1
Amperometric Biosensors

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1.1
Introduction

The scope of this chapter is to review the advancements made in the area of amperometric biosensors. It is intended to provide general background about biosensor technology and to discuss important aspects for developing and optimizing biosensors. A major concern of this chapter is also to critically review the benefits, limitations, and potential of the different approaches to biosensor research and its applications. An introduction to biosensor research is given (Section 1.1) before criteria of "good to excellent" biosensor research are outlined (Section 1.2), and a standard for characterizing biosensor performance is defined (Section 1.3). Endeavor has been made to define what "good to excellent" biosensor research represents.

Because of the volume of the literature regarding amperometric biosensors as well as space limitations it is not possible to cite any substantial contribution to the field. We selected – to the best of our knowledge – representative work that can be of use not only for beginners but also for advanced researchers in the field as a basis for discussion. Examples of success stories accomplished in biosensor research are given as case studies in Section 1.4. General milestones and achievements relevant to biosensor research and development are listed in Table 1.2. The final conclusions are given in Section 1.5.

A way to address the current impact of biosensor research on analytical chemistry, biochemistry, biology, and medicine is to have a look at the number of publications. Table 1.1 contains the number of articles and reviews with the keyword "biosensor" and related keywords published between 2005 and 2010. About 11 345 papers and 549 reviews have been published containing the keyword "biosensor."

Almost 2000 papers dealing with glucose or employing glucose oxidase as biological recognition element have been published during the last five years. Glucose sensing is one of the success stories of biosensing. The health and the quality of life of diabetes patients depend on the accurate monitoring of their blood glucose levels by means of glucose biosensors. [59–65] The widespread use of glucose

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Table 1.1 Numbers of papers published in important fields of biosensor research between 2005 and 2010.

Keywords	Number of papers published	Number of reviews published
"Biosensor"	11 345	549
"Biosensor" and "glucose"	1 974	96
"Biosensor" and "glucose oxidase"	1 331	37
"Biosensor" and "laccase"	109	7
"Biosensor" and "cellobiose dehydrogenase"	11	0
"Biosensor" and "DNA"	2 166	156
"Biosensor" and "disposable"	287	6
"Biosensor" and "amperometric"	1 931	86
"Biosensor" and "electrochemistry"	1 715	63
"Biosensor" and "reagentless"	177	3
"Biosensor" and "direct electron transfer"	570	25
"Biosensor" and "mediated electron transfer"	53	2
"Biosensor" and self-assembled monolayer"	389	12
"Biosensor" and "conducting polymer"	220	20
"Biosensor" and "osmium"	40	0
"Biosensor" and "PQQ"	26	6
"Biosensor" and "NADH"	190	5
"Biosensor" and "biofuel cell"	73	8
"Biosensor" and "microsensor"	45	2
"Biosensor" and "microelectrode"	185	16
"Biosensor" and "microarray"	257	26
"Biosensor" and "biochip"	155	12
"Biosensor" and "protein chip"	304	14
"Biosensor" and "microfabrication"	57	3
"Biosensor" and "microfluidics"	184	15
"Biosensor" and "scanning electrochemical microscope"	51	
"Biosensor" and "nano"	476	29
"Biosensor" and "nanobiosensor"	33	4
"Biosensor" and "nanomaterial"	56	16

Database search for publications of the latest five years was performed on 10 May 2010 with Web of Science (Thomson Reuters).

oxidase (GOx, EC 1.1.3.4) as analytical reagent has been reviewed in detail. [63, 66, 67] The success of GOx as biological recognition element for biosensors is not only due to the importance of its substrate glucose and its enzymatic performance but also to its outstanding high stability and relatively low price.

Thus, it is not surprising that GOx has also evolved into an initial testing tool for the primary evaluation of new biosensor architectures. It seems to be almost the indestructible "working horse" as a model system. However, one needs to be careful with the general applicability for transferring the findings from initial studies to other more challenging biological recognition elements without providing substantial experimental evidence. This highlights the importance of design-

ing smart electron transfer (ET) pathways allowing the use of a general biosensor design for more than one biological recognition element (and analyte).

The number of papers for the different keywords from Table 1.1 gives hints on the current trends in the field of biosensor research. As mentioned above, glucose sensing is an ongoing trend. Whereas "biosensing and DNA" (2166 papers, 156 reviews) is still a hot topic, especially for the area of low-cost diagnostic devices. The use of biosensor approaches to biofuel cells (about 80 papers in the last five years) is increasingly of interest, and the use of nanomaterials is just evolving and becoming a hot topic. Although, to the best of our knowledge, nanobiosensors not only fabricated out of nanomaterials but also with a transducer surface confined to nanometric dimensions have not yet been realized.

The number of publications, however, does not address the level of quality of the presented research. What, in fact, represents the outcome of all these publications? What represents the resulting scientific advancement? This may not be so easy to answer as it as first seems. Thus, we will first give a general introduction to amperometric biosensors in the rest of this section, before we address the quality issue of biosensor research in Sections 1.2 and 1.3 and before we present some of the success stories of biosensor research (Section 1.4) in order to address the questions mentioned at the beginning of this paragraph.

1.1.1 Definition of the Term "Biosensor"

The use of enzyme electrodes was reported for the first time in 1962 [68]. The term "biosensor" was introduced by Cammann in 1977 [6]. The IUPAC definition of a biosensor, however, was introduced as recently as 1999 to 2001 [3–5]. Figure 1.1 schematically summarizes the set-up of a biosensor. A biosensor is a device that enables the identification and quantification of an analyte of interest from a sample matrix, for example, water, food, blood, or urine. As a key feature of the biosensor architecture, biological recognition elements that selectively react with the analyte of interest (e.g., antibody–antigen or enzymatic reactions) are employed. It is important to note that the biological recognition element is either integrated within or in close proximity to the transducer. The transducer enables the transformation of the analyte recognition and/or catalytic conversion event into a quantifiable physical signal, for example, a current in an amperometric biosensor.

As outlined in Figure 1.1, a biosensor consists of different components. Examples of these components are given in Figure 1.2. It is obvious that there are many ways to design a biosensor architecture. A variety of biological recognition elements ranging from enzymes to antibodies can be employed. The compilation given in Figure 1.2 helps one to understand which parameters change during a biological recognition event in a biosensor. This knowledge is fundamental for developing and optimizing biosensors. The choice of the transduction process and transduction material is dependent on this knowledge as well as the chemical approach to construct the sensing layer on the transducer surface.

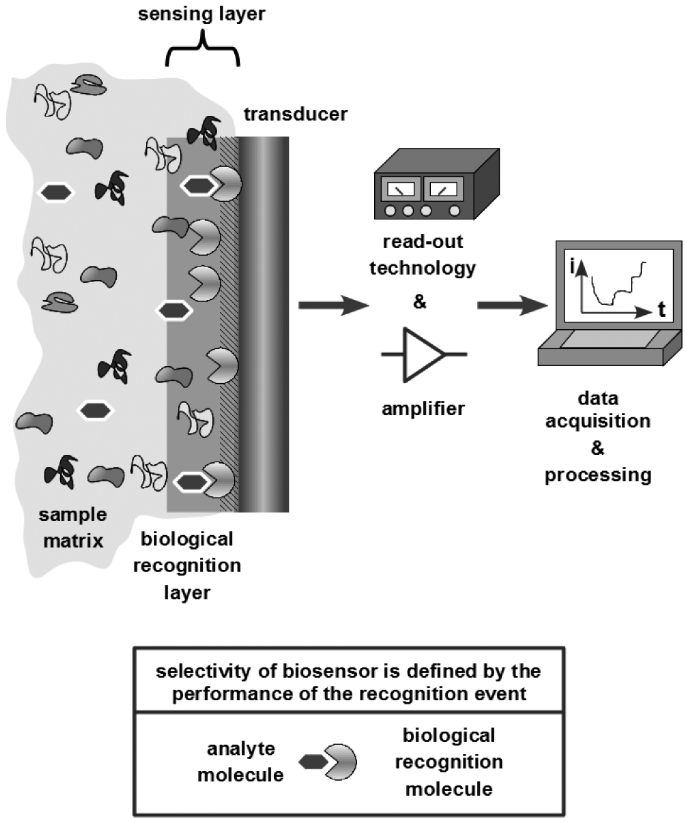


Figure 1.1 Typical biosensor set-up.

The choice of the biological recognition element is the crucial decision that is taken when developing a novel biosensor design. It is important to define criteria for, for example, a suitable redox enzyme for a specific biosensor. Most importantly, the enzyme needs to selectively react with the analyte of interest. The redox potential of the primary redox center needs to be within a suitable potential window (usually between –0.6 and 0.9 V vs. Ag/AgCl). The enzyme needs to be stable under the operation and storage conditions of the biosensor and should provide a reasonable long-term stability. It is advantageous if the chemical structure of the enzyme allows the introduction of additional functionalities for chemical modification with redox mediators, binding, or crosslinking with the immobilization matrix. In addition, the potential for tuning the properties of the redox enzyme by means of genetic or chemical techniques can be helpful for biosensor optimization. An important factor, especially with respect to potential commercialization, is that the redox enzyme is available at reasonable costs and effort.

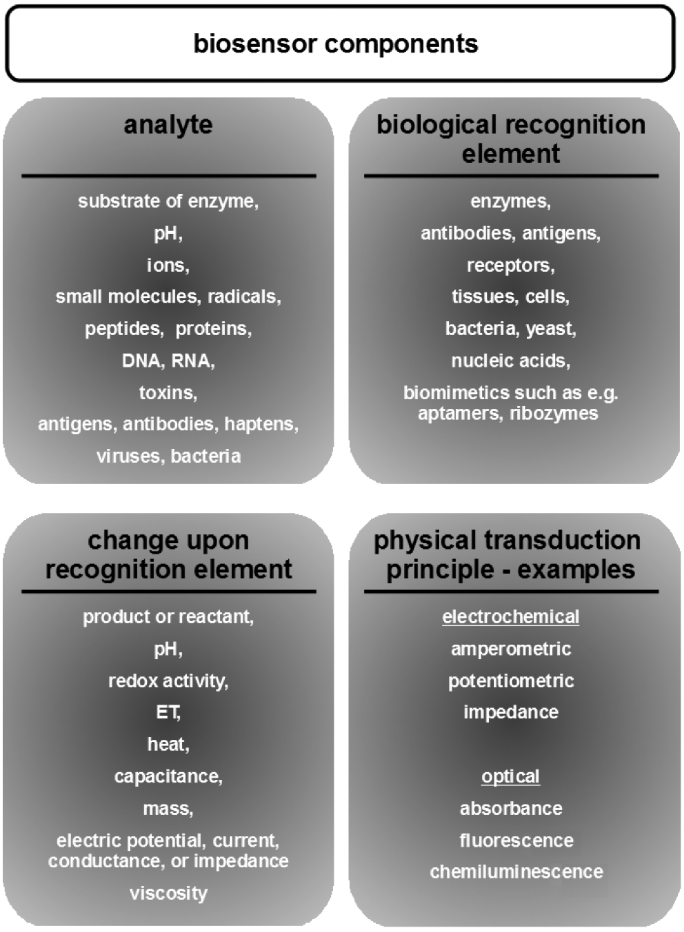


Figure 1.2 Examples for biosensor components.

The advantages of employing enzymes in biosensor architectures are the following:

i) They exhibit a very high catalytic activity with a turnover on a per mole basis which makes them not only exceptional bioelectrocatalysts for effective signal amplification in biosensors but also for biofuel cells. Good turnover frequencies k_{cat} are in the range of up to at least $100\,s^{-1}$.

ii) Typically, enzymes have a high selectivity for their substrates.

iii) In addition, the driving force, the redox potential that is needed to achieve enzymatic biocatalysis, is often very close to that of the substrate of the enzyme. Therefore, biosensors can operate at moderate potentials.

iv) In several cases, an improvement of the enzyme stability was found when enzymes were immobilized on transducer surfaces [25, 69].

The disadvantages of using enzymes in bioelectrochemical devices are the following:

i) Enzymes are rather large molecules. Thus, despite the high catalytic turnover at the active site of the enzyme, the overall catalytic (volume) density is low. As an example, at most about a few picomoles of enzyme molecules per square centimeter are contained in a monolayer of enzymes. Barton and coworkers calculated that the theoretical current density in such a monolayer is about $80\,\mu A\,cm^{-2}$ under the assumption that the "footprint" of the enzyme is about $100\,nm^2$ and the turnover frequency is about $500\,s^{-1}$ [70].

ii) Often the active site of the enzyme is deeply buried within the surrounding protein shell. Thus, direct ET is often not possible and artificial redox mediators are required.

iii) Enzymes have a limited lifetime and, therefore, biosensors exhibit only a limited long-term stability. So far, operational lifetimes of biosensors have been realized to up to 30 to 60 days [71, 72].

Mainly oxidoreductases have been employed for biosensors [73]. However, especially in the context of biofuel cell development, the spectrum of enzymes employed as bioelectrocatalysts is increasing [25]. For biosensor applications, it is important that the catalytic activity strongly depends on the substrate concentration which corresponds to an operating range of about the K_M value or below. This is important for obtaining a suitable dynamic range of the envisaged biosensor. In the case of blood glucose, for example, normal glucose levels are between 4 and 8 mM [74]. Typically, sugar-oxidizing enzymes have rather high K_M values (about 10 mM). Thus, if such enzymes are employed, the resulting biosensor can operate below substrate saturation. In contrast, in the case of biofuel cells the substrates are often present at concentrations well above the K_M value.

Electroanalytical techniques (also in combination with other techniques, e.g., optical techniques such as photometry and Raman spectrometry) can be employed to investigate many functional aspects of proteins and enzymes in particular. It is possible to study the biocatalytic process with respect to the chemistry of the active site, the interfacial and intramolecular ET, slow enzyme activators or inhibitors, the pH dependence, the transport of the substrate, and even more parameters. For example, slow scan voltammetry can be used to determine the relation of ET rates or of protonation and ligand binding. In contrast, fast scan voltammetry allows the determination of rates of interfacial ET. In addition, it is also possible to investigate chemical reactions that are coupled to the ET process, such as protonation. The use of direct ET for mechanistic studies of redox enzymes was recently reviewed by Léger and Bertrand [27]. Mathematical models help to elucidate the impact of different variables on the entire current signal [27, 75, 76].

1.1.2
Milestones and Achievements Relevant to Biosensor Research and Development

Biosensors have been studied extensively during the last fifty years. Hence, a number of milestones mark the progress made in biosensor research. Table 1.2 summarizes the main scientific milestones that are relevant to biosensor discovery and further development of this technology.

1.1.3
"First-Generation" Biosensors

Though many highly complex detection schemes can be found in biosensor designs, the simplest approach to a biosensor is the direct detection of either the increase of an enzymatically generated product or the decrease of a substrate of the redox enzyme. Additionally, a natural redox mediator that is participating in the enzymatic reaction can be monitored. In all three cases it is necessary that the compound monitored is electrochemically active. The use of GOx as biological recognition element for a "first-generation" biosensor design is the typical case and has been employed numerous times (Figure 1.3). Here, the increasing concentration of the product H_2O_2 or the decrease in O_2 concentration as natural co-substrate can be electrochemically detected in order to monitor glucose concentration [68, 103, 110, 150, 151].

The major drawbacks of the first-generation biosensor approach are the following: (i) if the O_2 concentration is monitored, it is challenging to maintain a reasonable reproducibility due to varying O_2 concentrations within the sample and (ii) working electrode potentials for either the oxidation of H_2O_2 or the reduction of O_2 are not optimal because these potentials are prone to the impact of interferences present in biological samples, such as ascorbic acid or dopamine.

1.1.4
"Second-Generation" Biosensors

In order to achieve biosensors which operate at moderate redox potentials the use of artificial redox mediators was introduced for the "second-generation" biosensors [135, 152–157]. Following the pioneering work by Kulys and Svirmickas [124, 125], Cass *et al*. were the first to show that an artificial redox mediator, ferrocene, could be employed for an amperometric glucose biosensor [135]. Figure 1.4 schematically explains how such a redox mediator can be used to read out the analyte concentration within a sample. The employed redox enzyme for the analyte of interest is able to donate or accept electrons to or from an electrochemically active redox mediator. It is important that the redox potential of this mediator is in tune with the cofactor(s) of the enzyme. Preferably, the redox mediator is highly specific for the selected ET pathway between the biological recognition element and the electrode surface. Note that the difference in potential between the different cofactors and the introduced artificial redox mediator should not be less than $\Delta E \sim 50\,\mathrm{mV}$

Table 1.2 Milestones and achievements relevant to biosensor research and development.

Year	Contribution
1800s	Alessandro Giuseppe Anastasio Volta (1745–1827) introduced modern electrochemistry, and found out at that the frog legs employed in the 1791 experiments of Luigi Galvani (1737–1798) to generate currents were not the true source for the stimulation. Actually, it was the contact between two dissimilar metals. He termed this type of electricity "metallic electricity" and demonstrated the first electrochemical battery using his voltaic piles [77].
1839	The principle of the fuel cell was discovered by Christian Friedrich Schönbein (1799–1868) presenting a hydrogen–oxygen fuel cell [78, 79]. Sir William Robert Grove (1811–1896) created one of the first fuel cells which he called a "gas battery" [80]. He also wrote one of the first books that stated the principle of conservation of energy in 1846. Grove is known as the "father of the fuel cell." Friedrich Wilhelm Ostwald (1853–1932), a founder of the field of physical chemistry, contributed significantly to the operation principles of fuel cells [81]. The term "fuel cell" became fashionable around 1889.
1889	Walther Nernst (1864–1941) introduced the Nernst equation [82].
1894	Emil Fischer (1852–1919) introduced the key-lock-principle (specific binding between enzyme and substrate) [83].
1913	Leonor Michaelis (1875–1949) and Maud Leonora Menten (1879–1960) developed the basis for enzyme kinetics and defined a mathematical model, the Michaelis–Menten kinetics [84].
1916	Immobilization of proteins (adsorption of invertase on activated charcoal) reported for the first time by Nelson and Griffin [85].
1922	Jaroslav Heyrovský (1890–1967) invented polarography and the use of the dropping mercury electrode for electroanalysis [86, 87]. Heyrovský and Masuro Shikata (1895–1965) developed a polarograph that was able to automatically record cyclic voltammograms and that was the first automated analytical instrument [88]. In 1959, Heyrovský received a Nobel prize for the development of polarography [89].
1925	George E. Briggs and John B.S. Haldane re-evaluated the Michaelis–Menten equation and contributed to the modern view on the steady-state treatment of enzyme-catalyzed reactions [90].
1926	Otto Warburg (1859–1938) discovered cytochrome c oxidase ("Warburg ferment"). This represents the basis for the description of the mechanism of cellular respiration (Nobel prize in 1931) [91]. Later, Warburg discovered the cofactors (NADH) and the mechanism of dehydrogenases. This leads to optical tests for NADH and NADPH which allows for testing the activity of dehydrogenases. This indicator reaction can be coupled with other enzyme reactions. These advancements were the basis of the work of Hans-Ulrich Bergmeyer (Boehringer Mannheim) promoting enzymatic analysis in the 1960s [92].
1950	Erwin Chargaff (1905–2002) discovered that the ratio of adenine to thymine and guanosine to cytosine is in all living creatures about 1 (Chargaff's rules) [93].
1953	James Dewey Watson (born 1928) and Francis Harry Compton Crick (1916–2002) developed a model for the structure of the double helix of DNA [94].
1955	Frederick Sanger (born 1918) determined the complete amino acid sequence of the two polypeptide chains of insulin. He received a Nobel prize in 1958 for his work on the structure of proteins, especially insulin [95].

