Chapter 14
Bio-nanostructured Interfaces Fabricated by Scanning Probe Nanolithography (SPN)

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14.1 Introduction

Biomolecular patterning at the nanoscale is currently a very active field that combines the know-how of two different fields: biology and nanotechnology. The interest in the preparation of nanoscale features at surfaces relies on their wide range of applications in different areas, including physical science and life science. In particular, the immobilization of biomolecules having nanometer resolution has been pursued for their interest in fundamental studies in molecular and cell biology or to prepare platforms with different biomedical applications such as molecular diagnostics just to mention a few of them.

In particular, as depicted by Christman et al. [1, 2], in the case of proteins, the ability to spatially orient and anchor them at the nanoscale affords useful materials in such applications as biosensors, biomaterials, and tissue engineering. Several major advantages are derived from this control of the protein distribution at the surface at the nanometer scale. For instance, protein nanoarrays offer the possibility to achieve greater sensitivity in diagnostic tests since the disease progression is often connected with single proteins [3]. In addition, further miniaturization from protein microarrays to nanoarrays may allow for the discovery of currently undetectable disease markers [3]. Moreover, provided the possibility to have multifunctional surfaces with a large variety of different proteins, the detection of thousands of biomarkers could be performed on one single chip [4–7].

Scanning probe nanolithography (SPN) has its origin in 1981 and since then this technique has been used to gain knowledge of surface structures and molecular organization in different fields, including physics, chemistry, and biology, and has been later expanded to other areas of science and technology [8]. In addition, SPN is today a largely extended technique for fabrication of nanoscale structures,

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especially for directly inducing selective modifications on a surface with a precise localization of the defined pattern [9].

SPN takes advantage of a sharp tip to either build or destruct the interface to produce different surface patterns. The tip is connected to one of the following equipments: scanning tunneling microscope (STM), atomic force microscope (AFM), scanning electrochemical microscope (SECM), or scanning near-field optical microscope (SNOM) [10]. These techniques offer remarkable versatility and permit both a precise spatial fabrication and simultaneously the possibility to image the patterned surfaces at the nanometer or molecular scale. Moreover, in addition to the ultrahigh resolution they do not require the use of particular masks or templates. Finally, their relatively low cost and capability to operate in ambient conditions is worth mentioning.

In this chapter, we will review the main strategies employed to produce nanometer-scale surface patterns by using scanning probe techniques focusing on the fabrication of nanostructured interfaces with biomolecules capable of acting in recognition processes.

14.2 Methodologies for Generating Nanoscale Features

A significant effort has been carried out to develop different methodologies in order to generate nanoscale features. These include bottom-up self-assembly approaches [11–18], redox control [19], conductive AFM [20], scanning-near field photolithography [21], and stamping techniques, such as nanoimprint lithography [22, 23], just to mention a few of them. Other alternatives resort to the combination of several patterning methodologies. For instance, Falconnet et al. produced protein patterns combining nanoimprint lithography and molecular self-assembly [24]. Within this context, in this chapter, we will highlight and analyze those methodologies that involve the use of AFM tips to create biomolecular nanopatterns.

In comparison with other methods, to pattern biomolecules at the nanometer scale, scanning probe approaches can be carried out under ambient conditions to permit the formation of high-resolution patterns, and the pattern to be fabricated can be easily modified and adapted for a particular demand. Moreover, in the case of dip-pen nanolithography (DPN), there exist a variety of “inks” that can be employed. Therefore, the surface chemistry can be easily modified. The major challenges remaining for these approaches are related to the small area that can be patterned and that the process can be rather slow. The latter can be, at least to some extent, overcome by using arrays of multiple tips that reduce the patterning time. In order to summarize the capabilities of SPN techniques that include DPN, nanografting, and nanoshaving, Table 14.1 contains the main advantages and limitations of directly related approaches: electron beam lithography, nanocontact printing, or nanoimprint lithography.

Table 14.1 Nanoscale-patterning methods using biomolecules. (Reproduced with permission-from reference [25])

Technique	Advantages	Limitations
Electron beam lithography(EBL)	Maskless, stampless High resolution Arbitrary pattering with different shapes and sizes	Slow (serial process) Complicated, expensive (requiring equipment, clean room and vacuum condition) Small area patterning
Nanocontact printing(NCP)	Simple (direct patterning) Parallel, cheap Fast process Large area patterning	Preparing nanoscale stamp with high feature density Mechanical stability of stamp Diffusion of SAM inks
Nanoimprint lithography (NIL)	Large area patterning with a high throughput and low cost Parallel	Stress and wear of mold Use of polymer Slow (molding, demolding, and etching process)
Nanografting/nanoshaving	High resolution, ambient. Quick change of fabricated patterns	Small area patterning
Dip-pen nanolithography(DPN)	High resolution, ambient Variety of inks usable Parallelization possible	Slow (serial process) Small area patterning

14.3 Tip-based Nanofabrication Approaches

Tip-based nanofabrication refers to those techniques that employ a functionalized cantilever-tip or an array of cantilever-tips to perform the surface patterning. Initially, these techniques resorted to what has been named "destructive" SPL, i.e., the materials at the surface are either removed or modified by applying external mechanical, thermal, electrical, or optical energy through the scanning probe [26]. Typical examples include plowing through the first of two resist layers on an arbitrary substrate and following with a development step [27–29], nanoshaving and nanografting [30, 31], thermomechanical indentation [32], thermochemical nanolithography (TCNL) [33–35], electrostatic nanolithography [36, 37], electrochemical oxidation [38–40], and scanning near-field optical lithography (SNOM) [41–43]. More recently, other alternatives considered the possibility to place materials onto a surface, "constructive" SPL for instance by using the probe wetted with a particular ink as a "pen." This methodology was first described by Mirkin et al. [44, 45] and today other variations have been developed further by combining the DPN with additional stimuli such as forces, [46], heat [47], or electric fields [48].

These techniques have been employed for different materials; however, the preparation of polymeric nanostructures has received particular attention. Thus, SPL has extended their use for multiple polymer-based applications including the preparation of polymer resists for nanofabrication, the preparation of polymeric carriers for functional materials, nanopatterning of electronically active polymers, or the fabrication of polymer brush nanostructures [10].

14.3.1 Dynamic Plowing Lithography

Dynamic plowing lithography (DPL) consists of surface modification by indenting the material with a vibrating tip using an AFM working in the tapping mode [27–29]. The tapping mode is required since a permanent contact with the substrate (contact mode of the SFM) restricts the tip movement to directions close to the cantilever axis due to frictional forces. Equally, other directions can lead to a cantilever torsion and thus produce irregularities in the final pattern. By using these conditions, this method provides a lithography technique that is nearly free from problems due to cantilever torsion and permits us to image the modified surface without any further modification.

DPL has been employed to pattern semiconductor crystals and oxide layers. However, the tip rapidly degraded since the hardness of both the material and the tip are in a similar range. Nevertheless, this approach appeared to be interesting when the pattern transfer and the lithography process are separated. More precisely, we can plow first a thin polymer layer (Fig. 14.1 left) and then transfer the pattern by a wet chemical etchant, which does not significantly affect the polymer thin film.

14.3.2 Nanoshaving/Nanografting

Nanoshaving works in a similar fashion as plowing lithography. However, in this case a resist material is mechanically removed by an AFM tip for creating nanometer-scale patterns on surfaces [30]. In this technique, the immediate removal of the displaced compound requires additionally the suppression of readsorption [31]. On the other hand, nanografting operates on a matrix monolayer immersed in a solution containing the desired molecules different from the matrix. In this situation, as the AFM tip plows through the matrix monolayer, the matrix molecules are removed and replaced by these reactive molecules in solution [30]. As an example Liu et al. [31] succeeded to pattern self-assembled monolayers (SAMs) using mixtures of thiols with various chain lengths. As a consequence, nanografting can create both positive and negative patterns, depending upon the relative chain length between the new and matrix adsorbates [49, 50] (Fig. 14.2).

