Anal Bioanal Chem (2009) 393:1563–1570
DOI 10.1007/s00216-008-2595-6

TRENDS

Affinity capturing for targeting proteins into micro and nanostructures

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Published online: 20 January 2009

Abstract Protein immobilization into micro and nanoscaled patterns opens exciting possibilities in fundamental and applied research. Developing efficient capturing techniques while preserving the structural and functional integrity of the proteins on surfaces is a key challenge for surface scientists. In this paper, current techniques for site-specific protein immobilization into engineered surface architectures are reviewed. Fundamental principles for functional protein immobilization on solid supports are discussed and popular affinity-based recognition pairs and their application for capturing proteins into nano and microstructures are presented.

Keywords Protein immobilization · Microstructure · Nanostructure · Patterning · Nitrilotriacetic acid · Biotinylation

Introduction

Protein immobilization on solid supports is rapidly gaining importance for analytical applications such as functional proteomics [1], diagnostics, and drug screening [2]. These applications require multiplexed immobilization of proteins into arrays in a functional manner. Moreover, the extraordinary broad spectrum of protein functions and their highly specific recognition of cognate ligands make them very attractive functional elements for constructing biotechnological sensors and actuators. For such applications, even more sophisticated targeting of proteins into microscopic and nanoscopic structures is required in order to obtain efficient integration of protein building blocks into functional assemblies. Immobilization of proteins is frequently achieved by direct adsorption or by chemical reactions with solid supports, and patterns are readily obtained by photolithography, microcontact printing, piezodispensing, or through the use of scanning probe microscopy-based techniques [3–5]. These applications are successful if either very robust proteins such as antibodies are involved, or if only a minor fraction of the protein needs to be active for further functional analysis. For many proteins and applications, however, functional attachment of proteins to solid supports is a key challenge because of the metastable nature of proteins and their wide-ranging physicochemical properties. Depending on the sensitivity of the detection scheme and the size of the structures, activity of 10–90% of the immobilized protein is desired. Two main issues have turned out to be of key importance for successful functional protein immobilization:

1. because protein interaction with the bare solid support frequently causes denaturation and loss of function, protein-compatible layers are required for shielding the surface; and
2. site-specific attachment of the protein to the surface is required in order to ensure homogeneous functionality with the intact protein activity.

For assembling proteins into micro and nanostructures, the protein coupling approach must be compatible with the respective patterning technique. This review will focus on surface engineering approaches, which aim to site-specifically

Dedicated to Prof. Günter Gauglitz on the occasion his 65th birthday.

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capture proteins to structured surfaces rendered biocompatible by appropriate surface modification. Moreover, promising techniques employed to pattern protein-capturing surfaces into micro and nano-scaled structures will also be introduced.

