

Strategies for Patterning Biomolecules with Dip-Pen Nanolithography

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Dip-pen nanolithography (DPN) is an atomic force microscopy (AFM)-based lithography technique, which has the ability to fabricate patterns with a feature size down to approximately 15 nm using both top-down and bottom-up approaches. DPN utilizes the water meniscus formed between an AFM tip and a substrate to transfer ink molecules onto surfaces. A major application of this technique is the fabrication of micro- and nano-arrays of patterned biomolecules. To achieve this goal, a variety of chemical approaches has been used. This review concisely describes the development of DPN in the past decade and presents the related chemical strategies that have been reported to fabricate biomolecular patterns with DPN at micrometer and nanometer scale, classified into direct- and indirect DPN methodologies, discussing tip-functionalization strategies as well.

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1. Introduction

Controlled micro- and nanopatterning of biomolecules on solid surfaces, while at the same time maintaining the activity and structural integrity of molecules, has attracted increasing attention in recent years because of its potential applications in biotechnology and medicine, including the production and preparation of biochips and biosensors, drug discovery, and fundamental studies of biological processes.^[1] There are various techniques to fabricate small-scale architectures, such as traditional photolithography,^[2] microcontact/nanocontact printing (μ CP/nCP),^[3] nanoimprint lithography (NIL),^[4] electron-beam lithography,^[5] and scanning probe-lithography (SPL).^[6] One scanning probe technique, atomic force microscopy (AFM), was invented in 1986 to use van der Waals interactions to study surface properties.^[7] AFM has become a very popular technique to elucidate the nanometer-scale topography of different materials, and in particular biological molecules in view of the ability of the technique to image in solution. Besides direct imaging, AFM has also been explored to manipulate atoms and molecules on surfaces.^[8] AFM-based lithography utilizes an AFM probe to create patterns chemically, mechanically, or electrically. AFM-based lithography techniques such as dip-pen nanolithography (DPN),^[9] nanografting and nanoshaving,^[10] conductive AFM lithography,^[11] and enzymatic lithography^[12] attract enormous interest due to their technical potential and relatively low cost. Among these techniques, DPN is a popular method for fabricating biomolecular patterns. The developments and applications of DPN have been thoroughly reviewed by Ginger et al.^[13] and Salaita et al.^[14] In addition, the broader topic of biopatterning has received significant attention.^[1a,b,15] Most of these review articles include many different lithographic techniques and chemical approaches used for patterning biomolecules, especially DNA and proteins. In this review, we specifically focus on two aspects: the development of DPN and the chemical strategies utilized for generating biomolecular architectures on different solid surfaces with DPN, which are classified into direct- and indirect DPN methodologies, including a discussion of tip-functionalization approaches.

2. Dip-Pen Nanolithography (DPN)

In this section, first, the development of the dip-pen nanolithography (DPN) technique is reviewed; subsequently the strategies of modifying AFM tip surfaces are discussed, and finally the two main strategies for immobilizing biomolecules with DPN are introduced.

2.1. Development of DPN

Dip-pen nanolithography was introduced by the Mirkin group in 1999 for transferring 1-octadecanethiol (ODT) and 16-mercaptohexadecanoic acid (MHA) from an AFM tip to a gold surface.^[9] The proposed idea of DPN is very similar to writing with a conventional quill pen and ink. **Figure 1**

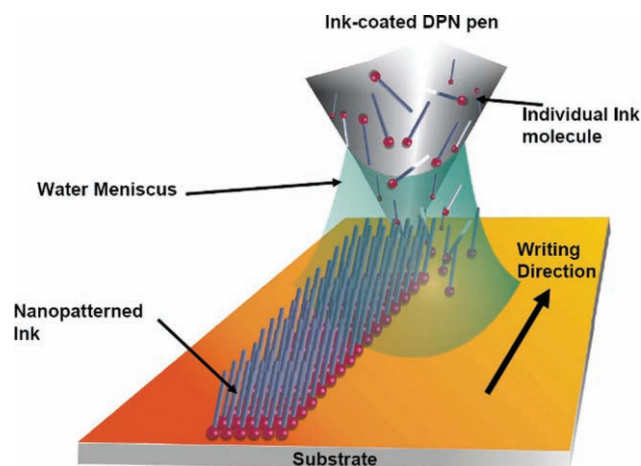


Figure 1. Schematic representation of the basic molecular transport in the dip-pen nanolithography (DPN) process. Reproduced with permission.^[16] Copyright 2005 Elsevier.

illustrates the general process of DPN.^[16] A water meniscus is formed while an ink-coated AFM probe is brought into contact with a substrate in an ambient environment, and the ink molecules are transferred to the surface via this water “bridge.” Depending on the precise nature of the molecule–substrate interaction, which can be either (electro-)chemisorption or physisorption, the ink molecules may become immobilized.^[9,13,14]

The precise mechanism of ink transportation is complicated and differs from one system to another. Over the past few years, several groups have reported factors that have an influence on the deposition processes, including the chemical properties of the ink molecules and the chemical nature of the tip and substrate, as well as the probe geometry, tip–surface contact time, writing speed, and ambient environment. These conclusions are mostly based on studies of ODT or MHA

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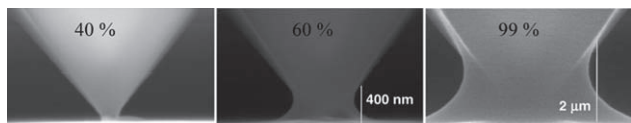


Figure 2. Environmental scanning electron microscopy (ESEM) images of the water meniscus formed between an AFM tip and a substrate. Sequential images were collected at 5 °C at relative humidities of 40%, 60%, and 99%, respectively. Reproduced with permission.^[19] Copyright 2005 American Chemical Society.

deposited on gold surfaces.^[16,17] For example, Haaheim et al. demonstrated that narrower MHA lines on gold surfaces were obtained by using sharper AFM tips or smoother surfaces.^[16] Many inks including inorganic molecules and biomolecules have been used to demonstrate that longer tip–surface contact times or slower writing speeds results in higher spatial resolution of patterns.^[9,18] Since the water meniscus is proposed as a bridge to transport ink molecules, the shape and size of the water meniscus is another crucial issue. Weeks et al. used environmental scanning electron microscopy (ESEM) to image the formation of the water meniscus between a silicon nitride AFM tip and a gold or silicon substrate at different relative humidities (RHs). It was found that the height of the meniscus is a function of RH as shown in **Figure 2**.^[19] Later, the same authors used ESEM to study the dynamic growth of the water meniscus and found that the water meniscus took many minutes to reach an equilibrium state when the tip was brought into contact with the surface. This observation suggests that the meniscus growth may account for the DPN ink transport.^[20] These ESEM results can be used to explain the observed increase in growth rate of MHA with increasing RH reported by Rozhok et al.^[17a] and Peterson et al.^[21] However, Sheehan et al. demonstrated that ODT was transferred onto Au surfaces at near 0% RH, where it is very difficult to form a water meniscus, and that the diffusion coefficients of ODT deposited at different RHs did not change much.^[17c] Rozhok et al. also reported that humidity did not influence the growth rate of ODT and deduced that different behaviors between ODT and MHA could be explained in terms of their different solubilities in the water meniscus.^[17a] In addition, it has been demonstrated by Lenhert et al. that phospholipids can be patterned under water.^[22] All of these results still led to the question whether the water meniscus plays an important role in the ink transportation process of DPN and also suggest that the nature of the ink influences ink transport. Several models have been proposed to explain the ink transport observed in various experimental DPN studies; however, none of these models could be used to explain all conditions. Therefore, a thorough understanding of the interplay of all the mechanisms will enable the development of more optimized ink–substrate combinations and achieve a higher spatial resolution.^[13]

DPN enables the preparation and registration of patterns from below 100 nm to a length scale of many micrometers. The highest resolution achieved so far is with alkanethiol patterns fabricated on gold surfaces with a size of ~15 nm. Moreover, the flexibility of DPN allows the preparation of different patterns on substrates. Since its invention, DPN has



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been performed with a large variety of combinations of inks and substrates. Although DPN can be regarded as an effective tool for nanofabrication, initially a major drawback, like all other scanning probe lithography systems, was that it is inherently a serial process, which limits the throughput. In order to increase the efficiency of DPN, a passive “duplicating” tip array system has been developed.^[14]

The first and simplest passive probe array implemented for parallel DPN was described by Hong et al. in 2000.^[23] The pens were brought simultaneously into contact with a slightly tilted substrate and scanned together. This produced the same number of patterns as the number of tips in the array. The results showed that the feature size of patterns had less than a 10% standard deviation in line-width, implying that line-widths in DPN experiments are not strongly dependent on the tip–substrate contact force at least within certain ranges.^[24] Recently, passive probe arrays have been developed to write multiple arrays at one time and the number of probes of each array has increased dramatically to ~55 000 which allows users to obtain as many as 450 000 000 sophisticated features at the sub-100-nm scale within 30 min.^[14,25] Several examples have been presented using passive probe arrays to fabricate biomolecular patterns in parallel, and are described in detail in Section 3.

