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Quartz crystal microbalance for immunosensing

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Abstract. An immunosensing system is described for the detection of specific antibodies in human sera using a piezoelectric immunosensor and a flow through system. A quartz crystal microbalance theory, regarding mass and viscoelastic effects in multilayer systems, which is derived from the Mason circuit, gives the theoretical background for the interpretation of measurement data obtained from an oscillator circuit. An acoustic model for the antigen/antibody reaction is described (Part I). An immunospecific detector was developed by immobilizing synthetic peptides or recombinant proteins, comprising major diagnostic epitops of HIV, on the surface of the transducer. Attempts have been made to use the sensor for the initial screening for antibodies, specific against the Human Immunodeficiency Virus (HIV), in human serum samples. Results are obtained within a few minutes and with a selectivity comparable to a licensed HIV ELISA (Part II).

Introduction

Based upon piezoelectric transducers, with a suitable receptor layer, it seems to be feasible to measure the level of a viral antigen or the correlated immune response in sera. In contrast to established diagnostic procedures, immunosensors are characterized by reduced analytical expenditure, short response times and the possibility of reaching a higher degree of automation.

The general application of immunosensor techniques to clinical diagnosis has been inhibited by the lack of immunosensor sensitivity and stability in comparison to established methods in serology.

Although it could be shown that an immunoassay using quartz crystals in the liquid phase is feasible [1–10], no reliable new product has yet been developed, which can compete with standard methods (as for instance, ELISA).

The effect of interfacial viscosity, as investigated by Thompson et al. [9–13], can influence the resonant frequency and the slip boundary condition [14] at the solid liquid interface. This interfacial structure can change if the liquid properties (e.g. ionic strength, pH value, buffer composition or serum composition) changes. The composition of human serum is the reason for many interferences (e.g. unspecific binding).

In part I of this paper, a theory is developed which is necessary to understand the measurement signals obtained from the oscillator. The theory explains viscosity and mass effects. An equivalent circuit model for an immunological coating and its effect on the oscillator frequency and gain control is presented.

The second part of this paper describes initial immunological experiments with precoated piezoelectric quartz crystals. A highly purified immunological model system was used to optimize the immobilization procedure and the flow through system. For detecting HIV specific antibodies in human sera, a recombinant gp 41 antigen of HIV was immobilized on the transducer's surface. Nonspecific binding could be eliminated by preconditioning the carrier buffer with an equivalent proportion of a nonspecific human serum.

Part I. Basic principles of QCM

QCM Theory

The ideal case of an Immuno Quartz Crystal Microbalance (IQCM) measurement in a real sample such as serum occurs when only the antigen-antibody reaction causes a signal. In reality the antigen-antibody reaction in sera appears to be a confusing mixture of viscoelastic interactions between quartz crystal, antibodies, antigens and serum matrix. Antigen-antibody reactions compete with unspecific binding reactions, with serum viscosity and ionic interactions at the antigen/antibody-serum interface. Is it possible to distinguish the recognition pro-

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cess from the other so called matrix effects? A theoretical knowledge of the response of quartz crystals to a change of physical parameters is one step towards such a distinction. In the following, the latest theories of QCM are initially discussed; a useful theory for IQCM is then postulated and the consequences on immunological measurements investigated.

