**IUPAC Technical Report**

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# Immunodiagnostics and immunosensor design

**Abstract:** This work compiles information on the principles of diagnostic immunochemical methods and the recent advances in this field. It presents an overview of modern techniques for the production of diagnostic antibodies, their modification with the aim of improving their diagnostic potency, the different types of immunochemical detection systems, and the increasing diagnostic applications for human health that include specific disease markers, individualized diagnosis of cancer subtypes, therapeutic and addictive drugs, food residues, and environmental contaminants. A special focus lies in novel developments of immunosensor techniques, promising approaches to miniaturized detection units and the associated microfluidic systems. The trends towards high-throughput systems, multiplexed analysis, and miniaturization of the diagnostic tools are discussed. It is also made evident that progress in the last few years has largely relied on novel chemical approaches.

**Keywords:** analytes; antibodies; antigens; diagnosis; enzyme-linked immunoabsorbent assay (ELISA); fluorescence-activated cell sorting (FACS); health; immunochemistry; immunosensors; IUPAC Chemistry and Human Health Division; multiplexes.

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# 1 Introduction

The first immunoassays were described in the late 1950s [1]. They were performed with radiolabels in an aqueous medium and called radioimmunoassays (RIAs). Shortly thereafter, modified techniques followed, such as the

enzyme-linked immunoassay (EIA) and the enzyme-linked immunosorbent assay (ELISA) (*cf*, [2]), immunohistochemical detection of molecules in tissues, and fluorescence-activated cell sorting (FACS), to name a few.

Today, immunochemical methods represent a core diagnostic technique that will be available for increasing numbers of applications [2–6]. Research in immunodiagnostics relies heavily upon analytical chemistry and nanotechnology [7]. More than 500 000 antibodies are presently available on the market, with information easily accessible through various online sources such as www.antibodypedia.com and www.antibodies-online.com. The development of diagnostically useful antibodies is progressing rapidly, based on both the modification of existing methods and also entirely new approaches such as yeast display.

Two other forces are driving instrumental innovation. On the one hand, increasingly sophisticated high-throughput diagnostic instruments that must be handled by trained experts are being developed for clinico-chemical laboratories. On the other hand, there is just as strong effort to develop small devices that are easy to use at point-of-care [8].

The progress of biosensor and biorecognition research in the past 10 years has been accompanied by the development of novel immunosensor techniques [9] that use improved methods to attach antibody or antigen molecules to sensor surfaces of intelligent microfluidic systems with integrated detectors. This development has facilitated miniaturizing diagnostic tests, allowing parallel measurements of different biomarkers in a single run, and speeding up the diagnostic procedures, all of which in turn enabled high-throughput analysis at reduced cost. As a consequence, an increasing number of novel and improved diagnostic tools are available in clinical laboratories and expand into many other health-related areas such as environmental pollution monitoring and residue analysis.

# 2 Advances in generating diagnostic antibodies

## 2.1 The immune system and antibodies

The immune system is a highly complex network of cells, enzymes, mediators, and immunoglobulins that become activated when foreign substances and microorganisms enter the body. The resulting effect is to neutralize the internalized entity and thus protect the organism [10, 11]. For the immune defence of the functioning organism, white blood cells and supporting enzyme systems (e.g., the complement system) are as crucial as immunoglobulins (i.e., antibodies). In immunoassays, antibodies are the basic recognition elements responsible for the capture of the desired analyte.

In the living vertebrate, internalized foreign macromolecules act as antigens and induce in the adaptive immune system selective production of immunoglobulins that are suitable to bind the antigen [10, 12]. The Y-shaped structures of the immunoglobulins are composed of the heavy and light chains. The ends of the arms carry two identical variable regions (Fv) that are responsible for antigen recognition, whereas the rod-like region containing constant sequences (Fc) is important for physiological functions of antibodies. Immunoglobulin G (IgG) is the most common, robust, and best-studied type of antibody but there are other immunoglobulins such as IgA, IgE, IgM, and IgD.

The variable region of each heavy and light chain has an antigen recognition site, consisting of three complementarity-determining regions (CDRs). These are variable peptide sequences, each with a highly specific spatial structure and chemical surface pattern. The six CDRs of each arm of an immunoglobulin molecule interact noncovalently with the epitope of the antigen.

## 2.2 Immunogenicity of large and small molecules

Immunization is the process whereby either a single or repeated exposure of an organism to a foreign biomolecule induces in the organism a response that neutralizes the intruding agent. Similarly, immunogenicity is

the ability of a (usually foreign) substance that is introduced into an organism to induce an immune response, which includes the production of antibodies. Many types of large molecules, notably proteins and glycoproteins, have generally good immunogenicity in animals. In order to increase the production of antibodies, the substances are usually injected together with an adjuvant that is a mixture of biological and non-biological substances that enhance the attraction of cells of the immune system to the injection site and thereby enhance the immune reaction. After one or several injections, those B lymphocytes of the immune system that are genetically able to produce antibodies against the administered antigen will start to proliferate and become plasma cells. These plasma cells then release soluble antibodies into the blood. The affinity of an antibody to its antigen often improves in the course of a prolonged immune response. This is called affinity maturation.

In contrast to large foreign molecules, injection of foreign molecules of small size (<1500 Dalton), such as pharmaceutical drugs, does not generally lead to antibody formation [13]. However, such small molecules (haptens) may gain immunogenicity when they are covalently attached to a macromolecule [4, 14–16]. When an animal is immunized with such a protein-hapten construct, a certain fraction of the resulting antibodies will be directed against epitopes of the macromolecule and another variable fraction against bound hapten. Altogether, the raising and isolation of antibodies against small molecules is often very time-consuming and has not always been successful.

## 2.3 Techniques to produce diagnostic antibodies

Antibodies used for diagnostic purposes should exhibit several key features such as high-affinity and specificity for their antigen, good binding behaviour when coupled to surfaces or to amplifying systems, and stability. Antibodies with such features must be raised and tested before they can be used reliably in an assay.

Instead of using the whole antibody molecule, it is possible to employ antibody constructs for diagnostic purposes. A precondition is that they possess the complementary binding region. A number of different types of fragments are routinely used today (Fig. 1). The newer approaches to antibody design often focus on smaller fragments, such as the antigen-binding fragment Fab or its dimer $F(ab')_2$. Small fragments with preserved variable regions often exhibit well-preserved antigen-binding behaviour. They may have advantages over whole antibody molecules, specifically in situations where diffusion in a matrix is important (e.g., in immunohistochemistry), or where a small mass is favourable to improve detection signals.

Thousands of different antibody molecules with a wide range of affinities to different antigens are present in the serum of healthy humans and mammals at rather constant total concentrations. But, when a specific

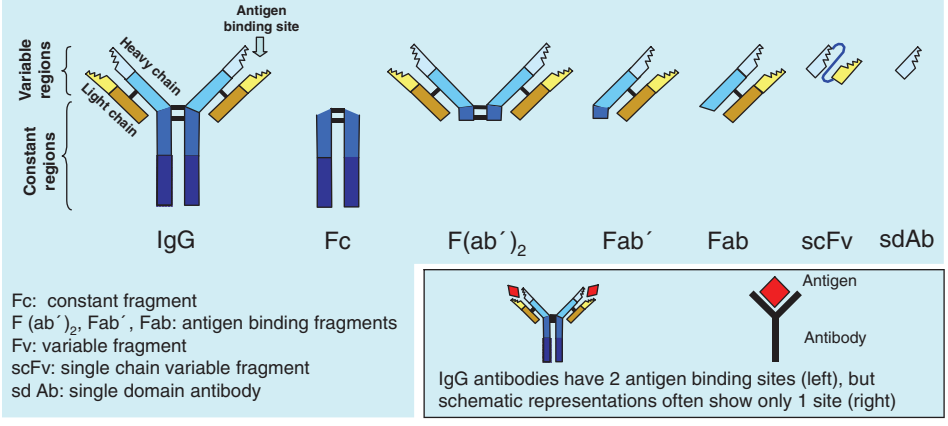


**Fig. 1 Immunoglobulin G and some of its fragments.** Immunoglobulin G is composed of two heavy and two light chains. The variable regions carry the antigen-binding site, which recognizes the epitope of the antigen. The figure shows some of the fragments that are used in the development of immunodiagnostic tests. The insert shows that though each IgG antibody can bind two antigen molecules, systematic drawings often depict only one binding.

antigen exposure occurs, the resulting immune response selects high-affinity antibodies from immunoglobulin structural libraries, thus enhancing the selective proliferation of the lymphocytes, which are genetically able to produce such antibodies (or T-cell receptors).

Different methods are available to produce selected antibodies for scientific and technical purposes (Fig. 2). The classical method of raising antibodies is the repeated injection of an antigen-preparation in animals. The immune system of the animal will respond by producing the different antibody isotypes (IgG, IgM, IgA, IgE, IgD) at various levels, but IgM and IgG are the most important for use in immunoassays. Normally, production of a number of antibody molecules with small differences in their peptide sequences in the variable region, able to react with one or more epitopes of the antigen, will be induced. Thus, raw immunoglobulin fractions isolated from immunized animals contain a mixture of related antigen-specific antibodies (**polyclonal**). They belong to different isotypes with partly overlapping binding behaviour toward the antigen. For immunochemical diagnosis, these polyclonal antibody mixtures can either be used as such, or they can be subfractionated to achieve better homogeneity of the purified antibody. An immunized animal can remain a source for antibody production during its lifetime. The raising of polyclonal antibodies is often time-consuming and, if not always in one animal species, may be successful in a different species.

**Monoclonal** antibodies differ from polyclonal antibodies in that each copy has an identical peptide sequence, and thus an identical antigen-binding site. Thus, each copy has the same affinity to the same epitope of the antigen. Techniques to raise monoclonal antibodies were first developed in the 1970s [17]. In the early form of monoclonal antibody production, antibody-producing B cells retrieved from the spleen of an immunized animal were fused with an immortal tumor cell line [18]. The resulting immortalized hybridoma cells were selectively cultured and allowed to proliferate and produce antibodies. All antibody molecules derived from a single parent cell are identical. Those hybridoma cells producing the most suitable antibody molecule were separated from other cells and allowed to proliferate on a larger scale where they produced the desired amount of monoclonal antibody. In its early development, this technique was labour-intensive, and although it has now been optimized, it has been largely displaced by methods that allow a higher-throughput production of antibodies.

Today, antibody-fragments can be produced on a large scale in a process called phage display [19, 20]. The process begins when mRNA molecules coding for antibodies or their variable fragments are isolated from B lymphocytes. The corresponding complementary DNA strands (cDNA) are generated and amplified in vitro. The cDNA is then integrated into the gene of a bacterial virus (phage) in a way that allows the expression of antibody fragments on the viral surface. This technique can be used to produce millions of different variable region fragments (each derived from a different B cell). Those phages producing scFv and Fab fragments that fit best to the antigen will be amplified. Yeast display [21] is a related but even more powerful technique, where antibody fragments are produced by yeast cells on a larger scale. However, one has to be aware that the microbial synthesis of immunoglobulins will lack posttranslational species-specific glycosylation, which may affect binding affinity.