The idea of DPN has been extended to several DPN-based techniques, including a nanopen reader and writer

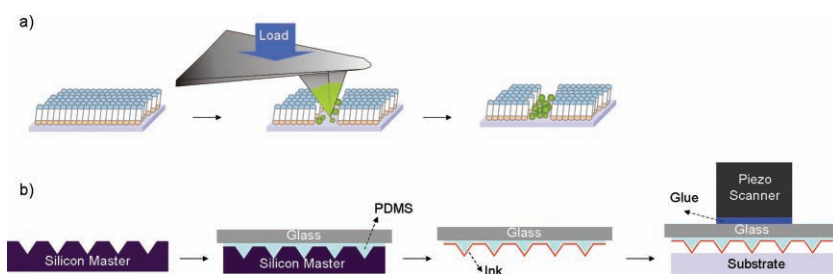


Figure 3. The schematic processes of a) a nanopen reader and writer (NPRW) and b) polymer pen lithography (PPL).

(NPRW),^[26] electrochemical DPN (E-DPN),^[27] scanning probe contact printing (SP-CP),^[28] and polymer pen lithography (PPL).^[29] The process of NPRW shown in **Figure 3a** is based on the combination of DPN and nanografting.^[26a] An AFM tip is inked with molecules different from the molecules of a self-assembled monolayer (SAM) on the substrate. Subsequently, the tip is brought into contact with the surface at higher load. The prefunctionalized SAM is removed mechanically, followed immediately by the deposition of molecules from the tip. E-DPN utilizes the water meniscus as a tiny electrochemical cell where metal salts precoated on the tip, e.g., H_2PtCl_6 , are dissolved and then reduced to metal that is deposited on the substrate.^[27] SP-CP utilizes sharp or flat-topped DPN tips made of poly(dimethylsiloxane) (PDMS) to transfer ink onto a substrate.^[28] More recently, PPL was developed by Huo et al. in the Mirkin group. **Figure 3b** schematically illustrates the process of PPL.^[29] A PDMS tip array is prepared from a silicon master with pyramid-shaped structures. Subsequently, the inked elastomeric tip array was brought into contact with a substrate to transfer inks. It was found that the transportation of ink molecules was time- and force-dependent. Polymer pen arrays are lower-cost and higher-throughput compared with passive DPN probe arrays. Around 11 million pens can be brought into contact with substrates simultaneously to generate patterns. In addition, by varying the extension of the piezo scanner (force), structures with feature sizes of nanometer- and micrometer-length scales can be achieved.^[29,30]

2.2. Surface Modifications of AFM Tip Surfaces

Different approaches of functionalizing tip surfaces have been reported. These approaches are usually utilized to facilitate the transportation of biomolecules from an AFM tip to a substrate. Demers et al. found that it was difficult to control the sizes and shapes of DNA patterns when using a bare silicon nitride tip. Therefore, they functionalized the tip surfaces with 3'-aminopropyltrimethoxysilane (APTMS) to improve the adhesion of DNA to the tips.^[18a] Lee et al. reported a method to functionalize Au-coated Si_3N_4 cantilevers. The process shown in **Figure 4** started with growing a monolayer of symmetric 11-mercapto-undecylpenta(ethylene glycol)disulfide (PEG) on the cantilever, followed by coating an Au/Ti layer on the tip side via thermal evaporation, and finally by incubating in thiotic acid to form a carboxylic-acid-group-terminated SAM on the tip surface. The PEG

layer prevents the adsorption of protein on the back side of the cantilever, and the hydrophilic carboxylic acid groups can facilitate the adsorption of proteins onto the tip surfaces.^[31] Lee et al. presented another method to functionalize AFM tip surfaces with carboxylic-acid-group-terminated SAMs. The process started with coating an Au thin film onto a Si AFM tip, followed by incubating the tip in mercaptoundecanoic acid (MUDA) solution to grow carboxylic-acid-group-terminated monolayers.^[32] Lim et al. modified an

AFM tip with 2-[methoxypoly(ethyleneoxy)-propyl]trimethoxysilane (PEG-silane) to facilitate the transfer of proteins from tip to surfaces and also to prevent the denaturation of proteins on the tip surface.^[33] Jiang et al. also used the PEG-silane-functionalized tip to generate peptide amphiphile (PA) nanofiber patterns. The PEG-silane was utilized to avoid the self-assembly of nanofibers on the tip surfaces.^[34] In addition, Nam et al. reported coating a thin nickel layer onto a Si_3N_4 AFM tip to facilitate the wetting and transport of histidine-tagged (His-tagged) proteins onto surfaces.^[35] To carry out multiplexed inking of 2D probe arrays with inkjet printing techniques and to avoid the capillary-action-driven adhesion of inked cantilevers to the substrates, Wang et al. functionalized the gold-coated tip areas of 2D probe arrays with 1-mercaptoundecanol and the remaining Si_3N_4 and Si/SiO_2 areas with OTS for patterning phospholipids. They also observed that the phospholipids ink droplet can be confined to the tip when the surfaces of tips were functionalized with ODT.^[36]

Porous matrix materials have been developed to functionalize the AFM tip surfaces to facilitate the transfer of biomolecules or to increase the total amount of coated ink molecules. Choi et al. fabricated hydrophilic nanoporous polyoxazoline-coated Si AFM tips by functionalizing the bare Si surface with 11-iodoundecyl-trichlorosilane as an initiator and performing the polymerization of 2-methyl-2-oxazoline monomers on the pretreated surface.^[37] The authors demonstrated that it is at least five times faster to generate protein nanoarrays with the nanoporous polyoxazoline-coated tip than with a conventional Si tip.^[37] We have presented a simple method by utilizing layer-by-layer (LbL) self-assembly techniques to grow a thin layer of a porous matrix on Si_3N_4 tips.^[38] Two different polymers, poly(4-vinylpyridine) (PVP) and poly(acrylic acid) (PAA), were affixed via hydrogen binding onto cantilever surfaces pretreated with *N*-[3-(trimethoxysilyl)propyl]-ethylenediamine (TPEDA) monolayer. Porous structures were

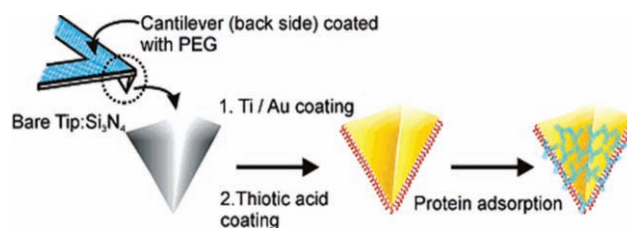


Figure 4. Schematic representation of the procedures of functionalizing AFM cantilever and tip surfaces, reported by Lee et al. Reproduced with permission.^[31] Copyright 2003 American Chemical Society.

