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Biosensor-Based Methods in Clinical Diagnosis

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1. Introduction

Despite the multitude of different parameters currently measured in the clinical laboratory, only a minor part of these are measured by means of biosensor-based methods. Within the group of biochemical sensors, enzyme or metabolic sensors as integrated devices in clinical analyzers are dominating. Economically most important are sensors for measuring blood glucose. The implementation of lactate and urea sensors in commercial analytical systems is just beginning and a few more analytes are being intensively investigated in research laboratories *(1)*. Thus, it seems that metabolic biosensors slowly leave the academic playground.

Much more complex to assess is the future of affinity-based biosensors for diagnostic routine applications. The prospect of simplified, faster, and cheaper analytical tools has forced extensive research efforts to bring immunosensors on the diagnostic market. First success could be achieved with the commercialization of affinity sensor systems for research applications by Pharmacia Biosensor (Uppsala, Sweden) and Fisons Applied Sensor Technology (Cambridge, UK). However, the technological and economic challenges to an immunosensor as a diagnostic tool are considerably larger, because of the extraordinary sensitivity of the established methods and the critical question of costs.

This chapter will introduce the most important principles for enzymatic biosensors. Furthermore, we will discuss the basic technologies for affinity sensing representing the fundamentals of the commercialized sensor systems and, probably, the basis for future technologies in clinical diagnostics.

1.1. Definition of a Biosensor

"A biosensor is the spatial unity of a physical transducer and a biological recognition system" *(2)*

From *Methods in Molecular Medicine, Vol 13 Molecular Diagnosis of Infectious Diseases*
Edited by U Reischl Humana Press Inc , Totowa, NJ

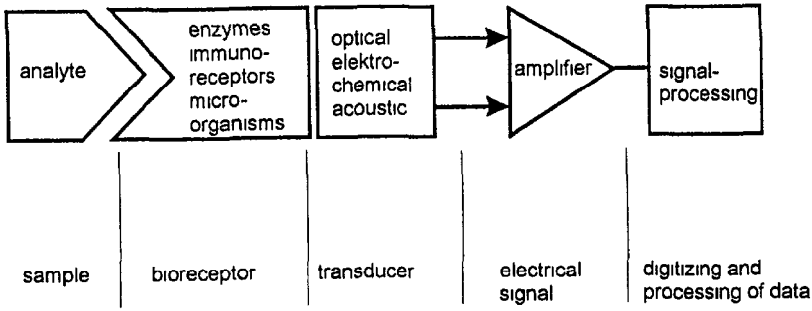

Fig. 1. Construction principle of a biosensor

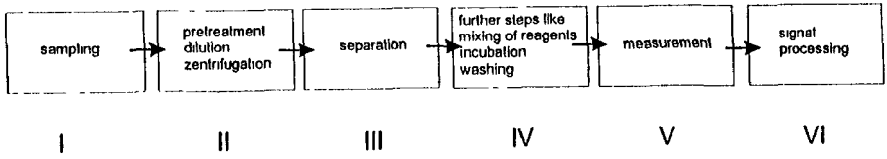

Fig. 2. Stages I–IV of an analytical procedure. A biosensor would comprise tasks III–V

This biosensor definition is widely accepted and visually specified in Fig. 1, where the main biochemical receptor molecules are indicated, as are the main physical transducers. Additionally, this drawing points out the possibility of combining a biochemical sensor with integrated signal amplification and processing devices to create a biochemical sensor with improved characteristics.

1.2. Advantages of Biosensor-Based Clinical Diagnostics

The potential advantages of biosensors in the clinical laboratory should be demonstrated on the basis of the human immunodeficiency virus (HIV) screening enzyme-linked immunosorbent assay (ELISA). Figure 2 shows diagrammatically the progress of an analytical procedure. This analytical procedure is specified in Fig. 3 for the analytical sequence of an ELISA. The introduction of an immunosensor-based analytical step would put together the separation of the analyte from the sample solution (task III, Fig. 2) and the signal transduction into an electrically measurable signal (task V, Fig 2). Additional reagents, washing, and incubation steps are eliminated (task IV, Fig. 2).

