Protein Biochips

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Chemical Strategies for Generating Protein Biochips

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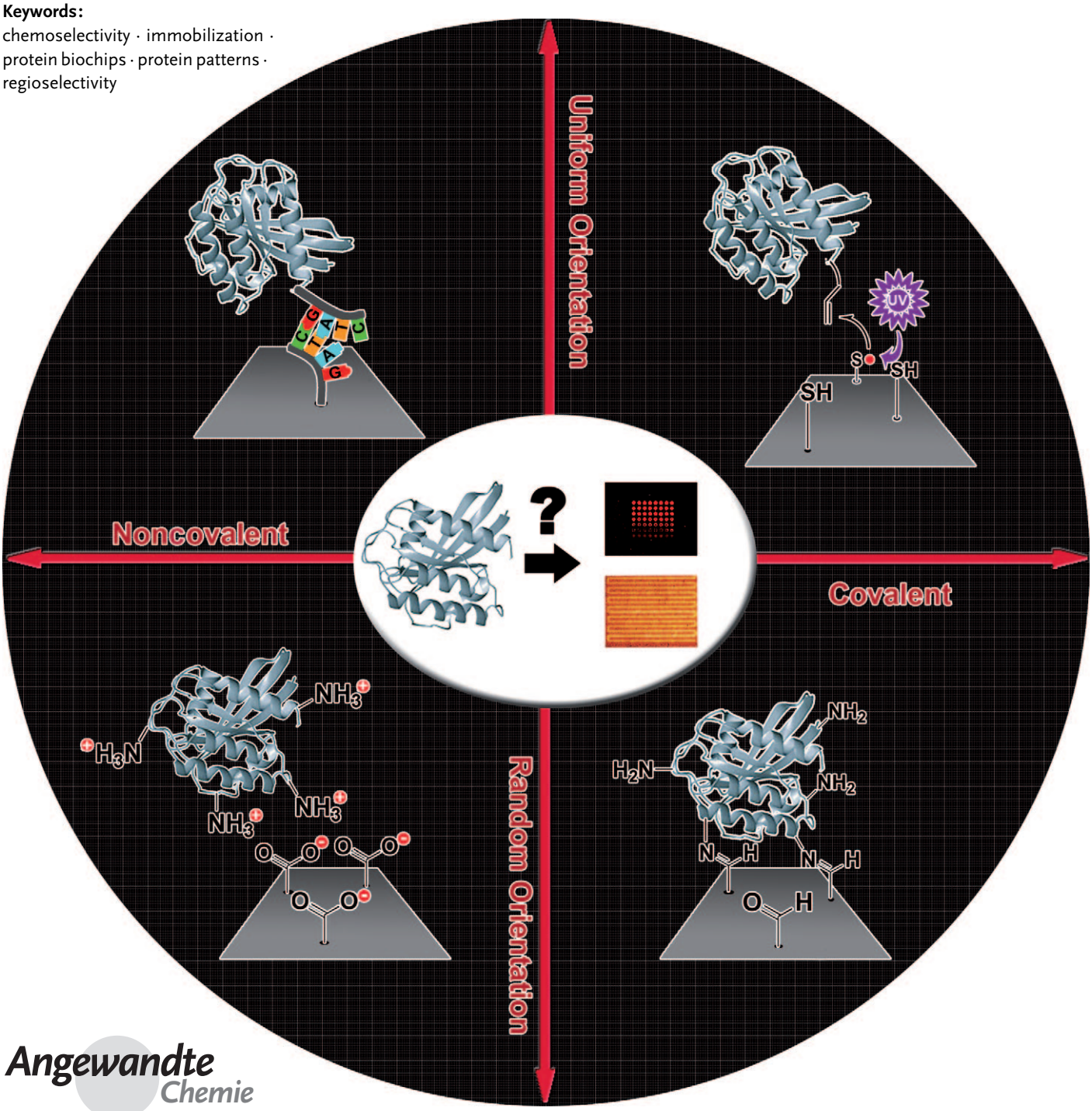

Protein biochips are at the heart of many medical and bioanalytical applications. Increasing interest has been focused on surface activation and subsequent functionalization strategies for immobilizing these biomolecules. Different approaches using covalent and noncovalent chemistry are reviewed; particular emphasis is placed on the chemical specificity of protein attachment and on retention of protein function. Strategies for creating protein patterns (as opposed to protein arrays) are also outlined. An outlook on promising and challenging future directions for protein biochip research and applications is also offered.

From the Contents

1. Introduction

An attractive approach to rapid profiling of entire proteomes has arrived with the concept of the protein microarray or protein biochip.[1] Using technologies developed for the production of DNA microarrays, thousands of proteins can be spotted onto a chip. Subsequently, a biological sample can be spread on the chip, and binding proteins can be identified. The protein of interest can then be analyzed using techniques such as fluorescence imaging, time-of-flight mass spectrometry, and peptide mass fingerprinting. Such protein biochips promise a fast, high-throughput means to profile disease-related proteins or to study protein–protein and protein–drug interactions, which was previously only possible using methods such as western blotting and enzyme-linked immunosorbent assays (ELISA). Despite the promise and potential of this concept, protein chips have become, with a few exceptions, only sparsely integrated into, for example, drug discovery research.[2–10]

Although the advantages offered by such protein microarrays parallel DNA microarray technology, in particular with respect to the requirement of only tiny quantities of precious and expensive samples and reagents, the analogy with DNA arrays is more apparent than real. Constructing protein arrays requires more steps and is more complex than the generation of DNA microarrays, in particular owing to the sensitive nature of proteins, which often results in (partial) denaturation upon chemical treatment and immobilization.[11,12]

An additional driving force for the development of protein biochips emanates from related biotechnological fields, such as the development and application of biosensors, biocatalysis, and bioanalytics. In these fields, there has been a recent shift towards generating surfaces that display proteins in highly controlled patterns, preferably with nanometer-scale precision.[13–16] The ongoing miniaturization of biodevices and biomaterials is required to address fundamental and applied problems related to complex interfaces, such as those encountered between biological entities and artificial systems and devices.[17,18]

One of the paramount challenges of manufacturing a viable protein chip is the correct choice of a solid surface and the development of surface chemistry that is compatible with a diverse set of proteins while maintaining their integrity, native conformation, and biological function.[19,20] Furthermore, protein attachment on the chip should be controlled with respect to chemical selectivity. In other words, it should be controlled which functional groups or tags of a protein take part in the immobilization. Moreover, it is desirable to control regioselectivity, that is, whether only one or several protein orientations are preferentially adopted on the surface.[21–25] In addition, one-step preparation schemes should be applied wherever possible as opposed to methods requiring a second step or more extensive protein modifications prior to immobilization.[9,26]

Typically, protein chips are prepared by immobilizing proteins on chemically activated glass slides using a contact spotter or a noncontact microarrayer, both of which became widely accessible in the high-throughput fabrication of DNA microarrays.[27,28] However, the drive to not only fabricate arrays of protein spots but to create patterns on a surface or to attain feature sizes in the nanometer regime has spurred the development of various structuring methods that are widely employed in nanotechnology and materials science.[29] For example, dip-pen nanolithography (DPN) provides access to the smallest features, which allows for a smaller chip size with many more reactive sites than conventional microscale robot spotting techniques.[30–32]

Another important challenge to the optimal use of protein biochips is the development of suitable detection strategies. A broad range of techniques has been developed, such as fluorescence imaging, surface plasmon resonance (SPR), and mass-spectrometry-based methods. These methods are described in a number of excellent review articles.[2–10,33,34] The aim of this Review is to bring together chemical, biological, and nanotechnological strategies for the generation of protein biochips. The first section presents general means for chemically tailoring organic and inorganic surfaces for protein immobilization. The following sections describe protein

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immobilization strategies facilitating either random or defined protein orientation.

2. Chemical Activation of Surfaces

The final performance of a protein biochip strongly depends on parameters related to the immobilization process itself. These include:

a) the chemical and physical properties of the surface, as they influence both specific and nonspecific binding of target and nontarget proteins;
b) the distance between the immobilized proteins and the chip surface;
c) the orientation of the immobilized proteins, which might impair binding, especially to large analytes such as other proteins; and
d) the density of the proteins on the surface, which determines the chip's sensitivity and limit of detection.

The selection of the solid surface employed for generating the protein chip depends on the intended application. For example, gold surfaces are often used for the development of biosensors with electrochemical and SPR read-out[34] because of their outstanding electrical conductivity and convenient functionalization by means of thiol chemisorption. In contrast, glass or silicon is typically preferred for optical sensors because of their transparency (in the case of glass) and low intrinsic fluorescence. In general, these surfaces are characterized by their chemical homogeneity and stability, their controllable surface properties (such as polarity and wettability), their ability to be modified with a wide range of chemical functionalities, and the reproducibility of surface modification.

2.1. Planar Chip Surfaces

2.1.1. Reactive Interfaces on Glass

Glass slides are the favored surface for DNA microarrays for a number of reasons such as availability, flatness, rigidity, transparency, amenability of the surface to chemical modification, and nonporosity.[35–37] Methodologies for functionaliz-

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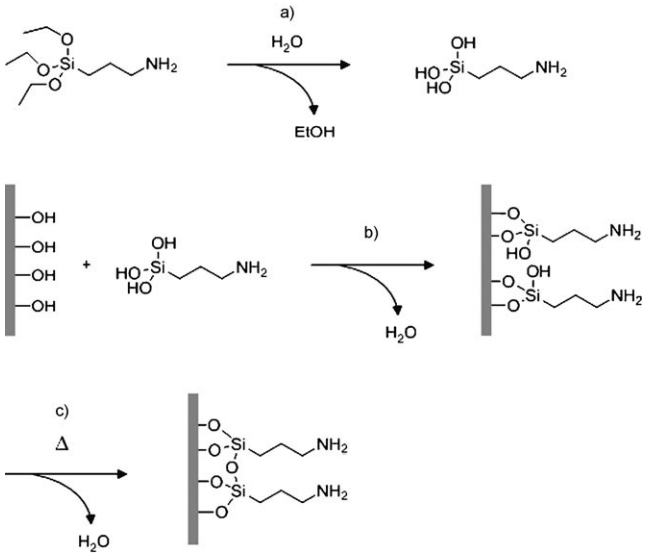
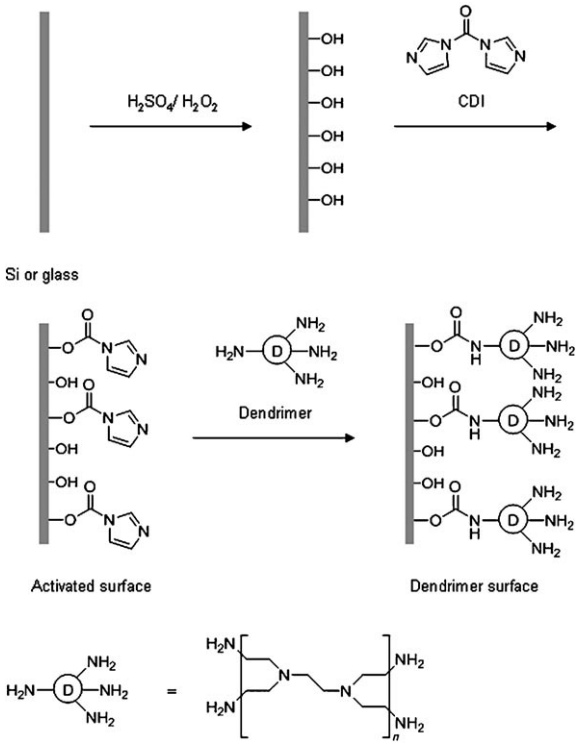
ing glass slides with chemical groups have been reported for the development of small-molecule and DNA microarrays.[28,38,39] The main method for functionalization of glass slides uses reactive silanol groups (Si–OH) on the glass surface. The silanol groups can be generated by pretreatment of the surface with, for example, piranha solution (H_2O_2/H_2SO_4) or oxygen plasma. Organofunctional silanes of the general structure $(RO)_3Si(CH_2)_nX$ or trichlorosilanes are then used to introduce a new functional group on the surface.[40] A large variety of silane reagents are commercially available, bearing amine, thiol, carboxy, epoxide, and other functional groups for subsequent modification steps.[40–42] Scheme 1 displays the proposed steps involved in this functionalization.[41–45] The initial hydrolysis step can occur either in solution or at the surface, depending on the amount of water present in the system.[43,45] An overabundance of water results in excessive polymerization in the solvent phase, while a deficiency of water results in the formation of an incomplete monolayer.[43,44] Various protocols for silanization can be found in the literature, employing deposition of silanes from organic solutions, from aqueous solutions, from the gas phase, or by chemical vapor deposition.[46]



Scheme 1. Proposed mechanism for the silanization with aminopropyltriethoxysilane (APTES): Hydrolysis of the reactive siloxanes (a), which can take place in solution or on the substrate surface, allows condensation with surface silanol groups (b). Thermal curing of the resulting film causes further cross-linking (c).[41–45]

Dendrimers are compounds with branched chemical structures that can carry a range of chemically reactive groups at their periphery. They have been applied for surface derivatization to create a larger functional surface area. The dendritic structure can either be synthesized in situ by derivatization of the surface with multifunctional linkers[47] or be generated by direct surface modification with a presynthesized branched structure, such as polyamidoamine,[46,48,49] phosphine,[50] or poly(propylene imine) dendrimers.[51] In general, the second strategy (Scheme 2) is preferred, as reactions on the surface suffer from lower yields

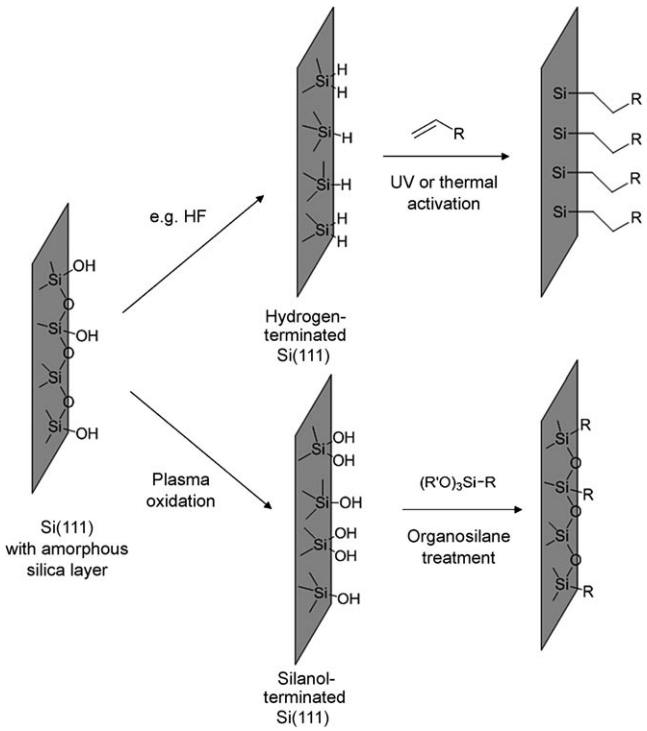


Scheme 2. Production of dendrimer-functionalized chips using poly(propylene imine) dendrimers: After cleaning with piranha solution, the glass or silicon surface can be activated with carbonyldiimidazole (CDI), for example. Dendrimers can then be immobilized through their peripheral amine groups.[51]

and thus decrease the number of activated groups. In a systematic study, Pathak et al. showed that by increasing the density of poly(propylene imine) dendrimers, the activity of alkaline phosphatase randomly immobilized on dendrimer-coated glass slides can be preserved.[51]

2.1.2. *Reactive Interfaces on Silicon*

Apart from glass, silicon has also been used as a surface material for fabricating biochips. Silicon wafers are used on a large scale in the semiconductor industry, because these chips possess high electric conductivity, great chemical resistance to solvents, good mechanical stability, and low intrinsic fluorescence. However, chemical functionalization of silicon surfaces is complicated by the fact that silicon spontaneously oxidizes in air to produce an amorphous silica layer. Therefore, surface modification strategies for the formation of covalent silicon–carbon bonds require, first, a special pretreatment of the silicon surface to remove the oxide layer and, second, an activation of the silicon surface for subsequent reaction with organic moieties (Scheme 3). This activation is typically achieved by treatment of the silicon surface with HF to generate a hydrogen-terminated Si(111) surface, which can further react with unsaturated ω-functionalized alkenes upon ultraviolet irradiation or thermal activation.[52] Alternatively, when silicon is oxidized with plasma (ionized gas), functionalization with organosilanes in analogy to glass slides can be carried out.[41] In recent years, various procedures have been



Scheme 3. Examples of Si(111) functionalization: Si(111) spontaneously forms an amorphous silica layer in air. Treatment with HF, for example, produces a hydrogen-terminated silicon surface that can react further, for example with ω-functionalized alkenes. Treatment with oxygen plasma provides silanols at the Si(111) surface, which can then react with organosilanes.[41,52]

developed to functionalize a range of alternative oxide surfaces that are of particular interest for specialized applications such as implants (titanium, tantalum, and niobium), electrical devices (indium tin oxide (ITO) and diamond), and others, such as silicate minerals (mica). Silane chemistry[53,54] and electropolymerization[55,56] procedures were applied in the case of ITO, while photoimmobilization was used to activate diamond.[57] To functionalize mica, (poly)electrolytes were used in addition to silane chemistry,[58–60] while for titanium, tantalum, and niobium[61,62] self-assembled monolayer (SAM) formation using thiols and phosphonates was also reported.[63,64]

2.1.3. *Reactive Interfaces on Gold*

Gold surfaces have been extensively used in biosensing applications and offer the advantage of being easily functionalized with SAMs[65,66] of ω-functionalized thiols, disulfides, and sulfides. The generation of SAMs on such surfaces strongly depends on the crystalline morphology of the underlying metal. Au(111) yields SAMs having the highest density and highest degree of regularity and is therefore most widely applied. Usually, gold films are applied onto polished glass, silicon, or freshly cleaved mica.[65,66] The thermal deposition of gold onto silicon wafers is the most feasible method for the preparation of gold surfaces. Using an intermediate layer of chromium or titanium promotes the adhesion of gold on the silicon wafers. The preparation of smooth gold surfaces can be carried out employing the so-called template stripping method of gold from, for example, mica, which is described elsewhere.[67] The chemistry of the gold–thiol interface is well known and is much easier to control than organosilane chemistry. Thiols, sulfides, and disulfides are dissolved in a sufficiently pure solvent (in general ethanol or water) and are then applied onto the cleaned gold surface. A typical thiol monolayer on gold is shown in Figure 1. Generation and applications of SAMs have recently been reviewed elsewhere.[65,66]

The terminal groups of heterobifunctional thiol compounds are important for the potential interaction of the SAM with proteins, and thus, a variety of functionalized thiols are commercially available and have been used in protein biochip applications. As long as the terminal groups are relatively small, they have little or no influence on the orientation of the monolayers. However, this is not the case for large terminal groups, such as proteins. Therefore, to circumvent the reduction of packing density upon incorporation of large headgroups, mixed SAMs are increasingly used for the immobilization of biomolecules.[68]

For a mixture of two thiol compounds that does not show demixing tendencies, a random attachment of both compounds to the surface can be assumed.[69] Thus, an ω-substituted alkane thiol can be mixed with short-chain

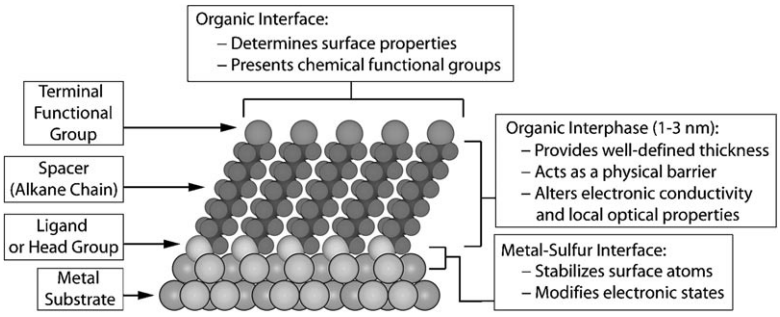


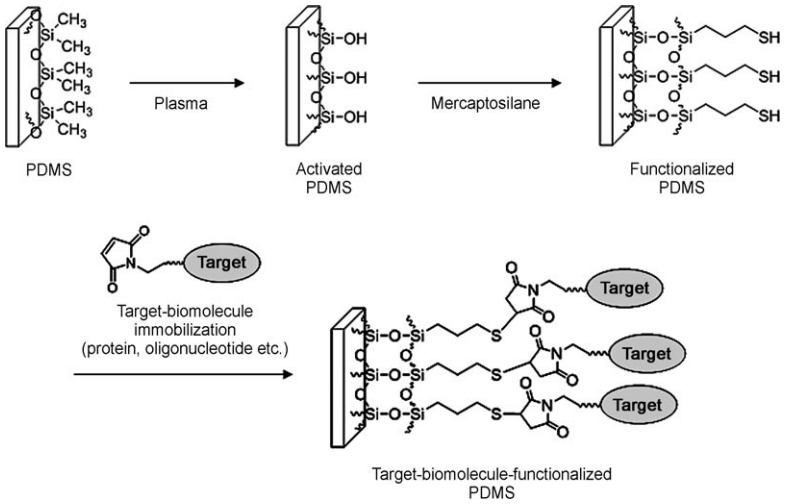
Figure 1. Ideal self-assembled monolayer (SAM) of terminally functionalized alkylthiolates bound to a Au(111) surface, showing the alkyl chains in the characteristically tilted orientation.[66] Reproduced with permission from ACS.

unsubstituted thiols. For the resulting presentation of anchor molecules, steric hindrance is minimized. The major drawbacks of using thiolates on surfaces are, however, their mobility on the solid surface, which limits the lifetime of the chips,[65,66] and their susceptibility to photooxidation.[70] This susceptibility, however, can be utilized to selectively pattern SAMs by taking advantage of lithographic techniques.[70]

2.1.4. *Reactive Interfaces on Polymer Surfaces*

Polymer support materials are potentially valuable alternatives to inorganic surfaces. The inexpensive production of

polymers makes their use in commercial applications particularly attractive. For example, in ELISA applications the most commonly used microtiter plate material is polystyrene,[71–74] and a wide range of microfabricated devices are also based on polymers. Currently these polymeric devices are becoming available for implementation in existing standard microarray equipment.[75–77] Prominent examples of such devices are microfluidic chips, which are usually manufactured from polymeric materials such as poly(dimethylsiloxane) (PDMS), poly(methyl methacrylate) (PMMA), and polycarbonate (PC). For protein immobilization, chemical surface modification of these polymers is required, as they lack suitable functional groups in their native form.[78,79] For example, PDMS can be treated by plasma oxidation, which allows functionalization with organosilanes (e.g. mercaptosilanes to generate thiol-terminated PDMS; Scheme 4).[78]



Scheme 4. Example for the chemical functionalization of PDMS: A PDMS substrate is activated by plasma oxidation and functionalized with a mercaptosilane to produce a PDMS surface displaying thiol groups, which can then be used for subsequent immobilization of target biomolecules, for example, maleimide-functionalized proteins or oligonucleotides.[78]

PMMA can be treated with 1,6-hexanediamine to obtain an aminated surface that is suitable for subsequent protein immobilization.[79] In the case of PC, sulfonation of the surface with sulfate groups has been described.[79]

