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The Art of Immobilization for SPR Sensors

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1 Introduction

Since the first seminal work on the use of SPR-based detection technology in bioanalytical applications [1], the field has seen a tremendous development.

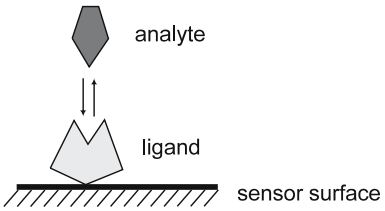

Fig. 1 One interaction partner is immobilized to the sensor surface. The analyte is free in solution and binds to the immobilized molecule (denoted "ligand" in this and subsequent figures)

This is manifested in numerous ways, including the growing use of SPR in research as well as in the many significant improvements in commercial instruments, which have opened up their use for a wide range of applications and user groups. Technical advances have been made in many areas, including the detection unit, fluid and sample handling, data treatment and not least, in the immobilization procedures for functionalization of the sensor surface. This chapter will deal with the progress made in surface modification techniques and approaches for immobilizing interacting partners on these surfaces (Fig. 1).

Although immobilization on solid surfaces or matrices has been described and practiced for several decades, SPR-based biosensors pose some unique requirements. A successful direct, label-free measurement of specific binding events will be facilitated by the best possible activity of the immobilized interactant. It is a general rule that all types of non-specific binding to the surface must be kept as low as possible in order to prevent irrelevant signals interfering with the interpretation of the specific interaction. Since SPR detection can be applied to a great variety of analytical applications, a correspondingly large range of methods for immobilization have been developed. Given the wide variation in molecular properties, no generally applicable immobilization method has emerged. Rather, even among proteins, different approaches may be needed in order to reach the required activity. Approaches for both covalent immobilization and for affinity-based capture methods will be reviewed.

The SPR phenomenon is ideally suited for miniaturization and for array format applications. Methods for the immobilization of the range of molecules that can be expected for array formats have also been developed and implications and issues related to this will be described. Finally, future trends and opportunities related to immobilization for SPR detection will be discussed.

2
Surface Modifications

Early descriptions of SPR technology for bioanalytical applications were based on simple physical adsorption of proteins to an active metal surface [1]. However, it was soon realized that a more sophisticated approach was needed in order to meet the challenges demanded by the range of potential applications involved. Commonly used metal substrates such as gold and silver show a high tendency for spontaneous adsorption of proteins and other molecules. This passive binding to the metal substrate results in a loss of the bioactivity. Similarly, studies on antibody binding activities in ELISA-type assays after their adsorption to plastic surfaces have shown levels as low as 2–10% of the adsorbed amount [2].

These effects can be explained by a reorganization of the immobilized molecule to attain the most favorable thermodynamic state. For example, adsorption to hydrophobic surfaces is driven by rearrangements that optimize contact of hydrophobic segments with the substrate. Passive binding to a surface substrate also opens possibilities for uncontrolled exchange of the immobilized molecule during an analysis cycle. If the modified surface is used for repeated analysis cycles, the probability of exchange will be further enhanced and lead to unreliable assays.

2.1
Coating of Surfaces with Self-Assembled Monolayers

Extensive efforts have been made to develop approaches for coating metal surfaces before immobilization. This serves to minimize non-specific adsorption, as well as to introduce reactive groups for specific immobilization. The most successful methods are based on the concept of molecular self-assembly of thiol- or disulfide molecules on the metal surface. The spontaneous formation of organic disulfide monolayers on gold was initially shown by Allara and Nuzzo in 1983 [3] in the context of models for interface studies. Monolayer formation is driven by a strong coordination of sulfur with the metal, accompanied by van der Waals interactive forces between the alkyl chains. With a sufficient chain length, the resulting monolayer forms a densely packed and very stable structure that is oriented more or less along the normal to the metal surface (Fig. 2).

These nanometer-thick layers are easily fabricated from commercially available substances, or can at least be synthesized with relative ease [4]. The first applications of self-assembled monolayers (SAM) for biosensor use were described in the late 1980s and originally developed for Biacore instruments [5, 6]. Hydroxyl-terminated long chain thiol alkanes were designed for the formation of the SAM on gold. Such layers can be activated for direct linkage of various molecules or further derivatized with different chemistries

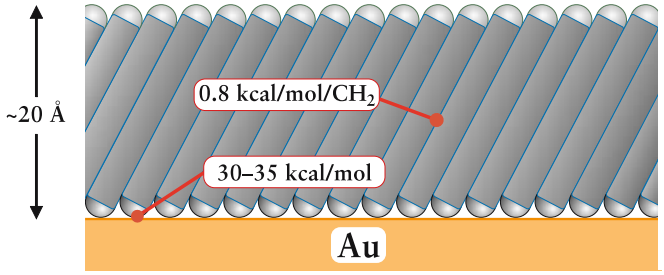

Fig. 2 Schematic illustration of a self-assembled monolayer structure on a gold substrate

for more advanced surface modifications, as will now be described in more detail.

The possibility for different functional end groups in the alkyl thiols creates a high degree of flexibility in terms of the types of surface properties that can be obtained. Extensive studies involving various types of coatings have been reviewed elsewhere [4, 7, 8]. For example, in early applications [5, 6], a terminal hydroxyl function was introduced to give the surface a highly hydrophilic character, while acting as a means for immobilization of various molecules, either directly or via suitable linkers. Direct covalent immobilization of proteins to various ω-terminated groups has also been described, although there are limitations with such approaches.

2.2 Development of the Dextran Hydrogel

Even if flat surface substrates are made hydrophilic, their rigid character may induce denaturation or impaired activity of proteins [9]. Furthermore, SPR senses mass-dependent refractive index changes a few hundred nanometers from the metal surface. Taking advantage of this, surface modification procedures were developed for sensor chips produced for the company, Biacore AB in which a thin hydrogel-like polymer layer based on dextran was introduced. The dextran polymer is composed of mainly unbranched glucose units, providing high flexibility and water solubility. Immobilization is facilitated via epoxy modification of the terminal hydroxyl SAM and subsequent nucleophilic reaction of the dextran under alkaline conditions [5, 6, 10] (Fig. 3).

The surface can be further activated with suitable linkers for subsequent immobilization, and here the introduction of carboxymethyl groups has proven a versatile alternative. By choosing different sizes of dextran, ranging from 10 kDa to over one million Da, surfaces tailored for specific applications can be created. This type of surface modification serves multiple purposes. The hydrogel-like layer provides a highly hydrophilic environment.

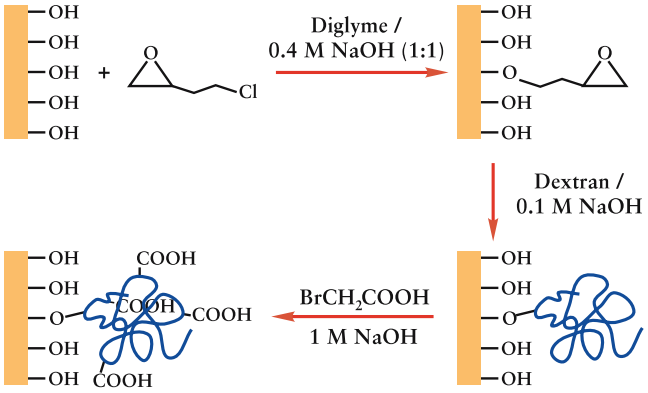

Fig. 3 Synthesis sequence for the construction of a carboxymethyl dextran-coated sensor surface

The glucose-based polymer is highly suited for well-defined covalent immobilization of proteins that rely on a wide variety of chemistries. Furthermore, the extended matrix structure has been shown to increase the binding capacity several-fold compared to flat surfaces. Finally, this thin layer extension is well matched with respect to the penetration depth of the evanescent wave [11, 12].

The linkage of dextran polymer chains to the sensor surface provides an open, non-cross-linked structure on which immobilized molecules can attain a solution-like state with a certain level of freedom to move around within the hydratized layer. This view is supported by the excellent agreement that has been obtained in comparisons of affinity data from Biacore's SPR-based platforms and solution-based methods [13]. The most commonly used carboxymethylated derivate of dextran surfaces also have the benefit of improved solubility properties. The degree of carboxymethyl modification can be modulated for different applications and sensor surface capacity requirements. These types of negatively charged layers may exhibit electrostatic background binding of basic compounds, which needs to be considered in the design of the immobilization procedure and the assay. However, working under physiological buffer conditions normally suppresses such effects by electrostatic shielding. Alternatively, lowering the degree of carboxymethylation can also be used to reduce this effect.

Other hydrophilic polymers have been conceived as alternatives to dextran [6]. For example, polyvinyl alcohol and polyacryl acid derivatives are feasible and graft combinations thereof have been shown to be applicable to SPR detection [14]. Poly-L-lysine has become popular for DNA microarray coatings, due to its highly positive charge. It has also been attached to SAM-derivatized gold surfaces for subsequent modification with thiol reactive groups [15].

2.3 Further Chemical Modifications to Optimize the Sensor Surface

Although flat or two-dimensional (2D) surfaces are used for various applications within the DNA and protein microarray area, practical uses for SPR detection were originally limited. This can be attributed to the sensitivity limitations of the technology, despite the relative ease in handling and the wide variety of developed chemistries based on flat surface structures. Thus, an immobilized monolayer may not give sufficient binding responses under certain conditions, especially if immobilization leads to compromised activity of the immobilized partner. As described previously, non-specific binding also needs careful consideration.

