1 Introduction

1.1 Overview

Immobilized biomolecules are involved in numerous industrial applications but are also the basis of many fundamental biological processes. Especially the immobilization of active proteins e.g. for biotechnological, biochemical or cell-based assays is a challenging task. The biochemical activity a protein will exhibit after immobilization on a surface is dependent on the location of the surface linker, on the surface surrounding the protein and its accessibility for interaction partners as well as its surface density. In addition the lateral molecule distribution pattern for example in gradients can be important e.g. in cell signaling experiments.
A wide range of specific and unspecific immobilization approaches have been introduced. For an optimal immobilization the best method to create a functional surface with a protein generally requires extensive experimental investigation and optimization. The variety of influencing parameters often hampers the prediction and generalization of advisable immobilization routes to gain a maximum number of fully active molecules on the surface. This work approaches the challenges of immobilization of active proteins from two directions: Surface functionalization and generation of defined protein patterns on surfaces.

For an optimal surface immobilization of an active protein a wide variety of chemical immobilization approaches has to be identified since it is dependent on each individual case. To enable a faster screening of immobilization conditions two approaches to create arrays of different surface functionalizations were assessed. The first approach can generate nine areas that can be individually functionalized. This newly developed approach includes the design and construction of a device based on mechanical sealing of surface areas and proof-of-concept experiments. In the second approach a particle-based technique is applied to create an array of surface functionalities. Using this technology the adsorption of a protein is characterized with a custom developed software for batch analysis of fluorescence assay images. To test the influence of surface functionalization on the orientation of the immobilized model protein IL-8 in a high-throughput on-chip assay seven monoclonal and one polyclonal antibody were characterized with regard to their

binding site within the protein by epitope mapping. The detection of orientation is based on masking of these epitopes that might occur upon covalent immobilization via such an epitope.

Defined artificial protein patterns can serve as an interesting tool to mimic biological microenvironments of cells or in biochemical assays. Especially the relative density of immobilized molecules e.g. to encode developmental or chemotactic migration patterns is complex to control. Many methods either focus on high lateral resolution or on high sample throughput but are not suitable to provide both simultaneously. To pattern large areas with subcellular resolution and complex patterns with varying densities a lithographic method using a digital micromirror display was established to create protein patterns. This approach should allow a fast and flexible generation of grayscale patterns. Natural environments typically consist of a three-dimensional surrounding with different surface properties. To mimic three dimensional cell niches the micromirror patterning approach was applied to polymer films that were thermoformed to yield three-dimensionally structured cell culture substrates that can be decorated with arbitrary lithography patterns.

1.2 Immobilization

The immobilization of biomolecules is an essential step in biotechnology and in life sciences in general. Applications of immobilized biomolecules can be found in diagnostics in the immobilization of nucleic acids on DNA-arrays for genetic profiling or gene expression analysis [1,2], as well as in pharmaceutical production e.g. in the application of protein A for affinity chromatography of antibodies [3] or glucose oxidase for the detection of blood sugar [4] or for biocatalysis. Enzymatic products that are produced in a tons-per-year scale include enantiopure alcohols obtained from racemic alcohols by the use of enantiospecific lipases, the low calorie sweetener aspartame involving a step catalyzed by thermolysine and the 6-amino-penicillanic acid generated from penicillin by penicilin acylase. [5] The greatest advantage of immobilization is a fundamentally improved process: the product does not have to be purified from added enzymes in solution but the precious catalyst can be applied in a continuous process when immobilized to a stationary phase. In other cases such as the DNA-array technologies the immobilization is the prerequisite for signal detection in an assay. The location of a detected binding event within an array can be traced back to a certain DNA probe because its position within the array is known. In such setups the defined position after immobilization encodes the probe identity and allows a high degree of multiplexing e.g. in DNA microarrays.

In nature surface bound molecules fulfill critical functions. At the interface of cells, sugars of the glycocalyx are involved in processes such as cell-cell recognition, endocytosis or pathogen-host recognition. As a part of the extracellular matrix fibronectin mediates cell adhesion and growth [6–9]. Some signal molecules such as chemokines can interact with the glycocalyx to form haptotacic gradients that direct the migration of leucocytes [10–12].
Tailored immobilization approaches can create functional surfaces by retaining and preserving the activity of immobilized signal molecules for *in vitro* experiments to study a variety of natural processes such as cell signaling and differentiation.

