Chemical Sensors

An Introduction for Scientists and Engineers

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such modules would be too sluggish. Also, additional preamplifier circuits based on special low-noise amplifiers have been proposed which would be used prior to the input of a common potentiostat (Fig. 7.26). In the input stage (OA1 in the figure), specific low-noise operational amplifiers with high-input impedance are used. The frequency response of RC elements for different measurement ranges must be adjusted very carefully. Considerable efforts has been devoted to blocking distortions from the power supply and from other sources.

Even with the latest low-noise amplifiers, a distortion-free function of picoampere amplifiers is only possible if the complete set-up is enclosed in a Faraday cage (made of metal sheets or grids) which must be grounded.

7.3 Sensors Based on Other Electrochemical Methods

Impedance measurement can be considered a third way to evaluate electrochemical sensors besides potentiometry and amperometry. Electrochemical impedance studies in a narrower sense deal with phenomena at the electrode surface. The overall impedance of a chemosensor also includes effects of charge carrier properties far from the electrode. This was visualized by equivalence circuits presented in Chaps. 2 and 5. By individual experimental design, the study can be focused more on processes at the electrode surface or otherwise on ion properties in homogeneous solution. Even the variation of the dielectric constant in a layer will affect the overall impedance. If impedimetry is designed only to acquire data corresponding to ionic properties or value of the dielectric constant, it is not really an electrochemical method, in a strict sense.

Among the class of impedimetric sensors are real electrochemical sensors where a chemical reaction is the source of information. Of special importance are biosensors with SAMs, which are considered in more detail in the following chapter (Knichel et al. 1995, Rickert et al. 1996).

7.4 Electrochemical Biosensors

7.4.1 Fundamentals

Biological Recognition as a Principle of Selectivity

Following the actual definition (Chap. 1, Sect. 1.2), biosensors are characterized by a receptor function which is implemented by biologically active substances that are able to recognize selectively other substances by a biological mechanism. This *biological recognition* is based on the *lock-and-key principle* in the majority of cases. It means that molecules are identified by their size.

The selectivity of biosensors is astonishing. Bioactive substances sometimes may identify reliably one specific substance among a matrix of millions of others.

Biosensors either work as *biocatalytic* or as *bioaffinity* sensors. In biocatalytic sensors, mostly *enzymes* are immobilized at an electrode surface to act as selective catalysts. Enzymes catalyse slow reactions. The reaction rate, under appropriate conditions, is a quantitative measure for the substrate concentration. If the sample is one of the reactants, i.e. if the sample itself is the substrate, then chemical sensors can be created on this basis. The reaction rate can be measured in terms of an electrolysis current if amperometric biosensors are applied. An alternative approach is to indicate the reaction product of the catalysed reaction selectively, as is done with potentiometric biosensors. In bioaffinity sensors, as the second group, commonly very stable complexes with sample molecules are formed and bound strongly to the sensor surface. The extent of this complex formation is a quantitative measure of the sample concentration. It can be measured indirectly, since many properties of the electrode are changed by complex formation. In bioaffinity sensors, mostly the antibody-antigen reaction is utilized.

Nucleic acid sensors can be classified as another group of biosensors.

Immobilization of Biologically Active Substances

Bioactive substances designed to act as receptors must be immobilized at an electrode surface. There are some differences depending on the question whether biocatalytic or bioaffinity sensors must be realized. Potentiometric and amperometric sensors also are characterized by different requirements. In principle, with potentiometric sensors a higher impedance can be tolerated, whereas amperometric sensors require a good electric conductivity. Nevertheless, immobilization techniques are similar for both groups.

Adsorption is the simplest way to fix bioactive molecules at an electrode surface. Commonly, adsorbed molecules are bound by weak forces, resulting in less durable sensors. Adsorption is useful to immobilize enzymes, antibodies and nucleic acids.

Frequently a simple contact between an electrode surface and a solution is sufficient to generate reasonable adsorptive layers without adding further reagents. This is valid in particular for carbon-containing surfaces. In some cases, however, an adsorptive bond with a carbon surface may affect the enzyme function to such a degree that it is deactivated. Sometimes even denaturation occurs. The majority of molecules is bound only weakly, and a part of them is later lost by progressive desorption. Electrodes with adsorptive layers preferably are used for tentative experiments. They are useful mainly for fast tests.

Enzymes, antibodies, nucleic acids and other bioactive substances alternatively can be bound by covalent chemical bond at solid surfaces. This kind of

immobilization commonly brings about monolayers that are located directly at the electrode surfaces, similar to many adsorptive layers. In contrast to adsorption, a very stable bond is achieved. Hence, electrodes with chemically bound molecules are robust and durable. Immobilization commonly is performed by a two-step procedure. First, the surface is prepared to generate tether groups. Normally, nucleophilic groups are formed, such as carboxylic, amino-acid, hydroxy, thiol, and phenolic groups. Following this chemical activation, active molecules are attached to the surface groups. Metallic surfaces require preparation steps which are different from those for surface groups and useful for carbon surfaces. The arsenal of synthetic methods to bind enzymes covalently is limited. Due to the time-consuming nature of this process, there are few commercial applications available.

A highly efficient way to bind enzymes covalently has been derived from SAMs, which were introduced in Chap. 2, Sect. 2.3. Originally, gold electrodes were functionalized in a complex series of reaction (Willner et al. 1993). An alkyl chain was fixed via an SH group at the gold surface. At the opposite end, the chain contained an amino group that was linked to the enzyme by means of a coupling reagent. On top of the resulting layer, multiple layers of other enzyme molecules were formed, which could even be linked finally with the mediator ferrocene by covalent bonding.

The first step in SAM formation is often given as adsorption, although in fact it consists in the formation of a very stable covalent bond. Enzyme electrodes on the basis of SAMs are robust in general. Useful sensors are manufactured on the basis of gold films made by thin-film techniques.

Imbedding in *carbon pastes* or in *conducting organic salts* is a useful immobilization method for many bioactive substances. Most of them, like enzymes and antibodies, are proteins. Complete biological cells and even microorganisms have also been embedded. Such substances and objects are sufficiently hydrophobic to be compatible with the ingredients of carbon pastes. Carbon pastes are prepared by mixing carbon powders (graphite, spectral carbon or glassy carbon particles) with an organic, water-insoluble binder like liquid paraffin or silicon oil. The paste is pressed into a tube equipped with a metallic contact at the remote end. Carbon pastes are useful for a large variety of applications. Even small *organs* of animals can be fixed in this way. The electrochemical function of carbon pastes is similar to that of compact carbon electrodes covered by an adsorptive film. Particles in the paste also normally exhibit at their surface an adsorptive bond with the active substance. By metallizing the particles, a more selective function can be achieved (Wang et al. 1995).

