TRENDS AND PERSPECTIVES IN IMMUNOSENSORS

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ABSTRACT

Immunosensors are devices that comprise both a biomolecular recognition system, such as an antibody and its corresponding antigen, and a transducer to translate the high affinity and specific binding event into a physical signal.

Antibodies are produced by an immunological response to the presence of a foreign substance called an antigen. Antibodies may be immobilised onto a variety of platforms including bulk planar surfaces and nanoparticles by either covalent or adsorption strategies. Different interfaces between the bio-components and the detector are available to monitor in ‘real-time’ the signal generated by biological interactions. The transducers detect, for example, the change in electron transfer, absorbance, fluorescence, refractive index, mass change or heat transfer as the antibody binds to the antigen of interest. Such analytical devices have allowed a wide range of analytes to be identified and quantified such as pathogens, toxins, environmental food contaminants and disease biomarkers.

The demand for sensitive, rapid, ‘on-site’ techniques has taken advantage of the latest advances in microfluidics and nanotechnology. This chapter will highlight current trends in immobilisation, micro/nano-fluidics/ and transducers utilised. A number of examples outlining the exploitation of these elements in immunosensors and their successful applications will be described.

1. INTRODUCTION

Conventional immunoassays such as enzyme-linked immunosorbent assay (ELISA) can be complex and laborious to perform. They are often time-consuming, need to be carried out by highly-trained staff with expensive and specialised equipment, and thus are difficult to automate. Long incubation steps are required for the analytes to diffuse from the solutions to the solid platform and for the antibody (Ab)-antigen interaction to reach equilibrium. In addition, they involve numerous washing steps to ensure the absence of any non-specific binding. The small ratio between the low platform surface and the large volume of the different solutions can also affect the sensitivity and efficiency of the assay.

Immunosensors are a good alternative to traditional immunoassays. They use a recognition element such as an Ab or antigen in combination with a transducer. They allow the observation and analysis of the Ab-antigen interaction in ‘real-time’, with or without the need for a label. The binding is detected on the platform, either directly or indirectly, by a transducer providing a signal when the reaction occurs. The two main formats used for immunosensors are sandwich and competitive assays. When immobilising the Ab, it is of crucial importance that the method of immobilisation maintains the stability and activity of

* ‡ CV and CL contributed equally
the Ab. Immunosensors can be used to monitor the presence of the Ab or antigen and either
the Ab or the antigen can be immobilised or labelled depending on the assay requirements.

The basic structure of an Ab is a Y-shaped molecule made of two heavy (H) and two light (L)
polypeptide chains held together by disulfide linkages (-S-S-). Each chain consists of
constant (C) and variable (V) regions (Fig. 1). The H chain is composed of a variable region
(VH), responsible for binding to the antigen, and three constant regions (CH1, CH2 and
CH3). The light chain is made of one variable region (VL) and one constant region (CL). The
antibody binds to the antigen through the variable light (VL) and heavy (VH) domains
forming the hyper-variable regions of the antibody, known as the complementarity
determining regions (CDRs). The selection of the Ab to be used on the immunosensor is a
crucial step to develop a sensitive and reliable assay. The chosen Ab must be specific, have a
stable interaction with its target and be able to detect the antigen at the required concentration
range. The development of specific Abs involves immunising living hosts by injecting a
foreign substance, the antigen, leading to an immunological response. Polyclonal,
monoclonal and recombinant Abs can all be used for the development of immunosensors.
Polyclonal Abs, generated by multiple plasma cells, are cheap and easy to produce and can
recognise a large number of epitopes. On the other hand, they can lack specificity.
Monoclonal Abs are specific to an individual epitope as they are generated using hybridoma
technology. Cheaper but less stable, recombinant Abs are the product of phage display
technology. Genetic engineering and biopanning of Ab libraries allow for the selection of
highly specific and sensitive recombinant Abs [1].

Fig (1). Schematic representation of an immunoglobulin G (IgG). The antibody structure is
made of two heavy and two light chains held together by disulfide linkages.
Immunosensors use optical, electrochemical and mass transducers. For each situation and each compound to be analysed, adaptation of the different types of immunosensor may be required. Several factors can affect the practicality of an immunosensor design. It will need to be optimised in order to reach a high level of sensitivity, efficiency, accuracy and reproducibility. Ease-of-use and low production cost targets need to be reached in addition to meeting the market demands and complying with regulatory authorities.

2. IMMOBILISATION OF RECOGNITION ELEMENTS

The attachment of a capture Ab or antigen to an assay surface requires careful consideration. Both the nature of the assay system and the substrate used will determine the strategies required for Ab immobilisation. Immobilisation of active recognition molecules at high-density to the sensor surface is one of the most critical steps in biosensor development. This aspect of immunosensor design will determine whether the immobilised Ab is correctly oriented to favourably interact with the target antigen with minimal steric hindrance.

![Diagram of antibody structures](image)

**Fig. (2).** Formats of recombinant Abs: The scFv fragment consists of the VH (red) and VL (green) chains connected by a flexible linker at their terminal ends. A scAb fragment incorporates an additional constant light chain added to the terminal of VL. The Fab fragment contains the VH (red) and VL (green) chains with both constant heavy (blue) and light (grey) chains. Figure adapted from [1].

Entire Ab’s have estimated molecular dimensions of 15 x 7 x 3.5 nm, while the smallest Ab fragments, i.e. single-domain Ab fragments, are only 4 x 2.5 x 3.5 nm (Fig. 2) [2]. Whole Ab’s when immobilised may tilt due to the presence of the Fc region, or lie on their side, leaving the antigen binding sites close to the assay surface [3]. Pronounced steric hindrance may result, in particular if the antibody reacts with a large-molecular-weight antigen. Ideally,
specific recognition of the target antigen should occur at the lowest possible concentration. Therefore, the smaller the Ab fragment, the more can be immobilised onto the surface. This increased Ab fragment density should theoretically result in enhanced detection sensitivity of the target antigen. Cho et al. [3] experimentally confirmed this when an enhanced binding capacity was reported from an assay using Fab fragments compared to full Abs (Fig. 2). The authors carried out a sandwich-type immunoassay for the cardiac biomarker, Troponin I (cTnI), where the detection limits were 1.01 and 0.13 ng mL\(^{-1}\), for the full Ab and Fab fragment respectively. The authors attribute the different analytical performances to variable Ab densities on the assay surface, caused by the differing packing ability of full versus fragmented Abs.

Unfortunately, proteins and Abs in particular, may lose their biological activity when immobilised on a surface. This can be due to a change in conformation when binding with the assay surface and/or unfavourable orientation of the active site. Non-specific adsorption of Abs on surfaces also frequently causes inactivation. Furthermore, Ab modification, such as engineering or labelling, can diminish the antigen binding ability. Binding may be particularly diminished if the modification occurs on antigen binding sites, which is often the case for random chemical tagging of Abs. The ideal choice of immobilisation method should not significantly change the ability of the Ab to bind its target antigen.

The spacer between the Ab and the immobilisation surface also plays a role in the final Ab–antigen interaction [4]. Direct conjugation of Abs to surfaces often hinders antigen binding (steric hindrances) and limits the mobility of the bound Abs. Immobilising Abs via flexible spacer chains can lead to target antigens being captured twice as efficiently as directly-linked Abs [4]. This technology is adapted by Biacore (GE Healthcare) where they use carboxymethylated dextran as a linker, which is attached to a gold sensor surface. Ab or antigen binding to the dextran layer results in a high binding capacity giving a high sensor response, which Biacore recommends as “advantageous for capture assays”.

