Chapter 2
Biochemical Components Used in Biosensor Assemblies

Over the past years, biosensor research has revolutionized clinical diagnostics, especially in diabetes management, and has had a significant impact on laboratory research. As was shown in Chap. 1, this was due to the extremely high importance of biochemical components that determine both the interpretation of the signal and the possibility to specifically detect a variety of the species, including metabolites, drug residues, toxic hazards, proteins, etc. It should be mentioned that from the very beginning, the development of biosensors has been mainly concentrated in the groups of biologists and medical staff who are involved in the development of medical equipment or investigation of molecular backgrounds of human health. For this reason, the behavior of enzymes, nucleic acids and microorganisms was considered to be self-evident so that the main efforts had been concentrated on transducers and signal transduction principles. The situation has been changing, and many chemists and engineers begin their scientific careers in medical equipment and compact sensing devices for environmental monitoring, point-of-care medical diagnostics and treatment and related areas. They are not as familiar with the biochemical backgrounds of biosensor performance as their predecessors and need broadened information about the advantages and limitations of biochemical reagents in biosensor design. Below, the main biochemical functions of principal biochemical components are considered, with particular emphasis on those most important from the point of view of biosensor development. The description of proteins, nucleic acids and microbial cells does not claim to be exhaustive and can easily be extended by monographs and student books on biochemistry. However, this chapter contains the minimum of information necessary for the beginning of biosensor development for young researchers and graduate students who are not specializing in the life sciences. The information is classified in such a manner that the knowledge most important for biosensor development is summarized and presented in a way that is acceptable for quick search and use. In addition to the main information on the structure and biochemical functions of appropriate biomolecules, some useful data are placed in tables. The description is supported by examples related to the most common situations or decisions related to biosensor manufacture or operation.

G. Evtugyn, *Biosensors: Essentials*,
Lecture Notes in Chemistry 84, DOI: 10.1007/978-3-642-40241-8_2,

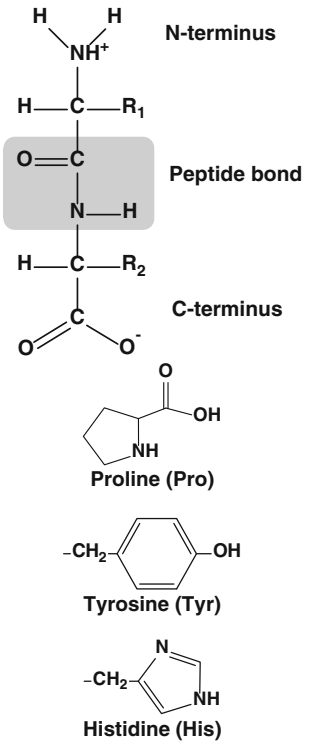
2.1 Enzymes

Let's start with enzymes that not only belong to the most common representatives of the biochemical systems of biosensors but were also the first to demonstrate the potential for the commercialization of biosensors and their application outside university laboratories.

Enzymes are proteins, i.e., biopolymers that consist of α-amino acid residues connected by amide linkages and which exert catalytic activity. In total, 20 α-amino acids are present in the protein structure (Fig. 2.1) (Copeland 2000).

All amino acids are important as building blocks of proteins and can take part in enzyme activity. But some of them are more frequently mentioned when the enzyme behavior or biosensor applications are discussed.

Thus, *cysteine* is the only amino acid that provides covalent disulfide bridging between protein sub-units or a protein and thiolated solid support. Cystine, a cysteine dimer, is important in establishing redox equilibrium (2.1). Cysteine residues are also used for the site-specific covalent attachment of enzyme molecules to the Au surface with covalent Au–S bonds simultaneously formed in the



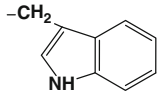
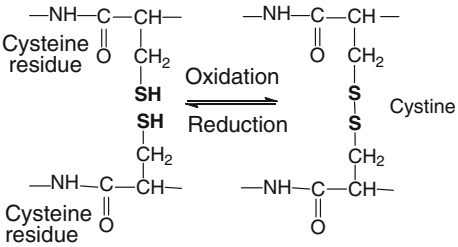
R	Amino acid
-H	Glycine (Gly)
$-CH_3$	Alanine (Ala)
$-CH(CH_3)_2$	Valine (Val)
$-CH_2CH(CH_3)_2$	Leucine (Leu)
$-CH(CH_3)C_2H_5$	Isoleucine (Ile)
$-CH_2CH_2-S-CH_3$	Methionine (Met)
$-(CH_2)_4NH_2$	Lysine (Lys)
$-CH_2C_6H_5$	Phenylalanine (Phe)
$-(CH_2)_3NHC(NH_2)NH_2$	Arginine (Arg)
$- CH_2SH$	Cysteine (Cys)
$-CH_2COOH$	Aspartic acid (Asp)
$-CH_2CH_2COOH$	Glutamic acid (Glu)
	Tryptophan (Trp)
$-CH_2OH$	Serine (Ser)
$-CH_2CH(OH)CH_3$	Threonine (Thr)
$-CH(NH_2)CH_2C(O)NH_2$	Asparagine (Asp)
$-CH(NH_2)CH_2CH_2C(O)NH_2$	Glutamine (Gln)

Fig. 2.1 Dipeptide structure and some amino acids forming primary sequence of proteins. A three-letter code is given in *brackets* after the amino acid

presence of dissolved oxygen. Some of the reagents, i.e., cystamine or thiourea, affect the cysteine–cystine equilibrium and hence the efficiency of the enzyme attachment to the solid support.



(2.1)

"Acidic" amino acids (*asparagine* and *glutamine*) contain a carboxylic group in the side chain that is not involved in the peptide bond formation. The dissociation of the group makes the amino acid sequence negatively charged. Similarly, the "basic" amino acids (*lysine*, *arginine* and *histidine*) positively charge the protein domains by protonation of their amino groups. The equilibrium with the amino acids bearing proton donating and proton accepting groups specifies the total charge of a protein globule in an aqueous solution. The pH changes in the protein microenvironment alter the charge of the protein globule caused by such interactions. This is one of the reasons for the pH dependence of the enzyme activity.

The point of the recharging of the protein globule is called the *isoelectric point*. It is equal to the pH value corresponding to the zero charge of a protein. Isoelectric points are determined not only for proteins but also for individual amino acids in electrophoretic measurements (Westermeier 2005).

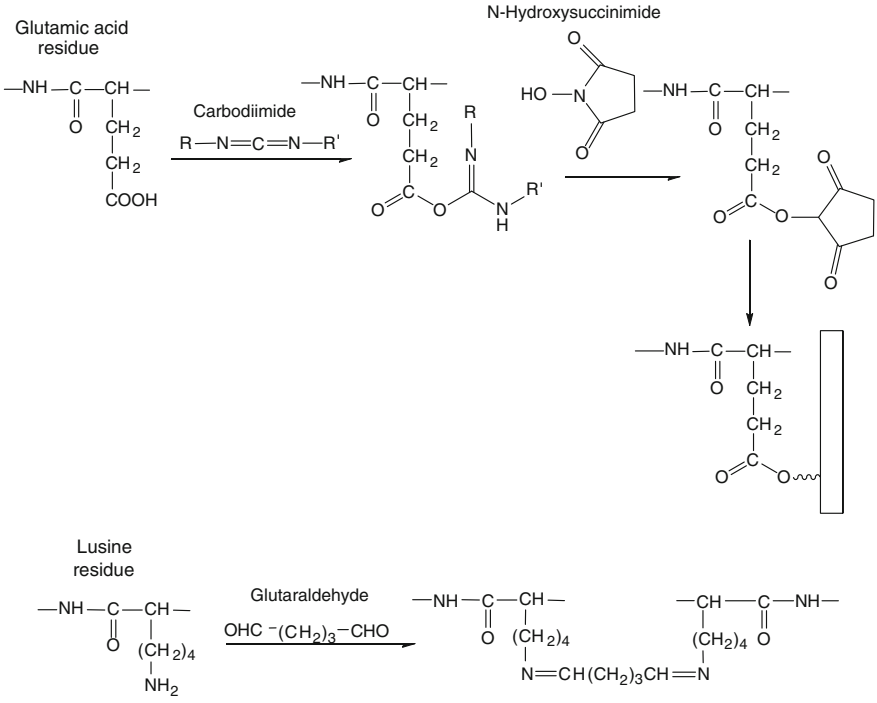
> Electrophoresis is the motion of dispersed particles relative to a fluid under the influence of a spatially uniform electric field. The direction and rate of the movement allow the charge of the particles to be determined.

If the pH of the solution is higher than the isoelectric point, the protein is negatively charged and, vice versa, if the pH is lower than the isoelectric point, the protein bears a positive charge.

Besides "acidic" and "basic" amino acids, the amino acids include other functional groups, e.g., hydroxyl (*serine*, *threonine*), aromatic (*tryptophan*, *tyrosine* and *phenylalanine*) or aliphatic (*glycine*, *alanine*, *valine*, *leucine* and *isoleucine*) units. As cysteine, the "acidic" and "basic" amino acids take part in the covalent binding with some species present in the solution.

Among others, there are the so-called *bi-functional reagents*. They are required for the modification of the enzyme molecules intended to vary their characteristics or covalent attachment to the solid carrier, e.g., the polymer support or transducer of a biosensor. The examples of such a covalent modification are presented below (2.2). The carbodiimide binding is commonly used for modification of carboxylic

and amino groups and can be performed in two stages. First, one azomethine fragment is attached to a carboxylic group to form an ester group. Then, *N*-hydroxysuccinimide is bonded to the second fragment. The final product is easily combined with aminated and carboxylic functional groups of proteins, polymeric supports, electrode materials, etc. The second stage accelerates the modification and increases its efficiency. The direct reaction of the second azomethine fragment with carboxylic groups without any *N*-hydroxysuccinimide treatment is rather slow for application in biosensor assembling (Cao 2006).



(2.2)

Glutaraldehyde can react with thiol, amine, carboxylic and hydroxide groups of the amino acid residues or plastic support. The relative reactivity of the fragments to be modified decreases in the above-mentioned order. The reaction results in cross-linking protein molecules and their attachment to the high molecular supports or the transducer surface. The reaction is complicated by polycondensation of the initial glutaraldehyde molecules and the reversibility of the Schiff base formation.

The sequence of appropriate amino acids in accordance with the way they bind is called a primary protein structure. It can be represented by a linear sequence of three letters (see Fig. 2.1) or single-letter codes assigned to each amino acid. The primary sequence of amino acids pre-determines the three-dimensional structure of a protein that depends on all kinds of interactions, including the variation of the

lipophilic–lipophobic balance, π-stacking interactions, donor–acceptor and electrostatic interactions and H-bonds. Besides amino acids, proteins can contain other species, e.g., carbohydrates in glycoproteins, covalently attached to the terminal amino acid residue.

Steric interactions highly depend on the metal ions and their complexes with organic ligands, on small organic molecules, and water kept in the protein globule but can, more or less freely, escape the globule. The presence of such small items is of primary importance for such biochemical functions as enzyme activity. This is especially true for enzymes catalyzing the redox conversion of a substrate. For them, the protein part is called an *apoenzyme*. The small molecule with its own redox activity that is responsible for the acceleration of the substrate oxidation is called a *cofactor* (Fig. 2.2).

The cofactors are subdivided into two groups, i.e., metal ions and organic molecules, *coenzymes*. The coenzymes, in turn, can dissociate from the enzyme molecule to act independently in a solution (*co-substrates*) or act in the protein globule (prosthetic groups). In the latter case, they are permanently associated with an enzyme, often by covalent bonds. The types of enzyme cofactors are presented in Fig. 2.3.

Nicotinamide adenine dinucleotide (NADH) is one of the most important co-substrates and is related to the functioning of more than 250 enzymes catalyzing the oxidation of organic species (Eq. 2.3). NADH is released in the solution and requires special efforts for re-oxidation to complete the cycle of the electron transfer of the target process. The phosphorylate derivative of NADH, NAD(P)H, is another common representative of co-substrates. In the reaction scheme, the co-substrates are recorded together with the enzyme substrate (see Eq. 2.3 as an example):

$$\text{L-Lactate} + \text{NAD}^{+} \xrightarrow{\text{Lactate dehydrogenase}} \text{Pyruvate} + \text{NADH} + \text{H}^{+} \quad (2.3)$$

Cofactor molecules, except for prosthetic groups, can be reversibly removed from the enzyme molecule. This results in the loss of its catalytic ability. The treatment of an apoenzyme with the cofactor molecule results in a partial or

Fig. 2.2 Reconstruction of an active enzyme from a protein part (apoenzyme) and a cofactor

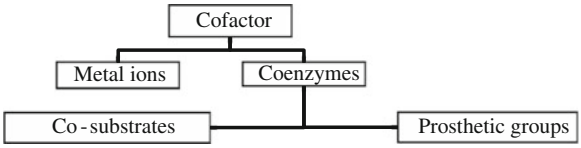
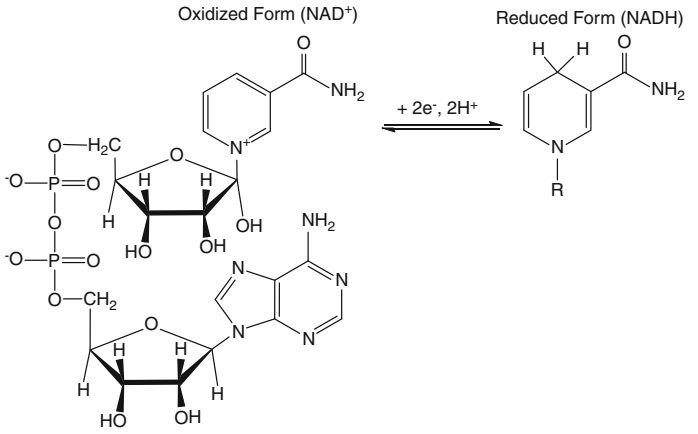


Fig. 2.3 Reconstruction of an active enzyme from a protein part (apoenzyme) and cofactor

full restoration of its catalytic activity. The enzyme obtained by interaction of an apoenzyme and a cofactor is called a *reconstructed enzyme* (*holoenzyme*).



(2.4)

The reaction of the apoenzyme with a cofactor is very specific and can be used for the selective detection of a cofactor. The inactivation of the enzyme caused by the removal of a metal cofactor is observed in some procedures of the enzyme isolation and purification assuming the use of such complexing agents as EDTA. This is an undesirable process that suppresses the catalytic efficiency and hence the sensitivity of the enzyme sensor toward the substrates. On the other hand, reversible removal of the cofactor can be necessary for establishing multi-enzyme cascades on the artificial support or for specific immobilization of the enzyme on appropriate support by covalent linkage incompatible with the cofactor. Many cofactors exhibit a reversible influence on the enzyme activity as well, which is used for the mild control of the chemical energy in metabolism.

In addition to apoenzyme and the reconstructed enzyme, the term "*proenzyme* (*zymogen*)" can be found in the literature (Plainkum et al. 2003). This is an inactive enzyme precursor that does not exert any enzyme activity but can be "activated" by a specific biochemical reaction. Contrary to the interaction in the "apoenzyme–cofactor" pair, the activation of proenzyme is highly specific, and a specific enzyme system is needed. In many cases, the activation of proenzyme is achieved by the specific removal of terminal groups by selective hydrolysis of P–O–C bonds. The phosphate group is of primary importance in many biochemical reactions related to oxidative metabolism and ATF synthesis and simultaneously controls the enzyme activity especially in substrate cycles (glycolysis–gluconeogenesis, etc.). The high specificity and necessity of additional enzymes with their own substrates complicates the use of proenzyme activation in analytical systems although the substrate cycles were successfully used for the detection of lactic acid, glucose, and a few other metabolites.

As other proteins, enzymes can self-associate into the dimeric and tetrameric forms involving subunits, either different or similar in their enzymatic activity, i.e.,

the efficiency of the catalysis. Enzyme subunits are bonded by weak multi-point interactions and, sometimes, with disulfide S–S bridges. The ions of alkali and alkali earth metals promote the association of the enzyme subunits due to electrostatic interactions. Contrary to apoenzymes, the enzyme subunits in a dissociated state retain most of their catalytic activity. The process of di- and tetramerization is spontaneous and sterically specific. If the subunits differ in their specific activity, the enzyme can form various oligomeric structures in accordance with the number of the associated particular subunits. This can result not only in the various catalytic properties but also in specificity toward particular substrates, pH and temperature influence, etc. Thus the enzyme lactate dehydrogenase is a tetramer that consists of two types of subunits. Their content varies with environmental conditions and this is used for the biochemical adaptation of trout toward low water temperature in mountain lakes and rivers. Together with minor changes in the amino acid sequence near the enzyme-active site, this fact provides a variety of the enzyme characteristics observed not only for various biological species but also within individuals belonging to the same population.

For this reason, the description of the enzyme application, for example, in biosensor assembly, requires not only its definition in accordance with the biochemical function, but also of its origin and, to some extent, purification protocol. The latter requirement does not cover commercial preparations with rather standard characteristics, although, especially at the beginning of biosensor development, the enzyme characterization was often considered an indispensable part of the investigation with appropriate efforts in kinetics quantification and purity examination.

As common chemical catalysts, enzymes accelerate conversion of selected species called enzyme substrates due to the decrease of the reaction energy. Hence the enzymes do not affect the equilibrium constant of the substrate reaction and function by the specific binding of the substrates into a very unstable reactive product, i.e., an enzyme–substrate complex. The great efficiency of catalysis at rather low temperatures (commonly below 50 °C) and the physiological pH interval (pH = 5.0–8.0) is one of the reasons for the wide use of enzymes in the biosensor assembly. Thus, the enzyme urease accelerates the hydrolysis of urea (Eq. 2.5) by a factor of about 10^{14}.

$$\mathrm{NH_2{-}\underset{\underset{\mathrm{O}}{\|}}{\mathrm{C}}{-}NH_2} + \mathrm{H_2O} \xrightarrow{Urease} 2\,\mathrm{NH_3} + \mathrm{CO_2} \tag{2.5}$$

The functional groups occurring in the reaction with a substrate molecule form an *active site* of the enzyme. As a result of such interaction, a new reactive compound, an *enzyme–substrate complex*, is formed. Later, the complex is converted to the final products with releasing the initial enzyme molecule that is able to bind to another substrate molecule. Each enzyme molecule can contain several active sites; their number usually corresponds to the number of subunits or cofactors. Thus the enzyme glucose oxidase is a dimer involving two identical subunits; each of them involves one cofactor, flavine adenine dinucleotide (FAD). Crude enzyme

preparations can contain the proteins partially dissociated into subunits with a various number of enzyme active sites and molecular mass of the fragments so that the specific enzyme activity is calculated against one active site but not one molecule of an enzyme. This is not very important for rather simple enzyme molecules but can have significance for newly isolated enzymes with the distribution of subunits not characterized and dependent on the isolation and purification procedures. The amino residues of the active site can stay rather far from each other in the primary protein sequence but are brought together in the spatial structure typical for the enzyme molecule. This means that any changes in the three-dimensional structure of the enzyme will affect the enzyme activity. Most such interactions in living beings are reversible and provide a flexible alteration of the enzyme activity in accordance with particular demands.

Apart from the active site, an *allosteric site* can be specified for some enzymes. It combines some functional groups that are able to specifically interact with particular species (Fig. 2.4).

Although such interaction does not block the access to the enzyme-active site, it affects the catalytic activity of an enzyme due to changes in the non-covalent interactions—hence, the spatial structure of the domain in the proximity of the enzyme active site. The species that influence the enzyme activity are called enzyme *effectors* and the variation of enzyme activity due to the allosteric site binding is called an *allosteric enzyme regulation* (Traut 2008). Together with changes in cofactor binding, allosteric regulation provides a specific way for directed changes of enzyme activity, i.e., its reduction (*inhibition*) or increase (*activation*). Allosteric regulators do not exhaust all the mechanisms of such changes in enzymatic catalysis. Thus, inhibition mechanisms involve the non-specific denaturation of the protein three-dimensional structure by high temperatures or strong acids (bases) as well.

A similar effect is exerted by compounds irreversibly modifying the functional groups of an active site. Activation can be achieved by the partial dissociation of an enzyme to separate subunits or by the addition of the excessive amounts of a cofactor. In some cases, enzyme–substrates or the products of their enzymatic conversion can be attached to allosteric sites and alter the enzyme activity. In most cases, this results in a decrease of the reaction rate (substrate/product inhibition). Not all the enzymes have an allosteric site in their structure, but they are all

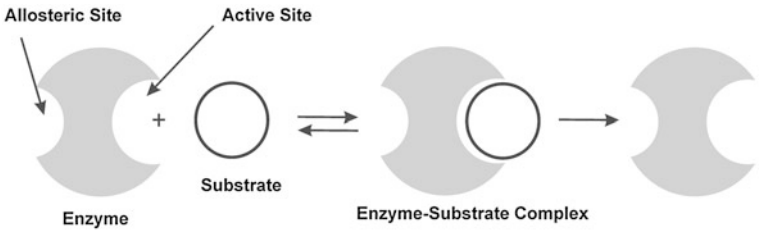


Fig. 2.4 Reconstruction of an active enzyme from a protein part (apoenzyme) and a cofactor

sensitive toward effectors, the number and specificity of the action of which depend on both the enzyme and substrate. In substrate detection, the addition of effectors in the reaction media provides stabilization of the signal and its increase against standard conditions. Allosteric inhibitors can be used for masking undesirable enzyme activity, for example, related to the competitive path of an analyte conversion. In a more complicated case, the effectors are obtained in accordance with the relative increase/decrease of the signal toward the constant concentration of a substrate. It should be noted that except in rare cases, the specificity of the response on an effector is much less than that on a substrate. This limits the application of such schemes in biosensor-based analysis.

The sequence of amino acid residues determines the three-dimensional structure of the protein due to numerous interactions by electrostatic and H-bonding, by van der Waals, hydrophobic and donor–acceptor interactions (Berg et al. 2007). The H-bonding between carbonyl and amide groups of amino acids results in the formation of regular domains, i.e., α-helix and the β-pleated sheet structures. Both of them determine the *secondary protein structure*. The α-helix is a spring-like structure with residues joined by a hydrogen bond between carbonyl oxygen and a nitrogen proton in peptide groups; about 3.6 amino acids are placed in each turn of a coil. The β-pleated sheet is stabilized by hydrogen bonds between peptide linkages to form distorted planes, one embedded in another one. The regular areas described are subdivided by amorphous parts so that the protein molecule usually has a rounded, sometimes elongated or kidney-shaped particle often called a globule.

The enzyme–substrate specificity is a most important feature of enzymatic catalysis. In accordance with the type of substrate conversion, six classes of enzymes are specified in the enzyme nomenclature approved by the Nomenclature Committee of the International Union of Biochemistry and Molecular Biology (Table 2.1).

