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Piezoelectric immunosensors: analytical potentials and outlooks

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Abstract. The state-of-the-art in the design of piezoelectric immunosensors is analysed. The analytical conditions, types of immunochemical reactions detected by piezogravimetric sensors and methods for immobilisation of bioreceptor molecules on the electrode surface of piezoelectric resonators are considered. Special attention is given to the use of immunosensors for the detection of microorganisms and low- and high-molecular-mass compounds in liquid media. The bibliography includes 172 references.

I. Introduction

In the past decade, the use of immunosensors has become one of the most attractive and promising trends in bioanalytical chemistry. A distinguishing feature of this type of analytical devices is their ability to determine compounds of interest in complex mixtures with a high degree of sensitivity and selectivity and without any sample pretreatment. Immunosensors, like any other bioprobes, are built up of two major closely interconnected operational units, *viz*., a bioselective material (a receptor) and a detector (a physical transducer). The receptor comes into contact with the tested substance, while the detector transduces the signal generated in the course of the biochemical reaction into an analytical response which can further be transformed and presented in a digital form.

High selectivity and high sensitivity of immunoassays are attained through the use, as key reagents, of antibodies (Ab), *i.e*., protective proteins formed in the organisms of higher animals in response to external stimuli (antigens, Ag).[1,2] The neutralising effect of Ab is based on their ability for highly specific recognition of complementary antigens and formation of immune Ag – Ab complexes with their subsequent elimination from the organism.

Traditional immunological methods enable determination of even minute concentrations of antibodies or antigens (high-molecular-mass immunogens and low-molecular-mass haptens); however, they require complex time-consuming procedures. In contrast, immunobiosensors detect antigen – antibody interactions within a course of tens of seconds. The processes accompanying the operation of immunosensors are very similar to those occurring in living organisms. The formation of immune complexes is concomitant with changes in the voltage, current, optical (*e.g*., refractive index) and acoustic characteristics, *etc*.; therefore, biochemical interactions can be recorded with the help of a vast array of physical, *e.g*., optical, electrochemical, acoustic (including piezoelectric), *etc*., transducers.

The interaction of an analyte with a piezoelectric sensor is associated with changes in the frequency (f), amplitude and phase of the transducer oscillations.[3] Piezoelectric sensors are highly sensitive to changes in both mass and some physicochemical properties (*e.g*., viscosity, density, elasticity, conductivity, *etc*.) of the receptor layer and the fluid in contact with the sensor.[4,5] Especially well-studied are immunosensors operating on a microbalance principle. In order to enhance the stability and accuracy of measurements and to reduce the acquisition time from sensors operating at high damping fluid loads, modern devices employ high-precision sensitive digital systems for measuring frequency signals and efficient noise filtration methods. However, this trend demands special consideration and is beyond the scope of the present review.

The role of analytical signals in gravimetric sensors is played by the decrease in the oscillation frequency of the sensor (Δf) caused by the increasing mass of the receptor layer in the course of selective immunochemical coupling.

Apart from very high (at the picogram level) sensitivity of gravimetric immunosensors, the latter enable direct on-line detection of biochemical reactions without the use of labelled (fluorescent, enzymatic, radioactive, luminescent, *etc*., probes) materials employed in classical solid-phase immunoassays, *e.g*., Enzyme-Linked Immunosorbent Assay (ELISA), and related processes. The presence of non-polar organic solvents, which usually diminish the sensitivity of procedures that employ labelled materials, is not critical for the direct interaction of antibodies with antigens sensed with piezoelectric detectors.[6] Other advantages of gravimetric immunosensors include fast response, handling ease, portability and possibility to be incorporated into multisensor and information gathering and processing systems.

Piezoelectric detection compares favourably and even exceeds in sensitivity many popular optical, spectrophotometric, electrochemical, fluorescent, immunoenzymatic, *etc*., procedures for monitoring immunochemical reactions and is only slightly inferior

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to reflexometry.[7,8] In contrast to solid-phase immunoanalysis based on the use of non-recoverable reagents, immunosensors can easily be regenerated for repeated use. This capability makes their broader application in routine assays more expedient, primarily because the cost of a single assay is significatly reduced.

Some theoretical aspects of performance of piezoelectric biosensors and their specific applications for the detection of a vast array of analytes were reviewed.[6,8–19] Two processes are taken into consideration in estimating analytical responses of biosensors, *viz*., the increase in the coating weight due to formation of heterogeneous immune complexes and the transformation of this weight increase into electrical signals.

The development of various types of gravimetric sensors (*e.g*., test systems, detectors for flow-injection analysis, *etc*.) based on highly specific interactions [antibodies – antigens, enzymes – substrates, receptors – ligands (hormones, cytokines), avidin – biotin, protein A – antibodies, DNA – DNA or RNA, transport proteins – various molecules and ions] will significantly expand the area of their application. Such sensors can find use in medicine, food industry, perfume production, pharmaceutical chemistry, agriculture (veterinary medicine, horticulture, plant growing), biotechnology, mining industry, military engineering and ecological monitoring of toxicants as well as soil and waste water pollutants of bacterial origin.

Unfortunately, in Russia the theoretical aspects of the design of mass-sensitive immunosensors are still very poorly investigated, whereas in other countries this trend of research is developing rapidly.

The present review is devoted to the description of different types of immunoassay based on the use of piezoelectric transducers with special reference to conditions for their implementation and methods for immobilisation of bioreceptor molecules. Special attention is given to the efficiency and outlooks in the application of gravimetric immunosensors for the detection of microorganisms as well as high- and low-molecular-mass biologically active substances in liquid media.

II. Some functional characteristics of gravimetric piezoelectric immunosensors in gaseous and liquid media

An acoustic microbalance is a piezoelectric quartz resonator, which operates on the bulk acoustic wave principle. It represents a thin disc from a natural or synthetic crystal of quartz with metal (gold, silver, aluminium, nickel, chromium) electrodes plated on both sides of the disk by thermal spraying.[18–21] To minimise thermal effects, the disc is cut up along the shear plane of the crystal (the so-called AT-cut) manifesting the highest thermal stability in the temperature range 10 – 50 °C.[12]

Only the area between the electrodes appears to be piezoelectrically active; the mass sensitivity of a piezoelectric resonator decreases appreciably with an increase in the distance from the centre. Especially popular are crystals with a quartz plate diameter of 10 – 16 mm and gold (less often, silver) electrodes operating at intrinsic oscillation frequencies from 5 to 15 MHz. Some literature reports describe the use of resonators with improved frequency characteristics of the order of 30 (see Refs 22 and 23), 70 and even 110 MHz.[24,25] Thin crystals resonating at high frequencies are more sensitive to mass fluctuations, but suffer from drawbacks of fragility and high noise levels.

The application of an external alternating electric potential to a piezoelectric material produces internal mechanical stress between the electrodes which leads to the deformation of the crystal. The resonance is achieved by including the crystal in an oscillation circuit where electric and mechanical oscillations are near to the fundamental frequency of the crystal. The intrinsic frequency of a resonator depends on many factors some of which are constant, *e.g*., size and structure of the plate (thickness, density, roughness, type and shape of the cut, *etc*.), while some are variable, *e.g*., masses of metal electrodes and external factors (density or viscosity of gaseous and liquid media in which the measurements are performed, composition, ionic strength and pH of test solutions, *etc*.).[4,5,10,11] The oscillation frequency of an AT-cut crystal decreases linearly with an increase in the mass of the materials deposited on the electrode surface in accordance with the Sauerbrey equation:[26]

$$\Delta f = -\frac{2.3 f_0^2 10^6 \Delta m}{A},$$

where Δf is the change in the oscillation frequency of the crystal (in Hz), f_0 is the intrinsic oscillation frequency of the crystal (in MHz), Δm is the mass of the receptor layer coated onto the electrode surface (in g) and A is the surface area of the electrode (in cm^2).