Although there are numerous applications and experimental setups in which immobilized biomolecules play a crucial role and a tremendous spectrum of dedicated reactions has been introduced, the immobilization of functional proteins remains a challenging task. Protein function is based on by their three-dimensional

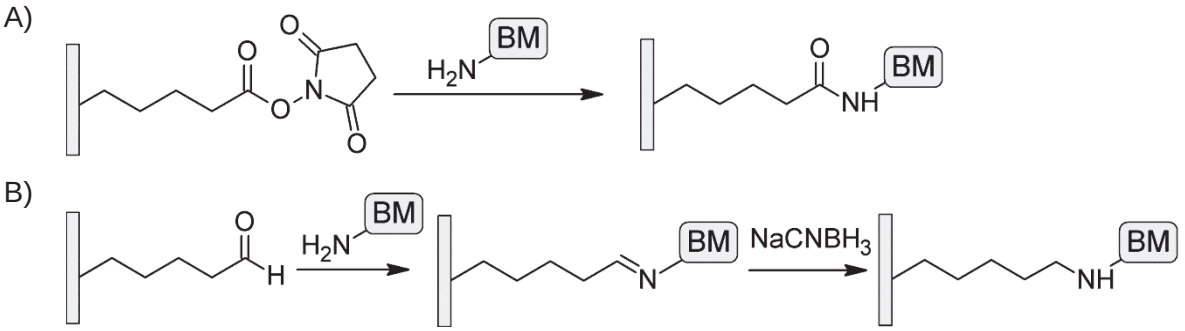
structure. The orientation and conformation a protein exhibits after immobilization determines its activity. If functional protein domains are altered during an immobilization reaction this can diminish the activity, in the worst case the protein completely loses its activity e.g. if a residue in the active site of an enzyme is covalently bound or access to the active site is blocked. A typical example is the oriented immobilization of antibodies to increase their binding activity.[13] Long linker molecules might allow a flexible orientation and good substrate access, while direct cross-linking to a surface can diminish protein activity to a minimum if the active site is no longer accessible. If a surface in a biological experiment promotes an incorrect orientation of a signal protein, the interaction e.g. with a membrane-receptor can be hampered resulting in a loss of function of the immobilized protein despite a correct conformation.

1.3 Protein immobilization approaches

Proteins expose a variety of functional groups on their surface and exhibit an enormous structural diversity. After surface binding protein denaturation e.g. by hydrophobic interaction on the surface has to be avoided and reactive sites have to remain accessible.

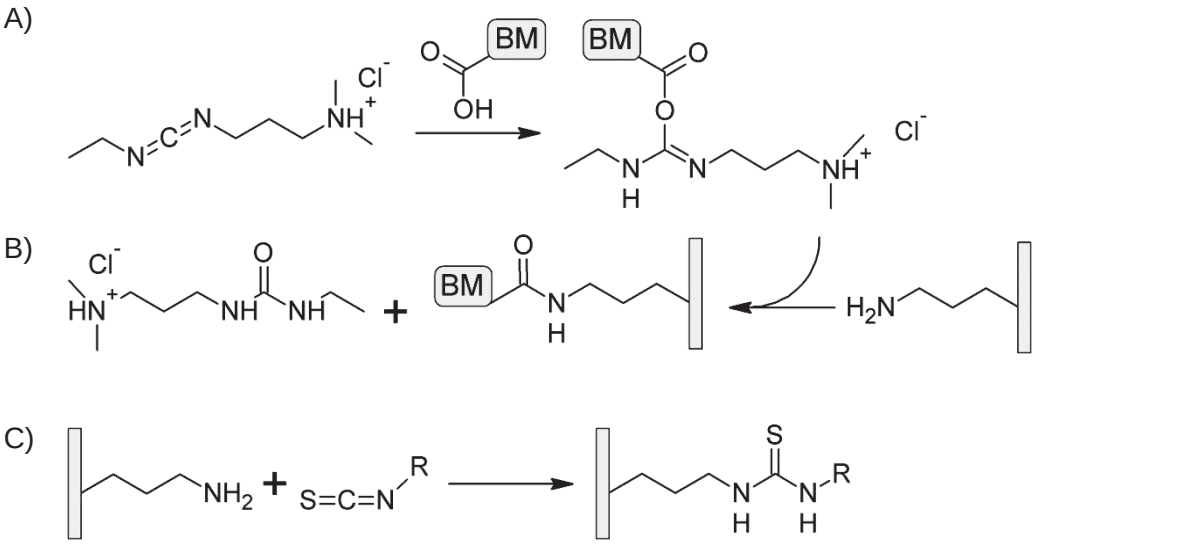
1.3.1 Unspecific immobilization

The easiest way to attach a protein to a surface is by adsorption. Such a non-covalent binding on hydrophobic surfaces generally leads to random orientations and is likely to cause conformational changes due to an unfolding of hydrophobic protein core domains over time that induce a loss of activity. [14,15] Examples include protein adsorption to the hydrophobic self-assembled monolayers (SAMs) of alkanethiols on gold [16] or antibody coatings on plastic ware such as polystyrene [14]. Nevertheless adsorptive coating is widespread, especially for less orientation-sensitive applications such as fibronectin or laminin to convey cell adhesion in cell cultivation. Similarly a random orientation can result from a covalent immobilization via reactive functional groups of proteins that are exposed on the protein surface in high abundance, such as amines (lysine, N-terminus), carboxylic acids (glutamic acid, aspartic acid) and hydroxy-groups (serine, threonine, tyrosine). They can be directly used for covalent immobilization e.g. via the reaction of epoxy groups with nucleophilic residues or *N*-hydroxy-succinimide-(NHS)-esters or aldehydes with primary amines [17] (Scheme 1).