Oxidoreductases catalyze the redox conversion of the substrates. This is the most popular enzyme class used in biosensor assembly because of the importance of

Table 2.1 Enzyme nomenclature

Class	Name	Examples of subclasses
EC 1	Oxidoreductases	EC 1.1 acting on the CH–OH group of donors
		EC 1.2 acting on the aldehyde or oxo group of donors
		EC 1.4 acting on the CH–NH_2 group of donors
		EC 1.6 acting on NADH or NADPH
		EC 1.11 acting on a peroxide as acceptor
EC 2	Transferases	
EC 3	Hydrolases	EC 3.1 acting on ester bonds
		EC 3.2 glycosylases
		EC 3.4 acting on peptide bonds (peptidases)
EC 4	Lyases	
EC 5	Isomerases	
EC 6	Ligases	

appropriate substrates that belong to metabolites, vitamins, disease indicators, etc. The biosensors based on oxidoreductases are demanded in biomedical diagnostics, food quality testing and other similar areas. The oxidoreductases are subdivided into several groups, depending on the nature of electron acceptors:

- *dehydrogenases* catalyze the reaction formally related to two hydrogen atoms transfer from the substrate to the acceptor differing from molecular oxygen (see reaction 2.3 for lactate dehydrogenase);
- *oxidases* catalyze a similar reaction of the hydrogen transfer to molecular oxygen with the formation of hydrogen peroxide (glucose oxidase);
- *monooxigenases* catalyze a similar reaction but water is obtained instead of H_2O_2;
- *peroxidases* catalyze the oxidation of the substrates with hydrogen peroxide or organic peroxides.

Transferases catalyze the transfer of a group (methyl, glycosyl, etc.) from one compound to another. *Hydrolases* promote the reaction of the hydrolysis of various bonds in aqueous media and the reverse reaction of esterification in predominantly organic media. Both processes have industrial and analytical applications especially regarding the detection of toxic species (organophosphorus nerve agents, carbamate pesticides) and derivates of organic acids (area, carbamates etc.). In accordance with their popularity for biosensor application, hydrolases are the second enzyme class after oxidorudectases. *Lyases* cleave to C–C, C–O, and C–N bonds by elimination. Eliminating a group can be specified in names like decarboxylase, aldolase, dehydratase (for eliminating carbon dioxide, aldehyde, or water). When important, the reverse reaction is expressed by the name *synthase*. *Isomerases* catalyze geometric or structural changes within one molecule. And finally, *ligases* are enzymes catalyzing two molecules joined together. The reaction is coupled with the hydrolysis of ATP or a similar triphosphate.

Each enzyme is uniquely specified with a code containing the information about the class and subclasses depending on the substrate converted and the reaction catalyzed. Thus, acetylcholinesterase, an enzyme widely used for the detection of organophosphate nerve agents, has the code EC 3.1.1.7. The code shows that the enzyme belongs to the class of hydrolases (EC 3), the subclass of enzyme catalyzing the hydrolysis of esters (EC 3.1), the subgroup of the one that hydrolyzes carboxylic esters (EC 3.1.1) and that related to choline esters hydrolysis (special number 3.1.1.7). The enzyme nomenclature combines the enzyme name from the substrate class and the reaction to be accelerated. For example, the glucose oxidase catalyzes the reaction of glucose oxidation, etc. Nevertheless, in the nomenclature, some common names, like urease (EC 3.5.1.5) or laccase (EC 1.10.3.2), are still used.

The urease and glucose oxidase mentioned above belong to the enzymes with individual substrate selectivity when only one compound acts as an enzyme substrate. More common is the situation where the enzyme catalyzes the conversion of a family of structurally relative compounds. Thus, tyrosinase and peroxidase catalyze the oxidation of a wide range of phenolic compounds and aromatic amines, and carboxyesterase accelerates the hydrolysis of uncharged esters. In some cases, the

enzymes can activate different substrates by various biochemical paths. Thus, cytochrome P_{450} catalyzes the reactions of *N*-demethylation and *C*-hydroxylation depending on the enzyme origin and substrate nature. It should be mentioned that cytochrome P_{450} is a complex of at least three different enzymatic centers that are involved in various stages of the substrate conversion, and their action differs depending on the substrate structure. To some extent, the substrate selectivity can also vary within one source of enzyme by changing the conditions of the reaction control. The attachment of the enzyme onto the solid support limits the steric access of bulky substrates in favor of smaller molecules. The pH and temperature changes can alter the contribution of various subunits in the resulting reaction rate. The use of lipophilic substances and hydrophobic solvents changes the equilibrium and can reverse the direction of the reaction against that in aqueous solution. All of these possibilities are compatible with the biosensor platform when the substrate specificity as such is insufficient for the detection of an analyte.

Table 2.2 summarizes the examples of the most common applications of various enzymes in the biosensor assembly. Besides substrate determination, the detection of inhibitors is also used by enzyme sensors even though the selectivity of the regulation of enzyme activity is inferior to the substrate detection (Byfield and Abuknesha 1994; Ispas et al. 2012).

The kinetics of enzymatic conversion of a substrate is formally described by the Michalis–Menten reaction (Eq. 2.6) (Keleti 1986).

$$E + S \underset{k_{-1}}{\overset{k_1}{\longleftrightarrow}} ES \xrightarrow{k_2} E + P \tag{2.6}$$

Here E, S and P denote free enzyme, substrate and product molecules, respectively. The k_i are the rate constants of the elemental steps of the reaction. The first step involves the reversible formation of the enzyme–substrate complex *ES,* which then irreversibly decomposes to the product and initial enzyme molecule ready to bind another substrate molecule. The formal kinetics provides the following expression of the reaction rate (2.7) for the Eq. (2.6) known as the Michaelis–Menten equation.

$$\upsilon = \frac{\mathrm{d}c_P}{\mathrm{d}t} = \frac{\upsilon_{\max} c_S}{K_m + c_S} \tag{2.7}$$

K_m is the Michaelis constant, which has the dimension of a concentration that also corresponds to the substrate concentration giving the reaction rate equal to half of its maximal value ($\upsilon = 1/2\upsilon_{\max}$); $\upsilon_{\max} = k_2 c_E$ is the maximal rate of enzymatic reaction. In most enzymatic reactions, K_m varies from 10^{-1} to 10^{-7} M. The higher the K_m value, the lower the affinity of an enzyme-active site toward the substrate.

The relation k_2/K_m quantifies the efficiency of the substrate conversion. It has a theoretical upper limit of 10^8–10^9 M^{-1} s^{-1}. Besides the maximal rate, the efficiency of enzyme reaction can be quantified by *enzyme activity*.

The term "enzyme activity" can refer to the enzyme quantity (mass of the enzyme preparation, mass of the support with an enzyme attached) or to the

Table 2.2 Application of various enzymes in the biosensor assembly

Enzyme class	Enzyme name	Analyte	Application area
Oxidoreductases	Glucose oxidase	Glucose	Health industry, medicine, food testing, biotechnology
	Glucose-6-phosphate dehydrogenase	Glucose, phosphate	Medicine, environmental monitoring
	Lactate oxidase, lactate dehydrogenase	Lactate	Health industry, medicine, biotechnology
	Alcohol dehydrogenase	Ethanol	Beverage testing, blood tests, microbe industry
	Tyrosinase	Phenols	Environmental monitoring, pharmaceutics industry
	Peroxidase	H_2O_2, phenols, aromatic amines	Auxiliary enzyme for enzyme sensors based on other oxidoreductases, medicine, pharmaceutics and microbe industry, environmental monitoring
	Cholesterol oxidase	Cholesterol	Medicine, food industry, dietology
	Uricase	Uric acid	Medicine
	Xanthine oxidase	Hypoxanthine, xanthine	Medicine
Hydrolases	Acetylcholinesterase	Acetylcholine, nerve agents	Medicine, environmental monitoring, chemical warfare detection
	Urease	Urea	Medicine, agriculture chemistry
	Alkaline phosphatase	Organophosphate esters	Medicine
	Cholesterol esterase	Cholesterol ester	Medicine, food industry, dietology
	Organophosphate hydrolase	Organophosphates	Environmental monitoring, chemical warfare detection

volume of an enzyme solution. The enzyme activity is measured in units (U). One unit corresponds to the amount of enzyme that catalyzes conversion of one

> *Enzyme activity* is an amount of a substrate converted in a time in standard reaction conditions.

micromole of a substrate per minute. In addition to that, the turnover number is used. This is a maximal number of substrate molecules that can be converted by one active site of enzyme per time unit.

In many manipulations with enzymes required for biosensor development, the K_m value increases. This might be due to limitation in the substrate access or changes in native conformation of the enzyme globule. Similar relations are used for the quantification of the enzyme kinetics with immobilized enzymes if the rate of substrate transfer exceeds that of its biochemical conversion. Such investigations are necessary for the establishment of the influence of biosensor assembling on the reactivity and selectivity of substrate detection that depends predominantly on the biochemical characteristics of the reaction. Instead of the reaction rate,

derivative functions directly measured with appropriate transducers can be used. Thus, for amperometric sensors combined with oxidoreductases, the current recorded can be considered a measure of the rate of an enzymatic reaction. This allows estimating the Michaelis constant by the direct substitution of the υ values with the current in the Eq. (2.6) and appropriate plots mentioned above. The specificity of the heterogeneous reactions of enzymes will be considered in greater detail below in the section devoted to enzyme immobilization.

The Eq. (2.7) can be simplified by the assumption of $K_m \gg c_S$. For low substrate concentration, the rate of an enzymatic reaction linearly depends on its value (2.8).

$$\upsilon \approx \frac{\upsilon_{\max} c_S}{K_m} = \frac{\upsilon_{\max}}{K_m} c_S \tag{2.8}$$

Some other equations related to enzyme kinetics and estimation of kinetic parameters are presented in the Appendix. They can be useful for the characterization of the enzyme-substrate system and its optimization in the biosensor development and operation.

The linear piece of calibration curves of a substrate usually covers about 30 % of the maximal shift of the reaction rate. A linear relationship is used in simplified methods of the substrate analysis that are called "*fixed time*" and "*fixed concentration*" methods. In the first case, the concentration of the product is measured in a definite time interval after the start of the reaction. The reaction period is chosen within the linear piece of the calibration curve so that the concentration measured is directly proportional to the reaction rate. In the fixed concentration method, the time period related to constant conversion of the substrate is measured as a value inversely proportional to the rate of the reaction. The idea of such measurements is to substitute the estimation of the rate, which should be based on a set of measurements performed in different time intervals with a single measurement to be made on the assumption of the linear dependence of the signal and a constant degree of the substrate conversion. Such a measurement mode is especially compatible with semi-quantitative techniques of substrate determination and the colorimetric detections that are often developed for filed applications (test strips, indicator tubes, etc.). Thus the fixed concentration method assumes the measurement of the time period necessary to reach a definite color shade provided by the product of the enzymatic reaction. For enzyme sensors, the fixed time approach substantiates the choice of the measurement period, especially in cases when the enzymatic reaction is not limited by the substrate transfer.

In steady-state conditions of biosensor operation, the enzymatic conversion of the substrate is compensated for by the diffusion flux in the direction orthogonal to the transducer surface. These conditions can be expressed by the superposition of the second Fick's Law describing the diffusion rate and Michaelis–Menten kinetics yielding the Eq. (2.9).

$$\frac{\partial c_S}{\partial t} = D_S \frac{\partial^2 c_S}{\partial x^2} - \frac{\upsilon_{\max} c_S}{K_m + c_S}$$
$$\frac{\partial c_P}{\partial t} = D_P \frac{\partial^2 c_P}{\partial x^2} + \frac{\upsilon_{\max} c_S}{K_m + c_S} \tag{2.9}$$

Here, D_S, D_P are the diffusion coefficients for the substrate and product, respectively, t is the reaction time, and x is the distance in orthogonal direction from the transducer interface to the bulk solution. If the concentrations of the reagents outside the surface layer are taken to be constant, the solution of equations results in the Eq. (2.10) describing the linear dependence between the surface and volume concentration of a substrate.

$$c_S = c_S^0 \exp\left(-\sqrt{\frac{\upsilon_{\max}}{K_m D_S}} t\right) \tag{2.10}$$

The enzyme sensors that meet the Eq. (2.10) function at a rather low substrate concentration corresponding to the linear piece of the Michaelis–Menten plots (see above). In this case, the diffusion inside the surface layer does not compensate for the decay of the substrate concentration due to enzymatic conversion. From the practical point of view, such behavior is typical for thin membranes with moderate enzyme activity. The substrate concentration profile within the surface layer is linear (Fig. 2.5a). In the opposite case, when the enzyme activity is low or the transport of the reactants is hindered by high viscosity (low permeability) of an enzyme support, all the active sites of an enzyme in the membrane remain saturated so that the signal of a biosensor depends on the rate of enzyme reaction but not on the concentration of a substrate in the bulk solution (Fig. 2.5b). These two extreme cases are also called the diffusional and kinetic regimes of biosensor functioning. In between, there is an area where both kinetics and mass transfer affect the substrate determination.

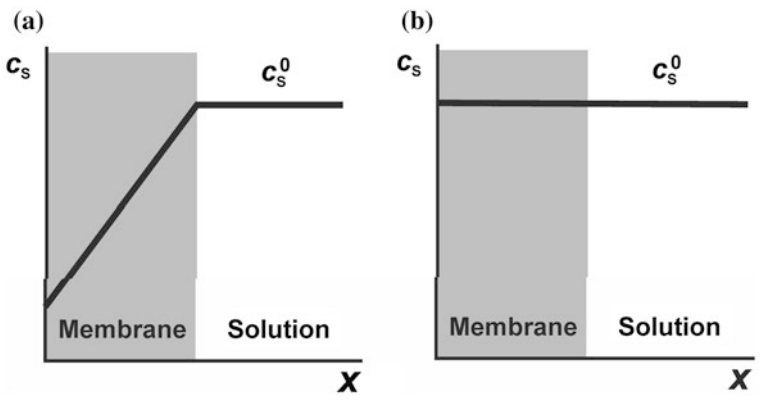


Fig. 2.5 Substrate concentration profiles related to diffusional (**a**) and kinetic (**b**) regimes of the enzyme sensor functioning and nature

Most of the calculations in the biosensor area originate from several assumptions. First, the kinetic parameters of enzymes implemented in the surface layer are supposed to be the same in solution. This might be true for gentle approaches to the formation of the surface layer that do not assume significant changes of the protein structure. However, this might not be true for extreme environments (hydrophobic supports, application of organic solvents for enzymatic membrane formation) or for the use of modified enzymes including specific tags and functional groups implemented for use in biosensor assembly. The second assumption involves the presentation of a surface layer as a uniform homogeneous media that differs from aqueous solution only by its higher viscosity. This is quite acceptable for ultra-thin layers, with enzymes directly attached to the transducer surface or for rather thick membranes with a low enzyme activity made from gelatin, chitosan, polysiloxanes, etc. Meanwhile, the use of nanosized carriers, lately very popular, complicates the behavior of biosensors. Carbon nanotubes, silver and gold nanoparticles provide different conditions for reactant transport, especially for charged species. In some cases, channels of increased permeability and pores that are different in size and accessibility to the substrate might appear.

Besides transport properties, the enzyme carrier can affect the kinetic parameters of an enzymatic reaction due to changes in the dielectric constant, acid–base equilibria regulation and electrostatic interactions. In some cases, the products of the substrate conversion can be accumulated in the layer and change its properties and possible influence on the substrate conversion. All of the factors presented make it very difficult to simulate the enzyme sensor behavior.

In addition to substrates, enzymes are affected by other species that do not take part in the reaction but influence its rate. In general, such compounds are called *enzyme effectors*. In nature, many enzyme effectors exert a reversible influence on the enzyme active site. Their contribution increases and decreases with their concentration and is observed only in the presence of a substrate, the reactions of which become faster or slower depending on the effector nature. The specificity of natural effectors and reversibility of their influence are due to their regulatory function in biochemical paths. Negative effectors, or inhibitors, decrease the enzyme activity. They can also protect living beings from pathogens or enemies such as predators or competitors for living space and other resources.

The interest in the detection of inhibitors by enzyme sensors is mainly related to the synthetic compounds belonging to xenobiotics. They have never been released from natural sources and appeared in the environment as a result of anthropogenic

> The inhibitor is a molecule that reacts with an enzyme-active site to prevent it from converting the substrate.

impacts. Pesticides are one of the most important examples of such analytes. Toxic metals and natural toxins can be determined by appropriate biosensors as well. The

Table 2.3 Application of various enzyme sensors for inhibitor determination

Analyte	Representative	Enzyme/substrate	Signal transduction
Pesticides	Organophosphates, carbamates	Acetylcholinesterase/ acetyl(thio)choline) (Evtugyn et al. 1999; Arduini et al. 2010)	Amperometric, potentiometric, optic (visual detection)
		Tyrosinase/catechol (Campanella et al. 2007)	Amperometric
	Triazines	Tyrosinase/catechol (Hipolito-Moreno et al. 1998)	Spectrophotometric
	Dithiocarbamates	Aldehyde dehydrogenase/ propanaldehyde (Noguer and Marty 1997)	Amperometric
Industrial pollutants	Cyanide	Peroxidase/ascorbic acid (Volotovsky and Kim 1998)	Field effect transistor
	Heavy metals	Urease/urea (Rodriguez et al. 2004; Yang et al. 2006; Ogonczyk et al. 2005)	Potentiometric, spectrophotometric, field effect transistor
		Peroxidase/catechol (Bogdanovskaya et al. 1994)	Amperometric
	Chlorophenols	Peroxidase/catechol (Solna et al. 2005)	Amperometric
	Fluoride	Tyrosinase/catechol (Asav et al. 2009)	Amperometric
Pharmaceuticals	Antidepressants	Monoamine oxidase/dopamine (Medyantseva et al. 2007)	Amperometric

list of the common inhibitors that can be determined with enzyme sensors is summarized in Table 2.3.

The mechanism of enzyme–inhibitor interaction depends on their nature. From a whole variety of inhibitors, some classes are specified. Thus, *specific inhibitors* have their structure adapted to the enzyme active site or some functional groups included in the active or allosteric site of an enzyme. They are subdivided in accordance with the reversibility of their interaction and the target group attacked. Non-specific inhibitors change the structure of a protein globule irrespective of the specific groups at the active site. To specify such species, the term "denaturation" is used instead of "inhibition." The non-specific inhibitors involve detergents, the compounds breaking disulfide bridges, polyelectrolytes, etc.

The classification of various inhibitors is presented in Fig. 2.6.

Irreversible inhibitors are highly toxic species that exert their influence on enzyme activity by direct interaction with an enzyme active site. To some extent, the structure of irreversible inhibitors is similar to that of the substrate. This provides the specificity of enzyme–inhibitor interaction which is commonly much higher than that of reversible inhibitors. In analogy to the *ES* complex, the reactivity of the *EI* (enzyme–inhibitor) complex formed by irreversible inhibitors is small enough to exclude a part of enzyme molecules from the catalytic cycle.

The mechanism of irreversible inhibition can be illustrated by acetylcholinesterase and organophosphate insecticides. The enzyme promotes the hydrolysis of acetylcholine, the neuromediator (2.11) (Skládal 1996).

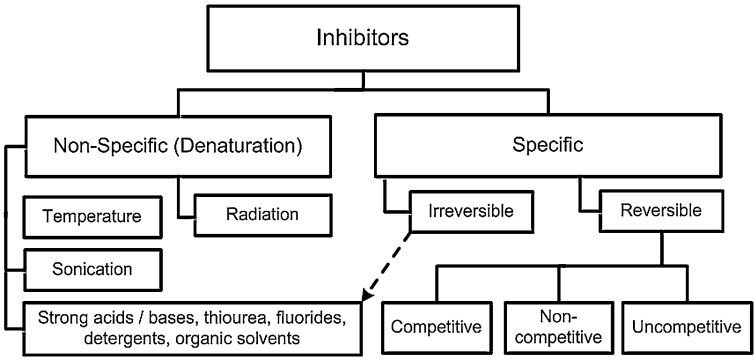
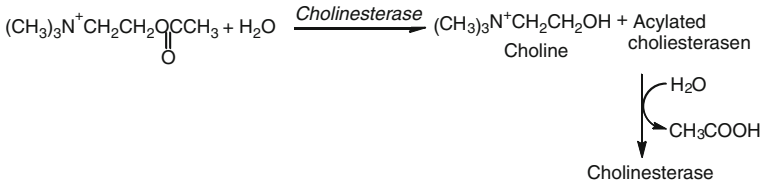


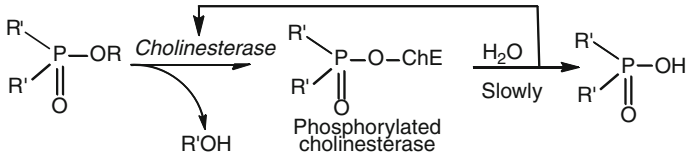
Fig. 2.6 Classification of enzyme inhibitors in accordance with the mechanism of action and nature



(2.11)

After the *ES* complex formation, the substrate breaks up into two parts, i.e., the acetic acid and choline. The latter remains in the active site due to the formation of the esteric bond with a hydroxide group in the serine residue of a protein. The intermediate product, called "acetylated cholinesterase," reacts with the final release of the acetic acid and regeneration of an active form of the enzyme. The reactivity of acetylated acetylcholinesterase is fast enough to establish a high turnover number of the enzyme.

Organophosphates are also able to form an esteric (phosphorylated) acetylcholinesterase so that the first step of the reaction is very similar to that of acetylcholine (2.12) (Giacobini 2000).



(2.12)

However, the hydrolysis of phosphorylated cholinesterase proceeds very slowly and the total rate of the substrate conversion decreases dramatically. The rate of the enzyme regeneration can be accelerated by special agents called *reactivators*, or antidotes. Thus, the reaction of the irreversible inhibitor with an enzyme is

followed by the partial destruction of its molecule. Therefore, the intermediate stage, a phosphorylated enzyme, cannot be reached from the side of the product, i.e., from alcohol R′OH, enzyme *E* and organophosphoric acid. This is one of the key features of irreversible inhibitors that distinguishes them from reversible inhibitors that meet the conditions of "true" chemical equilibrium.

The term "irreversible" in enzymology and biosensorics is also referred to the reactions that cannot be inverted in the time period comparable with the measurement duration. Such an empirical approach makes it possible to extend the number of "irreversible" inhibitors to those interacting with an enzyme reversibly but not flexibly. For example, mercury ions inhibit the enzyme activity of most enzymes due to their interaction with thiol groups of the proteins. The product of such interactions does not assume changes in the chemical structure of an inhibitor. However, the dissociation of the *EI* complex is extremely slow, so that the restoration of enzyme activity can be achieved only by treating the enzyme preparation with chelating agents. The kinetic consideration of irreversible inhibition differs from that the mechanism of which is implied. Below, a "true" irreversible inhibition with an inhibitor modification is described.

An irreversible inhibition can be monitored by the consecutive addition of an inhibitor and substrate to the enzyme preparation. The first step is called "incubation" and is performed in the absence of the substrate that diminishes the sensitivity of inhibitor detection. The inhibitory effect is quantified by the relative decay of enzyme activity. For the inhibitor concentration significantly exceeding that of the enzyme-active site, the Aldridge Eq. (2.13) is used (Aldridge 1950).

$$\ln \frac{\upsilon_o}{\upsilon_t} = k_{II} c_I \tau \tag{2.13}$$

Here, υ_o and υ_t are the rates of enzymatic reaction prior to and after the incubation step, c_I is the inhibitor concentration, and τ is the incubation time. The k_{II} bimolecular inhibition constant describes the efficiency of the enzyme-inhibitor interaction. This depends on the nature of the reactants and reaction conditions but not on the quantity of the enzyme and inhibitor as such. The bimolecular inhibition constant can be expressed by a combination of rate constants of various steps of the reaction.