Thus a biosensor-based analytical procedure promises:

1 Lower consumption of reagents;
2. Shorter analysis time;
3 Simplified test performance, and
4 Ease of automation

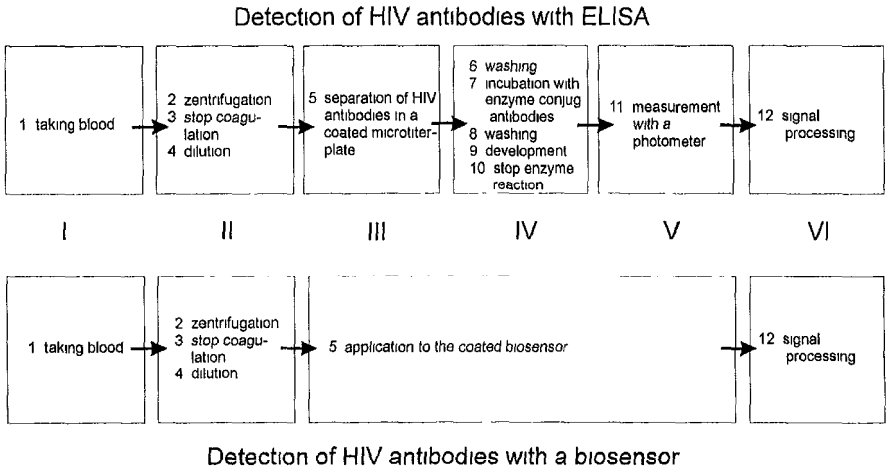

Fig. 3. The principle of Fig. 2 demonstrated on the basis of an HIV screening ELISA.

This list of advantages is certainly not complete, but explains the tremendous research efforts undertaken in this field.

1.3. History of Biosensors

The history of biosensors started when Leyland C. Clark and C Lyons presented their reusable enzyme electrode *(3)*. This invention led to a commercialization of enzyme electrodes for glucose, lactate, urea, and ethanol in our day. One well-known example is the portable mediated glucose electrode for home monitoring of glucose, from MediSense Inc. (USA). Several other companies are also in the market with their glucose sensors. An overview is given in ref. *1*. In 1993 the ISTAT Corp. (Princeton, NJ) introduced a multibiosensor using amperometric and ion-selective potentiometric and conductometric sensors for the simultaneous detection of different parameters (sodium, potassium, chloride, glucose, blood urea, and hematocrit) *(4)*.

Recently, nonmetabolic biosensors came into the market. One is the Threshold system from Molecular Devices (Menlo Park, CA), which is based on a light addressable potentiometric sensor (LAPS). This sensor is mainly used to monitor the physiological state of single cells under the influence of drugs or toxic compounds *(5)*.

The first direct affinity sensor system, called the BIAcore (*see* Chapter 31), is based on Surface Plasmon Resonance (SPR) and was commercialized in 1990 by Pharmacia Biosensor (Uppsala, Sweden) *(6)*. In 1993 Fisons Applied Sensor Technology (Cambridge, UK) launched an affinity sensor system with comparable properties *(7)*. Both sensor systems are mainly applied in pharmaceutical and immunochemical research laboratories to characterize the binding

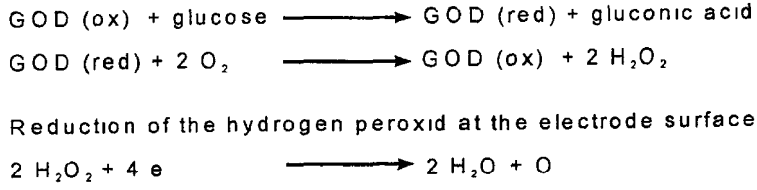

Fig. 4. Biochemical reactions on the surface of an amperometric glucose electrode (GOD = glucose oxidase)

between biochemical receptor molecules and their ligands. Typical applications are, for instance, the mapping of epitopes or the screening and simultaneous characterization of monoclonal antibodies (MAbs).