2.2. Chips with 3D Matrixes

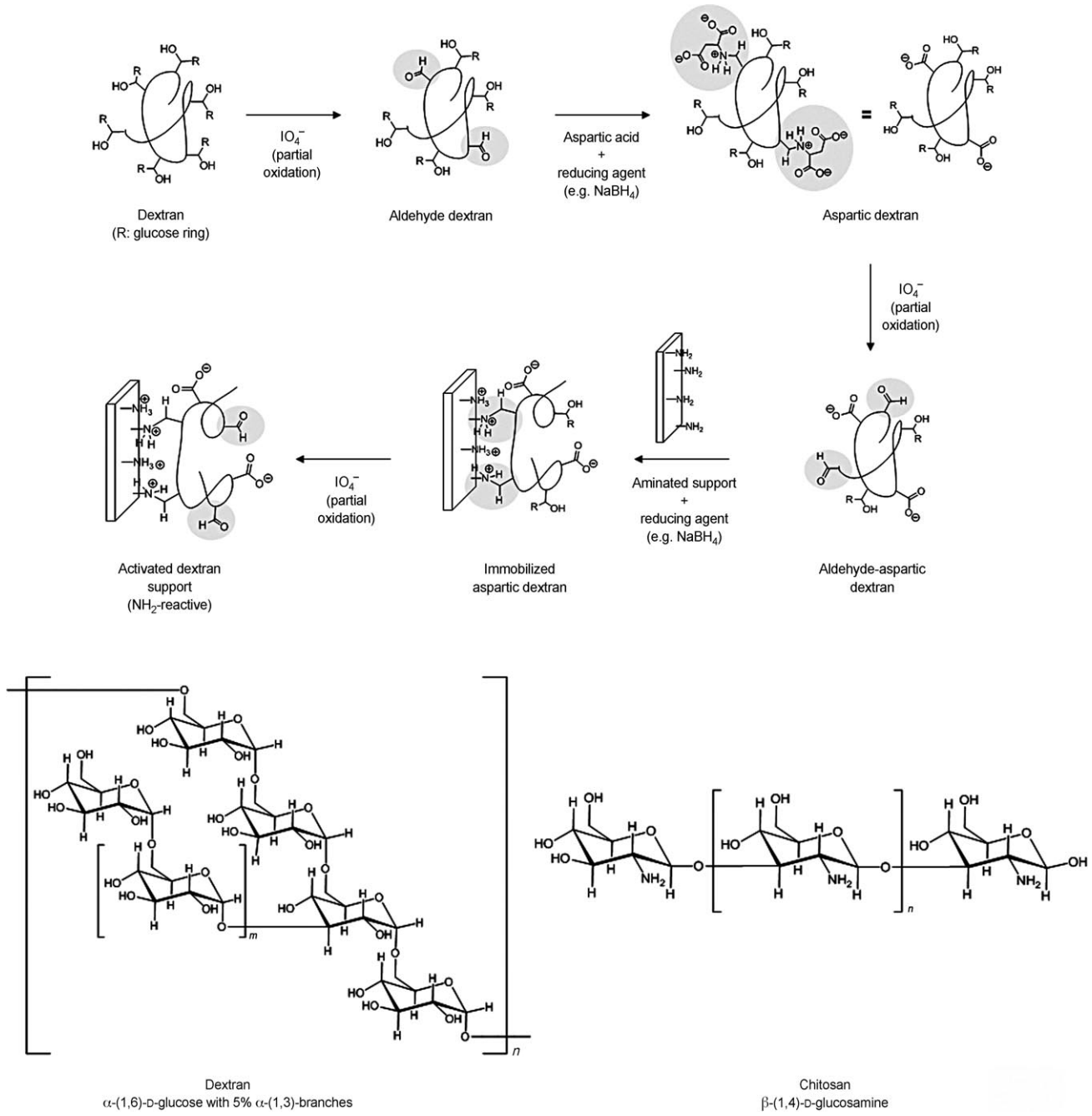
Instead of spotting proteins onto a two-dimensional solid surface, molecules can diffuse into a porous matrix formed by polymer membranes or hydrogels. These matrixes show a high capacity for protein immobilization and can provide a more homogeneous "natural" aqueous environment than flat surfaces, thus preventing denaturation of proteins. However, they suffer from problems related to mass transport effects and sometimes high background signals.

Traditional membrane materials that have been used are nitrocellulose and nylon, the latter providing greater physical strength and binding capacity. Protein attachment to nylon is also generally more stable than to nitrocellulose. Nylon allows for positive or negative electrostatic interactions or photocross-linking, while nitrocellulose is believed to bind proteins by means of hydrophobic interactions.[41] Casting of these membranes onto the surface of glass enhances their stability and thereby improves spot resolution from 0.5–1 mm to 25–200 μm, allowing for their application in microarray technologies.[36,80] Further improvement of mechanical stability is offered by anodically oxidized porous alumina. This material offers readily available surface chemistries, in particular silanization methods, which can lead to higher densities of biomolecular probes, and thus to higher sensitivity in array applications.[81]

Polymeric hydrogels represent hydrophilic matrixes into which proteins can diffuse, leading to an up to 100-fold higher capacity of immobilization than is found for planar surfaces.[82,83] Covalent attachment of the gels to solid surfaces allows for generation of stable microarray chips. For example, agarose and acrylamide can be photopolymerized onto a surface functionalized with acryl groups.[82,83] Subsequently, the polymer can be activated with hydrazine or ethylenediamine to generate amine groups on the surface.[84,85] Other examples of polymeric gel surfaces that can be used for the immobilization of proteins involve polysaccharides, such as chitosan or dextran. Chitosan is an amine-modified, natural, nontoxic polysaccharide, and it is biodegradable. Owing to its pH-responsive properties, it can simultaneously be immobilized onto glass supports and bind proteins through electrostatic interactions. Dextran is a complex branched polysaccharide consisting of glucose molecules joined into chains of varying lengths. Dextran hydroxy groups can be oxidized to aldehyde functionalities that can then be covalently immobilized onto amine-functionalized supports (Scheme 5). This combination forms the surface of commercial Biacore chips.[136] Unreacted aldehyde groups can be further used for protein immobilization. Figure 2 shows the use of lysine-functionalized poly(ethylene) glycol (PEG), which was spotted onto aldehyde-coated glass slides; subsequently, immunoglobin G (IgG) was immobilized.[86] Supramolecular hydrogels composed of glycosylated amino acids have recently been introduced as a surface material for protein arrays.[87] Biodegradable polyesters, such as poly(L-lactic acid) and its various copolymers with D-lactic acid and glycolic acid, have also been studied as surfaces for biological applications.[88]

2.3. Design Principles for Minimizing Nonspecific Adsorption

In contrast to DNA microarray applications, nonspecific binding represents a major obstacle in the development of protein microarray assays. As nucleic acids are uniformly negatively charged, spontaneous adsorption to a given surface



Scheme 5. Reaction of dextran with periodate produces non-ionic aldehyde dextran. Subsequent modification with aspartic acid and periodate results in aldehyde- and aspartate-modified dextran, which can be attached to amine-capped surfaces by reduction. To recover protein-reactive aldehyde functionalities, further oxidation with periodate is necessary, affording a negatively charged, protein-capturing polymer.[41]

is much easier to suppress than for proteins, which can adsorb through electrostatic, van der Waals, and Lewis acid–base forces as well as through hydrophobic interactions and conformational changes.[89,90] As mentioned above, nonspecific adsorption of proteins (e.g. antibodies) still represents the standard methodology for the immobilization of proteins in microtiter-plate assays. However, the quality of these assays is determined not only by the desired binding events between proteins but also to a large extent by the suppression of undesired, nonspecific binding of analytes and other components within the biological sample. Such nonspecific binding can give rise to background signals and thus to low signal-to-noise ratios. Achieving low degrees of nonspecific binding is therefore the most important factor not only in ELISA and related high-sensitivity protein detection assays[91] but also in the preparation of protein microarrays.

Effective reduction of nonspecific adsorption has been achieved by careful selection of the surface material, for instance by using naturally occurring surfaces such as elastin,[92] sarcosine,[93] agarose,[94] cellulose,[95] and polysac-

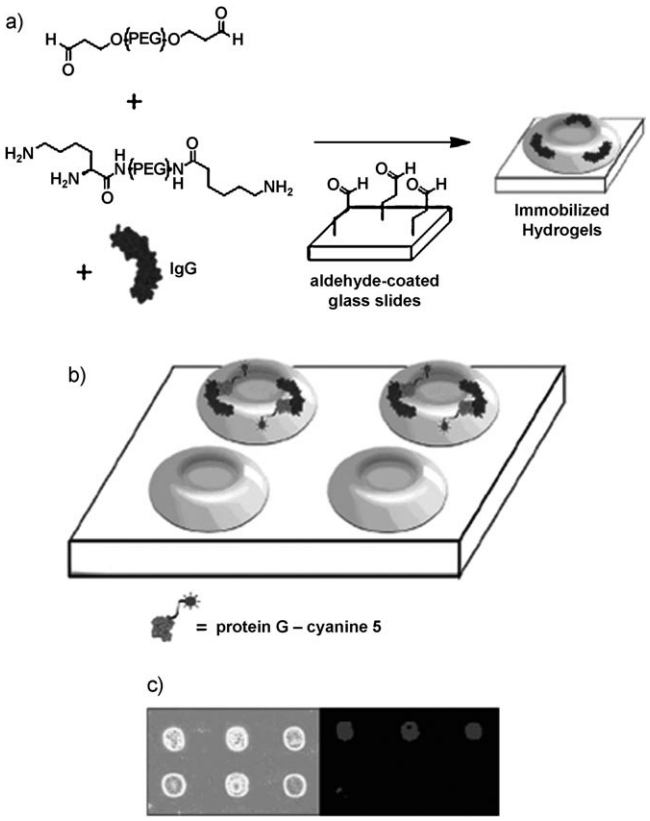


Figure 2. a) Hydrogels created from aldehyde- and lysine-functionalized poly(ethylene glycol) (PEG) and containing IgG were spotted onto aldehyde-coated glass slides to create gel pockets attached to the glass slides. b) Incubation of the IgG-containing hydrogel spot array with Cy5-labeled protein G allowed detection of IgG-containing hydrogel spots. c) Optical (left) and fluorescence images (right) of the hydrogel spots. Spot diameter is approximately 200 mm. Only the top row of hydrogel spots, which contain IgG, show fluorescence.[86] Reproduced with permission from ACS.

charides,[93,96] or by using synthetic polymeric surfaces such as fluorocarbon polymers and molecules,[97,98] polyethylene glycol,[99,100] poly(vinyl alcohol),[101,102] or polyelectrolytes.[100,103,104] One particularly versatile approach to suppressing nonspecific adsorption is based on surfaces that present oligo(ethylene glycol) derivatives.[105–107] An elegant study by Whitesides and Prime showed that crystalline helical and amorphous forms of SAMs of oligo(ethyleneglycol)-functionalized alkanethiolates on gold are resistant to protein adsorption.[108]

It is hypothesized that binding of interfacial water by the ethylene glycol layer is important for the ability of the SAM to resist protein adsorption.[109] However, the susceptibility of ethylene glycol chains to autoxidation limits their long-term application. Surface phospholipids also minimize nonspecific binding. Their strong hydration capacity, achieved by electrostatic interaction, is postulated to be responsible for this effect.[109] The zwitterionic properties of monolayers of, for example, oligophosphorylcholine SAMs result in suppression of kinetically irreversible nonspecific adsorption of proteins. Unfortunately, phosphorylcholine monolayers are not very stable. In an attempt to further rationalize the design of surfaces resistant to protein adsorption, Whitesides and coworkers formulated a hypothesis relating the preferential exclusion of a "solute" to its ability to render surfaces resistant to the adsorption of proteins (Figure 3). When

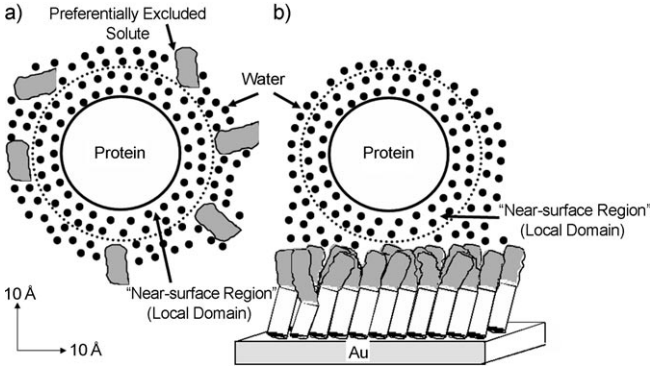


Figure 3. Protein-resistant surfaces: Solutes preferentially excluded from the "near-surface" solution domain of a protein cause substantial preferential hydration of the protein (a). Attaching such a solute to a surface minimizes (unspecific) protein adsorption (b).[110] Reproduced with permission from ACS.

elements of known osmolytes (organic compounds affecting osmosis) or kosmotropes (organic compounds contributing to the stability and structure of water–water interactions) were incorporated into alkanethiolates such as betaine, taurine, or hexamethylphosphoramide, SAMs of these compounds displayed improved protein repellency.[110] Although these elaborate approaches have proven to be effective for minimizing nonspecific adsorption, it must be clearly stated that the old-fashioned blocking of reactive surface sites by the addition of blocking agents such as the protein bovine serum albumin (BSA), skim milk powder, or other reagents and the presence of surfactants such as Tween-20 and sodium dodecyl sulfate (SDS) are usually indispensable to the suppression of nonspecific protein adsorption.[72]

2.4. Installing Functional Groups on Activated Surfaces

Reactivity of a chip surface is determined by the functional groups it displays. The density of the reactive groups is one important factor controlling the amount of protein that can be immobilized on a specific surface area and thus consequently influences the limit of detection attainable with the particular chip. The direct attachment of a protein to a surface without a spacer can cause steric constraint of the protein's reactivity or interaction capability compared to the protein in solution. Moreover, multiple direct contacts with the surface can induce denaturation or partial denaturation and thus a decrease in activity. By introducing a spacer between the protein and the reactive group on the surface, these effects can be minimized. The spacer can be of nearly any desired length and can possess a variety of chemical characteristics; it can be rigid or flexible, hydrophilic or

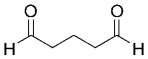
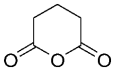
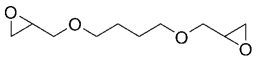
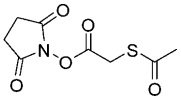
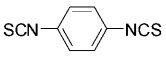
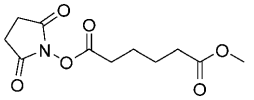
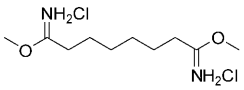
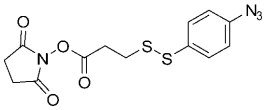
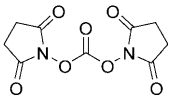
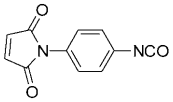
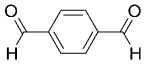
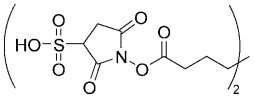
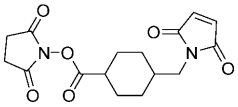
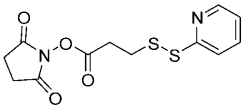
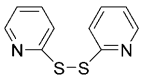
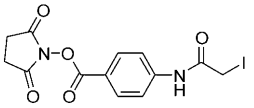
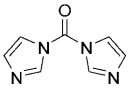
hydrophobic, charged or neutral.[42,111] It can even contain programmable information, as we will discuss in the context of nucleic acid linkers (see Section 3.2.1.4).

Surface functionalization with a spacer can allow for attachment of potentially any desired reactive group. Such spacers are often bifunctional, consisting of either two similar or two different groups. Most commonly used spacers are those carrying two similar groups that react with amine groups on surfaces generated as described in the previous sections. Commonly used examples are glutaraldehyde, 1,4-butanediol diglycidyl ether, 1,4-phenylene diisothiocyanate, dimethylsuberimidate, divinylsulfone, bis(sulfosuccinimidyl)-suberate, disuccinimidyl carbonate, or terephthaldialdehyde (Table 1). In the case of thiol-preactivated surfaces, 2,2′-dipyridyl disulfide can be used to initially generate a reactive thiopyridyl disulfide, which then readily forms disulfide bonds with cysteine groups of proteins. In the case of hydroxy-preactivated surfaces, *N*,*N*′-carbonyldiimidazole (CDI) can be used. Homobifunctional spacers have the disadvantage of potentially connecting two neighboring groups, either on the surface or on the biomolecule, thereby blocking the reactivity for further attachment of other molecules or inducing undesired cross-linking. To avoid this effect, ring opening or heterobifunctional spacers can be employed. Examples of such spacers (Table 1), which are commercially available, comprise glutaric anhydride or molecules carrying a thiol-reactive maleimide and an amine-reactive succinimidyl ester functionality such as SMCC and SIAB.

3. Protein Immobilization Strategies

The chemical strategy that is chosen to attach proteins to surfaces can largely determine the properties of the protein biochip. Conventional spotting of unmodified proteins onto reactive surfaces leads to statistical orientation of the proteins

Table 1: Structures of commonly used spacer/linker molecules (SATA, MSA, SADP, PMPI, SMCC, SPDP, SIAB from Pierce).

Spacers	
Homobifunctional	Heterobifunctional
Amine-reactive	**Amine-reactive**
glutaraldehyde 	glutaric anhydride 
1,4-butanediol diglycidyl ether 	SATA (protected thiol) 
1,4-phenylene diisothiocyanate 	MSA (protected acid) 
dimethylsuberimidate 	SADP (photoreactive) 
divinylsulfone 	**Amine- and hydroxy-reactive**
disuccinimidyl carbonate 	PMPI 
terephthaldialdehyde 	**Amine- and thiol-reactive**
bis(sulfosuccinimidyl)suberate 	SMCC 
Thiol-reactive	SDPD 
2,2′-dipyridyl disulfide 	SIAB 
N,*N*′-carbonyldiimidazole 	

on the underlying surface. Numerous examples of antibody arrays have shown that this strategy is sufficient to maintain the antibody's binding capability and thus enable the anticipated application. However, in certain cases when the chemical strategy invokes random protein orientation, the properties of the immobilized proteins may be partially or even completely lost.[5,10,112,113] To ensure accessibility of the protein's active site and thereby enable the detailed study of protein function, a homogeneous surface orientation of proteins on chips without affecting their conformation and function should be sought. This goal is generally met by adopting chemoselective protein immobilization strategies. This section surveys two chemical strategies for the immobilization of proteins. The first part addresses the use of nondirectional noncovalent (Section 3.1.1) and covalent (Section 3.1.2) coupling reactions to immobilize proteins on surfaces, characteristically resulting in randomly oriented proteins. The second part covers the use of more sophisticated methodologies based on either directional noncovalent interactions (Section 3.2.1) or covalent chemo- and regioselective coupling reactions (Section 3.2.2), both of which lead to uniformly oriented proteins on surfaces.

3.1. Randomly Oriented Proteins

3.1.1. Noncovalent Attachment

Proteins can adsorb on surfaces through ionic bonds and through hydrophobic and polar interactions. Which of these intermolecular forces dominate the interaction will depend on the particular protein and surface involved. Random protein physisorption on hydrophobic polystyrene microplate surfaces represents the by far most common method of protein immobilization. Protein immobilization can also be achieved through electrostatic interactions. Surfaces that are modified to contain positively charged amine or negatively charged carboxy groups are most suitable for this approach. The resulting adsorbed protein layer is likely to be heterogeneous and randomly oriented, since each molecule can form many contacts in different orientations, minimizing repulsive interactions with the surface and previously adsorbed proteins. Adsorption strategies are mainly used with three-dimensional materials. For example, polypropylene membranes modified with polyaniline allow adhesion through combined electrostatic and hydrophobic interactions and demonstrate high affinity for and compatibility with different proteins.[85] However, the most common polymer surfaces in protein chips are hydrogels on gold, which are used in Biacore and other technologies employing SPR read-out.[114,136] Nitrocellulose on glass is also widely applied, because protein binding at such surfaces is well known from western blots and diagnostic membrane-based systems.[115,116] Among hydrogels, sulfate-modified dextran performs much better than dextran modified with cellulose and aspartic acid because of the higher density of charged groups on the surface, which can lead to improved adsorption.

A clean gold surface can also be directly functionalized with thiol-containing molecules. Therefore, direct immobilization of proteins on gold surfaces can be achieved by simply exploiting the high affinity of cysteine residues in the protein for the gold surface, resulting in efficient chemisorption.[117,118] After immobilization, a passivation step should be carried out to block remaining gold areas. This method is advantageous, because no surface modification is necessary prior to protein attachment. However, the adsorption capacity of such surfaces is limited, and proteins tend to lose activity when in direct contact with metal surfaces. Protein activity and surface loading can be increased significantly when SAMs are used as an intermediate layer (Section 2.1.3). Although electrostatic interactions are not directive, they are often used when patterning antibodies onto negatively charged nanoparticles that are deposited on surfaces in specific patterns using DPN[119] etching,[120] or colloidal lithography.[121] Alternatively, hydrophilic domains of phase-separated block copolymers or directed deposition of hydrophilic polymers can be used to pattern antibodies on surfaces.[122–125]

3.1.2. Covalent Attachment

In general, proteins offer many functional groups, mainly in the amino acid side chains, that are suitable for immobilization purposes. Such functional groups can be used to covalently couple proteins to surfaces by a range of different reactions. Suitable complementary groups installed on the solid support are described in Section 2. Various chemically modified surfaces are commercially available for this purpose, and some of them are designed to suppress nonspecific adsorption. Many of these methods have been established and refined in the course of the development of Biacore technology, which provides a robust platform for in situ monitoring of binding events (e.g. protein–protein interactions). Such Biacore chips can also be adapted with noncovalent capture ligands, which are described in Section 3.2.[136] Table 2 shows some typical examples of compatible groups attached to surfaces and the functional groups they react with. It is important to note that protein attachment can occur simultaneously through many residues, thereby restricting degrees of conformational freedom (and thus possibly activity) and also increasing heterogeneity in the population of immobilized proteins.

3.1.2.1. Amine-Reactive Surfaces

By far the most common method to covalently attach proteins to surfaces uses the amine group of the lysine side chain. Unfortunately, the high abundance of these groups (greater than 10%) can lead to the aforementioned problems of increased heterogeneity and restricted conformational flexibility owing to multipoint attachment on a surface. Surface-bound *N*-hydroxysuccinimide (NHS)-activated carboxylic acids are most commonly used for coupling with protein amine groups, forming stable peptide bonds.[126–130] Immobilization efficiency depends on several parameters, for example, pH value, concentration, ionic strength, and reaction time, and in some cases conditions need to be optimized for several classes of proteins. Nonetheless, the NHS ester is sufficiently reactive and stable towards hydrolysis if standard reaction procedures are applied, such as phosphate-buffered

Table 2: Methods used for nonspecific covalent protein immobilization.