Scheme 1: Immobilization of biomolecules (BM) via primary amines. A) Amide bond formation on NHS functionalized surface. B) Reductive amination on aldehyde functionalized surface.

Instead of pre-activating the surface an approach with carbodiimide activation of carboxylic residues aimes at coupling of the activated protein to amino-functionalized surfaces (Scheme 2, A, B). The reaction of isothiocyanate conjugated dyes (e.g. FITC) is a popular reaction for fluorescence labeling of proteins via aminogroups but can also be applied to immobilize fluoresceinisothiocyanate (FITC) to an amino-surface (Scheme 2, C).



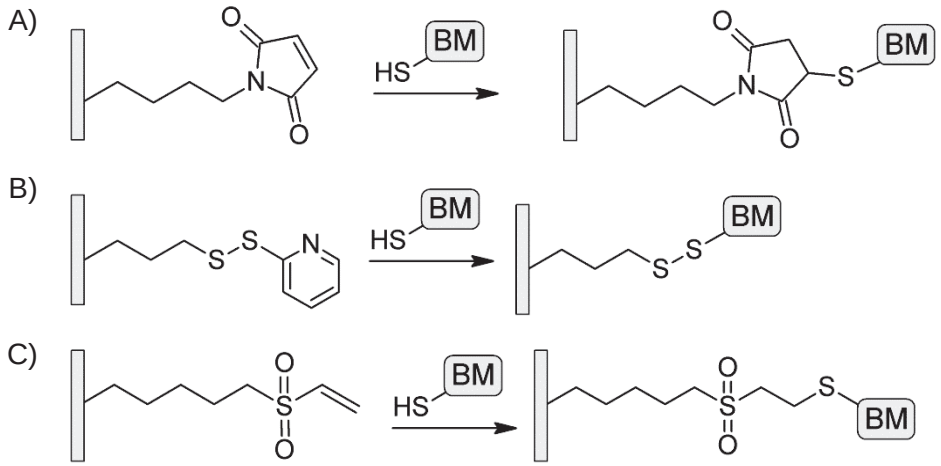
Scheme 2: Immobilization on amino functionalized surface of a biomolecule (BM) of an activated carboxyl-group and isothiocyanate. A) Activation of carboxylic groups with EDC (1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide). B) Immobilization to amine functionalized surface. C) Reaction of an isothiocyanate with an amine-surface yielding a thiourea linkage.

The high availability of such functional groups on the surface leads to random orientations of the protein molecules on the surface. If a region required for protein function is blocked this has a tremendous impact on protein activity. Hence a regiospecific immobilization is preferred if protein orientation is important for its function.

1.3.2 Regiospecific immobilization of modified proteins

A common strategy for regiospecific reactions is to introduce a tag, an additional, new functionality that can be exploited for an orthogonal immobilization reaction without interfering with other functional groups of the protein. Functional groups that are unique on the protein surface and can be reacted without side-reactions with other residues in a biological system are termed "bioorthogonal" [18].

In the most simple case, amino acids that do not naturally occur on the surface can be added to the protein sequence to allow a regiospecific immobilization. One example is the C-terminal addition of cysteine that allows bioorthogonal immobilization via maleimide, activated disulfides or vinyl sulfone groups (Scheme 3).

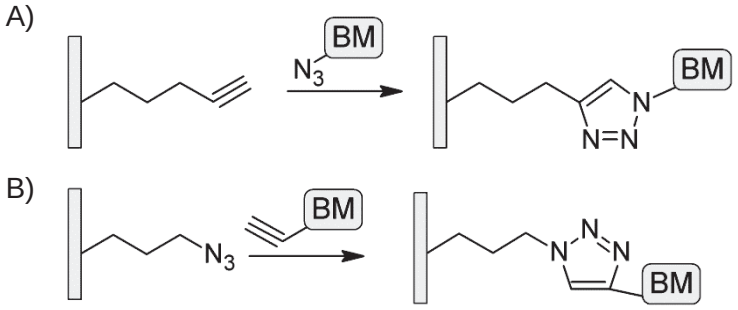


Scheme 3: Immobilization of thiol-containing biomolecules (BM). A) Maleimide functionalization; B) Disulfide functionalization; C) Vinyl sulfone functionalized surface.

Bioorthogonal functionalities can also be introduced by periodate oxidation of a N-terminal serine or threonine. The terminal 2-amino alcohol is oxidized by periodate creating an aldehyde that can be further reacted e.g. with a hydrazine forming a hydrazone. [19] Other approaches include reverse proteolysis of the C-terminus to introduce additional amino acids with bioorthogonal functionalities [20] or approaching individual aromatic side chains of tyrosine by electrophilic aromatic substitution with diazonium salts, iodine, or nitrous acid [21].