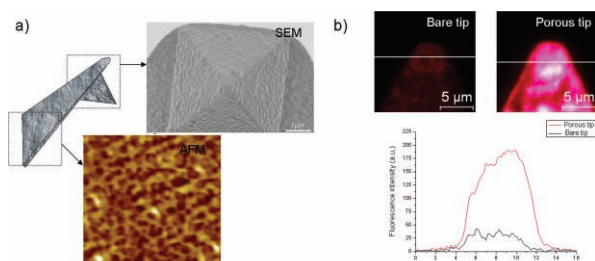


Figure 5. a) Scanning electron microscopy (SEM) and tapping-mode AFM height image of an LbL15-functionalized porous tip incubated in KOH solution for 15 min. The number “15” in the label LbL15 denotes the number of PVP–PAA bilayers. b) Fluorescence images and fluorescence intensity profiles of a bare AFM tip and an LbL15-functionalized porous tip inked with histidine-tagged tetrameric reef coral fluorescent protein. Reproduced with permission.^[38] Copyright 2009 American Chemical Society.

formed by immersing the LbL-created polymer coating into base solutions (**Figure 5a**). The pore size of the porous structure can be adjusted easily by changing the incubation time of LbL film in the base solution. The porous matrix enabled the tip to encapsulate more ink molecules, which was confirmed by comparing the fluorescence intensities between porous and bare tips loaded with visible fluorescence proteins (VFP) (**Figure 5b**).^[38]

Recently, colloid-terminated tips were developed by Kramer et al. particularly aimed at depositing specific molecules onto rough and soft surfaces instead of for high patterning resolution. The colloids attached were made from poly(methyl methacrylate) (PMMA) because their swelling behavior is dependent on environmental conditions such as humidity and pH.^[39]

2.3. Strategies to Immobilize Biomolecules with DPN

All the developments in DPN described in Section 2.1–2 enable many applications in chemistry, materials sciences, and biosciences.^[13,14] In this review, the focus is on the application of fabricating biomolecular patterns on solid surfaces. Generally, two strategies can be discerned to pattern biomolecules with the DPN technique, namely an indirect method and a direct method (**Figure 6**). The indirect method (Section 3) first places a suitable molecular functionality at a surface. For instance, first a patterned layer of biomolecule-adherent molecules is fabricated, followed by the immobilization of biomolecules from solution. The indirect method is also a common strategy utilized by many other lithographic techniques, i.e.,

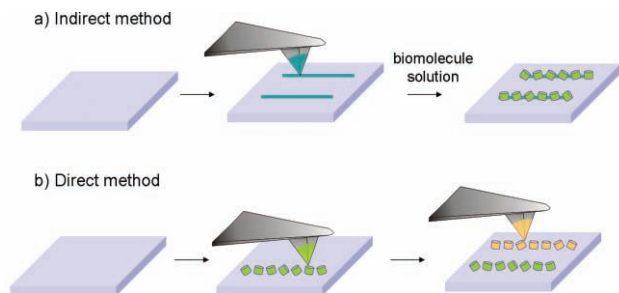


Figure 6. Schematic representation of a) indirect and b) direct dip-pen nanolithography (DPN) method for patterning biomolecules.

those mentioned in Section 1, for patterning biomolecules. The direct method (Section 4) comprises direct deposition of the biomolecules from an ink-coated tip onto a surface.^[1a,b]

Indirect DPN approaches offer an elegant way to fabricate biomolecular patterns and prevent denaturation of fragile biomolecules caused by direct writing.^[31] However, the preparation procedures are more time-consuming, and nonspecific binding effects of the biomolecules to be patterned may influence the fidelity of the patterned structures. Therefore, suitable passivation of those areas where patterning of biomolecules is not desired is a necessary step before immobilizing the biomolecules of interest, and is described in detail in Section 3. On the other hand, direct DPN is a more straightforward approach to generate biomolecular patterns without nonspecific binding.^[1b] In addition, the direct DPN strategy enables one to deposit different kinds of biomolecules on the same surface, which is relatively difficult to achieve using the indirect DPN strategy.^[36] However, it has several restrictions, such as slow transportation of high-molecular-weight biomolecules from an AFM tip to a substrate, difficulties of preserving the biological functionalities of biomolecules on the tips' surface during writing, and short operation time due to the limited ink volume.^[37] To overcome these restrictions, chemical modification of the AFM tip surfaces, which is described in Section 2.2, and adding carriers into the biomolecular inks are two commonly used approaches. Glycerin (glycerol) is frequently added to biomolecule solutions to slow down the evaporation of water and facilitate the transport of ink molecules from a tip to substrate. In addition, agarose and phospholipids have been demonstrated as biomolecular ink carriers.

Functional immobilization of biomolecules, especially proteins, onto solid surfaces is very challenging since the interaction of proteins with a bare solid substrate frequently results in denaturation of proteins.^[40] A variety of chemical strategies has been utilized in either indirect or direct DPN for patterning biomolecules. In general, two main types of substrates, gold- and silicon-based substrates have been used. Therefore, in each subsection of Sections 3 and 4, the chemical strategies utilized for patterning biomolecules are discussed based on the classification of the substrates used. In addition to the approaches summarized here, there are many other methods employed to fabricate biomolecule patterns onto a solid support using other lithography techniques, which have been described in several articles and may also be applied to DPN in the near future.^[15b,41]

3. Patterning Biomolecules with Indirect Dip-Pen Nanolithography Methods

3.1. Gold Surfaces

Several indirect DPN methods utilize MHA as ink molecules to generate dot or line patterns on gold surfaces. Subsequently, the nonpatterned areas are passivated with ODT to prevent the nonspecific adsorption of biomolecules. The carboxylic-acid-terminated groups of MHA are subsequently used to immobilize biomolecules via electrostatic interactions. Kwak et al. used this method to fabricate cytochrome c dot patterns

on gold surfaces with a feature size of ~ 200 nm. Multilayers of protein patterns and nonspecific binding effects were observed. Because of the electron transport properties of cytochrome c, these protein patterns have potential applications in biomolecular photodiode systems.^[42] Lee et al. generated lysozyme, Immunoglobulin G (IgG) and retronectin arrays with feature sizes of ~ 100 – 350 nm. The areas surrounding MHA patterns were passivated with 11-mercaptoundecyl-tri(ethylene glycol) (EG₃-SH) instead of ODT. The biological activity of IgG arrays was retained as confirmed by binding anti-IgG to IgG with a 1:1 reaction and nonspecific binding was negligible. Patterns of retronectin, the cell adhesion recombinant fragment of fibronectin, were used to adhere 3T3 Swiss fibroblast cells. This array was used for a study of cellular interactions with nanopatterned surfaces. The authors observed that the cells only attached to the printed region and were immobilized in a more flattened morphology.^[43] Later, they extended this approach to fabrication of arrays of antibodies (anti-p24) with well-defined feature sizes in the sub-100-nm range. The bio-activity on Au surfaces in highly sensitive and selective immunoassays to detect human immunodeficiency virus type 1 p24 antigen (HIV-1 p24) was preserved (**Figure 7**). The HIV-1 p24 protein in plasma obtained from HIV-1-infected patients was hybridized with the antibody array. The bound protein was hybridized to a gold antibody-functionalized nanoparticle probe in order to amplify the detected signal. The nanoarray features in the three-component sandwich assay were confirmed by AFM and field-emission scanning electron microscopy. The authors demonstrated that 0.025 pg HIV-1 p24 protein per milliliter of plasma can be detected, which indicates that such an immunoassay can exceed the detection limit of conventional enzyme-linked immunosorbent assays (ELISA) by several hundred times.^[44] Zhang et al. generated MHA patterns on an Au thin film thermally evaporated on Ti-coated oxidized silicon. The Au/Ti film without patterning with

MHA was removed by chemical wet etching. The exposed SiO_x area was subsequently passivated with *n*-octadecyltrimethoxysilane (OTS) to prevent the nonspecific adsorption of proteins. A single layer of rabbit IgG was immobilized on patterned MHA-coated, elevated Au nanostructures on SiO_x/Si substrates. Fluorophore-labeled anti-rabbit IgG was used to confirm the bioactivity of these proteins on solid supports.^[45]

Membrane protein complexes attached to protonated amino-terminated SAMs created by DPN via electrostatic interactions have also been reported by Valiokas et al. The authors deposited HS(CH₂)₁₆NH₂ onto a Au surface with a resolution of ~ 300 nm and subsequently blocked the nonpatterned region with HS(CH₂)₂CONH(CH₂CH₂O)₍₋₁₅₎CH₃ for immobilization of the selective recruitment of membrane protein complexes from mutant *Rhodobacter sphaeroides*. These contain photosynthetic core complexes that form the reaction center of the light-harvesting complex one (LH1).^[46]