Alternatively to carbon pastes, conducting organic salts have been used to imbed bioactive components, in particular to immobilize enzymes. The redox mediator tetrathiofulvalene acts as electron donor and forms a solid, conducting salt with the electron acceptor tetracyanoquinodimethane. This salt has a low melting point and can be mixed with proteins to give a conducting

paste. This 'binder' fulfils a second function, i.e. that of a mediator (Bartlett 1990).

Inclusion in polymers or hydrogels frequently is used to fix biologically active substances or microorganisms. Polymer layers are easily coated on solid surfaces. They are solvents for active substances, but they can also include large molecules, cells or microorganisms which are 'glued' in this way to the surface. A disadvantage is their low electric conductivity. To overcome this drawback, conducting particles can be added. Polymer layers commonly have only a low capacity to absorb water. This is a disadvantage with enzymes, which need water to exhibit their specific functionalities.

Hydrogels are well suited to immobilize enzymes. They can contain water up to 98 percent. Inclusion in hydrogels is one of the oldest methods of immobilization. It was applied first with potentiometric enzyme electrodes. The gelating agents used were gelatine and algines, the latter frequently attached with calcium-containing side chains. Synthetic gels like polyacrylamide and polyvinylalcohol have also been applied frequently. With amperometric sensors, besides the low conductance, slow diffusion of the sample inside the gel matrix presents additional problems. It has a negative influence on response time. A further problem is the continuous washout of enzyme. Such loss can be minimized by crosslinking enzyme and matrix. The resulting aggregates are less soluble and are kept in the matrix without lowering their efficiency. A widely used crosslinking agent is glutaraldehyde whose function follows the scheme given below:

$$\boxed{E}-NH_2 \quad + \quad OCH-(CH_2)_3-CHO \quad + \quad H_2N-\boxed{PVA}$$

$$\downarrow$$

$$\boxed{E}-N=CH-(CH_2)_3-CH=N-\boxed{PVA}$$

It is possible to generate a layer of crosslinked enzyme molecules alone, without binder, at an electrode surface.

To avoid washout of enzyme, permselective membranes can be clamped across the gel layer. Dialysis membranes (commonly foils of cellulose acetate) are well suited for that purpose. They can be penetrated easily by small molecules, but bulky protein molecules (among them enzymes) are retained.

It is not surprising that *electropolymerization* has been used to make functional layers for biosensors. A fruitful development of functional layers started with this application. The method was used preferably to immobilize enzymes, either by embedding in polymer layers or by linking with polymer surfaces which had been functionalized before by attachment of amino groups. The stability of embedded molecules has been further improved by crosslinking with glutaraldehyde.

An interesting kind of covalent fixing is the *avidin-biotine reaction*. It is used exclusively for biosensors. In this reaction, a small molecule is somehow 'enveloped' by a large molecule.

Figure 7.27. Biotin

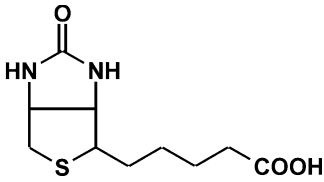


Figure 7.28. Scheme of avidin-biotin reaction

Avidin is a high-molecular protein found in egg white (albumen). Alternatively, *streptavidin* extracted from *steptomyces* can be used in place of avidin. Avidin has a strong affinity to *biotin*, which is found in egg yolk. Biotin, also known as vitamin H, is a low-molecular, water-soluble B-complex vitamin (Fig. 7.27). One avidin molecule can bind up to four biotin molecules. The bond is very strong and stable against extreme pH. Since avidin only requires the bicyclic system of biotin, the carboxylic group of the latter can be used for further linkages. For sensor application, commonly the transducer surface is modified by avidin. The probe molecule is *biotinylated* and linked to the transducer surface. Nucleic acids and proteins, among them enzymes, can be biotinylated easily.

The avidin-biotin reaction can be used to link several molecular monolayers which lie on top of each other with avidin and biotin molecules arranged in alternating mode. A large variety of configurations results from different combinations of the simple reaction depicted schematically in Fig. 7.28. The stability of the avidin-biotin complex is extremely high.

7.4.2 Classes of Electrochemical Biosensors

Enzyme Sensors

Potentiometric enzyme electrodes were the first biosensors. In such sensors, an IES was coated by an enzyme layer acting as a biocatalyst for reaction of a specific substance. The reaction product subsequently was detected by the ISE. This feature had been transferred soon to ISFETs. A special term, *ENFET*, has even been informally proposed for enzyme-modified ISFETs. Attempts were also made to utilize further biological interactions for recognition of analytes and construction of potentiometric biosensors. *Immunologic sensors* on the basis of *antigen-antibody reaction* may be called *IMFETs* if they are built on top of a MOSFET. Immunologic reactions, however, may be used much more efficiently in combination with other transducers besides potentiometric

Figure 7.29. Potentiometric urease sensor with enzyme entrapped in a gel. The gel layer, supported by a nylon tissue, covers the surface of an ammonium sensitive glass electrode

ones. To this day, potentiometric biosensors remain unique to a few enzymatic reactions.

Among the oldest biosensors is a urea sensor (Guilbault and Montalvo 1969). As depicted in Fig. 7.29, the enzyme urease is fixed in a hydrogel layer of polyacrylamide which is coated on the surface of a glass electrode with nylon gauze as support. Urea diffuses into the gel, where a catalysed hydrolysis takes place:

$$\mathrm{CO\left(NH_2\right)_2 + H_2O \xrightarrow{urease} CO_3^{2-} + 2\,NH_4^+}\ .$$

The reaction products carbonate and ammonia change the pH, which is detected by the glass electrode, thus allowing one to estimate the urea content. A better function can be achieved by utilization of a special ammonium-sensitive glass electrode, as proposed by the inventors.

Actual potentiometric biosensors are commonly designed on the basis of ISFETs, i.e. they are ENFETs. Inclusion in hydrogels is no longer done. Covalent bonding, preferably at carbon surfaces, is preferred. Alternatively, embedding in polymer layers is performed, today preferably in polymers generated electrochemically. Also widespread are PVC coatings with specific softeners (as solvents for active substances) as well as layers of silicon rubber and polyurethane.

Table 7.5 presents some examples of practically useful potentiometric enzyme sensors. It is no coincidence that a large variety of glucose sensors has been developed. The demand for glucose sensors in medicine is high, since easy-to-handle analytic procedures for blood sugar control are required to help people with diabetes. One of the sensors in Table 7.5 utilizes an enzyme pair which finally releases fluoride as a reaction product. Fluoride is detected efficiently by a fluoride-sensitive lanthanum single-crystal electrode. Other prominent products of enzymatic reactions are H_3O^+, which can be detected with a pH-sensitive glass electrode, and H_2O_2, detectable with a redox electrode containing noble metal or carbon particles.

