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Biosensors for Diagnostic Applications

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Abstract Biosensors combine a transducer with a biorecognition element and thus are able to transform a biochemical event on the transducer surface directly into a measurable signval. By this they have the potential to provide rapid, real-time, and accurate results in a comparatively easy way, which makes them promising analytical devices. Since the first biosensor was introduced in 1962 as an "enzyme electrode" for monitoring glucose in blood, medical applications have been the main driving force for further biosensor development. In this chapter we outline potential biosensor setups, focusing on transduction principles, biorecognition layers, and biosensor test formats, with regard to potential applications. A summary of relevant aspects concerning biosensor integration in efficient analytical setups is included. We describe the latest applications of biosensors in diagnostic applications focusing on detection of molecular biomarkers in real samples. An overview of the current state and future trends of biosensors in this field is given.

Keywords Biomarker · Biosensor · Detection · Diagnostics · Test format

Contents

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1 Introduction

Diagnostic applications include the detection of disease-related biomarkers, e.g., metabolites or proteins, in human body fluids. Biomarker concentrations can be used to define disease type, state, or progress as well as the patient's response to therapy. Research on disease-related biomarkers is an ongoing process [1]. It is undeniable that established biomarkers exist which are significant for certain diseases, such as increased levels of blood glucose typically indicate diabetes [2]. However, other biomarkers reported to be disease-related are still under investigation regarding their significance in clinical diagnostics [1]. This applies, for instance, for biomarkers which are used to specify subtypes of diseases, such as markers used for classification of a tumor into subsets, which could be a useful tool in a more efficient medical treatment [1, 3]. Furthermore, particularly in the latter case, more than one biomarker has to be determined to allow efficient diagnosis, leading to marker profiles or "molecular signatures" [3, 4]. Although these markers might not be fully established in clinical routine, owing to strict requirements regarding human health, the number of clinical tests is still rising, resulting in an increasing demand for suitable and effective analytical instruments [5].

Currently, testing for biomarkers is typically performed in centralized laboratories using large automated clinical analyzers based on DNA or protein microarrays including immunoassay methods [6, 7]. They usually allow multiplex detection of several analytes, but also require trained staff and increased time and effort [5]. On the other hand, portable analytical instruments for determining blood glucose levels [8, 9] or blood coagulation [10] for patient self-testing are available. They provide results within minutes, enabling independent dosage of medication in the patient's self-management. These devices usually determine only a single analyte or a few analytes, but this is sufficient for the applications they are designed for. However, between the multianalyte high-throughput arrays used in centralized laboratories and the single-analyte devices used by the patients themselves there is a huge gap. For detection of marker profiles, e.g., at a physician's office, user-friendly instruments are required. They should allow rapid detection and quantification of several analytes without the need for specialized staff. For this testing scheme outside the central laboratories, i.e., near the patient, the term "point-of-care testing" (POCT) has been adopted. Although many different technologies and systems have been developed in this area, a clear leader has not yet been established in clinical routine [5, 11].

Biosensor setups have also been considered as POCT devices. According to the definition by the International Union of Pure and Applied Chemistry (IUPAC), a biosensor is an integrated receptor–transducer device, i.e., it combines a biochemical recognition system (receptor) with a detector (transducer). A biosensor transforms the biochemical (biological) response into a measurable output signal [12]. Therefore, biosensors should not require additional processing steps or reagents between sampling and signal output, and hence are most promising when it comes to quantitative, fast, and economic measurements [3–5, 11, 13].

Biosensors based on electrochemical detection have already found their way into POCT devices, with most of them being used as glucose meters [2, 8]. However, compared with the numerous publications and patents available, there is a huge gap between scientific output and commercial success, the latter implying availability and acceptance in clinical routine [13, 14].

One reason for this discrepancy may result from the sample matrix, which is more complex in clinical samples (e.g., serum, containing numerous proteins) than in laboratory samples prepared to demonstrate the system's performance (e.g., buffer solution, containing only the target analyte). The complex sample matrix can interfere with the biosensor signal response, which therefore has to be investigated in addition to the system's performance shown with buffer solutions [13, 14]. Furthermore, considering commercialization, a powerful biosensor instrument requires both a high-performance biosensor component and a user-friendly and efficient instrumental housing [15]. However, in many cases only individual aspects of biosensor instruments were thoroughly investigated, i.e., only single components were optimized, disregarding potential system integration. Finally, not least owing to biomarker profiles still under investigation, there is a need for versatile sensing technologies which can easily be adapted to the respective application, including multiplex sensing as well as miniaturization and low costs [13, 14].

In the following we will describe biosensor transduction principles and biorecognition layers as well as test formats with regard to their potential use in diagnostic applications. We will outline relevant aspects concerning biosensor integration in an efficient analytical device. This will be followed by an overview of biosensor applications in diagnostics focusing on serum biomarkers in cardiovascular diseases (CVDs), cancer, and autoimmune diseases as well as biomarkers in cerebrospinal fluid (CSF) for neurodegenerative diseases. We will describe the latest developments and give an overview of future trends of biosensors in this field.

2 Biosensors and Biosensor Systems

A biosensor is an analytical device which combines a biorecognition element with a transducer [12]. Whereas the biorecognition element determines the degree of selectivity or specificity of the biosensor, the biosensor's ability to detect low concentrations is mainly influenced by the transducer, as it transforms the biological or biochemical response into a quantifiable output signal [16]. Biosensors can be classified by the transduction principle, i.e., the detection, or by the type of biorecognition layer, which defines the type of specific analyte binding [12]. Table 1 gives an overview of the most common types of biosensors following these classifications. It is obvious that, for instance, immunosensors can be designed using optical or electrochemical detectors, acoustic biosensors can be based on antibody or aptamer coating, etc. Terms such as "micromachined"

Table 1 Classification of biosensors

Biosensors by transduction principle	Biosensors by biorecognition element → alternative biosensor term[a] (if available)
Electrochemical	Enzyme → enzyme sensor
Optical	Antibody → immunosensor
Acoustic or gravimetric	Aptamer → aptasensor
Thermal or calorimetric	Oligonucleotide → DNA sensor, genosensor
Magnetic	Cell → whole (microbial) cell biosensor
	Molecularly imprinted polymer

[a] Instead of (biorecognition element) biosensor

biosensors are sometimes used to describe the manufacturing method and therefore may include several types of biosensors, regardless of the transduction or biorecognition principle [17].

2.1 Biosensor Transduction Principles

2.1.1 Overview

According to the categories typically used, the "enzyme electrode" introduced by Clark and Lyons [18] in 1962 was an amperometric biosensor. This milestone in biosensor development was followed by other electrochemical biosensors, but it was not until 10 years later that biosensors based on other transduction principles were reported. The first gravimetric biosensor was a quartz crystal microbalance (QCM)-based immunosensor for protein detection; it was reported in 1972 [19]. This was followed by the first thermal biosensor for enzyme–substrate studies, called an "enzyme thermistor", in 1974 [20]. The first optical biosensor based on total internal reflectance fluorescence (TIRF) used fluorescein-labeled antibodies for detection of small molecules (haptens), and was also reported at the beginning of the 1970s [21, 22]. The first fiber-optic-based biosensor—which was dedicated to glucose detection just like the first electrochemical biosensor—was reported not until a decade later [23]. The first biosensors exploiting magnetism as a transduction principle for a variety of analytes were introduced in the 1990s [24–26].

Biosensor development has been driven by analytical demands, but it was the technological progress in related fields, such as communication and optics, as well as in manufacturing, which enabled the state of development of the current devices [27]. Electrochemical biosensors, for instance, are now comparatively easy to miniaturize, which is one of the reasons for their widespread availability [28]. In fact, detectors for biosensors used today depend mainly on electrochemical transduction, followed by optical and acoustic effects [4]. Thermal transduction is less frequently used, as are magnetic effects. However, the latter are increasingly employed as a separation tool in bioanalytical assays [29]. The following sections will outline current transduction principles to give the reader an idea of potential methods which would allow particular diagnostic applications. For a detailed

description of the respective measurement setups, including detection limits and commercialization status of available devices, the reader may refer to the cited references.

2.1.2 Electrochemical Transduction Principles

Electrochemical biosensors are typically supplied as electrode setups. They are generally divided into three major categories, depending on the underlying measurable parameter. These are current for amperometric biosensors, potential or charge accumulation for potentiometric biosensors, and conductive or resistive properties for conductometric or impedimetric biosensors, where both of the latter are usually treated as one category [28, 30].