Recently, interest has focused on antibodies formed by camelids. Camels, llamas, and related species naturally form a special type of heavy-chain antibody devoid of light chains, which is quite stable, easy to handle, and exhibits only low immunogenicity [22]. Camelid antibodies are considered a promising starting

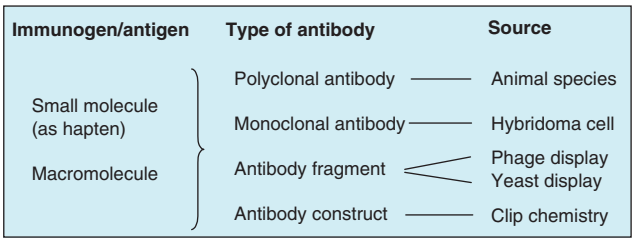


**Fig. 2 Types of antibodies.** Different methods for raising and constructing antibodies are available today. Depending on the sources, the products have different technical and chemical properties.

material for diagnostic and therapeutic antibodies. There are also efforts to use antibodies from chickens [23, 24] or to raise antibodies using plant viruses [25].

## 2.4 Raising antibodies against families of small molecules

Many natural and manmade substances are members of chemical families that share a common backbone but differ with regard to side chains or functional groups. Examples are steroid hormones and many pharmaceuticals and pesticides [14, 26]. For immunodetection of such chemical families, it would be favourable to have one test that measures the sum of all congeners (desirably in a single assay) and a second test that measures individual congeners. This requires availability of suitable antibodies. Antibodies against the common chemical group can be produced either by immunization of an animal with just the chemical backbone where the variable side groups have been split off or blocked, or by immunization of animals with a mixture of all congeners, in the hope that the affinities of the formed heterogenic antibodies will be adequate to measure the sum of all congeners. In contrast, to produce congener-specific antibodies, animals are immunized either with each of the purified congeners separately, or with modified congeners where the chemical-family-specific backbone has been blocked. In each case, the resultant antibodies must be tested for their specificity and cross-reactivity. Analytical results can be further optimized on the level of the assay system, for example, by capturing the new antibody either with the hapten used for immunization or with a modified hapten.

## 2.5 Advances in the chemical modification of diagnostic antibodies

Immunochemical assays today are characterized by multiple interactions between antibodies, antigens, and detection systems. A number of classical chemical methods exist that allow connection of a protein with other compounds. These include the use of carbodiimide, bromocyan, or hydrazide. In addition, immobilisation protocols using biomolecules to which immunoglobulins bind with high affinity have been developed. The best examples are protein A (from *Staphylococcus aureus*) and protein G (from *Streptococcus* sp.), both of bacterial origin, which are known for their ability to bind to the Fc region of IgG. ZZ protein is a synthetic Fc-region-binding domain, derived from protein A. Affinities differ, depending on the immunoglobulin isotype and animal species. These proteins are used for many purposes, such as detecting, cleaning, or attaching immunoglobulins.

In immunoassays, surfaces of assay devices may be pretreated with protein A or protein G so that an added antibody will be immobilized via its Fc region, with the distinct advantage of a freely accessible antigen-binding site. Another protein with similar use is protein L (from *Peptostreptococcus magnus*), which binds to a domain of the antibody light-chain variable region.

Another standard procedure makes use of the high affinity of the streptavidin-biotin complex (SABC). Biotin readily binds to several Fc locations of antibody molecules without affecting the antigen-binding behaviour. Avidin, streptavidin, and NeutrAvidin proteins have the ability to bind up to four biotin molecules. These avidin molecules can be readily conjugated with detection enzymes or fluorochromes. When a biotin–antibody conjugate comes in contact with streptavidin, a very strong noncovalent bond between biotin and streptavidin is formed. This chemical bridge allows attachment of an antibody to a surface or a detection device. It was also reported that NeutrAvidin had the highest degree of nonspecific adsorption to the surface, presumably due to its low carbohydrate content and near-neutral isoelectric point [27].

Epitope tagging is a comparatively new method. It uses the insertion of an artificial peptide sequence into a (primary) antibody molecule to create a selective recognition site for a secondary antibody that is directed against the primary antibody. Usually, a small peptide with a defined amino acid sequence is inserted into the antibody molecule. This sequence (e.g., six consecutive His residues) serves as an epitope for a tag-specific secondary antibody. This technique has dramatically expanded the possibilities for constructing amplifying systems on demand. Although genetic engineering is the most common method for introducing epitope tags into a protein, chemical methods are also in use.

Another approach to introduce chemical reactivity into an antibody molecule is the attachment of reactive groups to polysaccharide residues of the Fc fraction. When the movement of the Fc region is restricted by its surroundings, this may affect the stoichiometry and strength of the antibody-antigen binding [28]. In one study, a hapten linker increased sensitivity 100-fold [29].

# 3 Advances in immunoseparation

Many of the basic immunoseparation techniques were developed between about 50 and 100 years ago. These methods have since been continuously improved and diversified for specific applications. Some of the methods are basic components of immunodiagnostic tests and will therefore be described here.

## 3.1 Immunoprecipitation and particle aggregation

Each IgG antibody molecule has two identical binding sites for its antigen. At the same time, most antigen molecules have more than one epitope to which different polyclonal antibodies can bind. This is why mixing an antibody with its antigen leads to extensive crosslinking and visible precipitation of immune complexes if adequate concentration ratios prevail. In the immunoaggregation test the detecting antibody molecules are bound to the surfaces of suspended particles (e.g., latex). Addition of antigen then results in particle aggregation and visible turbidity (Fig. 3) that can be quantified, for example, with light-scattering methods. This conventional method still has applications today [30–32], notably in the form of chromatin immunoprecipitation (ChIP) [33].

## 3.2 Immunodiffusion

In the Ouchterlony immunodiffusion test, antigens are allowed to diffuse in a matrix such as agar-agar from an application point towards the diffusing antibody [34]. At the distance where antibodies and antigens concentration reach equilibrium, the precipitating antigen–antibody complex forms. The precipitation line often has the form of a circular arc and can be evaluated with the naked eye. This and related simple tests can be used to assess whether immunization of an animal has led to the desired formation of specific antibodies.

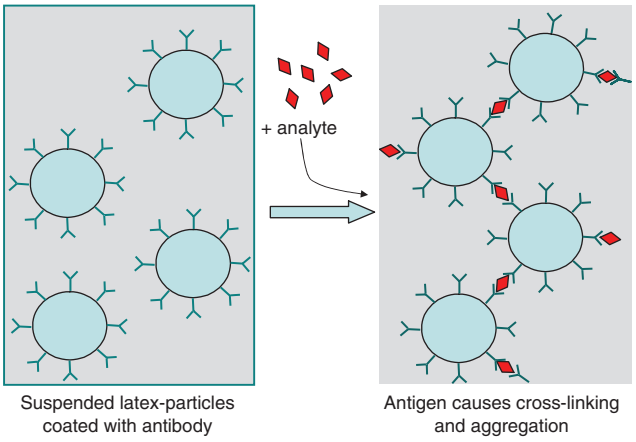


**Fig. 3 Latex agglutination assay.** The latex agglutination assay is a classical immunochemical test. Addition of the antigen leads to crosslinking of the particles and visible turbidity. The relative concentrations of the reaction partners affect the result.

### 3.3 Immunochromatography

In this approach, antigen is dissolved in a suitable aqueous solution and subjected to paper, thin-layer or column chromatography. The antigen moves towards an antibody layer where an antigen–antibody complex is formed. Although this is a comparatively simple and well-established technique [35], the thin-layer approach has turned out to be a platform of outstanding value in immunodiagnostics. In fact, this approach is one of the leading separation principles in immunodiagnostic assays today, where it is called a lateral-flow immunochromatographic assay (see below).

### 3.4 Immunoaffinity separation

This technique exploits the strong binding force between antibody and antigen with the aim of separating and purifying one of the reaction partners [36]. In one variant of the immunoaffinity technique, the antigen is chemically attached to a suitable solid matrix such as column-chromatography material. When a biological fluid containing the antibody is added, the antibody will be fixed by the matrix-bound antigen, whereas impurities will be washed out. An elution buffer then liberates the purified antibody from the antigen.

### 3.5 Immunoelectrophoresis

Immunoelectrophoresis [37] is a method by which proteins are separated in an electric field on a suitable matrix such as agarose or polyacrylamide gel and then identified on that matrix by immunoreaction with their recognizing antibodies. Electrophoresis can be done with native proteins that have their epitopes conserved, or it can be done with proteins that have lost their tertiary structure, for example, in a sodium dodecylsulfate (SDS)-containing buffer. In the latter case, the tertiary structure of the separated proteins must be reconstituted before immunodetection. This occurs by allowing the protein to diffuse from the SDS-containing matrix into a fresh matrix (**immunoblotting**) containing a reconstitution buffer, sometimes with the accelerating aid of an electric field (**electroblotting**). The transferred proteins are then identified with antibodies.

### 3.6 Fluorescence-activated cell sorting (FACS) separation and detection

FACS is a technique that allows separation of cells according to size and cell-specific molecular markers, and is also increasingly used to separate coated particles. FACS is a special form of flow cytometry (FCM) and will be described in connection with FCM below.

## 4 Architecture of immunoassays

Hundreds of different variants of immunoassays are in use. They are the result of optimization for individual antigen–antibody interactions. However, there are a couple of construction principles that can be considered as a general skeleton of immunoassays.

The centres of interest are primary antibodies and their specific binding to the antigen. The molecular scaffolds around this reaction have the purpose of stabilizing the reaction and measuring its products with high sensitivity and accuracy. Parts of the scaffold may include proteins A or G, the SABC method (described above), or related capture methods. Secondary antibodies that bind to the Fc part of primary antibodies are often used. In order to work, secondary antibodies must be species- and isotype-specific to the primary antibody. The same is true for tertiary antibodies and their affinity to secondary antibodies. The following types of antibodies can be differentiated:

| | |
|---|---|
| Primary antibody | Binds the antigen |
| Secondary antibody | Binds to primary antibody, functions as detection or amplifying antibody |
| Tertiary antibody | Binds to secondary antibody, often functions as detection antibody |
| Capture antibody | Anchors to a solid surface, binds to the antigen |
| Coating antibody | Coats a surface, often a capture antibody |
| Detection antibody | Carries a reporter system |

The arrangement of the construction elements differs according to the type of assay. Here we describe some common assay formats:

## 4.1 Immunosorbent and immunochromatographic assay

The **immunosorbent assay** [2, 6] is one of the best-known immunoassays (Fig. 4). The term "sorbent" means that the primary reactant of the assay is firmly adsorbed to a suitable surface. The chemical reaction is built up on the attached primary molecule, usually in a microwell (also called a microtiter) plate with a suitable surface such as polystyrol, or in some cases on particles [38]. The detection system is often an enzyme reaction (enzyme-linked immunosorbent assay = ELISA) or a fluorescent signal (fluorescence-linked immunosorbent assay = FLISA), but many other systems are also in use.

