

Review

Immunosensors—principles and applications to clinical chemistry

Peter B. Lippa^{a,*}, Lori J. Sokoll^b, Daniel W. Chan^b^a Institute for Clinical Chemistry and Pathobiochemistry, Klinikum rechts der Isar der Technischen Universität München, Ismaninger Str. 22, D-81675 Munich, Germany^b Department of Pathology, Division of Clinical Chemistry, Johns Hopkins Medical Institutions, 600 N. Wolfe Street / Meyer B-121, Baltimore, MD 21287-7065, USA

Received 22 March 2001; received in revised form 16 July 2001; accepted 20 July 2001

Abstract

Introduction: Immunosensors are affinity ligand-based biosensor solid-state devices in which the immunochemical reaction is coupled to a transducer. The fundamental basis of all immunosensors is the specificity of the molecular recognition of antigens by antibodies to form a stable complex. This is similar to the immunoassay methodology. Immunosensors can be categorized based on the detection principle applied. The main developments are electrochemical, optical, and microgravimetric immunosensors. In contrast to immunoassay, modern transducer technology enables the label-free detection and quantification of the immune complex. **Methods:** The analysis of trace substances in environmental science, pharmaceutical and food industries is a challenge since many of these applications demand a continuous monitoring mode. The use of immunosensors in these applications is most appropriate. Similarly, a series of clinical problems may be solved by continuous monitoring of certain analytes. **Conclusions:** Clinical chemists should take advantage of immunosensors in clinical diagnostics. There are many recent developments in the immunosensor field which have potential impacts. The future role of this technique in intralaboratory, as well as bedside testing, will become even more important as the clinical laboratory is faced with increasing pressure to contain costs. © 2001 Elsevier Science B.V. All rights reserved.

Keywords: Antibody technology; Biosensor; Chip technology; Immunoassay; Immuno-sensor; Label-free detection

Abbreviations: AFM, atomic force microscopy; aP, alkaline phosphatase; 4-APP, *p*-aminophenyl phosphate; BARC, bead array counter; BSA, bovine serum albumin; FCFD, fluorescence capillary fill device; FET, field-effect transistor; FIA, flow-injection analysis; FO, fractional binding site occupancy; FPW, flexural plate wave; GABA, γ -amino-4-butyric acid; hCG, human chorionic gonadotrophin; H-FABP, heart-type fatty acid-binding protein; HIV, human immunodeficiency virus; HLA, human leucocyte antigen; HRP, horseradish peroxidase; ISE, ion-selective electrode; ISFET, ion-selective field-effect transistor; LDH, lactate dehydrogenase; MA, methamphetamine; MALDI-TOF, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; MIP, molecularly imprinted polymer; NPGP, nonpenetrating glycoprotein antigen; OBA, optical biosensor assay; 7-OHC, 7-hydroxycoumarin; OWLS, optical waveguide lightmode spectroscopy; QCM, quartz crystal microbalance; RI, refractive index; RIA, radioimmunoassay; SAM, self-assembling monolayer; SAW, surface acoustic wave; SEB, Staphylococcal enterotoxin B; SELEX, systematic evolution of ligands by exponential enrichment; SH-APM, shear horizontal acoustic plate; SLB, supported phospholipid bilayer; SPR, surface plasmon resonance; STW, surface transverse wave; TE, electrical field wave; TIRF, total internal reflection fluorescence; TIRS, total internal reflection spectroscopy; TM, magnetic field wave; TRAW, thin-rod acoustic wave; TSH, thyroid-stimulating hormone; TSM, thickness-shear mode.

* Corresponding author. Tel.: +49-89-4140-4759; fax: +49-89-4140-4875.

E-mail address: lippa@klinchem.med.tu-muenchen.de (P.B. Lippa).

0009-8981/01/\$ - see front matter © 2001 Elsevier Science B.V. All rights reserved.

PII: S0009-8981(01)00629-5

1. Introduction

The specificity of the molecular recognition of antigens by antibodies to form a stable complex is the basis of both the analytical immunoassay in solution and the immunosensor on solid-state interfaces. The underlying fundamental concept of these analytical methods as ligand assays is based on the observation of the product(s) of the ligand-binding reaction between the target analyte and a highly specific binding reagent [1].

The development of immunoassay technology is a success story especially for the clinical laboratory and still continues to be a vibrant area of research [2]. Further development and automation will expand the possibilities of immunoassay analysis in the clinical sciences. Besides this, new areas for trace analyses using immunoassay were defined in the last decade: the environmental analysis of trace substances and quality control in the food industry. Since these applications also need a continuous monitoring mode, the idea of an immunosensor as a continuously working heterogeneous immunoassay system, covering these features, was conceived. The immunosensor is now considered as a major development in the immunochemical field. Despite an overwhelming number of papers in this field, there are only a few commercial applications of immunosensors in clinical diagnostics. The reasons are, in part, unresolved fundamental questions relating to immobilization, orientation, and specific properties of the antibodies or antibody-related reagents on the transducer surface. In addition, a key issue is which clinical applications may benefit most from immunosensor devices in the routine medical laboratory. Only if there is consensus on the clinical utility of this new technique can the gap between the high expectations of the developer and reality be closed. Designers of immunosensor devices must be aware of the general and special needs of laboratory medicine from new analytical techniques.

A new analyzer should be simple and “rugged” for the measurement of analytes. Measurements have to be performed precisely and accurately, even under STAT conditions. The analyzer must be fully automated and capable of performing rapid measurements with turnaround times of $\ll 1$ h. Additionally, the determination of an analyte should prefer-

ably be without sample pretreatment in matrices, such as serum, plasma, urine or cerebrospinal fluid. All parameters determined with a new analyzer must meet the following criteria, which are defined in various guidelines: low imprecision, small lot-to-lot variations, high analytical sensitivity, optimum analytical specificity and accuracy with long calibration stability, and low interferences by drugs or normal and pathological sample components.

In the clinical laboratory, a future substitution of immunoassays by immunosensors simply depends on the superiority and versatility of the new methodology. The applicability for point-of-care testing or when they are temporarily implanted into the patient additionally depends on the reliable and accurate analysis of the desired analyte, without drift problems or matrix interferences.

Due to the tremendously growing variety of developments, this review is not intended to be comprehensive. Hence, the main focus will be the description and assessment of reported clinical applications of immunosensors. For a more thorough understanding, we refer to several excellent reviews in the last 5 years on technical aspects [3–9] and the application of immunosensors [10–14] in various fields. Other related reviews deal with antibody engineering developments [15,16] and latest immunoassay technologies [17–19].

2. Antibodies as bioaffinity interface for both immunoassays and immunosensors

It should first be clarified that the specificity for the measurement of analyte(s) in all immunosensor systems, as in the case of immunoassays, is dependent on the application of binding molecules. This pivotal feature is shared by both technologies. New developments in protein engineering for immunoglobulins (antibody fragments, chimeric antibodies, etc.) or in substituting antibodies by alternative binding components (e.g., aptamers) or structures (molecular imprinting) will, therefore, be applicable to either technologies, if available. In particular, the possibilities in antibody engineering will enable changes in the affinity and fine specificity of antibodies [20,21], as well as the expression of

fragments as fusion proteins coupled to reporter molecules [22].

2.1. Immobilization procedures for antibodies

Antibodies have to be properly immobilized on the immunosensor surface, which is mostly part of a flow-through cell. The optimum density and adjusted (but not random) orientation of the antibodies are of paramount importance. Due to the different types of sensing surfaces, this manipulation can have benefits (e.g., improvement of the reaction kinetic parameters), but also unfavorable effects (e.g., increased nonspecific binding, partly destroyed paratope). There are four different types of oriented coupling of antibodies: binding to Fc receptors (such as protein A or G or recombinant A/G fusion protein) on the surface [23,24]; binding of other binding partners to structures, covalently linked to the Fc part of the antibody, e.g., the biotin residue on the Fc binds to surface-coated streptavidin [25,26]; coupling to the solid support via an oxidized carbohydrate moiety on the C_H2 Fc domain; and the binding of Fab or scFv (see Section 2.2) fragments to the surface of the device via a sulphhydryl group in its C-terminal region [27,28].

Numerous chemical reactions can be applied to the immobilization onto solid surfaces. Defined linkages between the antibody (or its carbohydrate moieties) and the solid phase material (silica, silanized silica, Ta- or Ti-oxides, plastics, sepharose, metal films) are being built by glutaraldehyde, carbodiimide, succinimide ester, maleinimide, periodate or galactose oxidase (EC 1.1.3.9) [29]. Moreover, photo-immobilization of antibodies using albumin, derivatized with aryl diazirines as photolinker, is applicable [30]. Physiosorption is not recommended due to the local instability of the layer caused by the mechanical stress in the flow-through cell. An exciting new method for antibody immobilization of a quartz surface of a piezoelectric sensor was presented by Nakanishi et al. [31]. It is based on the deposition of an ethylenediamine plasma polymerization film on the quartz crystal. This film is extremely thin and homogeneous, incorporating amino functions which may be further derivatized and linked to immunoglobulins, resulting in an orientation-con-

trolled and highly reusable sensing surface. Another recent development is the planar-supported phospholipid bilayer (SLB), which can be formed on solid supports by vesicle fusion and Langmuir–Blodgett methods. SLBs maintain two-dimensional fluidity and accommodate multivalent binding between surface-bound ligands and receptor molecules in solution [32].

For noble metal surfaces, such as gold, in particular, in optical immunosensors, self-assembling monolayer (SAM) techniques seem to be first choice. In general, a SAM is built of long-chained (C₁₂ and higher) *n*-alkylthiols with derivatized organic functional groups, which are easily linked to the gold film via the thiol groups by a mechanism still not fully understood. The functional groups of the SAM crosslink with the Fc portion of the antibody (e.g., via the biotin/streptavidin system [33,34]), whereas the self-organization of the matrix prevents the surface being subjected to nonspecific binding effects (see also Section 3.2.1 for capacitance immunosensors). In addition, the covalent coupling of IgG to a short-chain (e.g., thioctic or mercaptopropionic acid) SAM-modified metal surface has been shown to be an effective affinity-based layer for optical immunosensors [35].

2.2. Regeneration of antibody-coated sensor surfaces

Conventional homogeneous and heterogeneous immunoassays, respectively, work discontinuously. It is highly desirable, however, that immunosensor devices, applied in clinical diagnostics, are capable of quasi-continuous recording. The repeated use of disposable sensing elements may mimic a pseudo-continuous action, but this is not considered here. In true immunosensors, the analyte/antigen interaction on the sensor-coated surface is reversible. With the given short incubation times in the flow-through device, the reaction between antigen and antibody is far off the equilibrium state. Fast reversibility and high sensitivity are mutually exclusive of each other. This was made clear by Hock in a comprehensive theoretical explanation [15]. Consistently, an adequate analytical sensitivity is only warranted if antibodies with increased affinity ($> 10^{10} \text{ M}^{-1}$ or at least with highly improved on-rate) are applied.

The regeneration of the binding sites of the antibodies bound to the immunosensor surface needs stringent procedures. Antibody regeneration using acidic or alkaline solutions, guanidinium chloride, or ionic strength shock is potentially harmful to the binding ability and may lead to a diminished lifetime of the immobilized antibodies and insidious drift problems.

Besides this, it must be considered that with the short reaction times between the antibodies and soluble analytes in the flow-through system, the cross-reactivities of the antibody applied can be increased. A highly specific recognition of the antigen is a kinetic-controlled process due to the complexity of the conformational changes in the Fab portion of the antibody upon binding of the antigen.

There are different approaches to solve the “antibody regeneration problem”: one approach is to displace the antigenic analyte by a highly concentrated solution of a related antigen with weak affinity to the surface-bound antibody. However, this depends on the availability of a suitable antigenic surrogate. This is not always feasible and is only applicable to small analytes. A second approach is to use the techniques of antibody engineering to improve the chemical stability of antibodies as whole molecules or as Fab fragments. The phage display technique [36] is such a powerful tool. This can be helpful in the selection of antibody fragments with improved stability, as was recently shown by Jung et al. [37]. Libraries of mutants of single-chain Fv fragments (scFv; comprising the variable regions of the L and H chains, joined by a peptide linker) are generated by a combination of site-directed and random mutagenesis. The selection can be carried out under different physical or chemical pressures to produce thermodynamically more stable scFv mutants. An interesting third approach is a pseudo-regenerating procedure for immunosensors, described by Santandreu et al. [38]. An amperometric sensor is coated with a conducting immunocomposite, formed by a mixture of specific antibody with methacrylate monomer and graphite. After polymerization, the device is ready for use. Repeated measurements became possible if the polymer is polished thoroughly with abrasive paper. (These notes do not apply to immunosensors with a competitive configuration, in which antigenic compounds and not antibodies are surface-immobilized.)

2.3. Alternative analyte-binding compounds for immunosensor applications

2.3.1. Aptamers

Aptamers are single-stranded DNA or RNA oligonucleotide sequences with the capacity to recognize various target molecules with high affinity and specificity. These ligand-binding oligonucleotides mimic properties of antibodies in a variety of diagnostic formats. They are folded into unique overall shapes to form intricate binding furrows for the target structure [39]. Aptamers are identified by an *in vitro* selection process known as systematic evolution of ligands by exponential enrichment (SELEX) [39,40]. Aptamers may have advantages over antibodies in the ease of depositing them on sensing surfaces. Moreover, due to the highly reproducible synthetic approach in any quantities (albeit the affinity constants are consistently lower than those of antibodies and the stability of these compounds is still questionable), they may be particularly useful for diagnostic applications in complex biological matrices. The aptamer-based schemes are still in their infancy and it is expected that modified nuclease-resistant RNA (and DNA) aptamers will soon be available for a variety of therapeutic and diagnostic formats. The potential of aptamers for use in biosensors has been outlined by Lee and Walt [41], who designed a fiber-optic biosensor using an anti-thrombin DNA aptamer, immobilized on the surface of silica microspheres and distributed into microwells on the distal tip of the imaging fiber. With this device, the determination of thrombin at low concentration was possible. Exciting new possibilities are evolving by the introduction of signaling aptamers (with ligand-dependent changes in signaling characteristics) and catalytic active so-called “apta-zymes” (which would allow the direct transduction of molecular recognition to catalysis), recently reviewed by Hesselberth et al. [42].