Table 1.2 (*Continued*)

Year	Contribution
1956	Rudolph A. Marcus (born 1923) introduced a theory of electron transfer, named Marcus theory. He received the Nobel Prize in Chemistry for this achievement in 1992 [19, 29, 30].
1956	Leland C. Clark Jr. (1918–2005) presented his first paper about the oxygen electrode, later named the Clark electrode, on 15 April 1956, at a meeting of the American Society for Artificial Organs during the annual meetings of the Federated Societies for Experimental Biology [96].
	In 1962, Clark and Ann Lyons from the Cincinnati Children's Hospital developed the first glucose enzyme electrode. This biosensor was based on a thin layer of glucose oxidase (GOx) on an oxygen electrode. Thus, the readout was the amount of oxygen consumed by GOx during the enzymatic reaction with the substrate glucose [68]. This publication became one of the most often cited papers in life sciences. Due to this work he is considered the "father of biosensors," especially with respect to the glucose sensing for diabetes patients.
1957	The first crystal structures of proteins were resolved [97].
1959	Rosalyn Sussman Yalow (born 1921) and Solomon Aaron Berson (1918–1972) developed the radioimmunoassay (RIA) which allows the very sensitive determination of hormones such as insulin based on an antigen–antibody reaction [98, 99]. In 1997, Yalow received the Nobel Prize in Medicine for developing RIA. Today the RIA technology is surpassed by enzyme-linked immunosorbent assay (ELISA) because the colorimetric or fluorescent detection principles are favored over radioactive-based technologies.
1960s	General Electric (GE) developed a fuel cell-based electrical power system employing the so-called "Bacon cell" in order to maintain the Gemini and Apollo space capsules of NASA.
1963	Garry A. Rechnitz together with S. Katz introduced one of the first papers in the field of biosensors with the direct potentiometric determination of urea after urease hydrolysis. At that time the term "biosensor" had not yet been coined. Thus, these types of devices were called enzyme electrodes or biocatalytic membrane electrodes [100].
1964	For the first time, enzymes were used as fuel cell catalysts by Yahiro *et al.* in a glucose/O_2 biofuel cell [101].
1967	G.P. Hicks und S.J. Updike introduced the first practical enzyme electrode immobilizing the enzyme within a gel [102, 103].
1969	George Guilbault introduced the potentiometric urea electrode [104].
1970	Bergveld introduced the ion selective field effect transistor (ISFET) [105].
1970s	ELISA was introduced by Stratis Avrameas (Institut Pasteur, France) und G. Barry Peiers (University of Michigan, USA) and others [106, 107].
1972	Betso *et al.* showed for the first time that direct electron transfer (ET) of cytochrome c could be realized at mercury electrodes. This breakthrough suffers from nonreversible electrochemistry due to protein denaturation on this electrode material [108].
1973	Ph. Racinee and W. Mindt (Hoffmann La Roche) developed a lactate electrode [109].
1973	G.G. Guilbault and G.J. Lubrano introduced an amperometric glucose enzyme electrode that was based on the detection of the product of the enzymatic reaction, hydrogen peroxide [110].
1975	The first commercial biosensor (YSI analyzer) was introduced [60, 61]. A review by Newman and Turner summarized the commercial development of blood glucose biosensors used at home by diabetes patients [59].

(*Continued*)

Table 1.2 (*Continued*)

Year	Contribution
1976	First microbe-based biosensors [111–113].
1976	The first bedside artificial pancreas was introduced. The glucose analyzer allows one to control an insulin infusion system (the Biostator) [114–116].
1977	Karl Cammann introduced the term "biosensor" [6].
1977	First realization of reversible ET of cytochrome c employing tin-doped indium oxide electrodes [117] and 4,4′-bipyridiyl as a promoting monolayer on gold electrodes [118, 119].
1979	First steps towards biofuel cells were realized [120–123].
1979	Pioneering work by J. Kulys using artificial redox mediators [124, 125].
1980s	Self-assembled monolayers (SAMs) start to receive considerable attention in the scientific community and are employed in biosensor research [49–52].
1981	Oxidation of NADH at graphite electrodes is described for the first time [126, 127].
1982	First needle-type enzyme electrode for subcutaneous implantation by Shichiri [128].
1982	First biologically engineered proteins using site-directed mutagenesis, enabling work on specific mutants of enzymes [129–131].
1983	First surface plasmon resonance (SPR) immunosensor [132–134].
1984	First ferrocene-mediated amperometric glucose biosensor by Cass *et al.* [135]. The work led to the development of the first electronic blood glucose measuring system which was commercialized by MediSense Inc. (later bought by Abbott Diagnostics) in 1987.
1988	Adam Heller and Yinon Degani introduced the electrical connection ("wiring") of redox centers of enzymes to electrodes through electron-conducting redox hydrogels [47, 136]. This work was the basis for continuous glucose monitoring employing subcutaneously implanted miniaturized glucose biosensors [137–139].
1988	Direct ET by means of immobilized enzymes was introduced [22, 120, 122, 123, 140–142].
1990	Bartlett *et al.* introduce mediator-modified enzymes [143].
1980s to 1990s	Nanostructured carbon materials such as C_{60} and nanotubes were discovered [144, 145].
1997	IUPAC introduced for the first time a definition for biosensors in analogy to the definition of chemosensors [3–5].
2002	Schuhmann *et al.* introduced the use of electrodeposition paints (EDPs) as immobilization matrices for biosensors [17]. Following work enabled the incorporation of redox mediators into the polymer structure of EDPs [18, 146].
2003	An enzymatic glucose/O_2 fuel cell which was implanted in a living plant was presented by Heller and coworkers [147].
2006	The first H_2/O_2 biofuel cell based on the oxidation of low levels of H_2 in air was introduced by Armstrong and coworkers [148].
2007	An implanted glucose biosensor (Freestyle Navigator System) operated for five days [149].

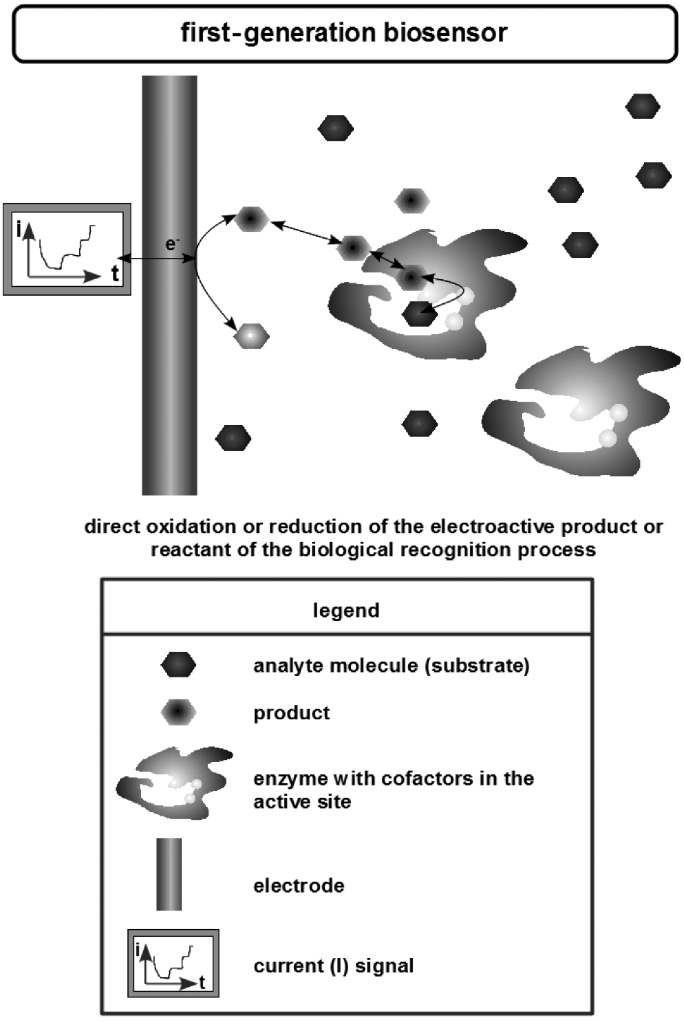


Figure 1.3 Schematic representation of the architecture of a "first-generation" biosensor.

in order to provide a reasonable driving force of the reaction. As free-diffusing mediators, a large variety of compounds such as ferrocene derivatives, organic dyes, ferricyanide, ruthenium complexes and osmium complexes have been used [158].

What are the most important properties of redox mediators suitable for biosensors? First of all, the electrochemistry has to be reversible and they need to be stable in the oxidized and reduced forms. No side reactions should occur. The redox potential needs to be compatible with the enzymatic reaction. It is helpful if the basic structure of the redox mediator also allows for chemical modifications

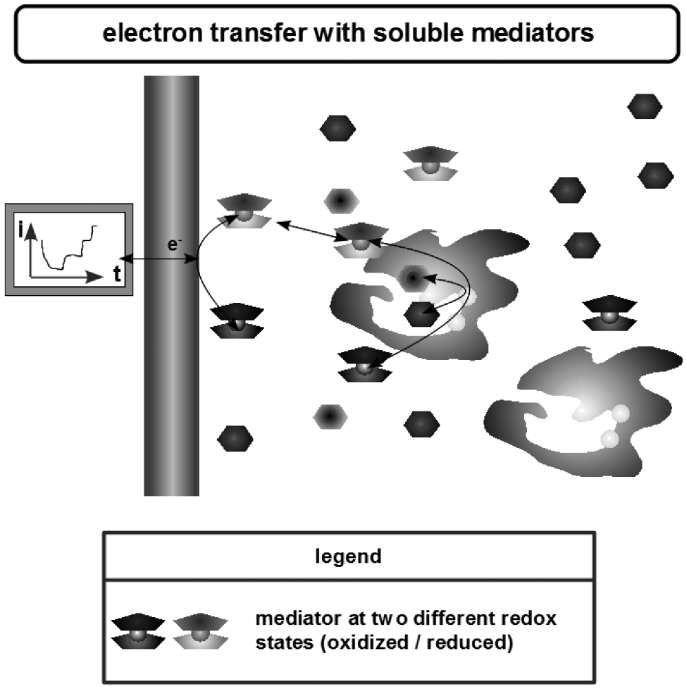


Figure 1.4 Schematic representation of a biosensor operating with soluble mediators.

enabling tuning of the desired redox potential. The immobilization of the redox mediator on the electrode surface and/or the redox enzyme needs to be possible. For instance, functional side chains for, for example, covalent binding to the polymer backbone, redox enzyme, or electrode surface need to be available. The redox mediator should not be toxic and available at reasonable cost and experimental effort. Note that the K_M value of the enzyme for a specific redox mediator also impacts the sensor response.

The major drawback of using either a natural or an artificial free-diffusing redox mediator in a biosensor design as illustrated in Figures 1.3 and 1.4 is that sufficient natural (e.g., O_2) or artificial mediator needs to be available to the active site of the enzyme and, subsequently, at the electrode surface for generating a detectable current signal. In addition, and of more importance to the accuracy and long-term stability as well as product safety, artificial mediator molecules that are not securely fixed within the sensing film can leak from the electrode surface [159]. This will change the sensor performance over time. In addition, not all redox mediators are biocompatible. The described problems with the use of free-diffusing redox mediators are not critical for single-use devices. For example, self-monitoring devices for monitoring blood glucose levels are very successfully used by diabetes patients at home [59, 61, 65, 160–163].

1.1.5 "Third-Generation" Biosensors

A different approach to realize biosensor architectures is the immobilization of a redox enzyme on the electrode surface in such a manner that direct ET is possible between the active side of the enzyme and the transducer [22, 140]. Thus, free-diffusing redox mediators are not necessary for these types of biosensors [164–169]. Biosensor designs based on direct ET have been investigated thoroughly and comprehensively reviewed [22–26, 170–182]. Figure 1.5 schematically illustrates how such direct ET can be realized within "third-generation" biosensors.

Proteins can spontaneously adsorb on many electrode materials [176] as schematically shown in Figure 1.5a. The interaction is mainly governed by hydrogen bonds as well as electrostatic, dipole–dipole, or hydrophobic interactions. It is important to take into account spontaneous adsorption on the electrode surface because it might also contribute to the overall current signal of a biosensor based on a more complex architecture. The impact of this effect can be evaluated by performing suitable control experiments.

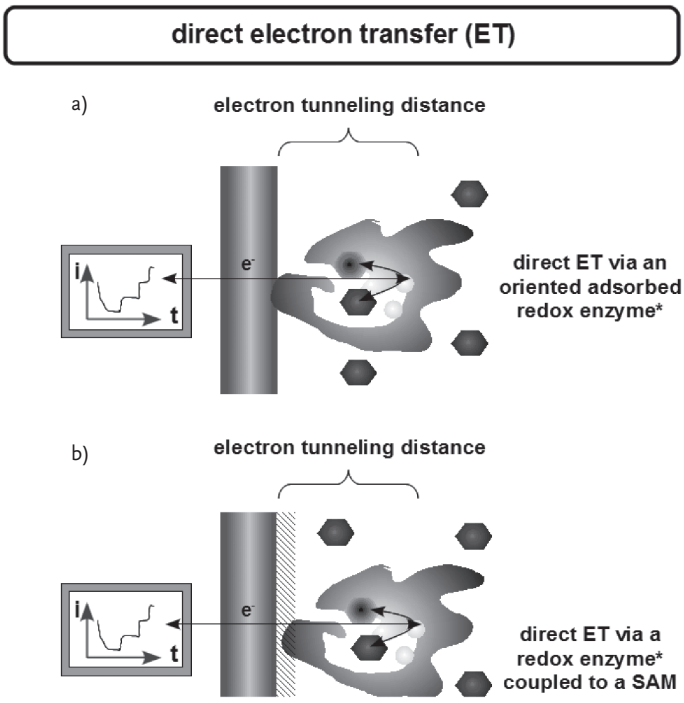


Figure 1.5 Schematic representation of biosensor architectures based on direct ET: (a) via an oriented adsorbed redox enzyme; (b) via a redox enzyme coupled to a self-assembled monolayer (SAM).

It is important to note that proteins tend to denature during such an adsorption process on noble metals or carbon electrodes. In addition, the stability of the adsorbed sensing layer is highly dependent on the pH value and ionic strength of the solution as well as the temperature, the electrode material, and other additional factors. For instance, as early as 1972 direct ET was observed on mercury electrodes employing cytochrome c as redox protein [108]. Reversible electrochemical behavior of cytochrome c was not observed because the protein denatured on the surface.

Therefore, it was a milestone when reversible ET of cytochrome c was achieved for the first time by employing tin-doped indium oxide electrodes [117] and 4,4′-bipyridiyl as a promoting monolayer on gold electrodes [118, 119]. Starting from the 1980s self-assembled monolayers (SAMs) have received considerable attention in the scientific community and have been successfully employed in biosensors [49–52]. The advantages of SAM-based biosensor architectures are the following: (i) the technology is easy and straightforward to use because the formation of SAMs is relatively fast [52]; (ii) enzymes can be adsorbed on SAMs providing the enzyme has an overall surface charge opposite to that of the SAM [176, 183]; (iii) the covalent attachment of redox enzymes and mediators is possible [184–186]; (iv) the design of alkanethiols can be tailored to particular needs (e.g., length of spacer, type of functional groups, mixture of alkanethiols with different properties for integrating different chemical functionalities, generation of multilayers); and (v) SAMs are sufficiently stable with respect to temperature and pH and can be operated in a rather broad potential range (about –1.4 to 0.8 V vs. SCE). This stability window with respect to applied redox potential can also be utilized to generate structured biosensor designs by, for example, stripping of confined areas within the SAM using a scanning electrochemical microscope (SECM) [187–189]. Thus, complex biosensor architectures even employing multiple redox enzymes or mediators can be realized.

It needs to be taken into account that direct ET between a redox enzyme in very close vicinity to the electrode surface (e.g., first monolayer) is normally very slow. It might even be impossible due to the shielding of the active side and/or redox-active cofactors of the enzyme by the surrounding insulating protein shell. Therefore, observation of direct ET has so far been restricted to either small redox proteins or redox enzymes that are characterized by the location of their cofactor(s) close to the protein shell. If the distance is significantly longer than 10 to 15 Å the chance for efficient direct ET is also significantly reduced according to Marcus theory.

Biology has solved this problem by introducing multi-cofactor enzymes in which the overall distance between two redox sites is divided into a number of shorter distances (multiple cofactors with different redox potentials instead of just one cofactor) or by introducing small redox shuttle proteins such as cytrochromes in the respiratory chain. Thus, ET cascades have been proven to be very efficient and useful. This principle was borrowed from nature not only for direct ET-based biosensors but also for mediated ET-based biosensors. Note that the overall efficiency of ET cascades depends on the entire architecture of a biosensor. A striking

option is to reduce the distance between the active site of the redox enzyme and the transducer surface. Figuratively speaking, one could bring the enzyme closer to the electrode surface or vice versa. For example, the enzyme could be genetically or chemically modified to reduce the impact of the protein shell on the ET distance between the electrode and the active site, or the orientation of the active site towards the electrode surface is controlled by chemically (or genetically) modifying the enzyme or the electrode surface. Strategies for such modifications have been extensively used and evaluated [186]. An example of bringing the electrode surface closer to the enzyme could be the introduction of conducting nanoparticles or nanostructures into the sensing layer in order to increase the probability of ET taking place [190].

However, ET efficiency is not only dependent on the distance of the involved redox relays but also on the properties of the electrode material, the nature of the enzyme, the properties of the immobilization matrix, and the redox mediator (if any) in a complex manner.

For the evaluation of a biosensor design based on direct ET, one needs to take into account that not all the enzymes immobilized on the transducer surface are at productive ET distance. A portion of the immobilized enzymes may be oriented in such a way that direct ET is not possible due to a longer distance between the catalytic side of the enzyme and the transducer in comparison to the ideal oriented distance. A certain percentage of the enzymes immobilized may lose or change their catalytic activity during the immobilization procedure. Therefore, one approach to estimate the ratio of enzyme molecules that communicate via direct ET and enzyme molecules that are fully functional but do not contribute to the overall current response of the biosensor is to measure the current in the absence and presence of a suitable free-diffusing redox mediator. Another approach would be to estimate the catalytically active enzyme concentration on the electrode surface by means of a standard optical enzyme activity test. This is also helpful in case no direct ET can be detected and it is not clear if the enzyme undergoes denaturation during the sensor fabrication and operating process.