14.3.3 Thermomechanical or Thermochemically Induced Nanopatterns [33, 34]

Thermomechanical writing was first reported by Mamin and Rugar [32, 51]. They employed an infrared laser focused on an AFM tip, which is in contact with a transparent polymethyl methacrylate (PMMA) substrate. The laser beam heated the AFM tip and the later softens the PMMA in the contact region. The heated tip causes very local heating of the polymer material above its softening temperature,

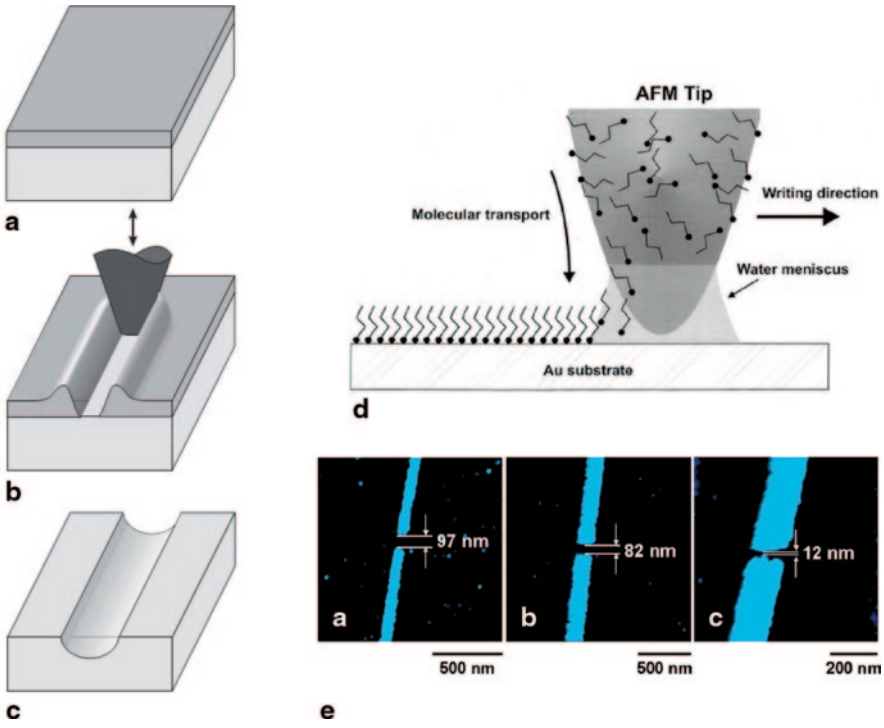


Fig. 14.1 *Left*: Example of "destructive" tip-nanofabrication. Scheme of a nanoscale line produced with a SFM: **a)** Substrate with a polymer coating of a few nanometers thickness. **b)** Plowing of a furrow into the polymer layer with the vibrating tip of the SFM along a desired line. **c)** Transfer of the line by etching the substrate in the furrow and removing the masking layer (shown here). Reproduced with permission from ref. [29]. *Right*: example of "constructive" tip-nanofabrication. **d)** A schematic diagram of the DPN process where the ink molecules transfer from an AFM probe to the substrate surface [44]. **e)** TMAFM topographic images of etched MHA/Au/Ti/SiOx/Si nanogaps [45].

which effectively acts as the switch to turn on the writing process [11]. As a consequence, applying a controlled pressured allowed to create a pit. The pits range in size from several hundred angstroms to 1 m, depending on the size of the laser pulse and the loading force on the tip. As a consequence, this technique has been proposed, among others for ultrahigh storage density permitting store, read back, and erase data in very thin polymer films [52]. More recently, thermomechanical writing has been improved and used for other purposes [53–56].

TCNL employs a resistively heated AFM cantilever to induce chemical reactions in a nanometer-scale position, thus, inducing changes on the surface functionality of thin polymer films or SAMs [35]. The basis for achieving a high degree of spatial resolution is the sharp thermal gradient in the vicinity of the warmed tip and the kinetics of the chemical reaction that are known to increase exponentially with

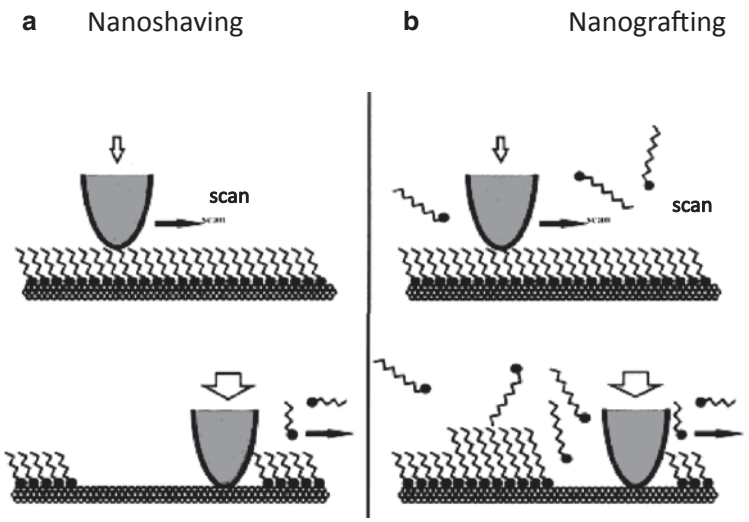
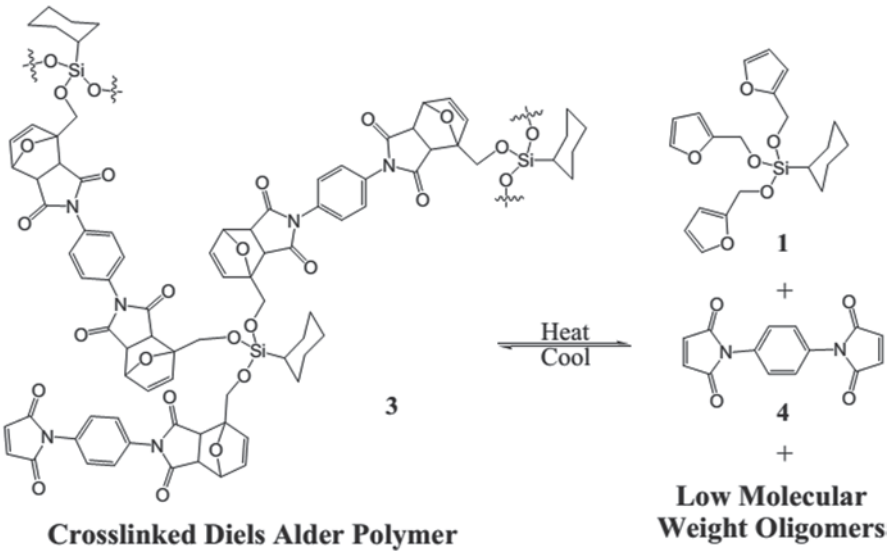


Fig. 14.2 Schematic diagrams to produce nanopatterns either by nanoshaving or by nanografting. (Reproduced with permission from reference [31])

temperature. An illustrative example of this approach has been reported by Gotsmann et al. [34] who employed a reversible cross-linking that allows the thin film to exist in two different chemical states: a low-temperature state of high-molecular-weight material and a high-temperature state of low-molecular-weight molecules (see Scheme 14.1).



Scheme 14.1 Reversible Diels–Alder (DA) reaction for depolymerization of cross-linked thin films on heating and re-cross-linking of the low-molecular-weight products on cooling. (Reproduced with permission from ref. [34])

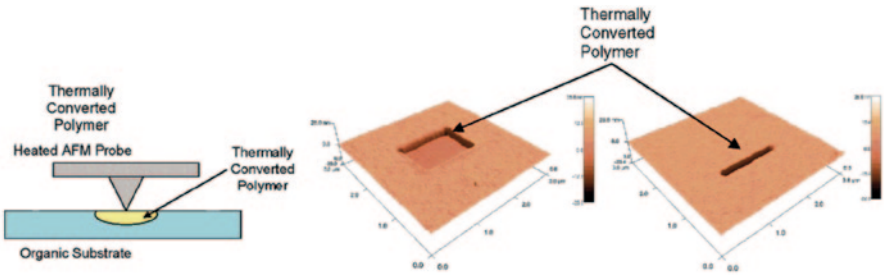


Fig. 14.3 Local thermal writing into the organic substrate using a heated AFM cantilever tip. *Left*: Thermal writing process. *Middle* and *right*: Thermally induced writing using a conjugated polymer made by click chemistry at the micrometer and submicrometer scale. The recessed regions are the locations where the heated AFM tip has scanned over the polymer film and locally induced cycloaddition. Reproduced with permission from ref. [33]. *AFM* atomic force microscope

Finally, thermochemical nanopatterning can be associated with thermomechanical lithography. As a consequence, both surface structure and functionality can be simultaneously varied. For instance, Bakbak et al. [33] described an interesting approach in which the heating of a tip directed the synthesis of conjugated fluorescent polymers. In particular, they employed the thermal and Cu-catalyzed 1, 3-dipolar cycloadditions of terminal diynes with aromatic diazides. In addition, they reported the fabrication of nanostructured organic semiconductors by nanoscale thermal processing of annealed diazide/dialkyne thin films with a heated AFM cantilever tip (Fig. 14.3).