In order to make surface biocompatible, molecular layers of bio-inert, hydrophilic polymers have been successfully employed. Next to dextran hydrogels, dense layers of poly (ethylene glycol) (PEG) have been proven efficient for shielding surfaces against proteins [6]. While relatively long PEGs with a molecular mass of 2000–5000 g mol^{-1} yield the most biocompatible surfaces, very dense layers of short oligo(ethylene glycol) chains with 3–6 monomer units as obtained by self-assembly of alkyl thiolates monolayers are also capable of very efficiently shielding of protein interactions with the surface [7]. Non-covalent assembly of lipids and lipid-like molecules into mono and bilayers has also been very successfully used for rendering surfaces biocompatible [8]. Lipid-based surface architectures, however, are significantly more fragile than covalently attached layers and are particularly sensitive to exposure to air. Protein-repellent surfaces are not suitable for protein immobilization by physisorption and, therefore, require further functionalization with chemical or biochemical anchoring groups for protein capturing. Covalent coupling of proteins to surfaces through reactive groups on amino acid side-chains is frequently used for tethering protein to surfaces, and numerous procedures have been described. Such chemical reactions are not very selective and for most proteins yield highly heterogeneous attachment. Moreover, these reactions can involve residues important for function and thus affect the activity of the protein. Therefore, protein capturing techniques are desired, which site-specifically tether proteins to surfaces. Monoclonal antibodies have been used very successfully for protein immobilization because these very robust proteins can be efficiently physisorbed by surfaces and they sometimes bind proteins very stably. This approach, however, requires a suitable monoclonal antibody which recognizes the protein of interest without affecting its function. Moreover, antibodies are large proteins and not ideally suited for high-resolution structured surface fabrication processes. A more generic strategy for site-specific tethering involves fusion of a protein or peptide tag to the protein of interest, which is used as a recognition unit for capturing to surfaces. This approach originates from affinity chromatography and several recognition pairs are already available [9]. One critical issue for protein immobilization, however, is that stable attachment of the protein to the surface is highly important for most analytical applications, while affinity chromatography requires reversible binding. Thus, only recognition pairs with high binding stability have found successful application in surface engineering. Moreover, efficient recognition elements are mostly proteins, most of which can only be handled under physiological conditions, whereas surface fabrication processes involving patterning techniques frequently require robust molecules. For these reasons, efficient recognition units for capturing proteins on surfaces are scarce. Currently most approaches rely on either the interaction between biotin and streptavidin or related proteins, or on the complexation of immobilized transition metal ions by oligohistidine tags. These conceptionally different approaches for protein immobilization and their application towards functional protein patterning in micro and nanometre dimensions will be described in detail in the following text.

Biotin-mediated protein immobilization

Capturing proteins on surfaces by interaction of co-factor biotin with the proteins avidin/streptavidin and their derivatives is a classic approach in solid-phase assays first described by Bayer and Wilchek [10]. Biotin rapidly binds to avidin/streptavidin in 4:1 stoichiometry in a quasi-irreversible, non-covalent interaction. Because avidin is rather positively charged, the neutral variants streptavidin or neutravidin are used for surface functionalization in order to minimize non-specific interactions. The binding stoichiometry of the biotin/streptavidin complex enables sandwich binding architectures on surfaces, where biotinylated surfaces are reacted with streptavidin, and the remaining biotin binding sites are used to capture biotinylated proteins (Fig. 1c). The stable chemical property of biotin makes it easy to go through relatively harsh fabrication conditions during the build up of a micro or nano-structure, while the high affinity of biotin/streptavidin interaction provides steady protein binding on the surface. For using this versatile interaction, biotin has to be attached to the protein of interest. Chemical biotinylation of proteins with standard reagents such as amine-reactive *N*-hydroxysuccinimide esters in most cases is a statistical process yielding heterogeneously and multiple labelled species. In contrast, enzymatic biotinylation elegantly enables site-specific attachment of biotin and biotin derivatives. For this purpose, the biotin ligase BirA from *E. coli* has been very successfully employed; it is capable of selectively ligating biotin to a 15 amino acid peptide tag (AviTag or AP-tag, Fig. 1d) [11]. Novel enzymatic biotinylation techniques employ phosphopantetheinyl transfer of biotinylated Coenzyme A derivatives to peptidyl or acyl carrier proteins [12] or even short recombinant peptides [13] (Fig. 1e). These reactions are highly specific and can be efficiently carried out without prior purification of the protein of interest, enabling capture of proteins from crude lysates. Also, fusion with an intein tag and subsequent reaction with a