1.1.6
Reagentless Biosensor Architectures

For a variety of applications it is useful to employ artificial redox mediators for biosensor architectures. In the case of mediated ET, redox mediators shuttle electrons between the active side of the enzyme and the electrode. The main advantages of employing mediated ET within a biosensor device are that the ET process is independent of the presence of natural electron acceptors or donors. For instance, oxygen is ubiquitously present in biological systems. Given that the redox mediator is appropriately selected, the influence of possible interfering compounds can be reduced because the working potential of the biosensor is defined by the formal potential of the redox mediator. In addition, the pH dependence of the sensor response can be better controlled. Furthermore, the sensitivity and overall current response can be increased. Multicomponent ET cascades can be designed.

Due to the drawbacks of free-diffusing redox mediators, especially with respect to continuous monitoring of the analyte of interest, the development of reagentless biosensors has become of importance over the last 15 years [191]. The outstanding feature of a reagentless biosensor is that all components required for the electroanalytical reaction are securely immobilized on a transducer surface. This is a nontrivial task because the immobilization approach needs to ensure a microenvironment within the biosensor film that facilitates the biological recognition reaction and an efficient ET cascade. The only free-diffusing component of the overall assay reaction is the analyte which is provided by the sample solution. The most appropriate approaches towards reagentless biosensor architectures are the use of an appropriate redox enzyme and an ET pathway that either uses direct ET or mediated ET via securely immobilized redox mediators within the biosensor film. There are several technologies available for immobilizing biological recognition elements on transducer surfaces: adsorption, microencapsulation, entrapment, covalent attachment, and crosslinking. These techniques have been comprehensively reviewed [173, 192–197].

What does a suitable immobilization matrix for a reagentless biosensor need to provide? First of all, the selected biological recognition element needs to be securely fixed at the electrode surface. Furthermore, it needs to provide a microenvironment that either maintains or tunes the enzyme activity at a desired level. This is important not only for the efficiency of the reaction with the analyte and the ET but also for the operation, storage, and long-term stability of the biosensor. Matching charges and hydrophobicity/hydrophilicity as well as hydrogen bonds, complexation, or covalent binding sites are important. It is advantageous if the nature of the immobilization matrix is intrinsically open for optimization, for example, by means of adapting the chemical structure, tuning the chemical and/or physical properties as well as the immobilization technique. With respect to miniaturization and automation of biosensor production as well as the reproducibility within the production process, it becomes of importance that the immobilization approach can be done in a way that enables exclusive addressing of the electrode surface. A certain flexibility of the backbone of the immobilization matrix has proven to be advantageous, especially with respect to the aspect of diffusion limitations. Note that one limiting case for biosensor performance can be the diffusion limit. In addition, suitable binding sites for redox mediators, spacers, or (multiple) redox enzymes are useful. Redox mediators also need to be securely immobilized within the biosensor film. If *in vivo* or *in vitro* use of the device is intended the film needs to be biocompatible and compatible with special needs such as sterilization.

Figure 1.6 describes what reagentless biosensor structures based on mediated ET can look like.

Designing appropriate and efficient ET pathways within a biosensor intrinsically requires that the generated environment is suitable for the chosen biological recognition element. Its catalytic activity and stability might be tuned to the desired performance by the immobilization matrix. The immobilization procedure itself needs to be compatible with the ET pathway strategy. For instance, the stress on

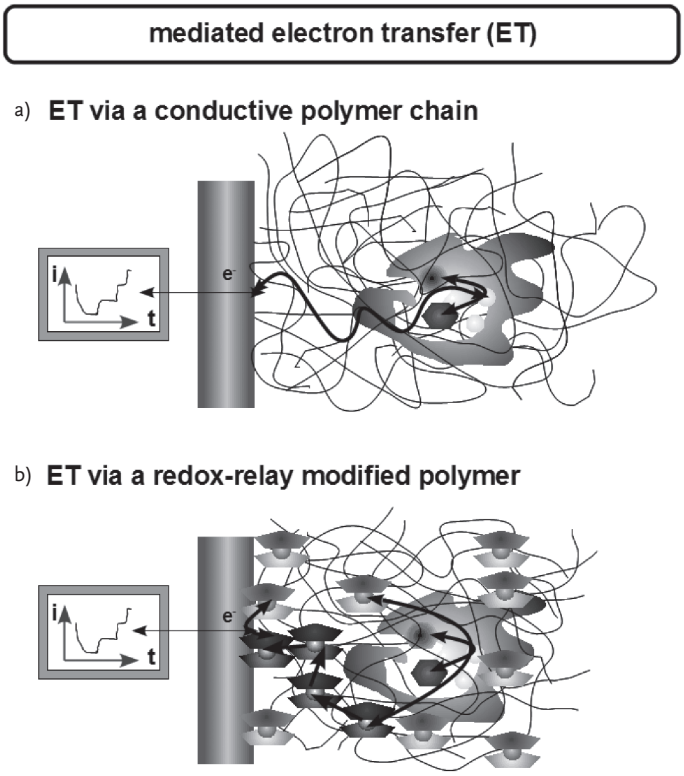


Figure 1.6 Schematic representation of reagentless biosensors: (a) ET via conductive polymer chains; (b) ET via redox-relay modified polymer chains.

an enzyme by either the chemical nature of the immobilization reaction or the applied potential for electrodeposition on the transducer surface may lead to a significant loss of enzyme activity (enzymatic sensor) or binding reaction (affinity sensor). Efficient ET between a redox enzyme and the redox relays in the immobilization matrix to the electrode surface is mainly controlled by the distance between the individual redox couples [198] participating in the overall ET reaction. Thus, for further optimization, it is crucial to elucidate the rate-determining steps of all involved processes.

For instance, if one would like to design a suitable redox polymer for a certain enzymatic reaction, it helps to think about the following factors. First, the redox polymer needs to create a three-dimensional network that allows secure immobilization of the enzyme with a reasonable pore size. In addition, fast diffusion of the analyte, products, or counter-ions and fast ET kinetics need to be ensured. The polymer film deposited as sensing layer creates a diffusion barrier which often prolongs the response time, shifts the linear measuring range, and decreases the sensitivity of the sensor. Second, the redox polymer should create a local

microenvironment that is beneficial for enzyme immobilization, functionality, selectivity, kinetics, and stability. Good interaction between the active site of the enzyme and the redox relays, especially for the first ET step, is vital. For example, ET transfer distances have to be reasonably short (<10 Å).

The general advantages of reagentless biosensor structures can be summarized as follows. Since all components of the assay are securely immobilized on the electrode surface, there is no or just a negligible loss of redox mediators, cofactors, and/or enzymes over the time of operation. This is of importance for the performance and safety of a device because the impact of free-diffusing possibly toxic substances is minimized. Therefore, reagentless biosensor architectures are often used for *in vitro* and *in vivo* measurements as outlined in Section 1.4.5.

Which advantage of a certain generation of biosensors outweighs the advantages of the other generations will, however, depend on the analytical task. The specifications of requirements (e.g., type and concentration range of analyte, composition of sample matrix and occurrence of possible interferences, official regulations for the final application, overall cost limitation for the device) need to be defined. A comprehensive review of the state of the art for the specific analytical task helps to develop a suitable strategy. Sampling and sample preparation are additional important aspects. After a preliminary testing of the envisaged biosensor design, it has to be thoroughly tested to determine if it is capable of identifying and quantifying the analyte of interest. For applications with high throughput and/or commercial interest, approaches have to be evaluated to properly deal with data collection, processing, interpretation, documentation, and reporting. It might be necessary to provide suitable instrumentation to operate the sensing device, and features such as a self-referencing system for calibration, temperature control, etc., might become assets to be considered.

1.1.7
Parameters with a Major Impact on Overall Biosensor Response

The choice of biosensor architecture depends to a major extent on the (bio)chemical processes involved in the biorecognition process. The processes in close proximity to the electrode surface that are involved in a typical biosensor reaction are rather complex. It is important to have an overview about the variables that affect the performance of a biosensor (Figure 1.7) and which of these parameters may have a major impact on the signal response. As a matter of fact, the design of appropriate sensor architecture depends on the specific demands arising from the particular analytical task. Thus, a sound understanding of the challenges of the analytical task with respect of the main reactions involved in the overall sensing process is mandatory. It is important to note that due to the complexity of a biosensor architecture one deals with a multiparameter space of which, most likely, only a limited number of parameters can be controlled. This implies that it may be helpful to visualize the analytical task and the envisaged biosensor design at molecular dimensions in order to better understand the processes taking place, thus being able to identify at an early stage potential pitfalls with

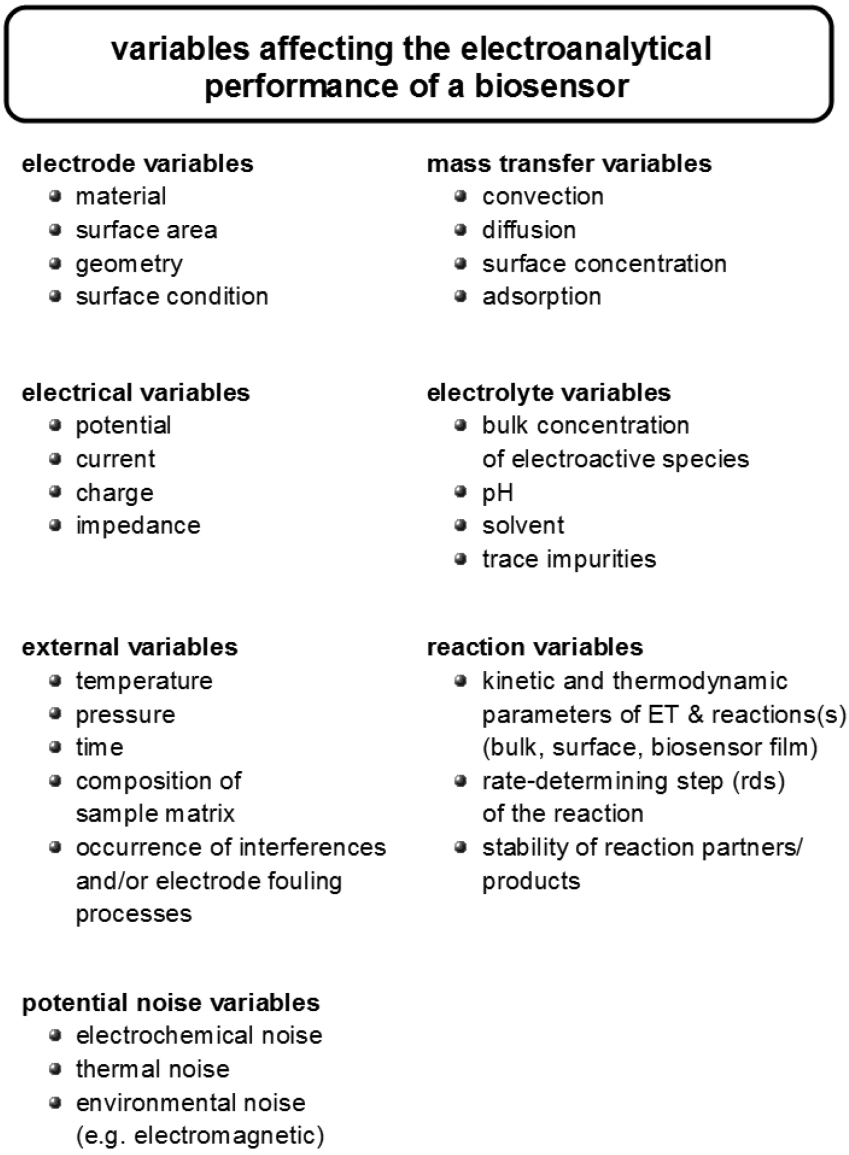


Figure 1.7 Parameters influencing the overall response of a specific biosensor architecture.

respect to general device layout as well as the interpretation of the obtained data. It is indispensable to always have in mind the impact of diffusion, enzyme, and ET kinetics. In addition, the impact of temperature is not negligible. Diffusion, kinetics, selectivity, and overall (bio)sensor performance are highly temperature dependent [199, 200].

A detailed list of variables affecting the electroanalytical performance of a biosensor (Figure 1.7) enables an awareness of the main "adjusting screws" of a particular system for further optimization.

Knowing the most influential parameters of a specific biosensor architecture is the basis to understand and fine tune the performance of these devices in a rational manner. Figure 1.8 summarizes the key features of typical biosensors and lists several that are of additional importance for commercial devices. Among these, selectivity, sensitivity, accuracy, response, and recovery time as well as operating lifetime are some of the most important key factors. Keeping in mind the needs of the specific analytical task of interest, it seems to be necessary to characterize at least the key parameters mentioned in Figure 1.8 in order to specify the analytical performance of a biosensor design.

It is indispensable to elucidate the rate-limiting steps of the overall reaction sequence in order to develop an appropriate optimization strategy. Thus, mathematical and chemometric approaches are expected to promote a deeper understanding of the processes involved. As a useful source of information for modeling biosensor responses, a related book chapter by Bartlett *et al.* [201] is recommended.

An important aspect of biosensor optimization is the elimination of interferences, or at least a reduction of the impact of interferences. In many samples there are components that either directly react at the electrode surface or the involved redox centers or interfere with the biological recognition reaction (e.g., inhibitors or other substrates for the enzyme). In addition, leakage from the sensing layer, loss in enzyme activity or electrode fouling may occur. Thus, changes in sensitivity and baseline drifts may occur during biosensor operation. Therefore, suitable strategies for calibration are needed to ensure reproducible and quantitative results. For real-world applications it is imperative to characterize and optimize the biosensor architecture under actual measuring conditions. A useful review by Phillips and Wightman [202] discusses critical guidelines for the validation of *in vivo* microsensors.

Electroanalytical methods and biosensor architectures offer various strategies to tune selectivity for the analyte of interest [203]. Among the diverse approaches to tackle problems arising from interferences and electrode fouling are the following. (i) Size exclusion and/or charge repulsion are utilized when additional films or membranes are placed as upper layers on top of the actual sensing layer. Typical membranes are polymethylcellulose, Nafion, hydrogels, polypyrrole, *o*-phenylenediamine, polyeugenol, and other electrodepositable films (conducting or nonconducting). However, this approach is used at the expense of biosensor response time. (ii) Use of suitable redox mediators allows operation at moderate potentials below the potential of abundant interferences such as ascorbic acid. (iii) The applied potential is a useful tool to discriminate between different electroactive species under the assumption that the redox waves are distinguishable. Electroanalytical techniques such as cyclic voltammetry (CV), fast-scan CV, differential pulse voltammetry (DPV), square wave voltammetry (SWV) or differential pulse amperometry (DPA) as well as multiple pulse amperometry [204, 205] are useful for determining several species in parallel and discriminating between them.

key features of a biosensor

key parameters of biosensor performance

- analyte
- employed biocomponent for recognition of analyte
- type & composition of sample matrices
- selectivity
- sensitivity
- dynamic range
- detection limit
- response time
- reproducibility
- precision
- stability (in use, long-term, storage)
- calibration approach (requirements, intervals, drift)
- duration to reach baseline

additional parameters of biosensor performance, e.g. relevant for commercialization

- cost per measurement
- duration of measurement
- turnover of measurements (time resolution)
- warm-up time
- sample volume
- measuring temperature
- size, weight & price of the device
- costs, size & weight of required instrumentation
- delivery time
- development status of the device/procedure (commercial product, standard procedure (in house or commercial, established research procedure, basic research, proof-of-principle)
- time between maintenance checks
- potential to be coupled with other analytical techniques
- data storage and processing

Figure 1.8 List of key characteristics of a biosensor.

(iv) The choice of the immobilization matrix can be important for the susceptibility to interferences. (v) Mathematical models and chemometrics can be employed. One approach utilized the impact of temperature on biosensor performance to improve selectivity [199, 200]. A recent review summarizes for example some of the strategies towards the elimination of interference of glucose biosensors [206].

1.1.8 Application Areas of Biosensors

Today biosensors are mainly used for healthcare applications, controlling industrial processes, and environmental monitoring, as outlined in Figure 1.9. In all cases the biosensor design, packaging, and instrumentation required are dependent on the purpose of the measuring approach. In several cases, the type of sample dictates the biosensor design. For example, if potentially harmful samples such as blood or contaminated waste water are of interest, disposable sensor formats are preferred. Samples can be analyzed off-line in a laboratory, such as glucose testing of patients' blood samples in a hospital laboratory or water samples from rivers. In addition, off-line analysis can also be performed close to the operation side of an industrial plant or process or glucose monitoring can be performed at home by patients themselves. For several applications, however, there is a need for on-line analysis in real time, such as quality control in the food or drug industries or metabolite monitoring at the bedside or during surgery. The requirements for

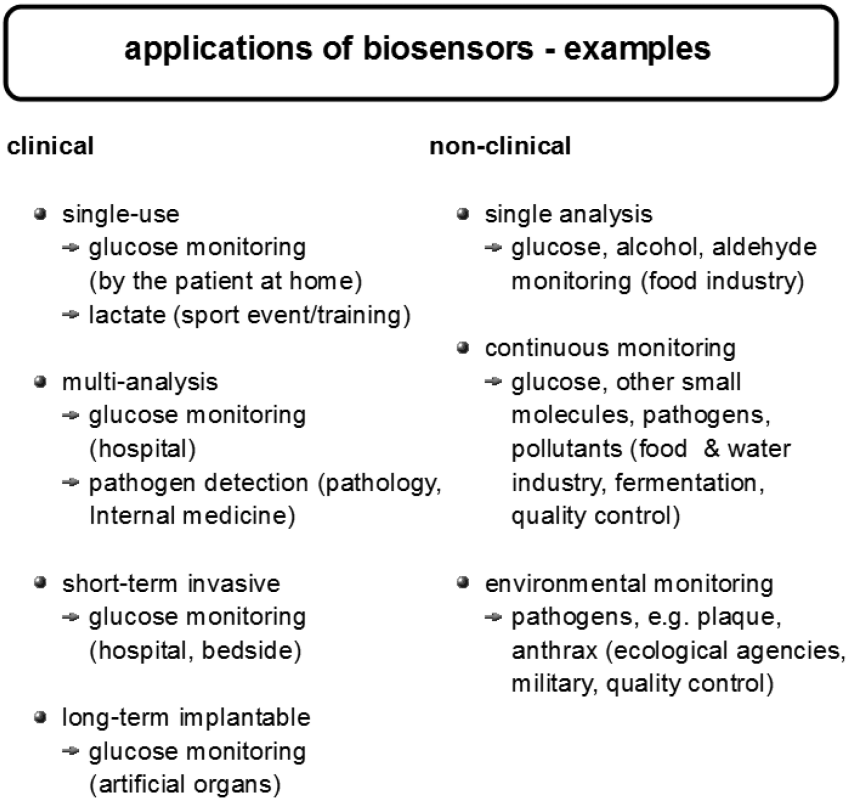


Figure 1.9 Areas of application for biosensors.

a single-use device differ from those for multi-analysis and continuous monitoring and have to be taken into account when considering overall biosensor architecture.