14.3.4 Electrostatic Nanolithography

As has been described among others by Heminghaus et al. [57] and Schaffer et al. [58], strong field gradients can produce forces that overcome the surface tension in thin liquid films, inducing an instability. In this situation, electrostatic and/or van der Waals pressure overcomes Laplace pressure; a film heated above the glass-transition temperature (Tg) becomes highly unstable with regard to small perturbations, thus leading to particular surface features [36]. More precisely, the dynamic instability of a dielectric liquid in a strong electric field (10^{7}–10^{8}Vm^{-1}) has been implemented for polymer melts, and a wide range of architectures with submicrometer features (~100 nm) have been created [58]. Within this context, AFM-assisted electrostatic lithography (AFMEN) generates nanometer-scale features (1–50 nm) by simply biasing (0–20 V) a highly conductive tip across a thin polymer film. The features of the nanopatterns created from polymer films depend on the type of polymer used, the applied bias, and speed of the AFM tip. Figure 14.3 shows the nanolines drawn on a PMAA thin film at different voltages varying from −4 to −11 V at an AFM tip speed of 0.5 ms^{-1}.The observed line widths are 62, 78, 113, 135, and 172 nm. Whereas at low negative potential, i.e., the first two lines drawn at −4 and −5 V, are all raised patterns, an increase in the bias applied induced the formation of grooved patterns (last two lines drawn at −9 and −11 V) (Fig. 14.4).

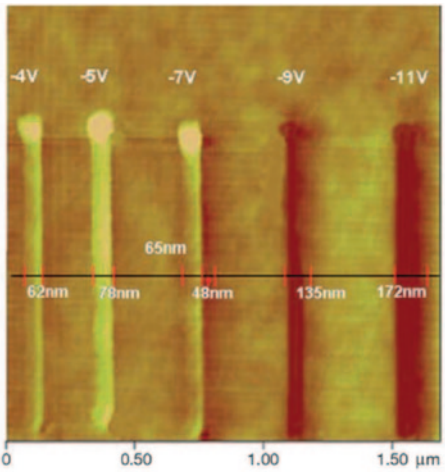


Fig. 14.4 AFM image of the nanolines drawn with a tip speed of 0.5 mms^{-1} at different applied voltages. (Reproduced with permission from ref. [37]). *AFM* atomic force microscope

14.3.5 Electrochemical Oxidation [38–40]

In a parallel fashion to what has been depicted below, Sagiv and co-workers were pioneer in the use of bias voltage pulses applied to a conductive SFM tip to chemically modify the surface by inducing the electrochemical conversion of surface-exposed terminal groups of an SAM. Thus, this strategy served to create chemical functionalities introduced directly onto a surface with precise distribution. The authors reported different chemical patterns using, for instance, nonadecyltrichlorosilane (NTS) monolayers with terminal vinyl groups on the surface or chemically inert *n*-octadecyltrichlorosilane (OTS) monolayers [59–61]. Particularly interesting is the latter where by applying a surface voltage the authors detected the presence of acid groups on the patterned monolayer and revealed that the monolayer was preserved during the electrooxidation process (Fig. 14.5). Moreover, these acid groups can be employed to carry out further functionalization steps [62]. A review covering further details of this technology has been already published [38].

14.3.6 Scanning Near-field Optical Lithography (SNOM)

The basic idea behind near-field optical methods is to avoid the normal diffraction limited far-field imaging by taking advantage of evanescent components of a point-like light source [41–43, 63]. Since evanescent waves decay exponentially with the distance to the surface and dominate in the near field zone, SNOM requires operation in a region where probe-sample distance remains below the excitation wavelength. By using these conditions, the lateral resolution achieved with this method is

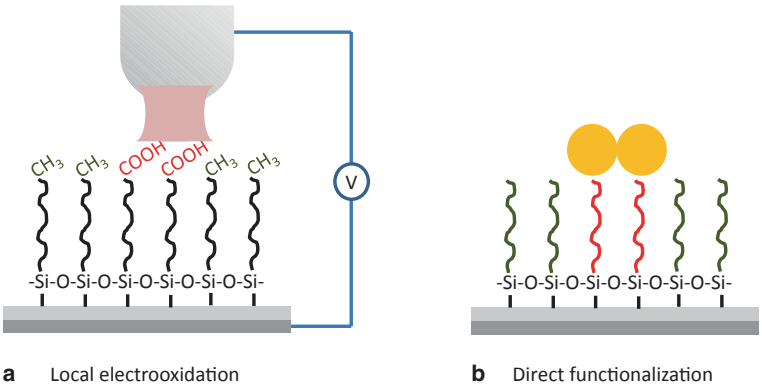


Fig. 14.5 **a** The local electrooxidation of *n*-octadecyltrichlorosilane monolayers induces the formation of carboxylic acid end groups, which can be used for **b** the direct attachment of functional materials (*yellow* circle; *red*: COOH-functionalized monolayer; *green*: nonfunctionalized monolayer). (Reproduced with permission from ref. [62])

largely below the diffraction limit for microscopic imaging. The shear-force method is suitable for conductive as well as for nonconductive samples, such as conventional photoresists.

14.3.7 Dip-pen Nanolithography (DPN)

DPN was introduced in 1999 by Mirkin and co-workers [44]. They reported that water can be either transported from an AFM tip to a substrate or from the substrate to the tip, depending on the properties of each [64]. Based on these findings, the authors developed strategies to deposit different compounds on solid surfaces. In DPN the AFM tip is coated with a liquid thin layer composed of solvent and what has been named "ink." The AFM tip delivers the molecular ink directly to a specific region of a target substrate by simple contact with the surface. Typically, the "inks" used in DPN form a monolayer on the substrate as a consequence of the chemical reaction between the ink and the surface [65] and thus the choice of a particular ink is determined by the substrate employed [9]. Inks can vary from organic reactive molecules, such as silanes, alkynes, thiols, and silazanes (e.g., 1-octadecane-thiol or ODT) to inorganics, such as oxides, metals, and magnetic compounds (e.g., gold nanoparticles) to biomolecules such as peptides, DNA, and proteins (e.g., thioredoxin) [66]. In comparison with previous methodologies DPN can be employed under ambient conditions. However, as depicted by Ginger et al. the optimal conditions for this technique require the use of a chamber with controlled humidity [65].

Table 14.2 Techniques developed to immobilize biomolecules at the micro-/nanoscale

Deposition method	Best resolution	Ink	Substrate	Reference
Microcontact printing	500 nm 19 m	IgG protein Lipid bilayers		[76] [77]
Optical lithography	8 m 500 nm	Alkanethiols Streptavidin		[78] [2]
Scanning probe lithography	30 nm lines 45 nm dots	Collagen IgG protein	Gold	[79] [80]
Surface-patterning tool	2–3 m dots 150 nm lines	Cy3-streptavidin Quantum dots conjugated to streptavidin		[81]
Microspotters	30 m dots	IgG protein and oligonucleotides	Glass	[82, 83]
Nanopipettes	440 nm dots 510 nm dots	IgG protein Biotylinated DNA	Glass	[84]
Nanofountain probe	40 nm lines 200–300 nm dots 200–300 nnm dots	MHA DNA IgG protein	Gold	[85, 86]

14.4 Immobilization of Biomolecules onto Surfaces Through Nanolithography

A large number of biomolecules such as DNA, RNA, virus, or proteins have been immobilized onto surfaces with nanometer precision by using different SPN approaches. The targeted applications pursued for the immobilized surfaces varied from proteomics and genomics, clinical-based diagnostics to studies devoted to protein profiling or the screening of drug candidates [66–69]. Other biological and biomedical applications include the elaboration of nanobiochips and nanobiosensors, tissue engineering, and molecular and cell biology [1, 70–75].