Surface functional groups	Protein functional groups	Product
NHS ester[126–130]	H_2NR	amide
aldehyde[7,9,131–138]	H_2NR	imine
isothiocyanate[99]	H_2NR	thiourea
epoxide[133,139,140]	H_2NR	aminoalcohol
amine[121,157] [a]	$HO(O)CCH_2R$	amide

[a] With coupling reagent (e.g. CDI).

saline at pH 7.3 and room temperature. Another widely used strategy employs surface-bound aldehyde groups that can react with amine groups of proteins to form labile imine linkages that can be subsequently stabilized by reduction with sodium borohydride to form secondary amines.[131–133]

MacBeath and Schreiber reported one of the first microarrays to successfully demonstrate the stable immobilization of proteins through amine groups (lysine residues or the NH_2 terminus) onto aldehyde-derivatized glass slides,[134] a procedure commonly used earlier in Biacore technology.[136] They showed the interaction between surface-bound protein G and IgG, between surface-bound protein p50 and an inhibitor, and between the FRB domain (FK binding protein-rapamycin-binding) and its protein binding partner.[134] Since then, aldehyde–amine chemistry has been extensively applied for protein immobilization on different surfaces.[7,9,135,136] For example, Gordus and co-workers used this methodology to generate protein microarrays of SH2/PTB for mapping a quantitative protein interaction network for the ErbB receptors (Figure 4).[137] Aldehyde derivatization has also been performed on SAMs on gold and has been successfully used to prepare micropatterned cytophilic protein surfaces.[138] Another method to immobilize proteins through lysine residues uses isothiocyanate-functionalized surfaces.[99] Epoxy-modified surfaces have been pursued owing to their stability at neutral pH values and under aqueous reaction conditions and because of their reactivity. Presumably the reaction with proteins occurs through a two-step mechanism, that is, fast adsorption and subsequent intermolecular chemical attachment. Some commercially available epoxy supports promote satisfactory immobilization only at high ionic strength, conditions that are not applicable to many proteins.[133,139,140] From a practical point of view, it is noteworthy that various commercially available chip surfaces have recently been systematically compared with respect to their performance in antibody-based microarray applications.[141–145]

Recently, different procedures have been adopted for creating locally activated carboxylic acid groups on surfaces, allowing for the site-selective attachment of proteins. Using reactive microcontact printing[146] and microstamping,[147] spatially defined patterns of activated carboxylic acid groups were fabricated to immobilize laminin and fibronectin. Precisely positioning mercaptoaldehydes in SAMs using atomic force microscopy (AFM) allowed for the creation of IgG and metalloprotein patterns.[148–151] Mirkin and co-workers used DPN to create activated carboxylic acids on defined nanometer-sized patches (Figure 5).[152–155] These amine-reactive templates were then used, for example, to immobilize protein A or protein G, which in turn binds to the Fc region of IgG.[152,153] An anti-β-galactosidase IgG nanoarray created in this way was treated with labeled β-galactosidase, and the captured enzyme was detected on the array by fluorescence, thus demonstrating biological activity of both the underlying IgG nanoarray and the captured target protein.[152,153]

In another example, alkyl thiols in SAMs on gold were selectively converted into weakly bound alkyl sulfonates using a near-field scanning optical microscopy (NSOM) technique.[156] The sulfonates were then replaced by carboxylated alkyl thiols activated with carbonyldiimidazole (CDI). Subsequent incubation with protein samples was used, for instance, to attach light-harvesting complex proteins, leading to nanopatterns of less than 100 nm feature size.[156]

3.1.2.2. *Carboxy-Reactive Surfaces*

Immobilization through carboxy groups may be an alternative to amine-based immobilization procedures, because aspartic and glutamic acid usually constitute the major fraction of surface groups on proteins. Thus, instead of adsorbing proteins by multipoint noncovalent interactions onto aminated slides, mild coupling methods can be used to attain covalent immobilization using CDI to activate protein-bound carboxylic acid groups.[157] This chemical strategy has the disadvantage that it can induce rapid cross-linking of the proteins, owing to reaction of the activated carboxylic ester with the ubiquitous amine groups of lysine residues. For example, site-specific patterning of fluorescent proteins onto amine-functionalized regions created by plasma enhanced chemical vapor deposition (PECVD) gave access to micrometer-sized squares.[121]

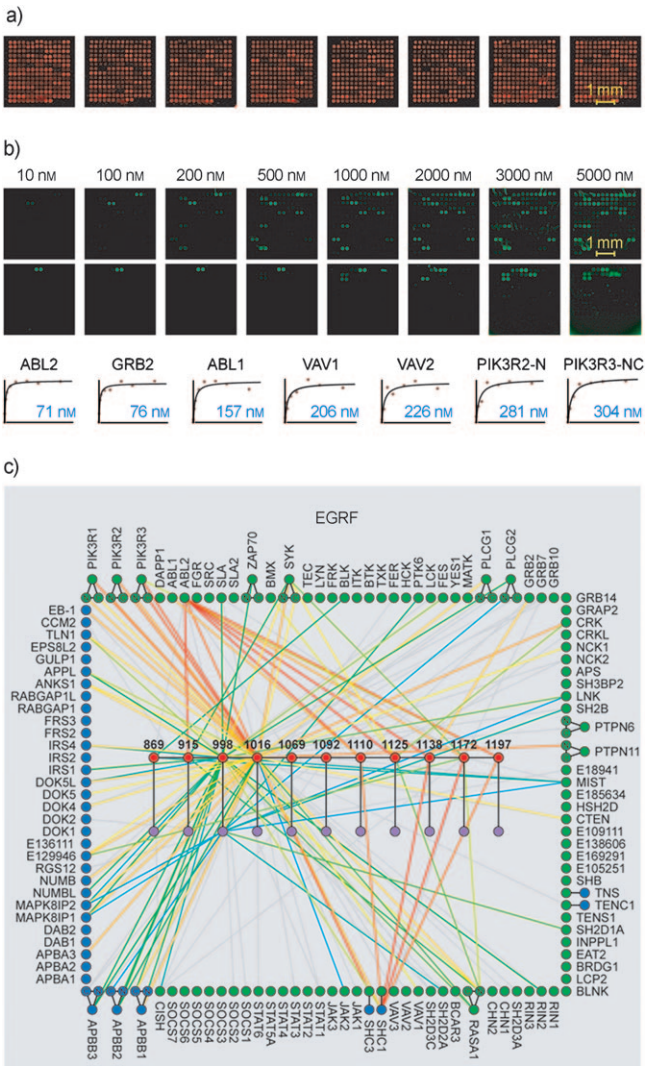


Figure 4. a) SH2/PTB protein microarrays for mapping a quantitative interaction network for the ErbB receptors: 159 SH2 and PTB domains were spotted on an aldehyde-modified surface to produce microarrays in wells of 96-well plates (spots visualized with 5% Cy5-labeled BSA, which was added before spotting). b) Subsequent incubation with 66 5- and 6-carboxytetramethylrhodamine (5(6)-TAMRA)-labeled phosphopeptides representing ErbB binding sites allowed for protein interaction analysis, and the results were fitted to a binding model (representative images). c) The quantitative data obtained allowed for the construction of quantitative protein interaction networks for the four human ErbB receptors (EGFR, representative image).[137] Reproduced with permission from Nature.

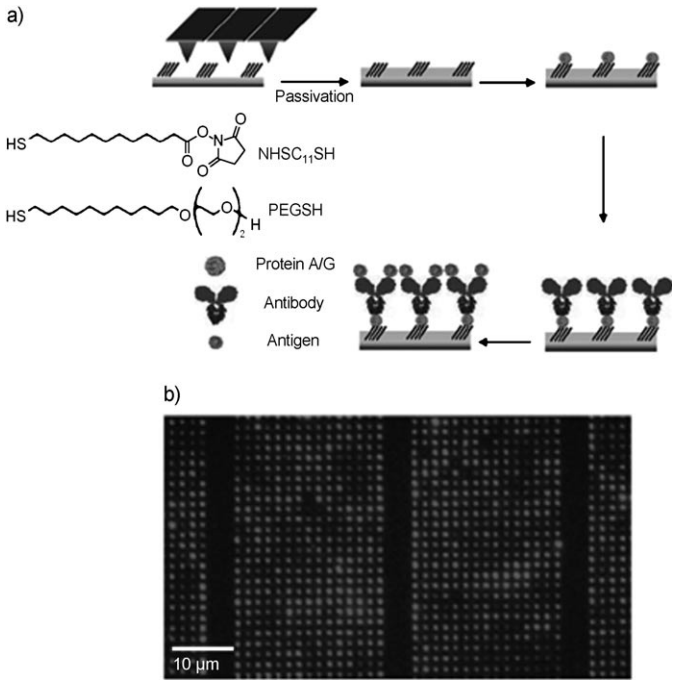


Figure 5. Generation of a functional antibody nanoarray using parallel DPN: a) Nanometer-sized spot arrays of amine-reactive alkyl thiols are created on Au with a 26-pen DPN array. The surrounding surface is pacified with thiolated PEG molecules; subsequently, protein A/G was passivated. Human anti-β-galactosidase IgG was then captured by the protein A/G nanospots, resulting in an IgG nanoarray. b) Read-out with Alexa-594-labeled β-galactosidase.[152]

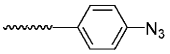
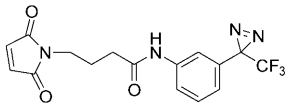
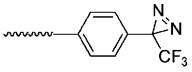
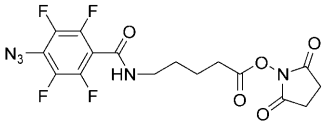
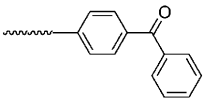
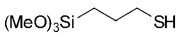
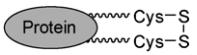
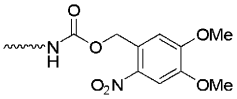
3.1.2.3. *Photoactive Surfaces*

A major improvement of genomic analysis was facilitated by Fodor et al., who adopted photolithography principles for the spatially directed synthesis of oligonucleotides on chips using photolabile protecting groups.[158] This approach was later used to produce high-density arrays of oligopeptides,[158,159] but extension to the synthesis of complete proteins appears virtually impossible. Nonetheless, researchers were challenged to generate protein chips using direct photochemical attachment of proteins to solid supports.[160]

Although photochemical immobilization methods are potentially subject to a variety of limitations, such as reaction conditions that possibly degrade or denature proteins, they have two important advantages over printing and spotting procedures: They provide fast access to protein chips without the need for prior chemical modification of the proteins, and they allow control over the shape of protein patterns with high lateral resolution. Most photochemical reactions can be carried out under mild conditions, independent of pH value and temperature, and are initiated through irradiation at $\lambda \approx 350$ nm, to which the majority of biomolecules are transparent. After light activation, the reagents undergo distinct chemical transformations that finally lead to the formation of covalent bonds between the photogenerated intermediates and the proteins.

Commonly used photoreagents, such as arylazides, diazirines, benzophenones, and nitrobenzyl groups (Table 3), which are known from photoaffinity labeling[161] or photocaging, are attached to the chip surface. Arylazide is activated by photolysis, resulting in a reactive nitrene, which can insert into C–H bonds. Unfortunately, nitrene intermediates undergo rapid intramolecular ring expansion reactions leading to highly electrophilic cyclic compounds, which show a relatively slow insertion rate. The undesired ring expansion reaction can be minimized using perfluorophenylazides (PFPA), since the substitution with fluorine decreases the rate of ring expansion reactions. Diazirines generate reactive carbenes upon exposure to light. Carbenes react within

Table 3: Overview of photoreactive groups and heterobifunctional linkers.[161–171]

Photoreactive groups		Heterobifunctional linkers
arylazide		
diazirine		
benzophenone		
azidophenylalanine		
disulfide		
nitrobenzyl[a]		

[a] Photocleavable protecting group.

microseconds with formation of covalent chemical bonds to proteins, generating an irreversible link between the chip and a protein. Sigrist and co-workers demonstrated the photoimmobilization of alkaline phosphatase and antibodies, which remained active after the photochemical attachment.[162,163] Using NSOM to locally irradiate diazirin-modified silicon surfaces allowed for the fabrication of 500 nm wide structures of BSA, of which the activity could not be tested.[164] Nitrobenzyl chemistry, often called caging chemistry, involves the attachment of a labile chemical group that can be broken down upon UV irradiation, thereby generating a ketone, carbon dioxide, and a liberated reactive group. Benzophenones are precursors to ketyl radicals, which easily react under formation of covalent bonds, as demonstrated by Fodor, Schultz, and co-workers in the immobilization of IgG onto benzophenone-modified glass slides using photomasks.[165] Alternatively, heterobifunctional photolinkers bearing one photoreactive group and one chemically reactive group can be used. Such linkers can be immobilized on the surface through irradiation and can then react with a protein through a reactive group or vice versa.[163,166–168] One such example is the immobilization of horseradish peroxidase (HRP) on films modified with perfluorotetradecanoic acids with heterobifunctional cross-linkers containing an NHS ester.[169] Ligler and co-workers devised an experiment in which thiol groups attached to glass surfaces were locally oxidized to sulfonates, allowing for the immobilization of IgG on the nonexposed areas.[170] In another example, Petersen and co-workers immobilized a variety of proteins into 1 μm spots using the photoinduced cleavage of disulfide bridges upon UV illumination of adjacent aromatic amino acids, which results in free thiols that readily react with thiol-reactive surfaces.[171]

3.2. Uniformly Oriented Proteins

3.2.1. Noncovalent Attachment

Orienting the site of interest of an immobilized protein away from the chip surface should facilitate interaction analysis, especially in the case of large interaction partners, such as other proteins. Strategies adopted from established capture reagent/fusion protein pairs, which were originally developed for protein purification by column chromatography, have been adopted to immobilize fusion proteins onto surface-bound affinity tags to uniformly orient proteins on a chip surface. Many biologically active fusion proteins are available, including popular fusion tags such as glutathione S-transferase (GST),[172] maltose binding protein (MBP),[173] FLAG peptide,[174] hexahistidine (His_6),[175] and dehalogenase.[176–178] The advantage over physisorption or covalent chemistry lies in the specificity and directionality of the supramolecular interaction and the tunability of the type and number of host–guest interactions. In addition to homogeneous and oriented attachment, the reversibility of immobilization can be very attractive from an economical point of view, because chip and sensor surfaces might be recyclable and suitable for repeated use.

3.2.1.1. Nickel Nitrilotriacetic Acid Surfaces

Using a tandem tagging approach, fusion proteins containing both polyhistidine His_x and GST tags can be purified using a glutathione column with subsequent immobilization onto a nickel nitrilotriacetate (Ni-NTA) chip. Although laborious, seminal work by Snyder and co-workers has shown the applicability of this approach by spotting 5800 yeast proteins fused to GST–His_6 at their N termini onto Ni-NTA slides. The immobilized proteins were then screened for their binding ability to other proteins and phospholipids.[22] From ten expected protein targets, only six were detected, but the results led to identification of a common potential binding motif of a set of protein interaction partners.[22] These results were not investigated further with respect to surface orientation of the proteins. Nevertheless, the superior signals observed in the case of the Ni-NTA approach, as opposed to results from randomly oriented protein immobilization on aldehyde slides, were attributed to a more uniform protein orientation.[22]

Currently, recombinant proteins bearing an engineered His_6 tag are produced by genetic engineering, thus enabling immobilization of His_6-tagged fluorescent proteins, antibodies, virus proteins, and growth factors on Ni-NTA chip surfaces.[179–185] NTA is a tetradentate ligand that forms a hexagonal complex with different divalent metal ions (usually Ni^{2+}), leaving two binding positions available for binding to a His_6 sequence (Figure 6). When His-tagged proteins are immobilized on Ni-NTA SAMs, it is possible to reverse the

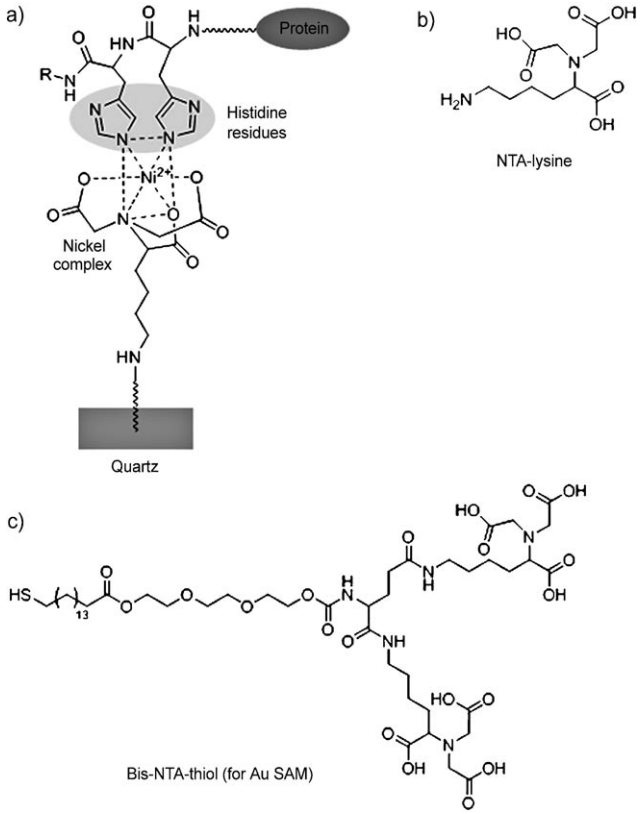


Figure 6. a) Binding of a His_6-tagged protein to a Ni-NTA-functionalized quartz surface. The protein binds through two of its histidine residues.[174] b) NTA-Lysine, typically used for preparing Ni-NTA surfaces. c) A multivalent, bis-NTA-thiol showing improved binding capabilities for His_6-tagged proteins over single NTA groups.[198]

immobilization by the addition of ethylenediaminetetraacetate (EDTA) or imidazole, as is the case in conventional Ni-NTA affinity chromatography.[179,181,186] NTA derivatives (Figure 6) can be covalently bound through active esters (*N*′-(3-dimethylaminopropyl)-*N*-ethylcarbodiimide (EDC), NHS) or maleimide derivatives to dextran surfaces or glass slides, for example. NTA was installed on gold after reaction of maleimide NTA with *N*-succinimidyl-*S*-acetylthiopropionate or directly through thiolated NTA derivatives.[179,182,187,188] The NTA system does have a few drawbacks, such as metal-dependent nonspecific protein adsorption to the surface and the relatively low affinity of the His tag to the Ni-NTA complex ($K = 10^7\,\mathrm{M}^{-1}$), potentially leading to unwanted dissociation of immobilized proteins. Improved stability of the complex can be achieved by taking advantage of the multivalency principle, which is based on the simultaneous interaction between multiple functionalities on one entity and multiple complementary functionalities on another entity.[189] While an increase of the binding affinity of the His_6 tag to NTA receptors by several orders of magnitude can be achieved by increasing the surface density of NTA groups, complex formation remains reversible, which has been demonstrated in several model protein immobilization studies.[185,187,190,191]

In a range of experiments, the Tampé group showed the benefits of being able to control the orientation of immobilized proteins. In the case of 20S proteasome carrying site-specific His tags either at the α or the β subunits, immobilization with Ni-NTA SAMs led to a preferred orientation (Figure 7).[192–194] In the case of immobilization at the α subunit, it was possible to elucidate the surface-association step of the protein degradation mechanism using surface plasmon resonance and single-molecule cross-correlation spectroscopy.[192–194] In another example, different conformations of the ligand binding domain of estrogen receptor (ER), induced by an agonist and an antagonist, were studied using conformation-specific peptides or proteins.[195,196] In the electrochemical sensor setup of Gothelf and co-workers, the His_6-tagged ER was immobilized onto Ni-NTA slides,[195] while Mrksich and co-workers employed GST-fused proteins immobilized onto glutathione thiol SAMs, which were analyzed by mass spectrometry.[196] Wegner et al. implemented NTA-functionalized SAMs within microfluidic devices, through which multiple His-tagged proteins were delivered to discrete locations on the chip surface using parallel microchannels.[188] Antibody–antigen binding interactions and sequence-specific interaction of double-stranded DNA with TATA box binding protein were monitored using SPR and fluorescence imaging. The results indicated the retention of activity of the immobilized fusion proteins.[188] In a very recent example, Mrksich

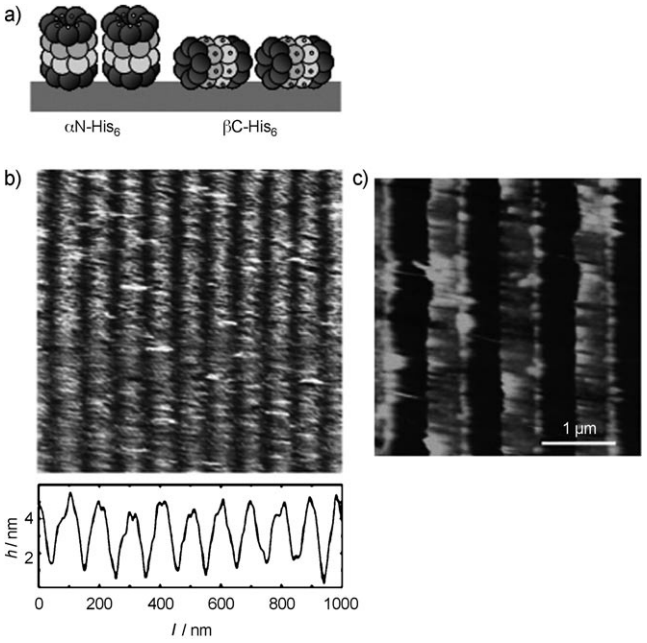
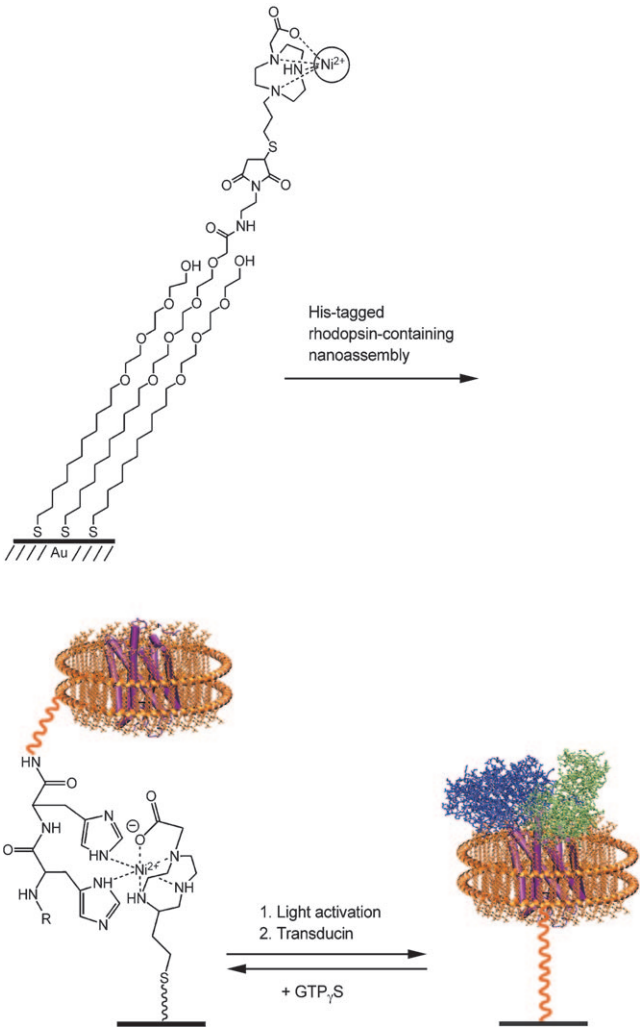


Figure 7. a) αN-His_6: Proteasome with α-subunit His tag (bound end-on); βC-His_6: Proteasome with β-subunit His tag (bound side-on). b) Topographic AFM image of lines of oriented αN-His-tagged proteasome complexes immobilized by electron beam lithography by locally generating amines on a nitro-functionalized SAM and subsequently introducing NTA receptors, which bind to the αN-His tag. Below the AFM image, a height profile across the line patterns is shown.[194] c) AFM image of His-tagged DsRed fluorescent protein attached to a chemical pattern made by nanoimprint lithography.[200]

and co-workers succeeded in immobilizing His_x-tagged rhodopsin proteins onto SAMs (Scheme 6).[197] Mass spectrometry was employed again to carry out a functional assay based