Amperometric biosensors are typically used to detect small molecules by means of an enzyme, e.g., a peroxidase, catalyzing a redox reaction [31]. The amperometric biosensor for the detection of glucose by means of glucose oxidase was not only the first biosensor principle introduced, it is still, after several improvements, the most widespread biosensor [28]. The principle of potentiometric measurements is familiar to most from the commonly used pH electrode. Similar to amperometric biosensors, potentiometric biosensors are best suited to detect small molecules [32]. Detectors for potentiometric biosensors include the family of field-effect transistors, which have been gaining interest in recent years owing to newly developed nanomaterials, such as carbon or silicon nanotubes and nanowires. They allow label-free detection, i.e., do not require labeling with enzymes or nanoparticles to get a signal response, which reduces the expense [28]. Impedimetric biosensors are the youngest among the electrochemical biosensors and also offer label-free detection of biomolecules. They allow the investigation of a large variety of analytes, ranging from small molecules to proteins and even cells [33]. Ongoing research in the field of electrochemical biosensors is mainly focused on new materials, e.g., graphene [34] and diamond [35], as well as on miniaturization and parallelization of the devices, e.g., by screen-printed electrodes [28, 36].

2.1.3 Optical Transduction Principles

Optical biosensor detectors can generally be divided into labeled and label-free. The labels are, e.g., fluorophores or nanostructured materials such as nanoparticles, quantum dots, or carbon nanotubes [37]. Detectors using labels typically use the evanescent field outside a waveguide resulting from total reflectance within the waveguide. This evanescent field can excite, for instance, fluorescent or chemiluminescent labels outside the waveguide [38]. An example of this is the first optical biosensor which was based on TIRF [22]. Chemiluminescence can also be induced electrochemically; it is then called electrochemiluminescence [37]. With labels, small molecules as well as large molecules, such as proteins, can be detected.

The evanescent field can also be used for label-free detection, which is then based merely on refraction. The penetration depth of the evanescent field is typically only a few hundred nanometers; the refractive index of the medium determines the propagation of the radiation. Hence, it is advantageous if large analyte molecules have to be detected, such as proteins, as they lead to a higher change in the refractive index. Furthermore, when the sensing layer is being prepared, it has to be ensured that analyte binding occurs within the penetration depth of the evanescent field, otherwise the binding cannot be detected by the transducer and therefore will not be transformed into a quantifiable signal. The best examined and most widespread evanescent field technique uses surface plasmon resonance (SPR) on gold surfaces [39]. The respective systems have been further developed continually [40], including related techniques, such as SPR imaging [41] and localized SPR [42]. Other waveguiding methods make use of grating couplers or prism couplers; the latter was introduced as a so-called "resonant mirror" [43]. Furthermore, evanescent field techniques have been combined with interferometric principles, as realized in Mach–Zehnder and Young interferometers [39, 44, 45]. Recently developed evanescent field techniques for label-free optical detection for biosensors include use of Bragg gratings, photonic crystals, and optical ring resonators [44, 45].

Apart from refractometry, label-free optical detection can also employ reflectometry. In this case, signal response changes result from changes in the optical thickness, i.e., the product of the refractive index and the physical thickness, where changes of the latter prevail. Hence, the evanescent field does not have an impact here, and binding events do not necessarily have to be near the surface [39]. Again, large molecules are preferred for detection as they lead to greater thickness changes. Detection methods using reflectometry include ellipsometry, which is based on polarized light, and reflectometric interference spectroscopy, which can be performed with white light, which is why reflectometric interference spectroscopy may be considered as a simplified version of ellipsometry [46].

In addition to the numerous optical detectors which are already available, ongoing research is motivated by the aim of introducing new label-free transduction principles for biosensors that allow, in particular, the label-free detection of low concentrations or small molecules or both. Currently under investigation are, e.g., terahertz spectroscopy [47] and surface-enhanced Raman spectroscopy [48].

2.1.4 Acoustic Transduction Principles

Gravimetric (mass-sensitive) transduction principles use mechanical acoustic waves which are typically generated by means of piezoelectric materials. Signal response changes mainly result from mass changes in the biorecognition layer; hence, it is advantageous if large molecules have to be detected [49, 50]. Acoustic detection is usually label-free, but the use of nanoparticles as mass labels to increase mass sensitivity has been reported [51].

The best known acoustic biosensors are QCM, surface acoustic wave (SAW), and cantilever biosensors [49, 50]. QCM-based biosensors were the first mass-sensitive biosensors reported and are still the most commonly used acoustic biosensors. They use thickness shear modes of the piezoelectric substrate and hence are bulk acoustic wave devices [50, 52]. In contrast, mechanical waves of SAW biosensors propagate along the surface of the piezoelectric substrates. This allows higher resonance frequencies than with QCM devices, which is beneficial, because the mass sensitivity increases with increasing frequency [53]. Cantilever biosensors originate from the cantilevered sharp tips used in atomic force microscopy. If they are operated in the dynamic resonant mode, mass increase on the cantilever surface leads to a decrease of the resonant frequency, similar to QCM biosensors. If they are operated in the static deflection mode, mass increase causes a deflection of the cantilever which can be measured, e.g., optically, or, provided that a piezoresistive coating was used, electrically [54, 55]. Aside from miniaturization and parallelization of the devices, ongoing research is focused on other nanomechanical resonators, e.g., trampoline-like resonators, as a future development aiming at higher mass sensitivity of the resulting biosensors [51].

2.1.5 Thermal Transduction Principles

Thermal transduction relies on the principles of calorimetry, which requires the measurement of temperature changes. Thermal biosensors typically use thermistors, i.e., temperature-dependent resistors, as the detector unit. Similar to the term "enzyme electrode" used for the first amperometric biosensor, the first thermal biosensor was called an "enzyme thermistor". Thermal transduction offers the possibility of label-free detection of small molecules, but measurements using enzyme labels have also been reported. Still, thermal biosensors have rarely been used for biosensor measurements. One reason for this may be the difficulty to deal with nonspecific temperature changes, e.g., arising from dilution or solvation effects, requiring additional means, such as reference channels [56, 57]. More recent research suggests the use of thermopiles instead of thermistors as this would simplify the ambient temperature control of the system and hence the miniaturization of the device [58].

2.1.6 Magnetic Transduction Principles

Magnetic transduction principles for biosensors usually require magnetic nanoparticles as labels, because the analytes to be detected are typically nonmagnetic. The most commonly used magnetic effect for signal transduction in biosensors is giant magnetoresistance. Magnetoresistance is the change in the resistance of a material due to the application of a magnetic field. If alternating layers of ferromagnetic and nonmagnetic metals are used, this change is much greater than expected owing to quantum interferences resulting in giant magnetoresistance [59–61]. However,

giant magnetoresistance biosensors—as well as other biosensors based on magnetic transduction principles—are less common than biosensors based on electrochemical,s optical, or acoustic detection. We think this is because the magnetic principles require more sophisticated readout electronics than the methods mentioned above. On the other hand, magnetic labels are very attractive for use as a separation and purification tool as there is usually no magnetic background in biological samples. Depending on the type of magnetic label, they can still be used for detection afterwards, e.g., using electrochemical or optical transduction principles [29, 59].

2.1.7 Radioactive Transduction Principles

The first immunoassay developed by Yalow and Berson [62] in 1959 was a radioimmunoassay (RIA) for detection of insulin. Radioisotopes were not only the first labels used, for a long time they also offered one of the most sensitive detection methods for immunoassays. As it is not possible to differentiate radioactivity of bound radionuclides from that of free radionuclides, they must be separated. Therefore, all RIAs follow heterogeneous test formats requiring an immobilization step on a surface [63]. Hence, transduction principles exploiting radioactivity should almost be predestined for use in biosensor measurements. However, using radioisotopes is also accompanied by several disadvantages, particularly regarding technical and safety issues [63]. This may be the main reason why—to the best of our knowledge—radioactivity has not found its way into transduction principles for biosensors.

