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Abstract

Immunosensors are solid-state devices in which the immunochemical reaction is coupled to a transducer. They form one of the most important classes of affinity biosensors based on the specific recognition of antigens by antibodies to form a stable complex, in a similar way to immunoassay. Depending on the type of transducer there are four types of immunosensor: electrochemical, optical, microgravimetric and thermometric. The most commonly used bioelements for the development of electrochemical immunosensors are antibodies (Ab), followed by aptamers (Apt) and, in the last five years, microRNA (miRNA). In order to perform an early diagnosis, a method that is able to measure peptides and proteins directly in a sample, without any sample pre-treatment or any separation, is preferred. This direct detection can be performed with methods making use of the specific interaction of proteins with Ab, Apt and miRNA. The recent developments made in the immunosensor field, regarding the incorporation of nanomaterials for increased sensitivity, multiplexing or microfluidic-based devices, may have potential for promising use in industry and clinical analysis. Some examples of assays for several commercially available biomarkers will be presented. The main application fields, beside biomedical analysis, are drug abuse control, food analysis and environmental analysis.

Keywords: Immunosensors, immunoassay, antibody, aptamer, clinical analysis, environmental monitoring
1. Introduction

1.1. Immunosensors and immunoassays

Immunosensors are solid-state devices in which the immunochemical reaction is coupled to a transducer. They form one of the most important classes of affinity biosensors based on the specific molecular recognition of antigens by antibodies to form a stable complex, in a similar way to immunoassays. In contrast to immunoassays, modern transducer technology enables the label-free detection and quantification of the immune complex [1]. The immune system possesses a great ability to distinguish self from non-self. Antibodies (Abs) with high specificity are synthesized by the organism in reasonable quantities after sensing the foreign species called antigens (Ag), as part of the immune defence system. The ability of organisms to recognize the presence of Ags and to respond rapidly by synthesizing Abs that exhibit high binding constants [2] was and continues to be exploited by scientists in order to create new specific analytical devices. Molecules generally designed as Abs include a number of classes and subclasses of immunoglobulins with high specificity towards various targets.

The high sensitivity and selectivity of the immunoreactions together with the availability of Abs or Apt for a broad range of molecules make immunochemical methods useful tools in a large variety of applications, including clinical analysis. The use of immunosensors instead of other immunochemical techniques simplifies the analysis considerably, making it rapid and reliable [3].

Lately, a plethora of biomolecules whose presence or level of expression is an indicator of some pathological condition (we refer here to biomarkers) are currently used in immunoassays. Those devices represent a convenient way of measuring the concentration of biomolecules in biological fluids (such as serum, urine, etc.) by means of immunological reactions.

Most clinical protein biomarker detection is done today by enzyme-linked immunosorbent assay (ELISA), but requirements for the relatively expensive test kits and the plate readers limit ELISA’s usefulness for fast diagnostics [4]. LC-MS-based proteomics is currently used for biomarker discovery, but is currently too expensive and technically too complex for routine clinical diagnostics [5, 6]. Microarrays developed on a 96- or 384-well plate format, Ab-coated with colorimetric detection are also being used in clinical diagnostic. These arrays are simple and highly selective and allow multiplexed measurement of proteins [7, 8, 9, 10]. Several commercially available automatic or semiautomatic analysers for multiplexed protein measurements, employing fluorescence (Luminex, Myriad RBM), electrochemiluminescence (ECL) (Roche Diagnostics, Mesoscale Discovery), or surface plasmon resonance (Horiba Inc., BIO-RAD; Biacore Life Sciences, GE Healthcare) measurement technologies are currently used in hospital laboratories [11]. Assay kits are available for measurement of up to 10 selected target proteins per sample with detection limits (LOD) of 1-100 pg/mL in serum [4]. These commercial instruments require specialized consumables, including sample well plates, chips and reagent kits [12], which are expensive and, thus, they have limited usefulness for point-of-care (POC) applications where resources are limited. Fluorescence-based detection strategies typically require laser sources and precise alignment of optical components, whereas electro-

Depending on the type of transducer there are four types of immunosensor: electrochemical (also classified into potentiometric, amperometric and impedimetric), optical, microgravimetric and thermometric. All of these types can either be run as direct (non-labelled) or indirect (labelled) immunosensors. The most commonly used labels are enzymes such as peroxidase, glucose oxidase, alkaline phosphatase, catalase or luciferase. Some other labels such as electroactive compounds (ferrocene or In²⁺ salts), fluorescent reagents (rhodamine, fluorescein, Cy5, ruthenium diimine complexes, phosphorescent porphyrin dyes, etc.) and since recently metallic nanoparticle (gold or silver produced in situ electrochemically) are also used [3]. Although indirect immunosensors are highly sensitive, mainly due to the analytical label, the concept of direct sensing represents a reliable alternative in the development of immunoassay systems [13]. The appropriate immobilization of the recognition element at the surface of the transducer element is of paramount importance. Different immobilization techniques, such as direct adsorption onto the electrode surface, self-assembled monolayers (SAMs), polymer matrices or magnetic beads (MBs), etc., can be used. Direct adsorption is simple, but the proper orientation of the recognition element for the immunocomplex formation is poor and passivation of the electrode surface frequently occurs. A more ordered, oriented immobilization is obtained with SAMs that are generally formed on gold surfaces, using either thiolated Abs or DNA, or compounds containing a thiol group at one end and a carboxyl or amino group at the other end able to form a covalent bond with the Ab. SAMs can also be obtained on graphite surfaces using diazonium salt chemistry. Polymers with different functional groups, such as chitosan, polysulphone or polyaminobenzoic acid, may also be used for the immobilization of these recognition elements [14]. MBs have gained popularity in the development of immunosensors due to their good stability, small size and spherical geometry with a high load of recognition elements, fast-reaction kinetics, and the ease of surface modification with functional groups, DNA, enzymes, streptavidin, protein A or G, etc. [15].

The role of electrochemical immunosensors in analysis has considerably increased in the last years, due to their low cost, high sensitivity, potential for miniaturization and automation, and low power requirements. Important developments have been secured, especially in sensor design concerning the type of bioelement (Ab, Apt, Affibody, miRNA) and the immobilization technique.

2. Label-free versus labelled immunosensors

The immunoassay is a device based on biochemical detection and measurement of biomolecules in a test solution using a specific Ab or immunoglobulin. Immunoassays rely on the specificity of the immunoreaction, and more precisely on the ability of an Ab or Apt to recognize and bind to a specific target, usually an Ag. The measurable signal proportional to the Ag concentrations is obtained with the aid of a label. Some of the commonly employed labels include radioisotopes [16], chromophores [17], fluorophores [18] and
enzymes [19]. Depending on the nature of the label, the immunoassay could be classified into radioimmunoassay, chromoimmunoassay, fluoroimmunoassay and enzyme immunoassay, respectively [20].

The enzyme immunoassay or enzyme-linked immunosorbent assay (ELISA) is the most commonly used technique employed as a diagnostic tool for clinical purposes. The working principle of ELISA consists in the immobilization of an unknown amount of Ag onto a solid surface, and then a specific Ab is added over the surface so that it can bind to the Ag. This antibody is usually labelled with an enzyme; the substrate of the enzyme is then added, which will be converted into a compound that can be quantified by means of optical methods. There are four types of ELISA: direct ELISA, indirect ELISA, sandwich ELISA and competitive ELISA [20].

The simplest protocol is the one used in the case of direct ELISA [21]. The Ag is adsorbed to a plastic plate, then an excess of another protein (normally bovine serum albumin) is added to block the free binding sites. An enzyme is linked to an Ab in a separate reaction and the formed enzyme-Ab complex is added to the well. The Ab interacts with its Ag, forming an Ag/Ab-enzyme complex, and the excess of unreacted Ab-enzyme is washed off. Afterwards the substrate of the enzyme is added, and is converted into a compound that gives a measurable signal directly linked to the Ag quantity.

Indirect ELISA is a two-step ELISA assay which involves the use of two Abs to bind the Ag: a primary capture Ab and a secondary signalling Ab. The primary Ab is incubated with the Ag followed by the incubation with the secondary Ab. However, this may lead to non-specific signals because of a cross-reaction that the secondary Ab may bring about. This technique has a high sensitivity because more than one labelled Ab is bound per Ag molecule.

Sandwich ELISA is a less common alternative, but is highly efficient in sample Ag detection. Moreover, many commercial ELISA pair sets are built on this sandwich ELISA.