As has been reported by Mendes et al. [26], the immobilization of biomolecules requires accomplishing several additional issues. Immobilized biomolecules require to be placed in a precise position (shape and distance between them has to be controlled) with nanometer-scale resolution. Equally, the immobilized biomolecules should not be degraded or damaged during the immobilization process so that the native biological properties remain identical. (Table 14.2)

As depicted above, several approaches using the tip-based technology are excellent candidates to immobilize biomolecules at the micro-/nanometer scale. Herein, we will focus on the most extended methodologies to immobilize biomolecules for biorecognition purposes that include DPN, nanoshaving, and nanografting. As will be depicted in detail, DPN and nanografting can be achieved by using two alternatives either by directly writing/grafting the desired biomolecule or by pre-patterning the surface with an organic molecule showing high biomolecule affinity. Moreover, as depicted in Fig. 14.6, the patterning process can be followed by a second patterning step where, for instance, free surface can be completed with a second molecule.

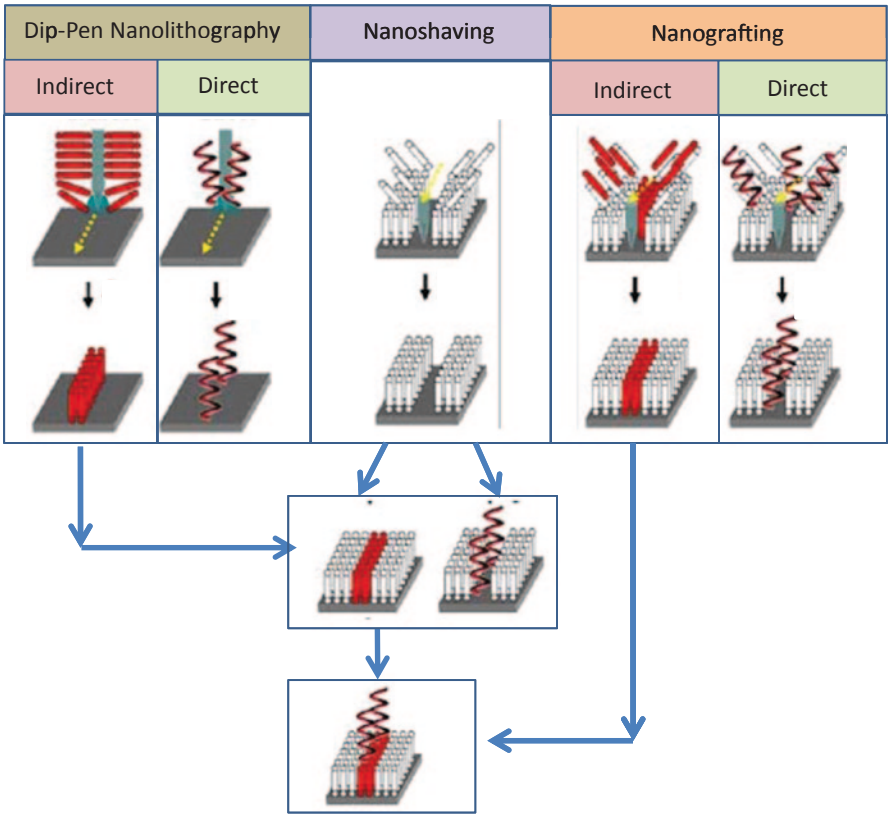


Fig. 14.6 Schematic representation of the different scanning probe lithographic techniques employed to indirectly or directly immobilize biomolecules on surfaces at nanometer-scale resolution. Whereas in the direct approach, the biomolecules are directly deposited onto the substrate, in the indirect approach, the nanopatterns created by the different lithographic techniques are used in a second stage (i.e., post-patterning process) as templates to immobilize the biomolecules onto surfaces. (Figure adapted from ref. [26])

14.4.1 Biomolecules Deposited by Dip-pen Nanolithography (DPN)

In DPN the tip is wetted with the biomolecule solution and is employed to deliver the material to a particular surface by bringing the tip in contact with the substrate. DPN utilizes the water meniscus formed between an AFM tip and a substrate to transfer ink molecules onto surfaces. As a consequence, patterns with resolutions ranging from few microns down to ~10 nm can be fabricated. The high resolution obtained offers a large increase in the areal density of the deposited motifs. According to Zhang et al. [65] (depicted in Fig. 14.7), the increase in density could reach 10,000- to 100,000-fold the values obtained with other methodologies such as

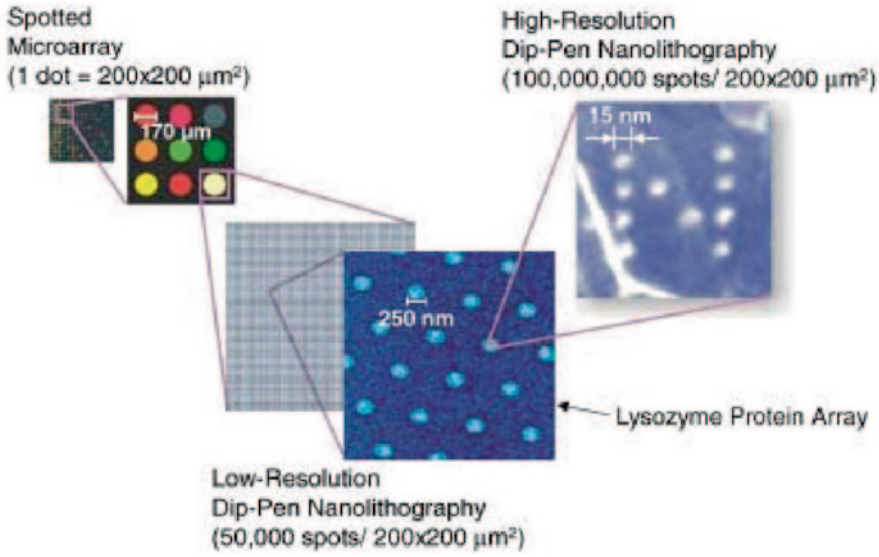


Fig. 14.7 Schematic illustration of the power of DPN resolution in the context of biomolecular nanoarray fabrication. (Reproduced with permission from ref. [65])

robotic spotting or photolithographic technology. Two main factors will influence the resolution of the final pattern obtained. On the one hand the size of the meniscus affects the size of the pattern created. Xie et al. [64] estimated that smaller meniscus allows higher resolution. However, when the meniscus is too small, it becomes unstable and fluctuates in shape, with fluctuations that are larger in magnitude than its average width. The meniscus size is not only dependent on the tip sharpness but also on other parameters including the humidity, wettability of the tip, and distance to the surface. On the other hand, the diffusion constants of the molecules being deposited also play a key role in pattern formation [44, 87]. For detailed information on the DPN methodology the reader is referred to several reviews that have covered the most recent advances [1, 26, 65, 88].

As depicted in Fig. 14.6, DPN can be carried out using two different methodologies. The direct method involves the deposition of the biomolecule directly onto the surface. In the indirect method, the immobilization of biomolecules is accomplished in two separate steps. In the first step biomolecule-adherent organic molecules are deposited from the solution. The second step concerns the adsorption of the biomolecule from the solution onto those areas where the organic molecule has been deposited (Fig. 14.8).

DPN has been employed for a variety of applications ranging from the construction of building nanostructured materials with DPN to the elaboration of DPN-patterned etch resists [65, 66]. Nevertheless, a major field of application of this technique concerns the controlled biomolecule deposition onto surfaces. In particular, DPN has been employed for the fabrication of micro- and nanoarrays of patterned biomolecules such as DNA or proteins in order to control the biorecognition pro-

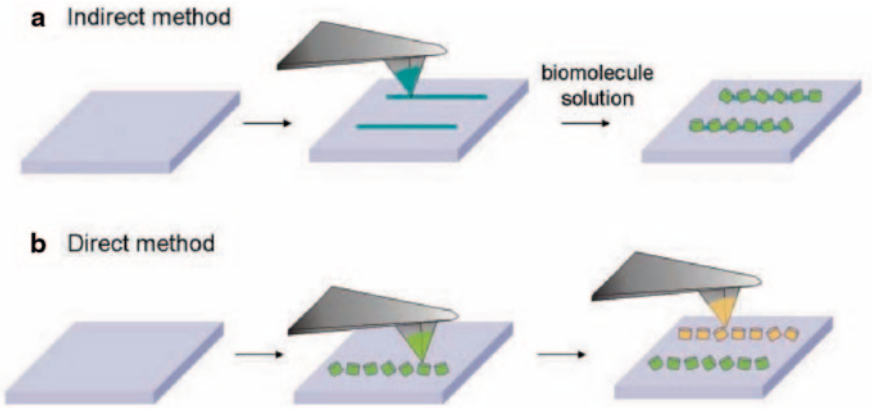


Fig. 14.8 Schematic representation of **a** indirect and **b** direct dip-pen nanolithography (DPN) method for patterning biomolecules [88]

cesses from the molecular to the cellular level. Equally, several studies have been concerned with the surface immobilization of virus and cells [89].