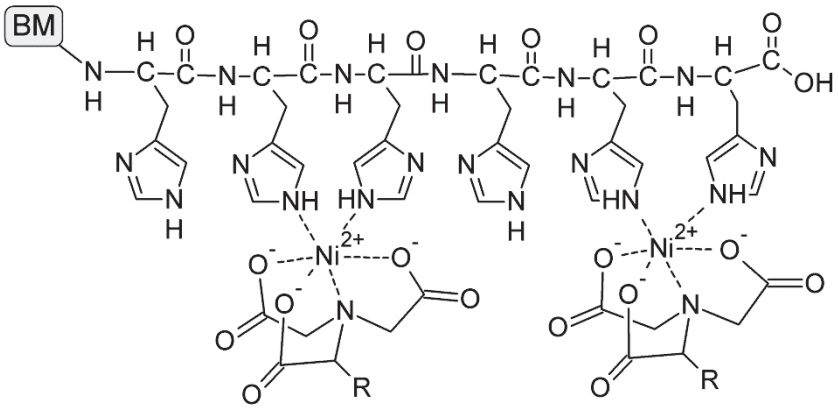
Nowadays, solid-phase synthesis of polypeptides and small proteins is possible up to a size of 100 residues. During this process artificial amino acids can be introduced at arbitrary positions to serve as a handle for following immobilizations. Alternatively heterologously expressed proteins or protein fragments can be complemented with synthetic peptides in protein semisynthesis. [22] The introduction of artificial amino acids to specifically modify a protein can also be performed by a biological approach

using amber suppression. Typically the rare amber stop codon TAG is applied in combination with modified tRNA to incorporate new amino acids into the genetic code [23]. Common examples for artificial tags are azides and alkynes [21] that allow bioorthogonal coupling reactions by the popular azide alkyne click reaction [24].



Scheme 4: Bioorthogonal reaction of azide and alkyne in a 1,3-dipolar cycloaddition. A) Alkyne functionalized surface and azide functionalized biomolecule (BM). B) Azide functionlized surface and alkyne functionalized biomolecule.

The introduction of an affinity tag can serve for specific non-covalent immobilization. A wide range of protein affinity tags has been developed. The FLAG-tag is an octapeptide [25] that can be tethered to surfaces by specific antibodies [26]. The His-tag, an oligomer of histidine -typically a hexamer- complexes immobilized bivalent cations such as Ni^{2+} or Cu^{2+} [27]. This tag is commonly applied in protein purification but can also be used for immobilization purposes.



Scheme 5: Schematic representation of a biomolecule (BM) with a His_6-tag bound to a nickel-nitrilotriacetic acid (Ni NTA) surface (R).

Enzymatic activity is also applied to immobilize proteins. The "SNAP-tag", a system that makes use of the enzymatic activity of O^6-alkylguanine transferase is an example for this. [28] Since this work is not focussed on enzymatic approaches the wide spectrum of literature will not be described in further detail.

A well established and widespread example for an affinity-based immobilization is the application of streptavidin in combination with biotin.

1.3.3 The streptavidin-biotin system

Streptavidin is a homotetrameric protein produced by the bacterium *Streptomyces avidinii*. Each subunit consists of 159 amino acids (uniprot ID P22629[25-183]) with a weight of 16.491 Da resulting in an weight of about 66 kDa for the full protein. The high affinity to biotin, also known as vitamin H or coenzyme R (244 g/mol), is believed to be a natural defense mechanism as it might inhibit the growth of other bacteria that depend on biotin as a cofactor [29]. One streptavidin tetramer can bind four biotin molecules with an association constant of 10^{15} L mol^{-1}. The non-covalent interaction is 10^3 - 10^6 times stronger than the affinity of specific antibodies to their antigens. [29] The strong interaction between streptavidin and biotin makes the complex resistant to changes in ionic strength, pH or washing once it is immobilized. In comparison to the egg-white derived avidin streptavidin provides less unspecific binding. These features make the streptavidin-biotin system popular for many applications and lead to the name of a "biological click system". For the covalent attachment of one or more biotin groups a wide spectrum of agents and approaches has been developed in analogy to common protein immobilization strategies [17]. Since the addition of biotin is a comparatively small modification for biomacromolecules such as proteins it does not significantly affect their physical or chemical properties [30]. To attach biotin to a protein the reaction with ε-amino groups of lysin residues with biotin-*N*-hydroxy-succinimid ester (NHS-ester) is a straightforward approach.
Streptavidin and many derivatives for localization and quantification purposes are commercially available. It is conjugated with organic dyes or quantum dots to be applied in fluorescence detection, conjugated with horseraddish peroxidase (HRP) or alkaline phosphatase for highly sensitive enzymatic assays in immunology or protein biochemistry and conjugated to gold particles for electron microscopy. The highly specific and robust interaction, its biocompatibility and the ease of use make the streptavidin-biotin system also popular for immobilization approaches.