The sandwich ELISA quantifies antigens between two layers of antibodies (i.e., capture and detection antibody). The Ag to be measured must contain at least two antigenic epitopes capable of binding to Ab, since at least two Abs act in the sandwich. Either monoclonal or polyclonal Abs can be used as the capture and detection Abs in sandwich ELISA systems. Monoclonal Abs recognize a single epitope that allows fine detection and quantification of small differences in Ag. A polyclonal is often used as the capture Ab to pull down as much of the Ag as possible. The advantage of sandwich ELISA is that the sample does not have to be purified before analysis, and the assay can be very sensitive (up to two to five times more sensitive than direct or indirect ELISA, but lower than ELISpot). The central event of competitive ELISA is a competitive binding process executed by original Ag (sample Ag) and add-in Ag. The procedures of competitive ELISA are different in some aspects compared with indirect, sandwich and direct ELISA assays. In competitive ELISA assays, the following successive steps are involved: 1) primary Ab (unlabelled) is incubated with sample Ag; 2) Abs-Ag complexes are then added to 96-well plates which are pre-coated with the same Ag; 3) unbound Ab is removed by washing the plate (the more Ags in the sample, the fewer Abs will be able to bind to the Ag in the well, hence "competition"); 4) the secondary Ab that is specific to the primary
Ab and conjugated with an enzyme is added; 5) a substrate is added, and the remaining enzymes elicit a chromogenic or fluorescent signal. For competitive ELISA, the higher the sample Ag concentration, the weaker the eventual signal is. The major advantage of a competitive ELISA is the ability to use crude or impure samples and still selectively bind any Ag that may be present.

In order to avoid confusion, the term ‘immunoassay’ is used for tests based on immunoreactions, while the term ‘immunosensor’ is specifically employed to describe whole instruments, i.e., immunoreaction-based biosensors. In the heterogeneous immunoassays, the Ab or Ag is immobilized at a solid substrate (e.g., microplate), while homogeneous immunoassays take place in solution [22]. Heterogeneous immunoassays are easier to design and construct, but they require a separation of the Ag and the Ab from the samples and their immobilization at a solid surface. In contrast, the homogeneous immunoassays usually involve the immobilization of the biomolecules on nano-/microbeads, thus allowing the integration of multiple liquid handling processes [23, 24]. The homogeneous immunoassays coupled with microfluidic devices are relatively inexpensive and efficient alternatives and can be used for the detection of complex samples, such as urine or blood, without any sample pre-treatment or large volume consumption. Microfluidic lab-on-a-chip technology also has the advantages of portability, integration and automation.

In the immunosensor design, the sensing element is formed by means of immobilization of Ags or Abs, and the binding event is transformed into a measurable signal by the transducer [25]. The performances of immunosensors are obviously related to the selectivity and affinity of the Ab-analyte binding reaction. The immunosensors can be either direct (where the immunochemical reaction is directly determined by measuring the physical changes induced by the formation of the complex) or indirect (where a sensitively detectable label is combined with the Ab or Ag of interest, Figure 1) [20]. The distinction has an entirely different meaning to the terms direct and indirect in the immunoassay fields, which are both tracer-related. They are distinguished by whether Ab binding is directly detected, for example, after an enzyme-labelled Ab is bound to an immobilized coating conjugate or an enzyme tracer to an immobilized Ab, or whether the detection only takes place after a secondary binding reaction, for example, if an enzyme-labelled secondary Ab is used to label the first Ab bound to an immobilized coating conjugate [21, 22, 23].

Immunosassays can be performed either in competitive or sandwich-type configurations. The competitive-type assay is based on the competition between the analyte in the sample which is unlabelled and a labelled analyte for a limited number of captured Abs. Two approaches can be employed: the first is based on immobilized Abs that react with free Ags in competition with labelled Ags; the second consists in immobilized Ags and labelled Abs. The sandwich-type ELISA-like assay is the most widely used format and employs a pair of matched, high-affinity Abs and appropriate labels, leading to an increased sensitivity and selectivity. The Ag of interest is captured between a pair of Abs and a labelled Ab is further used to obtain a detectable signal [26, 27, 28].

The reaction of proteins in solution with their complementary proteins immobilized on a surface leads to changes in, for example, refractive index, thickness and dielectric constant of
the immobilized layer. With proteins immobilized on a piezoelectric material, a change in resonance frequency is detected which is proportional to the mass change on the surface. These properties are exploited in optical, electrochemical and piezoelectric immunosensors. Ideally, immunosensors are devices with fast response, a high specificity and sensitivity. It is also preferable that they are regenerable, which means that they can be reused immediately after dissociation of the Ab-Ag complex, e.g., by using a chaotropic reagent [29].

To be able to detect the interaction, one of the immunoagents has to be labelled. Various labels have been applied, of which radioisotopes were among the first because of their inherent sensitivity. Other frequently used labels are chemiluminescent compounds and enzymes (e.g., alkaline phosphatase, horseradish peroxidase), which convert an enzyme substrate into a measurable product (Figure 2).

Many different configurations have been described and the four most commonly used formats are shown in Figure 3. In a homogeneous immunoassay Abs (Figure 3a), Ags and labelled Ags are mixed. Free labelled Ags and those bound to an Ab, can be distinguished by a change of the label's activity upon binding. Usually, immunoassays are heterogeneous, which means that either the Ab or the Ag is immobilized on a solid carrier and an immunocomplex is formed upon contact with a solution containing the other immunoagent.

The unbound proteins are removed by washing and the response obtained from the labels is proportional to the amount of protein bound. In a sandwich immunoassay, Abs are immobilized and after addition of the sample containing the Ag, a labelled secondary Ab is added (Figure 3b). Besides these non-competitive formats, competitive configurations can also be applied. In a competitive assay, competition takes place between free and bound Ag for a limited amount of labelled Ab (Figure 3c) or between Ag (the sample) and labelled Ag for a limited amount of Ab (Figure 3d) [30]. Electrochemical detection of immune interaction can
be performed both with and without labelling [31]. A frequently used format in electrochemical immunosensing is the amperometric immunosensor, where proteins are labelled with enzymes producing an electroactive product from an added substrate [32]. Direct detection
without labelling can be performed by cyclic voltammetry, chronoamperometry, impedance, and by measuring the current during potential pulses (pulsed amperometric detection). These methods are able to detect the electrode change in capacitance and/or resistance, induced by the binding of protein. These immunosensors have been developed using various supporting materials. Those built on silicon, silanized metal, or polypyrrole are often regenerable, while those based on SAMs, on silver or gold, are not. Chaotropic reagents, used to accomplish disruption of the Ab-Ag bond, have been reported to affect the SAM [33, 34] or to induce a decrease in the sensitivity of the sensor [34, 35].

3. Types of bioelement used in the development of immunosensors

The most common bioelements used in the development of electrochemical immunosensors are Abs, followed by Apt, and in the last five years the miRNA. In order to perform an early diagnosis, a method that is able to measure peptides and proteins directly in a sample, without any sample pre-treatment or any separation, is preferred. This direct detection can be performed with methods making use of the specific interaction of proteins with Ab, Apt and miRNA.

Abs are proteins produced in animals by an immunological response to the presence of a foreign substance (with a molecular weight larger than 1.5 kDa), a so-called Ag, and which have a specific affinity for this Ag [36, 37, 38, 39, 40]. Abs may be monoclonal or polyclonal. Polyclonal Abs have a specific affinity for the Ag, but they are directed to different binding sites (epitopes) on the Ag with different affinities. In immunoassays, often the use of monoclonal Abs is preferred. These Abs are formed by using identical cells cultivated by the hybridoma technology, and have optimized characteristics such as high affinity or high specificity. To conclude, polyclonal Abs are isolated after immunization of animals, while monoclonal Abs require hybridoma selection. Abs are structurally very similar. Of the five classes of immunoglobulins (IgA, IgD, IgE, IgG and IgM), which differ in glycosylation and the number and position of disulphide bridges, IgG (150 kDa) are mainly used for immunoassays. An IgG consists of two heavy and two light chains, which are interconnected by disulphide bridges (Figure 4). All chains have a variable and a constant region. The variable regions of the heavy and light chain combine in one interaction site for the Ag, which is called the antigenic site. Thus, an IgG molecule has two identical binding locations for the Ag [41, 42]. Abs often demonstrate strong binding features and high selectivity; however they have some intrinsic limitations related to their molecular properties. The most commonly used Ab type, the IgG molecule, is a large, bivalent, multidomain protein, dependent on disulphide bonds and with a complex glycosylation pattern. This explains its relatively poor heat stability and a difficult and expensive manufacturing process. In addition, Abs use only a minor part of the molecule for Ag recognition [43].