2.3.2. Anticalins

Lipocalins constitute a family of proteins for storage or transport of hydrophobic and/or chemically sensitive organic compounds. The retinol-binding protein is an example in human physiology. Schlehuber et al. [43] demonstrated that the bilin-binding protein, a member of the lipocalin family and origi-

nating from the butterfly *Pieris brassicae*, can be structurally reshaped in order to specifically complex potential antigens, such as digoxigenin, which was given as an example.

These binding proteins share a conserved β -barrel, which is made of eight antiparallel β -strands, winding around a central core. At the wider end of the conical structure, these strands are connected in a pairwise manner by four loops that form the ligand-binding site. The lipocalin scaffold can be employed for the construction of so-called “anticalins”, which provide a promising alternative to recombinant antibody fragments. This is made by subjecting various amino acid residues, distributed across the four loops, to targeted random mutagenesis. It remains to be shown that this class of proteins is applicable in diagnostic assays and in immunosensors. Critical points that still need to be defined include the synthesis and stability of the anticalins, the magnitude of the affinity constants, and the versatility for being crafted against the large variety of ligands.

2.3.3. Molecular imprinting techniques

This is a technique that is based on the preparation of polymeric sorbents with selectivity predetermined for a particular substance, or group, or structural analogs [44,45]. Functional and cross-linking monomers of plastic materials, such as methacrylics and styrenes, are allowed to interact with a templating ligand to create low-energy interactions. Subsequently, polymerization is induced. During this process, the molecule of interest is entrapped within the polymer either by a noncovalent, self-assembling approach, or by a reversible, covalent approach. After stopping the polymerization, the template molecule is washed out. The resultant imprint of the template is maintained in the rigid polymer and possesses a steric (size, shape) and chemical (special arrangement of complementary functionality) memory for the template. The molecularly imprinted polymer (MIP) can bind the template (= analyte) with a specificity similar to that of the antigen–antibody interaction.

Besides the main applications in solid-phase extraction and chromatography, molecularly imprinted polymers have already been employed as nonbiological alternatives to antibodies in competitive binding assays. A series of applications for analytes, such as

cyclosporin A, atrazine, cortisol, 17β -estradiol, theophylline, diazepam, morphine, and S-propranolol, were recently reviewed by Andersson [46], suggesting that molecular imprinting is a promising technique for immunoassays and immunosensors.

3. Immunoassay and immunosensor technologies

3.1. Definitions

3.1.1. Immunoassays

Immunoassays use antibodies or antibody-related reagents for the determination of sample analytes [18]. This analytical tool has experienced an evolutionary history since 1959, when Berson and Yalow first described the radioimmunoassay (RIA) principle. In the RIA, a fixed and limited amount of antibodies is reacted with a fixed and limited amount of radiolabeled antigen tracer and a variable concentration of the analyte [47]. The selectivity of the ligand-binding of antibodies allows these biomolecules to be employed in analytical methods that are highly specific even in complex biological matrices, such as blood, plasma, or urine. By combining the selectivity of antibody–analyte interactions with the vast array of antibodies preformed in immunization processes of host animals and the availability of numerous readily detectable labels (radioisotopes, enzymatically or electrochemically induced adsorbance or fluorescence or (chemi)-luminescence), immunoassays can be designed for a wide variety of analytes while with extraordinarily low detection limits [19,48,49].

3.1.2. Biosensors and immunosensors

A biosensor is an analytical device that integrates a biological element on a solid-state surface, enabling a reversible biospecific interaction with the analyte, and a signal transducer. The biological element is a layer of molecules qualified for biorecognition, such as enzymes, receptors, peptides, single-stranded DNA, even living cells are applicable. If antibodies or antibody fragments are applied as biological element the device is called immunosensor. Compared to conventional analytical instruments, biosensors are characterized by an integrated structure of these to components. Many devices are con-

nected with a flow-through cell, enabling a flow-injection analysis (FIA) mode of operation. Biosensors combine high analytical specificity with the processing power of modern electronics to achieve highly sensitive detection systems.

There are two different types of biosensors: biocatalytic and bioaffinity-based biosensors. The biocatalytic biosensor uses mainly enzymes as the biological compound, catalyzing a signaling biochemical reaction. The bioaffinity-based biosensor, designed to monitor the binding event itself, uses specific binding proteins, lectins, receptors, nucleic acids, membranes, whole cells, antibodies or antibody-related substances for biomolecular recognition. In the latter two cases, these biosensors are called immunosensors.

Biosensors are extensively used as diagnostic tools, predominately in point-of-care testing. Probably the most successful commercialization of biosensors today is the *in vitro* near patient measurement of capillary glucose using various hand-held systems with disposable reagent cartridges [50].

3.2. Immunosensor principles

The general immunosensor design is depicted in Fig. 1. There are four types of immunosensor detection devices: electrochemical (potentiometric, amperometric or conductometric/capacitative), optical, microgravimetric, and thermometric. The latter detection mode are neglected in this discourse due to a lack of considerable applications. All types can ei-

ther be run as direct (nonlabeled) or as indirect (labeled) immunosensors. The direct sensors are able to detect the physical changes during the immune complex formation, whereas the latter sensors use signal-generating labels which allow more sensitive and versatile detection modes when incorporated into the complex.

There is a great variety of different labels which have been applied in indirect immunosensors. Since they are in principle the same labels as used in immunoassays, the reader is referred to relevant references, dealing with modern immunoassay technology and automation [51–60]. Among the most valuable labels are enzymes such as peroxidase (EC 1.11.1.7), glucose oxidase (EC 1.1.3.4), alkaline phosphatase (aP), catalase (EC 1.11.1.6) or luciferase (EC 1.13.12.7), electroactive compounds such as ferrocene or In^{2+} salts, and a series of fluorescent labels (rhodamine, fluorescein, Cy5, ruthenium diimine complexes, phosphorescent porphyrin dyes, etc.). In particular, laser-induced fluorometric resonance energy transfer between two fluorophores offers methodological advantages [61] and can be extended to fiberoptic sensing (see Section 3.2.2) devices [62].

Although indirect immunosensors are highly sensitive due to the analytical characteristics of the label applied, the concept of a direct sensor device is still fascinating and represents a true alternative development to immunoassay systems. Its potential simplicity holds multiple advantages, making immunosensors progressive and future directed.

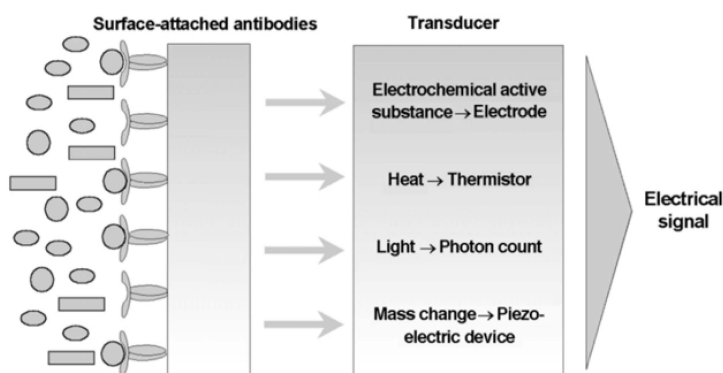


Fig. 1. Scheme of the general immunosensor design depicting the intimate integration of immunological recognition at the solid-state surface and the signal transduction.

3.2.1. Electrochemical sensors

3.2.1.1. Potentiometric immunosensors. The Nernst equation [63] provides the fundamental principle of all potentiometric transducers. According to this equation, potential changes are logarithmically proportional to the specific ion activity. Potentiometric transducer electrodes, capable of measuring surface potential alterations at near-zero current flow, are being constructed by applying the following methodologies:

Transmembrane potential. This transducer principle is based on the accumulation of a potential across a sensing membrane. Ion-selective electrodes (ISE) use ion-selective membranes which generate a charge separation between the sample and the sensor surface. Analogously, antigen or antibody immobilized on the membrane binds the corresponding compound from the solution at the solid-state surface and change the transmembrane potential.

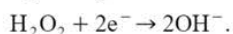
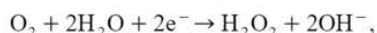
Electrode potential. This transducer is similar to the transmembrane potential sensor. An electrode by itself, however, is the surface for the immunocomplex building, changing the electrode potential in relation to the concentration of the analyte.

Field-effect transistor (FET). The FET is a semiconductor device used for monitoring of charges at the surface of an electrode, which have been built up on its metal gate between the so-called source and drain electrodes. The surface potential varies with the analyte concentration. The integration of an ISE with FET is realized in the ion-selective field-effect transistor (ISFET). This technique can also be applied to immunosensors.

An advantage of potentiometric sensors is the simplicity of operation, which can be used for automation, and the small size of the solid-state FET sensors. All potentiometric methods, however, are still suffering from major problems of sensitivity (being inferior to amperometric transducers) and nonspecific effects of binding or signaling (influences from other ions present in the sample). Especially, the signal-to-noise ratio causes analytical problems, which are difficult to circumvent [10,13]. Thus, a trend away from these techniques has been observed in the last few years. Only the ISFET may be seen as a candidate for ultrasensitive clinical immunosensor applications, in particular, when the

novel concept of differential ISFET-based measurement of the zeta potential is used. The streaming potential is a potential difference in flow direction, caused by the flow of excess ions resulting from a local distortion of the charge balance. The zeta potential, directly correlated to the streaming potential, reflects the potential changes in the diffuse outer layer at the solid–liquid interface. It efficiently reacts to protein accumulations onto sensor surfaces and, thus, is suitable for detecting immunocomplex reactions [64].

3.2.1.2. Amperometric immunosensors. Amperometric immunosensors are designed to measure a current flow generated by an electrochemical reaction at constant voltage. There are only few applications available for direct sensing, since most (protein) analytes are not intrinsically able to act as redox partners in an electrochemical reaction. Therefore, electrochemically active labels (directly or as products of an enzymic reaction) are needed for the electrochemical reaction of the analyte at the sensing electrode. Oxygen and H_2O_2 electrodes are the most popular. First described by Clark [65] in 1956, an oxygen electrode consists of an electrolyte-bearing chamber with a sensing Pt cathode, polarized at -0.7 V, and an Ag/AgCl reference electrode. The chamber is gas-permeable, covered by an O_2 -permeable membrane. The probable cathodic reactions are as follows:



The anodal reaction: $4\text{Ag} + 4\text{Cl}^- \rightarrow 4\text{AgCl} + 4\text{e}^-$ completes the electrical circuit. There is a series of enzymes with high catalytic reaction rates ($> 10^3 \text{ s}^{-1}$) in use for substrate transformation in amperometric systems, as reviewed by Ghindilis et al. [6]. In general, oxidizing rather than reducing potentials are applied.

Besides oxygen, generated by catalase from H_2O_2 , there are other amperometrically detectable compounds, such as ferrocene derivatives or In^{2+} salts [11]. A novel approach is the use of the redox polymer [PVP-Os(bipyridyl) $_2$ Cl], which is coimmobilized with specific antibodies [66]. Additionally, there are examples for enzymes with electrochemically active products. aP, for example, catalyzes the

hydrolysis of phenyl phosphate or *p*-aminophenyl phosphate (4-APP) compounds, which result in electrochemically active phenol or *p*-aminophenol [11]. Furthermore, enzymes, such as horseradish peroxidase (HRP), glucose oxidase, glucose-6-phosphate dehydrogenase (EC 1.1.1.49; with subsequent amperometrical oxidation of NADH) and others, have also been successfully applied as labels [12].

The main disadvantage for amperometric immunosensors of having an indirect sensing system, however, is compensated for by an excellent sensitivity. This is due to a linear analyte concentration range compared to a logarithmic relationship in potentiometric systems. Special attention must be directed to the system-inherent transport rate limitations for redox partners on the electrode surface.

3.2.1.3. Conductometric and capacitive immunosensors. These immunosensor transducers measure the alteration of the electrical conductivity in a solution at constant voltage, caused by biochemical (enzymatic) reactions which specifically generate or consume ions. The capacitance changes are measured using an electrochemical system, in which the bioactive element is immobilized onto a pair of noble metal (mostly, Au or Pt) electrodes. There are only few clinical applications available, as the high ionic strength of biological matrices makes it difficult to record the relatively small net conductivity changes caused by the signaling reaction (see, e.g., Ref. [67]). To circumvent this problem, recently, an ion-channel conductance immunosensor, mimicking biological sensory functions, was described [68]. The basis of this technique is the fact that the conductance of a population of molecular ion channels, built of tethered gramicidin A and aligned across a lipid bilayer membrane, is changed by the antibody–antigen binding event. Different applications using various antibodies, linked to the ion-channel complex, are given.

Another approach is the measurement of changes of the surface conductivity. Yagiuda et al. [69] developed a conductometric immunosensor for the determination of methamphetamine (MA) in urine. Anti-MA antibodies were immobilized onto the surface of a pair of platinum electrodes. The immunocomplex formation caused a decrease in the conductivity between the electrodes.