1.2 Criteria for "Good" Biosensor Research

It is obvious that science does not always lead to ground-breaking advancements that are worth publishing. This is also very much true for publications from biosensor-related research. The area of biosensor research is even more susceptible to publications that do not significantly contribute to the present state of the art, since the basic equipment for doing high-level biosensor research is comparatively cheap. Moreover, nearly any modified electrode with an immobilized biorecognition element will show a certain response upon the addition of a specific analyte, leading to a calibration graph, the possibility of determining the pH optimum, etc. This is not intrinsically a sign of low quality if the contribution is otherwise scientifically sound. An example would be a publication that only slightly changes an existing biosensor design, but otherwise is unambiguously supported by technically sound data and interpretation. However, there are also a large number of publications that suffer from technically wrong or biased data acquisition, processing, and interpretation or an insufficient amount of data for the hypothesis proposed.

To do research is basically to generate knowledge which is made available to the scientific community via publication. The main aim is that other scientists will be convinced by the scientific approach and they can adopt the strategy or scientific principle for answering their own research questions. Thus, criteria for "good to excellent" biosensor research have to be measured in terms of the following questions:

i) Does the research work introduce a novel sensing principle, a novel signal amplification strategy, a novel specifically adapted redox mediator with improved properties, a novel immobilization scheme, a novel sensor architecture with tunable parameters? Does the proposed research work contribute to an increase in fundamental knowledge, an in-depth evaluation of the signal transduction mechanism, or an in-depth physicochemical evaluation of the rate-determining steps and the interplay of the parameters in the complex parameter space?

ii) Does the research work introduce novel aspects to an already known sensing architecture, to an already known application, or does it extend a sensing principle to be more general? Does the work include the discovery of surprising results by combining a specific biological recognition element with an already known sensing principle and is there a rational way to understand this surprising result? Does the adaptation of an already known sensing principle to a specific application require innovative features?

iii) Is the proposed research work just a variation of an existing principle by varying the biological recognition element, the electrode material, the size and integration of the electrode? Is there any predictable contribution of the elements of which the sensing layer is composed to the expected signal generation, interference elimination, improvement of long-term or operational stability, etc.?

iv) Does the contribution solve a previously unsolved scientific question? Can the principle be the basis for improvements in sensitivity, selectivity, applicability?

v) Is the selection of the compounds used for creating the sensor architecture based on buzzwords such as nanomaterial, nanosensor, etc.? Is the effect of the material used correlated with the meaning of the buzzword or is it just used because of the buzzword?

vi) Does the complexity of the sensor architecture allow a rational investigation of the complex influence of all compounds used on the final sensor output? Is it an effect or a scientific result? If a novel effect is discovered, can it be explained by a scientifically sound argumentation chain? Is it possible to design control experiments to provide evidence for the hypothesis concerning the sensing mechanism?

vii) Is there any possibility of reproducing the measurements in the same laboratory at another time or even in a different laboratory? Are all results derived from one sensor? Is there any statistical evaluation of the repeatability of the sensor fabrication protocol, and of the obtained signals?

viii) If the results are sound and justified, do the authors try to benchmark the results with existing sensing strategies for the same analyte and in the same application?

Taking the above into account it seems to be straightforward to distinguish between fundamental biosensor research and biosensor development. For fundamental biosensor research, the discovery of novel sensing strategies or bioelectrochemical signal transduction schemes, the elucidation of the fundamental processes, and the understanding of the complex parameter interplay that finally leads to the observed sensor signal are the focus of the research. For biosensor development, an existing sensor principle has to be adapted to a specific application taking into account costs, storage time, reproducibility, calibration, validation, legal consequences, etc. Presently, many biosensor papers that predominantly deal with fundamental biosensor design try to include some application aspects by showing that some standard samples can be measured at a required quality. However, these results are most often obtained in the research laboratory using the standard addition method and well-trained personnel. On the other hand, papers on application-oriented research often try to include basic mechanistic studies at a limited depth. This is also reflected by the editorial policy of international journals accepting work on biosensors. Recently, fundamental studies are

more often published in physical chemistry journals while the biosensor journals have shifted to be more application oriented.

Ideally, a biosensor design needs to be adaptable to a certain application or analytical task. If the design is a general principle, the biosensor performance needs to be tunable to the needs of a specific analytical task and be open for modifications leading to a broader range of analytes or applications. If the mechanism behind the biosensor function is at least partially understood, a fine tuning of the sensing layer or a rational adaptation of the sensor design may become possible. As pointed out above, the parameters influencing and limiting the overall sensing process of a biosensor are often not fully understood. In a classical approach, it was assumed that the parameters are linearly independent. Thus, it was assumed that one may independently vary one parameter while the others are kept constant. For example, parameters such as transducer type and pretreatment, enzyme and mediator concentration and their ratios within the film, type of immobilization matrix, immobilization parameters, and film thickness are varied. A rational optimization approach includes that the main parameters affecting the overall ET pathway and hence the final sensor response have to be investigated in order to find reasonable tools for tuning the performance of the selected biosensor design. As a matter of fact, it is well known that it is impossible to keep parameters constant while changing others. For example, if the enzyme loading is increased, the film thickness, the diffusional properties for the substrate and the products, the counter-ion movement, possible ET reactions, etc., may be altered simultaneously. Thus, a "pseudo" rational approach has to be complemented by combinatorial approaches in which the overall parameter space is addressed by means of a large number of measurements after permutation of all possible influencing parameters. The knowledge gained should be comprehensively summarized in a related publication. The final consideration should be whether the sensor fabrication process described in a paper can be directly repeated successfully in another laboratory leading to similar sensor responses.

1.3 Defining a Standard for Characterizing Biosensor Performances

Considering the workflow as proposed in Figure 1.10 it becomes obvious that it is essential to characterize a specific set of key parameters mainly determining biosensor performance. A selection of suitable key parameters is given in Figure 1.8. With respect to the particular needs of a specific analytical task one needs to decide which performance parameters make sense to be evaluated at a certain stage of the process. For example, Phillips and Wightman evaluated guidelines for the validation of *in vivo* microsensors [202]. In the following, the most common and most important characteristics of biosensors are discussed.

Though a biological recognition reaction is typically very selective, interferences may occur due to substances other than the analyte of interest. Such interferences can be converted by the biorecognition element or at the transducer surface and

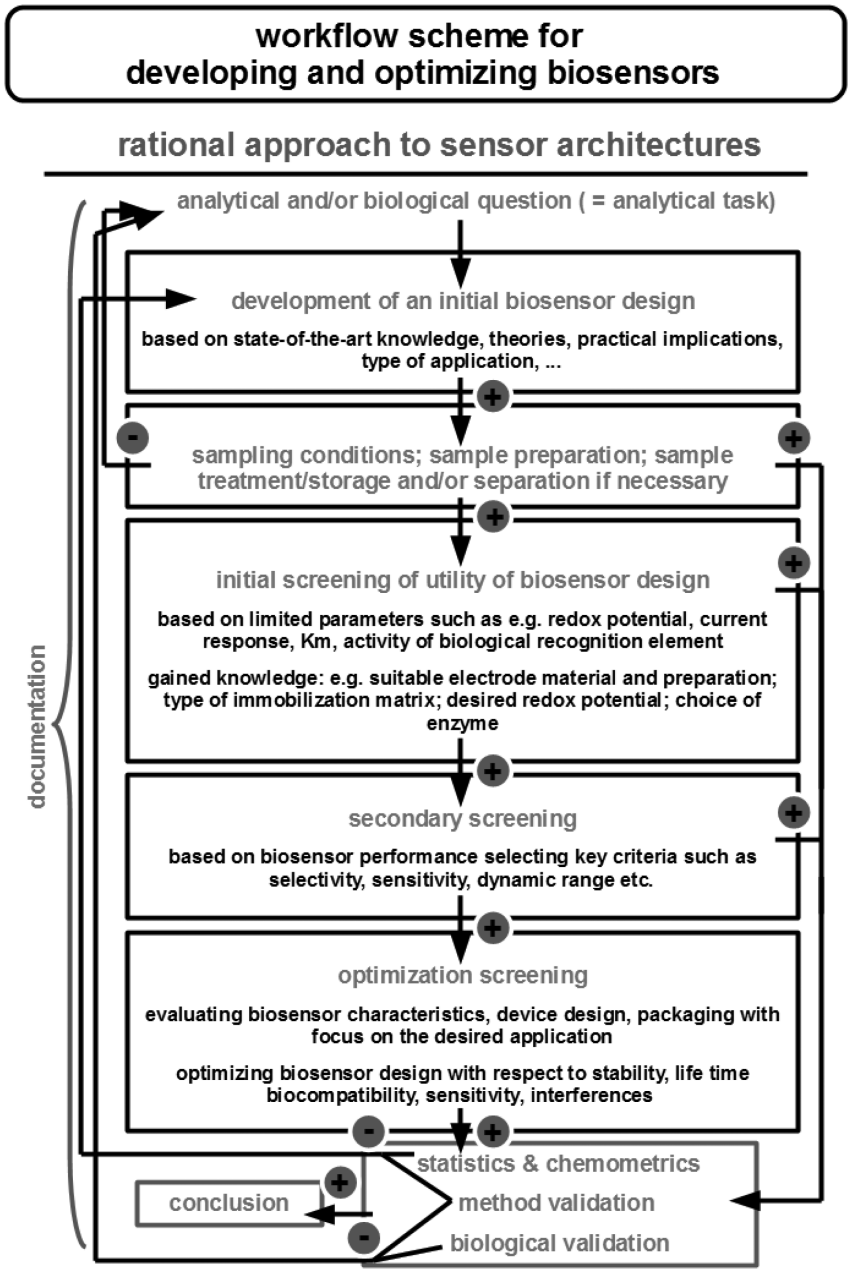


Figure 1.10 Workflow for successful biosensor research.

thus create false-positive results. The *selectivity* of a biosensor is characterized by the selectivity coefficient. The selectivity coefficient is defined as the quotient of the respective binding constants of the analyte of interest A and a potential interference I with the biorecognition element B:

$$B + A \rightarrow BA;\ K_{BA}$$

$$B + I \rightarrow BI;\ K_{BI}$$

$$K_{AI} = \frac{K_{BA}}{K_{BI}} K_{AI}$$

In addition to selectivity, *sensitivity* (S) is a vital parameter of the performance of a biosensor. Sensitivity is defined as the slope of change in signal with change in concentration:

$$S = \frac{\mathrm{d}(\text{signal})}{\mathrm{d}(\text{concentration})}$$

This is, however, only straightforward if the sensor response is linear.

The *linear range* of a sensor is defined as the range in which the sensor signal is proportional to a change in concentration. Linear range should not be confused with dynamic range. *Dynamic range* describes the range in which a change in concentration will lead to any sort of noticeable change in signal. Typically, the whole dynamic range will not yield a signal suitable to determine the analyte of interest in a reliable way. In most cases the *working range* of a sensor corresponds to the linear range.

The *limit of detection* (LOD) of a biosensor is one of the most important parameters to be determined. That holds especially true when disease markers have to be determined. The LOD is typically defined as

$$\mathrm{LOD} = k \times \mathrm{std}_{\mathrm{background}}$$

where k is the *signal-to-noise ratio* and $\mathrm{std}_{\mathrm{background}}$ is the standard deviation of the background signal. The value of k can be chosen deliberately depending on the desired accuracy of the LOD but is typically 3. Another definition describes the smallest detectable concentration of an analyte c_{LOD} as

$$c_{\mathrm{LOD}} = \frac{\mathrm{std}_{\mathrm{background}}}{S}$$

where S is the sensitivity.

It has to be pointed out that the LOD cannot be properly discussed without any knowledge of the binding constant of the primary biorecognition process. If the binding constant of the biorecognition process is, for example, in the nanomolar range, a detection limit far below seems to be thermodynamically impossible. Thus, it is very important to gain a solid understanding about the difference in which signal can be measured and amplified and which limit of detection can be achieved based on a distinct biological recognition process. Hence, it would be very helpful if together with a LOD an estimation of the binding constant was given.

Terms rarely mentioned in the biosensor literature are *accuracy* and *precision*. Accuracy describes the agreement between the average of the measured value and a reference value. To determine accuracy is relatively straightforward. Normally sensors are tested using solutions with well-known concentrations of the analyte ("true value"). Obtained values can easily be compared to the true value.

Precision describes the scatter of measured values around the average of the measured values. Precision is much more important for biosensor performance than accuracy. Accuracy can be influenced by systematic errors that can be corrected. However, a sensor producing values that are scattered will not be regarded as very reliable. Some confusion can be found in the use of terms that are measures for the precision of a sensor, *repeatability* and *reproducibility*. Many papers report work as highly reproducible even though reproducibility is defined as the *between-laboratory precision*. That simply means that a sensor's measurements are reproducible if the same results are obtained in different laboratories with the same sensor architecture [207]. This is, however, rarely tested. Most authors really test the repeatability of a sensor. Repeatability is the *in-laboratory precision*. In-laboratory precision means that the sensor yields the same value of, for example, concentration in repeated measurements. Repeatability also means that the same sensor architecture will yield the same result if manufactured in the same laboratory. It would hence make more sense to speak about the standard deviation of a single sensor and to analyze the repeatability of the respective sensor architecture than to speak about reproducibility if the latter has not been tested.

Another measure for sensor performance that is a potential source of confusion is the *stability* of a sensor. Sensor stability can mean different things including but not limited to *working stability*, *storage stability*, and *long-term stability*. Working stability (sometimes also called usage stability) describes the stability of the sensor during continuous operation. Storage stability obviously describes the stability of the sensor upon storage, while long-term stability describes the sensor stability during operation in a sample solution but not necessarily continuous operation. It already becomes clear that the "stability" of a sensor will rarely mean the same thing for different sensor architectures.

Sensor performance is also characterized by commercial means, most importantly *time per measurement* and *cost per measurement*. Again, there is no one-size-fits-all definition for these terms and one should emphasize on being transparent in the way in which these numbers are determined.

In conclusion, a number of parameters are helpful when characterizing biosensor performance. It is, however, extremely important to be precise in the use of these terms. A clear definition of the measured variable is mandatory in any case and needs to be reported in a transparent fashion.

1.4 Success Stories in Biosensor Research

This section aims at discussing how "good" biosensor research inspires the scientific community to achieve advancements that reach from novel basic concepts to

real-world applications. To do so, selected examples with a significant scientific impact are presented. This selection is not exhaustive, and there are many other possible success stories, but is just some examples selected by a very personal view.

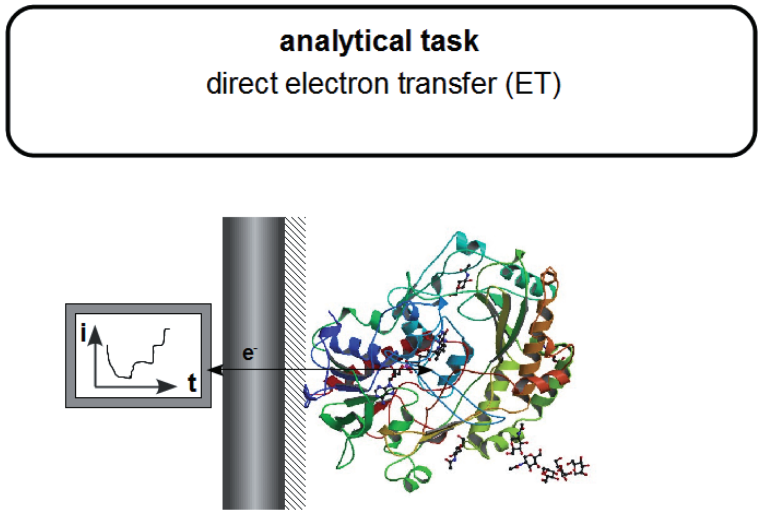
1.4.1
Direct ET Employed for Biosensors and Biofuel Cells

The fundamentals of biosensors that exhibit direct ET between biological recognition element and electrode have been discussed thoroughly in the section on third-generation biosensors (Section 1.1.5). This section concentrates on highlighting the major contributions made in the area of direct ET in biosensors and biofuel cells. Though the latter is not a sensing application it draws from the same concepts as third-generation biosensors, and biofuel cells are a striking example of the continued development of redox-enzyme electrodes.

The realization of direct ET poses some challenges that already have been outlined above. Figure 1.11 summarizes the key features and challenges of this approach. The protein shell may prevent ET processes due to a large distance between active site and electrode if the active site is deeply buried within the protein. Proteins with suitable characteristics for direct ET have to be securely fixed to the electrode surface in an orientation facilitating direct ET. The orientation of the redox enzyme towards the electrode surface is the main challenge in designing direct ET pathways. Basically, two cases can be distinguished: direct ET between the active site of the enzyme or direct ET via an internal electron pathway within the protein. The first case is relevant for rather small redox proteins or for redox enzymes exhibiting an active site closely located at the outer protein shell. The second case mainly applies to multi-cofactor enzymes. However, the orientation is critical in both cases since either the redox-active center or the cofactor closest to the protein shell has to be located within a productive ET distance [27, 208–211]. Recently, a study investigated the different orientations of recombinant horseradish peroxidases to gold surfaces [212].

Early work on the direct electrochemistry of redox proteins suffered from the poor stability of those proteins at electrode surfaces. Though a significant body of work demonstrates the direct electrochemistry of redox proteins at graphite electrodes [213], the major breakthrough came with the use of surface-modified electrodes that provided a substrate for the stable orientation and immobilization of redox proteins at the electrode surface [208, 214, 215]. Surface-modified electrodes allowed for the study of the direct electrochemistry of cytochrome c on 4,4′-bipyridyl-modified gold electrodes [216]. As previously mentioned, SAMs on electrode surfaces are useful tools to realize biosensors suitable for direct ET [52]. The creation of monolayers on electrode surfaces with immobilized recognition elements is not limited to SAMs; a review by Willner and Katz summarizes the different approaches to realize covalent binding of enzymes to functionalized electrode surfaces as well as strategies to employ modified enzymes (e.g., protein conjugates) [186].

Enzymes that have been much studied in direct ET configuration include peroxidases [217, 218], especially horseradish peroxidase [219, 220], laccase [120, 121],



key features & challenges for direct ET

- immobilization of the enzyme on the electrode surface (impact on the structure of the enzyme, on the enzyme activity, on the diffusion of the substrate, on the ET kinetics, ...)
- orientation of the active site of the enzyme to the electrode surface
- orientation of the cofactor(s) of the enzyme to the electrode surface
- distance(s) between the redox relays taking part in the direct ET pathway
- selection of a suitable redox potential for the measurement (driving force of the reaction, catalytic current should be observed at the redox potential of the redox relay (e.g. a cofactor) communicating with the electrode surface)
- efficiency of the overall ET process

Figure 1.11 Biosensors based on direct ET.

and dehydrogenases [221] including fructose dehydrogenase [222], cellobiose dehydrogenase [223–225], and quinohemoprotein alcohol dehydrogenase [226]. It is important to keep in mind that for characterizing biosensor responses it is important to check if the enzyme employed is still able to efficiently catalyze the physiological reaction at a rational range of redox potential.