However, this ratio is valid only for small mass increments. Overloading not only diminishes the sensitivity of assays, but also results in the stripping of signals; therefore, commercial piezoelectric microbalances are usually set to operate in the range from ~ 1 ng cm^{-2} to ~ 100 μg cm^{-2}.

1. Specificity of immunosensors in gaseous media

The first attempt to use antigens as electrode coatings for piezoelectric microbalances dates back to 1972;[27] however, the interest of investigators in this field was awakened some time later. A report [28] on a highly sensitive (at the ppb level) detection of the pesticide parathion in air, which was based on the use of anti-parathion antibodies immobilised on a piezoelectric crystal, was published in 1986. Traces of moisture present in the thin-film coating of a sensor favoured the occurrence of immunochemical reactions in the gaseous phase. Piezoelectric sensors based on immobilised proteins (antibodies) were successfully employed for the detection of atmospheric pollutants including sulfur dioxide, carbon monoxide, hydrogen chloride, nitrogen dioxide, ammonia, organophosphorus pesticides, *etc*.[28–32]

However, some literature reports refuted the occurrence of specific immunochemical reactions in antibody-based dry films used as biosensor coatings for detecting pesticide content in the air.[33] It is noteworthy that piezoelectric immunosensors are no longer employed for the analysis of gases due to the difficulty of analytical signal recording resulting from minute changes in the mass of the biosensor coatings during direct detection of low-molecular-mass compounds.

2. Some functional features of biosensor systems in liquid media

Early attempts to use piezoelectric biosensors for conducting measurements in liquid media were unsuccessful, since the crystals ceased to oscillate after being immersed into a solution. To overcome this problem, the mass gain was measured in the gaseous phase after incubation of the sensor with the test solution and subsequent drying. In this case, the frequency shifts were governed by the Sauerbrey equation. This procedure is commonly referred to as the dip-and-dry method.[10,27,34,35] An alternative approach includes the construction [36,37] of a different in principle excitation circuit for the analysis of fluids; this is based on the use of a flow detection cell where the test solution is exposed to only one side of the crystal.[5,21,38,39]

The resonance frequency of a crystal immersed into a solution is strongly affected by the properties of the liquid phase; in this case, the Sauerbrey equation is not fulfilled. There exists no general equation which would take into account the contribution of all environmental factors to the overall response of a piezoelectric sensor in a liquid medium. Such a signal depends on the structure of the interface between the sensor coating and the surrounding solution. Such boundary effects are levelled off when only one side of the resonator is exposed to the solution and if crystal optically polished are used.

With consideration of the above limitations, piezoelectric immunosensors are used either as analytical test systems or as detectors for flow-injection analysis.

The pioneering studies that used piezoelectric immunosensors for a comparison of activities and specificities of antibodies in liquid media were carried out using the dip-and-dry method.[27] Since the 1980s, this trend was developing more intensively culminating in the elaboration of several methods for the detection of different subclasses of antibodies[40] and an indirect/competitive procedure for identification of antigens.[41] In the 1990s, the range of detectable compounds increased considerably at the expense of biosensors for the detection of a vast array of microorganisms,[42–52] blood cells,[53–56] albumin,[38,57] atrazine,[17,58,59] human transferrin,[60] insulin,[61] cocaine[62] and immunoglobulin M (IgM).[63,64] The major problems in conducting routine assays by this method are related to the conditions of exposure of biosensors to test solutions and their subsequent drying as well as the nature of the solvent adsorbed onto the sensor coating.

The range of substances detected by piezoelectric sensors in liquid media is rather broad and includes high- and low-molecular-mass compounds and cells. An original procedure was developed[42] for the detection of microconcentrations of *Candida albicans* in the range from 10^6 to 5×10^8 cells ml^{-1}. High specificity of assay was confirmed by the absence of interactions of other yeast species or other antigen types with the sensor coating. Later, this approach was used for the detection of a vast array of pathogenic microorganisms (*Salmonella typhimurium*,[44] *Salmonella paratyphi*,[45] *Listeria monocytogenes*,[46,47] *Vibrio cholera*[48]), viruses (diarrhea,[49] herpes[50,51]) and blood cells (human granulocytes,[53] T-lymphocytes,[54] erythrocytes[55,56]).

Despite the relatively low reproducibility of experimental results due to hydration and the differences in the degree of the solvent adsorption on the receptor surface, the dip-and-dry method did not lose its significance to this day. Some recent publications describe different applications of this technique for the detection of microorganisms,[52,65,66] proteins[67–69] and toxicants.[70,71] Its main advantage is in the possibility to use immunosensors as convenient quick-operating test systems for extralaboratory diagnostics and screening of environmental objects. The reliability of determination increases, while the limit of detection decreases with an increase in the time of exposure of the biosensor to the test sample, proper washing and drying of the biolayer to the constant weight.

A two-component sensor system one component in which is a detector and the other one is a reference sensor was developed in order to minimise the non-specific interactions between the sample components and the receptor layer of the sensor.[69] In this system, immobilisation of bioreceptor molecules on the sensor surface is preceded by the blockage of the sensitive area of the reference sensor.

The use of piezoelectric immunosensors in flow-injection analyses seems to hold more promise. In this case, the time required for a single assay is reduced to several minutes and the signal is followed in real time.[72] This enables much higher reproducibility of the experimental data (measurement of relative changes in the frequency shift) and reduces the effects of non-specific interactions, since compounds adsorbed on the biosensor surface are easily removed by washing with the carrier solution. This, in its turn, allows re-usability of biosensors after regeneration of the biosensitive layer at the end of each measurement cycle.

The measurements are performed in a low-volume flow cell through which the test solution is pumped at a predefined speed. The cell volume varies from 35 to 100 μl.[5,39,73]

More recent developments include a vast array of flow-type immunosensors for the detection of high- (bacteria, viruses, phages; antibodies, protein antigens, nucleic acids) and low-molecular-mass analytes (drugs, metabolites, ecotoxicants).

III. Types of immunoassay

Immunoanalytical techniques based on piezoelectric immunosensors are classified according to the following criteria:

— nature of the receptor layer (antibodies, antigens, hapten–protein conjugates);

— type of biochemical reactions that induce the analytical signal (direct, competitive or displacement detection; mass amplified assays based on analyte modification);

— mechanism of generation of the analytical signal (increase or decrease in the mass of the bioreceptor layer);

— measurement technology (one- or two-step detection; dip-and-dry method, flow-injection analysis).

The classifications based on the type of the biochemical reaction and mechanism of generation of the analytical signal are especially popular. Among those, immunological methods based on the use of piezoelectric quartz transducers for conducting direct, indirect/competitive and displacement assays are among the most extensively studied techniques.[13,16] Sandwich assay and approaches aimed at increasing the masses of adsorbed molecules and, hence, the analytical signal of the sensor, were developed for augmenting the sensitivity of immunoassays.

1. Direct detection

This one-step procedure is the simplest among other methods and is based on the interaction of the receptor layer of the biosensor with the analyte. The decrease in the resonance frequency due to formation of an immune complex on the electrode surface is directly proportional to the concentration of the analyte (Fig. 1). This kind of analysis is performed both in dip-and-dry and flow formats. Sensor electrodes are modified by antigens, monoclonal antibodies or polyclonal antibody-containing sera.

To increase the selectivity of assays, the sensor is pretreated with a solution of a non-ionic detergent or an inactivated protein which does not cross-react with test sample components [bovine serum albumin (BSA), gelatin, *etc.*], but is bound to the non-specific sites localised on the receptor surface of the biosensor. For example, the receptor layer of a DNA-based biosensor only weakly reacts with BSA molecules to produce a feeble frequency signal. In contrast, the analytical signals generated by anti-DNA antibody-containing sera from patients with autoimmune disease amounted to hundreds of Hertz.[74]

Direct detection is the most efficient approach to identification of high-molecular-mass objects (somatic cells, microorganisms, protein macromolecules, antibodies, nucleic acids, glycoproteins, glycolipids and other antigens), since their sorption is accompanied by a significant increase in the mass of the receptor layer.