A number of techniques are known today for the in vitro generation of a wide range of molecular recognition bioelements besides Abs, from which binders with high affinity and specificity can be selected. Besides the favourable molecular recognition properties of Abs,
these alternative scaffold proteins often offer improved characteristics such as small size, high stability in rough conditions, absence of cysteine, high yield in bacterial expression and the option to use them for multispecific analysis.

Affibody molecules are a class of engineered affinity proteins with high potential for therapeutic, diagnostic and biotechnological applications. Affibody molecules are small (6.5 kDa), single-domain proteins that can be isolated for high affinity and specificity to any given protein target [43].

Affibody molecules have already been investigated as affinity probes in ELISA setup and protein microarrays. For example, in one such study, dimeric Affibody molecules with affinity for IgA, IgE, IgG, TNFα, Insulin and Taq polymerase were immobilized on thiol dextran microarray and an LOD of 70 fM was obtained [44]. Affibody molecules were also evaluated as capture agents in a sandwich array format with unlabelled target protein and monoclonal Abs for detection, demonstrating specificity in a complex serum or plasma sample. The use of Affibody molecules in sandwich assays for analysis of human samples might also be advantageous in a cross-reactivity perspective, since human anti-animal Abs are occasionally present which might lead to cross-linking of capture and detection Abs, resulting in elevated background signals, which are avoided when affibodies are used [44].

Apts, derived from the Latin word “aptus” (meaning to fit) are synthetic single-stranded DNA or RNA molecules that can bind with high affinity and specificity to their target molecules. These artificial “nucleic acid” ligands can be generated against proteins, small molecules, ions, whole cells, tissues and organs [45]. Those specific nucleic acids, selected from random
sequence pools, have been shown to bind various targets that do not have a nucleic acid structure. The development of *in vitro* selection and amplification techniques has allowed the identification of specific Apts, which bind to the target molecules with high affinity. Many small organic molecules with molecular weights from 100 to 10,000 Da have been shown to be good targets for selection. Moreover, Apts can be selected against difficult target haptens, such as toxins or prions. The selected Apts can bind to their targets with high affinity and even discriminate between closely related targets. Apts can thus be considered a valid alternative to Abs or other biomimetic receptors, for the development of biosensors and other analytical methods [46]. The process to select Apts, known as *in vitro selection and amplification* or SELEX (from Systematic Evolution of Ligands by Exponential enrichment), was first reported in 1990, independently by two research teams [42].

Apts offer several advantages over Abs. Being isolated through an *in vitro* procedure, they can be easily adapted to completely non-physiological conditions and against targets that would otherwise be invisible to the immune system. The conditions of their selection process can be adjusted to change their kinetic parameters (i.e., $k_{on}$ and $k_{off}$ rates). Other advantages include the ability of Apts to denature and refold easily, with better shelf life and ease of attachment and detachment of the bound molecule. Their resistance in harsh conditions (temperatures, pH) and their ability to distinguish between similar proteins (e.g., mucin class) are also appreciated by scientists developing aptasensors [47]. The advantage of having a recognition region that can repeatedly refold as been effectively demonstrated recently in a microarray application [48], where denaturing conditions (e.g., with 7M urea) were used to regenerate free Apts that were refolded into their active conformations in the binding buffer after the measurement was completed. Such a procedure allows for multiple measurements with the same Apt.

Discovered in 1993 [49], microRNAs (miRNAs) are 22 nucleotide-long non-coding RNA molecules that regulate the expression of target genes at the post-transcriptional level by either translational repression or degradation of miRNAs [50]. It has been estimated that the human genome may encode over 1000 miRNAs, which are thought to regulate about 60 % of the mammalian genes. Altered expression patterns of miRNAs have been associated with many diseases, including several types of cancer, cardiovascular disorders, diabetes, rheumatic disease and neurological disorders [51]. Due to their unique miRNA expression signatures that have been demonstrated in many pathological conditions, these molecules have been proposed as novel biomarkers in diagnostics. The miRNA profiles in clinical samples will not only signify the presence of a disease, but can also help in the prediction of its course and help to select personalized treatments. miRNAs are currently detected in blood serum, plasma, urine, saliva, sweat and other bodily fluids. The levels of circulating miRNAs in serum were estimated to be in the 200 aM to 20 pM range [52]. The field of circulating miRNA analysis is in its early stages, but it has great potential due to the fact that miRNAs are relatively stable molecules and can be easily accessible in a non-invasive manner.
4. Immunosensors for biomedical and environmental analysis

There is a strong and continuous demand for fast, easy and cheap analytical devices for trace detection of compounds of clinical and environmental interest, as well as for pharmaceutical and food industries. The high selectivity and sensitivity of immunosensors make them the most appropriate for the detection and monitoring of the above mentioned domains. The recent developments made in the immunosensor field regarding the optimal orientation of Abs, incorporation of nanomaterials for increased sensitivity, multiplexing and microfluidic-based devices may have potential for promising use in industry and clinical analysis.

Some examples of assays for several commercially available biomarkers are presented in Table 1.

<table>
<thead>
<tr>
<th>Target</th>
<th>Method</th>
<th>LOD</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mucin 1 (MUC1) Free-PSA</td>
<td>Ab-lectins sandwich optical immunoassays</td>
<td>1.2 U/mL, 0.4 U/mL</td>
<td>[53]</td>
</tr>
<tr>
<td>Mucin 1 (MUC1)</td>
<td>ZnO nanorods QCM sensor</td>
<td>1 U/mL</td>
<td>[54]</td>
</tr>
<tr>
<td>MUC1</td>
<td>Electrochemical aptasensors based on AuNP</td>
<td>EIS: 3.6 ng/mL, DPV: 0.95 ng/mL</td>
<td>[55]</td>
</tr>
<tr>
<td>Total Prostate specific antigen (PSA)</td>
<td>White-light reflectance spectroscopy</td>
<td>0.20 mg/mL, 0.15 mg/mL</td>
<td>[56]</td>
</tr>
<tr>
<td>PSA</td>
<td>3D label-free immunosensor based on crumpled graphene-Au nanocomposites</td>
<td>0.59 mg/ml</td>
<td>[57]</td>
</tr>
<tr>
<td>Carcinoembryonic antigen (CEA)</td>
<td>Electrochemical 3D origami microfluidic paper based immunosensor</td>
<td>0.08 pg/mL, 0.06 mL</td>
<td>[58]</td>
</tr>
<tr>
<td>Carcinoembryonic antigen 125 (CA125)</td>
<td>SPR immunosensor</td>
<td>31.0 ng/mL</td>
<td>[59]</td>
</tr>
<tr>
<td>Myoglobin</td>
<td>Electrochemical aptasensor based on planar hall magnetoresistive transducer and superparamagnetic labels</td>
<td>86 pM</td>
<td>[61]</td>
</tr>
<tr>
<td>Troponin 1 (cTnI) C-reactive protein (CRP)</td>
<td>poly(dimethylsiloxane)-AuNP-QD electrochemical sensor</td>
<td>cTnI: 0.004 μg/L, CRP: 0.22 μg/L</td>
<td>[60]</td>
</tr>
<tr>
<td>Thrombin</td>
<td>Electrochemical aptasensor based on methylene blue and ferricene</td>
<td>170 pM</td>
<td>[62]</td>
</tr>
<tr>
<td>LDL cholesterol</td>
<td>Impedimetric immunosensor based on AuNP-AgCl/polyaniline composite film</td>
<td>0.34 pg/mL</td>
<td>[63]</td>
</tr>
<tr>
<td>LDL cholesterol</td>
<td>Impedimetric immunosensor based on reduced graphene platform</td>
<td>5 mg/dL</td>
<td>[64]</td>
</tr>
<tr>
<td>Creatinine</td>
<td>Electrochemical immunosensor based on polypyrrole immobilization platform</td>
<td>0.46 mg/dL</td>
<td>[65]</td>
</tr>
<tr>
<td>Influenza virus</td>
<td>Electrochemical DNA sensor based on avidin-biotin affinity reaction</td>
<td>8.51·10^{-14} M</td>
<td>[66]</td>
</tr>
<tr>
<td>Influenza A virus</td>
<td>Surface acoustic sensor based on Love waves</td>
<td>1 ng/mL</td>
<td>[67]</td>
</tr>
<tr>
<td>Legionella pneumophila</td>
<td>Electrochemical immunosensor based on ZnO nanorods</td>
<td>1 pg/mL</td>
<td>[68]</td>
</tr>
<tr>
<td>Neisseria gonorrhoeae</td>
<td>Electrochemical DNA sensor based on polyaniline films on In-SnO plates</td>
<td>0.5·10^{-14} M</td>
<td>[69]</td>
</tr>
<tr>
<td>HIV</td>
<td>Capacitive immunosensor based on polytyramine films</td>
<td>7.9·10^{-14} pg/ml</td>
<td>[70]</td>
</tr>
<tr>
<td>Clobutinol</td>
<td>ECL sensor with incorporated AuNP and QD</td>
<td>8.4 pg/mL</td>
<td>[71]</td>
</tr>
<tr>
<td>Ketamine</td>
<td>Impedimetric sensor based on SAM</td>
<td>0.41 pmol/L</td>
<td>[72]</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>Label free polypyrrole based immunosensor</td>
<td>1.0 pg/mL</td>
<td>[73]</td>
</tr>
<tr>
<td>Acetaminophen</td>
<td>Electrochemical immunosensor based on graphene oxide</td>
<td>0.17 μM</td>
<td>[74]</td>
</tr>
</tbody>
</table>
4.1. Immunosensors as diagnostic tools