It is very important to define criteria to unequivocally proof a direct ET pathway between an immobilized redox protein and an electrode surface. The first important prerequisite is the occurrence of the direct electrochemistry of the redox cofactor inside the protein in the absence of the substrate. Hence, a reversible redox wave in a cyclic voltammogram of the protein-integrated cofactor has to be visible with a formal potential which clearly shows that the protein structure is not

disturbed during the immobilization process. The second and most important prerequisite is that upon addition of the substrate the catalytic current increases at the redox potential of the protein-integrated cofactor without any significant overpotential at least at slow scan rates. If these two features in the cyclic voltammogram are not seen, a direct ET pathway can be excluded.

The development of enzyme electrodes with immobilized redox enzymes in direct ET communication was the prerequisite for the design of enzyme-based biofuel cells. For representative recent reviews see [70, 227, 228]. A fuel cell generally converts chemical energy into electrical energy in a continuous process as long as fuel is supplied. Biofuel cells convert chemical energy by means of a biocatalytic process as can be seen in Figure 1.12. Typically, enzyme-based biofuel cells consist of at least one enzyme electrode on either the cathode or anode side of the fuel cell or enzyme electrodes on both the cathode and the anode sides. The high specificity of fuel conversion by enzyme electrodes allows for membrane-free

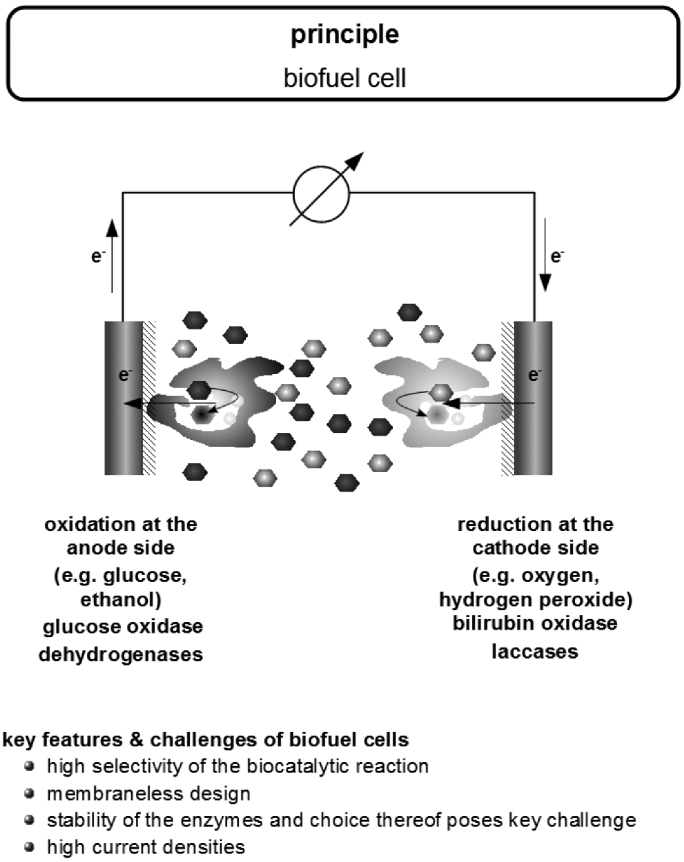


Figure 1.12 Principle of biofuel cells.

designs in which the cathode and anode reactions proceed in a single compartment.

The typical reaction on the cathode side is the reduction of oxygen at either a platinum catalyst or an electrode. The ET pathway at the enzyme electrode can be mediated (Section 1.4.3) or direct. Enzymes that have been employed in biofuel cells relying on direct ET include laccases [229, 230] which, however, suffer from a pH optimum in the acidic range and inhibition by halide ions. Thus, despite their favorable high potential for oxygen reduction they show poor stability in human tissue and fluids. Alternatively, bilirubin oxidase has been used as oxygen reduction biocatalyst in biofuel cell cathodes due to the better pH optimum and the insensitivity towards chloride ions. However, bilirubin oxidase has an about 200 mV lower reduction potential for molecular oxygen [231]. Alternatively, reduction of peroxide can be the cathodic reaction in biofuel cells. Relying on the experience with peroxidase-modified electrodes in biosensor research, electrodes modified with peroxidases have been shown to be highly efficient biocatalysts in biofuel cells [232, 233]. Microperoxidases that are truncated forms of cytochrome c have also been employed in biofuel cells [234] in which they convert hydrogen peroxide that is supplied by the enzymatic reaction of GOx with glucose and oxygen.

The most common reaction at the anodic side of biofuel cells is the oxidation of sugars which relies on the catalytic properties of oxidases. This class of enzymes has, however, usually poor potential for direct ET. Direct ET on the anodic site was, however, described for a number of hydrogenases [235, 236] and cellobiose dehydrogenase [225, 237, 238]. Enzymatic catalysis by means of direct ET was also realized on conducting graphite or TiO_2 particles [239, 240].

In conclusion, biofuel cells have a tremendous potential to be applied in, for example, implantable sensors or similar functional devices. They are a striking example of the continued development and application of the principles of biosensors employing direct ET.

1.4.2
Direct ET with Glucose Oxidase

Glucose sensors are *the* success story with respect to biosensor research and application. Today, diabetes patients are able to monitor their blood glucose levels on their own at home with commercial devices [59–65]. All these successful devices use a mediated ET pathway with natural or artificial redox mediators irrespective of whether GOx or other glucose-converting enzymes such as pyrroloquinoline quinone (PQQ)-dependent glucose dehydrogenase or nicotinamide adenine dinucleotide (NAD^+)-dependent glucose dehydrogenases are used. Also, there have been continuing attempts to demonstrate direct ET between the active-site integrated flavin adenine dinucleotide (FAD) cofactor of GOx and an electrode surface. However, after solving the crystal structure of GOx [241] it becomes clear that the ET distance from the protein-integrated FAD is large and hence fast ET kinetics are unlikely.

Despite the knowledge about the large ET distance, there is an ongoing attempt to propose direct ET between GOx and specifically prepared electrode surfaces (Figure 1.13). However, the specific nature of GOx, namely its reaction with its natural electron acceptor O_2 and the probably unwanted generation of H_2O_2, may lead to glucose-proportional current changes which are incorrectly attributed to direct ET reaction. Even if all traces of molecular oxygen are removed the source for the obtained current changes is often not clear. For example, if carbon nanotube-modified sensor surfaces are used, it is difficult to unequivocally confirm that no traces of the metal catalyst used for the growth of the nanotubes are left providing free-diffusing metal complexes which may serve as redox mediator for shuttling electrons between the active site of GOx and the electrode surface. As a

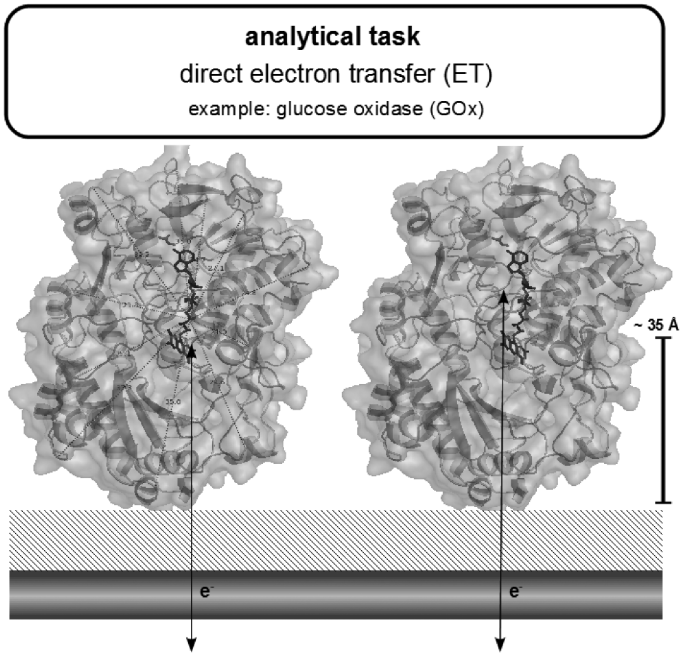


Figure 1.13 Direct ET between GOx and an electrode surface.

matter of fact, these potential sources for free-diffusing redox species become increasingly unpredictable the more different components are used to fabricate the sensor.

Thus, as already pointed out, there are clear presuppositions which have to be met before a potential direct ET pathway may be discussed. First, the FAD/$FADH_2$ redox wave in a cyclic voltammogram has to be visible at the potential which is characteristic for the cofactor bound within the active enzyme. Moreover, upon addition of glucose a clear oxidation current has to commence at this redox potential without any significant overpotential. In our opinion, this is one of the main sources for falsely assuming direct ET. It is known that FAD is not covalently bound to the enzyme and hence can become dissolved in the electrolyte during denaturation of the protein. The redox potential of free-diffusing or surface-adsorbed FAD differs from that of FAD located at the active site of the enzyme. Thus, due to the high surface area of the often-used sensor architectures and the comparatively large amount of GOx adsorbed on the electrode surface, the FAD/$FADH_2$ redox couples may often be due to released FAD. Upon addition of glucose, the catalytic current is then not closely related to the observed redox wave and hence is no criterion for a potential direct ET between GOx and the electrode surface. In the following paragraphs a number of recent publications are briefly mentioned in which the source of the observed glucose-proportional current is not completely clear and there may or may not be alternative possibilities to a direct ET process to explain the observed effects.

In the following, a number of recent papers proposing direct ET of GOx will be discussed. A uniformly porous TiO_2 material was synthesized using a carbon nanotube template-assisted hydrothermal method and GOx was adsorbed leading to glucose-proportional currents [242]. Similarly, three-dimensional macroporous inverse TiO_2 opals were synthesized from a sol–gel procedure using polystyrene colloidal crystals as templates. Glucose oxidase was successfully immobilized on the surface of an indium tin oxide electrode modified using inverse TiO_2 opals. Cyclic voltammetry showed stable and well-defined redox peaks for the direct ET of GOx in the absence of glucose. This redox peak increased upon addition of glucose [243]. Along the same lines, direct electrochemistry of GOx adsorbed on boron-doped carbon nanotubes/glassy carbon surfaces [244] or an oxidized boron-doped diamond electrode [245], nitrogen-doped carbon nanotubes [246], exfoliated graphite nanosheets [247], single-wall carbon nanotubes in combination with an amine-terminated ionic liquid [248], and GOx incorporated into polyaniline nanowires on carbon cloth [249] was proposed. Entrapping GOx at the inner wall of highly ordered polyaniline nanotubes [250] or chemically synthesized multi-walled carbon nanotube–SnO_2–Au composites [251], co-deposited GOx–NiO nanoparticles [252], and immobilization of GOx in a natural nanostructural attapulgite clay film-modified glassy carbon electrode [253] have been investigated. Biologically synthesized silica–carbon nanotube–enzyme composites displayed stable redox peaks at a potential close to that of the FAD/$FADH_2$ cofactor of immobilized GOx. The immobilized enzyme was stable for one month and retained catalytic activity for the oxidation of glucose [254].

Direct electrochemistry of GOx immobilized on a hexagonal mesoporous silica-modified glassy carbon electrode was investigated. A pair of redox peaks at a potential of –417 mV was obtained and a diffusion-controlled electrode process with a two-electron transfer coupled with a two-proton transfer reaction process was postulated [255]. However, despite of the well-defined $FAD/FADH_2$ redox process, biocatalytic oxidation of glucose was only possible in the presence of a free-diffusing redox mediator such as ferrocene monocarboxylic acid. This behavior is quite common and supports the assumption that the FAD causing the redox process may be free or surface-adsorbed FAD, which is no longer bound to the enzyme. There are a number of similar studies in which the first criterion, namely the visible voltammogram of the cofactor, seems to be met; however, no electrocatalytic current could be obtained upon addition of glucose. Another series of publications propose direct ET based on a decrease of the electrocatalytic response of the reduced form of GOx to dissolved oxygen [256, 257] or using complex multicomponent immobilization layers with integrated nanomaterials and binders such as GOx–graphene–chitosan [258], dispersed multiwalled carbon nanotubes in a gold nanoparticle colloid stabilized by chitosan and an ionic liquid [259], a carbon nanotube-modified glassy carbon electrode with GOx immobilized within a chitosan film containing gold nanoparticles [260, 261], CdTe quantum dot–carbon nanotube–Nafion films [262], a conductive cellulose–multiwalled carbon nanotube matrix with a porous structure using a room temperature ionic liquid as solvent and encapsulating GOx within this matrix [263], or carbon nanotubes in combination with platinum nanoparticles and chitosan [264].

In all the attempts mentioned above the enzyme was not modified, and hence its size and the large ET distance from GOx to the (nanostructured and high-surface-area) electrode remained constant. Despite the $FAD/FADH_2$ redox wave often being visible in the related cyclic voltammogram, the measured redox potentials varied largely between about –0.49 and –0.41 V which remained without large changes upon addition of glucose. Due to the limitations for direct ET as derived from Marcus theory, these observations are most likely not caused by a true direct ET process but alternative explanations have to be considered despite the observed and repeatedly obtained effects. Alternatively, the ET distance may be decreased by the formation of enzyme–nanoparticle hybrids in which the nanoparticle penetrates into the protein shell [265]. However, in these cases the catalytic current for glucose oxidation is often obtained at high overpotentials. Recently, a more rational approach aimed at decreasing the size of GOx either by preparing genetically modified GOx [266] or by wrapping off the glycosylation shell of the enzyme [267–269]. However, even then it is very hard to distinguish if the catalytic reaction is at the potential of the functional enzyme-integrated FAD or of FAD which may have been released from enzyme molecules.

Thus, despite the large number of publications and the steep increase in the number of publications about direct ET between GOx and modified electrode surfaces, one has to be extremely careful with the possible over-interpretation of the observed effects. The proposed sensors may work fine in dedicated applications; however, it is a fundamental difference if a sensor concept can be applied

and glucose concentrations can be reliably determined or if a basic physicochemical claim about a potential direct ET pathway is suggested.

1.4.3 Mediated ET Employed for Biosensors and Biofuel Cells

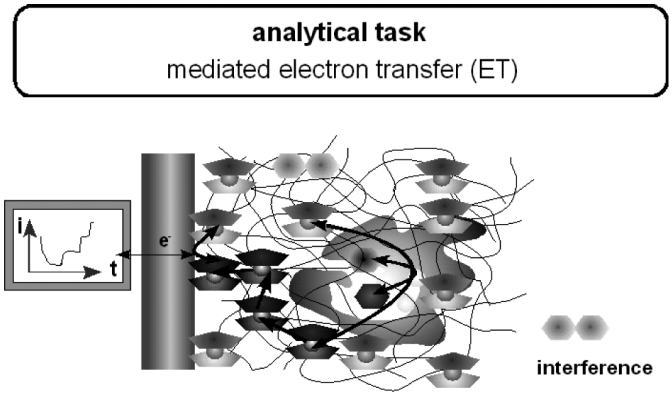
As already described in Sections 1.1.4 and 1.1.6 (mobile or immobilized) mediators and/or conducting polymers can also be employed to shuttle electrons between a redox enzyme and an electrode surface [11, 12, 16, 20, 21, 24, 25, 28, 270–273]. This approach is called mediated ET (see also Figure 1.6). Efficient ET throughout the entire sensing layer is envisaged in order to avoid only the enzyme layer in close vicinity to the electrode surface contributing to the overall current signal.

For many applications, soluble mediators are not suitable. Thus, redox hydrogels (hydrogels covalently modified with a redox-active mediator) are increasingly being used for reagentless biosensor structures and more recently also for biofuel cells. Heller and coworkers introduced osmium complex-modified redox hydrogels as matrices for biosensors [47, 137–139]. It was determined that the linker length between the osmium complex and the polymer backbone has an impact on the sensor response [274]. Most likely, the mobility of the osmium complex is affected by the length and, hence, flexibility of the polymer backbone and results in a higher efficiency of ET if optimized.

The polymer backbone of typical redox hydrogels is highly hydrophilic and is based on, for example, poly(vinyl pyridine) [48, 275–279], poly(vinyl imidazole) [280, 281], poly(acrylic acid) [282], or poly(allyl amine) [283]. Onto these backbones redox mediators, for example, osmium complexes or ferrocene derivatives, are covalently attached. The biosensors are typically realized by dropping a mixture of the redox hydrogel, a bifunctional linker, and the biological recognition element on the electrode surface. The obtained sensing film adheres well on the electrode surface in most cases and swells in aqueous solutions. Thus, the polymer is rather flexible which promotes the ET rate, the mobility of the counter-ions, and the diffusion of the substrate of the enzyme and the resulting reaction products within the sensing layer [284, 285]. The properties of a hydrogel may also provide an enzyme-friendly microenvironment, and even extend the lifetime of the involved biological recognition elements.

Electron hopping between redox relays covalently incorporated at the polymer backbone dominates the ET. Note, however, that often the first ET between the active site of the redox enzyme and the polymer-bound redox relay represents the rate-limiting step of the entire ET reaction. Biosensors have been miniaturized on the basis of redox hydrogels by employing manual dropping or dipping procedures and, for example, needle-type implantable glucose sensors have been fabricated [137, 286–289]. Properties of electron-conducting redox hydrogels were reviewed most recently in 2006 [272]. Figure 1.14 highlights the analytical task and the challenges involved for mediated ET-based devices.

The approach of employing redox hydrogels helps one to obtain higher current densities which are not only advantageous for, for example, long-term glucose



key features & challenges for mediated ET

- efficiency of the overall ET process
- enzyme-friendly formation of the sensing layer
- enzyme-friendly microenvironment (important for biocatalysis and stability)
- pore size of the polymer film should securely incorporate the enzyme
- mobility of substrate and products of the biocatalytic reaction, and counter ions
- incorporation of a suitable amount of biological recognition elements for amplification
- selection of a suitable redox relay (refer to ideal characteristics of a redox mediator) that is preferably covalently attached to the polymer
- The number of redox relays within the polymer chains should facilitate fast ET.
- The contribution of current signal obtained by plain adsorption of the enzyme and/or polymer directly on the electrode surface needs to be significantly smaller than the current signal generated by mediated ET
- reduction of the influence of potential interferences or biofouling processes by selecting suitable film compositions and redox mediators

Figure 1.14 Analytical task of developing and optimizing biosensors based on mediated ET.

determinations but also for biofuel cell applications. Higher current densities compared to those of conventional biosensors are a prerequisite for bringing fundamental studies on biofuel cells closer to real-world applications. Osmium complexes exhibit many properties of an ideal mediator as outlined in Section 1.1.4. For example, their coordination structure is not very much impacted by the oxidation or reduction of the complexes. By modifying the ligand structure the redox potential can be fine tuned to the desired range [272]. The same principle is true for redox-modified electrodeposition paints (EDPs) [146], which were introduced by our group in 2002 [17, 290, 291].