The reaction (2.13) can be converted into the form (2.14), which is more convenient for the description of the biosensor response. It expresses the dependence of the remaining enzyme activity (*a*) on the inhibitor concentration.

$$\log a = \log 100 - \frac{k_{II} \tau}{2.303} \tag{2.14}$$

In both cases, the inhibition effect depends on the product of the inhibitor concentration and the period of its interaction with an enzyme. An increase in the incubation period diminishes the concentration of an inhibitor exerting the same effect on enzyme activity. The incubation period is commonly limited by 20–30 min from taking into account the enzyme stability and the biosensor usability.

Besides the remaining enzyme activity and inhibition constants, some general characteristics of inhibition are used, e.g., the inhibition degree $I\,\%$ (2.15) and inhibitor concentration resulting in the decrease of the enzymatic activity by a half I_{50}. They are also applied for the description of reversible inhibition and even enzyme denaturation caused by such physical factors as sonication, radiation, or heating.

$$I\,\% = \frac{\upsilon_0 - \upsilon_t}{\upsilon_0} 100\,\% \tag{2.15}$$

The irreversible character of inhibition can be detected by several experiments. Thus, inhibition increases with the incubation period and does not depend on the substrate concentration. The addition of the substrate to the inhibitor on the incubation step decreases the inhibition effect. The confirmation of the irreversible mechanism of inhibition is important for the selection of the working conditions of biosensor functioning. Specifically, it allows choosing a high substrate concentration and performing the incubation step with no substrate in the reaction media.

Reversible inhibitors exert a great variety of interaction mechanisms that all refer to the true equilibria of the stages with the enzyme participation (Copeland 2000). The kinetics of reversible inhibition as well as the approaches to the establishment of inhibition constants are given in the Appendix.

The implementation of enzymes into a solid support on the surface of the signal transducer affects the canonical dependence of inhibition on an analyte concentration. This might be for various reasons, some of which belong to the mass transfer conditions and others to the enzyme-inhibitor interaction. In short, the fewer the limitations of the substrate/inhibitor access, the higher the sensitivity of an inhibitor determination in other comparable conditions. Other effects, like the charge of enzyme carriers as such, or the accumulation of the reactants in the surface layer, are of lesser importance, at least for common signal transduction principles. Some other considerations for cholinesterase inhibitors detection are presented in Chap. 4.

In most cases, inhibition should be measured in conditions of the saturation of the enzyme with the substrate (the kinetic regime). This statement has direct application for reversible inhibition with the inhibitor solution added after the substrate to the reaction medium. Meanwhile, this is valid for irreversible inhibition as well, although the inhibitor commonly reacts with enzyme-active sites in the absence of a substrate (Fig. 2.7).

The kinetic regime of the signal measurement should be confirmed by a separate measurement series performed with the increasing substrate concentration. The following measurement of the reduced signal after the incubation step should be performed with the substrate concentration on the plateau of the calibration curve.

If the enzymatic membrane is not saturated with the substrate, the decrease in the number of active sites due to inhibition is compensated for by the involvement of other active sites that were occupied by the substrate molecules in the first signal measurement (Fig. 2.13). As a result, the inhibition degree calculated on the

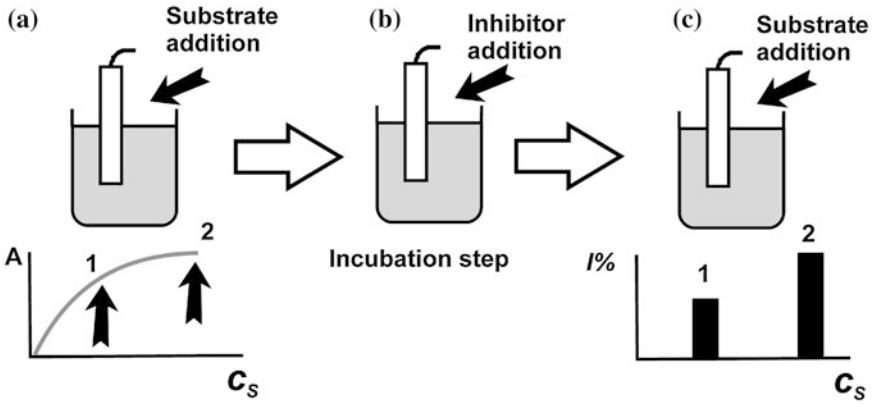


Fig. 2.7 Selection of the substrate concentration for irreversible inhibition measurement. **a** Signal measurement prior to the contact with an inhibition; **b** incubation step in the absence of the substrate; **c** the second measurement of the biosensor signal and calculation of inhibition degree *I* %. The choice of the substrate concentration on the progressive part of the substrate calibration *curve* (*1*) decreases the sensitivity towards the inhibitor in comparison with measurements with the saturating concentration of the substrate (*2*)

base of the biosensor signal shift will be lower than the actual shift of enzyme activity due to inhibition (Van Dyk and Pletschke 2011).

The use of the inhibition degree (*I* %) is a most popular approach of the biosensor signal processing, irrespective of the inhibition mechanism and biosensor functioning regime. The dependence of the inhibition degree on the inhibitor concentration is usually linearized in semi-logarithmic plots and commonly covers up to four orders of concentration magnitude. In some cases, the linear dependence $I\ \%—c_I$ is also described within one or two orders of magnitude. The proportional increase of the inhibition with the incubation time is observed for biosensors in a rather narrow interval of *I* %. If the inhibition exceeds 40–60 %, the influence of incubation becomes less than that expected from theoretical equations.

Although the inhibition degree is an experimental estimate of inhibition kinetics, it can be used for some conclusions about the mechanism of inhibition and the biosensor signal generation. Thus, for irreversible inhibitors, the increase in inhibition concentration should result in a 100 % inhibition, even though the inhibition is performed in the presence of a substrate. In some cases, the limited *I* % level is below 100 %. If the irreversible mechanism of inhibition has been confirmed by measurements with an enzyme solution, the underestimation of inhibition with an enzyme sensor can be due to the following reasons (Evtugyn et al. 2012):

- the chemosorption of an inhibitor on the enzyme support with a decrease in its concentration near the enzyme active site;

- the non-enzymatic reaction of the substrate conversion increasing the concentration of a product detected with the signal transducer irrespective of the enzyme activity;
- the reactivation of the inhibited enzyme active site promoted by a support component; and
- changes in the microenvironment of the enzyme in the surface layer suppressing the inhibitory effect of an analyte.

These changes might result from the pH shifts or the substrate/product accumulation in the surface layer.

In most cases, the decrease in the sensitivity of a biosensor signal toward the irreversible inhibitor caused by the above reasons can be overcome by introducing appropriate changes in the content of the surface layer and measurement protocol.

The reversible inhibition, vice versa, results in the limiting inhibition below 100 % and is theoretically independent of the measurement time. The latter condition is not obligatory and in many cases the biosensor should first be incubated in the reversible inhibitor solution prior to substrate addition. The obvious reason of such a step from the protocol of irreversible inhibition measurement is the promotion of steady-state conditions. Contrary to the theory, the reversible inhibition measured by the appropriate biosensor depends on the order of the reactant addition and is usually higher for preliminary inhibition than for the opposite order of reactant addition. For heavy metals, e.g., mercury, the behavior of an enzyme biosensor does not differ significantly from that of irreversible inhibition measurement.

For rather thin membranes, the inhibition degree can be regarded as a measure of the inhibited active sites of an enzyme. This allows the expression of inhibition parameters by the changes in the biosensor signal. Thus, for the amperometric detection of the current I related to the product of the substrate conversion, Eq. (2.16) can be used. The I_0 value corresponds to the maximal current reached with the enzyme sensor in the absence of an inhibitor, i.e., to the saturation of the enzyme with a substrate (Adeyoju et al. 1995).

$$I\,\% = \frac{c_{EI}}{c_{EI} + c_E} = \frac{c_I}{c_I + K_I} = \frac{\Delta I}{I_0} \tag{2.16}$$

The Eq. (2.32) assumes purely competitive inhibition, a most common mechanism of inhibition occurring in biosensor-based investigations. It should be taken into account that the kinetics of reversible inhibition determined for homogeneous conditions do not always coincide with those for immobilized enzymes. Moreover, the formal mechanism of inhibition observed for the biosensor signal can experience changes that depend on measurement conditions, e.g., the use of an organic solvent to increase the solubility of inhibitors.

The Eq. (2.34) can be linearized in the Scatchard plots $I\,\%/c_I$–$I\,\%$ (2.17) or by the Hill Equation (2.18) (Kurganov et al. 2001).

$$\frac{I\,\%}{c_I} = \frac{I\,\%}{K_I} + \frac{I_0}{K_I} \tag{2.17}$$

$$\frac{I\,\%}{100 - I\,\%} = \left(\frac{c_I}{K_I}\right)^x \approx \left(\frac{c_I}{I_{50}}\right)^x \tag{2.18}$$

In the Hill equation, the K_I value can be determined by the slope of the graph in double logarithmic plots. The Hill coefficient x is an empirical parameter introduced to take into account cooperative effects in the non-Michaelis–Menten kinetics description. The x value for the enzymes most popular in biosensor development and inhibition measurement is nearly one in aqueous solutions and can increase in the presence of polar organic solvents. Besides the Hill coefficient, the effect of measurement conditions can be quantified by the I_0/K_m value with the Michaelis constant determined from biosensor measurements.

2.2 Antibodies

Antibodies, also called *immunoglobulins*, or γ-globulins, are Y-shaped serum glycoproteins that are produced in the blood as a response to foreign substances called *antigens*. Mainly, antigens are related bacteria, viruses, and their parts (cell membrane fragments, deoxyribonucleic acid (DNA) (Blažková et al. 2009), carbohydrates (Huang et al. 2011), proteins (Minkstimiene et al. 2009), etc.). The interaction between an antigen (Ag) and antibody (Ab) is formally expressed by equilibrium (2.19) characterized by an association constant K_a, also denoted as an affinity constant.

$$\mathrm{Ag} + \mathrm{Ab} \overset{K_a}{\longleftrightarrow} \mathrm{Ag} - \mathrm{Ab} \tag{2.19}$$

The product of interaction, Ag–Ab, is called an *antigen–antibody complex*. Although the reaction is reversible, the equilibrium is shifted toward the complex so that one unbounded antigen molecule corresponds to 10^{15} and even more Ag–Ab complexes. The reaction (2.35) is very specific. Some antibodies can distinguish the enantiomers of the same organic molecule. For this reason, the immunochemistry approaches become popular in biomedical assays and other areas requiring a sensitive and specific detection of biologically active compounds. Both Ab and Ag molecules can be detected using immunoassay approaches, but more frequently the Ag molecules are considered a target.

It should be mentioned that the production, purification and characterization of antibodies remain an essential part of investigations on immunoassays and immunosensors. For this reason, many reports on immunosensors are devoted to the procedures related to the preliminary stages mentioned and the variety of target analytes as well as specific antibodies against them grows enormously. To some extent, the situation in the area of immunosensors development is similar to that

observed for enzyme sensors 30 years ago when the number of biochemical components increased faster than the number of specific techniques applied for signal transduction. Meanwhile, signal transduction in immunosensors is still mainly derived from conventional techniques applied in immunoassays developed for biomedical applications. Let's start with the consideration of biochemical backgrounds of immunoassays and immunosensors.

All antigens belong to high-molecular compounds. However, some of the low-molecular species called *haptens* can also initiate the production of antibodies and bind with them. The haptens first interact in living beings with some native biopolymers, such as proteins, to form a high-molecular adduct initiating the immune reaction. Such a mechanism of hapten action is taken into account in the production of specific antibodies against haptens and the signal measurement of appropriate biosensors.

The structure of an antibody is outlined in Fig. 2.8. It consists of four sub-units comprising two identical oligopeptides called "large" or "heavy" chains (H-chains), and two identical small, or "light" chains (L-chains). The heavy and light chains are held together by non-covalent forces and covalent disulphide bridges of cysteine residues. The carbohydrate residues in antibodies are covalently bonded to the *C*-terminal half of the molecule (Fc). Themolecular weights of H- and L-chains are 50 and 22 kDa, respectively. The total molecular weight of an antibody usually varies from 140 to 200 kDa (Berg et al. 2007).

The key part of the Ab molecule with the Ag binding sites is termed the Fab fragment. Thus, there are two Fab fragments in one Ab molecule and each one of them comprises an entire light chain and a segment of the heavy chain.

The binding versatility and specificity of antibodies toward the Ag molecules are achieved due to the variation in the chain region near the tips of a "Y-shaped" molecule (the *N*-terminal part of the sequences). Within these variable regions, three regions are hypervariable. They are called complementarity-determining regions. The opposite *C*-terminal end of the chain is constant for nearly all vertebrates. The L- and H-chains of Ab are folded into globular motifs of about 110 amino acids, formed by two β-pleated sheets. The L-chain contains variable light (V_L) and constant light (C_L) domains. The H-chain contains four domains

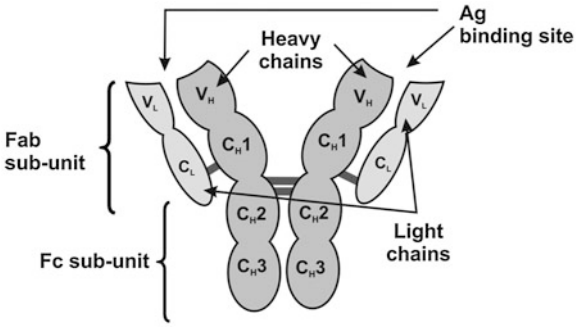


Fig. 2.8 The outline presentation of the antibody molecule

(denoted in Fig. 2.13 as V_H, C_H1, C_H2, C_H3) and the domains are assembled in pairs. The assembly of the variable domain of the H- and L-chains brings six complementarity-determining regions into a spatial proximity. They form the Ab combining site.

Antibodies are divided into five classes (IgG, IgA, IgM, IgD, and IgE) according to their heavy chain structure. Each class has some sub-classes determined by the structure of the H-chain domains. IgG is present in its highest concentration in the serum; IgM is the first to appear in the serum after the Ag exposure. IgA is the major Ab class in external secretions like saliva, tears, bronchial mucus, and intestinal mucus. IgE causes an allergic reaction, while the role of IgD is yet unknown.

Besides native Ab molecules, their derivatives can be used in the immunoassays and biosensor assembly. Thus, the treatment of Ab with dithiotreitol and some other reducing agents provides breaking disulphide bridges and formation of various subunits that retain the specificity of the Ag recognition (Stocklein et al. 1998). The thiol groups remaining on the *C*-regions of the sequences can be used for the attachment of the "half-Ab" molecules to gold or other supports. The Fab fragments can be isolated as well after treating the Ab molecules with papain. They can be used instead of the full molecules to recognize the same analytes with a lesser non-specific binding of interferences and the higher stability of immunopreparations.

The Ab production on a laboratory scale is mostly based on the immunization of a susceptible mammal (rabbit, goat, sheep, chicken, etc.) with a target Ag. For haptens, a conjugate of hapten with a high-molecular carrier is first prepared to increase the efficiency of immunization. Then the concentration of Ab in the blood gradually increases over several weeks. The blood is extracted from an animal and the Ab concentrated and purified from serum proteins. Various β-T-lymphocytes are activated by the same Ag to start producing Ab at the immunization step. Thus, the Ab pool (*polyclonal* Ab) is obtained with a different sensitivity towards various potential binding sites (*epitopes*) in the Ag molecule. Polyclonal antibodies exhibit an extended variability of the Ag binding that can vary from one animal (immunization step) to another. The Ab comprising the pool can also react to the molecules structurally relative to Ag and therefore decrease the specificity of the immunoassays. This ability is also called *cross-reactivity* and is quantified by the ratio of the concentrations of the target Ag and other species yielding the same relative binding of Ab in the sample. Cross-reactivity is highly dependent on the immunization protocol but can be altered to some extent on the stages of biosensor assembling and signal protocol optimization (Law 1996).

Another way to produce the Ab was provided by genotechnology. In this method, the cells producing antibodies are removed from the spleen and fused with tumor cells to form so-called "hybridoma cells." They can infinitely replicate and produce strictly the same Ab (called *monoclonal antibody*) as a parent cell. Being more expensive at the initial stages of research, monoclonal antibodies show a much better selectivity toward the target analytes and a lesser mass production cost than monoclonal antibodies. In a similar manner, recombinant Ab fragments (commonly Fab and single-chain variable fragments) are obtained from bacteria,

e.g., *Escherichia coli*, with an introduced specific gene (Rasmussen et al. 2007). This greatly facilitates the production and manipulation for biotechnological, medical or research applications.

Recombinant Ab technique is a third possibility for producing antibodies. In these protocols, the Ab genes are cloned, introduced and expressed in inexpensive and relatively simple organisms (yeast, plant and insect cells). *E. coli* is the most common host organism. The properties of antibodies obtained in various ways are summarized in Table 2.4 (Dankwart 2000).

A similar technique can be used for the production of the recombinant Ab fragments. Thus, the ScFv antibodies (molar mass about 27 kDa) consist of variable parts of heavy and light chains joined together with a peptide linker (Zeng et al. 2012). scFv is the smallest antibody fragment retaining the Ag binding specificity of the parent Ab. They can be genetically engineered to contain metal binding amino acids (cysteine, hystidine) involved in covalent immobilization of ScFv on metal surfaces. The linkers mentioned can participate in the formation of di-, tri- and tetrameric ScFv molecules with enhanced sensitivity of recognition.

The affinity of Ab is characterized by an equilibrium constant determined in a series of experiments using the different ratios of the Ab and Ag concentrations. Assuming the stoichiometry 1:1 of the Ag–Ab interaction, the following Scatchard equation can be derived from the equilibrium (2.20):

$$\frac{c_{\mathrm{Ag-Ab}}}{c_{\mathrm{Ag}}} = K_a c_{\mathrm{Ab}}^0 - K_a c_{\mathrm{Ag-Ab}} \tag{2.20}$$

Table 2.4 Properties of polyclonal, monoclonal and recombinant antibodies

Properties	Polyclonal Ab	Monoclonal Ab	Recombinant Ab
Supply	Limited and variable	Unlimited production possible	Unlimited production possible, immunization not mandatory
Uniformity	Properties change with different sera	Constant properties	Constant properties, variation by genetic manipulations
Affinity	Mixture of antibodies with different affinities	Uniformity high or low, can be selected by testing	Uniformity high or low, can be selected by testing and can be modified
Cross-reactivity	Results from different affinities	Different, depending on individual Ab	Different, depending on individual Ab, can be modified
Demands on Ag	High purity required for specific antisera	Impure Ag or mixture of antigens can be used for immunization, pure Ag necessary for screening	Impure Ag or mixture of antigens can be used for immunization, pure Ag necessary for screening, immunization not mandatory
Cost	Low	High	Once established, low

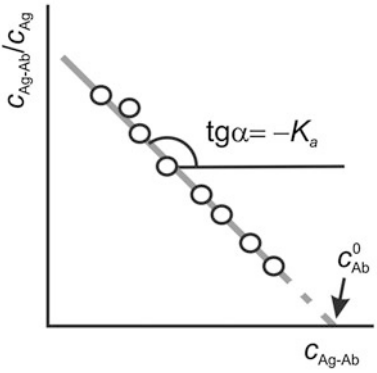


Fig. 2.9 Graphical presentation of the Scatchard equation

In accordance with (2.20), the ratio of the bonded Ag to free Ag linearly depends on the concentration of the Ag–Ab complex with a slope equal to the association constant K_a (Fig. 2.9). If Ab is bonded to nAg molecules, the Eq. (2.20) can be converted to (2.21):

$$\frac{c_{\mathrm{Ag-Ab}}}{c_{\mathrm{Ag}}}\frac{1}{c^0_{\mathrm{Ab}}} = nK_a - K_a\frac{c_{\mathrm{Ag-Ab}}}{c^0_{\mathrm{Ab}}} \tag{2.21}$$

In the plots of $\left(c_{\mathrm{Ag-Ab}}/c_{\mathrm{Ag}}\right)/c^0_{\mathrm{Ab}}$ against $c_{\mathrm{Ag-Ab}}/c^0_{\mathrm{Ab}}$, the linear graph intersects the x-axis at $x = n$.

Methods of the graphical presentation of the immune interactions based on the Scatchard equation are valid for the monoclonal antibodies and for the polyclonal ones with similar affinity toward Ag. If the affinity constant varies, groups of clone Ab can be specified from Scatchard graphs by specifying a narrow concentration range of the reactant corresponding to the high and low activity of antibodies. Besides, a full non-linear fitting of the progressive curve is used for assumed stoichiometry of interaction.

Although the reaction of Ab and Ag is reversible, the use of one of the reactants attached to the solid support does not guarantee a multiple use of the biosensor system. The dissociation of the Ag–Ab complex is normally too slow for spontaneous reactivation. The use of the concentrated solutions of inorganic salts with the addition of surfactants can accelerate the process. Of other measurement parameters, temperature is of particular interest. Commonly, the reaction of the sample with antibodies, either in solution or on a solid support, is performed at the ambient temperature or that close to the temperature of the host body (37 °C for human antibodies). Intermediate steps of washing and removal of excessive reagents can be performed at 40–60 °C. In general, immunoreagents are sensitive to the solution temperature to a lesser extent than the enzymes. The same applies to the presence of polar organic solvents that alter the affinity but at higher concentration and to a lesser extent than the activity of the enzymes. This might not be very important for the immunoassay format based on the measurement of enzyme

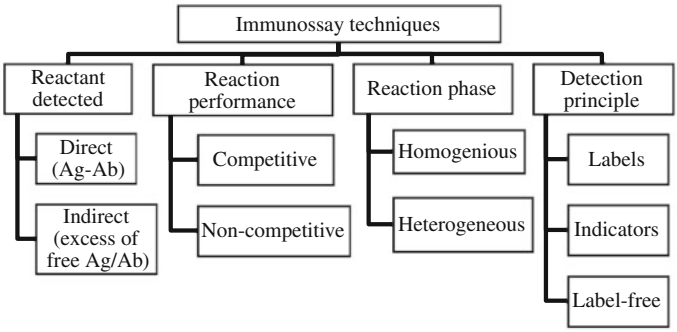


Fig. 2.10 Classification of immunoassay methods

activity like enzyme linked immuno sorbent assay (ELISA) but extends the opportunities of enzyme-free detection techniques.

All the methods of immunoassays are based on the detection of either the Ag–Ab complex or excessive amounts of reagents. The division on direct and indirect methods and homogeneous and heterogeneous types among other classification principles seems most important (Fig. 2.10).

Thus, the *direct* detection of Ag–Ab complexes can be performed by any mass-specific transducer, e.g., quartz crystal microbalances or cantilever-based techniques. Of other methods that are not compatible with the biosensor format, agglutination can be mentioned. In this method, the decrease of the solubility of the Ag–Ab complex against individual components is recorded by optical techniques. The sedimentation can also result in the surface accumulation of various species such as radioactive isotopes. Taking into account the sensitivity of appropriate detectors, radioimmunoassay belongs to one of the most sensitive detection methods with the femtomolar limits of detection. In *indirect methods*, the formation of Ag–Ab complexes is detected via the signal of auxiliary agents either covalently attached to the reactant or left free in reaction solution. In the first case, the compound or functional group measured by the appropriate transducer is termed a *label* and in the second case an *indicator*.

