Antibodies: production, functions and applications in biosensors

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The antibody–antigen interaction is characterised by high affinity and high specificity, which makes it an excellent candidate as an analytical tool for selective and sensitive determinations. This biochemical interaction is being increasingly detected by solid-state physical transducers, in devices which are termed 'biosensors'. The structure of the antibody molecule is central to its binding function. In addition, several techniques are available by which antibodies may be produced. An appreciation of both of these areas is essential in developing an immunosensor. The choice of the appropriate transducer is also important, and this short article also highlights some recent applications involving the use of electrochemical, optical, surface acoustic wave and semiconductor transducers.

1. Introduction

Immunological techniques are particularly suited to the detection of low level concentrations of analytes in biological matrices. In the determination of low level analytes there are two main requirements: (1) a sensitive assay, and (b) high specificity. Immunosensors take advantage of the high selectivity provided by the molecular recognition of antigen by antibodies. In principle, antibodies of high affinity may be produced against any antigen. Immunosensors are thus characterised by high selectivity, sensitivity, and versatility. In the design of immunosensors (or any molecular sensor) two phenomena must be combined:

- the selective molecular recognition of the target molecule: an important factor is the intrinsic specificity of the biological material involved in the recognition process, as problems of interference may occur in complex samples;
- the occurrence of a physical or chemical signal consecutive to the recognition, subsequently converted by the transducer into a second signal, generally electrical, with a transduction mode which can be either electrochemical, thermal, optical or based on mass vibration.

High sensitivity as well as high selectivity and reproducibility are essential requisites for immunosensors. This short review will deal with current antibody production technology and its application in the developing field of immunosensors.

2. Basic antibody structure

The structures of antibody molecules are varied, but are typified by the immunoglobulin G (IgG) subclass. This class of antibody is also the one most used in sensor applications. A basic four polypeptide chain model has been proposed. An IgG molecule consists of two identical heavy (H) and two identical light (L) chains (Fig. 1). The H chain is 450 amino acid residues and the L chain is 212 amino acid residues in length. The two H chains are held together by disulphide (–S–S–) linkages, two in the case of human IgG1. L chains are also attached to H chains by disulphide bonds so that

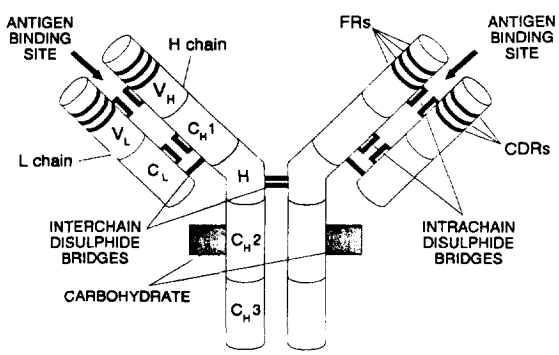

Fig. 1. Basic structure of the human IgG1 antibody molecule.

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one L chain associates with one H chain. These disulphide bonds are known as interchain disulphide bridges. Both the H and L chains are divided into structural domains based on amino acid sequence. An L chain has two domains, while an H chain generally has four. Two of the H domains and both L domains possess an intrachain disulphide bridge. These contribute to the globular nature of these proteins, hence the name immunoglobulins.

Both H and L chains are divided into constant (C) and variable (V) domains based on their amino acid sequence variability. The L chain has a single variable domain (V_L) and a single constant domain (C_L). An H chain has a single variable domain (V_H) and three constant domains (C_H1, C_H2 and C_H3). Pairs of domains associate with one another so that V_L and V_H, and C_L and C_H1 associate with each other. Two C_H2 regions pair with one another, as do two C_H3 regions. An additional domain that lies between the C_H1 and C_H2 domains is the hinge region. This area allows flexibility and some degree of freedom for the two arms of the antibody molecule.

