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Fabrication and applications of the protein patterns†

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Protein has been widely used for fabricating patterned structures since it is one of the most important macromolecules in living organisms, and protein patterns possess potential applications in many fields such as medical diagnosis, tissue engineering, biosensors, and medical screening. At present, there are two fashions to fabricate protein patterns: one is grafting the protein to the microstructure which is prepared by micro-fabrication techniques; the other one is achieving the patterned protein structures directly. Here we provide an overview on current status of the fabrication techniques and the applications of the protein patterns, and then give an outlook on the development of the fabrication techniques and the prospective applications of the protein patterns in future research.

protein pattern, microfabrication techniques, sensing, cell adhesion

1 Introduction

As an important category of macromolecules *in vivo*, protein plays an important role in structure constitution and performance of the tissue functions, such as tissue constitution and repair, constitution of the antibody, formation of the immunologic barrier, adjusting the osmotic pressure to maintain the balance between normal human plasma and tissue fluid, supply heat to keep the body's temperature. Thus protein performs an indispensable part of the most important macromolecules which help the body to maintain the ordered life activities.

The patterned surfaces, which are constituted of proteins, had caused great interest in recent years, and they performed a wide range of applications in the field of biosensors, biomedical interfaces and drug screening [1–5]. As an important class of macromolecules *in vivo*, protein has been widely used to fabricate the micro-patterned structures, and the patterns have many applications in the field of sensors, medical diagnostics, inducing the cell adhesion, regulating the cell morphology, micro-devices and preparation of the inorganic crystal patterned structures. Here we provide an overview on the status of the fabrication techniques and the applications of the protein patterns, and then give an outlook on the development of the fabrication techniques and the prospective applications of the protein patterns in the future research.

2 Methods of fabricating protein patterns

At present, there are mainly two methods to fabricate protein patterns, one is grafting the proteins to the microstructures which were prepared by microfabrication techniques, the techniques include photolithography, colloidal lithography, electron-beam lithography, micro-contact printing, nanoimprinting lithography; the other one is using the protein to achieve the patterned structures directly which is always combined with the atomic force microscopy tips, such as dip-pen nanolithography. As follows, we will give a description of these two methods on fabricating patterns and the applications of these techniques.

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2.1 Photolithography

So far, photolithography is the most successful and widely used technique to fabricate micropatterns [6]. Chen *et al*. [7] developed a simple approach to produce switchable superhydrophobic surfaces by the combination of photolithography and reactive ion etching method whose water contact angle could be switched from a superhydrophobic state to a completely wetted state. In the superhydrophobic state, the switchable superhydrophobic surfaces exhibited a protein resistant property similar to that of PEG surface. However, using an electric field, the same surfaces could convert to a wetted state which promoted the adsorption of protein molecules, and then the protein pattern achieved. Figure 1(A) illustrates the fabrication process of the switchable superhydrophobic surfaces which would be used to prepare the protein microarray. Firstly, a layer of photoresist was spun on the top of fluoropolymer which was coated on pre-patterned ITO electrodes and a photolithography process was used to define the superhydrophobic area on the photoresist. The superhydrophobic microarray was manufactured by oxygen plasma treatment. After plasma treatment, the remained photoresist was removed by rinsing the surface with acetone. So only the areas exposed to the oxygen plasma exhibited the superhydrophobic behavior. Then dropping the protein solution onto the top of the microarray and a 150 V voltage was applied to the selected ITO electrodes to induce the removement and absorption of proteins to the hydrophilic region. After washing the chip with PBS solution, protein microarrays with desired patterns were produced. Figure 1(B) shows the differential interference contrast (DIC) image of part of the chip where the areas inside the circles are the switchable superhydrophobic surfaces and the areas outside the circles are the flat Teflon surfaces, the fluorescence image of part of the patterned FITC-conjugated anti-chicken IgG chip, and the variation in fluosity from each spot is around 10% as indicated by the line rescence intenintensity profile. These results clearly demonstrate that it is possible to pattern uniform protein arrays using the switchable superhydrophobic surfaces. It is worthwhile to point out that using this method up to 100 × 100 protein spots could be deposited simultaneously within one second.

Dubey *et al*. [8] demonstrated the self-selective patterning of streptavidin and HaloTag™ into adjacent regions of biotin and chloroalkane respectively which were prepared onto a PEG based multicomponent hydrogel via selective photolithographic patterning from the same solution. The patterns were subsequently characterized by fluorescent microscopy and ToF-SIMS. Rapp *et al*. [9] reported the development of a lithography system using a digital mirror device which allows fast patterning of proteins by immobilizing fluorescently labeled molecules via photobleaching. Grayscale patterns of biotin with pixel sizes in the range of 2.5 mm are generated within 10 s of exposure to an area of about 5 mm^2. This maskless projection lithography method permits the rapid and inexpensive generation of protein patterns definable by any user-defined grayscale digital image on substrate areas in the mm^2 to cm^2 range.

2.2 Micro-contact printing

Twenty years ago, the Whitesides group [10–12] prepared protein patterned surfaces with feature size of micrometer scale by micro-contact printing. As a kind of soft- lithography, micro-contact printing is an efficient technique to fabricate patterned surfaces with low cost over large area, and the pattern resolution can reach submicrometer [13], so this method still very popular in these days.

With the development of fabrication techniques to produce the micro-contact printing stamp, nanometer scale contact printing (nano-contact printing, nCP) of single [14]

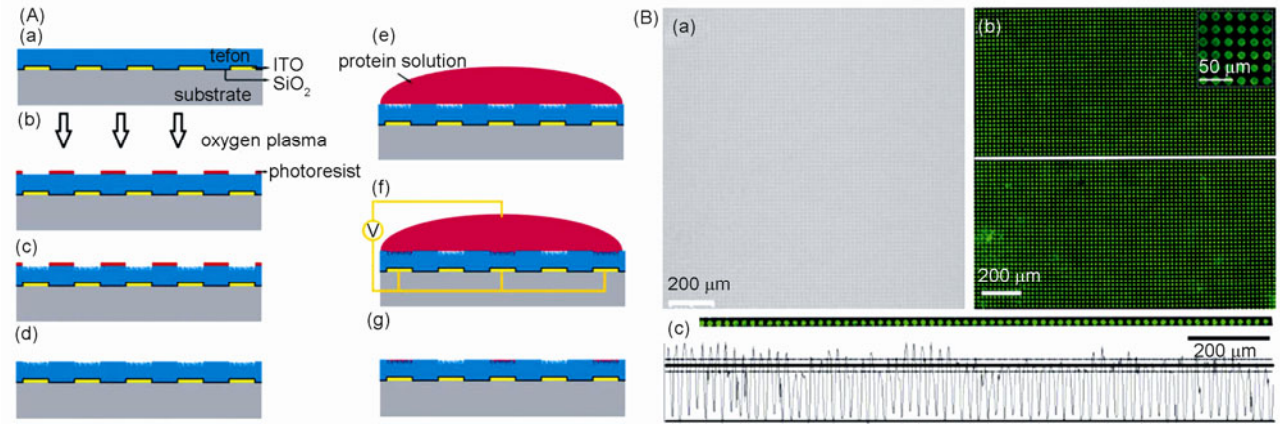

Figure 1 (A) Schematic of the patterning process of proteins. (B) Images of the microarrays and the protein pattern: (a) DIC image of the superhydrophobic microarray; (b) Fluorescence image of the patterned FITC conjugated antichicken IgG on the switchable superhydrophobic microarray, the applied voltage was 150 V, inset is the enlarged view; (c) Line image and intensity profile of the protein microarray measured along the white line in (b), the solid line is the averaged intensity and the dashed lines are the 10% variation range [7].