Previously, the term "markers" was used as well, but at present it is referred to as species indicating particular illnesses by the appearance or quantity changes in a human body. The reaction with the Ag bearing the label results in an increase of the signal due to the formation of immune complex.

Enzymes are the most popular 'labels' because of the possibility to detect their very low concentration by the amount of the product of the substrate conversion. The appropriate method involving the separation of the reactants on the solid support is termed ELISA. In addition to enzymes, colored particles, redox-active and fluorogenic compounds are used as labels and indicators.

Below are some of the most popular formats of heterogeneous immunoassay that are compatible with the biosensor format (Fig. 2.11) (Wild 2005).

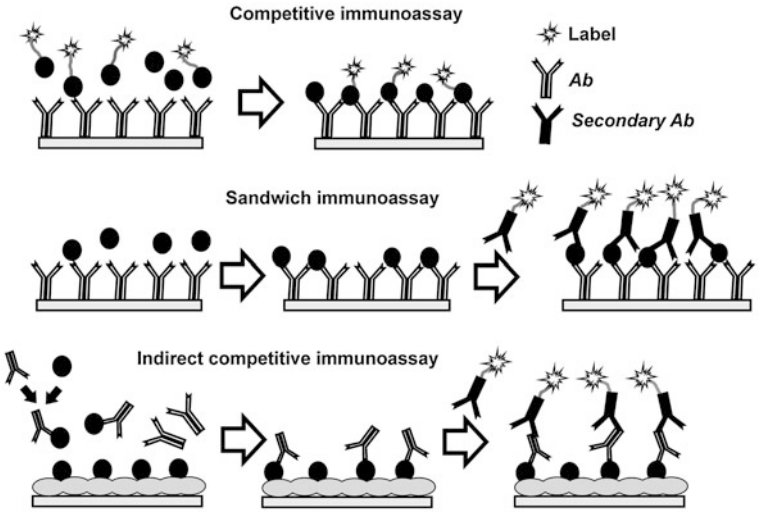


Fig. 2.11 Immunoassay formats

Direct competitive immunoassay. In this format, the sample to be tested is mixed with the solution of the appropriate immunoreagent bearing a label. The way of label introduction should be optimized to prevent its influence on the affinity of the labeled agent to its counterpart. Thus, for Ag detection, specific antibodies are attached to the solid support. The incubation with a mixture of labeled and non-labeled antigens results in their interaction with the layer of antibodies. The subsequent washing removes the non-bonded labeled reagent so that the signal of the label detected can be related only to the bonded label. Thus, the higher the signal, the lesser the content of non-bonded Ag in the sample tested. The direct competitive immunoassay is one of the simplest detection schemes that is quite suitable for the automated assay and immunosensor format. The measurement includes three steps (incubation, washing, and signal measurement) and hence a rather acceptable time interval is required. However, there are two principal limitations: First, the competition decreases the sensitivity of the Ag measurement because some of the binding sites are occupied by a labeled auxiliary agent. Second, at low target concentration, small changes of a maximal signal related to the label should be detected. This complicates the metrological characterization of the result and gives a high deviation of the signal against blank measurement.

Sandwich immunoassay. In this format, two types of antibodies are required. As in the previous case, the antibodies specific for Ag are attached to a solid support. The incubation with the samples allows accumulation of all the target analyte molecules on the solid support. After that, the support with the Ag–Ab complex attached is carefully washed and treated with secondary antibodies that bind to the analyte but not to the preliminary antibodies that remained unoccupied on the surface. Then antibodies are labeled so that the signal measured is directly

proportional to the number of the Ag–Ab complex onto the surface, i.e., to the molar concentration of a target in the sample.

In comparable conditions, the sandwich immunoassay is more sensitive than the competitive immunoassay. The detectable concentrations can differ up to ten times and even more. The minimal detectable levels depend on the signal-to-noise ratio but it is much simpler to observe a signal over the zero level than the decrease of maximal response in the competitive immunoassay. The only, yet significant limitation of sandwich immunoassay, consists of a multi-step protocol increasing the measurement time up to several hours and complicating its realization in the immunosensor format.

Indirect competitive immunoassay. This is a hybrid method that includes some steps performed on a solid support and others in the homogeneous solution. Indirect immunoassay was described for hapten detection because the traditional schemes of label-based immunoassay are not applicable to small molecules. In this case, the solid support is first covered with a high-molecular derivate of hapten; this might be the conjugate used for immunization (Ab production) or another one synthesized specifically for signal measurement. The potential carriers involve synthetic polyelectrolytes and proteins such as albumin from various sources or the soybean trypsin inhibitor. The sample is first mixed with an excess of specific anti-hapten Ab. After that, the mixture is placed onto the support bearing the hapten conjugate. It can react with the free Ab but not with the Ag–Ab complex present in the same solution. Thus the higher the concentration of the analyte, the lower the amount of Ab attached to the surface. The latter is detected with secondary antibodies bearing the label for sandwich immunoassay as described above.

The formation of a bulky Ag–Ab complex affects the optic and electric characteristics of the interface that can be recorded by appropriate techniques. Thus, total reflectance spectroscopy and surface plasmon resonance provide the information on the amount of the immunoreagent attached to the surface and the kinetics of the immune reaction. Being rather complex and cumbersome for mobile devices, such equipment deserved complementary estimates due to the high sensitivity and versatility of its response. And last but not least is that these measurement modes do not require any specific reagents, so that they are both label-free and reagent-free and can be used with minimal sample treatment.

The changes in the permeability of the surface layer caused by immunochemical reactions can also be traced by electrochemical techniques that provide an important tool for both the miniaturization and automation of the results. In the simplest way, the Ag–Ab complex limits the mass transfer of small ions like ferricyanides. This can be easily monitored by conductivity measurement and electrochemical impedance spectroscopy. In both methods, the changes in the content of the surface layer are quantified by the decreased rate of the charge transfer. In the case of the electrochemical impedance, the indicator ion is involved in the reversible electron transfer and the estimation of a mass transfer is supplemented by the influence of the charge distribution on the electrode interface. This increases the sensitivity of the method against the simple recording of the ion

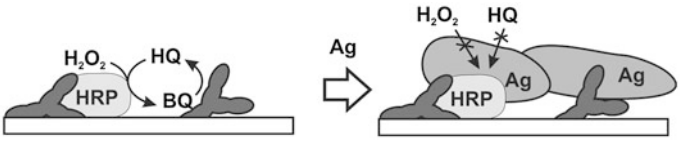


Fig. 2.12 Detection of Ag–Ab formation on the electrode by inhibition of the reaction of enzyme co-immobilized with specific antibodies. HRP horseradish peroxidase, HQ hydroquinone, BQ benzoquinone

distribution and allows drawing a number of conclusions on the steric rearrangement of the surface layer during the immunoreaction.

The use of enzymes as indicators of the mass transfer in the neighborhood of the Ab–Ag complex is reached by the co-immobilization of enzymes and antibodies onto the transducer surface (Fig. 2.12).

The binding of the Ag molecules suppresses the substrate access to the enzyme's active site. From the formal point of view, this phenomenon is described as similar to the competitive enzyme inhibition with a linear relationship between the relative decay of the enzyme activity and the Ag concentration. Acetylcholinesterase, alkaline phosphatase and peroxidase are mainly used both as labels in immunoassay and indicating enzymes in immunosensors. The high turnover number and the simple detection of enzyme activity based on optical or electrochemical transducers make them very attractive for the development of immunosensors. In Fig. 2.12, horseradish peroxidase (HRP) catalyzes the oxidation of hydroquinone (HQ) with hydrogen peroxide. The product of the reaction, benzoquinone (BQ), is reduced by the electrode to the initial HQ (Evtugyn et al. 2003).

Such a cycle, including the regeneration of a substrate in the electrode reaction, is called "the substrate recycling system;" it is analagous to similar biochemical paths, including the enzymes catalyzing the appropriate reactions of conversion and regeneration of a substrate.

The monitoring of the permeability of the surface layer, including Ab molecules, is less specific than the use of labeled immunoreagents as described above (see Fig. 2.11). Any changes in the surface layer, e.g., the non-specific adsorption of auxiliary reagents or sample components (serum proteins, lipids, nucleic acids for the assay of biological liquids) affects the permeability of the layer similarly to the target Ag–Ab interaction. The deterioration of the layer in the consecutive stages of washing, incubation and reagent addition decreases the sensitivity of the signal toward the Ag binding. This offers stricter requirements for assembling the surface layer, its durability and the stability of its main characteristics in the immunosensor manipulation.

ELISA-related immunoassay data are commonly quantified using empirical non-linear models, e.g., four-parameterlogistic-log, log-logtransforms, logistic-logtransforms, etc. The four-parameterlogistic-logmodel is used also for immunosensors based on immobilized immune reagents (Eq. 2.22) (Brady 2006)

$$y = \frac{A - D}{1 + \left(\frac{x}{C}\right)^B} + D \tag{2.22}$$

where x is the concentration of an analyte and y is the signal related to the Ag–Ab binding (absorbance for colorimetric end point determination, current for amperometric immunosensors, etc.). Certifications are determined for midpoint on the curve at a 50 % signal shift (IC_{50}), a maximum for the lower asymptote (A), and a minimum for the upper asymptote (D). B value corresponds to the slope of the mid-linear piece of the curve in semi-logarithmic plots, and C is a central point of this linear portion. The specific curve-fit constants vary from day to day and the accepted ranges of such variations should be determined. The reproducibility of the results for triplicate analysis is usually within 30 % but can be reduced to about 10 %, depending on the specific assay. The recoveries of positive controls typically range from 70 to 130 % or better. In similar conditions, the determination of haptens results in higher signal deviation than that of high-molecular compounds.

2.3 Protein/Peptide Receptors

Besides antibodies, some other proteins and peptides 'exert' the biorecognition properties demanded for the specific detection of biological targets, site-specific immobilization of biochemicals, or their labeling followed by signal transduction. Although most of them are still under study and will not 'assume' application in appropriate biosensors in the near future, the synthesis and characterization of protein-/peptide-based receptors is of increasing interest due to the opportunities they offer in the enhancement of the variety of biomolecular receptors and understanding their functioning both in living beings and in biosensor assemblies.

Synthetic peptides take 'intermediate' place between the amino acids and proteins. They are commonly synthesized by peptide synthesis from the randomized library of amino acids with separation of the target products by affine chromatography or solid-phase extraction (Tothill 2010). The progress in the chemistry and application of synthetic peptides is related to the attempts to avoid some of the problems often observed with antibodies and enzymes. They include low stability in extreme environment, poor performance in organic solvents, and high cost. The peptide receptors show increased affinity toward small organic molecules and are hence considered to be an alternative to antibodies in hapten analysis. The peptide-based biorecognition assumes multi-point interaction with an analyte molecule that is based on non-covalent interactions, e.g., hydrogen bonding, the formation of salt bridges, and hydrophobic and van der Waals interactions.

The peptide-based receptors consist of 5–10 amino acid residues. The short chain prevents winding the molecule and shielding its binding sites by self-aggregation. Although there are no strict limitations, most peptides applied to date include 20 essential amino acids that are similar to natural proteins. Several approaches have

been described for peptide synthesis and selection against target analytes, i.e., combinatorial library technique, phage display technology, rational design based on computational approach, and molecular imprinting. In combinatorial techniques, the diversity of peptides is first synthesized from a mixture of natural and unnatural amino acids, preferably on solid support. Then the peptides are tested for specific binding activity. Such approaches were successfully applied in the drug discovery. The screening for peptide receptors can be improved by the addition of target molecules that act as templates promoting the synthesis of the appropriate peptide that envelops the target (*molecular imprinting*). This can also be achieved by a combination of the primary synthesis of building blocks in aqueous media with their assembling on the interface in the presence of biological target followed by receptor isolation and stabilization by adsorption. *Computational modeling* predicts the molecular structure of the receptor and guides the selection of primary building blocks applied for peptide synthesis. On this stage, the interactions between a target analyte and potential receptor can be visualized and evaluated. In *phage display technology*, DNA fragments encoding random peptide sequences are inserted into bacteriophages. The peptides are expressed on the capsids and their affinity toward target molecules interest is tested by appropriate protocols.

Regarding the application of peptides in biosensors, the signal transduction with peptide receptors is mainly based on the same principles as conventional immunoassay. The binding of specific analytes can be detected by changes in optical or electrical properties of the interface or by direct mass changes measured with QCM technique. The list of analytes detected with peptide receptors involves mycotoxins (Tothill 2011), cAMP (Katayama et al. 2000) and dioxins (Mascini et al. 2004). The selection of peptides involved the formation of dipeptide combinatorial library, followed by the selection of most relevant dimers and their involvement in tetrameric structures.

A bioelectronic nose has been developed for the detection of seafood spoilage based on trimethylamine detection (Lim et al. 2013). A field effect transistor (FET—see details of signal transduction in Chap. 3) with the gap modified with single-walled carbon nanotubes was used as a signal transducer. Olfactory receptors that consisted of 15 residues were immobilized by self-assembling onto the surface of carbon nanotubes by preferably hydrophobic interactions of phenylalanine groups. The nose reacted on the triethylamine vapors by a significant shift of the conductance. The bioelectronics nose detected triethylamine on a femtomolar level and could distinguish the analyte from other similar amines. Besides seafood spoilage, the portable device can be applied to medical diagnostics and environmental pollution assessment.

Protein nanotubes are another example of the biorecognition elements derived from natural biopolymers (Seabra and Durán 2013). The synthesis of a new class of peptide receptors based on rationally designed eight alternating D- and L-amino acid residues of cyclo[-(D-Ala-Glu-D-Ala-Gln)$_2$-] was published in 1993. The internal pore diameter of the peptide nanotube is controlled by the size of the peptide subunit used, i.e., the number of amino acid residues within the cyclic structure. The cyclic peptide monomers can be modified with chromophore groups taking into

account their possible application in electronic and optical devices. Regular nanotubes can also be self-assembled from linear surfactants such as peptides enriched with aromatic amino acid residues. The peptide nanotubes are more reactive than carbon nanotubes, a conventional nanomaterial for biosensor development. Meanwhile they require additional modification to establish signal transduction, e.g., the introduction of metal porphyrines or nanocrystals (Matsui and MacCuspie 2001). Peptide nanotubes can be used as templates for the synthesis of silver nanowires (Reches and Gazit 2003) or copper nanospheres (Banerjee et al. 2003) and some other elements demanded in electronic devices. In sensor applications, the peptide nanotubes offer broad opportunities to construct nano-sized devices based on optical and electrical signals related to conformational changes of the peptide chains followed by alteration of the ionic transport (artificial ion channels) or optical properties of the interface (de la Rica and Matsui 2010).

Lectins are proteins of non-immune origin that interact with carbohydrates but do not modify them. First described in 1888 by Stillmark, lectins have received application a century later in cell biology as probes to investigate cell surface structure and functions (Rahaie and Kazemi 2010). Lectins are derived from plant, microbial and animal sources, and can be water-soluble or membrane-bound. The classification of lectins is based on their specific affinity toward various carbohydrates. Five groups are classified, i.e., lectins exerting affinity toward *N*-acetylglucosamine, galactose/*N*-acetyl-*D*-galactosamine, glucose/mannose, L-fructose and sialic acid. Concanavalin A is one of the most popular lectins in biosensor applications. It binds to polysaccharides and hence provides site-specific immobilization of glycoproteins to solid supports, e.g., signal transducers of a biosensor. In a similar manner, the specific binding of oligosaccharides in the walls of bacteria makes it possible to recognize them by mass sensitive sensors. Quartz crystal microbalance modified with lectin shows the reliable detection of about 1,000 cells in flow-injection mode for about 30 min. (Safina et al. 2008). Glycan-lectin interactions have been used for the sensitive detection of disease-related markers, e.g., cancer-associated T-antigens (β-D-Gal-[lf3]-D-GalNAc disaccharide) with electrochemical and fluorescent signal detection (Dai et al. 2006). Other analytical applications of lectin-based recognition involve glycosylation monitoring, glycoprotein separation and recognition (Bertok et al. 2013), drug delivery system assembling, (Gorityala et al. 2012) and the direct detection of cancer cells (Hu et al. 2013).

2.4 Nucleic Acids

DNA is the fundamental molecule of life. It is present in all living beings and determines the basic features of their reproduction and inheritance of the characteristics specific for appropriate biological species.

A single DNA molecule consists of a phosphate-sugar backbone bearing four different substituents at each deoxyribose ring of the chain (Fig. 2.13).

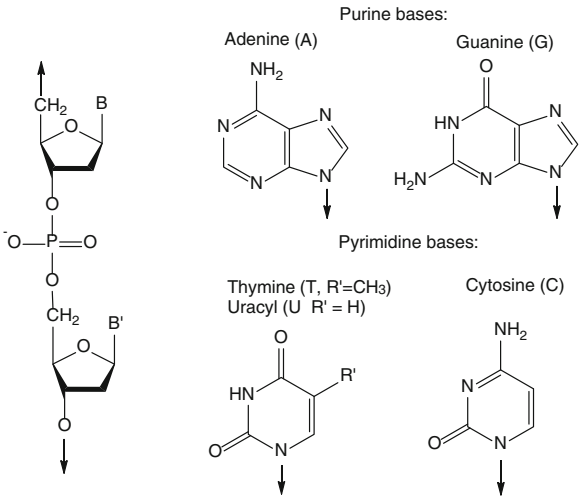


Fig. 2.13 Structure of the DNA backbone and of four DNA nucleic bases. *Arrows* indicate their bonds with the rest of the molecule

Uracil residue substitutes thymine in ribonucleic acid (RNA) and is present in aptamers, i.e., synthetic oligonucleotides obtained *de novo* by combinatorial chemistry. All the above-mentioned substituents are called *nucleic bases* (or simply *bases*) and are subdivided into two groups, i.e., purine and pyrimidine bases, which interact with each other by the formation of two and three hydrogen bonds (Berg et al. 2007).

The nucleic bases bonded to ribose (in RNA) or deoxyribose (in DNA) by *β*-glycosidic linkage are termed *nucleosides*. Their phosphorylation yields *nucleotides*. Thus, the DNA can be regarded as a polynucleotide and its fragment as an oligonucleotide. Although RNA and its fragments are very rarely used in biosensors, in some works, a more definite term, "*oligodeoxynucleotide*" (abbreviated "ODN") has been introduced. For the same reasons, the codes of nucleic bases and appropriate nucleotides (A, G, T and C for adenine, guanine, thymine and cytosine, respectively) are specified by dA, dG, dT and dC, where "d" means "deoxy." The necessity to distinguish between the derivatives of ribose and deoxyribose in the biosensor assembly is rather mild and can be easily omitted when the term "*DNA sensor"* is used. From this point of view, the short descriptions "poly(A)" and "poly(dA)" mean the same sequence consisting, only of adenine residues. Such sequences are called *homo-oligonucleotides* as well.

The sequence of deoxynucleotides bonded by phosphodiesteric bonds is a primary DNA sequence (chain, strand). Its direction proceeds from 5′- to 3′-ends, meaning the unoccupied positions of the terminal monomer units. In other words, a free hydroxyl group of ribose is placed at the beginning of the DNA chain and the monophosphoesteric group at its end (Fig. 2.14). The primary DNA sequence is recorded by codes referred to as nucleic bases. Thus, the oligonucleotide consisting of nine nucleotides is univocally described by the following record: 5′-ATTGCATTC-3′. It definitely differs from 5′-CTTACGTTA-3′ by its chain direction although the sequence of nucleic bases seems the same.

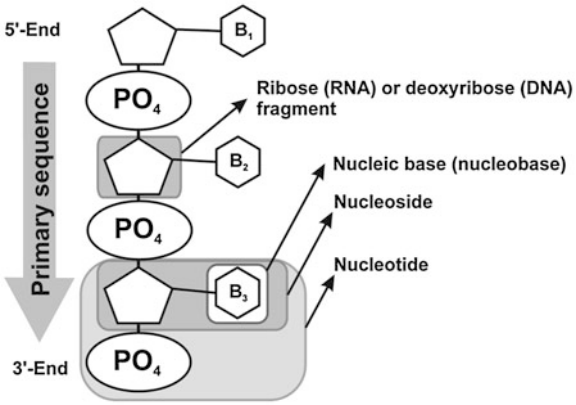
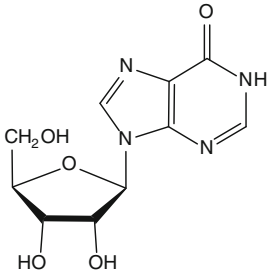


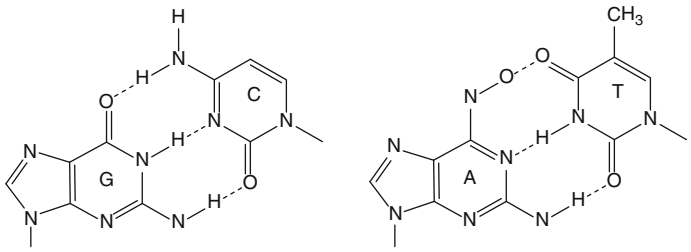
Fig. 2.14 Primary DNA sequence and its constituents

In synthetic analogs of native oligonucleotides, guanine can be substituted by inosine, which retains the ability to bind the cytosine fragment (Eq. 2.23). Some other nucleotide derivatives are known and used to increase the stability of the sequences demanded for biosensor preparation and operation.



(2.23)

A native DNA molecule consists of two antiparallel primary sequences wrapped around a central axis in the right-hand screw sense. From 10 to 12 base pairs are involved in each turn of the DNA helix. The spaces between the sequences are called *grooves*. One of them (the major groove) is broader than the other (the minor groove). The formation of the helix is due to specific interactions between all the nucleotides of each strand. Adenine interacts with thymine and guanine with cytosine with the help of hydrogen bonds (2.24).



(2.24)

The A–T and G–C base pairs fit exactly into the space between the backbones, so that the internal volume of the helix is filled with a parallel stack of the nucleic base pairs additionally stabilized by hydrophobic interactions of overlapping aromatic planes. The outer surface of the DNA has a high density of the negative charge of the phosphate groups.

Both nucleic base pairs and the grooves on the surface specifically coordinate some molecules. Thus, small aromatic molecules can penetrate the DNA helix (intercalation reaction). Proteins and polyelectrolytes are attached in the grooves of the molecule. Such interactions alter the volume and spatial structure of the DNA molecule and can be detected by appropriate changes in the charge distribution, optical properties, redox activity, etc.

The formation of the double-stranded (ds-) DNA from single sequences is called *hybridization*. In such a reaction, each nucleic base is bonded to a counterpart (A–T, G–C) in the second sequence. The feature of the hybridizing DNA strands to meet each other is called *complementarity*. The DNA sequences retain their ability for hybridization even if a few of the nucleic bases do not interact with each other. Such disturbance in full complementarity is called a *mismatch* (single-, double-mismatched DNA sequences, etc.). The selectivity of the DNA hybridization detection is characterized by the possibility to distinguish fully hybridized and mismatched sequences. In a simplified approach, homo-oligomeric nucleotides are used as fully mismatched sequences. In addition to mismatches in which the complementary oligonucleotide is substituted with another one, the *abasic sites* of DNA sequences are specified. This is a location in the DNA molecules in which there are neither purine nor pyrimidine bases. They are formed spontaneously or in the DNA damage (Lhomme et al. 1999). The detection of 'abasic' sites is one of the purposes of the DNA sensors on the potential hazards' estimation.