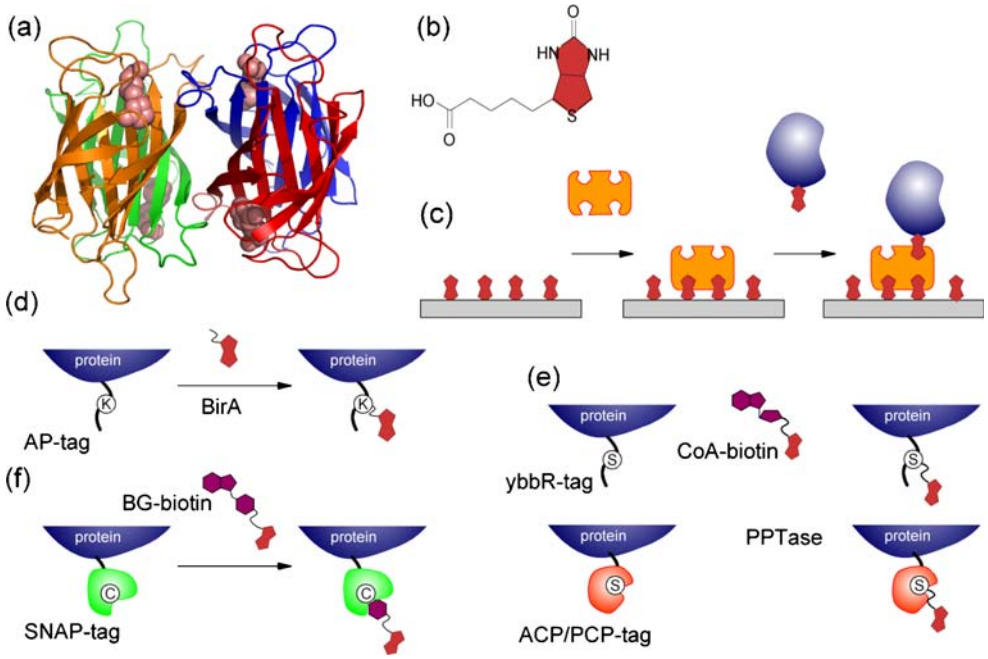

Fig. 1 Immobilization through biotin/streptavidin interaction. (**a**) Structure of tetrameric streptavidin (ribbon model) with four biotin molecules (spacefilling model). (**b**) Structure of biotin. (**c**) Immobilization of a biotinylated protein in a sandwich format. (**d**–**f**) Site-specific biotinylation through enzymatic reactions: (**d**) biotin ligase BirA-mediated biotinylation of the AP-tag; (**e**) phosphopantheteinyl-transferase (PPTase)-mediated transfer of phosphopantheteinyl-biotin to acyl or peptidyl carrier proteins or other peptide tags; (**f**) self-biotinylation of SNAP-tagged proteins using benzylguanin (BG)-biotin conjugates

biotinylated cysteine has been successfully employed for site-specific protein biotinylation for immobilization on surfaces [14].

Surface fabrication requires immobilization of streptavidin, which can be directly attached to the surface by physisorption or chemisorption. Alternatively, surfaces can be functionalized with biotin, which is a small, stable organic molecule highly compatible with surface manufacturing processes. Chemical linkage of biotin to surfaces through its carboxyl group is straightforward, and numerous biotin derivatives for surface modification including biotin-functionalized OEG-thiols and lipids are commercially available, yielding well-defined surface assemblies [15, 16]. Direct adsorption of neutravidin on to surfaces and its subsequent micropatterning by deep UV irradiation has been demonstrated to be an efficient technique for creating laterally structured surfaces for the immobilization of biotinylated proteins [17]. Patterning of streptavidin and derivatives directly adsorbed or coupled to surfaces by photolithography has been successfully employed for functional surface patterning; lateral resolution down to submicron dimensions has been achieved [18]. However, more elegant and defined approaches are based on surface biofunctionalization by chemical reaction with a suitable biotin derivative followed by self-assembly of the streptavidin layer. Different chemical approaches employing this method for functional surface patterning have been developed. Microcontact printing (Fig. 2a) of biotin on to different activated polymer surfaces has been described [19]. Various photochemical approaches have been developed for patterning biotinylation of surfaces. Chemical biotinylation of a PEG polymer brush after photo-deprotection of caged surface amines by UV-irradiation through a mask yielded patterns of streptavidin on a highly biocompatible surface [20]. Functional streptavidin patterns on polystyrene and nitrocellulose surfaces were successfully obtained by use of a photoactivable derivative of biotin [21]. More versatile surface patterning was achieved by direct, spatially controlled photochemical biotinylation by using a thiol-ene reaction [22]. This approach can, in principle, be used for spatio-temporally controlled protein immobilization on the stage of a confocal microscope. An inverse patterning approach was chosen by Majima and coworkers, who synthesized a nitroveratryl-based, cleavable biotin-farnesyl conjugate which was incorporated into phospholipid membranes. Thus, biotin could be removed from these membranes by UV irradiation [23], which can also be used for in situ protein patterning. Similar capabilities for controlling both the position and the concentration of protein immobilized on a surface in situ was recently described by Costantino and coworkers [24]. For this approach, which they termed laser-assisted protein adsorption by photobleaching (LAPAP), biotin-4-fluorescein was adsorbed by a BSA-coated glass substrate, via the generation of free radicals after fluorescein photobleaching, using a visible argon or diode laser, and used as a scaffold to bind proteins.