It is believed that direct binding of small molecules cannot be detected by piezoelectric immunosensors. For example, the sorption of a substance (4.4 ng cm^{-2}) onto a crystal with the intrinsic oscillation frequency of 10 MHz and the sensitivity of 0.227 Hz ng^{-1} cm^{-2} was accompanied by frequency changes that amounted to 1 Hz.[13] The antibody monolayer (capacity of 500 ng cm^{-2}) contained 7×10^{-12} moles of binding sites. The

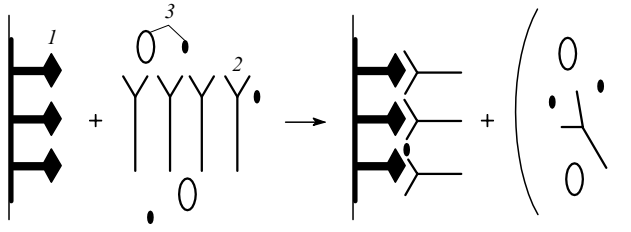


Figure 1. A schematic representation of a direct antibody assay. (*1*) Antigen (hapten), (*2*) antibody, (*3*) non-specific components of the mixture.

maximum binding of a low-molecular-mass ($\sim$1000 Da) antigen corresponds to 2.7×10^{-12} mol cm^{-2} and can induce only minor changes in the resonance frequency of the biosensor equal to several Hertz, which corresponds to the noise level. Nevertheless, the use of direct detection for identification of low-molecular-mass compounds, such as the herbicide atrazine [71] and 2,4-dichlorophenoxyacetic acid [13, 75] having the detection limits from 0.24 to 0.27 μg litre^{-1}, has been described. The discrepancies between the magnitudes of experimentally determined and calculated frequency shifts can be attributed to changes in the viscoelastic properties of boundary layers of the fluid as a result of complexation with low-molecular-mass analytes.

Indirect/competitive, sandwich and other assay types are routinely used for the detection of low-molecular-mass molecules.

2. Competitive analysis

The first single-step indirect/competitive analysis of haptens based on piezoelectric sensors was carried out [41] in 1980 and had a number of specific features. Whereas in routine immunochemical assays the competition for binding sites usually takes place between free and radiolabelled analytes, in the case of piezoelectric immunosensors the role of competing reagents is played by the free (non-bound) analyte, which is present in the solution, and the hapten – protein conjugate immobilised onto the bioreceptor layer (Fig. 2). The magnitude of the analytical signal of the sensor is inversely proportional to the hapten concentration in the test sample.

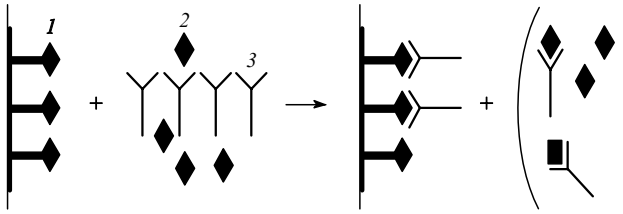


Figure 2. A schematic representation of an indirect/competitive assay. (*1*, *2*) Immobilised and free haptens, respectively; (*3*) antibody.

This approach is especially convenient for the detection of low-molecular-mass haptens, *e.g.*, vitamins, drugs, hormones, metabolites, herbicides, *etc*. Competitive assays exceed direct detection in sensitivity,[18, 58] however the range of measured concentrations is narrower by one or two orders of magnitude.

Not only hapten – protein conjugates, but also antibodies can be used for the formation of receptor layers in competitive assays. In the latter case, a fixed amount of the hapten – protein conjugate is added to each test sample; such modification has got the name 'alternative competitive analysis' (Fig. 3). The conjugated and free haptens are equiprobably bound to the active sites of the immobilised antibodies; therefore, the lower the concentration of the free analyte in the sample, the greater the amount of the conjugated hapten bound to the receptor layer of the sensor. In this way, sorption of a conjugated analyte contributes to a significant enhancement of the frequency response.

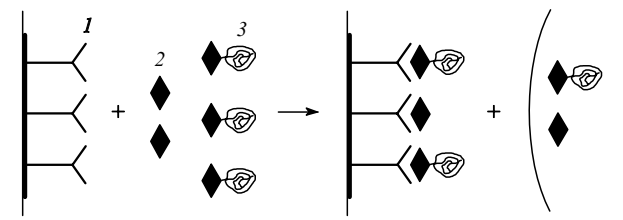


Figure 3. A schematic representation of an alternative competitive assay. (*1*) Antibody, (*2*) hapten, (*3*) hapten – protein conjugate.

This type of immunoanalysis was successfully employed [17] for the quantitation of triazine derivatives. The magnitude of the frequency shift induced by the binding of the triazine – protein conjugate to the receptor layer was inversely proportional to the triazine concentration in the range from 0.01 to 1 μg litre^{-1}. Despite its obviously lower sensitivity, the alternative method offers a broader concentration range in comparison with the competitive approach.

3. Two-stage sandwich assay

This assay is based on the use of secondary or species-specific antibodies which interact with the antibodies of the immune complex as with antigens specifically raised against them.[40, 76] Thus secondary antibodies enhance the analytical signal by forming sandwich complexes with test antibodies (Fig. 4), which significantly increases the selectivity and sensitivity of the assay. The sandwich technology was successfully employed [77] for determination of cholera toxin concentration in solutions (1×10^{-4} nmol litre^{-1}).

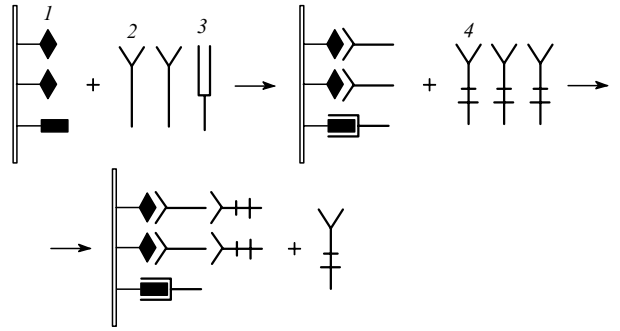


Figure 4. A schematic representation of a sandwich assay. (*1*) Antigen, (*2*) specific antibody, (*3*) non-specific antibody, (*4*) secondary antibody.

Sandwich assays are performed in two steps. In the first step, the antibodies (both specific and non-specific) are bound to an immobilised antigen. This is followed by an addition of secondary antibodies which react only with their specific antibodies to form sandwich-like complexes. The detection of allergen-specific immunoglobulin (IgE) in blood sera of patients with allergic dermatites is a classical example of clinical application of this technique.[68] The allergenic protein (Der p) isolated from dermatophagoids was immobilised on the electrode surface of a biosensor. The interaction of the sensor with an antigenic blood serum from an allergic patient resulted in the binding of different antibody classes to the receptor layer; this was followed by differentiation of adsorbed antibodies upon exposure of the sensor to secondary anti-human IgE. The ability of secondary antibodies for specific recognition enables the differentiation between bound allergen-specific antibodies and representatives of other classes of blood serum immunoglobulins (*e.g.*, IgG, IgM).

A method [78] for determination of human serum albumin (HSA) based on the complexation of HSA with two types of antibodies is yet another illustrative example of a successful application of sandwich analysis. Noteworthy, the analytical signal generated by the sensor exceeded that obtained by the direct detection method threefold.

If direct detection of antigens is performed by the competitive method, the sandwich assay is complete in one step. This approach was used to determine the testosterone content (13.6 μg ml^{-1}) in a biological fluid.[13, 76]

4. Mass amplified assay

In addition to sandwich assays, the sensitivity of low-molecular-mass assays can be enhanced through the use of other approaches.