and multiple [15] protein patterns have been achieved by Delamarche's group. Figure 2(a, b) shows the schematic process for the fabrication of a micropatterned stamp and how it was used to print proteins for the preparation of protein patterns. Firstly, they made an elastic polymer stamp, and immersed it in the "ink" (a solution of protein) and then the protein absorbed to the surface of the stamp through physical absorption. After that, they let the stamp contact to the substrate which will be used to make protein patterns tightly, then a monolayer of protein on the surface of the stamp which can be transferred to a target substrate after rinsing and drying by printing. Figure 2(c) shows the fluorescence image of the Rhodamine-labeled antibody pattern which was transferred to the substrate, and the result confirms that the patterned proteins remained bioactivity. After that, the author used different stamps which have different patterns and/or various inks to print several times onto the same substrate, used the microfluidic networks as microcontainers to define the areas of inking, and used the same stamp to print continuously, and they achieved different patterns with two (Figure 2(d)), sixteen (Figure 2(e)) and three (Figure 2(f)) kinds of proteins, respectively.

In the processes of fabricating protein patterns by micro-

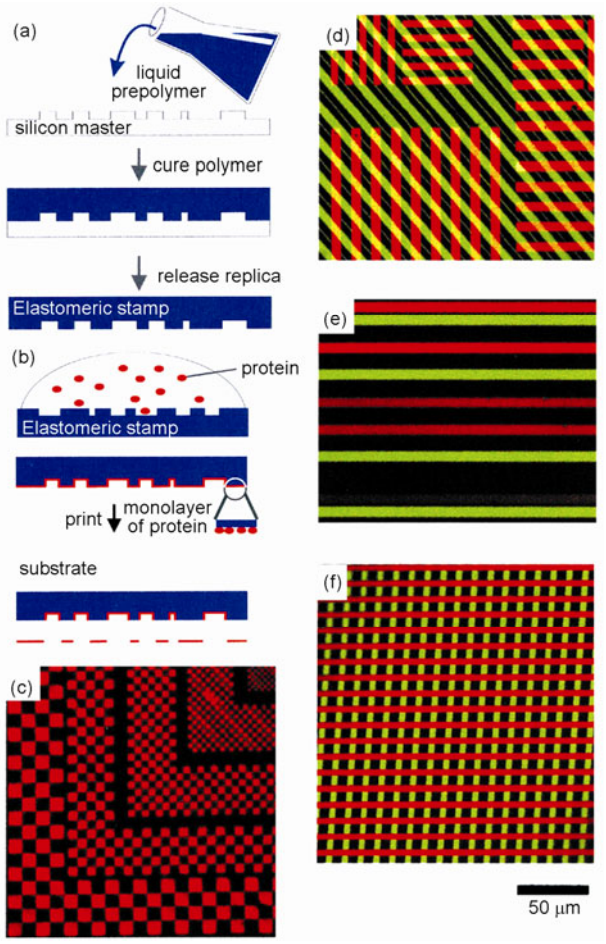

Figure 2 (a) Scheme of fabrication of a stamp and (b) its use in μCP; (c–f) Fluorescence images of different protein patterns [15].

contact printing, it is mostly depended on the unstable physical absorption between protein and substrates, and the patterns usually cannot be remained when they are under the strike of external forces. Thus, some groups used the reactive micro-contact printing to replace the physical absorption [16]. Since it needs to use the e-beam lithography to fabricate the stamp of the micro-contact printing, and the e-beam lithography is costly and can't achieve large area samples, researchers usually use the reactive micro-contact printing method to fabricate protein patterns, and it is always used at the initial stage when fabricating protein patterns. The basic process of this method is as follows. Firstly, prepare the stamp which will be used for printing, and coat the stamp with the protein solution which is aimed to be patterned. Then make the stamp contact with the substrate which have a reactive layer that can be reacted with the protein. After the protein covalently binding to the substrate through the reaction between the protein and the reactive layer, the protein patterns are achieved after removing the stamp.

Micro-contact printing process is simple, low cost and can achieve large area sample preparation, also the stamp of this method has a high degree of flexibility, then it can be used to achieve large area patterns with different proteins in a simple way. This method has many advantages, but when using it to fabricate protein patterns there usually have some external forces which have strong interactions with the proteins that can easily cause the protein denaturation, such as the tension and pressure. Thus, reducing the external forces and enhancing the biological activity of the protein molecules are still the challenges we must face in the future development of this technology.

2.3 Colloidal lithography

As a patterning method, colloidal lithography can also be used for fabricating protein patterned surfaces, and it is relatively novel. At present, there are two methods to prepare the colloidal lithography template which will be used for the immobilization of proteins: one is that the colloids deposited onto the substrates electrostatically in a mode with certain separation space; the other is that the colloids assembled into colloidal crystals which have a two dimensional hexagonal close packed structure. In the process of geometrical templating, Michel *et al.* [17] combined the SMAP method and colloidal lithography technology to fabricate TiO_2 nanopillars, and then the protein were absorbed to the dodecyl phosphate (DDP) while PLL-g-PEG were grafted to the surface of the SiO_2 which made the SiO_2 protein resistant. After that they achieved the streptavidin pattern through grafting the streptavidin to the nanopillars covalently with the feature size of 50 nm over a large area. A similar approach was reported by Agheli *et al.* [18]. In their approach polystyrene particles were assembled on the surface of SiO_2 substrate which was covered with an Au layer

to form a hexagonal close packed structure through electrostatically assemble, and then made the structure be unclose packed structure by particle annealing and use the structure as mask to etch the Au layer with SiO_2 background to make the Au layer into gold nanodiscs. After that alkanethiol was deposited onto gold, followed by the PLL-g-PEG layer on SiO_2. At last, Laminin, BSA and polyclonal/monoclonal anti-mouse laminin were immobilized onto the gold nanodiscs and were characterized by AFM height histogram and QCM. Cai *et al.* [19] prepared hexagonally arranged nanoarrays of lysozyme on 1 cm^2 substrate with a feature size of 120 nm. In their experiment, they made the 10-undecenyltrichlorosilane (UTS) deposited onto the Si substrate first, and then the colloidal template and PEG-silane. After removing the colloidal particles by ultrasonic, they deposited the lysozyme onto the UTS exposed surface and got the lysozyme pattern. At the same time Blatter *et al.* [20] also fabricated some submicron protein arrays by using similar methodology. Valsesia *et al.* [21] prepared patterned monolayer which have a chemical contrast component by using colloidal lithography, and then they studied the application of this pattern in fabricating protein arrays. Soon after this work, they prepared PAA nanoarrays and deposited PEG onto the surface, with the removing of the remained PS microspheres they achieved the patterned arrays with the feature properties of bioadherent and bioresistant [22], and this kind of polymer structure could be used for specific protein absorption to achieve the specific protein arrays. Recently, Kingshott *et al*. [23] invented a technique for the fabrication of binary protein patterns. They used the charged PS microspheres with different diameters to absorb different proteins, and then assembled the different microspheres into two-dimensional binary-colloidal crystals by the method of self-assembling, at the same time they got the binary protein patterns. From the images of fluorescence microscopy and atomic force microscopy, they evaluated that the protein patterns were successfully prepared and the nanoarrays performed an ordered arrangement in a large area.

