Chapter 2
Surface Sensitization Techniques and Recognition Receptors Immobilization on Biosensors and Microarrays

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Abstract The quality of a biosensing system relies on the interfacial properties where bioactive species are immobilized. The design of the surface includes both the immobilization of the bioreceptor itself and the overall chemical preparation of the transducer surface. Hence, the sensitivity and specificity of such devices are directly related to the accessibility and activity of the immobilized molecules. The inertness of the surface that limits the nonspecific adsorption sets the background noise of the sensor. The specifications of the biosensor (signal-to-noise ratio) depend largely on the surface chemistry and preparation process of the biointerface. Lastly, a robust interface improves the stability and the reliability of biosensors. This chapter reports in detail the main surface coupling strategies spanning from random immobilization of native biospecies to uniform and oriented immobilization of site-specific modified biomolecules. The immobilization of receptors on various shapes of solid support is then introduced. Detection systems sensitive to surface phenomena require immobilization as very thin layers (two-dimensional biofunctionalization), whereas other detection systems accept thicker layers (three-dimensional biofunctionalization) such as porous materials of high specific area that lead to large increase of signal detection. This didactical overview introduces each step of the biofunctionalization with respect to the diversity of biological molecules, their accessibility and resistance to nonspecific adsorption at interfaces.

Keywords Functionalization · Biofunctionalization · Surface chemical modification · Native biomolecules · Modified biomolecules · Staudinger ligation · Click-chemistry · Native chemical ligation · Expressed protein ligation · Silanization · Self-assembled monolayer · Entrapment · Nanoparticles · Sol–gel process · Adsorption · Chemisorption · Silica · Silicon · Gold layer · Streptavidin · Biotin ·

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Protein · DNA · Carbohydrate · Enzyme · Ligand capture · Protein capture · Site-directed immobilization · Site-specific immobilization

Abbreviations

μ-TAS	Micro-total analysis system
AAPS	N-(2-aminoethyl)-3-aminopropyltrimethoxysilane
ALD	Atomic layer deposition
APTS	Aminopropyltriethoxysilane
Asp	Aspartic acid
BSA	Bovin serum albumin
DIOS	Desorption/ionization on silicon
DMP	Dimethyl pimelimidate
DMS	Dimethyl suberimidate
DMSO	Dimethylsulfoxide
DNA	Desoxyribonucleic acid
DTT	Dithiothreitol
EDTA	Ethylenediamine tetraacetic
ELISA	Enzyme-Linked immunosorbent assay
ENFET	Enzymatic field-effect transistor
EPL	Express protein ligation
ET	Electron transfer
Glu	Glutamic acid
GOD	Glucose oxidase
GPTS	3-glycidoxypropyltriethoxysilane
IDA	Iminodiacetic acid
IPL	Intein-mediated protein ligation
ISE	Ion-selective electrodes
ISFET	Ion-selective field-effect transistor
ITO	Indium Titanium oxide
Lys	Lysine
M_2C_2H	4-(N-maleimidomethyl)cyclohexan-1-carboxylhydrazide
MALDI	Matrix-assisted laser desorption/ionization
MESNA	2-Mercaptoethansulfonate
MPAA	(4-carboxymehtyl)thiophenol
mRNA	Messenger ribonucleic acid
NCL	Native Chemical Ligation
NHS	N-hydroxysuccinimide
NTA	Nitrolotriacetic acid
ODN	Oligodesoxyribonucleotides
PAMAM	Poly(amino)amine
PCP	Peptide carrier protein
PCR	Polymerase chain reaction

PDITC	Phenylenediisothiocyanate
PDMS	Polydimethylsiloxane
PEG	Poly(ethylene glycol)
PNA	Peptide nucleic acid
SAM	Self-assembled monolayer
SIAB	Succinimidyl 4-(N-iodoacetyl)aminobenzoate
SMCC	Succinimidyl-4-(N-maleimidomethyl)cyclohexane-1-carboxylate
SMPB	Succinimidyl-4-(N-maleimidophenyl)butyrate
SPDP	N-succinimidyl-3(2-pyridyldithio)propionate
s-SIAB	Sulfosuccinimidyl 4-(N-iodoacetyl)aminobenzoate
TCEP	Tris(2-carboxyethyl)phosphine
TEOS	Tetraethoxysilane
TMOS	Tetramethoxysilane

2.1 Introduction

Sensors and further biosensors can be straightforwardly depicted as devices that convert a physical or biological event into measurable (electrical) signal. Biosensors exhibit two elementary parts; the sensitive part where specific biological events take place and a transducing system that mediates the biological events into quantifiable signal. The transducer work relies on various physical principles: mechanical (e.g. weight or topographic measurement), optical (e.g. fluorescence emission, absorbance of surface evanescent waves) or electrochemical (e.g. potentiometric or conductimetric measurements). Common feature of detection techniques is the biosensitive layer that confines the biological event in the very close vicinity of the transducer. A target biomolecule is detected when a specific interaction/recognition takes place in the sensitive layer where a bioreceptor has been attached (tethered probe, ligand or substrate). The specific interaction gives rise to a "chemical signal" at the surface that the transducer is sensitive to. Instances of chemical signals are variation of local concentration of ionic species or pH in the sensitive layer, formation of absorbing or fluorescent complex species, electron transfer to a conducting surface, variation of refractive index of the sensitive layer, etc. The performance of a biosensor comes from (1) its ability to immobilize receptors while maintaining their natural activity, (2) the bioavailability (accessibility) of the receptors to targets in solution, (3) a low nonspecific adsorption to the solid support. These specifications govern the specificity and sensitivity of such devices and can be tailored by an appropriate choice of the solid–liquid interface where the bioreceptors are immobilized. The chemical preparation of the surface is a key parameter; the physico–chemical properties of the interface play an important role in achieving optimal recognition of the target and limiting the nonspecific adsorption. Lastly, the stability of the sensitive part of a biosensor depends on the

sustainability of the surface functionalization. Immobilization of bioreceptors through robust and stable covalent bonds is a good means to gain stability. The optimum elaboration and use of biosensors calls for sure background knowledge about the surface preparation and immobilization process of biospecies.

This presentation gives a detailed overview of the individual steps of surface modification (functionalization) and immobilization of bioreceptors (biofunctionalization) onto solid supports. In the first section, the adsorption of biomolecules to solid–liquid interface is addressed. The simple utilization of adsorption and its main drawbacks are presented. A definite improvement in terms of reliability, selectivity, and sustainability brings in covalent immobilization as an alternative to adsorption. The main coupling strategies allowing covalent immobilization of native biomolecules are reviewed, together with the design of the surface properties aimed at improving the accessibility of analytes in solution by oriented immobilization of modified biomolecules. The last section introduces the chemical modification of a wide variety of solid supports used (flat solid supports, porous materials, polymer coatings and nanoparticles) as biosensing elements.

2.2 Adsorption, Chemical Grafting, and Entrapment

2.2.1 From Adsorption to Grafting, a Historical Perspective

The development of biological analysis techniques based on the detection of solid-supported biomolecular interactions began in the 1970's. Thus, ELISA (Engvall and Perlman 1971), Southern blot (Southern 1975) and Northern blot (Burnette 1981) techniques have been introduced and still remain widely used. The principle is simple and relies on the specific recognition between a molecule in solution and a partner molecule immobilized onto a solid support. Immobilization makes the detection easier and more sensitive because the molecules are concentrated on the surface and the recognition events to be detected are precisely localized. The molecular biologists familiar to the blotting techniques define the "unknown" molecule that is to be identified as the "target" and the well-characterized molecule that recognizes the target as the "probe." In the original blotting techniques, the target is immobilized on the support and probe is in solution. Immobilization of the molecules from the analyte solution proceeds through adsorption. Therefore, the surface where the recognition takes place is chosen such that most molecules of interest readily adsorb. Adsorption is not specific and there is no need for such techniques.

These standard blotting techniques consist of assaying a panel of probe molecules in solution by exposing them to the target immobilized on a solid support. This is conducted in parallel experiments; the number of individual tests corresponds to the number of available probe molecules. The reverse approach is much more suitable for large scale analysis. Modern blotting techniques consist in attaching known probes to a surface and contacting them to the target solution

as a single test. Several probes are attached to the same support as localized spots. A large number of different probes can be analyzed during a single test depending on the density of spots, therefore on the area of support in contact with the solution and on the spotting technology.

This method, where known chemical species are immobilized on a surface, takes after the so-called modified electrodes that electrochemists were developing simultaneously during the same period (Guilbault and Montalvo 1969, 1970). Thus, electrodes are made specific by immobilization of chemical species at their surface. The chemical reaction with the analyte at the electrode surface gives a chemical event (red-ox reaction, pH change, etc.) that is detected. The transduction is therefore limited to electrochemical detection.

In the 1980s, the blotting techniques evolved to multispots formats (multispot or dot) called arrays. These progresses are largely due to better control of the chemistry and physical chemistry of the support surface, synthesis of modified biomolecules, and manufacturing technologies of spot arrays. Early tests by blotting techniques were conducted on a small number of individual spots, giving "low-density arrays" or "macroarrays." The biological solutions were simply deposited as spots on glass slides or sheets of polymer materials (nitrocellulose or Nylon membranes for instance). More sensitive detection was achieved by using porous membranes of large available surface area that allowed larger loading capacities. The traditional process aimed at the immobilization of biomolecules is quite rough; it consists in depositing a drop of solution and letting it dry on the surface. Therefore, the biomolecules are presumably adsorbed.

There are several drawbacks related to the immobilization by means of adsorption only. Adsorption depends on the interaction of the biomolecule and the surface, so that the amount of adsorbed molecules may vary from spot to spot. Release to the solution is possible when the solid support is immersed in the analyte solution; this causes a loss of the signal and possible cross contamination of the spots. The reversal of the roles of probes and target (now probes are immobilized) allows the immobilization process to work again. Adsorption was used at the early beginning because it was very simple and could be readily implemented in the biology research laboratory. Since the probes are known molecules that are isolated and purified before their deposition by spotting, optimized chemistry can be done. Modified probe molecules are used to improve their immobilization. A decisive progress is made by replacing the adsorption by chemical grafting. Chemical grafting firstly avoids release to solution, which is the main drawback of adsorption. Other definite improvements are several. Chemical grafting allows reducing the background noise coming from nonspecific adsorption because optimized surface chemistry can be implemented for that purpose. Thorough cleaning of the surface (repeated rinsing) is possible in order to remove the nongrafted molecules and contaminants. Lastly, the device may be reusable if a correct regeneration process is devised. Possible reutilization is often a valuable benefit, even for disposable devices, because one wishes to repeat the analysis under the same condition in order to calculate a statistical mean value, the standard error of the mean, and to assess the variability of the sample. Therefore, the tendency replaces adsorption by chemical grafting.

Technological advances have made it possible to carry out chemical reactions at the micrometer scale. The full benefits of microtechnology or nanotechnology can be achieved if the chemical and physico–chemical properties of the solid surface are improved in parallel. The adaptation of immobilization technique to miniaturization is made while preserving the bioactivity and increasing the accessibility of target molecules in solution. For instance, the use of elaborated devices for the localized synthesis of oligonucleotides onto a solid surface at the micrometer scale allows the manufacture of DNA microarrays with several hundred thousand spots per square centimeter (Gershon 2002). Besides technological improvements, new methods are intimately related to progresses in the field of molecular biology and synthesis and/or modification of biomolecules (e.g. protein engineering, fusion proteins, tagging).

Definite improvements have involved new grafting methods to the surfaces but their implementation in the everyday practice of the users took much time. Although early attempts to oriented covalent linkage (one anchoring point preserving of the recognition sites) of DNA date back to 1964 (Gilham 1964), most biosensors or affinity tests up until the middle 1990s relied on simple adsorption or random grafting of the bioprobes (reaction through the DNA exocyclic amines (Millan et al. 1994) or phosphates (Sun et al. 1998)). Progresses in regio-specific grafting reactions emerged when the optimization of rough strategies reached their limits. Uncontrolled immobilization is only suitable for biological tests where the quantity of available biological products is not limited or for detection of a single species such as in ELISA tests.

Efforts have been focused on DNA analysis before the year 2000 which lead to the advent of DNA chips. Proteins chips came few years later (MacBeath and Schreiber 2000). Although the technology is currently able to achieve over 400,000 parallel measurements in a single test (DNA chip), the complexity of biological molecules leads to many artifacts that make the exploitation of these “high-density” microarrays troublesome. A tendency was to increase the amount of information (the density of spots) in order to collect redundant information that could be used to eliminate false responses. Bioinformatics appeared and became an independent discipline that the end-users could not control. Because of such difficulties, a current trend in proteins microarrays is lowering the number of parallel measurements to improve the robustness of the results.

Therefore, the increase of chemical signal allowed more sensitive analyses and miniaturization of the devices, giving “high-density arrays” (microarrays). This straight idea is to be tempered because the loss of selectivity is a limitation. Increasing the signal by improving the biochemical recognition sensitivity (higher density and bioavailability of attached probe molecules) often leads to the concomitant loss of selectivity. On the contrary, decreasing the background noise by preventing false recognition events improves the signal-to-noise ratio and the selectivity. Controlling the physical chemistry of surface preparation and grafting process is the way that helps improving the detection sensitivity and selectivity.

Lastly, the technologies derived from blotting techniques may fail in some specific cases because the confinement of the biochemical recognition at a

transducer surface is troublesome. An example is the recognition by carbohydrates and lectins that is very sensitive to the local environment. This requires looking for quite new strategy of analysis. As example, new strategies make use of molecular recognition in solution. Methods based on Biobarcode (Lehmann 2002; Nam et al. 2003; Winssinger et al. 2004; Diaz-Mochon et al. 2006; Bornhop et al. 2007; Chevolot et al. 2007) and nanoparticles are presented in the last part of this review.

Finally, new biosensor paradigms deal with miniaturization and integration of different features ranging from the preparation of the biological sample to the detection (micro-Total Analysis System (μ-TAS) or lab-on-a-chip). The adaptation of the surface chemistry is once again sought to tailor surfaces with different functionalities depending on the compartment system.

2.2.2 Adsorption and Grafting: Physical Chemistry and Thermodynamics

2.2.2.1 Surface Interactions or Chemical Grafting?

There is a confusion regarding the precise nature of adsorption, physisorption, chemisorption and chemical grafting because the words do not have the same acceptance in different scientific communities. It is difficult to eliminate the confusion coming from contradictory definitions. Explanatory definitions are given here in order to replace semantic disputes by scientific understanding. A more detailed discussion of the same type can be found in (Rouquerol et al. 1999).

Adsorption from solution takes place if the molecules interact with the surface by means of attractive interactions. Therefore, part of the molecules goes to the surface and a residual part remains in solution. A dynamic equilibrium is established where molecules in solution adsorb at the surface, while adsorbed molecules leave the surface. The system is equilibrated when the rate of adsorption and desorption are equal. The surface concentration and residual concentration are related, the larger the concentration in solution, the larger the adsorbed amount. The surface concentration is termed surface excess; it is expressed in mol/m^2. From a thermodynamic point of view, the standard chemical potential of the molecule at the surface is lower than that in solution, so that the molecules are in a more favorable environment at the surface. The interactions may be of various physicochemical origins and the standard free energy of adsorption may have enthalpic and entropic contributions of various relative magnitudes.

Grafted molecules are attached to the surface by means of a chemical bond (or several). Grafted molecules do not leave the surface, even if repulsive interactions are operated.

The difference between the adsorbed and grafted is really large and has important implications regarding sensor application. Adsorbed molecules are in

equilibrium with a residual concentration in solution. Molecules of the same nature are present in solution and should not be removed because it would shift the adsorption equilibrium. On the contrary, grafted molecules are irreversibly attached, so that the solution may be free of residual grafts in solution. Residual concentration can be safely removed from the solution by rinsing the surface.

Therefore, there are two major differences between adsorbed and grafted molecules:

- There is a residual concentration of molecules in solution in case of adsorption and such molecules are able to do the chemical recognition in bulk solution. Such unwanted molecules can be eliminated in case of grafting.
- Adsorbed molecules can be desorbed according to the reversible adsorption equilibrium, whereas grafted are irreversibly attached. In particular, rinsing out a surface-bearing adsorbed molecules causes washing them off.

The above view is oversimplified however. The difference between adsorption and grafting is a matter of binding energy. Adsorption involves interactions between molecules with interaction energies of the same magnitude as thermal energy. Therefore, Brownian motion can cause adsorption and desorption. The energy of a chemical bond is orders of magnitude larger, so that it is considered irreversible. But there are molecular interactions that are in between the two. The hydrogen bond can easily be broken. Polymers that bind to surface sites by several anchoring points cannot be desorbed by rinsing the surface with the pure solvent; adsorption looks irreversible, actually it is very slow (Fleer et al. 1993).The very strong biotin–avidin complex behaves like a chemical bond. A chemical bond can also be cleaved. For example, an ester bond hydrolyses when it is immersed in water and the hydrolyzed grafts can react again with the free surface sites. This process that looks like an adsorption–desorption process is so slow, however, that hydrolysis is called a chemical degradation. There is actually no fundamental difference between surface hydrolysis and desorption, this is just a matter of energy of activation that sets the release rate. The same indefinite difference is found between chemical reaction and complex formation in bulk solution.

The difference between physisorption and chemisorption is also a matter of interaction energy and this adds to the general confusion. Physisorption is adsorption by means of weak (physical) interactions while chemisorption involves stronger (chemical) interactions. Some authors consider chemisorption as irreversible attachment, therefore chemical grafting. The crossover from physical to chemical has never been precisely given in terms of energy (it depends on who is speaking), so that this is just a semantic discussion.

2.2.2.2 What are the Different Forces Acting on Molecules at the Vicinity of Surface and What About the Specificity?

The interactions that are responsible for adsorption are the same as in solution. Adsorption of an invited molecule to a host surface site can be viewed as a complex

formation where one of the partners is attached to the surface. The interactions are the same in this case. The presence of adsorption sites is not necessary however; attraction to the surface is enough. As example, adsorption of a hydrophobic molecule (surfactant) from aqueous solution to the oil–water interface does not involve localized adsorption sites. Generally speaking, specific adsorption often requires well-defined adsorption sites.

The interactions that are most relevant in the manufacturing and functioning of chemical sensors are the following:

- Hydrophobic interaction comes from the poor solvation of molecules or surface by water. Hydrophobic molecules are pushed one to each other by water solvent. This interaction is not specific since every nonpolar molecule or material is hydrophobic.
- Electrostatic interaction is either attractive or repulsive according to the respective signs of the electrically charged partners. This is also highly nonspecific since it only depends on the electrical charge and the ionic strength of the medium. The precise nature of the ionic species does not matter. Electrostatic interaction is strong and long-ranged.
- Polar interactions such as dipole–dipole interactions are electrostatic in origin but are not called as such. They are weaker and short-ranged, they can be either attractive or repulsive according to the orientations of the dipoles or multipoles.
- Hydrogen bond is an acid-base interaction that involves acidic and basic sites. It is oriented but not specific. The presence of both acidic and basic sites is enough for bonding.
- Complex formation involves sharing external electron orbitals of partners. It is highly oriented and closely depends of the chemical nature of the partner, so that it is specific.

Therefore, most interactions are not specific. Selectivity, especially chemical recognition comes from the combination of several localized interactions. Even the hydrophobic interaction can become specific when it is localized. As an example, proteins bear well-localized hydrophobic domains that can favorably interact with a patterned hydrophobic/hydrophilic surface if the separation between hydrophobic areas fit the distance between the hydrophobic domains of the protein. A single hydrogen bond, although oriented, is not specific; high selectivity is often brought about by combination of several oriented hydrogen bonds between the partners as for example between the bases of nucleic acids or in the biotin–avidin complex.

2.2.3 Kinetic Aspects of Adsorption, Desorption, and Grafting

- The adsorption rate is related to the concentration in solution. High concentrations are preferred and often compulsory, even if the fraction that remains finally adsorbed is small. In case of scarcely available materials, slow adsorption from

dilute solution is a problem. Drying the solution on the surface helps the adsorption of the dilute probe to be adsorbed but also forces the unwanted materials to the surface.

- Very slow desorption allows working quite safely in sensor applications. Adsorbed polymers desorb very slowly because of the multiple anchoring to the surface (Fleer et al. 1993). It is often considered irreversible. Therefore, polymer deposits are quite satisfactory and bear further surface treatments. Immobilization by means of entrapment of large species (proteins) in gels relies on the same idea. This is not a permanent attachment but tight entrapment which slows down enough the release with respect to the time scale of the utilization.
- Grafting is irreversible but involves a chemical reaction that is very slow if the probe is too dilute. Therefore immobilization by grafting materials of poor availability may be long. Long reaction time leaves opportunities for side-reactions, chemical degradations, etc. Adsorption causes a local increase of concentration at the surface that accelerates the grafting reaction. It is therefore often wise to perform the grafting reaction under conditions of strong adsorption and go back to weak adsorption conditions for recovering the selective biochemical recognition and low background signal.

2.2.4 Nonspecific Adsorption as a Source of Background Signal

In sensor applications, the target molecule should bind to the surface by specific interactions only in order to obtain a selective detection. Nonspecific interactions cause adsorption of unwanted molecules that induce an interfering signal. This is an important origin of background signal (noise). There are so many nonspecific interactions listed above that reducing the background signal is a difficult task.

The difficulties encountered with early devices relying on adsorption come from the uncontrolled nonspecific adsorption. Thus, sensitizing a surface by adsorption of a selective probe to the surface requires an interaction that remains active after the functionalization. A high adsorbed density aimed at obtaining a high detection level requires a strong interaction of the probe with the surface and consequently a high interaction of everything with the surface, leading to a high background signal. Typically adsorption of a negatively charged probe protein can be achieved to a positively charged surface; but every anionic species present in the analyte will bind to the surface by nonspecific interactions and the consequence will be a disastrous selectivity. Therefore, functionalization by adsorption does not allow to reach high sensitivity.

Grafting allows much more freedom because the physico-chemical characteristics of the surface can be changed after grafting the probe without wondering about probe release to the solution. Repulsion of grafted molecules out from the surface does not cause the loss of the attached probes and often appears

advantageous because the recognition site is pushed far from the surface, increasing the bioavailability.

2.3 Classification of the Main Immobilization Pathways

2.3.1 Specificities of Biomolecules for Chemical Coupling

Most of the molecules implied in biomolecular or cellular recognition mechanisms are water-soluble (nucleic acids, proteins, carbohydrates, etc.). Exceptions to this rule are integral membrane proteins that are permanently attached to biological membranes. Therefore, the coupling of biological molecules onto solid support should preferably proceed in aqueous solvent.

In addition to the solubility constraints, the preservation of the activity of immobilized molecules requires keeping nondenaturizing conditions. For example, temperature, salt and pH conditions are the important factors for proteins stability; the immobilization process should be adapted to such conditions. For instance, proteins are spotted to arrays surface in aqueous buffered solution containing large amount of low volatility moistening agent like glycerol to prevent evaporation of the droplets (MacBeath and Schreiber 2000; Lee and Kim 2002). The probes to be immobilized in their native (active) form are mainly coupled to the surface via reactive functional groups such as amino, hydroxyl, thiol, carboxyl and aldehyde groups. Covalent immobilization must avoid using the functional groups that are involved in the functional conformation of biomolecules (base stacking, protein folding) or/and in the recognition mechanisms.

Lastly, biological molecules are considered as chemical reagents during the immobilization process. Therefore, their concentration should preferably be that of conventional chemical reactions of organic chemistry, that is approximately 10 wt%. The concentration of reagents sets the reaction rate; it should not be calculated from the very small amount required for covering the surface with a dense layer. This is a difficult issue because biological molecules such as DNA, proteins or carbohydrates are available in small quantities owing to their expenses. So, the coupling step of biological molecules onto solid support is often done under unfavorable conditions with diluted aqueous solution in the micromolar range (Dugas et al. 2004).

Main strategies of immobilization of biomolecules rely on (1) physical adsorption onto solid support; (2) entrapment into polymeric networks (gels); (3) chemical grafting by means of covalent binding onto reactive groups of native molecules; (4) chemical grafting onto modified biomolecules (linker, polymerization); (5) biochemical approaches to conjugate modified biomolecules (e.g. tag expressed protein).

Obviously, immobilization chemistry must be specific, fast and provide stable chemical bonds. Large scale, high-throughput manufacturing of arrays also requires that the chemistry is simple and reproducible (Podyminogin et al. 2001).

