Protein Immobilization Strategies for Protein Biochips

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In the past few years, protein biochips have emerged as promising proteomic and diagnostic tools for obtaining information about protein functions and interactions. Important technological innovations have been made. However, considerable development is still required, especially regarding protein immobilization, in order to fully realize the potential of protein biochips. In fact, protein immobilization is the key to the success of microarray technology. Proteins need to be immobilized onto surfaces with high density in order to allow the usage of small amount of sample solution. Nonspecific protein adsorption needs to be avoided or at least minimized in order to improve detection performances. Moreover, full retention of protein conformation and activity is a challenging task to be accomplished. Although a large number of review papers on protein biochips have been published in recent years, few have focused on protein immobilization technology. In this review, current protein immobilization strategies, including physical, covalent, and bioaffinity immobilization for the fabrication of protein biochips, are described. Particular consideration has been given to oriented immobilization, also referred to as site-specific immobilization, which is believed will improve homogeneous surface covering and accessibility of the active site.

1. Introduction

A biochip is defined as a collection of miniaturized spots, also commonly referred to as a microarray, arranged on a solid substrate that permits many tests to be performed at the same time for achieving high-throughput screenings. In the past few years, protein biochips have become a booming field of research, witnessed by the large number of review papers and commentaries published on the subject.[1−16] DNA biochips were first generated in order to simultaneously monitor the expression level of several genes of an organism. However, mRNA expression level and corresponding protein abundance do not always correlate due to post-translational modifications. Protein-based microarrays offer the possibility to simultaneously analyze thousands of macromolecules for a specific property under investigation, allowing the step from genomics to proteomics.[17,18] In principle, enzyme activity analysis can be performed by screening the function of thousands of protein samples in parallel using a microarray. Zhu et al.[19] have proven this possibility creating the first proteome chip to monitor yeast proteins for several biochemical activities. Besides proteomics, a variety of attractive applications have been reported in literature,[20−24] broadening from drug discovery and development to diagnosis, from environmental monitoring to quality control. In particular, protein biochips show great potential in diagnosis by comparing the expression profiles of samples of healthy persons and patients, offering a tool for the reliable, rapid, quantitative, and multiple identifications of biomarkers. Miller et al.[25] prepared a microarray containing specific antibodies for serum profiling of potential prostate cancer biomarkers. Protein abundance from prostate cancer and control serum samples were compared, and five proteins having significantly different concentration levels were identified. Antigen arrays represent a powerful approach for antibody characterization and profiling for diagnostic and prognostic purposes, mainly for autoimmune diseases. The detection of antibodies involved in aberrant self-reactions is crucial in diagnosis and management of patients. Robinson and co-workers[26,27] characterized samples of serum of eight human autoimmune diseases including lupus erythematosus and rheumatoid arthritis, fabricating an antigen array containing 196 distinct biomolecules. Biochip technology has been used to develop allergen chips allowing to monitor the IgE reactivity profiles of patients.[28] The development of a multianalyte analysis system may lead to practical applicability of protein biochips to point-of-care devices, offering the advantages of fast quantification of the analyte concentration in the samples, obtaining quick and reliable responses.

An ideal protein biochip should have a suitable device material, mostly coated with a thin inorganic layer such as a noble metal to facilitate detection of binding events via imaging techniques and, on top, an organic thin film with protein-resistant functionalities (Figure 1). The organic surface needs to be modified with active groups that react with specific moieties of the protein to be immobilized. Proteins should be oriented on the surface in such a way that the binding sites are exposed to the sample solution.

Determining the optimal surface for each application is critical and influences the reliability of protein microarray experiments. A variety of microdevices have been fabricated, mostly based on substrates commonly used for DNA arrays, but the design of protein biochips raises some additional issues due to high sensitivity of the protein conformation to the surrounding environment. Glass slides derivatized with organosilanes are generally considered suitable substrates for immobilizing proteins. Since a microarray consists of a large number of molecules within a very small surface area ($<250\ \mu$m), density of the active surface groups is a key factor permitting the reduction of the amount of sample and influencing the sensitivity of the array.

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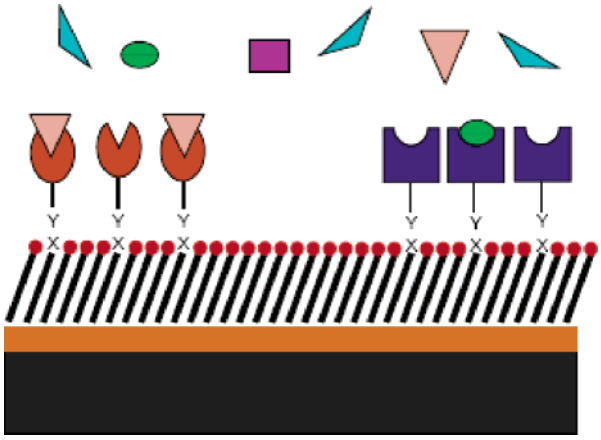


Figure 1. Scheme of an ideal protein array. A device material coated with an inorganic layer, bearing an organic thin film modified with protein-resistant functionalities (●) and different active groups (X, Y), each interacting with a different capture agent, able to bind a desired analyte within a complex protein mixture. Reprinted with permission from ref 3. Copyright 2001 Elsevier.

On flat surfaces, density can be maximized by building ordered monolayers where the immobilized molecules are separated by a minimal distance. Self-assembled monolayers (SAM)[29] are ordered molecular assemblies of different organic materials. SAM of organosilicon are currently used for coating glass slides, enhancing the biocompatibility of the surface and protecting proteins from denaturation during the immobilization step. Dendrimers, branched structures with a wide range of chemical functionalities constructed around a central core, have been applied for surface derivatization in order to create a three-dimensional structure yielding a larger surface area. Polyamidoamine (PAMAM) dendrimers, first proposed by Benters et al.[30] have been used for increasing the binding ability of surfaces.[31−33] Gold-coated substrates are quite widely used for monitoring protein interactions via imaging techniques. The spontaneous adsorption of organosulfur compounds onto gold leads to a highly ordered and densely packed layer, improving the availability of active surface groups. Successful interactions between ω-functionalities of SAM and side groups of proteins rely on the accessibility of the terminal groups, since steric hindrance can limit the rate of the reaction.[34] SAM of thiols on gold seem to be the most promising domain for protein biochips. The main drawback is nonspecific protein adsorption, causing background noise interfering with the signal. On the basis of the well-known protein repulsion capability of poly(ethylene glycol) (PEG), PEG-modified surfaces have been developed in order to avoid nonspecific protein adsorption.[35−38] Recently, a dual-functional biomimetic material based on poly(carboxybetaine methacrylate) (polyCBMA) was proven to be highly resistant to protein adsorption and, at the same time, suitable for immobilization via EDC/NHS chemistry due to the abundance of carboxylic groups.[39] The surface concentration of three different proteins (human fibrinogen, lysozyme, and human chorionic gonadotropin (hCG)) after adsorption was lower than 0.3 ng/cm^2, which is similar as found for a well-packed oligo(ethylene glycol) OEG SAM.

Instead of spotting proteins onto a two-dimensional solid surface, capture molecules can diffuse into a porous structure formed by polymer membranes or hydrogels. Three-dimensional supports show a high capacity of protein immobilization and a homogeneous aqueous environment preventing denaturation. Agarose and aldehyde−agarose have been derivatized with primary amino groups (MANA and DEAE−agarose) and poly(ethylenimine) (PEI) to improve immobilization performances.[40,41] Hydrogels represent hydrophilic matrices through which the biomolecules can diffuse, showing a 100-fold superior capacity of immobilization compared to that of planar surfaces. However, three-dimensional supports are plagued by problems relating to mass transport effects and high background signals, resulting in calculation of false kinetic rate constants during real-time protein−protein interaction measurements.

Microfluidic devices have evolved rapidly over the past decade, because they show great promise due to the small amount of sample required and the possibility of parallelization and high-throughput screening. Such devices are more complex than conventional microarrays, involving significant downscaling challenges. Many microfluidic systems are based on polymeric materials, like polycarbonate (PC), poly(methyl methacrylate) (PMMA), and poly(dimethylsiloxane) (PDMS) in which surface modifications are required since they do not contain any functional group in their native form.[42]

Despite that a number of substrates and surface modification methods have been proposed in the past several years, the best method has not yet been defined. The major aim of a microarray is to provide efficient binding between immobilized probes and ligands in a sample; therefore, the assay conditions and the detection performances need to be optimized. A suitable detection strategy should offer high throughput, high signal-to-noise ratio, good resolution, reproducible results, and high sensitivity and specificity. The optimal detection method meeting such criteria is still under consideration, but many label-free real-time detection methods for protein−protein interactions have been reviewed.[43] Surface plasmon resonance (SPR) has been highlighted as a leading technology for the study of real-time protein−protein interaction kinetics, providing feasibility for multichannel biosensing.[44−46]

Protein biochips have been considered to be the logical extension of DNA biochips, but present many different and more challenging issues, mainly due to the more complex chemistry. Moreover, proteins are often unstable and prone to denaturation, generating concerns about maintaining the native conformation. Thus, one of the most challenging tasks for protein biochip technology is immobilizing proteins onto surfaces in such a way that their three-dimensional structure, functionality, and binding sites are retained. Although a large number of papers on protein biochips have been published in recent years,[1−15] few have focused on protein immobilization technology. In this review, current protein immobilization strategies, which can be classified into three categories, i.e., physical, covalent, and bioaffinity immobilization, are described. Though immobilization of membrane proteins has been recently reported, the main focus of this review paper will be on water-soluble proteins.

2. Protein Immobilization

Immobilization can be defined as the attachment of molecules to a surface resulting in reduction or loss of mobility. The way in which proteins are immobilized will determine the properties of the microarray. In some cases, immobilization may lead to partial or complete loss of protein activity, due to random orientation and structural deformation. In order to fully retain biological activity, proteins should be attached onto surfaces without affecting conformation and function. Generally, the choice of a suitable immobilization strategy is determined by the physicochemical and chemical properties of both surface and protein. Many immobilization techniques have been developed in the past years, which are mainly based on the following three mechanisms: physical, covalent, and bioaffinity immobilization. The question of which strategy can be considered

as the best one is still open, but it is unlikely that one scheme will be optimal for all the protein families. Therefore, the extrapolation of immobilization strategies from one system to another for different classes of proteins is difficult and mostly unsuccessful due to the wide subset of characteristics and functional properties of proteins. Hence, several unsolved challenges are involved in immobilization techniques.