These potential limitations, however, have been largely eliminated. Early attempts describe modifications of the metal surface with thin insoluble layers, such as silica with subsequent functionalization via silane compounds [16]. The SAM approach created a tool for convenient introduction of various surface functionalities that can be used for immobilization [6]. Examples include SAMs that are ω-terminated with hydroxyl or carboxyl groups, which can be activated for covalent coupling via nucleophilic reactions. In addition, this modification with epoxy groups leads to activated surfaces that can be directly used for nucleophilic linkage.

A similar approach has been developed for biotin-based surfaces that can be further modified using streptavidin. Such structures provide a general capture tool by binding a wide variety of biotinylated compounds. Knoll et al. developed SAM-based surface modifications where ω-terminated biotinylated alkane thiols were utilized in different forms [17]. By mixing biotinylated molecules with hydroxyl-terminated thiols as diluting agents in different ratios, a 1 : 9 molar ratio was found to be optimal for binding monolayers of streptavidin. This is in contrast to a SAM composed of a single biotinylated thiol, where the biotin groups are sterically hindered from binding to streptavidin. By utilizing different alkyl chain lengths in the biotinylated thiols and the diluting molecules, the biotins can be exposed to more efficient binding of streptavidin. This strategy can also be used for other functional groups, such as combinations of carboxy- and hydroxyl-terminated thiols. The diluted biotin surfaces can also be generated by reaction of biotin derivatives with suitably functionalized SAMs, modifying a fraction of carboxy-terminated thiols with amine derivatives of biotin [18]. Both approaches have their limitations; the use of biotinylated or other modified thiols may be limited by the accessibility and cost of such molecules, while the surface modification strategy can be difficult to apply consistently.

When implemented correctly, however, both approaches yield streptavidin surfaces with good binding capacity and generally sufficient biocompatibility, even if there are reports that streptavidin has a tendency for unwanted binding of a range of compounds [19].

Several alternatives to biotin for mixed functionalized SAMs have been described. One of the more interesting approaches involves the use of short oligo ethylene glycol (OEG) units to increase the biocompability and to extend the functional alkyl thiol [8, 20]. Whitesides et al. have described mixed SAMs for use in SPR detection composed of *N*-hydroxysuccinimide activated carboxy-terminated OEG thiol alkanes and shorter hydroxyl-terminated analogs [21]. The OEG spacers constitute what can be considered as a very thin hydrogel like layer, creating surfaces with properties that resemble both 2D and 3D layers. The suppression of non-specific binding is similar or better than dextran-based surfaces, but capacities are limited to monolayer levels similar to other 2D surfaces. Extensive studies have investigated protein resistance effects by OEG-terminated SAMs, and these indicate that binding of interfacial water by the OEG moieties is important [22]. The susceptibility for oxidation and degradation of OEG-based structures may also present a practical problem, limiting storage stability and performance quality (see footnote 17 in [22]). Alternative spacing units that overcome these limitations have been evaluated and reviewed elsewhere [8, 23].

3 Immobilization Techniques

The development of SPR technology has encouraged the development of numerous strategies for the immobilization of different types of recognition elements. These have focused on proteins but also include methods for peptides, DNA, RNA, carbohydrate structures and organic molecules of various kinds including lipids and more complex natural products.

Immobilization methods have been successively developed from earlier adsorption processes, using highly controlled general covalent chemistries and specific alternatives of various kinds. Considerable experience has also

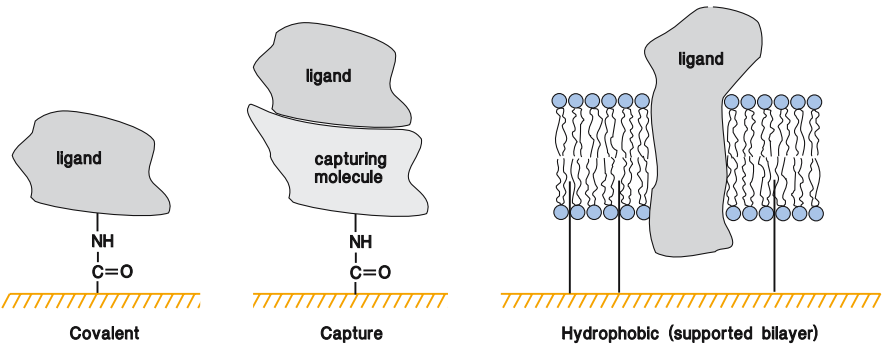

Fig. 4 Different approaches for immobilizing binding partners to the sensor surface

been gained from immunochemistry and affinity chromatography, where issues related to the maintenance of activity are also relevant [24, 25]. However, the miniaturization of the sensor areas and the true heterogeneous, interfacial conditions needed for immobilization are factors that have required novel solutions for satisfactory results. The availability of these methods has also been an important factor for the acceptance of SPR-based instrumentation as an established and widespread analytical tool.

The following section describes the most important immobilization techniques, including different covalent coupling alternatives, non-covalent capture techniques and more specialized methods for lipids and membrane proteins (Fig. 4).

3.1 Covalent Immobilization

Limitations in simple adsorption processes have led to the development of advanced surface coatings designed for controlled immobilization. Different functional groups have been introduced on the surface, enabling the formation of a stable linkage to another appropriate functional group. This may include an activation step of one or both of the functional groups, which results in transformation into a more reactive form. For proteins in particular, the chemistries utilized also need to be performed under relatively mild conditions and in aqueous solutions, placing certain limitations on the available repertoire.

The possibility of having a transformable functional group on the sensor surface is an attractive concept as a general starting surface for use with a range of coupling chemistries. The carboxymethylated dextran coating described in the previous section was designed to include the carboxylic acid residue as a functional group that can be used either for direct coupling, or

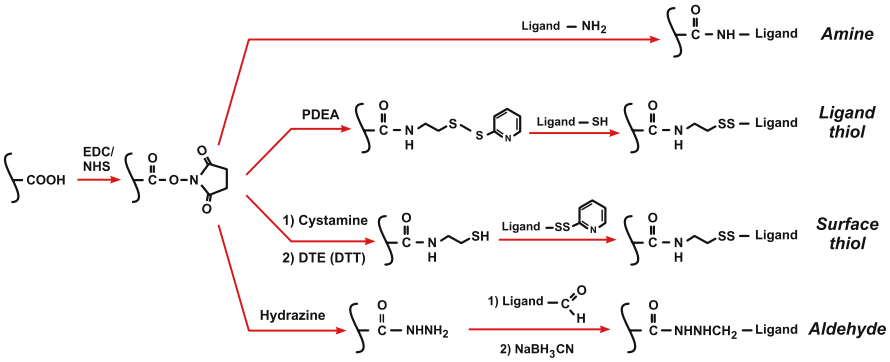

Fig. 5 Reaction sequences for different binding partner immobilizations based on couplings to the carboxylic acid group

switched to other functionalities. Figure 5 shows how the carboxylic groups can either be directly reacted with amine groups or converted for use in coupling chemistries based on thiol reactions, aldehyde and carboxylic acid condensations, and biotin capture techniques.

More detailed descriptions of the various covalent couplings will be given in the following section. Notably, a literature review indicates that the carboxymethyl dextran surface used in Biacore instruments in combination with the amine coupling method is by far the most widely used immobilization strategy [26].

3.1.1 Coupling of Nucleophiles to Carboxylic Groups

The most versatile and widely used approach involves coupling with reactive nucleophile functionalities to carboxylic groups on the sensor surface. The most common nucleophile utilized is the amine group in lysine residues, but hydroxyl groups can also be used. The carboxylic groups are readily introduced on dextran or other hydroxyl-containing surface layers via reactive haloacetic acids. In the case of SAM layers, alkane thiols that are ω-terminated with carboxylic groups can be utilized. To achieve the formation of a covalent amide or ester bond between the carboxylic and amine or hydroxyl groups, respectively, activation with carbodiimide reagents is most commonly used. Water-insoluble carbodiimides such as DCC (dicyclohexyl carbodiimide) are normally used in organic chemistry applications, but for reactions in aqueous solutions, alternatives like EDC (1-ethyl-3-(3-dimethylaminopropyl) carbodiimide) are preferred [27]. The purpose of the carbodiimide reagent is to create a reactive O-acyl isourea intermediate with the carboxylic group, which is then reacted with a suitable nucleophile. The coupling is normally performed in two steps, with activation followed by reaction, in order to avoid reaction between the carbodiimide and the immobilized molecule. However, in aqueous solutions the reactivity of the intermediate is so high that water hydrolysis rapidly transforms it back to carboxylic acid, if it is not trapped by another competing nucleophile. This side effect is conveniently overcome using a mixture of the carbodiimide and a reactive hydroxyl compound, forming an active ester derivative that is stable for several minutes to hours (Fig. 6).

