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Quartz Binding Peptides as Molecular Linkers towards Fabricating Multifunctional Micropatterned Substrates

By *Turgay Kacar, John Ray, Mustafa Gungormus, Ersin Emre Oren, Candan Tamerler,* and *Mehmet Sarikaya**

Protein microarray technologies, used in proteomics and clinic assays, require efficient patterning of biomolecules on selected substrates.[1–3] Fabrication of these microarrays is possible provided that the proteins are spatially immobilized on an inorganic surface via various lithography techniques, e.g., soft lithography,[4] dip-pen lithography,[5] and photolithography.[6] Within the last decade, protein immobilization became a key issue in bio-nanotechnology, 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.[7,8] The approaches for biomolecule immobilization on glass or metal substrates generally require surface functionalization by self-assembled monolayers (SAMs) of bifunctional molecules, such as aminoalkylalkoxysilanes for silica and carboxyl-terminated alkanethiols for gold substrates.[9,10] Despite their widespread utility, these traditionally available bifunctional molecules have certain limitations, such as causing random orientation of the protein on solid surface and requiring multistep chemical reactions, and the assembled monolayers can be unstable during immobilization.[11,12] To overcome these limitations, it is preferable to have molecules as direct linkers to the solid substrate of interest that not only have all the desired features of the conventional chemically prepared SAMs but also have specificity to a given solid substrate and assemble onto it efficiently. As we demonstrate in this work, biomolecules, in particular peptides, can provide the multifunctionality of such a desired linker with high structural stability. Molecular constructs based on peptides can be designed with bifunctionality, including a solid-substrate-binding ability and incorporating a target molecule aligned consistently to carry out a desired function.[13]

Recently, an alternative technique emerged in which the peptides with strong binding affinity to inorganic surfaces were used for the immobilization of nanoparticles and biomolecules.[13–15] Such peptides were either isolated from the hard tissues of the organisms, such as diatoms,[14] or selected against a specific inorganic target using combinatorial molecular biology protocols, such as phage[13,15,16] or cell-surface displays.[17,18] In the literature, there are numerous peptide sequences already selected for different inorganic substrates and used in proof-of-principle applications, e.g. GaAs[15] Pt,[16] Au,[17] Cu_2O,[18] ZnO,[18] quartz (SiO_2),[19] Ag,[20] TiO_2,[21] and zeolite.[22] There has been a surge of research activity utilizing these genetically engineered peptides for inorganics (GEPI),[13] which could be used for synthesis, binding, assembly, and linking of inorganic nanostructures, all under ambient conditions. These engineered peptides are also useful for functional electronics and optical devices.[13,15] Furthermore, the utility of genetically selected peptides are desirable in immobilization not only for their high inorganic-binding capability but also their high substrate and biomolecular specificity. For example, using gold binding peptides as linkers, functional proteins[12,23] and gold nanoparticles[24] were immobilized on gold surfaces. Using these peptides, synthesis of SiO_2,[14] Ag,[20] and Au[25] nanoparticles, and hybrid protein-Cu_2O nanostructures[26] have also been demonstrated.

Polydimethylsiloxane (PDMS) stamping,[4,27] used for patterning of SAMs of alkanethiols on gold surfaces, is a common soft-lithography technique for fabricating protein patterns.[28–33] PDMS stamping has been successfully used to prepare scaffolds for cell growth,[34] nanotube formation,[35] nanowire organization,[36] microfluidic systems,[37] and biosensing.[38] In general, silane- or thiol-based bifunctional molecules are used in these studies for attachments of biomolecules on solid supports such as glass or gold, respectively.[31,32,39–41] Using microcontact printing, we and others have demonstrated printing of gold-binding peptides on gold substrates for directed immobilization of gold nanoparticles,[24] streptavidin-functionalized quantum dots,[42] and proteins,[12] e.g., enhanced green fluorescent protein.

In this study, we show micropatterning of two different photoactive target molecules, fluorescent quantum-dot nanocrystals and fluorescein, on quartz surface, immobilized via quartz-binding peptides (see Experimental). The fabrication process incorporates a combination of microcontact printing and directed self-assembly processes successively. The patterned substrates were examined using fluorescence microscopy to demonstrate the bifunctionality and the quality of the pattern fabrication. Our results show that the attachment of quantum dots or photoactive molecules on a substrate is material-specific. For example, when silica is the substrate, a quartz-binding

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DOI: 10.1002/adma.200801877

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peptide (QBP) is used as the linker. To demonstrate the material-specificity of the QBP1, we show that it does not bind to a gold substrate.