2.1. Physical Immobilization. Proteins can adsorb on surfaces via intermolecular forces, mainly ionic bonds and hydrophobic and polar interactions. Which intermolecular forces exactly take part in the interaction will depend on the particular protein and surface involved. The resulting layer is likely to be heterogeneous and randomly oriented, since each molecule can form many contacts in different orientations for minimizing repulsive interactions with the substrate and previously adsorbed proteins.

The adsorption capacity of flat slices is limited by the geometric size of the immobilized proteins. High-density packing may sterically block active sites of proteins, interfering with functional properties. Surface loading is increased by constructing SAM that generally interact with proteins through hydrophobic or electrostatic low-energy interactions.

Adsorption mainly occurs with three-dimensional porous materials, like gel-pads. Polypropylene (PP) membranes modified with polyaniline (PANI) show an adhesion mechanism of combined electrostatic and hydrophobic interactions and demonstrate high affinity and compatibility toward different proteins.[41] The most used polymer substrates in protein biochip technology are hydrogels on gold, mainly due to the widespread success of the Biacore SPR technology. Among hydrogels, sulfate-modified dextrans were giving better performances than commercial carboxymethyl cellulose (CMC) when used to adsorb proteins contained in a crude extract of *E. coli*.[47] In comparison to aspartic-modified dextrans, sulfate-modified dextrans showed a higher density of surface charged groups, which leads to an improved adsorption capacity.

Drawbacks of the adsorption mechanism are random orientation and weak attachment, since proteins may be removed by some buffers or detergents when performing the assays. Moreover, problems relating to mass transport effects and high background signals emanating from nonspecific interactions can result in false calculation of kinetic rate constants during real-time SPR measurements.

2.2. Covalent Immobilization. More often proteins are covalently bound to the immobilization support through accessible functional groups of exposed amino acids. Covalent bonds are mostly formed between side-chain-exposed functional groups of proteins with suitably modified supports, resulting in an irreversible binding and producing a high surface coverage. The functional groups on the support are generated by chemical treatment, and several pretreated surfaces are already commercially available. Table 1 shows the functional groups potentially available in proteins for immobilization and the functionalities of the required surfaces.

Chemical binding via side chains of amino acids is often random, since it is based upon residues typically present on the exterior of the protein. Therefore, the attachment may occur simultaneously through many residues, enhancing heterogeneity in the population of immobilized proteins. Chemical attachment can also be guided in an orderly manner to attain oriented immobilization.[48] Well-defined immobilization procedures provide reproducible and oriented immobilization, avoiding denaturation, due to a reaction with amino acid groups not involved in activity or binding. Site-specific immobilization requires

Table 1. Commonly Available Functional Groups in Proteins and Functionalities of the Required Surfaces

side groups	amino acids	surfaces
$-NH_2$	Lys, hydroxyl-Lys	carboxylic acid active ester (NHS) epoxy aldehyde
−SH	Cys	maleimide pyridyil disulfide vinyl sulfone
−COOH	Asp, Glu	amine
−OH	Ser, Thr	epoxy

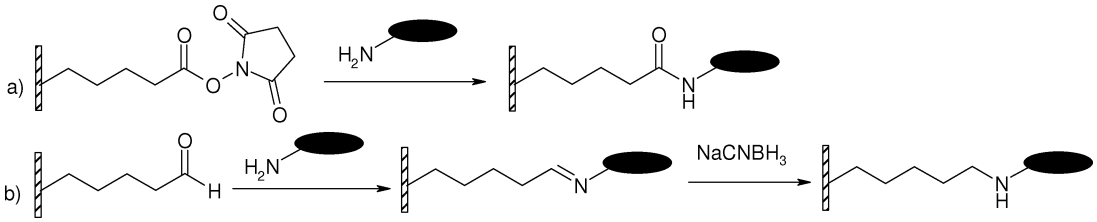
functionalization of the molecules or tailoring of the surface, or both. In some cases, oriented immobilization can be obtained if the protein possesses a single reactive amino acid in the structure. Theoretically, site-specific immobilization should be optimal, overcoming the insufficient exposure of functional domains due to unpredictable orientation. Hence, proteins can be attached to surfaces in a well-ordered manner, allowing reproducibility and conformational stability.

2.2.1. Nonspecific Immobilization. In principle, all of the functional groups of amino acids summarized in Table 1 can be used for direct chemical coupling with suitable types of derivatized surfaces via well-known bioconjugate techniques.[49]

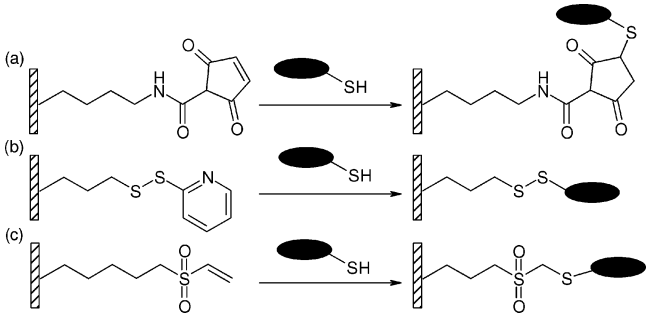
2.2.1.1. Amine Chemistry. Lysine residues are the most commonly utilized anchoring points since they are typically present on the exterior of the protein. Unfortunately, their abundance can create multipoint attachment on the support, increasing heterogeneity and restricting conformational flexibility. Scheme 1 illustrates the use of amine chemistry. *N*-Hydroxysuccinimide (NHS) is the most commonly used agent for coupling with amine groups, forming stable amide bonds (Scheme 1a).[33,34,50−54] Proteins need to be dissolved in a low-ionic-strength buffer and passed over the activated surface. NHS esters then react with nucleophilic groups of the proteins, creating a strong amide bond. Immobilization efficiency depends on a number of parameters, i.e., pH, concentration, ionic strength, and reaction time, but the conditions need to be optimized for each type of protein. The use of NHS chemistry on SAM has been reported by Patel et al.,[34] who assessed the effect of the accessibility of terminal carboxylated groups of SAM on the reaction with NHS and the immobilization of the protein catalase. The reactivity varied among homogeneous SAM of 11-mercaptoundecanoic acid (11-MUA) and 3-mercaptopropanoic acid (3-MPA) and a mixed SAM of 11-MUA/3-MPA. The immobilization efficiency decreased in the order mixed > 11-MUA > 3-MPA, due to the ordered arrangement of the homogeneous SAM sterically hindering the interaction with proteins. With increased disorder, the terminal carboxylated groups are more accessible for immobilization.

The interaction between amine and aldehyde groups leads to the formation of a labile Schiff's base that can be stabilized by reduction creating a stable secondary amine linkage (Scheme 1b).[55−57] The first microarray successfully and stably immobilizing proteins via an NH_2-terminus onto aldehyde-derivatized glass slides was reported by MacBeath and Schreiber.[58] Proteins dissolved in phosphate buffer were attached to the surface, and slides were subsequently immersed in bovine serum albumin (BSA)-containing buffer in order to prevent nonspecific binding. This immobilization method has proven to be appropriate for big proteins, since small ones can be obscured by BSA. Aldehyde−amino chemistry has been extensively used for years for protein immobilization on different surfaces.[59−61] Aldehyde

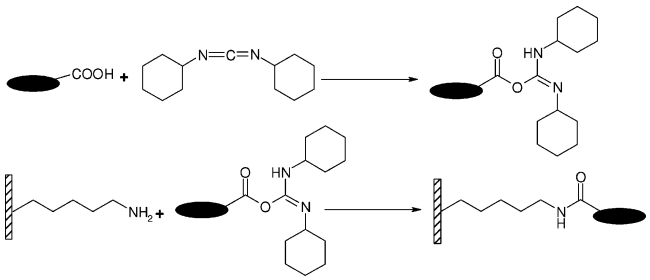
Scheme 1. Amine Chemistry on (a) NHS-Derivatized and (b) Aldehyde-Derivatized Surfaces



Scheme 2. Thiol Chemistry on (a) Maleimide-Derivatized, (b) Disulfide-Derivatized, and (c) Vinyl Sulfone-Derivatized Surfaces



Scheme 3. Carboxyl Chemistry Using Carbodiimide Activation



derivatization has been also performed on SAM and successfully used to prepare patterned collagen-type protein col3a1 surfaces to study cell adhesion.[62]

2.2.1.2. Thiol Chemistry. Cysteine contains a unique group, the thiol group, able to create internal disulfide bonds contributing to the stability of the protein. Since cysteines are not as abundant as lysines, random immobilization is less likely to occur. Scheme 2 shows the main coupling approaches involving thiol side groups of proteins.