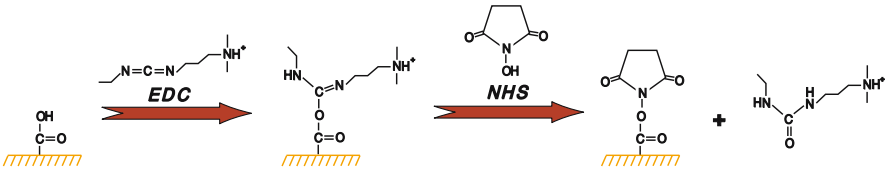

Fig. 6 Activation sequence of the carboxylic acid group with EDC/NHS

N-Hydroxy succinimide (NHS) has been found to be a very suitable reagent for these purposes and is normally mixed at high concentrations with EDC in water. Both the EDC and NHS act as buffering agents and a pH around 5–6 is obtained, providing conditions for an optimal reaction rate for NHS ester formation. Although other ester-forming compounds like nitrophenol and its derivatives are also possibilities, NHS is normally preferred due to its solubility in water, relatively low toxicity, and optimal reactivity for two-stage couplings. Extensive optimization studies have been performed on the activation and coupling conditions for protein immobilization to the carboxymethylated sensor surfaces developed by Biacore [27].

Coupling to the active esters can be carried out under various conditions depending on the molecular type. Displacements in aqueous solutions are normally done under slightly alkaline conditions, e.g., in carbonate or borate buffers around pH 8.5, where a normal alkylamine nucleophile is close to its pKa and can compete with water hydrolysis. This is also the preferred method for organic molecules and small peptides. These conditions have also been widely practiced when immobilizing proteins for affinity chromatography [24]. An alternative approach was developed for in situ immobilization of proteins to sensor surfaces [5, 6] where high-density modifications are desirable and has now become the standard method of choice. This concept relies on electrostatic attraction of the proteins to an NHS-activated carboxylated surface, on which a fraction of the carboxylic groups remain unreacted. Under low ionic strength buffer conditions, where the surface is negatively charged and the protein has a positive charge, a high local surface concentration of the protein is obtained. This greatly favors a reaction of the nucleophiles on the proteins over water hydrolysis of the esters. Suitable buffer conditions to achieve this are normally obtained by working in 10 mM acetate buffers at pH 4–6, where a large fraction of all proteins are positively charged [27]. Much lower protein concentrations than those normally used in coupling to solid phases can consequently be employed. The reaction times are also considerably shorter, in the range 1–10 min.

The electrostatic attraction approach can be applied to all types of surfaces that have a combination of reactive groups and residual charges. The most successful implementations, however, are found for 3D surfaces such as carboxymethylated dextran. Here, the attraction leads to multilayers of bound protein. Quantifications using radioactively labeled proteins that were also used to calibrate the SPR responses showed surface concentrations of up to 50 ng mm^{-2}, which represents several high-density packed monolayers for typical proteins [28]. Furthermore, covalent coupling occurs under very mild conditions, where only a small fraction of the nucleophilic groups on the protein are reactive (e.g., the amino groups on the lysine residues are unprotonated). This leads to very few immobilization points, little or no cross-linking and a high likelihood of preserving activity. Protein A immobilized under these conditions showed a binding capacity of more than three IgG

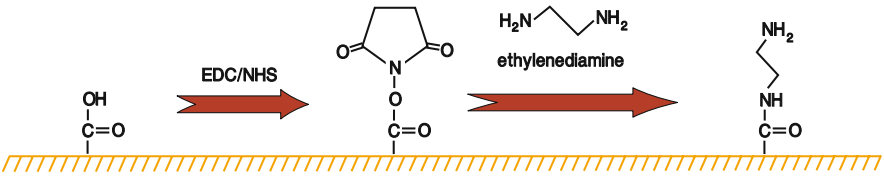

Fig. 7 Modification of the sensor surface with amine groups via EDC/NHS activation and ethylene diamine reaction

molecules per protein A molecule [27]. Likewise, immobilized IgG antibody molecules showed antigen binding capacities approaching 1.5 antigens per antibody (75% activity based on two antigen binding sites per antibody) [29]. These results stand in sharp contrast to results reported for immobilizations of monoclonal antibodies to chromatography supports, where low activities in the range 1–30% were obtained [30].

In some instances, the reverse approach may be preferred, with functional amine groups on the sensor surface and activated carboxylic groups. This strategy can be used when the molecule lacks appropriate reactive amines or other nucleophiles, or when the nucleophile is suspected to be close to the analyte binding site. The approach is particularly valuable when working with small organic molecules, as will be further described in Sect. 4.4. Amine groups can be introduced to the sensor surface in several ways. A convenient route involves the conversion of carboxylated surfaces via EDC/NHS activation and subsequent ethylene diamine reaction (Fig. 7).

Surfaces functionalized with primary amine groups should normally be further reacted directly after they are produced, as the amine groups rapidly lose reactivity when kept in normal aqueous buffer conditions [31]. This is believed to occur by carbamate formation via reaction with carbon dioxide, and also via oxidation phenomena.

3.1.2 Couplings to Thiol Groups

Although amine coupling to activated carboxylic groups is the most commonly used form of covalent immobilization, there are alternative approaches that may be preferable under certain circumstances. Amine coupling may occur at or near the active site, or the molecule may lack amine groups (which may not be possible to introduce due to chemical restrictions). A useful alternative is thiol coupling, which relies on reactive functionalities that are thiol-selective [32]. The thiol reactive groups most commonly used are active disulfides such as pyridyl disulfides or their derivatives, although maleimide and acyl halide derivates are common alternatives. The thiol groups can either be introduced on the sensor surface and reacted with molecules with thiol reactive groups (Fig. 8), or performed in reverse,

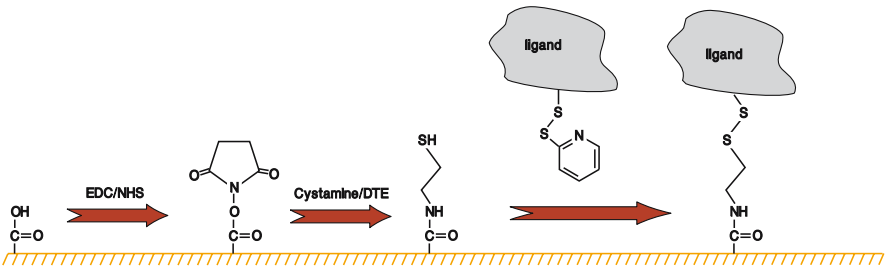

Fig. 8 Coupling of binding partners to thiol-modified sensor surface

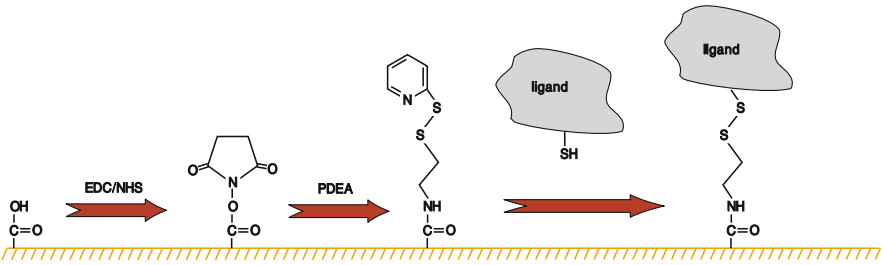

Fig. 9 Coupling of thiolated binding partner to pyridyl disulfide-modified sensor surface

with the active group on the surface and the thiol on the molecule to be immobilized (Fig. 9).

Various reagents for modifications based either on reactive disulfides, maleimides, or acyl halides are commercially available. One advantage of modifying a protein is the possibility of minimizing the number of coupling sites in order to avoid blocking the active site or to keep cross-linking low. Thus, even if the reagents are frequently directed toward amine groups, reaction conditions may be steered to preserve activity.

The disulfides can be coupled under very mild conditions, and in the case of pyridyl disulfides, even in acidic buffers [33]. The selectivity is also very high, with little or no interference from other nucleophiles. The disulfide bond can undergo exchange reactions with free thiol compounds that may limit stability under certain conditions. For example, buffers with added thiols such as mercaptoethanol may induce disulfide bond cleavage and dissociation of the immobilized binding partner. This effect has also been exploited for the reuse of modified surfaces. After cleavage with a reactive thiol under mildly alkaline conditions, the residual thiol groups on the sensor surface can be used for immobilizing disulfide-containing molecules [34].

The maleimide and acyl halide reaction proceeds via Michael addition and forms a thioether linkage to the thiol that is normally more stable than the disulfide bond (Fig. 10). The thioether bond is normally formed at pH 7.5

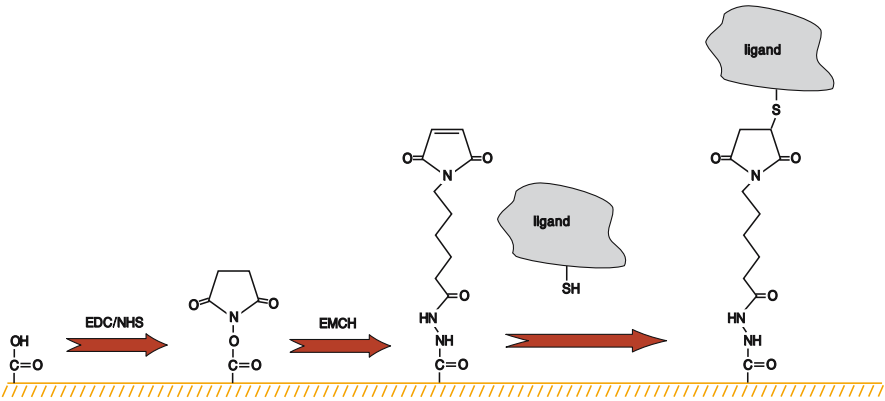

Fig. 10 Coupling thiolated binding partner to maleimide-modified sensor surface

to 8.5 but with somewhat lower selectivity than the disulfide reaction. Competition from other nucleophilic groups can occur under certain conditions and this needs to be considered in the choice of immobilization.