Scheme 6. Rhodopsin is immobilized on a self-assembled monolayer through a His-tagged membrane scaffold protein. Upon activation of rhodopsin with light, the receptor binds a transducin complex. GTPγS: guanosine-5′-(thiotriphosphate).[197]

on the photoactivation of rhodopsin. Photoisomerization of 11-*cis*-retinal, a lysine-bound chromophore, leads to a conformational change that allows for binding of the transducin protein complex.[197] Moreover, interaction with nonhydrolyzable nucleotides blocks the interaction of transducin with rhodopsin.[197]

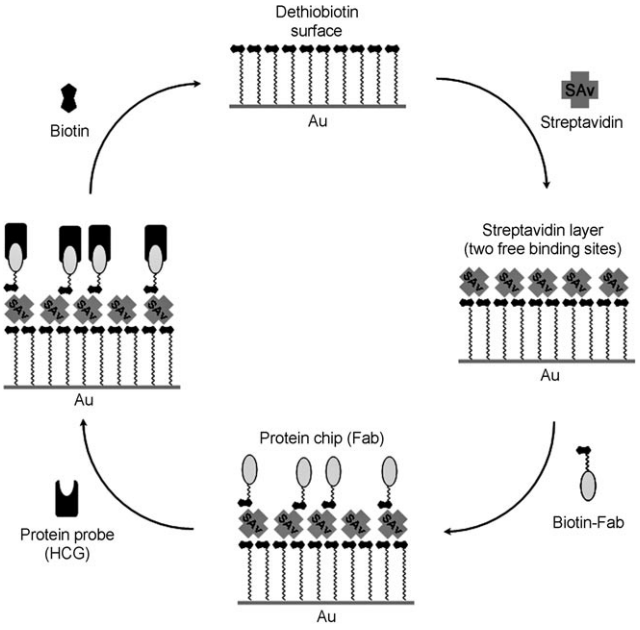
Micropatterns of NTA receptors have been prepared through microcontact printing of functionalized NTA molecules.[198,199] For example, Möller and co-workers printed amine-functionalized NTA on non-adhesive branched PEG films that contained amine-reactive isocyanate groups.[199] Addition of Ni^{2+} ions then led to the selective binding of His-tagged EGFP from crude cell lysates to such NTA patterns.[199] Huskens and co-workers were able to attach His-tagged DsRed onto 500 nm wide line patterns of NTA that were created by means of nanoimprint lithography (Figure 7).[200] By making use of the relative lability of NTA SAMs on gold, Tinazli et al. demonstrated the local displacement of α-His_6 proteasome by means of nanografting and subsequent local refilling with other His-tagged proteins.[201] Although distinct domains of uniformly oriented proteins were assembled at dimensions down to 50 nm, the fabrication process is laborious. A potentially faster fabrication process covering large areas was recently reported by Turchanin et al.[194] Using electron-beam lithography, nitro-functionalized SAMs were locally reduced to amines, to which NTA receptors were then attached.[194] Periodic protein lines of 100 nm width covering 1 mm^2 were obtained.[194] His-tagged ubiquitin and thioredoxin were deposited onto nickel oxide surfaces by DPN relying on the interaction between the nickel surface and polyhistidine residues. The resulting regular arrays with features as small as 80 nm were probed with antibodies.[202]

3.2.1.2. ***Biotin Surfaces***

Another strategy adapted from affinity chromatography is the specific binding of biotin to the proteins avidin or streptavidin (SAv). SAv comprises four identical subunits, each of which binds one biotin molecule. Owing to the high binding affinity between streptavidin and biotin ($K = 10^{13}$–$10^{15}\,M^{-1}$), the formation of this complex can be regarded as nearly irreversible,[203] on a scale nearly comparable to a covalent bond. Biotin–SAv bond formation is very rapid and is not affected by pH value, temperature, organic solvents, enzymatic proteolysis, or other denaturing agents. SAv is usually preferred over avidin, as glycoamino acids occurring on avidin can potentially cause unwanted nonspecific adsorption. This high-affinity biotin–SAv binding system has found many surface applications and has been reviewed recently.[204]

One design principle that is generally preferred for fabricating SAv monolayers is a stacked composition biotin/SAv/biotin instead of direct SAv immobilization, which potentially yields a lower degree of organization. For instance, the biotin layer directs the order in the SAv layer, with two biotin-binding sites facing the surface-bound biotin layer and the other two sites facing outward for capturing biotinylated proteins. This order can be combined with good control over the surface density of biotin groups in the SAMs, for example using mixed SAM monolayers composed of two thiol species, one of which is biotinylated and the other one not.[205] The biotin/SAv design principle was originally demonstrated in a notable contribution by Ringsdorf, Knoll, and co-workers, who complexed biotinylated concanavilin A and biotinylated antibody Fab fragments to biotin/SAv monolayers, thereby producing multilayers that present uniformly oriented proteins (Scheme 7).[206] Interaction of Fab fragments with hormones suggested a potential application as immunosensor.[206,207]

Saavedra and co-workers analyzed the orientation of biotinylated yeast cytochrome c in multilayered biotin/SAv surfaces using linear absorption dichroism and fluorescence anisotropy and found it to be in agreement with calculations and modeling.[208,209] Recently Holland-Nell and Beck-Sick-



Scheme 7. Creating protein chips using the biotin/SAv/biotin template approach: A SAM on Au displaying dethiobiotin is incubated with SAv, creating a SAv surface with two free biotin binding sites per SAv molecule. Incubation with a biotinylated target protein (here an antibody fragment, biotinylated anti-HCG Fab) results in the final protein chip, which was then used to detect a protein probe, human chorionic gonadotropin (HCG).[206]

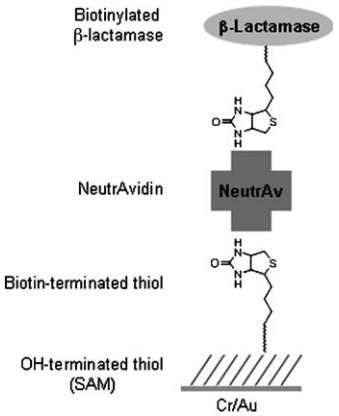
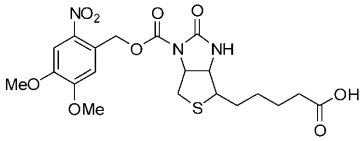
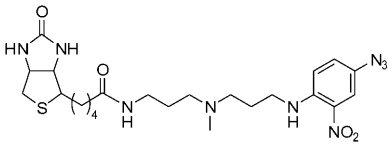
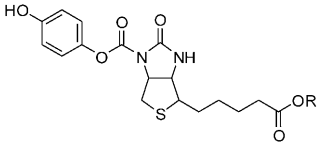
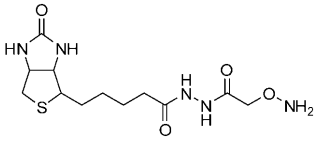
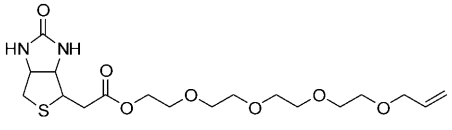


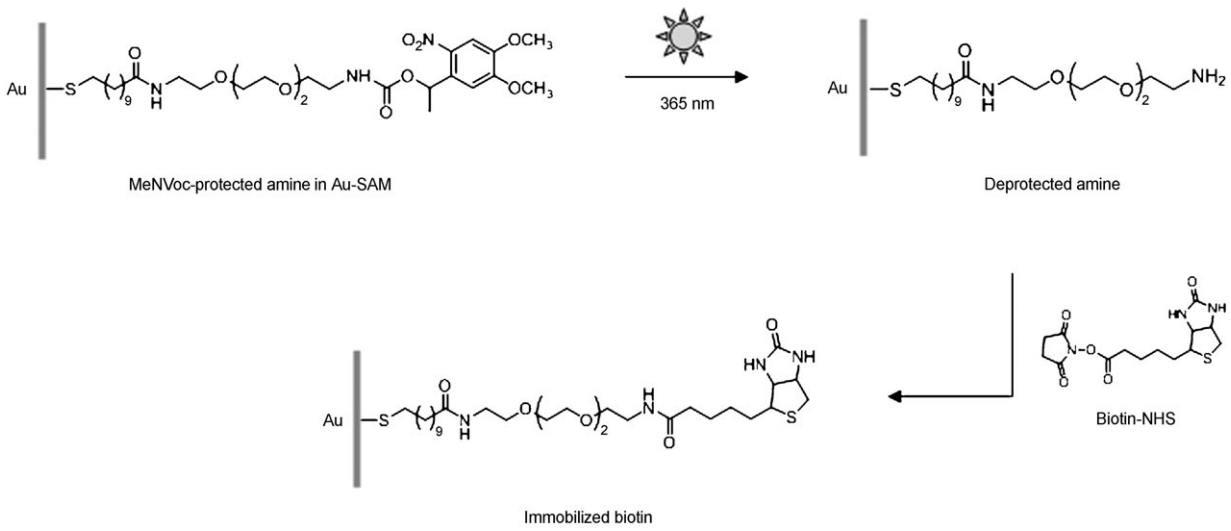
Figure 8. Lactamase chip setup used by Knoll and co-workers: Biotinylated β-lactamase was immobilized through a biotin-NeutrAvidin-biotin sandwich on a SAM created on a Cr/Au surface. The chip was then used to monitor enzymatic activity of the immobilized enzyme by SPR.[210]

inger immobilized a biotinylated reductase onto a biotin/SAv chip and showed convincingly the dramatic increase in activity of the uniformly oriented reductase compared to the randomly immobilized enzyme.[113] In another biosensor setup, Knoll, Textor, and co-workers monitored an enzymatic model reaction catalyzed by β-lactamase that was immobilized on gold in situ using the biotin assembly approach (Figure 8).[210] Möller and co-workers implemented the biotin/SAv template in their designed resistant poly(ethylene oxide) (PEO) surfaces to study the folding of RNase.[99] Application of the biotin/SAv concept to microfabricated channels to immobilize biotinylated kinesin enabled the study of three-dimensional tubilin transport,[211] whereas different biotinylated protein A/G were immobilized in parallel channels for use in sensor applications.[212]

With the motivation to create protein patterns instead of protein spot arrays using the biotin/SAv approach, one of the early goals was the local activation of initially deactivated biotin surfaces to obtain patterns of biotin available for binding. In a photochemistry approach, deactivated biotin contains a photolabile protecting group that can be selectively activated by photoirradiation through a photomask. When the surface is exposed to a solution of a target protein labeled with biotin/SAv, the protein is immobilized on the activated sites through SAv linkage.[160,213,214] To this end, the nitrogen atom of the imidazolidinone ring of biotin is acylated with a nitroveratryloxycarbonyl (NVoc) group (Table 4). Similarly, when all amine groups in SAMs are initially masked with methyl-6-nitroveratryloxycarbonyl (MeNVoc) groups, local release by photolysis allows for the incorporation of biotin (Scheme 8).[215,216] Alternatively, biotin can be locally installed on a surface by photoconversion of the headgroups of linkers attached to the surface. For example,

Table 4: Examples for biotin derivatives employed in light-directed protein immobilization.[215,216]

Name	Use	Structure
NVoc-Biotin	photocleavable protecting group[209]	
photobiotin	generation of active species using light	
hydroquinone-caged biotin	electrochemical release[55]	
ARP	aldehyde-reactive probe, invitrogen (hydrazine-modified biotin)[212,213]	
olefin-modified biotin	thiol–ene reaction[214]	



Scheme 8. Indirect biotin immobilization using a photocleavable protecting group.[215,216]

acetals can photochemically be converted into aldehyde groups that can then be treated with hydrazide-functionalized biotin.[217,218] Exposure to light through a mask yields micrometer-scale patterns, while exposure to the evanescent field associated with a NSOM even allows for constructing nanopatterns.

Jonkheijm et al. employed the thiol–ene reaction to pattern proteins onto a surface using the biotin/SAv approach (Figure 9).[219] A thiol-modified surface was coated with an olefin-modified biotin derivative and then exposed to UV light at 365 nm, leading to thioether formation and creation of biotin patterns. Incubation with Cy5-labeled streptavidin yielded highly homogeneous protein line patterns over centimeter-wide areas. Using an SAv sandwich approach, alkaline phosphatase and Ras GTPase were immobilized on such patterns, which retained their enzymatic activity and underwent protein–protein interactions qualitatively identical to the behavior in solution (Figure 9).[219]

Another method for the activation of a caged biotin surface is based on local electrochemical perturbation and has been recently described by Kim et al.[55] In this case, a hydroquinone protecting group, which can be electrochemically oxidized and then hydrolyzed, was attached to the nitrogen atom of the imidazole ring of biotin (Table 4). A novel electrochemical lithography technique was used to create patterns of biotin by local, heat-assisted release of surface protecting groups.[220] Other approaches made use of soft or probe lithography, either for direct local delivery of biotin or for creating suitable reactive patches that subsequently react with biotin derivatives.[124,199,220–230] The dimensions of the fabricated SAv patterns range from micrometers to a few tens of nanometers; some of these patterns have been used for the immobilization of fluorescent proteins and antibodies.

Alternatively, the bottom-up self-assembly of periodic DNA nanostructures has recently been used to create templates for programmable self-assembly of SAv and antibody arrays with controlled architectures and with feature sizes as small as 20 nm.[231,232] Although these methods provide control on the nanometer length scale, so far only relatively small areas have been covered.[231–236]

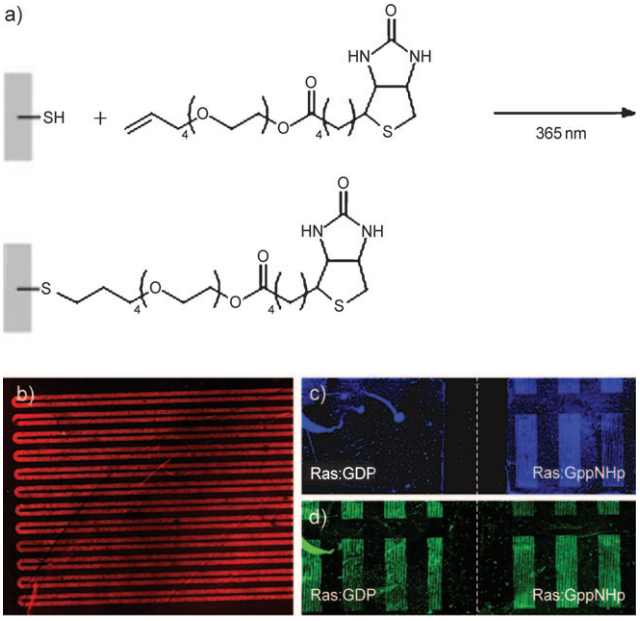
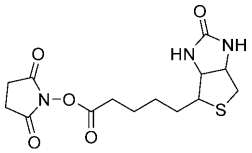
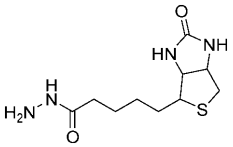
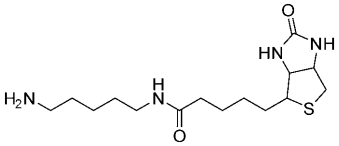
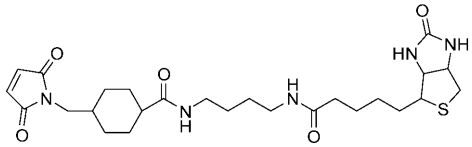


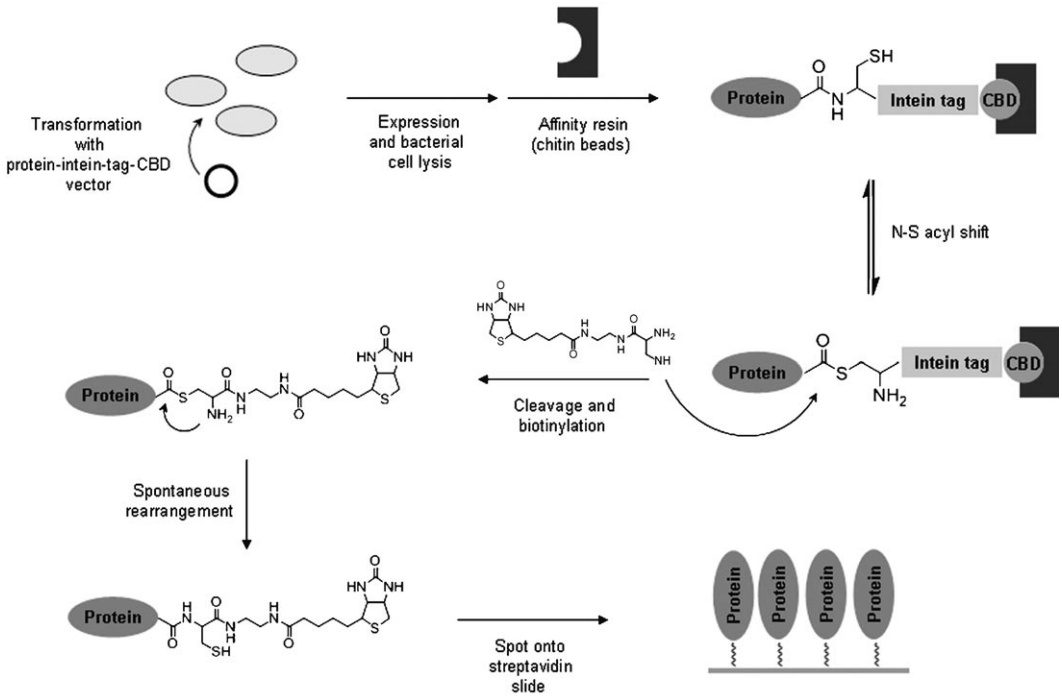
Figure 9. The thiol–ene reaction for protein patterning: a) Line patterns of biotin were created by exposing a thiol-modified surface coated with an olefin-functionalized biotin derivative to UV light at 365 nm through a photomask. b) Biotinylated alkaline phosphatase was then immobilized through a SAv sandwich and detected with fluorescently labeled antibodies. c) Inactive GDP-bound Ras and active GppNHp-bound Ras were immobilized by the same approach and incubated with the Ras-binding domain (RBD) of Raf conjugated to YFP. Only binding to active Ras:GppNHp was detected by fluorescence. d) Incubation with a Cy3-labeled anti-Ras antibody after incubation with RBD:YFP showed presence of both Ras forms (GDP: guanosine-5′-diphosphate; GppNHp: guanosine-5′-[(β,γ)-imido]triphosphate, a nonhydrolyzable GTP analogue).[219]

Synthetic ligand–receptor pairs with high affinity constants have recently been developed as alternative building blocks for the stepwise fabrication of protein arrays. To this end, cucurbit[7]uril-functionalized SAMs were used to capture ferrocenylated glucose oxidase, whereas the adamantyl–cyclodextrin interaction was used for the immobilization of cytochrome c and SAv.[223,237,238]

Historically, biotinylation of proteins has been carried out by standard bioconjugation techniques using chemically activated biotin derivatives (Table 5). To avoid random biotinylation and subsequent inactivation of proteins, site-specific labeling of proteins using biotin ligase strategies[239–243] or tag-free intein-based methods have been developed.[244–246] The latter method was recently reported by Yao and coworkers and relies on the chemoselective reaction between cysteine biotin and a reactive thioester group at the C terminus of a protein generated by intein-mediated expressed protein ligation (EPL, Scheme 9).[245] The advantage of this self-processing method is the reduction of potential perturbations that may arise when using some common large protein affinity tags. In an illustrative example, three model proteins (MBP, a fluorescent protein, and GST) were expressed in vivo, and after ligation with biotin in the crude lysate using this methodology they were spotted onto SAv slides.[245] The array was probed with the respective antibodies and demonstrated binding specificity and the retention of conformation and activity of the immobilized proteins.[245]

Table 5: Typical reagents used for protein biotinylation.[245]

Name	Use	Structure
biotin NHS	amine-reactive	
biotin hydrazide	carbohydrate-reactive	
pentylamine biotin	carboxylic acid reactive, Pierce—used with coupling reagent EDC	
biotin BMCC	thiol-reactive, Pierce	



Scheme 9. Biotin is introduced into a protein expressed with an intein tag by expressed protein ligation (CBD: chitin binding domain). Subsequently, the protein can be immobilized on SAv-functionalized surfaces.[245]

3.2.1.3. Immunoresponsive Surfaces

Taking advantage of nature's own protein capturing agents (e.g. antibodies) and antibody-binding proteins (e.g. protein A and protein G), microarrays have been generated by printing an array of monoclonal or polyclonal antibodies, antibody fragments, or synthetic polypeptide ligands.[247–250] However, a large number of antibodies must be identified prior to array fabrication. Furthermore, because antibodies are generally glycosylated and have large surface areas for interaction, they show significant cross-reactivity between target proteins. This lack of specificity can potentially lead to large numbers of false positives and negatives. While conventional antibody microarrays contain randomly oriented antibodies on the surface, control over orientation can be achieved by using, for example, protein A, an available natural IgG binding protein. Binding to protein A relies on the specific interaction with the Fc region of IgGs. Making use of this immobilization method ensures that the binding site of the antibody, located on the Fab variable region, remains well accessible for binding to antigens. Detailed reviews on oriented immobilization of antibodies and the application of such chips for immunoassays have been published elsewhere.[247–250] Nonetheless, current research is focused on further increasing control over the orientation of protein A attachment, which obviously influences the antibody orientation. To this end, several approaches have been reported;[251–253] for example, a chemoselective method of His_6 tagging protein A, which was then immobilized on NTA-modified surfaces.[254]

3.2.1.4. DNA-Modified Surfaces

Several attempts have been made in recent years to convert DNA microarrays into protein chips, taking advantage of the enormous specificity of Watson–Crick base pairing of two complementary single-stranded nucleic acids.[12,16,255] Oligonucleotide-directed immobilization provides exceptionally high stability and unique site selectivity and relies on well-established DNA chip production technology,[27,28] but illustrative examples are complicated by the demanding task of incorporating oligonucleotides into large proteins. To this end, syntheses were devised that couple thiopyridyl- or maleimido-modified oligonucleotides to cysteine residues of proteins, succinimide-modified oligonucleotides to lysine residues of proteins, or aldehyde-modified oligonucleotides to hydrazine-modified antibodies.[12,255–265] Semisynthetic conjugates of nucleic acids and proteins were site-specifically immobilized on DNA microarrays by Niemeyer and coworkers to yield bioactive protein chips for antibody-based immunodiagnostics.[266,267]

In a recent application of this concept, Heath and coworkers assembled hydrazone-linked DNA–antibody constructs on a DNA microarray.[268] With this chip, the simultaneous detection of a DNA sequence, a protein, and cells was accomplished on the same microarray slide.[268] However, looking towards more challenging targets, such as monitoring protein–protein interactions, it is mandatory to attach oligonucleotide tags to the protein regioselectively to control stoichiometry and orientation of the immobilized hybrid probes. To this end, the site-specific labeling of proteins with DNA oligonucleotides and peptide nucleic acid (PNA) strands was developed.[269,270] In these reports, cysteine-derivatized oligonucleotides were used in expressed protein ligation to attach DNA and PNA sequences to the C terminus of recombinant proteins.[269–274] Becker et al. recently applied this procedure to produce DNA–protein conjugates that were immobilized on standard DNA microarrays on silicon wafers to capture Ras proteins from crude cell lysates (Figure 10).[275] Chip-immobilized Ras-binding-domain (RBD)–DNA constructs were used to selectively detect active GTP-bound Ras protein using fluorescence imaging and MALDI mass spectrometry, thereby demonstrating the retention of biological activity of the immobilized constructs.[275]