The most important feature of immunosensors, the specificity of Ab–Ag reactions, makes them attractive candidates for developing diagnostic tools. Other advantages immunosensors may offer are: decreased medical cost by reducing, e.g., the hospitalization period due to their rapid analysis response; ease of use and miniaturization, making them suitable for “in-home” or “POC” use, thus improving the compliance of patients in follow-up therapy; and rapid
response, which can have a determining role in life-threatening diseases such as myocardial infarct or infectious diseases. Different molecules such as proteins, nucleic acids, metabolites, hormones, whole cells and various pathogens are bioanalytical targets for the development of diagnostic devices.

4.1.1. Immunosensors for detection of proteins

4.1.1.1. Cancer biomarkers

The high prevalence of cancer worldwide, together with its high recurrence and high mortality, creates an urge for rapid, reliable and sensitive detection methods, focusing the scientists’ attention on proteins that are frequently investigated as cancer biomarkers. Cancer biomarkers, like all other biomarkers, are generally defined as “a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacological responses to a therapeutic intervention” [97].

Their aberrant expression in biological fluids (serum, saliva) or cells and tissues can be an indicator of the presence of neoplastic processes. Because their abnormal levels appear from the first stages of cancer, they can play an important role in early stage detection, improving the survival rate of patients. Generally, more than one cancer biomarker is overexpressed for the same type of cancer. Therefore, in order to avoid false positive results, a panel for simultaneous detection of several significant cancer biomarkers is usually required.

Several strategies will be presented below for the detection of cancer protein biomarkers, such as Mucin 1 (MUC1, CA 15-3), prostate-specific antigen (PSA), cancer antigen CA125 and carcinoembryonic antigen (CEA), with an emphasis on electrochemical immunosensors reported in the last years.

Mucins are glycoproteins, either secreted or membrane bound, that have a specific pattern of expression altered during tumour progression, recently investigated as new potent biomarkers in epithelial cancers [98]. Among them, MUC1 has been more extensively studied, being involved in tumour proliferation and metastasis [99], as well as in chemoresistance and alteration of drug metabolism [100, 101]. Overexpression of MUC1 has been associated with breast, ovarian, pancreatic, liver, colon or lung cancer.

An Ab-lectin sandwich assay coupled with optical detection was proposed by Park et al. Abs against MUC1 were immobilized on a gold sensing surface and the protein was captured between Abs and lectins conjugated with fluoro-microbeads. Two different lectins were used: *Sambucus nigra* agglutinin and peanut agglutinin lectins. The fluoro-microbead guiding chip contained multiple sensing and fluidic channels and exhibited LODs of 1.2 and 0.4 U/mL, respectively, over the clinically important range of 1.25 to 25 U/mL [53]. Quartz crystal microbalance (QCM) sensors have gained increased attention lately due to their simple operating principles and the absence of labelling. A QCM sensor based on ZnO nanorods was developed by Wang et al. The label-free detection method exhibited a fast response time of 10 s and high sensitivity of 25.34±0.67 Hz/scale (1 U/mL) in the concentration range of CA15.3 from 0.5 to 26 U/mL [54]. Two simple aptasensors based on gold nanoparticle (AuNPs) modified screen-printed electrodes (SPEs) were reported for sensitive detection of MUC1. A
SAM of thiolated Apt was firstly formed on the surface of AuNP-modified disposable electrodes. The quantitative detection of MUC1 protein was achieved by electrochemical impedance spectroscopy (EIS), by measuring the changes in the charge transfer resistance after binding various concentrations of Ag, and by differential pulse voltammetry (DPV) using the signal of intercalated methylene blue (MB), which decreased after protein binding. LODs of 3.6 ng/mL by EIS and 0.95 ng/mL by DPV were obtained, in the linear range of 0-10 ng/mL [55]. Another Apt sandwich-based sensor for MUC1 detection was proposed, using Abs as capture probes and MB labels as detection probes. Specific Abs were immobilized on the poly(o-aminobenzoic acid) polymeric film (PABA) through the interaction between the COOH groups activated by the EDC/NHS chemistry and NH₂ groups of the Abs (Figure 5) [102].

Figure 5. Preparation of sandwich aptasensor for MUC1 detection: (a) o-ABA polymerization; (b, c) MUC1 monoclonal mouse Ab immobilization onto PABA-modified graphite SPEs by carboxyl activation; (d) PABA free binding sites blocking with 10 mM ethanolamine; (e) incubation with human MUC1 of different concentrations; (f) specific binding of Apt with human MUC1 Ag (g) interaction of MB with G bases in Apt; (h) DPV measurements [102]. “Reprinted from J. Electroanal. Chem. 717-718, Taleat Z, Cristea C, Marrazza G, Mazloum-Ardakani M, Săndulescu R. Electrochemical immunoassay based on aptamer–protein interaction and functionalized polymer for cancer biomarker detection, 119-124, Copyright (2014), with permission from Elsevier.”

After the reaction with Ag, the Apt binds to the immunocomplex which allows the accumulation of MB by interaction with the guanine bases of the Apt. DPV was then performed to measure the oxidation peak of MB. A linear response of the current with MUC1 concentration was observed in the range of 1-12 ng/mL with an LOD of 0.62 ppb [102]. Other strategies for
MUC1 detection include electrochemical aptasensors based on MB-labelled hairpin Apt, where the detection is based on the conformational changes of the labelled Apt after the binding of the protein [103] or on a dual signal amplification strategy, using poly(o-phenylenediamine)-AuNPs film for the immobilization of the captured Apt and AuNPs functionalized-silica/MWCNT core-shell nanocomposites as the tracing tag for the Apt detection [104].

PSA is produced by the prostate gland with normal levels ranging from 0 to 4 ng/mL, and it is overexpressed in prostate cancer. A label-free immunosensor based on white light reflectance spectroscopy for the determination of total- and free-PSA in human serum was reported. A two-site immunoassay was used, employing a biotinylated Ab in combination with streptavidin. High sensitivity was achieved with LOD of 0.2 and 0.15 ng/mL for total and free PSA, in the range of 0.5 to 100 ng/mL, and 0.5 to 20 ng/mL for total and free PSA, respectively [56]. Another interesting approach was used for the construction of a 3D label-free immunosensor for PSA-based crumpled graphene-gold nanocomposites (Figure 6). The graphene sheets decorated with AuNPs were prepared by aerosol spray pyrolysis. LOD of 0.59 ng/mL and a linear range of 0-10 ng/mL were achieved, due to the synergistic effect of graphene and Au, with increased biocompatibility, high level of bound Ag and fast electron transfer. The sensors also proved to be selective towards interferents such as alpha-fetoprotein, glucose, uric acid, bovine serum albumin and vitamin C [57].

Figure 6. Illustration of (a) 2D GR-Au and 3D GR-Au electrodes, (b) the formation of crumpled GR-Au composites via aerosol spray pyrolysis and (c) fabricating step of 3D label-free PSA immunosensor using crumpled GR-Au composites [57]. “Reprinted from Biosens. Bioelectron. 63, Jang HD, Kim SK, Chang H, Choi J-W. 3D label-free prostate specific antigen (PSA) immunosensor based on graphene-gold composites, 546-551, Copyright (2015), with permission from Elsevier”
CA125 is a glycoprotein that exhibits abnormal levels in ovarian, breast, liver, lung and gastric cancer, serving as biomarker for the diagnosis and therapeutic monitoring of these diseases. Since its discovery in the ovarian epithelia by Bast et al. [105], CA 125 has served as a target for the development of several label-free [106] or labelled electrochemical or optical sensors [107, 108]. CEA is another tumour-associated Ag used as a biomarker for clinical diagnosis of ovarian, breast and colon carcinoma, and was also widely investigated as a target for the development of optical or electrochemical sensors [109, 110, 111].