Coupling methods exploiting thiol groups can also be performed under electrostatic concentration conditions similar to those described for amine coupling [32]. As this step is normally carried out under acidic conditions, the method is best performed using reactive pyridyl disulfides.

3.1.3 Coupling to Aldehyde Groups

Schiff base condensation of aldehyde groups to amines and hydrazides has been utilized for glycoprotein immobilizations in chromatography applications [2, 35]. This method exploits the generation of aldehyde functionality by oxidation of carbohydrate residues in proteins. Standard protocols are available for mild oxidation of the sugars using sodium periodate solution. Sialic acid residues in particular, readily form aldehydes by cleavage of the exocyclic vicinal diol. The carbohydrates are not normally located near the active site and consequently, the resulting site-specific coupling may yield immobilized molecules with high binding activity. IgG antibodies in particular are well suited for aldehyde mediated immobilization.

Although Schiff base formation can be performed with amine groups, the low stability of the bond in aqueous conditions makes hydrazide a better alternative. Hydrazides can be introduced on the sensor surface via reaction of hydrazine or carbohydrazine to carboxylic groups after activation with EDC/NHS (Fig. 11) [32]. The hydrazide–aldehyde bond forms rapidly and is relatively stable in neutral to alkaline conditions, but disintegrates slowly in acidic buffers. If necessary, the bond can be further stabilized by reduction with sodium cyanoborohydride at pH 4.

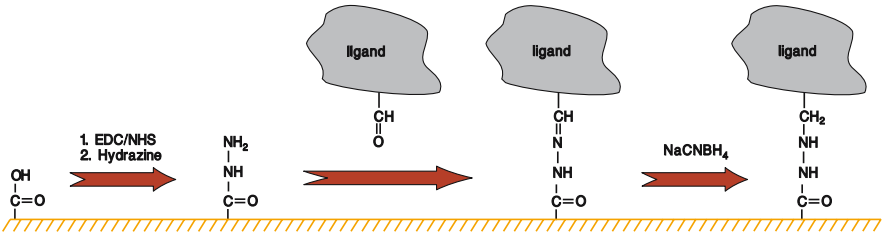

Fig. 11 Coupling of aldehyde containing binding partners to hydrazide-modified sensor surface, followed by cyanoborohydride reduction

As with covalent coupling methods, aldehyde coupling is well suited to electrostatic concentration conditions, provided the sensor surface holds residual negative charges.

In contrast to observations made in chromatography, the "site-specific" immobilization of antibodies via aldehyde groups to carboxymethyl dextran surfaces does not significantly improve activities [32]. This is probably related to the mild conditions that can be utilized in amine coupling (Sect. 3.1.1), which minimize multisite immobilization and thus preserve the binding activity, in combination with immobilization to the extended dextran polymer layer. Aldehyde coupling may be a good alternative for smaller proteins, as there may be a higher probability of masking the binding site through random coupling.

3.2 Capture-Based Coupling of Native and Tagged Molecules

As indicated in Sect. 3.1, covalent coupling techniques are limited under certain situations, for example, the molecule may be unstable under the required coupling conditions or the activity of the binding site may be impaired. Problems may also arise if the molecule is present in small amounts in cell lysates or other complex sample matrices. Immobilization based on non-covalent capture may be a good alternative in such cases. Capture is based on high affinity binding via a specific integral region or recombinant tag to a capturing agent on the sensor surface. An additional benefit of this approach is the possibility for removal of the immobilized binding partner after the analysis by an analyte-independent regeneration step, followed by renewed capture in the next assay cycle. The disadvantage with this alternative compared to covalent coupling is the significantly increased sample consumption. In addition, for applications with demands on quantitative data, the affinity must be sufficiently high that dissociation is insignificant. This can be achieved by the introduction of multiple tags to increase the strength by avidity binding to the capture agent on the sensor surface. A disadvantage here is that this may lead to decreased activity due to interference with the binding site and/or cross-

linking. For SPR detection, therefore, it is usually recommended to keep the degree of modification as low as possible.

The most commonly used capturing agents are specific antibodies directed towards tagged recombinant proteins. Frequently used tags include GST, Myc, FLAG, and poly-His residues. Integral residues in proteins may also be addressed, particularly for the capture of monoclonal antibodies (mAbs) from growth media. Here, antibodies specific for the Fc region of the mAb may be used. Other alternatives involve the use of protein A or protein G for selective IgG antibody capture.

The capture molecule is most frequently a protein (particularly an antibody), but can also be composed of organic molecules. His-tagged proteins can be selectively captured via the metal-chelating complex based on nitrilotriacetic acid (NTA) and nickel ions. NTA derivatives are immobilized to the sensor surface, either via coupling to carboxymethyl dextran [36, 37] or by use of SAM approaches [38]. The bond can easily be broken with a solution of a chelating agent like EDTA and the sensor surface is then reused after activation with a Ni^{2+} solution. The intrinsic affinity in the Ni/NTA–His bond, however, is relatively low (μM) [39] and this may be insufficient for robust assay performance. Variations of this method have therefore been developed, combining affinity-based NTA capture and covalent coupling via amine groups to NHS-activated carboxyl groups on the sensor surface [40].

Alternative capture systems have recently been described that involve peptide–peptide interactions. A heterodimeric coiled-coil peptide domain can be utilized by conjugation of an E-coil strand to the protein and reversible immobilization to the K-coil strand coupled to carboxymethylated dextran surfaces [41].

A special case of the capture approach involves the use of the avidin–biotin affinity bond. This very popular conjugation method is widely used and is also highly suitable for SPR sensors. In addition to their use as a linkage between biotinylated SAM layers and biotin-modified molecules [17], streptavidin and other avidin variants can also be conveniently immobilized to carboxymethylated dextran via amine coupling [32]. Biotinylation reagents of various kinds are commercially available, together with protocols for optimal modification. The high affinity of the biotin–avidin bond (10^{-12}–10^{-15} M) makes it practically impossible to break without destroying the immobilized avidin molecule and should therefore rather be considered as a covalent bond in its behavior. Chemical variants of the biotin structure and recombinant versions of avidin have been developed in order to diminish the high binding strength, but these are correspondingly less robust under capture assay conditions.

Antibody-based capture agents have also been used for both the His-tags and biotinylated molecules. Commercial antibodies are available, but in practice no general-purpose reagents that are optimal for all tagged proteins have been found. Depending on the type of molecule and method of tag introduc-

tion, the binding strength is affected on a case-by-case basis, and different antibodies may need to be tested to obtain the best performance.

Promising new approaches include the specific and covalent surface immobilization of fusion-tagged proteins. One recent example utilizes a fusion tag composed of a mutant of the human DNA repair protein O^6-alkylguanine-DNA alkyltransferase (hAGT) [42]. A derivative of O^6-bensylguanine was immobilized to carboxymethylated dextran surfaces and selective coupling of the fusion protein was obtained. Specific immobilization directly from crude cell extracts that expressed the hAGT fusion protein was also demonstrated.

3.3 Coupling Mediated via Lipid Layers

The interest in SPR-based detection of proteins interacting with lipids or in a lipid environment has steadily increased, particularly as many membrane-associated proteins are drug target candidates. Lipid membranes themselves can also be targeted, e.g., in the development of antibiotically targeted drugs. Lipids, being amphiphilic and normally without functional groups for covalent immobilization, are difficult to immobilize but approaches have been developed to overcome this problem. These approaches are based on the adsorption of lipid vesicles or liposomes to certain types of surfaces. In contact with planar surfaces, liposomes tend to unfold and create a well structured and densely packed lipid monolayer, in which the hydrophobic part of the molecule is oriented perpendicularly towards the surface [43]. The lipid head group faces towards the aqueous solution and can interact with analytes. The structure is sufficiently stable for SPR detection, but can easily disintegrate in the presence of detergents. Both hydrophobic and hydrophilic sensor surfaces have been shown to work, supporting the formation of SAMs from long-chain alkane thiols [44, 45]. The hydrophobic surfaces are particularly sensitive, however, to disturbances from minor impurities in the solutions used. The impurities can adsorb and interfere with the liposomes and care must be taken to obtain reliable results.