The reaction of DNA hybridization is reversible. Slow heating ("DNA melting") breaks the hydrogen bonds in the base pairs, and sharp cooling prevents the ss-DNA molecules from re-hybridization. Concentrated electrolytes, sonication and adsorption on the solid support result in partial de-hybridization as well. The ss-DNA molecules obtained have an amorphous structure; some of the pieces of the sequence can be hybridized to each other. This can result in the formation of loops, knots and some other unusual structures that affect the behavior of ss-DNA molecules in biosensor assembly.

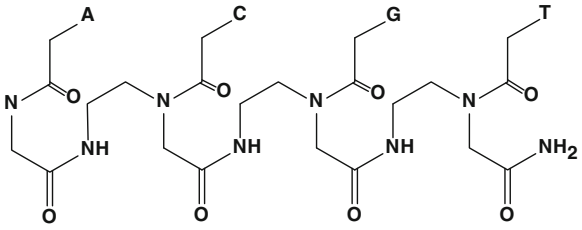
The ds-DNA helix described above exists in three major classes: DNA A, DNA B and DNA Z, which are different in their wrapping and screwing directions. The structure first analyzed by Watson and Crick belongs to the DNA B class (10 bp per turn). Native DNA is a rather flexible molecule in physiological conditions. Its geometry depends on the local nucleic sequence and microenvironment.

In DNA sensors, native DNA is not so often used because of its large size (molecular weight up to several million Da) and complications in reproducibility and specificity of the biosensor performance. More often, short sequences (ODNs, or simply oligonucleotides) are used. Many of them are selected from the polymerase chain reaction (PCR) of the DNA amplification used in a traditional DNA assay for establishing particular sequences specific for hosting organisms (pathogenic microorganisms or viruses). In analogy with the DNA assay techniques, such as ODNs are also termed *DNA probes*.

The ODN-based biosensors belong to the family of the DNA sensors. In some cases, the origin of oligonucleotides is mentioned ("gene related to sarcoma," or "HIV virus detection"). The length of the DNA probe does not exceed several dozen nucleotides. Modern technologies introduce modifying fragments into DNA probes, preferably by covalent modification of terminal groups. The list of modifiers involves fluorogenic labels like fluorescein and functional groups required for biosensor assembling (biotin, or thiol and amino groups).

Among native DNA molecules and derived oligonucleotides, some other biorecognition elements relative to DNA structure have become popular in the past decades.

Peptide nucleic acids (PNAs) are synthetic analogs of native DNA that have a similar distance between the nucleic bases and hence have hybridization ability. PNA consists of repeating *N*-(2-aminoethyl)-glycine units linked by amide bonds (2.25). PNAs do not contain any sugar moieties and phosphate groups and are much more stable toward hydrolysis and oxidation than DNA analogs (Wang 1998).



(2.25)

The application of PNAs in bioassay is based on their abilities to form hybridized PNA–DNA products similar to ds-DNA. In comparison with natural analogs, PNAs show excellent biological stability, higher binding affinity, and better specificity. Some of the advantages mentioned are related to the electrical neutrality of PNA molecules that excludes electrostatic repelling of negatively charged phosphate units of the DNA probe and target DNA sequence in

conventional hybridization event. The strong PNA–DNA duplexes result in a higher melting point, which is about 1 °C per base pair higher than that of corresponding ds-DNA duplexes. Single-base mismatches of the sequences exert a more destabilizing effect than that in DNA sensors. This makes it possible to detect them with high reliability and sensitivity. PNA probes are resistant to enzymatic degradation because the backbone of *N*-(2aminoethyl)-glycine is not recognized by nucleases and proteases. The same can be related to the stability of the PNA probes toward strong acids and high temperatures. The stability and high affinity of PNA–DNA interactions offers extended opportunities for application of the PNAs in sequence specific DNA detection and biomedical diagnostics (Singh et al. 2010). Besides, hybrid PNA–DNA, PNA–RNA duplexes can be used as building blocks for the assembling of biorecognition layers on the solid interface. The nanorods consisted of quadruplexes PNA_2–DNA_2 were reported in nanotechnology applications as building blocks for nanoscale bridges to conduct electrical charges (Armitage 2003).

Aptamers. Contrary to the DNA probes, *aptamers* are fully synthetic oligonucleotides obtained *de novo* from the DNA (RNA) library by a combinatorial chemistry approach (Mascini 2008; Song et al. 2008). The aptamers are selected from the randomly synthesized oligonucleotides by affinity chromatography, ultrafiltration, capillary electrophoresis, flow cytometry, centrifugation and other techniques against the target analyte. A chemically synthesized library consists of 10^9–10^{15} molecules of the ss-DNA, each possessing a random-sequence region positioned between specific primer sequences for amplification. For RNA aptamer selection, the primary ss-DNA library is first converted into a corresponding RNA library. The selected oligonucleotides are amplified by PCR for ss-DNA aptamers or reverse transcription-PCR for RNA aptamers. The properties of the aptamers

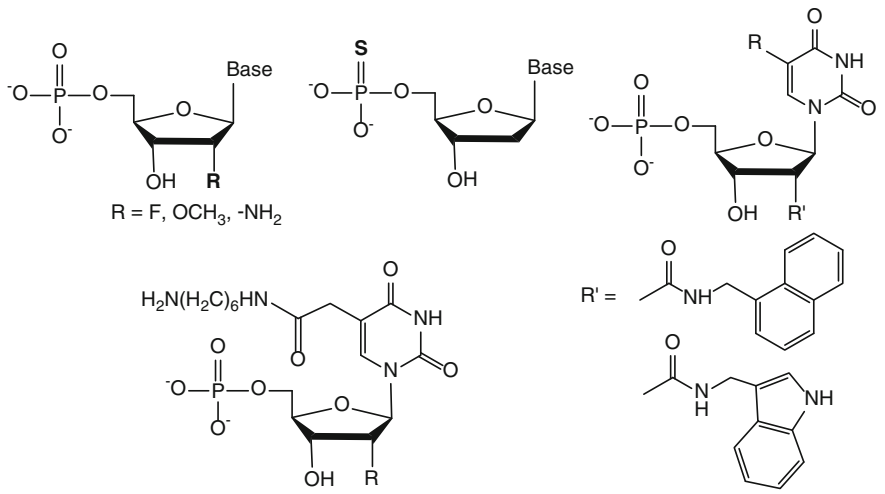


Fig. 2.15 Modified and unnatural nucleic acids used in the assembly of DNA/RNA aptamers

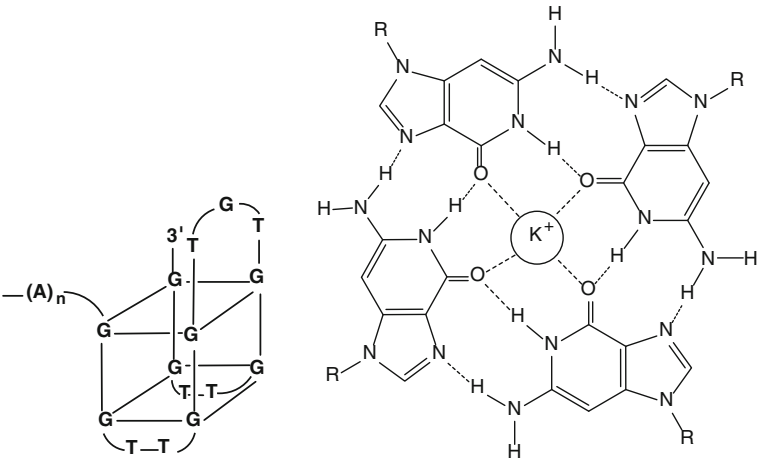


Fig. 2.16 Configuration of an aptamer against thrombin and the structure of G_4 quadruplex specifying three-dimensional structure of the aptamer. K^+ ions stabilize the quadruplex

can be sufficiently enhanced by the involvement of unnatural elements on the stages of synthesis of the primary library and product selection. The examples of modified natural and unnatural nucleic acids used for DNA\RNA aptamers are presented in Fig. 2.15.

The modification is directed to the covalent immobilization of aptamers via amino and carboxylic groups in the side-chains of the molecules as well as increased stability of aptamers toward hydrolysis and oxidation in aqueous environment and biosensor assembly. The incorporation of substituents to a 5′-position of uridine improved the selectivity of recognition of human proteins. The diversity of the response could be additionally increased by kinetic discrimination of binding (Gold et al. 2010). The technology based on such aptamers (Slow off-rate modified aptamer, SOMAmers) was successfully applied for biomarker discovery. The technique was tested on 813 proteins with the detection limits in picomolar range of 'conventration' and seven orders of magnitude of concentration determined (100 fM–1 mM). Fifty-eight proteins were identified as biomarkers of chronic kidney diseases.

The aptamers have a rather unusual primary and spatial structure that has no analogs in native DNA molecules. Thus, the aptamers can have loops of unhybridized sequences or guanine quadruplexes amalgamated in the stacks formed by the nucleotides belonging to the same or different sequences (Fig. 2.16) (Tucker et al. 2012).

The aptamers are also provided by linkers consisting of homo-oligonucleotides. They prevent a too-close position of the aptamer on the solid supports to avoid steric limitations of an analyte binding. The oligonucleotide linkers can form ds-DNA fragments that are rather rigid and duplicate the number of binding sites at

Table 2.5 Examples of aptamer applications in the assembly of aptasensors

Target analyte	Detection principle	Target analyte	Detection principle
Thrombin	Amperometric (Suprun et al. 2010; Degefa et al. 2009)	Interferon γ	Amperometric (Liu et al. 2010)
	Impedimetric (Radi et al. 2005; Li et al. 2008)	IgE	FET (Maehashi et al. 2007)
	QCM (Hianik et al. 2005)	Prostate cancer cell biomarker	Fluorescent (Farokhzad et al. 2004)
	Potentiometric (Numnuam et al. 2008)		
Adenosine	Electroluminescence (Zhu et al. 2011)	Cocaine	Amperometric (Zhang et al. 2011a)
Lysozyme	Amperometric (Cheng et al. 2007; Rodríguez and Rivas 2009	Glycoproteins	Amperometric (Xia et al. 2013)
HIV Tat protein	QCM (Minunni et al. 2004)	Adenosine	Amperometric (Feng et al. 2008)
Ochratoxin A	Amperometric (Bonel et al. 2011)	Trinitrotoluene	Fluorescent (Ehrentreich-Förster et al. 2008)
Acetamiprid	Impedimetric (Fan et al. 2013)	Breast cancer cells	Amperometric (Zhu et al. 2013)
K^+ ions	Amperometric (Radi and O'Sullivan 2006)	Chloramphenicol	Amperometric (Yan et al. 2012)
	Optical (Ho and Leclerc 2004)	Theophylline	Amperometric (Ferapontova and Gothelf 2009)

QCM quartz crystal microbalance; *FET* field effect transistor

the opposite ends of the sequences. Due to similarity of the geometry, it was suggested that such dimeric aptamers be called "aptabodies" (Hianik et al. 2008).

More than 200 aptamers are characterized against various 'analytes', but not all of them have been introduced in the biosensor assembly. The most common examples of aptamers applications into the biosensor format are presented in Table 2.5. They cover a great variety of the analytes from inorganic ions to large proteins. Certainly this does not mean the aptasensors could compete with the conventional techniques applied for potassium determination. This is proof of the concept confirming the great potential of aptasensors as universal measurement devices. Besides, the aptamers can be used as part of more complicated systems developed as prototypes of logic gates and other prototypes of biological computers.

DNAzymes and rybozymes are ds-ODNs which exert their own catalytic activity, i.e., oxidation or bond cleavage in DNA and RNA molecules, respectively (He et al. 2009; Kurata et al. 2000). Many of them have the structure of aptamers with a high affinity for the enzyme cofactor. Thus, the aptamer toward hemin exerts a peroxidase-like activity towards typical enzyme substrates (Yuan et al. 2011). Like aptamers, DNAzymes are selected from a randomized library with 10^{14}–10^{15} different sequences. The sensitivity of the target analyte detection is comparable or lower than that of antibodies with the lowest detection limits of about 45 fM.

DNAzymes were initially designed to detect Pb^{2+} and other divalent cations, but later on were adapted to other biomolecular targets (Zhang et al. 2011b). The interactions of aptamers very much depend on the microenvironment that promotes or hinders the required topology of the binding site. Thus, the attachment of the DNAzyme to the sd-DNA fragment switches the enzyme activity on, due to the folding of the aptamer side into the G_4 quadruplex followed by the hemin binding and the catalysis of the substrate oxidation. Thus, oligonucleotide 5′-ATT GGG AGG GAT TGG GTG GGC AC-3′ forms a stable G-quadruplex structure that binds hemin and then catalyzes oxidation of colorless 10-acetyl-3,7-dihydroxyphenoxazine to

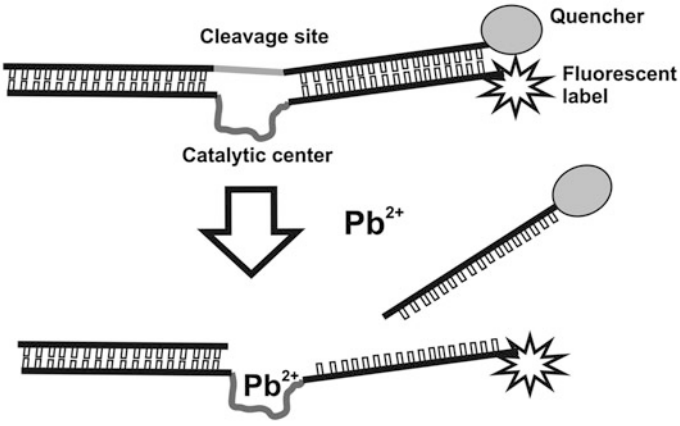


Fig. 2.17 Fluorescent detection of Pb^{2+} ions based on DNAzyme with fluorescent label

resorufin. The complex mimics the HRP activity and can easily be combined with glucose oxidase to detect glucose by the absorbance of the final product detected at 570 nm (Bo et al. 2013).

Other DNAzymes selectively cleave the sd-DNA sequences in the presence of selected metal ions. The principle of the signal detection with a fluorescent label is illustrated in Fig. 2.17. The Pb^{2+} ions activate the catalytic site of the ds-DNA-zyme and promote cleavage of the opposite part of the nucleotide sequence; this results in full dehybridization of the molecule. Prior to interaction, the terminal fluorescent label is shielded by a quencher group attached to the neighboring fragment of the sequence. Dehybridization results in removal of the quencher and initiation of the fluorescence measured by an appropriate detector. A similar approach can be used with electrochemically active labels for voltammetric or impedimetric detection of the target analyte (Xiao et al. 2007).

The DNAzymes have been successfully applied for the detection of infectious pathogens directly in the PCR products (Monsur Ali et al. 2011; Zagorovsky and Chan 2013). In these biosensing devices, the specific DNA sequence was used to assemble the catalytic site of the ds-DNA. This made it possible to switch on or switch off the fluorescence by the addition of catalytic sequences to the reaction mixture. Visual detection of the reaction is possible with the sequences labeled with Au nanoparticles.

2.5 Whole Cells as Biosensing Elements

The analytical application of whole cells in the biosensor assembly was begun in 1975. The determination of ethanol was described with *Acetobacter xylinum* immobilized in the cellulose membrane attached to the oxygen sensor. From this time, various approaches enhancing the number of analytes and improving microbial sensor performance have been elaborated, and they were recently summarized in reviews (Lei et al. 2006; Xu and Ying 2011; Ron 2007). Although many of them can be extended to other supramolecular structures, e.g., liposomes, organelles, mitochondria or fibroblasts, microbial biosensors have gained special attention due to the simplicity of operation with living cells and advantages related to their reproduction and self-maintenance in operational conditions.

To some extent, living cells can be considered to be a source of appropriate enzyme activity that can be utilized for the detection of specific substrates. In comparison with isolated enzymes, the use of living cells is complicated by the additional stage of the substrate transfer through the cell membrane. This makes the response of microbial sensors longer and less sensitive against conventional enzyme sensors. On the other hand, the target enzyme in a living cell exists in a natural environment including specific protecting and repairing systems and effective operation in a favorable medium. The selective transport of ions, especially that of H^+, specifies the functioning of enzymes bonded to the membranes or placed in periplasm and cytoplasm that remarkably differs from the conditions in

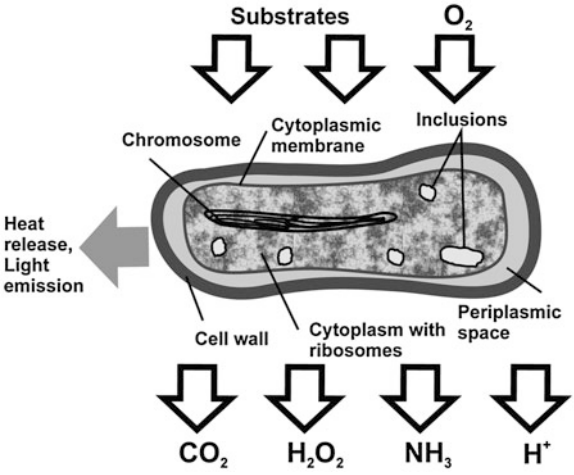


Fig. 2.18 Schematic representation of a bacterial cell and the material/energy fluxes in metabolic reactions

artificial membranes of biosensors. Genetic manipulations and gene fusion extend the biochemical paths of the substrate conversion and hence the ways to reach selectivity and sensitivity of detection in accordance with modern requirements of bioassay. Cells naturally provide cofactors and activators of enzymatic reactions that are normally added to enzyme sensors and are often unstable and rather expensive.

The detection of microbial reactions is based on general fluxes of the substances between a cell and the environment, as is schematically shown in Fig. 2.18.

The oxidation of the substrates results in oxygen consumption and release of the metabolic products, among which carbon dioxide corresponds to a full energy conversion. The efficiency of the destruction of the organic matter can be easily monitored by the O_2/CO_2 balance. In this respect, monitoring of microbial activity does not differ dramatically from oxidoreductase-based biosensors. In some cases, other products, e.g., ammonia for nitrate-/nitrite reducing microorganisms or H_2S for anaerobic species, can be detected to increase the specificity of the response. The pH shifts, as well as heat generation and biomass increase, also refer to general parameters utilizing the assessment of the bacterial activity both in suspensions, natural populations and in the biosensor assemblies. Their application for the measurement of biological oxygen demand (BOD) and antibacterial agents is described in more detail in Chap. 4. The examples of the substrate detection with microbial sensors are given in Table 2.6. Most of them are based on Clark-type oxygen sensors or pH shift measurements that are traditionally used in the monitoring of fermentation processes. Meanwhile, optic detectors have received increasing attention in the past decade. The measurement of light emission is achieved by specific proteins, e.g., luciferase or green fluorescent protein, which are displayed by the introduction of appropriate *lux* and *gfp* genes in the DNA of a host microorganism.

Table 2.6 Examples of microbial sensors for the detection of organic substrates

Substrate	Microorganisms	Detection principle
Ethanol, glucose	*Gluconobacter oxydans* (Tkac et al. 2002, 2003)	Amperometric (oxygen consumption)
Sucrose	*Gluconobacter oxydans/Saccharomyces cerevisiae* (Tkác et al. 1998)	Amperometric (oxygen consumption)
Paraoxon	Genetically engineered *Moraxella* sp. displaying organophosphate hydrolase (Mulchandani et al. 2006)	Amperometric (oxygen consumption)
Organophosphates	*Flavobacterium* sp. (Gäberlein et al. 2000)	Potentiometric
Formaldehyde	*Hansenula polymorpha* (Khlupova et al. 2007)	Amperometric (oxygen consumption)
Formate	*Cloatridium butyricum* (Ho and Rechnitz 1985)	Potentiometric
Phenolic compounds	*Pseudomonas putida* (Timur et al. 2003)	Amperometric (mediated oxidation)
Acetic acid	*Fusarium solani* (Subrahmanyam et al. 2001)	Amperometric (oxygen consumption)
Trichloroethylene	*Pseudomonasa eruginosa* JI104 (Han et al. 2002)	Potentiometric
	Pseudomonas putida (Hnaien et al. 2011)	Conductometric
Tetracyclines	Recombinant *Esherichia coli* with lux gene (Hansen and Sørensen 2000)	Luminescent

The determination of tetracyclines with bioluminescent microbial sensor is an example of such genetic engineering used for both enhancement of the selectivity and sensitivity of the assay (Hansen and Sørensen 2000). In this method, the tetracycline-related repressor is removed from *lux* or *gfp* genes fused in the DNA of *E. coli* so that the bioluminescence increases with the concentration of the target analyte contrary to inhibitory tests where the signal decays with the dose of toxic species. The application of microbial biosensors for the detection of toxic species is examined in Chap. 4.

The use of living cells instead of isolated enzymes complicates the interpretation of the signal and frequently shows moderate or low substrate selectivity, especially for oxidizable compounds like ethanol, glucose, organic acids, etc. 'Long' response affected by changes in the population of the cells on the biosensor transducer is another weak point of microbial sensors.

To some extent, the measurement time can be reduced by approaching the membrane-bonded enzymes, either in the whole cells or isolated together with a membrane piece. The influence of other enzymes, if necessary, can be suppressed by thermal treatment of the cell dispersion because membrane-bonded enzymes are more stable for such a procedure. However, the use of thermally killed cells deprives the biosensor of the advantages mentioned above, i.e., increased stability, repair mechanisms, etc. The immobilization of living cells and limitation of the transfer of auxiliary reagents (second substrates, cofactors) is another way to exclude the contribution of undesirable reaction of the target substrate.

2.6 Immobilization of Biochemical Elements of Biosensors

2.6.1 General Requirements for Immobilization Techniques

As was mentioned above, biosensor assembly involves a biochemical element closely attached to the surface of a signal transducer. The *immobilization* procedure involves the process of making the biochemical component insoluble and fixing it on an appropriate interface (solid support or transducer surface). Immobilization is a crucial stage of the biosensor development and it is required for the following reasons:

- Multiple uses of rather expensive biochemical reagents. This is particularly true for enzymes that can be repeatedly applied for the substrate determination without any intermediate regeneration. For DNA and immunosensors, the applicability of multiple application is not so obvious because of the problems related to additional treatment of the biosensors and changes in their characteristics;
- Stabilization of the biochemical components. At present, this purpose of immobilization seems even more important than multiple signal measurement. In many cases, immobilization improves the stability of the proteins and DNAs toward oxidants, organic solvents and digesting enzymes. This can be due to limited access of appropriate chemicals to the immobilized biomolecules in comparison with homogeneous conditions. The immobilization makes the three-dimensional structure more rigid as well, so that the enzymes or antibodies retain their biochemical function even when partially damaged;
- Involvement of the biomolecules in the assembly of a biosensor to meet specific requirements to signal transduction. In many cases, the immobilization is accompanied by changes in the native structure of a biomolecule. The product might be unstable in the solution but has some advantages over the parent molecule on the transducer surface. The Fab fragments mentioned in the Sect. 2.2 are an example of such an approach. They are produced by treating the antibodies with papain and stabilized by immobilization on the solid support, preferably by Au–S bonds. The product of immobilization is active in the binding of the Ag molecule; it is more stable and shows less cross-reactivity to the structures relative to an analyte in comparison with the initial Ab molecule. In other cases, the immobilization can be performed together with label implementation, partial opening of the protein globule, removal of the saccharide residues from the surface layer, etc. All of the operations are directed to reaching better conditions for signal transduction, i.e., accelerating the enzyme exchange between the cofactors of the enzyme and the electrode.