Quartz-binding peptides have been designed previously using a knowledge-based approach. Here, we specifically use QBP1 and QBP2 sequences that were chosen as strong and weak binders to quartz surfaces, respectively (see Experimental). The molecular binding characteristics of these two peptides have been quantitatively determined using various techniques, including surface plasmon resonance spectroscopy (SPR).[19] Based on the SPR data, the apparent adsorption rate constants ($k_{observable}$) of QBP1 and QBP2 were calculated to be $0.43\,s^{-1}$ and $0.068\,s^{-1}$, respectively. The $k_{observable}$ values represent the initial adsorption rates for each of the peptides on the silica surface (Supporting Information Fig. S1). For use as molecular inks in the PDMS stamping experiments, both peptides were chemically synthesized and were then either biotinylated, namely QBP-bio, or conjugated with fluorescein via the reactive isothiocyanate group, QBP-F. The QBP-bio conjugates were then labeled with fluorescent quantum-dot nanocrystals (QD) linked to streptavidin protein (SA-QD); here SA is known to have strong molecular recognition to biotin.[11]

Our first experiment demonstrates micropatterning of a functional target molecule (fluorescein) conjugated to QBP1 onto a quartz surface using microcontact printing (Fig. 1). Figure 1a shows a schematic illustration describing the PDMS stamping of the QBP1-F conjugate molecules used as ink. In this experiment, we chose fluorescein as the target molecule, due to its chromogenic property resulting in the emission of green light at $\lambda = 530\,nm$. As a control, we first attempted to stamp fluorescein on the quartz substrate alone. As shown in Figure 1b, we see no contrast when the patterned substrate was imaged

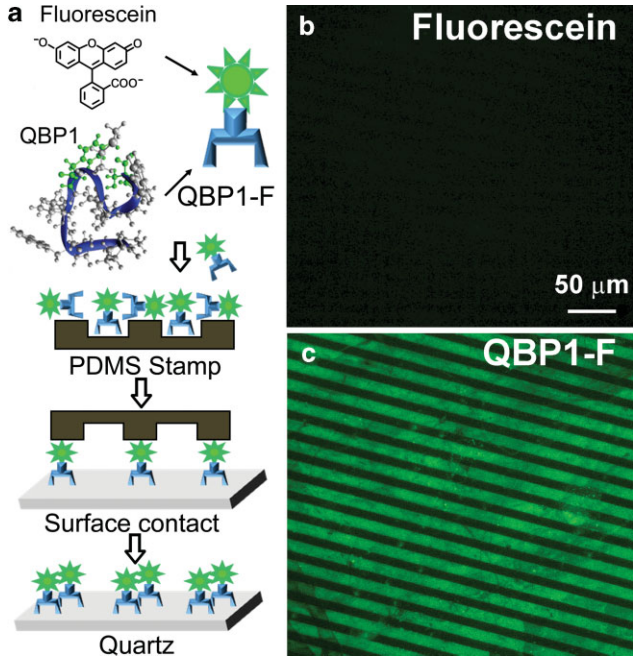

Figure 1. a) Schematic representation for PDMS patterning of QBP1-F on quartz. Fluorescence microscopy images of the substrates after micropatterning with b) fluorescein alone, as the control, and with c) QBP1-F.

through a fluorescein isocyanate (FITC) filter using a fluorescence microscope. The result of this control experiment indicates that the target molecule by itself has no affinity to quartz substrate. We then used fluorescein-conjugated molecule, QBP1-F, as the molecular ink to produce a pattern on the quartz substrate, following the stamping procedure shown in Figure 1a. The fluorescence microscopy image in Figure 1c clearly reveals high contrast, in which the green lines are indicative of the micropatterned QBP1-F molecules. Besides fluorescein, the procedure was repeated using an Albumin–fluorescein isothiocyanate conjugate, BSA-F, as another control molecule (Supporting Information Fig. S2). Comparison between the fluorescence intensity measurements recorded from these three samples (Fig. 1b and c, and Supporting Information Fig. S2) clearly shows that the QBP1-mediated fluorescein-molecule immobilization was an order of magnitude more efficient than that mediated by BSA (Supporting Information Fig. S3). This result demonstrates the utility of the inorganic-binding peptide, i.e., QBP1, as a linker mediating the immobilization of a fluorescent molecule, fluorescein, on silica using the microcontact-printing technique.