The protein on the nanoarrays which fabricated by the absorption method can be easily denatured since the absorption is uncontrollable, thus it limited the application of the biomaterials or devices. For this reason, it is important to invent a new method to prepare biomolecule patterns which can be used in the field of biomedical since the molecules in the patterns can maintain their activity. Lately, our group invented an approach to fabricate protein patterns which is based on the colloidal lithography [24], the schematic process is shown in Figure 3. Firstly, we patterned the atom-transfer radical polymerization (ATRP) initiator which were immobilized on the surface of the substrate by colloidal lithography, and then initiated the polymerization of PHEMA from the patterns of ATRP initiators, after that we grafted the protein onto the polymer chains covalently through the coupling reaction, then we got the protein patterned surfaces. Then the samples were investigated by fluorescence microscopy under blue light excitation. The uniformity of the IgG distribution were demonstrated by Figure 3(b), and the signal intensity across the entire area was almost the same. After enlarging the Figure 3(b), every protein dot could be seen clearly (Figure 3(c)). These results indicated that the proteins were not denatured during the immobilization process. Such protein nanoarrays may pave a way to fabricate high sensitivity and signal-to-noise ratio biosensor to detect antibody–antigen interactions. In principle, each kind of protein can be covalently grafting to the polymer chains to achieve protein patterns. Subsequently, FN patterns were used to perform experiments of cell culture. Cell experiments confirmed that mouse MC3T3-E1 osteblasts adhered well onto the multiscale patterns of FN resulting in organized cell patterns, and the cells maintained good biological activity. In our experiments, after being cultured for three days, the cell patterns were found to maintain well. Then we stained the skeleton, the results presented that most of the cells adhere onto the FN regions since the FN could promote cell adhesion. Moreover, localizing of single cell could be realized with the assistance of multiscale patterns with a diameter of 20 μm. More importantly, taking advantage of microstripe patterns of FN, the adhesion and elongation of cells could be controlled simultaneously [25].

As an advanced technique, by using colloidal lithography one can prepare large area protein patterns with low cost and without the auxiliary of expensive equipments. At the same time, the colloidal crystals can be easily prepared; the feature size of the pattern can be simply regulated by changing the colloidal particle's diameter which can as small as tens of nanometers, we can also modulate the feature size by adjusting the experimental parameters such as the pre-annealing of the colloidal crystals or slope vapor deposition to change the sreucture's feature size, and the protein pattern can maintain their biological activity. The

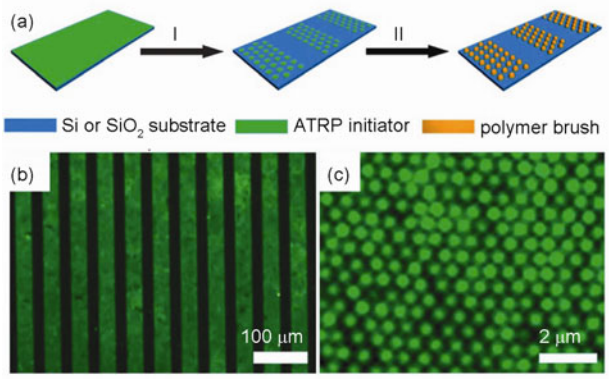

Figure 3 (a) Schematic process of preparing hierarchical polymer brush patterns; (b) The fluorescence image of the antibody patterns after bonding FITC conjugated secondary antibody; (c) The enlarged fluorescence image of the antibody patterns after bonding FITC conjugated secondary antibody [24].

same as other techniques, colloidal lithography has its own drawbacks since the mask used in this technique is colloidal spheres which limited the pattern's symmetry, and the defects can't be avoided during the assembling process of the microspheres. In general, colloidal lithography provides a new method to fabricate protein patterns and inject a new vitality for the development of the techniques.

2.4 Nanoimprinting lithography

Another way to fabricate protein patterns is nanoimprinting lithography which has the significant advantages such as rapid fabricating process, high throughput, low cost and so on. Nanoimprint lithography also uses a stamp to prepare physical features on the substrate which can be used for protein immobilization. In this case, the stamp is usually an Si template which is prepared by using the traditional lithography techniques. Then the stamp is made to contact with a polymer film and the polymer film was heated above its glass transition temperature, and achieve an imprint on the film. Then the imprinted substrate was modified to prepare protein patterns.

Hoff *et al.* [26] used a silicon stamp to imprint patterns onto the PMMA coated SiO_2 substrates. After removing the stamp, they used the reactive ion etching (RIE) to remove the residual PMMA and deposited a thin layer of CFx polymer residue on the SiO_2 substrate to make it be biological resistant. Then the PMMA was patterned into stripes and leaving patterns of SiO_2 and CFx polymer. At last, an amine-terminated silane were grafted to the oxide surface and then bond to a biotin–succinimidyl ester. Then when the surface was exposed to the solution which concludes streptavidin, it could be used to immobilize any kinds of target proteins which was biotinylated selectively. Using this method, they prepared the stripe patterns of the biotinylated BSA which has a feature size of 75 nm (Figure 4(b)), after they added the rhodamine-labeled streptavidin solution they got the fluorescence image of the protein pattern by using the fluorescence microscopy (Figure 4(c)).

Falconnet *et al.* [27] also used a Si stamp to imprint morphologies onto the PMMA coated substrates. The residual PMMA was removed by etching to create patterns of Nb_2O_5 in a background of PMMA. The substrates were then immersed into biotin functionalized poly (L-lysine)-graft-poly (ethylene glycol) (PLL-g-PEG/PEG-biotin). After removing the remained PMMA with acetone, the exposed substrate was passivated with PLL-g-PEG. Finally, streptavidin immobilized onto the patterned biotin regions, and achieved the protein patterns with a feature size of 100 nm.

Nanoimprinting lithography has been widely applied to solve different biologically related questions since it is a promising low cost method to fabricate nanoarrays for biosensing, and it has a great advantage since many biological researches require meaningful statistics. At present, there still remain some problems (e.g., alignment, demolding, fouling of the mold) need to be solved to increase the pattern's fidelity especially for a dense structure over a large area. However, nanoimprinting lithography may achieve the single molecule's immobilization onto a substrate in well defined areas. Nanofluidic devices prepared by nanoimprinting lithography may allow for the manipulation of biomolecules, which would be useful in the fabrication of lab-on-a-chips.

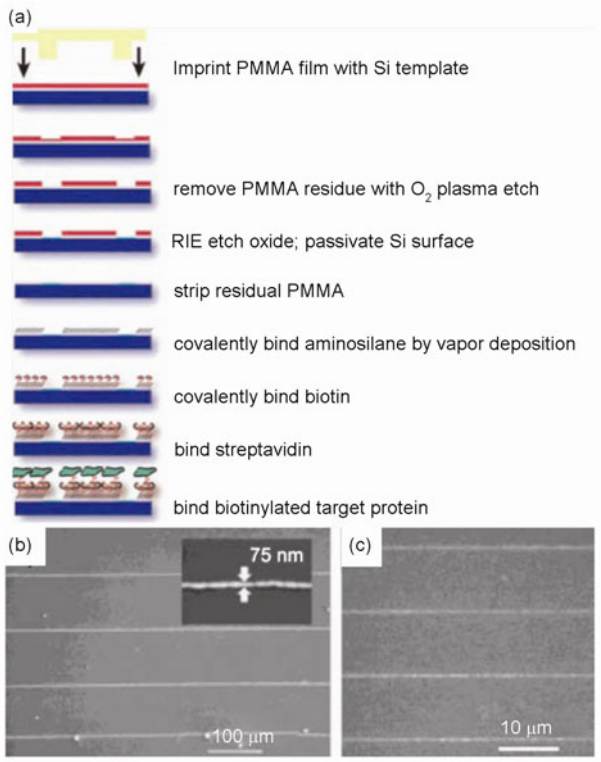

Figure 4 (a) Schematic process of substrate patterning and protein immobilization; (b) SEM image of oxide nanolines formed on a Si substrate; (c) Fluorescence image of nanolines after patterning with biotinylated BSA and binding rhodamine conjugate streptavidin [26].