1.4 Protein patterning approaches

Apart from the immobilization strategy which defines how a molecule is bound to a surface their spatial distribution is of critical importance. Biological surface patterns differ in size, shape or density contributing to the natural microenvironment of cells. It

is important to note that a functional surface will not show its maximum activity if the protein density is too low while an overpopulated surface can result in protein aggregation. Gradients of biological signal molecules influence essential processes. As part of the inflammatory response, cytokine gradients trigger the migration of leukocytes to fight invading pathogenic microorganisms [10,11,31]. In embryonic development differentiation and proliferation of neurons [32] or differentiation of stem cells to take up specific functions are controlled by patterns and gradients of signaling molecules [33].The effect of protein pattern size was shown in a study of Ingber et al. that demonstrated the requirement of cells for a minimum size of fibronectin patches to adhere and survive [34]. The shape of surface patterns was demonstrated to critically influence cell division [35]. A motif derived from systematic studies was later applied in cell based microarrays to study and control cell division by surface patterns [36,35,37] (Figure 1).

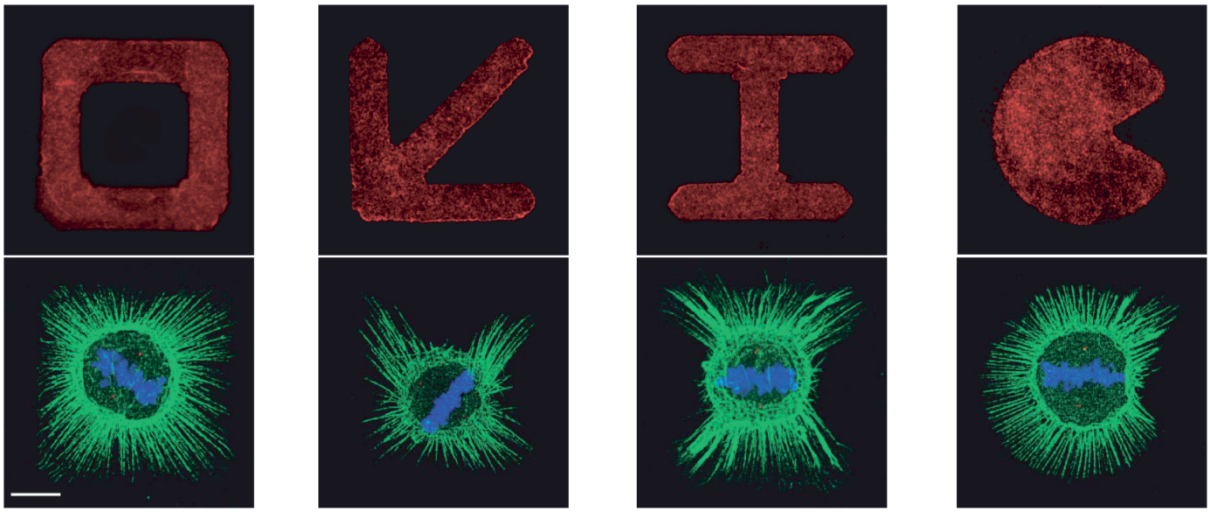


Figure 1: Surface pattern to control cell division. Mitotic cells on different fibronectin micropatterns (red, first row). Retraction fibres (green, second row), spindle poles (red) and the chromosomes (blue) were labelled. Scale bar, 10 µm. Adapted with permission from Macmillan Publishers Ltd: Nature [35], copyright 2007.

To create two-dimensional protein patterns a wide range of technologies have been introduced and discussed in literature. They differ in key features such as resolution, the color depth and patterning speed. The resolution is the size of the smallest creatable feature. The color depth defines the number of intensity levels that can be generated with an approach. While many technologies only allow the generation of binary patterns some are capable of creating "grayscale patterns" of varying molecule surface densities. The patterning speed is defined by the time that is necessary to create a pattern or to pattern a surface of a given area. This parameter

is sometimes hard to compare due to fundamental differences in patterning approaches.

1.4.1 Serial patterning approaches

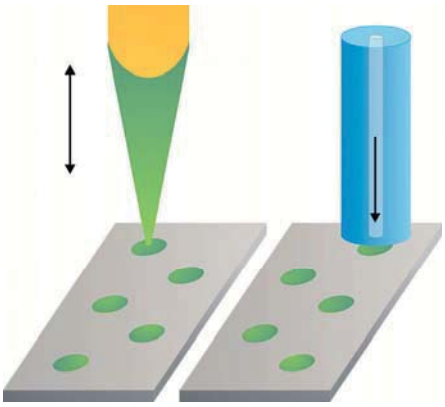
A straightforward approach to create binary patterns is direct spotting. For many applications, e.g. in microarray technology, a high density of protein spots is desired. In 1979 so-called dot blots were introduced that yielded spots in the mm size range on nitrocellulose membranes [38]. The switch to rigid substrates later on resulted in higher spot densities and application in discovery driven research in life sciences. [39,40] Classic contact printing approaches [1] in which a pin is dipped into the protein solution and then contacted with the target surface typically result in spot sizes of 150-200 µm in diameter (Figure 2, A, left). More recent contact free methods make use of capillaries with opening diameters as small as 100 nm (Figure 2, A, right). Applying a voltage between the capillary and the solution to be deposited feature sizes of ≥ 5 µm were produced. [41] A significantly higher resolution can be achieved if the probe of an atomic force microscope (AFM) is used for patterning (Figure 2, B). In dip-pen nanolithography (DPN) self-assembled monolayers (SAMs) of alkane thiols can be structured on a gold substrate. [42] After application of functionalized thiols to a surface, small molecules or proteins can be immobilized to this initial pattern. [43] Scratching a SAM with a thiol-inked AFM tip yielded features as small as 2 x 4 nm upon filling up the gap with a different thiol after scratching. [44]
Direct writing techniques such as spotting or DPN are serial processes. When the sample size or the pattern features are increased, patterning time will increase according to the size of the area to be patterned or the complexity of the given pattern.