**Lateral flow assay** or **immunochromatographic assay** does not fix the reaction to a surface but allows the analyte and part of the diagnostic molecule(s) to migrate on paper or other thin-layer chromatography material [39]. A sample of the analyte, such as urine or blood, is applied to the starting point of the diagnostic device (see Fig. 5). The fluid is allowed to migrate in one direction. On its way, the fluid mobilizes antibody-coated particles that are integrated into the matrix. While migrating, antigen can bind to its corresponding antibody on the particle surface. The antigen-carrying particles are fixed in a detection zone, where they form a coloured stripe that can be evaluated with the naked eye. Non-antigen-carrying particles move on to a control zone. The lateral flow assay is the basis of several common consumer tests, such as the pregnancy

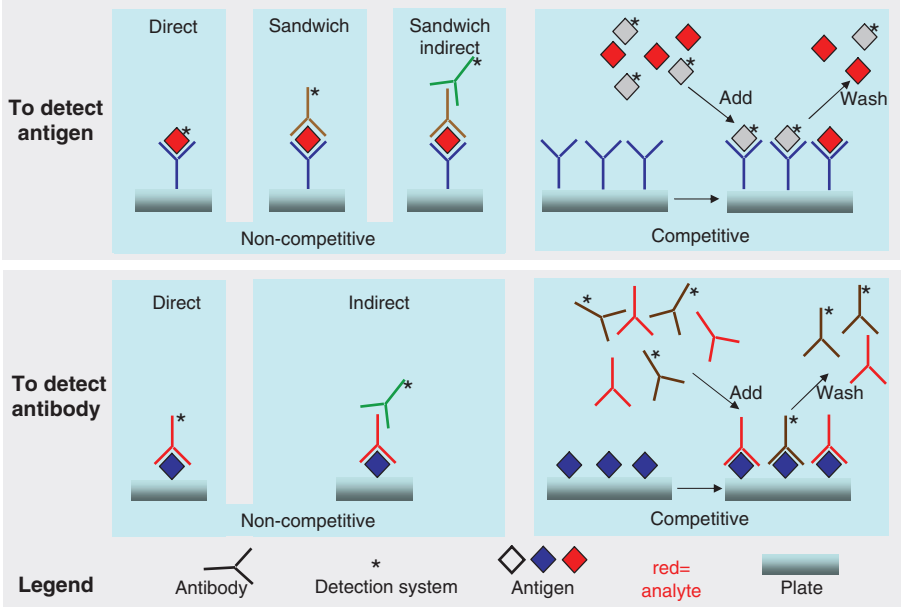


**Fig. 4 ELISA; examples for different formats.** ELISA and related assays can be used to measure either antigens or antibodies. The figure shows some of the common assay formats. "Direct" means that there is no secondary antibody or other secondary detection system. "Indirect" means that there is a secondary detection system. The "competitive" tests measure the result of multiple equilibria to assess the concentration of the analyte.

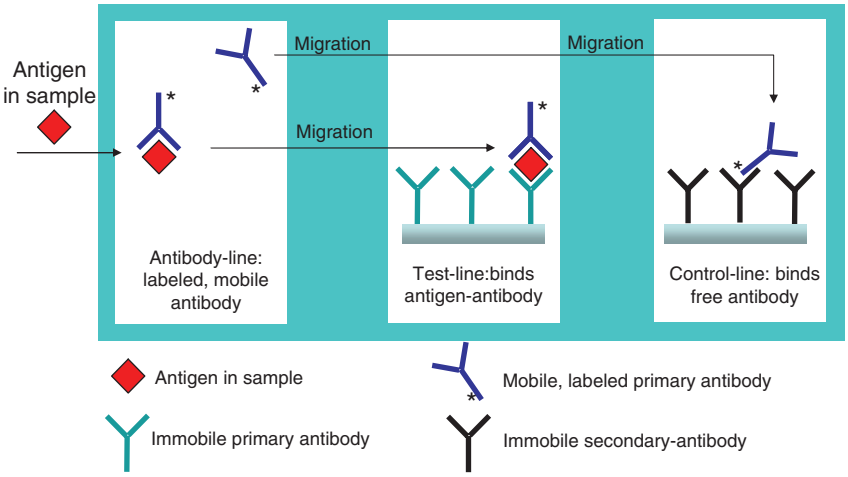


**Fig. 5 Schematic drawing of an immunochromatographic assay.** The steps of the immunochromatographic assay, also called lateral flow test, are shown. After addition of the sample fluid containing the antigen, the fluid migrates through three zones, where different reactions take place.

test, which simply differentiates between a positive and negative result for human chorionic gonadotropin in urine. Dipstick immunoassays [39] use the immunochromatography principle for on-site analysis.

### 4.2 Competitive and noncompetitive assays

Immunoassays can be used for the detection of both antigens and antibodies (Fig. 4). In the **non-competitive assay**, the test surface, such as a microwell plate, is coated with the primary (capture) agent. The biological material containing the analyte (antigen or antibody) is added and allowed to bind to the capture agent. If the analyte has been labelled, it can be detected directly (**direct assay**). Otherwise, detection requires the use of additional detection systems (**indirect assay**) such as an additional primary antibody that binds to a different epitope of the antigen (**sandwich** system) or additional augmentation systems such as secondary and tertiary antibodies.

A **competitive assay** (also called an inhibition assay) is applied, for example, when the antigen has only a single epitope, or if only a single antibody is available for binding to the antigen. The primary agent is coated on the microplate surface, and a defined amount of labelled analyte is added. Then the biological material containing the investigated analyte is added. This displaces labelled analyte from the antibody-binding site. The equilibrium concentration of the labelled analyte in the supernatant is then proportional to the analyte concentration in the biological sample, whereas the amount of plate-bound label is inversely related to the concentration. The competitive antigen can be either identical to, or just related to, the antigen used for raising the antibody; in the latter case it is referred to as a "heterologous hapten".

Immunoassays may be **homogeneous** or **heterogeneous**. In a homogeneous assay the reagents are in the liquid phase. In a heterogeneous assay the primary component is attached to a solid phase and the reaction occurs in an aqueous phase. The latter is presently the more common form for ELISAs and related systems.

## 5 Detection principles

Today, an increasingly large number of detection principles are available for immunoassays. Although the "E" in the acronyms EIA (enzyme immunoassay) or ELISA originally meant that the detection unit is an enzyme,

some authors today use the terms EIA or ELISA as "exchangeable", and even for immunoassays using other detection systems. One can differentiate between label-free assays and assays requiring labelled molecules. The latter have the disadvantage that labelling is an extra step, which has the risk of affecting the antibody–antigen reactivity. Labelling methods will be described in the section on immunosensors.

## 5.1 Detection with labels

**Radiolabel.** The radioimmunoassay (RIA) was the first elaborate immunoassay technique [1]. Originally it was performed in solution using precipitation methods. Although it is very sensitive and fast, it has gone out of common use, partly because of the safety problems associated with radiolabelled compounds. But in some tests (e.g., the study of thyroid hormone receptors) it can provide superior sensitivity when radiolabels with high specific radioactivity and high decay energy are used.

**Enzyme label.** Enzyme immunoassay (EIA) is an umbrella term for enzyme-linked detection. The rate of product formation is proportional to the analyte concentration in the sample and is usually detected by colour change. EIA can be combined with various separation and capture techniques. It can be used to detect antigen or antibody. It was originally performed as a homogeneous test. The enzyme-linked immunosorbent assay (ELISA) is a form of EIA where the scaffold is a solid surface upon which the analytical components are built, with an enzyme reaction as the final detector. It is a heterogeneous test.

**Fluorescence label.** The increasing variety and availability of stable fluorophores, and the advances in coupling them to a large number of functional groups, have enabled for fluorescence detection to become a leading method in immunoassays [40]. This has been favoured by recent advances in miniaturization of the equipment and the commercial availability of easy-to-use building blocks. The net result is a significant advantage over many other detection systems. Several types of fluorophore-reporter molecules can simultaneously be measured in parallel with good precision and sensitivity, allowing the user to make multiplexed analysis.

## 5.2 Label-free detection

This type of detection has the advantage that it does not require any labelling of molecules and thus avoids the extra effort for labelling and the danger of label-associated artefacts. Most of the current label-free detection techniques rely on detecting changes in refractive index, mass, heat, or electrical current when the target binds to its recognition element.

**Surface plasmon resonance (SPR).** An antibody is attached to a gold-coated surface. When antigen is added, the light emission behaviour of the gold surface will change in proportion to the mass increase of the antibody–antigen complex on the surface. This is a very powerful method [41], allowing for both the detection of antigen–antibody binding and also for studying binding kinetics and thermodynamics. More label-free techniques will be described in Section 6 (Immunosensors).

## 5.3 Amplified detection with label

### 5.3.1 Immuno-polymerase chain reaction (IPCR)

IPCR makes use of the fact that nucleic acids can be determined at much lower concentrations than proteins. IPCR is an extremely sensitive detection method, combining the specificity of antibody detection and the sensitivity of PCR [42, 43]. Here, the secondary antibody is connected to a marker DNA. The antigen–antibody binding induces a polymerase chain reaction, producing amplicons, which are detected. This technique can increase the sensitivity of immunoassays 1000-fold or more. By using several different marker DNAs, several antigens can be detected in a single assay, thus allowing multiplexed detection.

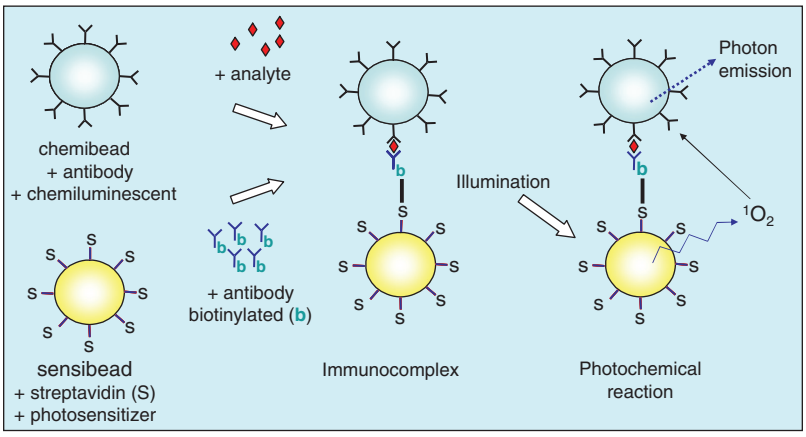


**Fig. 6 Luminescent oxygen channeling immunoassay.** This is a very elaborate assay which includes different particles and reagents in addition to the analyte. It can yield high sensitivity. The final detection is by light measurement.