2.2 Biorecognition Layers

2.2.1 Requirements

To turn a transducer into a biosensor, it has to be coated with a biorecognition element. Examples are given in Table 1. Enzymes [64], antibodies [65], oligonucleotides [66], and, more recently, aptamers [67, 68] are examples of biorecognition elements commonly used for diagnostic applications. Biosensor coatings consisting of whole cells (microbial biosensors) [69, 70] or of molecularly imprinted polymers [71] and other synthetic binders [72] as well as coatings using lipid bilayers or liposomes for embedding, e.g., membrane proteins [73], are currently only marginally used in this area and therefore will not be covered here.

The immobilization procedures for biorecognition elements depend mainly on the underlying device material and hence on the chemical environment available [74], but they are largely independent of the transduction mechanism. However, considering transduction processes near the surface, such as electrochemical principles based on electron transfer through electric double layers [33], evanescent field techniques [39], or SAWs [75], the thickness of the complete sensing

layer, i.e., biorecognition element including any intermediate layers above the transducer, must be taken into account. For instance, the thickness may be required not to be too great, particularly if diffusion through the layer is hindered. Otherwise, with increasing distance from the transducer surface, analyte binding to the biorecognition element might lead to a reduced effect on the near-surface signal transduction and hence to reduced signal changes [74].

Ideally, sensing layers should be stable, robust, and possess a certain lifetime. Surface-bound binding sites must be accessible and able to bind the analyte to be detected. On the other hand, nonspecific binding on the transducer surface has to be inhibited to prevent false-positive signals. This applies in particular for nonanalyte molecules in complex sample matrices, such as serum, as used in diagnostic applications. However, this also applies, for example, for secondary detection antibodies, which may be used for signal amplification (for details, see Sect. 2.3), i.e., for any nonanalyte molecule involved in the measurement which may interfere with the biosensor signal response. Strategies to avoid nonspecific binding include a high surface density of the surface-bound biorecognition element, if permitted by its type and availability, and additional intermediate layers with nonstick properties as provided, e.g., by hydrogels. Furthermore, the use of blocking solutions which contain surfactants, proteins (such as bovine serum albumin), or even analyte-free serum (if available) is recommended. These solutions are supposed to occupy the nonspecific binding sites; however, the binding efficiency might be reduced and the blocking proteins themselves might interact with the sample components. Another strategy is to dilute the serum sample to minimize nonspecific binding. However, this also dilutes the concentration of the analyte to be detected and therefore might not be suitable if the analyte concentrations which are expected are too low [76].

In the following sections typical immobilization strategies for biorecognition elements—including nonstick intermediate layers—will be outlined.

2.2.2 Noncovalent Immobilization

Direct adsorption of the biorecognition elements on the surfaces would be the easiest way; however, owing to unavoidable denaturation of the biomolecules and insufficient shielding against nonspecific binding of sample matrix components to the substrate, this method is generally not recommended. Electrostatic binding of, for instance, oligonucleotides might not be stable enough for use in biosensors, as in this case potential shifts in the solution's pH value or ion strength may lead to detachment of biorecognition elements from the surface. Therefore, immobilization protocols based on covalent coupling procedures are typically preferred [74]. To ensure the binding ability of enzymes, however, it has proven to be useful to encapsulate them, e.g., via hydrogel, sol–gel, or lipid bilayers, instead of coupling them covalently to the surface [28, 31].

2.2.3 Covalent Immobilization

The transducer surfaces typically provided are metals (e.g., gold), oxidic layers (e.g., metal oxide or quartz/glass), and polymers. First, functional groups have to be introduced. In the case of gold layers, suitably substituted thiols forming self-assembled monolayers (SAMs) are used as adhesion linking layers for further coupling. A chain length of at least ten carbon atoms is recommended to obtain well-defined and stable SAMs of high density. If oligonucleotides or aptamers provided with thiol substituents are available, they can be coupled directly to the gold surfaces, which will lead to complete biorecognition layers. On oxidic layers, SAMs can be formed by treatment with silanes. Frequently used silanes are 3-aminopropyl triethoxysilane and 3-glycidyloxypropyl trimethoxysilane, providing amino groups and epoxy groups, respectively, for further coupling procedures [74]. If the transducers expose polymer surfaces, they can be activated by oxidative treatment via wet etching or plasma treatment and processed directly [74] or silanized similarly to the oxidic layers [77]. In the next step, biomolecules or hydrogels serving as intermediate layers can be coupled to the transducer surfaces via the functional groups of the SAM.

As mentioned earlier, minimizing nonspecific binding on biosensor surfaces is crucial for biosensor measurements in complex media. In this context, it was shown that sensing layers consisting of antibodies coupled on gold surfaces by means of 16-mercaptohexadecanoic acid can be used for analyte detection in serum samples, because nonspecific binding was effectively reduced [78]. If gold surfaces are not at hand, it is often advantageous to couple the biorecognition elements via an additional hydrogel layer, as these layers are known to effectively prevent nonspecific binding on sensor surfaces. Furthermore, they enable mild reaction conditions, so the biomolecules will retain their physiological structure and hence their functionality in the subsequent modification step [74]. The most commonly used hydrogels featuring these properties are functionalized dextrans, particularly carboxymethyl dextran, and poly(ethylene glycol)s [39, 74].

If these hydrogels provide carboxyl groups, antibodies, enzymes, and other protein-based biorecognition elements can easily be immobilized covalently via their amino groups by means of carbodiimide. This coupling procedure is regarded as flexible, simple, and robust and provides high coupling yields, which is why it is the most frequently employed immobilization method [74]. If the transducer (hydrogel) surface provides amino groups itself, this method can still be applied, because the amino groups can easily be converted to carboxyl groups, e.g., by means of dicarboxylic acid anhydrides [74, 79]. One drawback of the carbodiimide coupling procedure is the fact that a protein's amino groups are distributed all over the molecule; hence, the protein will be coupled in a random orientation to the surface, resulting in a potential blocking of the protein's binding sites. A way to overcome this problem is the affinity binding of the biorecognition elements via corresponding capture molecules. Examples are the coupling of biotinylated species of biorecognition elements via streptavidin and the coupling of antibodies via protein A or protein G. However, these capture molecules might increase the

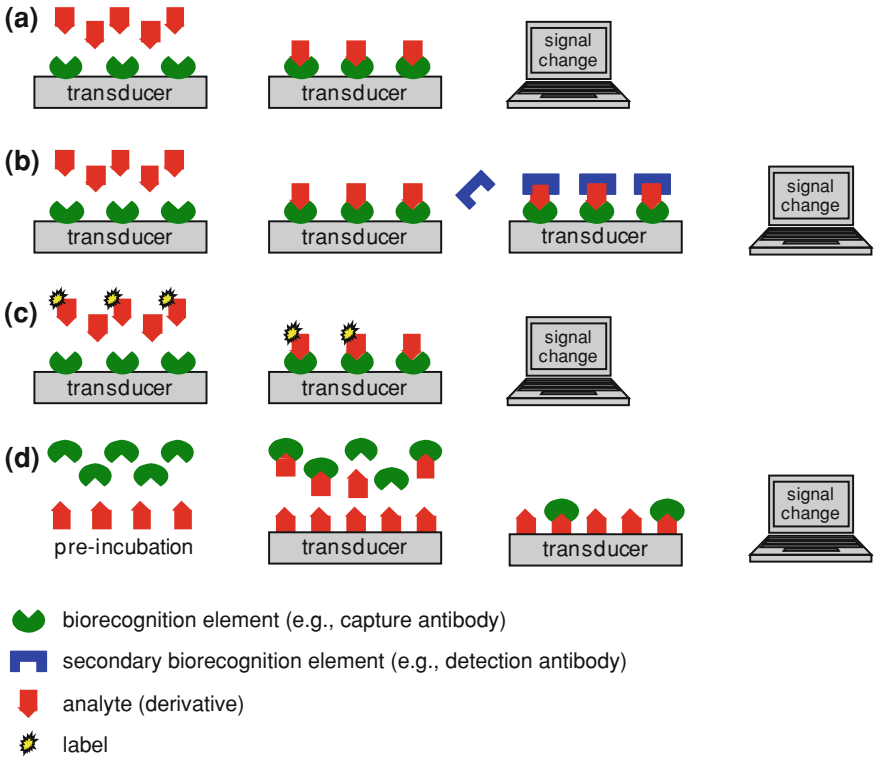

Fig. 1 Biosensor test formats. **a** Direct detection, label-free: quantifiable signal response obtained by binding of analyte. **b** Sandwich assay, label optional: quantifiable signal response obtained by binding of a secondary biorecognition element subsequent to analyte binding. **c** Competitive assay, label required: quantifiable signal response obtained by binding of a labeled analyte derivative competing with the analyte for the surface-bound binding sites. **d** Binding inhibition assay, label optional: after a preincubation step, in which equilibrium between analyte molecules and unbound biorecognition elements is achieved, the remaining free biorecognition elements are detected via immobilized analyte (derivative), allowing calculation of the original analyte concentration

degree of nonspecific binding on the sensing layer [74, 80]. For potential problems arising from the increased sensing layer thickness in the case of near-surface transduction principles, see Sect. 2.2.1.

