

Nanopatterning proteins and peptides

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Received 31st July 2006, Accepted 8th September 2006

First published as an Advance Article on the web 29th September 2006

DOI: 10.1039/b611000b

A variety of techniques have been developed to site-specifically immobilize biomolecules onto surfaces with resolutions below one micron. The ability to pattern proteins and peptides in particular has great potential for applications in biosensors, biomaterials, and tissue engineering. For example, immobilizing proteins at the nanoscale could lead to the development of diagnostic protein nanoarrays, while patterning peptides could lead to greater control over the cell/biomaterial interface. This review discusses the methods that have been reported for patterning proteins and peptides with submicron and nanometer resolutions.

1. Introduction

Over the past few decades, techniques to produce submicron and nanoscale features on surfaces have emerged. While such advances were initially applied to the electronics field, the fusion of biology and nanotechnology has begun to provide many opportunities for biotechnology and medicine. The ability to spatially orient and anchor proteins at the nanoscale affords useful materials for biosensors, biomaterials, and tissue engineering applications. For instance, since disease progression is often correlated with protein levels,¹ protein nanoarrays offer the prospect of greater sensitivity in diagnostic tests.² In addition, further miniaturization from protein microarrays to nanoarrays may allow for the discovery of currently undetectable disease markers.² Moreover, the detection of thousands of biomarkers could be performed on one chip, resulting in a more comprehensive biosignature for a patient or disease.^{3,4}

Patterning of proteins and peptides at the nanoscale is also biologically relevant. Cells recognize and adhere to cell adhesion receptors that are spatially organized on the nanoscale. The most common cell adhesion receptor, the integrin, is approximately 10 nm in diameter.⁵ Integrin clustering and formation of focal adhesions, which is necessary for complete signalling, occurs in the nanometer to micron regime.^{6–11} This suggests that presentation of cell adhesion ligands at the nanoscale is important to elicit a full cellular response. Therefore, nanopatterns of proteins and peptides could lead to a greater understanding of cell adhesion, as well as engineered biomaterials that are integrated with the host. This review focuses on current methods for the presentation of proteins and peptides into ordered patterns at the submicron and nanoscale. Wherever possible, reported advantages and limitations of the approaches are included for comparative purposes. Although DNA and other biomolecules have been attached to surfaces, the focus of this review is on protein and peptide patterning.

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2. Scanning probe microscopy

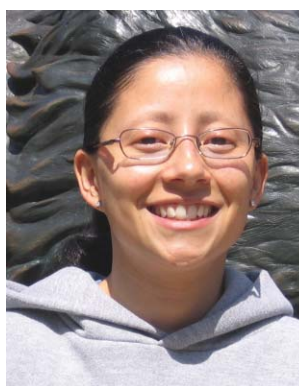
The first studies that demonstrated precise control over patterning proteins on the nanoscale used modified scanning



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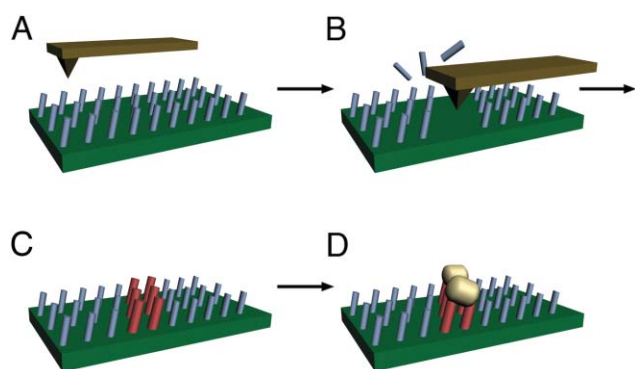


Fig. 1 Schematic of nanografting. An AFM tip is first used to scrape away patterns on an existing protein-resistant SAM (A–B), which are then replaced by new protein adherent SAM molecules from solution (C). Proteins then adsorb to the grafted patterns (D).

probe (SPM) or atomic force microscopy (AFM) techniques. AFM tips can be used to create patterns on a surface and also subsequently image them. Various techniques, including nanografting, dip-pen nanolithography, nanopen and conductive AFM, have exploited the use of these tips to spatially pattern proteins.

2.1 Nanografting

Nanografting involves the mechanical scraping of patterns into self assembled monolayers (SAMs). As the SAM molecules (Fig. 1A) are removed under the AFM tip (Fig. 1B), new molecules from the solution are adsorbed to the created patterns (Fig. 1C). By using molecules of known protein affinity, protein adsorption can be modulated and limited to the grafted areas (Fig. 1D).



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Wadu-Mesthrige *et al.* first demonstrated the utility of this technique for patterning proteins on the nanoscale.¹² They nanografted patterns of $\text{HS}(\text{CH}_2)_2\text{COOH}$ into a $\text{CH}_3(\text{CH}_2)_9\text{S}/\text{Au}(111)$ SAM. Following rinsing to remove any non-grafted COOH terminated molecules, the surface was incubated with lysozyme (a protein that lyses bacterial cell walls), which specifically adsorbed to the COOH terminated patterns. Likely, the driving force was electrostatic because of the positive charge of the protein. While proteins typically adsorb to hydrophobic surfaces such as methyl-terminated SAMs, the reaction conditions (low concentration and short incubation time) used in this study prevented adsorption on the background. AFM images confirmed an increase in height due to lysozyme; different heights were observed, indicating that this method results in various protein orientations on the surface. They also nanografted patterns of $\text{HS}(\text{CH}_2)_2\text{CHO}$ or $\text{HS}(\text{CH}_2)_{10}\text{CHO}$, which were subsequently used to attach immunoglobulin (IgG) and lysozyme, respectively, *via* imine bonds. Protein patterns as small as $10 \times 150 \text{ nm}$ and $40 \times 40 \text{ nm}$ were created (Fig. 2). Additional work by this group displayed patterns of BSA attached to 3-mercaptopropional features and confirmed the bioactivity of the patterned IgG.^{13,14}

Kenseth *et al.* used a similar approach by grafting a carboxylic acid terminated thiol into a methyl-terminated SAM;¹⁵ in this case, rabbit IgG was covalently bound to the carboxylic acid using 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) activation. In order to create a more protein-resistant background than provided by a methyl-terminated SAM, they also produced patterns in oligo(ethylene glycol) terminated monolayers. Following removal of the oligo(ethylene glycol) molecules at selected areas, either chemisorption of dithiobis(succinimidyl undecanoate) (DSU; an activated ester) or direct adsorption of thiol terminated Fab' antibody fragments was achieved. The oligo(ethylene glycol) SAMs effectively eliminated nonspecific protein adsorption; however, direct adsorption of the thiol terminated antibody fragments did not form a complete protein monolayer compared to covalent attachment to the DSU patterns. Submicron IgG patterns were demonstrated.