### 5.3.2 Catalyzed signal amplification (CSA)

This is another example of a complex detection system that allows staining with high sensitivity in immunohistochemistry [44]. In a first step, the antigen is labelled with a primary antibody, followed by a peroxidase-labelled secondary antibody. Then biotin-tyramide is added, oxidized by a peroxidase, and thereby precipitated near the antigen. A streptavidin-peroxidase conjugate is then added and binds to the precipitated biotin. This increases the peroxidase signal many fold. This method has several variations.

### 5.3.3 Luminescent oxygen channelling immunoassay (LOCI)

Various detection methods rely on luminescence [45], either for measuring cell signals, improving sensitivity, or reducing the workload of an immunoassay. LOCI is one of the well-known applications [46] (Fig. 6).

# 6 Immunosensors

## 6.1 Sensors

Much current chemical research is dedicated to the miniaturization of immunochemical detection systems using sensors. From a diagnostics point of view, a sensor is a small platform capable of measuring changes in a particular physical quantity, and then converting it into a signal. The basic prerequisite is that it can selectively measure molecules without influencing their properties. Sometimes a sensor is used to study the thermodynamics and kinetics of a chemical reaction [47, 48]. A sensor that can measure antigens with the use of antibodies, or vice versa, is called an immunosensor. Several very good reviews dealing with various measuring principles and advances in immunosensor design have appeared in the past few years, covering “lab-on-a-chip” technology [49, 50], the use of nanoparticles [51–53], wireless sensor networks [54], impedimetric principles [55], and point-of-care diagnostics platforms [56], among other topics. The major challenges in the design of an immunosensor are related to the need for miniaturization and for complex on-site analysis in medical diagnostics and other fields of human safety. A sensor is required to measure accurately the target analyte, typically present in very low concentrations in complex media, in the lowest possible volume and with minimum user manipulation. Therefore, the design and manufacture of successful immunosensor systems are multi-disciplinary in nature and require researchers to

apply knowledge from both life sciences and engineering. The components of an immunosensor are now increasingly related to chips used in computers, digital cameras, mobile phones, and inkjet printers. This opens up the possibility of fabricating immunosensors on a large scale with available chip technologies, which will reduce the price.

## 6.2 Advances in the surface chemistry of immunosensors

The detection principle of an immunosensor relies on the specific interaction between the desired analyte (antibody, nucleic acid, metabolite, etc.) and its recognition element, which is typically immobilized on a surface. To maximize the signal from the binding event, the capturing molecules must be immobilized in a mode that will enable them to retain their activity. This is one of the most critical parts of the biosensor, as the mode of immobilization will have a crucial effect on the sensitivity and accuracy of the device (Fig. 7). Interestingly, the recent advances in polymer science are shifting the trends in the development of immunosensors towards more disposable platforms. The real driving force is the need to develop inexpensive and disposable sensor substrates, with excellent physical properties, good chemical resistance, and ease of fabrication. New technological processes enabled the manufacture of the substrates with incorporated microstructures to accommodate various functional elements to pre-condition and handle a biosample, for example, metering, mixing, dilution or concentration in an appropriate buffered solution, plasma extraction, filtration, purification, and the interaction with other bulk or surface-confined reagents [57–59]. For these reasons, the trend is that new polymers and plastics are replacing more traditional surface materials such as glass, gold, and silica.

Fuelled by the increased interest in biomedical diagnostics, a number of scientific articles, reviews, and books dealing with useful immobilization strategies have appeared in the past few years [60–65]. They typically appraise methods mainly based on physical, covalent, and bioaffinity mechanisms of immobilization. The motivation behind these methods is to ensure the most favourable orientation of the immobilized biomolecules, in which the antigen-binding site is oriented away from the surface itself.

In general, the choice of a suitable immobilization strategy is determined by the physicochemical properties of both the surface and the biomolecules.

For example, Hu et al. [66] presented an interesting approach for covalent and oriented immobilization of scFv antibody fragments via an engineered glycan moiety. Their strategy was to immobilize the glycosylated scFvs at salt concentrations that precluded nonspecific adsorption of unglycosylated molecules, and the covalently attached antibody fragments then exhibited 4-fold higher functional activity than ionically adsorbed scFvs. Similarly, Walper and coauthors [67] compared the effect of an oriented immobilization method for single-domain antibodies (sdAbs). The format that provided the highest density of active molecules by at least a factor of two was an sdAb–streptavidin core tetramer. This was followed by the sdAb-alkaline phosphatase and then the site-specifically biotinylated monomer. The poorest performing immobilization methods were

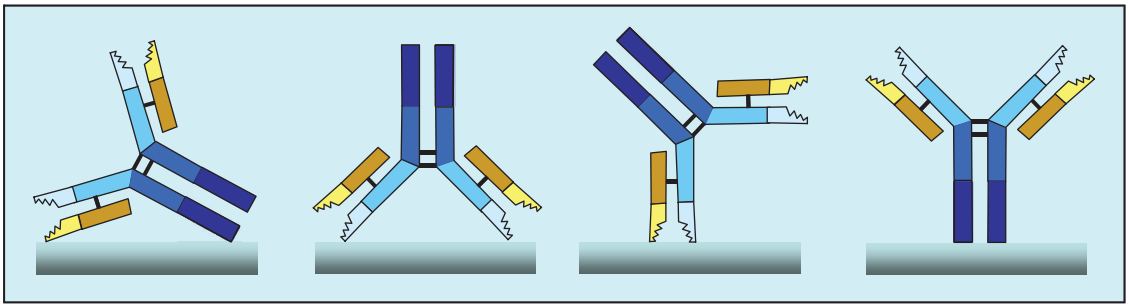

**Fig. 7 Possible orientations of IgG on a surface.** The schematic drawing shows that some of the orientations will lead to a partial or total blocking of the antigen-binding sites of the antibody. Therefore, it is important to be aware of this problem when designing an assay.

the two most common, direct covalent attachment and the randomly biotinylated sdAb attached via NeutrAvidin. Importantly, the oriented immobilization method also translated into improvements in limit of detection using a bead-based system. The sdAb–streptavidin core provided more than a 100-fold improvement in the limit of detection.

The differences between the immobilization modes and surface orientation of the full-size antibodies and antibody fragments were assessed by Balevicius et al. [68]. Total internal reflection ellipsometry (TIRE), a surface-specific technique, was used to investigate biological recognition layers of immunosensors. The data analysis showed that the immobilized active antibody fragments can specifically interact with a 2.5-times greater amount of antigen when compared to a randomly oriented layer of whole antibody. However, a uniform orientation of capture molecules does not necessarily translate into high-affinity interactions. Trilling et al. [69] used variable domains of llama heavy-chain antibodies (VHHs) as capture molecules to investigate which analyte properties contribute to sensitivity by orientation. SPR chips with randomly immobilized biotinylated VHHs were compared to streptavidin-coated SPR chips, on which similar quantities of oriented biotinylated VHHs were noncovalently immobilized. The authors suggested that orientation of the capture molecule hardly affects high-affinity interactions. Nonetheless, it leads to strong improvements in sensitivity for lower-affinity interactions.

Alternatively, metal ion affinity methods have been widely used to investigate the oriented immobilization of His-tagged proteins onto immunosensor chips covered with a functional stromatoid chelator, such as iminodiacetic acid (IDA) or nitrilotriacetic acid (NTA), via covalently bound films, polymer films, or both. Yang [70] and his colleagues reported on a strategy for oriented immobilization of functionally intact IgG on a polystyrene microwell plate via (IDA)–$Ni^{2+}$ and ZZ–His protein interaction. The authors immobilized a ZZ–EAP (*Escherichia coli* alkaline phosphatase)–His fusion protein, which exhibited Fc binding, His tag, and intrinsic alkaline phosphatase activities. The authors analyzed the interaction between rabbit IgG anti-horseradish peroxidase (anti-HRP) and its binding partner HRP to investigate the specificity and efficacy of their method. The His-tag-assisted method showed an enhanced signal, a 10-fold higher sensitivity, and a wider linear range. As an interesting alternative, the His-tagged binding surface layer can also be constructed by peptide self-assembled monolayer (SAM) as demonstrated by Bolduc and co-workers [71]. Significant reduction of nonspecific adsorption of non-analyte constituents of crude serum was reported using penta- and hexa-peptide monolayers.

Oriented covalent immobilization of antibodies onto heterofunctional metal chelate-glyoxyl supports (Ag–$Cu^{2+}$/G) was also the subject of a study by Batalla [72]. The immobilization of IgGs takes place in two steps: (i) the antibodies are conjugated to the support via His–metal coordination bonds, and (ii) their incubation under alkaline conditions then promotes an intramolecular covalent attachment between lysine residues at the Fc region and glyoxyl groups on the support surface. The optimal antibody distribution was achieved when these proteins were slowly immobilized on Ag–$Cu^{2+}$/G in the presence of imidazole. This bioconjugate was able to bind up to 1.5 mol of antigen per mole of antibody, only 1.3-fold less than the antibody in solution. Although their approach is more relevant to immunoaffinity chromatography, the resulting fairly inert solid surfaces and the optimally bound antibody bioconjugates showed high specificity towards the target antigens with reduced nonspecific binding of any other proteins.

While the simplest method of immobilization of the biorecognition elements on any surface is physical adsorption, most of the new materials are not always suitable for physical attachment of typically hydrophilic molecules to the often-hydrophobic plastic surfaces [73, 74]. The noncovalent binding mechanism in the physical adsorption process not only governs the random orientation of the captured proteins but also their denaturation on almost every type of material [75]. However, Zhao et al. [76] investigated the specific recognition between physically adsorbed and covalently captured monoclonal antibody to prostate-specific antigen (anti-hPSA) and its antigen (hPSA) for applications in prostate cancer diagnostics. In their study, the authors used spectroscopic ellipsometry and neutron reflection to investigate how solution pH, salt concentration, and surface chemistry affect antibody adsorption and subsequent antigen binding. The authors concluded that on all surfaces studied, the antibody predominantly adopted a “flat on” orientation. Perhaps contrary to general belief, the major finding was that a carefully executed antibody immobilization via appropriate

physical adsorption can actually replace elaborate interfacial molecular engineering involving complex covalent attachments. Similar results were previously reported by the group of Williams [77], who studied interfacial adsorption of a mouse monoclonal antibody (type 1 IgG, anti-βhCG) at the hydrophilic silicon oxide/water interface.