Parallel to the first experiment, we also used the biotinylated QBP as an ink, again using microcontact printing to generate a micropattern of a probe, e.g., biotin, conjugated peptide. This way, the fabricated pattern would be biofunctional against streptavidin, and could be used as a template for the assembly of streptavidin-coated QDs through biotin-streptavidin molecular recognition. In this case, we first used a PBS buffer (phosphate buffered saline) alone as the control experiment, stamping it on the quartz surface using PDMS, to produce a PBS pattern that was then incubated by SA-QD. We find that the PBS buffer alone used as an ink does not allow immobilization of QDs (Supporting Information Fig. S4). The utility of QBP-bio as a PDMS ink to generate a functional micropattern is shown schematically in Figure 2a. The micropatterned surface would then be incubated with SA-functionalized QDs, allowing its directed immobilization only on the stamped regions. We used both strong and weak quartz binding peptides, i.e., QBP1-bio and QBP2-bio, respectively, the latter being the negative control experiment. Figure 2b and c demonstrate the affinities of the quartz-binding peptides on quartz surface. We carried out the experimental procedure schematically described in Figure 2a using QBP1-bio as the molecular linker for the successful directed immobilization of SA-QD, as shown in Figure 2b. When QBP2-bio was used, instead, as the ink in PDMS patterning, because of its weak binding characteristics, no SA-QD immobilization was possible, as demonstrated in the negative control experiment displayed in Figure 2c. Finally, to demonstrate the material-specificity of QBP1-bio for quartz, we used gold as the substrate, and repeated the procedure. Again, as shown in Figure 2d and e, no SA-QD was immobilized on the patterned gold substrate, using either the strong- or the weak-binding peptide. These experiments indicate that QBP1 has a unique material-specific property, at least between quartz and gold substrates, in which it can immobilize probe molecules (e.g., biotin) on quartz, rendering the surface highly biofunctional.

In the last demonstration of solid-binding peptide as a molecular assembler, we use two different nano-entities, QDs and fluorescent molecules, and sequentially assemble them on a micropatterned surface utilizing the material-specificity of the

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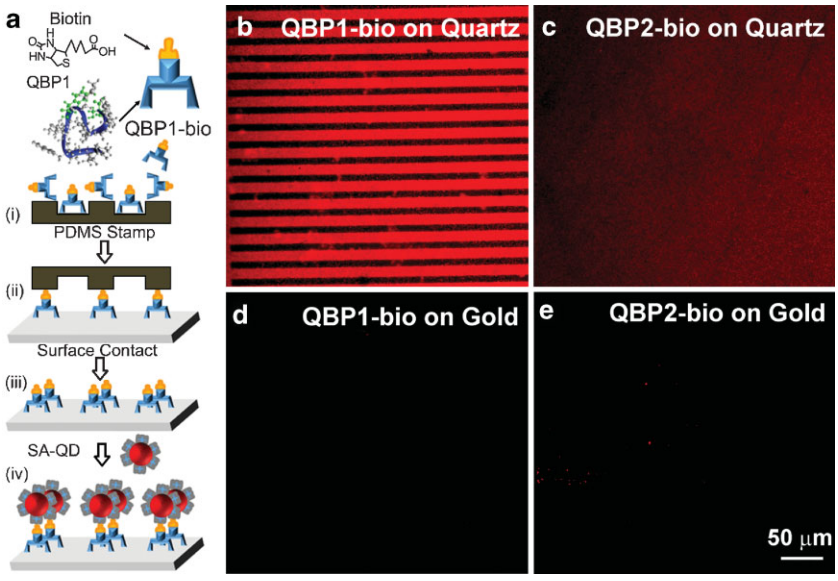

Figure 2. a) Schematic representation of PDMS patterning of QBP1-bio on a solid substrate. b) Fluorescence microscopy image of the micropattern formed through directed assembly of SA-QD upon the PDMS stamped QBP1-bio on quartz. c) The experiment was repeated, as a negative control, using the weak binder QBP2-bio as the ink, revealing no micropattern on the surface. In d,e), the procedures in b) and c) were repeated on gold surfaces, respectively (substrate as the negative control). The results demonstrate the specificity of the strong quartz-binding peptide as a true molecular linker, here used as a PDMS ink, on the quartz surface.