2.5 Electron beam lithography

As a maskless patterning method, electron beam (e-beam) lithography uses an electron beam to scan across the surface which makes it possible to produce high resolution patterns with feature sizes as small as 5–10 nm [28]. Thus, e-beam lithography is coming into fashion of patterning biomolecules. Since this method can not only achieve arbitrary protein nanopatterns with different shapes, sizes, and curvatures but also control the spaces and locations of the patterns precisely, it is possible to fabricate patterns with nanoscale interfeature spacing which can be used for preparing heterogeneous patterns.

Based on the background that mentioned above, Maynard *et al.* [29] prepared the first protein nanopatterns which contains multiple proteins by using the e-beam lithography. The schematic process of their experiment is

shown in Figure 5(a). Firstly, they modified the eight-arm poly-(ethylene glycol) (PEG) with one of the four protein reactive moieties: biotin, maleimide, aminooxy, or nickel (II) nitrilotriacetic acid (Ni^{2+}-NTA) (Figure 5(a)). Each of these groups can covalently bind the proteins at distinct sites. Then using the e-beam to etch the PEG to prepare patterns based on that when PEG is exposed to focused e-beams, it cross-links to itself and Si surfaces, but the unexposed area can be easily washed off, then the patterns of the protein-reactive PEG hydrogel come out which can react with protein effortlessly, after that they modified the exposed substrate via the chosen functionality to make it resist nonspecific binding and after the sample was immersed in the specific protein solution, they got the different protein nanopatterns (Figure 5(b–e)).

Besides, the three-dimensional arrangements of multiple proteins also performs to have many sophisticated applications [30] such as three-dimensional structures with micron size consisting of a single protein have been widely used to introduce the neuronal development [31], to trap bacteria [32], and for bioelectronics [33]. Maynard *et al.* also fabricated the first multicomponent, multilayer heterogeneous protein patterns which range from the micron scale to the nanoscale by using the e-beam lithography. And Howorka *et al*. [34] fabricated the high-resolution protein patterns which can react with different ligands by using the e-beam lithography. After bonding different ligands and characterized by the fluorescence microscopy, they achieved a simple gray-scale image of the medieval painting–"The Mona Lisa".

2.6 Dip-pen nanolithography

As a new patterning technology, Dip-pen nanolithography (DPN) has been widely concerned since Mirkin's group first reported in 1999 [35]. The main principle of this method is as follows. When the tip is close to the substrate, there will form a liquid bridge between the tip and the substrate since the tip is covered by the "ink", due to the physical or chemical absorption between the molecules in the "ink" and the

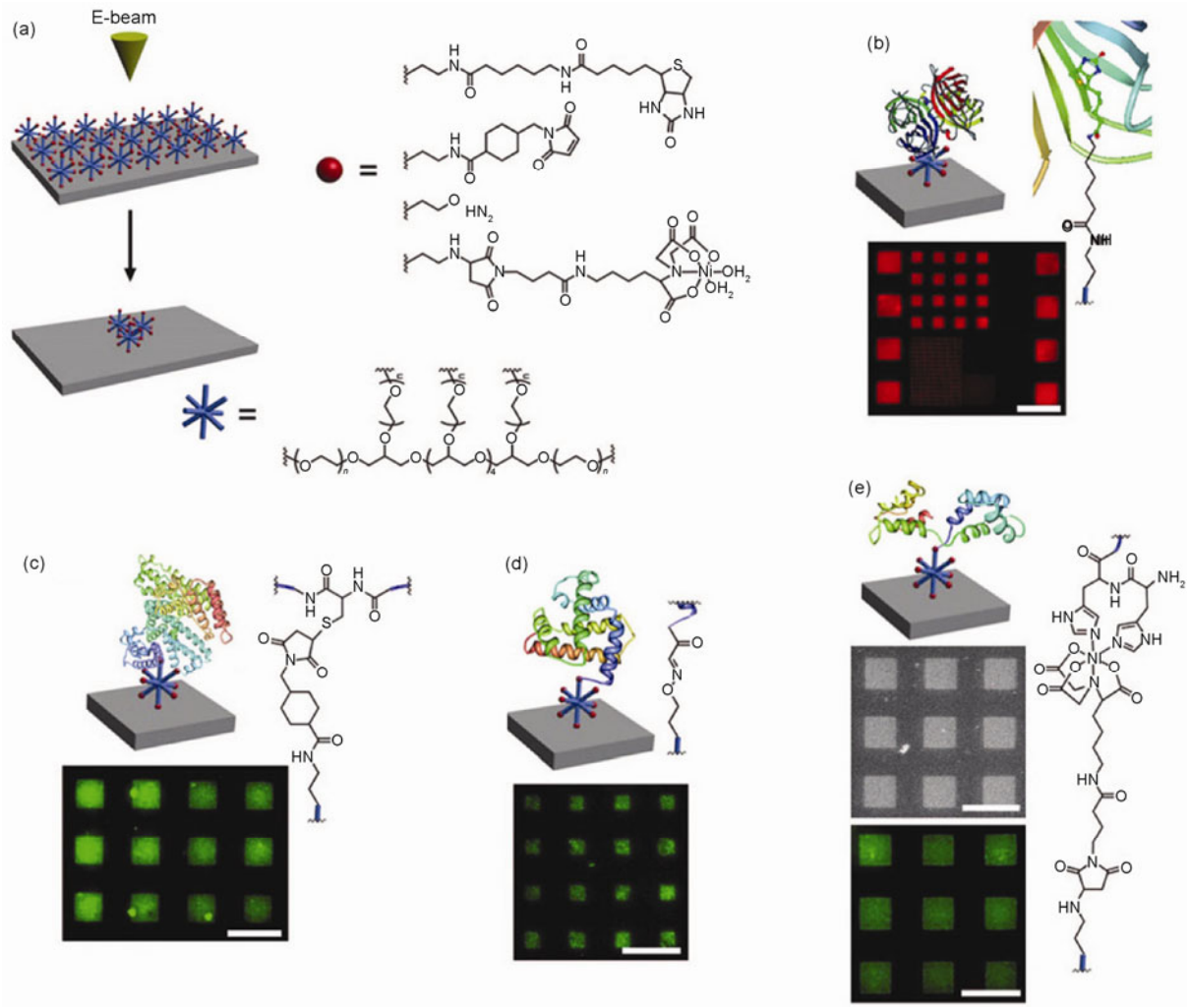

Figure 5 Electron-beam cross-linking of end-functionalized eight-arm PEG polymers for protein patterning [29].

substrate, the molecules in the ink will move to the substrate automatically, then one can get different patterned structures with the control of corresponding computer program [36]. For the specific molecules, the resolution of the patterns can be controlled by scanning speed, the chemical properties of the surface, temperature and humidity [37, 38]. By using the DPN technology, patterned structures of different materials can be easily prepared, such as conductive polymers, biomacromolecules, bacteria and so on [39–44]. Since the protein patterns of micron or nano scale, which was fabricated by DPN can be used to detect the target matter in a little sample sensitively, it performs great advantages for the applications in the field of biological recognition and medical diagnostics. Besides, the size of the protein patterns can be easily controlled through regulating the contact time between the tip and the substrate, and the protein patterns performs to be homogeneous and the biological activity maintains.