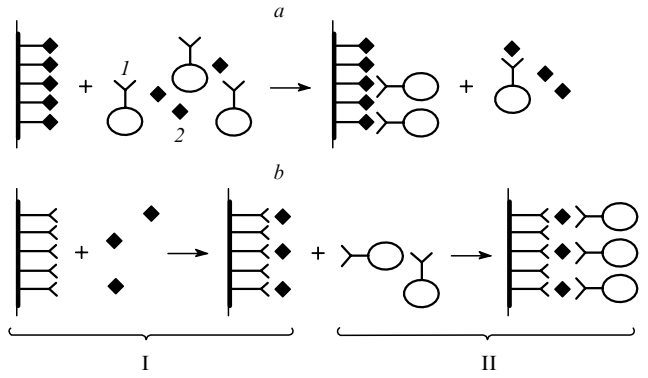


Figure 5. A schematic representation of a modified antibody assay. (*a*) Competitive assay, (*b*) sandwich assay, (*1*) modified antibody, (*2*) analyte.

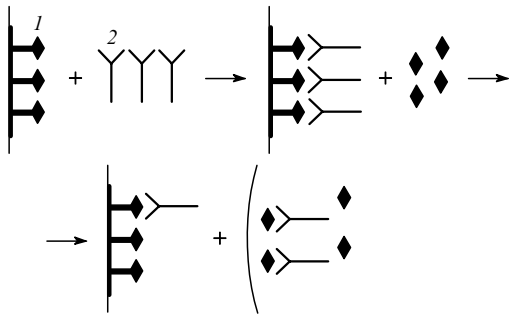


Figure 6. A schematic representation of a displacement immunoassay. (*1*) Antigen, (*2*) specific antibody.

One of them includes modification of sorbates by colloidal,[79,80] latex, *etc*., particles.[81–83] Such methods are singled out into an independent group termed as 'mass amplified assay' and are based on the increment in the analyte mass.[13] A schematic representation of such analyses is shown in Fig. 5.[82,83] Modification of antibodies by colloidal particles caused significant increases in the sizes and masses of the adsorbed molecules. The assays were performed in the competitive (Fig. 5 *a*) and sandwich (Fig. 5 *b*) formats.

In the former case, the antibody molecules modified by solid colloidal particles could bind either to free analyte molecules (in solution) or to analyte molecules immobilised on the surface of the sensor electrode. Thus, the binding of the antibodies to the receptor layer was inhibited by high concentration of the analyte. The magnitude of the analytical response was inversely proportional to the concentration of the test antigen.

In the latter case, the reaction was complete in two steps (I and II); the protein molecules modified by colloidal particles interacted, similarly to secondary antibodies, with the analyte molecules bound to the receptor layer. In both cases, the frequency signal of the sensor was strongly enhanced by the colloidal particles deposited on the sensor surface. Gold, lead, cadmium and zinc sulfide sols as well as chromium, titanium and iron oxide sols are generally used as colloidal particles.

Latex agglutination is a convenient procedure for ehnancing analytical signals. This approach was used[38] for the detection of *Escherichia coliform* species in solution within the concentration range 10^6-10^8 cells ml^{-1}. Latex particles able to modify *E. coli* specific antibodies were added to the reaction mixture.[84] After conjugation of the cells with antibodies immobilised on the sensor electrode, the anti-*E. coli* antibodies immobilised onto latex particles formed sandwich complexes with the cells bound to the antibodies on the electrode surface.

5. Displacement analysis

This format is especially suitable for determination of stabilities of affinity complexes (Ab–Ag, DNA–protein, DNA–RNA, *etc*.) in kinetic dissociation studies and for measurement of analyte concentrations. Whereas competitive analysis is a method of choice in determining minor concentrations of small molecules, macromolecules are more conveniently detected by displacement analysis. The method is based on the measurement of decrement in the mass of heterogeneous complex in the course of its dissociation induced by excess concentration of one of the reaction components. The analysis is performed in two steps (Fig. 6).

Determination[47] of *Listeria monocytogenes* in milk ($2.5\times10^5-2.5\times10^7$ cells ml^{-1}) is an example of this type of an immunoassay. The antibodies present in the sample were conjugated to a protein antigen isolated from *Listeria* sp., which was previously immobilised on the sensor electrode. This caused a decrease in the oscillation frequency of the sensor. Then, a displacement of the antibodies from the heteroimmune complex by *Listeria* cells added to the sample occurs. The amount of the displaced antibodies was directly proportional to the concentration of *Listeria* cells in the sample. A similar approach was used to determine the content of *Pseudomonas aeruginosa* in milk and drinking water.[85]

IV. Methods for immobilisation of biomolecules

The sensitivity, selectivity and reproducibility of the assays of high- and low-molecular-mass compounds based on piezoelectric immunosensors strongly depend on the method used for the immobilisation of receptor molecules on the electrode surface.

The main requirements to the quality of bioreceptor layers are as follows:[86]

— firm fixation of receptor molecules on the electrode surface;

— retention of high activity of immobilised biomolecules;

— uniform distribution of active binding sites on the electrode surface;

— physical and chemical stability (including regeneration stability) of the electrode layer in contact with the fluid during the measurement cycle;

— minimum mass of the receptor layer (the smaller the mass of the coating, the greater the mass increment of the analyte that can be established with the help of a microbalance).

The nature of the metal electrode should also be taken into consideration in deciding upon the immobilisation technique. Owing to their high chemical stability, piezoelectric resonators with gold electrodes are the most frequently used type; therefore, virtually all immobilisation techniques have been designed to operate with gold electrodes. However, in the past decade crystals with silver electrodes were gaining increasing attention by virtue of their low cost and higher sorptive capacity.[87,88] It was found[68] that immunosensors with silver electrodes modified by lipoic acid with *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride as a coupling reagent manifested 1.5 times higher sensitivity than sensors with gold electrodes. However, silver electrodes suffer from the disadvantage of being highly reactive and demands additional protection. This can be achieved, for example, through the use of polyethyleneimine supports able to form uniform layers and affording complete coverage of the electrode surface.

All currently known methods used for immobilisation of protein molecules may conventionally be divided into three groups:[13,16] physical sorption, incorporation into matrices and chemical sorption.

Each of these techniques has advantages and drawbacks of their own. Very often, different methods are used in combination, but the choice of a procedure best suited for meeting a specific goal is always arbitrary. The degree of immobilisation of biomolecules

and the stability of the receptor layer should always be checked experimentally.

1. Physical sorption

Physical sorption is the simplest and fastest method for immobilisation of protein molecules on the surface of crystal electrodes under mild conditions. This is ensured by non-specific interactions or hydrogen bonds,[89] which enable the preservation of high functional activity of protein molecules [70, 73, 90] and the formation of low-density layers.[91] The number of active antibody sites is usually kept constant, although in some cases [92–94] their functional activities can decrease as a result of conformational changes in the protein molecules. The amount of the adsorbed protein and, as a consequence, the sensitivity of the sensor are determined by the nature of the metal and the hydrophobicity of the protein, which, in its turn, depends on pH, ionic strength, temperature [95] and time of exposure of the sensor in the solution with antibodies. It was found [96] that after 4-day incubation the receptor layer could withstand up to 20 measuring cycles without any decrease in the analytical response.

Physical sorption is often used, *e.g.*, for the preparation of receptor coatings based on goat anti-ricin antibodies,[73] *Vibrio cholera* [48] and herpes virus antibodies,[49, 50] recombinant fragments of specific antibodies against human immunodeficiency virus (HIV), [97, 98] *etc*. The quantity of immobilised antibodies and cells can be estimated with a sufficient degree of accuracy from the difference between the frequency shifts in modified and pure piezoelectric quartz crystals.