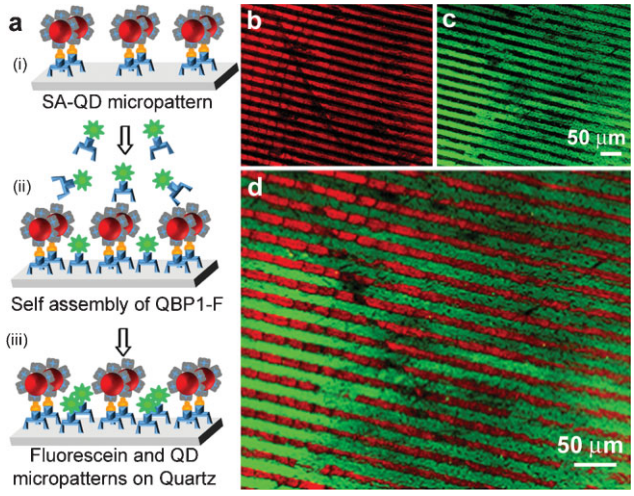

Figure 3. a) Schematics of QBP1-F assembly on a quartz substrate pre-patterned using QBP1-bio/SA-QD. b) Fluorescence microscopy image of a micropattern of directed immobilized SA-QD via the QBP1-bio patterned surface (similar to Fig. 2b). The image was recorded using a QD605 filter. c) Fluorescence microscopy image of the micropattern formed by the immobilization of QBP1-F conjugate on the unfilled microlines, using the same substrate as in b). The image was recorded using a FITC filter. d) Digital overlay of the images b) and c), demonstrating the utility of QBP1 both as a molecular ink for stamping, for directing the immobilization of QDs, and a mediated molecular assembler for a fluorescent molecule.

inorganic-binding peptide. In this case, directed immobilization of the QDs is followed by the GEPI-mediated assembly of the fluorescent molecule, using the procedure schematically illustrated in Figure 3a. The directed immobilization of SA-QD on a QBP1-bio-patterned surface is shown in Figure 3b as red stripes, imaged with a fluorescent microscope using a QD605 filter, revealing contrast similar to that in Figure 2b. Here, the dark stripes represent the regions originally unoccupied, exposing the bare quartz surface (Fig. 2b or Fig. 3a(i)). Next, following the procedure in Figure 3a, the assembly of the fluorescent molecule, i.e., fluorescein, is mediated using the QBP1-F molecular conjugate. The assembled conjugate molecules are imaged, as shown in Figure 3c, 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. Both images in Figure 3b and 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 sequence acts as an efficient molecular linker as well as a versatile PDMS ink. Furthermore, we demonstrate here the coassembly of two diverse nano-entities without the involvement of complex surface modification, often involved in silane-based procedures.[11,40]

In conclusion, we present an application of inorganic solid-binding peptides, used as ink for microcontact printing as well as linker for self-assembly. We demonstrate that the molecular constructs based on QBPs, e.g., QBP1, can be designed with bifunctionality, including both a solid-substrate-binding ability and incorporating a target molecule carrying out a robust function. Here, we use either fluorescein or biotin in QBP1-based molecular constructs with nanophotonic or biomolecular recognition functions. Furthermore, we demonstrate a procedure involving microcontact printing and self-assembly, both enabled using the inorganic-binding peptide. Using a combined procedure, one can coassemble diverse multifunctional molecular and nano-entities on micropatterned substrates, both at ambient conditions. The process described here has a potential utility in the efficient preparation of microarrays without the potential problems or limitations associated with the conventional silane-based chemical methods.

Experimental

Materials: SA-QD conjugates with an emission at 605 nm were purchased from Invitrogen, Co., USA. Quartz (111) crystal wafers were received from MTI, Co., USA.

In silico Peptide Design: In our previous study, we designed novel quartz-binding peptide sequences in silico, using a knowledge-based

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design protocol, developed by us [19]. In summary, in this knowledge-based design approach, we utilized the binding characteristics of a set of biocombinatorially selected peptides, and generated a set of new peptide sequences with enhanced binding functionalities. This new approach performs all-against-all comparisons of experimentally selected and characterized peptide sequences and scores the alignments using sequence similarity scoring matrices. Next, we derived a novel scoring matrix, QUARTZ I [19], which optimizes the similarities within the strong quartz-binding peptide sequences and the differences between the strong and weak quartz-binding peptide sequences. Using QUARTZ I scoring matrix, computer-generated random peptide sequences were compared to the strong quartz-binding peptide sequences; the ones with the highest and lowest similarity scores have been chosen as the strong (QBP1: PPPWLPYMPPWS) and weak (QBP2: CINQEGAGSKDK) quartz-binding peptides, respectively. Here, the similarity scores indicate that peptides with significant sequence similarity to strong quartz binders have strong affinity to quartz surface (e.g., QBP1), whereas the peptides with no sequence similarity to strong quartz binders have no affinity to quartz surface (e.g., QBP2). The experimental validation of the binding affinities of the designed peptides displayed excellent agreement with the predictions. Moreover, we also observed that the designed sequence has the highest affinity compared to the strongest phage display selected peptide [19].