<table>
<thead>
<tr>
<th>Immobilisation method</th>
<th>Advantages</th>
<th>Disadvantages</th>
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<tbody>
<tr>
<td>Adsorption</td>
<td>Simple method of immobilisation</td>
<td>Random orientation</td>
</tr>
<tr>
<td></td>
<td>No Ab modification</td>
<td>Ab denaturation</td>
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<td></td>
<td>High level of immobilisation achieved</td>
<td>Non-specific protein binding</td>
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<tr>
<td>Covalent coupling</td>
<td>Stable immobilisation</td>
<td>Random orientation</td>
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<td></td>
<td>Commercially available surfaces</td>
<td>Ab modification, possible denaturation</td>
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<tr>
<td>Ab fragment tag</td>
<td>Oriented immobilisation</td>
<td>Surface stability</td>
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<td>Ab-binding proteins</td>
<td>Oriented immobilisation</td>
<td>Surface stability</td>
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<td></td>
<td>No Ab modification</td>
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<tr>
<td>Ab fragment fusions</td>
<td>Oriented immobilisation</td>
<td>Compatibility between Ab fragment and fusion partner</td>
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<tr>
<td></td>
<td>Surface stability</td>
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Several strategies are used to immobilise capture Abs on solid surfaces including physical adsorption, covalent attachment, entrapment within a polymer matrix, directed immobilisation and Ab engineering for improved surface affinities (Table 1 and Fig. 3).

**Physical adsorption**
Although rather uncontrollable, physical adsorption is the simplest method of protein binding on a surface. It can occur through hydrophilic, hydrophobic or both types of interactions between Abs and the sensor surface. Surfaces used include glass and other silica particles, polystyrene and silicone plastics, nitrocellulose and nylon membranes, and also metallic surfaces such as gold and silver [5]. In addition, these surfaces may be further modified to improve adhesion including poly-L-lysine / nitrocellulose coating or organosilane modification of glass and plastic surfaces (e.g. cyanosilane or 3-aminopropyltriethoxysilane (APTES)). Such immobilisation processes can lead to random orientation of the absorbed Abs. In addition, Abs may interact with the adsorptive surface in such a way that the antigen-binding site is not available which impedes antigen detection. The hydrophobic interactions which drive passive adsorption of antibodies can reportedly reduce the number of functional sites or activities by more than 90% [6,7,8]. Using adsorption as the method of immobilisation also has the disadvantage that the biomolecule can easily desorb from the surface during use.

Another form of non-covalent immobilisation of Abs involves conducting polymer films such as polyaniline, polypyrrole and polythiophene. Immobilisation generally involves entrapment within the polymer matrix, where the Ab is co-immobilised with the polymer onto a sensor surface from a monomer solution. However, this can lead to denaturation of the Ab and also result in a large population of Abs that are inaccessible because they are buried deep within the polymer film. To solve this, many authors have used an electrochemical adsorption technique. This is achieved by applying a potential to the polymer electrode surface, which enhances electrostatic interactions with the polymer backbone and the Ab, thereby, facilitating immobilisation [9].

Adsorption methods, despite their drawbacks, have been used in many applications due to their simplicity with no modifications of Abs required. Novel surfaces for Ab adsorption with improved orientation have recently been reported. Surfaces covered with calixarene derivatives have been demonstrated to capture Abs with a proper orientation [10,11]. In addition, Zn\textsuperscript{II} metal complexes have been used for Ab adsorption [12,13]. Mercaptohexadecanoic acid (MHA) was patterned on a thin-film gold substrate followed by coordination of the carboxylic acid groups of MHA to Zn\textsuperscript{II} ions. The authors report how these Zn\textsuperscript{II} ions can immobilise Abs (e.g. chicken IgY and mouse IgM) that generally have a low affinity for Ab-binding proteins A, G, A/G, or L. However, they are unclear how the Abs are immobilised by the metal ions or even how these methods achieve partial orientation.

Covalent attachment

Covalent attachment of biomolecules to surfaces ensures sufficient immobilisation without leaching of the biomolecule from the substrate surface during use. Such covalent binding of proteins to a biosensor surface represents a rational and robust approach. One of the most commonly used methods for covalent immobilisation couples the Abs randomly via their free amino-groups to an activated sensor surface (Fig. 3). Chemical coupling agents, such as carbodi-imides and succinimidyl esters, may be used to activate carboxylic acids on sensor surfaces. Amines or alcohols can be activated by isothiocyanates, epoxides, glutaraldehyde or other aldehydes. Oxidation of alcohols is achieved with periodate to yield aldehydes, which react readily with amines (Fig. 3). In addition, conversion of alcohols to a highly reactive ester by cyanogen bromide allows for further reaction with amines present in the Ab. Another approach is to use what are termed hetero-bifunctional linkers. Hetero-bifunctional cross-linking reagents contain two different reactive groups, thereby, providing a means of covalently linking two dissimilar target groups (e.g. groups on the sensor surface with groups
on the protein). A wide variety of these linkers are commercially available to cover the broad range of functional groups necessary.

![Diagram](image)

**Fig. (3).** Scheme showing covalent immobilisation of antibodies to sensor surfaces via their free amino-groups. Reaction (a) involves activation of carboxylic acids (COOH), achieved with carbodi-imides and succinimyl esters. Reaction (b) shows amine surfaces (NH$_2$), which can be activated using isothiocyanates, epoxides or aldehydes. Reaction (c) shows alcohol surfaces which can be activated using periodate oxidation, isothiocyanates, epoxides, aldehydes and cyanogen bromide.

The chemistry for surface activation on glass and gold is also well established. In the case of glass surfaces, organosilanes are often employed. The silane part of the organosilane will react with the glass leaving the organo functional group available for conjugation to the protein. One of the most commonly used is the amino-silane 3-aminopropyltriethoxysilane (APTES), while other derivatives include aldehyde silane and epoxy silane [14]. Other authors have used amino-silanes combined with a bi-functional N-hydroxysuccinimide (NHS) or a mercapto-silane combined with maleimido-NHS [14]. In relation to gold surfaces, these are generally functionalised using a thiol or amino bi-functional cross-linker, which will attach to the gold, leaving another reactive group free to bind the protein of interest [15].

Although highly stable, Abs covalently-linked through amino groups are randomly oriented with their antigen binding capacities reported as 2–3-fold lower compared to those of well-oriented Abs [3,16]. Strategies available for the covalent site-specific oriented immobilisation of Abs exist using the carbohydrate chains of Abs for immobilisation [17]. One simple oriented immobilisation strategy involves chemically coupling the Ab directly onto the transducer surface, e.g. a non-coated gold surface and available thiol (–SH) groups on the probe [18,19]. However, these methods require chemical treatments of an Ab prior to immobilisation, such as carbohydrate oxidation and disulfide bond reduction, which may detrimentally alter it. Enzymatic digestion of an Ab followed by chemical reduction has yielded Fab fragments with exposed active sulfhydryl groups. Direct bonding between the sulfhydryl group and a gold surface, as well as thiol-specific cross-linking on non-metallic surfaces has been reported [20,21].

There are several advantages to using the directional immobilisation processes. Ideally, the surface-coupling site is at a distant position from the antigen-capturing site, thereby, allowing the antigen-binding domains accessibility to the analyte. Bonroy *et al.* [22] showed for a
particular Ab-antigen pair that the optimized fragment type combined with an oriented immobilisation of Fab fragments led to a greater than two-fold increase of the antigen-binding signals compared to randomly covalent immobilised full-size Abs.