The measurement of the reciprocal capacitance, performed at alternating voltage, is advantageous compared to conductometric devices, and serves two purposes. The first is to test the insulating monolayer on the sensor noble metal surface. Self-assembling monolayers, as described in Section 2.1, have insulating properties. Besides this, they prevent the immunosensor from being affected by nonspecific binding phenomena. Even minor desorption of the monolayer results in an essential increase in capacitance. Thus, the actual quality of the device can be checked. The second application is the measurement of changes in the effective dielectric thickness of the insulating layer during antigen binding, when antibodies are linked to the alkylthiol layer. Of course, this is on condition that the ω -substitution of the alkylthiol monolayer does not compromise the insulation. Hence, a marked decrease of the electrical capacitance is observed and is used to quantitate the analyte. Mirsky et al. [70] demonstrated that the determination of albumin in the range of 1–20 $\mu\text{g/ml}$ is made possible by such an immunosensor device. The destructive influence of lateral diffusion on nanostructured monolayers is prevented by use of the spreader-bar technique [71].

3.2.2. Optical sensors

Optical immunosensors are most popular for bioanalysis and are today's largest group of transducers. This is due to the advantages of applying visible radiation compared to other transducer techniques. Additional benefits are the nondestructive operation mode and the rapid signal generation and reading. In particular, the introduction of fiber bundle optics ("optodes") as optical waveguides and sophisticated optoelectronics offers increased versatility of these analytical devices for clinical applications [4,10,11, 13].

Changes in adsorption, fluorescence, luminescence, scatter or refractive index (RI) occur when light is reflected at sensing surfaces. These informations are the physical basis for optical sensor techniques. Usually, applied detectors are photodiodes or photomultipliers.

There are numerous applications of either direct label-free optical detection of the immunological reaction, of labeled immunospecies, or of the product(s) of enzymatic reactions. Most labels are fluorescent,

but bio- and chemiluminescence species are also possible. It is worth mentioning that the label-free evanescent wave-related sensors explicitly represent an elegant methodology, which is a valuable alternative to sophisticated immunoassays. Nevertheless, label-free systems are prone to unsolved problems, such as nonspecific binding effects and poor analytical sensitivity to analytes with low molecular weight. Kubitschko et al. [72] noted out that despite the efforts, all immunosensors are still one magnitude less sensitive than commercial immunoassays for determining analytes in human serum, particularly those with low molecular weight. They claim the use of mass labels, such as latex particles, in order to enhance the signal. The authors demonstrated the optimization of a nanoparticle enhanced bidiffractive grating coupler immunosensor for the detection of thyroid-stimulating hormone (TSH, MW 28,000 Da). The excellent performance characteristics of this sensor clearly showed how future devices should work. The problem of unspecific binding, however, can also be controlled by applying a reference sensing region on the chip, as described by Schneider et al. [73] for a Hartman interferometer device. The described sensor was highly sensitive even in human serum matrix. The BIAcore™ system also offers the possibility to use a flow cell as reference cell.

3.2.2.1. Total internal reflection spectroscopy (TIRS).

The common principle of the following analytical devices is that in an optical sensor with two materials with different refractive indices (RI), total internal reflection occurs at a certain angle of the light beam being directed through the layer with the higher RI towards the sensing interface. By this, an evanescent wave is generated in the material with the lower RI. This wave, being an electrical vector of the wavelength of the incident light beam, penetrates further into the medium with exponentially attenuated amplitude. Biomolecules attached in that portion of the medium will interact inevitably with the evanescent wave and, therefore, lead to a distinctive diminution of the reflected light. The resolution is directly proportional to the length of interaction. Infrared spectrometers, measuring attenuated total reflectance, are commonly built in the Kretschmann configuration: an optically absorbing film at the sensor's surface enables the measurement of the attenuated light intensity as a function of the wavelength of the incident beam. For total internal reflection fluorescence (TIRF), analytics benefit from the fact that incident light excites molecules with fluorescence characteristics near the sensor surface creating a fluorescent evanescent wave. The emerging fluorescence is finally detected. There are planar or fiber-optic TIRF systems; in Fig. 2 a fiber-optic device is

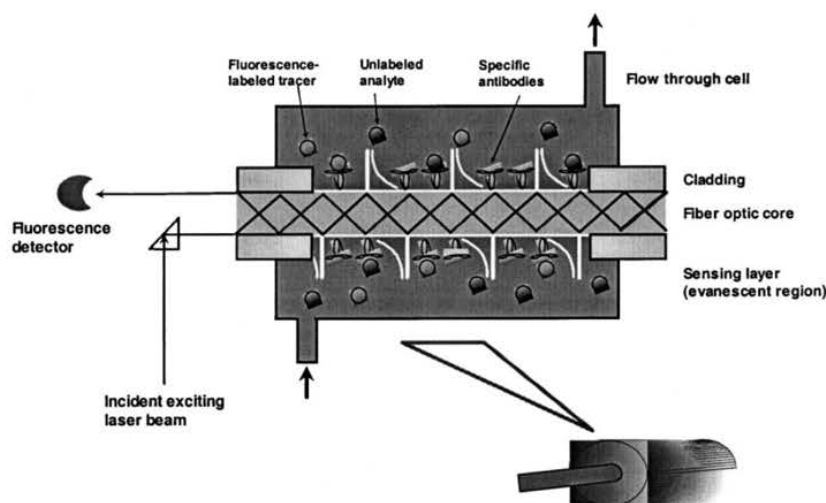


Fig. 2. Basic scheme of a fiber-optic TIRF immunosensor.

shown (scheme modified according to Ref. [5]). The technique has been developed mainly for an optical detection of fluorescence-labeled antibodies or antigens [74]. In the latter case, the fluorescence capillary fill device (FCFD) technique is worth mentioning. The FCFD is designed by using a planar optical waveguide and a glass plate separated from each other by a capillary gap. Fluorophore-labeled antigen is attached on the surface of the glass plate, whereas antibodies are immobilized on the surface of the optical waveguide.

Another phenomenon, the optical diffraction, is used by the optical biosensor assay (OBATM) system [75]: biomolecules are attached to the surface of a silanized wafer. The protein-coated surface is illuminated through a photo mask to create distinct periodic areas of active and inactive protein. Upon illumination with laser light, the diffraction grating caused by the ligand-binding process diffracts the incident light. An analyte-free (negative) sample does not result in diffraction because no antigen–antibody binding occurred creating the diffraction grating. The presence or absence of a diffraction signal differentiates between positive and negative samples. The intensity of the signal provides a quantitative measure of the analyte concentration.

3.2.2.2. Ellipsometry. If linearly polarized light of known orientation is reflected at oblique incidence from a surface, the reflected light is elliptically polarized. The shape and orientation of the ellipse depend on the angle of incidence, the direction of the polarization of the incident light, and the reflection properties of the surface. On adsorption of biomolecules onto a planar solid surface, phase and amplitude of the reflected light are altered and can be recorded by ellipsometric techniques. These changes in the polarization of the light are due to the alterations of the RI and the coating thickness. There are only few applications, such as the study of a cholera toxin-ganglioside GM1 receptor–ligand reaction, which were carried out using an ellipsometer [76].

3.2.2.3. Optical dielectric waveguides. Optical waveguides are glass, quartz or polymer films or fibers made of high RI material embedded between or in lower index dielectric materials. If a linearly polar-

ized helium-neon laser light wave, introduced into the high index film or fiber, arrives at the boundary at an angle which is greater than the critical angle of total reflection, it is confined inside the waveguide. Similar to surface plasmon resonance, an evanescent field develops at the sensor's surface. In this case, however, the evanescent field is generated by the excitation of the light itself in the dielectric layer. Most of the laser light is transmitted into the device and multiple reflections occur as it travels through the medium if a bioactive substance is placed over the surface. Some of the light, however, penetrates the biolayer. This light is reflected back into the waveguide with a shift in phase interfering with the transmitted light. Thus, changes in properties of the biolayer can be followed by detecting the changes in interference.

Waveguides are often made in the form of fibers. These fiber-optic waveguide systems offer advantages for sensors when being used for hazardous analysis. Planar waveguide systems are also applicable for interferometers. They use laser light directed towards the surface of the waveguide with the attached biomolecules, which is subsequently split into two partial electrical (TE) and magnetic (TM) field waves, perpendicular to each other. The interaction with the sample surface changes the relative phase between TE and TM by the different RI and surface thickness values. Various configurations, such as the Fabry-Pérot monomode channel interferometer, the Mach-Zehnder interferometer or the related two-mode thin-film waveguide difference interferometer, have been successfully established.

Another technique uses thin corrugations etched into the surface of a waveguide. This grating coupler device allows the measurement of the coupling angle of either the input or output laser beam. Both beams are correlated to the RI within the evanescent field at the sensor's surface. Recently, a long-period grating fiber immunosensor [77] has proven to be sensitive (enabling analyses down to the nanomolar range) and reproducible. Grating couplers are also used for optical waveguide lightmode spectroscopy (OWLS) [78,79]. The basic principle of the OWLS method is that linearly polarized light is coupled by a diffraction grating into the waveguide layer. The incoupling is a resonance phenomenon that occurs at a defined angle of incidence that depends on the RI of the

medium covering the surface of the waveguide. In the waveguide layer, light is guided by total internal reflection to the edges where it is detected by photodiodes. By varying the angle of incidence of the light, the mode spectrum is obtained from which the effective RIs are calculated for both TE and TM.

3.2.2.4. Surface plasmon resonance (SPR). Among the different detection systems, SPR is the most popular one. There are two leading systems on the market: the BIAcore™ systems from Biacore (Uppsala, Sweden) and the IAsys™ from Fisons Applied Sensor Technology (Cambridge, UK). Other systems (with small market positions) are the BIOS-1 from Artificial Sensing Instruments (Switzerland), the SPR-20 from Denki Kagaku Keiki (Japan), the SPEETA from Texas Instruments (USA), the IBIS from Windsor Scientific (UK) and the DPX from Quantech (USA). The first two commercial evanescent-wave devices are widespread in research laboratories due to the sophisticated apparatus and user-friendly control software. The BIAcore™, however, has the biggest market position.

The general principle of SPR measurement [80] is depicted in Fig. 3. Polarized light is directed from a layer of high RI towards a layer with low RI to result in total internal reflection. The sample is attached to the layer of low RI. At the interface between the two different media, a thin (approximately 50 nm) gold film is interposed. Although light does not propagate into the low RI medium, the interfacial intensity is

not equal to zero. The physical requirement of continuity across the interface is the reason for exciting the surface electrons (“plasmons”) in the metal film by the light energy. As a result, the electrons start oscillating. This produces an exponentially decaying evanescent wave penetrating a defined distance into the low RI medium, which is accountable for a characteristic decrease in the intensity of the reflected light. Hence, a direct insight in changes of the RI at the surface interface is made possible by monitoring the intensity and the resonance angle of the reflected light, caused by the biospecific interactions which took place there.

Whereas in the BIAcore™ system, the light affects the sensing layer only once, there are several propagation contacts in the IAsys™ due to the device’s resonant mirror configuration. The BIAcore™ SPR apparatus is characterized by a sensitive measurement of changes of the RI when polarized laser light is reflected at the carboxy-methylated dextran-activated device interface. The IAsys™ SPR device also uses a carboxy-methylated dextran-activated surface. Its dextran layer, however, is not attached to a gold surface, but to titanium, which forms a high refractive dielectric resonant layer. The glass prism is not attached tightly on the opposite side of the titanium layer, making space for an interposed silica layer of low RI. By this layer, the laser light beam couples into the resonant layer via the evanescent field. Therefore, the IAsys™ is seen as a combination of SPR resonant mirror with waveguide technol-

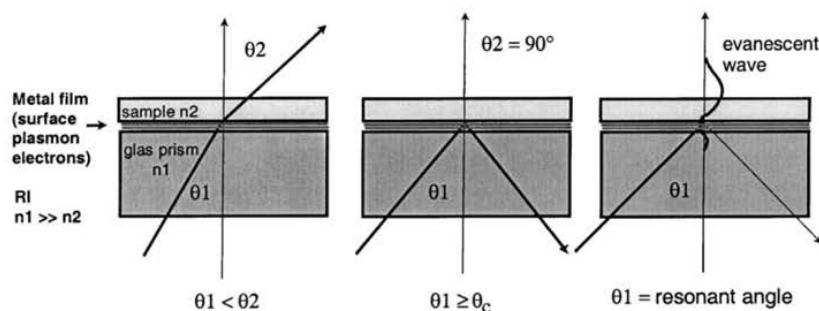


Fig. 3. Principle of SPR technology. Light is directed from a prism with a RI towards a layer with low RI, resulting in total internal reflection. Although light does not propagate into the medium, the interfacial intensity is not equal to zero. Physical requirements of continuity across the interface are the cause of excitation of surface plasmons in the metal film by the light energy, causing them to oscillate. This produces an exponential (evanescent) decaying, which penetrates a defined distance into the low-index medium and results in a characteristic decrease in reflected light intensity.

ogy. As a result, no decrease in the reflected light intensity at resonance is observed in this system. The specific signal is the change in the phase of the reflected polarized light.

A novel modification of a SPR immunosensor was given by Berger and Greve [81] in order to improve further the sensitivity of the sensor, the differential SPR, by applying a modulation of the angle of light incidence. The reflectance curve is measured with a lock-in amplifier and recorded in the first and second derivative.

3.2.3. Microgravimetric sensors

A direct measurement of mass changes induced by the forming of antigen/antibody complexes is also enabled by acoustic sensors. The principle of operation is based on the propagation of acoustic shear waves in the substrate of the sensor. Phase and velocity of the acoustic wave are influenced by the specific adsorption of antibody molecules onto the antigen-coated sensor surface. Piezoelectric materials, such as quartz (SiO_2), zinc oxide (ZnO) or others resonate mechanically at a specific ultrasonic frequency (in the order of tens of megahertz) when excited in an oscillating electrical field. The resonant frequency is determined by the distance between the electrodes on both sides of the quartz plate, which is equal to the thickness of the plate and the velocity of the acoustic wave within the quartz material [82]. In other words, electromagnetic energy is converted into acoustic energy, whereby piezoelectricity is associated with the electrical polarization of materials with anisotropic crystal structure. The most applied technique for monitoring the acoustic wave operation is the oscillation method. This means a configuration in which the device constitutes the frequency-controlling element of a circuit. The oscillation method measures the series resonant frequency of the resonating sensor.