For lateral organization of proteins in nanoscale dimensions, classic irradiation-based approaches are limited because of the diffraction-limited resolution of UV light. Templates with nm-sized structures such as bacterial S-layer or arrays of nanoparticles allow nanostructured surface patterning down to the molecular level [25]. However, these approaches lack the flexibility to freely define the geometry

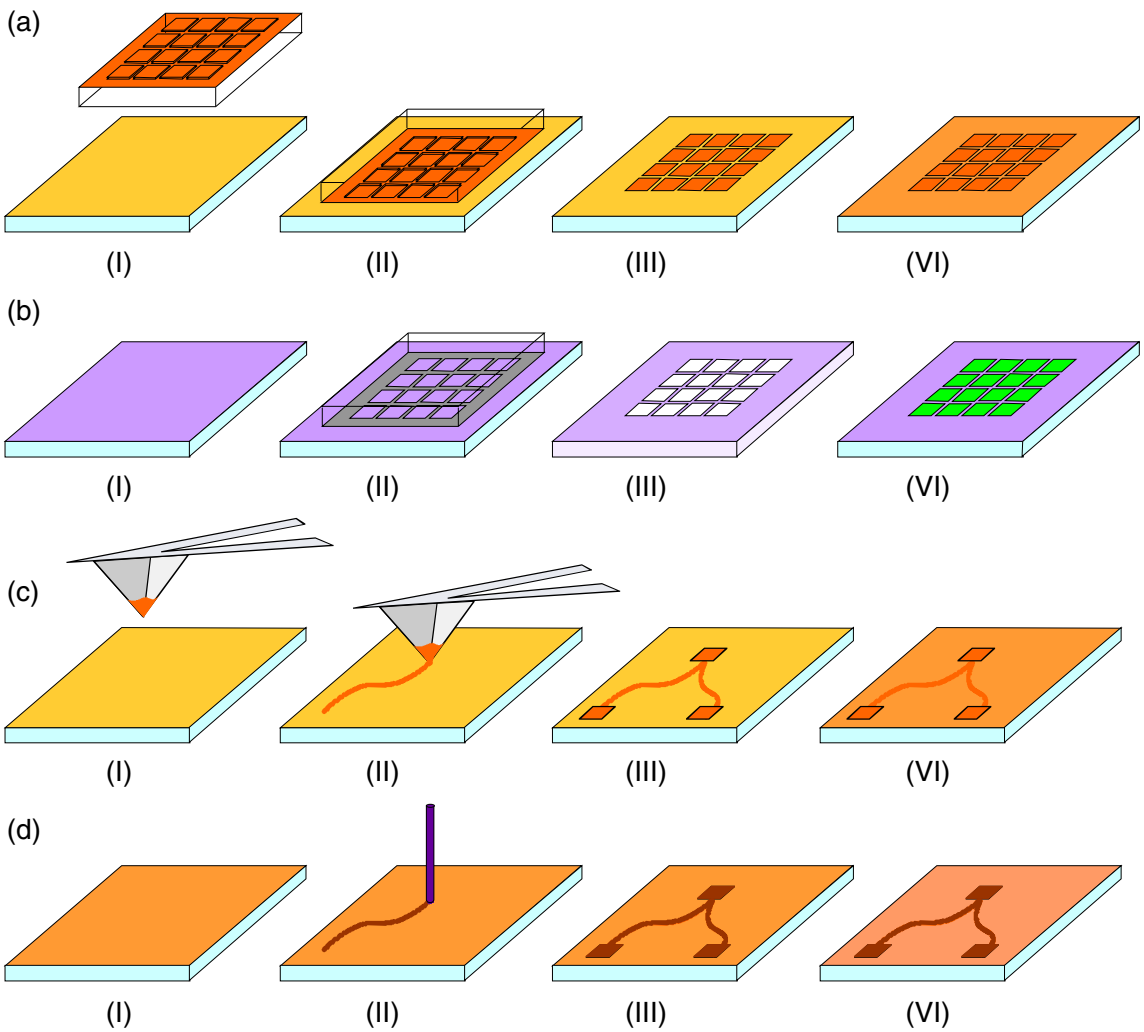

Fig. 2 Selected techniques for surface functionalization in micrometre and nanometre dimensions. (**a**) Microcontact printing. After inking a PDMS-stamp with a suitable ink such as an alkyl thiol (*I*), a monomolecular layer is transferred on to a planar substrate such as a gold surface only by the protruding regions of the stamp (*II*, *III*). By back-filling with a passivating compound, the remaining surface is protected against non-specific binding. (**b**) Surface patterning by UV-deprotection on surfaces. A chemically modified surface with functional groups caged by a photolabile protection group is illuminated through a mask (*II*) thus selectively uncaging the functional groups in the illuminated areas (*III*). Subsequently, uncaged regions are functionalized by chemical reaction (*VI*). (**c**) Dip-pen nanolithography. An AFM tip is inked with reactive molecules (*I*), which are subsequently transferred on to the surface into nanometre-sized structures (*II*, *III*). The remaining regions are then protected by back-filling with a passivating compound (*IV*). (**d**) Electron beam-induced chemical nanolithography. A surface coated with a suitable surface layer (e.g. a 