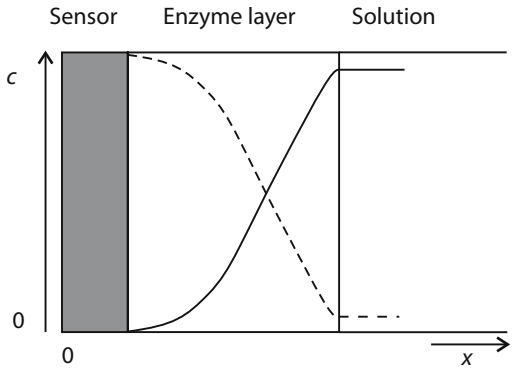


Figure 7.30. Concentration profile in stationary state at potentiometric enzyme sensor

The formation of a stationary concentration of reaction product at the electrode surface is a prerequisite for proper functioning of potentiometric enzyme sensors. Stable conditions are established after a certain settling time where reaction speed is equal to the diffusional transport of the analyte to the electrode (Fig. 7.30). This time is represented by the response time of the sensor. In stationary state, the maximum concentration of reaction product is located at the electrode surface (Vadgama 1990). The enzyme layer should be designed to ensure that this optimum state is reached again following a change in sample composition. Response times of potentiometric biosensors are different. Commonly they assume values of some minutes.

Table 7.5. Potentiometric enzyme biosensors

Sample	Enzyme	Reaction	Product detected
Urea	Urease	$CO(NH_2)_2 + H_2O \xrightarrow{\text{urease}} CO_3^{2-} + 2NH_4^+$	NH_4^+
Glucose	Glucose-oxidase	glucose + $O_2 \xrightarrow{\text{glucoseoxidase}} H_2O_2$ + gluconolactone	H_2O_2
Glucose	Glucose-oxidase;	glucose + $O_2 \xrightarrow{\text{glucoseoxidase}} H_2O_2$ + gluconolactone	F^-
	peroxidase	H_2O_2 + 4-fluoraniline $\xrightarrow{\text{peroxidase}} F^-$ + polymer products	
Neutral lipides	Lipase	lipide + $H_2O \xrightarrow{\text{lipase}}$ glycerin + fatty acids + H^+	H_3O^+
Lactate	Lact-oxidase	lactate + $O_2 \xrightarrow{\text{lactoxidase}} H_2O_2$ + pyruvate	H_2O_2

Amperometric biosensors to date are the largest and most important group of biosensors. Compared to potentiometric sensors, they are characterized by a much shorter response time.

The extreme selectivity of enzymatic reactions can be utilized best with enzymes catalysing electron exchange. Such enzymes are *oxidases* and *dehydrogenases*. The former catalyse redox reactions including oxygen [Eq. (7.21)], the latter such reactions with the participation of the coenzyme *nicotinamide adenosine dinucleotide* (NAD). Considering the oxidized form NAD^+, or the reduced form NADH, the general Eq. (7.22) is obtained:

$$\text{substrate} + O_2 \xrightarrow{\text{oxidase}} \text{product} + H_2O_2 \,, \tag{7.21}$$

$$\text{substrate} + NAD^+ \xrightarrow{\text{dehydrogenase}} \text{product} + NADH \,. \tag{7.22}$$

The substrate (i.e. the analyte) and its reaction products normally are not electrochemically active. The course of reaction is followed by evaluating the consumption or generation of species belonging to the redox couples O_2/H_2O_2 or NAD^+/NADH, respectively. The applied potential is adjusted such that the electrolysis current reflects either the consumption of the oxidizing substance or an increase of byproduct. Modified Clark electrodes are widely used to determine the oxygen loss caused by an enzymatic reaction. Also in use are redox electrodes which detect any H_2O_2 or NADH which formed during the reaction. Such electrodes work in a medium potential range. The redox potential of NAD^+/NADH amounts to ca. 0.8 V vs. saturated silver/silver chloride electrode. Hence sensors containing this coenzyme can operate in air-saturated solution.

All the enzymes mentioned in connection with potentiometric sensors can be used also with amperometric sensors. However, for the amperometric mode a much greater variety of useful reactions is available. Some examples are given in Table 7.6.

The function of simple amperometric enzyme sensors (first-generation sensors) includes the following steps (simplified): diffusion of substrate towards the electrode, reaction with immobilized enzyme, and regeneration of enzyme to give the original form by oxygen or $NADH^+$. The linear dependence of an electrolysis current on substrate concentration can be achieved only under the condition that diffusion is made the slowest step in the series of processes, since the slowest step determines the overall reaction rate. Diffusion control would not prevail if oxygen were depleted in the near-electrode region with the result that the enzyme could not be regenerated.

The selectivity of enzymes is based on the lock-and-key principle, i.e. the substrate molecules are included in a perfectly fitting cavity of the enzyme molecule. The redox-active centre of the molecule is positioned deep inside the molecule and barred from direct contact with the electrode surface. Regeneration cannot be executed by direct electron transfer, even if the enzyme

Table 7.6. Amperometric enzyme sensors

Sample	Enzyme	Reaction	Product detected
Polyphenol	Polyphenol-oxidase	polyphenol + O_2 $\xrightarrow{\text{PPO}}$ o-quinone	o-quinon
Cholesterol	Cholesterol-oxidase	cholesterol + O_2 $\xrightarrow[\text{ferrocene}]{\text{chOx}}$ cholestenone + H_2O_2	H_2O_2 /Fc
Ethanol	Alcohol-dehydrogenase	$C_2H_5OH + NAD^+$ $\xrightarrow{\text{EDH}}$ $CH_3CHO + NADH^+ + H^+$	$NADH^+$
Lactate	Lactatemono-oxygenase	lactate + O_2 $\xrightarrow{\text{LMOx}}$ acetic acid + CO_2 + H_2O_2	H_2O_2
Pesticides[a]	Acetylcholin-esterase	acetylcholine + H_2O $\xrightarrow{\text{ACE}}$ choline + acetic acid cholin + $2O_2$ + $H_2O \rightarrow$ betain + H_2O_2	H_2O_2

[a] by inhibition of acetylcholine esterase activity

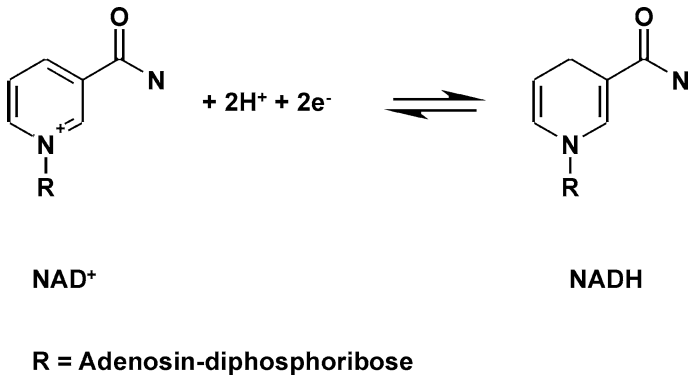


Figure 7.31. Redox equilibrium of nicotinamide adenine dinucleotide (NAD)

molecule is in touch with the electrode surface. This problem has been solved mainly by the introduction of *redox mediators*.