Instead of utilizing electrostatic interactions, Demers et al. generated MHA patterns on Au surfaces to couple with alkylamine-modified DNA via the formation of amide bonds.^[47] The nonpatterned area was passivated with ODT. The authors found that the MHA patterns could be fabricated again on the areas previously passivated with ODT during the initial writing procedure. Therefore, another sequence of alkylamine-modified DNA could be immobilized onto the newly created MHA patterns. The formation of two DNA patterns was confirmed by coupling DNA with complementary predesigned oligonucleotide-modified particles of different sizes. Different heights of DNA dot patterns and some stray particles nonspecifically bound to the areas surrounding the oligonucleotide patterns were observed in the AFM topography images.^[47] Nyamjav et al. also used the same approach to immobilize poly-L-lysine (PLL) onto the MHA prepatterned area. Such PLL/MHA patterns can

be used to attach motile *P. aeruginosa* bacterial cells.^[48] Rozhok et al. used a similar method to fabricate PLL/MHA patterns for immobilizing motile *E. coli* bacterial cells. The areas surrounding PLL/MHA patterns were passivated with 11-mercapto-1-undecano instead of ODT.^[49]

Smith et al. reported two ways to fabricate cowpea mosaic virus (CPMV) particles onto solid surfaces: one is via electrostatic interaction and the other is via covalent attachment.^[50] In the noncovalent method, they immobilized CPMV particles onto DPN-generated MHA patterns with the rest of the area passivated with EG₃-SH. Since nonspecific adsorption of proteins often results in substantial denaturation and hence loss of biological activity, these authors developed a covalent method to immobilize CPMV particles. They wrote mixed ink, including 11-mercaptoundecyl-penta(ethylene glycol) disulfide and a mixed disulfide substituted with one maleimide, onto gold surfaces. Subsequently,

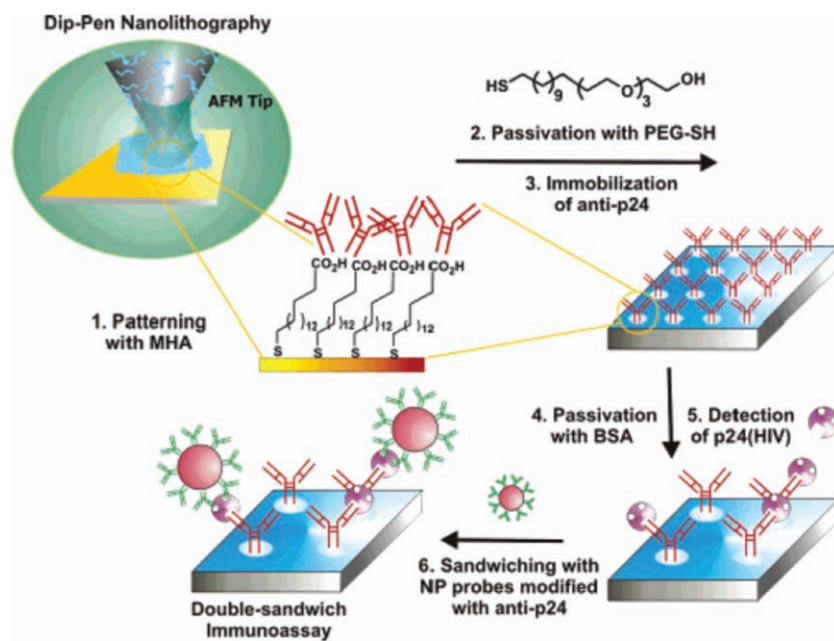


Figure 7. Schematic representation of the fabrication of immunoassay for detecting HIV-1 p24 antigen with anti-p24 antibody. Reproduced with permission.^[44] Copyright 2004 American Chemical Society.

cysteine mutant CPMV (Cys-CPMV) particles were attached to the patterned area via a covalent reaction of cysteine and maleimide. In both cases, AFM topography measurements confirmed the formation of single-layer CPMV particles patterns.^[50] In the same year, Cheung et al. also demonstrated patterning Cys-CPMV particles with the same chemical reaction. They used DPN and nanografting to create amine-terminated alkanethiol molecules on gold surfaces. The amine groups were subsequently reacted with 3-maleimidopropionate *N*-hydroxysuccinimide ester (MPS) to form maleimide-terminated groups, to which Cys-CPMV particles were bound.^[51]

Lee et al. demonstrated writing, with passive 26-probe arrays, of *N*-hydroxysuccinimide ester-terminated alkylthiols (NHSC₁₁SH) onto Au surfaces as a template for immobilizing the recombinant fusion protein, protein A/G, which combines the IgG binding domains of both Protein A and Protein G.^[52] The NHS ester groups are frequently used to couple with the primary amino groups of biomolecules via the formation of amide bonds. Protein A/G has affinity to the Fc, Fragment crystallized, region of IgG antibodies. By utilizing this affinity binding, massively parallel nanoarrays with biologically active antibodies can be generated.^[52] Hyun et al. also used NHS ester groups to fabricate biotin/streptavidin-terminated arrays. They first patterned MHA on Au surfaces with a feature size of ~200 nm. After obtaining the MHA patterns, they passivated the background with EG₃-SH and then activated carboxylic-acid-terminated groups of an MHA SAM with NHS and 1-ethyl-3-(dimethylamino)propyl carbodiimide (EDAC) to form NHS ester groups.^[53] Biotinyl-3,6,9-trioxaundecanediimine (biotin-NH₂) was used to generate biotin-terminated patterns. Subsequently, streptavidin, a tetrameric protein with very high affinity to bind four biotins ($K_a \approx 10^{13} \text{ M}^{-1}$), was adhered to the patterned biotin area from solution. Therefore these protein arrays can be utilized to bind a variety of biotinylated molecules. The authors used biotinylated bovine serum albumin (Biotin-BSA) to demonstrate the retained functionality of this streptavidin-biotin system.^[53] Later, Hyun et al. extended their approach to immobilize amine-containing stimulus-responsive elastinlike polypeptide (ELP) onto patterned NHS ester-terminated regions.^[54] The authors believe that ELP is end-grafted at the surface in a highly orientated manner because ELP only has two amino groups, one located at the N-terminus and the other on the side chain of the sole lysine residue. Such ELP patterns can be used as templates to capture an ELP fusion protein (Trx-ELP) above its lower critical solution temperature (LCST). The dissociation of Trx-ELP occurs when proteins become soluble in aqueous solution. This switch process is reversible, which offers the potential application of building reusable protein nanoarrays.^[54]

Rakickas et al. presented a platform to study protein-protein interactions and to form functional protein complexes at nanometer scale. For this purpose, multivalent chelator (MCh)-terminated templates were fabricated in a stepwise procedure combining dip-pen nanolithography and

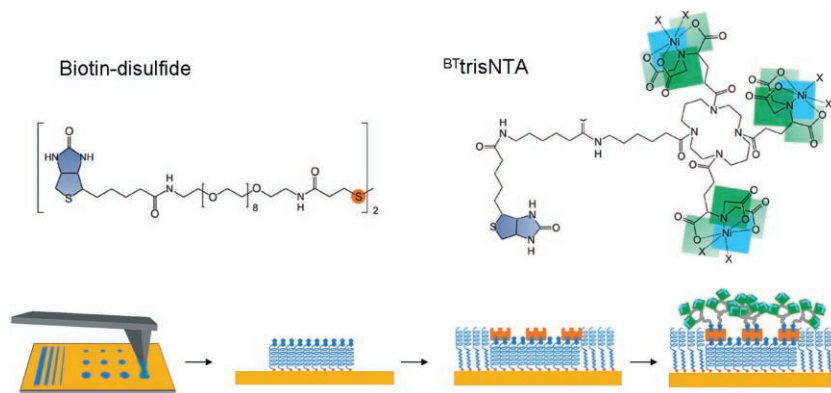


Figure 8. Schematic representation of the fabrication of ^{BT}trisNTA-terminated nanoarrays by DPN used for studying protein-protein interactions. Reprinted with permission.^[55] Copyright 2008 American Chemical Society.