4′-nitro-1,1′-biphenyl-4-thiol, SAM) is reduced and cross-linked by electron beam lithography (*I*, *II*). The non-exposed thiol is subsequently exchanged for a passivating compound, and the amine groups within the chemically modified region can be further functionalized

of the patterns, because the templates rely on self assembly. In contrast, lithographic techniques based on modifications with the tip of an atomic-force microscope have proven capable of functionalizing surfaces in nanometre dimensions. Nanopatterned streptavidin has been obtained by removing the protein-repellent PEG coating on a silicon wafer by the AFM tip, thus creating sites for physisorption of streptavidin [26]. A more general technique for nanostructured surface modification by the AFM tip developed by Mirkin and coworkers is dip pen nanolithography (DPN) [27] (Fig. 2c). This technique has been implemented for the functionalization of gold and glass-type surfaces with biotin [28–30]. These structures were visualized by AFM and fluorescence microscopy after binding of streptavidin. A very elegant, optical approach for nanopatterning was implemented by Leggett and co-workers, who used the scanning near-field optical microscope (SNOM) to generate nanometre scale patterns of reactive aldehyde groups on a protein repellent OEG terminated SAM. The aldehyde patterns were subsequently converted into a streptavidin nano-pattern by covalent reaction of amines on the protein with the surface aldehydes [31]. Similar nano-scaled resolution of biotin

patterning was also achieved by using a combination of nanoimprint lithography (NIL) and molecular assembly patterning by lift-off (MAPL) [32].