Robustness is also important because the chemistry is to be implemented in technology or biology laboratories that are neither equipped nor skilled for undertaking sophisticated chemistry under strict conditions.

2.3.2 *Strategy of Chemical Grafting*

Biomolecules cannot be coupled directly to the surface. Therefore, a chemical modification of the surface is performed before the biomolecule immobilization step itself. The surface modification often involves several steps, at least two. The most difficult step of the grafting process is the binding of the biomolecule. Indeed it has to accommodate the different constraints presented above: aqueous solvent, low concentration, low temperature, etc. The strategy consists in choosing the grafting reaction of the biomolecule first and adapting the surface functionalization to this choice. Once the chemical functionality of the surface is chosen, a process is devised for attaching this reactive function to the surface. The surface chemistry of the transducer is the next constraint to be managed. Indeed, the transducer in not chosen for its ability to be grafted but for its sensitivity to the chemical signal. Typical surfaces are inorganic materials such as silica, glass, gold, etc. There is not so much choice regarding reactions and reagents for grafting organic molecules to such inorganic surface. The involved chemistry is often quite vigorous, that is, not compatible with the functionality to be attached. For example, silanes cannot contain carboxylic acid or hydroxyl group; primary amino group is compatible with ethoxysilane but reacts strongly with chlorosilane. Because of this limitation, a coupling agent is often used in a second step after the first derivatization step performed directly on the transducer surface. A coupling agent is a difunctional molecule that reacts by one end to functionalized surface and leaves its second group for further reaction with the biomolecules. Coupling agents (Fig. 2.1) are

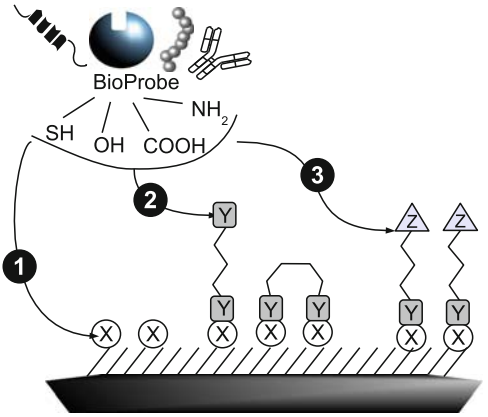


Fig. 2.1 Mains strategies of chemical grafting; (*1*) direct reaction to active surface, (*2*) activation of the surface with homodifunctional linker, and (*3*) activation of the surface with heterodifunctional linker

either homodifunctional if the two reactive functions are identical (e.g. phenylenediisothiocyanate) or heterodifunctional if they are different. The coupling agent is used for functionalization of the surface and/or for the chemical modification of the biomolecules as well.

The reactions of biomolecules to functionalized surfaces will be presented first, followed by a second part introducing the surface functionalization in several steps.

2.3.3 *Chemical Grafting of Native Biomolecules and Associated Surface Biofunctionalization*

2.3.3.1 Immobilization Through Amino Groups of Biomolecules

The most widely used route to conjugate biomolecules to solid supports makes use of reactions involving primary amino groups ($-NH_2$). The utility of amines stems from their high nucleophilic character and the existence of a wide variety of amine-base coupling chemistry suitable for use under aqueous conditions. The lysine residues of proteins have amino groups in ε-position. Figure 2.2 summarizes the main coupling routes of amines. Many reactions groups are acylation reactions leading to C–N bonds.

The unprotonated amino group is the nucleophilic (thus reactive) form of amines that exists in basic medium. The reactivity of amines in water depends on the pH according to the equilibrium between the protonated (acidic) and unprotonated (basic) forms given by the pK_a value. Increasing pH shifts equilibrium toward the unprotonated form and makes the reaction kinetics faster. A side-reaction occurring at high pH is the hydrolysis of acylation reagents that is observed for long reaction

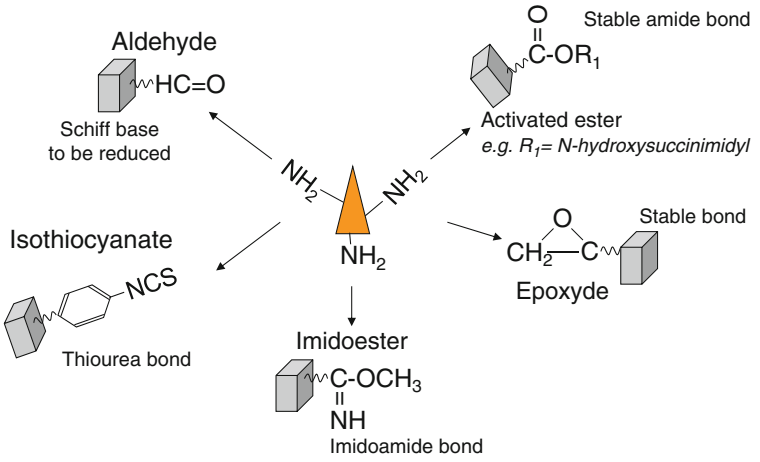


Fig. 2.2 Mains strategies of chemical grafting via amine groups

times in water. Anhydrous polar organic solvents are best suited if the biomolecules can resist. The competition between the acylation of amines and hydrolysis depends on the relative concentrations of amine and water. Hence, high concentration of amine is preferred.

2.3.3.1.1 Functionalization Using Epoxide

Surface immobilization of biomolecules onto solid support has been extensively studied for peptide synthesis (Anderson et al. 1964) and chromatographic separation since the 1960's (Sundberg and Porath 1974). Two efficient and suitable chemical routes (epoxy and activated ester) have emerged. They are currently used to immobilize molecules through their amine groups. As example, affinity chromatography columns are prepared by cross-linking reactive glycidyl groups to agarose (Sundberg and Porath 1974). Addition of biomolecules to epoxide ring involves nucleophilic primary or secondary amine, sulfhydryl groups or, less commonly hydroxyl groups (Wheatley and Schmidt 1999). The reaction rate of amine addition is optimal at pH = 11 (Sundberg and Porath 1974) because the hydrolysis reaction rate does not increase much in the pH range 7–11 (Wheatley and Schmidt 1999). The epoxy-activated phases have been criticized as being unreactive when compared to the other activated phases because of the low reactivity of amino groups at neutral pH (Ernst-Cabrera and Wilchek 1988). Milder conditions must be used in case of pH-sensitive molecules or solid support (Sundberg and Porath 1974). In particular silica or glass that are soluble in alkaline medium. Consequently, the use of strong alkaline solutions during immobilization of DNA to glass slides leads to inconsistent results because of the degradation of the underlying silane layer (Pirrung et al. 2000). Such activated supports give excellent results when favorable conditions are met with (Kusnezow et al. 2003; Oh et al. 2007).

Chemo-selective covalent coupling of hydrazide (R-$NHNH_2$) to epoxide-coated surface has been reported. The hydrazide group reacts more rapidly than thiol and amine functional groups (Lee and Shin 2005).

Epoxide functionalization of organic or inorganic supports is quite easy when compared to some other functional groups. Silica or glasses are derivatized by silane coupling agent (3-glycidoxypropyltriethoxysilane) or coated with epoxy-resins. Surface alcohol or amine groups of polymer substrates can be cross-linked by difunctional monomers (e.g. 1,3-butanediol diglycidyl ether) or by photografting glycidyl methacrylate monomers (Eckert et al. 2000).

2.3.3.1.2 Functionalization Using Activated Ester

Esters of N-hydroxysuccinimide (NHS-ester) are conveniently prepared from reaction of carboxylic acid and NHS under mild conditions in the presence of carbodiimide coupling agent (Cuatrecasas and Parikh 1972; Staros et al. 1986).

NHS-ester as other activated esters (e.g. the sulfonated ester derivative of N-hydroxysulfosuccinimide that has higher water solubility), N-hydroxybenzotriazole, p-nitrophenol, tetrafluorophenol allow acylation of primary amines in high yield. Aminolysis of these activated esters is notably much faster than hydrolysis, enabling reaction to be achieved in aqueous medium (Lee et al. 2003; Salmain and Jaouen 2003). Unlike other nucleophilic reactive groups, NHS-ester has low activity with secondary amines, alcohols, phenols (including tyrosine) and histidine. NHS-esters can be stored for long periods in cold and dry conditions. NHS-ester functional groups are thus versatile; they are widely used in peptide synthesis, preparation of biomolecules conjugates, and in surface immobilization (ligand affinity chromatography; biosensors and microarrays).

2.3.3.1.3 Functionalization Using Aldehyde

Aldehyde reacts reversibly with amines to form Schiff's base (imine group) (equilibrium 1 in Fig. 2.3). In order to get sustainable immobilization, imine groups must be reduced into stable secondary amines by borohydrides treatment, for example (reaction 2 in Fig. 2.3). It is worth noticing that this reaction deactivates residual unreacted aldehyde groups into alcohol groups that do not cause nonspecific adsorption. Aldehydes are moisture-sensitive and are easily oxidized into carboxylic acids (Zammatteo et al. 2000).

Functionalization of solid support by aldehyde reactive groups is classically performed by oxidation of alcohol function (Zammatteo et al. 2000) or reduction of alkenes (Hevesi et al. 2002) and can be also obtained by modification of aminated surface by glutaraldehyde (Yao 1983; Yershov et al. 1996).

2.3.3.1.4 Imidoester Functionalization

Difunctional imidoester (also called imidoether) reagents, dimethyl pimelimidate (DMP) or dimethyl suberimidate (DMS) (Fig. 2.16a) for example, react

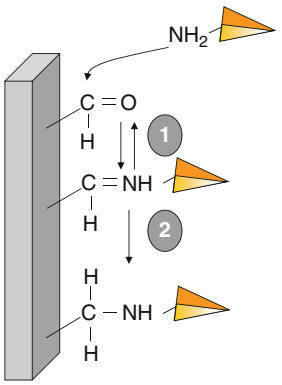
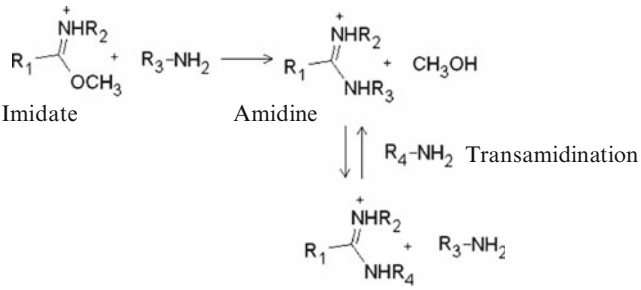


Fig. 2.3 Reaction between aldehyde and amine groups (*1*) and its subsequent stabilization by chemical reduction (*2*)



Scheme 2.1 Proposed mechanism of amidination and transamidination reaction (Adapted from Sundaram 1979)

rapidly with primary amines to form amidine bonds. Although imidoester homodifunctional cross-linkers have been used in biochemistry to study protein structure, only few examples concern the immobilization of ligands to solid support (Morris et al. 1975; Beier and Hoheisel 1999). Hydrophobic activated supports seem stable against wet storage (Sundaram 1979) when compared to soluble homologues which are extremely sensitive to moisture (half life of a few tenths of minute).

The reactive species are the neutral amino form of the primary amine and the protonated form of the imidoester (called "imidate") that both exist in different pH domains. Therefore, an optimum pH is determined between pH 7 and 10 (Sundaram 1979).

The amidine bond formed retains a net charge at neutral pH. It is important to note that amidine undergoes a subsequent transamidination in the presence of another nucleophile (Scheme 2.1). Thus immobilization through amidine bond may be reversible (Sundaram 1979).

2.3.3.1.5 Isothiocyanate Functionalization

Isocyanate (R-NCO) group is highly reactive but also amenable to deterioration during storage. Isothiocyanate (R-NCS) group is a less reactive alternative to isocyanate. It is quite stable in water and reacts with nucleophiles to yield thiocarbamate (reaction with thiol groups) or thiourea bonds (reaction with amines). Reaction of isothiocyanate on amine groups is well-known in biochemistry and serves to N-terminal peptide/protein sequencing. Despite this well-known reaction, immobilization of biomolecules to isothiocyanate functional groups proceeds mostly through reaction with thiol groups.

The preferred route to activate support with isothiocyanate functional groups implements modification of aminated support with phenylenediisothiocyanate cross-linker (PDITC) (Guo et al. 1994; Thiel et al. 1997; Beier and Hoheisel 1999). The thiourea bond is stable for use in microarray applications (Guo et al. 1994; Lindroos et al. 2001). It has been reported however that the antibody

conjugates prepared with fluorescent isothiocyanates showed degradation over time (Banks and Paquette 1995).

2.3.3.2 Immobilization Through Sulfhydryl Groups of Biomolecules

The chemical immobilization of complex recognition molecules like proteins onto the active layer of a biosensor is quite challenging. Specific concerns are: (1) attach these molecules by one anchoring point in order to obtain a repeatable, uniform and oriented layer; (2) avoid alteration of the active sites or denaturation that leads to loss of activity. The regio-specificity of immobilization using either amine or carboxylic acid moieties is difficult to control because there is large number of these hydrophilic residues (Lys, Glu, Asp) on the periphery of proteins where binding takes place. This leads to heterogeneity of the attachment and random orientation of the protein on the surface. Unlike amine or carboxylic groups, proteins possess a limited number of cysteine residues at their periphery. Covalent coupling strategies using thiol groups allow site-specific reactions. The sulfur atom of cysteine belongs to form a sulfhydryl (or thiol) group. The low likelihood of cysteine's presence or accessibility in protein restricts the use of thiol groups to immobilization of native molecules. This turns out advantageous in order to introduce solvent accessible reactive groups on protein surface. Cysteine residues involved in disulfide bonds can be chemically or enzymatically cleaved (Brogan et al. 2003) before a subsequent reaction with activated surfaces. A nondenaturizing method of cleavage of internal disulfide bridges of proteins by UV-light has recently been reported (Neves-Petersen et al. 2006). Proteins that do not bear cysteine residues can be genetically modified to engineered site-specific thiol groups distal from the active sites of the molecule (Firestone et al. 1996; Yeung and Leckband 1997; Backer et al. 2006).

Thiol groups allow very specific reactions because they are very reactive toward various chemical functions that are quite stable in water. The simplest approach is the reaction of thiol group onto supported thiol groups by formation of disulfide linkage. But disulfide bonds are unstable under reducing conditions. Alternatives involve formation of stable thioether bonds by Michael addition on maleimide groups or reaction with haloacetamide groups.

Thiols are prone to self-oxidation. The reactions of oxygen with thiols in aqueous solution give disulfides quite easily (pH 7–9). Radiolytic oxidation is also possible (Bagiyan et al. 2003). In order to regenerate the free reactive form, disulfide dimers are cleaved immediately before the coupling reaction (Chassignol and Thuong 1998). Dithiothreitol (DTT), β-mercaptoethanol or tris(2-carboxyethyl)phosphine (TCEP) are common disulfide-reducing agents. An additional step of elimination of the reducing agent from the solution before coupling the biomolecule to the support is required.

Phosphorothioates do not form dimers (Chassignol and Thuong 1998) and function as nucleophiles without prior reduction (Zhao et al. 2001). The elegant reaction

of phosphorothioates modified oligonucleotides to bromoacetamide activated support (Pirrung et al. 2000) avoids the oxidation side-reaction of thiols into disulfides.

2.3.3.2.1 Sulfhydryl Functionalization

The use of disulfide bond for immobilization of biomolecules is of particular interest for oriented immobilization of antigens and other proteins. A chemical reduction step induces cleavage of the disulfide bridge in the Hinge region of antibodies, yielding two fragments formed of a heavy and light chain and a free thiol group. The disulfide bridge is located distal from the antigen-binding site. Immobilization through this precise anchoring point allows oriented immobilization of antigen while maintaining its availability to antibody recognition. Reversible formation of disulfide linkage with thiol-functionalized surface have been investigated on glass slides modified with mercaptosilane coupling agent (Neves-Petersen et al. 2006).

N-succinimidyl-3(2-pyridyldithio)propionate (SPDP or sulfo-SPDP, Fig. 2.16) reacts with surface amino groups by the NHS moiety to form stable amide bonds (Gad et al. 2001; Hertadi et al. 2003). The 2-pyridyl disulfide group at the other end reacts with sulfhydryl residues borne by biomolecules to form a disulfide linkage (Ohtsuka et al. 2004). Interestingly, 2-pyridyl disulfide can be selectively reduced to thiol, even in the presence of other disulfide groups. It is worth to note that SPDP heterocross-linkers are also used to add thiol functionalities to nonthiolated proteins (Carlsson et al. 1978) for their subsequent specific immobilization.

2.3.3.2.2 Maleimide Functionalization

The double bond of maleimide undergoes a Michael addition reaction with sulfhydryl groups to form stable thioether bonds. It is also reactive toward unprotonated primary amines and hydroxyde anions (Shen et al. 2004). Interestingly, reaction of maleimide and sulfhydryl groups is specific in the pH range 6.5–7.5. The reaction of maleimide with sulfhydryl at pH 7 proceeds at a rate 1,000 times faster than the reaction with amines.

As depicted in Fig. 2.16, maleimide surface functionalization is achieved by coupling hetero-difunctional cross-linkers to surface terminal amino groups. During and after derivatization of surface amine groups by difunctional cross-linkers, maleimide groups are exposed to reaction with amino groups. Once grafted, the close proximity of the maleimide groups and residual amino groups remaining on the solid support may lead to a fast decrease of surface activity. A capping reaction of the amine groups with a large excess of highly reactive reagents such as isothiocyanate or succinimidyl ester groups is to be made in order to avoid the deactivation by residual amines. The main maleimide heterodifunctional cross-linkers aimed at immobilization of biomolecules (Chrisey et al. 1996; Strother et al., 2000a, b; Shen et al. 2004) are the succinimidyl-4-(N-maleimidomethyl)

cyclohexane-1-carboxylate (SMCC) and succinimidyl-4-(N-maleimidophenyl) butyrate (SMPB) or its sulfonated analogue sulfo-SMPB. Maleimide-activated solid supports cannot be stored for long time because of base-catalyzed addition of water to the imide bond and ring-opening the maleimide by hydrolysis (Shen et al. 2004; Xiao et al. 1997).

2.3.3.2.3 Haloacetyl Derivatives Functionalization

Condensation of thiol with haloacetyl moieties (bromoacetyl, iodoacetyl) creates stable thioether bond. A comparative study (Chrisey et al. 1996) of the iodoacetamide and maleimide reactive groups for oligonucleotide grafting indicated a strong dependence of immobilization performance with the solvent used. Functionalization of aminated supports with haloacetyl functional cross-linkers is currently done by succinimidyl-4-(N-iodoacetyl)aminobenzoate (SIAB) or its sulfonated analogues sulfosuccinimidyl-4-(N-iodoacetyl)aminobenzoate (s-SIAB). Direct functionalization of glass slides with bromoacetyl functional groups has been implemented by modification of clean glass with haloalkylsilanes (Firestone et al. 1996; Pirrung et al. 2000; Zhao et al. 2001). The very reactive iodoacetamido-functionality is sensitive to light and hydrolysis, so that the bromoacetamide group may be preferred.

2.3.4 Immobilization of Modified Biomolecules

The modification of biomolecules in perspective to their specific immobilization was considered for various purposes. These modifications are aimed at implementing a specific immobilization process such as electropolymerization (pyrole-derivatized biomolecules), or determining a site-specific anchoring point (protein tag, chemo-selective reactive site and/or site-specific reactive groups) for a uniform and oriented immobilization.

2.3.4.1 Covalent and Selective Immobilization of Biomolecules

The sensitivity and selectivity of biosensors depend on the quality of the interface and the activity of the tethered biomolecules. This activity is intimately correlated to the surface configuration of the immobilization, that is, the active site must remain accessible (Fig. 2.4).

Straightforward immobilization techniques rely on either adsorption, or direct covalent attachment of biomolecules to chemically activated surfaces (MacBeath and Schreiber 2000). The adsorption of proteins to interfaces causes possible denaturation and/or limits the availability of active sites. Adsorption is often associated to losses of activity and poor selectivity. Because adsorption is reversible,

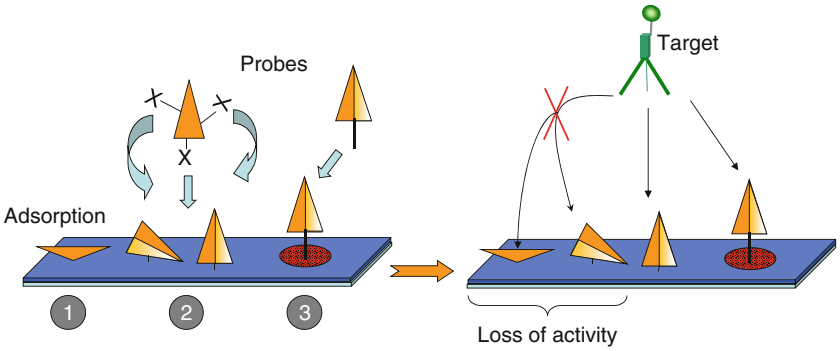


Fig. 2.4 Schematic representation of the different way of bioprobes immobilization. A probe can be adsorbed to the surface (*1*), immobilized by random reactions (*2*) and site-specifically immobilized to the surface. The adsorption and random immobilization results in loss of activity compared to the uniform orientation of site-specifically immobilized bioprobes

the surface does not withstand long-time reaction or washes aimed at enhancing sensitivity and selectivity. Unlike DNA, there is a large diversity of proteins of various size, physico-chemical properties, solubility or biological activities. Reactive amino or thiol groups of large protein molecules are scattered throughout the biomolecules. Hence, grafted proteins are randomly immobilized under various orientations. Protein immobilization after site-directed modification of proteins allow selective attachment of proteins to a solid support in a uniform orientation (Hodneland et al. 2002). Modifications are made by recombinant peptide tags, fusion proteins, or posttranslational modifications. Proper orientation of the immobilized proteins compared to random immobilization improve the biological activity (homogeneous-binding activity, kinetics of reactions) and thus measured signals (Firestone et al. 1996; Vijayendran and Leckband 2001; Zhu et al. 2001).

The diversity of biomolecules led to various specific strategies. The specific immobilization of DNA strands, antibodies, proteins and small molecules by means of oriented attachment is addressed in this overview.

2.3.4.2 DNA Probes

Nucleic acid stands are linear polymeric molecules formed by a chain of nucleotides borne by a sugar-phosphate backbone. The four nucleotides (Adenine, Guanine, Cytosine, Tyrosine) of the DNA sequence display internal amino-groups that are involved in the complementary base-pairing hybridization. These internal amino-groups must stay free and fully functional after immobilization.

Two main strategies are used to elaborate DNA microarrays. Prefabricated DNA strands such as long c-DNA fragments or synthetic oligonucleotide probes can be individually addressed on a solid support by micro-deposition techniques. The use of contact or noncontact deposition techniques allows manufacturing high-density

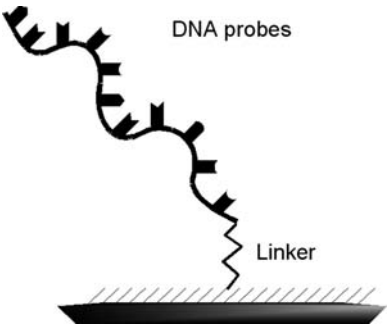


Fig. 2.5 Schematic representation of oriented DNA probe immobilization

DNA microarrays of up to 50,000 distinct spots on a glass slide. Another approach takes advantage of the progresses in DNA synthesis to fabricate each sequence *in situ* on the glass slide. The phosphoramidite chemistry of the standard DNA synthesis on cartridge has been modified and combined to spatially addressable techniques in order to synthesize in parallel up to 500,000 oligoprobes on one square centimeter(Fodor et al. 1991; Maskos and Southern 1992a, b). The DNA synthesis is beyond the scope of our discussion and we will focus on the immobilization of prefabricated DNA strands at the biosensor surface.

DNA strands can be uniformly and selectively immobilized by means of a reaction at one of their 5′ or 3′ end chain (Fig. 2.5). DNA molecules are conveniently end-modified by a reactive moiety called linker. A wide variety of linkers are available on synthetic oligonucleotides. Short DNA strands (oligonucleotides) modified by amino-, thiol-, hydrazide, phophorothioates or biotin-linkers are currently used for DNA immobilization. Similar modifications can be introduced into long DNA strands during PCR amplification using 5′ end-modified oligonucleotide primers (Saiki et al. 1989; Guo et al. 1994; Pirrung et al. 2000; Raddatz et al. 2002; Han et al. 2005).