Case *et al.* utilized the nanografting technique to specifically orient patterned proteins to the surface.¹⁶ Metalloproteins

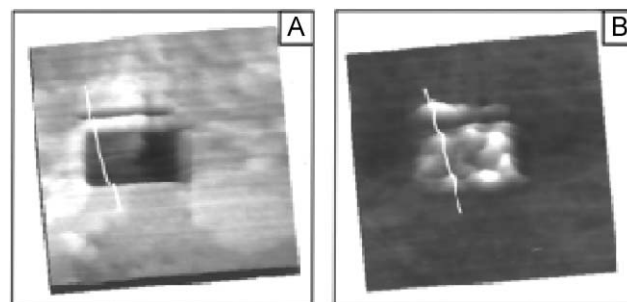


Fig. 2 Lysozyme patterns *via* nanografting. A $10 \times 150 \text{ nm}$ line and a $100 \times 150 \text{ nm}$ rectangle of $\text{HS}(\text{CH}_2)_2\text{COOH}$ were created in a $\text{C}_{10}\text{S}/\text{Au}(111)$ SAM (A). Lysozyme then adsorbed to the nanopatterns (B). Reprinted with permission from ref. 12. Copyright 1999 American Chemical Society.

containing C-terminal thiol groups were directly grafted into octadecanethiol SAMs. They demonstrated that the three-helix bundle metalloproteins were vertically oriented to the substrate with an increase in height similar to molecular model estimations. Patterns of 100×100 nm were created. This group also demonstrated attachment of a 4-helix bundle protein containing a glycine–glycine–cysteine tag at the C-terminus. However, protein height varied and nanografting was only successful one-third of the time; this was attributed to the presence of only a single thiol for attachment and refolding of the protein upon adsorption.¹⁷

The effect of surface charge and solution pH on protein adsorption was examined by Zhou *et al.*¹⁸ Three distinct areas containing either 6-mercaptohexan-1-ol (MCH), *N*-(6-mercapto)hexylpyridinium bromide (MHP), or 3-mercaptopropionic acid (MPA) were nanografted into hexa(ethylene glycol) terminated monolayers. The adsorption of lysozyme, rabbit IgG, and bovine carbonic anhydrase (II) were examined on the differently charged 400×400 nm patterns at a variety of pHs. All three proteins bound better to the negatively charged MPA and the positively charged MHP patches than the neutral MCH. As expected, protein adsorption varied greatly with pH. Using 200×200 nm patterns of MHP, they site-specifically adsorbed protein G. Following incubation with rabbit IgG they demonstrated a uniform height increase, rather than the typically observed multiple protein orientations. This was attributed to the use of protein G as an anchoring platform, which then caused the Fc binding domain of IgG to orient towards the surface, creating a uniform protein layer. Uniform orientation of bound anti-rabbit IgG was also reported. Such uniform presentation would be important in many applications, especially in biological sensors where protein orientation can affect bioactivity.

Nuraje *et al.* used nanografting to create proteins patterns for the assembly of carbon nanotubes.¹⁹ Trenches 400 nm $\times$ 4 μ m were first shaved into 1-octadecanethiol (ODT) monolayers. Mouse IgG was then adsorbed directly to the exposed gold regions. Anti-mouse IgG coated nanotubes were finally assembled onto the patterned IgG areas. They next demonstrated patterning two distinct features on the same substrate. Following patterning of mouse IgG, another set of trenches was shaved, and human IgG was grafted to the newly exposed gold. Anti-mouse IgG coated nanotubes and anti-human IgG coated peptide nanotubes specifically bound to the respective regions.²⁰ This group also directly attached the nanotubes. The 200 nm $\times$ 2 μ m trenches were shaved in alkylthiol monolayers, and nanotubes coated with thiol-terminated peptides then attached to the exposed gold.²¹ Multiple tubes could be aligned within larger trenches (1.5×3 μ m). This technique could potentially be used for the building of nanoscale devices containing hybrid biological–inorganic components.

2.2 Dip-pen nanolithography

Dip-pen nanolithography (DPN) is a scanning probe nanolithography technique that utilizes an AFM tip to deposit molecules onto a surface. AFM tips are first dipped in “ink,” which is typically an alkanethiol; the molecules then move from the tip to the surface (gold in the case of alkanethiols) via

diffusion through a water meniscus that forms between the tip and sample. Patterns ranging from 10 nm to several microns can be fabricated, with the resolution being limited by the tip sharpness and the diffusion constants of the molecules being deposited.^{22,23} Two methodologies exist for patterning proteins using DPN: indirect and direct. The indirect method involves first depositing protein-adherent molecules onto the surface, followed by protein adsorption from solution, while the direct method involves directly depositing the protein onto the surface (Fig. 3). Additionally, DPN can be performed with both contact and tapping mode AFM. Many groups have used contact mode; however, tapping mode is considered more gentle which may be more desirable with biological materials.^{24,25} This can be advantageous in both deposition as well as visualization.

2.2.1 Indirect DPN. Lee *et al.* (2002) first demonstrated the use of indirect DPN for patterning proteins at the nanoscale.²⁶ Using DPN in contact mode, 16-mercaptohexadecanoic acid (MHA) was deposited into dot and grid patterns (100–300 nm) on gold substrates. The background was then rendered protein resistant by incubating the substrates in 11-mercaptoundecyltri(ethylene glycol). Lysozyme and IgG were shown to specifically adsorb to the MHA patterns. To demonstrate that the proteins retained bioactivity, substrates were incubated with anti-IgG, which bound to the IgG patterns. They also adsorbed retronectin, the cell adhesion fragment of the protein fibronectin, and demonstrated cell binding to 200 nm dots separated by 700 nm. Kwak *et al.* reported a similar method using DPN patterned MHA (~ 200 nm features) and a ODT background to specifically adsorb cytochrome c.²⁷ Lee *et al.* (2004) expanded their original approach by detecting human immunodeficiency virus type 1 (HIV-1) in plasma using DPN generated nanoarrays.²⁸ In this case, anti-p24 antibodies, which are specific for the HIV-1 p24 antigen, were selectively adsorbed to MHA regions *via* electrostatic interactions resulting in ~ 80 nm arrays. BSA was subsequently used to passivate unmodified MHA. Samples were immersed in plasma from AIDS patients and p24 antigen binding was confirmed by AFM. The detection could be further amplified

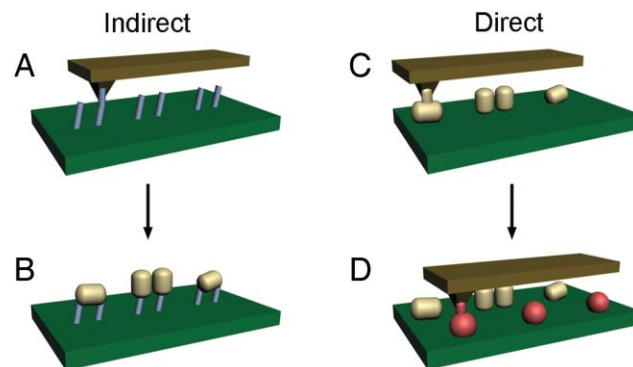


Fig. 3 Schematic of dip-pen nanolithography (DPN). Indirect DPN involves first depositing protein adherent molecules (A), then proteins in solution adsorb to the patterned areas (B). Proteins are directly deposited onto the surface from the AFM tip in direct DPN (C). By re-dipping the tip, another protein can be patterned (D).