Second-generation amperometric enzyme sensors are characterized by reversible, dissolved redox active substances (*mediators*) which are incorporated into the sensor matrix. Redox couples to be used as mediators should be reducible or oxidizable at moderate potentials, and their reactions should be fast. Mediators are mobile inside the sensor matrix and can react with spent enzyme molecules under regeneration of their original form. Mediators are 'electron shuttles' with a function following the simplified scheme given in Fig. 7.32. It remains unclear which processes occur inside the enzyme molecule during the course of electron transfer. According to one theory, inside the molecule there might be two contact sites, one reducing and one oxidizing, which are inter-

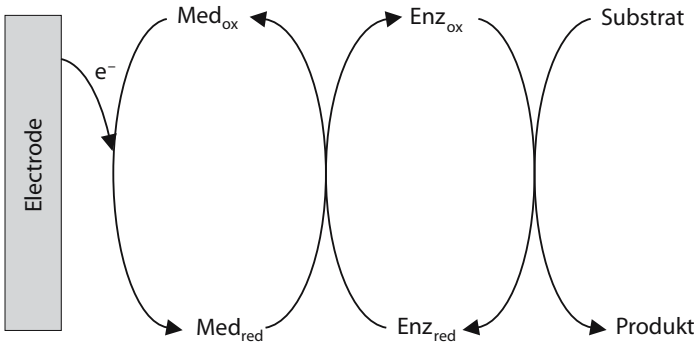


Figure 7.32. Reactions occurring in an enzyme electrode with mediator. Med_{ox} and Med_{red} are the oxidized and reduced forms of the mediator, respectively

connected by a conducting pathway. This would explain the fact that enzymes can take part in redox reactions without uptake or release of charges.

Some authors prefer to speak about a *third generation* of biosensors. This term is not used consistently. In some cases, it denotes a combination of sensor and its electronic evaluation unit, a so-called *biochip*. Also, the *reagentless biosensors* (sensors where all the active compounds are immobilized at an electrode) are sometimes referred to as third-generation biosensors.

Mediators should not be merely reversible redox couples, but they also should react rapidly with enzymes. Commonly used substances are listed in Table 7.7. Figure 7.33 presents the structure of the most important mediator, the ferrocene/ferrocenium couple.

The problem of all the soluble mediators is their tendency to be washed out of the matrix gradually. It would be useful if the mediator could also be immobilized. This seems to contradict the desired function, since the mediator molecules should be mobile independently. Nevertheless, it seems that both

Table 7.7. Common mediator substances and their redox potentials. E^* potential of an equimolar mixture of the couple at pH 7 vs. standard hydrogen eletrode

Mediator	E^*/V
$Os(bpy)_3^{3+}/Os(bpy)_3^{2+}$	0.84
Ferricinium/ferrocene	0.44
$Fe(CN)_6^{3-}/Fe(CN)_6^{4-}$	0.36
quinon/Hydroquinon	0.28
Methylene blue	0.01
Methyl viologen	− 0.44
Tetracyanoquinodimethane (TCNQ)	0.252
Tetrahiafulvalene (TTF)	0.216

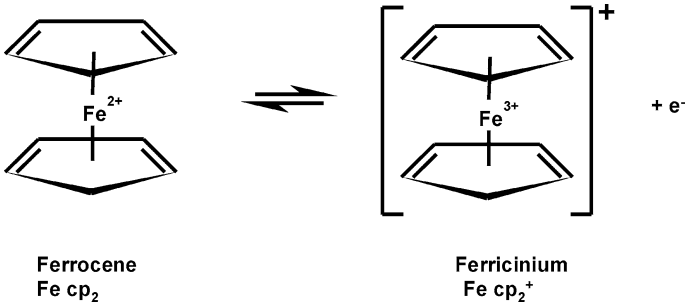


Figure 7.33. Redox equilibrium of ferrocene (dicyclopentadienyl-$Fe^{2+/3+}$)

requirements (immobilization, but mobility inside the matrix) are fulfilled by *redox polymers*. Most successful are electrodes where a mediator substance, the osmium bipyridyl complex, has been attached to a polymer structure forming a three-dimensional redox network (Fig. 7.34). Enzyme molecules are fixed at the surface of the polymer body (Heller 1990). The development status of enzyme sensors with redox polymers is very high. Up to four layers with different functionality have been attached to one sensor (Kenausis et al. 1997).

At present, amperometric glucose sensors are the most important and the most common biosensors. Somewhat less common, but also important, are lactate sensors. There is a large variety of shapes, but one can state the general ambitions for miniaturization and mass production. Sensors where the concentration of enzymatically generated oxygen is analysed are based on miniature forms of the Clark probe (Sect. 7.2.2). Frequently, thin sensors are encountered which can be stuck into a sample (Fig. 7.35). In this example, a small platinum anode is surrounded by a cylindrical silver body acting as cathode. Both are covered by a thin foil of cellulose acetate containing the enzyme. An outer layer of collodium or a dialysis membrane is used to protect the sensor (Wilson and Thévenot 1990). Different variations of this set-up are commercially available and widely used, although they belong to the group of *first-generation biosensors*.

Structures made by screen printing on ceramic or polymer board make up a large part of the commercially available enzyme sensors. Very often the

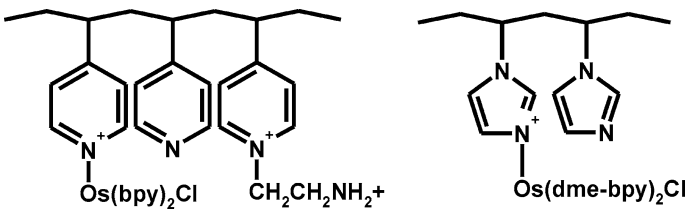


Figure 7.34. Structure of redox polymers with osmium species

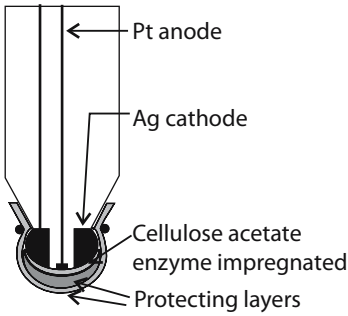


Figure 7.35. Enzyme sensor based on Clark probe

scheme given in Fig. 7.36 is encountered. A layer containing silver and silver chloride particles acts as reference electrode. In equilibrium with chloride ions in the membrane, this layer provides a constant reference potential. Other screen-printed layers act as working and counter electrodes. They contain particles of noble metals or glassy carbon. At present, ruthenium dioxide is also used as a material for the working electrode. This substance has been used widely in printed electronic circuits to form resistances. RuO_2 turned out to be useful for redox electrodes since it is catalytically active for many redox reactions. A solution of the enzyme glucose oxidase can be applied directly onto the printed electrode structures. The structures are stabilized then by crosslinking with glutaraldehyde. Finally, the surface is coated by a protecting layer, commonly a polymer membrane permeable for diffusion.