Important theoretical contributions to a better understanding of QCM have previously come from Sauerbrey [15], Lu and Lewis [16], Kanazawa and Gordon [17], Mecea and Bucur [18), Duncan-Hewitt and Thomson [19], Martin [20], Nowotny and Benes [21] and Nakamoto [22]. These are theoretical approaches for special cases under restricted conditions. Sauerbrey calculated the frequency shift under the condition of small changes of surface mass density and resonant frequency in air or gas phase. Lu considered elastic properties in a more general approach. The Sauerbrey formula is a linear approximation of the Lu formula. Kanazawa and Gordon considered viscous liquid loading and in a later publication from Reed and Kanazawa [23] the theory was extended to viscoelastic properties. Mecea and Bucur use the energy transfer model to explain the interaction between thin viscoelastic films and a quartz crystal. The model can also be used for finite electrodes. Duncan-Hewitt et al. suggest a four layer theory for a liquid, which wets the surface of a quartz crystal to different extents. A three-layer model for liquid and mass loading was presented by Martin et al. who also gave relationships between equivalent circuit network parameters and viscoelastic parameters of the films. The most general approach is the transfer matrix formalism from Nowotny and Benes describing multiple layered quartz crystals of any cut angle. Nakamoto's theory is based on the Mason equivalent circuit. Simultaneous mass and fluid loading can be explained by this theory. The Lu formula, extended with piezoelectricity, and the Sauerbrey, Kanazawa and Martin formulae can be obtained with this theory.

The theoretical model presented in this publication is based on the Mason equivalent circuit. The advantage of this model is that the acoustical properties of a multilayer quartz crystal can be treated using network methods. The one dimensional treatment is the only restriction of the model. Equivalent circuit elements can also be calculated very quickly from the mechanical properties of the layers. Multilayer systems can be transformed easily into an electrical network. The electrical network and the network elements can be analysed by impedance analysis. The electrical network model is convenient to design oscillator circuits; this simplifies biosensor application.

Discussion of the Mason model

The argumentation of Dieulesaint [24] has been followed; he treated piezoelectric and non-piezoelectric plates and their description with concentrated network elements due to Masons' suggestion. The description of the QCM with the Mason model is that of Nakamoto [22]. Some details concerning the depolarizing effects can be found in the monography of T. Ikeda [25]. All mathematical details can be found in these three publications.

A multilayer system consisting of a quartz crystal, several nonpiezoelectric layers and a viscous liquid as terminator can be described as a serial connection of acoustical T-networks and an acoustical termination impedance Z_T. The termination with air can be described as a short circuit. The piezoelectric quartz crystal possesses an additional port representing the piezoelectric coupling of mechanical (acoustic) and electrical properties (Fig. 1). The resonant frequency is reached when the imaginary part of the total impedance disappears. The real part influences the damping of the oscillation. For calculations of the total impedance, a linear approximation of the transcendent impedance is often used. This linear approximation is allowed in many cases, because the product of wave vector k and the layer thickness d of the quartz crystal ($k_Q d_Q$) is approximately π. $k_L d_L \ll \pi$ is valid for a thin layer. The linear approximations lead to the Sauerbrey and Kanazawa formulae, if a single layer or a single viscous liquid is treated. However using the second approximation of the quartz crystal impedance can lead to a more accurate description of the mass sensitivity, when the quartz crystal is simultaneous loaded with mass and liquid. As the loading with viscous liquid can cause strong detuning of the quartz crystal, the linear approximation of the acoustic quartz crystal impedance is too inaccurate.

Since the acoustic impedance is a complex function of mass density, elasticity and viscosity, these three physical material parameters influence the damping and the resonant frequency. For thin nonpiezoelectric layers, two cases are important:

- linear approximation of the layer impedance: in this case, there is no influence of viscous and elastic properties. The damping is not reduced,
- approximations of higher order: viscous and elastic properties have an influence on resonant frequency and damping.

If $k_L d_L$ is smaller than 0.6, a linear approximation of $\tan(k_L d_L/2)$ (Fig. 1) would cause an error of less than