The microgravimetric sensor devices are divided into quartz crystal microbalance (QCM) devices applying a thickness-shear mode (TSM), and devices applying a surface acoustic wave (SAW) detection principle. These sensors have reached considerable technical sophistication. A profound review on the technology of acoustic wave sensors was recently given by Čavić et al. [7]. These authors additionally describe the bioanalytical application of devices, such

as the flexural plate wave (FPW), the shear horizontal acoustic plate (SH-APM), the surface transverse wave (STW) and the thin-rod acoustic wave (TRAW), which will not be depicted in this review. Janshoff et al. [83] moreover point out that for various bioanalytic applications piezoelectroimmunosensors may be valuable alternatives to optical sensors.

In a fundamental work, Sauerbrey [84] showed for the gaseous phase that a weight change of the quartz surface is precisely determined by a shift in the resonating frequency. For a TSM device, he developed the following equation:

$$\Delta m = - \left(A \sqrt{\rho \mu} / 2 F_0^2 \right) \Delta F,$$

where F_0 is the resonant frequency, A the area of coating, ρ the density and μ the shear modulus of the quartz.

The equation can be interpreted that surface-added layers will extend the wavelength of the acoustic wave within the quartz device, i.e., the attenuation of the acoustic energy, visible as negative frequency shift, is directly related to the mass increase of the quartz surface.

Although theoretically unproven and contradicted by several authors, the Sauerbrey equation was also explains the utility of TSM sensor devices in liquid media. By exposing only one side of the crystal to the liquid surface, it was able to generate frequency responses to mass changes in liquid media comparable in sensitivity to gas phase depositions of ultra-thin films. The different theoretical models, describing microgravimetric measurements, however, have to be adapted to several wave damping factors, such as energy dissipation by viscous action of the medium, by energy storage, or by elastic deformation in the sensing film [7].

There are considerable similarities between the physical principles of QCM and SPR sensors, even when there are fundamental differences [85]. Both QCM and SPR are wave-propagation phenomena and show resonance structure. The elastic QCM wave and the surface plasmon wave are nonradiative, i.e., an evanescent wave exists. Changes of physical properties within the evanescent field lead to a shift of resonance. Thus, a linear approximation of the physical relationship is allowed for immunological application in immunosensors.

3.2.3.1. The TSM sensor. The TSM sensor consists of an AT-cut piezoelectric crystal disc, most commonly of quartz because of its chemical stability in biological fluids and resistance to extreme temperatures. The disc is attached to two metal electrodes on opposite sides for the application of the oscillating electric field. The TSM is run in a range of 5–20 MHz. The schematic design of a typical TSM device, used by Kaiser et al. [34], is shown in Fig. 4. Advantages are, besides the chemical inertness, the low cost of the devices and the reliable quality of the mass-produced quartz discs. Major drawbacks of the system are the insensitivity for analytes with a molecular weight < 1000 Da, and, as seen in all label-free immunosensor systems, nonspecific binding interferences. Nonspecific binding effects are hard to distinguish from authentic binding events due to the fact that no reference line can be placed in the sensor device. For a SH-APM device, however, Dahint et al. [86] showed that by appropriately selecting the device frequency, these spurious responses can be suppressed. This sensor is applicable for measurements in human serum matrix.

One of the first applications of TSM technology was an immunosensor for human immunodeficiency virus (HIV) serology [87]. This sensor was realized

by immobilizing recombinant viral peptides on the surface of the transducer and by detecting anti-HIV antibodies directly in human sera.

3.2.3.2. The SAW sensor. SAW sensors use thick ST-cut quartz discs and interdigitated metal electrode arrays that generate acoustic Rayleigh waves in both directions from the interdigital electrodes, their transmission being attenuated by surface-attached biomolecules. The oscillation frequency of a SAW sensor ranges from 30 to 500 MHz. The operation of SAW immunosensors with biological samples is compromised by the fact that the surface wave is considerably attenuated in the liquid phase. Thus, the domain of this technique is most likely restricted to gas phase operations.

4. Survey of new clinical, environmental and research applications of immunosensor systems

4.1. Clinical applications

Applications, dealing with “real” clinical samples, are still rare. Most of the papers describing the use of immunosensors in this field only have exem-

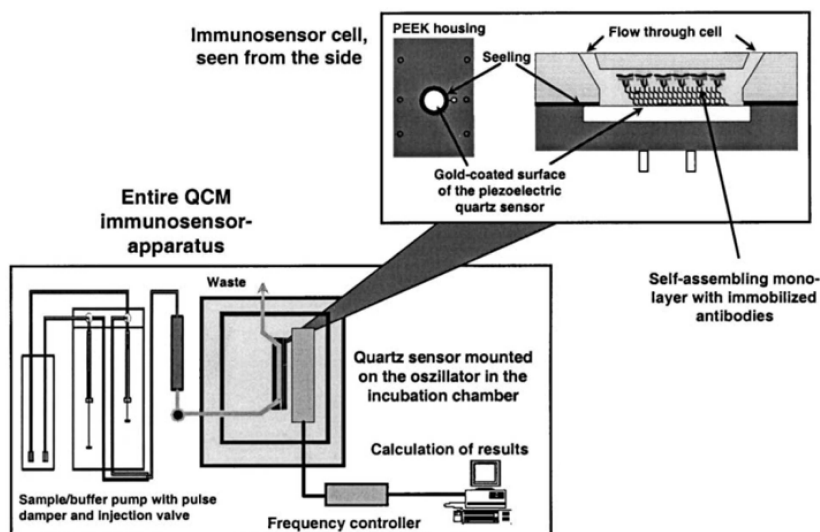


Fig. 4. QCM immunosensor device, used by Kaiser et al. [34].

plary character. Detailed data and evaluation protocols on performance characteristics, as well as comparisons with established methods for particular parameters, are missing, in most cases. The analytical potential of this novel technique, however, is evident. It is worthwhile mentioning that the applications, in particular, of optical immunosensor systems, which are recorded for the bioaffinity interaction analysis (described in Section 4.3), will most likely be of clinical interest in the next few years.

4.1.1. Applications using label-free detection

The formation of specific antigen–antibody complexes leads to changes in electrochemical, optical or other physical properties at the surface of the immunosensor flow-through cell. The detection of these changes is the basis of label-free immunosensors. Albeit it has to be emphasized that for all devices with label-free detection, mode nonspecific binding phenomena are a crucial point. It is impossible to distinguish the signal of an immunoreaction of a target molecule from that of a nonspecific adsorption of other sample components to the transducer if no target-specific label is used. Thus, all nonspecific effects have to be minimized prior to a meaningful clinical application.

4.1.1.1. Electrochemical systems. An interesting application is the determination of human chorionic gonadotrophin (hCG) by an amperometric immunosensor, described by Chetcuti et al. [88]. The authors used Nafion, a polyanionic perfluorosulfonated ionomer to immobilize anti-hCG monoclonal antibodies onto a glassy carbon electrode. Santandreu et al. [89] analyzed β -hCG using an amperometric sensor device based on a conducting immunocomposite (described in Section 2.2). Kelly et al. [90] described an immunosensor for lactate dehydrogenase (LDH, EC 1.1.1.27) isoenzyme 1 in serum. The LDH activity was measured amperometrically by the oxidation of NADH at the electrode surface. Reproducibility, as well as selectivity of the sensor, however, were insufficient for clinical use. Moreover, Liu et al. [91] used a similar approach with Nafion and a protein A coupled polyaniline on an amperometric sensor surface. A prototype amperometric immunosensor for rapid detection of plasma heart-type fatty acid-binding protein (H-FABP) as

early marker for myocardial damage, tested under routine conditions, was presented by Key et al. [92,93]. The authors used a monoclonal anti-FABP antibody, linked to aP, the enzyme catalyzing the hydrolysis of 4-APP to the electrochemically active phenol derivative. Meusel et al. [94] applied a heterogeneous amperometric immunosensor for the determination of apolipoprotein E in serum. The authors used a site-directed attachment of monoclonal anti-apo E antibodies to a disposable hydrazide-functionalized membrane surface. Bound analyte was then detected by use of a polyclonal antibody, labeled with aP, in conjunction with 4-APP as substrate for the carbon working electrode. The reliability of the disposable low-cost sensor chip recommends this technology for medical use. Feasibility studies are under way [94].

4.1.1.2. Optical systems. Kröger et al. [95] demonstrated a sensitive determination of the H-FABP by use of a direct optical immunosensor. As described in Section 3.2.2, Kubitschko et al. [72] applied a nanoparticle enhanced bidiffractive grating coupler immunosensor [96] for the detection of TSH in serum. The high sensitivity of this sensor is highly competitive to conventional immunoassay systems. Another clinical application field was entered by Nicholson et al. [97]. By use of a resonant mirror SPR sensor, the antibody responses to cancer vaccination in patient sera was monitored. The affinity maturation of anti-idiotypic tumor antibodies after vaccination of patients with advanced ovarian cancer could be followed by determining the kinetics of the antibody response, thus enabling an objective comparison of different immunization schedules. The detection of human β_2 -microglobulin (in buffer solutions, not serum) by use of a grating coupler sensor was described by Brynda et al. [98]. The authors applied a three-dimensional antibody network on the surface of the Ta_2O_5 grating coupler. A drug-monitoring application using the BIAcore™ system was presented by Keating et al. [99]. To monitor the anticoagulatory coumarin derivative 7-hydroxycoumarin (7-OHC), a 7-OHC-conjugate with bovine serum albumin (BSA) was immobilized onto a carboxymethylated dextran sensor chip. Serum samples containing the analyte were premixed with a polyclonal anti-7-OHC antibody and injected over the surface.

Excess antibody, bound to the immobilized conjugate, then generates a binding response, inversely proportional to the 7-OHC concentration.

4.1.1.3. Piezoelectric systems. Lu et al. [100] described a protein A-coated piezoelectric biosensor for the direct determination of human IgG. The special feature of this device is the favorable reusability of the surface, due to an indirect immobilization of protein A onto a poly-L-lysine-modified electrode surface by using a hydrophilic matrix of oxidized dextran as linker. Another analyte for a piezoelectric sensor analysis was IgE, as described by Su et al. [101]. The sensor surface was coated with an anti-IgE layer cross-linked via thioctic acid to form a highly ordered SAM. The device was able to detect IgE in the range of 5–300 IU/ml with good precision and may be regenerated five times without appreciable loss of activity. It also showed an excellent correlation to the CAP total IgE immunoassay from Pharmacia. Chu et al. [102] analyzed IgM in serum by use of a QCM with surface-attached goat anti-IgM antibodies. The immobilization was accomplished by a CNBr-activated copolymer coating procedure. IgM could be detected in the range of 5–93 $\mu\text{g/ml}$; the measurements were in satisfactory agreement with an established immunodiffusion procedure. Finally, a QCM system was also applied for the determinations of insulin [103] and cortisol [104] in buffer solutions and of IgA in saliva [105].

4.1.2. Applications using signal-generating labels

4.1.2.1. Electrochemical systems. Cook [106] used an amperometric immunosensor coupled to microdialysis, enabling an *in vivo* measurement of γ -amino-4-butyric acid (GABA) in sheep somatosensory cortex. The respective specific antibody is fixed to the platinum working electrode, the signal being generated in a competitive way by a HRP-labeled GABA tracer. The same author [107] presented also the real-time measurement of cortisol and corticosterone in animals using a similar antibody-based sensor. A flow displacement column-immunosensor for serum cortisol using HRP-labeled cortisol was established by Kaptein et al. [108]. A pH-FET immunosensor for the measurement of α_2 -interferon in cultivated broth

was described by Sergeyeva et al. [109]. α_2 -Interferon was immobilized at the gate of a pH-FET and brought into reaction with an anti- α_2 -interferon antibody linked to β -lactamase (EC 3.5.2.6). After addition of a specific enzyme substrate, a local change in pH at the surface of the transducer produced an electrochemical signal proportional to the conjugate concentration. The potentiometric sensor had a dynamic measuring range from 10 to 100 $\mu\text{g/ml}$. An interesting amperometric sensor for hCG in whole blood was demonstrated by Duan and Meyerhoff [110]. They employed gold-coated microporous electrodes and surface-bound capture antibodies binding hCG in a sandwich complex together with aP-labeled anti-hCG antibodies. The immunosensor performed separation-free in the biological matrix due to the fact that the surface-bound aP-activity is resolved from excess aP-labeled antibodies in the solution by introducing the 4-APP enzyme substrate from the reverse side of the membrane electrode. The enzymic reaction and the following oxidation of the formed *p*-aminophenol are in immediate proximity to the electrode surface.