A commonly used affinity-based method for orienting Abs specifically onto surfaces involves attachment of biotinylated Abs onto a (strept)avidin-modified surface. While technically this does not involve covalent binding, the dissociation constants of biotin-avidin and biotin-streptavidin interactions are some of the largest association energies observed for non-covalent interactions (of the order of $10^{15}$ mol L$^{-1}$). The use of this type of immobilisation crucially retains the biological function of the immobilised Ab [23]. Abs can be biotinylated randomly or by oriented labelling procedures. Much higher loading densities were achieved with randomly biotinylated scFvs than full size Abs, leading to improved biosensor sensitivity and specificity [24]. Site-directed biotinylation of Abs at their hinge region was developed to orient the immobilisation of Abs which showed consistently enhanced detection capabilities compared to randomly biotinylated Abs [25].

Several reports have demonstrated DNA-directed Ab immobilisation whereby single-stranded DNA-Ab conjugates were immobilised onto complementary DNA surfaces [26,27,28]. This allows directed immobilisation without the use of harsh chemicals and incubation procedures. Human chorionic gonadotropin (hCG) was measured to levels as low as 0.1 ng mL$^{-1}$ using DNA-directed Ab immobilisation on gold surfaces compared to an LOD of 5 ng mL$^{-1}$ using directly coupled Abs on gold surfaces. This is an example of where linker chemistry in assays is key: the long, stable, flexible linker of DNA between the Ab and the gold sensing surface was, according to the authors, a likely contributor to the observed high sensitivity.

Antibody-binding proteins
Oriented immobilisation can also be achieved via an intermediate layer as an alternative to direct chemical coupling (Fig. 4). An elegant example of this is to employ Ab-binding proteins such as protein A or G. These proteins bind specifically to Abs through their non-antigenic (Fc) regions, which leaves the antigen binding sites of the immobilised Ab available for binding. Such Ab-binding proteins have been extensively used to capture Abs on biosensor surfaces with their antigen-binding site maximally exposed to the solution and thus remaining fully functional. While the binding capacity of protein A is predominantly limited to three human IgG subclasses (IgG 1, 2 and 4), protein G has specificity for subclasses of Abs from many species. In addition, genetic engineering has enabled the production of a fusion protein, protein A/G that combines IgG binding domains of both protein A and protein G. Higher sensing abilities are regularly exhibited for immunoassays employing Ab-binding proteins for Ab immobilisation compared to those using conventional methods such as random covalent immobilisation [29,30]. Although there is the additional step of immobilising the Ab-binding protein on the surface prior to Ab immobilisation, Ab-binding proteins can be genetically engineered and easily prepared in large quantities, providing many options for immobilisation. For example, a protein G layer was engineered to contain cysteines, which formed a more ordered film on bare gold compared to a control protein G layer [31]. This genetically engineered protein G layer allowed the immobilisation of approximately 4-fold more Abs compared to the control.

Ab fragments can also be genetically fused to a range of proteins and subsequently immobilised onto an assay surface. Fusion of Ab fragments to a common immobilisation domain will allow for identical orientation of all Abs. Pleschberger et al. [32] fused a bacterial S-layer protein to a nanobody (single chain Ab). This fusion protein self-assembled
onto solid surface with the nanobody pointing outwards from the protein lattice surface into the solution. A similar approach was used to fuse scFvs with pIII surface protein, allowing a more sensitive detection to be achieved [33]. Fusion of Ab fragments with proteins such as beta-galactosidase, maltose-binding protein, calmodulin-binding protein, chitin-binding domain, cellulose-binding domain or glutathione-S-transferase is also possible. These fusion proteins (e.g. maltose-binding protein) on the Ab fragments allow for oriented immobilisation on surfaces containing the fusion partner (e.g. maltose).

In order to facilitate purification, most recombinant Ab fragments are expressed with an affinity tag. This tag may also be used for immobilisation onto a sensor surface (Fig. 4). Histidine (his-tag) fused Ab fragments may be non-covalently immobilised onto Ni\(^{2+}\) or gold surfaces. ScFv fragments may also be engineered to contain two histidines where the scFvs are bound in a proper orientation, can retain antigen-binding affinity, and in addition, may be coupled at a high surface concentration [34]. Replacing the standard single his-tag with a double his-tag on scFvs has led to significantly enhanced binding onto Ni\(^{2+}\)-nitrilotriacetic acid-coated substrates [35].

In biomaterial science, Abs with a high affinity for certain material types would be advantageous as they could be used to immobilise probes on biosensor surfaces. Ab fragments that bind to the biodegradable polymer polyhydroxybutyrate, have been generated by phage display, thereby, allowing site-directed oriented immobilisation of Ab fragments on this biomaterial [36]. By grafting material-binding peptides into loops of the complementarity determining regions of Abs, fragments with binding affinity and specificity for non-biological inorganic material surfaces (e.g. gold) have been generated [37].
Fig. (4). Schematic of different immobilisation strategies employed in immunosensors [2]. Reproduced with permission from Wiley-VCH Verlag GmbH.

3. IMMUNOSENSORS

Electrochemical

The oldest and most commonly used transducers are electrochemical. Electrical detection methods are appealing because of their low cost, low power consumption, ease of miniaturization, and potential multiplexing capability [38,39]. The basis of these detection systems stems from a binding-induced change in some electrical property of the circuit of which the sensor is a vital component. A number of attributes of electrochemistry make it popular as a detection technique in immunosensing. The high sensitivity of electrochemical transducers and sample independence of turbidity and colour make them the transducer of choice for many researchers. Since most Abs and antigens in immunosensors are unable to act as redox partners, a label is often conjugated to one of the components of the immunocomplex to promote an electrochemical reaction. The electrochemical signal produced is then quantitatively related to the amount of analyte present in a sample. Potentiometry, amperometry, and electrochemical impedance spectroscopy are among the electrochemical detection techniques most often used with immunoassay systems and immunosensors. The fundamental principles of these techniques are presented, followed by discussions based on some recent work and trends in these areas.

Potentiometric sensors

Based on the Nernst equation, potentiometric sensors measure a change in potential of a system. Potentiometric immunosensors depend on a change in potential between two electrodes due to a specific interaction between an Ab and its antigen [40]. One of the main disadvantages of this type of detection is the relatively small change in potential that arises from the interaction between an Ab and its antigen. Furthermore, interferences in the sample may prevent this small signal from being successfully detected. Such sensors, therefore, often have compromised sensitivity. A recent example of a potentiometric immunosensor involves using a polypyrrole coated screen-printed electrode [41]. Ab immobilisation on the polypyrrole layer was achieved by either direct adsorption or by binding a biotinylated Ab to streptavidin-coated polypyrrole. Incubation of this immuno-electrode was carried out in solutions containing either hepatitis B surface antigen or the cardiac marker, Troponin I. A sandwich immunoassay was completed by introducing a signal Ab labelled with horse radish peroxidase (HRP). Potentiometric measurements were performed in 0.01 M phosphate buffer saline (PBS) (pH 7.4) with o-phenylenediamine dihydrochloride added to initiate the enzymatic reaction. A deliberate separation of the immunoreaction from the detection step was employed to minimise matrix interferences. The change in potential was found to be proportional to the concentration of analyte in the sample. The sensitivities for hepatitis B surface antigen and troponin I were reported to be 50 fmol L⁻¹ and 0.4 pmol L⁻¹, respectively.