Early in 2002, Mirkin *et al.* [45] fabricated the protein microarray patterns by DPN. They fabricated the patterns of 16-mercaptohexadecanoic acid (MHA) on the substrate which was covered with a gold layer by using the tips which were covered by an MHA solution. Then they modified the exposed area with 11-mercaptoundecyl-tri(ethylene glycol) to make it protein resistant, after that they immersed the substrate into the protein solution and the protein grafted to the MHA targetly, then with a final treatment they got the protein patterned surface with sub-100 nm resolution. In addition, the nonspecific absorption would not happen in the resistant areas of the patterns even it was reacted with a multiple protein solution, and the patterns could be used for cell culture to study the cell adhesion and induce the actin skeleton grafting to the substrate.

At present, Mirkin group [46] fabricated multiplexed protein arrays by polymer pen lithography. The basic process of this experiment is shown in Figure 6(a): firstly, they fabricated the pyramid-shaped wells arrays in a Si substrate and filled them with protein solutions by inkjet printing. Then, the polymer pen array was treated with oxygen plasma to made the surface hydrophilic to minimize nonspecific adhesion of proteins. After that the pen array was placed in an NSCRIPTOR (NanoInk, Skokie, IL) nanolithography equipment and dipped in the wells to absorb the proteins onto the surface of the pen array. Then they took out the inked polymer pen array and used it to write directly on a Codelink slide which has a surface that have been modified with Nhydroxysuccinimide (NHS) ester functional groups. After the amine groups of the proteins reacted with the NHS esters completely through incubate the patterned slide at 4 °C for 8 h, they achieved the protein pattern with the slide was passivated by bovine serum albumin (BSA), rinsed with PBS buffer, and dried. Figure 6(b–d) shows the fluorescence images of a Si mould inked with three proteins (Figure 6(b)), a polymer pen array dipped into the Si mould (Figure 6(c)), and multiplexed proteins arrays made by PPL (Figure 6(d)), each pen in an array was used to make a 5 × 5 protein dot array with 5 μm spacing between the dots. During the process of fabricating protein patterns by DPN, they could control the feature size of the patterned structures from sub-100 nm to several-μm length scale through varying the tip-substrate contact time and contact force, even they could use 1600 tips to write directly to fabricate multiple protein patterns rapidly.

Recently, works in this field have attempted to overcome some drawbacks related to the technique, such as maintaining the biological activity of protein, the limited size of the pattern and the amount of protein deposited. Wu *et al.* [47] developed a tip modification method which references the principle of layer-by-layer to fabricate hydrophilic porous structures on the surface. This method increases the amount of protein solution that the tip can hold while maintaining the biological activity of the protein. Bellido *et al.* [48] reported the possibility of controlling the amount of proteins which deposited onto the surface by direct DPN. Nanoarrays of ferritin were fabricated directly on TEM grid and the amount of the molecules that deposited on the grid was well controlled through regulating the initial protein concentra-

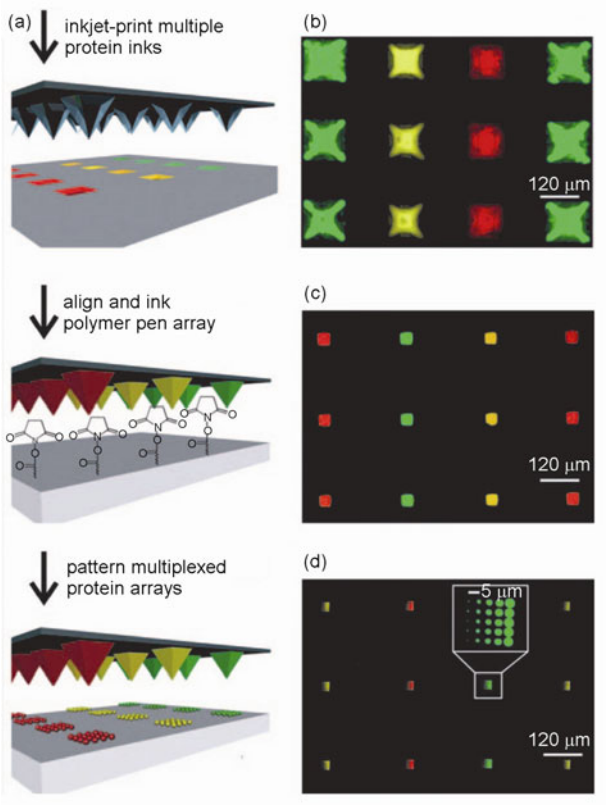

Figure 6 (a) Schematic of making multiplexed protein arrays by PPL patterning process; (b–d) Fluorescence images of (b) a Si mould inked with three proteins, (c) a polymer pen array dipped into the Si mould, (d) multiplexed proteins arrays made by PPL. Yellow: TRITC-conjugated anti-mouse IgG; Green: Alexa Fluor 488-conjugated anti-prostate specific antigen (anti-PSA); Red: Alexa Fluor 647-conjugated anti-cholera toxin beta (anti-CTb) [46].

tion, the diameter of the array dot and the contact angle between the protein solution and the substrate.

In conclusion, DPN is a versatile direct-writing nanopatterning technique which can not only be used for the fabrication of protein patterns but also for the preparetion of chemical templates for the study of biorecognition processes. Since this method can be used to fabricate protein patterns with small sizes but multi-dimensional, it is able to fabricate patterns with the size of individual biological macromolecules and offer the potential applications to study the interaction between the single protein and cell. Even though, since this method is based on the absorption of protein solution onto the tip, the precision of the pattern is greatly influenced by the tip's width, the relative humidity of the environment and the intrinsic properties of the protein solution; so it limited the practical application of this method for it needs perfect tips design, strict environmental conditions and high standard solution property request.

2.7 Other methods in protein patterning

Besides the microfabrication techniques that mentioned above, some groups have combined the microfliud technique, layer-by-layer method, DNA templating into the fabrication of the protein patterns, and all these methods provide new ideas for the research of this field. In the following, we will give a brief overview on these methods.

It has proved to be a useful method to develop Lab-on-Chip (LOC) devices by precisely control the microfluidic and regulation of the liquids in geometrically controlled microchannels [3]. Among the microfluidic platforms, fabrication of the multiplex detection interfaces by patterning different biomarkers or multiple stimuli in a single LOC device has attracted much interest for high throughput biological applications, such as biosensing [49–53], microbioreactors and cell detection [54–60]. For the aim to develop biofunctional LOC devices, the functional biomolecules' spatial position, orientation and binding to substrates should be accurately controlled. Localized and region specific patterning of multiple biomarker microarrays within microfluidic platforms is another important feature of a functional interface for the production of multiplex high-throughput devices [61, 62].

Based on the background mentioned above, Tabrizian *et al.* [63] fabricated multiplex protein patterns by using the microfliudic technique. The schematic presentation of the experimental procedure is shown in Figure 7(a–h). They grafted the (3-aminopropyl) triethoxysilane (APTES) to the surface of glass substrate to form a APTES monolayer by micro-contact printing method, then made the main channel contact with the substrate and made it be sealed, then introduced the parallel microfliudics into the main channel and different protein solutions were injected parally with the protein grafted to the substrate covalently and formed the multiplex protein patterns. Figure 7(i–k) shows the fluorescence images characterized from the surface of the main channel functionalized with FITC, Cy5 and Cy3 conjugated antibodies, respectively. The results proved that the integration of several microfliudics can be used to fabricate patterns with different proteins parallely. Figure 7(l) shows the superimposed image of the three images shown above, proves that the surface had been successfully functionalized with multiple antibodies. Figure 7(m) performs the fluorescence intensities of the functionalized surface. Using this method to fabricate protein patterns can not only overcome the questions caused by the physical absorption of the biomolecules, reduce the time of the fabrication process for protein patterns, but also can control the production of the protein patterns through modulating the flow condition of each microfliud and the concentration of the protein solution to achieve the patterns of mono or multiple protein, and ultimately fabricate surface of the single channel with multiplex detection which can be used in the field of biological high throughput.