Key parameter is to prevent interaction between the nucleobases and the surface, it has been reported that DNA molecule become totally inaccessible for hybridization when only 3% of its bases are involved in the covalent linkage (Bunemann 1982).

End modification of DNA allows not only introducing a site-specific group for covalent and oriented attachment, but allows inserting a spacer link between the nucleic acid probes and the surface. The spacer is intended to improve the mobility of the immobilized probes and thus their accessibility by the complementary strands, and move away the DNA probes from the surface to limit the adsorption and steric effects of the surface. Large improvements of hybridization yields have been reported by optimization of the spacer molecule and surface coverage (Guo et al. 1994; Herne and Tarlov 1997; Shchepinov et al. 1997). Indeed, accessibility is hampered by steric and electrostatic hindrances for too tightly packed surface-bound DNA strands.

After immobilization onto reactive surface, unreacted groups on the surface must be deactivated. In order to limit the adsorption of DNA molecules, deactivation involves hydrophilic molecules (e.g. alcohols, poly(ethylene oxide)). The widespread manufacturing of DNA microarray with DNA on glass slides involves amino-modified DNA probes. DNA probes are selectively tethered by their amino terminus because the terminal primary amino groups are much more reactive than the internal amino-groups of the DNA bases (Beier and Hoheisel 1999; Dugas et al. 2004).

Beside these standard covalent immobilization techniques some punctual works report on original DNA immobilization techniques such as silanized nucleic acids (Dolan et al. 2001), benzaldehyde-modified oligoprobes (Podyminogin et al. 2001). The direct and oriented immobilization of unmodified oligonucleotides onto zirconium-functionalized solid support takes advantage of the stronger interaction between the terminal phosphate group, $ROPO_3^{2-}$ compared to the phosphodiester groups of the backbone (Bujoli et al. 2005). This allows specifically tethered oligoprobes by means of oriented configuration. The incorporation of short segments of guanine oligomers as spacer, leads to a twofold increase in hybridization signal.

2.3.4.3 Antibodies

Owing to their high specificity, antibodies are widely used for purification/concentration of specific molecules in biological fluid by immunoaffinity chromatography or for diagnostic purposes in immunoassays. Whatever the nature of the attached molecular probe, uniform layers of well-oriented molecules are obtained by site-directed reaction, keeping the binding site accessible. Three specific reaction sites leading to three main strategies (Lu et al. 1996) of oriented immobilization are used for antibodies (Fig. 2.6).

The “biochemical approach” takes advantage of the selective binding of the Fc fragment of antibodies to specific receptors (protein A, protein G). This approach involves a first biofunctionalization of the solid support by Fc receptors (Vijayendran and Leckband 2001; Grubor et al. 2004; Briand et al. 2006) and subsequent binding of the antibody by its Fc fragment.

The two others approaches involve chemical immobilization of antibodies to solid support:

– The reduction of the disulfide bonds linking the heavy and light chain of the Fab fragment in the Hinge region is made by chemical, photochemical or enzymatic treatments. This creates site-specific thiol groups far from the binding sites (Brogan et al. 2003). Straightforward immobilization of antibody fragments containing thiol site-specific group can be realized directly onto gold surface (Brogan et al. 2003). However, higher immobilization efficiencies are obtained on thiol reactive surfaces.

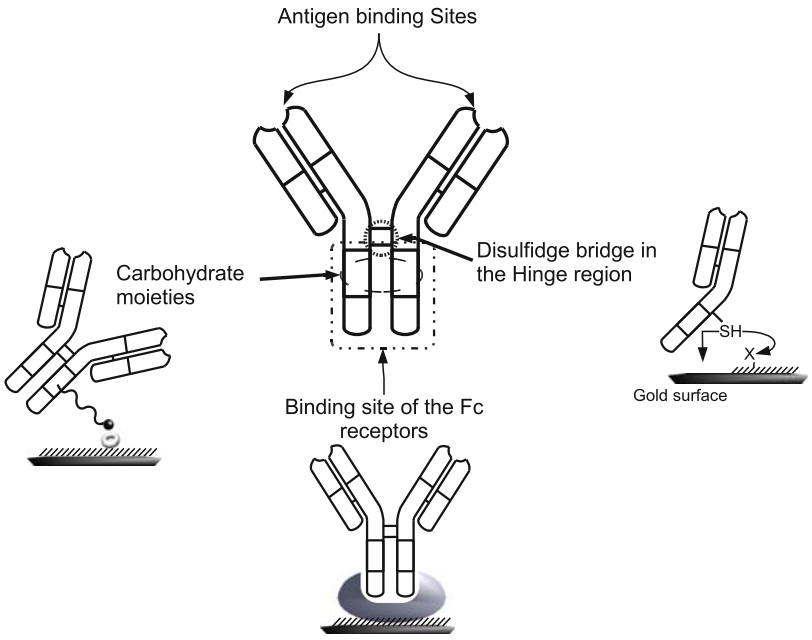


Fig. 2.6 Schematic representation of antibody molecule with the three sites dedicated for oriented immobilization

– A reactive site-specific group is created on the carbohydrate moieties located at the CH_2 domain of the Fc fragment. The carbohydrate is modified by specific ring-opening. For example, sodium periodate ($NaIO_4$) oxidizes vicinal diols of the carbohydrate into aldehyde reactive groups. Enzymatic oxidation (Solomon et al. 1990) with neuraminidase and galactose oxidase have also been reported for avoiding possible damages of the antigen-binding site during chemical oxidation. Direct grafting of antibodies is obtained by reaction of the aldehyde groups onto amino or hydrazide functionalized solid supports (Arenkov et al. 2000). Indirect grafting using heterodifunctional cross-linkers (4-(N-maleimidomethyl)cyclohexan-1-carboxylhydrazide (M_2C_2H), or Biotin-LC-hydrazide) also gave excellent results (Vijayendran and Leckband 2001). The hydrazide end group of the cross-linker reacts on the aldehyde carbohydrate to form a stable hydrazone bond.

2.3.4.4 Proteins

The production of proteins relies on the expression of genetically modified (cloned) host cells (e.g. *Escherichia Coli*, Yeast, phage). Uniform and specific attachment of proteins onto solid surface involves the incorporation of an accessible site-specific group onto the proteins. The processes for the immobilization the site-specific

reactive groups must be compatible with the functional groups of proteins and proceed under mild conditions in aqueous solution. Preferred modifications of native proteins are either genetic recombination of peptide tags or biochemical grafting of ligands (e.g. biotin).

2.3.4.4.1 Genetic Fusion of Peptide Tags

Fusion of site-specific peptide reactive moiety to native proteins is obtained with high selectivity by genetic recombination. Modifications are mostly done at the N-terminal or C-terminal extremity of native proteins in order to limit potential interference on the biological activity upon fusion of the reported group. Because oriented immobilization is intended to improve the biological activity, it must be ascertained that the genetically modified biomolecules display identical biological properties (folding, accessibility of the active site and the site-specific group for attachment, binding affinity) as the unmodified molecules or that cell expression is not underexpressed or truncated (Kindermann et al. 2003; Backer et al. 2006).

Various reported groups have been investigated for site-specific immobilization of proteins to functionalized solid supports. Straightforward approaches implement the simple incorporation of one amino acid (e.g. cysteine residue) or oligopeptide (e.g. polyhistidine tail). The thiol group of cysteine can be used for the thiol specific binding reactions to the surface as described in the previous section. Immobilization by means of a selective reaction to a thiol group limits the reaction of other naturally occurring nucleophilic groups of the protein (amino groups of lysines). Polyhistidine tails are selectively immobilized onto metal-chelate surface.

Other approaches involve species of larger size such as enzymatic domains or protein splicing elements. Proteogenic sequences have been used to covalently attach fused proteins to the solid surface or to introduce chemo-selective reactive sites or affinity tags in a subsequent posttranslational modifications step.

2.3.4.4.2 Metal-Binding Peptide Ligands

One-third of all proteins are metallo-proteins, and many of the reactions that are most critical to life are catalyzed by metallo-enzymes (Rosenzweig and Dooley 2006). Metal cations bound to specific peptide sequence or protein folding are active sites of many biological processes (oxygenic photosynthesis, nitrous fixation, replication and transcription). Selective adsorption of peptides and proteins comprising neighboring histidine residues was achieved to Ni^{2+} and Cu^{2+} ions bound to the surface (Giedroc et al. 1986; Hochuli et al. 1987). Metal ions form octahedral coordination complex with six ligands coming from the functional surface or the protein. Ligands such as iminodiacetic acid (IDA) (Fig. 2.7) or nitrolotriacetic acid (NTA) (Hochuli et al. 1987; Schmid et al. 1997; Cha et al. 2004) are subsequently covalently bound to solid surface and loaded with the metal cation. The tridentate IDA and quadridentate NTA chelating group form respectively three and four

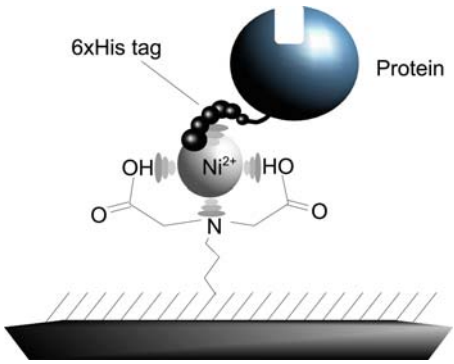


Fig. 2.7 Schematic representation of the metal-binding immobilization. The surface is chemically modified by iminodiacetic acid (IDA) moiety. The biomolecule is tag with a polyhistidine tail represented by the six black balls

coordination complexes. The remaining coordination sites filled by water molecules are readily displaced by stronger ligands (histidine) of the protein (Hochuli et al. 1987). Proteins genetically modified with polyhistidine tails (at least six histidines) at their N- or C-terminus were prepared for their oriented grafting by a chain end. Poly-His tag generally does not interfere with the structure or function of proteins (Cha et al. 2004). Uniform and oriented immobilization of 5,800 fusion proteins onto nickel-coated glass slides gave a protein microarray of higher sensitivity when compared to the protein immobilized through primary amines reactions on aldehyde coated slide (random orientation) (Zhu et al. 2001).

Limitations with respect to the immobilization through interaction between His-tagged proteins and metal complex attached to the surface come from the reversibility of the interaction (Schmid et al. 1997). The His-tag can be removed from the metal-bearing surface by exposure to reducing conditions, EDTA, detergents, large excess of ligands (imidazole).

2.3.4.4.3 Binding of Biochemical Affinity Ligands

Some very specific, stable and efficient biochemical interactions have been used to tether proteins to solid support by means of selective and oriented bond. Among the different partners investigated, the streptavidin–biotin system is the most widely used and known system to immobilize biomolecules onto solid support. Other recombinant affinity tag systems based on leucine zippers (Zhang et al. 2005) or split-intein systems (Kwon et al. 2006) efficiently attach recombinant proteins to support via uniform and oriented way.

2.3.4.4.3.a Streptavidin–Biotin System

Oriented immobilization of fused proteins can be made using affinity ligands of high selectivity. The very specific interaction of biotin and streptavidin is characterized by the strongest known noncovalent interaction ($K_a = 10^{15}\ M^{-1}$).

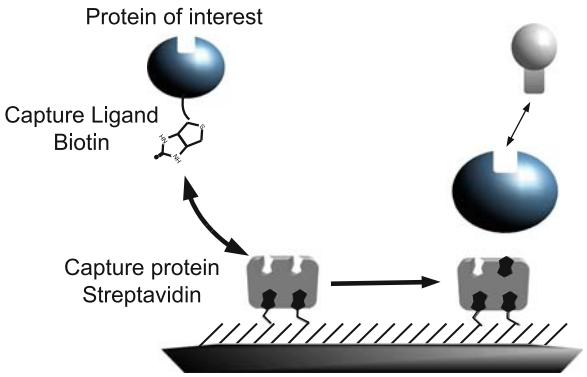


Fig. 2.8 Schematic representation of oriented immobilization of protein by affinity through the streptavidin/biotin interaction

Implementation of such affinity ligands involves the posttranslational modification of the recombinant proteins with a heterodifunctional cross-linker agent containing biotin prior to immobilization. The site-specific protein biotinylation have been developed through very specific reactions relying on peculiar biochemical activities (Lue et al. 2004a, b; Tan et al. 2004a, b; Yin et al. 2004). One technique takes advantage of the protein-splicing activity (inteins-mediated biotinylation) (Lue et al. 2004a, b; Tan et al. 2004a, b); another technique derived from the inhibition of protein synthesis by aminonucleoside antibiotic products (puromycin analogs); a last technique is based on non-ribosomal peptide synthetase activity peptide carrier protein tag (PCP tag) (Yin et al. 2004). PCP tags present several advantages when compared to the intein-mediated approaches. Firstly, the PCP tag made of a small protein domain (80 aminoacids) (Yin et al. 2004) is compatible with a wide range of proteins, making the PCP tag technique quite universal. Secondly, the high efficiency of the chemical ligation allows complete labeling reactions within 30 min requiring only micromolar concentration of reagents. The efficiency of intein-mediated biotinylation is highly dependent upon the intein fusions, so that a specific adaptation of the general scheme is required to each protein of interest.

Biotinylated proteins are immobilized onto avidin or streptavidin coated solid support (Fig. 2.8). Avidin (or streptavidin) is a tetrameric glycoprotein with four identical subunits. Avidin-coated supports were currently obtained by reaction of the natural amino groups of proteins to amine reactive surface. This biofunctionalization leads to randomly immobilized streptavidin. But, the specific interaction between biotinylated proteins and streptavidin leads to oriented immobilization of the molecule of interest. A possible trouble might be the control of the immobilization density and orientation. Oriented immobilization of the streptavidin has been performed by immobilization of the streptavidin on biotin functionalized solid support (Yeung and Leckband 1997; Vijayendran and Leckband 2001).

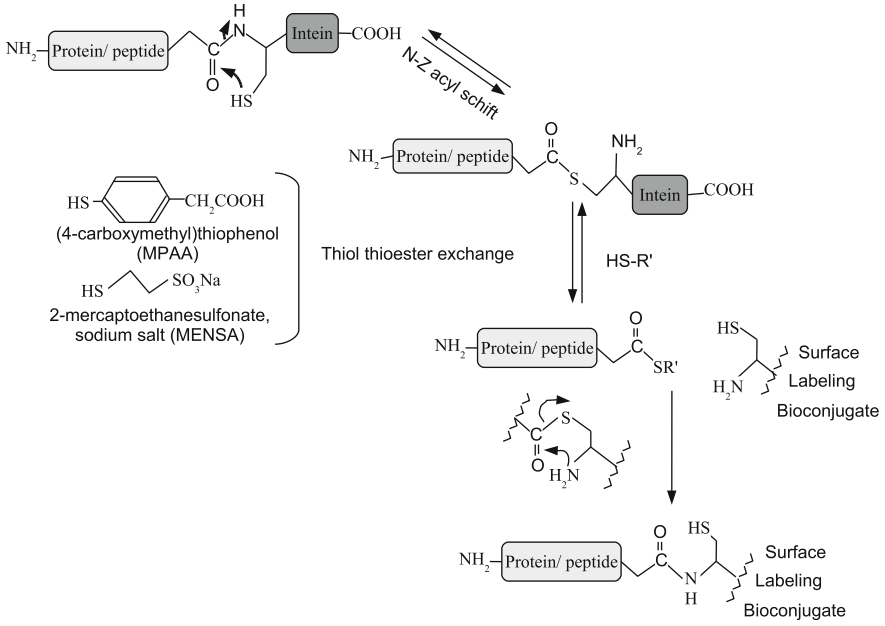


Fig. 2.9 Schematic representation of intein rearrangement and Native Chemical Ligation

2.3.4.4.3.b Other Affinity Tag Systems

On the same footing as for the well-known biotin–avidin pair, various affinity systems can be designed. Selective attachment of recombinant proteins through an artificial polypeptide scaffold system based on heterodimeric association of separate leucine zipper pair (Zhang et al. 2005). Recombinant proteins incorporate a basic component of the leucine system as affinity tag while an acidic component is attached to the surface. The heterodimeric association comes from efficient coiled–coil interaction (affinity constant of 10^{-15} M). The small size of the zipper tag (43 aminoacids) and the high specificity of the polypeptide scaffold are intended to be implemented in direct immobilization of various recombinant proteins from crude cell lysates without time-consuming purification steps.

Kwon et al. (2006) have developed a traceless capture ligand approach based on the use of protein trans-splicing mechanism. Intein fragments are known to promote protein splicing activity. These authors use a variant in which the intein self-processing domain is split into two fragments. One fragment is incorporate to the protein while the other one is attached to the surface. The specific interaction of these protein and ligand capture domains leads to a functional protein-splicing domain that, in parallel, splices the split intein out in the solution and attach by mean of covalent grafting the recombinant protein to the surface. This approach allows removing the affinity capture ligand after immobilization but suffers from long reaction time (up to 16 h) like the other intein-mediated approaches mentioned previously.

2.3.4.4.4 Enzymatic- and Chemo-Selective Ligation Reactions

Site-specific mutagenesis is used to introduce a unique cysteine residue (Firestone et al. 1996; Yeung and Leckband 1997) at the end of a long fusion tag of 15 aminoacids (N- or C-terminus) (Backer et al. 2006). The site-specific free sulfhydryl group can react directly on appropriately derivatized surface or conjugate with thiol-directed bifunctional cross-linker agent. Because thiol groups tend spontaneously to form disulfide bridges, the use of free sulfhydryl group involves a reduction step (with DTT for example) prior to the immobilization reaction.

2.3.4.4.4.a Chemo-Selective Ligation Reactions

Native chemical ligation. Native Chemical Ligation (NCL) involves the chemoselective reaction between a peptide-α-thioester and a cysteine-peptide to yield a native peptide bond at the ligation site. The carboxy-terminal α-thioester (COSR) group is generated with an intein vector (Fig. 2.10). Inteins are protein domains that catalyze protein splicing. More than 200 intein sequences (containing from 134 to 1,650 aminoacids) have been identified with a wide diversity of specific activity. Inteins can be one element or split domain that upon reconstitution allow protein splicing of the regions flanking by autocleavage (Perler and Adam 2000). Specifically engineered intein expression systems allow to perform single splice-junction cleavage (Fig. 2.9). Considering N-terminal intein expressed protein, the thiol group of cysteine side chain is involved in an S–N

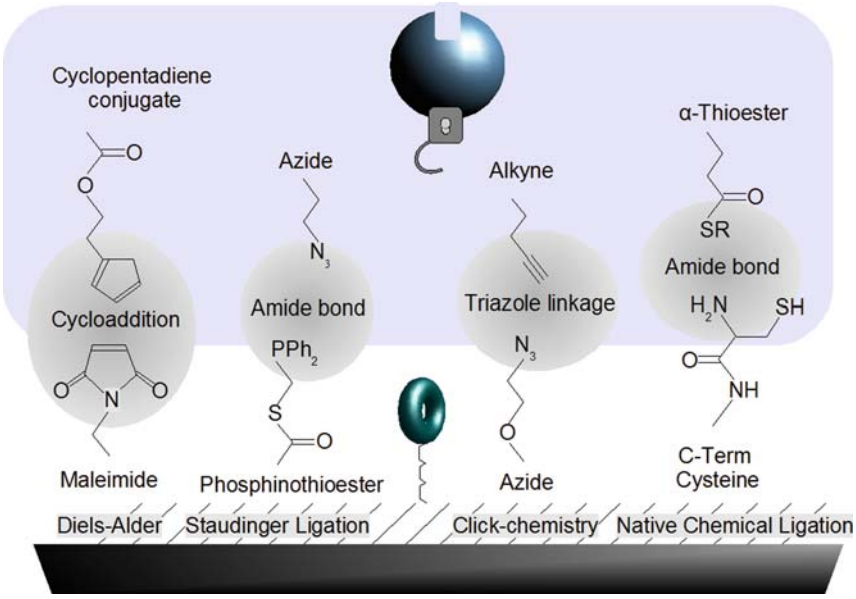


Fig. 2.10 Examples of immobilization of proteins by chemo-selective ligation

acyl shift to form a thioester bond. Unfortunately, the alkyl thioester residues generated at the C-terminal amino acid show extremely low rate of ligation. Trans-thioesterification with exogeneous thiols (e.g. MESNA or MPAA) have been reported to promote the *in situ* formation of more active thioester (Johnson and Kent 2006).

Peptide or protein thioester such as mercaptoethanesulfonic acid (MESNA) thioester reacts in neutral aqueous medium with an N-terminal cysteine residue, yielding a native peptide bond (amide bond) at the ligation site.

Intein-mediated protein ligation (IPL) or Express protein ligation (EPL) has been investigated for chemo-selective immobilization of proteins and peptides onto cysteine functionalized solid support (Camarero et al. 2004; Helms et al. 2007). Direct ligation to cysteine-modified biosensors surface required relatively high proteins concentration (100 μM) (Camarero et al. 2004). Whereas, NCL is extensively used for the preparation of proteins by synthesis or semi-synthesis as well as site-specific bioconjugation, few reports deal with direct immobilization onto solid support for biosensors or microarrays manufacturing.

In addition to direct immobilization, IPL/EPL has expanded the scope of chemo-selective ligations of proteins and peptides to solid support by incorporation of some unnatural functionality. The ligation process generates site-specific reactive groups that drive in a subsequent immobilization step onto appropriately functionalized solid support. EPL/IPL have mediated the incorporation of chemical (C-terminal azide, alkyne or cyclopentadiene) or biochemical reactive (e.g. biotin moiety) groups. Protein farnesyl transferase like EPL/IPL has been used to incorporate chemical reactive groups by modification of cysteine residue located at the C-terminus (Gauchet et al. 2006). The reactivity of these site-specific chemical reactive groups is orthogonal to that of biomolecules and allows specific chemical coupling in a second step. Most of the ligation reactions implemented by these two-step ligation strategies belong to the Staudinger ligation, click-chemistry or Diels–Alder ligation (Fig. 2.10). These chemo-selective reactions proceed under mild conditions and in aqueous solution, preferably in the absence of any potentially denaturing cosolvent (Dantas de Araújo et al. 2006).

Chemo-selective ligation. The Staudinger reaction allows conversion of an azide group into amine by phosphine or phosphite reducing agents. In the Staudinger ligation, an electrophilic trap (methyl ester or sulfhydryl group) undergoes an intramolecular rearrangement that leads to the ligation of the azide substituent by formation of a stable amide linkage. Staudinger ligation involves azido peptides or azido proteins and surface functionalized by phosphine groups. Oxidation of phosphine groups is a possible problem that can be limited by a suitable functionalized building block (Watzke et al. 2006). Azido-modified proteins are immobilized in 4h reaction at minimum concentration of 50 μM. For example, Soellner et al. (2003) have reported a traceless version of the Staudinger ligation using the fast and complete reaction of azido peptide or proteins to diphenylphosphinomethanethiol-modified surface (Fig. 2.10). Immobilization of azido peptide (5 nM in DMF/H_2O 50:50) reached 67% yield within 1 min. The use of wet organic solvent

for Staudinger ligation decrease the proteins nonspecific adsorption (Gauchet et al. 2006).

Chemo-selective covalent immobilization by click-chemistry involves reaction between azide-derivatized solid support and alkyne-derivatized proteins or peptides, yielding the chemically robust triazole linkage. Like NCL and Staudinger ligation, "click-chemistry" is fully compatible with the functional groups found in proteins. Maximum surface density for proteins immobilization by "click-chemistry" was achieved within 2 h for protein concentration of 20 μM (Gauchet et al. 2006).

The Diels–Alder reaction involves the condensation of a diene (a molecule with two conjugated double bonds) and a dienophile (an alkene) into a cyclic product. The Diels–Alder reaction (Yousaf and Mrksich 1999; Dantas de Araújo et al. 2006) has been investigated for immobilization of biomolecules modified by a site-specific diene (cyclopentadiene, hexadienyl ester) onto solid surface bearing the corresponding dienophile counterpart (respectively, 1,4-benzohydroquinone or maleimide functionalized surface). As a side-reaction that imparts selectivity, surface-bound maleimide reacts with the free thiol functions of the cysteine residues of proteins; a preprotection step by treatment with Ellmann's reagent avoids this undesired reaction (Dantas de Araújo et al. 2006). Reaction is amenable in aqueous solution of dilute proteins (8 μM).