The most important regions of the antibody with regard to the antibody–antigen binding interaction are the variable regions, consisting of the association of the V_H and V_L domains. Throughout these regions there is considerable amino acid variability from one molecule to the next. Within these domains, however, there are three distinct areas of even higher sequence variability and these are known as hypervariable regions. With three on the H chain and three on the L chain, these form six hypervariable loops, known as complementarity determining regions (CDRs). These CDRs are bounded by a total of eight framework (FR) regions. The CDRs are exposed at the end of the chains and constitute the area known as the antigen binding site. It is the generation of diversity in this area that allows many antibody molecules with different binding specificities and binding strengths (affinities) to be generated from one basic molecular structure. There are an estimated 10^8 antibody specificities possible from this system. Other areas of functional importance are the C_H1 region which binds complement C4b fragment. The C_H2 domains have carbohydrate binding sites (two in the case of human IgG1). The C_H3 domain possesses the effector functions of the immunoglobulin molecule that allow it to interact with the rest of the immune system.

In the immune system, a single B lymphocyte produces a single type of antibody molecule. In a typical immune response to an antigen, e.g. a foreign protein, many B lymphocytes produce many different antibody molecules all directed to different parts of the antigen. These areas of recognition on the antigen are known as epitopes. Such an antibody population is, thus, considered polyclonal. Polyclonal antibody serum has some limitations as it can vary from one animal to another or even from one immunization of the same animal to the next. A consistent source of antibody product cannot be guaranteed. A method was needed that would result in the production of a limitless supply of a single antibody molecule of defined specificity and affinity. Such antibodies are known as monoclonal antibodies.

3. Monoclonal antibody production

In 1975, Kohler and Milstein developed a system for the production of monoclonal antibodies [1]. B lymphocytes will only grow and divide for a short period in vitro. What was required was a means of immortalizing these antibody-producing cells. Myeloma cells are immortalized, tumorigenic B lymphocytes. These myelomas will grow and divide in vitro, but do not produce the antibody molecule required. Monoclonal antibody production involves the fusion of cells with the desired antibody-producing capability with myeloma cells to produce cell lines that produce specific antibodies.

For the production of monoclonal antibodies in mice (Fig. 2), the animal must first be immunized with the antigen of interest to provoke its immune system into producing antibodies to the antigen. The mouse spleen is then removed. This contains a high concentration of B lymphocytes (splenocytes) which can produce the required antibody. The spleen cells are fused with a suitable non-antibody producing myeloma cell with polyethylene glycol. This induces the membranes of both cells to fuse and merge. In the subsequent round of cell division, as the nuclear membrane breaks down, chromosome mixing occurs which may result in a hybridoma cell that possesses both the antibody-producing ability and the immortality of the parent cells. Following fusion, what remains is a mixed cell population of hybridomas and unfused splenocytes and myelomas. The splenocytes die off after a short period of growth. Unfused myeloma

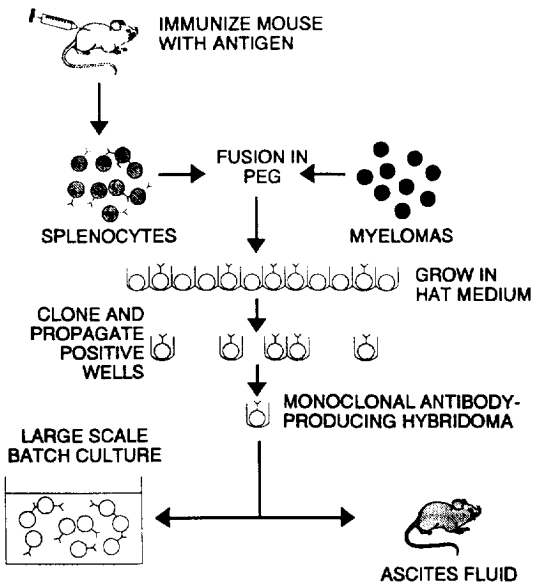

Fig. 2. Production of monoclonal antibodies from the mouse.

cells are removed using a selective growth medium called HAT (hypoxanthine, aminopterin, and thymidine) which prevents the synthesis of purines and pyrimidines in myeloma cells. What remains is a population of fused hybridoma cells.

