaning silicon nitride probes (DPN probes, type A, NanoInk) for 5 min to make the surface hydrophilic. Then the tips were inked for ≈3 to 5 s in peptides dissolved in either PC buffer (1 to 5 mM in PC buffer; 55 mM KH_2PO_4/45 mM Na_2CO_3/200 mM NaCl) or filtered DI water (filtered with a Milli-Q Plus Ultrapure Water System by Millipore, then with a vacuum-driven disposable filtration system with a 0.22-μm GP Express Plus Membrane by Millipore). The inked tips were immediately dried by blowing with compressed gas (Dust Off, Falcon) after each inking.

Microcontact-printing poly(dimethylsiloxane) (PDMS) stamps were covered with drops of engineered peptides dissolved in PC buffer (1 mM) for ≈1 h. The stamps were then blown dry with nitrogen gas.

Thermally oxidized silicon wafers (either 10 000 Å ± 5% or 5 000 Å ± 5% thermal oxide, (100), Silicon Quest International) or silicon wafers with a native oxide layer (100), (Mitsubishi Silicon America) were cut, then blown with a nitrogen stream to remove visible dust. The silicon substrates were sonicated in acetone and then isopropanol for ≈25 min each. For gold substrates, a 35–45-nm-thick layer of gold (from 99.999% pellets, K. J. Lesker) was thermally evaporated (Auto 306, BOC Edwards) onto the silicon substrates on top of a 1.5–4.5-nm-thick chromium adhesion layer (thermally evaporated from chromium-coated tungsten rods, Midwest Tungsten Service). Prior to patterning, the silicon substrates were plasma-cleaned for 5 min. DPN writing and AFM imaging (Type A probes, MSCT-AUHW, Veeco Instruments) were performed using a NanoInk Nscriptor scanning probe microscope in ambient conditions (temperature 21–24 °C, relative humidity 30%–50%).

The patterned biotinylated peptides were then exposed for 5–20 min to a solution of fluorescently labeled streptavidin (Alexa Fluor 594, Invitrogen; 0.01 mg mL^{-1} dissolved in 0.01 M PBS or Qdot 605nm Innovator's Tool Kit, Invitrogen). The fluorescent patterns were observed using an inverted epi-fluorescence microscope (TE-2000; Nikon) at 50× magnification (50× LU Plan; NA = 0.80; Nikon). A metal halide lamp (EXFO X-Cite 120) with appropriate optical filters was used for fluorescence illumination.

In the washing experiments, the patterned peptides were soaked in 0.01 M PBS with sodium azide or filtered DI water for a range of times while stirring with a stir bar at ≈1 600 rpm at room temperature (21–24 °C).

We did not observe significant differences between the thermally oxidized silicon and the silicon with a native oxide layer during the patterning. Both surfaces are very hydrophilic after 5 min of plasma cleaning (contact angles ≈5°). Previous

studies have shown that silicon surfaces are oxidized in ambient conditions, and oxygen-plasma cleaning increases the native oxide layer thickness after a few minutes.[60]

Keywords:
dip-pen nanolithography · gold · microcontact printing · peptides · silica

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