2.3.4.4.4.b Enzyme-Based Immobilization Reaction

Others methods of selective and covalent immobilization of proteins to surface are based on covalent bonding catalyzed by enzymes. Figure 2.11 depicts the reconnaissance of specific partners (capture protein and ligand) leading to selective biochemical immobilization. In such strategies, fusion proteins comprise a capture protein (enzyme) and the protein to be immobilized at the surface. The capture

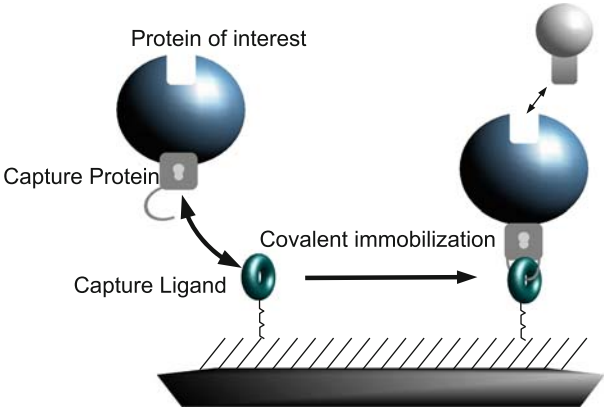


Fig. 2.11 Schematic representation of site-specific chemical or enzymatic immobilization of protein. The reactive group on the surface is named capture ligand while its counterpart link to the protein is the capture protein group

protein is a protein with a specific enzyme activity leading to the formation of a covalent link with the ligand. For example, serine esterase (cutinase) (Hodneland et al. 2002) or DNA repair protein (hAGT) (Kindermann et al. 2003) have been successfully fused to proteins and used to selectively graft them to, respectively, phosphonate or O^6-benzylguanine ligands attached to the solid surface. The highly specific interaction between both partners (e.g. hAGT fusion proteins) allows immobilization of recombinant proteins without time-consuming purification step (Kindermann et al. 2003). The covalent nature of binding ensures sustained immobilization of proteins. Fusion should not alter the properties of both partners and the enzyme is preferably small in order to minimize steric effects during the immobilization.

All these immobilization methods have been studied in the frame of proteomic applications. Large scale protein productions were required for protein microarrays manufacturing. The production and use of site-specific biotin labeling of proteins should not be a bottleneck with respect to these high-throughput technologies. Direct labeling of proteins from cell lysates and its subsequent use for microarray manufacturing without time-consuming and expensive protein purification have been successfully investigated (Lue et al., 2004a, b; Tan et al., 2004a, b; Yin et al. 2004). Due to potential problems that may arise during protein expression in a host cell and in order to simplify the process of protein microarray preparation, intein-mediated approaches (protein biotinylation or self-spliced split intein) in a cell-free protein synthesis have been also proposed (Lue et al. 2004a, b; Tan et al. 2004a, b; Kwon et al. 2006).

2.3.4.5 Small Molecules

An original approach for the generation of protein microarrays combines an original mRNA-peptide fusion synthesis and an addressable immobilization via hybridization to surface-bound DNA capture probes. The mRNA-peptide fusion process relies on the covalent linkage between the translated peptide and its own encoding mRNA (Lin and Cornish 2002; Weng et al. 2002). The DNA chip technology currently enables to produce microarrays with hundreds to thousands of different sequences (up to 60 bases length). The mRNA fusion method is amenable to the generation of large library size of fusion proteins (10^{15} distinct sequences). Limits will come from the selectivity of the hybridization. In addition, although there is no practical limit on the size of the mRNA use, limitations due to proper protein folding could be encountered in the large peptide sizes.

In this method, the fused peptides are addressed to the solid surface and are subsequently used to investigate interaction with specific ligands (targets) in solution. Several groups (Winssinger et al. 2004; Diaz-Mochon et al. 2006; Chevolot et al. 2007) have followed a similar approach, that is, by addressing DNA or PNA tagged biomolecules to DNA microarray. However, in these studies, they take advantage of this addressability to perform the binding reaction step directly in solution prior to addressing each compound onto the microarray. Emphasis is about

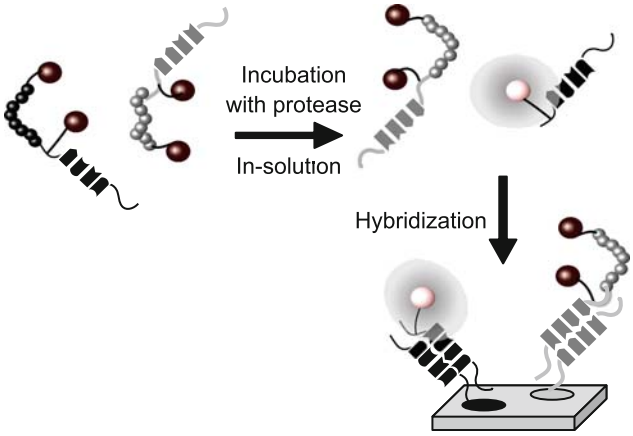


Fig. 2.12 Schematic representation of the specific immobilization of DNA encoded peptides

the higher activity in solution when compared to reaction where one partner is immobilized at solid surface leading to excellent sensibility. The production of chimerical biomolecules coupled to library of nucleic acid tags is a hurdle task.

DNA-based carbohydrate biochips were successfully realized by immobilization of oligonucleotide glycoconjugate molecules that present a nucleic acid sequence allowing hybridization to DNA chips (Chevolot et al. 2007). For example the screening of a library of protease substrates for global analysis of protease cleavage specificity was achieved (Fig. 2.12) by combining in-solution enzymatic reaction of fluorogenic protease substrate and binding the library to specific locations on DNA chips (Winssinger et al. 2004; Diaz-Mochon et al. 2006; Diaz-Mochon et al. 2007). It is worth noticing that a peptide nucleic acid (PNA) tag was bound to the substrate instead of an oligonucleotide because it was not cleaved by the enzymatic reactions. PNA is a nucleic acid analog in which the sugar phosphate backbone of DNA has been substituted by a synthetic uncharged peptide backbone of N-(2-aminoethyl) glycine units. PNA is chemically stable and resistant to enzymatic hydrolysis.

2.4 Surface Functionalization

Biosensors make use of a large panel of detection techniques ranging from electrochemistry to optics. The type and properties of the solid support do not matter so much for some detection techniques such as fluorescence. The support is said *passive*. Its properties may contribute to the sensitivity of the transduction of the chemical signal however. On the contrary, *active* supports or surface layers are absolutely required for transduction of the signal. For example, electronic detection requires conductive materials (vitreous carbon, gold surface, etc.). In addition, analytical properties such as sensibility, detection limit, specificity and lifetime

depend on the bulk and surface properties of the solid support and the sensitizing layer (functionalized surface). Sensibility and detection limit can be improved by (1) an appropriate design of the biomolecular coupling (i.e., uniform and oriented covalent coupling), (2) increasing the loading capacity of active molecule (surface coverage) while avoiding steric or electronic hindrance, and (3) limiting the adsorption of biomolecules that leads to denaturation of active species and/or to background signal in case of nonspecific adsorption.

The aim of the surface functionalization is to place detection biomolecules close to the transducer and in an environment similar to that of their natural medium where biological interactions take place. Thus, the functional solid support can be viewed as a mixed interface. The interface is composed of specific sites for immobilization overhanging an inert underlayer (Fig. 2.13).

The type and surface coverage of reactive sites are tuned according to the immobilization strategy, the nature of biomolecules (size, charge) and specificities of analysis (e.g., concentration range to be detected, ionic strength). Signal intensities increase with the number of receptors immobilized per unit surface, until an optimum. Beyond optimum surface coverage, steric and/or electrostatic hindrance lead to lower accessibility and loss of detection signal (Herne and Tarlov 1997; Houseman and Mrksich 2002).

Biological molecules (DNA, proteins) are electrically charged. The size and shape of these biomolecules are governed by intramolecular electrostatic forces that

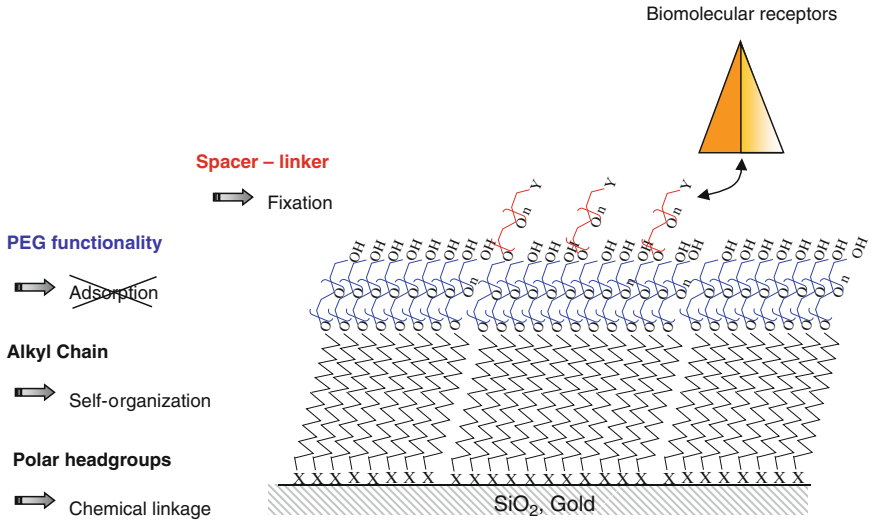


Fig. 2.13 Schematic representation of ideal surface modification for immobilization of biomolecules. Mineral support (silica, gold) are modified by organic layer. Amphiphile molecules with a polar head group and long hydrophobic tails arrange in dense layer. The inorganic polar head groups (silane or thiol) ensure a stable and robust anchorage to the support. The outer surface is terminated by hydrophilic PEG functionality to minimize nonspecific adsorption. Longer linkers bring site-specific groups for the immobilization of molecular receptors

can be modified by varying the ionic strength. In addition to the molecular surface area that controls steric hindrance, electrostatic forces also play an important role in immobilization and in biomolecular interactions. For example, Herne and Tarlov (1997) have reported the formation of densely packed single-strand DNA (ssDNA) monolayer by increasing the ionic strength conditions; but hybridization was prevented because of steric and electrostatic factors. Careful optimization of the DNA coverage and surface properties allowed improving the hybridization signal. Similarly, immobilization of small molecules (ligands) as dense monolayer (high surface coverage) limits their accessibility due to steric hindrance by larger biomolecules (Houseman and Mrksich 2002).

Improved accessibility of biomolecules is achieved by attaching them to the surface through a linker that moves them away from the solid interface. The linker must be sufficiently long to eliminate unwanted steric interference from the support and preferably hydrophilic enough to be swollen in aqueous solution (Guo et al. 1994; Hodneland et al. 2002).

The interface underneath the immobilized receptors is either the bare solid support or a layer of organic molecules that confers specific properties in relation to the biomolecular assay. This sublayer or surrounding matrix plays an important role in the functionalization and on the analytical properties of biosensors. The physico-chemical properties of the sublayer are tailored to limit the biomolecular adsorption. It may also be designed to protect the solid surface or the grafted surface from degradation.

Extensive literature deals with the adsorption of biomolecules at solid–liquid interface (Ostuni et al. 2001). Because adsorption is governed by dispersive interactions (Lifshitz–van der Waals), hydrophilic surfaces tend to resist the adsorption of biomolecules. But the hydrophilic character is not the sole relevant physico-chemical characteristic. Otsuni et al. (2001) reported that surfaces that resist protein adsorption present four common properties: they are polar, hydrogen-bond acceptors, not hydrogen-bond donors, electrically neutral. In biosensor applications, grafted poly(ethylene glycol) (PEG) is widely used to limit the adsorption of biomolecules to solid surface. Such surfaces grafted with PEG are often known as "PEGylated."

The variety of detection techniques and the complexity of parameters to be adjusted have lead to extensive literature about materials and their functionalization. It is described in three parts devoted to: (1) 2D surface functionalization; (2) 3D membranes and porous layers; (3) nanoparticles and nanostructures.

2.4.1 2D Immobilization: Grafting of Monolayers

Very few materials allow direct immobilization of biological molecules onto their bare native surface. Controlled immobilization that meets specificities of biosensor applications requires particular surface chemistry in order to maintain biological activity and minimize nonspecific adsorption. The formation of thin organic films

on mineral surface is widely used to promote the immobilization of biomolecules to solid surface. In addition to bringing adequate specifications to the solid/liquid interface, the organic film must fulfill several properties: reproducibility, homogeneity, thermal stability and chemical stability with respect to both environmental conditions (UV, biofouling, hydrolysis) and chemical reactions performed during the immobilization/functionalization process.

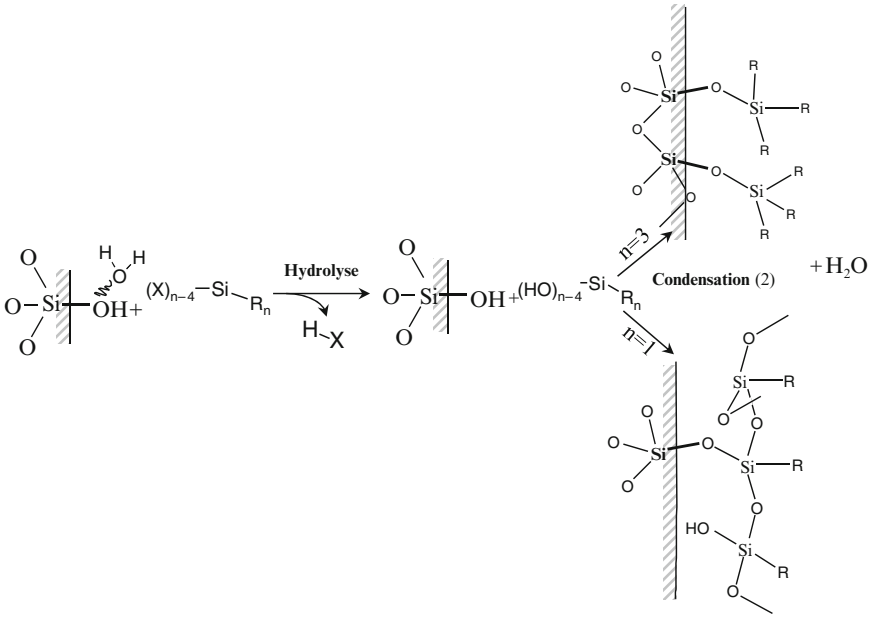
At the molecular scale, surface modification involves organic heterodifunctional molecules. One extremity is intended to react with the solid support while the other extremity brings appropriate functional end-groups for site-specific immobilization of bioreceptors. Various organic molecules and chemical reactions are involved depending on the nature of the solid support. For example, hydroxyl-terminated surfaces of inorganic oxides like silica, glass or titanium dioxide can be derivatized by silane coupling agents, gold surfaces are modified by adsorption of alkanethiolate reagents, hydride-terminated silicon surface undergo chemical modification by either hydrosilylation or coupling reaction with organometal compounds.

2.4.1.1 Alkylsilane Functionalization

The general formula of alkylsilane $R_nSiX_{(4-n)}$ shows a dual behavior, organic and inorganic. R is organic moiety and X is a hydroxyl or a reactive hydrolysable group (–Cl, –OMe, –OEt, etc.) that reacts onto hydroxyl-terminated supports. Reaction of silane molecules to surface silanol groups of silica leads to the formation of quite stable siloxane bonds (Si–O–Si). The siloxane bond is thermally stable and is relatively chemically stable. The siloxane bridge can be cleaved by strong alkaline solution and fluorhydric acid.

The functionality of silane is the number of reactive groups present at the silicon atom. Monofunctional silanes ($n = 3$) having only one reactive group at the silicon extremity bind to silica by means of a single bond. Multifunctional silanes ($n = 1$ or 2) bear several (2 or 3) reactive groups the silicon atom and can bind to the surface by several bonds. Binding by several bonds is stronger but the reaction is more difficult to be controlled in a reproducible manner.

An appropriate choice of the functional "tail group" (R) introduces the specific surface functionalization regarding the subsequent immobilization of bioreceptors. Some functional group cannot be used directly however because the silyl headgroup (alkoxy-, chloro-, dimethylamino-silane) is reactive toward several organic functions (carboxylic acid, alcohol, aldehyde, etc.) (Beier and Hoheisel 1999; Zammatteo et al. 2000; Dugas et al. 2004; Wang et al. 2005) or because the functional extremity can interfere with the film formation at the surface (e.g., amino groups) (Martin et al. 2005). Incompatible tail groups are either protected during grafting or chemically synthesized on the solid support after silane grafting. For example, a silane bearing a *tert*-butyl ester group has been grafted because activated acids and their acid precursors have not been chemically compatible with silanes; it has been subsequently converted into the corresponding acid and activated ester in a third step (Dugas et al. 2004). Aldehyde functional groups can be



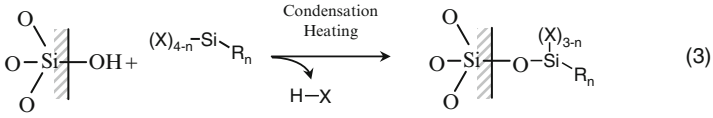
Scheme 2.2

obtained by reduction of alkenes (Hevesi et al. 2002). Several approaches have been proposed for grafting maleimido-terminated silane layer (Wang et al. 2005). As previously mentioned, many research applications involve surface modification by amino or epoxy groups followed by postgrafting modification by heterodifunctional linkers.

2.4.1.1.1 Reaction Mechanism

Two reaction mechanisms have been proposed for silane grafting (Boksanyi et al. 1976; Tripp and Hair 1995; Clark and Macquarrie 1998):

- The first mechanism "hydrolysis and condensation" is a two-step reaction. In the first step, substituents of the silicon atom are hydrolyzed to form silanols. The alkylsilanols establish hydrogen bonds with the free hydroxyl groups on the surface or with adsorbed water molecules on the surface. The covalent bonding is carried out by condensation and release of water molecule (Scheme 2.2). Water has a catalytic effect; its presence and concentration should be carefully controlled in order to ensure reproducibility.
- The second proposed mechanism corresponds to a direct condensation reaction between the silicon headgroups and the surface hydroxyls during a thermal treatment (Scheme 2.3).



(3)

Scheme 2.3

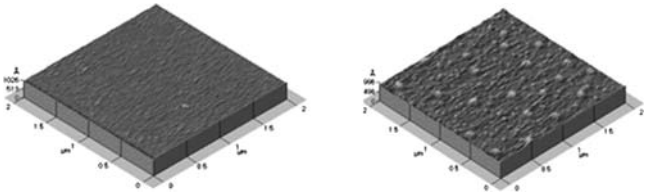


Fig. 2.14 AFM images of bare Si/SiO_2 wafers (right) and after 12 h of silanization with GPTS

The silane grafting mechanisms with surface oxide have not been completely characterized due to the extreme difficulty of following the chemical modifications that occur at the extreme surface of solid support. It is worth noting that no direct analysis (*in situ*) has characterized separately the hydrolysis step prior to the surface condensation. But some studies report low or no chemical grafting of silane molecules under strictly anhydrous conditions. But surprisingly, trimethylsilanol molecules are less "reactive" than their chlorinated homologues (trichlorosilane) (Azzopardi and Arribart 1994).

Water "catalyzes" the silane grafting reaction by causing the hydrolysis of silane leaving groups. Multifunctional silanes are hydrolyzed in solution into silanol intermediates that may undergo intermolecular polycondensation in solution. The small polymeric species can then react with the surface and lead to the formation an inhomogeneous layer.

AFM images in Fig. 2.14 are 3D representations of flat silicon dioxide surface after cleaning (left) and after silanization with the trifunctional glycidoxypropyltrimethoxysilane (GPTS) (right). The surface modified by GPTS exhibits nodules of 0.3 μm diameter and up to 150 Å height containing polycondensed silane.

There is vast literature dealing with *ex situ* characterization of alkylsilane derivatized support. The chemical grafting has been proven by infrared (Blitz et al. 1988; Angst and Simmons 1991; Azzopardi and Arribart 1994; Tripp and Hair 1995) and NMR spectroscopy (Sindorf and Maciel 1981, 1983; Kinney et al. 1993) on silica powders (high specific area). Nevertheless, some *in situ* studies performed on silicon dioxide allowed a more detailed description of the grafting mechanisms of various silanes in organic solutions (Azzopardi and Arribart 1994).

The choice of the type of silane is of importance. The silanization reaction is largely related to relevant experimental conditions. The nature of the silane and its concentration (Boksanyi et al. 1976; Szabo et al. 1984; Azzopardi and Arribart 1994; Tripp and Hair 1995), the nature of the solvent and its water content

(Tripp and Hair 1992; McGovern et al. 1994; Navarre et al. 2001), temperature (Boksanyi et al. 1976) and time (Gobet and Kovats 1984; Azzopardi and Arribart 1994) are the most important parameters.

The silane grafting reaction is a condensation of silane head groups to the free hydroxy groups of the surface. Thus the solid surface must be adequately prepared prior to the silanization reaction in order to create a constant density of such hydroxy groups (silanols) and improve the density of silane grafting and repeatability of the reaction (McGovern et al. 1994; Cras et al. 1999; Fadeev and McCarthy 1999). Surface cleaning removes contamination that may hamper the reaction to the surface hydroxyls. Surface cleaning steps under oxidizing conditions (e.g. "Piranha" solution (H_2O_2 + H_2SO_4), UV-light + ozone, oxygen-plasma treatment) and treatment in hot water are also intended to increase the hydroxyl surface density (e.g. on glass slides). After cleaning, the strongly hydrophilic hydroxylated surface is covered by several layers of hydration water molecules. It is therefore highly recommended to dry the freshly cleaned surfaces in order to get reproducible results.

The formation of monolayers at the surface of oxide surface proceeds through different ways depending that the silane is mono or multifunctional.

Trifunctional silanes such as trimethoxy or trichlorosilane react with surface hydroxyls by means of their three reactive groups, but this reaction is most often incomplete because it is not possible to attach the same silicon atom to the three oxygen's present at fixed positions at the surface, keeping the bonds lengths and angles close to reasonable values. Furthermore, the possible hydrolysis of silane taking place in case of incomplete drying of the silica surface leads to a concomitant polycondensation of the hydrolyzed silanes and condensation with the surface silanols; an ill-defined polycondensate of organosilane grows from the surface and a thick and rough final silane layer results (Tripp and Hair 1995; Yoshida et al. 2001). The formation of monolayers with trifunctional silane thus requires precise control of the experimental conditions (humidity, solvent, temperature).

Such troubles do not occur with monofunctional silanes that bind to the surface by means of a single bond. Even in the case of hydrolysis of the silane next to the surface, the polycondensation of hydrolyzed monomers lead to dimers that cannot react with the solid support. The grafting is directly related to the density of silanol groups on the surface. But monofunctional silanes have a lower reactivity than multifunctional ones (Evans and White 1968). This has to be compensated by the choice of a highly reactive leaving group such as dimethylamino (Szabo et al. 1984).

2.4.1.1.2 Alkoxysilane Film Properties

Three different silanization protocols can be described from the literature. Short or volatile silanes can be involved in gas phase silanization reaction. Clean and dry supports are exposed to vapor of silane molecules. This technique allows accurate control of experimental conditions in order to avoid hydrolysis of silane in solution. A variant of this technique called Atomic layer deposition (ALD) allowed the

preparation of homogeneous aminosilylated silica surfaces at low temperatures ($\leq$150°C) in a reproducible way by a solvent-free procedure (Ek et al. 2004).

Grafting of nonvolatile silanes can be carried out in two steps called "impregnation" and "condensation" (Chevalier et al. 2000; Dugas et al. 2004). A solution of the silane in anhydrous volatile solvent (e.g. pentane) is deposited on the surface and the solvent is evaporated under reduced pressure. This first "impregnation" step leaves a thin layer of pure silane on the surface. The grafting of silanes ("condensation") is performed by heating at elevated temperature (>140°C) under dry atmosphere. Highly reproducible grafting densities are obtained by this technique with monofunctional silane (Dugas et al. 2004).