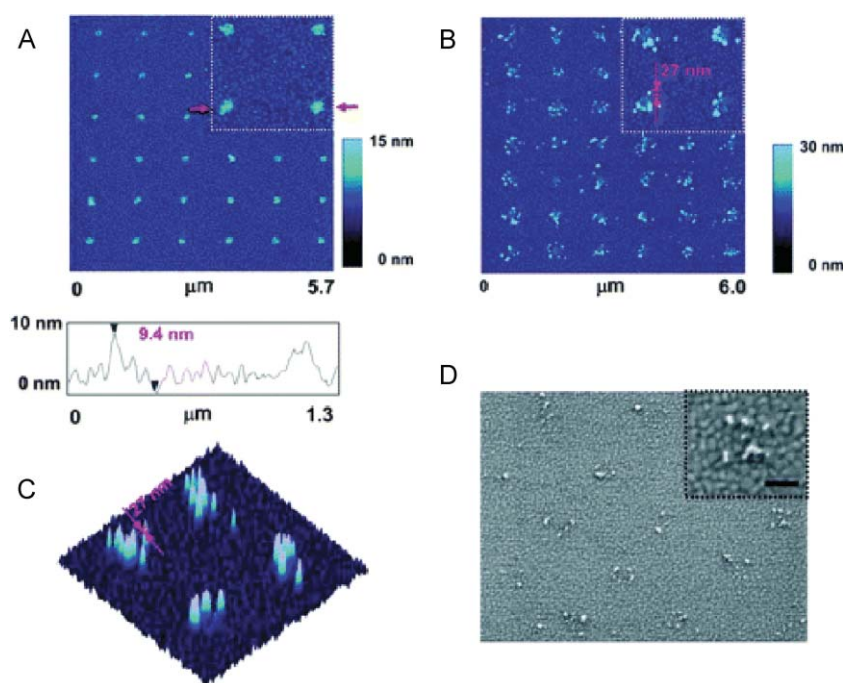


Fig. 4 Detection of HIV-1 p24 antigen in plasma. (A) p24 binds to anti-p24 IgG patterns. (B) The signal is then amplified by binding anti-p24 IgG coated gold nanoparticles. (C) Three dimensional sub-section of (B). (D) A FE SEM image from the same chip as (B). Scale bar = 150 nm. Reprinted with permission from ref. 28. Copyright 2004 American Chemical Society.

by incubating the samples with anti-p24 IgG coated gold nanoparticles (Fig. 4). The detection limit was more than 1000 fold more sensitive than the conventional enzyme-linked immunosorbent assay (ELISA) technique. These results are significant for disease detection applications. More recently, indirect DPN patterning of 350×150 nm MHA features spaced one micron apart was used to immobilize single virus particles through coordination to Zn^{2+} ions.²⁹ Valiokas *et al.* employed DPN to deposit patterns of an amine terminated SAM, which were then used for immobilizing membrane protein complexes on patterns with a resolution of 300 nm after passivation of the background with a poly(ethylene glycol) (PEG) SAM.³⁰

Hyun *et al.* (2002) utilized DPN in contact mode to create patterns of MHA with features ~ 200 nm.³¹ The background was then passivated with an oligoethylene glycol terminated alkanethiol. Instead of adsorbing proteins onto the MHA features, amine-terminated biotin was covalently attached to MHA after activation of the carboxylic acids with *N*-hydroxysuccinimide (NHS). Substrates were then incubated with the protein streptavidin, which conjugated to the biotin *via* molecular recognition. Streptavidin is a tetrameric protein that can bind four biotins with very high affinity ($K_a \sim 10^{13} \text{ M}^{-1}$). Therefore, these patterns could be used to link a variety of biotinylated ligands. To demonstrate this, surfaces were incubated with biotinylated BSA, which bound specifically to the patterned streptavidin. Pena *et al.* also exploited the interaction between biotin and a related protein, neutravidin. Using DPN, they deposited NHS-biotin onto polyethylenimine coated surfaces, and then demonstrated attachment of the protein.³² By using photolithography and wet-chemical etching, they also created DPN patterns next to

micron scale objects, which would be applicable to micro-electromechanical systems (MEMS).

Smith *et al.* used DPN in contact mode to pattern regions with functional groups for chemospecific immobilization of proteins rather than adsorption through hydrophobic or electrostatic interactions, which can result in various orientations and loss of biological activity.³³ They patterned 150 nm circular features using an “ink” consisting of 98% 11-mercaptoundecylpenta(ethylene glycol) disulfide and 2% of a mixed disulfide substituted with one maleimide group. The background was then passivated with a thiol terminated penta(ethylene glycol). A mutant cowpea mosaic virus capsid containing a free cysteine attached to the patterns *via* Michael addition. Lee *et al.* (2006) also presented a technique for orienting antibodies by indirect DPN.³⁴ Protein A/G, which has affinity for the Fc region of IgG, was immobilized to nanoscale features (100–650 nm) of 11-mercaptoundecanoyl-NHS ester on gold. A significant advance in this study was the use of a 26-pen array, which allowed for the creation of nanoscale features over centimeter-scale areas.

Zhang *et al.* used a combination of DPN and wet chemical etching to produce protein arrays with 340 nm features.³⁵ DPN was first used to pattern areas of MHA onto SiO_2 -Si substrates coated with thin layers of Ti and then Au. A ferri/ferrocyanide etchant and aqueous hydrofluoric acid (HF) was then used to remove the Au and Ti layers respectively without affecting the MHA patterns. The newly exposed SiO_2 was passivated with *n*-octadecyltrimethoxysilane and rabbit IgG adsorbed to the MHA patterns. Binding of anti-IgG demonstrated that bioactivity was retained.

Hyun *et al.* (2004) employed indirect DPN to create nanoscale protein switches. They utilized previously described

methods³¹ to attach elastin like polypeptide (ELP) to MHA patterns (200 nm) *via* NHS/EDC chemistry.³⁶ They demonstrated that binding of a thioredoxin–ELP fusion protein occurred above its lower critical solution temperature (LCST) when the polypeptide is desolvated and aggregated, while dissociation occurred below the LCST when it became soluble. This clever approach could be applied to any ELP linked protein; therefore, these biomolecular switches may have potential application for reusable protein arrays and other nanobiotechnology applications.