2.3 Biosensor Test Formats

2.3.1 Test Formats Derived from Assay Formats

Figure 1 gives an overview of test formats derived from assay formats applied in biosensor measurements. In principle, four categories of test formats are available:

1. Direct detection (Fig. 1a)
2. Sandwich assay (Fig. 1b)
3. Competitive assay (Fig. 1c)
4. Binding inhibition assay (Fig. 1d).

By definition, biosensors provide immobilized biorecognition elements; hence, biosensor test formats are per se heterogeneous. Therefore, they can generally be derived from test formats used for traditional immunoassays, such as RIA and enzyme linked immunoassay (ELISA), which include the use of labels [81, 82]. However, instead of a "passive" substrate, such as a microplate, a biosensor provides an "active" transducer on which the biorecognition element is immobilized. Hence, biosensors permit an additional test format, i.e., the direct and label-free detection of the analyte (Fig. 1a). In this case, provided a label-free transduction principle is used, the signal response changes as soon as the analyte binds to the biorecognition element immobilized on the transducer surface. It is a one-step process without any other reagents which even allows real-time monitoring of binding to the biosensor surface, if required. The simplicity, speed, and the inexpensiveness of this approach are the major advantages of this procedure and are among the driving forces for the development of label-free transduction principles. However, the performance in the detection of low concentrations or of small molecules (or both) may be limited. Furthermore, when one is working with complex sample matrices, such as serum, the simplicity of the direct approach might turn out to be a considerable disadvantage, because nonanalyte components from the sample matrix may also bind to the transducer surface, leading to false-positive results. This has to be strictly avoided [45, 83, 84]. To prevent nonspecific binding, specific procedures for the preparation of biosensor recognition layers are required which have to ensure that only the analyte to be detected will bind on the biosensor surface. Details are described in Sect. 2.2.

Widely used labels for biosensors involve enzymes, nanoparticles, and fluorescent or electrochemiluminescent probes [82, 83]. Details about corresponding transduction principles are described in Sect. 2.1. If labeled test formats are used, undesired effects resulting from nonspecific binding of sample matrix components are supposed to be reduced. This is because the final biosensor signal response is usually determined by the labeled compound, whose binding behavior is assumed to be largely independent of the matrix components. However, nonspecific adsorption of labeled compounds will also lead to false-positive results and therefore has to be avoided as well. An important advantage of labeled test formats (including the use of transduction principles requiring labels) is the higher potential for the detection of lower analyte concentrations due to the amplification afforded by the respective label or label-induced reaction, such as a fluorescent signal or an enzyme-catalyzed redox reaction [83]. However, a significant disadvantage of labels is that they can interfere with the analyte binding, leading to distorted results. In the case of TIRF or other fluorescence-based transduction principles, autofluorescence of biological samples can be a problem as well [85]. Furthermore, the need for labeled compounds

usually implies higher operation costs than for direct label-free detection, as the costs of these compounds have to be added. Another point to consider is the fact that real-time monitoring of the analyte binding to the surface is not possible [45, 82–84]. Finally, when it comes to deciding whether to use labels or not, the transduction principle also has to be taken into account, as some of the transduction principles require the use of labels. Examples are transduction principles based on fluorescence, which would require the use of fluorophores. On the other hand, a label-free gravimetric transduction principle, for instance, can be used without a label, but a compound labeled with a nanoparticle as a mass label may help to increase the signal respone, if required [51].

In a sandwich assay (Fig. 1b), the analyte-related signal response is obtained when a second biorecognition element binds to the analyte after the analyte has bound to the biosensor surface. This requires analyte molecules which are large enough for two independent biorecognition elements to bind. Whether the second biorecognition element has to be labeled or not depends on the transduction principle. It is also possible to use a third biorecognition element binding to the second one [51] ("indirect sandwich"). The use of more than three biorecognition elements is not common [81]. In a competitive assay (Fig. 1c), the analyte and a known concentration of the labeled analyte (derivative) compete for the surface-bound analyte-specific binding sites provided by the biorecognition element. This test format including the corresponding transduction principles requires labels. In the binding inhibition assay (Fig. 1d), a preincubation step, in which the analyte and a known concentration of the unbound biorecognition element equilibrate, precedes the actual measurement. Again, whether the biorecognition element has to be labeled or not depends on the underlying transduction principle. After equilibrium has been attained, the binding sites of the remaining free biorecognition elements are detected with a biosensor providing an immobilized analyte (derivative), allowing one to derive the original analyte concentration in the sample [82]. This method is particularly suitable for label-free detection of small molecules, as the signal response is achieved by binding of the corresponding biorecognition element. The latter is usually a protein of higher mass and size and hence should create a higher signal response [86].

2.3.2 Test Formats Based on Molecular Switches

Today, 85% of all biosensors sold are glucose sensors [8]. The reason for this success story might be that this is one of the very few biosensor types in which the interaction between the analyte and the biorecognition element creates a reagent which can easily be detected: in this process the chemical transformation of glucose by glucose oxidase results in the formation of hydrogen peroxide, which can be monitored amperometrically [2, 28, 87]. In contrast, other biosensor formats, such as those mentioned earlier, are mainly based on physical effects near the transducer surface, i.e., no reagent is released, and there are few significant conformal changes supporting the signal response. From a technical point of view, the

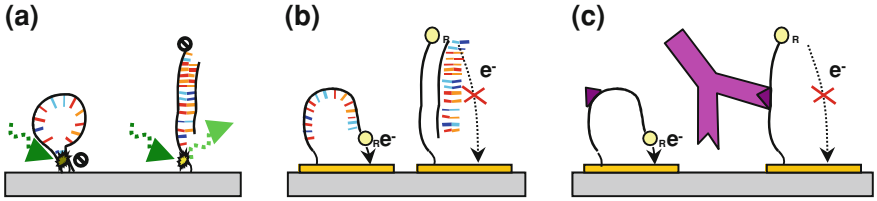

Fig. 2 Proposed sensing mechanisms of molecular switches. **a** The classic fluorescent molecular beacon. The proximity between a fluorescent dye and a quencher is broken as a consequence of a binding reaction (hybridization); thus, the quenched fluorescent signal becomes visible. **b** An electrochemical molecular switch. The proximity of a redox species *R* to a current-collecting electrode is broken as a consequence of a binding reaction (hybridization); thus, the electrons are not able to drift to the electrode anymore, resulting in a decrease of the current. **c** The same mechanism as in **b** can also be used to detect larger molecules, such as antibodies, by means of an aptamer which will change its conformation once the antibody is bound

fact that analyte binding to the biorecognition element does not create a measurable species per se, such as emission of electrons or photons or the like, is the main problem for biosensors. Still, there may be a change in conformation which can be used for the detection. On the basis of this thought, a new type of biosensor was developed in which the interaction between the analyte and the biorecognition element results in a significant conformation change. To allow simplified detection, the biorecognition element was designed in a way that the conformation change will induce an additional response which can easily be measured. These sensors are called "folding-based" biosensors, most of them making use of molecular switches (Fig. 2). They can be ranked as a further development of aptasensors. The easiest and earliest cases of such switches were reported for the detection of nonhybridized DNA by means of a complementary single-stranded DNA (ssDNA) bound to a surface (see Fig. 2a). These biosensors used the fact that ssDNA can be designed in a way, e.g., by matching complementary bases at both ends of the DNA strand, that a fluorescent dye and a quencher matching the dye are in close proximity. In this case, incoming light will not result in visible emission because of quenching. If the complementary ssDNA is detected from the sample, the DNA hybridizes and the proximity between the dye and the quencher is broken, thus allowing visible emission of light as a consequence of the binding. The first fluorescent biosensors of this kind were reported as "molecular beacons" in 1996 [88]. These principles were later transferred to electrochemistry [89, 90], suggesting the mechanism depicted in Fig. 2b. In this setup a redox species (e.g., methylene blue) is tagged to the end of the ssDNA, which is immobilized on a current-collecting electrode. In the nonhybridized state the redox label will dwell near the surface, allowing the electrons created during the oxidation process to drift to the electrode, resulting in a current. In the hybridized state the resulting double-stranded DNA (dsDNA) will stiffen, thus bringing the redox label away from the surface. As a result, the registered current will be

reduced. Similar mechanisms can also be used to detect affinity binding systems such as antigen–antibody reactions [91] or protein binding to a (small) recognition element [92], both cases using deformation of an aptamer carrying a redox label in a way that the measurable current will be changed (Fig. 2c).