Enzyme sensors in thick-film technology, according to the design in Fig. 7.36, are commercially available. Most common are glucose sensors of this type. The protecting layer allows one to use the sensors for glucose determination in blood samples without pretreatment.

Enzyme electrodes where the active molecules are chemically bound to a solid surface are not very widespread yet. One example is an amperomet-

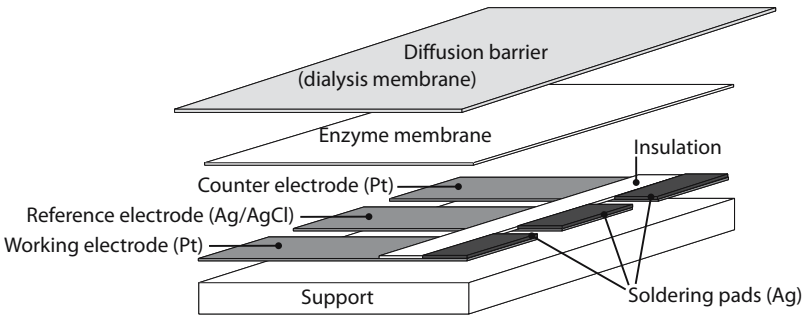


Figure 7.36. Amperometric sensor in thick-film technology. For working and counter electrodes, also layers containing RuO_2 are used

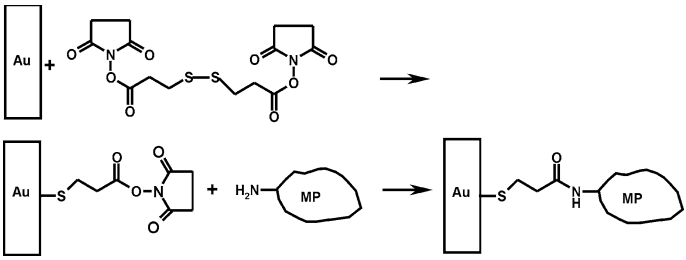


Figure 7.37. Adsorption of DTSP (dithiobis[*N*-succinimidylpropionate]) at a gold electrode and covalent bonding of horseradish peroxidase (HP) at active ester groups of SAM formed

ric sensor with horseradish peroxidase bound to a graphite surface that was functionalized by cyanur chloride (CC) (Cardosi 1994).

SAMs are used increasingly to immobilize enzymes. A simple example is sketched in Fig. 7.37 (Darder et al. 1999).

A specific problem related to enzyme sensors is their restricted shelf life, since enzymes are not very stable. In dry state, they can be stored for some time. Disposable sensors are activated when they are humidified but lose their activity soon after use. Efforts are being made to improve the stability of enzyme sensors by chemical treatment. It has been proposed that the active substance in polyelectrolytes be immobilized with polysugars (Gibson and Hulbert 1993). The enzyme-polyelectrolyte complex formed seems to be stabilized electrostatically by some kind of Faraday cage. In this way, the enzyme activity remains preserved somewhat longer.

Immunosensors Immunosensors are an alternative way to make use of the extreme selectivity of biological receptors. Living organisms can generate antibodies to dispose of nearly every existing substance. Consequently, nearly every substance can play the role of antigen. In immunosensors, antibodies are considered to be reagents which selectively form stable complexes with the antigen that constitutes the sample. After isolating and cleaning, the antigen is immobilized at an electrode surface. In this way, a probe is prepared which should be able to bind a single sample among thousands or even millions of different substances. Although this reaction is disturbed somewhat by parallel non-selective adsorption processes, immunosensors are characterized by a considerable selectivity. The next question is how to generate an electrochemical signal.

Immunosensors are bioaffinity sensors, i.e. antigen and antibody form a stable complex, but there is no decomposition of reactants or formation of further reaction products. Consequently, analytical information in amperometric immunosensors cannot be obtained directly by evaluation of an electrolysis current reflecting the reaction rate, as in biocatalytical sensors. The common way to obtain amperometric signals is chemical modification (*labeling*) of the

sample (the antigen) to be determined. The sample can be linked e.g. to an electrochemically active group or to an enzyme which can be detected after complex formation by means of its catalytic activity.

Alternative ways of obtaining electrochemical signals with immunosensors are based on the fact that different electric properties of an antibody layer are changed by the formation of the antibody-antigen complex. The complex formation changes the charge distribution at the sensor surface, which can be measured in terms of a potential shift, i.e. by potentiometry. Also, conductance variation can be evaluated by impedimetry.

For immobilizing the isolated and cleaned antibody, methods similar to those described in Chap. 2, Sect. 2.3 can be used as. There is, however, a peculiarity. Since the most common type of antibody molecules is Y-shaped (Chap. 2, Sect. 2.2.8), the molecules must assume a specific orientation at the sensor surface. The antigen-binding sites should be oriented towards the sample solution (Fig. 7.38). This orientation is best achieved if the molecule is linked covalently at its group opposite to the antigen-binding site. For that purpose, reactive groups are bound to carbohydrate groups at the region near the molecular 'hinge'. Alternatively, the antibody is modified by the *protein G* which binds specifically at the 'foot' of different antibodies. This protein can be immobilized easily at an electrode surface in advance. Very useful is binding of antibody molecules in the form of SAMs. It must be born in mind that antibodies are voluminous molecules with considerable space requirements. A common practice is to form first a monolayer composed only partially of bifunctional molecules (molecules carrying a group for surface binding at one end and for linking with the antibody protein at the other end). Together with the bifunctional molecules, shorter, monofunctional molecules are fixed at the surface. The latter 'dilute' the bifunctional layer.

A somewhat different method is modification of the antibody molecule itself by a 'linker' carrying a thiol group at its free end. Thiol groups readily bind to gold surfaces. Regardless of the advantages of SAMs, immobilization by simple adsorption is also used.

Potentiometric immunosensors have been assembled on the basis of a thin semiconducting titanium dioxide film. The latter was covered by an activated polymer membrane containing covalently bound antibodies. Complex forma-

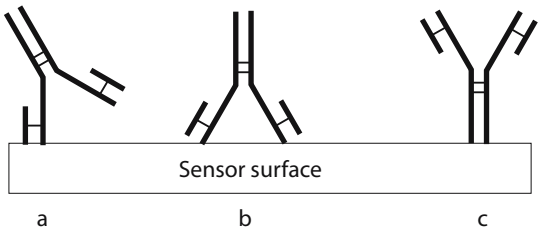


Figure 7.38. Orientations of antibody molecules at solid surface. Only position c is useful for sensor application

tion with the antigen caused a potential shift, which is a measure of the antigen content in solution (Yamamoto et al. 1983).