The main disadvantages of such immobilisation include low sorptive capacity and low stability of coated surfaces, which diminishes the reproducibility of immunoassays and reduces the service lives of immunosensors. As the method in question does not provide well-developed surfaces required for covalent immobilisation, the sensitivities of biolayers are rather low.[91]

The use of physical sorption is not confined to the direct formation of bioreceptor coatings of sensor electrodes; it also favours the formation of intermediate protein layers which provide strong binding to both the metal surface of the electrode and the immunoreagent molecules. Protein A,[61, 86, 87, 90, 98–100] which forms stable complexes with gold in neutral media (its binding constant is equal to 10^8 litre mol^{-1}) (see Ref. 101), is often used for the formation of intermediate layers. At the same time, protein A, which represents a polypeptide constituent of *Staphylococcus aureus*, can actively and specifically bind to immunoglobulins (to IgG, in particular) without affecting the active sites of antibodies which retain their high activities in the immobilised state.[90, 102] Such immobilisation ensures not only high sensitivity and high binding capacity of the bioreceptor layer, but also its high elasticity, which is especially important for stable performance of piezoelectric immunosensors.[103] Immunosensors designed for the detection of insulin [104] and some viral and bacterial species [105] the bioreceptor coating in which was formed by protein A molecules demonstrated high sensitivity and stable performance even after a period of several months.

Further improvements of this technique included structural modification of proteins aimed at increasing the strength of binding of protein molecules to the gold surface. To achieve this, amino acid residues (*e.g.*, cysteine) containing SH groups were incorporated into the structure of the recombinant protein A (B5C1) by genetic engineering methods.[102] The resulting intermediate layer based on the synthetic protein manifested higher binding capacity in comparison with the native protein A, which made it possible to increase the service life of the immunosensor without any loss in activity.

2. Incorporation into matrices

Incorporation of proteins into gels [106–108] or polymeric matrices,[51, 53, 54, 109, 110] which enables immobilisation at the expense of physical interactions, is rather a popular approach.

To obtain a polymeric matrix, a drop of a polymer solution in an organic solvent is placed onto the surface of a cleaned electrode and let dry until complete evaporation of the solvent. Immobilisation of antibodies is achieved by immersion of the sensor into an antibody-containing solution; then, antibodies are included into the polymeric layer. Such immobilisation was used [35] to determine the content of HSA. In earlier studies, biosensors based on polymeric matrices were actively employed for determination of blood cells, *e.g.*, human T-lymphocytes,[54] erythrocytes [55, 56] and granulocytes;[53] however, the analytical signals generated by them were unstable in repeated measurements. The strength of binding of biomolecules can be increased through additional use of glutaraldehyde. To obtain a receptor layer, a solution of polyethyleneamine (PEA) [70, 111] or polyethyleneimine (PEI) [70] in methanol is added dropwise to the surface of a crystal electrode and, after removal of the solvent, is incubated sequentially with glutaraldehyde and appropriate biomolecules, *e.g.*, antibodies or hapten–protein conjugates. In this case, the selective layer of the sensor retains its activity up to a period of 38 days.

Polymeric matrices prepared by electropolymerisation [45] or by the glow discharge method [21] (which excludes the use of toxic solvents) are more efficient, but are still seldom used. Protein molecules in such polymeric films are linked by covalent bonds.[21, 45, 112, 113]

Natural biopolymers (*e.g.*, proteins, polysaccharides) are also used for immobilisation of receptor molecules. A method for the formation, on the electrode surface, of hydrogels based on proteins (BSA) and antibodies has been described.[70] The biosensitive layer thus prepared was distinguished for high sensitivity and stability.

In recent years, polymeric films were especially often used as electrode modificators (base layer) increasing the adhesion of biomolecules, since hydrophobisation and functionalisation of receptor surfaces enhance the stability of the bioreceptor layer.

3. Chemical sorption

This method is used for the preparation of dense and strong saturated layers that manifest high stabilities in liquid media.[114] It is especially suitable for flow or flow-injection analyses. Covalent coupling provides firm fixation of biolayers on the electrode surface, which minimises the losses upon long-term exposure to fluid media, provides higher reproducibility of analytical signals and improves re-usability of immobilised solid phases after regeneration. Besides, chemical sorption allows preservation of functional activities of proteins over long periods of time (up to two years). However, in some cases the number of active sites in covalently coupled antibodies decreases under toxic effects of chemical reagents.[115] Chemisorption usually employs gold (less often, silver) electrodes, and never aluminium or chromium electrodes.

Cross-linking, which is singled out into a separate group by some authors,[108, 109, 116] represents a modified version of chemical sorption. It makes use of a vast array of mono- and heterobifunctional cross-linking reagents, such as hydrazide- and succinimide-containing derivatives. Glutaraldehyde, which reacts smoothly with amino groups to form additional methylene bridges in the protein molecule, is used more often than other reagents.[68–71, 77, 111, 117–120]

Cross-linking is performed using different procedures. After short-term incubation of antibodies with a cross-linking reagent at room temperature, a drop of the reaction solution is added to the surface of a crystal electrode and air-dried.[118] In the alternative approach, a drop of an antibody solution placed onto the electrode is exposed in a closed cell saturated with glutaraldehyde vapour at low temperature.[70] The fundamental difference between these two techniques is in different effects of the cross-linking reagent in homogeneous and heterogeneous media. Whereas after mixing of experimental solutions methylene bridges are formed throughout the whole bulk of the fluid, glutaraldehyde reacts exclusively with protein molecules on the surface of the liquid

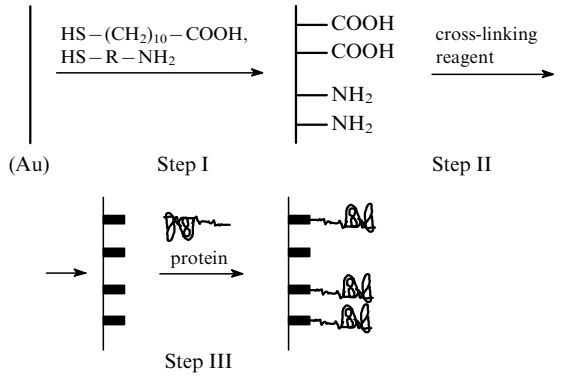


Figure 7. A scheme of immobilisation of biomolecules by chemical sorption.
Immobilisation steps: I, activation of the metal surface; II, modification of functional groups; III, immobilisation of the protein molecule.

phase. This enables preservation of the native conformation of the active sites of the antibodies inside the layer and increases the sensitivity of the receptor coating of the sensor.

Virtually all chemical immobilisation procedures are complete in three steps (Fig. 7).

The surface of sensor electrodes is activated by application of a thin film (base layer) which is firmly fixed on the metal surface and contains reactive functional groups essential for subsequent immobilisation of protein molecules. In addition, the film coating protects the surface of metal electrodes from oxidation and premature destruction by corrosive media.

The formation of base layers from self-assembled monolayers (SAM) is of particular interest. Its advantages include homogeneity of base layers, their uniform distribution and spatial accessibility of their active sites and functional groups for the binding to receptor molecules as well as high mechanical strength and chemical stability.[119, 120] An ideal monolayer consists of densely packed filaments firmly bound to smooth (*e.g.*, metal) surfaces.[121, 122]

Treatment with siloxane derivatives, *e.g.*, (γ-aminopropyl)-triethoxysilane (APTS) and (γ-glycidoxypropyl)trimethoxysilane (GOPS) containing reactive amino and hydroxy groups, respectively, is widely used [36, 71, 123–127] for the formation of SAM-based base layers. The surfaces thus modified bind biomolecules either directly (as in the case of GOPS) or after treatment with glutaraldehyde. The coupling of antibodies to siloxane films *via* amino and hydrocarbon groups of bifunctional cross-linking reagents [116] favours the formation of dense (74–220 ng cm^{-2}) surface layers. High (from 78% to 92%) binding capacity of the latter in reactions with antigens is responsible for the wide applicability of this immobilisation technique.