Furthermore, the sensor system of Artificial Sensing Instruments ASI AG (Zürich, Switzerland) *(8)* should be mentioned, which is based on grating coupler technology. A piezoelectric quartz crystal sensor system is offered by Universal Sensors Co. (New Orleans, LA) *(9)*. Whereas systems developed by Pharmacia and Fisons are supplied with an appropriate technology to fix biomolecules to the sensor surface, ASI and Universal Sensors sell the bare sensors. Customers must establish their own coating technology.

2. Metabolic Sensors

Metabolic sensors determine the concentrations of low molecular analytes by means of the specificity and biocatalytic activity of enzymes in combination with an appropriate electrochemical transducer. The two fundamental transducer principles for metabolic sensors—amperometric and potentiometric electrodes—could be explained on the basis of the glucose and the urea electrode.

2.1. Amperometric Glucose Electrode (10,11)

Amperometric electrodes are based on the reduction or oxidation of an electroactive species at the surface of a polarized electrode. The sensor signal is the current between the electrode (normally called working electrode) and a reference or counter electrode.

The simplest structure of an amperometric glucose electrode consists of a gold electrode and a thin layer of crosslinked glucose oxidase (GOD). Almost all of the glucose sensors developed to date utilize GOD, which oxidizes glucose into gluconic acid (Fig. 4). GOD is reduced but quickly reoxidized by oxygen present in the sample, forming hydrogen peroxide. The hydrogen peroxide produced by this reaction sequence can be detected by reducing it at the surface of the suited polarized amperometric electrode. The measurable electrode current is directly proportional to the glucose concentration in solution.

Because of two main disadvantages the indicated glucose sensor works well only in buffered glucose solutions. One problem is the dependence of the sensor signal on the oxygen concentration in the solution, which changes depending on the air pressure, temperature, and sample composition. At the potential needed to reduce the hydrogen peroxide at the electrode surface, several substances that normally occur in blood (most notably ascorbic acid) can interfere with the reduction process of hydrogen peroxide. These undesirable interferences had the consequence, that the second-generation electrodes utilize so-called mediators as electron shuttles, replacing the oxygen in the redox process (*see* Fig. 4). Simultaneously, mediators allow a lower reduction potential at the working electrode. Ferrocene derivatives are mostly used

The basis of the lactate sensor is the enzyme lactate oxidase (LOD) instead of GOD. Otherwise, the reaction sequence depicted in Fig. 4 is in principle the same.

2.2. Potentiometric Urea Electrode (10,11)

In contrast to amperometric electrodes, where electroactive species are consumed at the electrode surface, potentiometric electrodes measure the electrode surface potential, which is determined by small electrolyte ions (like protons, sodium, or potassium). In the case of the urea electrode protons were consumed by ammonia, which itself is the product of the urease enzyme-based hydrolysis of urea. Thus, a pH sensor coated with a layer of the urease enzyme will measure a pH change proportional to the concentration of urea in the sample. Alternatively, the ammonia concentration itself can be measured by a potentiometric ammonia electrode. For more details concerning amperometric and potentiometric enzyme sensors, *see* refs. *10* and *11*.