2.2.2 Direct DPN. Wilson *et al.* first demonstrated nanoscale protein patterning using the technique of direct DPN.²⁵ Thiolated collagen and collagen-like peptides were directly patterned into 30–50 nm lines on Au substrates using DPN in tapping mode.²⁵ They demonstrated that this method preserved the native triple-helical structure and bioactivity. Noy *et al.* also use the direct DPN technique to write lines of fluorescently tagged human chorionic gonadotropin antibody on glass pretreated with 3-glycidioxypropyltrimethoxysilane at the submicron scale.³⁷ The protein was covalently attached to the surface *via* reaction of the amines with the surface bound epoxides and subsequently imaged using an AFM and scanning confocal microscope. Lee *et al.* (2006) also used a pretreated surface to immobilize angiogenin and integrin $\alpha_v\beta_3$, using direct DPN in contact mode, into patterns as small as 120 nm. In this case, the gold substrate was coated with Prolinker[®], which contains crown ether moieties that can capture protonated amines by host–guest interactions.³⁸ Agarwal *et al.* used DPN in tapping mode to deposit the synthetic peptide MH2, which contains an N-terminal c-Myc tag and a C-terminal histidine tag, into line widths as small as 70 nm.²⁴ They reported that tapping mode was a gentler form of AFM and did not scratch the substrate; they had observed scratches with contact AFM. Rather than “writing” on inorganic substrates or SAMs, Li *et al.* (2005) deposited antibodies and streptavidin onto single stretched DNA molecules and achieved features sizes of ~ 40 nm.³⁹

Several groups have also examined modified AFM tips to facilitate diffusion of the proteins from the tip to substrate. Lim *et al.* modified tips with 2-[methoxypoly(ethyleneoxy)-propyl]trimethoxysilane (Si-PEG) in order to form a protein resistant surface to reduce the activation energy required to transport the protein to the surface and to decrease denaturation.⁴⁰ Using this approach, IgG patterns as small as 50 nm were written. This group also examined alternative modifications of the standard Si₃N₄ tips.⁴¹ The gold coated cantilever was immersed in 11-mercaptoundecylpenta(ethylene glycol) disulfide to produce a protein resistant coating on the reverse side of the cantilever. The tips were then coated with Au, followed by treatment with thiotic acid to create a hydrophilic tip, which aided the adsorption of the proteins on the tip surface. They then demonstrated the ability to sequentially write patterns of two distinct proteins, IgG and lysozyme, using the same tip on the same chip. Feature sizes of 45 nm were demonstrated. They reported that their feature sizes were largely determined by contact time, with longer times producing larger features. The results are important because

they pave the way to creating arrays of different proteins for applications in proteomics and sensors.

2.3 Nanopen

Taha *et al.* delivered proteins through cantilevered nanopipettes using a near-field scanning optical microscopy (NSOM)/SPM confocal system.⁴² Proteins flow through the nanopen and are delivered using AFM control. Patterns of protein G and green fluorescent protein (GFP) with resolutions of ~ 250 nm were fabricated using this approach. The protein patterns could be subsequently imaged with the same NSOM platform. The authors mention that an advantage of this approach is that the nanopen can be attached to standard high performance liquid chromatography (HPLC) instrumentation to deliver multiple proteins without the need to re-dip the tip.

2.4 Conductive AFM

Several groups have also examined the application of a potential through AFM tips in order to locally alter surface properties for protein attachment. Agarwal *et al.* applied voltage to an AFM tip in order to facilitate transport of proteins from the tip to surface.⁴³ Tips were first coated with histidine-tagged proteins or peptides. A negative bias was applied to the tip and a nickel-coated substrate, causing ionization of the Ni surface and localized binding of the His tag proteins *via* metal chelation. When zero potential was applied, deposition did not occur. Nam *et al.* also utilized Ni surfaces for direct-write DPN; however they demonstrated feature sizes of 80 nm without an applied field by writing on already oxidized Ni substrates.⁴⁴ AFM tips were first coated with Ni and then immersed in histidine tagged proteins (ubiquitin or thioredoxin). The proteins were then deposited and binding of anti-ubiquitin and anti-thioredoxin antibodies confirmed bioactivity.

Pavlovic *et al.* applied a potential between an AFM tip and a silicon oxide surface modified with thiols, resulting in local oxidation to thiolsulfinates/thiolsulfonates.⁴⁵ Immobilization of β -galactosidase, a thiol rich protein, into patterns as small as 200 nm *via* disulfide bond formation was demonstrated. Binding was only partially reversed by addition of dithiothreitol (DTT) due to inaccessibility of the disulfide bonds between the surface and the protein.

Yoshinobu *et al.* also applied voltage to the AFM tip, which resulted in anodic oxidation of Si surfaces.⁴⁶ Both negative and positive submicron protein patterns were demonstrated. In the negative patterning, a thick oxide pattern was drawn on a thin SiO₂ layer by applying the anodizing voltage between the tip and the surface. The remaining thin oxide layer was then removed with HF and the protein ferritin was adsorbed to expose Si regions. An alternative for negative patterning involved first coating the thin oxide layer with amino groups using an amine-terminated silane, and then writing thicker oxide patterns with the tip. Proteins were again immobilized outside of the oxide patterns. In positive patterning, the thin oxide layer was treated with octadecyltrichlorosilane (OTS); oxide patterns were again created by applying voltage from the tip to the surface. Amine groups

were then attached to the oxide patterns, which allowed for the adsorption of ferritin.

Gu *et al.* used conductive AFM on α -hepta(ethylene glycol)methyl ω -undecenyl ether monolayers formed on conductive silicon(111) H-terminated surfaces to create patterns of ~ 90 nm holes.⁴⁷ They suggested that the applied voltage resulted in end hydroxyl groups which could be converted to COOH groups with succinic anhydride. Avidin was attached to the patterns by activation with EDC. Patterned avidin was capable of binding biotinylated BSA selectively over native BSA. The authors noted that high density arrays could be written in approximately one minute, which is significantly faster than other SPM techniques. Naujoks *et al.* also employed conductive AFM to create protein patterns in thin layers of poly(methyl methacrylate) (PMMA).⁴⁸

The above mentioned studies nicely demonstrate nanoscale protein patterning using SPM-based techniques. The high resolution achieved is a significant advantage of these methods.⁴⁹ Another major advantage is that different shapes, for example dots or lines, are readily prepared. In addition, a wide variety of proteins can be delivered onto a number of surfaces under ambient conditions. A reported limitation is that, like other serial techniques, the speed is slow and the accessible area is restricted.⁴⁹ Additionally, although it is promising to use direct-write DPN to pattern multiple proteins without cross-contamination, the approach is still challenging because of slow transport of the protein from the tip to the surface and the need to re-dip the tip.⁴¹ Recent advances on multi-probe arrays,³⁴ tip modification,^{40,41} protein delivery (*i.e.* nanopens),⁴² and conductive AFM⁴⁷ are addressing these issues.