The fusion mixture can be conveniently divided up into many culture wells. The fused hybridomas are then allowed to grow. Periodically, supernatant from the wells can be analyzed for the presence of antibody to the desired antigen using a suitable assay system, normally an enzyme immunoassay. Positive wells can then be further subdivided. Eventually, some wells will contain clones of hybridomas derived from a single parent cell which produces a single antibody type. A monoclonal antibody-producing hybridoma has now been isolated.

When a clone has been suitably purified from the rest of the hybridoma population, it can then be propagated on a larger scale. This can be performed in vitro in culture flasks and larger bioreactors, or in vivo via ascitic fluids in mice. In vitro production results in high purity antibody, but the process is technically demanding, expensive and results in relatively low yields. Injecting the hybridoma into the peritoneum of a mouse allows the hybridoma to grow rapidly as a tumour. Large amounts of antibody are produced in the resulting ascitic fluid. Yields with this system are high, but so too is the level of contaminating antibody and other proteins from the mouse and further purification is necessary.

The resulting monoclonal antibody molecules can be assessed for their binding characteristics, specificity and cross-reactivity. Such molecules can then be applied to sensor systems.

The majority of monoclonal antibodies generated are of mouse origin. However, the generation of human monoclonal antibodies using basically the same system is also possible. Routine immunization of humans is not feasible except in the case of vaccination in the prevention of disease. The source of B lymphocytes is also different. The spleen is also not feasible as a source of B cells from humans. More often, tonsillar lymphocytes, peripheral blood lymphocytes or other lymphoid tissues are used. In the absence of the possibility of in vivo human immunization, in vitro techniques are available where human B lymphocytes are brought to maturation with an antigen in the presence of the relevant growth factors and cytokines.

A further problem with human monoclonal antibody production is the absence of any non-secreting human myeloma cells. Hybridization with available human myelomas results in a mixed antibody population. To circumvent this, human–mouse hybridomas are made using a mouse myeloma. Such hybridomas can be unstable but are maintained with regular subcloning.

Transformation of B lymphocytes is a natural result of their infection with the Epstein-Barr virus. This property has been applied to the in vitro transformation of human B lymphocytes as an alternative to fusion with a myeloma [2]. Although these techniques do make possible the production of human monoclonal antibodies, the work and difficulties encountered in using such a system are considerable. Modern molecular genetic approaches are now being applied to the production of monoclonal antibodies of human, mouse and other origins.

4. Combinatorial phage display libraries

A molecular genetic technique known as combinatorial phage display may be the answer to the problems of human monoclonal antibody production and also allows the flexibility to produce antibody fragments of both human, murine and other origins by genetic methods. Several systems have been described. In one [3], the heavy and light