Amperometric sensors

Amperometric sensors measure the current produced by the oxidation or reduction of an electroactive species (which is monitored at a specific potential). The magnitude of the current can be related to the analyte concentration. To promote an electrochemical reaction at the sensor surface, a label must be introduced to the immuno-complex since both Ab and antigen are not intrinsically electroactive. Enzyme labels including the oxidoreductase, HRP, and the hydrolytic enzyme alkaline phosphatase (AP) are frequently used. The magnitude of
the current from the redox reaction involving the enzyme can then be quantitatively related to the analyte concentration. A major advantage of enzyme labels is the amplification of the signal that can be detected as a result of the catalytic effect even when a minute quantity of enzyme is used. However, a drawback of the enzymatic signal-generating scheme described above is that it is an indirect detection technique.

In some amperometric immunosensors, the enzyme may be located some distance away from the electrode surface. In addition to this, the redox active site of an enzyme is usually covered by an insulating protein layer and therefore may not undergo direct electron transfer reactions at an electrode surface. In other amperometric immunosensors, the presence of interfering substances in biological samples may require using an alternative electron transfer pathway. These two issues may be overcome with a redox-active species called an electron transfer mediator. Often used in analytical chemistry, electron transfer mediation is a well-known technique for overcoming limitations of electron transfer at metal electrodes. Mediators act as a shuttle to transfer electrons between the redox centre of the electrode active materials (such as the peroxidase enzyme, HRP or glucose oxidase) and the surface of the electrode. A typical example of a mediator includes ferrocene and its derivatives. An electrochemical immunosensor using a mediator and single walled carbon nanotube forests as the electrode surface was demonstrated in a competitive immunoassay for detecting biotin-HRP and unlabelled biotin. In this competitive assay, labelled and unlabelled antigens competed for binding sites on the anti-biotin capture Ab. The mediator chosen for this system was hydroquinone, used to facilitate a catalytic reduction of hydrogen peroxide by transferring electrons between HRP and the peroxide. LODs of 2.5 nM and 16 μM were achieved for biotin-HRP and unlabelled biotin, respectively.

Generally, the development of electrochemical immunosensors involves the immobilisation of an immunocomplex on an electrode, followed by detection via the immunocomplex label at the same electrode. Interdigitated array (IDA) microelectrodes have gained popularity as transducers in electrochemical immunoassays. A simple IDA consists of a pair of interdigitated microelectrode “fingers”. When an IDA is used as a sensing electrode in a voltammetric experiment, the two interdigitated electrodes are usually held at different potentials to achieve redox cycling of the electroactive species to be detected.

![Fig. (5). Scheme showing how interdigitated electrodes work. Redox cycling between 4-AP and 4-QI occurs at adjacent electrodes (denoted Anode and Cathode).](image)

Redox cycling at interdigitated electrodes is illustrated in Fig. 5 where an oxidizing potential is applied to one of the two IDA electrodes (denoted Anode) to promote the oxidation of 4-aminophenol (4-AP) to 4-quinone imine (4-QI). Then, 4-QI diffuses to the adjacent electrode held at a more negative potential (denoted Cathode), where it is reduced to 4-AP, which can then undergo another oxidation at the next adjacent electrode. Major advantages of this redox cycling are lower LODs and improved sensitivity achieved by enhancing the Faradaic current relative to the background current, resulting in an improved signal-to-noise ratio. Thomas et al. used an IDA consisting of 25 pairs of platinum microelectrodes as a detector in
immunoassays for mouse IgG [43]. Four-fold signal amplification was obtained compared to single-electrode detection, with an LOD of 3.5 fmol mouse IgG.

Electrochemical impedance spectroscopy
Changes occur in the interfacial charge, capacitance, resistance, mass, and thickness at an immunosensor surface upon binding of an antigen by an immobilised Ab. Electrochemical impedance spectroscopy (EIS) is a method for probing the features of an electrode surface modified by such immunocomplexes. In EIS, sensing is accomplished by measuring changes in the resistance and/or capacitance of the electrode–solution interface upon binding of a target molecule to a receptor functionalized surface [38,39]. EIS does not require the use of enzyme labels to report binding events which contrasts with other electrical measurements, such as amperometry, potentiometry and voltammetry.

Recent developments in EIS report using alternative electrode materials such as electrically conducting polymers [44,45] and nanoparticles [46,47]. Additionally, electrode geometry has proven important with interdigitated electrode arrays providing improved sensitivity [39,48]. Impedance-based sensors have been used to monitor protein–carbohydrate [49] and protein–protein [50,51] interactions. For example, Yu et al. [52] reported multiplexed protein monitoring with an array of gold electrodes. Immobilised on the gold electrodes were human IgG (hIgG), rat IgG (rIgG), human globin and bovine serum albumin. This array was incubated with goat anti-hIgG Ab, goat anti-rIgG Ab, anti-human globin Ab and a mixture of the three Abs. Formation of an antigen-Ab complex significantly changed the electron transfer resistance of the electrodes allowing detection of the four Abs.

Trends in electrochemical immunosensors
Nanomaterial-enhanced electrochemical immunosensors are currently a hot topic with the rapid growth in this area being driven by a need for analytical devices requiring smaller sample volumes, decreased power consumption and improved performances. Due to their dimensions, nanostructured materials display unique properties not traditionally observed in bulk materials. Characteristics such as increased surface area along with enhanced electrical properties make them suitable for sensing applications. In addition, nanomaterials are comparable in size to many biomacromolecules. Therefore, their nanodimensions exploit the possibility of bringing these materials into close proximity with proteins, enabling them to act as one dimensional channels for electron transfer in proteins.

Based on their unique electrical and catalytic properties, nanoparticles can perform various roles in electrochemical sensing systems. They can be used for the immobilisation or labelling of biomolecules, the catalysis of electrochemical reactions, the enhancement of electron transfer, or even act as a reactant. Due to their excellent conductivity and catalytic properties metal nanoparticles are ideal “electronic wires” for enhancing the electron transfer between redox centres in proteins and electrode surfaces. For example, gold nanoparticles (Au NPs) can exhibit excellent catalytic activity due to their relative high surface area to volume ratio, and their interface-dominated properties, which significantly differ from their bulk counterparts [53] (Table 2).
Table 2. LODs of amplified electrochemical bioassays.

<table>
<thead>
<tr>
<th>Assay</th>
<th>Target</th>
<th>LOD(^a)</th>
<th>Ref.</th>
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</thead>
<tbody>
<tr>
<td>Au(III) ion</td>
<td>IgG</td>
<td>3 pM</td>
<td>[54]</td>
</tr>
<tr>
<td>Oligonucleotide (purine base)</td>
<td>IgG</td>
<td>13 fM</td>
<td>[55]</td>
</tr>
<tr>
<td>CNT-ALP(^b)</td>
<td>IgG</td>
<td>3 fM</td>
<td>[56]</td>
</tr>
<tr>
<td>CNT-HRP(^c)</td>
<td>PSA</td>
<td>100 fM</td>
<td>[57]</td>
</tr>
<tr>
<td>AuNP-Ab</td>
<td>IgG</td>
<td>7 aM</td>
<td>[58]</td>
</tr>
<tr>
<td>AuNP-Ab-hydrazine</td>
<td>IgG</td>
<td>670 aM</td>
<td>[59]</td>
</tr>
<tr>
<td>AuNP–polymer complex</td>
<td>IgG</td>
<td>1 fM</td>
<td>[60]</td>
</tr>
</tbody>
</table>

\(^a\) Limit of detection, \(^b\) CNT-ALP (carbon nanotube-alkaline phosphatase), \(^c\) CNT-HRP (carbon nanotube-horseradish peroxidase).