4.1.2.2. Optical systems. Astles and Miller [111] used a fiber-optic immunosensor for analysing free phenytoin in plasma. The analytical signal depended on the degree of energy transfer from B-phycoerythrin-labeled phenytoin to Texas Red-labeled anti-phenytoin antibodies. The immunoreaction, therefore, was signaled by fluorescence quenching. This attempt allowed continuous measurements without a separation step. PSA in whole blood was analyzed with good performance characteristics by Daniels et al. [112], applying allophycocyanin as fluorophore in an FCFD system. A fiber-optic TIRF system was used by Mahoney et al. [5] for the measurement of hCG in serum. The authors applied a secondary anti-hCG antibody, linked to allophycocyanin. Schult et al. [113] also detected hCG in serum by use of a disposable evanescent field fluorescence excitation chip with a remarkable analytical sensitivity. The label in the sandwich assay was a Cy5-labeled second anti-hCG antibody. This technique brings the immunosensor near to the goal of a routine medical diagnostic application. A competitive SPR-based immunosensor for morphine, using a morphine-BSA conjugate as tracer, immobilized at

the chip surface, is described by Sakai et al. [114]. Whether the system is able to measure the drug in serum and urine, was not tested. D-dimer measurement was performed by Rowe et al. [115] as part of the simultaneous detection of clinical samples using a fluorescence-based optical immunosensor (see also Tables 1 and 2). A patterned array of capture antibodies was coated on a planar waveguide. Bound analytes were subsequently quantified by fluorescently labeled detection antibodies, the specific pattern being recognized by a CCD camera. Papkovsky et al. [116] showed an optical immunosensor setup which used glucose oxidase as antibody-linked label in conjunction with a luminescence lifetime-based O₂-sensitive membrane; the device enabled the detection of the LDH isoenzyme 1. A self-contained fiber-optic device for measuring myoglobin was developed by Hanbury et al. [117]. Cascade Blue-labeled antibodies, entrapped with polyacrylamide gel at the distal face of the fiber, reacted with the protein analyte. The heme group of myoglobin then quenched the dye fluorescence, the signal deriving from this energy transfer.

4.1.2.3. Piezoelectric systems. Wang et al. [118] coupled a FPW gravimetric immunosensor with a novel mass-amplifying label. By this, the sensor became very sensitive for the detection of nonpenetrating glycoprotein antigen (NPGP), a breast cancer tumor marker. The competitive configuration of the sensor was as follows: a NPGP fusion protein antigen was coated to the SiO₂ surface, then the first antibody, linked to colloidal gold, was incubated, its binding ratio depending on the presence of NPGP as analyte. In a second step, the device surface was exposed to a silver developer solution, in which the gold colloids served as silver deposition nucleation sites. Unfortunately, the analytical characteristics of the FPW sensor were not evaluated using real serum samples.

4.2. Microbiological and environmental applications

Besides clinical diagnostic applications, the evaluation of potential application areas in bacteriology or virology is a fast-growing area in the immunosensors field. This includes serology, monitoring of food

Table 1
Applications of immunosensor techniques for **microbiological analyses**

Reference	Application	Method
[151]	<i>Escherichia coli</i> O157:H7	Flow-through immunofiltration sensor
[152]	<i>Chlamydia trachomatis</i> in urine	Piezoelectric IS
[153]	<i>E. coli</i>	FIA IS
[154]	<i>Salmonella</i> in foods	Amperometric IS
[155]	Protein A from <i>Staphylococcus aureus</i>	Fiber-optic-based biosensor
[156]	<i>Legionella pneumophila</i> serogroup 1	Near-IR spectral fiber-optic IS
[157]	Staphylococcal enterotoxin B (SEB)	Piezoelectric crystal IS
[158]	SEB	Automated fiber optic biosensor
[159]	SEB, bacteriophage M13, <i>E. coli</i>	Optical multichannel IS
[160]	<i>Herpes</i> viruses	Reusable piezoelectric IS
[161]	Botulinum toxin	Evanescent wave IS
[162]	<i>Bacillus anthracis</i>	Immunochromatographic IS
[163]	<i>S. aureus</i> in food	Amperometric IS
[164]	<i>Rubella</i> serology	Optical IS
[165]	<i>Enterobacteria</i>	Piezoelectric IS
[166]	<i>S. typhimurium</i>	Piezoelectric IS
[167]	<i>E. coli</i>	FPW IS
[115]	<i>Yersinia pestis</i> F1 antigen, SEB	Fluorescence-based optical IS
[168]	<i>Nitrobacter</i>	Amperometric enzyme-linked IS
[169]	<i>Treponema pallidum</i> serology	SPR IS
[170]	SEB	Portable fiber-optic IS
[171]	African swine fever virus	Piezoelectric IS
[119]	<i>Y. pestis</i> and SEB serology	Planar array IS

Abbreviation: IS, immunosensor.

Table 2
Applications of immunosensor techniques for **environmental analyses**

Author	Application	Method
[172]	Atrazine and terbutylazine	Amperometric immunomigration sensor
[173]	Pesticides	Grating coupler IS
[174]	Atrazine	Direct optical IS
[175]	Polychlorinated biphenyls	Continuous flow IS
[176]	Hexahydro-1,3,5-trinitro-1,3,5-triazine	Micro IS
[177]	Atrazine	Electrochemical IS
[178]	2,4-dichlorophenoxyacetic acid	Disposable amperometric IS
[179]	2,4-dichlorophenoxyacetic acid	Amperometric IS
[180]	Progesterone in milk	Immunochromatographic IS
[66]	Atrazine	Electrochemical IS
[181]	2,4-dichlorophenoxyacetic acid	One-way fluorescence IS chip
[182]	Organophosphate nerve agents	Fiber-optic microbial biosensor
[183]	Organophosphate nerve agents	Potentiometric enzyme electrode sensor
[184]	Fumonisin	SPR biosensor
[185]	Biological threat agents	Bidiffractive grating biosensor
[186]	Toxic substances	Electrochemical enzyme IS
[187]	Progesterone in cows' milk	Screen-printed amperometric IS
[188]	Organophosphate pesticides	Screen-printed amperometric biosensor
[189]	Pesticide residues	Conductive polymer-based IS
[190]	Xenoestrogens	Enzyme-linked receptor assay
[191]	Atrazine	Piezoelectric IS
[192]	Sulfamethazine residues in milk	SPR biosensor
[193]	SEB in cream	Rapid automated IS
[170]	SEB in diverse media	Portable fiber-optic biosensor
[194]	Cocaine in coca leaf extracts	Fiber-optic IS
[195]	Atrazine	Continuous flow IS
[196]	Environmental contaminants in water	Highly parallel affinity sensor
[197]	Herbicides	Quartz crystal IS
[198]	Benzoyllecgonine in urine	Flow IS

Abbreviation: IS, immunosensor.

(fermentation) and hygiene processes, and detection of biological warfare products. There are also reports of environmental analyses of pesticides, herbicides, and hormonal active compounds (e.g., xenoestrogens) in food products, drinking water or soil. Forward looking are array systems (see also Section 5.1 for microarray techniques) for a simultaneous analysis of multiple analytes, as shown by Wadkins et al. [119].

Although this field is closely related to the clinical sciences, in this review, only a selection of recent publications are given in tabulated form in Tables 1 and 2. This selection makes no differentiation between applications using label-free detection or signal-generating labels. Recent articles, presenting an overview of these applications, are given by Hock [15, 120], Karube et al. [121], Killard et al. [122],

Lynch [123], Scheper and Lammers [124], and Tran-Minh [125].

4.3. Research applications

4.3.1. Kinetic studies of biospecific interactions

Immunosensor techniques allow the performance of systematic kinetic studies of the biospecific interaction between ligands and antibodies in real time. In particular, the commercially available SPR systems IAsys™ and BIAcore™ are marketing highly automated laboratory affinity-binding devices, both in terms of detection mode and data processing. Although there are series of limitations for the derivation of affinity constants from the quantitative kinetic data in the sensorgrams [126], both SPR systems have proven to be extremely useful and,

thus, attractive for research involving affinity-binding processes. In particular, affinity data for antibodies, which are to be applied to immunoassays, are increasingly derived from SPR analyses [34,127,128]. There is already a surge of other applications of these affinity ligand-based systems for the investigation of ligand-binding events, not restricted to antibodies [129]. Without doubt, immunosensors are already established in the bioresearch laboratories. Updated lists of journal articles on evanescent wave biosensor technology and applications are available on the websites of <http://www.affinity-sensors.com> and <http://www.biocore.com>. In this context, three examples should be given: it was possible for Cheskis et al. [130] to monitor the modulation of the estrogen receptor- α interaction with DNA by different steroidal ligands using the BIAcore™ device. This device was also successfully applied by De Crescenzo et al. [131] for real-time kinetic studies on the interaction of transforming growth factor- α with the epidermal growth factor receptor extracellular domain. Morgan et al. [132] characterized the kinetics of assembly and dissociation of the human leucocyte antigen (HLA) class I heterotrimeric complex. They developed a model for investigating the binding of class I HLA complexes on whole cells to human β_2 -microglobulin, immobilized on an IAsys™ surface. Instructive sensorgrams are given in each of these three papers. However, also integrated optical grating couplers are applicable for the determination of kinetic rate constants of ligand-binding events, as shown recently by Polzius et al. [133].

4.3.2. Genomics and proteomics

Great expectations are generally placed on biosensors as analytical systems for DNA research for rapid detection of mutations or polymorphisms, for discovery of genes, and for expression monitoring. There is a tremendously growing number of publications in this field (see, for instance, Refs. [134,135]). An elegant approach for a rapid visual detection of nucleic-acid targets was recently described by Ostroff et al. [136]: after capture of complementary target sequences to surface-coated oligonucleotide probes, a biotinylated detector nucleic acid was added. By adding an anti-biotin antibody, linked to peroxidase, and substrate, the deposition of an insoluble product onto the silicon sensor surface gener-

ated a uniform thin organic film. The film changed the interference pattern of reflected light from the surface, being the specific signal suitable for quantification.

Moreover, the rapidly growing field of proteomics is benefiting from the abilities of bio- and immunosensors for investigating the functionality of peptides prior of being analyzed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF). Nelson et al. [137–139] reported on successful combinations of MALDI-TOF with SPR to analyze sequential biomolecular recognition events.

5. Discussion and conclusions

5.1. New technologies

5.1.1. Microarrays

Today, there is great interest in multiplexed, high-throughput assays, not only for clinical applications, but also, e.g., for drug discovery. The microarray technique has first been described by Ekins [1,55,140] for an antibody microspot array. This type of microarray has to be distinguished from the DNA chip technology, which is based on nucleic-acid recognition on a surface-immobilized template. DNA biosensor arrays are of considerable recent interest due to their promise for obtaining sequence-specific informations (for a recent review, see Ref. [141]). They are being rapidly developed commercially towards the goal of quick, simple and precise testing for susceptibility to genetic diseases. The integrity of functionally important biochemical pathways in tumors, for instance, will probably be evaluated by gene transcriptional profiling. Whereas for DNA chips the sensitivity is not a critical issue, the antibody microarray, capable for ultrasensitive multi-analyte determinations, needs novel analytical approaches. Even though the commercialization of the Microspot® array (Boehringer Mannheim) suffers from considerable delays, this elegant concept, called the “ambient analyte assay”, is still advanced and will hopefully soon be available. Since multiple antigens can only be tested on a microfabricated antibody array format, the determination of the fractional binding site occupancy (FO) of each individual anti-

body spot allows the simultaneous quantification of a mixture of various analytes in one sample [1]. The FO varies with the antibody amount, present in the assay, but becomes independent from it when the antibody quantities are near to zero, now reflecting only the ambient analyte concentration. Hence, a small amount of antibody, closely packed as a monolayer on the array's surface (10^4 – 10^5 molecules/ μm^2), is able to sense the analyte concentration, independent of the sample volume. Subsequently, the detection of the FO of the sensing antibody is managed in a competitive or noncompetitive way by using fluorescence labeled second antibodies against the unoccupied binding sites or against a second epitope on the analyte molecule. By use of a different fluorescence label of the sensing antibody, the ratio of the two reporter signals (α and β) allows the direct determination of the FO. In practice, the microspots of areas of 100–1000 μm^2 are interrogated by a confocal laser microscope as a sensitive method of determining the α - and β -fluorescence, emitted from each individual spot (see Fig. 5). A further multitesting approach was already mentioned in Section 4.1.2: Rowe et al. [115] simultaneously measured D-dimer together with staphylococcal enterotoxin B (SEB) and the F1 antigen from *Yersinia pestis* with a fluorescence array immunosensor.

Multianalyte applications may be clinically useful in the fields of cytokine testing, detection of allergen-specific IgE molecules, drug-monitoring (analysis of a drug parent compound together with its

major metabolites), drugs of abuse testing, determination of vitamins, rare blood group typing, etc.

5.1.2. Atomic force microscopy

The new techniques of atomic force microscopy (AFM) and optical trapping (optical tweezers) allow the location of individual atoms or molecules on solid-state surfaces. They are exceptional tools for the characterization of the atomic binding forces of ligand–receptor interactions [142]. In the AFM, a solid-state surface of interest is moved past a extremely small tip, attached to a flexible cantilever. When the tip is coated with specific molecules, the deflection of the cantilever caused by the rises and falls of the tip in response to the topology and specific interaction of the surface is registered and provides the direct molecular recognition of the binding partner on the solid-state surface. Schwesinger et al. [143] were able to detect the unbinding forces of single antigen–antibody complexes and to correlate them with their respective thermal dissociation constants. They used various anti fluorescein scFv fragments, immobilized on a gold surface, together with fluorescein as antigen on a Si_3N_4 tip of the AFM microscope. Baselt et al. [144] presented the concept of a novel Bead Array Counter (BARC) for measuring the forces that bind antibody–antigen, but also DNA–DNA or ligand–receptor pairs together. The system will use these interaction forces to hold magnetic microbeads to a solid substrate. First analytical results are still pending. Also, the optical tweezers-based immunosensor is able to measure the force required to separate antigen from the complex with the respective antibody. Its principle is the use of the radiation pressure when laser light (with power as great as 150 mW) is focused on a dielectric object. This pressure arises from the transfer of photon momentum when light is reflected or scattered by the object and can be used for trapping and (re)moving the object. Helmerson et al. [145] detected extremely small quantities of BSA as soluble antigen under competitive binding conditions, when trapping and pulling away a BSA-coated microsphere from anti-BSA antibodies attached to a solid-state surface. The minimum amount of force, applied by the tweezers to break the antigen–antibody complex, was measured. BSA as free analyte in the solution was detected by the reduction of this force when displac-

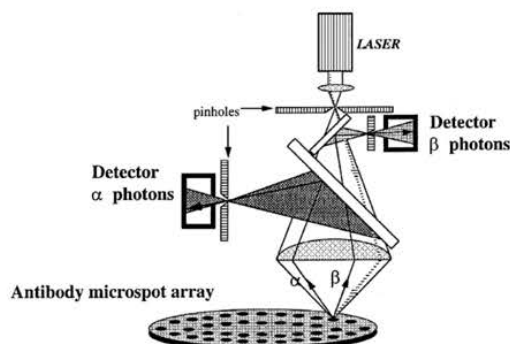


Fig. 5. Microspot multianalyte antibody array. Reproduced with permission from Ref. [1].

ing the BSA microspheres in binding to the surface-coupled antibodies.