It should be mentioned that all the advantages of immobilization are not compulsory and no one could rely on the immediate success of this procedure without any special efforts in investigating the conditions and testing the products

of immobilization with primary attention to the signal transduction system to be applied for biosensor development. Generally, the following parameters of investigation are of primary importance:

The support (carrier) of the biomolecules. The immobilization assumes the use of polymeric or viscous materials that provide a mechanical support for the proteins or DNAs implemented in the biosensor assembly. At the beginning of the biosensor history, while the enzymes were mainly used, the supports provided the replacement of a biochemical component after its inactivation. This made the biosensor less expensive because the price of many transducers was rather high. Plastic membranes produced for separation or filtering of biochemicals were adapted for enzyme immobilization. The first supports were based on common materials such as cellulose acetate or nitrate. To some extent, this remains true at the moment, although the arguments referred to the replacement of biorecognition elements become less significant. It seems more valuable that the use of replaceable membranes diminishes the probability of the technical mistakes that are rather common in conventional protocols of enzyme immobilization. On the other hand, some biomedical enzyme sensors are so cheap that their being disposable is preferable to the substitution of any elements.

From the parameters of the carriers, the permeability toward low-molecular analytes and durability are of primary importance for the biosensor performance. As in many other aspects of biosensor development, the final choice of the materials for immobilization is a compromise between many features that depends on the area of biosensor application, requirements of analytical and operational characteristics and preferences of a researcher. Thus, cellulose nitrate is represented by a variety of commercial membranes widely used in membrane technologies, such as protein plotting and even water desalination. Meanwhile, the use of a cellulose nitrate for enzyme immobilization is limited by its rather low elasticity and fragility. This complicates fixing the membrane onto the transducer surface, especially if its geometry differs from that of a flat plane. Gelatin and other hydrophilic supports (agar, chitosan, and [to some extent] chitin, and, lately, clay and zirconium phosphate) exhibit remarkable stabilizing effects and compatibility with almost all transducers and measurement protocols. The enzymes implemented in a gelatin matrix can be stored for several years without any loss of activity. Meanwhile, they swell in aqueous solution and change the signal within several hours during the first measurements. The material of support indirectly affects many other characteristics of biosensors, e.g., the interference of detergents, the pH dependence of the response, the reproducibility of main characteristics in mass production, etc. In comparison with the first (mainly "enzymatic") period of biosensor development, the focus is shifted to the materials obtained "in situ" with the immobilization of a biochemical component onto the surface by a self-assembling procedure. These materials are much more compatible with miniaturization and mass production requirements than the conventional membranes based on commercially available membranes.

The content of membrane components. To some extent, this group of optimization criteria follows from the choice of the carrier. Most often, the amount of

the biochemical component is specified. For enzymes, its quantity is expressed by the specific activity that takes into account not only the mass of enzyme itself, but also the substrate access conditions and the efficiency of immobilization. For immunoreagents, the titer can be used for the same surface. It is denoted as a dilution of the Ab solution resulting in a pre-determined shift of the parameter indicating the formation of the Ag–Ab complex. Derived from spectrophotometric measurements, the titer is mainly related to the shift of the optic density of the solution by one unit. Both titer and enzyme activity do not refer to the absolute amounts of biochemicals but rather to their biochemical function (substrate conversion and Ag binding). Their use dates to times when the determination of real concentrations of biochemical components was rather difficult and not obligatory. In simple cases, the effect of the content of the membrane is expressed by the graphs or equations describing the signal of the biosensor as a function of any parameter related to the biocomponent quantity. Thus, it might be a volume of the aliquot of DNA/enzyme solution added to the polymer for membrane preparation or the mass of a membrane per surface unit. The results of such optimization are hardly extended to other conditions because they do not allow estimating the real quantities of biochemicals used for immobilization.

From a worldly point of view, the more enzyme/DNA in the surface layer, the better for the biosensor signal. This is not true 'the more so that' for all the biosensors the maximal signal corresponds to a definite range of the biochemicals in the surface layer. This is due to various reasons, and the balance between the rate of biochemical reaction and mass transfer is of great importance. Thus, a lower enzyme quantity can give a higher signal because it provides a thinner membrane with a faster substrate access in comparison with bigger enzyme loading. A too- dense placing of DNA probes limits the number of bulky molecules attached to the binding sites on the surface unit, etc.

Other reasons to limit the quota of biocomponents in the surface layer involve the worsening of immobilization efficiency, increasing the price of the final device, and shortening the lifetime of a biosensor.

Of course, other components of the surface layer also affect the performance of a biosensor but their contribution seems less important than the amount of the biocomponent. When the surface layer is formed directly on the transducer, the ratio of binding reagents and matrix is of interest. Thus, the reactions of polymerization and polycondensation yield products varied in porosity, permeability, charge and even thickness depending on the monomer ratio and the amount and nature of polymerization initiators. Sol–gel technologies and molecular imprinting are mostly mentioned regarding this aspect of immobilization.

Immobilization efficiency. The criteria of the final success are mainly determined by the requirements to a particular biosensor. Some of them should retain their characteristics during a rather long period of storage but can be quite moderate in the accuracy of signal measurement (biomedical enzyme sensors for substrate detection). Other biosensors are expected to show a remarkable stability of the response (flow-through biosensors, enzyme sensors for inhibitor determination)

with no specific requirements towards sensitivity. These and other similar parts predetermine the strategy of immobilization and the estimation of its results.

The stability of a signal during the longest period of its use is certainly encouraged for all the biosensors. Together with the minimal amount of biocomponents necessary to reach the desired signal, that is an advantage attained. As in many other attempts to accomplish a goal, the price of the result is of greatest importance. In many cases it is probably simpler to limit the stability of the response in order to obtain reliable immobilization protocol or to reach a higher sensitivity of analyte detection at the expense of the biosensor's lifetime. Incidentally, the sensitivity of the inhibitor determination is frequently higher for the lower long-term stability of the response toward the substrate. For DNA sensors and immunosensors, the long-term stability of the response is less important than the reproducibility of the signal, because such biosensors are often intended for a single use. Nevertheless, some general estimations of immobilization protocol can be given.

For enzyme sensors, the *remaining enzyme activity* can be used. This is the relative decay of enzyme activity caused by immobilization (80, 20 %, etc.). The decrease of enzyme activity during immobilization is due to the inactivation of the enzyme by organic solvents or other reagents necessary for immobilization, or due to a limited access of the substrate caused by the shielding effect of the carrier, or a disturbance in the native protein structure. The remaining enzyme activity does not distinguish between these mechanisms so that the real decay of activity can be lower than the shift of the response after immobilization. The relative enzyme activity was also suggested for the quantification of an enzyme inhibition. At present, the absolute signal of an enzyme biosensor and its stability are preferable instead of the remaining enzyme activity in the characterization of the immobilization efficiency.

For immunosensors and DNA sensors, the consideration of immobilization is much milder than that for enzyme sensors. Many of them utilize the approaches already elaborated for solid-phase bioassay (ELISA- and DNA-based hybridization detection). For this reason, the transfer to the biosensor format does not imply any significant changes in the immobilization techniques. The only exception is the use of microdevices like FETs, which have no macroscale analogs. In this case, as well as for other biosensors developed *ab ovo*, the stability of the response and the sensitivity of the analyte detection are often used to characterize the biomolecule attachment. The latter parameter is quantified either by the slope of the calibration curve or the concentration resulting in a 50 % shift of the signal against its maximal value.

The immobilization of living cells (microorganisms, microalgae, etc.) is mainly based on their adsorption on the solid support and implementation in the gels. The use of polysaccharides, e.g., Sepharose, agarose or cellulose nitrate, provides very strong adhesion of the cells that can usually be removed from the support only with simultaneous deformations of the tectorial membrane. The use of gelating materials is preferable because of the higher surface concentration of the cells and milder conditions for transfer of the oxygen and metabolites to the cells. Gelatin, agar, Ca alginate gel, and polyacrylamide copolymers are described for this purpose. It should be mentioned that the adsorption of living and death cells can differ dramatically even though the cells inactivated by thermal treatment exert

Table 2.7 Characteristics of the biocomponent immobilization methods for biosensor development

Type	Immobilization method	Advantages	Drawbacks
Physical	Physical adsorption	Simplicity, accessibility of the binding sites, low cost	Reversibility, limited operation period, dependence of immobilization efficiency on the support pre-treatment
	Sol–gel immobilization	One-step synthesis, hydrophilicity of the matrix, mild limitations of the analyte access	Moderate stability toward hydrolysis, rather high viscosity of the matrix, swelling in aqueous solution and compression of the films in dry conditions
	Entrapment into polymeric films	Improved storage and operation stability, one-stage deposition, variety of the matrices used	Limited access especially of bulky analytes, necessity in special reagents for polymerization, rather long response time
	Electropolymerization	One-step synthesis, controlled thickness and mass of the coated film	Partial denaturation of biopolymers, reversed leaching of the biocomponents, dependence of the operation stability on the ionic strength and pH
	Layer-by-layer composites	Easy control of thickness and charge, self-organization of selected layers, compatibility with LB-film technology and SAMs, preservation of the native structure of biocomponents	Reversibility, limited mechanical durability, dependence of the characteristics on the ionic strength of the solutions, temperature and biopolymer structure, necessity in monitoring layers integrity
	Affine	High efficiency and specificity of the DNA/protein binding (site-specific immobilization), mimicking natural processes of biopolymer functioning	Requirements of additional modification of biopolymers and/or specific reagents used for signal transduction
Chemical	Covalent binding	High efficiency and prolonged storage stability, compatibility with the protocols elaborated for protein chemistry, stability toward oxidative cleavage and hydrolysis, fixing biopolymer structure onto the solid template	Necessity in toxic reagents, low conformational lability of the biopolymers attached to the solid support, possible distortion of their native three-dimensional structure

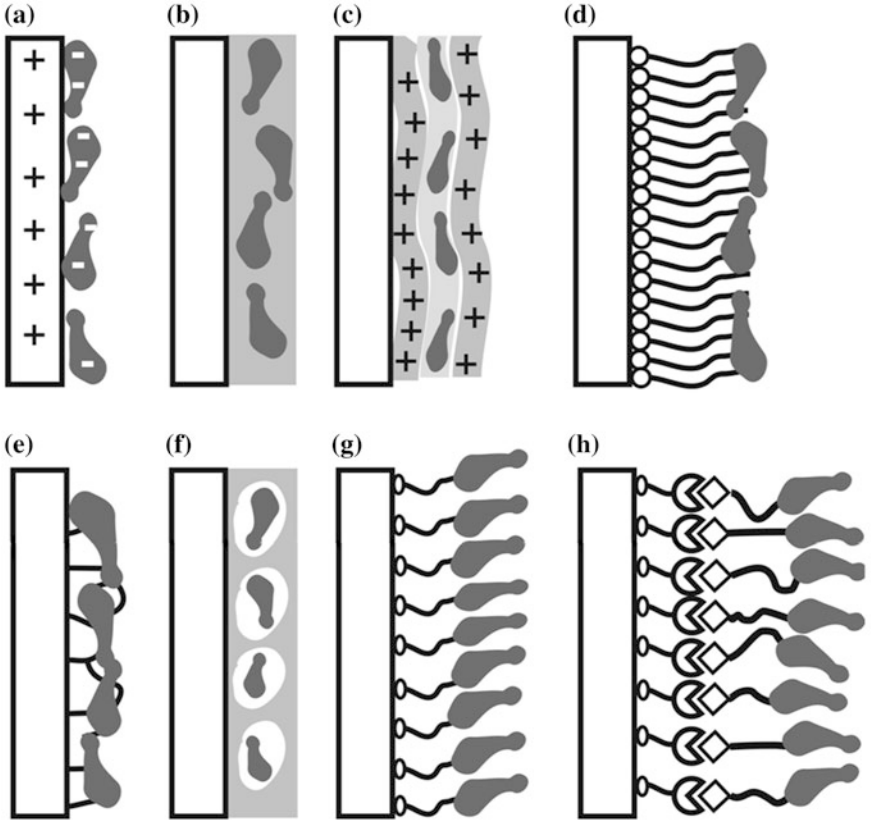


Fig. 2.19 Schematic presentation of immobilization protocols used for biosensor development. **a** Electrostatic adsorption. **b** Implementation in polymeric film. **c** Layer-by-layer immobilization in the polyelectrolyte complexes. **d** Non-covalent immobilization on the self-assembled monolayer. **e** Cross-linking by bi-functional reagents. **f** Immobilization in reversed micelles. **g** Covalent binding to the solid support (sensor surface); **h** Affinity immobilization

enzymatic activity for a certain period of time. This can be used for distinguishing living and dead cells in cytotoxicity biosensors. The immobilization of microorganisms is often followed by cell division so that the number and biomass of microorganisms changes within the period of the biosensor operation. The biomass changes can be controlled by the content of cultural medium, e.g., by decreasing the concentration of biogenic elements. However, such an approach affects the response of the microbial biosensor toward target analytes (commonly oxidized organic compounds).

All the immobilization protocols can be classified in accordance with the forces retaining the biomolecules on the surface and the way to the biorecogniton layer formation. The general principles of immobilization are presented in Table 2.7 and Fig. 2.19.

2.6.2 Physical Immobilization

Physical immobilization does not assume the formation of the covalent bonds between the biopolymer and the carrier (membrane material, mechanical support or transducer surface).

In the simplest case (immobilization via physical adsorption), the protein or DNA molecules are held on the solid surface due to multi-point interactions, preferably electrostatic (Pividori and Alegret 2005). The adsorption can affect the three-dimensional structure of the biopolymer, but normally such changes are negligible because the number of bonds providing the three-dimensional structure of a protein or DNA exceeds that of sites attached to the solid surface.

Protein globules are adsorbed as they are; the linear molecules of DNA probes are positioned along the surface plane. However, increasing density of the surface filling can result in a partial desorption of the molecule domains with formation of aggregates and even of multilayer coatings. In this case, the DNA oligonucleotides are differentiated by their strength of binding to the carrier, their spatial structure and hence the affinity toward a biotarget, e.g., complementary ss-DNA sequences or proteins.

The use of porous materials with pores that meet the size of biopolymers simplifies the adsorption and makes desorption less probable. The surface of the backsides of the pores or channels has the charge distribution, roughness and some other features that dramatically differ from those on the surface. Besides, the number of the bonds established by a single protein or DNA molecule with a carrier is much higher in pores than on the surface plane. This promotes the implementation of the bulky biopolymers even if they are weakly bonded to the surface of the carrier. Meanwhile, the implementation of the biopolymers in the internal volume of porous materials complicates their interaction with the analyte molecules. For this reason, these techniques are preferably used for the detection of small molecules (enzyme substrates and inhibitors, DNA intercalators, etc.). Even in this case, the regeneration of the sensor signal requires more effort than that of the same biocomponents placed on flat surfaces. The use of polar materials, e.g., carbon black, metal oxides, anodized alumina layers, etc., promotes the physical adsorption. It can also be amplified by the pre-treatment of the support to increase its charge or the number of polar functional groups by anodic oxidation of the electrode materials in the electrochemical sensors. Thus, the oxdative treatment of carbon materials results in the formation of hydroxyl, carbonyl and carboxylic groups on the surface. Electrochemical or chemical oxidation increases the internal volume of porous graphite and decreases the average size of carbon nanotubes. In most cases, this stabilizes the characteristics of biosensors and increases their signal due to the higher surface concentration of the biocomponents.

Self-regulation is one of the advantages of physical adsorption. The quantities of biopolymers attached to the surface are mainly determined by the real surface area and the number and distribution of the charged fragment in the binding. This means that the transducers treated in a similar manner adsorb approximately the

same amount of biopolymers regardless of their concentration in solution. This is especially convenient if the real concentration of the biocomponent is unknown and estimated by indirect parameters, such as the enzyme activity of the biological extracts. On the other hand, the same advantage offers very high requirements for the reproducibility of the surface used for physical adsorption. Any mechanical polishing, etching and treatment with a reactive species affects the roughness, pore distribution and polarity of the surface. This can alter the characteristics of physical adsorption, especially if no full coverage of the surface is reached.

The reversibility of physical adsorption is another drawback of this immobilization approach. Being placed in a solution with no biocomponent, the biosensor loses some part of the adsorbed components leaching from the surface layer by shifting the equilibrium of adsorption–desorption. This is a thermodynamically favorable process that cannot be stopped but can be slowed down. The higher the strength of the biopolymer adsorbed to the surface, the lower the desorption rate. In many cases, multi-point attachment, and especially immobilization in pores, makes desorption so slow—as compared with the biosensor lifetime—that it can be neglected. However, sometimes the rate of leaching suddenly increases. Thus, the increase in the ionic strength of the solution by sample injection can provoke the desorption of the proteins retained in the surface layer by electrostatic interactions. The same refers to any processes diminishing the charge of the carriers. For the groups participating in acid–base equilibria, the desorption can be initiated by a sharp pH shift. This was observed, for example, in hydrolases. The reaction of a substrate hydrolysis results in the formation of acids appearing in the microenvironment of the immobilized enzyme. Even though the absolute amount of the reaction product is usually small, the reaction with the carboxylate fragment of the carrier can sharply decrease the pH value in the close neighborhood of the enzyme and hence decrease the electrostatic forces between the enzyme and carrier.

Paradoxically, the physical adsorption of biopolymers calls for a specific treatment of the surface to increase its roughness and charge, whereas the reproducibility of the biosensor performance is better on minimally treated materials that have a very smooth surface established by some specific features of a crystallographic plane (grapheme, highly ordered pyrographite, doped diamond). For DNA sensors, there was an attempt to avoid this limitation by the application of polarizing voltage. Screen-printed electrodes made of a carbon paste have rather modest adsorptional capacity against other relative carbon materials but are well reproduced on a smooth working surface. The electrodes were immersed in the DNA solution and polarized at about 0.8–1.0 V to accumulate negatively charged DNA molecules on the working surface (Mascini et al. 2001). After that, the electrodes were moved to the analyte solution and the signal was measured. Such an approach was justified for the detection of hybridization, DNA damage, and even intercalation by small antitumor drug molecules. This makes the immobilization protocol very simple and reliable, especially taking into account the low cost of the transducers sufficient for their single use.

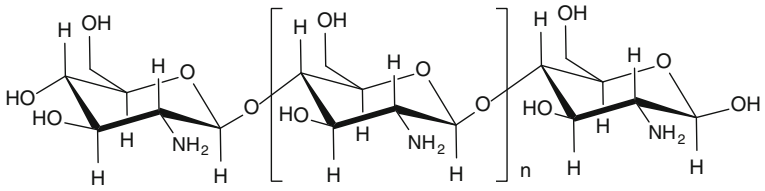
Two drawbacks limit the application of the approach described. First, the formation of the biorecognition layer is performed by the user, not the manufacturer.

This complicates the application of the biosensors, especially in field conditions. Second, any interruption of the circuit promotes desorption of a part of biopolymers. This also refers to the stage of transfer of the DNA sensor from the accumulating solution to the sample that has to be performed with the cell switched on. This can be performed only with several kinds of equipment without any consequences for the quality of the signal recorded.

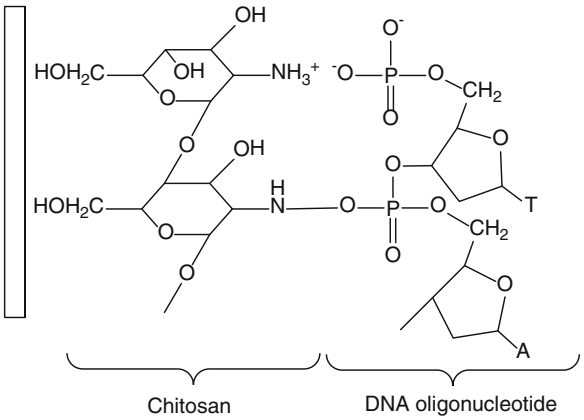
To avoid complications resulting from insufficient stability of the biorecognition layer obtained by physical adsorption, the biosensors can be covered with additional permeable membranes that mechanically prevent the leaching of the biomolecules from the surface area. The membranes are produced from polymers like cellulose nitrate and polycarbonate. Many of them are intended for electrophoresis and related laboratory techniques. The protective membranes can be also formed on the layer of adsorbed biomolecules by other methods described below (sol–gel technology, electropolymerization, chemical polymerization, and deposition from the organic solvent of the appropriate polymer). The protective membranes are widely used for commercial enzyme sensors where they offer some additional opportunities, e.g., selection of the substrates from interferences, mechanical protection, especially in flow, prevention of biofouling for invasive biosensors. The additional membranes should not decelerate the transfer of the analyte molecules and are applied mainly for enzyme sensors.

In some cases, the direct interaction of the carrier with biomolecules becomes irreversible and results in the formation of the chemical product of interaction. This process, called *chemosorption*, can be observed, for example, for proteins and thiolated oligonucleotides deposited on the gold nanoparticles or golden electrodes. The reaction of the Au with SH groups in the presence of dissolved oxygen is spontaneous and fast enough to strongly attach the biopolymer to the carrier. Previously, this way of immobilization was used only for the compact Au films obtained by electroplating (electrochemical transducers and optical devices based on transparent Au films). The application of nanoparticles obtained by the chemical reduction of $AuCl_4^-$ anions extended the application of chemosorption to other carriers such as carbon materials or even cellulose derivatives. First they are modified with Au nanoparticles by consecutive treatment with aurochloric acid and a reducer (citric or ascorbic acid) and then with biopolymers attached to the Au nanoparticles adsorbed on the carrier. The latter provides the stabilization of the Au nanoparticles as well and prevents their growth and amalgamation during deposition.

The difference between chemical and physical sorption in other cases is mild. Sometimes the rate of desorption is so slow that it cannot be observed in the time period comparable with the signal measurement duration. In other cases, the mechanism of adsorption can vary, depending on the particular conditions. Thus, chitosan (2.26) can electrostatically attract charged biomolecules such as DNA via protonated amino groups. Besides, the formation of esteric bonds has been discussed for the same molecules (2.27) (Mandong et al. 2007).



(2.26)



(2.27)

The *entrapment of the biomolecules into the polymeric film* is another way of physical immobilization, very close to that described above for adsorption followed by deposition of the upper polymeric film (Shtilman 1993).

The main difference is that the entrapment protocol assumes a uniform distribution of the biomolecules in the polymer. It was first suggested for the stabilization of enzyme activity. For this purpose, the enzyme-containing samples (extracts, tissue homogenates) are mixed with starch, gelatin or agar and left to gel. The product can be molded as a thin film by casting the viscous liquid on glass or placing it in the pores of an appropriate sorbent for better durability and mechanical stability. As mentioned, hydrophilic polymers swell in aqueous solutions and form rather thick membranes with extended response due to the slow substrate transfer. In dry conditions, they are fragile and thin but restore their size and shape after the contact with water. Similar materials based on polyacrylic acid, their copolymers and ethers are used in gel electrophoresis and related techniques developed for protein and DNA purification.