More common silanization protocols involve reaction in solution. Trialkoxysilanes are widely used for immobilization purposes. Aminopropyltriethoxysilane (APTES) and glycidoxypropyltrimethoxysilane (GPTS) are the most widely used silanes for chemical surface modification. Grafting reactions are currently carried out from aqueous alcohol solution (95% ethanol, 5% water) under slightly acidic conditions (pH = 4.5–5.5). This simple route for surface modification often leads to irreproducible results (Yoshida et al. 2001). As example, the analysis by ellipsometry of DNA layers immobilized by the intermediate of the trifunctional glycidoxypropyltrimethoxysilane (GPTS) has revealed the nonuniform grafting density and layer thickness that is larger than expected on the basis of the length of the oligonucleotides (Gray et al. 1997). In addition, the terminal functional groups can also interfere with the chemical grafting and reduce the uniformity of the monolayer. In particular, the amino group of APTES adsorbs by hydrogen bonding to silanols groups on the oxide surface (Horr and Arora 1997; Sieval et al. 2001) (Fig. 2.15).

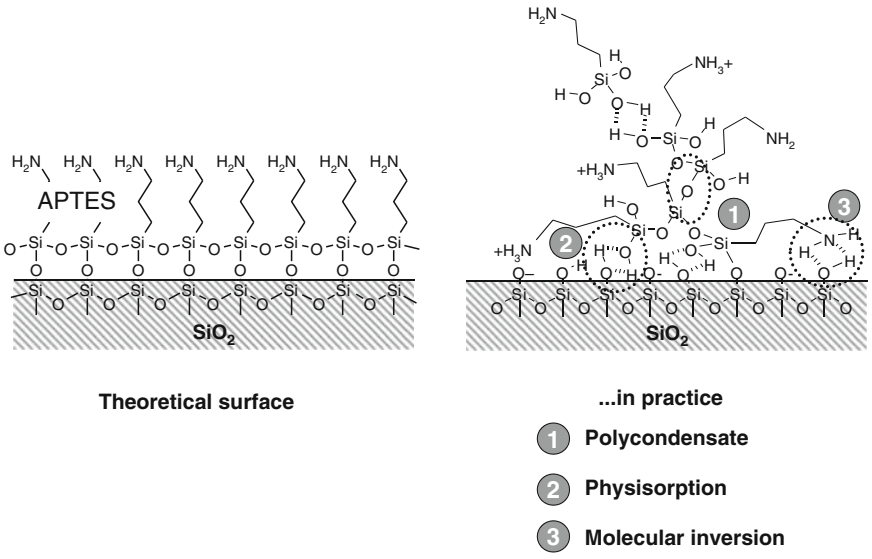


Fig. 2.15 Schematic representation of intended layer and effective layers formed with APTES

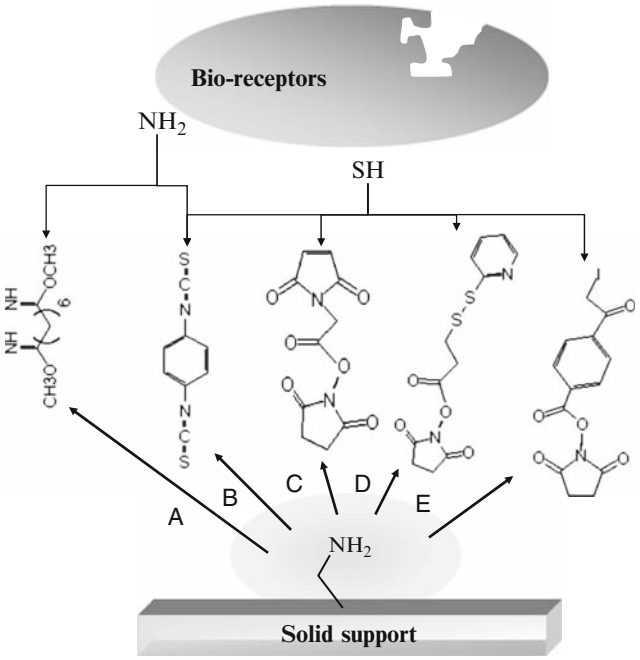


Fig. 2.16 Schematic representation of the versatile derivatization of aminated support. (**A**) dimethylsuberimidiate; (**B**) 1,4-phenylene diisothiocyanate (PDITC); (**C**) N-succinimidyl-3-maleimidopropionate (SMP); (**D**) N-succinimidyl-3-(2-pyridyldithio) propionate; (**E**) N-succinimidyl (4-iodoacetyl) aminobenzoate (SIAB)

Once a functional silane has been grafted, the functional group can be modified by reaction with a coupling agent. Amine functional groups are extensively used for surface functionalization because they are quite versatile. γ-Aminopropyltriethoxysilane (APTES) or N-(2-aminoethyl)-3-aminopropyltrimethoxysilane (AAPS) coupling agents are widely used to modify hydroxylated supports (silica's, glasses). Aminated supports are then treated with homo- or hetero-difunctional reagents (or cross-linkers) allowing to create a large panel of active surfaces toward nucleophilic groups (Fig. 2.16). Water-soluble cross-linkers (sulfonate analogues) give consistently higher coupling yields than their insoluble counterparts (Adessi et al. 2000).

As described previously, surface functionalization with epoxy, activated esters or aldehyde functions can also be prepared by reaction of homodifunctional molecules (Yershov et al. 1996; Volle et al. 2003).

The formation of well-controlled films from the short-chain functional silanes can be achieved by precise control of the experimental conditions (silane concentration, reaction time and water content) (Hu et al. 2001). Larger density can be reached by combining chemical grafting and self-organization of the grafts as follows. The elaboration of dense, homogeneous and stable monolayers can be prepared from long-chain trifunctional silanes by self-assembly. A self-assembled

monolayer, SAM, is formed when surfactant molecules adsorb as a dense and organized monomolecular layer on a surface. Dispersive interaction (van der Waals) along the long enough hydrophobic tails is the driving force of the self-organization. For example octadecyltrichlorosilane shows self-assembling behavior. The terminal functional group at the extremity of the alkyl chain must be protected in order to avoid interference during the film formation (Martin et al. 2005). In addition, this terminal "tail group" must be sterically small enough to limit interference during monolayer organization driven by alkyl chain interactions.

SAMs formation is realized under careful control of the solvent composition, moisture and temperature conditions. Trichlorosilanes compared to trialkoxysilanes are highly moisture sensitive.

SAMs of pure alkylsilane (R is nonfunctional alkyl) self organize quite easily. Terminal functional groups at the extremity of the alkyl chain tend to destabilize the monolayer. For example, long-chain of octadecyltrichlorosilane ($CH_3(CH_2)_{17}SiCl_3$) form dense monolayers while longer chains (21 CH_2) are required to obtain well-ordered monolayers with a hydrophilic "tail group" such as poly(ethylene glycol) or protected amino groups (Navarre et al. 2001; Martin et al. 2005).

True dense SAMs provide stable and well-oriented layers. The insulating polymeric surface coating helps to protect the Si–O–Si bonds between the coupling agents and the surface. SAMs can withstand harsher conditions than less ordered film tethered to the surface. It can serve to form a dielectric barrier for electronic detection devices. However the benefits coming from the SAM properties (dense packing, well-ordered monolayer) hamper the reactivity of the surface groups used for covalent coupling of bioreceptors or biomolecular interaction (Fryxell et al. 1996). Incorporation of the desired functional groups in an inert background monolayer (deposition of a mixed film) avoids the crowding effect of the functional substituents at the surface. Mixed films can be prepared either in one shot (Martin et al. 2005; Hoffmann and Tovar 2006) or in a two-step process (Harder et al. 1997).

2.4.1.1.3 Silane Coupling Applied to Various Materials

Silanes are used to modify hydrated surface materials ranging from glass slides to aluminum oxide materials:

- Glass is widely used in microarray techniques owing to its chemical stability, transparency, low-fluorescence background and flatness. Surface modification involves silane grafting.
- Miniaturization of biosensors has lead to use materials coming from microelectronics. This trend calls upon semiconductor materials like crystalline silicon wafers. Thermal silicon dioxide (SiO_2) or silicon nitride (Si_3N_4) are among the materials that are commonly used as insulating or passivating layer for electronic detection systems (e.g., field-effect transistors, GenFET, Genosensors). These materials are also implemented to fabricate miniaturized cantilevers that are used to detect biomolecular interaction by deflection of the cantilever.

Indium Titanium Oxide (ITO) (Moore et al. 2006) is a conducting material deposited as thin films in electrochemical detection system.
- Specific optical detection techniques rely on the use of high refractive index layer such as titanium oxide (TiO_2) (Trummer et al. 2001; Dubruel et al. 2006) that can be chemically derivatized by alkoxysilanes.
- Integrated system (Lab on chip or μTAS) involves hybrid materials composed of glass, silicon, silicon dioxide and/or often elastomer materials. Polydimethylsiloxane (PDMS) elastomers are widespread in such miniaturized systems. The surface of PDMS is modified by silane molecules in order to limit biomolecule adsorption and to promote bioreceptors immobilization.
- Porous materials like porous silicon (Bessueille et al. 2005) or anodized aluminum oxide (Lee et al. 2007) present hydroxyl that can be turned out to chemical surface modification.

2.4.1.2 Hydrosilylation of Silicon Support

Siloxane bridges that ensure anchoring of the alkoxysilane to hydroxylated materials like glass are alkaline sensitive chemical bonds. Silicon-based materials coming from the microelectronic industry are used to implement miniaturized system that can be thermally oxidized into a silica layer at their surface for undertaking the classical silanization reaction on silica surface. Alternatively, they can be used without surface oxidation. Raw bare silicon exhibits silicon–hydride surface groups (Si–H) that can be derivatized by difunctional organic molecules to form stable silicon–carbon bonds, potentially allowing the incorporation of a wide range of chemical groups. Silicon surfaces coated with a self-assembled alkyl monolayer are very stable. For example, derivatized porous silicon withstands to harsh alkaline conditions (pH $>$ 10) (Buriak et al. 1999).

Hydrogen terminated silicon surfaces are prepared just before modification because rapid oxidation in air leads to a thin silicon dioxide passivating layer. Silicon surfaces are prepared by a short hydrogen fluoride treatment just before the subsequent chemical modification. Different grafting routes were assessed, using reaction with alkenes, alkynes (Buriak and Allen 1998), peroxides (Linford et al. 1995) with organometallic agents (Tao and Maciel 2000). The well-known chemistry in solution encompasses thermal- (Sieval et al. 2001), radical-, Lewis acid- (Boukherroub et al. 1999) or metal complexes catalysis (Zazzera et al. 1997) and UV-light mediated (Boukherroub and Wayner 1999) hydrosilylation reactions.

Surface modification by long-chain alkenes or alkyne have been characterized by IR spectroscopy, ellipsometry, X-ray reflectometry to form dense and organized monolayers. The covalent Si–C linkages are very stable (UV-light, fluorhydric acid, alkaline conditions). The efficiency of molecular grafting largely depends on the steric hindrance of the molecules (Buriak et al. 1999). Insertion of alkenes or alkynes into surface Si–H groups yields alkyl or alkenyl termination, respectively. Both chemistries are remarkably tolerant to a wide range of chemical functionalities (Buriak et al. 1999). But as for the silane grafting reaction, the terminal functional

groups of the ω-alkenes have to be protected to prevent direct reaction with the Si surface which results in disordered monolayers (Strother et al. 2000a, b; Sieval et al. 2001). The formation of mixed monolayers with amino-terminated function has been reported with control over the surface density of the amino-groups (Sieval et al. 2001).

Silicon devices are now utilized for microelectromechanical systems (MEMS), microfluidics and micro-total analytical systems (μ-TAS), biosensors and other bioanalytical applications. The chemical modification of silicon surfaces have been applied to systems of various shapes; flat silicon substrates (Strother et al. 2000a, b), nanowires (Streifer et al. 2005), nanoparticles (Nelles et al. 2007) and porous layers (Mengistu et al. 2005). In addition, the great stability of the Si–C bonds when compared to the siloxane bridge allows to use the biosensors under harsher conditions or to reuse the biosensors without or with only minor losses of activity (Strother et al. 2000a, b).

2.4.1.3 Organo-sulfur Precursors for Surface Functionalization

Intensive literature deals with the formation, properties and applications of self-assembled monolayers of alkylthiolates on metals. The high affinity of thiols (R–SH), disulfides (R–S–S–R) and sulfides (R–S–R) for metals (gold, silver, copper, palladium, platinum, and mercury) drive the formation of well-defined organic surfaces with useful chemical functionalities displayed at the exposed interfaces for biosensor applications. Gold is usually the surface material of choice due to its chemical inertness and the well-defined structure of the obtained film (densely packed and ordered array of long chain molecules). Gold does not oxidize at room temperature, does not react with atmospheric oxygen and is biocompatible (compared to silver that oxidizes readily in air and is toxic to cells (Dowling et al. 2001)).

Self-assembled monolayers of organosulfur precursors spontaneously form on gold upon immersion of gold surfaces in a solution of precursors. Film formation and properties of SAMs on gold are out of the scope of this report and are exposed in details elsewhere (Ulman 1996; Love et al. 2005).

Flat gold surfaces are commonly prepared by physical vapor deposition (PVD) techniques, electrodeposition or electroless deposition on various supported materials (silicon wafers, glass, mica and plastic substrates). The strong adsorption of alkylthiols onto gold surface is able to displace miscellaneous impurities or contaminant already present at the surface (Love et al. 2005; Luderer and Walschus 2005). Cleaned surfaces are thus not absolutely essential to produce alkylthiolate SAM. Kinetics of the SAM formation are however affected because desorption of contaminants may be slow. Therefore, preparation of SAMs on gold is easy and reproducible. Common procedures for the cleaning of gold surfaces are chemical treatment with highly oxidizing solutions (e.g. “piranha solution”), electrochemical cleaning in sulfuric acid solution (Luderer and Walschus 2005; Lao et al. 2007) and thermal treatment, annealing in a flame for instance (Briand et al. 2006).

The formation of SAMs on gold surface with organosulfur compounds follows two distinct kinetics. The first very fast step corresponds to the adsorption of molecules from solution to the surface and the second slow step where monolayer organizes into a well-order state. Adsorption takes from millisecond or minutes in millimolar solutions to 1 h in micromolar solutions (Ulman 1996; Love et al. 2005). The second step is the reorganization of the initially adsorbed monolayer into a crystalline configuration. The 2D crystallization on the surface is slow and may require hours to several days until monolayer reaches tightly packed SAM with minimum defects (Ulman 1996; Luderer and Walschus 2005).

Film formed from alkylthiols or alkyl disulfides lead to identical film properties. Upon the adsorption process these organosulfur compounds form alkanethiolate species ($R–S^-$):

$$\mathrm{RSH} + \mathrm{Au} \rightarrow \mathrm{RS} - \mathrm{Au} + \mathrm{e}^- + \frac{1}{2}\,\mathrm{H_2}$$

$$\mathrm{RS} - \mathrm{SR} + \mathrm{Au} \rightarrow \mathrm{RS} - \mathrm{Au} + \mathrm{e}^-$$

The most common protocol for preparing SAMs on gold and other materials is immersion of clean gold surface into dilute (1–2 mM) solution of ethanolic alkylthiol (or disulfide) for 12–18 h at room temperature. Film properties (reproducibility, physico-chemicals behavior of the organic interface, SAM defects, density) depend on the nature of the sulfur compound (head groups, length of the alkyl tail, nature of the terminal group for heterodifunctional molecules), and on reaction factors (immersion time, concentration of adsorbate, temperature, solution composition) (Love et al. 2005).

For biosensor application purpose, mixed SAMs are easily prepared. Mixed monolayers allow to control the density of the reactive species (diluting), to increase its accessibility and to bring chemical inertness to the underlying surface.

2.4.1.3.1 Direct Biofunctionalization of Gold Surfaces

Straightforward immobilization of native proteins, peptide (via cysteine groups) or thiols modified DNA strands have been reported on gold surface (Steel et al. 1998; Brogan et al. 2003; Ohtsuka et al. 2004). Tightly packed monolayers were obtained with oligonucleotide but immobilization is less efficient with other molecules like oligopeptides (Ohtsuka et al. 2004). Misorientation is possible however when the other functional groups (amines) bind to the gold surface. Herne and Tarlov (1997) reported that thiol-derivatized DNA molecules mostly interact with gold surface through the sulfur atom of the thiol group. Small extent of adsorption by nucleobases of oligoprobes took place and resisted even hard washings conditions. But interestingly, displacement experiments, where the gold surface functionalized with HS-DNA was contacted with high concentrations of small thiolated molecule

(6-mercapto-1-hexanol), have resulted in nearly complete displacement of adsorbed molecules.

Supramolecular assembly of hindered thiolated molecules has been reported to form compact and homogeneous monolayers on gold electrodes. Collagen-like peptides exhibit rod-like conformation and have been used to prepare SAMs onto gold electrode (Yemini et al. 2006). Enzymes were covalently immobilized through the amino terminal group of the peptides. The collagen-like peptides confers biocompatibility properties to the sensors surface while ensuring a stable attachment of enzymes to the biosensors.

2.4.1.3.2 Functionalization of Gold Surface for Covalent Coupling

Various thiol precursors have been used to tailor the surface functionalities of gold surface. Bioreceptors are covalently immobilized through amine or thiols reactive groups (see Sect. 2.2). A large panel of difunctional thiol molecules is available for straightforward SAMs formation on gold surfaces. As for the alkylsilane mixed monolayers, different synthetic routes allow the formation of mixed monolayers (Love et al. 2005). The simplest one consists in coadsorption of mixtures of alkane thiol precursors.

In order to increase the accessibility and limit the influence of the underlying surface, the site-specific groups which serve to immobilize bioreceptors (–COOH (Briand et al. 2006), ligand protein (Hodneland et al. 2002)) are placed at the extremity of a spacer. These ω-substituted alkanethiols are mixed with shorter alkanethiols terminated by hydroxyl or oligo(ethylene glycol) groups.

In order to move away bioreceptors from the organic interface, immobilization strategies have been initiated through a "sandwich" configuration. A first receptor is immobilized (streptavidin (Grubor et al. 2004), protein G (Briand et al. 2006), S-layer protein (Pleschberger et al. 2004) and DNA (Lao et al. 2007)) and serves as a spacer to specifically immobilize the biological probe through oriented configuration. In addition to increase accessibility, these strategies allow to immobilize bioprobes under oriented and selective configurations.

Direct detection, label-free, measurements call upon "active" surface layers involved in the detection system. For example, electrodes are the sensitive elements of electrochemical detection techniques. Gold surfaces are also used to propagate surface plasmon waves in the conventional surface plasmon resonance (SPR) technique. Surface chemistry will be tailored in order to match the detection technique specifications. Achievement of dense and compact hydrophobic monolayers with low defects, allows formation of insulating layers of low dielectric permittivity, therefore small Faradaic background at the electrode biointerface regarding their electrical properties. The electrode/solution interface in potentiostatic measurements is described equivalent to several capacitors in series (Berggren et al. 2001). The electrical equivalent circuit is different for amperometric and voltametric detection techniques that rely on the detection of electroactive species (red-ox, electron transfer proteins). The hurdle consists in establishing an

electrical connection between the receptor and the signal transducers (electrode surface). Direct electron transfer (ET) through the organic interface represents the alternative approach to the use and incorporation of "electrochemical" mediators (Zhang and Li 2004). One strategy to the direct electron transfer is DNA-mediated protein ET (Lao et al. 2007). In this case, the interface helps to efficiently drive electrons to the conductive electrode.

2.4.1.4 Reactivity Issues

Slow kinetics of grafting reaction is the main concern of bioimmobilization. Slow reaction rates come from the very low concentrations of species to be grafted and detrimental close vicinity to the surface. The slow reaction rate cannot be dealt with using extremely long reaction time because the reactive surface is open to competitive side-reactions during the same time. Therefore highly reactive species are selected, so that the reaction rate of solid-supported molecules is largely controlled by diffusion. Of course, the concentration gradients that drive diffusion are low when the mean concentration itself is low. Diffusion can be supplemented by stirring and mixing the solution at the solid surface. Stirring inside miniaturized devices is a technological challenge. A common practice in array manufacturing using spotters is letting the deposited drops dry. Drying the drops improves immobilization rate because the reagents are concentrated *in situ*; but no reaction can take place when the drops are dry to completion because the reagents are no longer mobile. Therefore, drying is useful but it has to be controlled in a chamber with residual humidity.

Additional aspects related to the underlying surface also interfere onto reactivity. The close proximity of the outermost functional groups and the hard surface modifies the mobility behavior (and reactivity) when compared to reaction in solution. It is well-known that the rates of interfacial reactions are slower than in solution. There are obvious causes coming from geometrical reasons. One of the reagents is motionless because it is attached to the surface; therefore mutual diffusion coefficient is roughly reduced by a factor of 2. Secondly, diffusion is restricted to half a space, which causes a supplementary reduction of rate by a factor of 2. The actual losses of reaction rates with respect to solution kinetics are much larger than the rough geometrical factor of 4 however. There are several additional phenomena that are significantly operating. Words often found in the literature are "steric hindrance", "reduced mobility", "lack of bioavailability". This is difficult to go beyond the words as long as fundamental studies have definitely reached clear-cut conclusions.

The parameters affecting surface reactions are of two distinct types: one type pertaining to the accessibility to the reactive sites; the second type deals with the reaction itself that requires the correct mutual orientation of the reagents and the desolvation of the reaction centers. So-called steric effects have various origins. The underlying material contributes since buried and/or misoriented reactive groups are not reactive. The high surface concentration of reactive functional

sites affects the grafting kinetics at high conversion; lateral steric hindrance and crowding effects take place when the biomolecules of large size are immobilized up to high grafting densities.

The problem of accessibility and misorientation depends on the attack trajectory dictated by the reaction mechanism. This is the case for the SN_2 reaction where the terminal "tail group" attached at the surface undergoes "backside attack" by incoming nucleophilic reagents and the leaving group has to diffuse off the reaction site (otherwise the back-reaction takes place). The hindrance by the underlying layer dictates the reaction rate (Fryxell et al. 1996). Grubor et al. (2004) have compared the reactivity of two NHS-ester ω-functionalized thiols of different chain lengths (C_3 and C_{11}) adsorbed onto gold surface. SAMs made of short thiol precursor (3 $–CH_2–$) are of lower packing density than longer alkyl-chain thiol precursor (11 $–CH_2–$); the film is less ordered and less rigid. The hydrolysis and acylation reaction rates of the NHS end group at the top side of the SAM surface are strongly influenced by the presence of the SAM and the structure of the SAM appears of definite importance. The rate of base-catalyzed succinimidyl ester hydrolysis at the surface is about 1,000 times slower than in solution; the rate of hydrolysis and amidation is significantly lower for long-chain when compared to short-chain SAMs. Similar steric effects on SN_2 reaction on planar SAMs have been reported by several authors (Hostetler et al. 1998; Templeton et al. 1998; Dordi et al. 2003).

Moving the reactive groups away from the surface via hydrophilic spacer arms can improve the solubilisation of the receptors and minimize the background influences of the underlying interface.

In addition to the hindrance arising from the position of the reactive site relative to the surface, lateral steric effects or crowding effects take place (Chechik et al. 2000; Houseman and Mrksich 2002). This arises when bulky reactants are involved when compared to the supported reactive sites and their loading surface densities. Crowding at the surface may even contribute for small molecules in case of unfavorable interactions. Vicinal dipolar moieties such as carbonyl group of ester or carboxylic acid functionalized SAMs can establish hydrogen-bonds and/or undergo condensation reactions (e.g. condensation of carboxylic acids into anhydride (Fryxell et al. 1996)). Hydrogen bonding between polar functional tail groups (e.g. ester groups), lead to molecular segregation when these molecules are coadsorbed with "diluent" molecules to form mixed monolayer (Fryxell et al. 1996; Rao et al. 1999).

Positive contribution of the highly ordered and oriented reactive end-groups present at the surface of SAMs has also been reported. The apparent increase in reaction rate is attributed to the favorable orientation of the reactive groups (Chechik et al. 2000).

Lastly, electrostatic interactions, either attraction or repulsion, often contribute to the reaction rates when the surface is electrostatically charged because it bears ionic groups. Electrostatic interactions are long-ranged and depend on the ionic strength. This is clear that electrostatic interactions do not change the reactivity itself but attract to the charged surface ionic species of opposite charge or repel ions having charge of the same sign. The surface charge depends on the pH when the

ionic groups are either acidic or basic. The variation of the surface charge with respect to pH does not obey simple mass action law because electrostatic interactions attract or repel H^+ and OH^- ions. Such effect is often described in terms of apparent pK_a. The intrinsic acid-base properties of the surface groups are not changed however; electrostatic interactions only just increase or decrease the local concentration of ions in the close vicinity of the charged surface. Interactions between the biomolecule and the support are also under the influence of electrostatic forces (Yeung and Leckband 1997).