Currently, Lee *et al.* [64] reported a novel technique to fabricate three-dimensional (3D) protein nanostructures through the alternate layer-by-layer assembly of bacterial protein nanoparticles and DNA on the patterned gold dot arrays, and it achieved a development of the layer-by-layer assembling method and fabrication of the three-dimensional protein nanostructures (Figure 8). They invented an advanced technique which can finely control the assemble of specific protein nanoparticles and fabrication of the patterned arrays of multi-layer proteins through systematically employs the structural, functional and physicochemical properties of proteins and DNA. Since the *E. coli* Dps performs unique structure and functional properties, such as both the N- and C-termini of *E. coli* Dps nanoparticles points outside, and it has a highly positive surface charge which shows strong affinity for DNA, also it has a relative high stability of the structure, so they chose the *E. coli* Dps in their experiment. They fabricated gold nanodots arrays on the Si substrate which covered by a thin layer of nickel, and then modified the nickel surface with recombinant proteasome α particles which have hexahistidine peptides that have the nickel affinity, after that the polyadenine oligonucleotides were grafted to the surface of the gold dots. Next, they alternatively assembled the recombinant Dps particles and double-strand DNA onto the gold dots through layer-by-layer method reduplicative while with the pH shift between 8.0 and 5.0, resulting in the multilayer formation of Dps nanoparticles on the gold dots.

Since the application field of DNA becomes broader, using programmable self assembled DNA as a template for protein patterning has been attracting attention. Two-dimensional DNA template for protein patterning was first

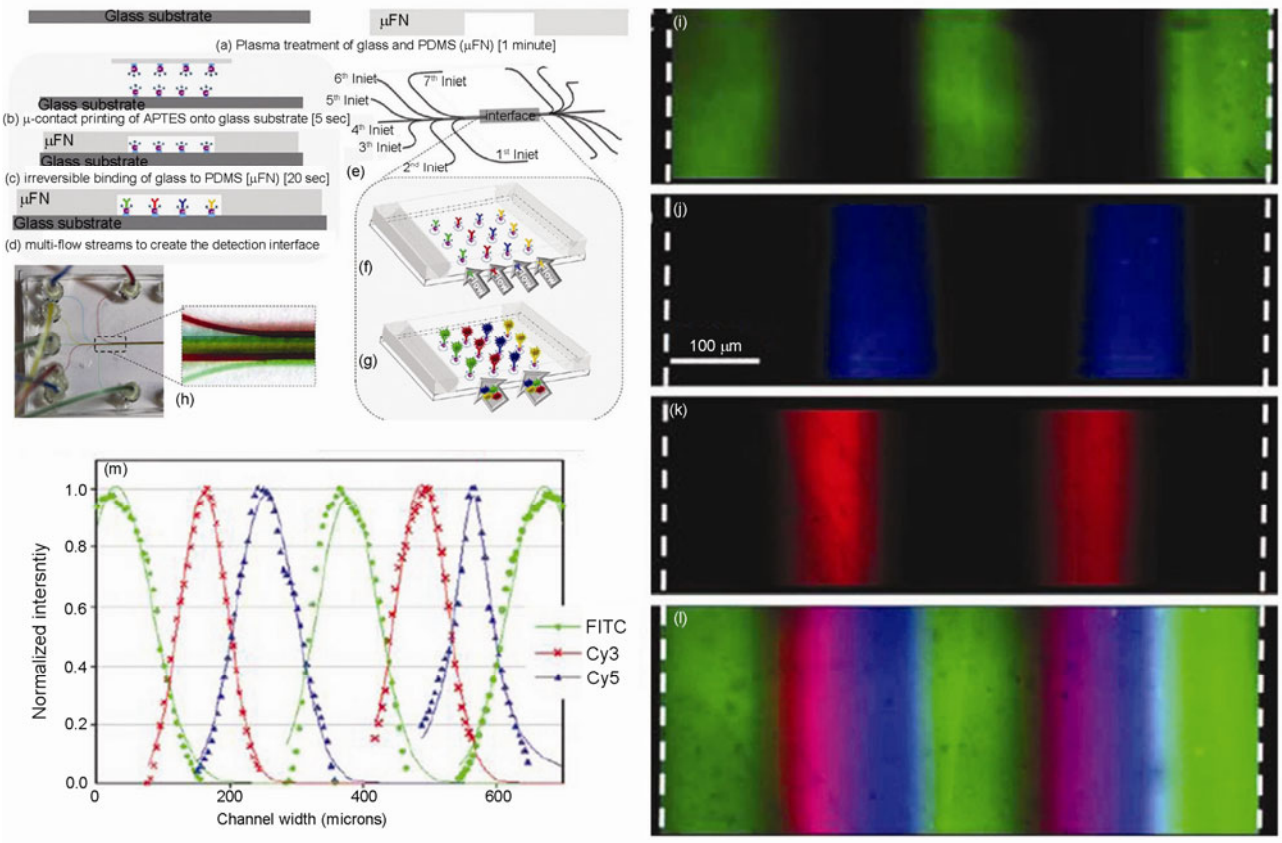

Figure 7 (a–h) Schematic presentation of the experimental procedure; (i–k) Fluorescence microscope images after microchannel surface functionalization, green, red and blue colors represent FITC, Cy3 and Cy5 conjugated IgG secondary antibodies respectively; (l) Superimposed fluorescence microscope image of the channel representing all three antibodies, the white dashed lines represent the microchannel walls; (m) Normalized fluorescence intensity of the surface across the channel width shown in Figure 7(l) [63].

reported by Yan *et al.* in 2003 [65]. They achieved streptavidin pattern by DNA self assemble through the reprogrammed sticky ends into 4 × 4 tiles of nanogrids which contains biotinylated oligonucleotides on each tile. Through selectively modified each tile with biotin and integrate two DNA tiles together, a variety of streptavidin patterns were achieved [66]. Cohen *et al.* [67] showed the method to prepare multiple arrangements of streptavidin with ~25 nm separations through the polyamidebiotin conjugation. In a similar way, aptamers modified by human-thrombin and platelet derived growth factor (PDGF) have been incorporated into the DNA template and achieved the patterns of the thrombin and PDGF. At present, DNA can be used to fabricate two-dimensional structure with nanometer precision programmable control which makes this technique an attractive candidate for protein patterning.