molecular recognition-directed assembly (**Figure 8**). The biotin-oligo(ethylene glycol) (OEG) disulfide molecules were first deposited on gold with line-widths below 100 nm, and then streptavidin was adhered to the biotin-terminated patterns from solution. Subsequently, the streptavidin groups were attached with tris-nitrilotriacetic acid biotin derivatives (^{BT}trisNTA) to form MCh-terminated templates for assembly of His-tagged proteins. Histidine tags are generally engineered onto protein sequences to facilitate protein purification in analytical procedures. The ^{BT}trisNTA coordinated with Ni(II) areas were used to immobilize His₁₀-ifn α 2 proteins, whose functionality was confirmed by coupling interferon alpha 2 (INF α 2) labeled with QD₆₅₅, quantum dots emitting at 655 nm. This process was reversible by adding imidazole solution to remove His-tagged proteins.^[55] This His-Ni(II)-NTA system is frequently used to immobilize biomolecules with controlled orientation on solid surfaces.^[15b]

Hoover et al. presented an indirect approach to produce peptide arrays for immobilizing cells (3T3 Swiss Albino mouse fibroblasts). They produced nanoarrays of hydroquinone terminated alkanethiol on glass substrates covered by a thin gold film. The nonpatterned regions were passivated with tetra(ethylene glycol) terminated alkanethiol. By electrochemically oxidizing the nanopatterned hydroquinone, the corresponding quinone was obtained, which can react selectively with water soluble oxyamine-terminated linear arginine-glycine-aspartate (RGD) peptide. These peptide patterns were used to study the polarity of adhered cells without the influences of cell-cell interactions. It was found that a cell was affected by its nano-environment to polarize toward the patterned higher-density peptide region.^[56]

Vega et al. relied on the ability of Zn(II) ions to bridge a surface patterned with MHA and the tobacco mosaic virus (TMV) with its carboxylate-rich surface. They demonstrated the possibility of isolating and controlling the orientation of TMV particles.^[57] Later, they used the same approach to immobilize antibodies, such as immunoglobulin Y (IgY) and immunoglobulin M (IgM). Although they did not know the precise chemistry behind the reaction between the antibodies and Zn(II) ions, this approach enables the further adhesion of protein A, G, A/G or L with functionality.^[58]

3.2. Silicon-Based Surfaces

Many indirect DPN experiments use the thiol-on-gold couple, where the chemisorption of the thiol molecules onto gold surfaces is the driving force of ink transfer from tips. Patterning of biomolecules with indirect DPN on silicon-based surfaces is also reported. Using silicon-based substrates, especially glass substrates, with DPN provides several advantages including the application of a large repertoire of chemical strategies and easy observation of deposited luminescent molecules.

Pena et al. reported one way to fabricate biotin-terminated arrays to immobilize fluorophore-labeled neutravidin on silicon-based substrates.^[59] They first sputtered polyethylenimine (PEI) onto SiO₂ surfaces, followed by depositing NHS-functionalized biotin. Some nonspecific binding was observed, but the authors claimed that the signal-to-noise ratio was sufficient. Nyamjav et al. fabricated poly(allylamine hydrochloride) (PAH), a polyelectrolyte, patterns on SiO_x wafers, demonstrating that these well-defined positively charged regions can be used to guide the stretching of long DNA molecules.^[60] Such stretched DNA adhered to patterned PAH areas and was subsequently used as a template to immobilize Fe₃O₄ nanoparticles by electrostatic interactions.^[61]

Jung et al. demonstrated that it is possible to fabricate alkoxy silanes patterns on glass substrates. They used 3'-mercaptopropyltrimethoxysilane (MPTMS) as the ink molecules. The MPTPS patterns were verified by reaction with biotin-maleimide via the formation of thioether bonds to immobilize Cy3-labeled streptavidin.^[62] Later, the authors extended this approach to fabricate (+)-biotinyl-3-maleimidopropionamidyl-3,6-dioxaoctanediamine (maleimide-PEO₂-biotin) directly onto MPTPS-functionalized glass substrates.^[18b] However, the authors observed that obtaining consistent patterns of the maleimide-PEO₂-biotin was very difficult even when DPN experiments were performed with a RH above 90%. Finally, they found that adding small amounts of the non-ionic surfactant Tween-20 (polyoxyethylene sorbitan monolaurate) to the ink can activate the transfer of the biotin linker molecules from the tip to the MPTMS-coated substrates, enabling biotin patterning routinely with sub-100-nm resolution at a moderate RH of 50–65%. Cy3-labeled streptavidin was again used to confirm the successful generation of maleimide-PEO₂-biotin patterns.^[18b] The same chemical strategy has also been utilized by Kim et al.^[63] They wrote maleimido-C₃-nitrilotriacetic acid (NTA) onto MPTMS-functionalized glass substrates for the effective immobilization of His-tagged enhanced green fluorescent proteins by utilizing coordination chemistry.^[63] We have presented another indirect DPN method by writing Ni(II) ions onto NTA-terminated monolayer-functionalized glass substrates as templates to immobilize His-VFPs at the

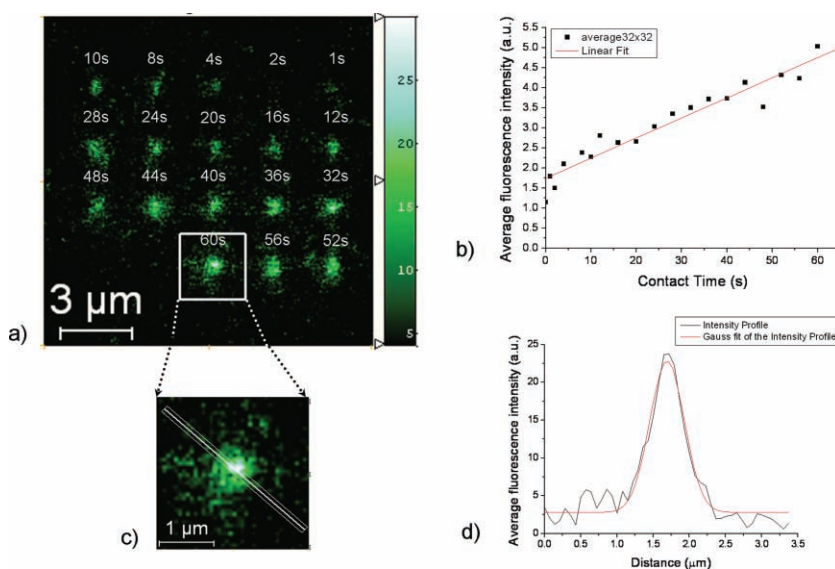


Figure 9. a) Fluorescence images of His-EGFP patterns acquired after incubating prepatterned Ni(II) area with the protein solution. The Ni(II)-coated tip–surface contact times are listed above the dot patterns. b) Plot of average fluorescence intensity of each dot versus tip–surface contact time. A linear fit to the data is plotted in red. c) Zoom-in of His-EGFP pattern generated with tip–surface contact time of 60 s shown in panel a. d) Average fluorescence intensity profile of the area inside the rectangle indicated in c (black line) can be fitted well with Gaussian fit profile (red line). Reproduced with permission.^[64] Copyright 2010 American Chemical Society.

submicrometer scale.^[64] The Ni(II) complexed His-VFP patterns can be removed by rinsing with an ethylenediaminetetraacetic acid (EDTA) solution. Therefore, protein patterns could be regenerated with different feature sizes, which had earlier been demonstrated by using μ CP. In addition, complexing with His-VFPs enabled us to investigate the dependence of the transfer of nickel ions on fundamental parameters of DPN, which cannot be directly observed by AFM topography measurements (**Figure 9**).^[64,65]

Sekula et al. demonstrated spatial patterns of supported phospholipid membranes by depositing 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) mixed with phospholipids coupled with nickel-chelating headgroups or biotinylated phospholipid in different stoichiometric ratios onto hydrophilic glass substrates as templates to immobilize Cy3-labeled streptavidin and His-tagged green fluorescent proteins (His-GFP), respectively. By using passive 26-probes arrays, integrated protein nanoarrays exploiting two different functionalities were generated. The authors also demonstrated that these biomimetic membrane patterns can be used as substrates for cell culture, demonstrated by the selective adhesion and activation of T-cells.^[66]

Zhou et al. reported an approach to fabricate nanopatterns of peptides of specific chain lengths. The preparation procedure started by depositing G4-NH₂ polyamidoamine (PAMAM) dendrimers onto Si/SiO_x substrates with feature sizes of ~180 nm via electrostatic interactions. The patterned dendrimer substrates were incubated in tryptophan-*N*-carboxyanhydride (Trp-NCA) solution for different times to initiate ring-opening polymerization (ROP), a well known method to obtain oligopeptides with variable chain lengths.^[67]