Immobilized transition metal ions

Although the biotin-streptavidin interaction enables highly stable tethering of proteins to surfaces, reversible affinity capturing not only provides oriented and homogeneous attachment but also the possibility of detaching the protein and thus repeated use of the same surface. For this purpose, complexation of histidine residues with immobilized transition metal ions offers powerful solutions. Originally developed for affinity chromatography of histidine-rich proteins, this approach has gained tremendous importance for purification of recombinant proteins fused to a tag of 6–10 histidine residues [33]. Transition metal ions such as Zn (II), Cu(II), Ni(II), or Co(II) are immobilized by chelating agents such an iminodiacetic acid (IDA) or nitrilotriacetic acid (NTA, Fig. 3a), thus maintaining 2 or 3 coordination sites free for histidines. This interaction can be very efficiently disrupted by competing coordinators such as imidazole, which selectively and rapidly remove the protein from the surface under mild conditions (Fig. 3c). For protein immobilization on surfaces, NTA has been favoured over IDA because it coordinates transition metal ions more stably. One key advantage of immobilized metal ions for capturing proteins on surfaces is the chemical nature of this recognition unit, enabling versatile and efficient surface chemistries. In a visionary paper published by Whitesides and coworkers in 1996 [34], they devised a mixed self-assembled monolayer of alkyl thiols terminated with a short OEG and an NTA moiety. Meanwhile a variety of procedures for NTA-functionalization of glass-type surfaces and gold surfaces [35, 36] have been described. As an alternative approach, non-covalent self-assembly of NTA-functionalized lipids on solid supports (solid-supported lipid mono and bilayers) has been successfully employed for capturing His-tagged proteins onto surfaces [37]. A key