1.4.2 Parallel patterning approaches

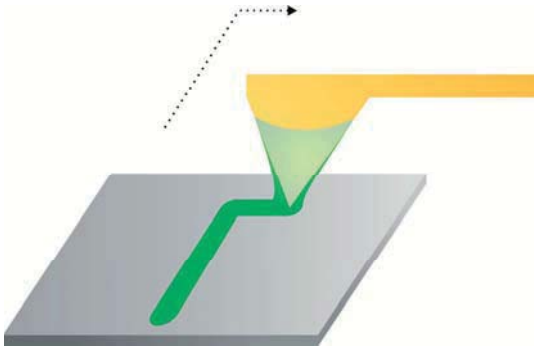
In parallel processes, a number of pattern features can be applied to a surface simultaneously. One of the most popular examples of parallel patterning is a technique referred to as microcontact printing (µCP). [45] Here a polymer stamp, most commonly made of polydimethylsiloxane (PDMS), is homogeneously coated with a protein solution and then pressed on the target substrate (Figure 2, C). Complex patterns can be transferred within seconds, and resolutions as small as several hundred nanometers can be achieved. [46] Polymer pen lithography (PPL) is a combination of DPN and µCP that uses an array of PDMS tips controlled by a DPN

device and has been applied to generate multiplexed protein patterns for cell adhesion experiments. [47]

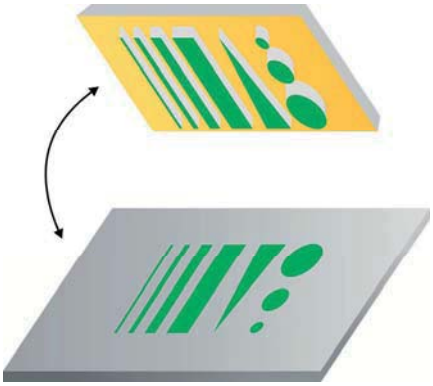
A)



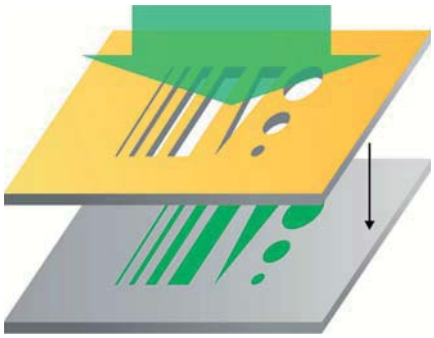
B)



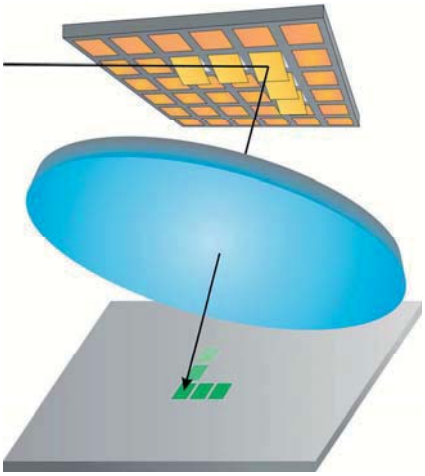
C)



D)



E)



F)

Technique	Pattern	Resolution
Microcontact printing	Binary	2 µm
Dip-pen nanolithography	Binary	55 nm
Resist based photolithograpy	Binary	1-2 µm
Digital mirror device	Grayscale	2.5 µm

Figure 2: Overview of common patterning technologies for biomolecules. Serial patterning approaches include spotting either with a classical printer or with a cantilever. A) left: contact printing with a metal pinhead, right: contact-free piezo printer with a thin glass capillary, B) writing spots or line patterns with dip-pen nanolithography. Parallel approaches that can create several features in one working step include C) micro-contract printing in which a pattern on a polymer stamp is pressed on the surface of interest and D) mask-based photolithography. A novel approach that additionally allows grayscale patterning is E) digital-mirror based patterning. F) The table presents minimum resolutions, data excerpt from [48].