3. Immunosensor Principles

Affinity or immunosensors use any kind of complementary molecules with high affinity and specificity as biological recognition systems. The primary effect of the binding event is a mass change or a change in the refractive index at the sensor surface. Therefore, suitable transducers for affinity sensing are acoustic and optical transducers. In both cases defined modes of acoustic or optical waves are guided close to the surface of the transducer to probe the surface mass density (microweighting) of the surface refractive index. The propagating wave forms a so-called evanescent field, which penetrates a definite distance into the surrounding fluid. The strength of the evanescent field is influenced by the adsorption of biological molecules within the penetration depth α (Fig. 5). Optical and acoustic transducers can be subdivided regarding the utilized wave mode and its constructive implementation. For an application overview *see* ref. *12*. For a comparison of the performance of the optical immunosensor systems *see* ref. *13*. Acoustic biosensors are reviewed in ref. *14*.

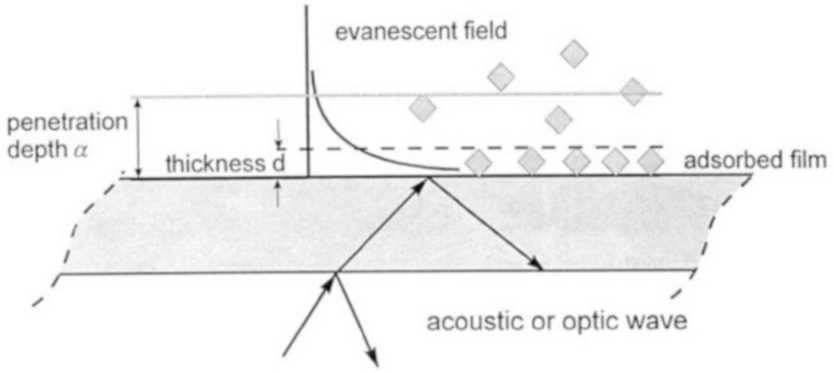

Fig. 5. Cross-sectional view to a optical/acoustic waveguide. The evanescent field penetrates into the surrounding medium.

3.1. Optical Immunosensor Principles

3.1.1. Surface Plasmon Resonance (SPR)-Based Devices

Surface plasmons are collective oscillations of electrons in a thin metal layer near the metal–dielectricum interface. A necessary condition for surface plasmons is that the dielectric constant is complex ($\varepsilon = \varepsilon + i\varepsilon''$) and has a negative real part. In addition, the real part must satisfy the condition $-\varepsilon' > n^2$, where n is the diffractive index of the dielectricum. The oscillation is coupled with a transversal magnetic polarized electromagnetic field (TM). This field penetrates the dielectricum as evanescent field.

One possibility to excite a surface plasmon is described by Kretschmann (Fig. 6) *(15)*. A TM-polarized laser beam reflects totally at a thin metal film (approx 100 nm). The metal film is deposited on a prism and at certain incident angles, when impulse and energy match the impulse and energy of the excited state of the collective electron field, the electron field absorbs light and excites a surface plasmon. Therefore, for a certain angle of incidence a minimum of reflected light occurs. If the surface condition at the gold surface changes, e.g., because of an immunologic reaction, the resonance condition changes based on the following formula:

$$\Delta k_R = (\omega / c) \cdot [(\varepsilon_d - 1) / \varepsilon_d] \cdot [|\varepsilon_r| / (|\varepsilon_r| - 1)]^2 \cdot [(|\varepsilon_r| + \varepsilon_d) / (|\varepsilon_r| + 1)] \cdot (1 / |\varepsilon_r|^2) \cdot (2\,\tau\, d / \lambda) + \Delta k_{RV} \quad (1)$$

Δk_R is the change of the k-vector of the surface plasmon inducing a change of the incident angle of the light beam with matching k-vectors. ω/c is the wave vector of the light in vacuum, ε_d is the dielectric constant of the layer, ε_r is the real part of the metal dielectric constant, d is the thickness of the layer, and λ is the wavelength of the light in the layer. In practice, the laser beam is rotated and the output intensity is measured as a function of the angle.