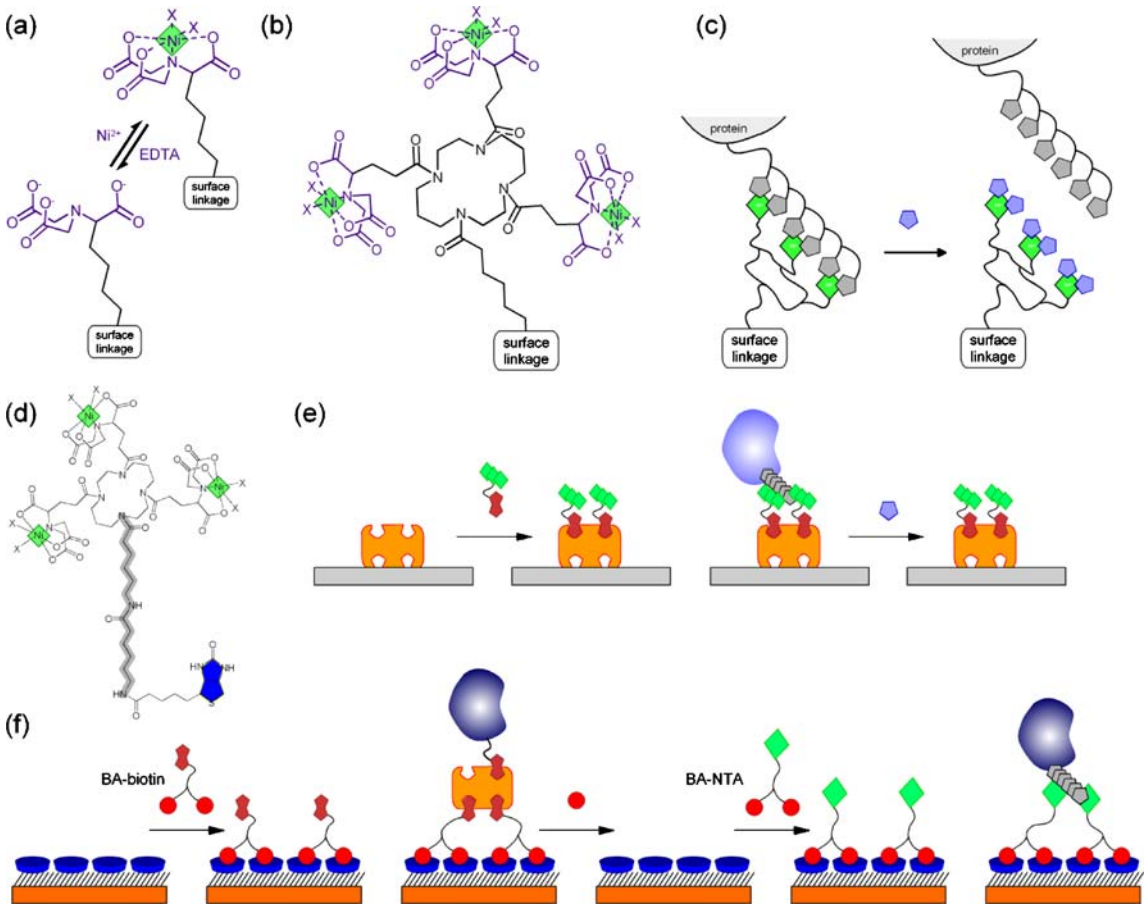

Fig. 3 Adapters for protein immobilization. (**a**) Reversible loading of Ni(II) ions on to an immobilized NTA moiety. (**b**) Structure of the MCH tris-NTA with complexed Ni(II) ions. (**c**) Reversible binding of a his-tagged protein to Ni(II)-loaded tris-NTA upon addition of imidazole (*blue*). (**d**) A tris-NTA/biotin conjugate for reversible tethering of proteins to streptavidin-functionalized surfaces (**e**). (**f**) Molecular printboards as a generic platform for protein immobilization. Multivalent interactions of bis-adamantyl (BA) conjugates (BA-biotin and BA-NTA) with immobilized β-cyclodextrin (*blue*) is employed for reversible functionalization of surfaces for protein capturing

problem, which was soon identified for many of these approaches, was that His-tagged proteins rather rapidly leaked from Ni-NTA surfaces. This problem was recently overcome by synthesis of multivalent chelator head (MCH) groups, supramolecular entities with 2–4 NTA moieties incorporated onto branched and cyclic scaffolds [38, 39]. These MCH, which recognize oligohistidine-tags by multivalent interactions (Figs. 3b, c), were shown to bind His-tagged proteins with sub-nanomolar binding affinities [38]. Bottom-up chemistries for efficient attachment of these MCH to glass type surfaces via a protein-repellent PEG polymer brush have been reported [40], and biocompatible MCH-SAM on gold have been described [36, 41]. Using conjugates of MCH with biotin, streptavidin-functionalized supports can also be used for capturing His-tagged proteins, lending this technique an even greater versatility [42].

Numerous approaches for Ni-NTA-based protein patterning by microcontact printing of reactive alkyl thiols followed by chemical coupling of NTA [43] and direct microcontact printing, or by backfilling of NTA derivatives, have been described [28, 36, 44, 45]. Next to multivalent NTA, also mono-NTA in high surface densities yields stable protein immobilization by multivalent binding on surfaces. Thus, the binding affinity can be varied by the density of NTA moieties on the surface. This feature was exploited for differential targeting of proteins into array elements with different NTA densities, which were obtained by combining microcontact printing with piezo-dispensing [44]. After selective elution of proteins from arrays elements with a low density of NTA moieties by imidazole, these array elements were selectively refilled with a different protein [45]. Thus, sequential assembly of binary protein microstructures was achieved. Like the patterning of biotin-functionalized surfaces, photochemical approaches have been used successfully to pattern NTA surfaces. One noticeable advantage for photochemistry-based patterning methods is that the patterning can be done on dimensions as large as a centimetre simultaneously [22]. In one work [20], photosensitive silanes containing nitroveratryl (Nvoc)-caged amine groups and protein-repellent tetraethylene glycol units were used for modification of silica surfaces (Fig. 2b). After masked irradiation by UV-irradiation and coupling with trisNTA, protein micropatterns were successfully generated. Recently, we have established a generic method for photochemical micropatterning of NTA-functionalized glass-type surfaces by highly selective photodestruction of the NTA moieties (unpublished results).