Alternative surface modifications have therefore been developed for the formation of lipid bilayers. Phospholipids modified in the head group with hydrophilic thiolated spacers have been utilized for anchoring lipid bilayers to gold surfaces [46]. Gold surfaces with hydrophilic polymers have also been modified with hydrophobic groups to which liposome structures can tether [47]. A surface based on carboxymethylated dextran modified with long alkyl chains was developed and provides a convenient support for immobilization of lipids [48]. Depending on the type of lipid and liposome preparation conditions, either intact liposomes or planar lipid bilayers are formed in contact with these types of surfaces [49]. Methods have also been developed for rapid and controlled formation of planar bilayers in flow-based systems [50]. As shown in Fig. 12, a mixture of lipids and detergents is first

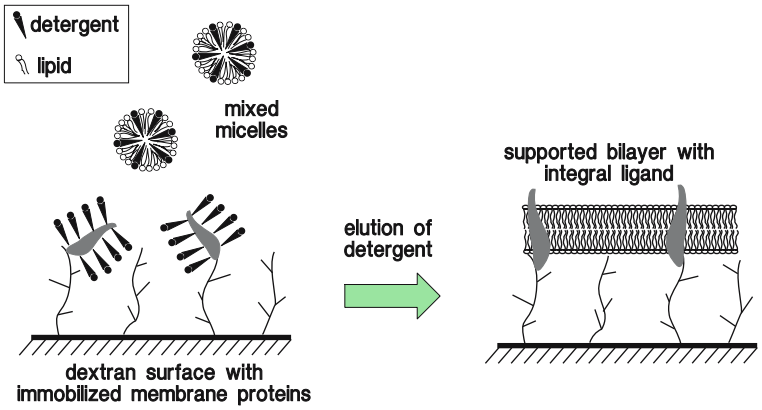

Fig. 12 On-surface reconstitution of immobilized membrane proteins with lipid bilayers by injection of mixed micelles and rapid detergent elution

injected, followed by a switch to pure buffer that depletes the detergent and leaves the lipid layer.

Alternative approaches for liposome assemblies have also been demonstrated, where histidine-tagged lipids have been introduced in vesicles, which were then anchored to chelator surfaces [39, 51]. Similarly, oligonucleotide-modified lipids can be incorporated in the vesicles and bind to complementary sequences immobilized on the sensor surface [52, 53]. The latter strategy can also be utilized for spatially resolved immobilizations.

Lipid bilayer surfaces are suitable for incorporation of membrane-associated protein receptors such as G protein-coupled receptors (GPCRs). This can be achieved by tethering vesicles with reconstituted receptors to the surface. Alternatively, solubilized proteins can be bound to the sensor surface followed by a rapid in-situ reconstitution by the lipid–detergent method [50] (Fig. 12). This method has the potential to produce receptor densities that are sufficient for use in SPR sensors. However, the widespread use of SPR in this field is still limited by the availability of membrane-associated proteins and their low stability, as well as by a lack of methods for handling them in a purified format.

3.4 Creating and Validating Functional Sensor Surfaces: General Comments and Practical Tips

Reliable SPR-based assays require solid foundations. In particular, it is important that the immobilization chemistry selected to couple a protein to the sensor surface does not interfere with its binding activity. The best immobilization strategy is one in which the immobilized partner is presented in

a conformation and orientation that allows as closely as possible the interaction to proceed as it would in vivo.

Although the structure of a protein may encourage the use of one type of immobilization chemistry in preference to others, the optimal strategy must be empirically determined. Efficient coupling, while important, must not be at the expense of activity. In this section, some immobilization chemistry options for potentially problematic interactants such as acidic proteins, are discussed. Secondly, novel ways to immobilize membrane proteins are presented and finally, some recently developed methods of thiol coupling are addressed.

3.4.1 Immobilization Strategies

3.4.1.1 Amine Coupling

Direct immobilization of proteins using amine coupling is the most commonly used strategy because most proteins contain many potentially reactive primary amine groups (Sect. 3.1.1). This method is cited in more than half of all published papers featuring Biacore systems. Proteins with an isoelectric point (pI) greater than approximately 3.5 can be efficiently preconcentrated close to the sensor surface by electrostatic attraction and immobilized in the presence of a buffer of around pH 5. A typical example of the SPR response during the course of immobilization of a protein via amine coupling is shown in Fig. 13.

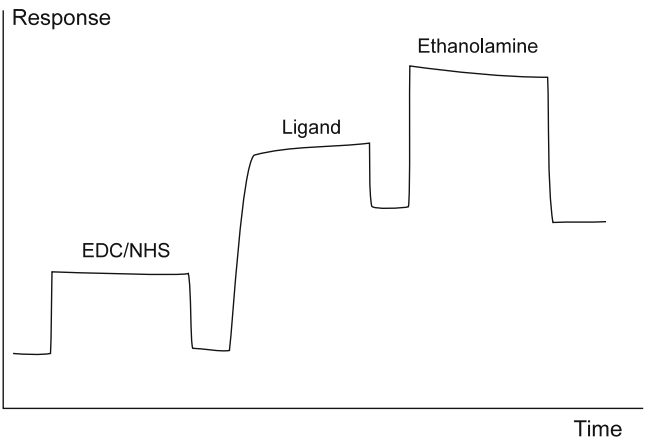

Fig. 13 Sensorgram for a typical amine coupling illustrating the distinction between the amount of protein bound and the amount immobilized

However, at this pH, highly acidic proteins (pI ~ 3 or less) carry a net negative charge and are repelled from the dextran layer. It is not possible to reduce the pH to accommodate the immobilization of highly acidic proteins because the dextran on the chip surface will protonate and become resistant to activation by EDC/NHS. In order to immobilize highly acidic proteins by amine coupling, it is necessary to modify the standard immobilization protocol. Some options are now described.

3.4.1.2 Micelle-Mediated Immobilization of Negatively Charged Proteins

A novel amine coupling method has been developed by Biacore in which an acidic protein is carried by a positively charged micelle (a cluster of oriented surfactant molecules). The micelle–protein complex carries a net positive charge at neutral pH, and is therefore attracted to the sensor surface (Fig. 14).

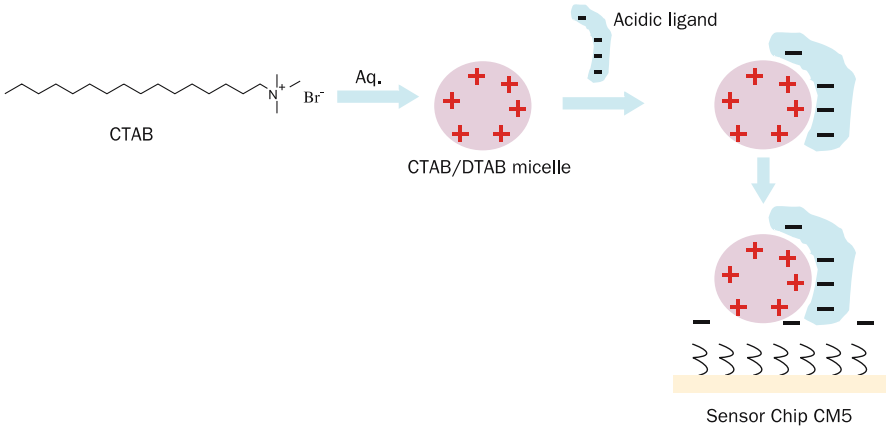

Fig. 14 Hexadecyl-3-methylammonium bromide (CTAB)/dodecyl-3-methylammonium bromide (DTAB) micelle-mediated immobilization of acidic proteins. CTAB/DTAB forms positively charged micelles in aqueous solutions. The micelles bind electrostatically to the negatively charged sensor surface and may thus be used as carriers for acidic proteins

3.4.1.3 Directed Amine Coupling: Protein Modification in Solution

Although immobilization of proteins by amine coupling does not usually inhibit the entropic freedom of macromolecules or significantly change their interaction properties [54], it is nevertheless desirable to present a protein to its binding partner in a directed and uniform orientation. This may be achieved by chemically modifying a specific region of the protein in solution

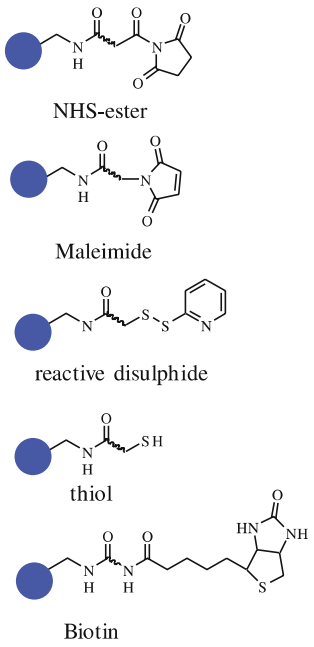

Fig. 15 Modification options. The *wavy lines* illustrate the presence of an undefined chemical structure between the amine group on the protein and the introduced functional group