The maleimide double bond undergoes an addition reaction with thiol groups to form stable thioether bonds (Scheme 2a).[63−66] The reaction is specific at a pH range of 6.5−7.5, while at higher pH values some cross-reactivity with $-NH_2$ has been observed. Maleimide reacts rapidly, but it undergoes slow hydrolysis at aqueous conditions, which may give problems in protein handling. Both homobifunctional (bis(maleimidohexane) (BMH)) and heterobifunctional (*N*-(ϵ-maleimidocaproyl)succinimide (EMCS) and sulfosuccinimidyl 4[*p*-maleimidophenyl] butyrate (sulfo-SMPB)) linkers have been developed. Disulfide reagents participate in disulfide exchange reactions with another thiol leading to the formation of a new mixed disulfide (Scheme 2b).[67,68] Reversibility of the linkage by exposure to reducing reagents can become a problem in attempting stable immobilization. Pyridyil disulfides are commonly used since they contain a leaving group which is easily transformed into a nonreactive compound. Unfortunately, these reagents are relatively insoluble in aqueous buffers, requiring the reaction to be performed in an aqueous/organic mixture. Vinyl sulfone reacts with −SH groups by a conjugate addition reaction, also known as Michael addition (Scheme 2c). It has shown to be suitable for selective modification of protein cysteine groups under mild and physiological conditions.[69] Reaction with thiol groups is effective within a pH range of 7−9.5, while reaction with amine occurs generally above pH 9, but at a slower rate. The reaction yield is almost quantitative. Thiol selectivity and water stability are the main advantages of this reagent, which has been extensively used for pegylation of proteins using vinyl sulfone−PEG derivatives.[70,71] Morpurgo et al.[71] showed that vinyl sulfone−PEG can provide selective attachment to cysteine residues of ribonuclease (RNase). The reaction was rapid and selective at pH 7.9; in contrast, reaction with lysine groups was slow and uncompleted and occurred only at pH 9.3. Reactivity and selectivity can be controlled by pH and the presence of charged amino acids in proximity of cysteine residues, which modulates the pK_a of the thiol groups. Since deprotonated thiols, rather than thiols, are the reactive species in the Michael addition reaction, the kinetics are influenced by the electrostatic environment of the thiol groups.[72]

2.2.1.3. Carboxyl Chemistry. Immobilization via carboxyl groups may be an interesting opportunity since aspartic and glutamic acid usually constitute the major fraction of surface groups on proteins. Thus, instead of adsorbing proteins via multipoint interactions onto aminated slides, mild coupling methods may be used to attain covalent immobilization (e.g., carbodiimide activation in Scheme 3).

An effective strategy to covalently couple proteins via carboxyl groups using carbodiimide (CDI) for activating −COOH was proposed by Fernandez-Lafuente et al.[73] using β-galactosidase from *Aspergillus orizae* as a model protein. Innovative MANA−agarose gels containing very low p*K* primary amine groups were prepared and utilized for adsorbing the enzyme onto the support, in a low-ionic-strength medium. After addition of CDI, ionic adsorption was transformed to covalent attachment. These low-p*K* aminated supports allow covalent immobilization using low concentrations of CDI (1−10 mM). This is advantageous because it has been shown previously that a high concentration of CDI dramatically decreases enzymatic activity.[74]

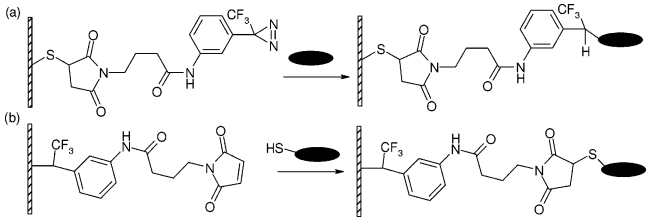
2.2.1.4. Epoxy Chemistry. Epoxy chemistry seems to be an appropriate system for developing easy protocols, due to its stability at neutral pH values, wet conditions, and reactivity with several nucleophilic groups to form strong bonds with minimal chemical modification of the protein. Though covalent reactions between proteins and epoxy supports are extremely slow, previously adsorbed proteins were shown to react at high rate with nearby epoxy groups in the same support.[75] Therefore, a two-step mechanism has been hypothesized, which consists of fast adsorption followed by intramolecular chemical attachment, promoted by the higher "apparent" concentration of epoxy groups on the surface. Commercially available epoxy supports unfortunately reveal several experimental limitations. Epoxy−agarose shows negligible immobilization both at high and at low ionic strength conditions, due to the lack of a hydrophobic core for the adsorption step. Epoxy−Sephabeads and epoxy−EupergitC promote satisfactory immobilization only at high ionic strength, and not all the proteins can be exposed to such harsh

conditions. Multifunctional supports with two moieties, one containing groups able to promote physical adsorption and the other having epoxy groups in sufficient amount to enable covalent immobilization, have been designed to improve the efficiency of immobilization and stability of the immobilized proteins.[76,77] Epoxy−amino group supports presenting a layer of ethylenediamine promote physical adsorption via amine groups, and then covalent linkage via epoxy groups. Lipase from *C. rugosa* and β-galactosidase from *A. oryzae* were immobilized both on conventional epoxy supports and amino-modified support, the latter demonstrating a faster immobilization rate. Moreover, this new support increased the half-life of immobilized glutaryl acilase, invertase, and β-galactosidase from *Thermus* sp. by 3−5 times. Thiol-modified epoxy supports have also been proposed in order to immobilize proteins via thiol−sulfide exchange followed by covalent immobilization via epoxy chemistry.[76]

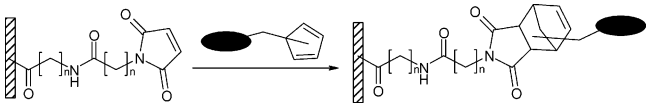
2.2.1.5. Photoactive Chemistry. Photoimmobilization demands the presence of mediating photosensitive reagents, generally activated by incident light of an appropriate wavelength. After light activation, the reagents undergo distinct chemical processes that finally lead to the formation of covalent bonds between the photogenerated intermediates and the biomolecules. Photoimmobilization offers several advantages for surface immobilization purposes, as highlighted in a few review papers.[78,79] The photoreaction is a single-step reaction, which is efficient and fast and does not require functionalization of the target molecules. Therefore, it can be utilized for immobilizing biomolecules lacking active functional groups. The reaction can be carried out at mild conditions, independent of pH and temperature. Biological compatibility is perhaps the most important advantage of this technique, since the commonly used photoreagents, i.e., arylazides, diazirines, benzophenones, and nitrobenziles, are activated by irradiation at wavelengths $\geq$350 nm for which most part of biomolecules are transparent. Arylazide is activated via photolysis resulting in 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 (PFTA), since the substitution with fluorine on aromatic rings decreases the rate of ring expansion reactions. Diazirines generate reactive carbenes. Nitrobenzyl chemistry, often called caging chemistry, involves the attachment of a labile chemical group which will be broken down upon UV irradiation generating a ketone, carbon dioxide, and the freed reactive group. Benzophenones are precursors of ketyl radicals, which easily react with covalent bonds.

Photolinker-mediated chemistry is based on the use of heterobifunctional photolinkers having one photoreactive group and one chemical reactive group. Such linkers can react via irradiation to the surface and chemically to the protein or vice versa. Horseradish peroxidase (HRP) was immobilized on films modified via PFTA chemistry with heterobifunctional crosslinkers containing NHS−ester.[52] The reaction was carried out at pH 8.2 to prevent NHS hydrolysis and to maintain the reactive amine unprotonated. Another heterobifunctional linker combining photoreactivity with thiol selectivity has been designed by Collioud et al.[80] The reagent *N*-[*m*-[3-(trifluoromethyl)-diazirin-3-yl]phenyl]-4-maleimido butyramide (MAD) can perform a two-way chemistry: chemical coupling to thiolated surfaces followed by photochemical reaction to biomolecules and/or photochemical coupling to an inert surface followed by

Scheme 4. Photoactive and Covalent Chemistry of Mad Linker



Scheme 5. Immobilization of Diene-Functionalized Protein onto Maleimide-Modified Slides



covalent reaction to thiol groups of biomolecules (Scheme 4).

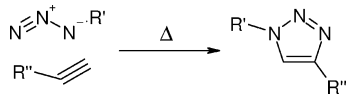
1-Fluoro-2-nitro-4-azidobenzene (FNAB)[81] has been used for the activation of polystyrene surfaces, generating a fluorinated surface reactive to amino residues. HRP was loaded onto FNAB-treated supports, and a 3-fold increase of immobilization efficiency was observed compared to that of untreated supports. Photoreagents can also be used to develop hydrophilic organosilane films resistant to protein adsorption.[82] 2-Nitro-5-[11-(trimethoxysilyl)undecyl]oxybenzyl-methoxy PEG (NMPEG-silane) films produce surfaces which reject proteins prior to irradiation and allow site-specific protein immobilization after irradiation. The solvent used for deposition has been proven to be a key parameter in determining the amount of nonspecific binding and covalent attachment.

2.2.2. Site-Specific Immobilization. In order to ensure that all the biomolecules are oriented in such a way that their binding sites are exposed to the sample solution, uniform alignment onto the surface should be achieved. Different site-specific immobilization techniques have therefore been developed in the past few years. Such approaches often require functionalization of the target molecules or derivatization of the surface or both.

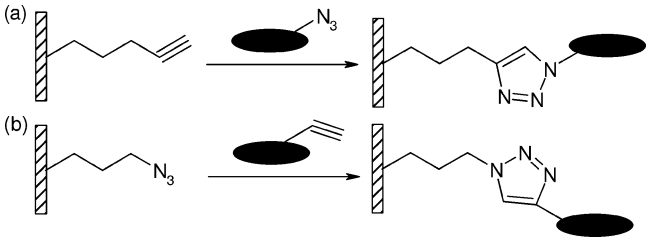
2.2.2.1. Diels−Alder Cycloaddition. Diels−Alder cycloaddition usually takes place between an electronically matched pair of a dienophile and a conjugate diene to form a six-membered unsaturated ring. In Scheme 5, the dienophile is the maleimide-modified surface and the diene is ligated to the protein to be immobilized. Diels−Alder-mediated immobilization has been used as a straightforward method for the preparation of a peptide chip[83] with the kinase substrate AcIYGEFKKKC−NH_2 immobilized onto a SAM on gold to be used for the characterization of enzymatic activity. First the peptide of interest was conjugated to a cyclopentadiene linker during solid-phase synthesis. Then, the Diels−Alder reaction with a benzoquinone-modified SAM took place, resulting in rapid and site-selective immobilization. Since Diels−Alder reactions 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.[84] Streptavidin was chosen as a model protein and was first ligated to a cyclopentadiene derivative. Diene-modified streptavidin and unmodified control were dissolved in pH 6 double-distilled water and spotted onto the maleimide slide. Slides were then treated with labeled biotin and no signal was recorded for the negative control proving that the immobilization is due to Diels−Alder ligation.