3. Stamping

3.1 Micro/nanocontact printing

Microcontact printing (μ CP) was originally demonstrated by Whitesides and coworkers for creating spatially resolved patterns of alkanethiols on Au.^{50,51} This technique has been called nanocontact printing (nCP) when the features are nanosized. Poly(dimethylsiloxane) (PDMS) stamps are created from a master, which is typically fabricated from Si wafers patterned by photolithography or by electron-beam lithography. The stamps are first dipped in the “ink” (protein solutions in the examples in this review) and then the stamp is brought into contact with the surface to transfer the ink (Fig. 5).

Renault *et al.* demonstrated the use of nCP for printing proteins. They created 100 nm patterns of IgG and green fluorescent protein (GFP) on glass substrates by decreasing the

feature sizes on the PDMS stamps and the contact areas with the substrate, while diluting the protein ink solutions.⁵² Li *et al.* (2003) also demonstrated nanoscale protein patterning using nCP. By using an AFM V-shaped test grating as the original master for hard PDMS stamps, they were able to create 70 nm features of a titin multimer protein.⁵³ Csucs *et al.* introduced new polyolefin elastomer stamps for μ CP which fabricated continuous patterns of fibrinogen in the 100 nm range.⁵⁴

3.2 Nanoimprint lithography

Nanoimprint lithography also employs a stamp; however, the stamp is used to create physical features in the substrate, which then can be employed for protein immobilization. In this case, the stamp is typically an Si template that is fabricated using standard lithographic techniques. The stamp is brought into contact with a polymer film heated above its glass transition temperature, resulting in an imprint in the polymer. The imprinted substrate can then be modified to produce protein adherent patterns. Hoff *et al.* used a silicon mold to imprint patterns into PMMA coated SiO₂ substrates.⁵⁵ Following separation of the stamp, O₂ reactive etching (REI) removed residual PMMA and CHF₃ REI removed the exposed oxide while depositing a thin layer of CF_x polymer residue. The PMMA was stripped, leaving patterns of SiO₂ and CF_x polymer. An amine-terminated silane was used to coat the oxide surface and then bind to a biotin–succinimidyl ester. Streptavidin and biotinylated BSA were subsequently attached to the 75 nm patterns.

Falconnet *et al.* also used a silicon stamp to imprint features into PMMA coated substrates.⁵⁶ Residual PMMA in the patterns was similarly removed by etching from the coated Nb₂O₅ substrates, creating patterns of Nb₂O₅ in a background of PMMA. The samples were then dipped into biotin functionalized poly(L-lysine)-graft-poly(ethylene glycol) (PLL-g-PEG/PEG–biotin). After removal of remaining PMMA with acetone, the background was passivated with PLL-g-PEG. Finally, streptavidin was immobilized onto patterned biotin regions, creating 100 nm protein patterns.

A significant advantage of μ CP/nCP and nanoimprint lithography compared to serial techniques is that large areas can be patterned quickly.^{57,58} Other reported advantages include high throughput and low-cost.⁵⁵ A challenge of both techniques is that patterning many different proteins on the same substrate would be difficult due to alignment issues.²² Reported challenges for nCP are lateral diffusion of the “ink” molecules and pattern distortion as a result of the soft material of the stamp and nonuniform pressures applied during printing.⁵⁷ These issues have successfully been addressed by employing large molecules including proteins, diluting the “inks” and modifying the stamp.^{52–54,57}

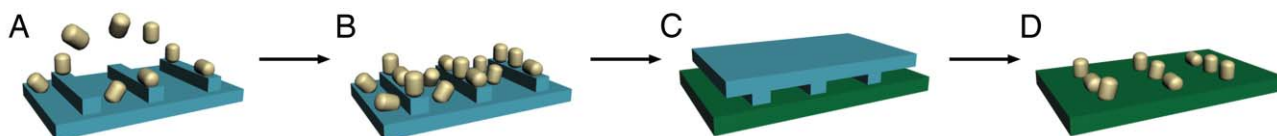


Fig. 5 Schematic of micro/nanocontact printing. Stamps are first incubated in a protein solution (A), which results in deposition on the stamp (B). Stamps are next pressed against a substrate (C) and proteins are transferred to the substrate at locations of contact (D).

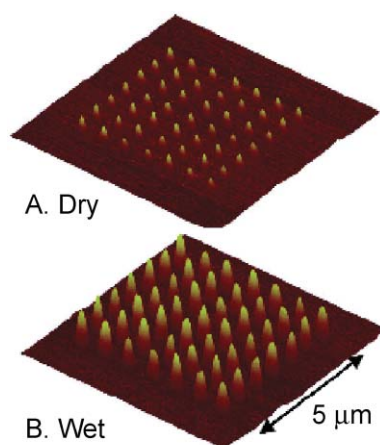


Fig. 6 AFM images of amine-terminated PEG nanopatterned hydrogels. (A) dry. (B) hydrated. Reprinted with permission from ref. 63. Copyright 2004 American Chemical Society.

4. Electron beam lithography

Electron beam (e-beam) lithography, which is maskless and instead uses an electron beam to scan across the surface, is capable of producing very high resolution patterns with features sizes as small as 5–10 nm.⁵⁹ As a result, e-beam lithography is becoming increasingly popular for patterning biomolecules.

Harnett *et al.* ablated patterns into monolayers of MHA or ODT on Au using e-beam radiation.⁶⁰ The patterns were then filled with an amine-terminated SAM, followed by conjugation of biotin using NHS–biotin. Avidin coated beads or fluorescent anti-biotin IgG bound to 300 nm wide lines. Rundqvist *et al.* also ablated patterns into a monolayer; neutravidin coated nanospheres adsorbed to patterns as small as 40 nm, which were created in a PEG thiol SAM.⁶¹ Borini *et al.* irradiated porous silicon with e-beam to create submicron patterns of adsorbed glucose-binding protein, glutamine-binding protein, and sugar-binding protein from *P. horikoshii* with varying depths depending on dosage.⁶²

In a clever approach, Hong *et al.* utilized e-beam lithography to site-specifically crosslink dry amine or hydroxy-terminated PEGs to a PEG–silane monolayer.⁶³ Upon exposure to an aqueous environment, this technique resulted in nano-hydrogels (~200 nm wide) attached to the Si substrates (Fig. 6). BSA was then attached to the hydrogel pads *via* EDC chemistry. A photoactivatable crosslinker was linked to the BSA, which upon exposure to ultraviolet (UV) light, reacted to conjugate laminin and fibronectin to the patterns.

Textor and coworkers employed e-beam lithography to create patterns within a PMMA resist, which was spin-coated onto a layer of SiO₂ on indium tin oxide (ITO)-coated glass slides.^{64,65} Reactive ion etching was then used to transfer the pattern to the underlying SiO₂. Dodecyl phosphate (DDP) was adsorbed to the ITO surface, while PLL-g-PEG bound to exposed SiO₂. Streptavidin subsequently adsorbed to the hydrophobic, 130–140 nm sized DDP patterns.