As stamping leads to binary patterns, gradients have to be simulated by increasing line or spot densities.[49] Alternatively one- and two-dimensional gradients have been created using elastomer stamps of variable thickness that deliver thiols to surfaces in densities corresponding to stamp thickness.[50] Linear gradients were also created by diffusion of proteins into flat agarose stamps and transferred directly onto functionalized surfaces.[49]

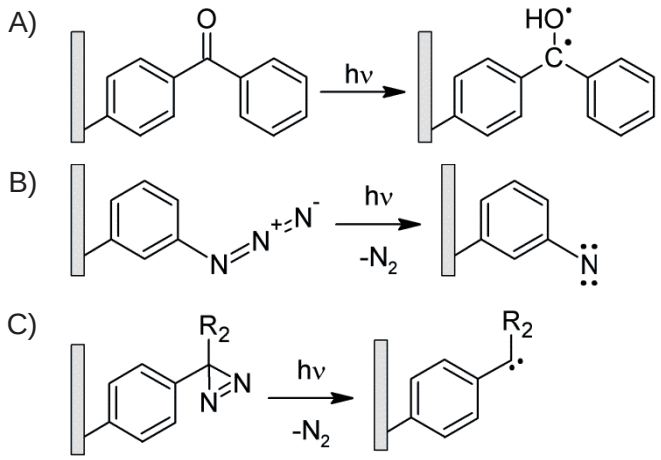
An alternative to μCP is the use of microfluidic networks (μFN) to deposit proteins and protein gradients on a substrate. In this technique an elastomer is mounted to a solid support to form micrometer scale channels. When the channels are filled with protein solution the surface is patterned according to the shape of the fluidic channels and the applied protein concentrations lead to different surface densities. This technology is intrinsically restricted to patterns that can be implemented with fluidic approaches. [51]

1.4.3 Photolithography

Photolithographic processes have emerged in the field of protein patterning within the last years. Although they benefit from decades of research in material and equipment most processes need to be adopted to avoid protein damage e.g. by high temperatures or intense UV irradiation. Derived from classical resist based approaches "bioresists" were introduced. They can be developed under mild conditions to retain protein structure. [52] For cell based applications all compounds that contain potentially hazardous chemicals should be avoided as they might bleed from the bulk in following steps after curing. Frequently unsaturated compounds such as acrylates are used in photoinduced radical polymerization either in classical lift-off [53,54]or etching processes [55]. Light-responsive surface groups allow the generation of grayscale protein patterns. A number of photochemical approaches that use adsorbed or covalently bound compounds activated by light have been introduced. Common photocleavable protecting groups are nitrobenzyl, [56] nitroveratryloxycarbonyl, [57] and *o*-cinnamoyl groups. Here coupling is performed after dose-dependent deprotection by irradiation. Direct coupling can be achieved with benzophenone, [58] phenylazide, [59] or diazirine [60] derivatives that form radicals, nitrenes or carbenes upon irradiation, respectively. [61–63]

Typically a homogeneous coating of such photolinkers is locally irradiated to create a pattern of activated functional groups that bind the protein. Different gray values and

gradients can be created by varying the irradiation intensity or time. In opposition photoresists usually yield binary patterns.



Scheme 6: Photolinkers. All molecules are excited by UV irradiation. A) Benzophenone. Irradiation yields a biradical that results in C-C bonds. B) Arylazide. Excitation yields an active nitrene that can insert into C-H, C-C, C=C, N-H, O-H or S-H bonds. C) Aryldiazirine. Upon irradiation an active carbene is formed which can insert into C-H, C-C, C=C, N-H, O-H or S-H bonds.

In 2003 Holden and Cremer introduced a simple concept for protein patterning on adsorbed proteins using a mask-based and laser excitation [64]. In their approach called protein adsorption by photobleaching (PAP) they patterned a protein coated surface with biotin functionalities using mask-based lithography (Figure 3). Initially the surface of interest is coated with a protein by adsorption (Figure 3, A). In the second step the sample is immersed in a solution of fluorescein-biotin and irradiated. Upon excitation with high energy dye molecules are bleached.