Overall, the new and emerging polymeric, disposable materials used for sensor substrates introduce some technological advantages in the production of immunosensors, such as ease of fabrication, low cost, machinability, and facile large-scale production. However, while the specific chemical nature of such materials can sometimes be seen as an advantage, it also introduces a specific feature and an undesired challenge in the limited choice of the surface chemistry used for the chemical activation of the plastic sensor substrates.

## 6.3 Detection systems of sensors

In general, a detection system can be either label-free or labelled. Label-free systems have the advantage that the additional second step of introducing a labelled reporter molecule or an enzyme is not required. In label-free systems the sensor's detection system measures reaction-related physicochemical changes on the sensor surface. The label-free transducer principles are typically based on a variety of responses, including increase of mass-induced mechanical deflection (cantilever) [78, 79] shown in Fig. 8, mass spectrometry [80], mass-induced change of gold/silver surface light reflection surface plasmon resonance [81], electrical potential changes [82, 83], change of the inner heat (calorimetry) [84], acoustic or surface acoustic wave detection [85], surface-enhanced Raman scattering [86], and piezoelectric or piezoresistive platforms [87, 88], among others (Table 1). Label-free detection methods have been used primarily for in-depth characterization of biomolecule interactions. There are often misconceptions about the accessibility of these platforms, since they usually require specialized training. A new wave of more cost-effective, robust, and accessible platforms has appeared. One of the recent emerging technologies is the dotLab System, which uses diffractive optics system (dot) to detect biomolecular interactions and can be used for a variety of applications in the study of a spectrum of biologically relevant analytes, including proteins, DNA, and even microorganisms [89].

Alternatives to label-free approaches are detection systems requiring an optically visible or electrochemically responsive label in order to visualize the binding event. Owing to their simplicity, high potential for miniaturization, and relative ease of operation, these methods are very popular despite the obvious potential disadvantage that one or more additional chemical steps are required, each with a risk of failure.

In ELISA and FLISA, the classical labels are enzymes or fluorescent dyes. In both cases, it is essential to maximize the signal-to-noise ratio to achieve clinically relevant sensitivity and limits of detection in the

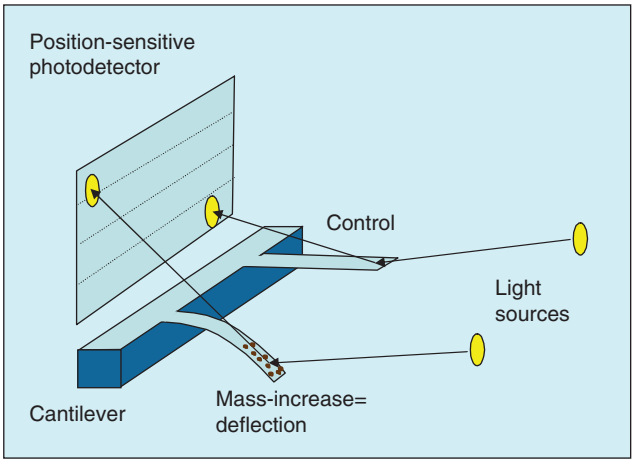


**Fig. 8 Cantilever, schematic drawing.** The cantilever is a micromechanical device. When a reaction takes place on the lever, leading to a mass increase, a deflection occurs, which can be measured with an optical device.

**Table 1** Novel immunosensor detection systems (examples). The table depicts some of the developments, which are aimed at high sensitivity, miniaturization, and multiplexing.

| |
|---|
| Surface plasmon resonance |
| Evanescent wave |
| Fluorescense polarization |
| Elliptometry |
| Acoustic surface wave |
| Cantilever mass |
| Cantilever piezo |
| Electrochemical detection |
| Thermophoresis |
| Calorimetry |
| PCR reporter |
| Fibre optics for remote detection |

immunoassay devices [90]. Simple assay designs involve first capturing an antigen onto a surface using one antibody, then measuring the surface concentration by visualizing the captured antigen through its reaction with a second, labelled antibody. Recently, organic dyes were replaced with nanoparticles (NPs), doped with tens of thousands of dyes, thus providing an intense signal. Fluorescent NPs are useful as labels since they can be measured directly, without the need for any amplification step [52, 91, 92]. NP labels can, however, suffer from disadvantages in comparison with simple molecular labels, most notably effects due to particle aggregation [93] and the related effect of nonspecific binding [94] to the capture surface. The benefits of using NPs can only be realized if they are efficiently coated with detection antibody and the fraction of the coupled antibody that is in fact active or available for reaction with antigen [95] is sufficiently large.

Lin et al. [96] recently presented variations of ELISA by using enzymes that release oxygen radicals and thus initiate oxygen-triggered fluorescence reactions (Fig. 6). These can be measured with a photomultiplier or with a small and comparatively inexpensive light-sensitive CMOS chip that is also used in digital cameras.

MacCraith and Ruckstuhl were pioneers in concepts of efficient collection of the fluorescent light emitted by the reporting molecules in assays. They demonstrated the advantages of collecting the emitted light under the supercritical angle fluorescence (SAF) in biomedical diagnostics [97, 98] and in optical imaging [99]. This new technology enables monitoring of analyte binding in real time with high discrimination relative to background fluorescence. The SAF technology is an inexpensive and sensitive platform adaptable to scanners and multiwell plates, and it seems to have a great potential for replacing the work-intensive and time-consuming ELISA.

Electrochemical methods are also increasingly applied to immunoassays, because they overcome problems associated with other modes of detection. In particular, when compared to conventional immunoassays, electrochemical immunosensors show versatility, reliability, and fast analysis time. A review by Laschi [100] presents some interesting examples of immunochemical assay developed using magnetic beads as a solid phase coupled with electrochemical detection techniques, in particular, using electrochemical arrays as transducers.

## 6.4 Microfluidic systems

Microfluidic systems tailored for analyte separation and detection have attracted increasing research activity over the past decades. In particular, the prospect of integrating all steps from sample preparation and reagent storage to assay readout in a single microfluidic device bears great promise to leverage next-generation diagnostic products. Modern sensors are the core part of microdevices [101]. Currently, the standard control of the reagent flow is done by intelligent microfluidic systems with micropumps and valves. However, the need for novel, low-resource fabrication and assembly methods for creating disposable detectors leads to the development of alternative microfluidics concepts such as hybrid paper-polymer devices [102], centrifugal micro-

fluidics platforms [103], and regeneration-free immunosensors [104]. The recent surge in the development of microfluidics systems was fuelled by research advances in polymer science. In particular, the availability and the great choice of new classes of polymers and co-polymers sparked an enormous interest from scientists to use them as disposable substrates for diagnostic devices [105]. These materials have been engineered to meet key criteria such as low autofluorescence, ease of fabrication, and manipulation. Importantly for microfluidics development, they also have excellent mouldability and good machinability to form complex microfluidic features such as channels [106, 107], valves [108], and other features enabling samples to be preprocessed with relative ease: dilution or concentration in an appropriate buffered solution, filtration, purification, and the interaction with other bulk or surface-confined reagents.

The new, state-of-the-art microfluidics systems often need to measure in less than 1 μL of sample volume, with the analyte concentration in pmol/L range and with minimum user manipulation. While the prospect that even a very small sample of blood or of biological material is sufficient to make many different analyses could be seen as a distinct advantage, it also bears some technological challenges for microfluidics systems. For example, because the concentration of the target molecule(s) is often very low, it is critical to maintain that concentration while delivering the analyte-containing fluid through the microfluidic preprocessing features to the active assay area of the chip substrate. Antibodies are common examples of analytes in diagnostic devices, and can randomly adsorb onto the surface of the plastic substrate [109]. Therefore, their final concentration over the detection area can be quite different from the original crude sample, which can contribute to false negatives or inaccurate quantitation.

Though promising, there are nonetheless major challenges with which the new microfluidics systems will have to cope. Some are related to the need for miniaturization and trends for multiplexing [110–112], which means that the parallel measurements of different analytes are performed in one set and with the same fluidic system. These two specific requirements must be addressed along with the basic requirement for minimum user manipulation to ensure high precision and accuracy [113] of the immunosensor. However, this means that the space-demanding microfluidics features such as sample preprocessing, dilution, reagent storage, purification, and the actual sensor area must all be incorporated into one single substrate. The factor of the "small footprint" or limited "real estate" on most of the microfluidics systems that operate along a single axis or plane will need to be reconsidered, particularly in those that are aimed for production of single-use, point-of-care diagnostics devices.

## 6.5 Role of chip technology

The emerging market of modern chip technology of mobile phones, cameras, and inkjet printers, and their use in the health sector, is rapidly expanding. An appealing advantage is realized in telemedicine, with connection to even the remotest areas of the world. Some recent logical innovations include saving and subsequently distributing diagnostic images over the mobile network for knowledge sharing, feedback or quality control, integration of the mobile phone's images with bioassay microchips, or simple biomedical optical analyses using available photodiodes or CMOS sensors.

Wang et al. reported on a simple and inexpensive microchip ELISA-based detection module that employs a portable detection system, i.e., a cell phone/charge-coupled device (CCD) to quantify an ovarian cancer biomarker, HE4, in urine. In their study, the sensitivity of microchip ELISA coupled with a cell phone or a CCD was 89.5 % at a specificity of 90 %, which compared favorably with that obtained in a previous study using conventional microplate ELISA [114].

Recently, Balsam et al. exploited the possibility of biomedical optical analysis using a low-sensitivity webcam. They captured hundreds of low-sensitivity images using a webcam in video mode, as opposed to a single image typically used in a cooled CCD. Then they used a computational approach consisting of an image-stacking algorithm to remove the noise by combining all of the images into a single image [115].

Tuijn and co-workers have performed feasibility studies in Uganda of using mobile phones for capturing microscopy images and transferring these to a central database for assessment, feedback, and educational

purposes. Clear images were captured using mobile phone cameras of from 2 megapixels (MP) up to 5 MP. Images were sent by mobile Internet to a website where they were visualized and feedback could be provided to the sender by means of text messaging [116].

Pioneering studies such as those mentioned here are prime examples of where connecting mobile technology to diagnosis has a considerable potential to improve diagnostic services and to bring current biomedical diagnostic approaches to the majority of the world's population in resource-poor settings with remote clinical centres. It is anticipated that at the current pace of integration of mobile technology and immunosensors, we are close to products that will reach the stage of point-of-care field-testing. This is particularly true for many biomedical devices based on optical detection ranging from microarray analysis to FCM.

# 7 Immunochemical visualization of tissues and cells

## 7.1 Immunohistochemistry with tissue slices

Two aspects can be distinguished in cell biology: structure and function. Histochemistry is a method to detect and localize specific structures in tissues, cells, or subcellular specimens. In its traditional format, it makes use of chemical affinities to biological molecules of dyes or other reporters, or visualizes enzyme activities. It is often performed on tissues that have been fixed with substances like formaldehyde, in order to arrest living processes and to "freeze" the tissue in its original structure. Immunohistochemistry uses antibodies to detect specific molecules and subcellular structures, with which they are associated. Thin tissue slices are cut and incubated with a solution containing the antibody. The attachment sites of the antibody are visualized with an enzyme reaction or with a fluorescent label. Hundreds of fluorophores are available today, which differ with regard to their affinities to biomolecules, their stability, and their excitation and emission wavelengths. The slices are then analyzed under a (fluorescence) microscope to detect the localization of colour or fluorescence in the tissue.