Such electrochemical immunosensors proved meaningful only where the signal was obtained by impedance measurements. A first example was a sensor for determination of methamphetamine in urine (Yagiuda et al. 1996). The antibody *anti-methamphetamine* was immobilized at two adjacent platinum electrodes and crosslinked by treatment with glutaraldehyde. In contact with the sample solution, conductivity of the antibody layer between the electrodes decreased as a result of antigen binding. This sensor is a member of the group of chemoresistors.

Layers of synthetic peptides (molar mass ca. 3000 Da) which represent a specific site of an antigen have been bound via 'linkers' or 'spacers' at a gold surface. As a result, a SAM-based impedimetric biosensor has been realized (Rickert et al. 1996). The signal was obtained by evaluation of the impedance spectra recorded in the presence of the reversible redox system ferrocyanide/ferricyanide. The redox reaction of this 'indicator' is blocked when the antigen binds with the antibody. For this sensor, the term *impedimetric sensor* is appropriate. It has been applied to detect the foot-and-mouth disease virus. Such application justifies the considerable efforts expended on the preparation of synthetic peptides.

Sensors with Whole Cells, Microorganisms and Organs Living organisms have always been used to observe changes in the environment, i.e. as *biomonitors*. Biosensors result from combining organisms with transducers in such a way that measurable signals are obtained. Commonly, enzyme action is also the determining source of information in sensors based on whole organisms, cells or organs. Often it is useful to leave enzymes in their natural environment. In this way, a better stability of the biocatalysts is achieved and costs are minimized. Disadvantages are longer response time, lower selectivity and worse reproducibility.

The most elementary biosensors are fruit pulps or slices which have been combined with amperometric electrodes. A well-known example is the 'bananatrode' (Wang and Lin 1988). This sensor, most useful for demonstration experiments, contains a paste mix of banana pulp, nujol and carbon powder which has been pressed into a glass tube with an electric contact (Fig. 7.39). The mass contains the enzyme polyphenolase, which catalyses the oxidation of polyphenols, among them important biological messengers like dopamine. The sensor can be tested by means of simple compounds like catechol, which can be detected in beer. As a result of air oxidation, *o*-quinone is formed. The latter is an electrochemically active compound which can be detected e.g. by differential-pulse voltammetry.

With fruit pulps of eggplant, apple and potato, sensors similar to that with banana pulp have been assembled. In all these cases, the enzyme polyphenoloxidase is the active agent. Products of oxidation are also *o*-quinones.

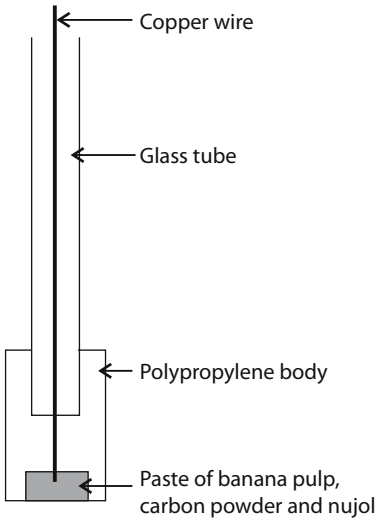


Figure 7.39. The 'bananatrode', an amperometric biosensor with banana pulp

Biosensors have also been set up with plant tissue slices rather than pulps. As an example, a potato slice spiked with glucose oxidase has been used to detect phosphate and fluoride (Schubert et al. 1984).

Isolated biological cells or cell fragments (e.g. membrane particles) have been used as receptor layers in biosensors. Such compositions allow interesting experiments, but they are not suited as a basis for commercial sensors.

Biosensors with living organisms can be realized with bacteria, algae, fungi and protozoae. Commonly, the function is based on the enzymes contained in the organisms. Often it is simpler and cheaper to grow a culture of bacteria or fungi and immobilize the complete culture than to isolate the enzymes, a tedious process. Here, the microbe plays the role of enzyme container.

Microorganisms can be immobilized in the same way as biologically active substances. Common ways are imbedding in gels or polymers as well as inclusion behind a membrane. Modified Clark electrodes are widely used (Sect. 7.2.2). The biological activity of many microorganisms is connected with consumption or formation of oxygen. Bacteria consume molecular oxygen by respiration, whereas some microalgae generate oxygen by photosynthesis. Pollutants in the sample will affect these activities and can be detected by a changed sensor signal. A typical arrangement is shown in Fig. 7.40. The microbes are immobilized on a membrane positioned between a dialysis membrane and the oxygen-permeable membrane of the Clark sensor, mostly of macroporous PTFE.

Examples of microbial biosensors on the basis of Clark electrode are sensors containing the microalga *Chlorella vulgaris*, which was covered by an alumina membrane (Pandard et al. 1993). Also bacteria cultures of *Bacillus subtilis* and

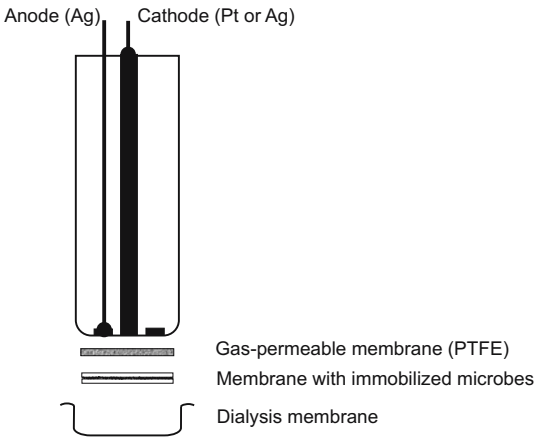


Figure 7.40. Microbial biosensor on basis of Clark probe

Bacillus licheniformis entrapped by a polycarbonate membrane have been used (Li and Tan 1994).

Biosensors with microorganisms are good monitors for toxic substances in natural waters. Also the *biological oxygen demand* (BOD) can be determined by means of biosensors.

Probes with organelles or with whole organs clearly perform like biological recognition mechanisms. Among the examples described was a sensor containing the immobilized antennae of a potato beetle (*Leptinotarsa decemlineata* Say) (Schroth et al. 2001). It was connected via an electrolyte bridge with the gate terminal of a field effect transistor. Traces of guajacol in air caused a detectable change in gate potential. Traces of guajacol and related compounds are released when foils of the potato plant are injured. The potato beetle is able to recognize such traces over a distance of many kilometres. A 'bio-FET' has even been assembled by entrapping an intact living beetle. A similar object has been built up with the antennae of small crabs. Such sensors respond to traces of trimethylaminoxide.

Nucleic Acid Sensors Molecules of nucleic acids exhibit extraordinary potentials. They are electrochemically active due to oxidation of the base guanine, and they can act as ligands which are able to bind many foreign substances. Due to these properties, they should be interesting receptors for chemical sensors. On the other hand, it would be useful to have analytical sensors for nucleic acid determination. The most important sensors in connection with nucleic acids are *hybridization sensors*. These allow one to search in solution for a specific DNA type and to identify in this way an individual biological species, a group of individuals or even a single individual. Hybridization sensors implement a specific form of the *genetic fingerprint*.