For a rational design of biosensor devices, it is advantageous to aim for non-manual fabrication processes. Electrochemical techniques provide advantages as many polymers can be electrochemically formed or deposited such as conducting

polymers and EDPs, for example. The sensing layer is formed by applying potential cycles or sequences of suitable potential pulses while the biological recognition element is present in the solution [17, 292]. The advantage of this approach is that the films are formed exclusively on the electrode surfaces due to the electrochemical initiation of the deposition process. Thus, miniaturization of model biosensor architectures is straightforward and mass production of devices even at small dimensions is feasible. In addition, by automating the fabrication process, the reproducibility of the obtained biosensors should be improved.

One successful strategy to improve ET rates between enzyme and electrode is the modification of conducting polymers with redox mediators in order to obtain reagentless biosensors [11, 270, 271, 292–299]. The drawback of electropolymerization of conducting polymers is that the reaction is sensitive to oxygen, which complicates fabrication at the industrial scale.

Mediated enzyme electrodes were also realized on combined microscale and nanoscale supports [300]. Bioelectrocatalytic hydrogels have also been realized by co-assembling electron-conducting metallopolypeptides with bifunctional building blocks [301]. More recently, redox-modified polymers have been employed to build biofuel cells [25, 70, 302, 303]. In 2003, an enzymatic glucose/O_2 fuel cell which was implanted in a living plant was introduced [147].

The main potential of mediated ET lies in the increase of current densities, as the essential challenge of designing biofuel cells is to increase the biocatalytic power of these devices. Biofuel cells presently reach a power output in the range of about 10^{-6} to $10^{-3}\,W\,cm^{-2}$. Practical conventional fuel cells operate in the range of about 1 to $10^{8}\,W\,cm^{-2}$ [303]. Taking the calculations from Barton and coworkers into consideration [70], in which, as mentioned above, the theoretical current density of a monolayer was estimated to be about $80\,\mu A\,cm^{-2}$, one would require thousands of layers to obtain a current density above $10\,mA\,cm^{-2}$.

To summarize, the advent of redox-relay modified polymers, such as redox hydrogels, conducting polymers, or EDPs, enabled the development of biosensors that even made it to commercial applications such as implantable glucose sensors. In addition, this approach is now increasingly used for the development of biofuel cells.

1.4.4 Nanomaterials and Biosensors

Without any doubt, nanotechnology has had and is still having an enormous impact on science. When speaking of nanotechnology one typically assumes that structures are used with at least one dimension being in the sub-100 nm range. The advantages of and new possibilities offered by nanotechnology are manifold. Materials exhibit new properties when scaled down from bulk material to nanometric dimensions. These properties can be precisely fine tuned, thus allowing for the fabrication of defined structures and materials optimized for a certain purpose. Consequently, nanomaterials and concepts from nanotechnology have been much employed in biosensor development. Several reviews on the topic [182,

304–306] provide a detailed overview of the possibilities of nanotechnology in the field of biosensor research. The following summarizes the most important trends.

The main challenges in the application of nanomaterials for biosensor designs are the definition of the material properties, the reproducible synthesis of materials with suitable properties, and the meaningful application of nanotechnological concepts to biosensors. Definition of material properties and, thus, the choice of materials are common to other areas of biosensor research and have been discussed earlier in more general terms. The question of how to synthesize or otherwise access these materials will not be answered exclusively by the biosensor expert. Instead, multidisciplinary effort will be necessary to obtain nanomaterials with properties as required for a novel biosensor design. The seemingly most challenging task of applying nanotechnology to biosensors is to really make use of "nano features" and not simply using nanomaterials without them adding value to the biosensor architecture. In the area of biosensor research some features of nanostructures become important in addition to pure material properties. For instance, in nanometric structures diffusion lengths become very short and hence mass transport is highly efficient. Since mass transport is crucial in many biosensor designs, an increase or at least a change in sensor performance can be expected from using nanometric structures.

There are basically three broad categories of approaches towards nanobiosensors and in particular in electrochemical nanobiosensor development. The modification of a (macroscopic) transducer with nanomaterials is the first of these approaches. In electrochemical biosensors, this would translate into large electrodes modified with nanomaterials. The second approach is the miniaturization of the transducer, namely the use of nanoelectrodes [307] or other miniaturized circuitry of nanometric dimensions. The modification of biomolecules with nanomaterials or coupling of biomolecules and nanomaterials is the third category of approach towards nanobiosensors. Of course the lines between these approaches are blurred and some sensor designs may draw from more than one of these concepts.

1.4.4.1 Modification of Macroscopic Transducers with Nanomaterials

There is an enormous variety of nanomaterials that can potentially be employed in biosensor architectures. The most prominent among them are metal nanoparticles [304], quantum dots [308], and carbon nanotubes [309–311]. All of them have been employed in biosensors though not necessarily exclusively electrochemical biosensors. Quantum dots (QDs) offer unique absorption properties making them highly suitable for the construction of biosensors with optical readout. The most diverse electrochemical nanobiosensors are, however, obtained from carbon nanotubes (CNTs) which offer a wide range of different applications.

CNTs were discovered in the early 1990s [312]. CNTs have a tubular structure of closed topology and consist of hexagonal honeycomb lattices made up of sp^2 carbon units. A schematic of the structure of CNTs is shown in Figure 1.15. The diameters of CNTs are typically several nanometers. The length of CNTs can be up to several micrometers. Two basic forms are distinguished, single-walled

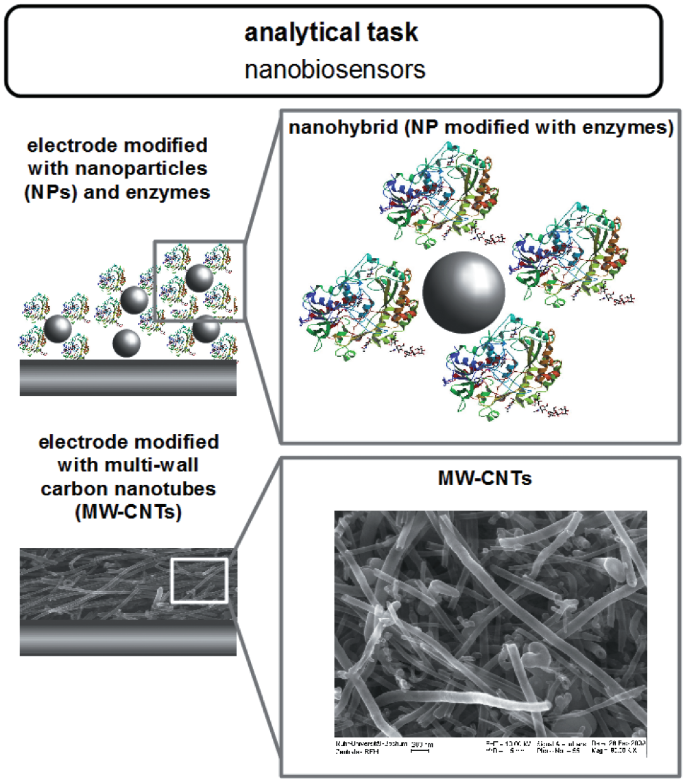


Figure 1.15 Analytical task of nanobiosensors.

carbon nanotubes (SWCNTs) and multiwalled carbon nanotubes (MWCNTs). Besides their chemical stability [313], one of the most interesting characteristics of CNTs for electrochemical biosensors are their ET properties [314]. The ET properties of CNTs can be modified by surface groups such as oxygen, NO_2, or amino groups. Electrodeposition and other forms of growth of metal nanoparticles on CNTs result in another class of nanomaterials with high application potential in electrochemical biosensors [315]. The suitability of CNTs as immobilization matrices retaining or even enhancing the activity of the respective biomolecule has been discussed [316]. In addition, the large surface area of CNTs results in a large active electrode area and CNTs can prevent electrode fouling such as caused by NADH oxidation [317].

The fabrication of electrodes (often glassy carbon or gold electrodes) modified with CNTs typically suffers from the low solubility of CNTs in most commonly used solvents. Hence, CNTs are in many cases dispersed within solvents or polyelectrolytes and drop-coated onto the electrode to be modified. Alternatively, CNTs are incorporated within composite binding materials such as Teflon [318]. Another route to CNT-modified electrodes is the direct growth of CNTs on the electrode material [319]. Electrochemical biosensors based on CNTs have been used in the determination of a wide variety of analytes including glucose, fructose, cholesterol, lactate, catechols, hydrogen peroxide, alcohols, cholines, and organophosphates, as recently reviewed in [310], as well as DNA and proteins [320]. With the level of pioneering work left behind, the powerful combination of biorecognition and extraordinary ET properties and material properties of CNTs can be expected to yield even more high-performance electrochemical biosensors in the near future.

1.4.4.2 **Nanometric Transducers**

This section highlights two trends in nanobiosensing. First, the use of nanofluidics [321, 322] in biosensing and, second, the use of nanoelectrodes [307] and nanoelectrode arrays [323] will be briefly discussed.

Nanofluidics is part of the field of so-called lab-on-a-chip analytical devices which integrate all essential tasks of an analytical problem into a chip-based format [45]. Lab-on-a-chip devices originate from microsystems technology and have several of advantages over conventional instrumental analysis, such as cost-effectiveness due to small material amounts used, time-effectiveness due to small diffusion lengths and therefore extremely efficient mixing of reagents, and other transport phenomena that can be employed to efficiently separate reagents. Furthermore, lab-on-a-chip devices are ideally suited for automated analysis allowing for high-throughput screening. These advantages become even more pronounced when the devices are of nanometric dimensions. In nanofluidic devices, at least one dimension of the device is close to the Debye length and hence transport phenomena not known at the macroscale and even microscale predominate. Biosensing employing nanofluidics includes immunoassays [324] among other analytical schemes such as reviewed in [322].

The electrochemical properties of nanoelectrodes differ significantly from those of macroelectrodes. Like microelectrodes [325], nanoelectrodes are characterized by a hemispherical diffusion field (whereas at macroelectrodes, linear diffusion dominates in amperometric measurements and voltammetric experiments at slow and moderate sweep rates). Consequences of the hemispherical diffusion field are the fast establishment of a stationary diffusion current, high current densities, and a favorable signal-to-noise ratio. Hence, nanoelectrodes have proven to be highly sensitive probes of biorecognition reactions. Often nanoelectrodes are employed as nanoelectrode arrays. Interdigitated nanoelectrode arrays allowed for the label-free detection of DNA using redox mediators [326]. Just as microelectrodes were much employed as probes in scanning electrochemical microscopy [327, 328] to study the immobilization processes of biomolecules on surfaces [329, 330],

nanoelectrodes have the potential to allow for an even more detailed mapping of biological activity.

1.4.4.3 Modification of Biomolecules with Nanomaterials

The direct modification of biomolecules with nanomaterials resulting in biomolecule–nanomaterial hybrids offers interesting possibilities for biosensing. Gold nanoparticles can be used to immobilize enzymes or other biorecognition elements on electrodes or other supports. However, in this case the nanoparticles often just function as a linker and the sensor architecture does not benefit from a unique property due to nanometric dimensions. In contrast, the already mentioned unique optical properties of QDs make these materials well suited as fluorescent labels in optical sensors [331, 332] really taking advantage of a nanofeature.

The use of nanoparticle–enzyme hybrids has been recently reviewed [333, 334] as has been the use of nanotechnology in the manipulation of redox systems at an earlier stage [335]. The wiring of enzymes by redox hydrogels or osmium-modified EDPs is the subject to another part of this chapter. Such an establishment of electrical contact between a redox enzyme and an electrode can also be achieved by nanoparticles. Standing out in this field of research is the wiring of redox enzymes by gold nanoparticles [265]. In this work, GOx was reconstituted with a gold nanoparticle (1.4 nm in diameter, corresponding to the size of the redox center of the enzyme) that was functionalized with the enzyme's cofactor FAD. Such enzyme–nanoparticle hybrids were assembled on gold electrodes leading to exceptionally good electrical contact between the enzyme redox center and the macroscopic electrode.

In conclusion, nanotechnology has contributed significantly to recent developments in biosensor research. Modification of macroelectrodes with nanomaterials has resulted in new exciting ET properties and biocompatibility. Nanometric transducers have been used to obtain new classes of biosensor devices. Finally, biomolecule–nanoparticle conjugates show a promising application potential in biosensor development. An aspect of nanotechnology that is rarely mentioned is the potential harm of nanomaterials towards health. Though a significant effort has been put into the research of this field [265, 336, 337], the consequences of the use of nanomaterials in everyday life are not yet fully understood. It seems, however, that the potential risks of nanomaterials are by far outweighed by the possibilities offered by nanotechnology.

1.4.5 Implanted Biosensors for Medical Research and Health Check Applications

As depicted in Figure 1.16, (electrochemical) biosensors are either placed in laboratory animals for fundamental (patho-)physiological and neurochemical *in vivo* measurements or implanted in the human body for health check purposes and metabolite monitoring. In the field of *in vivo* medical research, enzyme-based

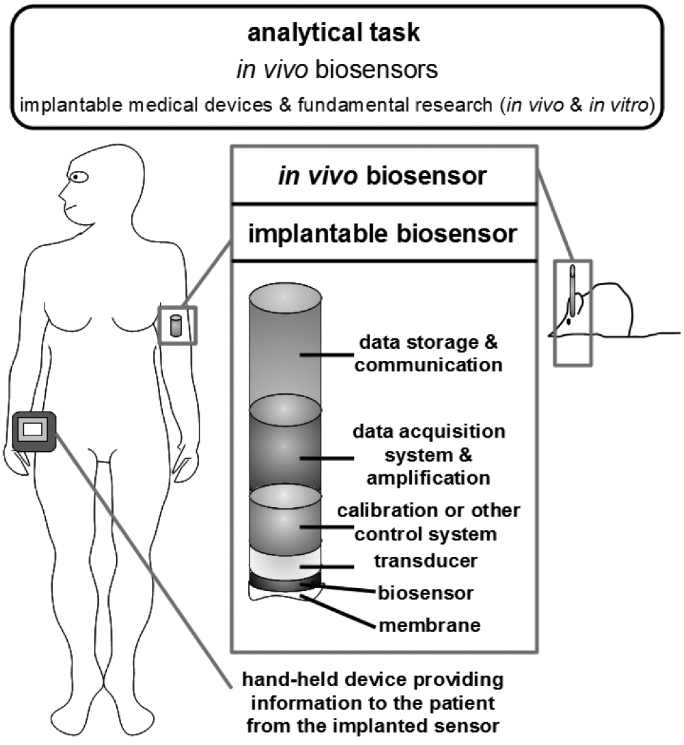


Figure 1.16 Analytical task of developing and optimizing *in vivo* biosensors.

analytical tools are often used for spatially confined measurements of their corresponding target species in preselected regions of living test subjects.

The main challenges in the field of implantable sensors are the stability of the sensor, the selectivity of the sensor, and the biocompatibility of the sensor. First of all, the sensor must not be rejected by the body. When implanted, the sensor should operate for a prolonged time to justify any surgical procedure necessary for the introduction of the sensor into the body. Even when these two challenges are met, the sensor has typically to deal with a very complex sample matrix, most commonly blood. *In vitro* sensors have to cope with the same demands in terms

of their selectivity while long-term stability is usually not such a critical issue. However, *in vitro* sensors also have to be biocompatible in such a way that their presence should not influence the biological environment in which they operate.

At present, the highly heterogeneous rat or mouse brain environment is probably most prominently addressed by *in vivo* and *in vitro* biosensors. Nevertheless, other parts of the rodent central nervous system, the many secretory glands of the regulatory endocrine system, or the tissue of muscles are sites of interest for implantable biosensors. Fixed in a particular brain region for fundamental cognitive, pathological, and pharmacological investigations, the sensing tips of, for instance, tapered voltammetric enzyme microbiosensors have demonstrated their ability to directly record up- and down-regulations of neurochemicals that may appear in response to premeditated external stimuli such as feeding, drug administration, or gratification at the local level with high time resolution. Biosensors that are implanted in humans, on the other hand, are supposed to report the dynamics of the levels of metabolites related to mental or physical disease states. To reach this challenging goal they may be placed subcutaneously, just beneath the carrier's skin, or at deeper body locations close to target organs such as the kidney, liver, and pancreas or the muscles of the extremities. Specific applications of biosensors in the human body include clinical point-of-care testing in hospital settings and personal diabetes management using handheld monitoring devices. However, the main focus in this success story of biosensor development is not on the description of a number of examples from *in vivo* and healthcare measurements but advancements that were reported in the last five years in terms of the design and quality of electrochemical biosensors for successful analysis in a firmly implanted configuration, be it in animals or humans. For in-depth information on specific examples of both classical *in vivo* (neurochemical) studies and standard human metabolite monitoring with implanted glucose, glutamate, lactate, acetylcholine, or peroxide biosensors the reader is referred to several recently published comprehensive review articles on the two subjects [65, 163, 289, 338–351].

The clever involvement of new enzymes or adapted enzyme blends in the design of implantable biosensors was used to detect physiologically or pathologically relevant biochemical compounds other than the five conventional ones already mentioned. The release of the well-known purine ATP as potent extracellular signaling molecule was, for instance, demonstrated *in vivo* for the *Xenopus* tadpole spinal cord during motor activity with implanted biosensors that had co-immobilized glycerol kinase, glycerol-3-phosphate oxidase, and phosphocreatine kinase [352]. Miniaturized carbon fiber-based biosensors for *in vivo* measurements of acetylcholine and choline have been prepared by means of a co-immobilization of acetylcholine esterase and choline oxidase [353]. The gliotransmitter D-serine, well-known for a long time to modulate neurotransmission at the glutamatergic synapse, has been monitored in the rat brain striatal extracellular fluid with implanted biosensors employing mammalian D-amino acid oxidase as the indicating biological recognition element [354]. The common neurotransmitter dopamine is typically measured *in vivo* in particular brain sections with direct fast CV at the solid graphite disc of polished glass–epoxy insulated carbon-fiber microelectrodes

[355, 356]. In an attempt to improve the selectivity of local dopamine measurements in the complex extracellular matrix of brain fluid, an implantable enzyme-based dopamine microbiosensor has been constructed based on the immobilization of tyrosinase in a thin-film chitosan coating of carbon-fiber disc microelectrodes [357]. *o*-Dopaquinone, which is the product of the tyrosinase reaction with dopamine, was monitored via its reduction at the modified microelectrode surface. The application of these cathodic tyrosinase dopamine microbiosensors was reported for the continuous real-time *in vivo* visualization of electrically stimulated dopamine release in the brain of anesthetized laboratory rats. Remarkably, due to the cathodic potential the sensor response was not significantly disturbed by the presence of typical interferences such as ascorbic and uric acid, serotonin, norepinephrine, and epinephrine.

As with any conventional electrochemical biosensor, an implanted biosensor should also have an exceptional selectivity and sensitivity for the target compound, a low detection limit, and a fast response time that is well tailored to the time course of the expected dynamic changes in the concentration of the target analyte in the surrounding tissue. There are, however, important additional properties to look for when the ambition is for long-term stable electrochemical biosensor performance in the complex matrix of the bodies of animals or humans. It is very important for *in vivo* brain biosensor analysis, but also valid for other situations, to obtain a sensitive acquisition of a strongly localized signal from the molecule in question. In this case, a sensor design is needed that offers a positionable tapered sensor of small total tip dimension which often equals the diameter of an electroactive disc plus twice the thickness of its insulating sheath. Small sensor tip size will of course also be beneficial for placement with minimal (brain) cell and surrounding tissue damage. The exploration of glass- or polymer-insulated needle-type carbon or metal microelectrodes as diminutive precursor structures for biosensors offered an appropriate solution for this problem and no real innovation in this aspect arose in the period under consideration.