5.1.3. Nanotechnology

Advancement in nanotechnology will help for a further development of immunosensors [146], particularly in proteomics and cellomics. Optical fiber-based nanosensors depend on the construction of nanometer-sized optical fibers. There are two different techniques already available to produce fibers' tips approximately 50 nm in diameter: a CO₂ laser-based micropipet-puller or the chemical-etching technique with hydrofluoric acid [146].

Miniaturization will allow the integration of all steps of the analytical process into single-sensor devices ("lab-on-a-chip"), which will be partly disposable. In particular, the fluidics parts of the sensor will benefit from this new technology and allow a reduction of sample volume < 1 µl [147]. However, pumps, valves, and electronic control circuitry are also probable building parts of a miniaturized and integrated sensor [57]. Thus, cheap and even implantable immunosensors [107,148] will be available and possible clinical applications will be expanded considerably.

5.2. Future perspectives for clinical applications

There is a great opportunity for introducing immunosensor devices, since clinical diagnostics represent a huge, well-established and important analytical field. Hence, sophisticated immunosensor systems may be successful in the clinical laboratory. Worthwhile future goals for immunosensors are, in our opinion, microdialysis-based continuous analyses in the fields of blood coagulation, therapeutic drug monitoring (including area-under-curve measurements), cytokine and cardiac markers in intensive-care units, as well as point-of-care systems for fertility and other endocrinological monitoring, type I allergy and other immunological analyses for patients in ambulatory settings. It should be remembered for future developments, however, that the diagnostic market is highly competitive and dominated by powerful biotech companies. Moreover, this field is dictated by clearly defined medical laboratory standards and guidelines; comprehensive and tight regulations are set by governmental institutions

worldwide. Additionally, further improvements of laboratory services and cost-effectiveness are seen mainly in the implementation of a modular-designed laboratory automation [149]. Until today, these circumstances have impeded significant implementation of immunosensor devices in clinical settings on a successful commercial basis. It is a must for every biosensor designer to look at the special demands of clinical chemistry prior to making new developments. As already stated by Connolly [150], several critical issues, such as cost per test, regulatory requirements, quality control, instrumentation setup and test parameter panel, need to be taken into consideration when evaluating immunosensor projects. Also, a short overview of the economic impacts and diagnostic technologies, which are currently used in centralized clinical laboratories, is given by the author [150] and strongly recommended for non-insiders.

In conclusion, the technical developments of both immunoassays and immunosensors are ongoing efforts. In particular, optical, electrochemical, and microgravimetric immunosensors have proven to be potent and future directed analytical tools. Their sensitivities are comparable to immunoassay methods. This can be concluded from selected applications, cited as references in this review. Advantages, such as cheap mass production of disposable chips, the possibility for real-time measurements even *in vivo* (due to miniaturization) and the option for multianalyte analysis, will probably bring this technique into the realm of practicability for diagnostics. However, the way to that goal is still uncertain. Meanwhile, sophisticated immunoassays are still indispensable for the clinical chemist. It is conceivable that in clinical diagnostics, immunosensors will be able to occupy only an analytical niche for online-monitoring purposes in the near future. This is in contrast to the situation in environmental analysis, bioprocess control and research applications concerning biomolecular interaction analysis, where the novel technique will surely play a major role. To support this development, more attention should be focused on improving the specific and regenerable bioaffinity interface: this includes, among other issues, specific binding protein engineering, molecular imprinting, and optimized solid-state surface chemistry. On the other side, laboratory medicine is called upon to define more stringently the clinical chemistry stan-

dards of evaluation of new methods and the possible application fields, such as point-of-care testing, where this potent novel technique may be meaningfully applied. This will help to focus on areas where the commercialization will probably be successful.

Acknowledgements

We thank O.S. Wolfbeis, Regensburg, for critical reading and helpful discussions.

References

- [1] Ekins RP. Immunoassay and other ligand assays: from isotopes to luminescence. *J Clin Ligand Assay* 1999;22:61–77.
- [2] Lowe CR. Analytical biotechnology. *Curr Opin Biotechnol* 1996;7:1–3.
- [3] Aizawa M. Immunosensors. *Bioprocess Technol* 1991;15:249–66.
- [4] Gizeli E, Lowe CR. Immunosensors. *Curr Opin Biotechnol* 1996;7:66–71.
- [5] Mahoney WC, Luderer AA, Brier RA, Lin JN. Real-time immunodiagnosics employing optical immunobiosensors. In: Chan DW, editor. *Immunoassay automation. An updated guide to systems*. San Diego: Academic Press; 1996.
- [6] Ghindilis AL, Atanasov P, Wilkins M, Wilkins E. Immunosensors: electrochemical sensing and other engineering approaches. *Biosens Bioelectron* 1998;13:113–31.
- [7] Čavić BA, Hayward GL, Thompson M. Acoustic waves and the study of bio-chemical macromolecules and cells at the sensor–liquid interface. *Analyst* 1999;124:1405–20.
- [8] O'Sullivan CK, Vaughan R, Guilbault GG. Piezoelectric immunosensors—theory and applications. *Anal Lett* 1999;32:2353–77.
- [9] Mullett W, Lai EP, Yeung JM. Surface plasmon resonance-based immunoassays. *Methods* 2000;22:77–91.
- [10] Morgan CL, Newman DJ, Price CP. Immunosensors: technology and opportunities in laboratory medicine. *Clin Chem* 1996;42:193–209.
- [11] Aizawa M. Immunosensors for clinical analysis. *Adv Clin Chem* 1994;31:247–75.
- [12] McNeil CJ, Athey D, Renneberg R. Immunosensors for clinical diagnostics. *EXS* 1997;81:17–25.
- [13] Pearson JE, Gill A, Vadgama P. Analytical aspects of biosensors. *Ann Clin Biochem* 2000;37:119–45.
- [14] Rogers KR. Principles of affinity-based biosensors. *Mol Biotechnol* 2000;14:109–29.
- [15] Hock B. Antibodies for immunosensors. A review. *Anal Chim Acta* 1997;347:177–86.
- [16] Fitzpatrick J, Fanning L, Hearty S, et al. Application and recent developments in the use of antibodies for analysis. *Anal Lett* 2000;33:2563–609.
- [17] Price CP. Progress in immunoassay technology. *Clin Chem Lab Med* 1998;36:341–7.
- [18] Price CP. The evolution of immunoassay as seen through the journal *Clinical Chemistry*. *Clin Chem* 1998;44:2071–4.
- [19] Hage DS. Immunoassays. *Anal Chem* 1999;71:294R–304R.
- [20] Borrebaeck CAK. Antibodies in diagnostics—from immunoassays to protein chips. *Immunol Today* 2000;21:379–81.
- [21] Söderlind E, Ohlin M, Carlsson R. Complementarity-determining region (CDR) implantation: a theme of recombination. *Immunotechnology* 1999;4:279–85.
- [22] Casadei J, Powell MJ, Kenten JH. Expression and secretion of aequorin as a chimeric antibody by means of a mammalian expression vector. *Proc Natl Acad Sci U S A* 1990;87:2047–51.
- [23] Dubrovsky T, Tronin A, Dubrovskaya S, Vakula S, Nicolini C. Immunological activity of Ig G Langmuir films oriented by protein A sublayer. *Sens Actuators, B* 1995;23:1–7.
- [24] Quinn J, Patel P, Fitzpatrick B, et al. The use of regenerable, affinity ligand-based surfaces for immunosensor applications. *Biosens Bioelectron* 1999;14:587–95.
- [25] Pearson JE, Kane JW, Petraki-Kallioti I, Gill A, Vadgama P. Surface plasmon resonance: a study of the effect of biotinylation on the selection of antibodies for use in immunoassays. *J Immunol Methods* 1998;221:87–94.
- [26] Schettler H. Avidin and streptavidin in clinical diagnostics. *Biomol Eng* 1999;16:73–8.
- [27] Lu B, Smyth MR, O'Kennedy R. Oriented immobilization of antibodies and its applications in immunoassays and immunosensors. *Analyst* 1996;121:29R–32R.
- [28] Domen PL, Nevens JR, Mallia AK, Hermanson GT, Klenk DC. Site-directed immobilization of proteins. *J Chromatogr* 1990;510:293–302.
- [29] Brynda E, Houska M, Kvor J, Ramsden JJ. Immobilisation of multilayer bioreceptor assemblies on solid substrates. *Biosens Bioelectron* 1998;13:165–72.
- [30] Gao H, Sanger M, Luginbuhl R, Sigrist H. Immunosensing with photoimmobilized immunoreagents on planar wave guides. *Biosens Bioelectron* 1995;10:317–28.
- [31] Nakanishi K, Muguruma H, Karube I. A novel method of immobilizing antibodies on a quartz crystal microbalance using plasma-polymerized films for immuno-sensors. *Anal Chem* 1996;68:1695–700.
- [32] Yang T, Jung S, Mao H, Cremer PS. Fabrication of phospholipid bilayer-coated microchannels for on-chip immunoassays. *Anal Chem* 2001;73:165–9.
- [33] Gizeli E, Liley M, Lowe CR, Vogel H. Antibody binding to a functionalized supported lipid layer: a direct acoustic immunosensor. *Anal Chem* 1997;69:4808–13.
- [34] Kaiser T, Gudat P, Stock W, Pappert G, Luppa PB. Biotinylated steroid derivatives as ligands for biospecific binding studies with monoclonal antibodies using immunosensor technology. *Anal Biochem* 2000;282:173–85.
- [35] Disley DM, Cullen DC, You HX, Lowe CR. Covalent

- coupling of immuno-globulin G to self-assembled monolayers as a method for immobilizing the inter-facial-recognition layer of a surface plasmon resonance immunosensor. *Biosens Bioelectron* 1998;13:1213–25.
- [36] Winter G, Griffiths AD, Hawkins RE, Hoogenboom HR. Making antibodies by phage display technology. *Ann Rev Immunol* 1994;12:433–55.
- [37] Jung S, Honegger A, Plückthun A. Selection for improved protein stability by phage display. *J Mol Biol* 1999;294:163–80.
- [38] Santandreu M, Céspedes F, Alegret S, Martínez-Fabregas E. Amperometric immunosensors based on rigid conducting immunocomposites. *Anal Chem* 1997;69:2080–5.
- [39] Jayasena SD. Aptamers: an emerging class of molecules that rival antibodies in diagnostics. *Clin Chem* 1999;45:1628–50.
- [40] Tuerk C, Gold L. Systematic evolution of ligands by exponential enrichment: RNA ligands to bacteriophage T4 DNA polymerase. *Science* 1990;249:505–10.
- [41] Lee M, Walt DR. A fiber-optic microarray biosensor using aptamers as receptors. *Anal Biochem* 2000;282:142–6.
- [42] Hesselberth J, Robertson MP, Jhaveri S, Ellington AD. In vitro selection of nucleic acids for diagnostic applications. *Rev Mol Biotechnol* 2000;74:15–25.
- [43] Schlehuber S, Beste G, Skerra A. A novel type of receptor protein, based on the lipocalin scaffold, with specificity for digoxigenin. *J Mol Biol* 2000;297:1105–20.
- [44] Ansell RJ, Ramström O, Mosbach K. Towards artificial antibodies prepared by molecular imprinting. *Clin Chem* 1996;42:1506–12.
- [45] Sällergren B, Andersson LI. Application of imprinted synthetic polymers in binding assay development. *Methods* 2000;22:92–106.
- [46] Andersson LI. Molecular imprinting: developments and applications in the analytical chemistry field. *J Chromatogr. B: Biomed Sci Appl* 2000;745:3–13.
- [47] Berson SA, Yalow RS. Assay of plasma insulin in human subjects by immunological methods. *Nature* 1959;184:1648–9.
- [48] Gosling JP. A decade of development in immunoassay methodology. *Clin Chem* 1990;36:1408–127.
- [49] Ngo TT. Developments in immunoassay technology. *Methods* 2000;22:1–3.
- [50] Turner APF, Chen B, Piletsky SA. In vitro diagnostics in diabetes: meeting the challenge. *Clin Chem* 1999;45:1596–601.
- [51] Sokoll LJ, Chan DW. Choosing an automated immunoassay system. In: Wild D, editor. *The immunoassay handbook*. 2nd edn. New York: Nature Publishing Group; 2001. p. 261–5.
- [52] Sokoll LJ, Chan DW. Clinical analyzers. *Immunoassay. Anal Chem* 1999;71:356–62.
- [53] Anonymous. Automated immunoassay analyzers. *CAP Today* 2000;14:33–64.
- [54] Gajda A, Hawthorn M. Laboratory technology. Simultaneous multiple analyte testing: the next 'revolution' in lab testing? *Adv Lab* 1999;8:69–70.
- [55] Ekins RP. Ligand assays: from electrophoresis to miniaturized microarrays. *Clin Chem* 1998;44:2015–30.
- [56] Mendoza LG, McQuary P, Mongan A, Gangadharan R, Brignac S, Eggers M. High-throughput microarray-based enzyme-linked immunosorbent assay (ELISA). *Biotechniques* 1999;27:782–6.
- [57] Felder R.A., Kost G.J. Automation: Part 1. Modular stepwise automation and the future of diagnostic testing. *MLO Med. Lab. Obs.* 1998 Apr;30(4):22–4, 26–7.
- [58] Kricka LJ. Miniaturization of analytical systems. *Clin Chem* 1998;44:2008–14.
- [59] Bock JL. The new era of automated immunoassay. *Am J Clin Pathol* 2000;113:628–46.
- [60] Li DJ, Sokoll LJ, Chan DW. Automated chemiluminescent immunoassay analyzers. *J Clin Ligand Assay* 1998;21:377–85.
- [61] Oswald B, Lehmann F, Simon L, Terpetschnig E, Wolfbeis OS. Red laser-induced fluorescence energy transfer in an immunosystem. *Anal Biochem* 2000;280:272–7.
- [62] Wolfbeis OS. Fiber-optic chemical sensors and biosensors. *Anal Chem* 2000;72:81R–9R.
- [63] Buerk DG. *Biosensors. Theory and applications*. Lancaster, PA: Technomic Publishing; 1993.
- [64] Koch S, Woias P, Meixner LK, Drost S, Wolf H. Protein detection with a novel ISFET-based zeta potential analyzer. *Biosens Bioelectron* 1999;14:413–21.
- [65] L.C. Clark, US Patent #2,913,386, 1956.
- [66] López MA, Ortega F, Dominguez E, Katakis I. Electrochemical immunosensor for the detection of atrazine. *J Mol Recognit* 1998;11:178–81.
- [67] Berney HC, Alderman J, Lane WA, Collins JK. Development of a capacitive immunosensor: a comparison of monoclonal and polyclonal capture antibodies as the primary layer. *J Mol Recognit* 1998;11:175–7.
- [68] Cornell BA, Braach-Maksvytis VLB, King LG, et al. A biosensor that uses ion-channel switches. *Nature* 1997;387:580–3.
- [69] Yagiuda K, Hemmi A, Ito S, et al. Development of a conductivity-based immunosensor for sensitive detection of methamphetamine (stimulant drug) in human urine. *Biosens Bioelectron* 1996;11:703–7.
- [70] Mirsky VM, Riepl M, Wolfbeis OS. Capacitive monitoring of protein immobilization and antigen-antibody reactions on monomolecular alkylthiol films on gold electrodes. *Biosens Bioelectron* 1997;12:977–89.
- [71] Mirsky VM, Hirsch T, Piletsky SA, Wolfbeis OS. A spreader-bar approach to molecular architecture: formation of stable artificial chemoreceptors. *Angew Chem, Int Ed* 1999;38:1108–10.
- [72] Kubitschko S, Spinke J, Bruckner T, Pohl S, Oran N. Sensitivity enhancement of optical immunosensors with nanoparticles. *Anal Biochem* 1997;253:112–22.
- [73] Schneider BH, Dickinson EL, Vach MD, Hoijer JV, Howard LV. Highly sensitive optical chip immunoassays in human serum. *Biosens Bioelectron* 2000;15:13–22.
- [74] Domenici C, Schirone A, Celebre M, Ahluwalia A, De Rossi D. Development of a TIRF immunosensor: modelling