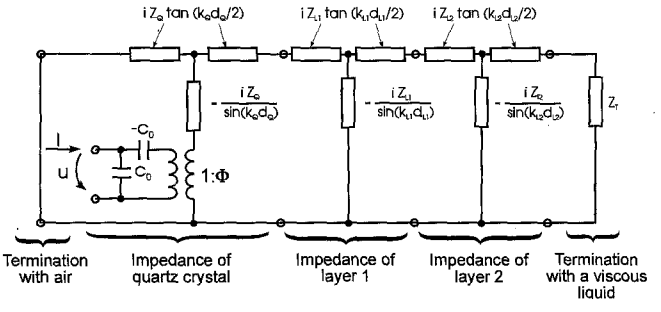

Fig. 1. The Mason Model of a piezoelectric quartz crystal with two layers of finite thickness, a termination with a viscous liquid and a termination with air. The piezoelectric effect transforms the mechanical parameters (force, particle velocity, acoustic impedance) into electrical parameters (voltage, current, electrical impedance) via a transformation factor. C_0 is the electrode capacity and $-C_0$ is caused by the depolarizing effect. Z_x is the acoustic impedance of the material. k_x is the wave vector of the acoustic wave within the layer. d_x is the thickness of the layer. Z_T is the acoustic impedance of a viscoelastic liquid. Z_x and Z_T are complex and depend on the materials viscosity and elasticity

1%. (I.e. the influence of viscous and elastic properties on the resonant frequency and damping is less than 1%).

Consequences for immunological measurements

It is not possible to describe all chemical effects during an immunological experiment in serum samples with the model described above. The question of the possibility of creating experimental conditions which allow unambigous measurement results with controlled matrix effects will now be investigated.

The specific immunological reaction forms a thin protein layer during the contact period with the serum matrix. The resonant frequency is influenced by the viscoelastic properties of the antigen – antibody layer, by the viscous bulk properties of the serum and by a confusing mixture of unspecific binding processes of serum proteins and ions, which take place at the protein layer/serum interface. These processes can be summarized as interfacial viscoelastic effects. In a first attempt, the immunological reaction can be modelled with three layers, the proteins, the interfacial effects and the serum. In reality these three layers are not strictly defined. Furthermore, the effects of serum viscosity and interfacial viscoelasticity during online monitoring of an antibody/antigen reaction can be much larger.

With a *Differential Flowinjection Analysis* (DIFIA) some of these problems can be eliminated. It is possible using flow injection analysis to obtain two reference measurements with a well defined matrix, in this case Phosphate Buffered Saline (PBS) with pH 7.4. These two reference measurements were performed before and after the serum sample was on the sensor. Assuming all matrix effects which are independent of the antigen/antibody reaction are reversible, the matrix effects can be eliminated by a differential measurement of the two reference measurements. The bulk viscosity of serum and buffer solution, as well as the interfacial viscosity, are visible as transient effects. The assumption of the reversibility of independent matrix effects has to be verified by negative tests.

The measurement results of serum experiments, illustrated in Fig. 3 and explained later in more detail, show no significant change of the damping. In Fig. 4 the sample serum produces a measurable but reversible change of damping. This indicates that the first approximation of the acoustical impedance is accurate enough to lead to the Sauerbrey mass – frequency dependency. The reversibility of independent matrix effects could be verified with negative tests (Figs. 2 and 3). Irreversible matrix effects, which could be detected with unspecific human sera, did not produce frequency shifts larger than 20 Hz. This value is the detection limit for positive sera; weak positive serum can not be distinguished from a strong unspecific reaction. There was no single serum in the test series, which showed positive result in the ELISA test and negative result in the QCM.

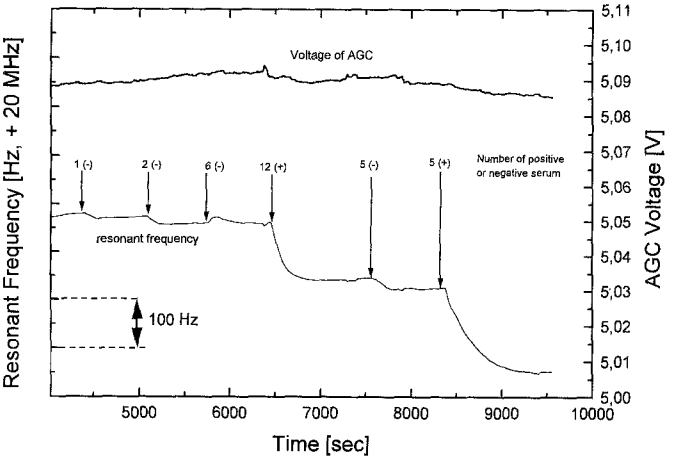

Fig. 3. Measurement of antibodies in serum with a quartz crystal immunosensor. A recombinant HIV antigen (gp41) was immobilized and several HIV positive and negative sera were injected