The use of sulfur-containing compounds (*e.g.*, thiols and disulfides) that are adsorbed irreversibly on gold surfaces by coordination bonds between sulfur and gold atoms is yet another popular approach to the preparation of SAM. Alkanethiol-based films are among the most extensively studied types of SAM. As the orientation and stabilisation of monolayers is provided by van der Waals interactions between the methylene groups, the use of alkanethiol chains containing more than ten methylene groups permits one to obtain highly ordered monolayers of the crystal type.[121]

A similar approach was used to prepare SAM based on poly-L-lysine,[128] hydrophobic lipids,[129, 130] cysteamine,[131] cysteimine [72] and *p*-aminophenol,[45] which provide amino groups for modified layers; thiopropionic and lipoic acids;[68, 72, 121] mercaptoundecanol [132] containing reactive carboxy and hydroxy groups and dithiobis(succinimidylpropionate) [72, 132] the NHS groups in which are directly involved in the binding of biomolecules.

High elasticity, which imparts conformational mobility to receptor molecules during their interactions with test objects and ensures successful transmission of signals to piezoelectric transducers, is a crucial requirement to piezoelectric sensor coatings. Therefore, in the second chemisorption step the activated surface of the sensor electrode is modified with a cross-linking reagent, such as glutaraldehyde, cyanogen bromide, thiol acids, hydrazine, 1,4-diepoxypentane, *etc.*[115] To prepare more elastic biolayers with enhanced ligand-binding properties, high-molecular-mass biopolymers, *e.g.*, proteins A and G,[70] BSA[70] or polysaccharides: dextran [132] or periodate-oxidised dextran,[72] are used as cross-linking reagents. The formation of a receptor layer where covalently linked dextran is used as a spacer is schematically represented in Fig. 8.

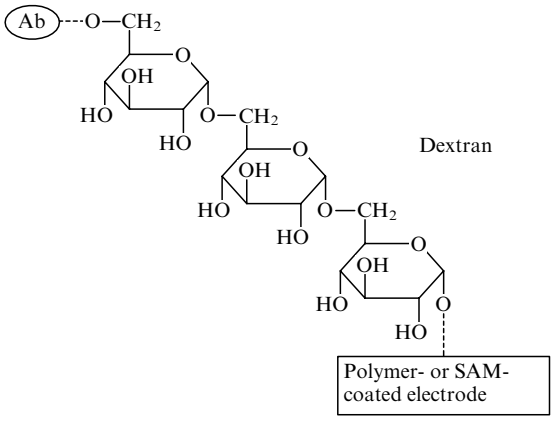


Figure 8. A scheme of formation of a receptor layer where dextran is used as an intermediate layer between the electrode coating and the protein molecule.

Carbohydrate chains covalently attached to the protective coating on the sensor confer conformational 'flexibility' on the biomolecules that are bound to them.[109, 115] Dextrans activated by periodate oxidation were used [72, 133] to prepare three-dimensional dense base layers with enhanced ligand-binding properties. However, this method has a number of disadvantages: it is time- and labour-consuming and requires the use of toxic reagents.

Biomolecules (protein A, lectins, avidin–biotin complex) used for modification of electrode surfaces present additional interest as flexible spacers. Therefore, the assignment of immobilisation methods to different groups is always arbitrary and combinations of various methods are used more and more often for the preparation of bioreceptor layers. The formation of the avidin–biotin complex is described by an affinity constant equal to 10^{15} litre mol^{-1}, which points to its stronger ($\sim 10^3$ -fold) binding in comparison with immunochemical fixation. Moreover, biotin is endowed with the ability to bind easily and firmly to antibodies without any appreciable loss of activity. Therefore, bioreceptor layers based on the avidin–biotin complex, which retains stability over a broad range of pH upon long-term exposure to liquids, are widely employed in immunochemical studies.[57, 132, 134]

The third step includes direct covalent binding of protein molecules to reactive groups of spacers.

To salient advantages of chemical sorption of biomolecules one can relate the great diversity of immobilisation techniques and the possibility to use them in combination, which, in its turn, favours the development of strategic approaches to the preparation of highly selective layers with predefined characteristics and the design of biorecognising receptors for the detection of analytes.

The disadvantages of the above-considered immobilisation methods (cross-linking, covalent binding, incorporation into

polymeric matrices, immobilisation on biomolecules) include relatively high masses of receptor layers and, as a consequence, the need for step-by-step control over mass increases in order to prevent the losses in the assay sensitivity and to provide stable performance of piezosensors. In some cases, continuous monitoring of receptor layers may be required, since no versatile procedure exists allowing the attainment of high immobilisation levels with simultaneous preservation of activities of immobilised antibodies. Therefore, immobilisation conditions should be optimised individually for each specific measurement.

V. Conditions for regeneration of bioselecting coatings

Re-usability of biolayers after each measurement cycle is a valuable feature of immunosensors, especially those designed for flow-injection screening of large panels of test samples where the same crystal has to be used many times.

It is known that formation of heterogeneous immune complexes is a reversible process; therefore, regeneration is confined to their dissociation under the influence of various factors that cause a shift in the reaction equilibrium towards the initial compounds. The increase in the ionic strength is one of such factors. Considering that immune complexes are stabilised by hydrogen bonds and van der Waals interactions, any increase in the ionic strength brings about conformational changes in the active sites of antibody molecules with simultaneous desorption of the analyte from the bioreceptor layer and restoration of the initial number of binding sites in the immobilised biomolecules. Acid or alkaline solutions [0.2 M glycine/HCl buffer (pH 2.5),[58, 67, 68, 134] 0.2 M glycine,[6] 0.001 M HC1,[13, 67, 68] 0.1 M HC1,[6] 0.5 M $H_2Cr_2O_7$,[13] 1 M HCOOH,[71] 0.001–0.1 M NaOH (see Ref. 6)] as well as 30%–50% methanol[6] and denaturing reagents [6–8 M $(NH_2)_2CO$ (see Ref. 44) and guanidine[6]] are generally used for regeneration; their action suppresses the interactions of the active sites with the analyte. It is found that dilute solutions sometimes fail to induce complete dissociation of immune complexes, whereas more concentrated reagents can diminish the sensitivity of biosensors as a result of damage of the layer consisting of immobilised ligands. From this standpoint, restoration of their initial sensitivities and preservation of mass after each measurement are the main requirements to regeneration of biolayers. The regenerating effect of 0.15–3 M KSCN, which depends on the nature of the immunoreagents and enables up to 20-fold utilisation of the bioreceptor layer without any detriment to its activity, has been described.[126, 127]

Selective biochemical interactions, which yield more stable complexes than those formed upon immunochemical coupling, can be used, along with chemical reagents, for decomposition of immune complexes. Thus synthetic peptides able to react with adsorbed antigens (*e.g.*, viruses) and to be easily eliminated from the receptor surface can be used for regeneration of biolayers.[51] In this case, the biosensors retained their activities over 18 measurement cycles. A sorbed protein analyte can be eliminated on treatment with a proteinase. For example, a solution of non-specific proteinase K induced hydrolytic elimination of antibody molecules in a neutral medium without any effect on the immobilised ligands of the biolayer.[6]

When long-term performance causes partial inactivation and destruction of the sensor biolayer as a result of which the reproducibility of analytical signals diminishes, the metal surfaces of sensor electrodes should be cleaned and washed properly with highly concentrated solutions of alkali and acids, *e.g.*, 0.5–1.2 M NaOH (pH 11–13); 0.5–1.2 M HCl (pH 1–2), or concentrated hydrochloric acid. The choice of conditions for complete regeneration of the sensor surface depends on the nature of the metal electrode and the method used for the formation of the base layer. For example, if antibodies are immobilised on a gold electrode using protein A, the protein layer is completely eliminated by sequential washing of the sensor with 1.2 M NaOH, 1.2 M HC1 and concentrated HC1.[58] Siloxane films are easily destroyed by treatment with concentrated HCl. A solution of $H_2Cr_2O_7$, which allows complete purification of gold electrodes of piezoelectric resonators from thiol within a course of 15 min, can be used as an efficient regenerating reagent. The elimination of (γ-aminopropyl)triethoxysilane-containing base layers requires long-term incubation of the sensor in a hot solution of $H_2Cr_2O_7$.[13] (γ-Aminopropyl)triethoxysilane- and tetraethoxysilane layers are easily eliminated from the surfaces of silver electrodes with 0.1 M HC1 (pH 2), which acts directly on the oxide film of the metal.[13, 125–127, 135] After washing and drying, piezoelectric resonators can be repeatedly used for the formation of biosensitive layers of another specificity.