- the equilibrium behaviour of a competitive system. *Biosens Bioelectron* 1995;10:371–8.
- [75] Tsay YG, Lin CI, Lee J, Gustafson EK, Appelqvist R, Maggini P, Norton R, Teng N, Charlton D. Optical biosensor assay (OBA). *Clin Chem* 1991;37:1502–5.
- [76] Stenberg M, Nygren H. A receptor–ligand reaction studied by a novel analytical tool—the isotope ellipsometer. *Anal Biochem* 1982;127:183–92.
- [77] DeLisa MP, Zhang Z, Shiloach M, et al. Evanescent wave long-period fiber bragg grating as an immobilized antibody biosensor. *Anal Chem* 2000;72:2895–900.
- [78] Tiefenthaler K. Integrated optical couplers as chemical wave guide sensors. In: Turner APF, editor. *Advances in biosensors*. London: JAI Press; 1992. p. 261–89.
- [79] Lukosz W. Integrated optical chemical and direct biochemical sensors. *Sens Actuators, B* 1995;29:37–50.
- [80] Liedberg B, Nylander C, Lundström I. Biosensing with surface plasmon resonance—how it all started. *Biosens Bioelectron* 1995;10:i–ix.
- [81] Berger CEH, Greve J. Differential SPR immunosensing. *Sens Actuators, B* 2000;63:103–8.
- [82] Aberl F, Kösslinger C, Wolf H. Molecular diagnostics of infectious diseases. In: Reischl U, editor. *Methods in molecular medicine*, vol. 13, Totawa: Humana Press; 1997. p. 519–27.
- [83] Janshoff A, Galla HJ, Steinem C. Piezoelectric mass-sensing devices as biosensors—an alternative to optical biosensors? *Angew Chem, Int Ed Engl* 2000;39:4004–32.
- [84] Sauerbrey G. Verwendung von Schwingquarzen zur Wägung dünner Schichten und zur Mikrowägung (Application of quartz oscillators for weighing of thin films and for microweighing). *Z Phys* 1959;155:206–22.
- [85] Kösslinger C, Uttenhaller E, Drost S, et al. Comparison of the QCM and the SPR method for surface studies and immunological applications. *Sens Actuators, B* 1995;24–25:107–12.
- [86] Dahint R, Bender F, Morhard F. Operation of acoustic plate mode immuno-sensors in complex biological media. *Anal Chem* 1999;71:3150–6.
- [87] Aberl F, Wolf H, Kösslinger C, Drost S, Woias P, Koch S. HIV serology using piezoelectric immunosensors. *Sens Actuators, B* 1994;18–19:271–5.
- [88] Chetcuti AF, Wong DK, Stuart MC. An indirect perfluoro-sulfonated ionomer-coated electrochemical immunosensor for the detection of the protein human chorionic gonadotrophin. *Anal Chem* 1999;71:4088–94.
- [89] Santandreu M, Alegret S, Fàbregas E. Determination of β -HCG using amperometric immunosensors based on a conducting immunocomposite. *Anal Chim Acta* 1999;396:181–8.
- [90] Kelly S, Compagnone D, Guilbault G. Amperometric immunosensor for lactate dehydrogenase LD-1. *Biosens Bioelectron* 1998;13:173–9.
- [91] Liu CH, Liao KT, Huang HJ. Amperometric immunosensors based on protein a coupled polyaniline-perfluorosulfonated ionomer composite electrodes. *Anal Chem* 2000;72:2925–9.
- [92] Key G, Schreiber A, Feldbrugge R, Glatz JF, Spener F. An immunosensor for rapid estimation of the early heart infarction-marker FABP. *Acta Anaesthesiol Scand, Suppl* 1997;111:289–92.
- [93] Key G, Schreiber A, Feldbrugge R, et al. Multicenter evaluation of an ampero-metric immunosensor for plasma fatty acid-binding protein: an early marker for acute myocardial infarction. *Clin Biochem* 1999;32:229–31.
- [94] Meusel M, Renneberg R, Spener F, Schmitz G. Development of a heterogeneous amperometric immunosensor for the determination of apolipoprotein E in serum. *Biosens Bioelectron* 1995;10:577–86.
- [95] Kröger D, Katerkamp A, Renneberg R, Cammann K. Surface investigations on the development of a direct optical immunosensor. *Biosens Bioelectron* 1998;13:1141–7.
- [96] Spinke J, Oran N, Fattinger C, Koller H, Mangold C, Voegelín D. The bi-diffractive grating coupler: application to immunosensing. *Sens Actuators, B* 1997;38–9:256–60.
- [97] Nicholson S, Gallop JL, Law P, Thomas H, George AJT. Monitoring antibody responses to cancer vaccination with a resonant mirror biosensor. *Lancet* 1999;353:808.
- [98] Brynda E, Houska M, Brandenburg A, Wikerstål A, Skvor J. The detection of human β_2 -microglobulin by grating coupler immunosensor with three dimensional antibody networks. *Biosens Bioelectron* 1999;14:363–8.
- [99] Keating GJ, Quinn JG, O’Kennedy R. Immunoassay for the determination of 7-hydroxycoumarin in serum using “real-time” biosensor analysis. *Anal Lett* 1999;32:2163–76.
- [100] Lu HC, Chen HM, Lin YS, Lin JW. A reusable and specific protein A-coated piezoelectric biosensor for flow injection immunoassay. *Biotechnol Prog* 2000;16:116–24.
- [101] Su X, Chew FT, Li SF. Self-assembled monolayer-based piezoelectric crystal immunosensor for the quantification of total human immunoglobulin E. *Anal Biochem* 1999;273:66–72.
- [102] Chu X, Lin ZH, Shen GL, Yu RQ. Piezoelectric immunosensor for the detection of immunoglobulin M. *Analyst* 1995;120:2829–32.
- [103] Suri CR, Jain PK, Mishra GC. Development of piezoelectric crystal based microgravimetric immunoassay for determination of insulin concentration. *J Biotechnol* 1995;39:27–34.
- [104] Attili BS, Suleiman AA. A piezoelectric immunosensor for the detection of cortisol. *Anal Lett* 1995;28:2149–59.
- [105] Tajima I, Asami O, Sugiura E. Monitor of antibodies in human saliva using a piezoelectric quartz crystal biosensor. *Anal Chim Acta* 1998;365:147–9.
- [106] Cook CJ. Monitoring on-line of extracellular gamma-amino-4-butyric acid using microdialysis coupled to immunosensor analysis. *J Neurosci Methods* 1998;82:145–50.
- [107] Cook CJ. Real-time measurements of corticosteroids in conscious animals using an antibody-based electrode. *Nat Biotechnol* 1997;15:467–71.
- [108] Kaptein WA, Zwaagstra JJ, Venema K, Ruiters MHJ, Korf J. Analysis of cortisol with a flow displacement immunoassay. *Sens Actuators, B* 1997;45:63–9.
- [109] Sergeyeva TA, Soldatkin AP, Rachkov AE, Tereschenko MI, Piletsky SA, El’skaya AV. β -Lactamase label-based

- potentiometric biosensor for α -2 interferon detection. *Anal Chim Acta* 1999;390:73–81.
- [110] Duan C, Meyerhoff ME. Separation-free sandwich enzyme immunoassay using microporous gold electrodes and self-assembled monolayer/immobilized capture antibodies. *Anal Chem* 1994;66:1369–77.
 - [111] Astles JR, Miller WG. Measurement of free phenytoin in blood with a self-contained fiber-optic immunosensor. *Anal Chem* 1994;66:1675–82.
 - [112] Daniels PB, Fletcher JE, O'Neill PM, Stafford CG, Bacarese-Hamilton T, Robinson GA. A comparison of three fluorophores for use in an optical biosensor for the measurement of prostate-specific antigen in whole blood. *Sens Actuators, B* 1995;26–27:447–51.
 - [113] Schult K, Katerkamp A, Trau D, Grawe F, Cammann K, Meusel M. Disposable optical sensor chip for medical diagnostics: new ways in bioanalysis. *Anal Chem* 1999;71:5430–5.
 - [114] Sakai G, Ogata K, Uda T, Miura N, Yamazoe N. A surface plasmon resonance-based immunosensor for highly sensitive detection of morphine. *Sens Actuators, B* 1998;49:5–12.
 - [115] Rowe CA, Scruggs SB, Feldstein MJ, Golden JP, Ligler FS. An array immuno-sensor for simultaneous detection of clinical analytes. *Anal Chem* 1999;71:433–9.
 - [116] Papkovsky DB, O'Riordan TC, Guilbault GG. An immunosensor based on the glucose oxidase label and optical oxygen detection. *Anal Chem* 1999;71:1568–73.
 - [117] Hanbury CM, Miller WG, Harris RB. Fiber-optic immunosensor for measurement of myoglobin. *Clin Chem* 1997;43:2128–36.
 - [118] Wang AW, Kiwan R, White RM, Ceriani RL. Silicon-based ultrasonic immuno-assay for detection of breast cancer antigens. *Sens Actuators, B* 1998;49:13–21.
 - [119] Wadkins RM, Golden JP, Pritsiolas LM, Ligler FS. Detection of multiple toxic agents using a planar array immunosensor. *Biosens Bioelectron* 1998;13:407–15.
 - [120] Hock B. Advances in immunochemical detection of microorganisms. *Ann Biol Clin* 1996;54:243–52.
 - [121] Karube I, Yano K, Sasaki S, Nomura Y, Ikebukuro K. Biosensors for environmental monitoring. *Ann N Y Acad Sci* 1998;864:23–36.
 - [122] Killard AJ, Smyth MR, Grennan K, Micheli L, Palleschi G. Rapid antibody biosensor assays for environmental analysis. *Biochem Soc Trans* 2000;28:81–4.
 - [123] Lynch JM. Terrestrial biotechnology. *Curr Opin Biotechnol* 1998;9:247–51.
 - [124] Schepers TH, Lammers F. Fermentation monitoring and process control. *Curr Opin Biotechnol* 1994;5:187–91.
 - [125] Tran-Minh C. Biosensors in flow-injection systems for biomedical analysis, process and environmental monitoring. *J Mol Recognit* 1996;9:658–63.
 - [126] Nieba L, Krebber A, Plückthun A. Competition BIAcore for measuring true affinities: large differences from values determined from binding kinetics. *Anal Biochem* 1996;234:155–65.
 - [127] Adamczyk M, Gebler JC, Gunasekera AH, Mattingly PG, Pan Y. Immunoassay reagents for thyroid testing: 2. Binding properties and energetic parameters of a T₄ monoclonal antibody and its Fab fragment with a library of thyroxine analog biosensors using surface plasmon resonance. *Bioconjugate Chem* 1997;8:133–45.
 - [128] Van Regenmortel MH, Altschuh D, Chatellier J, et al. Measurement of antigen-antibody interactions with biosensors. *J Mol Recognit* 1998;11:163–7.
 - [129] Malmqvist M. Biacore: an affinity biosensor system for characterization of bio-molecular interactions. *Biochem Soc Trans* 1999;27:335–40.
 - [130] Cheskis BJ, Karathanasis S, Lytle CR. Estrogen receptor ligands modulate its interaction with DNA. *J Biol Chem* 1997;272:11384–91.
 - [131] De Crescenzo G, Grothe S, Lortie R, Debanne MT, O'Connor-McCourt M. Real-time kinetic studies on the interaction of transforming growth factor α with the epidermal growth factor receptor extracellular domain reveal a conformational change model. *Biochem* 2000;39:9466–76.
 - [132] Morgan CL, Newman DJ, Cohen SBA, Lowe P, Price CP. Real-time analysis of cell surface HLA class I interactions. *Biosens Bioelectron* 1998;13:1099–105.
 - [133] Polzius R, Dießel E, Bier FF, Bilitewski U. Real-time observation of affinity reactions using grating couplers: determination of the detection limit and calculation of kinetic rate constants. *Anal Biochem* 1997;248:269–76.
 - [134] Abel AP, Weller MG, Duveneck GL, Ehrat M, Widmer HM. Fiber-optic evanescent wave biosensor for the detection of oligonucleotides. *Anal Chem* 1996;68:2905–12.
 - [135] Zhai J, Cui H, Yang R. DNA based biosensors. *Biotech Adv* 1997;15:43–58.
 - [136] Ostroff RM, Hopkins D, Haerberli AB, Baouchi W, Polisky B. Thin film biosensor for rapid visual detection of nucleic acid targets. *Clin Chem* 1999;45:1659–64.
 - [137] Nelson RW, Krone JR, Jansson O. Surface plasmon resonance biomolecular interaction analysis mass spectrometry: 1. Chip-based analysis. *Anal Chem* 1997;69:4363–8.
 - [138] Nelson RW, Krone JR, Jansson O. Surface plasmon resonance biomolecular interaction analysis mass spectrometry: 2. Fiber optic-based analysis. *Anal Chem* 1997;69:4369–74.
 - [139] Nelson RW, Nedelkov D, Tubbs KA. Biomolecular interaction analysis mass spectrometry. BIA/MS can detect and characterize proteins in complex biological fluids at the low-to subfemtomole level. *Anal Chem* 2000;72:404A–11A.
 - [140] Ekins RP. Microarrays: their origins and applications. *Tibtech* 1999;17:217–8.
 - [141] Wang J. From DNA biosensors to gene chips. *Nucleic Acids Res* 2000;28:3011–6.
 - [142] Kricka LJ. Optical tweezers and immunoassay. *Clin Chem* 1997;43:251–3.
 - [143] Schwesinger F, Ros R, Strunz T, et al. Unbinding forces of single antibody-antigen complexes correlate with their thermal dissociation rates. *Proc Natl Acad Sci* 2000;97:9972–7.
 - [144] Baselt DR, Lee GU, Natesan M, Metzger SW, Sheehan PE, Colton RJ. A biosensor based on magnetoresistance technology. *Biosens Bioelectron* 1998;13:731–9.
 - [145] Helmersen K, Kishore R, Phillips WD, Weetall HH. Optical