Immunohistochemistry is a leading method in cell biology [117]. It provides information about localizations and dynamics of cellular components. It is an important tool of inter- and post-surgical diagnosis, allowing the pathologist to provide a rapid result concerning type and extent of pathologic structures in a patient's tissue. In industrial toxicology there is a need for histological examinations of virtually all organs that must be tested for pathologic parameters. This has led to the development of a highly standardized and sometimes automated immunohistochemical methodology [118]. Organ slices are cut by a slicing machine, automatically incubated with the antibody-containing reagent, and transferred to the microscope where the histological picture is automatically evaluated with specific software. The procedure provides the required information about the normal histology and toxicant-induced pathologic changes.

There are cases where antigens in the tissue are masked and thus inaccessible to diagnostic antibodies. In such situations, heat- or proteolysis-induced epitope retrieval can be applied to improve the signal [119, 120].

## 7.2 Immunohistochemistry of suspended cells

Related techniques can be performed in single-cell suspensions, such as living white blood cells. Depending on the technique, the cells may be permeabilized to allow entry of a fluorescence-labelled antibody, which binds to structures carrying the antigen. At the end of incubation, the fluorescence emission of each cell is recorded. Use of more than one antibody, each carrying a different fluorochrome, enables simultaneous detection of multiple cellular structures and can provide information on how they interact. High-throughput automated analysis can be achieved using microwell plates, for example, with 384 or more wells [121]. Here, numbers and intensities of fluorescent areas can be analyzed with a modern fluorescence plate reader.

## 7.3 Immuno-electron microscopy

Immuno-electron microscopy is used to study the presence of the antigen within the ultrastructure of a tissue. After tissue fixation and embedding, ultrathin tissue slides are made and incubated with gold-colloid labelled antibody. Under the electron microscope, the gold particles are visualized as round, electron-dense spots [122, 123].

## 7.4 Enzyme-linked immunosorbent assay (ELISPOT)

ELISPOT visualizes proteins, which have been released by living cells in vitro. It is often used to detect antigen-specific T lymphocytes, as they secrete cytokines upon specific stimulation. Practically, microplate wells are treated to attach a capture antibody on the solid well surface. Cells of interest are added on top and stimulated so that they release specific proteins (e.g., a cytokine). Released molecules are captured by the immobilized antibody. After a wash, a detection antibody is added. When the microwell plates are observed from above, the areas around those cells that have released the specific cytokine, can be visualized as colour spots (Fig. 9). The method has a unique strength for T-cell diagnostics [124]. It has been used for interferon gamma measurements in connection with transplantations for more than 20 years [125] and is used for measuring human immune responses to vaccines [126].

## 7.5 Lymphocyte transformation test (LTT)

In allergology, it is possible to differentiate between four different allergic mechanisms. A major difference is between allergy type 1, which is mediated by an IgE antibody, and allergy type 4, which is mediated by the T-cell receptor (TCR) molecules of T cells. The IgE molecules of type 1 allergy can be measured reliably with ELISA. But the detection of a type 4 allergy is much more elaborate. It can be done with the LTT, which measures the reactivity of T cells that possess the proper TCR for an allergen. When stimulating a larger number of suspended living T cells with the appropriate antigen, those (few) cells that carry the TCR molecules specific for the antigen will transform and proliferate. Proliferation can be measured as an increase of the DNA content during a several-day incubation (Fig. 10). Increased proliferation compared to control suggests that the blood contains T lymphocytes that are reactive to the antigen and may be the cause of a type 4 allergy.

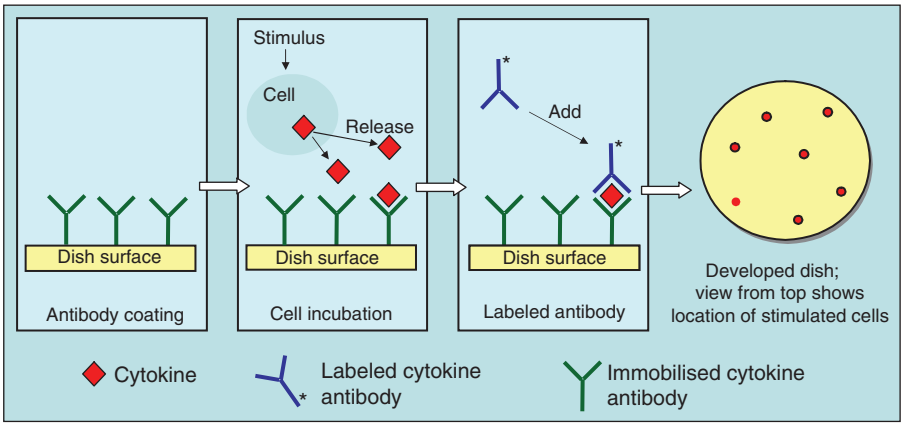


**Fig. 9 Visualization of cytokine releasing cells with ELISPOT.** Enzyme-linked immuno spot assay (ELISPOT) is a technique that allows one to study in a cell mixture those cells that produce and release specific antigens (e.g., cytokines) after a stimulus. The cells are seeded on antibody-treated culture dishes. The antibody captures antigens that are released by the cells. A detection system allows the areas of releasing cells to be localized.

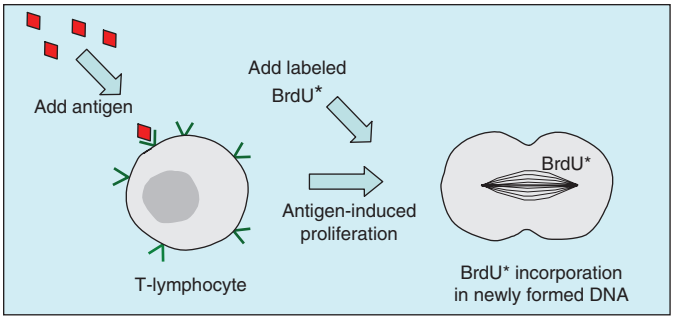


**Fig. 10 Lymphocyte transformation test (LTT).** This test is used to find out whether the lymphocytes of a patient with a type 4 allergy are reactive to the suspected antigen (e.g., a drug or metal). Lymphocytes are taken from the patient's blood, exposed to the suspected antigen, then allowed to grow in culture in the presence of an indicator for DNA replication (here labelled 5-bromo-2′-deoxyuridine, BrdU) for a couple of days. Antigen-sensitized cells will be induced by the antigen to proliferate (divide). The extent of proliferation is indicated by the amount of BrdU that is incorporated in DNA.

Although the LTT (also called lymphocyte proliferation test) does not employ antibodies, it is a common immunological test for the diagnosis of metal and drug allergies [127].

## 7.6 Detection of cell types by flow cytometry (FCM)

FCM is classically used to study the numbers of lymphocytes and other leukocytes in the blood. These cell types and their subtypes carry characteristic surface proteins called cluster of differentiation (CD) antigens. Antibodies to CD antigens, labelled with different fluorochromes, are added to the cell suspension. Each of them binds to its respective surface protein and thereby differentially labels a specific cell type. The cell mixture is then allowed to flow cell-by-cell through a capillary system and its optical detection unit. The fluorophores attached on each cell are excited by lasers of defined wavelengths, and the fluorescence emission is measured at two angles (Fig. 11). This allows characterization of each cell according to size and surface markers. Microspheric beads of defined size are used to calibrate the absolute numbers and sizes of cells.

Although originally developed for counting immune cells in the blood (e.g., in connection with leukemia), FCM is now also applied to study other samples, such as single-cell suspensions which have been prepared from organs [128, 129], bacteria, organelles, and bead-attached proteins. With three lasers for excitation and some 10 fluorescence channels plus 2 light-scattering channels in advanced high-end devices, more than 100 parameters can be differentiated in a single run. Depending on cell type and conditions, between 1000 and 50 000 cells or microspheres can be measured per second. This allows the detailed sub-differentiation of cell types that were previously considered to be identical, but can now be differentiated due to the availability of antibodies to many antigens on the cell surface. This enormous success in multiplexing has turned the method into a very powerful technique in clinical diagnostics (e.g., subtyping of leukemias) and research. However, it should be noted that such methods produce a very large amount of data that must be properly analyzed and interpreted.

FCM methodology is increasingly used as a detection method for antigens using the particle immunoassay (PIA). In the first step, magnetic particles of µm dimensions are coated with one or more differing antibodies. The particles are incubated with the analyte, containing the respective antigen. Using antibody/antigen-specific fluorescent labels, those particles carrying a specific antigen can be identified by FCM. Using different particle sizes and different fluorescent labels with characteristic fluorescence emission peaks, some 100 antigens can be determined in a single run. This is a novel approach for multiplexed analysis of a large set of disease markers in a single patient, and the FCM technique is becoming an increasingly important method for multiplexed immunodiagnostics. It can be used in specialized form in intensive care units to diagnose a

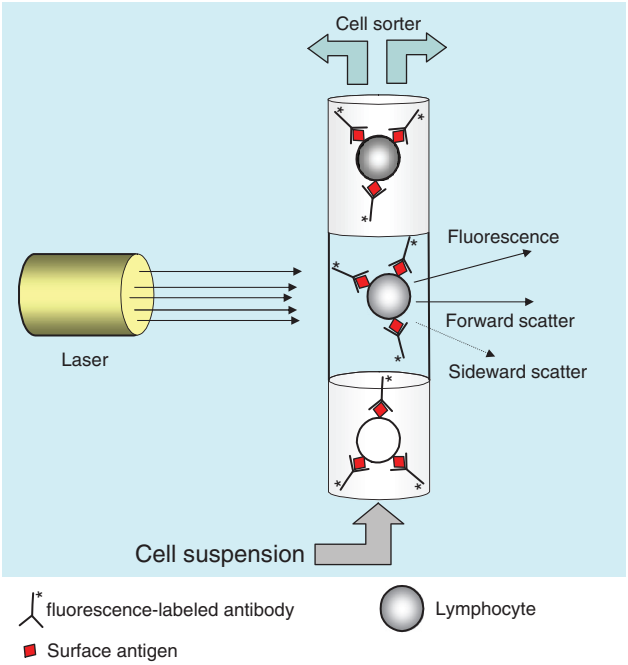


**Fig. 11 Flow cytometry.** Flow cytometry is classically used to identify lymphocyte subpopulations in blood, but can also be used for other cell types, bacteria, or microparticles. Fluorescence-labelled antibodies that are specific to surface molecules of a cellular subtype are added to the cell mixture. The cell suspension is then moved through an optical device, where fluorescence emission and light scatter is measured of each cell. This gives information about the size and subtype of each cell. Cells can also be sorted according to their features.

patient's immune status [130]. It allows discovery of changes in cell physiology (e.g., in combination with microscopic screening [131, 132]), and also screening for bacteria [133] or enzyme activities [134]. A limitation is the high cost of these advanced instruments and the fact that they require a highly trained operator and strict laboratory conditions. Adequate quality control is obviously essential [135].