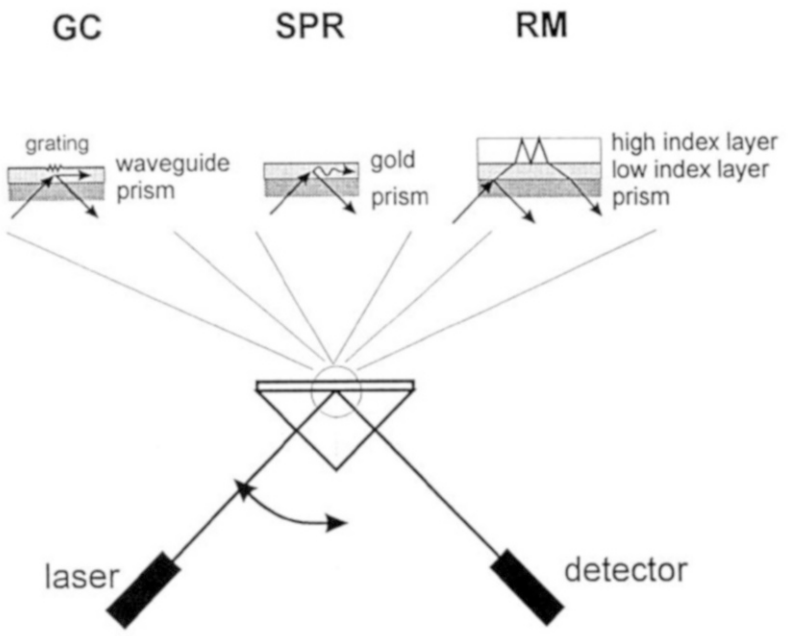

Fig. 6. The different types of optical waveguide structures: GC, grating coupler; SPR, surface plasmon resonance; RM, resonant mirror.

A further instrumental configuration utilizes a fixed incident angle and a focused beam, comprising a range of angles. The intensity distribution of the reflected light is measured by a sensor array. Furthermore, the end face of an optical fiber can be metallized. The radiation of the metal layer with white light leads to an excitation of surface plasmons. In the reflected light, absorption lines can be detected *(16)*.

3.1.2. Grating Coupler (GC)

Grating couplers use, instead of gold, waveguiding dielectricums like TiO_2 ($n = 2.4$) with a high refractive index (Fig. 6). The light beam is coupled into the wave-guiding film by means of a diffraction grating. According to the diffraction theory *(17)* a maximum of input coupling of light intensity occurs, if:

$$\sin\alpha = (N - l \cdot \lambda/\Lambda \,/\, (n_{air}) \tag{2}$$

where N is the effective refractive index of the waveguide and the surface adlayer, l is the order of diffraction, λ is the wavelength, Λ is the grating period, and n_{air} is the refractive index of air.

Commonly, diffraction gratings are molded into the dielectric waveguide. They can act as input grating couplers as well as output grating couplers or as

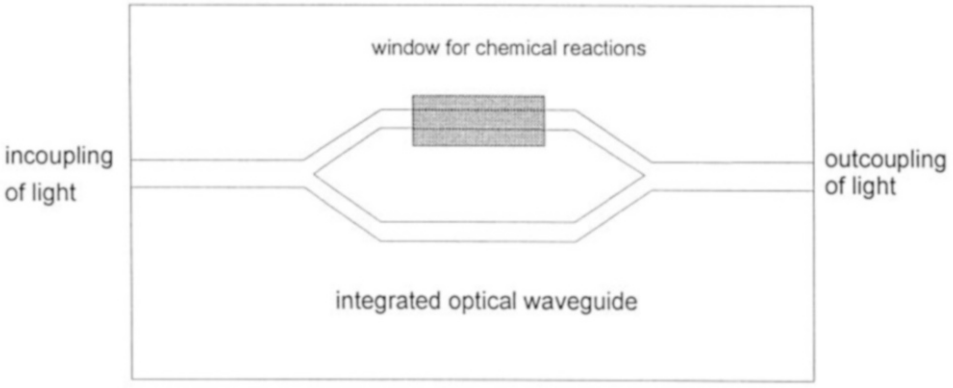

Fig. 7. Simplified drawing of a Mach-Zender Interferometer.

a combination of two gratings on one chip to couple light into and out from a waveguiding film. The formation of an adlayer onto the sensor surface changes the effective refractive index N and therefore the coupling efficiency. Either the intensity of the emitted light depending on the position of the detector or the intensity of the incoupled light as a function of the incoupling angle can be measured.