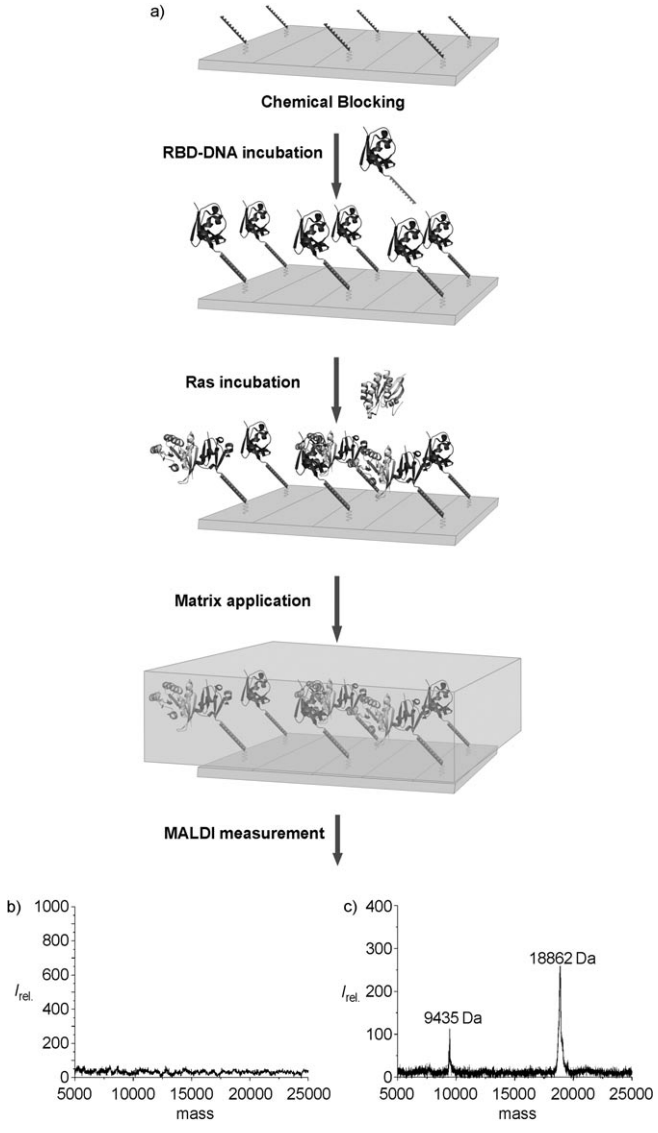


Figure 10. a) An Ras-binding-domain–DNA (RBD-DNA) conjugate was immobilized by DNA-directed immobilization using a surface displaying a complementary single DNA strand. After incubation with active (GppNHp-bound) Ras and MALDI matrix application, captured Ras can be detected by MALDI. b) MALDI background of RBD-DNA chip. c) MALDI of RBD-DNA chip with captured Ras).[275]

A further case in point is the combination of multiple orthogonal DNA assembly processes with the stable biotin/SAv template, which avoids laborious DNA labeling of proteins.[243,255,276–279] In several studies, reviewed elsewhere,[16,280,281] Niemeyer and co-workers employed the complexation of biotinylated proteins with SAv–DNA to form protein–oligonucleotide preconjugates (Figure 11). These

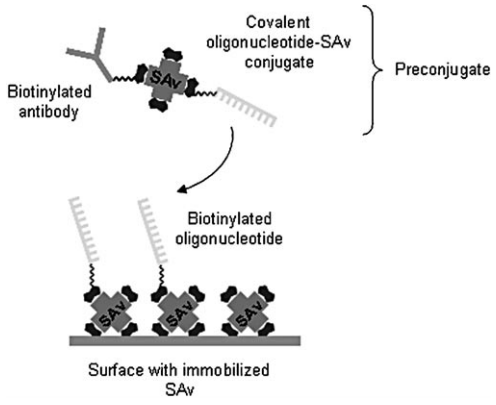


Figure 11. Creating multiple protein arrays through DNA-directed immobilization using the biotin–SAv interaction: Biotinylated proteins are preconjugated to a set of SAv–DNA conjugates (UA to UC), thus "coding" them. Incubation with an array of complementary single-strand DNA (A′ to C′) leads to the directed immobilization of each protein.[255]

preconjugates were then immobilized onto a surface displaying complementary oligonucleotides. In this way FMN:NADHH oxidoreductase, luciferase, antibodies, and horseradish peroxidase (HRP) were immobilized.[243,255,276–279,282]

LaBaer and co-workers developed a method to accelerate the high-throughput screening of protein arrays. To this end, they immobilized biotinylated expression plasmid DNA, which encoded target proteins with C-terminal GST tags, together with an anti-GST antibody.[283] A protein microarray was obtained after in vitro translation of the proteins and their immobilization by the antibodies. After initial validation of their approach, the detection of protein–protein interactions was demonstrated for the human DNA replication complex.[283] This method was also used by others.[284–286]

3.2.2. *Covalent Attachment*

Chemical attachment through side chains of amino acids is often random, because it is based on residues typically present more than once on the exterior of proteins. Therefore, attachment may occur simultaneously through several residues, potentially creating heterogeneity in the population of immobilized proteins.[21] Most methods used for chemoselective immobilization of proteins are based on ligation methods originally developed for the synthesis, semisynthesis, and selective derivatization of proteins by chemical means.[287,288] All these methods involve the derivatization of a protein with a unique chemical group at a defined position that can later react chemoselectively with a complementary group on a solid support.

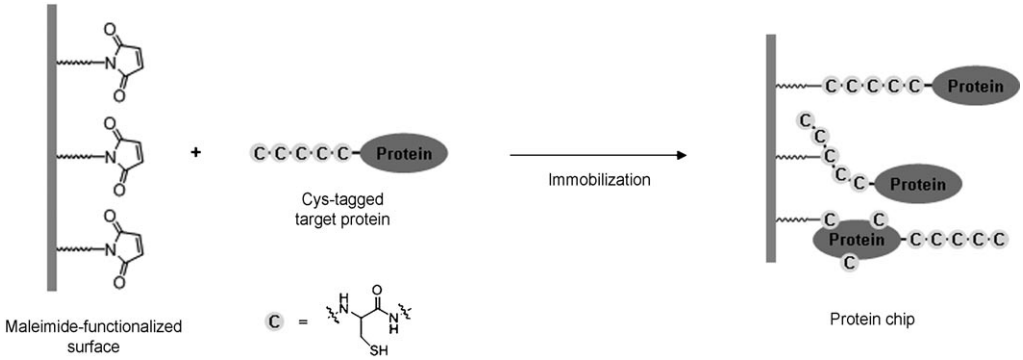
3.2.2.1. *Thiol-Reactive Surfaces*

In some cases oriented protein immobilization can be achieved if the protein possesses a single, accessible, reactive amino acid. Cysteine (Cys) is the only naturally occurring amino acid containing a thiol group in its side chain, and its relative abundance in proteins is small (less than 1%). Thiols have a pK_a of around 8.5 and are sufficiently nucleophilic at pH 7 to react selectively with chemical functionalities such as α-haloacetyl- and maleimide-modified surfaces (Section 2.4), forming a stable thioether bond.[267,289–291] Reactive Cys residues used for coupling and immobilization must not be involved in any structural element, but they should be exposed in a solvent-accessible region of the protein. Cys residues can be introduced to a protein through site-specific mutation of, for example, Ser or Ala residues, preferably in a remote solvent-accessible part of the protein. Gaub and co-workers genetically modified a lipase from *Candida antarctica* to carry an accessible C-terminal cysteine residue, which was then shown to selectively immobilize on a maleimide-functionalized surface in the presence of another free cysteine on the protein itself.[291] An alternative was shown by attaching an oligo-Cys tag to the protein, providing higher reactivity (Scheme 10).[290] Genetically modified cow pea mosaic viruses with unique cysteine residues at specific locations on their capsomers were assembled on a maleimide surface in patterns of 30 to 50 nm width using probe lithography.[292–294] Morpurgo et al. showed the selective attachment of cysteine-modified ribonuclease (RNase) to vinyl sulfone surfaces by a conjugate addition reaction.[295]

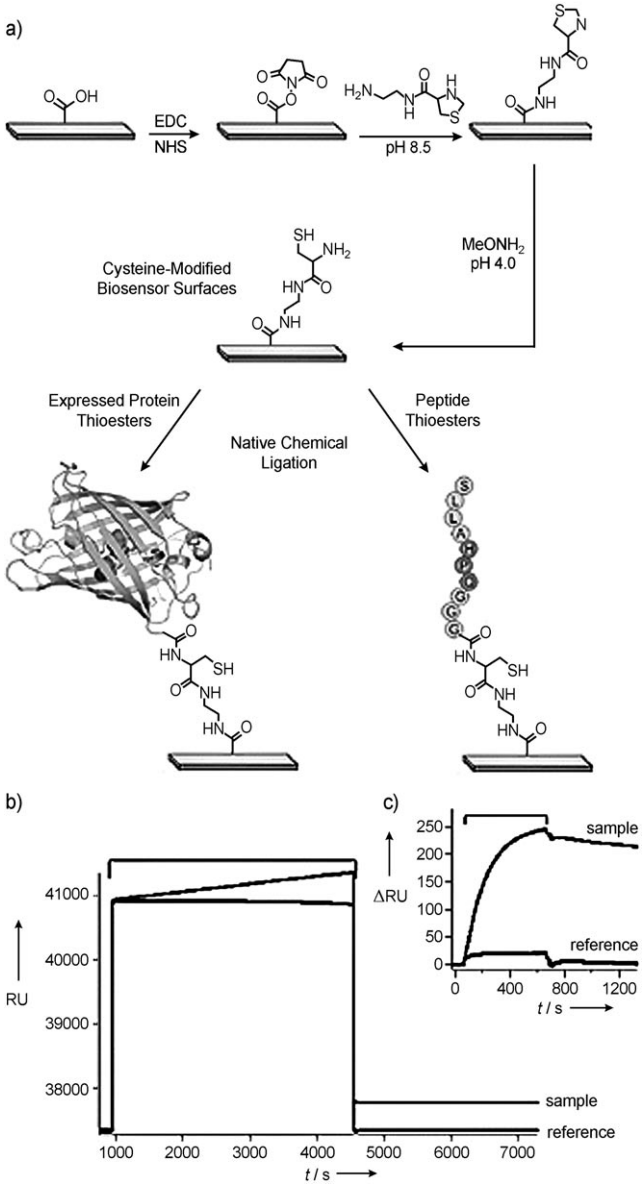
3.2.2.2. *Native Chemical Ligation to Surfaces*

One of the most efficient ways to chemospecifically immobilize proteins is through native chemical ligation (NCL). For example, Camarero et al. produced two fluorescent proteins with C-terminal thioesters that were immobilized onto N-terminal Cys-modified glass slides and detected through their native fluorescence.[296] As a control, a solution of unmodified protein was spotted. The results showed that specific peptide ligation was responsible for the immobilization.[296] In another report by Yao and co-workers, N-terminal cysteine-containing fluorescent proteins were generated using intein-mediated protein splicing and spotted onto thioester-modified slides. Successful immobilization was confirmed by incubation with a specific antibody and by the native protein fluorescence.[297] A recent report from Meijer and co-workers monitored the immobilization of a thioester-modified fluorescent protein onto a thiazolidine-capped surface with SPR (Scheme 11).[298]

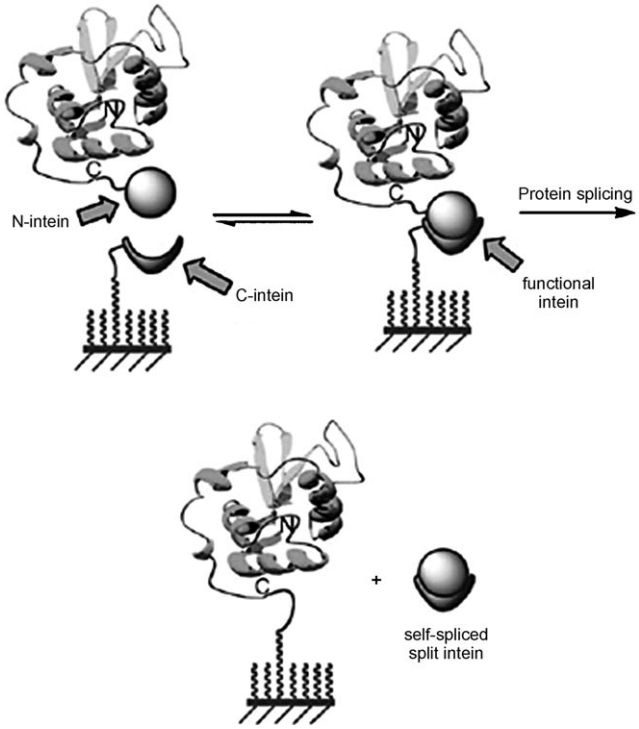
Camarero and co-workers proposed a traceless ligand strategy using protein trans-splicing (Scheme 12). In this case, the intein domain is split into two fragments (N-intein and C-intein).[299] The C-intein fragment is covalently immobilized on a glass surface, while the N-intein fragment is fused to the C terminus of a protein that is to be attached to the surface. When the two intein fragments interact, they form an active intein domain that binds the protein to the surface while releasing the split intein into the solution. The naturally split



Scheme 10. Protein immobilization by cysteine residues and the oligocysteine tag: A target protein (EGFP) is functionalized with an oligocysteine tag in a solvent-accessible position. Spotting onto a maleimide-functionalized surface leads to protein immobilization at any cysteine residue of the oligocysteine tag as well as, less probably, at internal cysteine residues of the protein (as a side reaction).[290]



Scheme 11. Protein immobilization using native chemical ligation: a) A fluorescent protein (green fluorescent protein, GFP) expressed as thioester is incubated with an SPR chip surface displaying cysteine. Cysteine attacks the thioester, leading to immobilization of the protein. b) SPR trace showing protein immobilization occurring during injection of the protein thioester. c) SPR trace showing capture of the immobilized GFP by an anti-GFP antibody.[298]

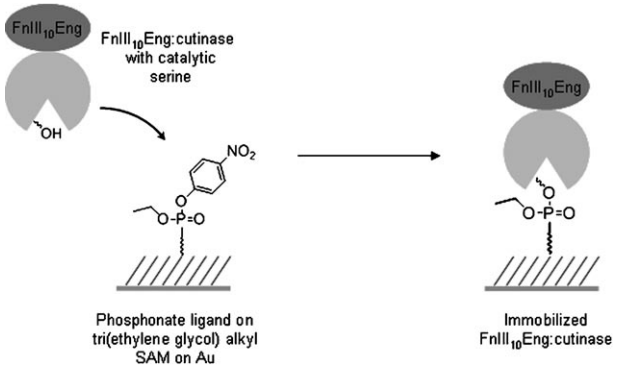


Scheme 12. Protein immobilization through split-intein-mediated protein trans-splicing. Interaction of an immobilized C-intein fragment with an N-intein fragment fused to a target protein leads to formation of a functional intein with subsequent self-splicing and protein immobilization.[299]

DnaE intein possesses C- and N-intein fragments able to self-assemble spontaneously ($K = 10^{-5}\,\text{M}^{-1}$). Intein-fused MBP and fluorescent proteins were immobilized, and their (immuno)analysis showed no significant loss of fluorescence, thus demonstrating the retention of protein conformation.[299] Control experiments with proteins lacking the intein fusion showed no protein attachment.

3.2.2.3. Enzymatically Reactive Capture Surfaces

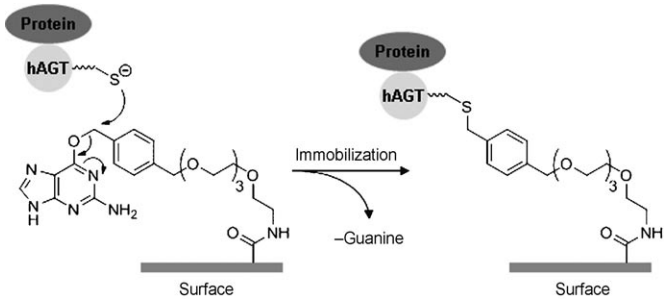
Inspired by the use of reactive ligands to capture proteins in solution and by the use of small-molecule microarrays,[38,300–302] Hodneland et al. recognized the possibility to selectively immobilize proteins on surfaces with total control over their orientation.[68] They used the protein calmodulin fused to the enzyme cutinase as a capture protein (Scheme 13).[68] Cutinase is a serine esterase that forms a



Scheme 13. Enzyme-directed protein immobilization approach using cutinase fused to a target protein (here the tenth domain of fibronectin III): A monolayer displays phosphonate groups that react irreversibly with a serine residue in the cutinase active site, thus immobilizing the cutinase fusion protein.[68]

site-specific covalent adduct with phosphonate ligands. When a phosphonate binds to the active site of the enzyme, the hydroxy group of the catalytic serine residue reacts to yield a stable covalent adduct that is resistant to hydrolysis. However, the strategy is presumably not applicable when a variety of hydrolytic enzymes are present. When the cutinase-binding inhibitor was incorporated into SAMs on gold, the immobilization of cutinase from cell lysate could be monitored with SPR spectroscopy.[68] This approach was also used by the same group to array cutinase-fused antibodies.[303]

A different example reported by Johnsson and co-workers makes use of chemoenzymatic site-specific immobilization of proteins using a mutant *O*6-alkylguanine-DNA alkyltransferase (hAGT; Scheme 14). This modified enzyme can efficiently



Scheme 14. Protein immobilization using a mutant *O*6-alkylguanine-DNA alkyltransferase (hAGT) fusion protein: When presented with a surface displaying *O*6-benzylguanine derivatives, hAGT transfers the substituted benzyl moiety to itself, thus immobilizing the hAGT fusion protein.[304–306]

transfer a benzyl group to itself when presented with *O*6-benzylguanine (BG) derivatives.[304,305] The mutant enzyme is tolerant to bulky substituents at the benzyl group, thus allowing for coupling to solid supports. The concept for immobilization was demonstrated with AGT-fused GST, and subsequent anti-GST binding was investigated with SPR[304] and atomic force microscopy.[306] Other groups have reported the immobilization of alkaline phosphatase using microbial transglutaminase,[307] of a variety of proteins using sortase,[308] and of fluorescent proteins with the surface-binding domain (SBD) of poly(hydroxyalkanoate) (PHA) depolymerase.[309]

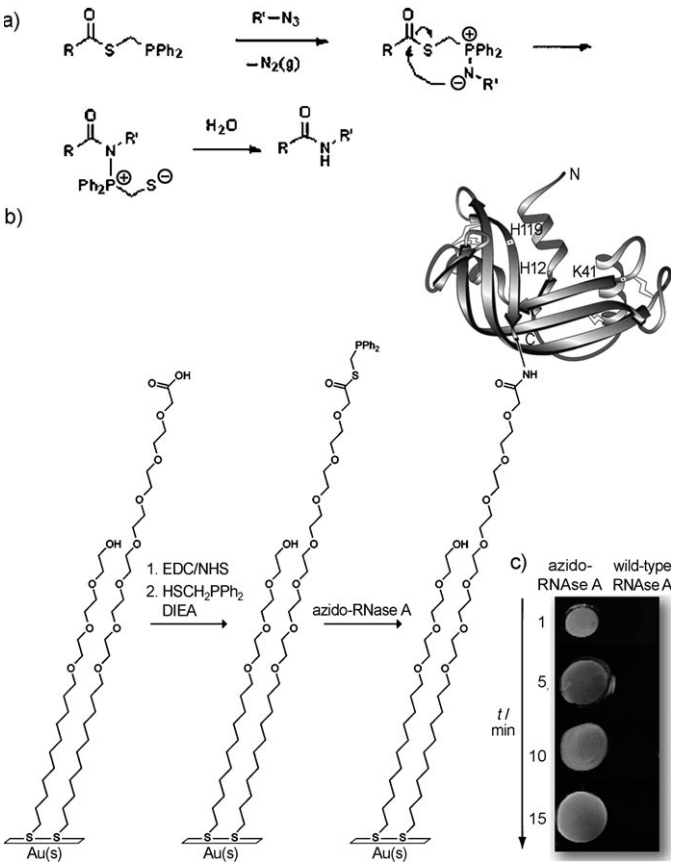
3.2.2.4. Immobilization by Staudinger Ligation

The Staudinger ligation requires an azide group and a phosphine-containing ester or thioester. The mechanism involves the formation of an iminophosphorane intermediate and subsequent nucleophilic attack of the iminophosphorane nitrogen atom on the thioester via a five-membered ring leading to an aminophosphonium salt. Subsequent hydrolysis yields an amide bond. The Staudinger ligation proceeds under mild conditions, in aqueous solution, almost quantitatively, and without noticeable formation of any side-chain products. The first application of the Staudinger ligation to couple proteins to a solid support was proposed by Raines and co-workers, although in this first study the immobilization was indirect.[310] An azide-modified peptide fragment of a truncated RNAse, RNAse S′, was immobilized on a surface modified with phosphinothioester groups.[310] Subsequent incubation with the corresponding protein fragment led to reconstitution of active RNase S′ through complex formation.[310] In a recent follow-up study, the azide-modified ribonuclease A protein was chosen as a model for direct immobilization by the Staudinger ligation and was also spotted onto a phosphinothioester-modified surface (Scheme 15).[311] Control experiments indicated that binding occurs only by the Staudinger ligation. Furthermore, the catalytic activity and the ability to bind a natural protein ligand of the immobilized enzyme was demonstrated.[311]

Waldmann and co-workers demonstrated the direct immobilization of azide-modified N-Ras protein through the Staudinger ligation onto phosphane-modified surfaces (Figure 12).[312,313] The proteins were spotted at pH 7.4–7.6, and clear, reproducible fluorescent signals were recorded after an immobilization time of 4 h with a minimum protein concentration of 50 μM. The immobilization reaction implies chemoselectivity of the method. The retention of protein activity was demonstrated with an anti-Ras antibody recognizing a helix belonging to the active site of the protein.[312,313] The azide function, however, is not present in any naturally occurring protein, but Bertozzi and co-workers have reported a novel method for the incorporation of azide groups into recombinant proteins.[314]

3.2.2.5. Immobilization by Cycloaddition Reactions

Another powerful chemical strategy that makes use of the availability of azide-functionalized proteins is the Huisgen 1,3-dipolar cycloaddition of an azide and an alkyne to form a



Scheme 15. Top: Mechanism of the Staudinger ligation. Bottom: Azide-modified RNAse A is immobilized onto phosphine-modified slides by Staudinger ligation (DIEA = diisopropylethylamine). Fluorescently labeled antibody read-out of RNAse A spots shows increasing immobilization over time, while wild-type RNase A shows no immobilization in the timeframe depicted.[311] Reproduced with permission from ACS.