The second relevant issue for success with biosensors in the chronically implanted configuration is sufficient sensor stability over the extended time of data acquisition throughout a trial. For a lot of significant behavioral studies but basically in the general implantation case the desired period is days if not weeks of measuring time. The long-term quality of the sensor performance is of course impeded by the gradual loss of proper signal generation caused by the foreign body response and contaminating contact of functional sensor entities on the electrode surface, the immobilization matrix, and the immobilized biological recognition element with protein and lipid contents of the immediate physiological measuring environment [358–362]. Among the issues that can be adverse to long-lasting sensor functioning are (i) the fouling of the immobilization layer in the form of a delamination or loss of porosity which is essential for substrate (analyte) diffusion, (ii) the degradation or denaturation of the biological recognition element, (iii) the passivation of the electrode surface by nonspecific adsorption of proteins and lipids, and (iv) the slow formation of a barrier for substrate diffusion through an ongoing fibrous encapsulation of the biosensor tip. In view of

these considerations, both the optimization of immobilizing top coat and an advanced morphological and chemical design of the transducing electrode surface of enzyme-based *in vivo* biosensors set the scope for the development of new concepts that offer a well-thought-out prevention against the listed set of detrimental effects of sensor tip implantation and the preservation of the analytical response. Several reports in this context have dealt with the adaptation of redox hydrogels employed for the entrapment of the enzymes used via either a mild nondegrading biocompatible environment for the active macromolecule or the creation of hydrogel surfaces that are less prone to the adsorption of contaminating (protein) species. Suggestions include a self-cleaning nanocomposite hydrogel membrane [363], biomimetic hydrogels [364], the involvement of surfactants in the formation procedure of redox hydrogels [365], and hydrogels with optimized type and ratios of individual polymerizing components [366, 367]. Taking advantage of the fact that nitric oxide effectively inhibits platelet and bacterial surface adhesion, Shin and Schoenfisch proposed advanced biosensor interfaces with a high potential to resist biofouling via the implementation of an additional nitric oxide-releasing top coating made of *N*-diazeniumdiolate-modified polymers [368]. Self-assembling polyelectrolyte–poly(ethylene glycol)-based nanofilm multilayers have been demonstrated on porous alumina supports as effective diffusion-controlling and protein adsorption-resistant coatings and were reported as optimized dual-function immobilization matrices for implanted biosensors [369]. Also recommended as surface modifications with promising biocompatibility properties were apparently low-fouling zwitterionic carboxybetaine methacrylate coatings [370], microporous collagen scaffolds that minimized unfavorable tissue reactions while stimulating angiogenesis in the vicinity of biosensor tips [371], porous poly(L-lactic acid) coatings to reduce fibrosis and promote new blood microvessel formation in the tissue surrounding the implanted biosensor surface [372], intentionally pre-adsorbed coatings of constructive proteins capable of inhibiting bad foreign body responses [373], new hydrophilic poly(ethylene glycol)-based redox copolymers bearing electrochemically active ferrocene and thiol/disulfide functionalities for anchoring to a gold electrode surface [374], and special nanoporous membranes [375, 376].

At present, roughly a quarter of a billion (!!!), a still steeply increasing number, worldwide cases of diabetes are reported. Situations of hypo- and hyperglycemia in patients have to be avoided and thus effective blood glucose measurement and control is a top analytical task in medical diagnostics and healthcare, respectively. Already prior to the period covered by this section, personal self-monitoring of internal glucose levels became routine in small-volume blood samples obtained, for instance, by piercing the fingertip or arm. The required commercial tools and information on both their technology and on the glucose meter marketplace is available, for instance, in [59, 65, 163, 343]. Glucose meters typically take advantage of sophisticated single-use screen-printed arrays of electrodes one of which is designed as the glucose sensor via specific immobilization of mostly GOx as the biological recognition element and the involvement of artificial free-diffusing redox mediators. Upon placement of a microliter droplet of whole blood, the electronics of the glucose measuring device assesses and digitally displays a

glucose equivalent in reasonably short time. Dependent on the quality of the measured value in relation to the accepted normal level, insulin injection or dietary carbohydrate uptake should be performed. Even if carried out several times a day, timed glucose monitoring with external sensors activated at user-chosen intervals obviously has the shortcomings that it fails to report irregular up and downs in between assessments and cannot utilize trends associated with daily habits of diabetics for an instant therapeutic action. In this context a better, albeit more challenging, route of blood glucose analysis and management is the operation of permanently implanted glucose sensors for a continuous direct detection of the analyte either in the bloodstream or the interstitial fluid of the subcutaneous tissue. The advantages and disadvantages of continuous glucose sensing as well as the difficulties in and steps forward to the establishment of the approach have been discussed in depth elsewhere [65, 338, 340, 341, 344, 346, 348, 377, 378].

Worth mentioning here as an excellent example of the remarkable achievements of focused joint academic and industrial glucose biosensor R&D is the appearance of the Freestyle Navigator® continuous glucose monitoring system from Abbot Diabetes Care/TheraSense, which recently got approval by the US Department of Health and Human Service, Section Food and Drug Administration (FDA) and became commercially available for diabetics in 2008 [65, 341, 379]. Other similar systems seem to be on their way, and include, as an example, a device that has already been tested successfully for stable long-term glucose monitoring in diabetic and nondiabetic animal models [380] and currently is awaiting FDA approval for GlySens Incorporation.

Electrochemical enzyme biosensors for *in vivo* studies and human body metabolite monitoring have in recent years been brought to quite an advanced level. A clear proof of the achievements is the good number of successful biosensor recordings of brain activity and the enterprise of marketable continuous blood glucose monitoring. Further improvements in the spatial and time resolution of *in vivo* measurements would need further sensor miniaturization and tapered nanobiosensors that should be similar to their microelectrode analogues in terms of the proper conductor embedment and resistance against sensor fouling. However, they should be equipped with a reduced total tip dimension for better positioning and nanometric sensing areas for fast and highly localized recording. *In vivo* biosensor measurements at the single-cell level or at least a very small cell cluster level could then add novel information to the fundamental insights that were and still are gained through *in vitro* single-cell electrochemistry with isolated cells out of their native matrix [381]. Though tapered enzyme-based nanobiosensors with small total tip radii have already been reported [382], these fragile needle-like versions of biosensors have, to the best knowledge of the authors, not yet been successfully operated *in vivo*. Here, there is definite room for future innovative research activity. Another area worth working on is the further extension of the lifetime of sensors for continuous blood glucose monitoring and the transfer of the principles of well-working GOx-based implanted biosensors to those incorporating other enzyme systems for broadening the scope of target analytes. The

related possible enhancements and expansion of vital health and disease marker monitoring could open up the individualized and portable medication and care plan that is envisioned by clinicians and patients around the globe.

1.4.6
Nucleic Acid-Based Biosensors: Nucleic Acid Chips, Arrays, and Microarrays

Biosensors and high-throughput electrochemical screening devices based on DNA, ribonucleic acid (RNA), and peptide nucleic acid (PNA) gain their outstanding sensitivity and selectivity from the very strong base pair affinity between complementary sections of lined-up nucleotide strands, which are the evolutionary genetic code maps of living beings. In fact, all of the genetic information required for body development and functioning as well as details on disease prevalence and states of an organism are smartly made available in the programmed sequence of the nitrogenous bases adenine (A), cytosine (C), guanine (G), thymine (T; in DNA), and uracil (U; in RNA). To create so-called nucleic acid (NA; or gene) chips (or (micro-)arrays), physicochemical transducers (e.g., the surface of an electrode, a microscope glass slide, or a quartz crystal microbalance) are decorated with regular patterns of spots of synthetic single-stranded oligonucleotides, each of them being associated with an intentionally designed A, C, G, and T/U order. Subsequent to the exposure of the sensor surface to a (clinical) sample the remaining analytical task is the identification of all NA spots that underwent hybridization. Definite localization of the collection of immobilized "probe" NA strands that undoubtedly found their matching binding partner (the "target") in a pre-prepared complex blend of genetic material is at the center of the analysis of an apparent gene expression.

The major challenge for an (electrochemical) NA biosensor is the full exploration of the massive amount of information that is buried in the totality of the genomes of mammalian species. Currently, only the very tip of the "genetic iceberg" is revealed and a vast amount of effort has to be invested to finally make the best out of the technology for health science and clinical diagnosis and medication. Figure 1.17 visualizes the analytical task for electrochemical NA sensors and assays.

The main challenge for designing NA-based biosensors is that the hybridization event needs to be monitored correctly. Mismatches need to be distinguished from matches. The assay procedure needs to be compatible with the conditions of hybridization and to allow tuning of the binding specificity. For this, assay strategies employing labels such as redox-active dyes or intercalators as well as enzymes or nonlabeled detection schemes can be used. Each approach has its own benefits and limitations. The design of the capture probe is critical for overall assay performance. If one is employing electrodes, one always has to take into account the impact of the electric field on the orientation of the NA molecules, because NAs are highly negatively charged molecules. In cases where the biosensor design should be compatible with commercialization of the device, the NA chip fabrication procedure needs to take production effort and costs into account.

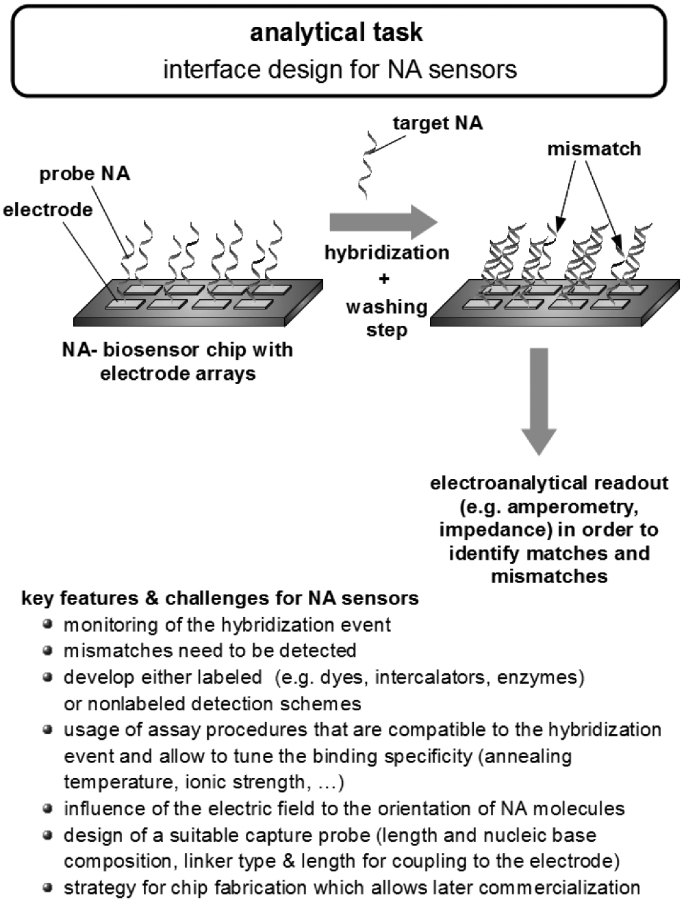


Figure 1.17 Analytical task of NA-based biosensors.

The most common electrode material for electrochemical NA chips is gold; however, other metals, carbon, and certain semiconductors have been used as well. The immobilization of NA probe strands can be achieved, for instance, via simple physisorption, the chemisorption of thiol-modified NA (when gold is the transducer material), the covalent binding of, for example, biotinylated NA to (strept)avidin-modified surfaces, and NA fixation into ultrathin polymeric surface coatings. For ultrasensitive electrochemical recognition of hybridization on NA chips, reagentless, labeled, and label-free schemes have been reported. Enzymes, for instance, may be attached to the endings of target NA fragments and trace detection of double-strand formation can be established via adapted electroreduction or electrooxidation of a product of an enzyme interaction with intentionally added substrate. Other successful schemes exploit redox-active intercalating or groove-binding mediator molecules that can enter the tubular and twisted

structure of the double helix of fused probe and target NA strands to become available for the generation of electrical current. Hybridization detection strategies may use redox mediators that are tethered to either probe or target strands for the creation of configurations favorable for the induction of a Faradaic sensor response, or utilize the distinguished electrostatic properties of immobilized single- and double-stranded NA for impedimetric or voltammetric hybridization detection. An already quite matured NA chip technology in its various facets has entered almost all fields of biology and medicine and a thorough analysis of gene screening data is currently an extensively explored specialty for genotyping, pharmacogenomics, pathogen classification, gene expression profiling, drug discovery, and molecular medical diagnostics. The very obvious indication of the prominent role of state-of-the-art NA biosensing is the noticeable explosion in the number of publications on the subject. A scientific literature screening with the search terms "DNA chip, DNA array, DNA microarray, gene chip, gene array, gene microarray genosensor, DNA biosensor," all combined with the Boolean operator OR and tested for topic or title appearance gave 19 227 and 1148 hits, respectively, for the five-year period 2006–2010. A search refinement with the phrase combination "electrochemical OR voltammetry" reveals close to 1000 topical publications, which, of course, are still far too many to have all been summarized in the constraint of the NA subsection of an overview article on electrochemical biosensors. Hence, the focus was placed on a selection of issues that – from the viewpoint of the authors – provide promising threads for practical advancements of NA-based diagnostics and reflect emerging trends in the field. Several recently published methodical review articles are, however, recommended as excellent additional sources of information about the basic concepts of NA immobilization on single or arrayed electrodes, for the fundamental details of the many existing analytical schemes for electrochemical detection of hybridization, and for the particulars of the variety of published designs of (electrochemical) NA chips [36, 38, 80, 95, 109, 128, 140, 145, 153, 207, 302, 383–386]. Good summaries on the state of the art of the specialties of aptamer-based NA sensing [70, 96, 106, 144, 300, 387] and on impedance/capacitance-based hybridization detection [388–390] are also available in the literature.

Aiming at an improvement of the analytical performance of NA chips/arrays in general and targeting a more sensitive detection of clinically relevant point mutations in particular, immobilized probes made of synthetic PNA have been brought into play as promising alternative biological key components of gene assays. In contrast to "normal" biological NA strands with negatively charged phosphate groups contained in their filamentary polymeric structure, PNA has an entirely uncharged backbone. In contact with single-stranded DNA or RNA target strands of a sample, PNA probe strands obey the rules of NA hybridization. However, as there is no negative polarity in PNA backbones, electrostatic repelling forces between hybridizing PNA and complementary DNA or RNA pieces are absent and the strand bonds in PNA–DNA/RNA hybrids are thus stronger than in the conventional case [391–393]. Benefits related to this effect are improved hybridization properties in terms of affinity, specificity, and sensitivity against single-base mis-

matches, a better chemical stability of the obtained duplex structures and resistance against enzyme cleavage by, for example, nucleases and proteases, and, last but not least, a reduced impact of the ionic strength of the measuring buffer on the outcome of a hybridization screening. An early demonstration of the potential of PNA probes was their successful immobilization onto a quartz crystal microbalance transducer and the subsequent application of the construct in hybridization experiments for the discrimination of perfect matches and single-base mismatches [394]. Sensitive single nucleotide mismatch detection with PNA-modified electrode transducers was the subject of a number of studies in the past five years. Representative examples are the exploitation of the impedance characteristics of a PNA–metal ion interaction for the identification of an individual C–T mismatch in a 15-mer PNA–DNA hybrid [40], the discrimination of completely complementary from mismatched double helices via specifically acting redox-active diviologen indicator molecules [49], the application of osmium mediator end-labeled PNA and stripping voltammetry for single-base mismatch detection [110], the use of ferrocene-labeled PNA for full match/mismatch identification [94], a single nucleotide polymorphism detection via joint employment of electroactive chitosan nanoparticles and PNA strings [97], and hybridization and mismatch recognition via the diverse electrostatic interaction of a cationic ruthenium(III) mediator with neutral PNA capture probes and anionic target DNA backbones [301]. Involvement of PNA probes in work on samples with clinical relevance includes, for instance, the detection of short sequences of the hepatitis C 3a virus [35], implementation in silicon nanowire biosensors for highly sensitive and rapid detection of Dengue virus [395], the application with a PNA-modified electrode for *Mycobacterium tuberculosis* pathogen detection [93], and the development of a PNA array for the identification and quantification of the cancer gene c-Ki-ras [396]. Another form of uncharged synthetic DNA is the morpholinos. With a comparable motivation as for PNA they have been explored as components of (label-free) surface hybridization assays [34, 50, 101].

Common electrochemical DNA chips/arrays are fabricated either with automated spotting procedures and controlled dispensing of small-volume droplets of NA solutions into a regular pattern or via a microlithography-controlled patterned (bio-)chemical on-chip synthesis. Both methodologies work well but they are associated with expensive apparatus and have their practical limits when the desired characteristic array dimensions such as probe spot diameters and distances approach the nanometric level. In view of that, cheap and spatially more accurate NA patterning techniques are sought after. In this context NA origami, the folding of longer filamentary NA structures into nanoscale two- and three-dimensional surface features [37, 51, 104, 397–400], has recently been suggested in the field of molecular NA sensing technology as a tactic for manufacturing nanoscopic NA chips [39, 98, 169, 401]. The potential of a combination of NA origami with existing nanoelectrode array fabrication technology has not yet been explored; however, this is an attractive theme that could lead to the development of powerful novel electrochemical tools for scaled-down NA hybridization screening in ultrasmall sample volumes.

Usually the electrode surface in an electrochemical NA biosensor acts as the physicochemical transducer that responds to exposure to target NA-containing sample solution and the formation of probe–target hybrids with a change in current flow, capacitance, or impedance. An interesting atypical configuration of a NA probe-carrying electrode is to use alternating electrode potential variations for controlled modulation of the structural conformation of the surface-tethered probe and fused probe–target strands and establish what is named a switchable DNA interface [402–404]. Hybridization with this method is not determined as usual via the acquisition of electrode properties but by means of an extra optical scheme that is susceptible to situations in which NA strands extend away from or fold onto the electrified sensor surface. At frequencies in the kilohertz region, the combination of electrode potential variation and an optical readout revealed distinct switching kinetics for changes from the upright to the flat surface position of NA strands, and the sensitivity was reported good enough to allow single-mismatch detection.