Electrostatically driven adsorption is a major origin of nonspecific adsorption that causes background noise in the detection signal. Careful control of surface charge is quite an important issue because nonionic hydrophilic layers are generally preferred. However, one can take advantage of adsorption, and particularly electrostatic adsorption, for improving or getting a better control over the chemical grafting. Thus, it has been noticed that aminated surfaces were highly reactive because their charge was positive in moderate pH conditions. Indeed, adsorption helps the reaction from very dilute solutions because it creates a local concentration of reagents in close vicinity of the surface. Let us point out that most biomolecules are anionic and strongly adsorb to cationic surfaces. Regarding aminated surfaces, the cationic form, $–NH_3^+$, of the primary amino group causes adsorption whereas the reactive species is the neutral amine form, $–NH_2$. A compromise is to be found between favorable adsorption and the surface density of reactive species; therefore there is an optimum pH for the grafting reaction. Strong adsorption helps grafting but also causes nonspecific adsorption during the utilization for the detection of negatively charged biospecies. Therefore, the presence of residual amino groups at the surface is detrimental. A "capping" step consisting in the deactivation of amino groups by reaction of strong electrophilic reagent after grafting is advisable. Capping yields either a neutral derivative, preferably hydrophilic, in order to prevent hydrophobic adsorption and biofouling, or an anionic derivative that creates electrostatic repulsions for anionic species (the reaction of succinic anhydride makes the conversion of primary amines into anionic monosuccinimidyl amides).

The overall issues affecting interfacial reactions may account for the apparent variability of results when different strategies are compared. The chemical reactivity for a given immobilization reaction is related to the chemical reaction itself and the interfacial properties of the underlying support. The influence of the support is consequential because the reaction conditions are quite different of those usually used in chemical synthesis; in particular the concentrations of reagents are very low.

2.4.2 3D Immobilization: Thick Layers, Entrapment Methods

The performances of biosensor devices are expressed in terms of detection specifications such as sensitivity (lowest detectable quantity), concentration range of

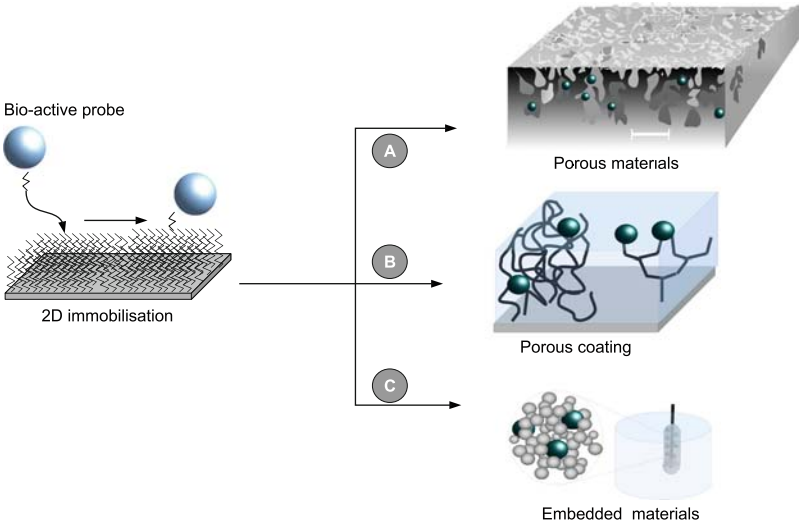


Fig. 2.17 Schematic representation of the three different approaches used to elaborate high specific area support for immobilization. (**A**) Immobilization of bioprobes onto porous materials, (**B**) immobilization of bioprobes onto polymeric layers deposited or synthesized onto flat support, and (**C**) entrapment of bioprobes during porous materials elaboration (sol–gel process)

linear response, and stability properties. Stability of the sensitive layer is mainly ensured by covalent grafting between reactive biospecies and the surface.

For a given appropriate surface preparation, signal intensity for most of biosensor devices depends on the surface density of immobilized species. The loading capacity of flat support (2D) is limited by the external surface area. A way to increase the sensitivity is increasing the specific area of the surface by elaboration of thick immobilization layers named 3D supports (Beattie et al. 1995; Fidanza et al. 2001). Different approaches have been envisaged to take advantage of 3D layers in the field of biosensors (Fig. 2.17). A straightforward approach is based on the biofunctionalization of porous materials with high specific area. A second common approach involves the formation or deposition of thick polymeric matrices onto flat supports. The active species are immobilized (chemically attached or simply entrapped) inside a gel made of, cross-linked polymer materials. A last approach to 3D biofilms is the direct entrapment or encapsulation of biocomponents inside porous materials during the synthesis (sol–gel strategy).

The activity on such layers depends on, (1) the mass transfer resistance (diffusion of analytes to receptors inside the 3D network); (2) the influence of the surrounding surfaces onto the biomolecules adsorption and denaturation (high surface area makes the biointerface more sensitive to the physico-chemical properties); (3) stability and/or leaching of the immobilized or embedded biocomponents into the liquid.

2.4.2.1 Porous Materials

Biologists are accustomed to use thick polymeric supports since long times (e.g. nitrocellulose membranes). The biomolecules are immobilized by deposition onto the membranes. Their huge binding capacity allows for large signal amplification. Widespread at macro-scale, the advent of glass slides coated with this kind of membrane has opened the way to membrane-based microarray technology. These porous materials suffer from nonspecific binding (Kusnezow et al. 2003) and release of the adsorbed probes. In order to improve the stability while maintaining a high binding capacity, the covalent immobilization of biomolecules has been investigated into various inorganic porous matrices.

Porous layers have been elaborated by means of several methods. Electrochemical etching is used to prepare porous silicon (p-Si), nanocrystalline silicon support (nc-Si) (Zhu et al. 2007) or porous aluminum oxide (Lee et al. 2007). Deposition of colloidal silica particles onto flat glass supports and subsequent thermal sintering yields either porous silica (Fidanza et al. 2001) or a porous glass containing 3D network of ordered micro channels, also called micro channel glass (Benoit et al. 2001).

The immobilization of bioreceptors makes use of the same strategies according to surface chemistry of the support as described above. Freshly prepared p-Si surface exhibits silicon–hydride termination. Oxidation by thermal or chemical treatment yields hydroxylated silicon dioxide layer which can be functionalized through alkylsilane derivatization. Functionalization with silanes also applies to porous silica, aluminum oxide and micro channel glass.

The covalent immobilization of biomolecules into these inorganic porous layers improves the stability of the biointerface with respect to washings and repeated utilizations. For instance, oligoprobes immobilized onto p-Si or nc-Si sustained up to 25 cycles of hybridization/stripping (Bessueille et al. 2005; Zhu et al. 2007). In addition, porous glass or p-Si bring about large increases of detection signal (up to tenfold) when compared to flat glass support (Fidanza et al. 2001; Bessueille et al. 2005). The greater loading capacities obtained on such porous layer have allowed to extend the linear dynamic detection range (Zhu et al. 2007) and increase the sensitivity (Cheek et al. 2001).

As a drawback, slow diffusion of molecules inside the 3D matrix causes an increase of response time related to the time required to reach equilibrium (Fidanza et al. 2001). A compromise has to be found to improve the diffusion of biomolecules while maintaining as great a specific surface as possible. Parameters to be optimized are the density and specific area of porous material, thickness of the deposit. In case of anodic etching of silicon substrate, the porosity and thickness of the porous layer can be adjusted by suitable choice of the initial silicon support (doping type and concentration of charge carriers, crystal planes orientation) (Ronnebeck et al. 1999) and/or by optimizing the etching conditions (Janshoff et al. 1998).

Diffusion inside 3D matrix can be forced by making the analyte flow through the porous layer. The most advanced solution to the diffusion resistance problem encountered in 3D matrices is to flow the analyte solution through free-standing porous membrane. As an example, a “flow-thru” device (Cheek et al. 2001) consists

in a 0.5 mm thick micro channel glass support made of a parallel and ordered stacking of 5 μm diameter micro channels oriented perpendicular to the surface plane. The liquid is allowed to flow through each micro channel assimilated to an individual micro reactor. Kinetics of chemical recognition is accelerated because the mass transport distance is greatly reduced when compared to flat surfaces. In addition, the increase of available surface area gives a 96-fold increase in signal intensity when compared to flat glass.

In addition to the high specific area and loading capacity, these materials have attracting specific properties that can be used for detection purposes in biosensor applications. For instance, the electronic or optical properties of porous silicon make it suitable as a transducer in biological sensors (Bayliss et al. 1995; Thust et al. 1996; Janshoff et al. 1998; Schoning et al. 2000; Archer et al. 2004; Lie et al. 2004). Several photonic porous silicon–based structures have been investigated as label-free detection systems. These systems take advantage of optical interferometric methods (Bragg mirrors, rugate filters or optical microcavity) for very sensitive detection of small molecules, DNA and proteins interactions (Tinsley-Bown et al. 2005; Koh et al. 2007; Rendina et al. 2007). Another porous silicon–based method for detection of label-free biomolecules relies upon surface-enhanced laser desorption/ionization mass spectrometry (Wei et al. 1999). Compared to the widespread Matrix-assisted laser desorption/ionization (MALDI) technique, desorption/ionization on silicon (DIOS) is a matrix-free desorption technique that offers high sensitivity, no matrix interference (Zhouxin Shen et al. 2004). This technique is amenable for small molecules including peptides, glycolipids or carbohydrates (Wei et al. 1999), DNA, proteins (Mengistu et al. 2005), and enzyme activity analysis (Zhouxin Shen et al. 2004). It is worth to note the outstanding use of porous silicon layers for label-free detection of biomolecules. This opens the frame to new developments in optically active materials for biosensor applications.

New trend for high-throughput screening of biomolecular interactions implement a "bar-code strategy." Indeed, shortcomings of flat microarray substrates are related to the extremely slow diffusion process of analytes throughout the surface and more generally in confined domains of small volume. Improvement of reaction rates by mixing small volumes of dilute solutions is a technological hurdle (Edman et al. 1997; Adey et al. 2002; Hui Liu et al. 2003; Toegl et al. 2003; McQuain et al. 2004).

One way to overcome the diffusion limitation of flat surface immobilization is to remote the biorecognition step in solution. Thousands of different probes that are attached on the labeled particles are dispersed in solution of analyte; the recognition event and the label are detected simultaneously. To meet the detection requirements, encoded micrometer-sized porous-silicon particles have been used to screen fluorescence-tagged proteins (Cunin et al. 2002).

2.4.2.2 Organic Polymer Coatings and Gels

A widely used technology makes use of polymer coatings. Macromolecules are either grafted to the surface, or cross-linked for avoiding their leakage into solution.

Water-insoluble polymers are often used in chemical sensors sensitization. This method is derived from the usual technology of the Ion-Selective Electrodes membranes. There may be some applications of water-insoluble polymers to biosensors. A well-known example which may be considered as biosensor is the potassium selective electrode and the ISFET sensors derived from it where the cyclic peptide complexing agent valinomycin is entrapped in a PVC membrane. But most cases require aqueous environment in the surroundings of the biochemical recognition site. Selected polymers are hydrophilic; the polymer layer is swollen by water in order to allow the diffusion of biospecies to the recognition site inside the aqueous environment of the polymer layer. Therefore, grafted water-soluble polymers or hydrogels are used as immobilization matrices on the sensor surface. Polymeric layers can be obtained by grafting prefabricated polymers (brushes, dendrimers) or synthesizing the polymer layer *in situ* on the surface by chemically initiated polymerization, photopolymerization, or electropolymerization (Korri-Youssoufi et al. 1997). The 3D architecture can be made of macromolecular chains tethered to the solid surface by one chain end. The attached macromolecules look like long spacers bearing one or several reactive group per chain (brushes (Pirri et al. 2006; Schlapak et al. 2006)). More complex architecture can be found with branched polymers and starburst structures (dendrimers). Star polymers and dendrimers are branched polymers of well-defined architecture of the polymer backbone that should be considered on the same footing as branched polymers. Such a precise structure probably does not bring about a significant difference with respect to the less ordered architecture of randomly branched polymers. It may not be worth implementing the complex chemistry required for the synthesis of dendrimers for immobilization purposes. A possible advantage of the (over)controlled chemistry of dendrimer is the possibility of attaching the reactive groups at the periphery of the dendrimer macromolecules. Hydrogels made of hydrophilic cross-linked polymers are another class. The classification into two different classes as grafted macromolecules and hydrogels, is difficult and arbitrary however since the differences between the two may appear quite slight. The wide variety of polymeric structures allows depositing layers of thicknesses ranging from few 10 nanometers to few micrometers, giving rise to different surface behavior and analytical properties. The deposition, grafting or synthesis of polymer layers have been realized onto chemically modified surfaces that ensure strong adhesion, chemical grafting or surface induced polymerization.

2.4.2.2.1 Polymer Coatings, Brushes, and Dendrimers

These coatings lead to relatively thin layers (<100 nm) when compared to inorganic porous materials or gels that can easily reach up to few micrometers thickness (Kato et al. 2003). Various polymer architectures as brush, grafted branched polymers, 3D dendritic polymers give technological answers to the several queries of the analyst (Fig. 2.18). “Polymer brush” refers to linear macromolecules adsorbed or grafted by one of their chain end. This term was introduced by Alexander and de

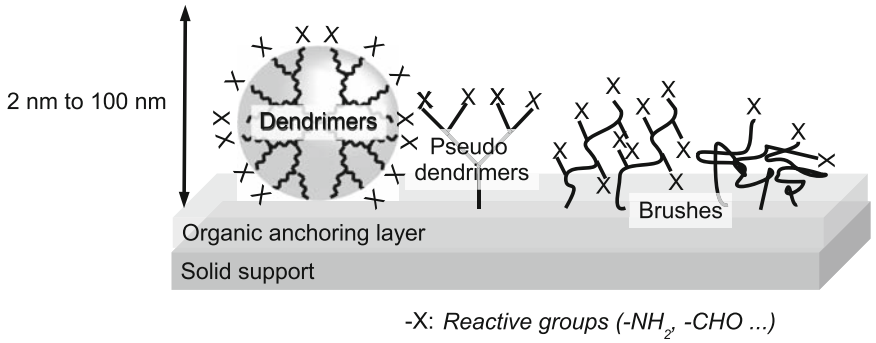


Fig. 2.18 Schematic representation of various surfaces modified with thin polymeric layers

Gennes for describing the dense regime of tethered chains where the density is such that the chains are constrained to stretch perpendicular to the surface (De Gennes 1987). The meaning of the word "brush" is currently extended to end-tethered polymer chains, whatever the grafting density.

There are two main strategies for chemical grafting of polymers: grafting reactive polymers to surface groups ("grafting to") or initiating the living polymerization from surface bound initiators ("grafting from") (Zhao and Brittain 2000). Grafting from process is currently developing fast thanks to the recent discovery of new living polymerization reactions that do not require the extreme dryness conditions of ionic polymerization reactions (Advincula 2006). In particular, controlled radical polymerization allows the *in situ* synthesis of vinyl polymers (acrylates, methacrylates, vinyl ethers) (Bergbreiter and Kippenberger 2006; Buchmeiser 2006; Tsujii et al. 2006). Photo initiated grafting is also promising (Dyer 2006; Matsuda 2006).

The primary aim of polymer coating is to place the bioprobes away from the solid interface in order to limit hindrance phenomena and make the probes available to the targets in solution. The chemical grafting of PEG polymers onto flat glass surface (appropriately functionalized) brings on very beneficial properties regarding the biological interactions. The hydrophilic PEG polymer chain is well-known for its resistance to protein adsorption. The PEG polymer acts as a hydrophilic noncharged spacer improving the solubilisation of the biospecies, avoiding the steric hindrance and limiting surface effects such as adsorption onto hydrophobic patches of the surface that may lead to denaturation or loss of activity (Shchepinov et al. 1997; Wang et al. 2002). Each PEG molecule is tethered by one end and leaves only one reactive group toward biomolecules immobilization. In case of DNA immobilization and hybridization, the PEGylated surface yielded fourfold increase in signal hybridization compared to 2D silanized support; another benefit was lowering the nonspecific adsorption by a factor of 13 (Schlapak et al. 2006). Additional advantage concerns the macroscopic improvement of the surface homogeneity since the soft the PEG layer of 3.5 nm thickness masks inhomogeneities (roughness) of the underlying silane anchoring layer.

Polymer brushes with multiple reactive groups increase the density of recognition sites. As example Piri et al. (2006) reported a "grafting from" approach where each macromolecule of the brush contained several reactive groups (~100) giving rise to larger signal compared to flat glass slides functionalized with the same reactive group (epoxy-functional silane). Despite the direct synthesis of polymer chain onto the solid support that would allow dense layers of parallel polymer chains, the surface concentration of polymer chains was fairly low (0.24 pmol/cm^2). In addition, the corresponding DNA grafting density (0.3×10^{13} molecule/cm^2) stood in the range of most grafting densities reported for 2D surface functionalization (Dugas et al. 2004). By comparison, the surface preparation by immobilization of prefabricated dendrimers (poly(amino)amine, G4 PAMAM) onto silanized glass slides gave DNA grafting density of 9×10^{13} DNA strands/cm^2 (150 fmol/mm^2).

Various approaches to elaborate polymer linkers (Beier and Hoheisel 1999) or chemically grafted functional polymers including dendrimers (Benters et al. 2001; Le Berre et al. 2003) have been reported. While the binding capacity of such nanometric structures are no necessarily higher the excellent accessibility of probes grafted on such dendrimer coatings lead to higher immobilization and hybridization efficiencies than those of 2D functionalized glass slides (Le Berre et al. 2003).

Linear polymer brushes and branched polymer coatings allow showing up the bioprobes in a configuration that resembles that of free solution (Pirri et al. 2006) and provide an excellent accessibility of the target. In addition, such hydrophilic polymer coatings show lower non-specific adsorption and hence low background signal and improved detection sensitivity (10–100-fold higher than 2D functionalized glass slide) (Le Berre et al. 2003). It is worth to note that no trouble regarding resistance to diffusion was reported, probably due to the relatively small thicknesses of the layers.

Lastly, polymer coatings allow improving the uniformity of the functionalized layer when compared to silane functionalized layers for example (Schlapak et al. 2006). The spots of microarrays obtained with polymer coating are uniform and homogenous (Benters et al. 2001; Le Berre et al. 2003). This result is particularly important since the ability to extract meaningful information from microarray experiments depends considerably on the spot quality (Diehl et al. 2001; Rickman et al. 2003; Dugas et al. 2005; Derwinska et al. 2007).

2.4.2.2.2 Gels, Hydrogels

The entrapment inside a thick layer of hydrogel is the first immobilization technique that was used for proteins (Guilbault and Montalvo 1969, 1970). This is a historical technology that still finds wide acceptance because of its simplicity. Thus, an aqueous solution of protein and water-soluble polymer is mixed with a cross-linking agent and drying at the surface of the sensor. The surface is ready to be used after it has been washed with water. As typical example, proteins are immobilized

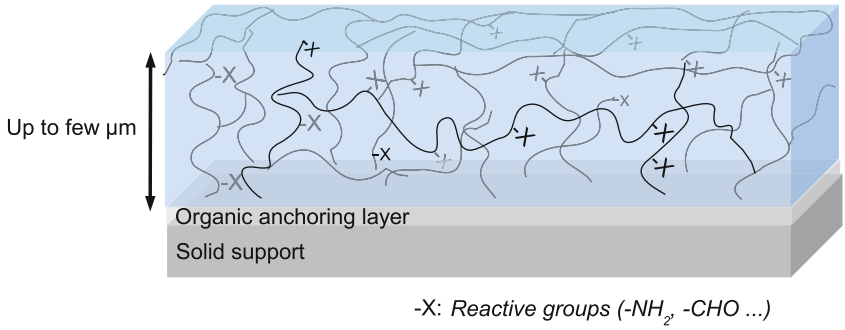


Fig. 2.19 Schematic representation of hydrogel coating

in a gel of bovine serum albumin cross-linked with glutaraldehyde. Bioprobes are either entrapped inside the gel but may also have reacted with the cross-linking agent, so that they are chemically attached to the polymer network constituting the gel. Of course there is only poor control over the chemical groups that have reacted and the orientation of the active centers.

An important benefit of the hydrogel technology lies in the immobilization of fragile biomolecules that should be kept in aqueous media for remaining active. One drawback inherent to spotting nanolitre droplets of protein solution on glass slides for microarray fabrication is the easy evaporation of water. Even in 100% humidity atmosphere, preventing drying very small drops is difficult. Preservation of protein stability and functionality in hydrated state involve the conservation of homogeneous water environment. Moisturizing additives are often added to the buffered immobilization solution: glycerol (MacBeath and Schreiber 2000), carbohydrates (trehalose, sucrose) (Kusnezow et al. 2003), or low molar mass poly (ethylene oxide) (Lee and Kim 2002). The use of highly hydrated polymers (hydrogels) provides a suitable liquid-like 3D environment for keeping the activity and allowing biological interactions. In addition hydrogels allow high loading capacities. Hydrogels (Fig. 2.19) are cross-linked hydrophilic polymers with interstitial spaces that contain as much as 90–99% w/w water (Hynd et al. 2007; Ulijn et al. 2007). Hydrogel matrices used in the field of biosensors are mostly based on polyacrylamide, polysaccharide (e.g. agarose) or substituted polysaccharide (e.g. carboxymethylated dextran) that are cross-linked. These hydrated coatings provide hydrophilic surfaces that prevent nonspecific adsorption of proteins. The manufacturing of hydrogel coatings can be realized by grafting prefabricated polymers (Zhou et al. 2004), UV-light or chemically initiated polymerization onto chemically derivatized solid support (Guschin et al. 1997; Wang et al. 2002). The coating can be realized collectively by casting the gel over the whole area of the support, or localized by photolithography (Guschin et al. 1997) or by dispensing gel drops onto the solid support (Rubina et al. 2004). The thickness of hydrogel films ranges from 200 nm (Zhou et al. 2004) to 30 µm (Arenkov et al. 2000; Angenendt et al. 2002).

The chemical immobilization of biomolecules into precoated 3D hydrophilic films uses a range of established chemistry. Carboxymethylated dextran films present carboxylate groups that readily react onto aminated biomolecules via an activation step with N-hydroxysuccinimide for example (Löfås and Johnsson 1990). Agarose gels are activated by a periodate oxidation ($NaIO_4$) step that leaves aldehyde reactive groups (Wang et al. 2002; Wei et al. 2004). Like agarose gel, prefabricated polyacrylamide gels can be chemically modified in order to immobilize biomolecules (Arenkov et al. 2000). Modifications involve incorporation of aldehyde groups or hydrazide groups that react respectively onto aminated biomolecules (proteins, DNA) or aldehyde groups that can be site-specifically introduced onto the polysaccharide portion of antibodies. Proteins can be entrapped by cogelation with Bovine Serum Albumin (BSA) using glutaraldehyde as cross-linking agent (Wan et al. 1999). Glutaraldehyde is a short homodifunctional cross-linker reagent widespread for protein conjugating. The aldehyde functionality reacts readily with amino groups and creates a network of cross-linked proteins. Coupling is not oriented however; proteins inside the thick layer display nonhomogeneous binding activity.

Taking advantage of the flexible synthesis of polyacrylamide gels by changing or modifying the monomers composition of the starting solution, it was possible to tailor the porosity of the gel matrices in order to improve access of macromolecules inside the gel (Arenkov et al. 2000). Copolymerization with acrylic-labeled biomolecules (Brueggemeier et al. 2005) or biomolecules bearing nucleophilic groups (–SH, $–NH_2$) (Dyukova et al. 2005) is a straightforward means to immobilize biomolecules with high efficiency (Rubina et al. 2004).

Gel coating–based microarrays are produced by spotting dilute solution of bioprobes onto the gel layer leading to uneven distribution of probes and leaving most material immobilized on the top layer of the gel. By contrast, copolymerization allows manufacturing gel drop with homogeneous distribution of probes within the gel and with high immobilization efficiency. For example, up to 87% of oligonucleotides modified with methacrylamide groups present in the starting solution have been immobilized (Dyukova et al. 2005).

Control of the porosity and loading capacity allows the immobilization of well-spaced (unhindered) biomolecules in aqueous environment and improves the diffusion of analyte through the gel matrix.