2.2.2.2. "Click" Chemistry. 1,3-Dipolar cycloaddition of an azide and an alkyne to form 1,2,3-triazole has been called "click" chemistry because it requires only simple workup and purification steps, rapidly creating new products (Scheme 6).[85] Since

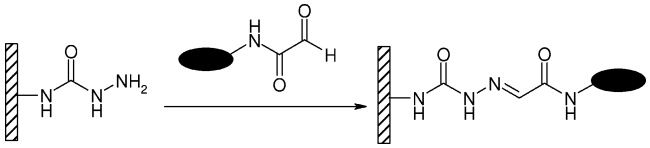
Scheme 6. Product of 1,3-Dipolar Cycloaddition



Scheme 7. (a) Immobilization of Azide-Functionalized Protein onto an Alkyne-Modified Slide and (b) Immobilization of Alkyne-Functionalized Protein onto an Azide-Modified Slide



Scheme 8. α-Oxo Semicarbazone Immobilization Chemistry



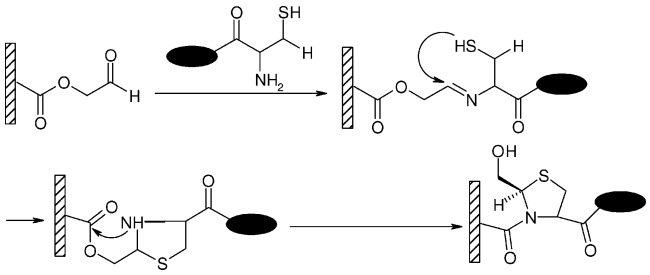
the introduction of azides and alkynes into macromolecules is easy and does not affect their stability and because triazole formation is irreversible and quantitative, azide/alkyne "click" chemistry is expected to provide a useful strategy for uniform, high-density surface immobilization of biomolecules in a covalent selective fashion. Duckworth et al.[86] performed the immobilization of azide-modified farnesyl transferase onto alkyne-derivatized agarose beads via overnight cycloaddition at room temperature.

Preparation of acetylene-terminated (Scheme 7a) as well as azide-modified (Scheme 7b) surfaces has been reported by different research groups.[86−88]

In situ preparation of azide-terminated monolayers has been performed for creating an ideal surface for immobilization, meeting the ordered structure of SAM with the specificity of "click" chemistry. Sun et al.[89] described a method to combine 1,3-dipolar cycloaddition with a Diels−Alder reaction. *N*-(ϵ-Maleimidocaproyl) (EMC)-derivatized glass slides were prepared and coupled via a Diels−Alder reaction to the cyclodiene 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 side products.

2.2.2.3. α*-Oxo Semicarbazone Ligation.* The fabrication of peptide microarrays using α-oxo semicarbazone ligation was reported by Olivier and co-workers (Scheme 8).[90−92] The preparation of semicarbazide slides was performed either via silanization and reaction with Fmoc−$NHNH_2$ or via direct silanization of the glass slide with semicarbazide silane. A glyoxyl group was inserted in a peptide-modified biotin via solid-phase synthesis, and the slides were incubated with streptavidin to test the specificity of interaction. The reaction between the semicarbazide slide and the aldehyde group led to the formation of a stable semicarbazone bond. A comparative study with the widely used aldehyde slides revealed a gain in sensitivity of about 18 fold. This method has proven to be applicable also to antibody immobilization.[90] Oxidized donkey IgG was immobilized using the same printing, washing, and incubation conditions used for the peptide arrays, proving the possibility of fabricating mixed peptide−protein microarrays.

Scheme 9. Peptide Ligation between N-Terminal Cysteine and Ester Glycoaldehyde to Form a Thiazolidine Ring



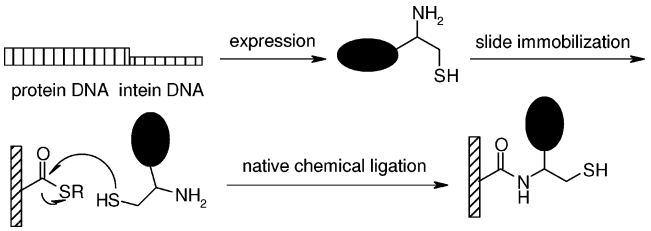
More recently, the same group[93] reported the preparation of semicarbazide-functionalized silicate nanoparticles for α-oxo semicarbazone ligation of peptides. First, a semicarbazide coupling reagent was reacted to the surfaces; then grafting of the peptides bearing a −COCHO functionality was performed in acetate buffer pH 5.5 at room temperature. The influence of chemical treatments on size, morphology, and porosity of the nanoparticles were controlled at each step of the preparation procedure.

2.2.2.4. Peptide Ligation. Peptide ligation[94] is a specific method to chemically couple unprotected peptides or proteins, via a variable chemoselective capture step followed by an intramolecular acyl transfer reaction. The chemoselective capture requires a nucleophile or electrophile proximally placed at the N-terminus segment and another compatible electrophile or nucleophile also proximally located at the C-terminus. The chemoselective capture of the nucleophile and electrophile pair brings the N- and C-termini into such a close proximity to permit an intramolecular acyl transfer reaction forming an amide bond. N-Terminal cysteine, serine, histidine, and threonine, containing weak-base nucleophiles such as thiol, amine, or hydroxyl groups spatially separated by two atoms from the α-amine, have been found to be the most suitable. For the C-terminus, an ester or a thioester is required. The ligation between N-terminal cysteine and ester glycoaldehyde leads to the formation of a thiazolidine ring, followed by an acyl migration to form a proline mimic (Scheme 9).

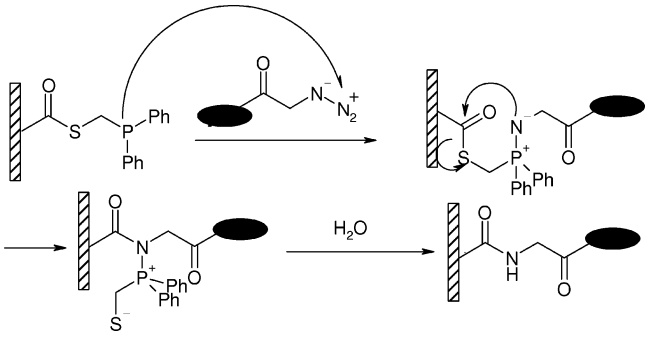
The first example of chemoselective ligation applied to immobilization on surfaces has been reported by Falsey et al.[95] The immobilization occurred via formation of a thiazolidine ring between glyoxyl-derivatized glass slides and N-terminal cysteine of biotin and peptide ligands. After chemical ligation, the microarrays were subjected to fluorescence detection of protein binding and cell adhesion, proving the suitability of this system for functional properties and cell-signaling analysis. Another approach based on native chemical ligation has been proposed by Lesaicherre et al.[96] Thioester-containing slides were prepared and spotted with cysteine-modified Janus tyrosine kinase (JAK) peptide substrate. After subsequent incubation with the corresponding kinase, the interaction was successfully detected by fluorescence. To evaluate the suitability of such slides for protein microarrays, cysteine-containing fluorescein was dissolved in PBS buffer at pH 7.4 and effectively spotted onto the slides. A more recent work of the same research group[97] combined intein-mediated protein splicing and peptide ligation for achieving site-specific immobilization (Scheme 10). N-Terminal cysteine-containing enhanced green fluorescent protein (EGFP) was spotted onto thioester-modified slides. Successful immobilization was confirmed by incubation with an anti-EGFP antibody. The native fluorescence of EGFP confirms the proper folding after immobilization.

The suitability of peptide ligation in creating stable and oriented protein microarrays has been explored in recent years.

Scheme 10. Protein Immobilization Using a Native Chemical Ligation Reaction of N-Terminal Cysteine-Containing Proteins Expressed by Intein-Mediated Protein Splicing



Scheme 11. Staudinger Ligation



A combination of SPOT synthesis and peptide ligation has been proposed by Toepert et al.[98] for the creation of arrays of WW protein domains. WW domain, whose name is derived from two conserved tryptophan residues, is known to be involved in protein−protein interactions of numerous degenerative diseases. A library of different WW domains containing N-terminal cysteines was produced by automated SPOT synthesis. Ligation was achieved by dissolving the protein domains in phosphate buffer at pH 7.5, followed by incubation with a peptide-modified cellulose slide. Ligation proceeded in 2 h with good yield; no starting material could be detected after 16 h.

The approach of Camarero et al.[99] combined expressed protein ligation with the formation of a stable peptide bond to create a protein array. Proteins α-thioester were generated via recombinant engineering and were covalently attached through their C-termini to a N-terminal cysteine PEG-modified glass surface. Two fluorescent proteins, DsRed and EGFP, were used as models to test whether the native structure is kept intact during engineering and attachment, since the fluorescence can be observed only with tertiary and quaternary folding. As a control, a solution of EGFP with no α-thioester function was also spotted on the same slide. The results showed that only specific peptide ligation is the responsible mechanism for the immobilization.

2.2.2.5. Staudinger Ligation. Staudinger ligation[100] can be considered as a peptide ligation method requiring an azide group and a functionalized phosphine-containing (thio)ester. The mechanism involves the formation of an iminophosphorane intermediate, followed by nucleophilic attack of the iminophosphorane nitrogen on the thioester via a five-membered ring leading to an aminophosphonium salt, hydrolyzed to produce the desired coupling (Scheme 11). The first application of Staudinger ligation to couple proteins to a solid support has been proposed by Soellner et al.[101] Glass slides were first amino-derivatized, then treated with an excess of PEG having succinimidyl ester termini (NHS−C(O)−PEG−C(O)−NHS) and diphenylphosphinomethanethiol, in order to produce a surface-bound phosphinothioester. Azido-modified ribonuclease S′ (RNase S′) was chosen as a model protein and spotted onto the slide, thoroughly washed with phosphate buffer (pH 7.2). Control experiments indicated that binding occurs only via Staudinger ligation. Recently, *N*-azide-modified ras protein has been immobilized via Staudinger ligation onto glass slides derivatized by PAMAM dendrimers to allow maximum surface covering.[102] An aminocaproic linker was introduced to generate distance between the phosphane groups and the surface. Azide-modified ras proteins were spotted onto the phosphane-modified substrates at pH 7.4−7.6. Clear and reproducible fluorescent signals were recorded after an immobilization time of 4 h with minimum protein concentration of 50 μM. Unfortunately, the data could not provide a formal proof of the orientation of the proteins on the surface. However, there is evidence that the immobilization reaction is regioselective. Moreover, Staudinger ligation proceeds under mild conditions, in aqueous solution, almost quantitatively, and without noticeable formation of any side-chain products.