Jonas and coworkers also fabricated patterns into a PMMA resist using e-beam radiation. An alkylsilane monolayer was then deposited onto the exposed substrate *via* gas phase

silanation.⁶⁶ Upon removal of the resist, the background was passivated with an oligo(ethylene oxide)silane. They demonstrated that by adjusting the width of the alkylsilane patterns, the orientation⁶⁷ and alignment⁶⁸ of adsorbed proteins could be modulated. Senaratne *et al.* used a lift off process to create gold posts.⁶⁹ The gold was then covered with a thiol terminated PEG containing a dinitrophenylcaproate (DNP) cap. Anti-DNP antibodies bound specifically to the 600 nm features. Rather than removing the PMMA resist, Powell *et al.* passivated the PMMA surface with BSA and adsorbed IgG coated gold nanoparticles (AuNP) onto exposed 500 nm wide SiO₂ patterns etched into the PMMA.⁷⁰ By varying the size of the features and incubating with successively smaller nanoparticles, multiple antibodies could theoretically be patterned on the same chip. Kumagai *et al.* utilized a commercially available positive e-beam resist; upon revealing 50–200 nm patterns of the underlying SiO₂ substrate, a layer of 3-aminopropyltriethoxysilane (APTES) was created using vapor deposition.⁷¹ Ferritin was then adsorbed to the features by adjusting the pH and ionic strength of the solution after resist lift-off.⁷² By decreasing the APTES patterns to 15 nm with 100 nm spacing, they were able to immobilize single ferritin molecules.

Like SPM techniques, high resolutions are possible with e-beam lithography and arbitrary patterns of different shapes and sizes are readily drawn on the surface.^{58,59,73} Both are major advantages of this technique. Compared to stamping techniques, e-beam lithography is significantly slower and of lower throughput.^{58,73} Projection electron lithography methods may address this issue.⁷⁴ Another challenge is that multiple proteins would be difficult and time consuming due to alignment issues and the required vacuum chamber. However, the above mentioned approach of etching different sized features and incubating with smaller and smaller nanoparticles was reported as one potential way to array multiple proteins.⁷⁰

5. Self-assembly

5.1 Particle lithography

A few groups have used both micro- and nanosphere particles to produce nano-sized protein patterns, a technique that is termed particle or colloidal lithography. The process relies upon the self assembly of spherical particles into periodic structures on surfaces in order to create a colloidal mask (Fig. 7). After removal of the spheres, proteins are adsorbed. Cai *et al.* produced hexagonally patterned lysozyme arrays with features sizes of ~120 or ~60 nm using this technique.⁷⁵ The polystyrene nanospheres created a mask on top of a 10-undecenyltrichlorosilane (UTS) monolayer; PEG–silane was then deposited on the surface except in areas where the beads were touching the substrate. Upon ultrasonic removal of the particles, lysozyme adsorbed to regions that were formally covered by the sphere mask, creating ordered nanoarrays. Valsesia *et al.* created a mask of polystyrene nanobeads on a mercaptohexadecanoic acid (MHD) monolayer, and the surface was exposed to oxygen plasma.⁷⁶ This reduced the particle size and etched any unprotected MHD. Exposed Au was filled with a CH₃ terminated SAM after removal of the residual beads. Antibodies then preferentially adsorbed onto

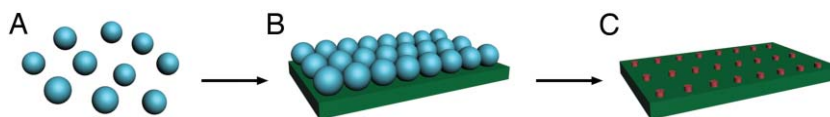


Fig. 7 Schematic of particle lithography. Micro or nanobeads (A) are first self-assembled on to the substrate (B), which creates a mask. Patterns are created where the beads were touching the substrate (C).

100 nm MHD patterns. Batra *et al.* also utilized polystyrene beads as a template for cross-linked poly(vinyl alcohol). Following removal of the beads, GFP was adsorbed to the more hydrophilic wells created in the polymer network, producing hexagonally ordered submicron protein arrays.⁷⁷ Agheli *et al.* created a layer of latex nanoparticles on gold films; dry etching then produced gold features on top of the SiO₂ substrate. An alkanethiol monolayer bound to the gold patterns, while PLL-g-PEG coated the SiO₂, allowing for specific adsorption of laminin to the ~125 nm features.⁷⁸

5.2 Polymer assisted templating

Various groups have examined nanopatterning techniques using polymer arrangements and modifications in an effort to elucidate the critical sizes and physical separations of cell adhesion ligands needed for focal adhesion formation and biochemical signalling. The majority rely upon estimations and mathematic models for pattern size and spacing,^{79–83} however Spatz and colleagues have developed a system that produces ordered patterns. They utilized diblock copolymer micelles containing Au dots (<8 nm), which self-assembled into a close-packed hexagonal template, to pattern cell adhesion ligands at the nanoscale.⁸⁴ Specifically, after the template was formed, the polymer was removed by gas plasma treatment. The exposed SiO₂ was then passivated with PEG–silane and thiol terminated peptides were subsequently attached to the Au dots. By varying the molecular weights of the polystyrene and poly[2-vinylpyridine(HAuCl₄)_{0.5}] components of the diblock copolymer, variable spacing between the single 8 nm Au dots was achieved. They reported that spacing less than 73 nm was necessary to induce focal adhesion assembly (Fig. 8). This corroborates with other studies, which suggest nanoscale presentation and clustering of ligands is critical for cell adhesion. By employing the same patterning technique along with magnetic tweezers, this group also reported that decreasing the spacing from 145 to 58 nm resulted in an increase in the initial cellular unbinding forces from ~6 to over 200 pN.⁸⁵ Groll *et al.* also employed this system to create hexagonal patterned gold particles. In their study, the background was passivated with a star PEG, which only allowed the top of the gold dots to be exposed for thiol terminated biotin and avidin binding.⁸⁶

5.3 DNA templating

Yan *et al.* utilized DNA templates to create protein arrays.⁸⁷ 4 × 4 Tiles of DNA with sticky ends self-assembled into lattices forming nanoribbons or nanogrids. By incorporating biotin into the center of the tiles, periodic arrays of streptavidin (~5 nm) with separations of ~19 nm were fabricated (Fig. 9). In a follow up study by Park *et al.*, they

incorporated two distinct tiles of DNA: one which contained biotin and one which did not.⁸⁸ The two tiles formed ordered patterns in the nanogrid by specifically designed sticky ends that promoted organized self-assembly. Streptavidin arrayed to every other tile. They also demonstrated patterning of the two biotin containing tiles, which created denser streptavidin arrays.