3D hydrophilic gel films are intended to provide a stable, low-fluorescence background, low nonspecific adsorption and high loading capacity for immobilization (Arenkov et al. 2000; Dyukova et al. 2005). The most valuable improvement of hydrogel films is holding biomolecules inside a solution-like environment far away from detrimental interfacial effects. This is crucial to maintain fragile biomolecules in active from upon immobilization and utilization conditions.

For example, Wang et al. (2002) have compared the efficiency of peculiar molecular beacons oligoprobes immobilized onto solid support regarding the discrimination of single nucleotide mismatches. Molecular beacons are hairpin-shaped oligoprobes obtained through a self-hybridization of complementary sequences present on a DNA strand. A bound fluorophore has its fluorescence quenched in

the hairpin structure; fluorescence is recovered when the hybridization of complementary DNA strands in solution opens the beacon. Immobilization of molecular beacons onto aldehyde-activated glass slides (2D chemistry) gives high fluorescence background and relatively low increase in fluorescence upon hybridization because the hydrophobic and electrostatic interactions between molecular beacons and the underlying surface destabilize the stem loop. Molecular beacons immobilized inside agarose film provide improved quenching efficiency and excellent discrimination of single-nucleotide polymorphism when compared to flat supports (Wang et al. 2002).

The loading capacity of immobilized probes per unit area is two to three orders of magnitude higher than that for flat support. Combination of the high loading capacity and the low nonspecific adsorption on hydrogel films leads to higher sensitivity (Angenendt et al. 2002; Brueggemeier et al. 2005). The large linear range of detection of hydrogel microchips allows quantitative assessment of antibody–lectin interactions (Dyukova et al. 2005), or enzymatic activities (e.g., inhibition of tyrosine kinase activity) in complex biological medium (Brueggemeier et al. 2005).

Diffusion inside thick porous membranes is slow however, which leads to quite long response times (Wang et al. 2002; Zubtsov et al. 2006). It would be wide to increase the area of gel spots to accelerate the diffusion (Rubina et al. 2004); of course, this is done at the expense of the spot density of the arrays. Interestingly, stirring the target solution on top of the surface not only accelerates the mass transport of targets to the hydrogel surface but also contributes to the penetration of targets inside the gel. Mechanical stirring (by flowing with peristaltic pump) leads to approximately fivefold acceleration in reaction–diffusion kinetics for hydrogel-based microchips (Zubtsov et al. 2006).

Hydrogel arrays have been investigated to immobilize and analyze oligonucleotides, proteins, antibodies, glycans and living cells (Proudnikov et al. 1998; Dyukova et al. 2005). Hydrogel microchips are compatible with various detection techniques. Colorimetric techniques (chemiluminescence) were implemented to monitor enzymatic activities (Brueggemeier et al. 2005). Investigation of protein–protein, enzymatic activity or enzyme–inhibitor interactions have been performed on hydrogel protein arrays by MALDI-MS (Gavin et al. 2005). Surface plasmon resonance techniques were used with relatively thin hydrogel films (200 nm) (Löfås and Johnsson 1990; Zhou et al. 2004). Adaptation of the detection to thick layers may be necessary in some instances. Thus, hydrogel coated slides of microarrays may show rather high variability of spot geometry and homogeneity (Afanassiev et al. 2000; Angenendt et al. 2002) which makes confocal detection of fluorescence worse than a robust classical detection (Derwinska et al. 2007).

Finally, bioresponsive hydrogels materials open the way to original label-free detection modes. Bioactive materials undergo macroscopic changes of the hydrogel network (swelling/collapse or solution-to-gel transition) in response to selective biological stimuli ((Ulijn et al. 2007) and references within).

2.4.2.2.3 Structured Coatings

The internal structure of thick coatings allows the control of the diffusion inside. This is of primary importance for indirect transduction modes where the signal does not come from the biorecognition itself but from secondary-products of the bioreaction. For example, the historical urea-sensitive enzyme electrode (Guilbault and Montalvo 1969, 1970) detects the local rise of pH when urea is enzymatically decomposed into ammonia. Electrochemical devices often require the accumulation of mobile species in the vicinity of the transducer surface: conductometric sensors detect variations of local concentration of ionic species; amperometric sensors often require electron transfer by mean of a red-ox mediator.

2.4.2.2.3.a Functional Multilayer Coatings

It has been recognized that the direct transfer of the ion-selective electrodes (ISE) membrane technology to chemical sensors has not been satisfactory. ISE membranes are self-supported polymer films where ion-sensitive chemical species are incorporated. Such species are mobile and partition between polymer membrane and solution. Coatings on solid surface do not require being self-supported and their thickness and internal structure may be optimized with respect to diffusion issues. An additional issue is the adhesion of the coating to the surface which can be ensured either by chemical grafting the materials or by a grafted sub layer (silane layer). The hydrophilic character of the membrane is essential for limiting nonspecific adsorption and fouling. Therefore, quite complicated multilayer coatings appeared for the manufacture of ion-sensitive field-effect transistor (ISFET), giving a definite improvement of the signal and reduction of background. The following example helps understanding the issues and benefits.

An interesting two-layers coating was proposed for the urea-sensitive enzymatic field-effect transistor (ENFET) and conductometric sensors made of interdigitated electrodes on a solid support (Shulga et al. 1993; Soldatkin et al. 1993, 1994; Jdanova et al. 1996). Thus, the hydrolysis of urea by urease yields ammonia that increases the pH at the surface of the FET device. Glucose oxidase catalyzes conversion of glucose into gluconolactone that yields gluconic acid upon hydrolysis; therefore, the local pH decreases in the presence of glucose. Urease is immobilized at the surface of the FET inside a classical gel of BSA cross-linked with glutaraldehyde. The detection signal comes from the ionization of the surface groups of the silica or silicon nitride insulator, which persists as long as the acidic or basic species stay close to the surface. The buffer in the analyte solution cancels out the signal. A definite increase of the signal is obtained when the diffusion of the acidic/basic species is blocked by an over layer deposited on top of the cross-linked BSA layer aimed at the immobilization of the enzyme. The external layer made of a polyelectrolyte (Nafion, polyvinylpyridine) opposes ion transport but does not slow down too much the diffusion of the neutral analyte (glucose, urea) to the internal layer.

2.4.2.2.3.b Structured Organic–Inorganic Particle Coatings

A gel of inorganic particles can be used as an immobilization matrix in the same way as the polymer gels described above. Organic particles could be used as well. The inorganic particles functionalized with organic grafted or mixed with organic biomolecules form a hybrid organic–inorganic material that is deposited as a thin film onto the solid support. The hybrid material stays at the surface of the support if the inorganic and organic compounds are stuck together as a water-insoluble and nondispersible material. Both organic and inorganic materials are held together by means of physical forces. This process is a simple and mild variant of the sol–gel method for the preparation of porous silica matrix described in the next section. The process is mild since it does not involve chemical reaction in the presence of the biomolecules.

Deposition of thin films of inorganic particles, mainly clays, has been introduced by Kotov (Kotov et al. 1997) and implemented further for the manufacture of "clay electrodes" (Mousty 2004). Basically, clay particles (laponite) and enzymes are precipitated on the support (Poyard et al. 1996). The clay gel consists in a concentrated oriented phase of clay swollen by water; macroscopic orientation (nematic phase) is achieved as thin films at the surface of electrodes (Chevalier et al. 2002). The laponite particles in the gel are held together in concentrated enough electrolyte medium; clay particles delaminate because of electrostatic repulsions in case of low ionic strength. Cationic species (surfactants, polymers) are also added possibly for inducing the sustained coagulation of the particles (Besombes et al. 1997; Coche-Guérente et al. 1998; Coche-Guérente et al. 1999). Fast coagulation conditions leave a loose network of particles that immobilizes enzymes and keeps the possible diffusion of analyte to the biomolecules. Tighter immobilization in a dense gel can be achieved by a slower coagulation rate. As for any immobilization process in a gel, optimization of the properties is a compromise between the sustainable immobilization of biospecies and the necessary diffusion of the analytes to the recognition site.

2.4.2.2.3.c Layer-by-Layer Alternate Polyelectrolyte Coatings

Electrostatic adsorption of electrically charged biospecies is quite easy to carry out but suffers the limitations of adsorption: poor control of the orientation of biospecies and massive nonspecific adsorption that leads to high background in sensor applications. In spite of these drawbacks, electrostatic adsorption can be utilized as a ready deposition process of thick polymer coatings. One simple way consists in heterocoagulation of negatively and positively charged species that precipitate onto the surface. This is identical to the well-known "complex coacervation" process used in the field of microencapsulation; it leads to high loadings of each charged partners inside a gel swollen by water (Gibson et al. 1996).

Better control of the thickness of the polymer coating is achieved by the layer-by-layer deposition of oppositely charged polyelectrolytes that leads to alternate polyelectrolyte layers. Such materials can be favorably used in many

coating applications (Jaber and Schlenoff 2006), including sensors applications (Caruso et al. 1997; Chluba et al. 2001; He et al. 2002; Ferreyra et al. 2003; Ferreyra et al. 2004; Rodriguez and Rivas 2004; Coche-Guérente et al. 2005; Miscoria et al. 2006). The structure of the deposit is a well-ordered alternate stack of both polyelectrolyte layers in favorable case. Actually, the structure is most often quite disordered but the thickness of the deposit can be nicely controlled by the number of elementary deposition steps; the surface charge of the full layer is that of the last deposited polyelectrolyte (Ferreyra et al. 2003; Ferreyra et al. 2006).

Alternate polyelectrolyte-nanoparticle multilayers are fabricated in the same way. Various particles such as gold nanoparticles (Santos et al. 2005), CdS semiconductor nanoparticles (Kotov et al. 1995), organic polymer particles (Kong et al. 1994)andclay (laponite as in the previous section but deposited layer-by-layer) (van Duffel et al. 1999).

2.4.2.3 Entrapment in Inorganic Gels

Bioactive species can be embedded within the inorganic nanostructure made of oxide materials using the sol–gel process. This technology involves mixture of alkoxysilane precursors or ionic salts (e.g., sodium silicate) in solution with functional biocomponents. *In situ* synthesis of a porous inorganic support is a straightforward and convenient way to entrap (immobilize) functional biocomponents.

2.4.2.3.1 Silica Sol–Gel Mechanism

In general, the sol–gel reaction of metal oxides precursors such as tetramethoxysilane (TMOS) or tetraethoxysilane (TEOS) proceeds at room temperature through three steps:

(1) "activation" of the metal oxides precursors by acid or base-catalyzed hydrolysis (Fig. 2.20).

It is worth noticing that by-products of the hydrolysis of alkoxysilanes are organic molecules (methanol or ethanol from methoxy or ethoxysilane precursors respectively).

(2) Polycondensation of the activated precursors or "monomers" to oligomers leading to formation of transparent and stable nanometric dispersions of silica particles (the "sol") (Fig. 2.21). The solid content of the dispersions of nanoparticles ranges from 4 to 20 wt% (Böttcher 2000).

The size of the sol particles and cross-linking within the particles depend upon the pH and solution concentration and composition. The low viscosity of sols make

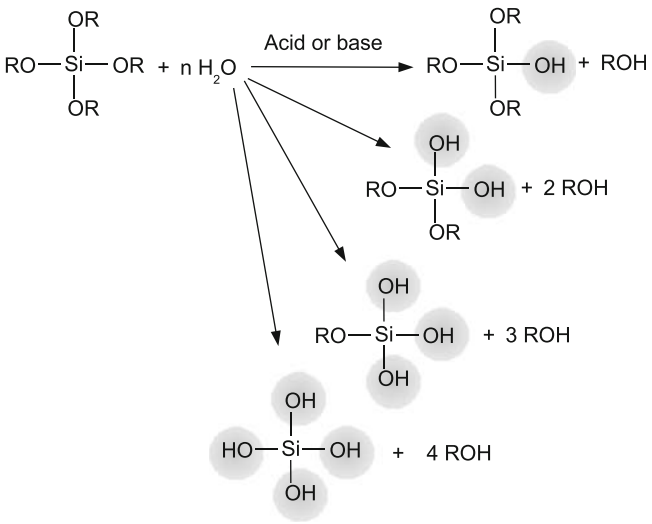


Fig. 2.20 Products of the hydrolysis of the alkoxysilane precursors in solution

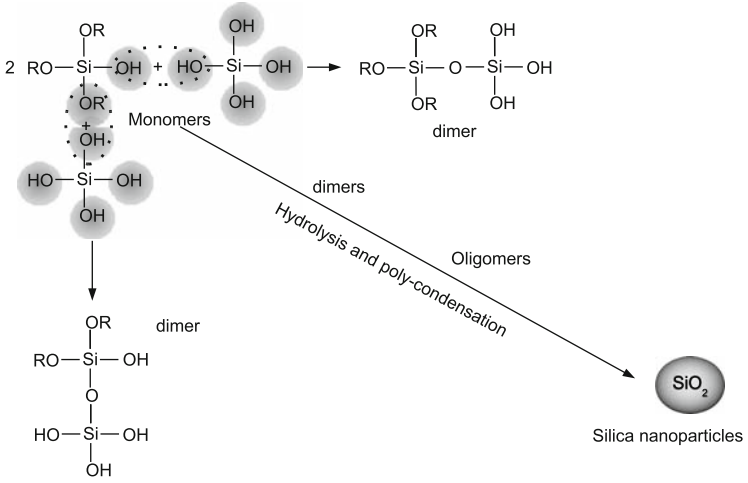


Fig. 2.21 Condensation and polycondensation of alkoxysilane and hydrolyzed alkoxysilane and formation of the silica nanoparticles in solution (sol)

them compatible with various deposition techniques (spin coating, spray drying, mold casting) in order to produce thin film or bulk materials.

(3) Formation of a three dimensional silica network by further polymerization and cross-linking (chemical sintering) of inorganic particles. This solidification of the sol leads to a solid network holding the liquid (the "gel") (Fig. 2.22).

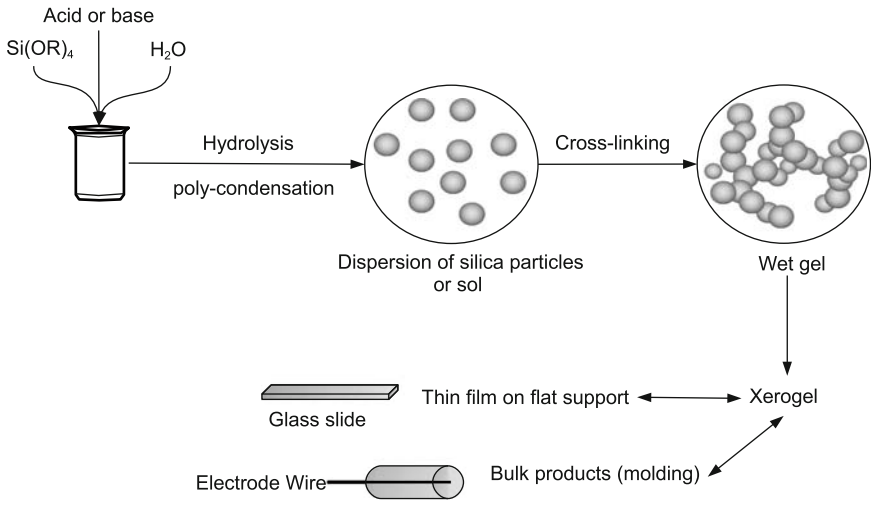


Fig. 2.22 Schematic representation of the sol–gel process and formation of xerogels

Subsequent drying of the gel affords xerogels. The physical properties of the 3D matrix depend on the size of particles and the extent of cross-linking occurring before gelation. The solvent evaporation after deposition of the wet gel as thin films or after mold casting leads to the formation of thin films of xerogels (0.1–2 μm) or bulk xerogels that replicate the shape of the mold. For example, sol–gel mixture can be cast into test-tubes with copper electrode wire; the resulting electrode will support silica sol–gel with embedded biomolecules (Thenmozhi and Narayanan 2007).

2.4.2.3.2 Bioactive Sol–Gel Silica Layers

Immobilization of a wide range of viable biomolecules ranging from enzymes to whole cells was made in silica gels (Avnir et al. 1994; Böttcher 2000). The silica sol–gel process is a simple and efficient fabrication process of inorganic porous materials. The preparation of bioactive sol–gel materials is realized through simple addition of the bioactive components either before or after hydrolysis of the precursors to the sol–gel mixture (Fig. 2.23). It is carried out at room temperature and is well-suited to the direct encapsulation of temperature sensitive biomolecules. Owing to the good mechanical, thermal and photochemical stability of the silica matrix, entrapped biomolecules are protected from external harsh conditions encountered in some applications (e.g. environmental analyses).

Organic solvents may cause proteins denaturation or affect the viability of cell systems. They are either introduced intentionally for solubilizing the precursors or they are by-products of the sol–gel reaction. Aqueous route for sol–gel preparation involving sodium silicate precursors or evaporation of the alcohol by-products before addition of proteins, avoid the unfavorable effects caused by the organic

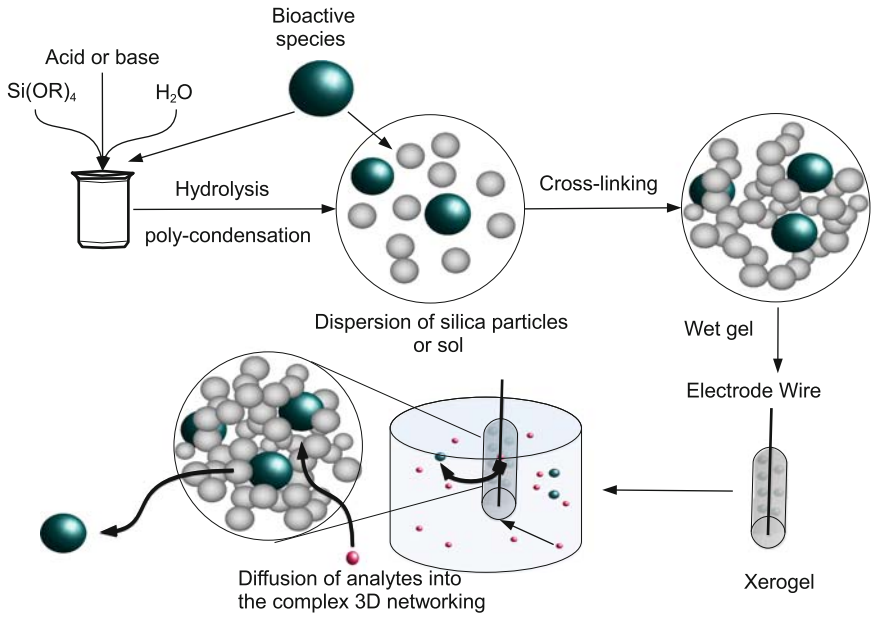


Fig. 2.23 Schematic representation of the sol–gel process and bioreceptors embedding

molecules (Carturan et al. 2004; Lin et al. 2007). Acidic or basic environments also are severe limitations with regards to cell immobilization (Carturan et al. 2004). Various stabilizing agents have been proposed to improve the stability of biomolecules during the sol–gel reaction; loss of activity was often observed however (Besanger and Brennan 2003). Utilization of functional silanes that release such stabilizing agents upon hydrolysis is an elegant alternative (Besanger and Brennan 2003). Functional silanes also allow the preparation of hybrid sol–gel materials with the optimum hydrophilic/hydrophobic balance that provides a suitable medium for enzymatic activity (e.g. lipases that possess hydrophobic domains) (Thenmozhi and Narayanan 2007). Similarly, membrane proteins can be immobilized (stabilized) in the phospholipid bilayer of liposomes prior to silica sol–gel entrapment (Besanger and Brennan 2003).

2.4.2.3.3 Activity and Stability of Biomolecules Doped Sol–Gel Silica

Performances of sol–gel biofilms with regards to biosensor applications depend on the activity of the bioactive receptors within the inorganic matrix and the storage stability. The catalytic activity of bioreceptors entrapped inside a 3D network is related to (1) the diffusion of analytes inside the 3D network where receptors are immobilized, (2) the extent of denaturation of receptors during the sol–gel process and (3) the leaching or release of the immobilized molecules during use.

2.4.2.3.3.a Leaching

Leaching occurs in the early use of biosensors. After an initial burst release, the remaining biomolecules may be strongly immobilized, allowing long-term stability if the matrix does not get physically damaged (Böttcher 2000). Leaching affects the reproducibility and sensitivity of biosensors.

Process parameters leading to increase the pore sizes (soluble additives, pH) increase the leaching of biomolecules (Lin et al. 2007). For instance, glycerol or glycerated silane precursors used for stabilizing proteins and improving silica–protein interactions, tend to increase the pore sizes and thus the leaching (Lin et al. 2007). Favorable electrostatic interactions between silica and proteins can be used to limit leaching (Rezwan et al. 2005).

Of course leaching can be prevented by covalent immobilization of the entrapped biomolecules (Thenmozhi and Narayanan 2007).

2.4.2.3.3.b Diffusion

Surface reactions are often diffusion-controlled. Diffusion in porous materials is mainly function of the porosity and the viscosity of solution. Porosity of porous materials is defined as the opens pores' space in a material according to IUPAC recommendations (Sing et al. 1985). Pores are classified into three categories: (1) macropores with widths exceeding 50 nm, (2) mesopores of widths between 2 and 50 nm and (3) micropores below 2 nm. Immobilization of biomolecules takes place in the mesoporosity. Porosity of the sol–gel materials can be controlled by the pH or the water/silane ratio of the sol–gel mixture. The experimental conditions are adjusted to limit leaching while optimizing the diffusion of analytes within the silica sol–gel matrix.

2.4.2.3.3.c Intrinsic Biological Activity

Intrinsic biological activity is referred to the activity of the immobilized biocomponents when compared to the nonimmobilized ones. Proteins can undergo denaturation. Cell systems may disrupt upon sol–gel reaction or be poisoned by experimental conditions. As consequences, activity of such bioactive components is altered.

Proteins activity measured by the Michaelis constant K_m varies strongly according to the protein type. Glucose oxidase (GOD) entrapped in sol–gel layers exhibits K_m values similar to free GOD in solution, while lipase activity is only 3–29% of the free enzyme (Böttcher 2000).

While some bacteria and yeast (e.g., *Saccharomyces cerevisiae*) survive the experimental conditions of sol–gel reaction, more global approaches allowing entrapment of functional microorganisms are challenging. Various solutions are protection of cell by alginate microencapsulation prior to sol–gel process (Heichal-Segal et al. 1995), retarded addition of cells just before the sol–gel

transition, and gas-phase synthesis of sol–gel prior to its deposition on top of the cells (Carturan et al. 2004).

2.4.2.3.4 In Conclusion

Biocompatible silica sol–gel processes are the convenient way to prepare bioactive films. By controlling the processing parameters and the sol–gel solution a wide panel of biological species can be embedded ranging from nucleic acid to cell system while maintaining their viability. A one-step immobilization method allows the formation of films or bulk products of various shapes (e.g., electrodes, thin film). Biosensors with excellent long-term stability (up to 120 days) are prepared with biomolecules immobilized inside mechanically and photochemically stable inorganic matrix (Thenmozhi and Narayanan 2007).

The silica sol–gel can be transparent in case of optical detection systems (Yamanaka et al. 1992; Lin et al. 2007) or enclose electronic mediators such as ferrocene, or graphite powders to improve response and reproducibility of electrochemical biosensors (Gun et al. 1994; Kunzelmann and Bottcher 1997; Li et al. 1997; Thenmozhi and Narayanan 2007).