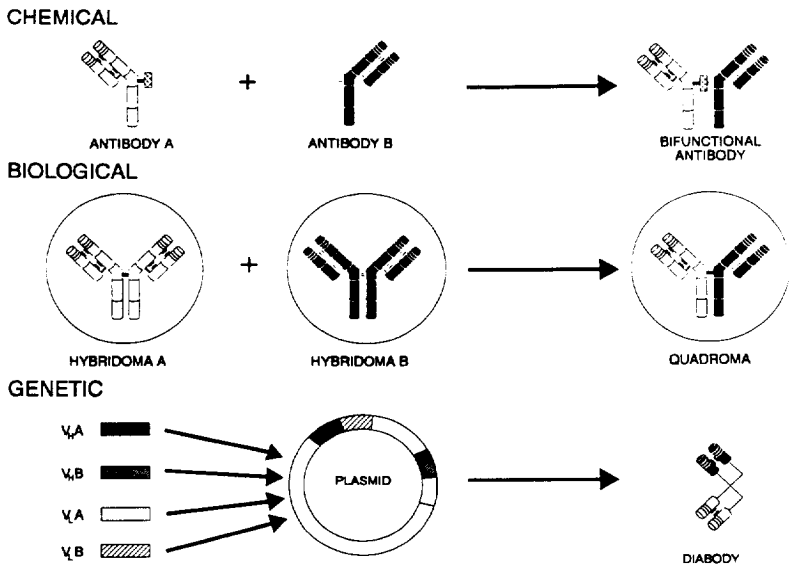

Fig. 3. Strategies for the production of bifunctional antibodies.

chain antibody genes are isolated from cells of the immune system of the animal of interest, e.g., mouse spleen or human blood cells, lymph nodes or bone marrow. These genes can then be combined and expressed as antibody proteins on the surface of a phage particle (a virus that infects bacterial cells). Antibodies of suitable binding affinities can be selected from weaker binding antibody molecules. When the highest affinity antibodies have been selected, they can then be produced by bacterial cells for ease of production and purification. Whole antibody chains are impractical for use in phage display libraries. Instead, only the part of the antibody required for antigen binding is used, i.e., the V_L, V_H, C_L and C_H1 domains which comprise what is known as the Fab fragment (fragment of antigen binding).

The Fab fragments generated can thus be used in much the same way in sensor applications as whole antibody molecules. They also have the added advantage of being safe for human in vivo applications if human lymphocyte-derived material was used to generate the library. It is also a very useful alternative to conventional monoclonal antibody production in the generation of mouse antibodies.

5. Bifunctional antibodies

As can be seen from Fig. 1, the antibody molecule has two binding sites for the same antigen. This is necessary for the formation of immune complexes and for the triggering of antibody effector functions. Chemical, biological and genetic methods have been developed for the production of antibodies which recognize a different antigen at each of the two antigen binding sites, creating a 'bifunctional' antibody (Fig. 3). The chemical methods first involve breaking the disulphide bonds joining adjacent H chains. Chemical cross-linking agents can then be used to join two heterologous L–H chain pairs. One popular agent is *o*-phenylene dimaleimide. Biological production can be achieved by fusing two hybridomas that produce different antibodies. This results in mixed populations of bifunctional antibodies along with other combinations which reduces the yield of bifunctional molecules [4].

The V_H and V_L domains of one antibody have been joined using genetic techniques to the V_L and V_H domains of a second, respectively. These were connected by amino acid linkers, forming a 'diabody'. This small bifunctional molecule maintains its antigen recognition properties [5].

Bifunctional molecules have many novel applications [6]. Many immunoassay arrangements can be created using these molecules. They avoid the need to conjugate enzymes chemically to antibody so circumventing any possible deleterious effects to either species. They are also useful in the area of targeted drug delivery to tumours, again avoiding chemical modifications. One arm of the anti-