A major advantage of these self assembly processes, particularly of the polymer assisted and DNA templating approaches,^{84,87,88} is that very high resolutions are obtainable. In addition, although there are often defects, medium to large pattern areas are possible, particularly with colloidal lithography.⁷⁵ Challenges common to most self assembly systems is that the pattern is usually repetitive, the feature shape is predetermined by the particles or templating molecules, and

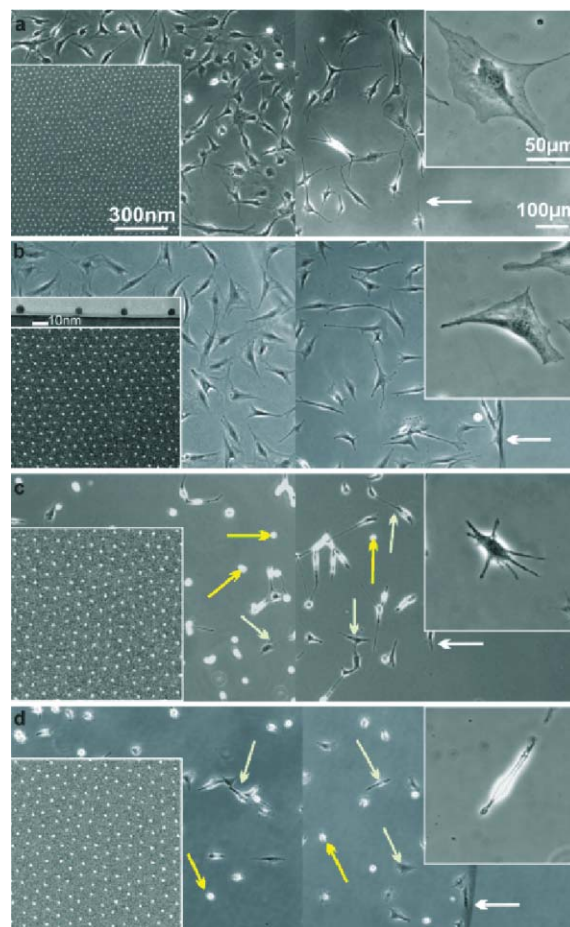


Fig. 8 Nanopatterned RGD surfaces for MC3T3 osteoblast adhesion. SEM images of Au dots are shown in the left insert. Dot spacing is varied between 28 (A), 58 (B), 73 (C), and 85 nm (D). Reprinted with permission from ref. 84. Copyright 2004 Wiley-VCH.

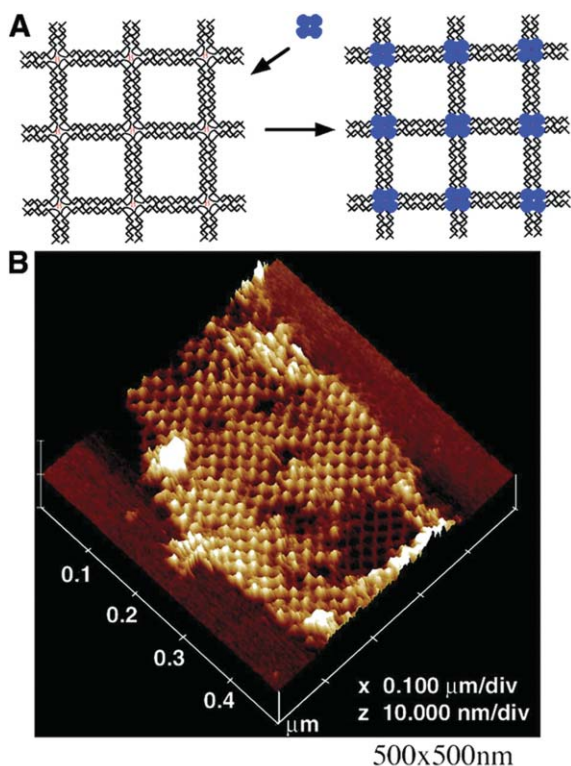


Fig. 9 Protein arrays generated by self-assembling 4×4 DNA nanogrids. Biotin is incorporated into one of the loops at the center of each tile. Streptavidin (blue) then binds to biotin, creating protein nanoarrays. (B) AFM image of the streptavidin array assembled on the DNA nanogrid. Reprinted with permission from ref. 87. Copyright 2003 AAAS.

patterning more than one ligand would be difficult.⁸⁹ However, these could potentially be addressed in combination with other techniques.⁸⁶

6. Other approaches

While the methodologies described above are more abundant in the literature, there also exist a handful of other techniques that researchers have employed to selectively pattern proteins. Bergman *et al.* fabricated arrays of 60 nm pits in gallium arsenide and silicon wafers using a finely focused ion beam.⁹⁰ Human serum albumin was immobilized only to the inner rims of the pits in gallium arsenide. The selective adsorption was attributed to surface changes as a result of ion bombardment. Bruckbauer *et al.* created protein patterns using a focused ion beam and scanning ion-conductance microscopy (SICM).⁹¹ Nanopipettes created from glass capillaries were filled with IgG solution and delivered to a glass substrate by applying a voltage. In order to create nano-sized patterns of the protein, they first created ~ 300 nm wells in a gold coated glass substrate using a gallium ion focused ion beam microscope.⁹² The gold was coated with a hexa(ethylene glycol) SAM, while the holes were filled with a 3-mercaptopropionic acid SAM to promote IgG immobilization. Using the nanopipet, IgG and anti-IgG were sequentially delivered to the holes.⁹²

Patolsky *et al.* attached an aldehyde terminated silane SAM to silicon nanowires.⁹³ Antibodies specific for anti-hemagglutinin (influenza A) and anti-adenovirus group III were then attached to the aldehydes *via* reductive amination. Virus binding could subsequently be detected through the change in conductance of the ~ 20 nm silicon nanowires.

Zhu *et al.* employed mechanical mismatching of films to create patterns for protein adsorption.⁹⁴ PDMS substrates were treated with O_2 plasma which created silica like thin layer films. The surface was then passivated with (tridecafluoro-1,1,2,2-tetrahydrooctyl)-1-trichlorosilane *via* deposition, followed by incubation with a Pluronic F108 solution. Due to the mechanical mismatch between the top brittle film and the underlying elastic bulk, parallel arrays of cracks were created by applying uniaxial tensile strains. Proteins could then be immobilized to the exposed under layer at the location of the cracks. Crack spacing, width (120 nm–3.2 μ m), and depth could be modulated by adjusting the mechanical properties of the system. They demonstrated fibronectin adsorbed to the cracks, which then mediated myoblast and neuroblast adhesion and growth. Crack widths and subsequent cell morphology were altered by changing the strain.

Welle *et al.* utilized a combination of electrospray ionization and Coulomb force-directed assembly to pattern both BSA and avidin into 200 nm wide patterns.⁹⁵ They used electrospray ionization to create a gas phase of charged protein molecules. A global electric field was then applied, which brought the proteins close to the oppositely charged surface. The surface consisted of charged patterns, which were formed by a conductive electrode, in a thin PMMA layer. Proteins were subsequently adsorbed to the charged patterns.