14.4.1.1 Direct DPN Nanolithography

Direct DPN has been employed to construct among others protein and DNA nanostructures which have exhibited a potential for different purposes such as screening of drug candidates and studying of protein expression profiling. DPN is a simple and straightforward way for creating protein and DNA patterns since features can be directly written onto the surface. Significantly, this method avoids the complications associated with nonspecific binding.

However, in general, the use of DPN to direct-write biomolecules requires the modification of the surface of commercially available AFM tips obtained upon several steps. For instance, as depicted in Fig. 14.9, this functionalization has been achieved by immersing the gold-coated cantilever in a solution of a symmetric 11-mercapto-undecylpenta(ethylene glycol)disulfide (PEG) [80] in order to prevent protein adhesion on the backside of the tip. After this step, the tip is coated with gold by means of thermal evaporation methods. The cantilevers with the gold-coated tips were immersed in thiotic acid. As a result, the hydrophilic tips with the carboxylic acid-terminated SAMs favor the protein adsorption on the tip surface. Upon functionalization of the tip and protein adsorption, the biomolecule can be directly deposited onto the solid surface. By using this methodology, Lee et al. [68] succeeded in the preparation of nanostructures of lysozyme (Lyz) and rabbit immunoglobulin-gamma (IgG) nanodot arrays constructed in a direct-write fashion. In further studies, the same group employed DPN for the construction of an angiogenin protein nanoarray. In particular, they studied the immobilization of angiogenin and their recognition activity with integrin $\alpha_v\beta_3$ used as model protein.

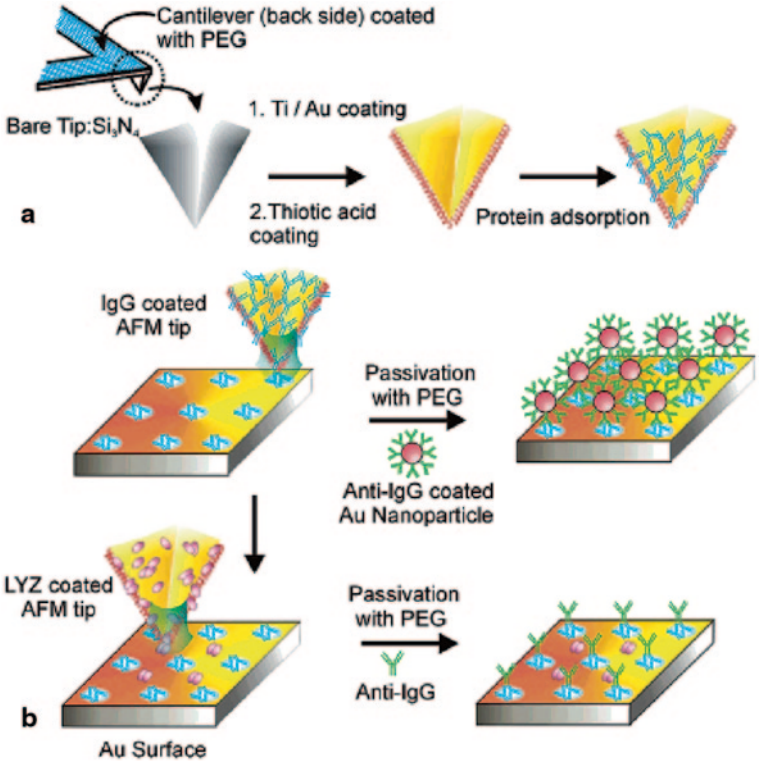


Fig. 14.9 Strategies for: **a** Tip Modification using a Ti/Au coating followed by a thiolic acid and the protein adsorption. **b** Scheme for the protein patterning using two different alternatives. On the first approach, upon coating with the IgG the surface is passivated and the biorecognition permits the immobilization of the anti-IgG-coated nanoparticles. The second strategy refers to a multistep procedure to pattern different proteins, i.e., IgG and lysozyme (Lyz) [80]

Other examples in which proteins were directly deposited on surfaces include thiolated collagen and collagen-like peptides onto gold surfaces [79]. The authors were able to print collagen and a collagen-like peptide down to 30–50 nm line widths. Moreover, the direct deposition methodology preserved the triple-helical structure and biological activity of collagen. In addition, this approach permits the formation of characteristic higher levels of structural organization. The sensitivity of these types of biological molecules to thermally induced structural changes (e.g., denaturation) makes the DPN lithographic method ideal in comparison to harsher techniques such as ion-beam-based lithography. According to the authors, the specific nanopatterned arrays of collagen might be used to induce an assembly network of collagen scaffoldings to mediate cell attachment processes, organized as optical gratings because of their ability to form liquid crystalline phases, and as guest–host systems for other biological or nonbiological components. The “direct-write” capability of biologically relevant molecules, while preserving their structure and

functionality, provides tremendous flexibility in future biological device applications and in proteomics arrays, as well as a new strategy to study the important hierarchical assembly processes of biological systems.

More recently, De Yoreo's group has shown that it is possible to generate nanoscale patterns of human chorionic gonadotropin (HCG) antibody [90]. In their approach, the protein was tagged with a specific fluorophore, so that the pattern can be easily identified and verified using optical imaging. More precisely, they fabricated patterns of HCG antibody tagged with tetramethylrhodamine (TMR) dye on the glass surface. The glass surface was pretreated with 3-glycidoxypropyltrimethoxysilane to introduce the epoxy groups that facilitate protein adhesion to the surface.

Direct dip-pen nanolithography was employed also by Demers et al. [65, 91] to pattern modified oligonucleotides on metals (gold) and insulators (silicon oxide). The first step requires the surface modification of a silicon nitride AFM cantilever with 3'-aminopropyltrimethoxysilane that promotes reliable adhesion of the DNA ink to the tip surface thus improving control over DNA patterning. In particular, the authors employed hexanethiol-modified oligonucleotides to directly pattern gold substrates with features ranging from 50 nm to several micrometers in size. The thiol group assures the chemisorption of the DNA into the underlying Au surface. Further hybridization experiments by using the anchored DNA require the passivation of the surface towards complementary DNA. For that purpose, the patterned surfaces with oligonucleotides were immersed in an ethanol solution of 1-octadecanethiol that coats the unpatterned gold surface with a hydrophobic monolayer. Finally, by using oligonucleotide-modified gold nanoparticles, the authors demonstrated that the immobilized DNA retained its highly specific recognition properties.

Due to the nanometric precision of DPN, this approach is an interesting alternative to fabricate not only patterns with nanometer-size resolution (as depicted above) but also go a step further in order to create biological nanoarrays. DPN is one technique that has shown particular promise in this area, allowing one to prepare standardized multicomponent arrays of biomolecules that can retain their biorecognition properties once transferred to a surface [44, 65, 80, 89, 92–96]. These arrays have been employed, among others, for the detection of proteins, DNA, and other small molecules and exhibit several advantages over typical microarrays. First, as mentioned above, nanoarrays created by DPN are 10,000–100,000 times denser that microarrays. Therefore, smaller areas are required to identify the same number of targets and smaller sample is required [66]. Moreover, a larger number of targets can be screened in a shorter period of time and due to the minimum area of the nanoarray a target can be detected at very low concentrations. In addition, cDNA and oligonucleotide arrays permit to quantify both gene expression and genomic structure (e.g., through single-nucleotide-polymorphism (SNP) detection). This unique feature has induced their extensive use among others in oncology [97], for the elucidation of the roles of genes in the pathogenesis of infectious diseases [98], in neurology for the evaluation of a large amount of genes [99], and the study of genomic structures and gene expression [98, 100].