Efforts have been made to improve cost effectiveness and miniaturize diagnostic devices, as well as in multiplexing detection of several cancer biomarkers, which would reduce analysis costs and increase diagnose accuracy. Special attention has been given to microfluidic devices that reduce the volume of used reagents, are easy to use, transport and store, and give a fast analytical response. Paper-based platforms have also been investigated as low-cost and simple to use materials. For example, a 3D origami microfluidic paper-based immunosensor for simultaneous detection of CA125 and CEA using metal ions as electroactive labels was developed (Figure 7).

![Figure 7](image_url)

**Figure 7.** Origami EC immunodevice [58]. “Reprinted from Biosens. Bioelectron., 63, Ma C, Li W, Kong Q, Yang H, Bian Z, Song X, et al, 3D origami electrochemical immunodevice for sensitive point-of-care testing based on dual-signal amplification strategy, 7-13, Copyright (2015), with permission from Elsevier”

The support for the immobilization of captured Abs was constituted by a paper working electrode, modified with Au nanorods to ensure a large surface area and superior conductivity. For signal amplification, Au/BSA nanocarriers were used for loading Pd²⁺ and Cd²⁺, which were conjugated with anti-CA125 and anti-CEA for a sandwich assay. DPV measurements
were recorded and the quantification of the two different Ags was obtained, based on the different stripping peaks obtained for the oxidation of the two metal tracers. LOD of 0.08 pg/mL and 0.06 mU/mL were obtained and the developed microfluidic POC device was successfully applied for the quantification of the biomarkers in human serum [58].

An interdigitated electrode-based capacitive sensor was reported for multiple biomarker detection: CEA, epidermal growth factor receptor (hEGFR) and CA15-3, based on AuNP’s amplification. CEA and epidermal growth factor receptor (hEGFR) could be successfully detected in the concentration range of 20-1000 pg/mL, while cancer antigen 15-3 (CA15-3) was detected in the range of 10-200 U/mL, with LODs of 20 pg/mL and 10 U/mL respectively [112].

4.1.1.2. Cardiac biomarkers

Cardiovascular diseases include diseases of the heart and blood vessels and are a leading cause of death globally. The WHO estimated that deaths due to cardiovascular diseases in 2008 accounted for 30 % of worldwide deaths, and by 2030 the number of deaths per year will reach 23.3 million [113]. The main risk factors include smoking, alcohol abuse, hyperlipidaemia, high blood pressure, diabetes, physical inactivity and unhealthy diet. Myocardial infarct and stroke are acute events caused by obstructions of blood vessels or rupture of brain blood vessels. Rapid diagnosis in this case is crucial for the survival of patients. Beside signs and symptoms, current diagnosis of myocardial infarction is based on ECK and measurements of cardiac enzymes, while stroke is usually diagnosed using brain computed tomography, magnetic resonance imaging or carotid ultrasound. Other methods for diagnosis of cardiac conditions include echocardiography, chest X-ray, angiography or catheterization. Although ECK remains the main management method, some patients show normal ECK even in the presence of cardiopathies, and thus ECK may be inconclusive. Several markers exhibit elevated blood levels in the presence of heart conditions serving as diagnostic tools with important prognostic significance. As in the case of cancer, simultaneous multianalyte detection is preferred.

Myoglobin is released from injured muscle tissues and is a potential marker used in the diagnosis of myocardial infarction. However myoglobin is not specific for cardiac damage, and therefore it is usually used together with other biomarkers such as troponin or creatinine kinase MB subform (Ck-MB). Troponin I or T are proteins with important roles in muscle contraction that also detect cardiac damage as they are released in blood in elevated levels in case of cardiac muscle cell death. Ck-MB is an enzyme of the creatinine phosphokinase family specific to the heart cell; increased levels are present in case of heart muscle injuries. Serum levels of these markers rise rapidly within the first hours after a heart attack (myoglobin being the first to increase) reaching a maximum after several hours, depending on the size of the infarct [114]. Due to the fluctuation of the levels of cardiac markers, real-time monitoring is important for accurate measurements.

An SPR-based sensor for continuous monitoring of myoglobin levels in human serum was reported [59]. A recyclable Ab was immobilized at the surface of a label-free sensor that can quantify Ab-Ag reaction in real time. Two flow channels were used, one with specific Ab and the second, serving as control, with non-specific Ab. Buffered solutions and spiked serum
samples of different concentration were then injected for the formation of immunocomplexes. The problems associated with the continuous sensing of myoglobin in human serum were overcome as follows: the non-specific binding of the analyte was avoided by pre-treating the sample with a mixture of detergents, specifically sodium dodecyl sulphate and P20, and adjusting the micelle size and net charge; and the aggregation of serum components was avoided by inactivating certain serum components by chelation of heavy metals. The sensor was able to detect the varying concentration of myoglobin over about 8 h with periodic one-point calibration every 3 h, with an LOD of 31.0 ng/mL, in the range from 31 ng/mL to 2000 ng/mL [58]. An electrochemical sensor for simultaneous detection of cardiac markers Troponin I (cTnI) and C-reactive protein (CRP), a known marker for inflammation and cardiac damage, has been reported based on a poly(dimethylsiloxane)-AuNP composite microfluidic system. Captured Abs for the two proteins were conjugated with CdTe and ZnSe QDs and square-wave anodic stripping voltammetry was used to detect the metal ions from the QDs in order to quantify the amount of bonded proteins. A linear range was obtained between 0.01 and 50 μg/L and 0.5 and 200 μg/L, with a very low LOD of approximately 0.004 μg/L and 0.22 μg/L, for cTnI and CRP, respectively [60]. Another target biomarker for cardiovascular disease is thrombin, a serine protease involved in blood coagulation, which acts as both a procoagulant (converting soluble fibrinogen in insoluble fibrin) and anticoagulant factor. It is also involved in the process of atherosclerosis and its cardiovascular complications [115]. The first Apt for thrombin, synthesized via SELEX in 1992, has a G-quadruplex structure and binds exosite I of thrombin [116]. A highly specific aptasensor was developed in a sandwich-type assay using planar Hall magnetoresistive transducer in combination with superparamagnetic labels. The sensor surface was modified with thiolated Apt to capture the thrombin added in the next step. Finally, a biotin-labelled secondary Apt was added to bind the thrombin-primary Apt complex and streptavidin-magnetic nanoparticles were used to measure the magnetoresistance signal. A linear response to thrombin concentration in the range of 86 pM-8.6 mM and an LOD as low as 86 pM was achieved using low sample volumes of only 2uL [61]. Interestingly, a simple Apt-target-Apt sensor was elaborated using the signal obtained after the electro-oxidation of intercalated MB and ferrocene for the quantification of thrombin. MB-tagged Apts were introduced by hybridization with complementary DNA sequences immobilized on the surface of the electrode. Thrombin was then added and its interaction with the Apt led to conformational changes of MB-Apt, driving the MB tag far from the electrode surface. A second Apt, tagged with ferrocene on both sides, was added to interact with thrombin, and the Apt’s conformational changes allow the two Fc tags to be close to the electrode surface. These conformational changes resulted in a decrease in the oxidation peak current of MB (I_{MB}) and an increase in that of Fc (I_{Fc}), and the logarithmic value of I_{Fc}/I_{MB} was shown to be linear with the logarithm of thrombin concentration. An LOD of 170 pM was achieved, in the linear range of 1 nM to 600 nM [62]. Several label-free aptasensors for thrombin detection coupled with electrochemical [117, 118] or optical detection [119, 120] were reported.