By means of direct electrochemical detection of Au NPs, Ko et al. [61] have reported a multiplex immuno-biosensor to detect cancer biomarkers including prostate specific antigen (PSA). Au NPs act as catalysts in the reduction of silver ions to metallic silver, which is deposited onto the gold nanoparticles, thereby enlarging the particles. The growing size of the particles connects them to each other, forming an electrical bridge; thus, decreasing the resistance between electrodes. This biosensor could detect PSA at an LOD of 10 pg mL\(^{-1}\).

Another immuno-biosensor for the rapid detection of PSA uses a different technique of integrating an immuno-chromatographic strip with screen-printed electrodes (SPE). This acts as an electrochemical transducer for stripping analysis of metal ions released from captured quantum dot (QD) labels. It was found that this biosensor showed high sensitivity with a LOD of 20 pg mL\(^{-1}\) [62].

Carbon nanotubes (CNTs) can also be used to develop highly sensitive and specific nanoscale immunoassays [63]. Examples of where CNTs were used to amplify detection of PSA in serum include CNTs carrying numerous horseradish peroxidase (HRP) enzyme labels and secondary Abs [64,65]. The secondary Ab and HRP label were covalently linked to CNTs at high ratios of 1:200 in place of singly labelled secondary Abs in a sandwich immunoassay [66]. This amplification strategy improved the LOD 100-fold to 4 pg mL\(^{-1}\) and the sensitivity by 800-fold, compared to conventional ELISA. These results highlight the excellent promise CNTs show in ultrasensitive immunoassay research in proteomics and systems biology.

In addition to electrochemical sensors using CNTs as an electrode substrate, sensors based on transistor arrangements using CNTs have been developed [67]. Single wall carbon nanotubes (SWNTs) are the most likely candidate for miniaturising electronics beyond the micro-electromechanical scale currently used in electronics. They exhibit electrical properties not shared by their multi-walled counterparts and certain sizes of SWNT act as semiconductors. The intrinsic bandgap in semiconducting SWNTs (typically 0.5 eV, but this is diameter-
dependent) allows them to be used as nanosized semiconducting channels in field-effect transistors (FETs) (Table 3) [68]. Since FET-based biomolecular detection does not employ fluorescence, electrochemical, or magnetic tags, it has been termed as “label-free” methodology [69,70,71]. FETs generally consist of a substrate (gate), two microelectrodes (source and drain), and a SWNT (or SWNT network) that bridges the electrodes. Usually SWNTs are grown directly via chemical vapour deposition (CVD) or cast from a dispersion onto a substrate either before or after the electrodes are patterned [72]. Single-nanotube FETs require arduous screening of devices to eliminate metallic SWNTs. This need is obviated for nanotube networks cast from dispersions, where the 2 to 1 ratio of semiconducting to metallic SWNTs renders the likelihood of forming a continuous metallic pathway between source and drain unlikely. Sensing is based on the fact that the current flow in SWNT FETs is extremely sensitive to the binding of biomolecules and produces a detectable signal. A wide variety of applications for CNT FETs have been investigated including the detection of proteins, Ab-antigen interactions, glucose, DNA and DNA hybridization. The LOD for the sensing of proteins or protein-protein interactions has generally been in the range of 100 pM to 100 nM [73].

An SWNT-FET immunoassay typically involves first immobilising an Ab, thus providing a recognition site for the target antigen. The current–voltage characteristics or conductance of the Ab-modified SWNT-FET are measured prior to antigen binding. Following exposure to the antigen, the conductance of the SWNT-FET device is again measured [74]. To detect IgE 50-amino-modified aptamers immobilised on a CNT-FET were used [75]. The net current change increased with the IgE concentration and an LOD for IgE of 250 pM was reported. Li et al. [76] studied the detection of PSA with an FET comprised of a network of SWNTs. The authors measured the electronic interaction of an anti-PSA Ab capturing PSA antigen. The interaction is thought to be a charge-transfer mechanism with a reported limit of detection of 14 pM, at a signal-to-noise ratio of 2. Table 3 summarises SWNT-FET biosensors showing how pM LOD for Ab–antigen binding [73,77] have been achieved.
Table 3: SWNT-FET biosensor configuration tabulated against receptor Ab, target antigen, LOD and signal amplification [68].

<table>
<thead>
<tr>
<th>Receptor</th>
<th>Target</th>
<th>SWNT configuration</th>
<th>CNT fabrication</th>
<th>SD fabrication</th>
<th>Gate</th>
<th>LOD</th>
<th>Signal amplification</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ab (SpA, hCG)</td>
<td>Antigen (IgG, β-hCG)</td>
<td>Mat</td>
<td>CVD (Fe)</td>
<td>Cr-Au</td>
<td>Liquid</td>
<td>1pM</td>
<td>SB area increase</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Shad-Mask/Evap</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ab (SpA, BT, U1A)</td>
<td>Antigen (IgG, SP, mAbs)</td>
<td>Mat</td>
<td>CVD (Fe)</td>
<td>Ti-Au</td>
<td>Liquid</td>
<td>100nM-100pM</td>
<td>N/R</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Shad-Mask/Evap</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ab (BT)</td>
<td>Antigen (SP)</td>
<td>Single/few</td>
<td>CVD (Fe)</td>
<td>Ti-Au</td>
<td>Bottom</td>
<td>N/R</td>
<td>N/R</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>PhotoL/Evap</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ab (anti-CEA)</td>
<td>Antigen (CEA)</td>
<td>Single/few</td>
<td>CVD (Fe, alumina)</td>
<td>Ti-Au</td>
<td>Liquid</td>
<td>300fM</td>
<td>N/R</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>PhotoL/Evap</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

CVD: chemical vapour deposition; PhotoL: photolithography; Evap: evaporation; Shad-mask: Shadow mask; SB: Schottky barrier; N/R: not reported; SpA: specific protein A, derived from Staphylococcus aureus; hCG: human chorionic gonadotropin; IgG: rabbit immunoglobulin G; β-hCG: mouse Ab; BT: biotin; SP: streptavidin; U1A: human autoantigen, a prototype target of the autoimmune response in patients with systemic lupus erythematosus and mixed connective tissue diseases; mAb: monoclonal Ab; CEA: carcinoembryonic antigen; anti-CEA: CEA Ab.
Due to ever improving technologies, the field of optical immunosensors is progressing rapidly. The methods involved are based on detecting the interactions between antigens and their specific Abs through changes in optical characteristics such as absorption, fluorescence, chemiluminescence and refractive index among others. Optical immunosensors can be classified into two categories according to direct or indirect detection of the binding event. Direct/label-free sensors monitor Ab-antigen interactions which generate a measurable signal. These methods do not require any additional reagents and can work in complex matrices. However, they may be limited by low molecular weight analyte detection and by being prone to non-specific interactions. On the other hand, indirect optical methods necessitate the incorporation of suitable labels such as fluorophores onto the Ab or antigen to monitor the binding event. This leads to assays with higher specificity and sensitivity but the labelling steps can be labour-intensive, the reagents involved expensive and the reaction may subsequently affect Ab binding reactions. Optical immunosensors often use an evanescent wave generated at the interface between two media with different optical properties, the sensor platform and the sample, to sense the binding events. The signal measured is correlated with the concentration of the analyte in the sample. A variety of transducers are available such as surface plasma resonance (SPR) and total internal reflection fluorescence (TIRF).