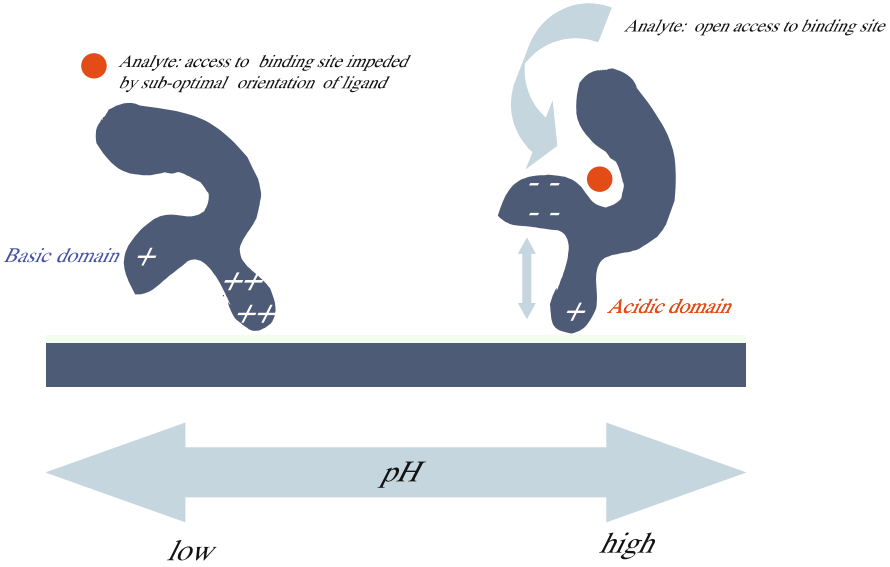

Fig. 16 A protein will have a net positive or negative charge according to the pH of the buffer in which it is dissolved. The distribution of the charges on the protein surface will influence the orientation in which it will approach and immobilize on a charged surface

in order to control the degree of modification or to steer the immobilization process. Some of the most commonly introduced modifications are shown in Fig. 15. Modification in solution may be considered, for example, when handling proteins with high or low pI in order to bring the pI close to the pH at which amine coupling proceeds efficiently.

Coupling at a specific pH may also affect activity, for example, it is possible to predict the orientation in which a protein will immobilize on the chip surface at a particular pH if the spatial distribution of charged amino acids is known (Fig. 16). Alternatively, the protein may be prevented from immobilizing on the surface via sites important to the interaction under study by having analyte present during immobilization, as discussed below [55].

3.4.1.4 Stabilization after Immobilization

Certain proteins are unstable and may deteriorate once they are on the sensor surface, even if the immobilization procedure has worked efficiently. HIV protease, for example, dissociates into its component monomers over time and the gradual reduction in molecular weight on the sensor surface is seen as a baseline drift on the interaction profile. This makes the kinetic analysis of interactions, particularly those involving small molecules, problematic. An extra post-immobilization activation/cross-linking step with EDC/NHS may be considered in order to achieve a stable baseline. The use of cross-linking must be empirically determined for each interaction and the process must not interfere with the activity of the protein.

3.4.1.5 Preservation of Activity During Immobilization

Stabilization during immobilization has been reported, where, for example, the protein kinases, p38α and GSK3β, were immobilized using amine coupling in the presence of a specific reversible inhibitor [56]. This treatment resulted in a more stable sensor surface with much higher specific activity for binding partners (Fig. 17).

3.4.1.6 Protein Stabilization After Capture

Capture protocols may be the most effective way to immobilize proteins, e.g., the protein may contain a molecular tag (Sect. 3.2). Consider, for example, Fig. 18, which depicts a capture system for measuring the binding of ATP or ATP inhibitor to an immobilized kinase.

The kinase in this case was tagged with histidine (His) and was captured on the sensor surface via an anti-His antibody. A capture system does not

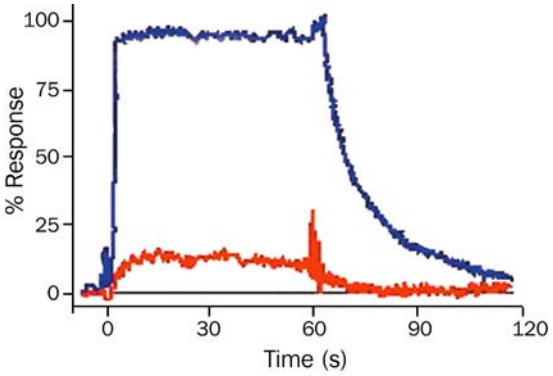

Fig. 17 Comparison of the surface binding capacity of unprotected and protected (stabilized) p38α. Inhibitor (1 μg) was injected over two flow cells containing p38α. One surface contained p38α immobilized using a standard amine coupling procedure (*lower trace*) while the second surface (*upper trace*) contained p38α stabilized by the inhibitor during immobilization

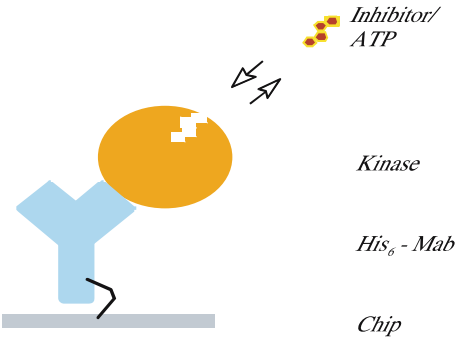

Fig. 18 Capture of a His_6-tagged kinase via an immobilized anti-His antibody

in itself guarantee a stable baseline but, by performing a subsequent cross-linking step, the kinase here was stabilized and remained active, yielding a surface that was open to detailed binding studies of ATP and ATP inhibitors. It is important to realize, however, that the capture surface may not be reused after stabilization.

Large baseline drifts caused by unstable proteins or poor capture may be overcome by using EDC/NHS as a cross-linking step. This step, however, may compromise protein activity if active sites are involved in the cross-linkages. The effect of cross-linking on activity, therefore, must be empirically tested for each system. In general, cross-linking should be as brief as possible; 15 s is often sufficient to achieve acceptable baseline stability, without compromising activity.

3.4.1.7 Immobilizing Membrane Proteins

Membrane spanning proteins are difficult to handle in vitro because they are often functional only when their hydrophobic transmembrane domains are maintained in a native structure. This conservation of structure requires close association with a hydrophobic milieu and presents a practical handling problem in the typical aqueous microenvironment of most in vitro systems.

However, the study of hydrophobic proteins using Biacore systems is possible using protocols specially designed for the purpose. For example, proteins may be firstly incorporated into liposomes and then immobilized on hydrophobic sensor surfaces (Sect. 3.3). It has been reported that lipid bilayers have been tethered to a sensor surface via hydrophilic spacers immobilized on a plain gold chip into which membrane-spanning proteins are then inserted [57]. The emphasis of this technique rests on encasing sensitive protein domains within a lipid microenvironment in which they can assume a native, functional structure.

A recently developed protocol for handling membrane proteins on Biacore's amphiphilic Sensor Chip L1 is *on-surface reconstitution* (OSR). In this process, the membrane protein is firstly solubilized in detergent. Then, it is immobilized on the sensor surface using amine coupling (although OSR may also readily be used in combination with capture). The next step is to inject mixed micelles, which bind to the lipophilic tails on the sensor surface and to the protein. Finally, the detergent is removed, inducing the lipids to form a plasma membrane-like bilayer, linked to the immobilized membrane protein via the natural affinity of lipids for the hydrophobic regions of the proteins (refer to Fig. 12). In this way it is possible to present a uniform, oriented field of plasma membrane proteins within a lipid bilayer on a sensor surface and to study how they interact with binding partners.

3.4.2 Thiol Coupling

Thiol coupling may be considered when using acidic proteins that do not preconcentrate at the chip surface at the pH required for optimal amine coupling, in cases where the protein contains few amino groups or where those present occupy the analyte binding site. Thiol coupling, like amine coupling, may also be considered as a means to control orientation on the sensor surface. Although thiol coupling is an established immobilization procedure, its versatility may be increased by introducing thiol groups to proteins, for example by using the thiolating agent, 2-iminothiolane (Traut's reagent). Here, the sensor surface is prepared for thiol coupling by firstly activating with EDC

and NHS followed by PDEA to introduce reactive disulfides. Primary amine groups on the protein are then modified to thiol groups using Traut's reagent. Thiol derivatization can be controlled to produce sensor surfaces with a very high binding capacity.

3.4.2.1 Surface Thiol Coupling

In additional to conventional thiol coupling, the same process may be followed by derivitizing carboxyl groups on the protein (instead of those on the sensor surface) with reactive disulfides. In this way, the protein may then be immobilized to a surface derivatized with thiol groups by treatment with Traut's reagent.

3.4.2.2 Thiol Coupling by Derivatization of Carbohydrate to Maleimide Groups

In this process, protein *cis*-diols or carbohydrates are firstly oxidized to aldehydes using sodium metaperiodate. The aldehyde groups are then modified to thiol-reactive maleimide groups using the bifunctional reagent, *N*-[ε-maleimidocaproic acid] hydrazide (EMCH). Immobilization using standard aldehyde coupling (via the conversion of diols to aldehydes, which then react with hydrazide groups introduced on the sensor surface) is well established. However, thiol coupling may enable the investigator to more tightly control the number of immobilization points between the surface and the immobilized partner. The maleimide-modified protein may then be immobilized on a thiol surface. Thiol coupling to a maleimide surface may also be performed by derivatization of carboxymethyl groups on the sensor surface. In this case, a maleimide surface is generated on the sensor surface, followed by immobilization of a protein possessing thiol groups via a thioether linkage. Proteins immobilized using maleimide coupling are more stable under reducing conditions than those immobilized using thiol coupling via disulfide formation because no thiol-disulfide exchange can take place.