- tweezers-based immunosensor detects femtomolar concentrations of antigens. Clin Chem 1997;43:379–83.
- [146] Cullum BM, Vo-Dinh T. The development of optical nanosensors for biological measurements. Trends Biotechnol 2000;18:388–93.
- [147] Song MI, Iwata K, Yamada M, et al. Multisampling analysis using an array of microreactors for an alternating-current field-enhanced latex immunoassay. Anal Chem 1994;66:778–81.
- [148] Turner AP. Immunosensors: the next generation. Nat Biotechnol 1997;15:421.
- [149] Markin RS, Whalen SA. Laboratory automation: trajectory, technology and tactics. Clin Chem 2000;46:764–71.
- [150] Connolly P. Clinical diagnostics opportunities for biosensors and bioelectronics. Biosens Bioelectron 1995;10:1–6.
- [151] Abdel-Hamid I, Ivnitski D, Atanasov P, Wilkins E. Flow-through immuno-filtration assay system for rapid detection of *E. coli* O157:H7. Biosens Bioelectron 1999;14:309–16.
- [152] Ben-Dov I, Willner I, Zisman E. Piezoelectric immunosensors for urine specimens of *Chlamydia trachomatis* employing quartz crystal microbalance microgravimetric analyses. Anal Chem 1997;69:3506–12.
- [153] Bouvrette P, Luong JH. Development of a flow injection analysis (FIA) immunosensor for the detection of *Escherichia coli*. Int J Food Microbiol 1995;27:129–37.
- [154] Brooks JL, Mirhabibollahi B, Kroll RG. Experimental enzyme-linked amperometric immunosensors for the detection of salmonellas in foods. J Appl Bacteriol 1992;73:189–96.
- [155] Chang YH, Chang TC, Kao EF, Chou C. Detection of protein A produced by *Staphylococcus aureus* with a fiber-optic-based biosensor. Biosci Biotechnol Biochem 1996;60:1571–4.
- [156] Daneshvar MI, Peralta JM, Casay GA, et al. Detection of biomolecules in the near-infrared spectral region via a fiber-optic immunosensor. J Immunol Methods 1999;226:119–28.
- [157] Harteveld JL, Nieuwenhuizen MS, Wils ER. Detection of staphylococcal enterotoxin B employing a piezoelectric crystal immunosensor. Biosens Bioelectron 1997;12:661–7.
- [158] King KD, Anderson GP, Bullock KE, Regina MJ, Saaski EW, Ligler FS. Detecting staphylococcal enterotoxin B using an automated fiber optic biosensor. Biosens Bioelectron 1999;14:163–70.
- [159] Koch S, Wolf H, Danapel C, Feller KA. Optical flow-cell multichannel immuno-sensor for the detection of biological warfare agents. Biosens Bioelectron 2000;14:779–84.
- [160] König B, Gratzel M. A novel immunosensor for herpes viruses. Anal Chem 1994;66:341–4.
- [161] Kumar P, Colston JT, Chambers JP, Rael ED, Valdes JJ. Detection of botulinum toxin using an evanescent wave immunosensor. Biosens Bioelectron 1994;9:57–63.
- [162] Long GW, O'Brien T. Antibody-based systems for the detection of bacillus anthracis in environmental samples. J Appl Microbiol 1999;87:214.
- [163] Mirhabibollahi B, Brooks JL, Kroll RG. A semi-homogeneous amperometric immunosensor for protein A-bearing *Staphylococcus aureus* in foods. Appl Microbiol Biotechnol 1990;34:242–7.
- [164] Parry RP, Love C, Robinson GA. Detection of rubella antibody using an optical immunosensor. J Virol Methods 1990;27:39–48.
- [165] Plomer M, Guilbault GG, Hock B. Development of a piezoelectric immunosensor for the detection of enterobacteria. Enzyme Microb Technol 1992;14:230–5.
- [166] Prusak-Sochaczewski E, Luong JH, Guilbault GG. Development of a piezoelectric immunosensor for the detection of *Salmonella typhimurium*. Enzyme Microb Technol 1990;12:173–7.
- [167] Pyun JC, Beutel H, Meyer JU, Ruf HH. Development of a biosensor for *E. coli* based on a flexural plate wave (FPW) transducer. Biosens Bioelectron 1998;13:839–45.
- [168] Sanden B, Eng LH, Dalhammar G. An amperometric enzyme-linked immunosensor for *Nitrobacter*. Appl Microbiol Biotechnol 1998;50:710–6.
- [169] Sevars AH, Schasfoort RB, Salden MH. An immunosensor for syphilis screening based on surface plasmon resonance. Biosens Bioelectron 1993;8:185–9.
- [170] Tempelman LA, King KD, Anderson GP, Ligler FS. Quantitating staphylococcal enterotoxin B in diverse media using a portable fiber-optic biosensor. Anal Biochem 1996;233:50–7.
- [171] Uttenthaler E, Kösslinger C, Drost S. Characterization of immobilization methods for African swine fever virus protein and antibodies with a piezoelectric immunosensor. Biosens Bioelectron 1998;13:1279–86.
- [172] Bäumner AJ, Schmid RD. Development of a new immunosensor for pesticide detection: a disposable system with liposome-enhancement and amperometric detection. Biosens Bioelectron 1998;13:519–29.
- [173] Bier F, Schmid R. Real-time analysis of competitive binding using grating coupler immunosensors for pesticide detection. Biosens Bioelectron 1994;9:125–30.
- [174] Brecht A, Piehler J, Lang G, Gauglitz G. A direct optical immunosensor for atrazine detection. Anal Chim Acta 1995;311:289–99.
- [175] Charles PT, Conrad DW, Jacobs MS, Bart JC, Kusterbeck AW. Synthesis of a fluorescent analog of polychlorinated biphenyls for use in a continuous flow immunosensor assay. Bioconjugate Chem 1995;6:691–4.
- [176] Charles PT, Kusterbeck AW. Trace level detection of hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) by microimmunosensor. Biosens Bioelectron 1999;14:387–96.
- [177] Keay RW, McNeil CJ. Separation-free electrochemical immunosensor for rapid determination of atrazine. Biosens Bioelectron 1998;13:963–70.
- [178] Kalab T, Skladal P. A disposable amperometric immunosensor for 2,4-dichloro-phenoxyacetic acid. Anal Chim Acta 1995;304:361–8.
- [179] Kröger S, Setford SJ, Turner AP. Immunosensor for 2,4-dichlorophenoxyacetic acid in aqueous/organic solvent soil extracts. Anal Chem 1998;70:5047–53.
- [180] Laitinen MP, Vuento M. Affinity immunosensor for milk progesterone: identification of critical parameters. Biosens Bioelectron 1996;11(12):1207–14.
- [181] Meusel M, Trau D, Katerkamp A, Meier F, Polzius R, Cammann K. New ways in bioanalysis one-way optical

- sensor chip for environmental analysis. *Sens Actuators, B* 1998;51:249–55.
- [182] Mulchandani A, Kaneva I, Chen W. Biosensor for direct determination of organophosphate nerve agents using recombinant *Escherichia coli* with surface-expressed organophosphorus hydrolase: 2. Fiber-optic microbial biosensor. *Anal Chem* 1998;70:5042–6.
- [183] Mulchandani P, Mulchandani A, Kaneva I, Chen W. Biosensor for direct determination of organophosphate nerve agents: 1. Potentiometric enzyme electrode. *Biosens Bioelectron* 1999;14:77–85.
- [184] Mullett W, Lai EP, Yeung JM. Immunoassay of fumonisins by a surface plasmon resonance biosensor. *Anal Biochem* 1998;258:161–7.
- [185] O'Brien T, Johnson III LH, Aldrich JL, et al. The development of immunoassays to 4 biological threat agents in a bidiffractive grating biosensor. *Biosens Bioelectron* 2000;14:815–28.
- [186] O'Daly JP, Zhao J, Brown PA, Henkens RW. Electrochemical enzyme immuno-assay for detection of toxic substances. *Enzyme Microb Technol* 1992;14:299–302.
- [187] Pemberton RM, Hart JP, Stoddard P, Foulkes JA. A comparison of 1-naphthyl phosphate and 4-aminophenyl phosphate as enzyme substrates for use with a screen-printed amperometric immunosensor for progesterone in cows' milk. *Biosens Bioelectron* 1999;14:495–503.
- [188] Rippeth JJ, Gibson TD, Hart JP, Hartley IC, Nelson G. Flow-injection detector incorporating a screen-printed disposable amperometric biosensor for monitoring organophosphate pesticides. *Analyst* 1997;122:1425–9.
- [189] Sandberg RG. A conductive polymer-based immunosensor for the analysis of pesticide residues. *Am Chem Soc Symp Ser* 1992;511:81–8.
- [190] Seifert M, Haindl S, Hock B. In vitro analysis of xenoestrogens by enzyme linked receptor assays (ELRA). *Adv Exp Med Biol* 1998;444:113–7.
- [191] Steegborn C, Skladal P. Construction and characterization of the direct piezoelectric immunosensor for atrazine operating in solution. *Biosens Bioelectron* 1997;12:19–27.
- [192] Sternesjo A, Mellgren C, Bjorck L. Determination of sulfamethazine residues in milk by a surface plasmon resonance-based biosensor assay. *Anal Biochem* 1995;226:175–81.
- [193] Strachan NJ, John PG, Millar IG. Application of a rapid automated immunosensor for the detection of *Staphylococcus aureus* enterotoxin B in cream. *Int J Food Microbiol* 1997;35:293–7.
- [194] Topozada AR, Wright J, Eldefrawi AT, Eldefrawi ME, Johnson EL, Emche SD, Helling CS. Evaluation of a fiber optic immunosensor for quantitating cocaine in coca leaf extracts. *Biosens Bioelectron* 1997;12:113–24.
- [195] Vianello F, Signor L, Pizzariello A, et al. Continuous flow immunosensor for atrazine detection. *Biosens Bioelectron* 1998;13:45–53.
- [196] Weller MG, Schuetz AJ, Winklmair M, Niessner R. Highly parallel affinity sensor for the detection of environmental contaminants in water. *Anal Chim Acta* 1999;393:29–41.
- [197] Yokoyama K, Ikebukuro K, Tamiya E, Karube I, Ichiki N, Arikawa Y. Highly sensitive quartz crystal immunosensors for multisample detection of herbicides. *Anal Chim Acta* 1995;304:139–45.
- [198] Yu H, Kusterbeck AW, Hale MJ, Ligler FS, Whelan JP. Use of the USDT flow immunosensor for quantitation of benzoylecgonine in urine. *Biosens Bioelectron* 1996;11:725–34.