2.3. Bioaffinity Immobilization. Biochemical affinity reactions offer a gentle oriented immobilization of proteins, providing an important advantage over other immobilization techniques. Moreover, not only oriented and homogeneous attachment is obtained, but it is also possible to detach proteins and make repeated use of the same surface.

2.3.1. Avidin−Biotin System. The use of avidin−biotin technology in generating biocompatible surfaces has been reviewed in great detail.[103] The approach exploits one of the strongest noncovalent bonds ($K_d = 10^{15}$ M^{-1}) ever known allowing the use of harsh conditions during biochemical assays. The specificity of the interaction permits uniformly oriented protein immobilization.

The main characteristics of the binding couple avidin−biotin can be found in the book "Avidin−Biotin Chemistry: A Handbook".[104] Avidin is a tetrameric glycoprotein soluble in aqueous solutions and stable over wide pH and temperature ranges. It can bind up to four molecules of biotin. The bond formation is very rapid and unaffected by pH, temperature, organic solvents, enzymatic proteolysis, and other denaturing agents. Streptavidin is a closely related tetrameric protein, with similar affinity to biotin, but differing in other aspects, such as molecular weight, amino acid composition, and pI. The properties of avidin and streptavidin have been improved using chemical and recombinant methods providing enhanced stability and/or controlled biotin binding. For example, NeutrAvidin displays highly specific binding to biotin, whereas NitrAvidin gives a pH-dependent biotin binding with strong interaction at acidic conditions (pH 4−5) and dissociation at higher pH.

Biotin or vitamin H is a naturally occurring vitamin found in all living cells. Only the bicyclic ring is required to be intact for the interaction with avidin; the carboxyl group on the valeric acid side chain is not involved and can be modified to generate biotinylation reagents used for conjugation with proteins. Since biotin is a small molecule, its conjugation to macromolecules does not affect conformation, size, or functionality. Biotinylation reagents can be classified depending on their reactivity toward diverse functional groups. The NHS ester of biotin is the most commonly used biotinylation reagent to target amine groups,[105] whereas biotin hydrazide can be used to target either carbohydrates or carboxyl groups.[106]

Both biotin and avidin/streptavidin may be attached to a variety of substrates. Direct immobilization of avidin occurs either via adsorption or covalent coupling. It has been reported that covalent attachment of NeutrAvidin onto carboxyl-derivatized polymers produces a higher coating density than immobilization via a biotinylated interlayer.[107] However, coupling via carbodiimide chemistry may affect its binding activity. A typical biotin/avidin/biotin multilayer is composed by biotin

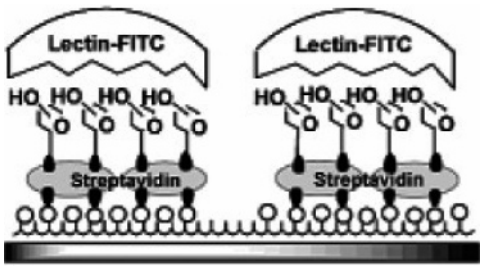
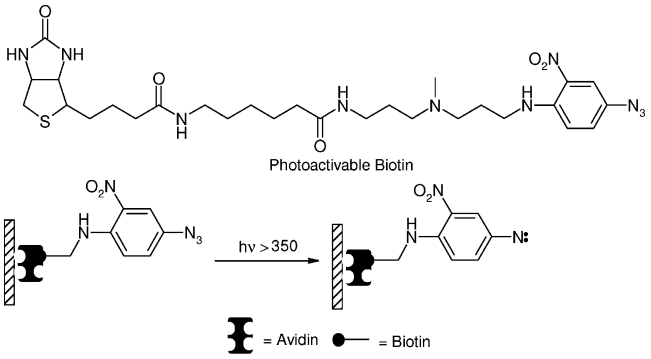


Figure 2. Glycopolymer-derivatized surface via a biotin−streptavidin−biotin layer (ref 108).

Scheme 12. Immobilization of Photobiotin Followed by Exposure to Light in Order to Form the Nitrene Group for Reaction with Alkyl Residues of Proteins



directly immobilized and avidin creating a secondary layer for binding biotinylated molecules (Figure 2). This approach is generally preferred due to the higher organization obtained in comparison to that of the direct immobilization of avidin. For instance, the biotin layer promotes an ordered avidin overstructure with two biotin-binding sites facing the surface and the other two facing outward. A glycopolymer-derivatized surface over the biotin−streptavidin−biotin layer has been created for surface glycoengineering.[108]

For attachment of biotin or biotinylated molecules, surfaces need to first be activated. Silanized glass fibers can be treated with acrylamide or 4-aminophenylmercuric acetate (APMA) to generate free amino groups reacting with NHS−biotin for attaining surface biotinylation.[105] Specific interactions have been shown between a SAM of biotinylated thiols and streptavidin, suggesting the possibility of combining a well-ordered SAM structure with the avidin−biotin system.[109] Studies with different SAM showed very low binding of avidin for the close-packed layers and significantly higher binding for the more loosely packed ones. A good control over the surface density of biotin groups is then extremely important, and it can be obtained using mixed SAM monolayers composed of two thiol species: one biotinylated and one not. The same conditions seem to be favorable for the construction of layers of biotinylated PEG.

Protein patterning can be performed using biotin photochemistry, divided into two different techniques: photoactivable and photocleavable chemistry. Photobiotin, composed of a biotin group, spacer arm, and an aryl azide moiety, has been used by Pritchard et al.[110] for immobilizing rabbit IgG and rat IgG (Scheme 12). Gold slides were first modified with a thiol monolayer onto which avidin was covalently bound and then incubated in a solution of photobiotin. Selected areas of the surface were exposed to light, resulting in the activation of the photobiotin. The so-formed aryl nitrene can reacted with alkyl residues of proteins in the sample. Photocleavable biotin, also called caged-biotin, is an analogue in which the nitrogen atom of the imidazole ring is acylated with a photolabile group, blocking biotin from the binding with avidin.[111−113] Upon UV exposure, the photolabile group is cleaved, allowing interaction with avidin. Photocleavable chemistry is based upon photolabile groups, such as nitroveratryloxycarbonyl (NVOC), shown in Scheme 13. A recent application of caged-biotin for patterning of proteins was reported by Kim et al.[114] Biotin was covalently coupled to a hydroquinone moiety, electrochemically oxidized to be converted into a benzoquinone cation, and subsequently broken down in water by nucleophilic acyl substitution releasing active biotin.

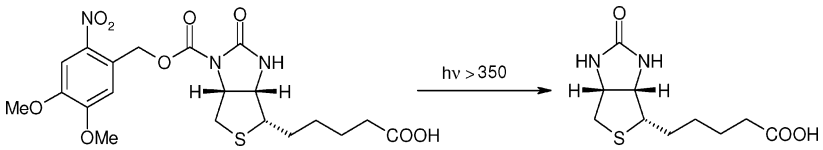
The avidin/biotin system offers numerous advantages: widespread availability of biotinylated proteins, separate irradiation and immobilization steps, avoiding direct exposure of proteins to UV light that might damage them, and the possibility of controlling surface density of binding sites by carrying out a partial photolysis step.

2.3.2. His-Tag System. For providing a handle to allow site-specific immobilization, recombinant proteins produced by genetic engineering can be expressed with affinity tags, whose applications have been highlighted in several research papers.[115−117] Tags are placed at defined positions on proteins, preferably sufficiently far away from the active site in order to achieve optimal accessibility of the ligands. Poly(His) is the most popular tag due to the advantages of small size, compatibility with organic solvents, low immunogenicity, and effective purification under native and denaturing conditions. Proteins with a $(His)_6$ tag at the C- or N-terminus can be immobilized via a nickel-chelated complex, like Ni−nitriloacetic acid (NTA). NTA is covalently bound to the surface and then loaded with a divalent metal cation, usually Ni^{2+}.

The specific chelating interaction between NTA and His-tagged proteins involves the octahedral coordination of the nickel ion with two valences occupied by two imidazole groups from the His-tag and four ligands donated by the NTA molecule (Figure 3). Different strategies have been developed for the fabrication of NTA microarrays. NTA can be simply covalently bound via EDC−NHS on carboxy−dextran surfaces or via maleimide chemistry on silanized glass slides.[115,118] More stable binding due to multipoint attachment has been noticed on surfaces densely covered with NTA. A high density of metal chelator at the surface has been achieved via multivalent chelator heads (MCH) enabling stable and stoichiometric immobilization and full retention of activity.[119] Scheme 14 shows the chemical structure of MHC immobilized on the surface with either one (Scheme 14a) or three (Scheme 14b) NTA areas.

Coupling MCH onto glass surfaces modified with PEG polymer provides reduction of nonspecific binding.[120] The combination of stability, robustness, and a broad range of applications of SAM on gold with MCH has been presented as a suitable surface for protein chip applications.[121] Gold surfaces

Scheme 13. NVOC Caged-Biotin Activation by Light



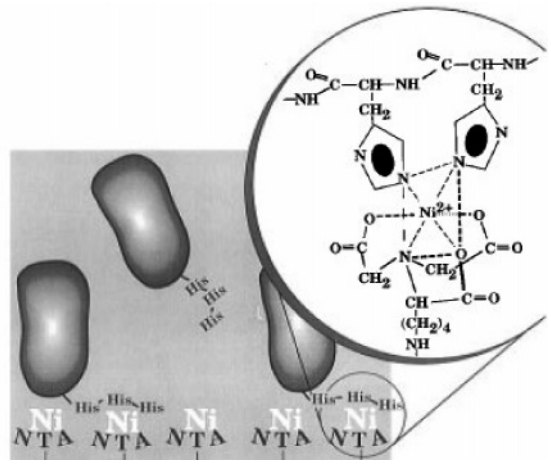


Figure 3. Schematic representation of the binding of His-tagged proteins on a Ni−NTA surface (ref 115).