**Direct optical detection**

The main types of direct optical immunosensor detectors are based on grating-couplers, Mach-Zehnder interferometers and SPR [78,79,80,81]. Some of the most sensitive optical immunosensors involve SPR, an optoelectronic phenomenon, first observed by Wood in 1902 [82]. SPR immunosensors use electromagnetic waves, also called surface plasmons, to probe interactions between the analyte in solution and its recognition element immobilised on a thin metal film usually made from gold. The wave’s oscillation occurs and propagates at the interface between two transparent media of different refractive index such as glass and a metal. When plane polarised light, of appropriate wavelength and angle, illuminates the metal film, via a hemispherical optical prism, the light is reflected back into the metal layer and partly refracted to the sample solution. The energy from the incident photons is absorbed and generates the surface plasmons (Fig. 6). Above a critical angle of incidence, no light passes into the sample solution and total internal reflection (TIR) occurs. When the light is totally internally reflected, an evanescent wave is created at the interface close to the metal layer. The evanescent wave penetrates the thin film and excites the electromagnetic surface plasmon wave, with the intensity of the wave depending on the angle of incidence [83,84]. SPR occurs as the surface plasmons absorb some of the light energy from the evanescent field, which corresponds to a match between the energy and momentum of the incident light and the energy and momentum of the surface plasmons. The energy of the incident light is transferred to the plasmons, which can be observed as a marked drop in the total internal reflection light intensity reflected from the sensor surface at a specific angle (SPR angle θ). The resonance angle is sensitive to the refractive index of the solution adjacent to the sensor surface. Interactions between the recognition elements and their analytes change the mass at the surface of the chip but also the refractive index. Those variations affect the velocity, and, therefore, the momentum of the surface plasmons, causing a shift of the resonance to a longer wavelength and a change in the incident angle at which resonance occurs.
Different formats of SPR immunosensors exist. Direct, sandwich and competitive inhibition assays have all been developed using the technology (Fig. 6). In direct immunoassays, the recognition element is the Ab immobilised onto the metal layer and the change in mass and in resonance angle due to its interaction with an antigen is correlated to the concentration of the analyte. Guidi et al. [85] demonstrated the detection of insulin-like growth factor-1 in milk using a SPR direct assay. Since milk spoils quickly, real-time monitoring of the milk samples is preferable to time-consuming methods such as ELISA. SPR sandwich assays involve an additional Ab to the one immobilised on the sensor surface, improving its sensitivity compared to direct assays as the antigen is then recognised and captured by two Abs. Teramura et al. [86] developed an SPR sandwich assay to detect alpha-fetoprotein, a major tumour marker for hepatocellular tumour, in human plasma at the ng level necessary for clinical diagnosis. However, competitive inhibition assays are the method of choice for small-molecule detection. Such assays can sometimes involve the conjugation of the antigen to a carrier protein for immobilisation on the sensor surface. The Ab is pre-incubated with the antigen and the mixture injected over the sensor surface. The free Ab is then separated from the bound Ab. The concentration of the free Ab is inversely proportional to the concentration of the antigen. Competitive inhibition assays have been used to detect a wide range of small-molecule antigens as small as 250-500 Da [87].

The advantages of SPR immunosensors are that the same chip can be used several times; these SPR assays do not necessitate any labelling and can monitor in ‘real-time’ the binding events of small molecules with a very high sensitivity. They can give information on kinetics, affinity and concentration of the target analytes. However, the equipment required can be bulky and expensive, but emerging technologies will lead to significant reductions in size. SPR assays have found many applications in different fields such as biomedical diagnostics [88,89], and drug discovery [90,91].
Fig. (6). SPR phenomenon representation. The binding interaction between the Ab and the antigen results in a mass change at the surface of the sensor leading in a change in refractive
index (θ1 to θ2). The three main assay formats are: (A) direct assay, (B) sandwich assay and (C) competitive inhibition assay.

Indirect optical detection

Immunosensors with indirect optical detection involve the labelling of recognition elements, used to sense the Ab/antigen interaction. Those labels will fluoresce, luminesce or absorb the light. Different strategies have been developed to use such labelled biomolecules with the most popular being the addition of a fluorophore.

Total internal reflection fluorescence (TIRF) is based on total internal reflection happening between optically dense media joined to the recognition elements and optically less dense sample solutions containing the target molecule. At large angles of incidence, the excitation beam reflects into the glass and generates an evanescent wave at the interface with the sample. The wave can only excite the elements present on the TIRF surface. It has a maximum intensity at the surface, which is reduced with increasing distance from the surface [92]. These sensors demonstrate high sensitivity and can avoid separation and extraction steps from complex matrices such as body fluids [93,94]. As an example, studies were carried out using TIRF immunosensors to evaluate the concentration of bovine progesterone, a reproductive hormone present in raw milk [95,96]. The assay was designed as a competitive-inhibition test with progesterone attached on the sensor surface and a monoclonal anti-progesterone Ab used as the biological recognition element. The LOD was 0.04 ng mL\(^{-1}\) and this immunosensor for progesterone could be utilised as a reproductive management instrument. Albrecht et al. [97] developed an assay for the detection of C-reactive protein (CRP), an inflammation marker using TIRF. A binding-inhibition and a sandwich assay were developed. The concentration of CRP in normal blood is usually between 1 to 10 mg L\(^{-1}\) but can reach 40 mg L\(^{-1}\) with a viral infection and 500 mg L\(^{-1}\) with a bacterial infection. The immunosensor had an LOD of 0.44 mg mL\(^{-1}\) and 0.13 mg mL\(^{-1}\) for the sandwich and inhibition assay respectively. Thus, they have the potential to contribute to the realisation of a diagnostic test for CRP.

There are also examples of optical immunosensors using evanescent wave effects coupled to optic fibres. The optic fibre is a perfect transducer to combine with fluorescent signals increasing the efficiency of light collection. When light illuminates an optic fibre, a small proportion escapes from the surface of the fibre generating an evanescent wave interface between the optic fibre and the sample of interest. The evanescent zone extends along the length of the fibre sensor and decreases in intensity exponentially from the sensor surface. At an appropriate wavelength, fluorescent entities, such as labelled Abs present in the evanescent wave, can absorb energy and fluoresce. Part of the fluorescence is refracted into the fibre and detected. Both direct and sandwich assay formats have been used. For the sandwich assay, the antigen is recognised by both a capture Ab immobilised onto the fibre surface and a second labelled detection Ab added to the solution. If there is no antigen present, no fluorescence is observed. However, if there is antigen present, the detection Ab fluoresces due to its excitation by the evanescent wave. Long et al. [98] developed a fluorescent fibre-optic immunosensor for the detection of microcystin-LR in water samples. The inhibition assay was carried out with the level of Abs binding to microcystin-LR-
ovalbumin conjugate covalently immobilised to the fibre optic surface depending on the concentration of microcystin-LR in the water samples. The LOD was 0.03 μg L⁻¹ and the quantitative detection range 0.1-10.1 μg g⁻¹. These immunosensors have advantages in that they are sensitive, specific and inexpensive. In addition, they are not prone to electromagnetic interference, offer ‘real-time’ monitoring of the binding events and can be easily miniaturised [99,100].