3 Applications of the protein patterns

3.1 Sensing

With the scientific research going deep, protein patterns have been widely used in the field of sensing. Unlike nucleic acids which have a predictable sequencespecific hybridization chemistry, proteins perform incredible differences in their functional groups, affinities, secondary and tertiary structure. Besides, proteins usually have multimerization and post-translational modification after the translation process which make the protein structure more diverse, such as acetylation, glycosylation, and phosphorylation. Thus, it is difficult to achieve the protein replication and amplification with current equipments, and limiting the protein-arrays' sensitivity. In addition, antibodies only bind to a small part of the target protein which called epitope. Since each protein has an incredibly complex structure, antibodies always bind to epitopes with identical or similar structure in off-target proteins in protein arrays [68, 69], then produce nonspecific cross-reactive signals. Based on this overview, Wang *et al.* [70] designed a simple and sensitive nanosensor to characterizing antibody cross-reactivity which contains high density arrays of giant magnetoresistive (GMR) [71, 72] nanosensors and magnetic nanotags as shown in Figure 9. In their experiment, they made the antibody and magnetic nanoparticles in the same solution to bind on the surface of

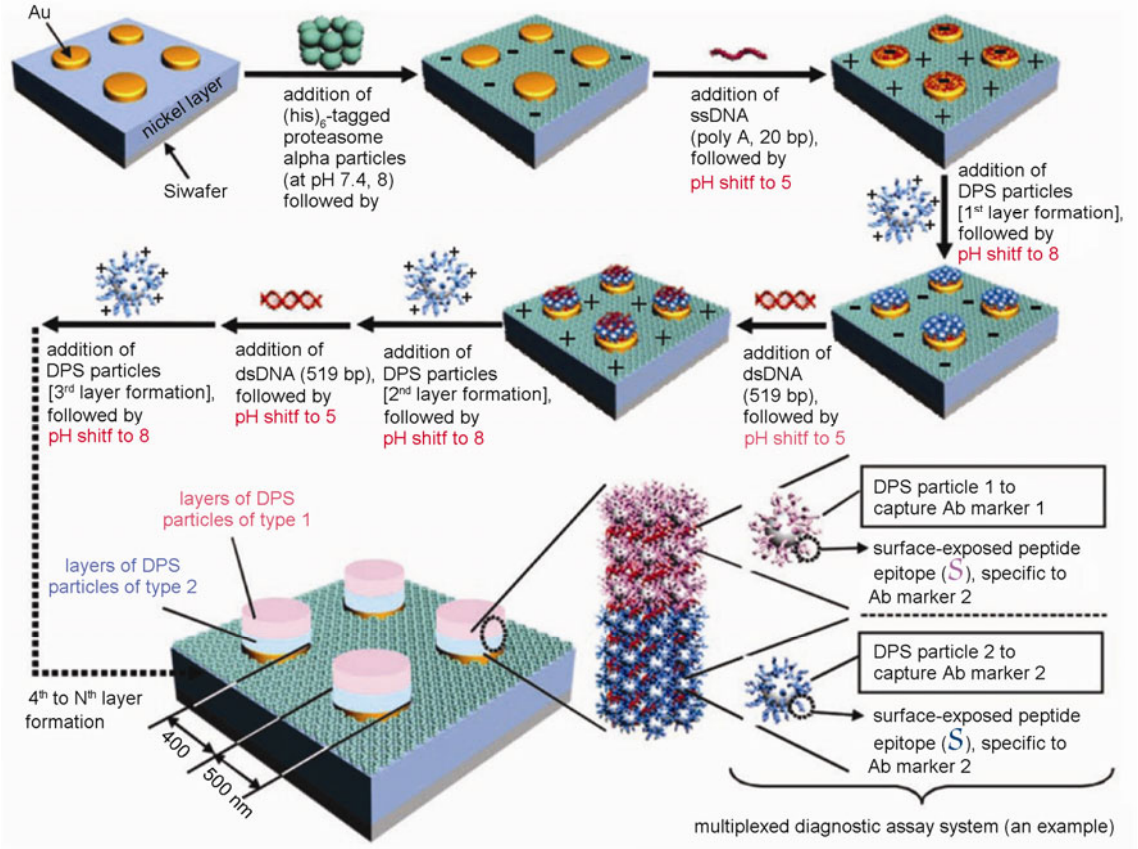

Figure 8 Schematic illustration of layer-by-layer assembly of an array of multilayered protein nanostructures [64].

the sensor which get rid of the wash steps that needed for traditional sandwich assays. The basic process of the assay works as follows: they made the GMR arrays which have the function of targeting the biomolecules of interest through modified the arrays with capture antibodies, and the biomolecules were selectively captured by antibodies that immobilized on the GMR sensor after the sample were treated in the reaction well. Besides, they used piezoelectric robotic spotter technology to spot capture antibody droplets with a volume of 350 picoliter onto individual GMR nanosensors in the array (Figure 9(b) insert). Then they added the magnetic nanotags solution modified by streptavidin to the reaction well. There was no reaction happened because the complementary reaction chemistry between the captured antigen and magnetic nanotag was not yet present in the well (Figure 9(a)). After that the antibodies modified with biotin were introduced, and the antibodiy diffused throughout the reaction well and linked the magnetic nanotags to the analyte that immobilized to the surface with a signal produced in the underlying GMR sensor (Figure 9(b)). In this method, the antibodies cross-reactivity can be assessed with only one wash-free step. By using this method, the author achieved the detection limit down to 50 fmol/L in a 25 μL sample. Besides, this method can be widely used in practical detections because the process is simple to implement which lacks tedious washing steps, using the common salt buffers which will not denature the biomolecules, it also achieves a high sensitivity in less than 15 min.

At the same time, Rossi *et al.* [73] used the patterned protein as an assay platform to compare the immune efficiency of the reaction between antigen and antibody with the homogenous protein substrate, the results showed that the immune efficiency was higher, the activity of the immobilized protein maintained and increased the ability of

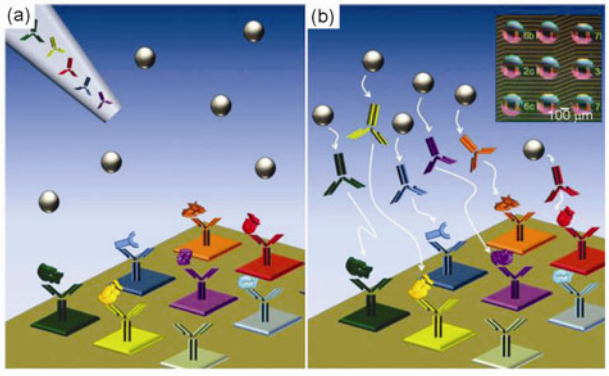

Figure 9 Schematic representation of the autoassembly immunoassay where each square represents a 100 μm × 100 μm GMR nanosensor and each color represents a unique target antibody and antigen [70].

the protein to form a complex. Wolinsky *et al.* [74] immobilized the antibodies that against the human immunodeficiency virus type 1 (HIV-1) p24 antigen on the gold surface to form the active antibody patterns with the feature size less than 100 nm through DPN method, and they used this antibody pattern to determine the presence of the human immunodeficiency virus type 1 (HIV-1) in blood samples. In their experiment, they achieved the limit of detection of 0.025 pg/mL, which can exceed the limit of detection of conventional enzyme-linked immunosorbent assay (ELISA)-based immunoassays (5 pg/mL of plasma) by more than 1000-fold.