Without a doubt, NA-based biosensing and the application of the related arrayed gene chips for studying mRNA levels and examining gene expression profiles in human, animal, and plant samples are among the hot topics in current analytical chemistry. The prominent role is of course strongly related to the accumulated success of the many running genome projects. In particular the previous disclosure of the human genome [405] and the expectations from specific gene identification for disease diagnostics are fostering the exclusive position of hybridization assays. Electrochemical hybridization detection has been proven competent and competitive enough to be an attractive alternative to the more expensive and often technically more complex optical options in certain circumstances. However, handling the massive genetic data material of the human genome is a huge challenge for NA chip technology no matter what detection scheme is employed. So far, only an extremely small percentage of the wide range of possibilities has been accomplished. Significant advancements in both NA array fabrication and electrochemical, mass, or optical readout strategies and equipment are indispensable to enable personalized medical care based on individual gene profiles become part of daily life.

1.4.7 Immunosensors

Immunosensors rely on the extremely high binding affinity of antibodies towards their respective antigens. Apart from the very specific biorecognition reaction, antibodies and antigens can be produced to obtain a specific binding partner for a target of interest. A comprehensive overview of antibody production is given in [406]. However, the challenge of producing antibodies should not be underestimated. Apart from the production of the actual biorecognition element, the challenge in the design of (electrochemical) immunosensors lies in the development of a suitable detection scheme. Basically two types of immunosensor approaches can be distinguished: labeled and nonlabeled approaches.

1.4.7.1 Labeled Approaches

Labeled immunosensors typically make use of a reporter molecule attached to the respective antibody. Apart from the electrochemical detection schemes discussed below, immunosensors employ fluorescent labels, radioactive labels, or nanoparticles, among other reporter systems [407–410]. Labeled immunosensors normally operate using either a direct or indirect sandwich procedure or a competitive format as depicted in Figure 1.18.

Sandwich assays rely on secondary antibodies binding to the target antigen or antibody after the primary biorecognition reaction. In a competitive assay format, labeled and nonlabeled antibodies (or antigens) compete for the binding sites of

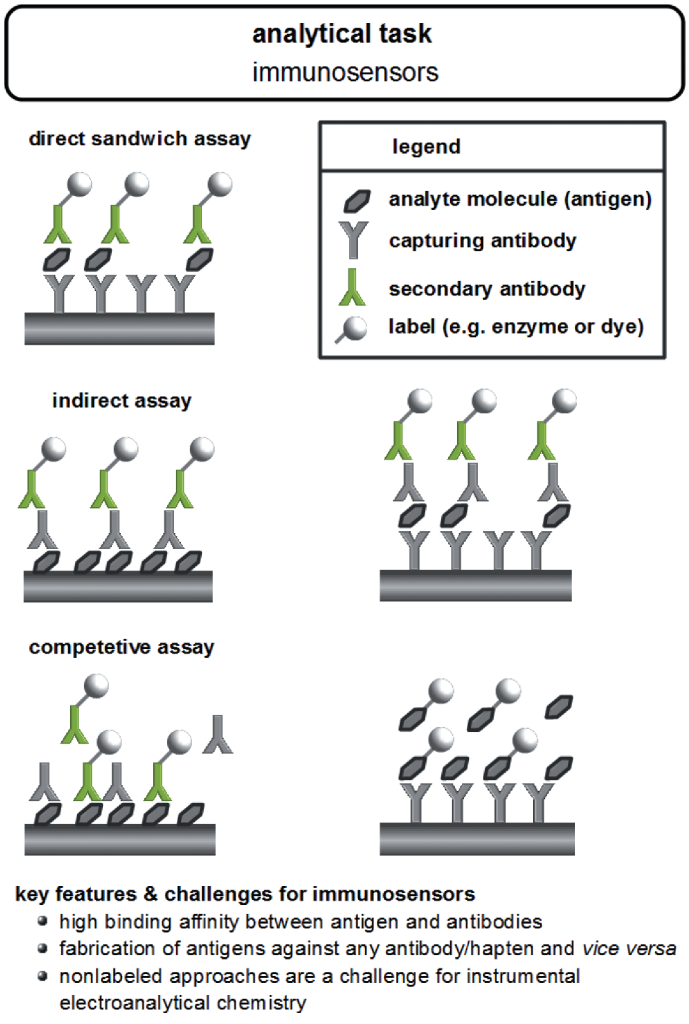


Figure 1.18 Analytical task of immunosensors.

the biorecognition layer. In direct assay formats basically all binding events of the target analyte can be addressed by the secondary antibody leading to typically higher sensor signals than in competitive assays. Competitive assays, however, require less working steps leading to potentially more cost- and time-effective sensors.

Electrochemical immunosensors have been the subject of a large number of reviews [409, 411–413]. By far the most prominent electrochemical immunosensor is the enzyme-linked immunosorbent assay (ELISA) [98, 99]. This class of sensor uses enzyme labels that produce or consume an electroactive substrate or cofactor which can be monitored at an electrode interface. The most common enzyme labels are horseradish peroxidase, GOx, and alkaline phosphatase. The latter opens up a tremendous potential for powerful immunosensors. Alkaline phosphatase cleaves the non-electroactive *p*-aminophenylphosphate into p-aminophenol. *p*-Aminophenol undergoes a reversible oxidation at moderate working potentials of 150 mV (vs. Ag/AgCl) [414]. This feature does allow for the amplification of the obtained signal. If an electrode system is used comprising two closely spaced electrodes such as interdigitated electrodes (IDEs) [415, 416], *p*-aminophenol can be recycled (reduction of *p*-benzoquinonimine) at the second electrode after primary detection at the first electrode (oxidation of *p*-aminophenol). The reporter molecule is cycled between the two electrodes leading to a significant amplification of the sensor signal. This detection scheme has hence not only introduced an alternative enzyme as reporter system but has inspired a whole class of amplified electrochemical sensors including but not limited to immunosensors. Analytes that have been detected using this detection scheme as reviewed in [409, 411–413] include a wide range of bacteria, viruses, tumor markers, and others.

1.4.7.2 **Nonlabeled Approaches**

Detection of biological recognition reactions between antibodies and antigens omitting labels and reporter systems typically relies on sophisticated instrumental analysis. Among others, mass spectrometry and chromatographic approaches can be used to detect immunoreactions. Among optical methods, surface plasmon resonance (SPR) spectroscopy is an interesting way to detect antibody–antigen binding events [58]. Mass-sensitive biosensors based on vibrating cantilevers [417] or the quartz crystal microbalance [418, 419] offer a straightforward way to detect antibody–antigen binding.

Instrumental electroanalysis offers some alternatives for the detection of biological recognition events in immunosensors such as capacitive immunosensors and sensors based on electrochemical impedance spectroscopy. Both techniques are universal platforms for the detection of immunoreactions and hence bacteria, viruses, tumor markers, and more, as described above, have been detected with these methods.

Capacitive biosensors [390] detect changes in the capacitance of an electrode upon the occurrence of a binding event. The capacitive structure comprises a series of components such as the electrochemical double layer including the diffuse layer from ions in solution, the grafting layer, and the biorecognition layer. Since the contribution of the biorecognition layer to the overall capacitance is typi-

cally large compared to that of the other components, changes in the biorecognition layer upon the binding of antibodies or antigens can be probed by measuring the changes in the capacitance of the biosensor. This is often accomplished by potential-step experiments that require relatively cost-effective electrochemical equipment.

Electrochemical impedance spectroscopy (EIS) [420, 421] measures the complex resistance of an electrochemical system. An electrochemical system in equilibrium is perturbed by a low-amplitude sinusoidal perturbation, typically in the range 5–10 mV around the equilibrium potential. The different components of the electrochemical system will react at different speeds resulting in a frequency-dependent shift in magnitude and phase between perturbation and response of the electrochemical system. A detailed analysis of the response of the electrochemical system and its components such as double-layer capacitance, charge-transfer resistance, or even diffusion coefficients of the molecules involved can be quantified separately. The components of an electrochemical system including a biorecognition layer are altered upon a biorecognition event and can thus be probed by EIS. The occurrence of an immune recognition reaction typically results in an increase of the charge-transfer resistance of an electrochemical system. Consequently, EIS detection schemes have been widely employed in biosensors and in immunosensors in particular, for the quantification of target analytes [422, 423].

In conclusion, electrochemical immunosensors are a useful class of biosensors that have taken advantage of some major developments during the past decades. The use of enzyme labels in ELISA-type immunosensors and simple amperometric detection schemes resulted in simple and cost-effective alternatives to fluorescence immunosensors. In particular, the use of alkaline phosphatase as enzyme label allowed for the fabrication of advanced immunosensors with signal amplification by means of redox cycling, which has been a success story of its own. This detection scheme has been used in immunosensors and other biosensors and has stimulated significant developments in electrode fabrication. Instrumental electroanalysis, namely capacitance measurements and EIS allow for label-free detection of immunoreactions.

1.5 Conclusion

The full potential of amperometric biosensors has not yet been tapped, especially with respect to the versatile and broad range of applications for which biosensors can be used. Many contributions to the field of biosensors and biofuel cells still are at the “proof-of-concept” stage. Thus, the authors hope that this chapter will promote lively and valuable discussions in order to generate new ideas and approaches towards the development and optimization of biosensor architectures.

This field of research has a true multidisciplinary, “boundary-crossing” nature which is actually one of the driving forces in science in general and a great impetus for the biosensor field in particular. From the perspective of the later application

of a biosensor design, for example, physicians, biologists, or engineers are involved in order to provide the specifications for a certain analyte of interest and to define the analytical challenge. Chemists and engineers are involved in the packaging of the biosensor device and additional instrumentation for readout of the signals. With respect to the actual sensing layer on the transducer, not only analytical chemists such as electrochemists but also material chemists, organic chemists, polymer chemists, biochemists, and biologists may well work hand in hand in order to achieve the desired performance of the biosensor.

There has been more than four decades of developing amperometric biosensors. Of the many approaches towards biosensor architectures reported in the literature, a vast majority of the possibilities introduced still remain restricted to the realm of academic papers. This is mainly due to the still unsolved problem of unspecific adsorption which affects biosensor performance in many cases, degradation of the sensing layer in *in vivo* applications, limited long-term stability of biological recognition elements, etc. In addition, in complex biosensor architectures comprising a large number of different components for immobilization, for a designed ET pathway, anti-interference layers, amplification systems, etc., the extremely complex multiparameter optimization procedure still does not provide sufficient information for a rational design of sensors. Thus, many papers provide recipes leading to a concentration-dependent change in a sensor signal without a full understanding of the underlying physicochemical properties of the sensor components and the interplay of the most significant influencing parameters. Thus, especially the knowledge gained for a particular sensor design cannot be easily transferred to other biological recognition elements, other transducer materials, etc. Having this in mind, we feel that cooking-book-type mixing of many components on a transducer surface, recording of a calibration graph, and using the same sensor for the quantification of an artificial sample do not provide substantial progress in the field. Therefore, we would like to propose, on the one hand, an application-driven approach in which a real analytical task is finally accomplished taking also into account the repeatable and reproducible fabrication of the developed sensor concepts and statistical evidence for the applicability of the biosensor. On the other hand, a more in-depth understanding of the complex influence of the different parameters has to be acquired and a general understanding about the functioning of sensors at a molecular level is required to allow for a rational optimization of the sensors. Many routes are still open to novel approaches in biosensor design and to bridge the gap between academic model studies and real-world applications.

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Abbreviations

CV	cyclic voltammetry
DNA	deoxyribonucleic acid
DPV	differential pulse voltammetry
E	electrode potential
ET	electron transfer
FAD	flavin adenine dinucleotide
GOx	glucose oxidase
k_{cat}	turnover rate
K_M	Michaelis constant
MA	microarray
NA	nucleic acid
NAD^+	nicotinamide adenine dinucleotide
NP	nanoparticle
PQQ	pyrroloquinoline quinone
SAM	self-assembled monolayer
SCE	standard calomel electrode
SECM	scanning electrochemical microscope

Glossary

This glossary explains many of the technical terms relevant to amperometric biosensors. For additional terms not listed here, the reader is referred to, for example, the *Electrochemical Dictionary* [1].

bioelectrochemistry	a scientific discipline describing ET reactions between biological redox-active entities such as enzymes, redox-labeled proteins, living cells, etc., and suitable modified electrode surfaces.
biofuel cell	a device that converts chemical energy to electrical energy by means of biocatalysis.
biosensor	an analytical device that consists of a biological recognition element for the analyte of interest either integrated within or in close proximity to a transducer. The transducer enables the transformation of selective and quantitative information on the presence of the analyte into a quantifiable signal [2–7].
– first generation	direct oxidation or reduction of natural and freely diffusing electroactive reactants, cofactors, or products of a biological recognition reaction at an underlying electrode.
– second generation	oxidation or reduction of an artificial electroactive mediator that transfers electrons between the enzyme and the electrode surface.

– third generation	direct ET between the enzyme and the electrode surface excluding any intermediate ET reactions with redox mediators.
chemical sensor	a device that identifies and/or quantifies an analyte-of-interest as a result of a chemical reaction or interaction of the analyte with a sensor components. This chemical information is transduced into a physical signal which can be amplified.
cofactor	the terms "prosthetic group" and "coenzyme" are used for tightly bound, specific non-polypeptide units required for the biological activities of proteins. The term "cofactor" is often used synonymously with "prosthetic group" or "coenzyme." However, it is important to note that enzymes containing more than one prosthetic group are usually called multi-cofactor enzymes. Here, the term "cofactor" is exclusively used for redox-active non-polypeptide substructures in an enzyme which are tightly but not necessarily covalently bound within the protein. Free-diffusing compounds such as NAD^+/NADH are not included in this meaning of the term "cofactor." They are called "coenzymes" or "mediators."
conducting polymer	polymers or "synthetic metals" that are intrinsically conducting or semiconducting. Examples are, among many others, polypyrrole, polyaniline, and polythiophenes [8–16].
diffusion	random movement of a species under the influence of a concentration gradient.
– linear	diffusion that is found at electrodes with an electroactive surface that is large in comparison to the thickness of the diffusion layer at the electrode surface. Commonly associated with macroelectrodes.
– hemispherical	diffusion that is found at electrodes with an electroactive surface that has a similar dimension to the thickness of the diffusion layer. Commonly associated with disc-shaped microelectrodes.
electrodeposition	a process in which a film of metal, polymer, oxide, or another composite is formed on an electrode surface by electrochemically induced oxidation or reduction of a precursor reagent.
electrodeposition polymer	a polymer that can be electrodeposited. For example, protons or hydroxide ions can be electrochemically generated within the diffusion zone of an electrode. This enables a pH-induced modulation of the solubility of the electrodeposited polymer, leading to a precipitation of the polymer and the formation of a polymer film on the electrode surface [17, 18].

electron transfer (ET)	a process in which an electron is transferred from one redox center to a second. ET according to Marcus theory is mainly dependent on the distance between the electron donor and acceptor, the driving force for the overall process (i.e., the potential difference), the reorganization energy of the involved redox centers, and the intervening medium (i.e., the nature of the protein matrix) [19, 20].
– direct ET in biosensors	ET from the active center of the biological recognition element to an electrode without the involvement of a mediator or cofactor [20–27].
– mediated ET in biosensors	ET transfer from the active center of the biological recognition element to an electrode via intermediate ET processes with a redox mediator [20, 21, 24, 25, 28].
enzyme electrodes	electrodes modified with an immobilized enzyme.
flow injection analysis	an analysis technique which is based on the injection of a defined volume of a liquid sample into a continuous flowing carrier stream that at one point passes a detector.
immobilization, electrochemically induced	a process in which a composite or reagent is altered from a free-moving state to a fixed state by means of an electrochemically induced modulation of molecular properties of the compound that has to be deposited.
impedance	complex resistance of an electrochemical system.
interdigitated array	interdigitated arrays normally consist of two electrodes with finger-like structures which intertwine like a zipper structure.
interferences	substances that can electrochemically react with transducer surfaces or redox centers, which are not the substrate of the biological recognition element in a biosensor. In addition, compounds can interfere with the biological recognition reaction (e.g., inhibitors) or substrates other than the analyte of interest if the enzyme does not exhibit narrow substrate selectivity.
ion-sensitive field effect transistor (ISFET)	a semiconductor device in which the current between two electrodes (source and drain) is controlled by a third electrode (gate) which is ion-sensitive. Changes in ion activity in the surrounding electrolyte result in a change of the potential at the gate and hence in a modulation of the current between source and drain.
label-free	an assay strategy or a biosensor architecture in which no artificial reporter molecules have to be attached to one of the assay components.
Marcus theory	theory for single-electron transfer reactions [19, 29, 30].
microarray (MA)	electrochemical MAs are electrode structures that consist of ensembles of at least four or more microelectrodes that are typically arranged in an orthogonal grid. Electrodes can be either interconnected or individually addressable [31–33].

microfabrication	techniques that lead to well-defined structures and patterns in the micrometer range or below. Includes techniques such as physical or chemical vapor deposition and photolithography [34–40].
microfluidics	discipline that deals with devices in which fluids are confined and moved in micrometer-sized spaces and channels [41–46].
multi-electrode array	electrode structure that consists of four or more electrodes that are arranged in an orthogonal grid. Electrodes are typically individually addressable and miniaturized (see microarray).
multiplexing	the signals from individual sensors are sequentially read out by a single measuring device.
nanomaterials	materials in which at least one of the spatial dimensions of the material is in the sub-100 nm range.
nanosensors	a sensor that makes use of either nanomaterials or transducers with at least one spatial dimension in the sub-100 nm range.
protein conjugate	a synthetic molecule linking a protein with a partner molecule. The linked molecule can be either a small molecule such as a linker, a dye, or biotin or larger molecules such as antibodies or enzymes or other materials such as nanoparticles or quantum dots. The resulting hybrid molecules combine properties of both of the linked molecules.
protein chips	analytical devices to probe protein–protein interactions. A protein chip typically comprises an array of sites capable of carrying out an analytical task.
protein modification	chemical or genetic modification of a protein to adapt its properties to a certain task.
reagentless biosensor	a biosensor which comprises all components required for the analytical reaction securely fixed on the transducer surface. The only free-diffusing component of the sensing process is the analyte.
redox hydrogel	a hydrogel consists of a polymer that is highly dispersed in water and hence provides fast diffusion for molecules through the polymer network. In addition, a redox hydrogel is modified with redox-active groups such as redox mediators [47, 48].
redox mediator	a redox mediator shuttles electrons between two molecules or between a molecule and an electrode (homogeneous or heterogeneous charge transfer).
redox relay	see redox mediator.
selectivity	the ability to discriminate between different substances.
self-assembled monolayer	molecular monolayers spontaneously formed at interfaces (e.g., electrode–solution) by self-assembly. The monolayer is

	characterized by a high degree of orientation, molecular order, and packing [49–53].
scanning electrochemical microscope (SECM)	a tool for imaging of local electrochemical activity and topography of a sample by means of an ultramicroelectrode (tip) with the tip scanning either at constant height or constant distance. A variety of detection modes, such as feedback, generator–collector, direct, alternating current, and redox competition modes, are available [54–57].
surface plasmon resonance (SPR)	surface-sensitive optical technique in which the changes in the oscillation of surface electromagnetic waves are employed to detect changes on the surface of a metallic substrate [58].
thin-film electrode	an electrode that is modified with a thin layer of a substance that is not the basic electrode material. Includes metal films, polymer films, and self-assembled monolayers.

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