Fluorescent capillary fill immunosensors have also been developed. They consist of two sheets of glass held apart at a distance of 100 μm and between which the reagents are immobilised and the binding reactions take place (Fig. 7). The sample enters the device by capillary action. Once inside, the analyte present in the sample matrix has to compete with the fluorescently-labelled antigens for the binding sites of the Abs that are immobilised on the glass. When the fluorescently-labelled analyte is trapped on the glass plate in the sample matrix, the fluorophores within the evanescent field are excited. The fluorescence formed in the capillary is quantified by scanning the capillary with light to excite the fluorophore labels. The photons, emitted at angles smaller than the critical angle, are trapped in the capillary walls and the light leaving the capillary is detected and the level of photons correlated to the concentration of the analyte. Sensitivities of 3 ng mL⁻¹ and 0.5 ng mL⁻¹ have been obtained for hCG in serum and estrone-3-glucuronide in urine, respectively [101,102,103]. More recently, Petrou et al. [104] developed a similar system to simultaneously determine the level of three hormones, follitropin, hCG and prolactin, in human serum, which levels are important for the diagnosis of fertility and development disorders. In that case, the immunosensor was made from a plastic capillary. The LOD was 1.3 μg L⁻¹, 2.3 IU L⁻¹ and 3.6 IU L⁻¹ for prolactin, follitropin and hCG, respectively, which are appropriate levels for the determination of these analytes in human serum.
**Fig. (7).** Schematic representation of the fluorescence-based detection capillary fill immunosensor.

Planar waveguide immunosensors employ evanescent illumination to excite fluorophores at the surface of a waveguide. They differ from fibre optic sensors by collecting the emitted light perpendicular to the waveguide while fibre optic immunosensors collect the emitted light at the end of the fibre. Planar waveguide immunosensors have been used to perform analysis on sample assays in parallel. Rowe-Taitt *et al.* [105] demonstrated using a cocktail of fluorescent Abs on a single sensor substrate the simultaneous detection of six biohazardous analytes: cholera toxin (from *Vibrio cholerae*), *Staphylococcus aureus* enterotoxin B, ricin, *Bacillus anthracis*, *Francisella tularensis*, and *Brucella abortus*.

Another type of fluorescence-based immunosensor employs fluorescence polarization; it is based on the increase in fluorescence polarisation of small labelled antigens (tracer) added to the sample (Fig. 8). When the tracer is free, its volume is small, its rotation fast and its fluorescence polarisation low. The fluorescence increases when it interacts with its specific Ab leading to a larger volume of the tracer-Ab complex and to a slower rotation. However, in the presence of the target antigen, the antigen competes with the tracer for binding with the Ab and the polarization signal decreases. The higher the concentration of unlabelled antigen present in the sample, the less bound tracer is present and, consequently, the lower the polarization of the fluorescent emitted light. These sensors use fluorophores and are very precise with a coefficient of correlation below 5 %. However, they can be prone to light scattering interferences [106]. Gachoz *et al.* [107] developed a fluorescence polarization immunoassay for the detection of mycotoxins such as ochratoxin A, aflatoxin B1 and zearalenone. The LOD of the mycotoxins was 1 ng mL$^{-1}$, thus, below the acceptable maximum residue limit in food. Wang *et al.* [108] developed an immunoassay for the detection of the antibiotic, sulfamethazine (LOD of 41 ng mL$^{-1}$), with a value available in just 5 minutes.
Chemiluminescence and quantum dots (QDs) have also received increasing interest for labelling Abs in the development of immunosensors. Chemiluminescent immunosensors use enzyme labels. The light emitted from the bound enzyme-labelled to the Ab/antigen is transmitted to a photomultiplier. Chemiluminescent detection possesses several advantages such as the relatively simple instrumentation required, very low LODs and a wide dynamic range \[10^9\]. Chemiluminescent optical fibre-immunosensors have been developed for the detection of Brucella with a LOD of Brucella smooth-A-O-chain antigen of 1.098 ng mL\(^{-1}\) corresponding to about 305 cfu mL\(^{-1}\) Brucella cells [110]. Salama et al. [111] reported an assay for the detection of Abs specific to GIPC-1, a protein involved in ovarian and breast cancer. The antigen was immobilised onto the optic fibre and a standard curve was built using a human monoclonal anti-GIPC-1 Ab purified from breast cancer patients, with a LOD of 30 pg mL\(^{-1}\). This chemiluminescent fibre-optic immunosensor had an LOD 50 times lower than a chemiluminescent ELISA, and approximately 500 times lower than a colorimetric ELISA for the detection of GIPC-1.

QDs are ultrasensitive colloidal nanocrystals with unique photophysical properties [112]. They offer advantages over molecular dyes by being brighter, resistant to photo bleaching and amenable to multiplexed detection [113]. They can be conjugated to Abs by replacing thiol acids present on the QD surface with the Ab or using linker molecules such as biotin and streptavidin. They have been used, for example, to label Abs specific for total prostate
specific antigen (PSA) [114]. QD sensors have great potential as a diagnostic tool for prostate cancer detection with an LOD of 0.25 ng mL\(^{-1}\) in human serum samples.

**Mechanical Immunosensors**

**Mass-Detecting Immunosensors**

Piezoelectric immunosensors are based on the analysis of binding events between antigens and Abs though mass increase at the surface of piezoelectric materials such as quartz crystal (Fig. 9). The quartz crystal resonates at a particular oscillation frequency when subjected to an electric field. Changes in resonance frequency are measured, which correspond to the mass increase on the chip surface during the interaction. The relationship between the mass and frequency was established by the Saurbrey equation; the higher the mass, the lower is the frequency [115].

![Fig. (9). Basic principle of piezoelectric transduction in an immunoassay.](image)

Two major types of piezoelectric transducer exist: the quartz crystal microbalance (QCM), also called bulk acoustic wave and the surface acoustic wave (SAW) transducers. With the first type, resonance happens in the whole crystal mass while for the latter resonance only occurs on its surface. The QCM can resonate at a frequency up to 30 MHz due to the stability of the crystal mechanical structure while the SAW frequency can go up to 200 MHz and they are thus expected to be more sensitive to the Ab-antigen interaction than QCM [116,117]. The first piezoelectric transducer was developed in 1972 by Shons and Dorman for the detection of IgG in bovine serum [118]. Such transducers show real potential for ‘point-of-care’ diagnostics. Recent studies reported the development of a QCM immunosensor to detect CRP based on an indirect competition assay. It aimed to detect CRP, a biomarker for heart disease, hypertension and inflammation. The sensor detected a wide concentration...
range of 0.130–25,016 ng mL\(^{-1}\) of CRP with an LOD of 0.130 ng mL\(^{-1}\). Also, the intensity of the signal could be increased by introducing a streptavidin coated gold nanoparticle increasing the sensitivity of the assay [119,120]. Lee et al. [121] developed an immunosensor using a SAW transducer to demonstrate the detection of hepatitis B surface Abs that are present during the late phase of the virus infection. The immunosensor showed binding-specificity to the Ab and a LOD below 10 pg \(\mu\)L\(^{-1}\). It could also detect the Ab in blood samples.

Piezoelectric immunosensors are popular as they are affordable and easy to use. They do not require the need to label Abs or antigens and can detect antigens down to the pg range. However, with piezoelectric systems, non-specific binding on the surface of the crystal can lead to erroneous results.