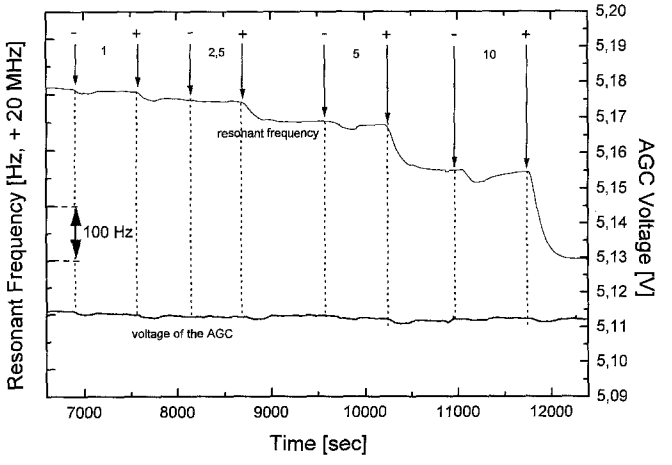

Fig. 2. Resonant frequency versus time of a QCM measurement. The quartz crystal electrode was precoated with a synthetic HIV peptide antigen derived from the core protein p24. At the indicated points of time, different dilutions of specific (+) and unspecific (–) monoclonal antibodies were injected into the carrier stream. The concentrations are given in µg/ml

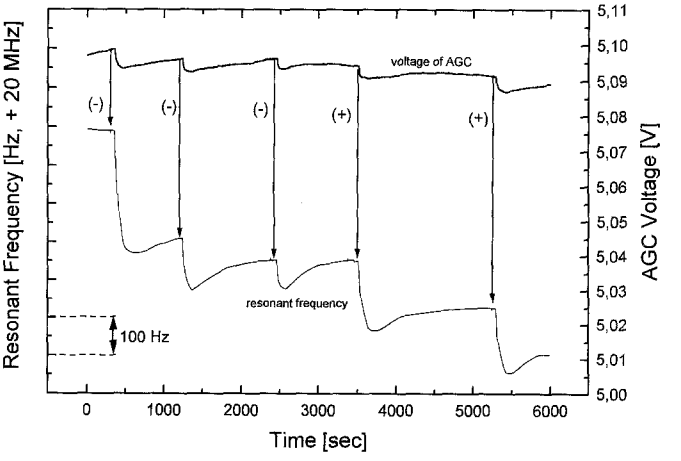

Fig. 4. Measurement of HIV antibodies with a non optimized measurement procedure. The addition of positive and negative sera is indicated by a (+) or a (–)

Part II. Application of the quartz crystal microbalance (QCM) as an initial screening test in HIV serology

Materials and methods

Detection of HIV specific antibodies

Although delayed antibody responses to HIV infection have been reported, most infected persons develop antibodies to HIV proteins within a few weeks. As virus determination is expensive and time consuming and viral antigens are not always detectable in the blood of infected persons, most of the established tests determine HIV specific antibodies. The most widely used serological test for HIV antibody screening is the Enzyme-Linked-Immunosorbent-Assay (ELISA), a discontinuous, multistep solid phase immunoassay. Sera or plasma of persons positively reactive in ELISA are retested for HIV antibodies with a supplemental test, such as the WESTERN BLOT or the indirect immunofluorescence assay.

Specificity and sensitivity of HIV antibody tests depend to a large extent on the target molecule immobilized on the solid surface. Current generation ELISA use recombinant viral proteins or synthetic peptides as antigen targets. The same recombinant viral proteins or peptides can be used as immunological receptor molecules for a direct immunosensor.