4. Patterning Biomolecules with Direct Dip-Pen Nanolithography Methods

4.1. Gold Surfaces

Fabricating nanostructures with DPN is usually performed by operating AFM in contact mode. However, the use of tapping-mode AFM for lithography and imaging of biomolecules may be more suitable, because of the sensitivity of biomolecules to the shear and friction forces required to control the surface in the contact mode. Two examples of utilizing tapping-mode AFM to deposit biomolecules via gold–thiol interaction were reported. The first was reported by Wilson et al., who delivered thiolated collagen and collagen-like peptides onto gold substrates with line-widths as small as 30–50 nm and showed that collagen still preserved its native triple-helical structure and bioactivity.^[68] Later, Agarwal et al. used DPN in tapping mode to deposit synthetic peptides (MH2) containing an active cysteine (Cys) residue onto a gold surface with a feature size as small as 70 nm.^[69]

Cho et al. demonstrated that thiol-containing TAT peptides, derived from the HIV-1 TAT protein, can be directly written onto Au substrates via gold–thiol interactions.^[70] More recently, Wei et al. deposited 3-repeat GBP1 peptide with biotin incorporated (biotin-3RGBP1) onto Au surfaces with the smallest feature size at ~80 nm. Alexa Fluor 594-labeled streptavidin was subsequently used to visualize the biotinylated peptide patterns.^[71] Both Cho et al. and Wei et al. also demonstrated peptide patterns on Si-based surfaces. Demers et al. used APTMS-functionalized tips to write DNA bearing hexanethiol groups onto gold at sub-100-nm scale via gold–thiol interactions.^[18a] Lee et al. reported using hydrophilic carboxylic-terminated SAM-functionalized tips to fabricate lysozyme (Lyz) and rabbit IgG arrays at the same time with the smallest feature size of ~45 nm in diameter without cross-contaminations.^[31] Not only can the hydrophilic carboxylic acid groups facilitate the adsorption of proteins onto the tip surfaces, but the Cys residues of rabbit IgG can also provide a strong driving force to adhere proteins onto gold surfaces due to thiol–Au reaction. The biorecognition properties of two-component protein arrays were confirmed by the observation of anti-rabbit IgG binding to the rabbit IgG features. The authors found that optimum patterning results were obtained when performing DPN at a RH of ~80–90% at room temperature.^[31]

Lee et al. used MUDA-functionalized AFM tips to deposit angiogenin and integrin $\alpha_v\beta_3$ on a gold substrate coated with a layer of Prolinker with pattern sizes as small as 120 nm. The Prolinker contains crown ether moieties that act as a host cavity for protonated amino groups on protein surfaces.^[32] Later, the authors used the same approach to generate anti-angiogenin arrays on the Prolinker-coated Au surfaces. In addition, the AFM tip functionalized with Prolinker was inked with angiogenin and then brought to contact with anti-angiogenin to study the interaction force.^[72]

Bellido et al. explored patterning horse spleen ferritin onto different kinds of surfaces including Au, silicon oxide, aluminum, and niobium surfaces and TEM grids.^[73] The applications of ferritin patterns are described in detail in the following section.

4.2. Silicon-Based Surfaces

Lim et al. used PEG silane-functionalized tips to write single layers of (anti-)rabbit IgG onto a negatively charged and aldehyde-terminated SiO₂ surface via respectively electrostatic interaction and covalent bonding down to a length scale of ~50 nm.^[33] Jiang et al. also utilized PEG silane-functionalized tips to write two kinds of PA nanofibers mixed with glycerin onto Si substrates at micrometer scale. Glycerin can enhance the transfer of PA and also avoid the aggregation of the PA molecules before writing onto the substrate.^[34] Choi et al. reported using hydrophilic nanoporous polyoxazoline-coated Si AFM tips to fabricate single layers of human IgG and prostate specific antigen (PSA) protein arrays onto *N*-2-amino-ethyl-11-aminoundecyltrimethoxysilane (AUMS)-functionalized Si substrates with feature sizes as small as ~250 and ~85 nm in diameter, respectively, via electrostatic interactions. The authors demonstrated that it is at least five times faster to generate protein nanoarrays with the nanoporous polyoxazoline-coated tip than with a conventional Si tip. In addition, they claimed that the mechanism of ink deposition was not dependent on the diffusion of materials because the feature sizes of the generated patterns did not increase with tip–surface contact time.^[37] We have reported utilization of porous multilayer-functionalized Si₃N₄ tips to deposit His-tagged VFPs onto TPEDA or Ni-NTA-terminated monolayer-functionalized glass substrates via electrostatic interactions and formation of metal ion–imidazole bonds, respectively. The smallest feature size of protein patterns was ~250 nm, and multilayers of ink were observed.^[38]

Noy et al. patterned lines of human chorionic gonadotropin (HCG) antibody tagged with tetramethylrhodamine (TMR) at sub-micrometer scale on a glass substrate pretreated with 3-glycidypropyltrimethoxysilane via the reaction of amines with epoxides. The epoxy-terminated monolayer facilitates the adhesion of proteins to surfaces. The resulting patterns were examined by an instrument that combines AFM and scanning confocal microscopy.^[74] However, the biological activity of the resulting patterns was not demonstrated.

Demers et al. also used APTMS-functionalized AFM tips to write oligonucleotides containing 5'-terminal acrylamide groups onto MPTPS-modified silicon oxide substrates at the sub-100-nm scale. The authors demonstrated the possibility of generating two-component DNA arrays on SiO₂ surfaces while retaining their biological activities, which was confirmed by hybridization with complementary fluorophore-labeled DNA.^[18a] Recently, Kramer et al. demonstrated the use of colloid terminated tips to generate collagen binding peptide and laminin patterns on SiO_x surfaces modified with two layers of polyelectrolytes, respectively.^[39]

A ribosome display is a cell-free system for in vitro selection of proteins and peptides from large libraries that can produce protein-ribosome-mRNA fusion molecules by coupling individual proteins (phenotypes) to their corresponding mRNA (genotypes). Kim et al. demonstrated deposition of acrylamide-modified oligonucleotides mixed with deionized water and *N,N*-dimethylformamide (DMF) onto MPTMS-coated glass substrates. The volume percentage of DMF used to facilitate the ink transport was very important for obtaining consistent patterns.

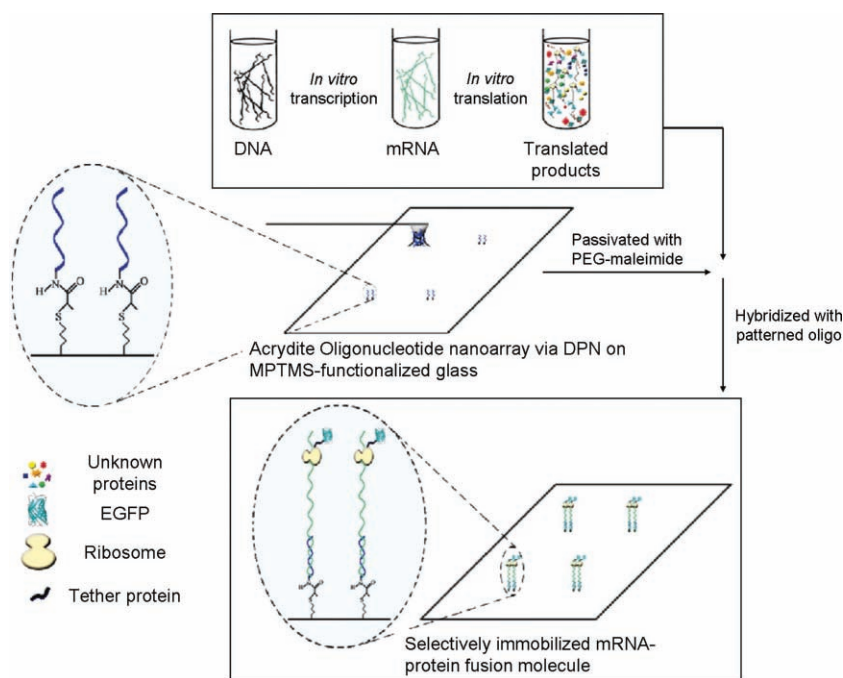


Figure 10. Schematic illustration of the ribosome display technique and the procedure of fabricating protein arrays by directly depositing oligonucleotide as templates. Reproduced with permission.^[75]