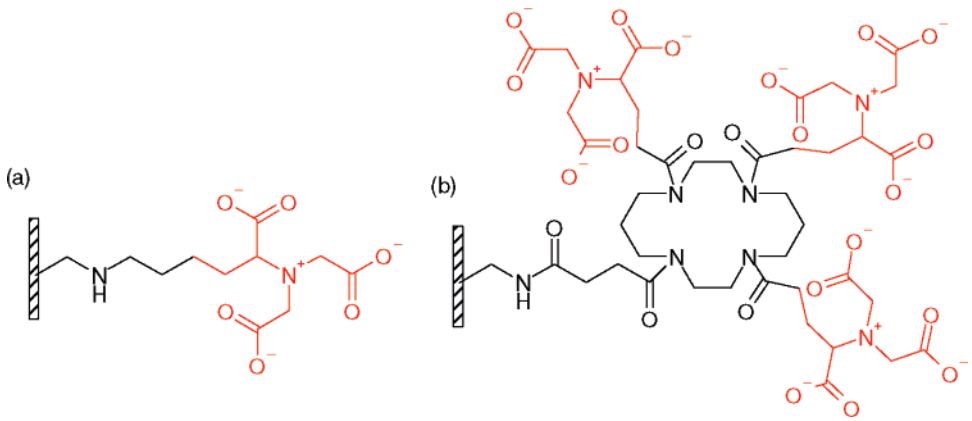
have also been derivatized with mixed SAM,[122,123] one terminated with NTA and the other one with ethylene glycol (EG), leading to specific binding of His-tagged proteins and negligible adsorption due to the presence of the EG group. A T-cell receptor (scTCR) construct was used as a model protein to test the immobilization capability and the activity retention. SPR was used to compare the functionality of scTCR immobilized either by bioaffinity to Ni−NTA or by chemical coupling to carboxylated dextran surface. Despite the larger immobilization capacity of dextran, scTCR on Ni−NTA showed a higher retention of antibody activity. The use of mixed functionalized SAM allows a flexible design of the surface, with the possibility of controlling the surface density and charge and steric effect in His-tagged protein interactions. The binding is highly specific and entirely reversible upon addition of a competitive ligand, such as histidine or imidazole, or a chelating agent, such as EDTA, able to remove the metal from the complexing agent NTA. Reusability is often considered as one of the main advantages, together with the possibility of efficiently orienting proteins onto surfaces. Moreover, the formation of the complex Ni^{2+}−His-tag is rapid and reversible. Last but not least, His-tags are commercially available for a large number of functional proteins. However, the NTA system does have a few drawbacks, like metal-dependent nonspecific protein adsorption to the surface and low affinity of the His-tag to the Ni^{2+}−NTA complex ($K_d = 10\ \mu M$).

2.3.3. DNA-Directed Immobilization. DNA arrays are routinely fabricated and, unlike protein arrays, are stable and robust. In recent years, several attempts have been made to convert DNA arrays into protein arrays using DNA hybridization. Actually, severe problems in protein microarrays result from the difficulty of site-specifically addressing many macromolecules under mild chemical conditions. Oligonucleotide-directed immobilization provides a solution due to the exceptionally high stability of DNA oligomers and the unique site-selectivity of the specific Watson−Crick base pairing. Surface fabrication is based on well-established DNA chip technology, able to produce high-density microarrays by successive attachment or spatially separated synthesis. Once the surface is patterned with the appropriate probe sequence, specific hybridization will take place, sorting out the target conjugates and directing them to the appropriate spot on the surface. In an attempt to create a suitable protein immobilization strategy, microarrays based on DNA hybridization have been developed in recent years.[124,125] For achieving DNA-directed immobilization, biomolecules of interest need to be coupled with ssDNA moieties, providing a specific recognition site for complementary oligonucleotides and thus a molecular handle for selective immobilization on DNA arrays. The incorporation of oligonucleotides into large proteins is still poorly developed. Currently, DNA−protein conjugates can be prepared by the following four strategies, i.e., direct covalent attachment, bifunctional linkers, interaction via a streptavidin bridge, and expressed protein ligation.[126,127]

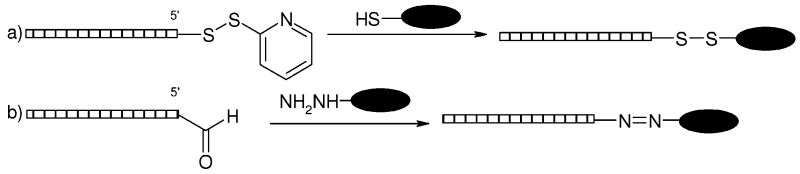
Direct attachment can be achieved via a disulfide exchange reaction between oligonucleotides containing a thiopyridyl sulfide to the reactive cysteine of a protein (Scheme 15a). In the approach proposed by Howorka et al., 5′-thiol-modified DNA oligonucleotides were activated with 2,2′-dipyridyil disulfide to yield 5′-*S*-thiopyridyl oligonucleotides for coupling to the protein.[128] Although this strategy produces homogeneous proteins carrying one ssDNA tag at a specific position, the inherent chemical instability of the disulfide bond in the presence of reducing agents may restrict its use. An efficient coupling method by chemically modifying antibodies with an hydrazide group and 5′-oligonucleotides with an aldehyde moiety has been proposed (Scheme 15b).[129,130] Conjugation results in the formation of a hydrazone bond stable at a wide pH range.

The second strategy relies on the use of homobifunctional or heterobifunctional linkers (Scheme 16). Both types of linkers were studied using oligonucleotides modified with a primary amino group at the 5′ end.[130] A 100-fold excess of the homobifunctional linker bis(sulfosuccinimidyl)suberate (BS) was reacted to the oligonucleotide and the ϵ-amino group of lysine of aequorin (AEQ) generating a covalent link. The heterobifunctional linker sulfo-succinimidyl 4-[*N*-maleimidomethyl]-cyclohexane-1-carboxylate (sulfo-SMCC) was reacted to the amino group of the oligonucleotide and to previously thiolated aequorin. The use of *N*-[γ-maleimidobutyryloxy]succinimide

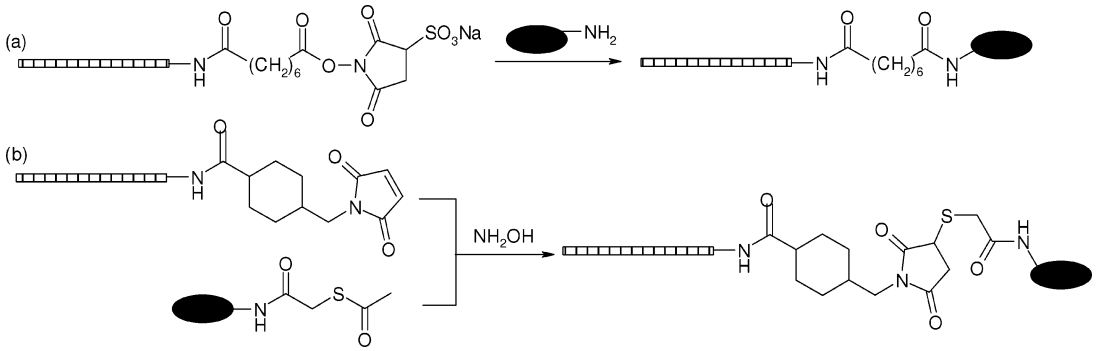
Scheme 14. Multivalent Chelator Heads (MCH) for Surface Modification: (a) Mono-NTA and (b) Tris-NTA



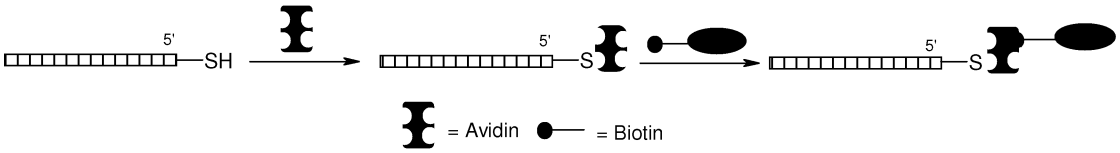
Scheme 15. DNA−Protein Conjugates Formed by Direct Covalent Attachment: (a) via Disulfide Exchange Reaction and (b) via Hydrazone Bond Formation



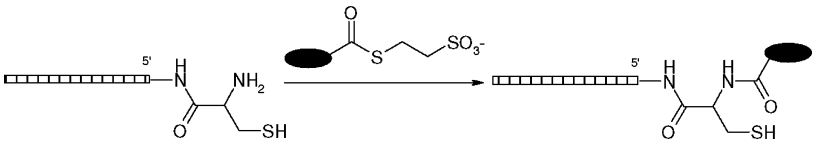
Scheme 16. DNA−Protein Conjugates after the Linker Has Been Coupled to the DNA: (a) Homobifunctional Linker (BS) and (b) Heterobifunctional Linker (sulfo-SMCC)



Scheme 17. DNA−Protein Conjugates Formed via Streptavidin−Biotin Affinity System



Scheme 18. DNA−Protein Conjugates Formed via Protein Ligation



ester (GMBS) and sulfo succinimidyl 4[*p*-maleimidophenyl]-butyrate (sulfo-SMPB) cross-linkers has also been reported.[131−133]

SsDNA−streptavidin complexes are versatile connectors for site-selective immobilization of biotinylated proteins (Scheme 17). These conjugates were synthesized from 5′-thiolated oligonucleotides and recombinant streptavidin and then immobilized onto surfaces via DNA hybridization.[134] Although the end result is the conversion of a DNA chip into a protein chip, this process requires extra steps, biotinylation of proteins and direct adsorption of streptavidin on the surface.