The flow through system and the quartz crystal microbalance

The quartz crystals used for the experiments described in the following are oscillating in a thickness shear mode with a fundamental frequency of 20 MHz. The diameter and the thickness of the quartz plates are 8 mm and 84 µm, respectively. In order to obtain a significant decoupling of the crystal oscillation from fringe effects, the gold electrodes have a diameter of only 2.5 mm. Shortcircuits between the quartz electrodes are avoided by exposing only one quartz electrode to the test liquid. The resonant frequency of the oscillator is measured with a frequency counter (model 6030, Kontron, FRG). The measurement data were evaluated by a PC via a GPIB interface (IEEE 488 specification). Figure 5 shows the schematic structure of the signal processing and the applied flow through system.

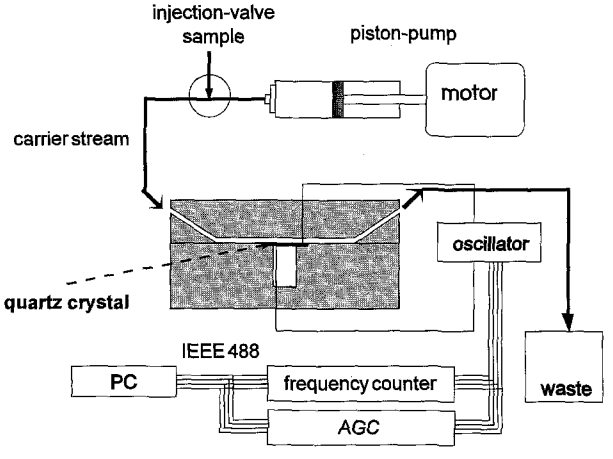

Fig. 5. Flow through system with integrated QCM immunosensor. A frequency counter measures the resonant frequency and a digital multimeter detects the voltage of an automatic gain control of the oscillator

With a characteristic flow rate of 31 µl per minute, the carrier buffer (Phosphate Buffered Saline, PBS, pH 7.4) was transported by a self developed microliter piston pump through the injection valve to the precoated acoustic immunosensor mounted in a flow-through cell. A conventional, inert HPLC injection valve with a 100 µl sample volume was used for injecting the sample solutions into the carrier stream.

The quartz crystal plates are integrated in a thin layer, flow through cell especially designed with respect to mass transport constraints of the heterogeneous antigen – antibody reactions. Carrier and sample solutions flow with a laminar streaming profile through a rectangular channel over the sensor surface.

The oscillator is a self constructed electrical circuit with a minimum total phase shift, so that the quartz crystal always works with phase zero. The resonant frequency of the oscillator follows the resonant frequency of the quartz crystal, provided that the damping is small enough to allow a zero phase of the quartz crystal. The change of the control voltage of the automatic gain control (Δ AGC) provides a signal which is proportional to the change of damping.

The coating of the piezoelectric quartz crystals

A simple and non pathogenic model system, comprising a synthetic p24 oligopeptide (aa 307–336 of a gag precursor) and a peptide specific monoclonal antibody was used in early experiments to optimize the immobilization procedcure, the immunosensor flow through system and the measurement procedure [26, 27].

For detecting HIV specific antibodies in human sera, a recombinant fragment of the transmembrane protein gp41 of HIV was used. This antigen has shown, in previous ELISA tests, a specifity of almost 100% and is commercialized in HIV screening tests by the BIOTEST AG (Dreieich, Germany) [28].

As the electrodes on the surface of the transducer consist of gold, the immobilization of a specific immunological receptor layer is simplified, because protein molecules adsorb strongly on gold surfaces due to hydrophobic and thio-gold interactions.