The strength of this approach is in the fact that the actual binding itself triggers the signal, thus the reference to a molecular switch: if no binding occurs, the switch will not be triggered. This principle is robust even in very complex backgrounds (cell culture medium, serum, etc.) as it is not affected by nonspecific adsorption on the transducer surface. As will be shown in the next part of this chapter, applications of biosensors in real (serum) samples are comparatively rare, despite the need for easy-to-use protein detection systems and despite the potential of biosensors in this field. This new biosensor type relying on a new test format has already been used for antibody detection and for protein detection in serum samples [91, 92]. Vascular endothelial growth factor is a regulator of both physiologic and pathologic angiogenesis. With a folding-based biosensor, a detection limit of 5 pM (190 pg/ml) in 50% blood serum could be achieved, which is in the clinically relevant concentration range [92].

2.4 Biosensor System Setup and Microfluidic Integration

The biosensor is the heart of every biosensor system. Without this component no signal can be generated. However, in most cases this is not sufficient. To be suitable for handling by the user, the biosensor needs to be provided in a way that ensures the device's integrity during handling and prevents damage to the device during operation. This is usually guaranteed by means of embedding the device into a biosensor housing. The combination of a biosensor in its housing is typically referred to as "biosensor chip". The physical embedding comes with the disadvantage that the biosensor may no longer be directly accessible for sample delivery or signal readout. This is why the biosensor housing usually has to be supplied with features other than mere physical protection [15].

There are several aspects to keep in mind when developing a housing strategy for a biosensor. Foremost, ease of handling the component has to be ensured. However, it may be equally important to keep in mind the additional costs caused by the biosensor housing and the processes required for the embedding. If the biosensor system is to be commercialized for the mass market, compatible manufacturing techniques have to be chosen. In such a scenario, the biosensor housing would usually be designed as a low-cost component created in an industrially available polymer such as poly(methyl methacrylate) or polystyrene. Furthermore, polydimethylsiloxane has become popular in recent years, especially if active microfluidic structures (such as

microvalves or micropumps) are to be embedded next to the biosensor [15]. Polymers allow the use of cheap mass-market replication techniques, such as hot embossing and injection molding, at low cost. Keeping manufacturing costs low will also allow a potential use of the resulting biosensor chips as single-use components, which are usually preferred in biomedical and clinical applications [13].

Another important aspect to consider for biosensor integration is the fact that usually more than one analyte may have to be detected, and thus the combination of several biosensors into biosensor arrays would be beneficial to allow multiplex detection in one measurement cycle. This is a task that can usually be fulfilled by the biosensor housing. Two potential application scenarios have to be considered here: more than one analyte (target) is to be detected in one sample ("single sample–multiple target" biosensor array) or one marker is to be detected in multiple samples ("single target–multiple sample" biosensor array). In both cases the physical protection of the individual biosensors may be provided by the same biosensor housing, but the individual biosensor chips have to be separated fluidically. This is required not only for the experiment but also for the application of the sensing layers.

The most commonly found example for the housing strategy in single sample–multiple target arrays is the creation of more than one transducer structure on a single substrate and their (potentially permanent) fluidic separation by means of a polymeric flow cell. Such examples can be found amply in the literature [93]. This strategy is also used for numerous commercialized biosensor systems, such as the SPR biosensor system of Biacore [94] and the SAW biosensor system of SAW Instruments [95]. This setup is especially advantageous if the physical structures required for biosensor elements (such as electrodes) can be manufactured by cheap techniques on planar substrates. Potential manufacturing techniques include screen-printed electrodes [28, 36] or electrodes made by spotting or printing of conductive polymers [96].

If the biosensor has to be created as a single component (maybe because a nonplanar substrate, such as a fiber, is used), the biosensors are embedded individually and assembled in chip format into biosensor arrays. Recently, a strategy was suggested allowing the embedding of individual biosensors into separate polymer housings and assembling them into versatile biosensor arrays [97]. For this, a disposable polymer microfluidic chip is used which defines the number of individual biosensors in the array. In the same work, it was also demonstrated that such a strategy can be complemented by integration of such an array into a microfluidic environment [97]. Especially suitable for such applications is the use of indirect microfluidic systems that allow an overall biosensor system array to remain an "all-disposable" setup, as the active components responsible for driving the fluids in the system, i.e., pumps and valves, will not come into contact with the sample [98].

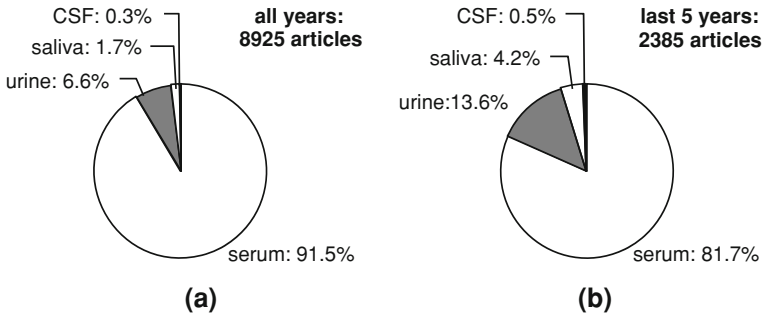

Fig. 3 Number of publications on biosensors in combination with different sample matrices, ISI Web of Knowledge, August 15, 2011. The keyword combination was "biosensors AND [sample type]". The article counts are shown as a percentage of the sum resulting from the four keyword combinations. **a** All years (database starts 1926); **b** last 5 years

3 Biosensors for the Detection of Biomarkers in Real Samples

3.1 Real Samples for Diagnostic Applications

When it comes to the choice of material for disease diagnosis, human body fluids instead of tissue are generally preferred, mainly because of the lower invasiveness. This goes well together with biosensor measurements, which typically require liquid samples. Provided that the disease markers to be detected are contained, samples seem the more attractive the easier they can be collected and processed with minimum time and effort [1]. The most commonly used sample type for biosensors in diagnostic applications is serum, but in the last 5 years other body fluids, such as urine and saliva, have increasingly been investigated (Fig. 3), which is because they can also be collected by noninvasive sampling. Furthermore, they allow a closer investigation of the state of their origin. Urine samples, for instance, may provide more information about the current state of kidney and bladder than serum samples [99]. Salivary samples, aside from detection of viruses, drug abuse, and some cancer biomarkers, allow, in particular, the monitoring of oral diseases [13, 100]. Biosensor measurements using CSF have also been gaining interest in recent years owing to potential biomarkers in CSF allowing early detection of neurodegenerative diseases, such as Alzheimer's disease [101]. All the real sample matrices have in common that they supply a complex protein background which may nonspecifically bind to the biosensor surface, resulting in false-positive results. For strategies to overcome this problem, see Sect. 2.2.

In the following sections the use of biosensors for the detection of disease-related biomarkers is demonstrated. Sample dilution has often been reported as means to reduce nonspecific binding, despite the fact that mostly small biomarker concentrations have to be detected. This leads to high demands regarding the biosensor systems.

Four application areas were chosen to show the performance of biosensors dealing with real samples:

- Cardiac biomarkers in serum. They may be a sign of, e.g., myocardial infarction and therefore require a fast and reliable detection.
- Serum biomarkers for cancer and autoimmune disorders. Chronic diseases require early diagnosis as well as regular control of certain biomarkers, i.e., a reliable and low-cost detection method would be beneficial.
- Biomarkers in CSF for neurodegenerative diseases. Early diagnosis and treatment would allow delay of severe symptoms, i.e., a reliable and low-cost detection method would be beneficial.