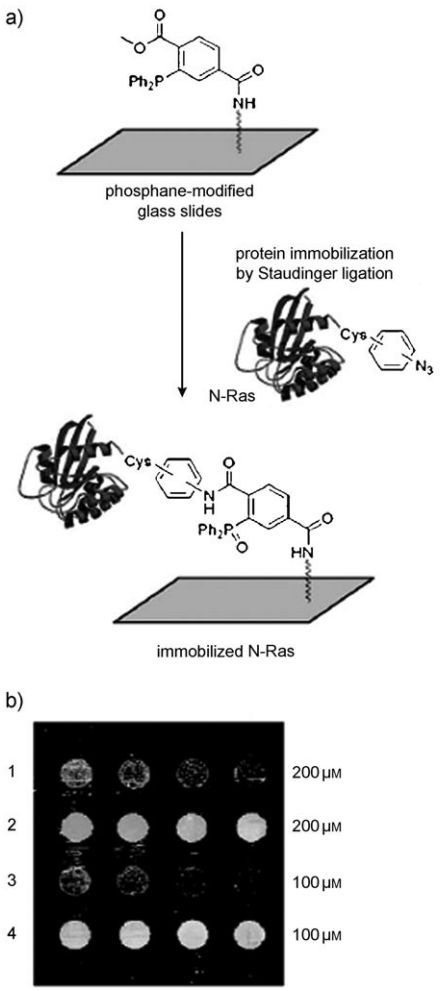


Figure 12. a) Azide-modified N-Ras was spotted onto phosphane-modified glass slides, where it was immobilized through the Staudinger ligation. b) Read-out with a Cy5-labeled antibody after 4 h showed successful immobilization of azide-modified N-Ras at concentrations of 100 mM and 200 mM (lanes 2 and 4) and close to no binding of wild-type N-Ras (lanes 1 and 3).[312]

1,2,3-triazole, also popularized as "click" chemistry. A potential disadvantage could be the required use of a copper(I) catalyst in the case of sensitive proteins. However, Bertozzi and co-workers recently published a copper-free variant of this reaction, but it has not yet been applied for protein immobilization.[315]

Distefano and co-workers performed the immobilization of azide-modified farnesyl transferase onto alkyne-derivatized solid supports in an overnight cycloaddition reaction at room temperature.[316] The chemical procedure can also be applied to azide-terminated chip surfaces for reaction with alkyne-modified fluorescent proteins. Lin and co-workers prepared alkyne- and azide-functionalized fluorescent proteins that were attached to slides modified with azide groups or alkynes, respectively (Figure 13). They showed that native proteins were not immobilized, as indicated by the absence of fluorescence. In their hands, the oriented immobilization of MBP led to increased binding activity compared to random immobilization.[112]

Very recently Waldmann et al. employed the "click sulfonamide reaction" (CSR) between sulfonyl azides and terminal alkynes to immobilize biotin, carbohydrates, phosphopeptides, and proteins. In the most prominent example, a terminally alkyne-modified Ras-binding domain (RBD) of cRaf1 was immobilized on a sulfonyl azide modified surface and consecutively incubated with either active GppNHp-bound Ras or inactive GDP-bound Ras and a fluorescently labeled antibody. Only binding of Ras:GppNHp to the RBD

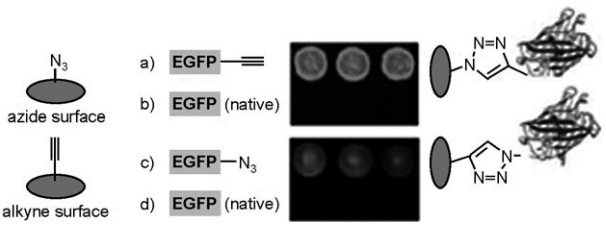


Figure 13. Immobilization of EGFP by click chemistry: In the presence of copper(I), alkyne- or azide-functionalized EGFP is spotted onto an azide- or alkyne-functionalized surface, respectively, to produce EGFP microarrays. Native EGFP was also spotted as a negative control. After 12 h at room temperature, fluorescence read-out shows functionalized protein on the surface.[112]

of cRaf1 was detected, demonstrating retention of biological activity of the RBD after immobilization by the CSR (Figure 14).[317]

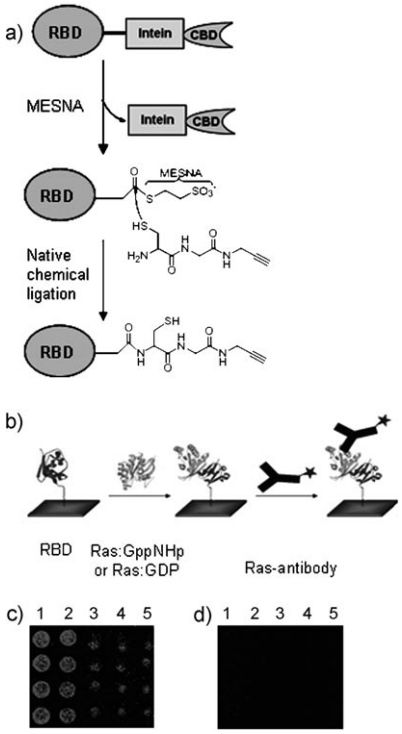


Figure 14. Immobilization of the cRaf1-RBD by the "click sulfonamide reaction" (CSR): a) RBD is terminally alkyne-functionalized by EPL (MESNA = sodium 2-mercaptoethanesulfonate). b) Two sets of spots of alkyne–RBD on a sulfonylazide-modified surface were incubated with active, GppNHp-bound Ras or inactive GDP-bound Ras followed by a fluorescently labeled anti-Ras antibody. c), d) Fluorescence images show only binding of active Ras:GppNHp (c) and not Ras:GDP (d) (1–5: 50, 25, 12.5, 6.2, 3.1 mM alkyne–RBD on sulfonylazide surface).[317]

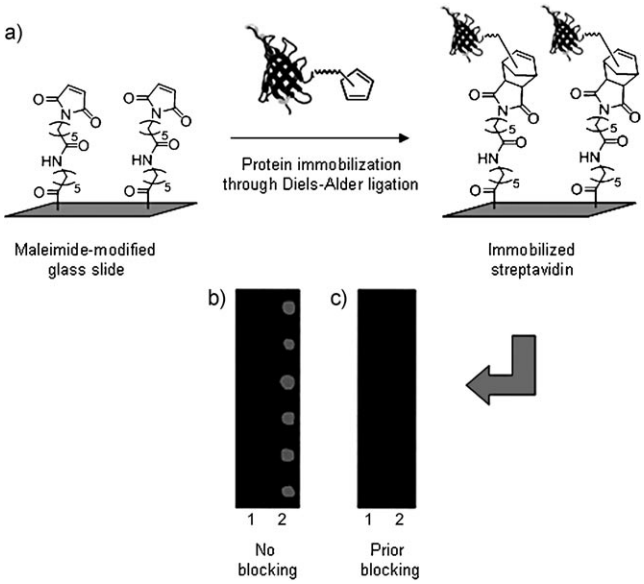


Figure 15. Protein immobilization through the Diels–Alder cylcoaddition: a) Diene-modified streptavidin (lane 2 in (b) and (c)) and wild-type SAv (as a negative control, lane 1 in (b) and (c)) were spotted onto maleimide glass slides b) without blocking; c) blocking with 5% 2-mercaptoethanol. After 8 h at room temperature the slides were analyzed with Cy5-labeled biotin.[321]

The Diels–Alder cycloaddition reaction usually takes place between an electronically matched dienophile and a conjugate diene to form an unsaturated six-membered ring. The Diels–Alder cycloaddition was applied for peptide immobilization by Mrksich and co-workers.[318–320] Since the Diels–Alder reaction can proceed in water at room temperature with a higher rate and selectivity than in organic solvents, the possibility to use it for efficient protein immobilization was recently explored by Waldmann and co-workers (Figure 15).[321] SAv was chosen as a model protein and was first ligated to a cyclopentadiene derivative. Diene-modified SAv and an unmodified control protein were dissolved in pH 6 doubly distilled water and spotted onto a maleimide slide. The slide was then treated with labeled biotin, and the immobilization of SAv was demonstrated using fluorescence. No signal was recorded for the negative control, indicating that immobilization was due to the Diels–Alder reaction.[321] Sun et al. described a method to combine the Huisgen 1,3-dipolar cycloaddition with a Diels–Alder reaction.[322] Maleimide-derivatized glass slides were prepared and coupled through a Diels–Alder reaction to the diene terminus of a bifunctional PEG linker carrying an alkyne group at the opposite side. A diverse series of biomolecules, including biotin, lactose, and a recombinant thrombomodulin, have been stably immobilized without formation of byproducts.[322] The Diels–Alder reaction has also been used for the selective immobilization of SAv to electroactive SAMs on gold, thereby potentially providing an exciting range of tailored surfaces.[319]

3.2.2.6. ***Other Examples***

In a new approach, Corgier et al. demonstrated the use of electrochemistry to site-specifically immobilize proteins and other biomolecules on surfaces.[323] They introduced aniline functionalities to oligonucleotides, antibodies, and horseradish peroxidase. After oxidation of the aniline moieties to aryl diazonium groups, they could be covalently bound to a graphite electrode in a spatially controlled electrodeposition. All biomolecules were shown to be active in follow-up assays, and work is currently underway to employ the technique for biochip fabrication.[323]

Another approach by Zhang et al. employs the non-natural amino acid azidophenylalanine for photopatterning.[324] The authors constructed an artificial polypeptide scaffold expressed in *E. coli* comprising an azidophenylalanine-containing elastin mimic domain and one domain of a heterodimeric leucine zipper pair, which was site-specifically immobilized on an alkyl-functionalized surface by exposure to UV light at 254 nm, leading to cross-linking of the arylazide group with the surface.[324] Subsequent incubation with fusion proteins (GFP and GST) carrying the second partner of the

leucine zipper pair led to noncovalent immobilization of the target proteins owing to a zipper pair affinity of 10^{-15} M.[324]

4. Conclusions and Outlook

New chemical methods offer attractive opportunities and technologies for generating protein biochips. As summarized in this Review, it is possible to chemically attach proteins to solid supports in a chemo- and regioselective manner, thereby enabling control over protein orientation on the surface and minimizing problems that result from random protein orientation, for example, insufficient accessibility of active sites. Furthermore, appropriate chemical methods allow for control of shape and lateral dimensions of protein patterns on chip surfaces.

These tailor-made chemical strategies are likely to have a major impact on the properties of protein chips, for example in the analysis of protein–protein interactions. The combination of chemo- and regioselective immobilization and the ability to control geometry and dimensions of protein patterns will give rise to new opportunities in proteomics science. Moreover, significant contributions to biomaterial sciences can be foreseen, but there are still a number of goals that must be achieved. On the one hand, chemical immobilization methods must be further optimized to allow simple immobilization of a large variety of different proteins in a highly controlled fashion. Ideally, this should be attainable in one step and without prior purification after expression. On the other hand, realization of reduced patterning sizes down to the nanometer range applied to hundreds and thousands of different proteins by means of a fast and robust process remains an unsolved challenge.

The recent progress of nanotechnological methods[29,325,326] promises to provide solutions to these problems, as elegantly shown by Delamarche and co-workers, who used a nanoprinting technique to deliver antibodies to surfaces.[327] Another appealing example was reported recently by Gaub and co-workers, who described a DNA surface assembly protocol to prepare extraordinarily precise nanoscale patterns of biomolecules.[17]

Ongoing progress in synthetic methodologies brings completely new opportunities for immobilizing proteins to the fore.[219,323,324] Insertion of the non-natural amino acid azidophenylalanine into proteins, for instance, allows the protein to be covalent linked to modified glass slides by UV cross-linking.[324] Another elegant method is the recent photopatterning of biomolecules using the thiol–ene reaction, which allows for the one-step chemoselective attachment of functional biomolecules, such as phosphopeptides.[219]

From a chemical point of view, it is necessary to join the disciplines of organic chemistry, biochemistry, materials chemistry, and physics. This combination will allow for chemical efficiency and selectivity to be applied to semisynthetic proteins and other biomolecules to fabricate high-quality protein biochips with high efficiency. The establishment of high-throughput screening proteomics facilities and the recent progress in obtaining full control over nanofabrication indicates that a next generation of functional protein chips possessing unique architectures through molecular design will be available for use as biochips and devices in the near future.

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[1] E. Phizicky, P. I. H. Bastiaens, H. Zhu, M. Snyder, S. Fields, *Nature* **2003**, *422*, 208–215.
[2] P. Mitchell, *Nat. Biotechnol.* **2002**, *20*, 225–229.
[3] A. Talapatra, R. Rouse, G. Hardiman, *Pharmacogenomics* **2002**, *3*, 527–536.
[4] J. LaBaer, N. Ramachandran, *Curr. Opin. Chem. Biol.* **2005**, *9*, 14–19.
[5] H. Zhu, M. Snyder, *Curr. Opin. Chem. Biol.* **2003**, *7*, 55–63.
[6] J. S. Merkel, G. A. Michaud, M. Salcius, B. Schweitzer, P. F. Predki, *Curr. Opin. Biotechnol.* **2005**, *16*, 447–452.
[7] D. S. Wilson, S. Nock, *Angew. Chem.* **2003**, *115*, 510–517; *Angew. Chem. Int. Ed.* **2003**, *42*, 494–500.
[8] M. F. Templin, D. Stoll, M. Schrenk, P. C. Traub, C. F. Vohringer, T. O. Joos, *Trends Biotechnol.* **2002**, *20*, 160–166.
[9] K.-y. Tomizaki, K. Usui, H. Mihara, *ChemBioChem* **2005**, *6*, 782–799.
[10] B. Schweitzer, P. Predki, M. Snyder, *Proteomics* **2003**, *3*, 2190–2199.
[11] P. F. Predki, *Curr. Opin. Chem. Biol.* **2004**, *8*, 8–13.
[12] C. M. Niemeyer, *Trends Biotechnol.* **2002**, *20*, 395–401.
[13] G. M. Whitesides, E. Ostuni, S. Takayama, X. Y. Jiang, D. E. Ingber, *Annu. Rev. Biomed. Eng.* **2001**, *3*, 335–373.
[14] M. M. Stevens, J. H. George, *Science* **2005**, *310*, 1135–1138.
[15] I. Willner, E. Katz, *Angew. Chem.* **2000**, *112*, 1230–1269; *Angew. Chem. Int. Ed.* **2000**, *39*, 1180–1218.
[16] C. M. Niemeyer, *Nanotoday* **2007**, *2*, 42–52.
[17] K. Blank, T. Mai, I. Gilbert, S. Schiffmann, J. Rankl, R. Zivin, C. Tackney, T. Nicolaus, K. Spinnler, F. Oesterhelt, M. Benoit, H. Clausen-Schaumann, H. E. Gaub, *Proc. Natl. Acad. Sci. USA* **2003**, *100*.
[18] N. Sniadecki, R. A. Desai, S. A. Ruiz, C. S. Chen, *Ann. Biomed. Eng.* **2006**, *34*, 59–74.
[19] W. Kusnezow, J. D. Hoheisel, *J. Mol. Recognit.* **2003**, *16*, 165–176.
[20] W. Kusnezow, A. Jacob, A. Walijew, F. Diehl, J. D. Hoheisel, *Proteomics* **2003**, *3*, 254–264.
[21] S. V. Rao, K. W. Anderson, L. G. Bachas, *Mikrochim. Acta* **1998**, *128*, 127–143.
[22] H. Zhu, M. Bilgin, R. Bangham, D. Hall, A. Casamayor, P. Bertone, N. Lan, R. Jansen, S. Bidlingmaier, T. Houfek, T. Mitchell, P. Miller, R. A. Dean, M. Gerstein, M. Snyder, *Science* **2001**, *293*, 2101–2105.
[23] Y.-Y. Luk, M. L. Tingey, K. A. Dickson, R. T. Raines, N. L. Abbott, *J. Am. Chem. Soc.* **2004**, *126*, 9024–9032.
[24] P.-C. Lin, S.-H. Ueng, M.-C. Tseng, J.-L. Ko, K.-T. Huang, S.-C. Yu, A. K. Adak, Y.-J. Chen, C.-C. Lin, *Angew. Chem.* **2006**, *118*, 4392–4396; *Angew. Chem. Int. Ed.* **2006**, *45*, 4286–4290.

[25] A. A. H. Talasaz, M. Nemat-Gorgani, Y. Liu, P. Stahl, R. W. Dutton, M. Ronaghi, R. W. Davis, *Proc. Natl. Acad. Sci. USA* **2006**, *103*, 14773 – 14778.
[26] F. Rusmini, Y. Zhong, J. Feijen, *Biomacromolecules* **2007**, *8*, 1775 – 1789.
[27] C. M. Niemeyer, D. Blohm, *Angew. Chem.* **1999**, *111*, 3039 – 3043; *Angew. Chem. Int. Ed.* **1999**, *38*, 2865 – 2869.
[28] M. C. Pirrung, *Angew. Chem.* **2002**, *114*, 1326 – 1341; *Angew. Chem. Int. Ed.* **2002**, *41*, 1276 – 1289.
[29] *Nanobiotechnology, Vol. I & II*, Wiley-VCH, Weinheim, **2004**, **2007**.
[30] K. Salaita, Y. Wang, C. A. Mirkin, *Nat. Nanotechnol.* **2007**, *2*, 145 – 155.
[31] D. S. Ginger, H. Zhang, C. A. Mirkin, *Angew. Chem.* **2004**, *116*, 30 – 46; *Angew. Chem. Int. Ed.* **2004**, *43*, 30 – 45.
[32] R. D. Piner, J. Zhu, F. Xu, S. Hong, C. A. Mirkin, *Science* **1999**, *283*, 661 – 663.
[33] N. Ramachandran, D. N. Larson, P. R. H. Stark, E. Hainsworth, J. LaBaer, *FEBS J.* **2005**, *272*, 5412 – 5425.
[34] H. J. Lee, Y. Yan, G. Marriott, R. M. Corn, *J. Physiol.* **2005**, *563*, 61 – 71.
[35] A. J. Holloway, R. K. van Laar, R. W. Tothill, D. D. Bowtell, *Nat. Genet.* **2002**, *32*, 481 – 489.
[36] E. Southern, M. Mir, M. Shchepinov, *Nat. Genet.* **1999**, *21*, 5 – 9.
[37] V. G. Cheung, M. Morley, F. Aguilar, A. Massimi, R. Kucherlapati, G. Childs, *Nat. Genet.* **1999**, *21*, 15 – 19.
[38] G. MacBeath, A. N. Koehler, S. L. Schreiber, *J. Am. Chem. Soc.* **1999**, *121*, 7967 – 7968.
[39] P. J. Hergenrother, K. M. Depew, S. L. Schreiber, *J. Am. Chem. Soc.* **2000**, *122*, 7849 – 7850.
[40] J. Sagiv, *J. Am. Chem. Soc.* **1980**, *102*, 92 – 98.
[41] A. del Campo, I. J. Bruce, *Top. Curr. Chem.* **2005**, *260*, 77 – 111.
[42] C. Heise, F. F. Bier, *Top. Curr. Chem.* **2006**, *261*, 1 – 25.
[43] J. A. Howarter, J. P. Youngblood, *Langmuir* **2006**, *22*, 11142 – 11147.
[44] A. V. Krasnoslobodtsev, S. N. Smirnov, *Langmuir* **2002**, *18*, 3181 – 3184.
[45] P. Silberzan, L. Léger, D. Ausserré, J. J. Benattar, *Langmuir* **1991**, *7*, 1647 – 1651.
[46] An example for silanization with aminopropyltriethoxysilane (APTES) could be as follows: a cleaned and plasma-treated silica substrate is stirred in a mixture of ethanol/water/APTES (95:3:2 by volume) for four hours at room temperature and subsequently cleaned with ethanol and acetone. R. Benters, C. M. Niemeyer, D. Wöhrle, *ChemBioChem* **2001**, *2*, 686 – 694.
[47] M. Beier, J. D. Hoheisel, *Nucleic Acids Res.* **1999**, *27*, 1970 – 1977.
[48] R. Benters, C. M. Niemeyer, D. Drutschmann, D. Blohm, D. Wöhrle, *Nucleic Acids Res.* **2002**, *30*, 10e.
[49] P. K. Ajikumar, J. K. Ng, Y. C. Tang, J. Y. Lee, G. Stephanopoulos, H. P. Too, *Langmuir* **2007**, *23*, 5670 – 5677.
[50] V. Le Berre, E. Trevisiol, A. Dagkessamanskaia, A.-M. Caminade, J. P. Majoral, B. Meunier, J. François, *Nucleic Acids Res.* **2003**, *31*, 88e.
[51] S. Pathak, A. K. Singh, J. R. McElhanon, P. M. Dentinger, *Langmuir* **2004**, *20*, 6075 – 6079.
[52] T. Strother, W. Cai, X. S. Zhao, R. J. Hamers, L. M. Smith, *J. Am. Chem. Soc.* **2000**, *122*, 1205 – 1209.
[53] G. M. Harbers, K. Emoto, C. Greef, S. W. Metzger, H. N. Woodward, J. J. Mascali, D. W. Grainger, M. J. Lochhead, *Chem. Mater.* **2007**, *19*, 4405 – 4414.
[54] C. S. Tang, M. Dusseiller, S. Makohliso, M. Heuschkel, S. Sharma, B. Keller, J. Vörös, *Anal. Chem.* **2006**, *78*, 711 – 717.
[55] K. Kim, H. Yang, S. Jon, E. Kim, J. Kwak, *J. Am. Chem. Soc.* **2004**, *126*, 15368 – 15369.
[56] K. Kim, J. Hwang, I. Seo, T. H. Hwan, J. Kwak, *Chem. Commun.* **2006**, 4723 – 4725.
[57] W. Yang, J. E. Butler, J. N. Russell, R. J. Hamers, *Langmuir* **2004**, *20*, 6778 – 6787.
[58] L. Szyk-Warszynska, A. Trybala, *J. Colloid Interface Sci.* **2007**, *314*, 398 – 404.
[59] A. Minarik, M. Humenik, S. Li, Y. Huang, G. Krausch, M. Sprinzl, *ChemBioChem* **2008**, *9*, 124 – 130.
[60] H. Okusa, K. Kurihara, T. Kunitake, *Langmuir* **1994**, *10*, 3577 – 3581.
[61] N. P. Huang, R. Michel, J. Vörös, M. Textor, R. Hofer, A. Rossi, D. L. Elbert, J. A. Hubbell, N. D. Spencer, *Langmuir* **2001**, *17*, 489 – 498.
[62] K. De Groot, R. Geesink, C. P. A. T. Klein, P. Serekian, *J. Biomed. Mater. Res.* **1987**, *21*, 1375 – 1381.
[63] S. Pallu, C. Bourget, R. Bareille, C. Labrugere, M. Dard, A. Sewing, A. Jonczyk, M. Vernizeau, M. Christine Durrieu, J. Amedee-Vilamitjana, *Biomaterials* **2005**, *26*, 6932 – 6940.
[64] C. R. Kessel, S. Granick, *Langmuir* **1991**, *7*, 532 – 538.
[65] A. Ulman, *Chem. Rev.* **1996**, *96*, 1533 – 1554.
[66] J. C. Love, L. A. Estroff, J. K. Kriebel, R. G. Nuzzo, G. M. Whitesides, *Chem. Rev.* **2005**, *105*, 1103 – 1169.
[67] P. Wagner, M. Hegner, P. Kernen, F. Zaugg, G. Semenza, *Biophys. J.* **1996**, *70*, 2052 – 2066.
[68] C. D. Hodneland, Y.-S. Lee, D.-H. Min, M. Mrksich, *Proc. Natl. Acad. Sci. USA* **2002**, *99*, 5048 – 5052.
[69] C. D. Bain, G. M. Whitesides, *J. Am. Chem. Soc.* **1989**, *111*, 7164 – 7175.
[70] J. M. Brockman, A. G. Frutos, R. M. Corn, *J. Am. Chem. Soc.* **1999**, *121*, 8044 – 8051.
[71] R. Jakob, *BioEngineering* **1994**, *10*, 16 – 19.
[72] J. R. Crowther, *ELISA: Theory and Practice*, Humana, New Jersey, **1995**.
[73] R. S. Matson, R. C. Milton, M. C. Cress, T. S. Chan, J. B. Rampal, *Methods Mol. Biol.* **2007**, *381*, 339 – 362.
[74] U. B. Nielsen, M. H. Cardone, A. J. Sinskey, G. MacBeath, P. K. Sorger, *Proc. Natl. Acad. Sci. USA* **2003**, *100*, 9330 – 9335.
[75] F. Fixe, M. Dufva, P. Telleman, C. B. V. Christensen, *Nucleic Acids Res.* **2004**, *32*, 9e.
[76] E. Delamarche, A. Bernard, H. Schmid, B. Michel, H. Biebuyck, *Science* **1997**, *276*, 779 – 781.
[77] H. Zhu, J. F. Klemic, S. Chang, P. Bertone, A. Casamayor, K. G. Klemic, D. Smith, M. Gerstein, M. A. Reed, M. Snyder, *Nat. Genet.* **2000**, *26*, 283 – 289.
[78] M. Nishikawa, T. Yamamoto, N. Kojima, K. Kikuo, T. Fujii, Y. Sakai, *Biotechnol. Bioeng.* **2008**, *99*, 1472 – 1481.
[79] S. A. Soper, A. C. Henry, B. Vaidya, M. Galloway, M. Wabuyele, R. L. McCarley, *Anal. Chim. Acta* **2002**, *470*, 87 – 99.
[80] S. C. Popescu, G. V. Popescu, S. Bachan, Z. Zhang, M. Seay, M. Gerstein, M. Snyder, S. P. Dinesh-Kumar, *Proc. Natl. Acad. Sci. USA* **2007**, *104*, 4730 – 4735.
[81] S. Lemeer, R. Ruijtenbeek, M. W. H. Pinkse, C. Jopling, A. J. R. Heck, J. den Hertog, M. Slijper, *Mol. Cell. Proteomics* **2007**, *6*, 2088 – 2099.
[82] E. N. Timofeev, A. D. Kochetkova, A. D. Mirzabekov, V. L. Florentiev, *Nucleic Acids Res.* **1996**, *24*, 3142 – 3148.
[83] D. Guschin, G. Yershov, A. Zaslavsky, A. Gemmel, V. Shick, D. Proudnikov, P. Arenkov, A. D. Mirzabekov, *Anal. Biochem.* **1997**, *250*, 203 – 211.
[84] C. Mateo, O. Abian, R. Fernandez-Lafuente, J. M. Guisan, *Biotechnol. Bioeng.* **2000**, *68*, 98 – 105.
[85] S. Piletsky, E. Piletska, A. Bossi, N. Turner, A. Turner, *Biotechnol. Bioeng.* **2003**, *82*, 86 – 92.
[86] M. M. Dominguez, M. Wathier, M. W. Grinstaff, S. E. Schaus, *Anal. Chem.* **2007**, *79*, 1064 – 1066.
[87] S. Kiyonaka, K. Sada, I. Yoshimura, S. Shinkai, N. Kato, I. Hamachi, *Nat. Mater.* **2004**, *3*, 58 – 64.
[88] J. J. Yoon, Y. S. Nam, J. H. Kim, T. G. Park, *Biotechnol. Bioeng.* **2002**, *78*, 1 – 10.