3.4.3 Summary

Alternatives to amine coupling may be considered under a number of circumstances, e.g., when working with acidic proteins or with proteins that may be compromised in their biological activity due to immobilization via primary amino groups located in the analyte recognition site. Membrane spanning proteins may require a hydrophobic microenvironment at the sensor surface in order to remain biologically active and therefore must also be specially

Table 1 Immobilization alternatives to amine coupling

Immobilization protocol	Application examples
Micelle-mediated immobilization	When using highly acidic proteins
Protein modification in solution	To control and favorably orientate proteins on the chip surface for optimal analyte binding
Protein stabilization after direct immobilization or capture	When using unstable proteins, e.g., multimeric or autoproteolytic proteins or an unstable capture system
On-surface reconstitution (OSR) as an alternative to liposome-mediated coupling	When working with membrane-spanning or membrane-embedded proteins that require a hydrophobic microenvironment for their biological activity
Thiol coupling (protein or chip surface)	When using highly acidic proteins, proteins that contain few primary amine groups or where those present may be involved in analyte binding
Maleimide coupling (protein or chip surface)	When thiol coupling is indicated but when the assay is to be run under reducing conditions

handled. Table 1 lists the immobilization alternatives discussed in this section and some of their possible applications.

4 Molecular Recognition Elements

As should be evident from the preceding sections, different types of molecular structures create specific demands to achieve optimal coupling. It is therefore appropriate to describe the specific properties of the respective molecular classes used as interacting partners and to suggest the most suitable immobilization methods for each of them. A more extended general review of the modification and conjugation of different classes of interacting partners may also be found in [25].

4.1 Proteins

Proteins are the most widely used immobilization partners in SPR-based assays but, as a very diverse class of molecules, they are not amenable to a common immobilization strategy. The common denominator is the polypeptide

backbone where the individual amino acids supply the functional moieties that can be utilized for immobilization to the sensor surface. The most useful amino acids, together with their corresponding functional groups are: lysine (– NH_2), cysteine (– SH), asparagine and glutamine (– COOH), serine and tyrosine (– OH), and histidine (imidazole). The *N*-terminal amino acid residues also constitute a potential linkage moiety via their end amine group. Despite these similarities, the different physical properties related to charge balance and distribution, size, and thermodynamic stability make almost every protein unique with respect to the ease and success of immobilization.

Antibodies are the most homogenous protein class and are also the most frequently used recognition elements for different types of applications. Characterizations of binding properties are important in the selection of therapeutic and diagnostic antibodies and their derivatives. Antibodies are also used for capture of various molecules, or as binders in concentration assays. IgG-type antibodies are composed of an F_c subunit and two Fab′ subunits, constituting in total a molecular weight of 150 kDa [58]. The active antigen-binding regions are localized in the Fab′ subunits and so immobilization to surfaces should ideally be made via the F_c region [59]. Given the size of antibodies this is not normally a problem and they are among the easiest molecules to immobilize. A typical IgG antibody contains 50–70 lysine residues and, when using covalent coupling to an electrophilic functionality, the probability for immobilization via the F_c region is high. There are also alternatives utilizing the carbohydrate residues attached to the F_c region. Mild oxidation of these sugars with periodate generates aldehyde groups, which can react via hydrazide functionalities on the sensor surface. Another way for covalent immobilization involves digestion and reduction of the antibodies into Fab′ fragments, exposing a sulfydryl group from the cysteine residue in the *C*-terminal region. The sulfydryl group is oriented away from the antigen-binding region and can be used for covalent coupling to selective groups such as reactive disulfides and maleimide groups. Certain subclasses of antibodies can also be non-covalently immobilized via the F_c region to protein A or protein G molecules. Antibodies, therefore, constitute a class of proteins that do not normally create any problems in the immobilization step.

Other soluble proteins behave much more heterogeneously than antibodies. The general approach is similar, and covalent immobilization via nucleophilic residues like amine and sulfydryl groups are normally the first method of choice. However, a small protein may be more susceptible to deactivation due to a higher probability that functional groups involved in coupling are close to the interaction site. The most commonly used alternatives involve the introduction of recombinant tags, such as oligo-histidines and other short peptides. The proteins can bind to immobilized antibodies or other capturing agents that specifically recognize these tags. Similarly, recombinant proteins containing larger fusion domains such as GST or Myc can also be generated.

4.2 Peptides

Given their structural similarity to proteins, the principles governing peptide immobilization are comparable. Depending on the composition of the peptide, electrostatic attraction can be utilized in a similar manner as for proteins. Amine- or thiol-based coupling can also be performed under slightly alkaline conditions, using millimolar concentrations of the peptide. Although this straightforward coupling procedure works well in many cases, immobilization of small peptides may need alternative strategies. For example, there is a significant risk that amine groups originating from the lysine residues or the *N*-terminal amino acid are involved in the binding of small peptides to an interaction partner. A thorough analysis of the peptide structure and an evaluation of possible immobilization sites are therefore recommended. Synthetic peptides can be extended with suitable coupling groups. Extra lysines, for example, can be introduced to a region of the peptide that is not involved in the interaction. Spacers that include cysteine residues for use in thiol-based couplings are also favorable alternatives [60]. Another preferred alternative is to biotinylate the peptide in a specific position, optionally followed by chromatographic purification. A well-defined derivative is thereby obtained, which can then be immobilized to streptavidin-modified sensor surfaces.

4.3 Oligonucleotides

Oligonucleotides are composed of negatively charged nucleotide groups that are relatively resistant to covalent coupling under mild aqueous conditions. Although the phosphate ester groups can be used in condensation reactions with nucleophiles such as primary amines, the reaction is slow and water hydrolysis competes unfavorably with the desired reaction. Further, the nucleotide bases are weak nucleophiles and cannot be utilized under conditions normally used for coupling.

The most common alternative for immobilization of oligonucleotides involves the use of biotinylated derivatives. These are conveniently made with standard reagents for oligonucleotide synthesis and biotin can be added at both the 3′ and 5′ ends. Immobilization of the biotinylated oligonucleotide to avidin-modified sensor surfaces is performed in neutral buffer conditions and is normally efficient.

An alternative method has recently been developed that uses 3′ or 5′ amino-derivatized oligonucleotides for coupling to activated carboxymethylated sensor surfaces. Electrostatic repulsion between negative charges on both the surface and the oligonucleotides normally slow this type of reaction. Here, however, the oligonucleotide is mixed with a positively charged

detergent like hexadecyltriammonium bromide (CTAB). Under conditions of micelle formation, this leads to a complex that can neutralize the repulsion effects, with the positive micelle acting as a carrier of the oligonucleotide, which is attracted to the negative surface, greatly increasing coupling efficiency [61] (see also Sect. 3.4.1). When applied to carboxymethylated dextran surfaces, the oligonucleotide densities reach levels twice those of streptavidin surfaces.

4.4 Small Organic Molecules

Although applications have been dominated by interactions involving immobilization of proteins, the use of small organic molecules such as hormones, vitamins, and drug candidates with molecular weights typically lower then 700 Da is increasing. Normally, these types of molecules need to be treated differently to those previously described. The type and number of suitable functional groups available for coupling to the sensor surface is unique for each molecule and general immobilization procedures are not applicable. It may even be necessary to synthesize derivatives of the molecule with functional groups in desired positions. This may also be a necessary step in order to minimize interference between the analyte and the immobilized molecule. Also, many organic molecules have very low solubility in aqueous solutions and need to be handled in organic solvents such as DMSO and DMF (optionally in water mixtures) during immobilization. Derivatives of molecules to which groups are introduced to increase water solubility are therefore attractive options. Approaches using electrostatic attraction as described previously are not normally applicable for small molecules.

Molecules with functional groups like aliphatic amines, thiols, aldehydes, or carboxylic groups can normally be covalently linked to suitable corresponding active groups on the sensor surface, as described in Sect. 3.1. Amine coupling is normally performed under aqueous buffer conditions at a concentration between 1 and 50 mM at pH 7–8.5. Thiol coupling proceeds efficiently in near-neutral buffer conditions, while aldehyde condensation to hydrazide-modified sensor surfaces can be performed in a slightly acidic buffer or in the presence of a reducing agent such as cyanoborohydride. Following their activation to reactive esters, molecules with carboxylic groups can likewise be immobilized to sensor surfaces with amines or hydrazide groups. The activation step can either be performed before coupling, utilizing EDC/NHS, or in situ, in the presence of EDC or some other condensation agent.

Small molecules without suitable functional groups need to be derivatized, a procedure normally requiring significant synthetic organic chemistry efforts. This approach is advantageous as the tailoring of the molecular

structure creates conditions necessary for a successful outcome of the assay. In particular, the choice of type of functional group and position in the molecule can be optimized in order to avoid interference with other groups or parts of the molecule. The introduction of a spacer is also normally of value, both in order to reduce steric interferences and to increase water solubility. Details of various modification approaches can be found in the literature [25]. The introduction of general tags such as biotin is also applicable to small organic molecules. This route is attractive due to the commercial availability of different derivatives of biotin, optionally with spacer groups.