Apart from its diagnostic power, FCM technology is also capable of sorting and separating cells according to their features in a method called fluorescence-activated cell sorting (FACS). Each fluorescence signal triggers a shutter, by which the different subtypes of cells can be separated and enriched.

In magnetic cell separation, surface antigens are labelled with antibody conjugates that are attached to magnetic nanospheres. Cell types can then be separated according to their labels by use of a magnetic field.

# 8 Progress and limitations in applied immunodiagnostics

## 8.1 Types of samples and matrices

Immunochemistry is a highly dynamic field. Its rapid development is often in competition with that of instrumental analytics, and sometimes in cooperation with it. In both areas, there are similar attempts to miniaturize the devices and speed up analysis time. The application areas are expanding, and the need for multiplexed and high-throughput solutions is evident [136–140]. The term "immunochemistry" was introduced about 100 years ago in connection with observations in the blood [141], and until recently, immunochemical applications mainly dealt with antigens and antibodies in the blood and other body fluids. These serological examinations were directly health-related. In the past decade, immunochemical analyses have increasingly expanded and included extracorporeal agents, such as food residues and environmental samples from water, soil, and air (Table 2). Aiming at the protection of the human environment, these newer

**Table 2** Matrices and analytes for which immunochemical analysis is increasingly applied (examples).

| Matrix | Analytes |
|---|---|
| Blood | Disease markers, drugs, autoantibodies |
| Tissue sample | Organ structure, cell structure, cancer diagnosis |
| Biotechnology sample | Antigen and/or antibody |
| Soil | Environmental chemicals, microorganisms |
| Air | Allergens, workplace chemicals |
| Drinking water | Organic contaminants |
| Open waters | Microorganisms, toxins, contaminants |
| Food | Biocide residues, contaminants, additives |
| Dust | Allergens, environmental chemicals |

The table gives an overview on the different fields in which immunochemical assays are applied. While in its beginning, immunochemical analysis was confined to blood components, the number of application fields has meanwhile rapidly increased.

applications can be considered indirectly health-related. In the following section, major immunochemical application fields are described.

## 8.2 Investigation of physiological regulation processes

In the healthy state of an organism, biochemical parameters in blood and urine tend to stay within relative small concentration limits (homeostasis). When the balance is disturbed, additional regulatory processes come into play. An example is cytokine release from cells of the immune system as a consequence of immunological defence mechanisms. The release from cells can be measured by ELISPOT and the concentrations in blood by ELISA. The relative concentrations of different cytokines reflect a certain regulatory status of the immune system and are used for the understanding of their role in inflammatory disease [142, 143]. Since increasingly more test reagents are available for the different cytokines and their receptors, such approaches contribute to a better understanding of the events during immune response in health and disease.

Another example is that of phosphorylated proteins. Phosphorylated proteins and their unphosphorylated counterparts play a key role in cellular regulation, but their differential measurement with traditional biochemical methods is difficult and not suited for routine implementation. The development of separate antibodies against unphosphorylated proteins and their various phosphorylated forms will give more insight into normal regulation and deviations in disease [144, 145].

One of the best-known examples for the measurement of hormonal upregulation is the pregnancy test. It measures the level of human chorionic gonadotropin in the urine, which is elevated in pregnancy [146]. As noted above, the lateral flow pregnancy test is one of the most used point-of care-tests today. It can be performed by anybody and delivers reliable and accurate results [5].

## 8.3 Drug monitoring

Some pharmaceutical drugs, such as cyclosporin A or digoxin, have a very narrow therapeutic range between ineffective low dosing and toxic overdosing, especially if patients have an unusual elimination (e.g., due to kidney disease). In such cases, drug monitoring is important. Classically, this is done by analytical chemical methods. However, these require a specialized laboratory and personnel. For routine diagnosis, immunochemical assays make it easy to monitor the levels of therapeutic drugs, but also addictive drugs in suspected persons or in persons participating in a withdrawal program. Although a urine sample [147] can provide the result within minutes, a possible interference from other pharmaceutical drugs may lead to incorrect results [148, 149].

## 8.4 Organ disease markers

Hundreds of disease markers are measured in the daily routine of the clinical laboratory. Some of the more traditional disease markers are enzymes that are released from an injured organ, such as α-amylase from injured pancreas or γ-glutamyl-transpeptidase from damaged liver. Such enzymes then circulate in the blood and can be measured in a serum sample by studying the conversion of their substrate to the product. However, in some cases there are several isoenzymes released from different organs, which will all contribute to the enzymatic turnover. Lactate dehydrogenase is a prime example. In such cases, the use of selective immunoassays for each of the isoenzymes is a very reasonable diagnostic approach to identify the injured organ. There are also nonenzymatic disease markers, such as troponin, which is released from cardiac cells during a heart attack [150]. This is measured by sensitive immunochemical methods.

The hormone insulin was one of the first analytes for which an immunoassay was used [1]. Insulin measurement has since been important for diabetes research, but less for routine diagnosis of diabetes. Due to the development of more sensitive immunochemical methods [151] and the trend towards very small blood volumes (e.g., from children) [152] insulin measurement may play a more prominent role in individualized medicine either to monitor endogenous insulin levels or to control the levels of therapeutic insulin [46]. Steroid hormones such as progesterone [153] are also typically measured with sensitive immunoassays.

## 8.5 Markers for autoimmune disease

One can generalize that those cells of the immune system that are directed towards foreign molecules are encouraged to proliferate, whereas cells directed towards "self" molecules of the body will be destroyed. The latter results in "immunological tolerance". The system is efficient but not perfect. As a consequence of disturbance of the self-tolerance mechanisms, the immune system may treat self-molecules as if they were foreign, causing immune reactions that result in autoimmune disease [154–156]. This is often directed towards only one or a few types of molecules of just one organ. Clinically, it is accompanied by settlement of inflammatory cells in that tissue and gradual loss of organ function [157, 158]. Rheumatoid arthritis and Hashimoto thyroiditis are common examples of autoimmune diseases. Immunohistochemical methods to detect antigens in tissues combined with immunoassays to detect the autoantibodies have led to a continuous improvement in diagnosis [159, 160].

Celiac disease is an example of a quite common intestinal disease that was widely undiagnosed until recent dramatic improvements in immunodiagnostic markers [161–164]. Celiac disease is a hypersensitivity to the wheat protein gluten. It is a disorder where allergic and autoimmune reactions are both involved. The availability of potent tests to measure IgA autoantibodies against tissue transglutaminase or endomysium of connective tissue, and antibodies against the wheat protein gliadin is a great diagnostic advance and will probably replace the invasive duodenal biopsy as a standard for diagnosis.

Some further examples of development of potent disease markers are autoimmune hepatitis [165], autoimmune urticaria [166], protective autoimmunity in cancer [167], and autoimmune lymphoproliferative syndrome [168].

## 8.6 Markers for allergic disease

In general, four types of allergies can be differentiated based on their immune mechanisms. All four involve a hyper-reactivity of the immune systems, although the mechanisms and symptoms are very different. Immunodiagnostic approaches are important for all four. As an example, type 1 allergy is caused when foreign substances, notably proteins (like grass pollen proteins) get in contact with mucosal surfaces such as the respiratory and gastrointestinal tracts and are recognized by the immune system as antigens. After the first exposure and a lag time, the immune system starts to produce antibodies of the IgE type. The antibodies

circulate and bind to the histamine-storing mast cells in the mucous membranes and to histiocytes in the blood. Upon subsequent exposures, the antigen will bind to its IgE and thus evoke an explosive histamine release from the histamine-storing cells. Released histamine is a hormone-like mediator that causes the known symptoms of type 1 allergies, characterized by local edema, pain, and inflammation, and after entering the circulation, lowering of blood pressure. Diagnostic tools for a type 1 allergy are the skin prick test, the oral food challenge test, and immunodiagnostic measurement of allergen-specific IgE in the blood [169]. High-specific IgE levels are associated with a higher risk for an allergy. Immunodiagnostic tests are also used to identify the triggering antigen in the environment (e.g., house dust mite proteins, cat hair, mould components, grass pollen, latex proteins, etc.).

The steadily improving and expanding diagnostic possibilities are very helpful for patients. However, some offered allergy tests measure immunological parameters that have not been proven to be associated with disease. An example is the measurement of IgG4 antibodies in connection with food intolerances [169]. Although it is known that positive results are not correlated with clinical symptoms, such an incorrect interpretation is quite common, and may give the patient a false belief that he/she has to avoid essential nutrients, with the consequence that a normal life becomes almost impossible.

Type 4 allergies are often triggered by small molecules such as metal compounds or drugs, which act as haptens to which T cells become sensitized. When there is a suspicion for a metal or drug allergy that would often be expressed as a skin rash, the causative agent may be detected with the help of a T-cell specific test. The patient's T cells are isolated and exposed to suspected antigens. Sensitized T cells may show increased reactivity to an allergen or its peptides that can be measured either by detecting cytokine release using ELISPOT [170] or by the proliferation of lymphocytes in the LTT [127], see above.

## 8.7 Cancer markers

Cancer cells are characterized among others by a change in protein expression. The appearance of fetal proteins or non-organ specific proteins, as well as an uncharacteristic distribution of proteins, is quite common. Moreover, previously sequestered proteins may present to the immune system and may induce the formation of tumour-specific autoantibodies. Atypical, tumour-specific proteins may be detectable in biopsies with immunohistochemical methods, or may be detectable following release into the blood [171, 172]. The increasing availability of such tumour markers and their antibodies has supported the idea that many tumour types occur in subtypes. These can be differentiated with immunochemical methods and will increasingly be the basis for individualized treatment.

Not all the known tumour markers fulfil the promise of specific and sensitive diagnostic utility [173]. An example is prostate-specific antigen (PSA). Serum of men with healthy prostates contains small quantities of PSA. Elevated PSA is often associated with prostate cancer and other prostate disorders [174]. It was believed that PSA should be used to monitor middle-aged men for a possible prostate tumour, but it has become evident that PSA measurement is associated with false positives and therefore unnecessary treatment [175]. The U.S. Preventive Services Task Force (USPSTF) recommends that "for men of any age…doctors and patients do not screen for prostate cancer because the potential benefits do not outweigh the harms" [174]. Nevertheless, PSA still has its role as parameter, for example, for monitoring progress during treatment.