A main advantage of the grating coupler sensors is that different polarization modes (TE or TM) can be coupled into the planar wave guide. This enables us to measure thickness and refractive index of a growing adlayer simultaneously. Furthermore, the propagation length of a light beam in a dielectric waveguide is much higher (several millimeters or centimeters instead of microns), so that the distance of light–adlayer interaction is much longer *(17)*. In theory, this makes dielectric waveguide sensors more sensitive than SPR-based transducers *(17)*.

3.1.3. Mach Zender Interferometer (MZ)

By splitting an integrated waveguide into two arms (Fig. 7), a guided light wave is separated into two parts. One arm of the waveguide is covered with a biological recognition layer. A change of the refractive index within the evanescent field of the guided wave (e.g., caused by an immunologic reaction) alters the wave velocity. Both light waves, whether perturbated or unperturbated, interfere at the crossing point of the waveguide arms and a measurable change of the light intensity at the outcoupling point appears. The intensity of light is given by *(18)*:

$$(I / I_0) = [1 + \cos(\Delta n_{eff} \cdot k_0 \cdot L)] / 2 \qquad (3)$$

where I is the measured light intensity at the outcoupling point, I_0 the light intensity at the incoupling point, Δn_{eff} the effective refractive index, k_0 the wave vector, and L the length of the window for the biochemical surface reaction.

3.1.4. The Resonant Mirror (RM)

The resonant mirror technology was developed by Fisons Applied Sensor Technology and is the basis of the IAsys system *(19–21)*. It represents a combination of an incoupled guided wave (SPR or GC) and an interference measurement. The device configuration is similar to the incoupling configuration of a SPR sensor. The metallic layer is replaced by a sandwich of a dielectric layer (thickness about 1 μm) of low refractive index (silica) followed by a high index resonant layer (titanium dioxide, 100 nm). The low index layer causes total internal reflection, but at certain angles light can couple into the high index layer, propagate some distance, and couple back into the prism The resonance angle is very sensitive to refractive index changes at the surface of the high index layer (Fig. 6).

The light is reflected totally at all angles, but under resonance conditions the phase undergoes a 2τ phase shift. Because this phase shift occurs on different angles for TE- and TM-polarized modes, each can act as reference beam for the other The light intensity of the interfered beams is measured as a function of the angle *(19)*.

3.2. Acoustic Sensor Principles

Only acoustic waves with a shear motion parallel to the sensitive surface can be utilized for the design of acoustic devices. In most cases quartz is used as piezoelectric material. Wave modes with an elongation normal to the sensor surface undergo a rapid loss of energy through radiation into the surrounding liquid.

3.2.1. Acoustic Resonator Structures

The acoustic resonator, also called bulk acoustic wave device (BAW), commonly uses AT-cut quartz crystals with resonance frequencies in the range of 5–30 MHz. The shear oscillations are excited with two gold electrodes (Fig. 8). The most general theoretical approach for this kind of sensors, based on the Mason equivalent circuit, is provided by Nakamoto *(22)*. From his theory, the historic Lu formula *(23)* extended with piezoelectricity as well as the Sauerbrey *(24)*, Kanazawa *(25)*, and Martin formula *(26)* can be derived. Simultaneous mass and fluid loading can be explained by this theory. On the basis of an equivalent electrical circuit (Fig. 9) and under the assumption that viscosity, stiffness, and dielectrical constants are not important, a mathematical correlation between the measurable shift of the quartz crystal resonance frequency and the thickness of an growing adlayer can be obtained·

$$\Delta F_R = -\left[(2 \cdot F_R^2) / c_{66} \cdot \rho_q)^{\frac{1}{2}}\right] \cdot \rho_f \quad d_f - \Delta F_{Rv} \tag{4}$$