Micro-cantilever Immunosensors

Benefitting from atomic force microscope (AFM) technology, microcantilever biosensors are an emerging sensor technique. This technique utilises bending of the microcantilever (which changes its resonant frequency) due to specific biomolecular interactions and recording of either optical or electronic signals. Vibrational frequency changes that are caused by molecules adsorbed on a microcantilever are referred to as “curling” due to the adsorption stress on one side of the cantilever. Molecular binding events on cantilevers may be transduced via deflection of the cantilever [122] or a change in the resonance frequency of the cantilevers (Fig. 10) [123]. The simplest method is a change in deflection, which is measured by reflecting a laser beam off the back of the cantilever and measuring its position with a photodiode. Cantilever deflection has been applied for detection of the pesticide DDT with an LOD of 10 nM [124]. A competitive immunoassay was carried out using a synthetic hapten conjugated with BSA and its specific Ab. Clinically relevant concentrations of myoglobin with a LOD of 85 ng mL\(^{-1}\) was detected by Raiteri et al. using a monoclonal anti-myoglobin Ab [125]. Using micro-cantilevers Wu et al. [126] detected PSA with an LOD of 0.2 ng mL\(^{-1}\) in serum containing 1 mg mL\(^{-1}\) albumin and plasminogen. The same research group, using very large arrays of up to 960 individually readable microcantilevers, demonstrated sensitive protein detection [127]. Monitoring the change in the resonance frequency on binding of an analyte is the principle behind resonance-based transduction of microcantilevers [123]. This is by far the most sensitive method, even if significantly more complex than that for deflection-based detection [122]. Using this more sensitive resonance-based measurement strategy, PSA with an LOD of 10 pg mL\(^{-1}\) was detected [128].
The incorporation of piezoelectric materials into microcantilevers can also be used to monitor the presence of biomolecules. Binding events on the cantilever surface are transduced by bending of the cantilever, which then generates an electric current in an attached piezoelectric element \[129\]. Piezoelectric-modified microcantilevers may be operated in both deflection-based and resonance-based modes. Analyte binding to the cantilever surface causes the cantilever to deflect, consequently depolarising the material and generating a current. A piezoelectric nanomechanical cantilever was fabricated by Lee et al. for the label-free detection of PSA with an LOD of 10 pg mL\(^{-1}\) \[130\].

Currently microcantilever-based sensors are an appealing method for sensitive, label-free detection of biomolecules. The major advantages of microcantilever sensors over traditional transducers such as the QCM exist in their superior sensitivity to analytes. LODs are at least comparable to those for SPR. The ease of cantilever functionalisation allows for systems as varied as single molecules to whole cells to be studied. In addition, the ability to microfabricate compact arrays of cantilevers facilitates simultaneous and high throughput measurements \[128,131\].

4. MINIATURISATION OF IMMUNOSENSORS

Intensive work has been directed toward the miniaturisation of immunosensors. The miniaturisation of assays will lead to the development of cheaper and more portable instruments, which can be used as point-of-care devices for diseases diagnosis. It will also facilitate research applications in areas such as environmental monitoring with the opportunity to carry out analysis in the field. Microfluidic platforms offer great potential for
the integration of immunoassays. Microfluidics will aid the reduction of sample volumes, some of which are not always available in large quantities (e.g. bodily fluids), and of expensive reagents such as Abs and enzymes [132]. Both samples and reagents can be added to the structures down to the nanolitre scale and transferred from one chamber to the next as they are going through a series of reactions. These steps can involve separation, mixing, incubation, washing steps and enzyme reactions with substrates necessary for the immunoassay [133]. It may also ease the automation of reactions alleviating the human error involved in the loss of accuracy and lowering the energy requirements of the assay [134].

The use of microfluidic devices will also lead to improved analytical efficiency by shortening the time required to carry out the reaction. This is due to larger surface-to-volume ratios and improved mass transport efficiency. However, this advantage can also increase the level of non-specific binding of the antigen and of its Ab outside the detection area. This problem can be alleviated by carrying out appropriate surface modifications depending on the choice of chip fabrication material. Microfluidic platforms can be made from various substrates such as silicon, glass and polymers. Early devices were mainly fabricated with silicon. Polymers are now more commonly used due to their cheaper material and fabrication cost as well as their superior optical properties. Polymers, such as polydimethylsiloxane (PDMS), polycarbonate (PC), polymethylmethacrylate (PMMA), and cyclo-olefins such as Zeonor, show considerable advantages such as their low cost and ease-of-use. PDMS allows for the rapid fabrication of prototypes and can be sealed and bound to other structures made from different materials [135,136]. With the aid of injection moulding technology, PMMA, a thermoplastic polymer, is even faster to use for chip fabrication, while cyclo-olefin polymers show excellent optical properties [137].

To remove any non-specific binding, surface modifications such as blocking steps with BSA can be necessary depending on the material selected [138]. Abs can be immobilised on hydrophobic substrates by adsorption. Due to the instability of Abs immobilised by adsorption, other surface chemistries have been developed to covalently bind the Abs to the platform [139,140,141,142,143,144,145]. The Abs and antigens can also be immobilised onto micro-beads added to the device. The beads offer a large surface area where added functional groups allow for the conjugation of Abs or antigens. They also allow for the easy transportation of Abs/antigens over the platform using pressure or electric fields [146,147].

Microfluidic platforms can vary in size and shape but are typically made of channels and reaction chambers of small dimensions. Fluids have to be mixed efficiently and transferred between the different channels and reaction chambers of the structure in a controlled way. Flow behaviour of fluids inside a microchannel is laminar but pumps and valves can be used to control the circulation of the samples and reagents [148,149]. In centrifugal microfluidic immunosensors (e.g. centrifugal devices (CD)), the circulation of the fluid relies on the hydrophilicity of the surface substrate. The sequential release of the fluids depends on the centrifugal and capillary forces; when spinning the device, the centrifugal force overcomes the capillary force in the valves [150,151]. An advantage of the CD is that centrifugal pumping eliminates the need for pumps requiring large power supplies to drive the fluids. Structures such as siphons and capillary burst valves can also be added to the design to regulate the microfluidic flow of the different solutions [152]. For example, capillary burst
valves prevent the circulation of the fluid forming a barrier when the channel section expands abruptly. Sandwich, direct and competitive immunoassays have been developed on CDs [153,154,155]. This makes these devices perfect candidates to be used in the field.

The successful development of an immunoassay involves a signal with a strong enough intensity to be detected. Microfluidic sensors mainly use optical and electrochemical transducers. Fluorescence optical systems are preferred over light absorbance ones due to their higher sensitivity on small platforms [156]. There are also reports of small immunosensors using SPR [157,158], chemiluminescence [159], light scattering [160] and electrochemical systems [161].

**CONCLUSION**

In conclusion, over the last 30 years, emerging technologies have led to the development of rapid immunosensors available for food quality, health and environmental monitoring. These assays demonstrate high sensitivities and specificities due to the progress in Ab development and allow the real-time monitoring of Ab-antigen interactions. This in conjunction with the diversity of transducers available has led to a rapid growth of research in immunoassay techniques. However, the assays available to date are largely more suited to research laboratories than to commercialisation. Novel microfluidic techniques permit the future miniaturisation of existing immunosensors which should lead to faster, cheaper and more sensitive assays. This will help to feed the need for automation of the tests developed and to a larger utilisation of immunosensors in modern applications such as point-of-care diagnostics.
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