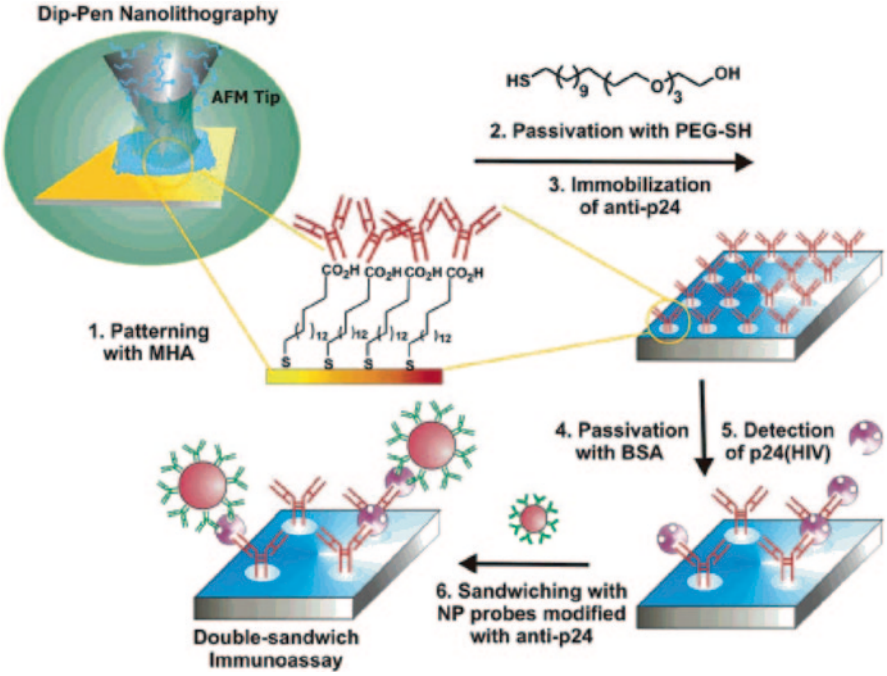


Fig. 14.10 Schematic representation of the immunoassay format used to detect HIV-1 p24 antigen with anti-p24 antibody nanoarray. (Reproduced with permission,reference [101])

Nanoarrays have been applied, for instance, in the detection of human immunodeficiency virus type 1 in plasma [101]. Lee et al. used DPN to generate nanoscale patterns of antibodies against the HIV-1 p24 antigen on a gold surface. They succeeded in the preparation of feature sizes that were less than 100 nm, while preserving the activity of the antibody. As depicted in Fig. 14.10, HIV-1 p24 antigen in plasma was hybridized to the antibody array in situ, and the bound protein was hybridized to a gold antibody-functionalized nanoparticle probe for signal enhancement. According to the authors, measurable amounts of HIV-1 p24 antigen in plasma obtained from men with less than 50 copies of RNA per ml of plasma (corresponding to 0.025 pg/ml) were achieved, which is evidence that the nanoarray-based assay can exceed the limit of detection of conventional enzyme-linked immunosorbent assay (ELISA)-based immunoassays by more than 1000-fold.

14.4.1.2 Indirect DPN Nanolithography

As mentioned, indirect DPN requires first the immobilization of small organic molecules with high affinity for the biomolecule of interest. Thus, in a second step, the biomolecule can be immobilized onto the organic molecules through specific interactions. Examples of the use of this approach are the use of electrostatic interactions

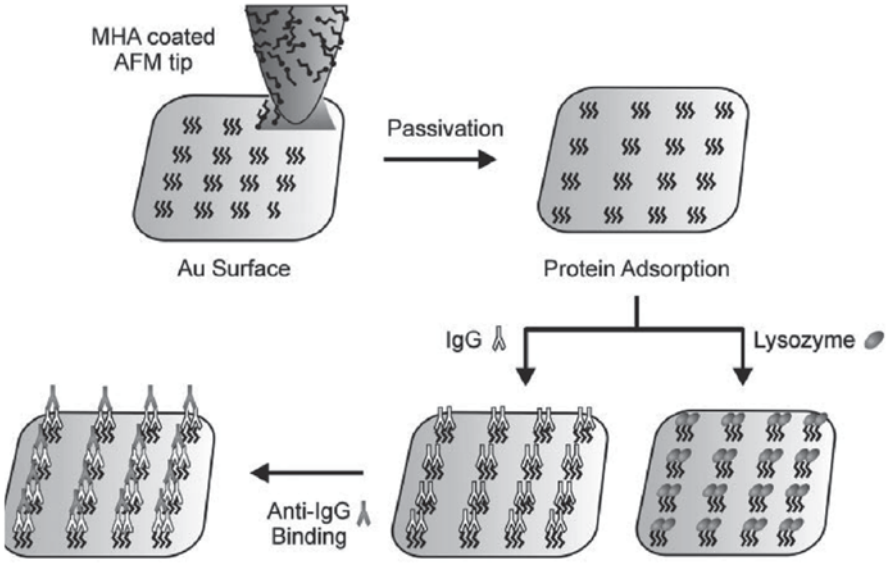


Fig. 14.11 Diagram of proof-of-concept experiments in which proteins were adsorbed on DPN-generated MHA patterns (1) MHA deposited from AFM tip onto surface, (2) passivation, (3) protein adsorption, (4) antibody recognition. (Reproduced with permission from ref. [105])

that have been successfully exploited to immobilize the negatively charged DNA [102, 103] and negatively charged membrane protein complexes onto protonated amino-terminated nanotemplates generated by DPN [104].

The indirect approach was first employed by Lee and coworkers [105] following the procedure illustrated in Fig. 14.11. In order to fabricate the protein array in a first step, the patterning 16-mercaptohexadecanoic acid (MHA) was carried out on a gold thin-film substrate in the form nanometer-size dots or grids. Then, the areas surrounding these features required to be passivated with 11-mercaptoundecyl-tri(ethylene glycol) by placing a droplet of the surfactant on the patterned area. Finally, the proteins were absorbed on the preformed MHA patterns by immersing the substrate in a solution containing the desired protein. The final pattern is a consequence of the high affinity for carboxylic acid-terminated monolayers at pH 7 and a relatively weak affinity for surfaces coated with 11-mercaptoundecyl-tri(ethylene glycol).

Moreover, this group evaluated the biorecognition capabilities of the nanoarrays by treating the surface with anti-IgG. Their findings show that regardless of the orientations of the IgG within the nanoscopic features, the proper orientation can be adopted under these conditions to react with the anti-IgG in a complex protein solution. Thus, the proteins maintained biological activity after adsorption.

In addition to protein arrays, other groups have been involved in the preparation of DNA nanoarrays by indirect DPN. Nyamjav and Ivanisevic [102, 103], based on the conventional molecular combing technique, succeeded in the preparation of long DNA molecules onto derivatized surfaces with three different shapes: dots,

squares, and lines [106, 107]. For that purpose, they fabricated surface templates composed of positively and negatively charged regions by pattering a polyelectrolyte "ink" (poly(allylamine hydrochloride)—PAH) on SiO_x. The affinity of the DNA for the positively charged areas allowed them to deposit the DNA strands on the areas modified with PAH.

The examples above describe employed electrostatic interactions to immobilize biomolecules onto the surface. However, molecular recognition-mediated coupling can be also applied for the same purpose. In this concern, Hyun et al. [93] also described an indirect approach for the fabrication of patterned protein nanostructures with feature sizes of the order of 200 nm based on molecular recognition processes. For that purpose, the authors immobilized first an SAM of 16-mercaptohexadecanoic acid (MHA) onto gold and the unpatterned regions were passivated with a protein-resistant oligoethylene glycol-terminated alkanethiol SAM. The carboxylic functional groups served to, in a following step, anchor covalently an amine-terminated biotin derivative with the chemically activated MHA SAM nanopattern. The authors described also the biorecognition capabilities of the surface by using streptavidin. The surface was incubated with streptavidin to form streptavidin nanostructures, mediated by molecular recognition between biotin and streptavidin. The resulting streptavidin nanopattern provides a universal platform for molecular recognition-mediated protein immobilization because of the ubiquity of biotin-tagged molecules.

14.4.2 Biomolecules Deposited by Nanoshaving

Nanoshaving, involves the precise removal by using an AFM tip of a pre-deposited monolayer. The removed areas can be used in a second step to anchor the desired biomolecule. Thus, in general, nanoshaving is combined with SAMs as nanometer thickness resists indirect immobilization of biomolecules onto surfaces [108].

Nanoshaving was used by Banerjee et al. [109] to direct the assembly of thiolated peptide nanotubes. In their strategy (depicted in Fig. 14.12), 1-octadecanethiol was used as a mask to prevent the nonspecific nanotube attachment and it was first self-assembled on the flat Au substrate. Then, the octadecanethiol SAM was shaved by an Si_3N_4 tip, thus, exposing Au regions. After the Au regions were drawn, the substrate was extensively washed. Then, the thiolated nanotubes were attached to the patterned Au pads on the substrate. After these substrates were washed thoroughly, the thiolated nanotube was observed to attach selectively to the Au areas via the thiol–Au interaction.