[89] V. Hlady, J. Buijs, *Curr. Opin. Biotechnol.* **1996**, *7*, 72 – 77.
[90] J. J. Gray, *Curr. Opin. Struct. Biol.* **2003**, *13*, 110 – 115.
[91] C. M. Niemeyer, M. Adler, R. Wacker, *Nat. Protocols* **2007**, *2*, 1918 – 1930.
[92] N. Nath, J. Hyun, H. Ma, A. Chilkoti, *Surf. Sci.* **2004**, *570*, 98 – 110.
[93] E. Ostuni, R. G. Chapman, R. E. Holmlin, S. Takayama, G. M. Whitesides, *Langmuir* **2001**, *17*, 5605 – 5620.
[94] J. Ponten, L. Stolt, *Exp. Cell Res.* **1980**, *129*, 367 – 375.
[95] S. B. Carter, *Nature* **1965**, *208*, 1183 – 1187.
[96] Y. Y. Luk, M. Kato, M. Mrksich, *Langmuir* **2000**, *16*, 9604 – 9608.
[97] T. G. Vargo, E. J. Bekos, Y. S. Kim, J. P. Ranieri, R. Bellamkonda, P. Aebischer, D. E. Margevich, P. M. Thompson, F. V. Bright, J. A. Gardella, Jr., *Biomed. Mater. Res.* **1995**, *29*, 767 – 778.
[98] K. S. Ko, F. A. Jaipuri, N. L. Pohl, *J. Am. Chem. Soc.* **2005**, *127*, 13162 – 13163.
[99] J. Groll, E. V. Amirgoulova, T. Ameringer, C. D. Heyes, C. Röcker, G. U. Nienhaus, M. Möller, *J. Am. Chem. Soc.* **2004**, *126*, 4234 – 4239.
[100] R. Michel, S. Pasche, M. Textor, D. G. Castner, *Langmuir* **2005**, *21*, 12327 – 12332.
[101] T. Sugawara, T. Matsuda, *J. Biomed. Mater. Res.* **1995**, *29*, 1047 – 1052.
[102] D. Batra, S. Vogt, P. D. Laible, M. A. Firestone, *Langmuir* **2005**, *21*, 10301 – 10306.
[103] J. D. Mendelsohn, S. Y. Yang, J. A. Hiller, A. I. Hochbaum, M. F. Rubner, *Biomacromolecules* **2003**, *4*, 96 – 106.
[104] C. M. Yam, M. Deluge, D. Tang, A. Kumar, C. Cai, *J. Colloid Interface Sci.* **2006**, *296*, 118 – 130.
[105] M. Mrksich, G. B. Sigal, G. M. Whitesides, *Langmuir* **1995**, *11*, 4383 – 4385.
[106] J. Hoffmann, J. Groll, J. Heuts, H. Rong, D. Klee, G. Ziemer, M. Möller, H. P. Wendel, *J. Biomater. Sci. Polym. Ed.* **2006**, *17*, 985 – 996.
[107] S. J. Dilly, M. P. Beecham, S. P. Brown, J. M. Griffin, A. J. Clark, C. D. Griffin, J. Marshall, R. M. Napier, P. C. Taylor, A. Marsh, *Langmuir* **2006**, *22*, 8144 – 8150.
[108] K. L. Prime, G. M. Whitesides, *J. Am. Chem. Soc.* **1993**, *115*, 10714 – 10721.
[109] S. Chen, J. Zheng, L. Li, S. Jiang, *J. Am. Chem. Soc.* **2005**, *127*, 14473 – 14478.
[110] R. S. Kane, P. Deschatelets, G. M. Whitesides, *Langmuir* **2003**, *19*, 2388 – 2391.
[111] K. M. Rusin, T. L. Fare, J. Z. Stemple, *Biosens. Bioelectron.* **1992**, *7*, 367 – 371.
[112] P.-C. Lin, S.-H. Ueng, M.-C. Tseng, J.-L. Ko, K.-T. Huang, S.-C. Yu, A. K. Adak, Y. J. Chen, C.-C. Lin, *Angew. Chem. Int. Ed.* **2006**, *45*, 4286 – 4290.
[113] K. Holland-Nell, A. G. Beck-Sickinger, *ChemBioChem* **2007**, *8*, 1071 – 1076.
[114] R. L. Rich, D. G. Myszka, *Curr. Opin. Biotechnol.* **2000**, *11*, 54 – 61.
[115] B. A. Stillman, J. L. B. Tonkinson, *BioTechniques* **2000**, *29*, 630 – 635.
[116] M. Reck, F. Stahl, J. G. Walter, M. Hollas, D. Melzner, T. Scheper, *Biotechnol. Prog.* **2007**, *23*, 1498 – 1505.
[117] D. L. Wilson, R. Martin, S. Hong, M. Cronin-Golomb, C. A. Mirkin, D. L. Kaplan, *Proc. Natl. Acad. Sci. USA* **2001**, *98*, 13660 – 13664.
[118] K.-B. Lee, J.-H. Lim, C. A. Mirkin, *J. Am. Chem. Soc.* **2003**, *125*, 5588 – 5589.
[119] H. Zhang, K.-B. Lee, Z. Li, C. A. Mirkin, *Nanotechnology* **2003**, *14*, 1113 – 1117.
[120] C. Wang, Y. Zhang, *Adv. Mater.* **2005**, *17*, 150 – 153.
[121] J. M. Slocik, E. R. Beckel, H. Jiang, J. O. Enlow, J. S. Zabinski, Jr., T. J. Bunning, R. R. Naik, *Adv. Mater.* **2006**, *18*, 2095 – 2100.
[122] N. Kumar, J.-I. Hahm, *Langmuir* **2005**, *21*, 6652 – 6655.
[123] L. M. Lee, R. L. Heimark, J. C. Baygents, Y. Zohar, *Nanotechnology* **2006**, *17*, S29 – S33.
[124] J.-M. Jung, K. Y. Kwon, T.-H. Ha, B. H. Chung, H.-T. Jung, *Small* **2006**, *2*, 1010 – 1015.
[125] J. Doh, D. J. Irvine, *J. Am. Chem. Soc.* **2004**, *126*, 9170 – 9171.
[126] C. M. Yam, M. Deluge, D. Tang, A. Kumar, C. Cai, *J. Colloid Interface Sci.* **2006**, *296*, 118 – 130.
[127] N. Patel, M. C. Davies, M. Hartshorne, R. J. Heaton, C. J. Roberts, S. J. B. Tendler, P. M. Williams, *Langmuir* **1997**, *13*, 6485 – 6490.
[128] B. Johnsson, S. Loefaas, G. Lindquist, *Anal. Biochem.* **1991**, *198*, 268 – 277.
[129] S. V. Rao, K. W. Anderson, L. G. Bachas, *Biotechnol. Bioeng.* **1999**, *65*, 389 – 396.
[130] K. Jiang, L. S. Schadler, R. W. Siegel, X. Zhang, H. Zhang, M. Terrones, *J. Mater. Chem.* **2004**, *14*, 37 – 39.
[131] S. F. D'Souza, S. S. Godbole, *J. Biochem. Biophys. Methods* **2002**, *52*, 59 – 62.
[132] H. J. Choi, N. H. Kim, B. H. Chung, G. H. Seong, *Anal. Biochem.* **2005**, *347*, 60 – 66.
[133] C. Mateo, R. Torres, G. Fernandez-Lorente, C. Ortiz, M. Fuentes, A. Hidalgo, F. Lopez-Gallego, O. Abian, J. M. Palomo, L. Betancor, B. C. C. Pessela, J. M. Guisan, R. Fernandez-Lafuente, *Biomacromolecules* **2003**, *4*, 772 – 777.
[134] G. MacBeath, S. L. Schreiber, *Science* **2000**, *289*, 1760 – 1763.
[135] N. V. Avseenko, T. Y. Morozova, F. I. Ataullakhanov, V. N. Morozov, *Anal. Chem.* **2001**, *73*, 6047 – 6052.
[136] a) R. L. Rich, D. G. Myszka, *J. Mol. Recognit.* **2007**, *20*, 300 – 366; b) R. L. Rich, D. G. Myszka, *J. Mol. Recognit.* **2006**, *19*, 478 – 534; c) R. L. Rich, D. G. Myszka, *J. Mol. Recognit.* **2005**, *18*, 431 – 478; d) R. L. Rich, D. G. Myszka, *J. Mol. Recognit.* **2005**, *18*, 1 – 39; e) R. L. Rich, D. G. Myszka, *J. Mol. Recognit.* **2003**, *16*, 351 – 382; f) R. L. Rich, D. G. Myszka, *J. Mol. Recognit.* **2002**, *15*, 352 – 376; g) R. L. Rich, D. G. Myszka, *J. Mol. Recognit.* **2000**, *13*, 388 – 407; h) R. L. Rich, D. G. Myszka, *J. Mol. Recognit.* **2001**, *14*, 223 – 228; i) S. Löfas, B. Johnsson, A. Edström, A. Hansson, G. Lindquist, R.-M. Müller Hillgren, L. Stigh, *Biosens. Bioelectr.* **1995**, *10*, 813 – 822.
[137] R. B. Jones, A. Gordus, J. A. Krall, G. MacBeath, *Nature* **2005**, *439*, 168 – 174.
[138] D. I. Rozkiewicz, Y. Kraan, M. W. T. Werten, F. A. de Wolf, V. Subramaniam, B. J. Ravoo, D. N. Reinhoudt, *Chem. Eur. J.* **2006**, *12*, 6290 – 6297.
[139] C. Mateo, O. Abian, G. Fernandez-Lorente, J. Pedroche, R. Fernandez-Lafuente, J. M. Guisan, A. Tam, M. Daminati, *Biotechnol. Prog.* **2002**, *18*, 629 – 634.
[140] V. Grazu, O. Abian, C. Mateo, F. Batista-Viera, R. Fernandez-Lafuente, J. M. Guisan, *Biomacromolecules* **2003**, *4*, 1495 – 1501.
[141] P. Angenendt, J. Glokler, J. Sobek, H. Lehrach, D. J. Cahill, *J. Chromatogr. A* **2003**, *1009*, 97 – 104.
[142] S. L. Seurynck-Servoss, C. L. Baird, K. D. Rodland, R. C. Zangar, *Front. Biosci.* **2007**, *12*, 3956 – 3964.
[143] S. L. Seurynck-Servoss, A. M. White, C. L. Baird, K. D. Rodland, R. C. Zangar, *Anal. Biochem.* **2007**, *371*, 105 – 115.
[144] B. Guilleaume, A. Buneß, C. Schmidt, F. Klimek, G. Moldenhauer, W. Huber, D. Arlt, U. Korf, S. Wiemann, A. Poustka, *Proteomics* **2005**, *5*, 4705 – 4712.
[145] C. Wingren, J. Ingvarsson, L. Dexlin, D. Szul, C. A. K. Borrebaeck, *PROTEOMICS* **2007**, *7*, 3055 – 3065.
[146] C. L. Feng, A. Embrechts, I. Bredebush, J. Schnekenburger, W. Domschke, G. J. Vancso, H. Schönherr, *Adv. Mater.* **2007**, *19*, 286 – 290.

[147] S. M. Bhangale, V. Tjong, L. Wu, N. Yakovlev, P. M. Moran, *Adv. Mater.* **2005**, *17*, 809 – 813.
[148] G.-Y. Liu, N. A. Amro, *Proc. Natl. Acad. Sci. USA* **2002**, *99*, 5165 – 5170.
[149] K. Wadu-Mesthrige, S. Xu, N. A. Amro, G.-y. Liu, *Langmuir* **1999**, *15*, 8580 – 8583.
[150] M. A. Case, G. L. McLendon, Y. Hu, T. K. Vanderlick, G. Scoles, *Nano Lett.* **2003**, *3*, 425 – 429.
[151] Y. Hu, A. Das, M. H. Hecht, G. Scoles, *Langmuir* **2005**, *21*, 9103 – 9109.
[152] S. W. Lee, B.-K. Oh, R. G. Sanedrin, K. Salaita, T. Fujigaya, C. A. Mirkin, *Adv. Mater.* **2006**, *18*, 1133 – 1136.
[153] K.-B. Lee, S.-J. Park, C. A. Mirkin, J. C. Smith, M. Mrksich, *Science* **2002**, *295*, 1702 – 1705.
[154] K.-B. Lee, E.-Y. Kim, C. A. Mirkin, S. M. Wolinsky, *Nano Lett.* **2004**, *4*, 1869 – 1872.
[155] R. A. Vega, D. Maspoch, K. Salaita, C. A. Mirkin, *Angew. Chem.* **2005**, *117*, 6167 – 6169; *Angew. Chem. Int. Ed.* **2005**, *44*, 6013 – 6015.
[156] N. P. Reynolds, S. Janusz, M. Escalante-Marun, J. Timney, R. E. Ducker, J. D. Olsen, C. Otto, V. Subramaniam, G. J. Leggett, C. N. Hunter, *J. Am. Chem. Soc.* **2007**, *129*, 14625 – 14631.
[157] R. Fernandez-Lafuente, C. M. Rosell, V. Rodriguez, C. Santana, G. Soler, A. Bastida, J. M. Guisan, *Enzyme Microb. Technol.* **1993**, *15*, 546 – 550.
[158] S. P. Fodor, J. L. Read, M. C. Pirrung, L. Stryer, A. T. Lu, D. Solas, *Science* **1991**, *251*, 767 – 773.
[159] K. R. Bhushan, *Org. Biomol. Chem.* **2006**, *4*, 1857 – 1859.
[160] A. S. Blawas, T. F. Oliver, M. C. Pirrung, W. M. Reichert, *Langmuir* **1998**, *14*, 4243 – 4250.
[161] S. A. Fleming, *Tetrahedron* **1995**, *51*, 12479 – 12520.
[162] I. Caelen, H. Gao, H. Sigrist, *Langmuir* **2002**, *18*, 2463 – 2467.
[163] A. Collioud, J. F. Clemence, M. Saenger, H. Sigrist, *Bioconjugate Chem.* **1993**, *4*, 528 – 536.
[164] C. Philipona, Y. Chevolot, D. Léonard, H. J. Mathieu, H. Sigrist, F. Marquis-Weible, *Bioconjugate Chem.* **2001**, *12*, 332 – 336.
[165] L. F. Rozsnyai, D. R. Benson, S. P. Fodor, P. G. Schultz, *Angew. Chem.* **1992**, *104*, 801 – 802; *Angew. Chem. Int. Ed. Engl.* **1992**, *31*, 759 – 761.
[166] P. Nahar, N. M. Wali, R. P. Gandhi, *Anal. Biochem.* **2001**, *294*, 148 – 153.
[167] D. A. Nivens, D. W. Conrad, *Langmuir* **2002**, *18*, 499 – 504.
[168] D. J. Pritchard, H. Morgan, J. M. Cooper, *Angew. Chem.* **1995**, *107*, 84 – 86; *Angew. Chem. Int. Ed. Engl.* **1995**, *34*, 91 – 93.
[169] M. Yan, S. X. Cai, M. N. Wybourne, J. F. W. Keana, *J. Am. Chem. Soc.* **1993**, *115*, 814 – 816.
[170] S. K. Bhatia, J. J. Hickman, F. S. Ligler, *J. Am. Chem. Soc.* **1992**, *114*, 4432 – 4433.
[171] M. T. Neves-Petersen, T. Snabe, S. Klitgaard, M. Duroux, S. Petersen, *Protein Sci.* **2006**, *15*, 343 – 351.
[172] M. R. Martzen, S. M. McCraith, S. L. Spinelli, F. M. Torres, S. Fields, E. J. Grayhack, E. M. Phizicky, *Science* **1999**, *286*, 1153 – 1155.
[173] P. Riggs, *Mol. Biotechnol.* **2000**, *15*, 51 – 63.
[174] B. A. Bouchard, B. Furie, B. C. Furie, *Biochemistry* **1999**, *38*, 9517 – 9523.
[175] J. Schmitt, J. Hess, H. G. Stunnenberg, *Mol. Biol. Rep.* **1993**, *18*, 223 – 230.
[176] S. Svendsen, C. Zimprich, M. McDougall, D. Klaubert, G. Los, *BMC Cell Biol.* **2008**, *9*, 17.
[177] G. V. Los, K. Wood, *Methods Mol. Biol.* **2007**, *356*, 195 – 208.
[178] Y. Zhang, M.-K. So, A. M. Loening, H. Yao, S. S. Gambhir, J. Rao, *Angew. Chem.* **2006**, *118*, 5058 – 5062; *Angew. Chem. Int. Ed.* **2006**, *45*, 4936 – 4940.
[179] E. L. Schmid, T. A. Keller, Z. Dienes, H. Vogel, *Anal. Chem.* **1997**, *69*, 1979 – 1985.
[180] E. Gizeli, J. Glad, *Anal. Chem.* **2004**, *76*, 3995 – 4001.
[181] G. Zhen, D. Falconnet, E. Kuennemann, J. Vörös, N. D. Spencer, M. Textor, S. Zurcher, *Adv. Funct. Mater.* **2006**, *16*, 243 – 251.
[182] P. D. Gershon, S. Khilko, *J. Immunol. Methods* **1995**, *183*, 65 – 76.
[183] R. Gamsjaeger, B. Wimmer, H. Kahr, A. Tinazli, S. Picuric, S. Lata, R. Tampé, Y. Maulet, H. J. Gruber, P. Hinterdorfer, C. Romanin, *Langmuir* **2004**, *20*, 5885 – 5890.
[184] K. Kato, H. Sato, H. Iwata, *Langmuir* **2005**, *21*, 7071 – 7075.
[185] M. J. W. Ludden, A. Mulder, K. Schulze, V. Subramaniam, R. Tampé, J. Huskens, *Chem. Eur. J.* **2008**, *14*, 2044 – 2051.
[186] N. Haddour, S. Cosnier, C. Gondran, *J. Am. Chem. Soc.* **2005**, *127*, 5752 – 5753.
[187] A. Tinazli, J. Tang, R. Valiokas, S. Picuric, S. Lata, J. Piehler, B. Liedberg, R. Tampé, *Chem. Eur. J.* **2005**, *11*, 5249 – 5259.
[188] G. J. Wegner, H. J. Lee, G. Marriott, R. M. Corn, *Anal. Chem.* **2003**, *75*, 4740 – 4746.
[189] M. Mammen, S.-K. Choi, G. M. Whitesides, *Angew. Chem.* **1998**, *110*, 2908 – 2953; *Angew. Chem. Int. Ed.* **1998**, *37*, 2754 – 2794.
[190] S. Lata, A. Reichel, R. Brock, R. Tampé, J. Piehler, *J. Am. Chem. Soc.* **2005**, *127*, 10205 – 10215.
[191] I. T. Dorn, K. Pawlitschko, S. C. Pettinger, R. Tampé, *Biol. Chem.* **1998**, *379*, 1151 – 1159.
[192] I. T. Dorn, R. Eschrich, E. Seemueller, R. Guckenberger, R. Tampé, *J. Mol. Biol.* **1999**, *288*, 1027 – 1036.
[193] S. Hutschenreiter, A. Tinazli, K. Model, R. Tampé, *EMBO J.* **2004**, *23*, 2488 – 2497.
[194] A. Turchanin, A. Tinazli, M. El-Desawy, H. Großmann, M. Schnietz, H. H. Solak, R. Tampé, A. Gölzhäuser, *Adv. Mater.* **2008**, *20*, 471 – 477.
[195] J. A. Hansen, V. V. Sumbayev, K. V. Gothelf, *Nano Lett.* **2007**, *7*, 2831 – 2834.
[196] W.-S. Yeo, D.-H. Min, R. W. Hsieh, G. L. Greene, M. Mrksich, *Angew. Chem.* **2005**, *117*, 5616 – 5619; *Angew. Chem. Int. Ed.* **2005**, *44*, 5480 – 5483.
[197] V. L. Marin, T. H. Bayburt, S. G. Sligar, M. Mrksich, *Angew. Chem.* **2007**, *119*, 8952 – 8954; *Angew. Chem. Int. Ed.* **2007**, *46*, 8796 – 8798.
[198] R. Valiokas, G. Klenkar, A. Tinazli, R. Tampé, B. Liedberg, J. Piehler, *ChemBioChem* **2006**, *7*, 1325 – 1329.
[199] J. Groll, W. Haubensak, T. Ameringer, M. Möller, *Langmuir* **2005**, *21*, 3076 – 3083.
[200] P. Maury, M. Escalante-Marun, M. Péter, D. N. Reinhoudt, V. Subramaniam, J. Huskens, *Small* **2007**, *3*, 1584 – 1592.
[201] A. Tinazli, J. Piehler, M. Beuttler, R. Guckenberger, R. Tampé, *Nat. Nanotechnol.* **2007**, *2*, 220 – 225.
[202] J.-M. Nam, S. W. Han, K.-B. Lee, X. Liu, M. A. Ratner, C. A. Mirkin, *Angew. Chem.* **2004**, *116*, 1266 – 1269; *Angew. Chem. Int. Ed.* **2004**, *43*, 1246 – 1249.
[203] N. M. Green, *Adv. Protein Chem.* **1975**, *29*, 85 – 133.
[204] C. L. Smith, G. S. Milea, G. H. Nguyen, *Top. Curr. Chem.* **2006**, *261*, 63 – 90.
[205] J. Spinke, M. Liley, F. J. Schmitt, H. J. Guder, L. Angermaier, W. Knoll, *J. Chem. Phys.* **1993**, *99*, 7012 – 7019.
[206] W. Muller, H. Ringsdorf, E. Rump, G. Wildburg, X. Zhang, L. Angermaier, W. Knoll, M. Liley, J. Spinke, *Science* **1993**, *262*, 1706 – 1708.
[207] J. Spinke, M. Liley, H. J. Guder, L. Angermaier, W. Knoll, *Langmuir* **1993**, *9*, 1821 – 1825.
[208] P. L. Edmiston, J. E. Lee, S. S. Cheng, S. S. Saavedra, *J. Am. Chem. Soc.* **1997**, *119*, 560 – 570.
[209] L. L. Wood, S.-S. Cheng, P. L. Edmiston, S. S. Saavedra, *J. Am. Chem. Soc.* **1997**, *119*, 571 – 576.
[210] F. Xu, G. Zhen, F. Yu, E. Kuennemann, M. Textor, W. Knoll, *J. Am. Chem. Soc.* **2005**, *127*, 13084 – 13085.