The bioactivity of written oligonucleotides was confirmed via hybridization with fluorophore-labeled complementary oligonucleotides or protein-ribosome-mRNA fusion molecules. The authors demonstrated that this approach (**Figure 10**) can be used to fabricate protein arrays without prior protein purification.^[75]

Cho et al. not only demonstrated direct patterning of thiol-containing TAT peptides onto an Au surface, but also onto Si substrates functionalized with a maleimide-terminated monolayer via the formation of thioether bonds.^[76] For patterning TAT peptides on surface-modified Si substrates, ~80% RH was required in order to obtain nanostructures. This was attributed to the high hydrophobicity of the maleimide-terminated monolayer.^[76] Besides fabricating biotin-3RGP1 peptide patterns on Au surfaces, Wei et al. also deposited two kinds of biotinylated peptide (biotin-QBP1 and biotin-QBP3) onto Si surfaces with the smallest feature size at ~80 nm and confirmed the formation of the peptide patterns with Alexa Fluor 594-labeled streptavidin.^[71] Sistiabudi et al. investigated how surface properties affected the direct writing of polyarginine and poly(glutamic acid) polypeptides. Three different surfaces were utilized including hydrophilic bare silicon oxide and 3-aminopropyltriethoxysilane (APTES)-functionalized Si substrates and hydrophobic trimethoxypropylsilane (TMPS)-functionalized Si substrates. Their results demonstrated that both polypeptides were not transferred onto hydrophobic TMPS-functionalized surfaces even when performing DPN experiments under high RH conditions. Furthermore, they found that the transport of polypeptides was facilitated by adding Tween-20 surfactant, because of the increased wettability of the collagenous

surface. Deposition of ink was therefore able to be operated at lower contact force and RH.^[77]

Senesi et al. presented another way (**Figure 11a,b**) to encapsulate proteins (IgG) and oligonucleotides onto AFM tip surfaces using agarose as a “universal” carrier to generate biomolecular patterns. They wrote with the smallest feature size of ~50 nm on *N*-hydroxysuccinimide-ester-activated glass substrates via the formation of amide bonds. By a reaction with fluorophore-labeled anti-IgG and complementary oligonucleotides, respectively, the functionality of the IgG and oligonucleotides patterns were confirmed. Combined with passive probe arrays, Cy3-labeled oligonucleotide arrays were fabricated in a parallel manner (**Figure 2, 11c**). The authors reported that the process of agarose-assisted DPN for patterning biomolecules is very fast and can be systematically adjusted by controlling the concentrations of accelerating agents, e.g., tricine buffer, used in agarose matrix.^[40]

Bellido et al. demonstrated patterning horse spleen ferritin mixed with glycerol onto silicon oxide substrates.^[73a] Ferritin is a biological scaffold that can be used to synthesize iron oxide nanoparticles. Therefore, the authors exposed the prepatterned protein arrays to a heat treatment at 450 °C for 1 h in an inert atmosphere to remove the organic protein shell. Subsequently, time-of-flight secondary-ion mass spectrometry (TOF-SIMS) was used to confirm the presence of iron oxide nanoparticles.^[73a]

Phospholipids, a main component of biological membranes, can self-assemble into 2D bilayers on solid surfaces and keep their property of lateral mobility. Therefore, patterned phospholipids are often utilized as model systems to mimic the behavior of nature biomembranes. Lenhart et al. used 2D cantilever arrays consisting of 55 000 tips to deliver fluorophore-labeled DOPC onto silicon wafers and glass slides with or without the treatment of oxygen plasma (**Figure 12a,b**).^[78] The feature sizes of DOPC patterns varied from ~100 nm to several micrometers. In addition, DOPC doped with two different fluorophores can be generated in a serial manner by using different tips (**Figure 12c**).^[78] Later, Lenhart et al. demonstrated direct patterning of DOPC mixed with phospholipids coupled

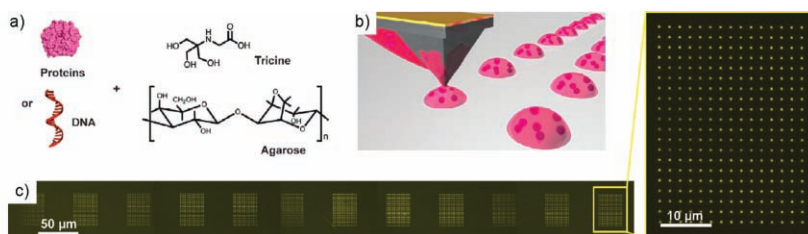


Figure 11. a) Schematic representation of ink and matrix components; b) an illustration showing the process of agarose-assisted DPN; c) epifluorescent microscope image of a 15 × 20 array of 500 nm Cy3-labeled oligonucleotide features generated in parallel from a 12-tip cantilever array. Reproduced with permission.^[40] Copyright 2009 American Chemical Society.

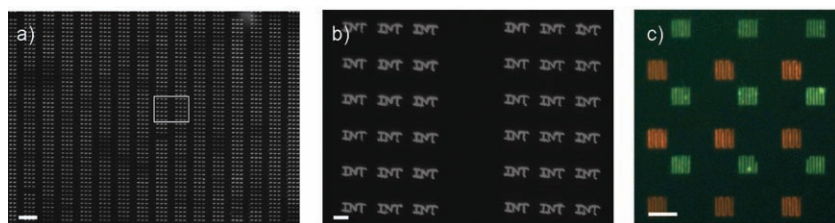


Figure 12. a) Fluorescence image of fluorophore-labeled DOPC pattern fabricated with a 2D cantilever array. b) Zoom-in of the area outlined by the white rectangle shown in panel a. c) Fluorescence image of DOPC labeled with two different fluorophores patterns serially generated by using two different tips. The scale bars shown in a, b, and c are 100, 10, and 5 μm , respectively. Reproduced with permission.^[78]

with Ni(II)-chelating or biotinylated headgroups onto glass substrates for subsequent immobilization of His-GFP and streptavidin, respectively, which is described in Section 3.2.^[66] Recently, they demonstrated that it was possible to transfer water-insoluble DOPC to hydrophilic glass coverslips under water.^[22] This approach enabled the authors to observe the spread of DOPC bilayers in situ.

4.3. Other Surfaces

In addition to fabricating DOPC patterns on silicon and glass substrates (described in Section 4.2), Lenhert et al. also reported patterning fluorophore-labeled DOPC onto substrates evaporated with metal films and hydrophobic polystyrene cut from petri dishes.^[78] More recently, the authors used the same approach to fabricate lipid-based photonic structures.^[79] Diffraction grating patterns of DOPC with controllable multilayer

heights and line-widths were generated on poly(methyl methacrylate) surfaces. Therefore, it is possible to measure in situ the feature sizes by observing the light diffracted from the DOPC patterns. These lipid-based photonic structures remained stable for a few days in an appropriate aqueous solution, and, therefore, the lipid multilayer grafting approach can be used as label-free biosensors to study specific lipid–protein interactions in solution.^[79] Bellido et al. also fabricated ferritin onto the surface of TEM grids. The hydrated iron(III) oxides

of ferritin can be detected by TEM; therefore, this approach can be utilized to quantify the amount of protein deposited by DPN.^[73b] Nam et al. used a Si_3N_4 AFM tip with a thin nickel layer coating to write His-tagged ubiquitin or thioredoxin onto nickel oxide surfaces with a feature size down to ~ 85 nm.^[35]

Most DPN experiments have relied on writing ink onto hard solid supports. It is particularly challenging to deliver molecules in a controlled manner onto soft matter, and therefore only a few examples have been reported. Li et al. wrote Cy3-antibody and Cy3-streptavidin onto a stretched DNA, which was predeposited onto a APTES-functionalized mica surface. The smallest feature size achieved was ~ 40 nm.^[80] Sistiabudi et al. not only deposited polyarginine and poly(glutamic acid) polypeptide onto Si-based substrates, but also onto the inner collagenous layer of Brunch's membrane (ICLBM). More recently, they presented the deposition of biotin-collagen-binding peptide (CBP) molecules onto the ICLBM surface and verified their bioactivity through the binding of Cy3-labeled streptavidin.^[81]

Table 1. Overview of reported chemical strategies utilized for indirect DPN for patterning biomolecules on solid surfaces.