Expressed protein ligation has also been employed for coupling oligonucleotides with fusion proteins, but protein splicing and DNA conjugation steps are inefficient and time-consuming. A new approach based on expressed protein ligation for modifying recombinant proteins specifically at their C-terminus has been proposed by Takeda et al. (Scheme 18).[135] In order to apply the native chemical ligation to proteins containing a C-terminal α-thioester with N-terminal cysteines, oligonucleotides containing a cysteine moiety were prepared. A new reagent for solution-phase coupling, i.e., Fmoc-protected dipeptide carrying NHS−ester, was designed and coupled to amine-modified oligonucleotides. Advantages are the ease of preparation of the cysteine-modified oligonucleotides and the availability of 5′ and 3′-amino oligonucleotides, allowing conjugation at both sides of ssDNA.

DNA probe surfaces have been extensively studied in order to establish the requirement for obtaining the highest surface coverage.[136] However, protein resistance has not been tested because these surfaces were designed for use with DNA. Recently, Boozer and co-workers[131,132,137] reported the preparation of stable and versatile biosensor surfaces to eliminate background noise interference during real-time analysis and to ensure proper placement of the protein on the surface.

A mixed SAM composed of ssDNA thiols and OEG-terminated thiols was prepared (Figure 4).[131] The ssDNA thiol concentration was held constant at 100 nM for all experiments, while the OEG thiol concentration ranged from 0 to 100 μM. The resulting ssDNA/OEG SAM surface selectively hybridizes with complementary ssDNA and resists nonspecific binding of

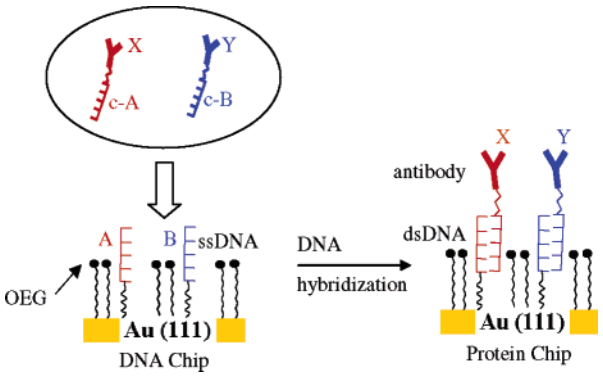


Figure 4. Schematic representation of the general construct of the DNA-directed sensor platform (ref 132).

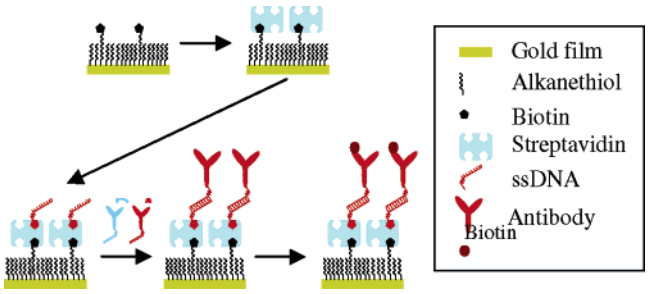


Figure 5. Schematic representation of the general construct of the DNA-directed sensor platform via a streptavidin bridge (ref 137).

proteins, achieving better control over protein orientation and conformation. Since such a platform can be considered as a suitable candidate for multichannel sensors, thanks to the exceptional specificity of DNA hybridization, it was used for the simultaneous detection of a set of three different proteins (Figure 4)[132] Briefly, thiolated ssDNA A, B, and C were used as a probe surface, while the corresponding complementary oligonucleotides were linked to anti-hCG, anti-hLH, and anti-FSH antibodies, respectively. Simultaneous detection of a set of three analytes was performed confirming the specificity of DNA-directed immobilization. The DNA sequence used for hybridization should display not only high hybridization efficiency for maximum conjugate immobilization but also no cross-reactivity for maximum specificity. To test the specificity of hybridization, the SPR response to both complementary and noncomplementary oligonucleotide strands was measured showing no detectable binding of the control sequence.

Figure 5 schematically represents the general construct of a sensor surface based on biotinylated ssDNA immobilized via a streptavidin bridge.[137] Streptavidin is immobilized on a mixed biotinylated/OEG SAM, and then biotinylated ssDNA is bound to streptavidin. Antibodies conjugated to a complementary ssDNA (red) are immobilized via hybridization in the correct location onto the surface. Antibodies conjugated to a noncomplementary ssDNA (blue) do not bind. The bound antibodies are then detected.

DNA-directed immobilization proceeds with high immobilization efficiency and results in excellent site-specificity, due to the formation of the rigid, double-stranded DNA spacer arm between surfaces and proteins. This technique allows functionalization of surfaces with a reproducible amount of biomolecules and thus improves the opportunities of fabricating reliable biosensor chips. Since the immobilized proteins can be completely removed by alkaline denaturation of the DNA double helix, a complete regeneration of the surfaces can be achieved. This is advantageous for the generation of a reusable chip but potentially problematic in some applications. For example, nucleases present in analyte samples may degrade DNA. However, probably due to short exposure time and also mild conditions, even sensitive molecules seem to retain full biological activity. Moreover, the DNA-directed strategy permits the simultaneous immobilization of multiple compounds in a single reaction, which is promising and suitable for multianalyte detection.

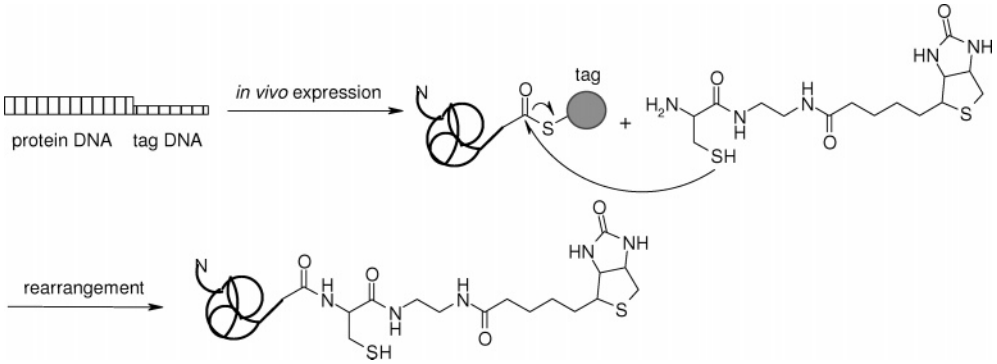
2.3.4. Affinity Capture Ligand System. Protein immobilization using affinity capture ligands involves the use of fusions of the proteins of interest with another protein that chemoselectively reacts with a substrate present at the surface. The use of recombinant affinity tags addresses the issues of orientation and surface density, which are difficult to accomplish in many of the other methods used to fabricate arrays.

Expressed protein ligation is generally used to modify the C-terminal of proteins with chemical tags. The first application of such an expression system for the construction of protein arrays has been reported by Lesaicherre et al.[138] Three model proteins, maltose binding protein (MBP), enhanced green fluorescent protein (EGFP), and glutathione S-transferase (GST), were expressed in vivo as fusion protein with a C-terminal tag (Scheme 19). Successively, they were biotinylated and spotted onto avidin-functionalized slides together with the corresponding nonbiotinylated proteins. The array was probed with their individual antibodies, proving the specificity of binding and the retention of conformation and activity.

Another affinity capture strategy is based on the covalent interaction between cutinase and its suicide substrate. Cutinase is a serine-esterase that forms a site-specific covalent adduct with phosphonate ligands, mimicking the transition state of the hydrolysis. In the work of Hodneland et al.[139] 4-phenylphosphonate was used as inhibitor and incorporated into SAM. The immobilization of cutinase, characterized by SPR spectroscopy, was performed in PBS buffer at pH 7.4 and shown to be irreversible. More recently, the same group[140] reported on the expression and immobilization of different cutinate-fused antibodies. The immobilization was completed within 5 min with antibody solutions at 10 μM concentration. A high fraction of the immobilized antibodies retained binding activity. In contrast, antibodies adsorbed to glass substrates retained only 20% of specific activity. A potential limitation of the cutinase-fused proteins method is the reactivity of the phosphonate inhibitor toward a variety of hydrolytic enzymes, which might affect the selectivity of the immobilization.

Immobilization of protein genetically linked to mutants of the human DNA repair protein O^6-alkylguanine-DNA alkyltransferase (AGT) was reported by Johnsson and co-workers.[141,142] AGT fusion proteins react selectively to O^6-benzylguanine (BG) derivatives allowing specific, covalent, and quasi-irreversible immobilization. BG derivatives were coupled

Scheme 19. Expression of Protein with a C-Terminal Tag and Biotinylation



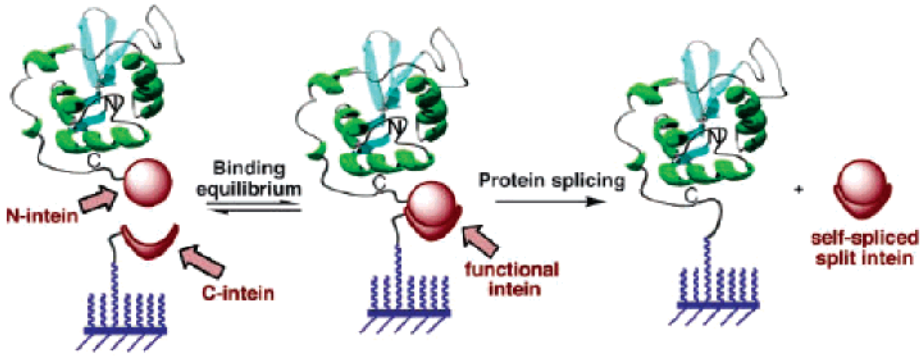
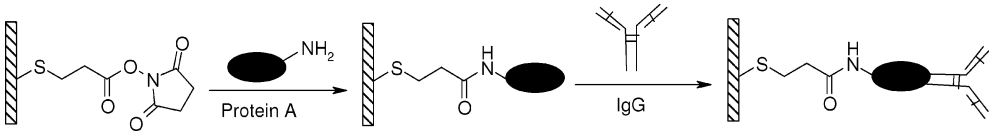


Figure 6. Site-specific immobilization of proteins onto a solid support through split-intein-mediated protein trans-splicing. Reprinted with permission from ref 145. Copyright 2006 Wiley.