4.1.2. Immunosensor for detection of metabolites

The detection of metabolites has gained increasing attention in recent years for the diagnosis of various diseases, metabolites being correlated with different pathologies. Glucose, for
example, exhibits high levels in patients with diabetes and its monitoring plays a crucial role in the management of the disease. The devices used for the detection of glucose are mainly constituted by enzymatic sensors. The well-known glucometer accounts for 85% of the market of commercially available biosensors [121]. Other metabolites of great interest are cholesterol and triglycerides, due to their involvement in atherosclerosis and in the pathology of cardiovascular diseases. Creatinine is a metabolite of creatine, eliminated by kidneys and excreted in urine, and it has become the preferred clinical marker for the diagnosis of renal filtration dysfunctions. The development of POC diagnostic devices could have an important impact on the improvement of the quality of life of patients suffering from metabolic disorders. Several POC devices for the detection of creatinine are already commercially available: IRMA TRUpoint (ITC, Edison, NJ), Radiometer ABL800 FLEX (Radiometer A/S, Bronshøj, Denmark), StatSensor (Nova Biomedical, Waltham, MA), and i-STAT (Abbott Diagnostics, East Windsor, NJ).

An interesting approach for detecting low-density lipoprotein (LDL) cholesterol was proposed based on immobilization of a large number of Abs on AuNP-silver chloride/polyaniline composite film. Abs specific to apolipoprotein 100 were chosen that recognized and bound apolipoprotein B present in the phospholipidic coats of the structure of LDL. EIS studies were performed to monitor the formation of Ab-Ag complex using $\text{Fe(CN)}_6^{4-/3-}$ redox probe. An increase in the charge transfer resistance was observed after the binding of LDL, due to its low conductivity coupled with the repulsion between negatively-charged phospholipid units and the negative charge of the redox probe. This simple, label-free immunosensor allowed for the detection of low concentrations of LDL of 0.34 pg/mL, in the linear range of 0-33.5 pg/mL [63]. With a similar approach, an impedimetric label-free sensor was developed using aminated reduced graphene oxides for the immobilization of anti-apolipoprotein B on the electrode surface. The aminated graphene oxides were synthesized chemically and deposited on the surface of the transducers through electrophoretic deposition. EDC/NHS was then used for covalent attachment of Abs via amide bond formation, followed by the addition of LDL solutions. The change in the resistance after the formation of Ab-Ag complexes allowed for the detection of higher concentrations of LDL of 5 mg/dL [64].

Besides metal nanoparticles and carbon-based nanomaterials, conductive polymers have been widely applied, for many years, as immobilization platforms for the development of immunosensors, due to their low cost, ease of preparation, and most importantly good biocompatibility. A simple electrochemical sensor was developed for the detection of creatinine in whole blood obtained from patients with renal transplant. The surface of a 16-gold electrode array was covered with a film of polypyrrole via electropolymerization, by applying a cyclic square-wave electrical field. Creatinine was embedded in the film in the same step. Anti-creatinine Abs labelled with HRP were mixed with a low volume of blood sample containing creatinine for the formation of immunocomplexes and added to the array for competitive reaction with the immobilized Ag. The decreased current of the HRP-Ab is proportional to the level of creatinine in the samples in the linear range of 0 mg/dL to 11.3 mg/dL with an LOD of 0.46 mg/dL. The method has the advantage of short response time <5 min and low sample volume (40 μL) [65].
4.1.3. Immunosensors for detection of pathogens

Infectious diseases have increased rapidly in recent years, causing high fatality rates due to incorrect diagnosis, delay in treatment and various complications. Worldwide, infectious diseases account for nearly 40% of the total 50 million annual estimated deaths, and represent the major cause of death in many developing countries [122].

Infections are caused by pathogenic microorganisms, such as bacteria, viruses, fungi or parasites. Most of the infections are highly contagious and can spread rapidly with the risk of causing epidemic or pandemic. Rapid and portable devices for the detection of pathogens are of paramount importance. Rapid screening for differentiation between viral and bacterial infection is extremely important for diagnosis and correct management of disease, limiting the overuse of antibiotics and reducing antibiotic resistance. A correct, rapid diagnosis may reduce the need for or period of hospitalization, with a high impact on medical costs. Current diagnosis is mostly based on cell culture and microscopic methods, serology or nucleic amplification testing, which are time-consuming. Most of the existing POC tests consist of immunoassays: agglutination, immunochromatographic and immunofiltration tests [123]. The development of portable devices that can be used at home or in doctors’ offices may permit a rapid diagnosis of various infections, allowing quick establishment of treatment and fast recovery by the patients. POC devices also have the advantages of being used in biological fluids with no need for complex sample pre-treatment.

Most of the sensors reported in the literature for the detection of pathogens are based on electrochemical techniques, due to their simplicity, low cost, rapid response and suitability for developing in small portable devices. The majority of these devices employ nucleic acids as recognition elements for the pathogenic agent, based on hybridization processes with a complementary DNA strand immobilized onto transducers, intercalation of redox mediators in double-stranded DNA, or label-free detection based mainly on impedimetric measurements.

Influenza is the most common acute respiratory infection, accounting for 52,294 deaths in the USA in 2011 [113]. It is caused by influenza type A, B and C viruses; the type A virus being responsible for the annually recurring epidemics. Mutations can also occur (e.g., avian or swine flu), raising health concerns.

A simple electrochemical DNA sensor was proposed based on avidin-biotin reaction for the immobilization of DNA capture probes on the surface of glassy carbon electrodes (GCEs). The surface of the electrode was firstly functionalized with carboxylic groups using 4-carboxyphenyl diazonium salts. Avidin was then bound, after ethylene carbodiimide/N-hydroxysuccinimide activation. Afterwards biotinylated DNA probes were immobilized through avidin-biotin interaction followed by the hybridization with target DNA. The hybridization process between the probe (5-biotin-ATGAGTCTTCTAAACCAGGTCGAA-3) and the target DNA (5-TTCGACCTCGGTTAGAAGACTCAT-3, a fragment of influenza virus gene sequence) was investigated by CV. A dynamic range between 10^{-13} M and 10^{-10} M was achieved with an LOD of 8.51 10^{-14} M [66].
Another label-free electrochemical immunosensor was reported for swine flu influenza virus (SIV), based on a self-assembled SWCNT network modified with anti-SIV Abs. The change in resistance upon binding of the Ag permits LODs of 180 TCID 50/mL (50 % Tissue Culture Infective Dose) to be achieved [124].

Piezoelectric techniques have attracted interest lately. A surface acoustic sensor based on Love waves was recently developed for the detection of influenza virus type A. SiO$_2$ coated piezoelectric LiNbO$_3$ wafers were used to bind Ags against haemagglutinin (HA) of influenza A H1N1 subtype. After a silanization process of the wafers using tri-ethoxy-silyl-butyrpaldehyde and tri-ethoxy-silyl-undecanal ethylene-glycol-acetal, and the reaction of the silan groups with EDC/NHS, the substrates were immersed in a solution containing anti-HA Ags. Phycoerythrin-conjugated Abs were immobilized for fluorescence microscopy analysis in order to check the successful binding of HA. Non-conjugated Abs were used for surface acoustic wave testing giving an LOD of 1ng/mL [67].

*Legionella pneumophila* is a bacterium that causes Legionnaires’ disease and Pontiac fever, life-threatening diseases with high mortality. An electrochemical immunosensor based on ZnO nanorods was reported on its detection. The nanorods were prepared by hydrothermal method at low temperature, and used to immobilize primary Abs by electrostatic interactions. The Ag was then captured by the recognition of its peptidoglycan-associated lipoprotein. A sandwich ELISA-like assay was performed by adding secondary Abs labelled with HRP in the next step, and the electroactivity of the product of the enzymatic reaction was investigated. A dynamic range of 1-5000 pg/mL was obtained in this case with an LOD of 1 pg/mL [68].

Sexually-transmitted diseases (STDs) are viral or bacterial infections transmitted from one human to another through unprotected sexual contact. The most common viral STDs are HIV/AIDS, hepatitis B, herpes and human papillomavirus, while for bacterial infections the highest numbers of cases are diagnosed for gonorrhoea, chlamydia and syphilis. The incidence of these diseases is worryingly high; approximately 20 million people will contract an STD each year in the USA, and half of the infections occur in the age range 15-24 [125].

A simple device was developed for the detection of *Neisseria gonorrhoea* by electropolymerization of polyaniline films on indium-tin-oxide plates. Avidin was then immobilized on the films after EDC/NHS activation to bind biotinylated ssDNA. The hybridization process with the target DNA and the amount of bound DNA was investigated by DPV and EIS measurements. DPV measurements have been performed using two methods: the first is based on monitoring of the oxidation peak of guanine in the capture probe, which decreased after the binding of complementary DNA; the second approach used the oxidation peak of intercalated MB for the monitoring of the hybridization process. The MB peak disappears after hybridization with the complementary oligomer, due to steric inhibition of MB packing between the double helix of the hybrid. EIS measurements confirmed the hybridization: an increase in the impedance was observed due to electrostatic repulsion between DNA and the redox probe. An LOD of 0.5 $10^{-15}$ M was obtained [69].