Thus, the choice of conditions for regeneration of bioreceptor layers and complete purification of metal surfaces of resonator electrodes depends on the nature of the metal and the method used for immobilisation of biomolecules; in each case, the conditions are selected experimentally. The possibility of performing two-level regeneration of resonators is a salient advantage of piezoelectric immunosensors, since it enables their repeated application and versatile performance, which eventually reduces the cost of the analysis.

VI. Applications of piezoelectric immunosensors

The areas of application of piezoelectric immunosensors for the detection of high- and low-molecular-mass analytes in liquid media are extremely diverse and include biochemical, clinical and analytical studies; certification of foodstuffs and pharmaceutical substances; monitoring of environmental objects and so on (Table 1).

The majority of modern piezoelectric immunosensors are designed for the detection of 'large-sized' analytes, such as protein molecules, lipid–protein complexes, nucleic acids and viruses as well as for identification of somatic and bacterial cells in biological fluids, drinking water and foodstuffs. Determination of concentrations of such objects with the help of piezoelectric immunosensors proved to be more convenient in comparison with traditional microbiological or immunological methods. Piezoimmunosensors are used in the clinical diagnostic practice for identification of blood constituents (erythrocytes, T-lymphocytes, granulocytes) and the most common pathogenic bacteria.[5, 6, 11, 13, 16, 17] Design of sensors for detecting pathogenic viruses (*e.g.*, herpes,[50, 51, 139] hepatitis,[136–138] human immunodeficiency,[97, 98] *etc.*) is yet another promising trend. A good correlation with the results obtained by ELISA was established upon screening of blood sera for the human immunodeficiency virus.[67, 68, 97] Piezoelectric immunosensors are used in veterinary medicine for identification of infectious pathogens in animals[65, 96] and poultry[52] and monitoring the quality of agricultural products.[13, 96]

An important role in the variability of microorganisms residing in human body and environmental objects is played by bacteriophages. Therefore, phages are presently used for the solution of many practical tasks including deciphering of the human genetic code, prophylaxis and treatment of infectious diseases and detection of pathogenic microorganisms in environmental objects. The most recent trends are towards the development of highly sensitive piezoimmunosensors based on resonators with intrinsic oscillation frequencies from 39 to 110 MHz; they are designed for the direct detection of phages M13 in liquids[25] and screening of phage libraries.[140, 141]

New, in principle, applications for piezoelectric immunosensors are described in the review.[142] A highly selective immunosensor was proposed based on immobilised monoclonal antibodies for discrimination of serogroups A, B and D of *Salmonella*. Such sensors manifest extremely high specificities in the recognition of antigens differing structurally at the level of immunodominant monosaccharides, *viz.*, optically isomeric 3,6-dideoxyhexoses (paratose, abequose, tyvelose). These findings open up fresh opportunities for the use of gravimetric immunosensors for

Table 1. Use of piezoelectric immunosensors in clinical diagnostics and ecological monitoring.

Analyte	Type of assay, method for immobilisation of biomolecules	Detection limit	Ref.
Microorganisms			
Hepatitis A and B viruses	direct assay, Ab were immobilised by protein A	10^5 particles ml^{-1}	136, 137
Hepatitis C virus	hepatitis C virus RNA was immobilised on cysteamine SAM through avidin (streptavidin)	–	138
Herpes virus	direct assay, Ab were immobilised by protein A	5×10^4 particles ml^{-1}	50, 51, 139
Potato X-virus	direct assay, chemisorption of Ab on a siloxane substrate	$4 \times 10^{-8} - 6 \times 10^{-3}$ mg ml^{-1}	125
Phage library	displacement analysis, biotinylated antigens were immobilised by streptavidin	$(1-5) \times 10^{11}$ particles ml^{-1}	140
Bacteriophages M13	direct assay, antibodies were immobilised by a bifunctional cross-linking reagent	10^9 particles ml^{-1}	25
Bacteriophages	–	5×10^{12} particles ml^{-1}	141
Salmonella sp. from serogroups A, B and D	direct assay, antibodies were immobilised by a bifunctional cross-linking reagent	$10^5 - 10^8$ cells ml^{-1}	142
Proteins			
Immunoglobulin G (IgG)	direct assay, Ab were bound to protein A	$10^{-6} - 10^{-2}$ mg ml^{-1}	143
Human IgG	direct assay, chemisorption of the protein complex IgG – Ab on a polystyrene substrate	5 μg ml^{-1}	144
HSA	Ab were immobilised on an ethylene diamine substrate, obtained by the glow discharge method	16 – 128 μg ml^{-1}	21
	direct assay, Ab were immobilised on polystyrene	5 – 100 mg ml^{-1}	35
Immunoglobulin M	direct assay, chemisorption of Ab on poly(2-hydroxyethyl methacrylate)	10 ng ml^{-1}	145
Antibodies to human immunodeficiency virus	direct assay, immobilisation of a synthetic HIV-peptide or recombinant human proteins	tests yes/no	97, 98
Staphylococcal enterotoxin B (SEB)	competitive method, Ab were adsorbed on gold	0.1 μg ml^{-1}	146
Cholera toxin	sandwich method enhanced by ganglioside-modified liposomes	10^{-4} nmol $litre^{-1}$	147
C-Reactive protein	latex agglutination	20 – 66.7 μg $litre^{-1}$	81
Ricin	direct assay, Ab were immobilised by physical sorption	0.5 μg $litre^{-1}$	73
Complement factors C1, C6	direct assay, chemisorption of Ab on a polyethyleneimine substrate	0.83 nmol $litre^{-1}$	148, 149
Ferritin	direct assay, Ab were immobilised on a cysteamine monolayer by cross-linking	0.1 – 100 ng ml^{-1}	131
α-Fetoprotein	direct assay, Ab were immobilised on a cysteimine monolayer by cross-linking	1.5 nmol $litre^{-1}$	150
Surface antigen of hepatitis B	the same	4.7 nmol $litre^{-1}$	151
Immunoglobulin E	direct assay, physical sorption of the protein antigen	0 – 300 IU ml^{-1}, 0.15 – 17.5 IU ml^{-1}	67, 68
	direct assay, affinity attachment to an aptamer based on single-stranded DNA	0.05 nmol ml^{-1}	152
Anti-DNA antibodies	direct assay, native and denatured DNA were immobilised by chemisorption	0.1 – 25 μg ml^{-1}, 0.03 – 8 IU ml^{-1}	74
Insulin	sandwich format, adsorption of Ab	0.17 nmol $litre^{-1}$	104
Transferrin	agglutination enhanced by poly(ethylene glycol)	2.1 nmol $litre^{-1}$	153
Immunoliposomes	competitive assay, immobilisation of hapten	$10^{-8} - 10^{-5}$ mol $litre^{-1}$	154
Low-molecular-mass compounds			
Ocadaic acid	competitive assay, Ab were covalently immobilised on polyethyleneamine substrate	1.9 μg ml^{-1}	70
	Ab were immobilised into a BSA-based hydrogel	50 ng ml^{-1}	70
2,4-Dichlorophenoxyacetic acid	competitive assay, Ab were covalently immobilised on an aminobenzenethiol substrate	0.24 – 0.27 μg $litre^{-1}$	155
Cortisol	direct assay, Ab were bond to protein A	36 μg $litre^{-1}$ (100 μmol $litre^{-1}$)	117
3-Indolylacetic acid	competitive assay	3 nmol $litre^{-1}$	156
Atrazine	the same	0.025 ng ml^{-1} (5×10^{-4} μmol $litre^{-1}$)	58, 59
	direct assay	0.01 ng ml^{-1}	71
Cocaine	direct assay, Ab were bond to protein G	33 μmol $litre^{-1}$	62
	competitive assay, the hapten was attached to the monolayer of mercaptoundecanoic acid	10^{-4} μmol $litre^{-1}$	157
β-Estradiol	immobilisation of an estrogen component capturing the receptor – estradiol complex	8 nmol $litre^{-1}$	158

Table 1 (continued).