2.4.3 Immobilization onto Colloidal Particles

Since many years, the colloids (latexes, magnetic particles, silica particles, fluorescent particles, etc.) offer multiple potentialities of applications, in particular in the fields of pharmaceutical, medical and biological sciences (Arshady 1993; Elaissari et al. 2003a, b, c). The major interest of these dispersed materials (simple or composite) is related to their use as support of biomolecules in the biomedical diagnosis. The first applications of colloidal particles in biomedical diagnostic tests are based on the agglutination process in which latex particles sensitized by antibodies or antigens are used (Fig. 2.24). The biological molecules immobilized,

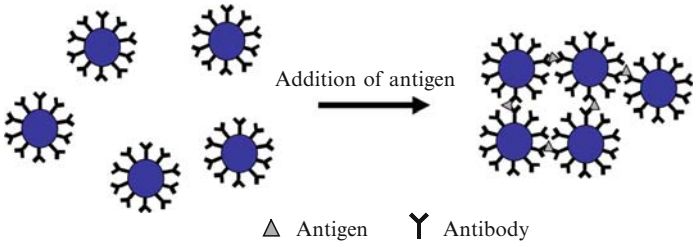


Fig. 2.24 Antibody-coated particles agglutinated by the specific antigen molecules (Stoll et al. 1993; Ouali et al. 1995)

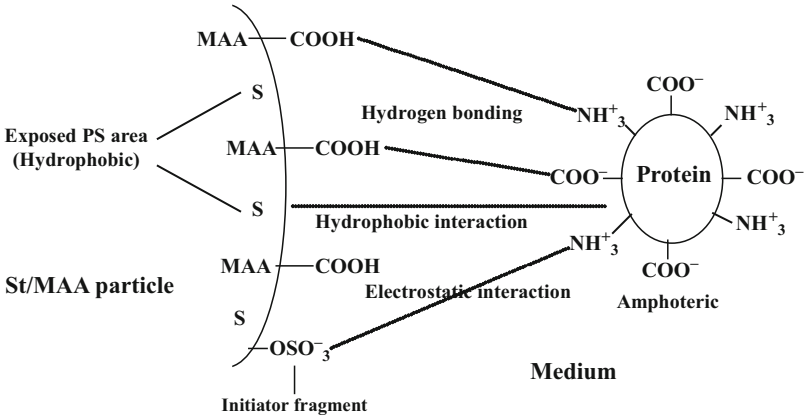


Fig. 2.25 Schematic representation of the possible interaction forces between proteins and polystyrene latex particles bearing sulfate and carboxylic groups. (St/MAA for styrene Methacrylic Acid particles). The interaction forces involving the adsorption process are classified into four interactions, that is, (*1*) hydrophobic interaction, (*2*) electrostatic interaction, (*3*) hydrogen bonding, and (*4*) Van der Waals interaction. The hydrophobic interaction has a major role in protein adsorption phenomena, especially the adsorption of proteins onto the low-charge particle surface

on latex particles can be antigens or antibodies. The immobilization of these molecules is mainly via covalent grafting or physical adsorption.

The specificity and the sensitivity efficiency of such traditional diagnostic are directly related to the surface particles properties of the latex particles and to the accessibility of the receptor. The interactions between receptor and reactive particles are strongly dependent on the colloidal and surface properties of the dispersion, and on the physical-chemistry properties of the receptor (Fig. 2.25). The immobilization (adsorption and/or covalent grafting) of receptor is generally studied by taking into account the influence of physical-chemistry parameters such as; pH, salinity, buffer nature, temperature, surface nature and the presence of competitive adsorbing agent. The immobilized receptors onto colloidal particles are characterized with respect to their conformation and biological activity. In this direction, various polymer-based colloids have been prepared for *in vivo* and *in vitro* biomedical applications (Elaissari et al. 2003a, b, c).

To reach those first conventional applications, the receptors can be antibodies, oligonucleotides (single-stranded DNA fragments), peptides and proteins. The use of particles bearing well appropriate reactive groups and surface properties may confer more stability to the elaborated conjugates (particle–receptor). In addition to the stability aspect, the reactivity of the receptor can be enhanced when the conformation was adequately designed. The needed reactive particles are elaborated using many heterophase processes (emulsion, dispersion, precipitation, self-assembly, physical processes) (Elaissari et al. 2003a, b, c).

Nowadays, colloidal particles are of great interest not only in conventional diagnostic, but also in microsystems based on microfluidics in which the sample preparation and the detection of the targeted disease label. Among, these colloids, only the magnetic particles contribute to the realization of the systems of diagnoses or analytical automated. In these novel technological applications, the specifications list of the desired particles is heavy to answer and still a challenging research area.

With the development of nano-biotechnologies, sensitive colloidal particles have received increasing attention due to their exhaustive panel of specifications:

(1) The colloidal particle offer high specific surface (6–60 m^2/g) compared to flattened surfaces such as silica wafer. Such high surface in dispersed media enhances the kinetic of the capture of the target. Indeed, the diffusion phenomena are limited in disperse media compared to flat support.
(2) To their possible guidance when they have magnetic property and to their possible intrinsic properties (i.e. fluorescent, conducting stimuli-responsive, etc.). In fact, colloidal particles bearing well appropriate receptor are used as solid support of biomolecules in various biological applications such as in immunoassay and DNA diagnostic, cell detection, and also in drug delivery area.

The combination of Microsystems technology and biosensor is the key point answering the above-mentioned criteria. In this direction, the use of nanocolloids has been recently explored.

The main objective of biomedical diagnostic research is to develop microsystems making it possible to analyze complex mediums such as the biological fluids and matrixes. Indeed, because of volumes of very reduced samples, of analyses in a significant number and requirements in terms of speed and low cost, an extreme miniaturization of the analytical systems is made necessary today (Fig. 2.26).

In brief, the formation of the new chemical bond, linking the macro-biomolecule to the particles, requires well defined water chemistry, a maximization of receptor–particle interactions in order to bring both counterparts within a shorter distance than the length of a chemical bond (otherwise no reaction takes place) and control of the colloidal stability of the particles and particle/receptor during and after the functionalization process.

To perform the covalent grafting of biomolecules onto colloidal particles is more complicated when compared to the use of flat solid surface. In fact, the general problem to solve is mainly related to colloidal stability of the particles during the covalent grafting process. In addition, the activation step of reactive groups of both polymer based particles and biomolecules (or receptors) should be performed in water phase. In fact, in order to avoid the alteration of the activity of proteic-based biomolecules, the immobilization onto particles is incontestably performed in water-based process. Whereas, in the case of single-stranded DNA fragments (i.e. oligonucleotides) or peptides, the use of polar and miscible solvents with water during the activation and chemical grafting is tolerated in some conditions.

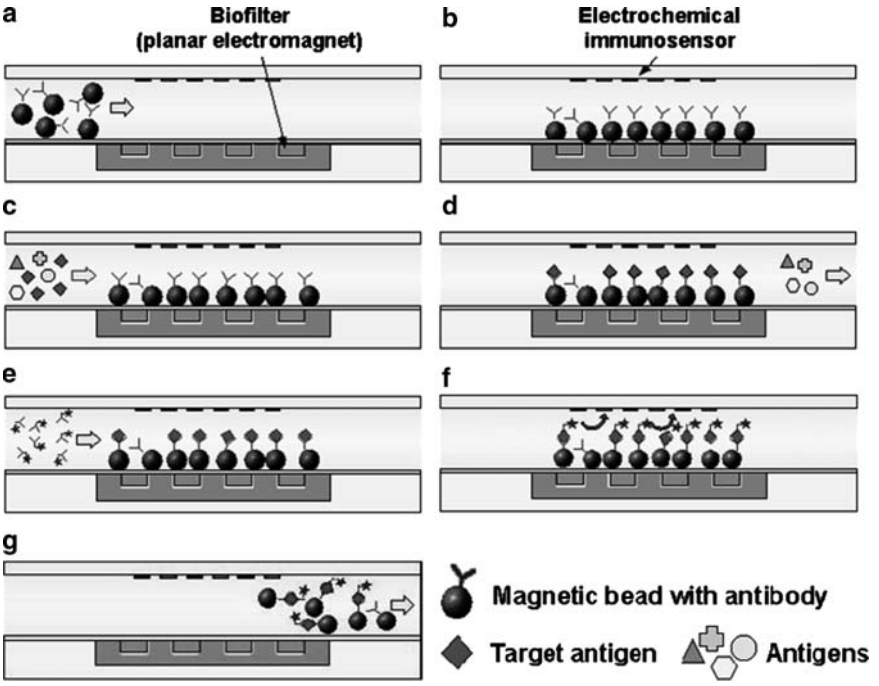


Fig. 2.26 Principle of the microchip assay developed by Choi et al. **a** Introduction of the magnetic beads into the microchannel. **b** Immobilization of the beads on the surface of the biofilter due to magnetic field. **c** Introduction of the sample. **d** Interaction between the bead and the target analyte. **e** Introduction of the secondary labelled antibody. **f** Electrochemical detection after the introduction of the substrate. **g** Washing (With permission from Choi et al. (2002).)

In the following parts, some basic chemical grafting processes are presented in order to provide the readers the well-established approaches.

2.4.3.1 Chemical Grafting of Biomolecules onto Activated Groups of the Particles

The covalent binding of macromolecules (or biomolecules) bearing primary amine group has been widely performed onto carboxylic containing particles. In this case, the activation of the carboxylic groups is first realized before adding the macromolecules at suitable pH, salinity and temperature. This covalent coupling method has been used for the immobilization of proteins, antibodies, modified peptides and then extended to expensive biomolecules such as oligonucleotides bearing amino-link spacer arm at its 5′ position (Fig. 2.27). This approach is due to easy automated synthesis of amino-link oligonucleotides.

The general problem of the immobilization process of receptor onto surface charged particles (i.e. carboxylic containing particles) is related to the colloidal

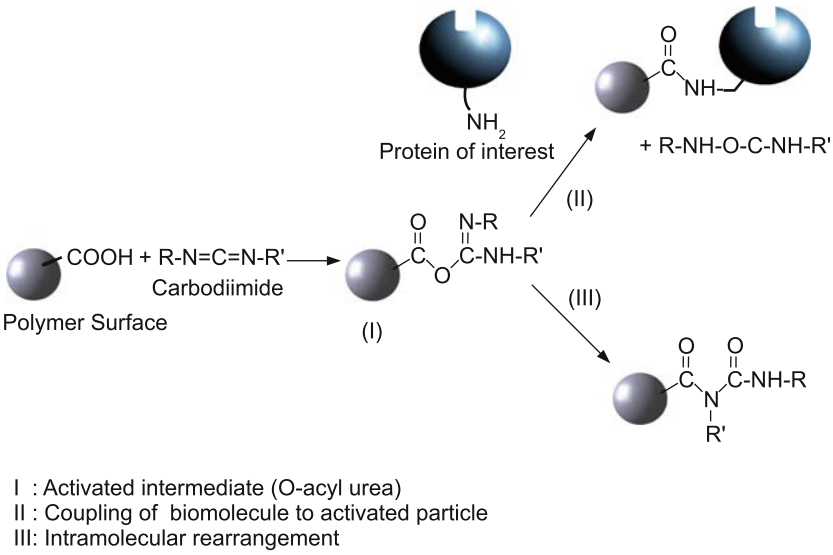


Fig. 2.27 Activation of carboxylic containing particles using charged activating agent 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDAC)

stability of the particles during the activation step. Indeed, the activation step (using water-soluble activating agent) of the particles leads to low charged surfaces and in some cases to amphoteric surface character. Consequently, the colloidal stability of the activated dispersion is highly sensitive to the salt concentration used during the receptor immobilization process. To avoid the loss of the colloidal stability of the particles during the activation step, high concentrations of noncharged surfactant are used. Other than the use of surfactant, it may dramatically reduce the covalent grafting yield of the receptor due to the screening effect of the surface-active groups. To prevent those phenomena, the amount and the nature of the used surfactant should be well selected or to use activated receptors onto active stable colloidal particles.

2.4.3.2 Chemical Grafting of Activated Biomolecules onto Reactive Groups of the Particles

In order to avoid the above-mentioned problems related to the colloidal stability and to the screening effect induced by the use surfactant, the activation of biomolecules followed by the grafting onto reactive particles was generally favored. In this case, biomolecules are activated using water-soluble homobifunctional activating agent. Such approach needs the purification step so as to isolate the activated biomolecules before their chemical grafting process. In this case, only small surfactant amount is needed and in some cases, surfactant free process is used. The general problem of such methodology is related to (1) the possible denaturation

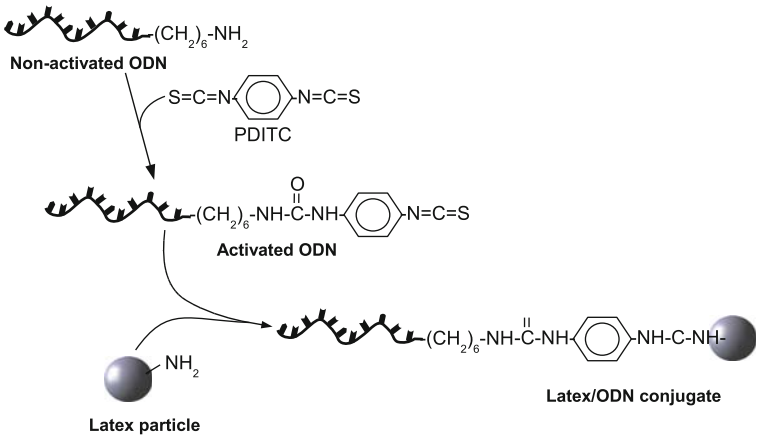


Fig. 2.28 Covalent immobilization of ODNs onto aminated-latex particles. Step 1: activation reaction of the ODNs by PDITC. Step 2 : coupling of the activated ODNs onto aminated-latex particles (Ganachaud et al. 2000)

or activity reduction of the activated biomolecules, and to (2) the separation process of activated and nonactivated biomolecules.

For illustration of such approach, the immobilization of oligonucleotides onto amino-containing latex particles is presented. The immobilization of oligonucleotides (ODNs) onto colloidal particles can be performed through various methods. To perform an irreversible immobilization of ODNs bearing amino-containing spacer arm onto particles surfaces, a chemical grafting has to be considered onto carboxylic, amine and aldehyde containing particles. The covalent grafting of single-stranded DNA fragment bearing amino-link spacer arm at its 5′ position (oligonucleotide) onto amino-containing particles (i.e. polystyrene latexes) is presented and discussed since such immobilization it is not well-known.

This chemical immobilization process is performed in various steps as depicted in (Fig. 2.28):

(1) The activation of the 5′ amino group of the oligonucleotide molecules using homobifunctional reagent such as phenylenediisothiocyanate (PDITC) (Ganachaud et al. 2001). The use of high amount of PDITC may lead to ODN–ODN conjugates.
(2) The chemical reaction between the activated oligonucleotide and the amine group of the particles. In order to avoid the aggregation phenomena, the reaction is performed at pH $>$ 8 and in the presence of noncharged surfactant. High pH is needs in order to have amine group rather ammonium and the surfactant is preserve the colloidal stability of the particles.
(3) The ODN/particle conjugates are washed using classical surfactant free and moderate salinity buffers (10 mM borate buffer, pH 10.7, 0.2 M NaCl, 0.3 wt% Triton X-405). To some extend, the final particles are hairy like particles.

The pertinent result that can be deduced from such particular system is related to the surface charge density. In fact, the surface reactive group density play non-negligible role in the adsorption process and consequently in the residual grafted amount of oligonucleotides after washing step. The grafted amount was found to decrease with increasing the incubation pHs. The observed behavior revealed that the adsorption is the key parameter that controls the final immobilization (via chemical grafting) process of activated ODN. In addition, the residual grafted amount of ODN increased with increasing the initial activated oligonucleotide molecules concentration until reaching a plateau conditions. The reached plateau values correspond to possible surface saturation, which is principally limited by steric hindrance. Whereas, the chain length and the microstructure of oligonucleotide are reported to be totally marginal in nature (Ganachaud et al. 2000; Elaissari et al. 2003a, b, c; Ganachaud et al. 2003).

In order to enhance the accessibility of chemically grafted biomolecules or receptors onto particles surface, the chemical binding methodology has been oriented to the use of selective chemistry or to the use of polymer-like spacer arm as coupling agent.

2.4.3.3 Chemical Grafting of Functional Biomolecules onto Active Particles

The only chemical grafting, which can be directly performed without any addition of surface-active agent is based on the use of high reactive moieties, such as covalent grafting of amine onto aldehyde compound (Fig. 2.3). Such approach has been used for instance, for performing the chemical grafting of amine containing oligonucleotides onto latex particles bearing aldehyde function (Charreyre et al. 1999). In order to maintain the colloidal stability of such reactive aldehyde containing latex particles charged initiators (e.g. potassium persulfate) are generally used in the polymerization process. Consequently, the final particles contain both charged initiator fragments and aldehyde groups. It is interesting to notice that the aldehyde function can be easily deduced from the periodate oxidation of saccharide compound (i.e. lipomaltonamide) (Charreyre et al. 1999; Beattie et al. 1995).

2.4.3.4 Receptor Covalent Grafting Mediated by Reactive Polymer

In some specific biomedical applications, the direct chemical grafting of receptors onto particles surface, leads to low stable sensitive particles and to drastic reduction of the sensitivity. This sensitivity problem is mainly related to the interfacial conformation of the immobilized receptor and to the loss in the reactivity induced by the mobility reduction. In order to favor the interfacial mobility of the immobilized molecules, three approaches have been used: (1) the chemical modification of the used receptor (i.e. single-stranded DNA fragment bearing amino-link spacer

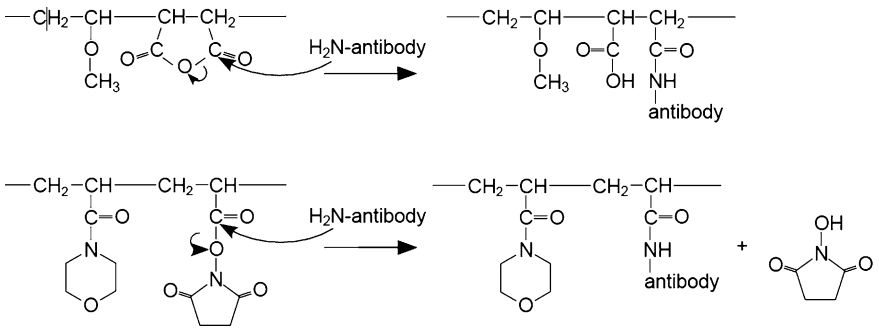


Fig. 2.29 Chemical grafting of antibody onto reactive anhydride or active-ester-based copolymers

arm), (2) the use of reactive hairy like particles and (3) the use of reactive polymers as macro-coupling agent rather than classical small molecules as above discussed.

The use of reactive polymer was found to be exiting methodology leading to interesting results. This approach has been used for the covalent binding of single-stranded DNA fragment bearing amino-link spacer arm (Erout et al. 1996a, 1996b) and antibodies (Rossi et al. 2004). The used reactive polymers are active-ester or anhydride based copolymers (Yang et al. 1990; Erout et al. 1996a, b) (Fig. 2.29).

Experimentally, the suitable biomolecule was first grafted on the reactive polymer using DMSO–water mixture in order to enhance the chemical grafting yield by reducing the hydrolysis kinetic (Erout et al. 1996a, b; Ladaviére et al. 2000). The polymer–biomolecule conjugates were extracted using HPLC technique. Before the total hydrolysis of the reactive groups of the used polymer, the chemical grafting of the conjugates was performed in the presence of surfactant free amino-containing latex particles (Rossi et al. 2004; Veyret et al. 2005).

The total hydrolysis of the residual reactive esters (or anhydride) leads to hairy like and hydrated sensitive particles (Rossi et al. 2004).

2.4.3.5 Immobilization of Cis-diol-Containing Receptor onto Particles Bearing Boronic Acid

Various works took advantage of this specific and strong specific complexation reaction by designing several synthetic phenyl boronic acid–containing materials (polymers and particles) (Camli et al. 2002; Uguzdogan et al. 2002; Elmas et al. 2004) (Elmas et al. 2002) mainly for biomedical application purposes. In fact, the specific recognition of cis-diol function by the boronic acid derivatives was used for controlling the reversible immobilization of proteins, enzymes or any biomolecules bearing glucose compounds. Indeed, this specific interaction is sensitive to the pH of the medium. The boronic acid exhibits a trigonal structure at low pH and tetragonal one at basic pH ($pH > pKa = 8.8$). Thus, the specific complexation reaction is favored at basic pH, because of the stability of boronate form. The shift to acidic medium leads to the decomplexation and thereafter to the release of the

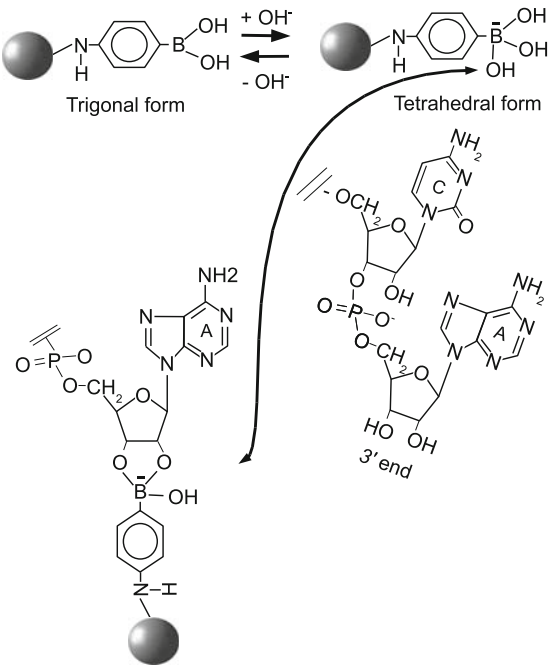


Fig. 2.30 Immobilization of RNA bearing cis-diol onto phenyl boronic acid containing support

cis-diol-containing molecules. It should be noted that the yield of the complexation reaction between the boronic acid and the polyols compound depends mainly on the availability of the cis-diol functions on the selected receptor. The use of such approach for RNA molecules immobilization onto latex particles bearing boronic acid groups was performed using surfactant free condition (Camli et al. 2002) (Fig. 2.30).

2.4.3.6 Dipol–Dipol Interaction

Biomolecules can be tethered onto polymer particles either by covalent coupling or physical adsorption. Standard chemical immobilization techniques or more specific adsorption do not allow addressing the final activity issue, as these methods have no control on the orientation of biomolecules on the support.

One alternative way to avoid such a disadvantage is to perform the immobilization process via regio–selective interactions, which can take place at a well-defined site of the biomolecule, not involved in the molecular recognition properties. In this direction, dipole–dipole interactions can be considered a promising approach because some biomolecules bear polar groups at a specific position, which could interact with polarizable groups containing particles surface (Fig. 2.31). So far, cyano groups have been recognized to specifically interact with sugar moieties

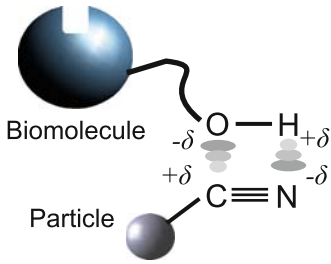


Fig. 2.31 Dipol–dipol cyano-sugar moieties interaction illustration

located in some biomolecules (e.g. antibodies). As a strongly polar group, cyano-sugar moieties interaction can be considered strong adsorption in terms of energy level. Such specific interaction is principally sensitive to the pH of the medium.

2.5 Concluding Remarks

Biofunctionalization is first a matter of defining an appropriate strategy according to the type of target molecules and the transducer technology. Thus, the detection mode (in solution of at the surface) is to be selected early; the type of functionalization as a thin 2D layer or a thick porous membrane. The criteria for this preliminary choice are the required sensitivity and the possible want for quantitative analysis.

Most coupling strategies for immobilizing native biomolecules make use of reactions with their amino and thiol groups. The chemical reaction can be selective by an appropriate choice of reactive group and experimental conditions (e.g. pH). However, it is a difficult task to keep the biological activity homogeneous over a population of biomolecules owing to the random nature of the coupling. The advent of miniaturized and highly parallelized reliable tests (microarrays for example) requires a good accessibility of the target in solution. The surface properties and the linkage between the solid support and the bioreceptors must be tailored to reach these analytical properties. Homogeneous immobilization is attained by introduction of a site-specific reactive group distal from the bioactive sites by modification of the biomolecules.

The wide variety of transducing techniques has lead to implement the immobilization step on a large panel of solid support and under different shapes (ranging from thin monolayer to thick porous membranes). Despite this wide diversity, homogeneous and uniform microenvironments at the solid/liquid interface are the most relevant parameters that govern the quality of a biointerface. The steric effects (receptors binding sites near the surface, tightly package of neighboring proteins) are among others parameters to be taken into consideration. The manufacturing of well-controlled 2D nanostructures (vertical and horizontal configuration) can be obtained by combining the SAM technology with selective site-directed strategy of biomolecules immobilization.

Thick interfaces have been considered in order to increase the surface loading of biomolecules and thus the detection signal. The 3D-embedding matrix can be simply considered passive membranes with high specific surface, or as active membranes with protective properties toward immobilized biomolecules or conferring specific properties for the detection. But it is often considered that porous materials limit the diffusion of target molecules.

As presented in this chapter, there are several strategies of biomolecule attachment to solid supports. The complex nature of biology, the ever growing demand of highly sensitive device asks for the development of new specific strategy to each new application.

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