The coating procedure consists of cleaning and incubation steps. The gold electrodes were extensively rinsed with acetone to remove adsorbed components which may inhibit the adsorption of the receptor molecules. The cleaned surface was then incubated for at least 12 h at room temperature with 10 µl of the corresponding antigen solutions, the synthetic p24 peptide or the recombinant gp41. The antigen molecules were dissolved in phosphate buffered saline (PBS, pH = 7.4) to a final concentration of 1 mg per ml. Sites for nonspecific binding were saturated with a nonspecific protein after rinsing the precoated quartz crystals with PBS (e.g. by using bovine serum albumin (1% w/v) incubated on the transducer's surface for 1 h at room temperature). After rinsing the

crystal surface again, the sensor was ready for serological applications.

The measurement procedure

The coated QCs were mounted in the flow through system and rinsed continuously with the carrier buffer (PBS) until the resonant frequency has stabilized in the flow. The time between two consecutive measurements is determined by the dispersion of the sample peak.

In the case of the p24 peptide antigen, different dilutions of nonspecific and specific monoclonal antibodies were allowed to flow through the cell as indicated in Fig. 2.

To determine the antibody status of human sera, constant dilutions (1 : 99) of different negative and positive sera were injected in the flow through system. For an efficient suppression of nonspecific effects in sera experiments, the carrier buffer was mixed with an equivalent proportion of a negative serum.

Results and discussion

The determination of specific monoclonal antibodies

Figure 2 shows the time dependence of the resonant frequency and the voltage of the automatic gain control (AGC) of a piezoelectric quartz crystal. The immunosensor has been precoated with the p24 peptide antigen and continuously rinsed with pure PBS. At the indicated times, dilutions of monoclonal antibodies were injected into the carrier stream. A certain time later, defined by the dead volume of the flow through system, the sample reaches the surface of the transducer. A measurable shift in the resonant frequency may be registered by the frequency counter. This frequency shift is correlated with the specificity and the concentration of the added antibody.

An unspecific antibody leads to a very small and reversible frequency shift. This can be explained by nonspecific adsorption and desorption processes on the transducer's surface. In contrast, the specific monoclonal antibody leads to a much higher and irreversible sensor signal due to the specific antigen-antibody reaction on the sensor surface.

The AGC voltage shows a small reversible change. This is caused by the higher viscosity of the sample liquid.

The detection of anti-HIV antibodies in human sera

Because of the complex consistency of serum, the direct detection of viral antibodies is complicated by nonspecific adsorption. In the case of QCM, viscoelastic changes at the liquid/solid interface with the mass accumulation during the binding of the antibody. Figure 4 shows a nonoptimized measurement cycle with human sera. Very high unspecific and irreversible reactions are detectable, which cannot be distinguished precisely from the specific binding. The AGC voltage shows irreversible changes, which are ten times larger than in Fig. 2.

In conventional heterogeneous immunoassays, several washing steps, for instance in combination with a competitive analytical routine, are installed to remove unspecific serum components. In direct immunosensing procedures either the receptor layer can be manipulated, the ingredients of the carrier varied or the sample dilution buffer changed.

Figure 3 illustrates experiments with different human negative and positive sera. In accordance with monoclonal antibody experiments described above, the serum samples of non infected patients (not containing specific antibodies) cause frequency shifts smaller than 20 Hz, whereas the testing of diluted sera of infected patients results in a shift up to 450 Hz. The increase in specificity is based on the application of a diluted human negative serum (in PBS) as carrier buffer in contrast to pure PBS in the experiments with the monoclonal antibodies. The dilution of the serum ingredient is exactly the same as the dilution of the samples, which have been tested. Therefore, the transducer faces a nearly constant level in viscosity, pH, ionic strength or protein composition. Certainly there are differences in the physical and chemical properties of human sera but, as shown in Fig. 3, this is visible in the QCM experiments with the negative samples. These samples cause very small signals due to their slightly different composition.

The AGC voltage shows no significant change. The viscoelastic influences can be neglected.

As long as the specific receptor layer is not saturated, the immunosensor can be used in serology (Fig. 3), thus the immunosensor is usable several times, if the antibody titer is not too high.