Indirect DPN: noncovalent attachments		
Mechanisms	Examples	Surfaces ^{a)}
Electrostatic interaction	Cytochrome c, ^[42] lysozyme, ^[43] antibodies (e.g., IgG ^[43,45] and anti-p24 ^[44]), retronectin, ^[43] and CPMV nanoparticles ^[50] on carboxylic-acid-terminated surface	Au
Electrostatic interaction	Membrane protein complexes ^[46] on protonated amino-terminated surface	Au
Electrostatic interaction	Negatively charged DNA ^[60,61] on PAH	Si-based
Biotin–streptavidin interaction	Biotin-BSA ^[53] on streptavidin-terminated surface	Au
Biotin–streptavidin interaction	Streptavidin ^[53,55,66] and biotin-terminated surface	Au
Biotin–streptavidin interaction	Neutravidin ^[59] and streptavidin ^[18b,62,66] on biotin-terminated surface	Si-based
Coordination chemistry	TMV ^[57] on Zn(II)-carboxylic-acid-terminated surface	Au
Coordination chemistry	His-tagged ifnar2 ^[55] on Ni-BTisNTA-terminated surface	Au
Coordination chemistry	His-visible fluorescent proteins ^[63,64,66] on Ni-NTA-terminated surface	Si-based
ELP fusion	ELP fusion proteins ^[54] on elastinlike polypeptides	Au
Other (not specified)	Antibodies ^[58] (e.g., IgY, IgM) on Zn(II)-carboxylic-acid-terminated surface	Au
Indirect DPN: covalent attachments		
Mechanisms	Examples	Surfaces ^{a)}
Thioether bond formation	Cys-CPMV nanoparticles ^[50,51] on maleimide-terminated surfaces	Au
Thioether bond formation	Biotin-maleimide ^[18b,62] on thiol-terminated monolayer	Si-based
Amide bond formation	Amine-containing DNA, ^[47] (PLL) ^[48,49] on carboxylic-acid-terminated monolayer	Au
Amide bond formation	Protein A/G ^[52] and amine-containing biotin, ^[53] peptide (ELPs) ^[54] on NHS-ester-terminated monolayer	Au
Amide bond formation	NHS-functionalized biotin on PEI-sputtered surfaces ^[59]	Si-based
Oxime formation	Oxyamine-terminated linear peptide ^[56] on quinone-terminated surface	Au
Ring-opening polymerization (ROP)	Trp-NCA ^[67] on G4-NH ₂ PAMAM dendrimer-deposited surface	Si-based

^{a)} Si-based surfaces include silicon, silicon oxide, and glass.

Table 2. Overview of reported chemical strategies utilized for direct DPN for patterning biomolecules on solid surfaces.

Direct DPN: noncovalent attachments		
Mechanism	Examples	Surfaces ^{a)}
Electrostatic interaction	IgG ^[33] on negatively charged SiO ₂ surface	Si-based
Electrostatic interaction	Collagen-binding peptide ^[39] on clean SiO _x surface	Si-based
Electrostatic interaction	IgG ^[37] and His-VFPs ^[38] on amino-terminated surface	Si-based
Electrostatic interaction	Laminin ^[39] on clean SiO _x surface coated with two layers of polyelectrolytes	Other
Crown ether to protonated amine interaction	Angiogenin, ^[32] anti-angiogenin, ^[72] and integrin $\alpha_v\beta_3$ ^[32] on Prolinker-coated surface	Au
Coordination chemistry	His-VFPs ^[38,63,66] on Ni-NTA-terminated surface	Si-based
Coordination chemistry	His-ubiquitin ^[35] and thioredoxin ^[35] on nickel oxide surface	Other
Others (not specified)	3RGBP1 peptides ^[71] on Au surface	Au
Others (not specified)	Biotin-QBP1, ^[71] biotin-QBP3 ^[71] peptides, and PA nanofibers ^[34] on Si surface	Si-based
Others (not specified)	Phospholipids ^[22,66,78] on Si and glass substrates with or without the treatment of oxygen plasma	Si-based
Others (not specified)	Horse spleen ferritin ^[73] on Au, silicon oxide, aluminum, and niobium surfaces and TEM grid	Au/Si-based/others
Others (not specified)	Phospholipids ^[78,79] on metal film, polystyrene, and poly(methyl methacrylate)	Others
Others (not specified)	Antibody ^[80] and streptavidin ^[80] on DNA	Biosurface
Others (not specified)	Biotin-collagen-binding peptides onto inner collagenous layer of Brunch's membrane (ICLBM) ^[81]	Biosurface
Direct DPN: covalent attachments		
Mechanisms	Examples	Surfaces ^{a)}
Thioether bond formation	TAT peptide with cysteine residues ^[76] on maleimide-terminated surface	Si-based
Au–thiol interaction	Thiol-containing collagen, ^[68] DNA, ^[18a] IgG, ^[31] lysozyme, ^[31] TAT peptide, ^[70] and synthetic peptides (MH2) ^[69] on Au surface	Au
Michael addition	Acrylamide-containing DNA ^[18a] and oligonucleotide ^[75] on thiol-terminated surface	Si-based
Amide bond formation	Amine-containing DNA ^[40] and IgG ^[40] on NHS ester- terminated surface	Si-based
Imine bond formation	Amine-containing IgG ^[33] on aldehyde-terminated monolayer	Si-based
Epoxy-to-amine reaction	Amine-containing HCG ^[74] onto epoxy-terminated surface	Si-based

^{a)}Si-based surfaces include silicon, silicon oxide, and glass.

5. Summary and Outlook

Different chemical approaches, sorted by noncovalent and covalent reactions that have been utilized in indirect and direct DPN strategies for patterning biomolecules, are summarized in **Table 1** and **2**, respectively; the different types of surfaces used are indicated. Both indirect and direct DPN strategies appear to have found equal favor during the years, reflecting the unique and complementary advantages of each approach. Indirect DPN enables the patterning of a single layer of biomolecules while avoiding denaturation of fragile biomolecules caused by direct writing. Direct DPN is, on the other hand, a more straightforward way to generate biomolecular patterns without nonspecific binding.^[1b]

When comparing the types of substrate used in indirect and direct DPN strategies, Table 1 and 2 reveal that gold substrates are used more frequently than silicon-based substrates in indirect DPN, but that the opposite is true for direct DPN. This observation can be attributed to the well-established study of gold–thiol interactions. In indirect DPN, suitable biomolecule-adherent molecules have to be placed on a surface first, and thiol molecules with different terminal

functional groups have been extensively used with DPN and are good candidates for this application. However, in direct DPN, the key parameter is the direct interaction between the proteins and the underlying substrates, which enables the use of multiple substrates types. The flexibility of using different surfaces and chemical immobilization strategies allows the exploration of various experimental conditions.

In conclusion, DPN is a relatively inexpensive and high-resolution AFM-based lithography technique. Although the patterning efficiency of DPN was low during the early stages of development of the technique, the successful design and fabrication of active and passive probe arrays during the past few years has dramatically improved the patterning efficiency. These probe arrays allow users to deposit different ink molecules, from small thiol molecules to large proteins, simultaneously and in a massively parallel manner at sub-micrometer scale. Different chemical strategies have been used to fabricate biomolecular nanostructures on solid supports with either direct or indirect DPN. Both approaches are equally popular and have their own advantages and disadvantages. To overcome the limitations of direct DPN, different chemical modifications of AFM tip surfaces have also been explored.

The combination of nanofabrication and biomolecules enables the study of fundamental interactions between biomolecules and cells and nanostructured surfaces, and the development of high-density bio-nanoarrays with potential for several applications including fast screening of routine diagnostic tests. Recently, orthogonal surface modification has been demonstrated using microcontact printing (μ CP) to immobilize specific molecules at micrometer-length scale,^[82] and this is of interest for DPN applications as well. Compared with other lithography techniques, DPN enables the preparation of arbitrary patterns on surfaces at sub-micrometer length scales with a relatively fast speed. In addition, by changing the coating of the tips, different ink molecules can be deposited on the same surface; therefore, the fabrication of smaller-scale surface modifications with arbitrary patterns and functional groups will be a powerful application of DPN in the future.

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