4.5 Carbohydrates

Interactions that involve carbohydrate structures are important in many biological events, including cell adhesion, apoptosis, and immune responses. Their interactions with proteins are normally weak in affinity and traditional techniques may be difficult to use. SPR detection can therefore be favorably utilized for these studies and the need for such analytical methods is expected to grow within the emerging field of glycomics. However, immobilization of carbohydrates may be a challenge, depending on the explicit nature of the substance.

The primary potential coupling sites for sugars are hydroxyl groups, although there is a high likelihood that the hydroxyls of small sugar compounds (e.g., mono- to oligosaccharides) are important for binding activity. Alternative strategies, similar to those used for other types of small molecules must therefore be applied. The most common approach is to use the anomeric aldehyde group for direct immobilization, or for modification in solution before immobilization. Aldehyde coupling as described in Sect. 3.1.3 is a good alternative in such cases. Alternatively, linker molecules with reactive groups such as thiols can be introduced [62]. The introduction of a biotin derivative is also a highly suitable route.

Polysaccharides can be immobilized in various ways depending on which functional groups are present. Glycosylated proteins can be considered as a special case, as described in Sect. 4.2. The main difference between "pure" polysaccharides and glycoprotein structures is that electrostatically mediated enrichment of the coupling cannot normally be used, depending on the presence of charged groups. For covalent immobilization via nucleophilic or electrophilic groups (e.g., reactive amines, aldehydes, or activated carboxyls) high concentrations of the polysaccharide need to be used and the resulting densities are still relatively modest. A better alternative is to biotinylate a suitable functional group before immobilizing the derivative to streptavidin surfaces, as shown for heparin [63].

5 Spatially Resolved Immobilizations

SPR detection is highly adaptable to multiplexed configurations in miniaturized formats. The flow cells in the original Biacore systems had four measuring spots positioned within a few millimeters (Fig. 19). Prototype systems with eight parallel flow channels have also been described and applied to food analysis applications [64].

In these systems, both the immobilized partner and analyte in solution are delivered to the sensor surface via an integrated microfluidics device. All the steps in the immobilization procedure can therefore be monitored by SPR detection and serve as an important guidance and quality check. The channels for fluid delivery and flow cell structures are made by micromolding of elastomeric materials and are produced with cell widths down to a few hundred micrometers [65]. Spatial distribution of immobilized partners is achieved by addressing the distinct flow cells individually.

The measuring spot density on several subsequently commercialized biosensors has greatly increased, allowing arrays to be probed and generating parallel interaction data. One recent development involves microfluidics systems with hydrodynamic addressing (HA) of the solutions (Fig. 19). By the

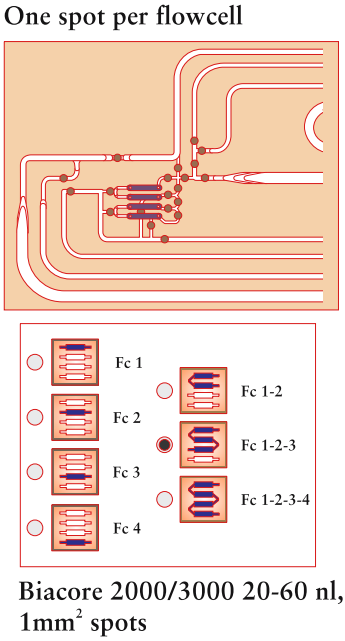

Fig. 19 Configurations and dimensions of various Biacore instrument flow cells. The left-hand flow cell is designed for hydrodynamic addressing

use of two parallel fluid inlets, solutions can be directed over different areas of a sensor surface in a single flow cell and can therefore be used for spatially resolved immobilizations [66]. Besides the obvious advantage of higher sample throughput, this flow cell system allows a more precise and detail-rich analysis to be performed. Firstly, as all interactions are measured simultaneously, highly accurate reference subtraction allows the measurement of very rapid kinetics. Further, by immobilizing several targets in one flow cell, interaction profiles may be directly compared under identical conditions.

In the Biacore A100 system containing four HA flow cells with five immobilization spots per flow cell, assays can be designed either for the maximum number of samples, or to deliver the maximum information per sample. In the first instance, identical immobilizations may be performed in all four flow cells, allowing four different samples to be analyzed in parallel during each analysis cycle, while in the latter, up to 20 different interactants may be immobilized across all four flow cells with one sample per cycle injected in parallel.

What types of application areas may best benefit from a high throughput array system that also delivers high quality kinetic data? As one example, the development of therapeutic mAbs, for example, is a complex and time-consuming process, involving generation, maintenance and above all, screening of thousands of hybridoma clones. Early identification of those hybridomas that produce the best candidate antibodies is a critical step in successful, cost-efficient development. Efficient screening of many hundreds of hybridomas would enable selection of candidates with the best prognoses for clinical success based on their kinetic properties. Secondly, even the most carefully designed and constructed biotherapeutics may be sensed as foreign proteins by the patient, causing an unwanted antibody response. The immunogenicity of newly developed drugs and vaccines is one area that could benefit from sensitive detection of potentially clinically relevant low/medium affinity antibodies, generating data on isotype, subclass specificity, and kinetics from a single system using low quantities of sera.

For larger 2D arrays, various approaches have been employed. Techniques developed during the 1990s, principally for DNA arrays have also been evaluated for SPR detection [67]. Depositions by contact or non-contact means have become the most common alternatives and several commercial arrayers are available. A general review of protein arrays can be found in [68]. One possible limitation with these approaches is that relatively high concentrations need to be employed, due to the requirement of high surface density of active molecules needed for SPR detection. The drop deposition of the solution must therefore be optimized to meet these needs. Ink-jet or piezoelectric printing devices that were originally employed for DNA applications can also be used for proteins, both in aqueous and organic solvents. However, careful optimization is normally needed when these are used for proteins in buffered solutions, as deposits and clogging of the ink-jet heads tend to occur, particular for solutions of high protein concentration. Tendencies for

smeared spots and an uneven spread of the density of the immobilized partner across the spot have also been reported.

Another important issue related to deposition of proteins is the tendency for denaturation. When spotting droplets of protein solution in the nanoliter range, evaporation of the solvent will quickly lead to air exposure. Alternative strategies have been employed to minimize evaporation, including printing in high humidity and using 40% glycerol solutions.

Photolithography has become a popular method for DNA arrays, with the Affymetrix approach for site synthesis of DNA probes. Similar approaches can be applied to spatially synthesize combinatorial libraries of peptides and other organic compounds [69]. However, applications within the protein array field are still in a preliminary state. Examples can be found in the literature [70, 71] where photomasks have been used to define the area for immobilization. After exposure to UV light in the presence of an immobilized partner, the surface is washed and the procedure repeated with a new mask and a new preparation of the molecule to be immobilized. The method may be limited by the risks for non-specific binding and the extended time needed to generate large arrays. Although it is tempting to perceive that functional protein arrays will be constructed on a similar scale to DNA arrays, the natural sensitivity of protein structure – essential for function – will almost certainly limit their size; it is simply not possible to control the biological activity of thousands of proteins extracted from their natural microenvironment and immobilized in vitro after lengthy and stringent amplification and purification processes. The functions of proteins are often dependent on domain integrity and demand precisely defined microenvironments. For example, the conformation of transmembrane receptors are difficult to predict in vitro, when the predominantly hydrophobic membrane-spanning domain is dissolved in an aqueous solution, rather than integrated in the protective and stabilizing milieu of the cell membrane. Variables such as immobilization conditions, orientation, and the possibility that the immobilization process may impede or conceal the very binding site of interest are further important considerations. Finally, unwanted adsorption to both the array surface and other proteins in a complex mixture may complicate the interpretation of results from a multiplexed array.

6 Outlook

The development of tools for the immobilization of molecules to sensor surfaces has been dramatic over the last two decades. This has been driven by the availability of better surface analysis techniques, including the commercialization of sensitive and reliable systems, as well as better tools for manipulation of proteins and other biomolecules. Growing interest in under-

standing biomolecular interactions on a quantitative level have also driven this development. These trends will almost certainly continue to drive the field of immobilization. The availability of large protein libraries for proteomics applications is increasing, which will drive the need for larger scale protein interaction analysis. This leads to a demand for higher throughput systems and more parallel and streamlined immobilization strategies. Better tools and techniques for the creation of 2D arrays will likely emerge, with the twin objectives of improving control over immobilization levels and retaining the activity of the protein.

Another field of development relates to improved tags for surface coupling and other conjugation steps. Fusion tags have great potential as immobilization sites to surfaces and can be used for general parallel coupling methods of arrays. However, several of the presently available tags are based on protein or peptide structures that significantly increase the size, which may cause expression problems during the protein fusion step. An additional consequence here is that the tag size may affect the activity of the protein. The limited stability in the bond between the tag and the capturing molecule can also be a problem. Consequently, it can be hoped that smaller tags with better binding properties to their capture partners will be developed in the coming years.

Considering the importance of membrane-associated proteins (particularly GPCRs) as drug targets, the limited range of options for sensor surface immobilization will certainly stimulate continued efforts to find better methods. Increases in the attainable surface densities of membrane proteins with sustained drug binding activity are necessary for their interrogation using label-free SPR detection methods. Some promising advances have been described during recent years [50, 52, 53] and these methods are likely to be further improved. The techniques to express, purify, and reconstitute membrane-associated proteins will also advance, increasing the options for their immobilization.

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