3.2 Detection of Cardiac Biomarkers

3.2.1 Cardiovascular Disease Diagnostics

Globally, CVDs are the main cause of death [102]. Among acute coronary syndromes, myocardial infarction, commonly referred to as "heart attack," is one of the most life-threatening forms, as it quickly causes irreversible damage to the heart. Therefore, it has to be diagnosed as soon as possible, e.g., by means of an electrocardiogram or by determination of concentrations of cardiac specific biomarkers, such as troponin and myoglobin. C-reactive protein (CRP) may also be detected; however, this marker is rather used for monitoring the risk of recurrence of myocardial infarction [103]. As previously reviewed, commercially available POCT devices exist which are able to determine the acute phase biomarkers within 5–20 min assay time in the required concentration ranges. Most of them are based on immunoassay or immunochromatographic test methods using fluorescence or chemiluminescence detection allowing the determination of several cardiac biomarkers within one measurement cycle. However, the integration of those devices in clinical routine—and thus their acceptance—is still low, mostly due to cost restraints and because clinical cutoff values have not yet been established [103]. Therefore, as biosensors provide the possibility for low-cost analysis, they could still find their way in this field of application. This will be demonstrated in the following by examples of biosensors for the detection of the cardiac biomarkers troponin, myoglobin, and CRP. The biosensors presented are immunosensors, i.e., antibodies were immobilized as analyte-specific biorecognition elements on the transducer surfaces.

3.2.2 Cardiac Biomarkers in Serum

Troponin and Myoglobin

Cardiac troponins occur in human blood only when the myocardium is directly damaged. Although increased levels are not observable before 3–4 h after the myocardial infarction, because of the lack of other markers, cardiac troponin I (cTnI) and cardiac troponin T (cTnT) are currently considered as the "gold standard" in myocardial infarction diagnosis. The cutoff levels are in the ranges 0.01–0.1 ng/ml (cTnI) and 0.05–0.1 ng/ml (cTnT), respectively. In contrast to the cardiac troponins, increased levels of myoglobin can be detected 1–3 h after the myocardial infarction; the cutoff levels were specified in the range 70–200 ng/ml. However, increased levels of myoglobin are not specific for myocardium damage. Therefore, in the case of a suspected myocardial infarction, it would be beneficial to monitor the concentrations of both troponin and myoglobin during the first few hours for early and efficient diagnosis [103].

Commercially available POCT devices using electrochemical biosensors are provided by Abbott Point of Care (i-STAT series), including a test cartridge for cTnI. This amperometric biosensor setup allows the determination of cTnI in the range 0–50 ng/ml. In contrast to the POCT devices mentioned in Sect. 3.2.1, this biosensor setup detects only a single biomarker [104]. Direct detection of cTnT in human serum samples obtained from cardiac patients could also be performed with a label-free capacitive immunosensor [105] and an SPR immunosensor [106]. In both cases, the concentrations were determined prior to the biosensor measurements by means of commercially available electroluminescence immunoassays. cTnT in serum could be detected with the capacitive biosensor in the concentration range 0.07–6.83 ng/ml [105], and with the SPR biosensor in the range 0.83–3.16 ng/ml [106].

An interesting approach was reported for direct detection of myoglobin with an impedimetric biosensor. The sensing layer was based on half-antibody fragments which could be immobilized in a well-defined orientation, resulting in very dense layers on the biosensor surface. Serum was spiked with myoglobin over the concentration range 10^{-14}–10^{-7} M, corresponding to 0.178 pg/ml to 1.78 μg/ml (M_r myoglobin 17,800) and could be detected with the biosensors in this wide concentration range [107]. Finally, an SPR immunosensor using a SAM based on 16-mercaptohexadecanoic acid was successfully used to detect myoglobin and cTnI in undiluted serum. (Actually, bovine serum was used for the measurements; nevertheless a complex serum sample matrix was represented.) The limit of detection of myoglobin was reported to be 0.9 ng/ml, and that of cTnI was 0.7 ng/ml; in both cases linearity was reported to be up to 50 ng/ml [78].

C-reactive protein

CRP is a classic acute phase protein; however, it is a nonspecific biomarker which indicates all kinds of tissue damage, bacterial infections, and other acute

inflammatory events, including even autoimmune diseases. Furthermore, CRP is a predictive marker of future risk of CVD. There is no defined clinical cutoff value, but concentration ranges for risk prognosis are suggested: CRP concentrations below 1 μg/ml mean low risk, concentrations in the range 1–3 μg/ml indicate intermediate risk, and concentrations in the range 3–15 μg/ml show a high risk [103]. Owing to the comparatively high concentrations in blood, CRP is one of the most commonly used model analytes for testing and demonstrating the performance of a biosensor. Therefore, numerous approaches with all kinds of transducer principles and biosensor coatings have been reported. CRP biosensors were recently reviewed in connection with other cardiac biomarkers, [103].

Furthermore, CRP was detected in human serum by means of TIRF using two test formats, i.e., sandwich assay and binding inhibition assay. Samples were diluted 1:100. The working range of the sandwich assay was 0.044–2.9 μg/ml (detection limit 0.13 μg/ml), and the working range of the binding inhibition assay was 0.13–22.9 μg/ml (detection limit 0.055 μg/ml). These values represent concentrations in undiluted serum, i.e., both methods allow the detection of CRP in the low/intermediate risk range [108]. An impedimetric biosensor using a so-called biogenic nanoporous silica membrane as a layer for each electrode was designed for direct and label-free detection of CRP and another cardiovascular biomarker, myeloperoxidase, in human serum. In human serum samples, for both proteins a large dynamic range from 1 pg/ml to 1 μg/ml was obtained [109]. Admittedly, in the case of CRP, quantification of concentrations below 1 μg/ml is usually not required. However, in this case sample dilution for minimizing the potential risk of nonspecific adsorption could be performed without further ado.

3.3 Detection of Cancer Biomarkers

3.3.1 Cancer Diagnostics

Besides CVD, cancer is globally a major cause of death [110]. There are more than 200 cancer-related diseases and all have one thing in common, i.e., an early detection and classification is crucial for a positive outcome for the patients [4, 7]. Since 1958, tumor staging has been standardized via the TNM system, which based on information about tumor size or depth (T), lymph node spread (N), and the presence or absence of metastases (M) [3, 111]. This anatomical-based staging system has increasingly been complemented by molecular markers to allow further classification of the tumor into subsets, targeting improved diagnosis, prognosis, or prediction of therapy [3]. This includes monitoring of cancer treatment or remission cycles, which is easier to perform if serum biomarkers can be used instead of tissue-related biomarkers [1, 13].

In the case of cancer, efficient diagnosis demands the detection of more than one single biomarker. Instead, a complete marker profile (“molecular signature”) has to be determined, both on the genomic and on the proteomic level [3, 4],

Table 2 Cancer biomarkers

Marker	Type	Source	Cancer type	Threshold	References
HER2/neu	Protein	Serum, tissue	Breast	15 ng/ml	[111, 112]
PSA	Glycoprotein	Serum	Prostate	4 ng/ml	[7]
			Breast	1 pg/ml	[113]
CEA	Glycoprotein	Serum	Colon	3 ng/ml	[7, 111]
hCG	Glycoprotein	Serum, urine	Testicular, ovarian	5 IU/l	[7, 111]

HER2 human epidermal growth factor receptor 2, *PSA* prostate-specific antigen, *CEA* carcino-embryonic antigen, *hCG* human chorionic gonadotropin

consisting of 30–100 biomarkers [83]. This rough estimation of the required number of biomarkers results from the fact that these marker profiles have not been established yet and are still under investigation [1, 4]. Still, a great number of biomarkers have already been linked to cancer types, which leads to enhanced diagnostic information than obtained using the TNM system only [3, 111]. Currently, clinical, quantitative detection of tumor markers is typically performed by means of immunoassay based methods [7]. Considering reduced time and effort, biosensors could offer an alternative, provided that the demands resulting from the respective diagnostic application are met. This means that the biomarkers have to be detected in clinically relevant concentrations in real samples. Furthermore, the determination of multiple concentrations in one measurement cycle would be preferable. In the following, examples of the current state of biosensor detection of cancer biomarkers will be shown. The biosensors presented are immunosensors, i.e., antibodies were immobilized as analyte-specific biorecognition elements on the transducer surfaces. The cancer biomarkers chosen are listed in Table 2, including the properties required because of the respective diagnostic application. The biomarkers chosen are FDA-approved [111].