Human immunodeficiency virus (HIV) causes acquired immunodeficiency syndrome (AIDS) and is transmitted mainly by unprotected sexual intercourse or use of unclean syringes [126].
A rapid, label-free capacitive immunosensor has been developed for real-time analysis of p24 Ag, a HIV-1 capsid protein that is detectable in serum shortly after infection. Silicon wafers were nanopatterned with gold via photolithography. A polytyramine film was then electrodeposited on the surface of the transducer, which chemisorbed AuNPs through the amine groups. Anti-HIV-1 p24 monoclonal Abs were then immobilized to capture the Ag from injected buffered or plasma samples, and the capacitance change was investigated. A linear relationship was observed from $2.4 \times 10^{-6}$ to $2.4 \times 10^{-3}$ pg/mL with an LOD of $7.9 \times 10^{-8}$ pg/ml [70].

A simple electrochemical ELISA-type sensor for the detection of HIV-1 and HIV-2 Abs was recently reported. HIV-1 gp41 and HIV-2 gp36 Ags were immobilized at the surface of the transducers followed by the addition of anti-HIV Abs and IgG labelled with alkaline phosphatase. The substrate p-aminophenyl phosphate was added to produce the redox-active product, p-aminophenol. It was observed that its oxidation peak increased linearly over a wide Ab concentration range (0.001-1μg/mL), with an LOD of 1 ng/mL (6.7 pM) for both HIV-1 and HIV-2 [127].

Hepatitis is an inflammatory condition of the liver affecting many people worldwide, caused by various types of hepatitis viruses (A-E). Hepatitis B and C are chronic diseases causing cirrhosis and liver cancer [128].

An optical immunosensor for the detection of hepatitis C virus Abs was developed using the photoimmobilization method [129]. For this purpose, a photoactivatable electrogenerated polymer film was deposited on the surface of conductive fibre optics in order to link the bioreceptor to the fibre tip through light mediation, and a solution containing HCV-E2 envelope protein Ag was added and illuminated with UV light. Due to the photochemical reaction, a thin layer of the Ag is covalently attached to the polypyrrole-benzophenone-modified surface that is able to bind anti-E2 Abs from samples. Chemiluminescence measurements allowed anti-E2 Ig detection at a low titer [129].

A disposable impedimetric sensor was reported for the detection of *Streptococcus*, based on gold SPEs modified with polytyramine film. Avidin was bound through the polymer amine groups, and the biotinylated Ab was then immobilized. The sensor was tested in (50 % (v/v) human saliva, resulting in a detection range of 100 to 105 cells per 10μL and 100 to 104 cells per 10 μL of bacteria, for cumulative and single-shot incubations, respectively [130].

4.2. Immunosensors for drug safety

Therapeutic monitoring is important for drug adjustment to achieve optimum efficiency and minimal toxicity of the drug. Ideally, the concentrations of drugs should be measured at the site of the action - the receptor - but due to its inaccessibility, drug concentrations are measured in bodily fluids such as serum, plasma, saliva, urine or cerebrospinal fluids [131]. The most commonly used techniques for establishing drug levels for drug monitoring such as HPLC, GC-MS, LC-MA, radioimmunoassay, chemiluminescence, etc., are time- and reagent-consuming, require qualified personnel and complex sample pre-treatment, and are expensive. Therefore, there is an urge to develop new, minimally invasive devices for drug detection that...
could be easily used in homes, laboratories and clinics. Special attention should be given to targeting drugs with a narrow therapeutic range, which may be easily over- and under-dosed.

A simple ECL sensor was developed for the detection of clenbuterol, a drug used for pulmonary diseases, incorporating AuNPs and QDs for enhanced signals. The AuNP/ovalbumin-clenbuterol/anti-clenbuterol-QDs sensor was characterized using EIS and ECL emission achieving LOD of 0.0084 ng/mL in the range of 0.02-50 ng/mL [71]. An impedimetric sensor based on 3-mercaptopropionic acid SAMs was reported for the sensitive and selective detection of ketamine, an anaesthetic drug. The immobilization of Abs via SAM and the recognition of the target Ag were evaluated by the change in the charge transfer resistance, allowing for the detection of ketamine levels of 0.41 pmol/L [72]. EIS has also been employed for the detection of ciprofloxacin by a label-free immunosensor, which is widely used for the treatment of pulmonary, urinary or digestive infections. Polypyrrole-NHS electrogenerated films were used to immobilize anti-ciprofloxacin Abs, with an LOD of 10 pg/mL being obtained [73].

Developing new methods for drug quantification is also important in the quality control of drug formulations in the pharmaceutical industry. A label-free immunosensor based on the graphite oxide immobilization platform was proposed to quantify acetaminophen from pharmaceutical formulations. Anti-acetaminophen Abs were linked via amide groups by NHS/EDC chemistry on graphene oxide-modified SPEs. The preparation steps were investigated by several electrochemical techniques such as SWV, EIS and EQCM, and the LOD found by SWV measurements was 0.17 μM [74].

Another important social and health issue is drug abuse and doping [132]. The development of fast, sensitive and specific screening tests is gaining increasing interest, mainly in sport, with special attention being given to the development of portable on-site detection methods for doping agents. Several devices for the detection of such drugs from saliva are already commercially available, for example, a gold particle-based lateral in-flow immunoassay named Oratect is available for the detection of marijuana (THC), cocaine, amphetamines, opiates, methamphetamines including MDMA (ecstasy) and either phencyclidines or benzodiazepines. Some of the most commonly used drugs for sport doping are beta-blockers, steroidal hormones, growth hormones, theophylline and derivatives, peptides and methamphetamines.

A simple and sensitive electrochemical sensor was developed for the simultaneous detection of two illicit drugs: morphine and methamphetamine. The simple approach employed SAM of 3-mercaptopropionic acid on a gold electrode that served to bind Abs specific for the targeted drugs. CV and EIS were employed as electrochemical techniques, revealing a decrease in the current together with an increase in the impedance after Ab binding and immunocomplex formation, due to the hindrance of electron transfer from the redox probe to the surface of electrodes. The sensors were able to detect morphine and methamphetamine in the linear range of 4 to 80 pg/L and 20-200 pg/L, respectively, with an LOD of 0.27 pg/L and 10.1 pg/L. The sensor is stable, reusable and may also be used in serum samples [133]. Another anti-doping device has been reported for the detection of human growth hormone, which is usually associated with anabolic steroids used to improve sporting performance. An SPR platform-based sandwich assay was developed using avidin-biotin coupling for the proper orientation.
of primary Abs, and the obtained sensor allowed the simultaneous detection of both hCG isoforms (20K and 22K) in concentrations as low as 0.9 ng/mL [134].

4.3. Immunosensors for food safety

Detection of food contaminants is a major issue, since these contaminants may have serious consequences for food safety and human health, as well as for medical and economical costs. The rapid and sensitive detection of food contaminants is important to prevent intoxication and provide efficient treatment [135]. Diseases caused by food contaminants may vary widely from several million cases to 81 million cases in the USA, with bacterial foodborne outbreaks accounting for 91 % of the total outbreaks [122].

The most common foodborne pathogens include *Escherichia coli* O157:H7, *Salmonella typhimurium*, *Listeria monocytogenes*, *Staphylococcus aureus*, *Clostridium perfringens*, *Enterobacter sakazakii*, *Campylobacter jejuni* and *Yersinia enterocolitica*. The most common methods for their identification, such as the conventional cell culture and microscopy, as well as the more recently applied methods such as ELISA and PCR, are time-consuming, and therefore immunosensors, through their advantages, are promising tools. An electrochemical immunosensor for the simultaneous detection of *Escherichia coli* O157:H7 (E. coli O157:H7) and *Enterobacter sakazakii* (E. sakazakii) was reported using carbon screen-printed low-density arrays modified with MWCNTs/sodium alginate (SA)/carboxymethyl chitosan (CMC) composite films. The film served for the immobilization of HRP-labelled Abs against the two pathogens. The array exhibited a linear response from $10^4$ to $10^{10}$ cfu/mL with LOD of 4.57 $10^4$ cfu/mL for *E.coli* and 3.27 $10^4$ cfu/mL for *E. sakazakii* [136].