The mixing of biocomponents with a polymeric matrix is a critical stage of the immobilization. Except for materials that are able to gel, this can be achieved by the application of polar organic solvents. The polymers are first dissolved in them and then mixed with an aqueous solution of protein or DNA. If the solvent is continually mixed with water, this can result in the formation of a homogeneous system that becomes dense and rigid while the solvent and water are evaporated. If the polymeric arrangement of the protein is too dense and this prevents the contact

with a low-molecular reagent (enzyme substrate, inhibitor, etc.), additives exerting a pore-forming effect can be used.

The low compatibility of the biopolymers with polar organic solvents is a main drawback to the immobilization into polymer films. Although many enzymes are still active in non-polar solvents (Campanella et al. 2001; Zaks and Klibanov 1985), the use of the solvents mixing with water irreversibly decreases the activity of enzymes and the affinity of antibodies toward target molecules. The organic solvents "extract" water molecules from the inner area of the protein and disturb the three-dimensional structure of the protein globule. On the first stages, the decay of activity is reversible and the immobilization efficiency is increased by shortening the contact period of an enzyme with organic solvents. However, even in this case, the variation of the enzyme activity can be observed for immobilized preparation for a rather long time. For DNA molecules, the compatibility with organic solvents is higher than that with enzymes. Thus, DNA is purified by recrystallization from phenol. Besides, the detection of analytes with the DNA sensors assumes much stronger requirements toward the accessibility of the binding sites so that the entrapment of DNA probes in a polymeric carrier is not currently used for the DNA sensors' design.

Careful optimization of the immobilization conditions makes it possible to avoid the limitations related to enzyme inactivation caused by polar organic solvents. Thus, for some oxidoreductases it was observed that the use of high concentrations of solvents decreases the enzymatic activity to a much lower extent than the more diluted aqueous solutions (Sekretaryova et al. 2012). Moreover, acetonitrile even increased the activity of lactate oxidase, peroxidase, glucose oxidase and acetylcholinesterase, at least within the short period of time required for enzyme immobilization. The phenomenon observed extended the number of materials used for enzyme immobilization by Nafion, i.e., an ion exchangeable polymer soluble only in organic solvents (Dimcheva and Horozova 2005).

To some extent, the negative influence of organic solvents can be lessened by the application of protecting compounds. When dissolved in water, they decrease the inactivation of proteins by protecting the globule from water-leaching, or by the substitution of water molecules in hydrogen bonds, stabilizing the protein conformation. Hydrophilic water-soluble polymers, polyols (polysaccharides, sorbitol, glycerin), and ionogenic detergents can be applied as protectors. The fact that crude enzyme preparations and minced biological tissues are less sensitive to organic solvents than purified enzyme preparations is due to the protecting influence of native components. Although the concentration of the additives is small enough (several percent of the matrix polymer), they affect both the enzyme activity and its sensitivity toward inhibitors.

The low solubility of the polymers in water can result in the formation of microemulsions when mixed with aqueous polymer solution. As a result, the enzyme remains in microdrops preferably with an aqueous content which is distributed in a hydrophobic polymer. The structure of the final product of immobilization is similar to that realized in reversed (oil–water) emulsions applied in the synthesis of nanosized polymer particles or extraction intensification. If the relative content of

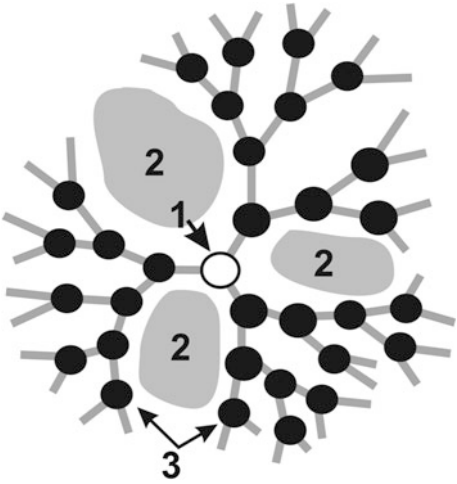


Fig. 2.20 Dendrimer molecule outline. *1* Core; *2* internal cavities; *3* branching points

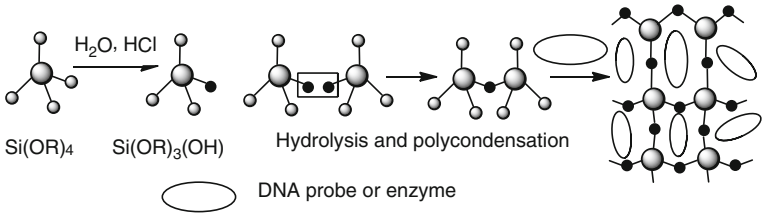
an enzyme-containing phase is high, the microdrops can exchange with the bulk solution by low-molecular compounds or ions. This provides both the functioning of an enzyme and the biosensor signal measurement. The low mechanical durability of the composite membranes and complications related to the formation of reproducible polymeric layers limit the use of such techniques in biosensor development.

The effect close to emulsification can be achieved by the application of *dendrimers*, hyperbranched polymers involving a core with repeated branching elements (Fig. 2.20) (Klajnert and Bryszewska 2001).

The dendrimer molecules are large enough to serve as "nanodrops," or molecular nanocontainers that keep the biomolecules in internal cavities. Besides, the electrostatic interaction with surface-charged groups and covalent attachments to them are realized. Polyamidoamine (PAMAM) dendrimers are mostly used (Satija et al. 2011). The generation of a dendrimer is determined as a number of "shells" (branching points) placed on increasing distance from the core. The molecules of the fourth generation provide the size and binding ability sufficient for interaction with most biopolymers. The formation of the dendrimer–protein (DNA) complexes is mostly spontaneous and can be controlled by varying the terminal functional groups of a dendrimer molecule (its generation) and reaction conditions. Like common polymers, dendrimer swells, but to a much lesser extent.

The entrapment of the biomolecules into the polymeric films has several variations more adapted to the biosensor design than the mechanical mixing of the component described.

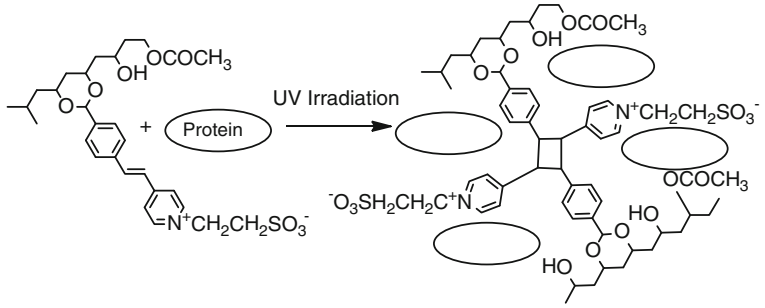
Sol–gel immobilization is based on the polycondensation performed directly on the surface of a sensor transducer in the presence of the biopolymer (Kandimalla et al. 2008). Organosilicates and siloxanes are mainly used for this purpose. The reaction is initiated with a minimal volume of water or aqueous solution of a strong acid added to the immobilization mixture (Reaction 2.28).



(2.28)

In a similar manner, the product of polycondensation can entrap small hydrophobic molecules required for signal transduction (mediators, fluorogenic or chromogenic agents, etc.). Leaching is prevented by their low solubility in water. The reaction rate and the characteristics of the product (viscosity, swelling, permeability for charge carriers, durability) depend on the concentration of the reactants, organic solvent and temperature. Organosilicate layers are quite compatible with glass and quartz, and for this reason are used with optical and piezometric detectors. For some materials, the formation of solid products is initiated by freezing the mixture (*cryogels*). Fully dried products (*xerogels*) can be mechanically dispensed and used in combination with any other polymer (Doretti et al. 1997). The organosilicate shell prevents the biopolymers from the negative influence of the other reagents mentioned. Similarly, inorganic polymers of hydrated metal oxides and zirconia phosphate can be obtained and applied in the DNA sensors' assembly.

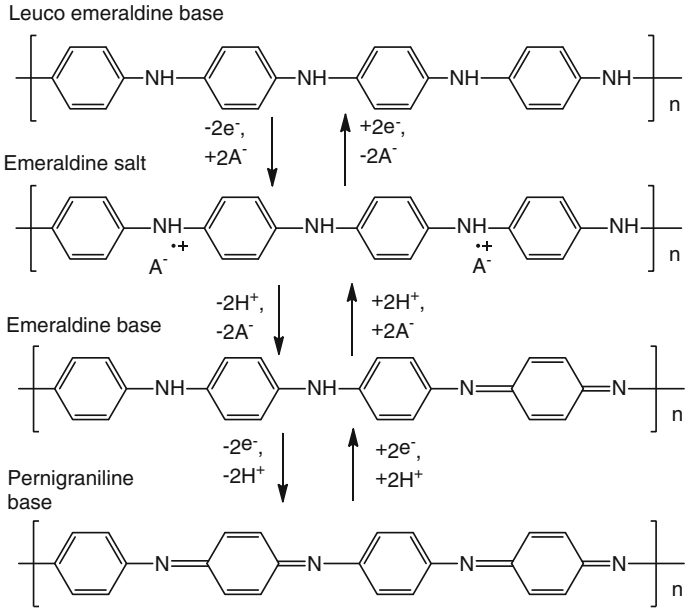
A similar effect is achieved by the application of photopolymerization (2.29). Contrary to sol–gel techniques, photopolymerization can be run directly in an aqueous solution of appropriate monomers in the presence of biopolymers under UV irradiation. Neither polycondensation nor photopolymerization involve the covalent binding of the biochemical components and hence preserve the native structure most favorable for the biorecognition of target analytes (Andreescu et al. 2002).



(2.29)

Electropolymerization is a process of the formation of insoluble oligomeric and polymeric products that deposit from the solution during the oxidation of monomers. If the electrolysis is performed in the presence of biopolymers, they are

entrapped in the growing polymer film and adsorb on the surface of the commonly positively charged final product. The advantages of the approach are the one-step protocol and simple regulation of the polymerization by choice of the potential and current. Some of the polymers show the electroconductivity comparable with that of semiconductors and even metals. This makes possible the electric wiring of the active sites of oxidoreductases and simplifies the signal transduction for other biopolymers. As an example, the synthesis of polyaniline provides the formation of several redox active forms of polyaniline, of which emeraldine salt exerts a rather high electroconductivity (Eq. 2.30) (Prakash 2002).



(2.30)

The electropolymerization of aniline requires strong mineral acids that inactivate enzymes. For this reason, the residual activity of immobilized enzymes does not exceed 2–3 %. In addition to polyaniline, polypyrrole and polythiophene also demonstrate electroconductivity but their polymerization is preferably conducted in polar organic solvents because of the low solubility of intermediates and initial species. Some other products of electropolymerization, such as polyphenazines and polyphenols, can serve as mediators of electron transfer.

The deposition of *polyelectrolyte complexes* was first suggested for the immobilization of bacterial cells on glass and then extended to other materials and biochemicals (Evtugyn and Hianik 2011).The immobilization is performed on the charged support by the consecutive addition of oppositely charged polyelectrolytes and removal of their non-bonded amounts by washing (Fig. 2.21). The protocol also called “Layer-by-Layer” (LbL) immobilization is especially suitable for the

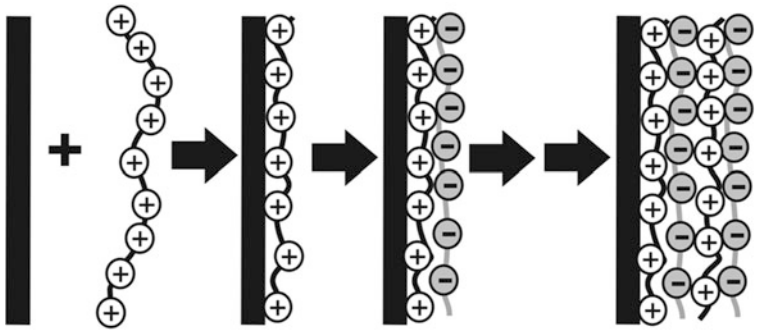


Fig. 2.21 Schematic representation of LbL immobilization

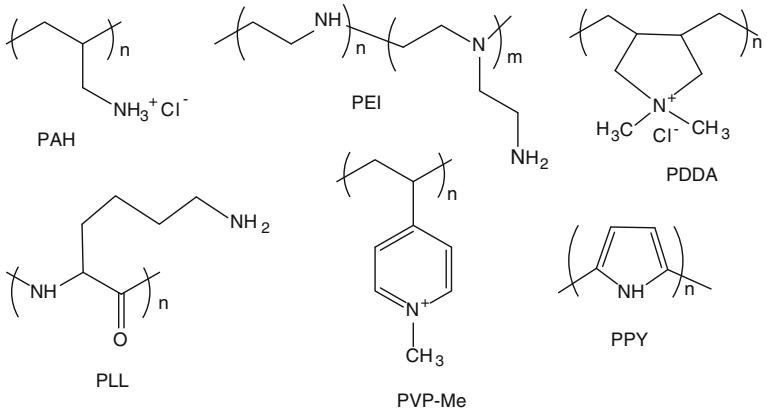


Fig. 2.22 Components of polyelectrolyte complexes used for biocomponent immobilization in biosensor design. *PAH* poly(allylamine hydrochloride), *PEI* poly(ethylene imine), *PDDA* poly(dimethyldiallylammonium chloride), *PLL* polylysine, *PVP-Me* partially methylated polyvinylpyridine, *PPY* polypyrrole

DNA oligonucleotides with a high density of charged phosphate groups of the backbone.

Synthetic polyelectrolytes typically implemented in the polyelectrolyte complexes are presented in Fig. 2.22.

Some of them become charged after protonation. Polypyrrole and some other products of electropolymerization change their charge during reversible oxidation on the electrode. Thus, assembling the complexes with such components can be controlled by external stimuli, i.e., pH changes or electrode polarization. The LbL immobilization can include positively charged chitosans (see Eq. 2.27) and negatively charged pre-oxidized carbon nanotubes as well.

The polyelectrolyte complexes have a definite number of layers easily regulated by the number of reagent additions. Weak, non-covalent interactions retain the

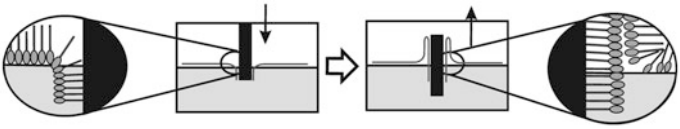


Fig. 2.23 The formation of a self-assembled monolayer and Langmuir–Blodgett film from the aqueous solution of amphiphilic compounds

access of small molecules that can move apart the ionic components while attaching the binding site of the biomolecule. This offers a fast and reproducible response toward specific analytes, e.g., intercalators or reactive oxygen species oxidizing the DNA molecule, or enzyme substrates and inhibitors (Decher and Schlenoff 2002).

The variation of polyelectrolytes manipulates the hydrophobic–hydrophilic balance on the inner interfaces of the composite layer and hence allows tuning the interactions of the DNA with various substances.

To some extent, other multilayered constructions are related to LbL composites. Thus, Langmuir–Blodgett (LB) films are formed by amphiphilic molecules consisting of a polar head bearing a long hydrophobic tail. They are self-assembled on the water–air interface to reduce the area of the contact of hydrophobic parts with a liquid phase. Then LB films can be gently collected from the surface and transferred to the solid support (Fig. 2.23) (Girard-Egrot and Blum 2007).

For this purpose, the spontaneous transfer of the monolayer from the water–air interface to the hydrophobic material is used. A thin plate is immersed in the solution and then vertically moved to pick up the film. Changes in the movement direction allow amplifying the number of layers deposited LbL on the solid support.

The DNA or protein molecules can be implemented in between the amphiphilic monolayers or positioned on the front of the LB-film if it contains charged groups. The low mechanical durability complicates the use of LbL constructions and LB-films in the biosensors, especially those intended for field applications. The defects in the structure of the mechanical support and the films themselves (cracks, ruptures, layer crossings) appearing during transportation or operation stages are self-repaired by hydrophobic forces. The use of supports capable of chemosorption or affinity interactions or implementation of the films in the pores of solid films improve the mechanical characteristics and stability of such films.

Self-assembled monolayers (*SAMs*) are a particular case of such constructions. They are formed by thiolated amphiphilic compounds that interact with Au as described above and form regular monolayers due to hydrophobic interactions between the long chains positioned in a way to minimize the contact with the aqueous environment (Chaki and Vijayamohanan 2002). The structure of SAMs is similar to half of the LB-film in which the ends of molecules are tightly fixed onto the surface by chemical bonds. Single- and multi-walled carbon nanotubes provide a mechanical support for supramolecular multilayers as well. Their efficiency is achieved by oxidative treatment resulting in the formation of negatively charged

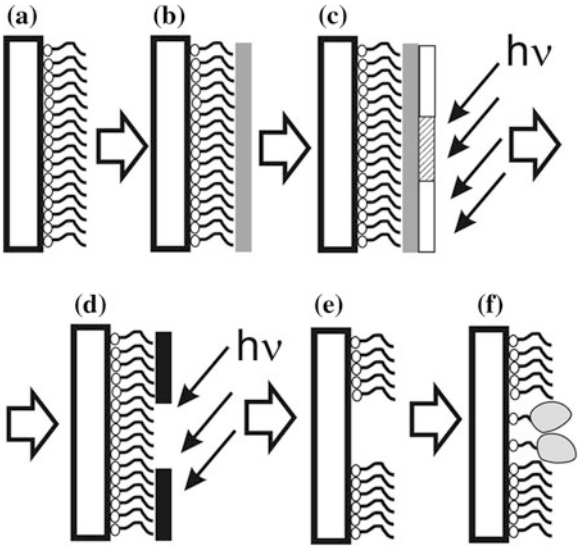


Fig. 2.24 Stepwise specific immobilization of biomolecules using SAM-based technology. **a** SAM formation; **b** photocurable polymer deposition; **c** mask deposition and UV irradiation; **d** removal of non-reacted components; **e** removal of SAM from the template sites (pore formation); **f** immobilization of thiolated biomolecule on naked Au spots

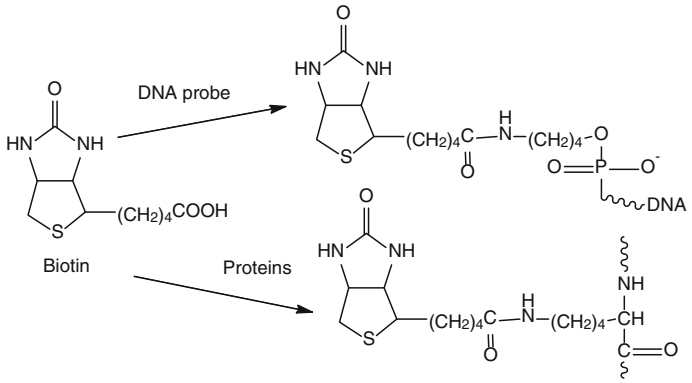
carboxylic groups at both ends of the tubes and on the defects of the side walls (Rahman and Umar 2009).

What is important for SAMs is that they provide a very accurate positioning of the biocomponents on the transducer surface and hence can be used for the preparation of miniaturized biosensors with dimensions comparable with those of microelectronic devices. Photolithography is applied for making the binding sites that are also called "spots." The principal scheme of such a process is shown in Fig. 2.24.

First the SAM obtained on the Au surface is covered with a photocurable polymer. Then, the negative mask is plotted onto the transparent material and placed on the polymer layer. This is a drawing of the future biosensor surface with the spots for protein/DNA binding marked in black. The surface is irradiated from a UV source to harden the photocurable polymer. The area protected by the black sections of the mask remains soft and is removed together with the mask by an appropriate solvent; after that, the SAM is chemically removed from the future spots by oxidation with oxygen to open the bare gold surface. Then thiolated target molecules can be attached accurately into the spot areas. The size of several microns and the density of the thousands and thousands of spots per mm^2 can be obtained by such a process. Of other applications of the SAMs, the prevention of undesired adsorption of interferences on the transducer surface should be mentioned.

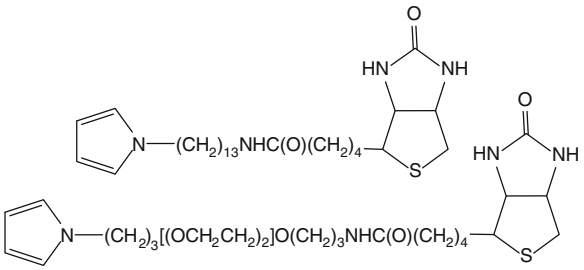
Affine immobilization is ranked as intermediate between physical and chemical immobilization protocols. The combination of biopolymers with a solid support is achieved by natural receptors providing a very specific and durable binding of the counterpart. Thus, biotin (Eq. 2.31) is able to specifically bind avidin or streptavidin with high efficiency (dissociation constant about 10^{-14}–10^{-15} M) (Dupont-Filliard et al. 2004). Due to the residue of valerian acid in the side chain, biotin can

be easily attached to the terminal phosphate groups of the DNA backbone or to the amino groups of the amino acid residues of the protein molecules, commonly via various spacers intended to provide a steric access for biological targets.



(2.31)

Both avidin and streptavidin bind up to four biotin molecules and hence serve as bridges to combine several biotinylated parts in the surface layer. Simple structure and modification protocols, as well as high stability, make it possible to introduce biotin into many commercially available biochemical molecules and supports. This simplifies the composition of the surface layers involving the DNA probes. Avidin or streptavidin can also be immobilized onto the solid support by other methods elaborated in protein chemistry, e.g., cross-linking with glutaraldehyde and implementation into the bovine serum albumin (BSA) matrix. Biotin can also be introduced into the electropolymerized layers by modification of the monomers used on this stage of modification. The examples of the molecules providing the formation of polypyrrole with covalently attached biotin are presented below (2.32) (Cosnier 1999).

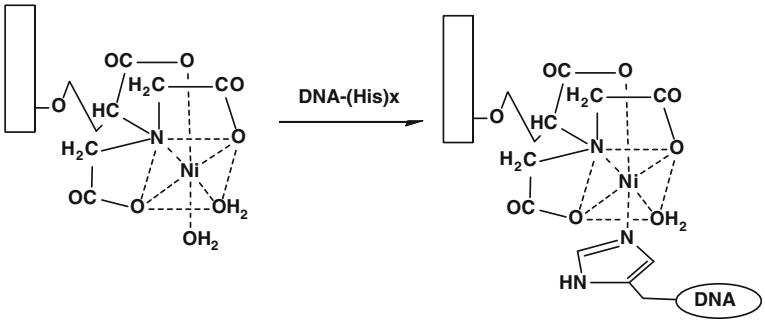


(2.32)

Long-chain linkers between the pyrrole moiety and biotin are required to give steric access of the target analyte to the binding site of a bioreceptor and retain the permeability of the biorecognition layer by charge carriers necessary for electrochemical signal transduction (Cosnier 2005).