3.2 Modulation of cell adhesion behaviors

In recent years, cell adhesion has got attractive attention since it is an important process in several biological phenomena such as embryonic development, angiogenesis, and metastasis. Cell adherent to surfaces is regulated by some integrate proteins and complexes with the size ranging from 10 nm to 10 μm. Focal adhesions (FAs) contain an extracellular matrix protein, a transmembrane protein and intracellular proteins. They play an important role in outside-to-inside signaling ports and help cells function properly [75], and the organization of surface proteins with nanometer- and micrometer-scale play a crucial role in the formation and function of the adhesion complex. Cell adhesion and cellular organization have been widely studied as a function of the available adhesive area and shape using micrometer-scale patterns [76, 77]. Haviland *et al.* [78] incubated the endothelial cells on the fibronectin nanorings and nanodots on a K-Casein background to study how different structures affect the cell adhesion. In their experiment, cells adhered to surfaces and formed protrusions toward both the nanodots and nanorings, but it performed a more pronounced manner on the nanoring patterns. In order to confirm the formation of the FA complex on nanodots and nanorings, they stained for vinculin (Figure 10), cells adhered to nanodots showed circle-shaped vinculin staining, while on nanorings performed more prominent and ring-shaped staining which reflected the underlying fibronectin pattern clearly. When incubated on nanodots, cells formed fibrillar, presumably cytoskeletal, structures originating from the nanodot. On the other hand, cells on nanorings performed diverging fibril bundles extending from the nanorings. The results guaranteed that different patterns of the extracellular matrix protein can regulate the FA complex organization and have a great influence on the organization of the adjoining cytoskeleton.

Besides, with the help of protein pattern, Kim *et al.* [79] successfully induced the fabrication of the mesenchymal stem cell patterns and studied the cell adhesion behavior. Smith *et al.* [80] researched the cell adhesion and cell surface carbohydrate expression by using exogenous lectin arrays, and provides a proof-of-principle demonstration of the direct qualitative profiling of cell surface carbohydrate expression. Sutherland *et al.* [81] investigated the cell adhesion and spreading on protein patterns of either FN or vitronectin (VN) in the range of 100–3000 nm and revealed the important differences in focal adhesion development on the two proteins nanopatterns. Suh *et al.* [82] designed a topographically fibronectin pattern used as cell culture substrate of variable local density and anisotropy as a facility and efficient platform to guide the organization and migration of cells in spatially desirable patterns. Besides, protein patterns also have been successfully used to direct vesicles to well defined areas on the surface [83], guide neuron and axon growth [84, 85], fabricate bioactive patterned surface and cell arrays [86, 87], modulate the spatial distribution of the extracellular matrix which secreted by cell and control the orientation of cell polarity [88, 89].

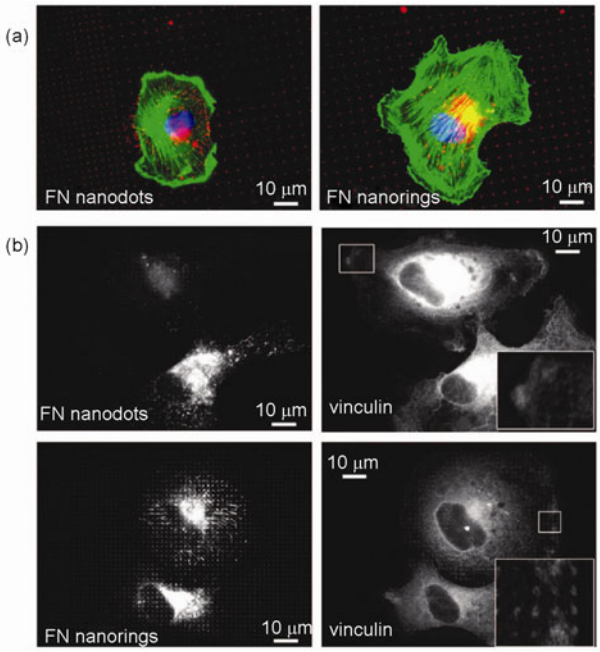

Figure 10 Endothelial cells adhere to nanopatterns of Fn on a K-Casein background. (a) Cells contact Fn nanopatterns and form stress fibers, red, green and blue represent Fn, actin and nucleus respectively; (b) Vinculin staining mirrors the Fn patterns as dots or rings. Adhesions are stronger on nanorings, which present more Fn molecules. Vinculin stainings reflect the underlying surface patterns of dots or rings (insets) [78].

3.3 Other applications

Besides the applications mentioned above, protein patterns also performed to have potential applications in other fields. Beritling *et al.* [90] combined different peptides with the CMOS device (Figure 11) to fabricate multiple peptides patterned surface, and used it in peptide synthesis *in situ*. By using this method, they achieved the parallel synthesis of

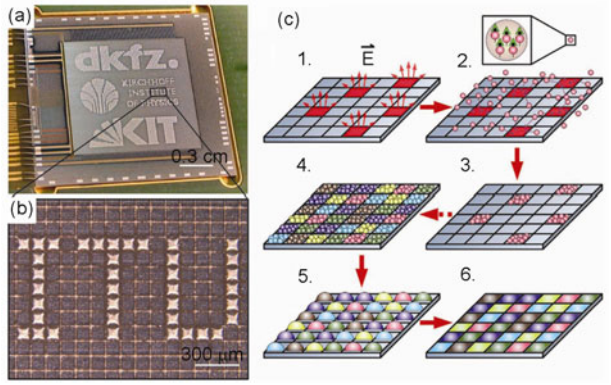

Figure 11 Microelectronic CMOS chip with deposition pattern. (a) Chip; (b) A magnifi ed detail; (c) Schematic deposition process [90].

16384 different peptides (10000 spots cm^{-2}) onto the surface of a microelectronic chip which is first reported in this field.

Hansford *et al.* [91] used the patterned proteins and peptides to induce the deposition of gold nanoparticles on the substrates. Moreover, they made it come true to fabricate gold patterned surface with the biomolecules' induction, and it provides a new idea to prepare the inorganic patterned surface by the biomolecular induction. Ward *et al.* [92] fabricated the patterned calcium oxalate monohydrate crystals on the substrate by using the patterned protein and phosphatidylserine bilayers which offer a new way in the field of fabricating crystal patterns of conductor or semiconductor and multiple electronic devices. Besides, protein patterns also have potential applications in the field of genomics, proteomics and diagnosis [93], drug discovery and synthetic biology [94], bioreactor and biotransfermation [95].

4 Conclusion and outlook

In this article, we overview the basic techniques that widely used to fabricate protein patterns and the applications of the protein patterns in sensing and modulation of cell adhesion, which provides us a new idea for the discussion of the specific properties and questions faced in large scale of single molecules, it even has a great influence on scientific research. Firstly, in the field of biosensing, since these nanopatterning techniques can increase the information density, it provides the possibility to develop a new generation of biosensors which based on the localized surface plasmon resonance and it allows to prepare well defined single biomolecule arrays. Secondly, protein patterns are expected to help us to have a good understanding of cell-surface interactions and give the opportunity to the development of better biomaterials and tissue engineering. Though these techniques have remarkable progress, each protein patterning process has its own drawbacks. At present, the parallel patterning techniques can provide large area homogenous samples with repetitive patterns while the serial writing techniques are more flexible with the type of pattern but usually slow when preparing large area samples. In the future, the fabrication techniques of patterning protein will mainly include the following aspects. (1) Developing original protein patterning techniques to overcome the drawbacks such as the expensive consumption of the apparatus, the complexity of the technology, high equipment requirements and difficult to prepare large area samples, which was aimed at making the facrication process simple, fast and low consumption. (2) Exploring the real three dimentional fabricating technique to achieve the true three-dimentional control to mimic the complex environment of cells and to raise questions such as "what is the effect of certain signals on the differentiation, proliferation, or apoptosis of cells". Although, such three dimensional control is not yet foreseen, the existing patterning techniques overviewed in this review supply us with many possibilities to learn more about biology in a novel fashion. (3) Integrating a series of surface analytical techniques in order to achieve a thorough characterization of the patterned surface which leads to better understanding its biological response for many surface analytical techniques are carried out in a vacuum environment which cannot represent the native state of the biological components.

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