Analyte	Type of assay, method for immobilisation of biomolecules	Detection limit	Ref.
Atropine	–	0.05 nmol litre^{-1}	159
Polynuclear aromatic hydrocarbons	competitive assay, Ab were covalently immobilised on a siloxane substrate	0.01 μmol litre^{-1}	160, 161
Nonylphenol	the same	0.8 ng ml^{-1}	162
Cotinine	"	0.1 μmol litre^{-1}	74
Salicylic acid	"	2.9 μmol litre^{-1}	126
Sulfamethoxazole	"	6×10^{-4} μmol litre^{-1}	127

validation of structural data for carbohydrate-containing biopolymers and design of diagnostic systems.

Piezoimmunosensors are used for identification of protein molecules, *e.g.*, of antibodies in blood serum (IgG, IgM, IgE). In order to promote efficient treatment of allergic and autoimmune diseases, patients' blood sera are screened for immunoglobulins (IgE) [67, 68, 152] and anti-DNA antibodies. Receptor layers containing native or denatured DNA were used [74] in direct flow-injection assays of anti-DNA antibodies in blood sera of patients with systemic lupus erythematosus.

Piezoimmunosensors were used as valuable tools for the detection of a vast variety of clinically important analytes, such as α-fetoprotein [150] and ferritin,[131] the tumour-associated protein markers. Unfortunately, the recently developed sensors cannot be used for the analysis of human blood samples because the range of quantifiable ferritin concentrations is very narrow. Further experiments should be aimed at expanding the quantifiable concentration range, since normal blood levels of this protein in healthy females and males are usually equal to 90 and 200 ng ml^{-1}, respectively.

DNA-Based piezoimmunosensors are used for hybridisation of nucleic acids.[163–165] A recently developed gravimetric immunosensor [166, 167] proved to be the instrument of choice in the estimation of genetically modified organisms.

Not only protein molecules and DNA, but also carbohydrate-lipid biopolymers [lipopolysaccharides (LPS)], are used as receptor layers. Biologically active LPS, which represent the major outer membrane constitutents of Gram-negative bacteria, are responsible for the O-antigenic specificity and toxicity of bacteria. A method for the study of interactions between immobilised bacterial endotoxins (LPS) and synthetic LPS-binding peptides has been developed. [135] The data obtained point to a high potential of gravimetric immunosensors based on immobilised LPS in estimating the efficiency of antitoxic agents (antidotes).

The widening assortment of competitive immunoanalytical techniques based on piezoelectric sensors gave a new impetus to the appearance of publications devoted to the analysis of low-molecular-mass substances (drugs, hormones, alkaloids, pesticides and their metabolites, ecotoxicants, vitamins, *etc.*).[168, 169]

Residual concentrations of pesticides, which belong to one of the most widespread classes of low-molecular-mass toxicants, can also be controlled with the help of piezoimmunosensors.[6]

Considering that veterinary antibiotics are used as widely as pesticides and that accumulation of drug components and their toxic metabolites in agricultural products is a potential hazard to human health, the use of piezoimmunosensors for the monitoring over the content of toxic substances in foodstuffs (milk, meat, eggs) and environmental objects (waste waters; rivers, brooks, subterranean waters) is a currently central task. Immunosensors for detecting antimicrobial sulfamide drugs in aqueous solutions and milk have been developed.[127, 170] In addition, piezosensors can be used in drug tests for adulterants.

A gravimetric immunosensor for measuring the content of cotinine (a nicotine metabolite and a specific marker of the functional status of smokers in sociological tests) in urine samples has been designed.[74] Screening of analytes revealed that cotinine content in the urine of active smokers varied from 4 to 6 mg litre^{-1}, while that in non-smokers was as low as 0.5–0.7 mg litre^{-1}. The sensitivity of the new piezosensor was comparable to those attained in polarisation immunofluorescent studies.

The operating characteristics of piezoelectric immunosensors for detecting hydrophobic ecotoxicants (*e.g.*, polyaromatic hydrocarbons [160, 161]) were improved in the presence of organic solvents.[6, 155]

The broad application of nonylphenol ethoxylates as detergents and emulsifying agents in the commercial production of cleansers, dyes, some herbicides and tanning agents led to the pollution of the environment with their degradation products. Especially hazardous and toxic is nonylphenol possessing carcinogenic, estrogenic, *etc.*, activities. A flow-injection method for measuring the nonylphenol content in water aimed at monitoring industrial effluents and water reservoirs has been developed.[162]

The possibility of ecological monitoring of toxicants (*e.g.*, ocadaic acid) produced by some algae species has been demonstrated.[70]

The ever increasing number of publications illustrates the strong inclination of investigators to expand the area of application of piezoelectric immunosensors. It is of note that flow-type immunosensors are efficient tools not only for the detection of analytes of different molecular masses, but also for monitoring the changes in the viscoelastic properties of boundary layers in immune reactions carried out in solutions [75] and for following biochemical reactions in real time. This unique capability of piezosensors enables kinetic studies of affinity reactions, such as antigen–antibody and protein–nucleic acid interactions, hybridisation of complementary DNA chains, receptor–ligand interactions, *etc.* Recent investigations in this field are documented in the reviews.[6, 15, 126] Besides, piezoimmunosensors hold much promise as potent tools for quantitation of cross-linking reactions between structurally related antigens and antibodies,[170] hybridisation of DNA [163–165] and epitope mapping.[171]

Combination of piezoelectric microbalancing with impedance measurements is a useful approach to the study of viscoelastic properties of films and fluids exposed on the surface of piezoelectric crystals. In this case, the alternating voltage having a frequency close to the intrinsic frequency of the resonator is applied to the piezoelectric crystal and the auxiliary electrode. The electrode potential and the current passing through the crystal are measured simultaneously. Their ratio (impedance) permits real-time monitoring of degradation processes in thin polymeric films in the presence of biomolecules; conformational changes; phase transitions, swelling and other immunochemical reactions.[22, 172]

Moreover, the possibility to combine piezoelectric microbalancing with other (*e.g.*, optical and electrochemical) methods including surface plasmon resonance and atomic force microscopy will further extend our knowledge of the actual events related to biochemical interactions at the cell level.

VII. Conclusion

The analysis of publications of the past decade demonstrates that the novel scientific trend related to the development and practical applications of piezoelectric immunosensors in bioanalytical chemistry has aroused an ever increasing interest of investigators all over the world.

The combination of a low cost with enhanced sensitivity, selectivity and re-usability makes piezoelectric immunosensors a valuable alternative to existing optical, electrochemical, *etc*., devices for conducting routine tests. As regards long-range outlooks, piezoelectric immunosensors can occupy a more prominent place as analytical tools for flow-injection detection of analytes of different molecular masses and genetically modified products, monitoring of immunochemical interactions and control over the growth of bacterial populations and changes in viscoelastic properties of liquid media. The development of diagnostic kits that are based on piezoimmunosensors and are specifically designed for early diagnosis of infectious, autoimmune and oncological diseases is still a topical task in modern clinical and veterinary medicine. Especially promising is the use of piezoimmunosensors for detecting drugs (doping control) and screening of urine, saliva and other biological fluids for alkaloids, hormones and other abused drug components.

In Russia, investigations aimed at the development of mass-sensitive immunosensors are still in their infancy. It remains to be hoped that the data presented herein will shed additional light on the broad prospects for future applications of piezoimmunosensors and will demonstrate their major advantages including real-time monitoring of molecular interactions without the use of any labels and complicated sample pretreatment procedures.

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