The biotin–avidin binding is a very popular one but not the only way for the affine immobilization. Concanavalin A, a plant protein of the lectin family, can specifically bind carbohydrates; protein A binds immunoglobulines. Their application is mainly related to the immobilization of enzymes and antibodies.

The term "affine immobilization" is also used for artificial receptors like Ni or Cu chelate complexes. Thus they can be spontaneously formed by nitrilotriacetic acid derivatives and Ni(II) or Cu(II) ions in aqueous solution or on the solid interface. The complex coordinates histidine residues in the equatorial position of a complex plane (Eq. 2.33) (Baur et al. 2010).



(2.33)

The biomolecules are modified with histidine residues ("tags") at the terminal functional groups. Commonly, the 5–10 histidine fragments are attached to the DNA probe or protein molecule to reach the efficiency of the binding necessary for biosensor development. The silicate sorbent bearing nitrilotriacetic acid group is commercially available.

Affine immobilization is site-specific and hence exactly determines the structure of the final product. Single-point binding offers a maximal access of the biological targets to the active sites and a minimal influence on the conformation of the protein or DNA molecule.

Methods based on the immunoreagents also refer to the affine immobilization, but are called "immunoimmobilization" techniques (Jin et al. 2008). The chemistry of such processes does not dramatically differ from that considered above for immunoassay approaches. Immunoimmobilization is often used for attaching the bacteria cells to the solid supports followed by their investigation by optical or piezoelectrical methods. In a similar manner, aptamers covalently attached to the solid support can bind to receptors on the surface of the cell wall and hence bear microorganisms in the biorecognition layer of a biosensor. Peptide receptors are displayed on the bacteriophage surface. Although the latter case is not yet realized in biosensor assembly, it also shows the advantages of site-specific affine immobilization.

A very high efficiency of binding makes the affine immobilization similar to the covalent binding described below. Meanwhile, the reaction is reversed and the free DNA oligonucleotides or proteins can be removed from the support by a sharp pH change and/or electrolyte addition.

2.6.3 Covalent Immobilization

Covalent immobilization presumes the formation of covalent bonds between functional groups of a carrier and a biopolymer. Covalent bonds are much stronger toward any outer influence than electrostatic and donor–acceptor interactions and H-bonds, and provide maximal stability of the product. The covalent binding may affect the affinity of the bioreceptors due to the alteration of their spatial structure and steric DNA structure and the affinity toward target biomolecules, but in most cases such an influence is insignificant. To some extent, steric limitations can be avoided by the introduction of the spacers, i.e., long fragments between the support and biopolymer. The reagents used in covalent binding are called *bifunctional reagents* (Fig. 2.25).

Bifunctional reagents can bind protein molecules to each other (*cross-linking*). The product becomes insoluble and is precipitated on the sensor surface or plastic support.

Glutaraldehyde and carbodiimide binding is most often used (Dugas et al. 2004; Lucarelli et al. 2004). The appropriate reagents attack the amino and carboxylic groups, respectively. The mechanism of protein binding is considered in Sect. 2.1 [Eq. (2.2)]. Glutaraldehyde can chemically modify thiol, amino and hydroxyl groups of proteins and the same terminal groups of the DNA oligonucleotides. The reactivity of glutaraldehyde decreases in the above range and is complicated by the chemical instability of the glutaraldehyde in the reaction of polycondensation and partial cyclization during its storage and use. Up to 70 % of glutaraldehyde is present in the aqueous solution in these forms (Eq. 2.34) (Migneault et al. 2004). The polycondensation diminishes the efficiency of immobilization by glutaraldehyde and makes the properties of the product less predictable and reproducible.

The influence of polycondensation can be suppressed by the application of glutaraldehyde vapors instead of the aqueous solution. These techniques were first suggested for the preparation of enzyme sensors based on FETs and other microelectronic devices. Due to the small size of the working area, the amounts of

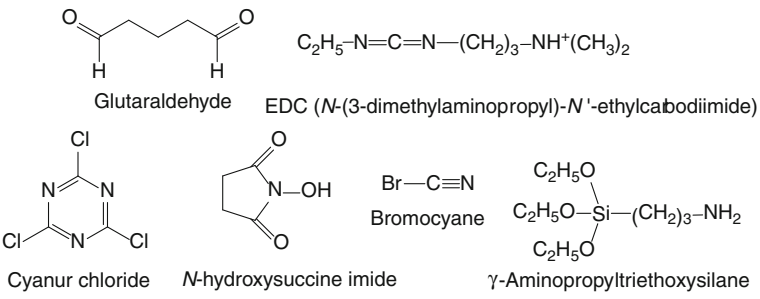
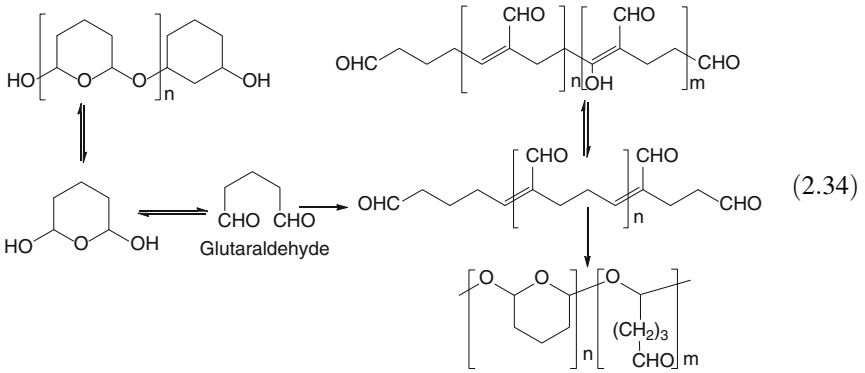


Fig. 2.25 Bifunctional reagents commonly used for the covalent immobilization of biomolecules

enzyme loaded onto the surface were much lower than those for conventional sensors. The FETs were fixed at the distance of several millimeters over the 5–15 % glutaraldehyde solution and the system was vacuumed.



(2.34)

The volatile monomer of glutaraldehyde crossed the gap between the solution and sensor surface and cross-linked the protein molecules as described. The period of treatment was certainly longer than that for the aqueous solution and the efficiency of immobilization is lower, but in many cases the result was adequate enough from the point of view of the signal stability and reproducibility.

The enzymes are mixed with inert proteins like BSA to decrease the loss of the enzyme activity often observed at cross-linking with bi-functional agents. The appropriate procedure is also called "implementation in the BSA matrix." It should be mentioned that both the amount of the enzyme and BSA are the subjects of careful optimization together with the glutaraldehyde concentration and treatment period, because these parameters determine the thickness of the protein film obtained and its permeability for the substrate or inhibitor of an enzyme. For carbodiimides, the decay of activity is not serious and no BSA or similar "thinners" are used. The same refers to the covalent immobilization of antibodies and the DNA oligonucleotides.

Both carbodiimide binding and treatment with glutaraldehyde of amino groups preferably result in the formation of Schiff bases with the >C = N- group sensitive to hydrolysis. This is not particularly important for the laboratory samples of biosensors but can be critical for commercial applications. Additional measures, e.g., the chemical reduction with $NaBH_4$ and the saturation of unreacted groups with glycine are often suggested to stabilize the immobilization products.

Some of the examples of covalent binding are given in Fig. 2.26. The carboxylated supports are easily prepared by a partial oxidation of carbon materials, i.e., glassy carbon and carbon black often used in electrochemistry. The oxidation of carbon nanotubes also results in the removal of terminal cups substituted with carbonyl and carboxylic functional groups. For multi-walled carbon nanotubes, the defects in the side walls are expected to give the hydroxyl, carbonyl and carboxylic groups active in covalent binding. From other sources, polyaminated and

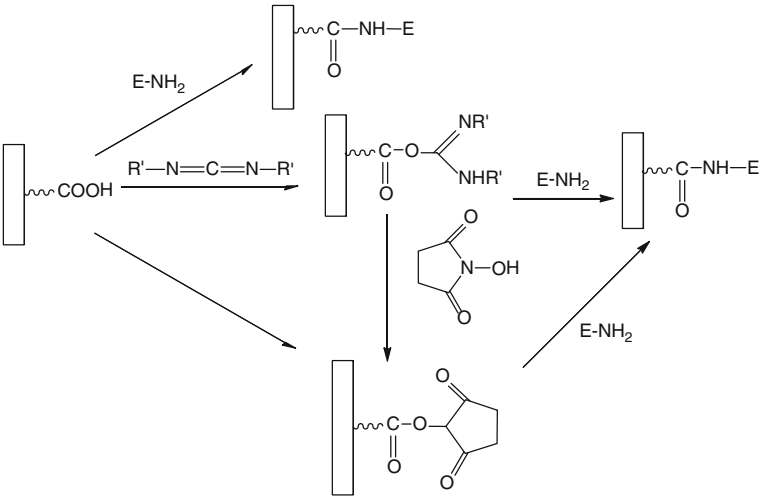
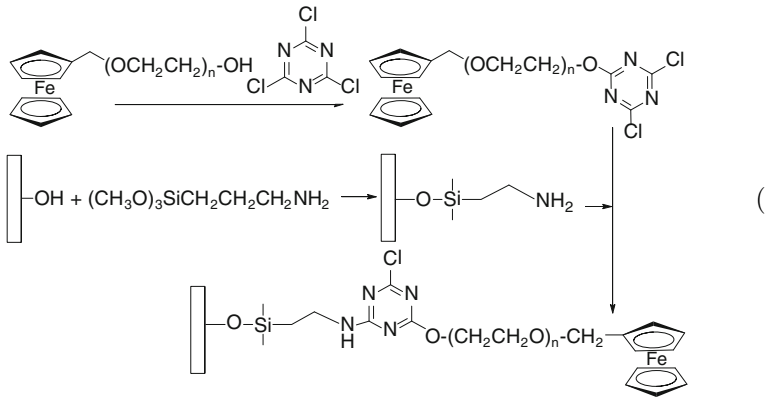


Fig. 2.26 Covalent immobilization of proteins (E-NH_2) on carboxylated carriers

carboxylated ion-exchange resin can be used. However, their efficiency in covalent immobilization is less than that of carbon materials due to the electrostatic repulsion and steric hindrance of biopolymer binding (Lucarelli et al. 2004).

Covalent immobilization is also used for the attachment of other auxiliary reagents, either to bioreceptors or to a solid support (Eq. 2.35). Thus, the mediators of electron transfer or fluorogenic substances can be fixed by covalent bonds for the following measurement of electrochemical and optic signals, respectively. Covalent immobilization is also applied for attaching the labels to the DNA or protein molecules already immobilized by the other protocols described. This makes covalent immobilization a universal tool for biosensor development.



(2.35)

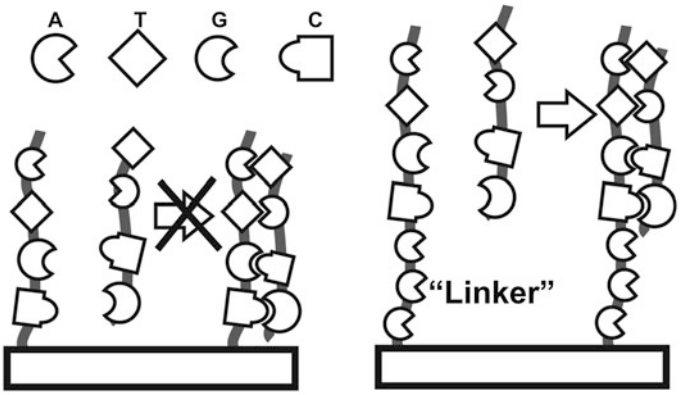


Fig. 2.27 The hybridization detection using a homo-ODN linker for avoiding spatial limitations of interactions

The optimization of covalent immobilization protocol is mainly directed to the minimization of the losses of biochemical functions, i.e., enzyme activity, affinity toward immunoreagents, etc. The formation of rather rigid constructs around the binding site by covalent interactions limits the flexibility of the immobilized biomolecule. Because of that, the immobilization product is less adjusted to an analyte structure than that in its native environment. For this reason, the rate of an appropriate biochemical reaction decreases, even though the quantity of the biomolecules in the surface layer of the biosensor remains constant.

As mentioned earlier, this limitation can be diminished if long-chain bridging molecules (also called *spacers* or *linkers*), are introduced in between the biopolymer and solid support. Besides alkyl radicals, polyoxyethelene and polyethylene imine linkers are used as shown in schemes (2.32, 2.35). In the case of the DNA probes, the functions of linkers are realized by a specific ODN sequence (Fig. 2.27). A homo-oligonucleotide strand is introduced between the recognition sequence and the terminal functional group so that the hybridization takes place not on the surface but spatially separated from the transducer. This scheme constrains the detection system but exhibits advantages of higher sensitivity and selectivity of the target sequence detection.

The use of linkers is a routine step in the covalent immobilization often combined with the SAM technology. The necessity of assembling monolayers with linked ODNs is related to the prevention of the DNA lodging. When DNA probes are placed along the support surface, the advantages of linkers become insignificant. Both linked and short sequences are expected to have a similar influence on steric factors while the hybridization of the target analyte takes place.

The choice and optimization of the linker is a part of the biosensor development that significantly depends on the signal transduction mode and the analyte nature. The bigger the analyte molecule, the more attention paid to this problem. Thus, the covalent modification of biorecognition elements is mostly considered in the

immunosensor development. The immobilization of the Ab cannot rely on the localization of the binding point because many of the protein functional groups are involved in the interactions with conventional bi-functional reagents. The application of Fab fragments and separation of the Ab molecules into identical parts, followed by their attachment on the golden supports, can help in reaching a high reproducible response.

For enzymes, covalent immobilization often results in the losses of their activity due to the limitations in the mobility of protein domains required for *E-S* complex formation. To suppress this unfavorable influence, the covalent immobilization can be performed in the presence of a substrate. While included in the active site of an enzyme, the substrate "freezes" the favorable spatial conformation of a protein globule. The same result can be reached by treating the enzyme with a reversible inhibitor. The covalent immobilization can be combined with preliminary implementation of an enzyme into an inert gel, e.g., gelatin, BSA or agar. The following treatment with carbodiimide or glutaraldehyde provides not only the necessary covalent binding but also decreases the swelling of the matrix and improves its storage stability especially at room temperature. The protecting effect of matrix components that interact with reactive species used in covalent immobilization is one of the reasons for the phenomenon previously observed in the enzyme sensor development. The use of crude enzyme preparations, e.g., extracts and homogenates, sometimes yielded better immobilization results than those after purification and isolation of the active components. For the same reason, the determination of inhibitors and substrates is affected by the stabilizers and antioxidants added to commercial products to prolong their storage periods. Certainly, this is not the only, and not even the primary reason for the immobilization influence on the biosensor performance, but it should be taken into account especially when a new enzyme source (or enzyme preparation) is considered for biosensor production.

Glycoproteins can be immobilized by the partial oxidation of the saccharide moieties yielding the aldehyde groups active in various condensation paths (Zaborcky 1974). Thus, peroxidase or glucose oxidase can be pre-oxidized by Os(VIII) oxide or periodate salt. The latter yields aldehyde groups that can react with aminated supports or even give oligomeric products of self-condensation similar to that described for glutaraldehyde. The oxidation changes the substrate specificity of the enzyme. Thus, the oxidation of glycolic fragments increases the rate of peroxidase oxidation of bulky organic substrates that have ready access to the hemin fragment deeply installed in the protein moiety. Besides enzymes, the oxidative immobilization is applied for the Ab immobilization.

As in the other cases of covalent immobilization, multi-layered films can be assembled on the transducer surface. Similar to the LbL immobilization, they have a regular and good reproducible structure of their inner layers but are more stable towards concentrated solutions of electrolytes and high temperatures that destroy the complexes coupled only with electrostatic interactions.

There is great evidence of the advantages of such an approach. As one example, the LbL immobilization of pre-oxidized glucose oxidase on the golden support is

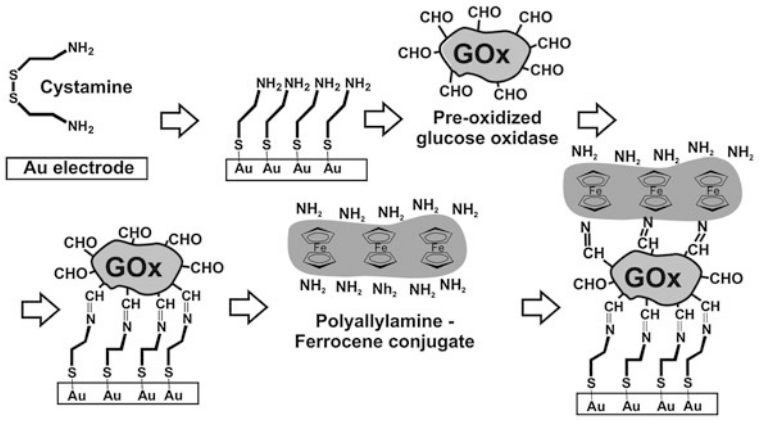


Fig. 2.28 Multilayered immobilization of pre-oxidized glucose oxidase on the cystamine modified Au electrode together with ferrocene containing polyallylamine

presented in Fig. 2.28 (Yang et al. 2004). The aldehyde groups of the enzyme are first coupled with the electrode modified by cystamine; then aminated ferrocene derivatives are attached to the protein layer. An alternate treatment makes it possible to build up a number of layers. Meanwhile, the electric wiring of the enzyme is retained through the whole construct due to intimate contact of the enzyme-active site and ferrocene moieties provided by the immobilization protocol.

The classification given here is rather arbitrary and many protocols, especially those recently developed, can exploit several approaches. Even simple cases, like the implementation of DNA probes on the zirconium phosphate layer, can be considered to be both the physical process of the mobility limitation and the affine reaction of phosphate groups of the DNA sequence with vacancies in the crystal net of the carrier. The interaction of DNA with chitosan, a natural polysaccharide obtained by partial hydrolysis of chitin, can be considered an electrostatic interaction and covalent binding with the formation of phosphate ester (see 2.27).

In other cases, different immobilization techniques are used on various stages of the formation of the biorecognition layer to reach the spatial separation of its components playing different roles in signal generation. An example of such hybrid immobilization techniques is given in Fig. 2.29 for DNA probe immobilization.

The electrode is first covered with a copolymer of pyrrole and its derivative-bearing biotin (Dupont-Filliard et al. 2004). Then the polymeric film obtained is consecutively treated with avidin as a bridging agent and a biotinylated DNA probe. This provides site-specific immobilization with a strictly controlled thickness and permeability of the surface layer. After hybridization detection, the DNA sensor is treated with a concentrated electrolyte solution to remove all the layers over the polypyrrole film and the deposition of another DNA probe can be performed on the same support.

Other similar schemes have been described to increase the sensitivity of the biological targets' detection. Thus, in addition to avidin–biotin bridges, the layers

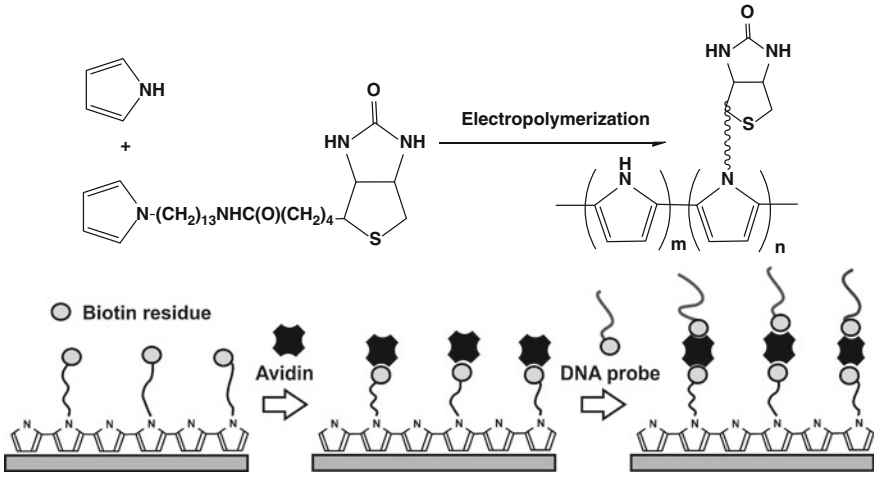


Fig. 2.29 Immobilization of the DNA probe by avidin–biotin binding coupled with covalent attachment of biotin residues to a polypyrrole layer

of biocomponents and auxiliary reagents can be coupled with Au nanoparticles and carbon nanotubes able to covalently bind thiol and amino functional groups, respectively. As in the previous case, regularity of the composition is provided by the order of the reactant addition and specificity of covalent binding.

In Fig. 2.30, alternating layers of carbon nanotubes and Au nanoparticles are obtained by intermediate oxidation and treatment of the reactants with cystamine. This provides the implementation of thiol groups on the sidewalls of the carbon nanotubes bearing carboxylate functions. The procedure makes it possible to deposit several dozen monolayers with their final assembling on thiolated DNA probes. As a result, both electric wiring and steric accessibility of the biotargets are achieved (Zhang et al. 2009).

The short summary of the immobilization techniques shows a great variety of possibilities available for each biocomponent. In many cases, different immobilization protocols result in quite comparable results, i.e., selectivity and specificity of the analyte detection. Some of the techniques acceptable in the laboratory are not convenient in mass production. And vice versa—most complicated protocols with careful maintenance of the order of reagent addition and intermediate washing and drying steps are better fulfilled in an automated regime developed for the biosensor manufacture than manually.

In many cases, the immobilization of biocomponents for biosensor development started from the methods that already existed in related areas, i.e., in affine chromatography, protein purification techniques, bioreactors and biofilm manufacture. Since first results are obtained, the protocols are modified to meet the specific requirements of biosensor performance. In most cases, they are directed to the longer lifetime and more stable signal within the whole biosensor operation.

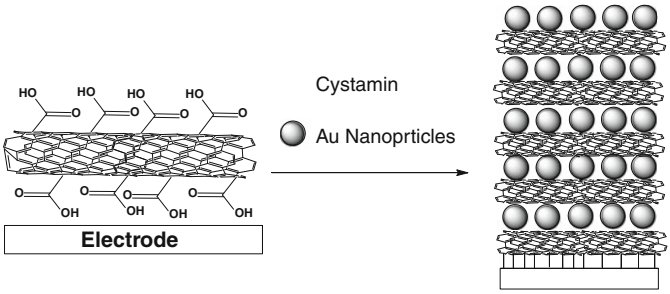


Fig. 2.30 Multiple assembling of the layers of carbon nanotubes and Au nanoparticles for the following immobilization of thiolated DNA probes

The influence of immobilization on the affinity of interactions and hence selectivity of the biosensor signal should also be taken into account.

It is interesting to note that microelectronics and micromachining techniques greatly influenced the development of new immobilization techniques in the past decade. Many of the technologies developed for the production of the microelectronic devices were adapted to biochemicals. In some cases, the success was obvious enough to be extended to a macro scale. Thus, the use of screen-printing techniques with a millimeter scale of the parts (conductor pads, electrode areas) can be considered to be a simplified realization of the photolithography methods used for the creation of multilayered structures based on silicon and metal oxide supports with micron resolution of linear dimensions of the structural elements (Albareda-Sirvent et al. 2000).

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http://www.springer.com/978-3-642-40240-1

Biosensors: Essentials

Evtugyn, G.

2014, X, 265 p. 133 illus., Hardcover
ISBN: 978-3-642-40240-1