Scheme 20. Schematic Description of Antibody Immobilization Using a Layer-by-Layer Architecture



to commercially available carboxymethylated dextran chips suitable for SPR measurements, used for monitoring both the immobilization and the interaction steps. Immobilization of AGT-fused GST and subsequent anti-GST binding were investigated. A strong signal of antigen−antibody binding was recorded only on sensor chips displaying the AGT−BG interaction. This immobilization strategy has been applied to "nonfouling" poly[oligo(ethylene glycol) methacrylate] (POEGMA) brushes.[143] To assess the nonfouling character of the polymer brushes, unspecific adsorption of AGT-fused proteins was studied and compared to that on polystyrene and glass surfaces controls. The relative fluorescence intensity indicated the suppression of nonspecific adsorption on POEGMA brushes.

A novel fusion protein system employing the substrate-binding domain of poly(hydroxyalkanoate) (PHA) depolymerase was developed by Lee et al.[144] Enhanced green fluorescent protein (EGFP) and red fluorescent protein (RFP) were used as model proteins and fused to the C-terminus of the substrate-binding domain. As a negative control, native EGFP was incubated with PHA microbeads, wherein no immobilization was observed. In contrast, fusion proteins are not only selectively immobilized but also retain their conformation, proven by the fluorescent activity, and the capability to interact with cognate antibodies.

The above-mentioned approaches suffer of a common drawback: the capture ligands usually remain attached to the surface after the immobilization process is complete. A new traceless capture ligand system has been recently proposed by Kwon et al.[145] The approach is based on the use of protein trans-splicing, in which the intein domain is split up into two fragments (N-intein and C-intein). The C-intein fragment was covalently immobilized onto a glass surface through a PEGylated peptide linker, whereas the N-intein fragment was fused to the C-terminal of the protein. When the two fragments interact, they form an active intein domain, which binds the protein to the surface (Figure 6).

The naturally split DnaE intein possesses C- and N-intein fragments able to self-assemble spontaneously. Maltose binding protein (MBP) and enhanced green fluorescent protein (EGFP) were chosen as model proteins and modified with the DnaE N-intein fragment at their C-termini. No significant loss of fluorescence was detected after immobilization, proving the retention of protein conformation.

2.3.5. Protein A/Protein G-mediated Immobilization. The immobilization of antibodies by use of protein A/protein G relies on the specific interaction with the F_c constant region of IgG molecules. Using this immobilization method ensures that the binding site of the antibody, located on the Fab variable region, remains well accessible for binding with the antigen. Detailed reviews on oriented immobilization of antibodies and applications in immunoassays and affinity chromatography are already available.[146,147] In the following, the recent use of this immobilization way for antibody microarrays will be discussed.

Wang and Jin used protein A for the immobilization of human IgG on a silicon surface. Silicon surfaces modified with dichloredimethylsilane (DDS) were used as control. The amount of bound IgG was deducted by the thickness of the antibody layer, measured by imaging ellipsometry. A homogeneous IgG layer was observed.[148]

A drawback of protein A-mediated immobilization is the lack of control on the orientation of protein A itself; therefore, different approaches were presented in order to achieve higher orientation control upon immobilization. In the work of Johnson et al.,[149] a hexahistidine tag was added at the C-terminus of a truncated version of protein A. The tag orients protein A on NTA-modified surfaces and lets the binding site be free to interact with the F_c region of the antibodies. Lee et al.[150] fabricated an ordered layer-by-layer architecture, with a self-assembled *N*-succinimidyl-3-(2-pyridyldithio)propionate (SPDP) layer reacting with the primary amine of protein A (Scheme 20). The formation of the protein A layer and the immobilization of IgG molecules were monitored by SPR spectroscopy and AFM topography.

Schmid et al.[151] reported the creation of a uniform sterically accessible antibody layer using dithiobisuccinimide propionate (DSP)-modified gold as the linker for protein A immobilization. AFM images showed a consistent uniformity of the protein A layer. The method was proven to be suitable for antibody binding.

Another disadvantage of the protein A-mediated immobilization is that only certain classes of antibodies are able to bind protein A. Streptococcal protein G shows a broader binding activity for immunoglobulines of different species and subclasses. Tanaka et al.[152] developed a fusion protein E72G3, containing 3-repeats of the protein G binding domain and 72-repeats of the pentapeptide derived from elastin, in order to

achieve self-adherence on hydrophobic surfaces. E72G3 provided self-adherence, as confirmed by AFM imaging, and retained Ab binding ability, as proven by immobilization of fluorescently labeled goat IgG.

2.3.6. Targeting the Carbohydrate Moiety. Many glycoproteins, including antibodies, hormones, and enzymes, can be immobilized through their carbohydrate moiety without affecting their biological activity, since the carbohydrate region is generally located in areas not involved in specific activity. Moreover, coupling of antibodies via carbohydrate moiety, located on the F_c region, was proven to enhance steric accessibility of the binding sites.[153] Immobilization of MAbs against horseradish peroxidase (HRP) and carboxypeptidase A (CPA) on hydrazide-modified substrates, through oxidation of the carbohydrate moiety, was already commonly used in the 1990s[154,155] for the preparation of multienzyme reactors. Immobilization of enzymes via a carbohydrate moiety has been widely used in industry to prevent deactivation of enzymes by deglycosylation. The use of concanavalin A, legume protein having specific saccharide-binding sites, for enzyme immobilization has been reviewed.[156]

A carbohydrate targeting approach has also been employed for fabrication of biosensors. SAM of conjugates of dithioaliphatic and aminophenylboronic acid on gold display sugar recognition. The interaction is based on the formation of cyclic esters with diols in both aqueous and nonaqueous media. This method was used to immobilize glycoproteins as glucose oxidase, HRP, and $NAD(P)^+$-dependent lactate dehydrogenase, maintaining their biological activities.[157,158] To overcome the instability of the boronate−carbohydrate interaction, the use of heterofunctional epoxy−boronate SAM to immobilize HRP was explored.[159] Immobilization through the carbohydrate moiety was recently shown to provide a higher retention of the activity of lipase from *C. rugosa*, as compared to that of lipase covalently attached to epoxy or glutaraldehyde supports.[160]

3. Conclusions and Future Perspectives

The past few years have witnessed impressive progress in protein biochip technology, taking further steps toward diagnostic and proteomic applications and opening up possibilities to gain insights into cellular processes. The step from genomics to proteomics will be accomplished with the high-throughput screening of all the protein functions. The creation of point-of-care devices for multianalytes diagnosis will bring to reality the concept of personalized medicine. The major challenge has been the lack of optimal ways to immobilize proteins. Many efforts have already been taken in the design of different immobilization strategies attempting to obtain a full coverage of the surface and the best detection performances. SAM of alkanethiols on gold have been highlighted as the most promising surface due to the ease of real-time detection via imaging techniques and a higher level of surface loading compared to other flat supports. The formation of mixed thiol/PEG monolayer generates a surface displaying protein resistance and adequate surface coverage without creating steric hindrance among immobilized molecules. Site-specific immobilization strategies mostly via bioaffinity and DNA-directed methods have been developed in order to entirely exhibit the incredible potential of protein microarrays. However, new strategies allowing site-specific and stable immobilization of proteins in such a way as to retain biological activity are urgently needed. Only by combining such immobilization approaches with improved arraying and detection devices, can the potential of microarrays to perform high-throughput protein studies be exploited.

Abbreviations

3-MPA, 3-mercaptopropanoic acid; 11-MUA, 11-mercaptopropanoic acid; ACP, acyl carrier protein; AEQ, aequorin; AFM, atomic force microscopy; AGT, O^6-alkylguanine-DNA alkyltransferase; APMA, 4-aminophenylmercuric acetate; BG, O^6-benzylguanine; BMH, bis(maleimidohexane); BS, bis(sulfosuccinimidyl)suberate; BSA, bovine serum albumin; CDI, carbodiimide; CMC, carboxymethyl cellulose; CPA, carboxypeptidase A; DDS, dichloredimethylsilane; DSP, dithiobisuccinimide propionate; EGFP, enhanced green fluorescent protein; EMCS, *N*-(ϵ-maleimidocaproyl)succinimide ; EG, ethylene glycol; FNAB, 1-fluoro-2-nitro-4-azidobenzene; FSH, follicle stimulating hormone; GMBS, *N*-[γ-maleimidobutyryloxy] succinimide ester; GST, glutathione S-transferase; hCG, human chorionic gonadotropin; hLH, human luteinizing hormone; HRP, horseradish peroxidase; JAK, Janus tyrosine kinase; MAD, *N*-[*m*-[3-(trifluoromethyl)diazirin-3-yl]phenyl]-4-maleimidobutyramide; MBP, maltose binding protein; MCH, multivalent chelator heads; MeNPOC, α-methyl-6-nitropiperonyloxycarbonyl; NHS, *N*-hydroxysuccinimide; NMPEG-silane, 2-nitro-5-[11-(trimethoxysilyl)undecyl]oxybenzyl-methoxy PEG; NTA, nitriloacetic acid; NVOC, nitroveratryloxycarbonyl; OEG, oligo(ethylene glycol); PAMAM, polyamidoamine; PANI, polyaniline; PC, polycarbonate; PDMS, poly(dimethylsiloxane); PEG, poly(ethylene glycol); PEI, polyethylenimine; PFTA, perfluorophenylazide; PHA, poly(hydroxyalkanoate); PMMA, poly(methyl methacrylate); POEGMA, poly[oligo(ethylene glycol)methacrylate]; (polyCBMA), poly(carboxybetaine methacrylate); PP, polypropylene; RFP, red fluorescent protein; RNase S′, ribonuclease S′; SAM, self-assembled monolayers; scTCR, T-cell receptor construct; SPDP, *N*-succinimidyl-3-(2-pyridyldithio)propionate; SPR, surface plasmon resonance; ssDNA, single-strand DNA; sulfo-SMCC, 4-[*N*-maleimidomethyl]-cyclohexane-1-carboxylate; sulfo-SMPB, sulfosuccinimidyl 4[*p*-maleimidophenyl]butyrate; XPS, X-ray photoelectron spectroscopy.

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