Sensors for DNA and with DNA. Traditional methods for electrochemical determination of nucleic acids, preferably of t-RNA (transfer ribonucleic acid) and DNA

(deoxyribonucleic acid), are based commonly on voltammetric stripping analysis. The nucleic acid is adsorbed at a carbon electrode from stirred solution with a constant potential imposed for a definite time period. Subsequently, the deposited material is oxidized. The current flowing during the oxidation step is the source of the analytical signal. Such procedures are not selective. They require tedious sample pretreatment steps and numerous reagents. The term 'chemical sensor' is not really applicable for the electrodes used in such processes.

Adsorptive bonding of DNA at carbon surfaces is relatively strong. Molecules in the resulting monolayers act as ligands for heavy metals and other substances, which can be determined by voltammetry after adsorptive deposition.

DNA Diagnostics and 'Genetic Fingerprint'. The general interest in DNA diagnostics is growing due to the rapid advances in knowledge of the human genome. Progress in microtechnologies has facilitated broader application of nucleic-acid-manipulation techniques. At present, the most important applications of DNA diagnostics are methods for recognition of polymorphism and genetic mutation. It is necessary to detect reliably only one base-pair mismatch in the double helix. Similar methods can be used with the *genetic fingerprint*, i.e. to identify one specific individual based on its DNA.

Commonly, the diagnostic process starts with the generation of a sufficient stock of nucleic acid material, since the original DNA sample commonly is present only in trace amounts. By means of the polymerase chain reaction (PCR) a small amount of DNA can be amplified exponentially. This reaction allows enzymatic replication of nucleic acid molecules outside a living organism. In the material produced, the genetic sequence of interest must be present. Indeed, only a tiny section of the molecule is necessary to detect sufficiently the defect site or to identify the individual of interest.

Actual versions of DNA diagnostics are:

- *Electrophoresis* on agarose gel. DNA is cut enzymatically into short sections which are separated by electrophoresis. As a result, a characteristic image is generated. It is used for identification.
- *Hybridization*. An *oligonuclotide* is immobilized at a surface and used as a *probe*. Oligonucleotides ('oligos') are modules of DNA strands. The oligonucleotide chosen must reflect a characteristic section of the large, single-stranded molecule. The sample solution contains the DNA sample which has been resolved into single strands (the DNA double string has been 'molten') by thermal treatment in advance. In contact with the probe, the complementary strand forms a 'duplex' (the double strand) if the exactly matching counterpart of the immobilized oligos is present in the sample solution. This process is called *hybridization*. It is associated with property changes that are the source of a useful signal. Frequently, signals are obtained optically by measurement of *fluorescence* or *chemiluminescence*. Optical techniques are laborious, since the sample DNA must be marked

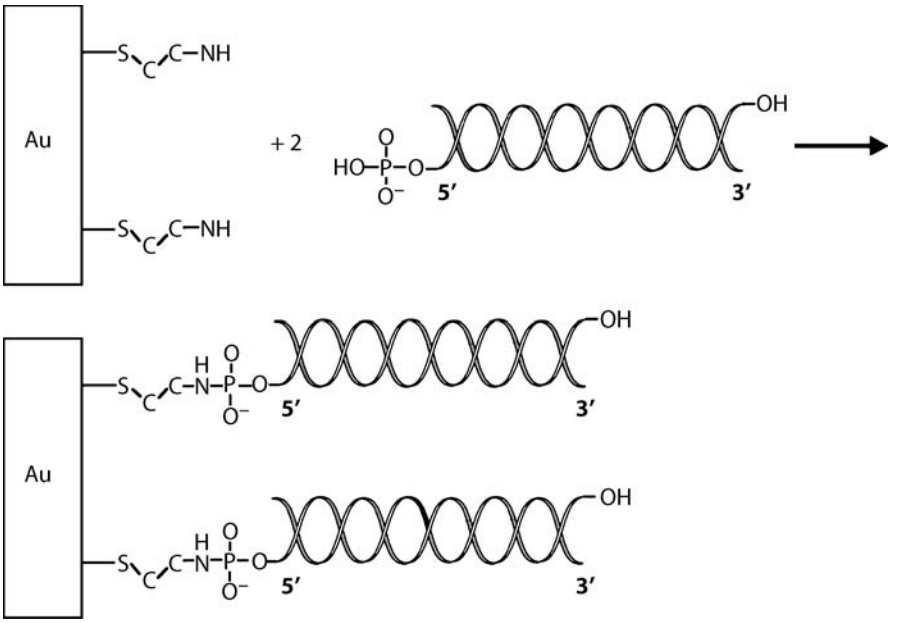


Figure 7.41. Covalent attachment of dsDNA at gold surfaces via SAMs

prior to analysis, e.g. by attachment of a fluorescing group. In contrast, electrochemical techniques allow one to detect hybridization without prior chemical modification of the sample.

Immobilization of DNA. Nucleic acids can be immobilized by means of methods equal to that used for protein molecules, i.e. adsorption, crosslinking, inclusion in gels or polymers, covalent chemical bonding with formation of SAMs, and finally, also the avidine-biotin complex formation (Sect. 7.4.1). SAMs with nucleic acids have also been used on silicon, where patterns have been generated by photolithography. Immobilization of DNA or oligonucleotides on glass or nylon surfaces is the basis of so-called DNA chips which combine numerous single probes. They are useful for simultaneous determinations (Ramsay 1998).

A characteristic procedure for immobilization of dsDNA or corresponding oligonucleotides is shown schematically in Fig. 7.41 (Zhao et al. 1999). The SAM on a gold surface was prepared in advance. It is allowed to react with DNA in the presence of the reagent 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide-hydrochloride (EDAC).

Electrochemical Hybridization Sensors. Hybridization detection means recognizing the degree to which an immobilized single strand has formed hybrids (duplexes) with the studied complementary counterpart. The following electrochemical methods for detection of hybridization are currently in use:

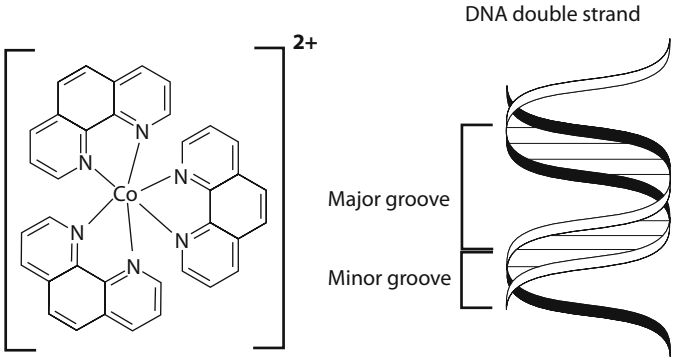


Figure 7.42. Cobalt-phenanthroline $Co(phen)_3^{2+}$ (*left*). Incorporation into *minor groove* of DNA (*right*)

- *Hybridization detection by means of reporter molecules*

Reporter molecules (indicators) are electrochemically active substances which are oxidizable or reversibly reducible. Most commonly, the cobalt phenanthroline complex $Co(phen)_3^{2+}$ is used. The molecule is attached in the *minor groove* of the DNA molecule (Fig. 7.42). There exist more redox systems which are incorporated in this way (intercalated). They must carry a positive charge (Millan et al. 1992, 1994, Millan and Mikkelsen 1993). Polynucleotides have been immobilized to form probes by means of covalent chemical bonding at the surface of glassy carbon and carbon paste electrodes. Cyclic voltammograms have been recorded before and after exposure to sample solution.