F_R is the resonant frequency of the unloaded quartz crystal, C_{66} is the quartz elastic constant, ρ_q is the quartz density, ρ_f is the density of the deposited film,

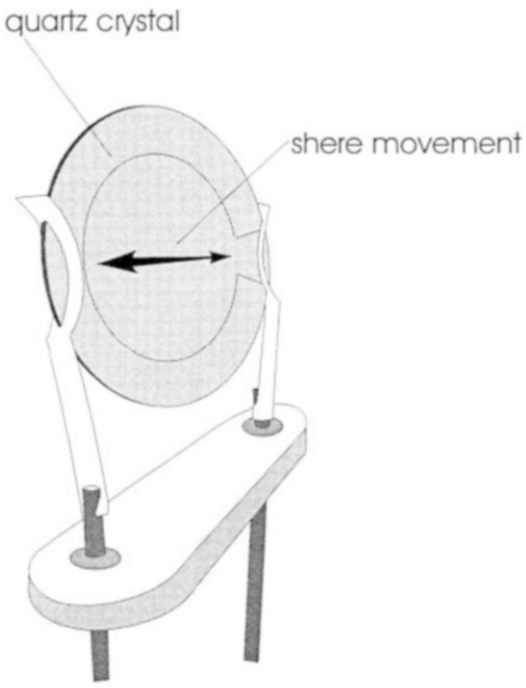

Fig. 8. Piezoelectric quartz crystal for applications in the gas phase. The inner circle indicates one of the electrodes.

and d_f is the film thickness. Δ_{FRV} is the frequency shift resulting from viscosity effects of the sample. In general, Eq. 3 is valid for the deposition of thin films like mono- or bilayers.

3.2.2. Acoustic Delay Line Structures

The characteristic feature of delay lines is that an elastic wave is excited in a piezoelectric substrate by an interdigital transducer. This elastic wave propagates through the piezoelectric substrate and is detected by a second interdigital transducer (Fig. 10). The adsorption of a thin film to the surface of the transducer influences the phase velocity and the amplitude of the propagating wave. The most important material for biosensor applications is quartz, although sometimes $LiNbO_3$ or $LiTaO_3$ are used *(27,28)*.

A variety of wave modes can be excited by choosing the appropriate cut of the piezoelectric crystal and the arrangement of the interdigital transducers. Since a good coupling between wave and surface is desired, the elastic wave should be fixed at the surface. Only Rayleigh waves (not suitable in liquids because of their vertical elongation) and Bleustein Gulyaev *(29)* waves are pure

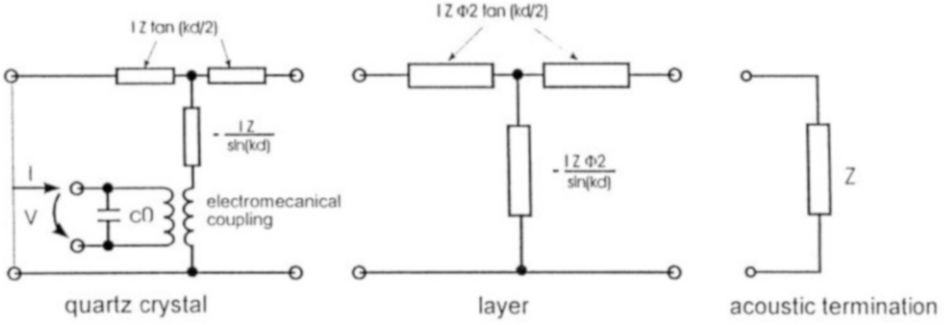

Fig. 9. Equivalent electrical circuit of a quartz crystal.