Our group has recently developed a photolithographic approach using a pH-responsive polymer to pattern proteins at the submicron scale.⁹⁶ Poly(3,3'-diethoxypropylmethacrylate) (PDEPMA) along with a UV sensitive photoacid generator (PAG) was spin-coated onto Si-SiO₂ substrates. Following exposure to UV light through a mask, acetal groups within the polymer were converted to aldehyde functionalities. Aminoxy-functionalized biotin was subsequently attached to patterned aldehydes without the addition of other reagents, forming oxime bonds (Fig. 10). This then allowed for the precise patterning of streptavidin. This methodology was first validated on the micron scale by creating 18×18 micron patterns of red fluorescent streptavidin with a deep UV exposure tool.⁹⁷ We have more recently fabricated submicron patterns of streptavidin within a protein resistant PEG background using an i-line stepper.⁹⁶ Streptavidin patterns were then used to form protein assemblies of biotinylated anthrax toxin receptor and protective antigen, a moiety of anthrax toxin, with 500 nm resolution (Fig. 11). A significant advantage of this technique is that hundred or thousands of features are generated simultaneously and large areas can be patterned. A limitation of this technique is that the resolution is restricted, and thus compared to the other above mentioned techniques the feature sizes are larger. Another advantage is that multiple proteins could potentially be patterned using a step and repeat process.

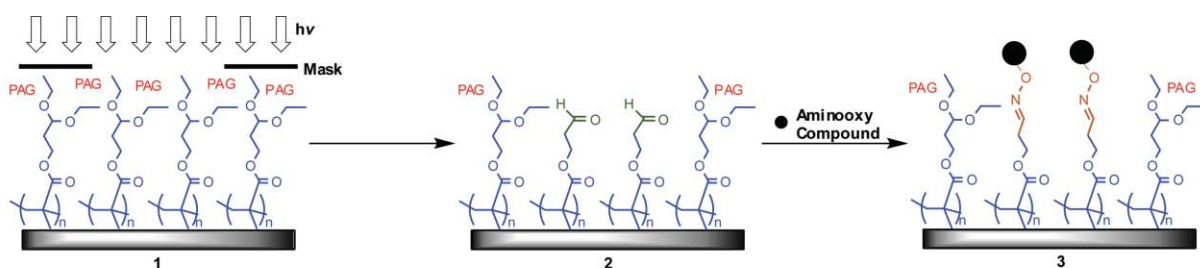


Fig. 10 Photolithographic patterning using a pH-responsive polymer. Upon exposure to UV light through a mask (1), acetals are hydrolyzed to aldehydes (2). Aminoxy-terminated compounds can then bind to patterned aldehydes through oxime bonds (3). Adapted with permission from ref. 97. Copyright 2005 American Chemical Society.

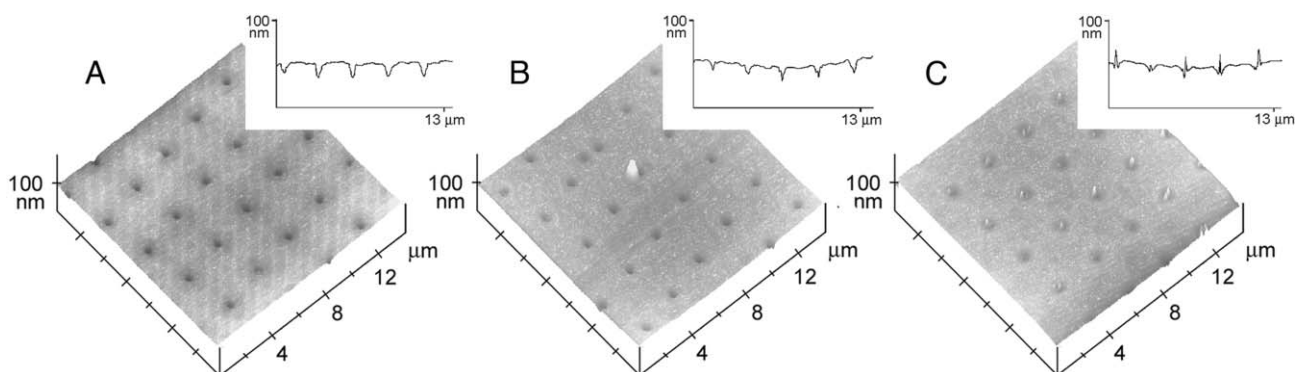


Fig. 11 Sub-micron protein patterning using photolithography. Tapping mode AFM image of patterned assemblies of streptavidin (A), biotinylated anthrax toxin receptor (B) and protective antigen (C). Features sizes are ~ 500 nm. Reprinted with permission from ref. 96. Copyright 2006 American Chemical Society.

7. Summary

Interdisciplinary work in biology and nanotechnology has begun to provide useful techniques for patterning peptides and proteins at the nanoscale (Table 1). High resolution patterns over large areas have been demonstrated. Application in

sensing disease markers and as cell adhesion substrates has also been shown. A major and exciting challenge for the future is to pattern tens or hundreds of different proteins or peptides into arrays. This, along with the advent of new characterization tools and detection methods, may allow for protein nanoarrays to become routine diagnostic tests in clinical settings. Furthermore, nanoscale patterning of cell adhesion proteins and peptides will continue to aid in the fundamental understanding of cell–matrix interactions, and thus allow for the creation of more robust biomaterials.

Acknowledgements

The NIH NIBIB (EB 005838-01) and the NSF through SINAM (DMI-0327077) is thanked for providing funding. KLC thanks the NIH NHLBI for a NRSA postdoctoral fellowship. Hewlett-Packard and CNSI are also thanked for funding part of the authors' research described herein.

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Table 1 Resolution for nanopatterning techniques

Technique	Maximum reported resolution/nm	Reference
Scanning probe microscopy		
Nanografting	10	12
Indirect dip-pen nanolithography	~ 80	28
Direct dip-pen nanolithography	~ 30	25
Nanopen	250	42
Conductive atomic force microscopy	20	43
Stamping		
Micro/nanocontact printing	~ 70	53
Nanoimprint lithography	75	55
Electron Beam	50	72
Self Assembly		
Particle lithography	60	75
Polymers chain arrangement	< 8	84
DNA templates	~ 5	87, 88
Focused ion beam	60	90
Scanning ion-conductance microscopy	~ 300	92
Chemical vapor deposition—nanowires	~ 20	93
Mechanical mismatch—cracks	120	94
Electrospray ionization and Coulomb force directed assembly	200	95
Photolithography	~ 500	96

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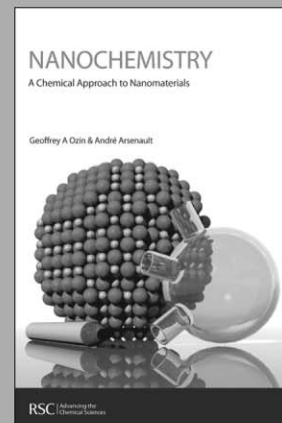
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