*Salmonella* is another dangerous foodborne pathogen causing infections with various clinical symptoms depending on the serotype, ranging from diarrhoea, fever and abdominal cramps to more severe illnesses such as Reiter’s syndrome that can lead to chronic arthritis. About 1.4 million salmonellosis cases occur annually in the United States, resulting in about 16,000 hospitalizations with nearly 600 deaths [137].

An impedimetric immunosensor was developed for the detection of *Salmonella typhimurium* in milk samples. Gold electrodes were functionalized with: single 11-aminoundecanethiol (MUAM); a mixture of MUAM and 6-mercaptop-1-hexanol (MCH); and a mixture of MUAM, MCH in the presence of triethylamine (TEA) to prevent formation of interplane hydrogen bonds among amine-terminated thiols. Specific Abs were then immobilized via SAM that served to capture the Ag from samples, resulting in changes of the resistivity of the solution. Signal amplification was obtained by performing the measurements in the sample media, which offered several advantages such as: higher response in shorter time due to simultaneous proliferation of the viable bacteria cells in the tested samples, insensitivity to the presence of dead cells, and elimination of the centrifugation and washing steps that are normally used to isolate bacterial cells. The results showed the importance of MCH in SAMs while the addition of TEA had rather negative effects. For a detection time of 2 h, the MUAM-MCH-based sensor provided reliable analytical signals for a concentration of three orders of magnitude lower than the infectious dosage of *S. typhimurium* [138].
Listeria monocytogenes can be transmitted to humans through consumption of contaminated food, such as smoked fish, poultry, meat and dairy products, and ready-to-eat foods [139, 140]. An impedance immunosensor for the detection of Listeria monocytogenes using TiO₂ nanowire bundle microelectrodes as the Ab immobilization platform was reported. The TiO₂ nanowire bundle was prepared through a hydrothermal reaction of alkali with TiO₂ powder and connected to the gold microelectrodes by mask welding. Impedance changes were investigated after the formation of nanowire-Ab-Ag complexes and correlated to bacterial number. The immunosensor was able to specifically detect 10 cfu/mL Listeria monocytogenes [141]. Ochratoxin A (OTA) is a thermostable mycotoxin produced by species of Aspergillus or Penicillium fungi, which contaminates a high variety of food stuffs, such as cereals, dried fruits, coffee, grapes, wine and beer, with nephrotoxic and carcinogenic effects on mammalians [142].

An Apt-based biosensing approach based on an evanescent wave all-fibre (EWA) platform with embedded dethiobiotin-modified fibres was recently reported. In a simple target-capturing step using Apt-modified magnetic microbeads, signal probes (represented by Apt-complementary oligonucleotide) conjugated with streptavidin are released and further detected by an EWA biosensor via facial dethiobiotin streptavidin recognition. A reusable, sensitive sensor was thus obtained that can maintain its response after being reused 300 times, with an LOD of 3 nM in the linear range of 6 nM to 500 nM [75].

OTA was also detected in wine samples using two indirect competitive ELISA-type immunoassays coupled with electrochemical methods. pAb and mAb were investigated for the capture of the Ag and different enzymatic labels, horseradish peroxidase (HRP) and alkaline phosphatase (ALP) were used and compared. The results showed that although similar performance was observed in buffer solution for the HRP-Ab-based strategy and the ALP-conjugated Ab sensor, when it comes to wine samples, the signal for the ALP-based sensor was affected by interferences. LODs of 0.7 ng/mL and 0.3 ng/mL were obtained for HRP- and ALP-labelled sensors [143].

4.4. Immunosensors for environmental control

Environmental air pollution and water control monitoring have become areas of intense interest in the research field due to the serious consequences for public health. Gas sensors are generally used for the detection of indoor and outdoor air pollutants such as carbon monoxide, formaldehyde, ethylene oxide, nitric oxide, hydrogen sulphide or chloride. Many contaminants infiltrate the soil, arriving in wastewaters and drinking water. These contaminants belong to various classes such as hormones, antibiotics, surfactants, endocrine disruptors, human and veterinary pharmaceuticals, X-ray contrast media, pesticides and metabolites or explosives, and are typically detected at concentrations ranging from 1 ng/L to 1 μg/L, reaching in some cases as much as 100 μg/L [144]. Water pollution is harmful to human, animal and water life, resulting in death of aquatic animals, disruption of food chains and diseases. The effects of consuming contaminating water can range from mild effects (diarrhoea, vomiting and nausea) to more severe diseases or even death. Pesticides, for example organochlorines, organophosphates, carbamates, pyrethroids and atrazine, can cause neurological effects, asthma, allergies and endocrine disruption, and even cancer and reproduction problems.
Other contaminants like nitroaromatic explosives, found in soil and groundwater near military zones, are toxic and carcinogenic to living organisms, causing aplastic anaemia, toxic hepatitis, hepatomegaly, cataract and skin irritation.

Standard methods for water quality analysis involve intensive sampling regimes and multistep sample preparation, requiring manual inputs which prohibit their integration into continuous monitoring systems, resulting in the need for developing new monitoring methods that can be easily integrated into water flow systems [145] such as electrochemical immunosensors.

Kanso’s group [76] reported on the development of electrochemical immunosensors based on magnetic beads (MBs) for the detection of oestradiol and ethinyl oestradiol in wastewaters. Oestradiol and ethinyl oestradiol are water contaminants found at levels of nanomolar range, which act as endocrine-disrupting chemicals, interfering with the endocrine systems and causing breast and testis cancers in humans. MBs were chemically modified with four different types of synthetic oestrogen derivative that contained carboxylic or amino groups. The assay was based on the competition between free oestrogen in the sample and immobilized oestrogen using a primary Ab labelled with ALP. Using SWV, an LOD of 1 and 10 ng/mL was obtained [76]. An interesting approach for a high-throughput microfluidic immunosensor for the detection of explosives in water has been presented by Adams et al. Immunoaffinity and fluorescence detection assays were coupled on a microfluidic device containing 39 parallel microchannels with immobilized Abs against TNT. With a total volume throughput of less than 6 mL/min, the assay is performed 60 times faster than the usual immunoassay, achieving an LOD of 0.01 ng/mL for TNT [77].

An electrochemical immunosensor for the simultaneous detection of pesticides, endosulphan and paraoxon was reported. Microcontact printing was used to pattern an SWCNT forest on the surface of transducers using diazonium salt chemistry. Redox probes of 1,1’-di(aminomethyl) ferrocene (FDMA) and pyrroloquinoline quinone (PQQ) were linked to the nanotubes, followed by Ab immobilization. Pesticides were quantified based on the current change of the redox probes upon the formation of immunocomplexes: the electrochemical signal of FDMA allowed the detection of endosulphan in the range of 0.05 to 100 ppb with an LOD of 0.05 ppb, while PQQ signal allowed paraoxon detection in the range from 2 to 2500 ppb with an LOD of 2 ppb [78].

5. Conclusions and perspectives

New approaches using magnetic nanoparticles, quantum dots, carbon nanotubes, noble metal nanoparticles or hybrid nanomaterials, either as labels or immobilization platforms, have increased the number of electrochemical immunosensors and their applications. These materials offer important advantages, such as good biocompatibility, which maintains bioreceptor activity, a high surface-to-volume ratio, which increases the amount of immobilized bioreceptor, and unique conductivity and optical proprieties. Their high selectivity and sensitivity, as well as the possibility to miniaturize these systems, have made the use of electrochemical immunosensors for in vivo applications possible.
Although numerous developments have been made in the field of immunosensors, novel techniques are still needed to improve the selectivity, sensitivity and simplicity of these devices, ready to meet the exigent requirements of clinical diagnosis or industry. The need for miniaturization and integration on an electronic platform able to detect the biomarkers or pathogens in real time are other challenges in this active area of research. In the past decade, remarkable progress in nanotechnology techniques has been made, together with new strategies using nanoparticles and nanostructured surfaces for electrochemical detection of a large variety of proteins. Low-cost, reliable, portable, multiplexed protein detection devices, especially those coupled with microfluidics, could ensure accurate detection of panels of cancer biomarkers in blood, urine or saliva. The application of immunosensors in drug abuse or doping as well as in the quantification of pathogens was achieved using various immobilization nanoplatforms for immobilization, and also using multienzyme labelling on metal nanoparticles, magnetic beads and carbon nanotubes or graphene, allowing for the ultrasensitive detection of target analytes.

The integration of electrochemical immunoassays into microfluidic platforms will create a versatile platform for the fabrication of devices for clinical diagnostics. The development and deployment of these systems could ultimately lead to more rapid clinical decision-making, reducing patient stress and health care costs.

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References