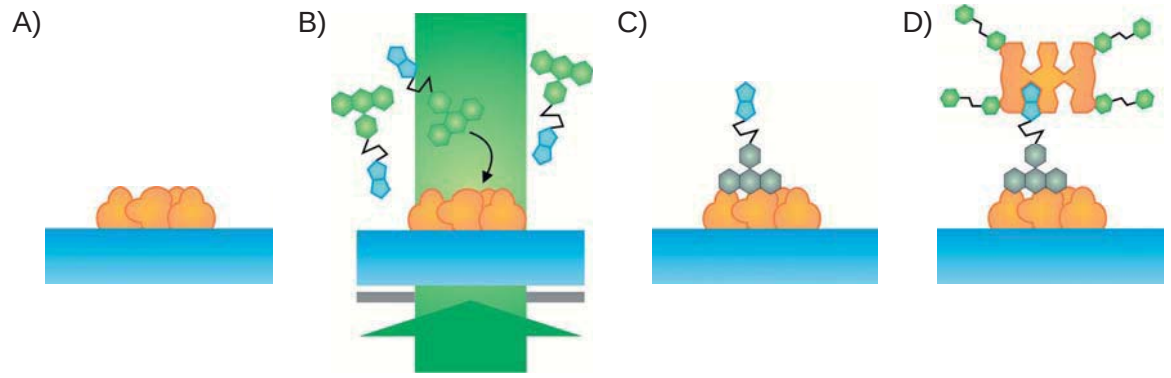


Figure 3: Scheme of protein adsorption patterning by photobleaching. A) The first step is coating of a surface with protein by adsorption. B) The surface is then immersed in a dye-conjugate solution. Bleaching of the dye through a mask (gray) induces deposition by covalent binding of activated dye-molecules to surface-exposed protein residues. C) After bleaching unreacted dye molecules are washed away revealing a primary pattern of immobilized molecules. D) The primary pattern of dye-conjugates can be decorated e.g. with fluorphore-labeled proteins.

During this step excited dye molecules that are near the surface are covalently attached to it (Figure 3, B). If a mask is used to pattern the excitation light beam the deposition reaction is restricted to irradiated surface areas. The density of immobilized molecules is thereby dependent on the irradiation intensity which allows the creation of intensity-dependent "gray values". After the deposition a primary pattern of bleached fluorescein-biotin is obtained on the surface that remains stable after the following washing step (Figure 3, C). In subsequent steps the photochemically generated primary pattern can be decorated with other molecules. Fluorescently labeled streptavidin can be added to reveal the pattern by fluorescence read-out (Figure 3, D). The exact mechanism of the photoreaction has not been elucidated, however the enhanced deposition in oxygen-enriched buffer suggests a radical mechanism. [64] The PAP approach has been successfully applied to pattern microfluidic channels coated with BSA, lysozyme, fibrinogen or IgG [65]. The approach has also been applied to chemically more defined surfaces such as methacrylate silanes. [64] The key advantage of PAP compared to most conventional photoreactive compounds is the applied wavelength. The excitation of conventional organic fluorophores does not harm proteins and allows to choose from a wide selection of compounds even for a multiplexed deposition. The process was adopted by Belisle et al. to create "grayscale" protein patterns such as density gradients. [66] Using a translucent liquid microcrystal display they created more complex and multiplexed patterns with three different dyes [67]. Such spatial light modulators (SLM) overcome the limitations of slow serial processing with a laser beam by irradiating a complete pattern based on digital data in one working step. At the same time the transmission based modulator absorbs a part of the transmitted light and limits the wavelengths available for irradiation. Protein gradients with an area of 150 to 200 µm² were created with this approach. Compared to laser writing the processing time was reduced from 80 min to about 5 min. Despite this significant increase in processing speed the method is still not fast enough to pattern bigger areas in the cm² range with the desired patterns for surface assays.

If biological responses to a variety of protein patterns are to be examined, techniques are preferable that allow the quick translation of a desired pattern from a digital data set to a protein pattern in a short time without a need to create intermediate components such as masks. At the same time patterning speed should be high

enough to create patterns in the cm² range to cover great parts e.g. of microscopy slides or other cultivation surfaces.

1.4.4 Digital mirror arrays

In parts of this work a digital micromirror device (DMD) was used for protein patterning with a PAP approach (Figure 2, D). These devices consist of thousands of micromirrors that can be individually controlled. In the on-state the incident light hits the target while it is reflected to a light dump when they are tilted to their off-state. Different light intensities (gray scales) can be created by controlling the rapid switching between both states via pulse width modulation. Individual pixel gray values are set according to a gray scale digital master image and its individual gray values. The reflection from these mirrors generates a structured light beam that reproduces the intensity distribution of the digital master. The master image can be transformed into a surface pattern of biomolecules e.g. by PAP or other photochemical approaches. Compared to translucent spatial light modulators the reflection based micromirror system does not suffer from attenuation of light passing through the SLM. In addition the irradiation maximum intensity is higher and the accessible range of wavelengths is wider. DMDs are common compounds in projectors and their use in maskless projection lithography has been described [68,69].

The use of commercially available systems in lithography is nevertheless limited, since their application is restricted to light sources of limited energy to avoid irreversible damage by overheating. Special DMDs for applications in lithography have been developed. However these systems are usually available as individual parts and have to be implemented in a custom build device. Such a system has been applied to create patterns on an immobilized protected thiol, that was subsequently decorated with biotin or proteins. [70] The DMD was used without projection optics to result in patterns in the range of several 10 µm and the device allowed the generation of binary patterns. In recent studies DMDs were also applied for a flexible fabrication of microfluidic devices also creating binary patterns. [71]
In this work maskless projection lithography applied in combination with protein adsorption by photobleaching (MPL-PAP) is introduced as a versatile tool to create protein patterns. Since fluorescence staining and detection play an important role in the described experiments the basics of fluorescence detection will briefly be reviewed.