Carcinoembryonic antigen (CEA) is a glycoprotein normally found in the embryo. In adults, its presence is associated with cancers such as lung tumours. Its blood level can be used as an indicator for the activity of a malignacy. There are efforts to further improve the immunoassays and immunohistochemical tests for CEA [176]. Some further examples are:

- carcinoembryonic antigen in lung cancer [177]
- tumour markers [178]
- gynecological cancer [179]
- pancreatic cancer [180]
- breast cancer [181, 182]

- breast cancer differential diagnosis [183]
- undifferentiated tumours [184], leukemias [185, 186], and soft-tissue tumours [187]
- renal neoplasms [188]
- oral cancer [189]

## 8.8 Markers for infectious diseases

Recent advances in the immunodiagnosis of infectious diseases have been discussed in several review articles [190–192]. The classical method for identification of bacteria is their isolation and growth on selective culture media. Final detection is usually performed by staining, microscopy, biochemical assays, and immunochemical procedures. In recent years, these classical identification methods have been supplemented and sometimes replaced by the advent of genetic fingerprinting with polymerase-chain reactions [193].

However, another strong detection strategy is based on the concept that intruding infectious microorganisms usually lead to reactions of the immune system, resulting in the production of specific antibodies. These can be measured in the blood. Such antibodies are studied in immunoassays with the aid of suitable antigens that have previously been isolated from the cultured pathogenic microorganisms. Positive findings can prove that an infection has taken place even when the infectious agent cannot be detected. The relative time courses and levels of specific IgM and IgG antibodies in the blood provide information about the onset and intensity of the infection. The combined serological results often give reasonable information for the further treatment of a patient. Good examples are hepatitis C [194] and HIV diagnosis [195]. The methods to study disease subtypes and their association with therapeutic success have been improved. It is hoped that there will also be a shift towards point-of-care diagnostics. Rapid tests are required especially for diagnosis in rural areas with poor medical infrastructure, for example, to diagnose tropical diseases [8]. Instantaneous on-site results are also valuable in clinical settings, because they allow treatment of bacterial infections with microorganism-specific antibiotics from the time of diagnosis. Moreover, there is a great need for immunoassays to detect parasitic diseases, such as malaria [196]. The problem is that the immune system does not readily develop antibodies against parasites, and, despite many efforts, the quality of available immunoassays is often insufficient. Nonetheless, given the immense interest and investments in immunochemical research in the past decade, we will certainly see suitable solutions in the future.

## 8.9 Markers in food hygiene

Food regulations are monitored by industrial and public food chemists. While some time ago only a few parameters, such as the visual detection of parasites in meat, were monitored, today the spectrum has expanded immensely. The newer analytes include natural ingredients, chemical residues, additives, microbial contaminations, parasites, and others. The increasing number of parameters would overcome the capacity of the controlling agencies, unless the classical methods were replaced by multiplexed, high-throughput methods. Therefore, there is a natural pressure for developments in this direction. An example for the advantage of immunoassays in this field is the monitoring of botulinum toxin in food, previously studied with a mouse bioassay, which can now be detected with a lateral flow device as an alternative [197].

Further examples of surveying for infectious agents, residues and contaminants are:

- pathogenic microorganisms on food [198]
- *Staphylococcal* enterotoxin B in milk [199]
- pesticides in fruits [200] and rice [201]
- human exposure to pyrethroid pesticides [202]
- melamine in milk [203]
- antibiotics in milk [204]
- wheat proteins in milk powder [205]

## 8.10 Markers for environmental chemical exposure

Monitoring environmental media such as air, soil, and water is a key activity that has contributed to the successful sanitation of environmental media in many countries in the past 30 years. Meanwhile, methods in analytical chemistry have improved beyond expectations. Hand-held analytical devices help to monitor and reduce levels of toxic chemicals at the work place. GC and GC-MS have been developed to an unexpectedly high sensitivity. Instrumental analytical methods will always be required as a final proof for identification but such instruments require intense maintenance and highly specialized operators, and may be quite costly. This is one reason for the development of immunoassays even for such chemicals, despite the existing, sensitive, and accurate instrumental analysis. Immunochemical methods make it possible for contaminants to be measured with less sophisticated equipment by less-skilled workers at lower cost in less time. Immunoassays have been developed for environmental contaminants such as 1,1,1-trichlor-2,2-bis-(4-chlorophenyl)ethan (DDT), 2,3,4,5,6-pentachlorophenol (PCP), polychlorinated biphenyls (PCBs), polycyclic aromatic hydrocarbons (PAHs), and many other chemical families.

When using such immunochemical assays, one must be aware that the results will not be identical with those of GC-MS; one cannot expect that the antibodies (e.g., for polychlorinated dibenzodioxins) will react with each congener proportionally to their mass concentration in the sample, and this has to be taken into account when interpreting the results. Some examples of applications involving small and environmentally important toxic substances are:

- cadmium [206]
- PAHs, PCBs [207], dioxins [208]
- organophosphorus pesticides [209, 210]
- pyrethroid pesticides in water [211]
- mycotoxins [212] and bacterial toxins [213]
- the wood preservative pentachlorphenol (PCP) in soil [214]
- explosives [215]
- chemical warfare agents [216]

## 8.11 Examples from veterinary medicine

Notably in farm animals, the monitoring of animal health and prevention of infectious disease is of major importance. Although the primary responsibility for hygiene lies with the owner, animal health and avoidance of infectious disease is of public interest. The veterinarian should have analytical tools available that provide rapid, reliable information on the presence of diseased animals on a farm. Immunochemical assays will thus increasingly be used as point-of-care diagnostic systems. The test systems are similar to those we know from human applications for studying infections, organ disease, reduced growth, or residues of environmental contaminants or pharmacological agents in animals. Beyond that, species-specific diseases such as bovine mastitis must be monitored, where ELISA can be used for early diagnosis [217]. One of the most recent and commercially available examples is a microfluidic sedimentation cytometer for milk quality and bovine mastitis monitoring, reported by Garcia-Cordero et al. [218]. Their detection system consists of 12 independent microfluidic devices, essentially flattened funnel structures, fabricated on the footprint of a single plastic compact disc (CD). Their device features rapid, low-cost, portable microfluidic sedimentation cytometer (SeCy) for assessing the somatic cell count and fat content of milk in 15 minutes using a “sample-in, answer-out” approach. Apart from bovine mastitis, it is also important to measure *Staphylococcal* enterotoxin in milk [24] to survey possible infections of cows.

The use of growth hormones to increase the yield of milk in dairy cattle has been widely banned. Methods are required to survey compliance with this ban and have been developed, for example, to detect insulin-like growth factor in cattle [219].

### 8.12 Limits and problems of interpretation

Each immunochemical diagnostic method requires a specific strategy that allows for pre-separation of matrix components and interfering substances so that the antibody–antigen reaction and detection will take place in a clean environment. To exclude the possibility of false results due to impurities, cross-reactions, or unsuitable analyte concentrations, it is necessary to run intra-lab quality controls and if available also inter-lab controls [135, 220]. Moreover, any new immunochemical test should prove its suitability in comparison with other analytical methods.

Immunoassays are developed to measure the analyte at low concentrations with high specificity and accuracy. One might therefore assume that from the point of view of clinical chemistry, results will always be clear and correct. However, immunoassays are complex constructs. Unless the prescribed reaction conditions are carefully followed, there is a danger of false results. Another and perhaps even more relevant problem is that of interpretation of the results. Many tests measure specific parameters with good sensitivity and accuracy. Nevertheless, there are cases where the results are of little or no diagnostic value. An example is the IgG4 measurement in connection with food allergies mentioned above. Despite some tests showing elevated levels of IgG4 antibodies, this is not a reliable indication of a food allergy. Relying on this kind of test, thousands of patients have been warned not to eat food for which they were tested positive, albeit the test has no reasonable predicative relevance. Another example is PSA, which for many years has been proposed as a predictive marker to survey for possible prostate cancer, but now the recommendation is changing [174]. While measurement of a diagnostic parameter must always have a rationale and validation, the extreme sensitivity of immunologically based methods increases the potential for abuse. Thus, it is important for researchers to consider the limits of immunoassays [221].

## 9 Future developments

Health-related applications of immunological detection methods and immunosensors will continue to develop, and two developments in particular can be anticipated. Due to the improvement of immunosensor techniques one can expect that point-of-care diagnostics will expand, notably in areas with a weak medical infrastructure. On the other hand, the professional labs will be provided with high-throughput, multiplexed automated systems that can measure perhaps dozens of immunological parameters with high sensitivity and accuracy. In both areas, good quality control will remain the basis for correct results.

## 10 List of abbreviations

| | |
|---|---|
| CCD | charged coupled device |
| CD | cluster of differentiation |
| cDNA | complementary DNA strain |
| CDRs | complementarity-determining region |
| CEA | carcinoembryonic antigen |
| ChIP | chromatin immunoprecipitation |
| CMOS | complementary metal oxide semiconductor |
| CSA | catalyzed signal amplification |
| DDT | 1,1,1-trichlor-2,2-bis-(4-chlorophenyl)ethan (insecticide) |
| EIA | enzyme immunoassay |
| ELISA | enzyme-linked immunosorbent assay |
| ELISPOT | enzyme-linked immunospot assay |
| Fab | antigen-binding fragment |

| | |
|---|---|
| FACS | fluorescence-activated cell sorting |
| Fc | constant fragment of antibody |
| FCM | flow cytometry |
| FLISA | fluorescence-linked immunosorbent assay |
| Fv | variable fragment of antibody |
| HIV | human immunodeficiency virus |
| HRP | horseradish peroxidase |
| IDA | iminodiacetic acid |
| IgG | immunoglobulin G (a major class of antibody) |
| IPCR | immuno-polymerase chain reaction |
| LOCI | luminescent oxygen channelling immunoassay |
| LTT | lymphocyte transformation test |
| NP | nanoparticle |
| NTA | nitrilotriacetic acid |
| PAH | polycyclic aromatic hydrocarbon |
| PCB | polychlorinated biphenyl |
| PCP | 2,3,4,5,6-pentachlorophenol (pesticide) |
| PSA | prostate-specific antigen |
| RIA | radioimmunoassay |
| SABC | streptavidin-biotin complex |
| SAF | supercritical angle fluorescence |
| SAM | self-assembled monolayer |
| scFv | single-chain variable fragment |
| sdAb | single domain antibody |
| SeCy | sedimentation cytometer |
| SPR | surface plasmon resonance |
| TCR | T-cell receptor |
| TIRE | total internal reflection ellipsometry |
| VHH | variable domains of the heavy chain of llama heavy-chain antibodies |

## 11 Glossary

A full glossary of terms can be found in [222].

## 12 Membership of sponsoring body

Membership of the IUPAC Chemistry and Human Health Division Committee for the period 2014–2015 is as follows:

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