Comparison of the immunosensor results with a licensed ELISA

Experimental results demonstrate the ability of piezoelectric immunosensors to measure the interaction between antibodies and antigens even in real samples. To overcome problems with nonspecific binding, the composition of the carrier buffer was changed as described previously.

In Fig. 6 the results gained with the piezoelectric immunosensor are compared with the results of a licensed

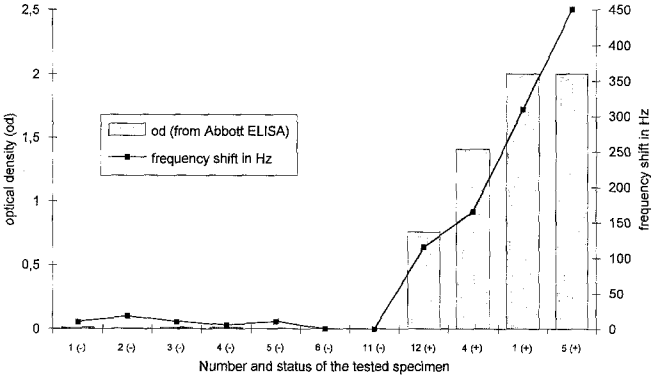

Fig. 6. Comparison of the results measured with the QCM immunosensor (frequency shifts depicted with a line) and with a licensed ELISA (Abbott, optical density depicted as bars). The tests were performed with 11 specimens (4 positive sera (+), confirmed by WESTERN BLOT, and negative (−))

ELISA (Abbott Recombinant HIV-1/HIV-2, 3rd generation). The positive sera utilized were all confirmed by a WESTERN BLOT test.

The maximum frequency response, measured with piezoelectric immunosensors after specific adsorption in human sera, is about 500 Hz. Non specific sera lead to signals of lower then 20 Hz. Therefore frequency shifts of more than 30 Hz can be correlated significantly with the presence of HIV specific antibodies. The determined signal heights in the experiments described in Figs. 3 and 6 range between 120 and 450 Hz; this is significantly above the lower detection limit of the QCM immunosensor.

11 specimens from different patients (7 negative and 4 positive samples) were tested. All samples recognized by the Abbott ELISA as positive were also recognized by the QCM as positive. However the overall time demand for testing one serum with a QCM in a flow through system is 10 min whereas the ELISA lasts for about two hours.

Experiments described in this paper were performed *without* including problem samples demonstrating a high degree of nonspecific binding or crossreactivity. However, as this piezoelectric immunosensor utilizes the same antigen target molecules as the established ELISA screening test, it seems obvious that the immunosensor can not provide a better selectivity.

The sensitivity of an immunosensor depends strongly on the design of the flow through system, in particular on the relation between convective mass transfer and surface reaction kinetics. These aspects are still under investigation.

The detection principle of a QCM is not limited to the determination of HIV specific antibodies. Provided that an antigenic target molecule is available, comprising the diagnostically important epitops, the detection principle seems to be applicable to other infectious diseases. Antigen molecules can be detected in the same manner if the transducer is precoated with a suited antibody.

However HIV is an excellent model system for developing new serological techniques and has been chosen for strategic reasons. This pathogenic agent is well characterized on the molecular level and the established screening tests are highly selective and sensitive because no false negative results or false positive results can be allowed.

Fundamental investigations, for instance concerning the immobilization of the immunological receptor molecule or the housing procedure of the piezoelectric transducer, were performed with non pathogenic agents (e.g. monoclonal antibodies or animalian antisera). Results presented here are based on initial experiments with clinical samples allowing an evaluation of the quality of the piezoelectric immunosensor.

Conclusions

Clearly the results for clinical samples are only preliminary, involving a small set of samples, much work remains to be done to reach the necessary reliability for serological analysis.

These results do, however, demonstrate the possibility of establishing a highly sensitive, rapid, simple and quantitative test for infectious diseases based on the direct measurement of molecular interactions.

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