3.3.2 Cancer Biomarkers in Serum

Human Epidermal Growth Factor Receptor 2

Breast cancer is the most common cancer among women worldwide. Human epidermal growth factor receptor 2 (HER2)/neu, an epithelial transmembrane protein, is mainly regarded as prognostic or as a therapy-predictive marker for this type of cancer [114]. The extracellular domain of HER2/neu is shed from cancer cells into the circulation and is therefore measurable in serum. Critical values for HER2/neu are above 15 ng/ml [112].

Immunosensors for direct and label-free detection of the HER2 extracellular domain in serum samples were recently reported. For detection by optofluidic ring resonators, serum samples were spiked with HER2 biomarker. The device was able to detect HER2 concentrations in serum in the range 13–100 ng/ml [112]. For detection by means of microcantilevers, diluted patient samples were used

(dilution 1:40), and the real concentrations were determined by a commercially available ELISA kit. The microcantilever allowed quantification of final serum concentrations above 2 ng/ml [115]. Both label-free biosensors allowed HER2 detection in the range of clinically relevant concentrations.

Prostate-Specific Antigen

Prostate-specific antigen (PSA), a serine protease produced by the prostate epithelium, is clinically used for the detection and screening of prostate cancer [116]. Critical values for PSA are above 4.0 ng/ml total PSA in blood [7, 116]. Actually, specificity of PSA for prostate cancer is low, resulting in the need for a marker profile to allow accurate diagnosis [4, 116]. Still, owing to its widespread use as screening parameter, PSA was commonly used as a model analyte for testing the performance of biosensors designed for diagnostic applications, which has led to numerous publications. Biosensors for PSA detection were recently reviewed [116]. The latest developments include mainly methods for enhancing the biosensor signal response to allow determination of low PSA concentrations, preferably in the picogram per milliliter range. This should allow early diagnosis of prostate cancer recurrence [116]. Furthermore, increased levels of PSA (above 1 pg/ml) are considered as a parameter for the detection of breast cancer [113].

An amperometric biosensor was used for PSA detection, where signal enhancement was obtained by labeling the secondary antibody (in addition to the peroxidase label) with nitrodopamine-functionalized iron oxide nanoparticles. Serum samples containing 1–10 ng/ml were tested with this biosensor sandwich assay and with ELISA. The correlation coefficient was 0.992, i.e., the results were in good agreement. A comparison of the performance of several electrochemical immunosensors for PSA detection was also given in this work [117]. A QCM biosensor was used for PSA detection in 75% serum. Signal enhancement was obtained by labeling the secondary antibodies with gold nanoparticles. In this sandwich assay, a linear range for PSA concentrations between 0.29 and 150 ng/ml was obtained, corresponding to 0.39–200 ng/ml in 100% serum [118]. Finally, a so-called localized surface plasmon coupled fluorescence fiber-optic biosensor was used to detect PSA in tenfold diluted normal women's serum. This sandwich immunoassay combined with the localized surface plasmon technique allowed the detection of 1.8 pg/ml PSA in diluted serum [113].

Carcinoembryonic Antigen

The glycoprotein carcinoembryonic antigen (CEA) has less specificity to a certain type of tumor than reported for HER2 or PSA (see earlier). Hence, it is associated with several types of cancer, in particular colorectal cancer, but also pancreatic cancer, liver cancer, and gastric cancer to name but a few [3, 7]. Critical values for CEA are above 3.0 ng/ml [7].

An SPR biosensor using signal enhancement by a secondary antibody (sandwich assay) was reported. Samples were spiked with the respective CEA

concentrations and diluted 1:10. CEA serum concentrations were determined in the range 25–800 ng/ml, i.e., only increased CEA levels could be detected [119]. Furthermore, two amperometric immunosensors were used for CEA detection via sandwich assay. The first amperometric biosensor did not use additional means for signal enhancement. Serum samples from colon cancer patients were tested, i.e., the serum CEA concentration was at least 3 ng/ml. The results were in good agreement with those from ELISA tests performed with the same samples [120]. The second biosensor used signal enhancement by labeling the secondary antibody with a gold nanoparticle carrying several peroxidase molecules. In spiked serum samples, concentrations in the range 1–40 ng/ml CEA could be determined. This means that CEA concentrations near and below the threshold value could be detected. Again, the results were confirmed with ELISA tests [121].

Human Chorionic Gonadotropin

The level of the glycoprotein hormone human chorionic gonadotropin (hCG) is increased in the case of pregnancy, and the threshold value recommended for a reliable diagnosis is 14.3 mIU/ml in serum [122]. In the field of cancer diagnosis, hCG is used as a biomarker for testicular and ovarian cancers. In this case, the threshold value is 5.0 mIU/ml, i.e., it is lower than in the case of pregnancy [7].

Direct detection of hCG was performed with a label-free capacitive immunosensor. Clinical serum samples were diluted 1:2 and tested both with the immunosensor and with RIA. Serum concentrations were in the range 18–450 mIU/ml hCG. The results obtained with the immunosensor were in good agreement with those obtained with RIA tests [122].

3.4 Detection of Biomarkers for Autoimmune Disorders

3.4.1 Autoimmune Disease Diagnostics

Autoimmune disorders are pathological conditions in which the immune response is directed against the patient's own cells and tissues. They can become manifest in a single organ or tissue (localized or organ-specific autoimmune diseases), such as type 1 diabetes, or can affect multiple organs and tissues (systemic autoimmune diseases), such as systemic lupus erythematosus (SLE). More than 80 autoimmune diseases have been described [123, 124]. All of them have in common that they are typically chronic, and an early diagnosis of disease type or disease flare-up is required to enable early treatment and avoid the risk of severe symptoms [125]. Autoimmune diseases are mainly characterized by autoantibodies, i.e., antibodies which are directed against self-proteins. Epitope specificity of autoantibodies as well as disease-related molecular profiles to identify the disease type and state have been widely investigated [123, 126].

3.4.2 Disease-Related Autoantibodies in Serum

Type 1 Diabetes

In type 1 diabetes, the autoimmune response is directed against the insulin-producing β-cells of the pancreas. Diabetes-related autoantibodies include insulin autoantibodies (IAA) and antibodies to tyrosine phosphatase-like protein IA-2 [76, 127]. For both autoantibodies, SPR biosensor assays were developed. For IAA detection, an "indirect competitive immunoassay" was performed using biosensors with immobilized insulin. Each serum sample was divided into two portions. One half was spiked with insulin, similar to the preincubation step in a binding inhibition assay, as the insulin would block potentially available IAA binding sites. The other half of the sample was just diluted (buffer), so the sample dilution in both portions was 1:2. In a measurement cycle, first the sample without insulin was applied on the biosensor surface. Autoantibody potentially bound on the biosensor surface was detected with a second antibody to enhance the signal response (sandwich assay). After a regeneration step, the sample with insulin was applied, including the subsequent detection with a secondary antibody. The difference between the signal responses was used as a measure of the IAA concentration in the sample. The cutoff value of the biosensor was determined to be 6 U/ml, i.e., IAA concentrations below that level would be detected as IAA-negative. This value was reported to be similar to the RIA cutoff value. We think this method describes an interesting alternative to deal with nonspecific effects, which may alter from serum to serum. By blockage of potentially present IAA in one half of the sample with insulin, an inherent reference was created which is consistent with the respective real sample [76]. A more traditional way for biosensor assay was used for the detection of IA-2 autoantibodies in human serum with QCM and SPR biosensors. In both cases a synthetic peptide of 15 amino acids derived from IA-2 molecules was immobilized on the biosensor surfaces. Measurements were performed using samples diluted 1:100 and spiked with antibody. A minimal detected antibody concentration of 0.2 mM in 1% serum was obtained with the SPR biosensor; the QCM biosensor was not sensitive enough [127].