Assuming that at first the electrode surface is covered by a dense layer of ssDNA molecules, the transport of reporter molecules towards the surface is strongly inhibited, and as a result, the electrochemical signal is low. After hybridization, the signal increases since the double strand in general carries a larger negative surface charge. Consequently, the indicator cation displays a stronger interaction with DNA. It cumulative and can reach the electrode surface more readily. The difference in both signals provides information about the degree of hybridization. Figure 7.43 depicts the process.

- *Indicatorless and catalytic detection of hybridization by means of synthetic polynucleotides*

The inherent electrochemical activity of DNA is utilized in processes without reporter molecules. It is well known that the guanine base of DNA can be oxidized at moderate potentials. This property can be utilized if the molecules constituting the probe are not oxidizable themselves. For that purpose, oligonucleotides are synthesized which contain inosine instead of guanine base molecules. Inosine is practically inert at potentials where guanine is oxidized. If the target molecule combines with the synthetic probe during

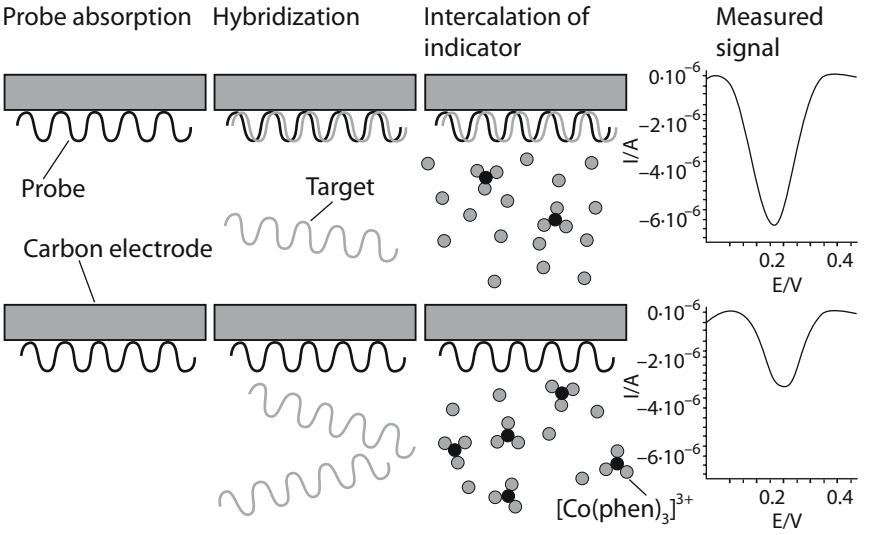


Figure 7.43. Hybridization detection by means of reporter molecule cobalt phenanthroline

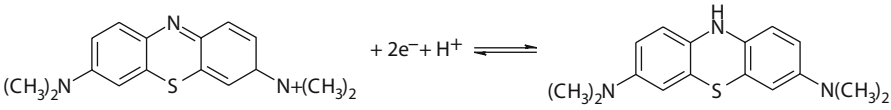
hybridization, due to the guanine content of sample molecules, an oxidation current can be measured (Wang et al. 1998). The method has been advanced by utilization of catalytically active metal complexes like the bipyridyle complex of ruthenium(II/III). In this case, the soluble complex transfers electrons from the guanine of dsDNA towards the electrode surface (Thorp 1998).

- *Hybridization detection by charge transfer along dsDNA axis*

Some results suggest that electrons can move along the axis of an intact dsDNA molecule. Electronic conductivity of single strands is much lower. Charge transfer is inhibited if the hybridization is imperfect, i.e. if a double strand with base mismatches is formed. Only one mismatch in a molecule can disturb the charge transport severely. On this base, a detection method for hybridization can be established (Kelley et al. 1999).

An electrochemical hybridization sensor can be designed using special intercalator substances. Useful intercalators are electrochemically active reversible redox couples which are able to be inserted (intercalated) into the so-called π-stack of the dsDNA molecule. They are flat molecules of planar size such as e.g. methylene blue. The redox reaction of methylene blue is reversible. It can be reduced to give its colourless form (leukomethylene blue), which is readily reoxidized (Fig. 7.44).

If one intends to utilize the conductivity of the dsDNA for hybridization detection, it is important to generate a well-ordered SAM of DNA double strands. This is achieved best with oligonucleotides of moderate length, which correspond to a molecular section of native nucleic acids. Most commonly an oligonucleotide of 15 base pairs is used with an alkyl chain containing an SH



Methylene blue Leuko methylene blue

Figure 7.44. Redox equilibrium of methylene blue and its colourless leuko form

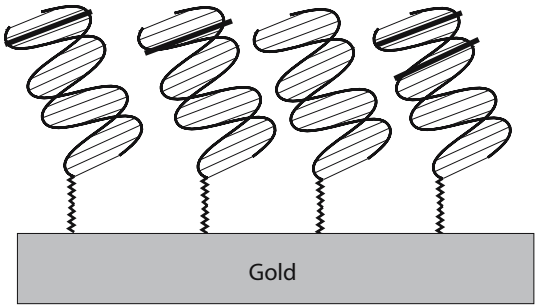


Figure 7.45. Intercalation of planar redox active molecules into double strand of DNA

group attached at the 5′ end as linker. SH groups readily bind covalently at a treated gold surface. As a result, a SAM of perpendicular oligonucleotides is formed (Kelley et al. 1997). The intercalator is inserted always in an upper position, far from the electrode surface (Fig. 7.45).

Electrons must cover the entire distance between electrode surface and intercalator molecule in order to undergo a redox reaction. A single base mismatch can interrupt the electron transport, hence the electrochemical signal is reduced drastically. The preferred electrochemical detection methods have been cyclic voltammetry and chronocoulometry. Intercalated molecules of methylene blue are reduced. Mismatches in the duplex formed after hybridization result in much lower signal intensity compared to perfect double strands.

Non-specific adsorption is a problem with all electrochemical hybridization detection methods. Molecules of nucleic acids are adsorbed at free sites of the electrode surface, i.e. at 'holes' in the DNA monolayer, the so-called pinholes. Such molecules contribute to the electrochemical signal, i.e. they simulate hybridization events.

7.5 References

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