[211] G. Romet-Lemonne, M. VanDuijn, M. Dogterom, *Nano Lett.* **2005**, *5*, 2350 – 2354.
[212] M. J. W. Ludden, X. Y. Ling, T. Gang, W. P. Bula, H. J. G. E. Gardeniers, D. N. Reinhoudt, J. Huskens, *Chem. Eur. J.* **2008**, *14*, 136 – 142.
[213] M. C. Pirrung, C.-Y. Huang, *Bioconjugate Chem.* **1996**, *7*, 317 – 321.
[214] S. A. Sundberg, R. W. Barrett, M. Pirrung, A. L. Lu, B. Kiangsoontra, C. P. Holmes, *J. Am. Chem. Soc.* **1995**, *117*, 12050 – 12057.
[215] D. Ryan, B. A. Parviz, V. Linder, V. Semetey, S. K. Sia, J. Su, M. Mrksich, G. M. Whitesides, *Langmuir* **2004**, *20*, 9080 – 9088.
[216] A. Buxboim, M. Bar-Dagan, V. Frydan, D. Zbaida, M. Morpurgo, R. Bar-Ziv, *Small* **2007**, *3*, 500 – 510.
[217] K. L. Christman, H. D. Maynard, *Langmuir* **2005**, *21*, 8389 – 8393.
[218] K. L. Christman, M. V. Requa, V. D. Enriquez-Rios, S. C. Ward, K. A. Bradley, K. L. Turner, H. D. Maynard, *Langmuir* **2006**, *22*, 7444 – 7450.
[219] P. Jonkheijm, D. Weinrich, M. Köhn, H. E. Engelkamp, P. C. M. Christianen, J. Kuhlmann, J. C. Maan, D. Nüsse, H. Schroeder, R. Wacker, R. Breinbauer, C. M. Niemeyer, H. Waldmann, *Angew. Chem.* **2008**, *120*, 4493 – 4496; *Angew. Chem. Int. Ed.* **2008**, *47*, 4421 – 4424.
[220] Y. Chang, Y. S. Ahn, H. T. Hahn, Y. Chen, *Langmuir* **2007**, *23*, 4112 – 4114.
[221] J. Lahiri, E. Ostuni, G. M. Whitesides, *Langmuir* **1999**, *15*, 2055 – 2060.
[222] J. Hyun, S. J. Ahn, W. K. Lee, A. Chilkoti, S. Zauscher, *Nano Lett.* **2002**, *2*, 1203 – 1207.
[223] M. J. W. Ludden, M. Peter, D. N. Reinhoudt, J. Huskens, *Small* **2006**, *2*, 1192 – 1202.
[224] R. Dong, S. Krishnan, B. A. Baird, M. Lindau, C. K. Ober, *Biomacromolecules* **2007**, *8*, 3082 – 3092.
[225] J. Gu, C. M. Yam, S. Li, C. Cai, *J. Am. Chem. Soc.* **2004**, *126*, 8098 – 8099.
[226] M. Lee, D.-K. Kang, H.-K. Yang, K.-H. Park, S. Y. Choe, C. Kang, S.-I. Chang, M. H. Han, I.-C. Kang, *Proteomics* **2006**, *6*, 1094 – 1103.
[227] J. Rundqvist, J. H. Hoh, D. B. Haviland, *Langmuir* **2006**, *22*, 5100 – 5107.
[228] L. A. Ruiz-Taylor, T. L. Martin, F. G. Zaugg, K. Witte, P. Indermuhle, S. Nock, P. Wagner, *Proc. Natl. Acad. Sci. USA* **2001**, *98*, 852 – 857.
[229] D. Falconnet, D. Pasqui, S. Park, R. Eckert, H. Schift, J. Gobrecht, R. Barbucci, M. Textor, *Nano Lett.* **2004**, *4*, 1909 – 1914.
[230] J. D. Hoff, L.-J. Cheng, E. Meyhöfer, L. J. Guo, A. J. Hunt, *Nano Lett.* **2004**, *4*, 853 – 857.
[231] S. H. Park, P. Yin, Y. Liu, J. H. Reif, T. H. LaBean, H. Yan, *Nano Lett.* **2005**, *5*, 729 – 733.
[232] H. Li, S. H. Park, J. H. Reif, T. H. LaBean, H. Yan, *J. Am. Chem. Soc.* **2004**, *126*, 418 – 419.
[233] H. Yan, S. H. Park, G. Finkelstein, J. H. Reif, T. H. LaBean, *Science* **2003**, *301*, 1882 – 1884.
[234] C. M. Niemeyer, M. Adler, S. Lenhert, S. Gao, H. Fuchs, L. Chi, *ChemBioChem* **2001**, *2*, 260 – 264.
[235] C. M. Niemeyer, M. Adler, S. Gao, L. Chi, *Angew. Chem.* **2000**, *112*, 3183 – 3187, *Angew. Chem. Int. Ed. Engl.* **2000**, *39*, 3055 – 3059.
[236] Y. He, Y. Tian, A. E. Ribbe, C. Mao, *J. Am. Chem. Soc.* **2006**, *128*, 12664 – 12665.
[237] A. Fragoso, J. Caballero, E. Almirall, R. Villalonga, R. Cao, *Langmuir* **2002**, *18*, 5051 – 5054.
[238] I. Hwang, K. Baek, M. Jung, Y. Kim, K. M. Park, D.-W. Lee, N. Selvapalam, K. Kim, *J. Am. Chem. Soc.* **2007**, *129*, 4170 – 4171.
[239] J. E. Cronan, *J. Biol. Chem.* **1990**, *265*, 10327 – 10333.
[240] D. Samols, C. G. Thornton, V. L. Murtif, G. K. Kumar, F. C. Haase, H. G. Wood, *J. Biol. Chem.* **1988**, *263*, 6461 – 6464.
[241] P. J. Schatz, *Biotechnology* **1993**, *11*, 1138 – 1143.
[242] J. Yin, F. Liu, X. Li, C. T. Walsh, *J. Am. Chem. Soc.* **2004**, *126*, 7754 – 7755.
[243] C. M. Niemeyer, J. Koehler, C. Wuerdemann, *ChemBioChem* **2002**, *3*, 242 – 245.
[244] R. Y. P. Lue, G. Y. J. Chen, Y. Hu, Q. Zhu, S. Q. Yao, *J. Am. Chem. Soc.* **2004**, *126*, 1055 – 1062.
[245] S. Chattopadhaya, L.-P. Tan, S. Q. Yao, *Nat. Protocols* **2006**, *1*, 2386 – 2398.
[246] M.-L. Lesaicherre, R. Y. P. Lue, G. Y. J. Chen, Q. Zhu, S. Q. Yao, *J. Am. Chem. Soc.* **2002**, *124*, 8768 – 8769.
[247] B. Lu, M. R. Smyth, R. O'Kennedy, *Analyst* **1996**, *121*, 29R-32R.
[248] J. Turkova, *J. Chromatogr. B* **1999**, 722, 11 – 31.
[249] A. Lueking, D. J. Cahill, S. Muellner, *Drug Discovery Today* **2005**, *10*, 789 – 794.
[250] a) C. A. K. Borrebaeck, *Immunol. Today* **2000**, *21*, 379 – 382; b) Y. Jung, J. Y. Jeong, B. Y. Chung, *Analyst* **2008**, *133*, 697 – 701.
[251] Y. Jung, J. M. Lee, H. Jung, B. H. Chung, *Anal. Chem.* **2007**, *79*, 6534 – 6541.
[252] O. Melnyk, X. Duburcq, C. Olivier, F. Urbes, C. Auriault, H. Gras-Masse, *Bioconjugate Chem.* **2002**, *13*, 713 – 720.
[253] X. Duburcq, C. Olivier, F. Malingue, R. Desmet, A. Bouzidi, F. Zhou, C. Auriault, H. Gras-Masse, O. Melnyk, *Bioconjugate Chem.* **2004**, *15*, 307 – 316.
[254] C. P. Johnson, I. E. Jensen, A. Prakasam, R. Vijayendran, D. Leckband, *Bioconjugate Chem.* **2003**, *14*, 974 – 978.
[255] C. M. Niemeyer, T. Sano, C. L. Smith, C. R. Cantor, *Nucleic Acids Res.* **1994**, *22*, 5530 – 5539.
[256] S. Howorka, S. Cheley, H. Bayley, *Nat. Biotechnol.* **2001**, *19*, 636 – 639.
[257] K. Glynou, P. C. Ioannou, T. K. Christopoulos, *Bioconjugate Chem.* **2003**, *14*, 1024 – 1029.
[258] I. A. Kozlov, P. C. Melnyk, K. E. Stromsborg, M. S. Chee, D. L. Barker, C. Zhao, *Biopolymers* **2004**, *73*, 621 – 630.
[259] R. Schlapak, P. Pammer, D. Armitage, R. Zhu, P. Hinterdorfer, M. Vaupel, T. Fruehwirth, S. Howorka, *Langmuir* **2006**, *22*, 277 – 285.
[260] C. Boozer, J. Ladd, S. Chen, S. Jiang, *Anal. Chem.* **2006**, *78*, 1515 – 1519.
[261] C. Boozer, J. Ladd, S. Chen, Q. Yu, J. Homola, S. Jiang, *Anal. Chem.* **2004**, *76*, 6967 – 6972.
[262] P. Kumar, K. C. Gupta, *Bioconjugate Chem.* **2003**, *14*, 507 – 512.
[263] F. Kukolka, M. Lovrinovic, R. Wacker, C. M. Niemeyer, *Bioconjugation protocols: Strategies and methods*, Humana, New Jersey, **2004**.
[264] F. Kukolka, C. M. Niemeyer, *Org. Biomol. Chem.* **2004**, *2*, 2203 – 2206.
[265] R. Wacker, C. M. Niemeyer, *Current Protocols in nucleic acid chemistry, Vol. 21*, Wiley, Hoboken, NJ, **2005**.
[266] R. Wacker, C. M. Niemeyer, *ChemBioChem* **2004**, *5*, 453 – 459.
[267] R. Wacker, H. Schroder, C. M. Niemeyer, *Anal. Biochem.* **2004**, *330*, 281 – 287.
[268] R. C. Bailey, G. A. Kwong, C. G. Radu, O. N. Witte, J. R. Heath, *J. Am. Chem. Soc.* **2007**, *129*, 1959 – 1967.
[269] S. Takeda, S. Tsukiji, T. Nagamune, *Bioorg. Med. Chem. Lett.* **2004**, *14*, 2407 – 2410.
[270] M. Lovrinovic, R. Seidel, R. Wacker, H. Schroeder, O. Seitz, M. Engelhard, R. S. Goody, C. M. Niemeyer, *Chem. Commun.* **2003**, 822 – 823.
[271] T. W. Muir, *Annu. Rev. Biochem.* **2003**, *72*, 249 – 289.
[272] M. Lovrinovic, C. M. Niemeyer, *Biochem. Biophys. Res. Commun.* **2005**, *333*, 943 – 948.

[273] M. Lovrinovic, M. Spengler, C. Deutsch, C. M. Niemeyer, *Mol. BioSyst.* **2005**, *1*, 64 – 69.
[274] M. Lovrinovic, C. M. Niemeyer, *ChemBioChem* **2007**, *8*, 61 – 67.
[275] C. F. W. Becker, R. Wacker, W. Bouschen, R. Seidel, B. Kolaric, P. Lang, H. Schroeder, O. Müller, C. M. Niemeyer, B. Spengler, R. S. Goody, M. Engelhard, *Angew. Chem.* **2005**, *117*, 7808 – 7812; *Angew. Chem. Int. Ed.* **2005**, *44*, 7635 – 7639.
[276] J. Ladd, C. Boozer, Q. Yu, S. Chen, J. Homola, S. Jiang, *Langmuir* **2004**, *20*, 8090 – 8095.
[277] C. M. Niemeyer, R. Wacker, M. Adler, *Nucleic Acids Res.* **2003**, *31*, 90e.
[278] C. M. Niemeyer, L. Boldt, B. Ceyhan, D. Blohm, *Anal. Biochem.* **1999**, *268*, 54 – 63.
[279] L. Fruk, J. Müller, G. Weber, A. Narváez, E. Domínguez, C. M. Niemeyer, *Chem. Eur. J.* **2007**, *13*, 5223 – 5231.
[280] M. Lovrinovic, C. M. Niemeyer, *Angew. Chem.* **2005**, *117*, 3241 – 3246; *Angew. Chem. Int. Ed.* **2005**, *44*, 3179 – 3183.
[281] A. Sassolas, B. D. Leca-Bouvier, L. J. Blum, *Chem. Rev.* **2008**, *108*, 109 – 139.
[282] U. Feldkamp, R. Wacker, W. Banzhaf, C. M. Niemeyer, *ChemPhysChem* **2004**, *5*, 367 – 372.
[283] N. Ramachandran, E. Hainsworth, B. Bhullar, S. Eisenstein, B. Rosen, A. Y. Lau, J. C. Walter, J. LaBaer, *Science* **2004**, *305*, 86 – 90.
[284] S.-C. Tao, H. Zhu, *Nat. Biotechnol.* **2006**, *24*, 1253 – 1254.
[285] A. Lueking, Z. Konthur, H. Eickhoff, K. Bussow, H. Lehrach, D. J. Cahill, *Curr. Genomics* **2001**, *2*, 151 – 159.
[286] C. Gutjahr, D. Murphy, A. Lueking, A. Koenig, M. Janitz, J. O'Brien, B. Korn, S. Horn, H. Lehrach, D. J. Cahill, *Genomics* **2005**, *85*, 285 – 296.
[287] J. A. Camarero, *Biophys. Rev. Lett.* **2006**, *1*, 1 – 28.
[288] T. W. Muir, *Annu. Rev. Biochem.* **2003**, *72*, 249 – 289.
[289] V. Gauvreau, P. Chevallier, K. Vallieres, E. Petitclerc, R. C. - Gaudreault, G. Laroche, *Bioconjugate Chem.* **2004**, *15*, 1146 – 1156.
[290] T. Ichihara, J. K. Akada, S. Kamei, S. Ohshiro, D. Sato, M. Fujimoto, Y. Kuramitsu, K. Nakamura, *J. Proteome Res.* **2006**, *5*, 2144 – 2151.
[291] K. Blank, J. Morfill, H. E. Gaub, *ChemBioChem* **2006**, *7*, 1349 – 1351.
[292] C. L. Cheung, J. A. Camarero, B. W. Woods, T. Lin, J. E. Johnson, J. J. De Yoreo, *J. Am. Chem. Soc.* **2003**, *125*, 6848 – 6849.
[293] J. C. Smith, K.-B. Lee, Q. Wang, M. G. Finn, J. E. Johnson, M. Mrksich, C. A. Mirkin, *Nano Lett.* **2003**, *3*, 883 – 886.
[294] C. Staii, D. W. Wood, G. Scoles, *J. Am. Chem. Soc.* **2008**, *130*, 640 – 646.
[295] M. Morpurgo, F. M. Veronese, D. Kachensky, J. M. Harris, *Bioconjugate Chem.* **1996**, *7*, 363 – 368.
[296] J. A. Camarero, Y. Kwon, M. A. Coleman, *J. Am. Chem. Soc.* **2004**, *126*, 14730 – 14731.
[297] A. Girish, H. Sun, D. S. Y. Yeo, G. Y. J. Chen, T.-K. Chua, S. Q. Yao, *Bioorg. Med. Chem. Lett.* **2005**, *15*, 2447 – 2451.
[298] B. Helms, I. van Baal, M. Merkx, E. W. Meijer, *ChemBioChem* **2007**, *8*, 1790 – 1794.
[299] Y. Kwon, M. A. Coleman, J. A. Camarero, *Angew. Chem.* **2006**, *118*, 1758 – 1761; *Angew. Chem. Int. Ed.* **2006**, *45*, 1726 – 1729.
[300] A. J. Chmura, M. S. Orton, C. F. Meares, *Proc. Natl. Acad. Sci. USA* **2001**, *98*, 8480 – 8484.
[301] N. Winssinger, S. Ficarro, P. G. Schultz, J. L. Harris, *Proc. Natl. Acad. Sci. USA* **2002**, *99*, 11139 – 11144.
[302] N. Winssinger, J. L. Harris, B. J. Backes, P. G. Schultz, *Angew. Chem.* **2001**, *113*, 3254 – 3258; *Angew. Chem. Int. Ed.* **2001**, *40*, 3152 – 3155.
[303] Y. Kwon, Z. Han, E. Karatan, M. Mrksich, B. K. Kay, *Anal. Chem.* **2004**, *76*, 5713 – 5720.
[304] M. Kindermann, N. George, N. Johnsson, K. Johnsson, *J. Am. Chem. Soc.* **2003**, *125*, 7810 – 7811.
[305] I. Sielaff, A. Arnold, G. Godin, S. Tugulu, H.-A. Klok, K. Johnsson, *ChemBioChem* **2006**, *7*, 194 – 202.
[306] S. K. Kufer, H. Dietz, C. Albrecht, K. Blank, A. Kardinal, M. Rief, H. E. Gaub, *Eur. Biophys. J.* **2005**, *35*, 72 – 78.
[307] N. Kamiya, S. Doi, Y. Tanaka, H. Ichinose, M. Goto, *Soc. Biotechnol. Jpn.* **2007**, *104*, 195 – 199.
[308] L. Chan, H. F. Cross, J. K. She, G. Cavalli, H. F. Martins, C. Neylon, *PLoS ONE* **2007**, *2*, e1164.
[309] S. J. Lee, J. P. Park, T. J. Park, S. Y. Lee, S. Lee, J. K. Park, *Anal. Chem.* **2005**, *77*, 5755 – 5759.
[310] M. B. Soellner, K. A. Dickson, B. L. Nilsson, R. T. Raines, *J. Am. Chem. Soc.* **2003**, *125*, 11790 – 11791.
[311] J. Kalia, N. L. Abbott, R. T. Raines, *Bioconjugate Chem.* **2007**, *18*, 1064 – 1069.
[312] A. Watzke, M. Koehn, M. Gutierrez-Rodriguez, R. Wacker, H. Schroeder, R. Breinbauer, J. Kuhlmann, K. Alexandrov, C. M. Niemeyer, R. S. Goody, H. Waldmann, *Angew. Chem.* **2006**, *118*, 1436 – 1440; *Angew. Chem. Int. Ed.* **2006**, *45*, 1408 – 1412.
[313] A. Watzke, M. Gutierrez-Rodriguez, M. Köhn, R. Wacker, H. Schroeder, R. Breinbauer, J. Kuhlmann, K. Alexandrov, C. M. Niemeyer, R. S. Goody, H. Waldmann, *Bioorg. Med. Chem. Lett.* **2006**, *16*, 6288 – 6306.
[314] K. L. Kiick, E. Saxon, D. A. Tirrell, C. R. Bertozzi, *Proc. Natl. Acad. Sci. USA* **2002**, *99*, 19 – 24.
[315] J. M. Baskin, J. A. Prescher, S. T. Laughlin, N. J. Agard, P. V. Chang, I. A. Miller, A. Lo, J. A. Codelli, C. R. Bertozzi, *Proc. Natl. Acad. Sci. USA* **2007**, *104*, 16793 – 16797.
[316] B. P. Duckworth, J. Xu, T. A. Taton, A. Guo, M. D. Distefano, *Bioconjugate Chem.* **2006**, *17*, 967 – 974.
[317] T. Govindaraju, P. Jonkheijm, L. Gogolin, H. Schroeder, C. F. W. Becker, C. M. Niemeyer, H. Waldmann, *Chem. Commun.* **2008**, 3723 – 3725.
[318] B. T. Houseman, J. H. Huh, S. J. Kron, M. Mrksich, *Nat. Biotechnol.* **2002**, *20*, 270 – 274.
[319] M. N. Yousaf, M. Mrksich, *J. Am. Chem. Soc.* **1999**, *121*, 4286 – 4287.
[320] M. N. Yousaf, B. T. Houseman, M. Mrksich, *Angew. Chem.* **2001**, *113*, 1127 – 1130; *Angew. Chem. Int. Ed.* **2001**, *40*, 1093 – 1096.
[321] A. D. de Araújo, J. M. Palomo, J. Cramer, M. Koehn, H. Schroeder, R. Wacker, C. Niemeyer, K. Alexandrov, H. Waldmann, *Angew. Chem.* **2006**, *118*, 302 – 307; *Angew. Chem. Int. Ed.* **2006**, *45*, 296 – 301.
[322] X.-L. Sun, C. L. Stabler, C. S. Cazalis, E. L. Chaikof, *Bioconjugate Chem.* **2006**, *17*, 52 – 57.
[323] B. P. Corgier, A. Laurent, P. Perriat, L. J. Blum, C. A. Marquette, *Angew. Chem.* **2007**, *119*, 4186 – 4188; *Angew. Chem. Int. Ed.* **2007**, *46*, 4108 – 4110.
[324] K. Zhang, M. R. Diehl, D. A. Tirrell, *J. Am. Chem. Soc.* **2005**, *127*, 10136 – 10137.
[325] W. T. S. Huck, *Angew. Chem.* **2007**, *119*, 2810 – 2813; *Angew. Chem. Int. Ed.* **2007**, *46*, 2754 – 2757.
[326] A. del Campo, E. Arzt, *Chem. Rev.* **2008**, *108*, 911 – 945.
[327] S. R. Coyer, A. J. García, E. Delamarche, *Angew. Chem.* **2007**, *119*, 6961 – 6964; *Angew. Chem. Int. Ed.* **2007**, *46*, 6837 – 6840.