Following a similar approach in later studies, they extended the concept to the preparation of patterned surfaces based on biological molecular recognition. The authors introduced a new type of building block, antibody nanotubes, and demonstrated anchoring them to complementary antigen arrays via antibody–antigen recognition [110]. The array of antigens was written by nanoshaving the alkylthiol SAM-coated Au substrates using the tip of an AFM. Then, the antigens were immobilized onto the shaved regions of the alkylthiol SAMs with the AFM tip. Finally,

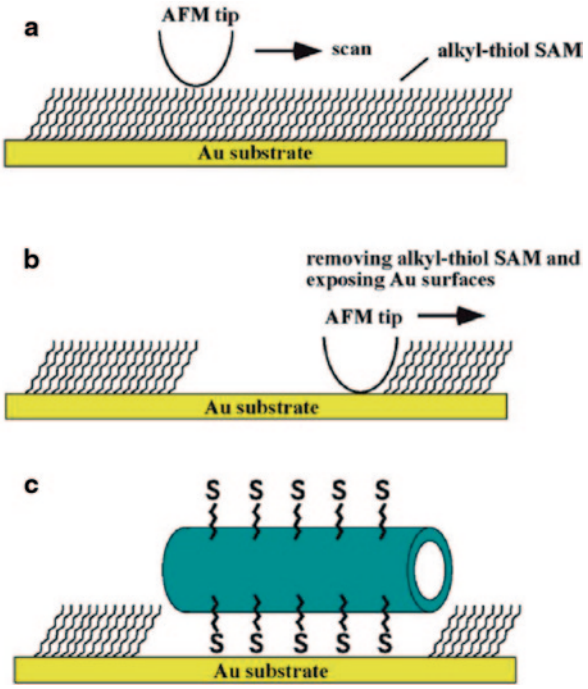


Fig. 14.12 Schematic diagram of thiolated peptide nanotube assembly on the Au trench arrays. **a** Self-assembly of alkylthiols on Au substrates. **b** Shaving trenches on the alkylthiol SAM by using the AFM tip. **c** Location-specific immobilization of the thiolated peptide nanotube onto the patterned Au trenches. (Reproduced with permission from ref. [109])

the antibody nanotubes were selectively attached onto the antigen regions. Later, to test the feasibility of antibody nanotubes for real applications in device fabrications by assembling them into more complex configurations, they explored the possibility to anchor multiple types of antibody nanotubes onto their respective complementary binding areas. To prove this hypothesis, they anchored selectively two types of nanotubes coated with different antibodies onto their complementary antigen areas, patterned by AFM tips (Fig. 14.13).

14.4.3 Biomolecules Deposited by Nanografting

Nanografting which is, at least to some extent, an extension of the above depicted nanoshaving, has been also extensively employed to create nanometer-size patterns onto SAMs. Similar to dip-pen nanolithography, nanografting can be carried out either using a direct or an indirect strategy. The direct approach involves the immobilization of the biomolecules onto the areas in which the SAM has been removed. This strategy requires the modification of the biomolecules with the appropriate functional groups (typically thiol) to be anchored to the surface. DNA and proteins have been deposited on gold surfaces using direct nanografting.

The indirect nanografting approach is schematically depicted in Fig. 14.14 [1]. By using the AFM tip, molecules anchored to particular positions at the surface are first removed (B). Then, the addition of new molecules can be absorbed on the

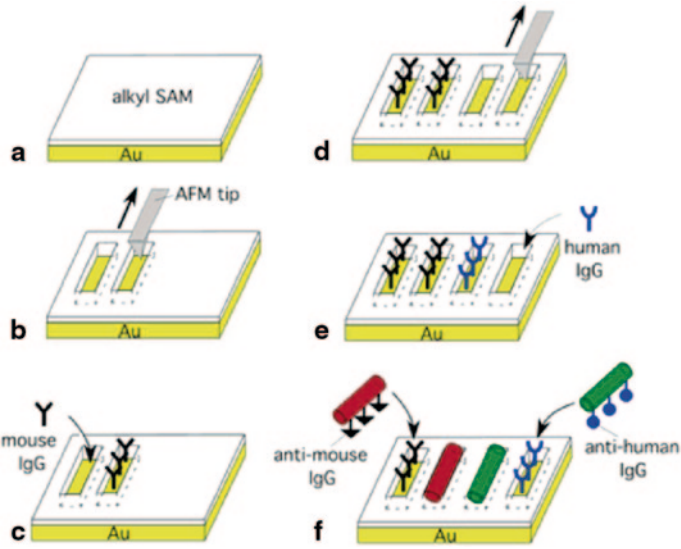


Fig. 14.13 Schematic diagram to assemble anti-mouse IgG-coated nanotubes and antihuman IgG-coated nanotubes onto their antigen-patterned substrates via biological recognition. **a** Self-assembly of alkylthiol monolayers on Au substrates. **b** Shaving trenches on the alkylthiol SAM by using the AFM tip. **c** Deposition of mouse IgG on the shaved trenches. **d** Shaving another array of trenches on the alkylthiol SAM by using the AFM tip. **e** Deposition of human IgG on the shaved trenches. **f** Location-specific immobilization of Alexa Fluor 546-labeled anti-mouse IgG nanotubes onto the mouse IgG trenches and FITC-labeled antihuman IgG nanotubes onto the human IgG trenches via their biological recognition. (Reproduced with permission from ref. [111])

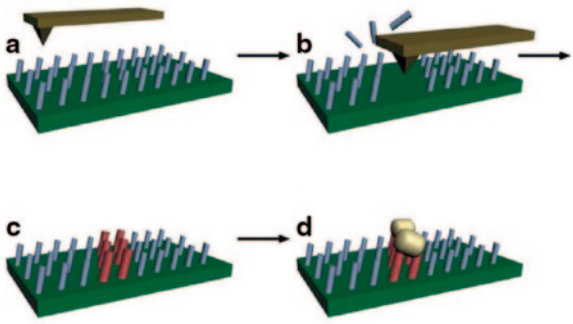


Fig. 14.14 Schematic of nanografting. An AFM tip is first used to scrape away patterns on an existing protein-resistant SAM *(A–B)*, which are then replaced by new protein-adherent SAM molecules from solution *(C)*. Proteins then adsorb to the grafted patterns *(D)*. (Reproduced with permission from [1])

available areas. The new molecules can have, for instance, affinity for a biomolecule so that the latter can be immobilized in a further step. This approach has been, among others, employed to pattern proteins by partial substitution of a protein-resistant SAM by a protein-adherent SAM.

The direct nanografting approach has been employed by Liu et al. [112] who assessed the application of nanografting in patterning single-stranded DNA (ssDNA).

They demonstrated that both the orientation and packing of the oligonucleotides within the patterns can be directly determined in situ using AFM. More interestingly, in their experiments they evaluated the accessibility of the oligonucleotides within the patterns by using DNase I enzyme digestion.

Proteins have also been deposited on solid surfaces using direct nanografting, typically using gold surfaces by means of thiol–gold interactions. The use of nanografting to pattern proteins was introduced by Liu in 1997. They demonstrated the advantages of this technique that permits, among others, the coupling of a protein to a surface without disturbing its tertiary structure [30]. The basic idea of their work relies on the use an AFM tip to disturb the original molecules (e.g., thiols on gold) from a specified area of a monolayer, thereby enabling different thiol molecules from a contacting solution to self-assemble by diffusion or exchange into the exposed gold sites. This strategy has been later followed by Hu and coworkers [113] and Case et al. [114] to pattern gold surfaces with proteins. Whereas Hu et al. [113] employed parallel three-helix bundle metalloproteins to orient vertically on a gold surface, Case et al. [114] reported the nanografting of de novo four-helix bundle proteins engineered to contain a Gly-Gly-Cys linker at its C-terminus.

Indirect nanografting involves the modification of the SAM monolayer, with other molecules exhibiting either higher or lower affinity by the biomolecules that will be absorbed in a further step. Several groups have employed this alternative to prepare nanopatterns on solid surfaces [30, 50, 115–119]. One of the most illustrative examples has been reported by Zhou et al. [116]. In their work, the authors tested three differently charged nanoscale features prepared by sequential nanografting of 6-mercaptohexan-1-ol, N-(6-mercapto)hexylpyridinium bromide, and 3-mercaptopropionic acid into a SAM. By using these charged/neutral surfaces, they studied the immobilization of three proteins (lysozyme, rabbit IgG, and bovine carbonic anhydrase (II)) onto these differently charged nanopatches as a function of the surface charge, additionally depending on the pH. At pH 4.5, all three proteins adsorbed onto the charged nanosurfaces. At higher pH, the proteins behaved differently, depending on the pH and relative surface charge of the nanosurface. Moreover, they developed an approach (depicted in Fig. 14.15) that combines electrostatic immobilization and specific protein–protein interactions to fabricate multiple layered (protein G/rabbit IgG/anti-IgG) three-dimensional (3D) protein nanostructures, demonstrating that the combination of nanografting, electrostatic immobilization, and specific protein interaction is a powerful tool for construction of novel 3D protein surface nanostructures.