Several approaches have demonstrated nanoscaled protein assembly by immobilized transition metal ions. The reversible nature of the His-tag/Ni-NTA interaction was exploited for a novel nanopatterning technique termed native protein nanolithography, which is based on selective removal and exchange of NTA-bound proteins by the tip of an atomic force microscope (AFM) [46]. This was achieved by use of a novel AFM mode, which enabled removal of the protein without affecting the integrity of the NTA surface. Thus, functional immobilization of different cell-surface receptors into nanometre-sized structures was achieved. Moreover, nanostructured NTA was achieved by nanoimprint lithography [47], by functionalization of nanopatterned streptavidin with a biotin-MCH conjugate [28], and by electron beam-mediated chemical nanolithography [48] (Fig. 2d). Very elegantly, AFM based metal ion loading of immobilized NTA was also used for nanopatterning surfaces with proteins [49]. DPN has also been used to spatially pattern NTA lipid into nanostructures. Selective adsorption of functionalized proteins based on streptavidin and His-tag were realized on these biomimetic membrane patterns with a resolution of 200 nm [30].

The reversibility of the interaction between oligohistine and immobilized transition metal ions has the advantage that once the template is patterned, it can be regenerated and repeatedly loaded with fresh proteins. For some cases, however, stable attachment of proteins is desired, and several attempts to stabilize these NTA-Ni complexes

Table 1 Patterning techniques used in protein affinity capturing

Patterning technique	Resolution	Other characteristics
Microcontact printing	1–16 μm (NTA hydrogel) [61], (biotin patterning) [19]	
Photolithography	5–100 μm (biotin surface) [22]	Patterning areas can be centimetres in width
Deep UV photolithography	400 nm (193 nm UV, biotin patterning) [18]	
Scanning near-field photolithography	150 nm (biotin patterning) [31]	
Nanoimprint lithography	75 nm (biotinylated BSA surface) [32], 700 nm (NTA monolayer) [47]	
e-Beam chemical nanolithography	1 μm, potentially 10 nm [48]	Currently limited to gold substrates
Native protein nanolithography: DPN, nanoshaving/nanografting	50 nm (bisNTA surface) [46], 200 nm (biotin-lipid and NTA-lipid patterning) [30]	Patterning areas limited by AFM scanning area

have been made, for example affinity mediated chemical crosslinking [50].

Perspectives

In the past ten years, tremendous progress has been made in surface engineering for protein immobilization, and powerful patterning techniques for the nanoscale have been devised. Table 1 is a list of the different protein-patterning techniques summarized in this review. For self-assembly of functional protein micro and nanostructures, multiplexed protein capturing will be critically important. Molecular printboard approaches [42, 51, 52] (Fig. 3f) and multiplexed DPN [23, 30] have already paved the way towards multiplexing protein capturing on micro and nanoscale based on the recognition pairs described above. However, for real applications, more orthogonal recognition pairs will be required for protein capturing, and potential candidates have to be thoroughly explored. Several novel techniques are currently being explored for site-specific covalent protein immobilization into microarrays including highly specific biochemical reactions such as native chemical ligation [53], and Staudinger ligation [54]. "Click chemistry" utilizing azide alkyne Huisgen cycloaddition has found usage in protein immobilization [55]. Self-immobilizing fusion proteins have been developed, employing enzymes which covalently bind to surface-coupled substrate analogues [56, 57]. Although some of these techniques—e.g. microcontact printing [58]—have already been used for lateral patterning, tailored surface modification is still missing. Also non-covalent recognition of peptide tags remains attractive, and promising solutions may be offered by oligonucleotide aptamers selected for high-affinity binding of proteins and peptides [59]. Aptamers have already been employed for protein immobilization into microarrays [60], and are likely to replace antibodies in many fields. In this promising field, surface chemistries and patterning techniques still need to be devised.

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