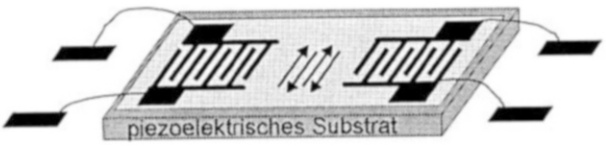

Fig. 10. Surface acoustic wave device (SAW). The acoustic shear wave and two interdigitated electrodes for the in- and outcoupling of the acoustic wave are depicted.

surface acoustic waves. Other wave modes, which are not localized to the surface but were found to be suitable for immunosensing, include flexural plate waves (Lamb Waves) *(30)*, acoustic plate waves *(27,31)*, love waves *(32,33)*, and surface transverse waves (STW) *(34,35)*.

For a mathematical description of the correlation between the phase velocity of an acoustic wave and the thickness of a surface adlayer, Auld found *(36)*:

$$(\Delta V / V) = -[(V \cdot h) / (4 \cdot P)] \{(\rho - (\mu / V^2)] \cdot |v_x|^2 + \rho \cdot |v_y|^2 + [\rho - (4 \cdot \mu) / (V^2)(\lambda + \mu) / (\lambda + 2 \cdot \mu)] \cdot |v_z|^2\} \quad (5)$$

V is the phase velocity in the substrate, h is the layer thickness, P is a normalizing constant, ρ is the specific gravity of the layer, μ and λ are elastic constants of the layer, and v_x, v_y, and v_z are particle velocities at the surface of the substrate. The formula is valid for all kinds of waves and for layers without losses.

3.3. Affinity Based Sensors—Summary

Existing analytical test systems are characterized by extreme sensitivity, short test times, and a very high degree of automation. The lower limit of detection is in the range of $10^{-14}M$, with measurement times down to a few minutes and prices per test lying in the range of a few US dollars *(37)*.

Table 1 gives an overview on the most important immunosensor principles, their sensitivities as cited in the literature and some model applications. The

Table 1
Immunosensor Principles, Investigated Applications, Found Sensitivities for Human IgG Molecules, and Literature Sources

Transducer	Applications	Lower detection limit, *M*	Refs	Remarks
Surface plasmon resonance	HIV, IgG, DNA, BSA, and more	10^{-10}	*38–41*	For further applications see the BIAcore literature and application list
Grating coupler	IgG, .	10^{-9}	*17,42*	For further applications see the ASI literature and application list.
Resonant mirror	IgG, . .	3×10^{-10}	*19–21*	For further applications see the Fisons literature and application list
Mach-Zender interferometer	IgG	10^{-10}	*18*	
Quartz crystal microbalance (BAW)	HIV, IgG,HSA, EBV, Herpes virus, bacteria, DNA	10^{-9}	*41,43–47*	
Piezoelectric delayline (SAW)	IgG, BSA, DNA, Atrazin, $C(NANP)_6Y$	3×10^{-10}	*27,31–35*	Atrazin is detected indirectly by means of an antibody-competition reaction.

concentration-sensitivity for immunosensors in mol/L, as indicated in Table 1, results from experimental investigations with the human IgG model system and is not below $10^{-10}M$. It can be stated that there is no significant difference in the sensitivity of optical and acoustic sensor systems. The chip prices range between $30 and $180 US at present *(13)*.

Most of the affinity sensor systems currently available on the market or currently under investigation can not compete with established laboratory methods because of sensitivity and cost demands. We believe that biosensor-based analytical systems would first be applied in niche markets, e.g., small laboratories, doctors' offices, or decentralized testing (intensive health care, bedside monitoring), and for parameters where the concentration is not critical. Whether immunosensors have the prospect of replacing the different types of immunoassays currently applied in the clinical laboratory will mainly depend on the sensitivity and the fabrication costs associated with this technology.

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