14.4.4 Other Alternatives

In addition, other alternatives to pattern bioactive molecules on planar surfaces have been reported that result from the combination of at least two of the approaches depicted above. As an illustrative example we would like to mention the work of Sun et al. [21] who fabricated of biological nanostructures by scanning near-field

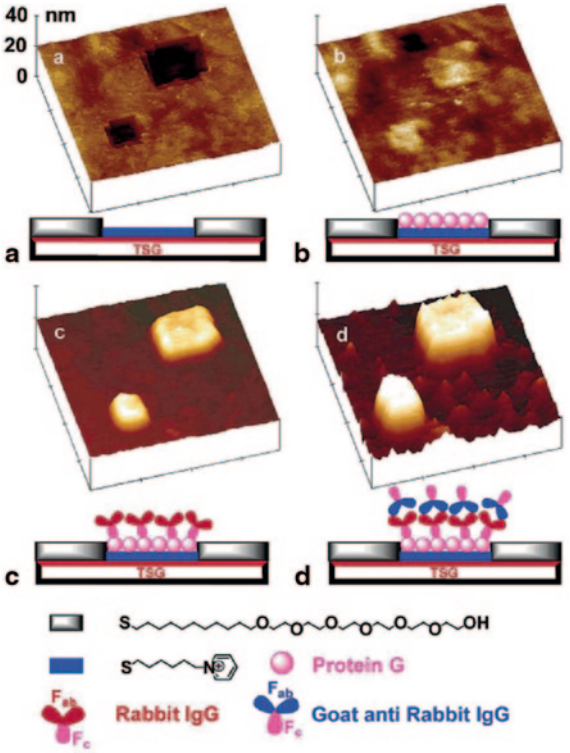


Fig. 14.15 3D AFM topographic images and schemes (below) showing the process of creating and growing the 3D protein nanostructures by electrostatic immobilization and biospecific interaction. All of the images have the same scan sizes (2×2 μm^2) and have the same z-scale. **a** Two nanopatches (200×200 and 400×400 nm^2) created by nanografting of MHP into the SAM of $C_{11}EG_6$ had a depth of 2 nm. **b** After incubation of the surface with protein G (0.1 mg/mL in PBS, 20 min), the patches were filled by protein G and transformed into plateaus with an average height of ca. 1 nm. **c** After a treatment of the surface with rabbit IgG (0.1 mg/mL in PBS, 30 min), the height of the protein nanofeatures increased to 8–9 nm. **d** After subsequent treatment of the surface with goat anti-rabbit IgG (0.1 mg/mL in PBS, 30 min), the height of the protein nanofeatures increased to 19–20 nm. (Reproduced with permission from [116])

photolithography of chloromethylphenylsiloxane (CMPS) monolayers. They evidenced that this approach can be applied to other systems, and demonstrated its utility for the fabrication of functional molecular nanostructures (Fig. 14.16). More precisely, the process developed by the authors involved 244-nm exposure of the CMPS SAM to create nanoscale patterns of surface carboxylic acid functional groups. Then, the carboxylic groups were employed to attach different biomolecules by their conversion to the N-hydroxysuccinimidyl ester and reaction of the active ester. According to the authors, the resulting patterns were activated readily under ambient conditions using simple, widely applicable coupling chemistries to facilitate the formation of patterned nanoparticle, protein, and DNA structures.

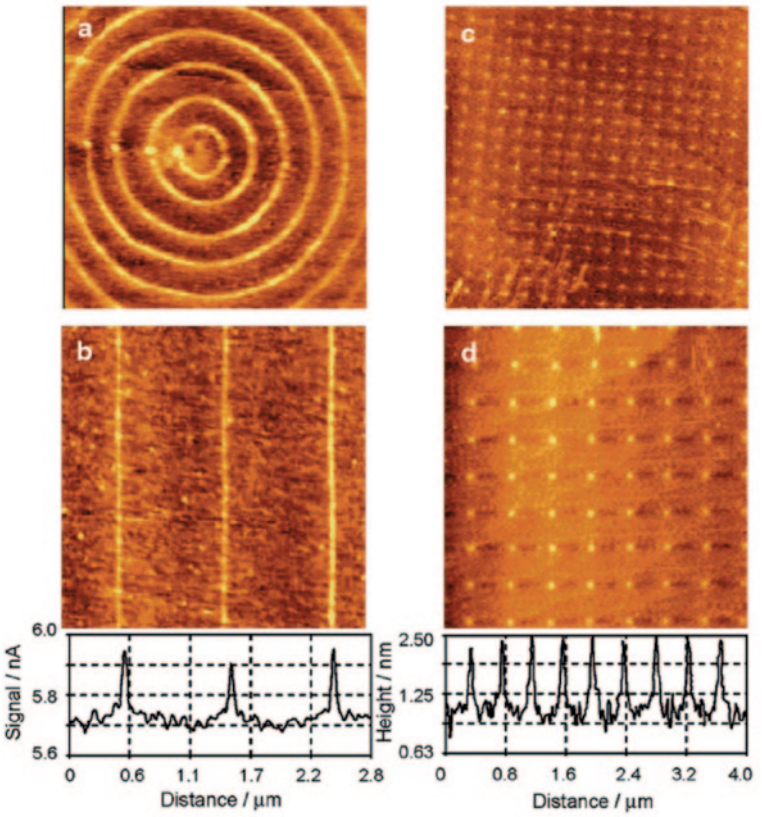


Fig. 14.16 *Left*: friction force microscopy images of nanopatterns. *Right*: topographical images of nanopatterned surfaces following incubation with calf thymus DNA. Image sizes: **a** 4.0×4.0 m^2; **b** 2.8×2.8 m^2; **c** 8.0×8.0 m^2; **d** 4.0×4.0 m^2. Reproduced with permission from [21]

14.5 Summary and Conclusions

In this chapter, we provided a general overview of the scanning probe nanopatterning (SPN) approaches focusing on their capabilities to fabricate biopatterned surfaces. First, we introduced the different approaches developed in order to obtain nanoscale resolution patterns and placed SPN within this context highlighting their advantages and limitations. Then, the basic principles of each technique developed from a common tip-based approach have been discussed. Finally, we selected those approaches that have been employed to a larger extent to pattern biomolecules on surfaces and pay special attention in those cases in which the patterned biomolecule is able to participate in biomolecular recognition processes. In particular, DPN, nanoshaving, and nanografting due to their unique resolution and the possibility to be carried out under ambient conditions have been extensively used to fabricate bio-nanopatterned surfaces. Table 14.3 summarizes the most extended approaches

Table 14.3 Summary of the biomolecules employed and the highest resolution obtained by using scanning probe lithography and references. (Reproduced with permission from [26])

	DPN		Nanoshaving	Nanografting	
	Indirect	Direct		Indirect	Direct
Patterned biomolecules	DNA, peptides, proteins, virus	DNA, peptides, proteins	Peptides, proteins	Proteins	DNA, proteins
Highest resolution	~85 nm	30 nm	150 nm	10 nm	10 nm
Examples	IgG protein dot features [120]	Collage-like peptide lines [79]	IgG protein lines [111]	Lysozyme protein lines [117, 118]	DNA lines [112, 121]

to pattern biomolecules by using scanning probe microscopes, highlighting the type of molecule immobilized onto the surface, the highest resolution achieved for each technique, and we gave particular examples.

SPN with their simplicity and unique resolution is currently an interesting tool to study the new properties of materials at the nanoscale. Their range of applications in a variety of fields includes microelectronics, optics, medicine, and biology. In particular, bionanopatterning of surface which is an active field in the biotechnology and nanotechnology interface has been highlighted for its potential applications ranging from molecular diagnostics to fundamental studies in molecular and cell biology [26]. However, in order to enlarge the range of applications, several challenges still need to be resolved including the possibility to pattern multiple biomolecule nanoarrays or the patterning speed.

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