Peptide Synthesis: Peptides, QBP1 and QBP2, are prepared on Wang resin via automated Fmoc peptide synthesis employing a CSBio 336s peptide synthesizer and HBTU activation. The biotinylated peptides are prepared using pre-activated biotin (Biotin-OPN, EMD Biosciences). Biotin is dissolved in 1:1 DMF:DMSO and incubated with the resin-bound peptide overnight. Addition of biotin was confirmed by Ninhydrin test. The resulting dry resin-bound peptides are cleaved, side-chain-deprotected using Reagent K (TFA/thioanisole/H_2O/phenol/ethanedithiol 82.5:5.0:5.0:5.0:2.5), and precipitated by cold ether. The obtained crude peptide is purified by Reverse-phase high-performance liquid chromatography (HPLC, Gemini 10u C18 110A column). The purified peptide was checked by mass spectroscopy (MS) using matrix-assisted laser desorption/ionization–time of flight (MALDI-TOF). See Supporting Information for HPLC and MS data for pure QBP1-bio. QBP1-F was commercially synthesized (United Biochemical Research, USA).

PDMS Stamping of Peptides: The PDMS stamps, kindly received from M. T. Zin (UW), were fabricated by molding a mixture of polydimethyl siloxane and curing agent (10:1, Sylgard 184, Dow Corning, USA) on the surface of a patterned silanized master for 2 days at ambient conditions. The stamps are then washed several times with ethanol, heptane, and last with ethanol, and dried with inert gas before being used.

The patterned side of the PDMS stamp is incubated with a peptide conjugate or fluorescein (54 μM) in PBS buffer for 5 min. The peptide solution is removed by pipette from the surface of the stamp, which is then dried with inert gas following brief washing with DI water. Quartz and gold substrates are cleaned using isopropanol and ethanol, and dried with inert gas. The clean substrate is then applied to the surface of the stamp and pressed using force for 10 s. and left on the stamp surface for 1 min. The substrate is removed from the stamp and the patterned side is washed with DI water for 2 min and dried with inert gas.

Following the stamping of biotinylated peptides, SA-QDs (20 nM) in PBS buffer is drop-coated onto the substrate and incubated for 15 min. The substrates were then rinsed with DI water for 2 min and dried with inert gas. In order to produce micropatterns of two different target molecules, i.e., QDs and fluorescein, following SA-QD labeling, the quartz substrate was drop-coated with QBP1-F (54 μM) in PBS buffer and incubated for 15 min. The substrate was again rinsed with DI water for 2 min and then dried with inert gas.

Cleaning and Reuse of PDMS Stamps: Detergent and sonication are effective methods for removal of peptide molecules bound to the PDMS stamp, making the stamp reusable. After the PDMS experiments, the stamp was washed with DI water and placed in a small Petri dish containing detergent prepared with 0.2% Triton X-100 in PBS buffer. Stamps were then sonicated for 10 min in order to remove unwanted particles from the surface. After sonication, stamps were again washed with ethanol and dried with inert gas.

Fluorescence Microscopy: The quartz substrates on glass slides were mounted on a Nikon Eclipse TE-2000U Florescence Microscope (Nikon, Japan) coupled with a Hamamatsu ORCA-ER cooled charge-coupled device (CCD) camera. FITC (exciter 460–500, dichroic 505, emitter 510–560, Nikon) and QD605 (exciter 320-460, dichroic 475, emitter 605/40nm Chroma Technology Co, Rockingham, USA) filter sets are used for QBP1-F and SA-QD detection, respectively. The images are recorded through Metamorph Software (Universal Imaging, USA).

Acknowledgements

This research was funded by NSF through an MRSEC at the University of Washington and partially by BioMaterial program. Partial support (TK and CT) was also provided by TR-SPO. We thank M. T. Zin, C. Maremma and U. O. S. Seker (UW) for technical help. Supporting Information is available online from Wiley InterScience or from the authors.

Received: July 4, 2008
Revised: August 14, 2008

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