Rheumatoid Arthritis

Rheumatoid arthritis is characterized by chronic inflammation of the joints; sometimes, systemic complications may be involved. Currently, antibodies against citrullinated peptides (citrulline-containing peptides) are the most specific serological markers for diagnosing rheumatoid arthritis [128]. Carbon nanotubes were used to detect those antibodies. First, a QCM biosensor was coated with single-walled carbon nanotubes to allow higher binding capacity owing to the three-dimensional structure, then citrullinated peptide was immobilized. Measurements were performed with serum samples diluted 1:300. Serum samples obtained from rheumatoid arthritis patients could be distinguished from serum

samples obtained from healthy individuals . The observed accuracy obtained with the QCM biosensor was even greater than that obtained by ELISA, i.e., with the QCM biosensor more rheumatoid arthritis patients with antibodies to the citrullinated peptide were identified [129]. In another approach, composites of multiwalled carbon nanotubes and polystyrene were used in an amperometric biosensor setup. Multiwalled carbon nanotube–polystyrene mixtures were applied on metal electrodes. The peptide was covalently coupled on these composite-based electrodes. Samples were diluted 1:100 and 1:200, respectively. Peroxidase-labeled secondary antibodies were used to allow detection (sandwich assay). Only a few patient samples were tested in preliminary tests, but they could be distinguished from samples from healthy donors [128].

Antiphospholipid Syndrome

Antiphospholipid syndrome is an autoimmune disease which is mainly characterized by recurrent thromboses. According to the name, antibodies against phospholipids are produced, such as anti-β2-glycoprotein I [126]. SPR biosensors with covalently immobilized β2-glycoprotein I were used to test serum samples which were diluted 1:100. Distinguishing between antiphospholipid syndrome patients and healthy individuals was possible [130].

Systemic Lupus Erythematosus

SLE is a systemic autoimmune disease which may affect almost any organ as well as connective tissues and blood vessels. Furthermore, SLE may lead to neurological and hematological disorders [126]. Several autoantibodies are used for diagnosis, mostly anti-chromatin, antibodies against nuclei, i.e., antinuclear antibodies (ANA), and anti-dsDNA. In particular, the latter may also be important as a diagnostic parameter for other autoimmune diseases. An amperometric biosensor was reported to detect anti-chromatin antibodies. Electrodes were screen-printed on a poly(vinylidene fluoride) membrane impregnated with chromatin. Serum samples were diluted 1:50 and applied on the sensors, followed by peroxidase-conjugated secondary antibody (sandwich assay). Serum samples obtained from SLE patients could be distinguished from serum samples obtained from healthy individuals [131]. For the detection of ANA in serum, a fiber-optic evanescent wave biosensor modified with colloidal gold has been developed. Extractable nuclear antigens were immobilized on the fiber. The ANA content detected in samples diluted 1:100 was in good agreement with that determined by ELISA tests [132]. Detection of anti-dsDNA in serum samples has been performed with potentiometric [133, 134], QCM [135], and SPR [136, 137] biosensors. Potentiometric biosensors were made of glassy carbon electrodes coated by electropolymerization of aniline [133] or phenothiazine dyes (methylene blue and methylene green) [134] and subsequent immobilization of dsDNA. Serum sample dilution in the range 1:20–1:50 was found to be appropriate to achieve the best

results. Sensor responses obtained with samples from SLE patients could be distinguished from those of healthy donors [133, 134]. Furthermore, a QCM biosensor was modified with dsDNA. Serum samples were diluted to a final protein concentration of 0.1 mg/ml. As serum contains 60–80 mg/ml protein [76], this equates to a dilution factor in the range 1:600–1:800. The results regarding antibody content were reported to be comparable to those from ELISA measurements; however, the QCM results were more affected by nonspecific binding, i.e., the biosensor signal response for the healthy donor sample was increased [135]. Finally, an SPR biosensor modified with oligodeoxynucleotide allowed serum samples obtained from SLE patients and serum samples obtained from healthy individuals to be distinguished [136]. Furthermore, the results were reported to be satisfactory compared with classic immunoassay methods [137].

Celiac Disease

Celiac disease, also referred to as celiac sprue, is an autoimmune disease which is characterized by a disorder of the small intestine triggered by wheat gluten. Serological markers include autoantibodies directed against tissue transglutaminase [138]. Screen-printed gold electrodes were coated with transglutaminase and used as impedimetric immunosensors. Sample signal responses were enhanced by a peroxidase-labeled secondary antibody with subsequent biocatalytic oxidation of the corresponding substrate (sandwich assay). Patient samples could be distinguished from samples of healthy donors [139]. Furthermore, an amperometric immunosensor was developed for detection of anti-transglutaminase. Transglutaminase was covalently coupled on gold electrodes. Antibodies in serum (dilution factors 1:100 and 1:400) were detected via a peroxidase-labeled secondary antibody (sandwich assay). Patient samples could also be distinguished from samples of healthy donors [140].

Cancer Autoantibodies

Circulating antibodies to cancer-related antigens are considered to be present in early stages of disease; therefore, autoantibodies are also increasingly being investigated as cancer biomarkers [141]. Autoantibodies against CEA (see Table 2), for instance, were detected with an SPR biosensor modified with CEA. Both direct detection and detection via a secondary antibody (sandwich assay) allowed patient samples to be distinguished from negative controls which were obtained from healthy individuals [142].

3.5 Detection of Biomarkers for Neurodegenerative Diseases

Neurodegenerative diseases, such as Alzheimer's disease and Parkinson's disease, are mainly characterized by progressive loss of functions of the nervous system.

The diagnosis of such a neurodegenerative disease is required in an early stage to allow early treatment and thus delay of severe symptoms. Current research in this field includes the investigation of disease-related biomarkers in CSF. Sampling of CSF is less invasive than sampling of brain tissue, and owing to its proximity to the brain, it should be better suited than serum to reflect processes in the brain [101].

SPR biosensors have been used to investigate biomarkers in CSF aiming at diagnosis of Alzheimer's disease. Amyloid β peptides in CSF have been reported as potential biomarkers for Alzheimer's disease. A sandwich assay based on an SPR immunosensor was developed to determine the concentrations of two different amyloid β peptides in CSF. The secondary antibody was required as a means of signal enhancement because of the low (subnanomolar) concentrations of amyloid β peptides in CSF. CSF samples from patients diagnosed with Alzheimer's disease could be distinguished from CSF samples from healthy donors [143]. Furthermore, it was suggested that the mitochondrial enzyme 17β-hydroxysteroid dehydrogenase type 10 (17β-HSD10) binds to amyloid β peptides. Therefore, the 17β-HSD10/amyloid β peptide complex could also serve as an Alzheimer's disease biomarker. To detect concentrations of this complex, antibody against amyloid β peptide was immobilized on SPR biosensors. After patient CSF samples (dilution factor 1:2) had been applied, antibody against 17β-HSD10 was applied as a secondary antibody (sandwich assay). An increase in signal response should only be obtained when the 17β-HSD10/amyloid β peptide complex binds to the surface, and not when the pure amyloid β peptide binds. The results were in good correlation with results obtained from ELISA tests performed with the same samples. Samples from patients diagnosed with Alzheimer's disease could be distinguished from control samples obtained from patients suffering from noninflammatory neurological diseases [144].

4 Conclusion

Biosensor development started around 50 years ago with the development of an "enzyme electrode" for monitoring glucose in blood. Since then, biosensor development has been ongoing, with clinical applications still being one of the main driving forces. However, despite the fact that biosensors are promising devices when it comes to fast and easy detection of analytes, their use has not yet been established in clinical routine, in contrast, for instance, to immunoassay techniques.

There is no doubt that numerous publications and patents dealing with biosensors have been published. However, looking at the material published in the last few years more closely, we realized that only a comparatively small part of the work reported was performed using real samples. One reason for this discrepancy might be that the problem of nonspecific binding arising from complex sample matrices has not been completely solved. Strategies commonly used to deal with nonspecific binding in a typical biosensor measurement usually have to be adapted

for the specific application and hence are not easily transferable to other applications. We think that biosensors based on molecular switches offer a promising tool to solve this problem, because in this case it is only the analyte binding which is able to generate a signal response (provided that cross-reactivity is excluded). Molecular switches have been reported for optical and electrochemical transduction principles, which are currently the most commonly used transduction principles for biosensors.

A powerful biosensor system requires a high-performance biosensor component as well as a user-friendly instrumental setup. However, biosensor setups have to be adapted to specific applications. As disease-related marker profiles are still under investigation, specifications regarding a suitable biosensor instrument can hardly be given at the moment. But, almost certainly, such a device should permit multiplex analysis to determine a marker profile in a sample in one measurement cycle. Therefore, a biosensor array would be required. At least as long as these profiles are not established, it could be useful to keep these arrays flexible. This would be supported by a packaging strategy in which each biosensor element is integrated in a single, array-compatible housing allowing user-defined combination. This would make the arrays potentially adaptable to the respective application and hence make the underlying biosensor instrument more versatile.

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