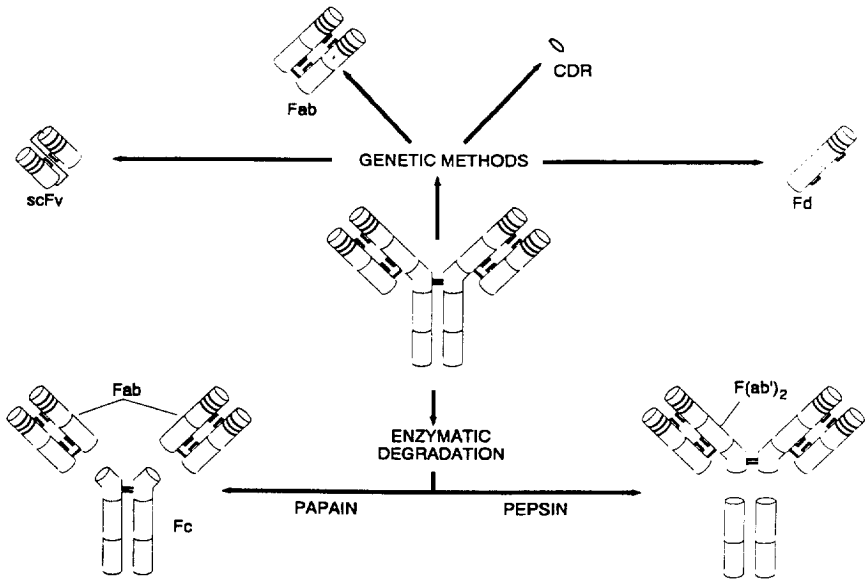

Fig. 4. The generation of antibody fragments.

body binds the drug while the other is free to seek out and bind to the tumour. They also have great potential for use in novel sensor applications.

6. Antibody fragments

Many portions of the whole antibody molecule have been identified as having various applications (Fig. 4). The Fc fragment of the antibody molecule is rarely required for such applications. This is only used if stimulation of the immune system by the antibody is required. Enzymatic digestion can remove this portion of the antibody. Pepsin degrades the H chains below the interchain disulphide bridges, leaving two Fab portions still connected via the hinge region [$F(ab')_2$]. Papain cuts above the disulphide bonds and results in two separate Fab fragments.

Other fragments of the antibody have also been generated by genetic methods (Fig. 4). The Fv fragment has been identified as the smallest fragment required for complete antigen binding. Due to the ease of manipulating these smaller sequences in vitro, much work has concentrated on such fragments. They may also possess better characteristics with regard to tissue penetration and pharmacokinetics than whole antibody. The resulting L–H chain pairing is unstable because the disulphide bond is missing in the Fv portion. To circumvent this problem, some workers have developed methods to link the two chains together, including glutaraldehyde cross-linking, addition of cysteine residues to form disulphide bonds or the inclusion of a flexible amino acid linker resulting in a single chain Fv (scFv) [7]. This latter method has proved extremely useful as V_H and V_L chains can be cloned into vectors with a linker in place. An scFv has been developed with a diagnostic application [8]. This scFv recognizes placental alkaline phosphatase (PLAP), a tumour marker which is used to screen ovarian and testicular cancers. A 'humanized' version of this mouse antibody has also been developed where the CDRs from the mouse antibody were used to replace the CDRs of a human Fv, making the antibody safer to use in humans.

Another small fragment, the Fd fragment, has been used in antigen binding. Most of the antigen binding was due to the H chain which had binding affinities 100-fold higher than the L chain [9], and binding still occurs in the absence of the L chain. This has led some to experiment with the Fd portion alone without the added complication of two chains [10].

Although the H chain dominates the L chain with regard to the binding affinity with antigen, L chains have also been successfully used alone as antigen-binding components, both in a monomeric [11] and a dimeric [12] form, the monomer directed against a cell surface antigen of the melanocyte cell lineage and the dimer directed against a CD4 antigen. This latter dimer had a binding affinity of

$2.5 \cdot 10^9\ M^{-1}$ which is comparable with H chain and even whole antibody affinities. No affinity was quoted for the monomeric light chain fragment.

One of the smallest antibody fragments to be used is a single CDR. An H chain CDR from an anti-HIV gp120 V3 loop antibody was capable of competing with whole antibody for the V3 peptide and in the cyclized form was also capable of inhibiting HIV replication [13]. CDRs have been directly used in sensor applications. CDRs from anti-lysozyme [14] and anti-herpes simplex virus glycoprotein [15] antibodies have both been used in combination with surface plasmon resonance sensors. As well as using the whole CDR, fractions of it are also being used to see whether these smaller peptides result in significant antigen binding without the need for longer peptide chains. Such short chains could be simply and cheaply synthesized.

7. Antibody-based sensors

Antibody properties for biosensor applications have been reviewed [16], together with future trends and future aspects of the research and development of immunosensors [17].

Along with these there have been a number of other reviews concerning antibody-based biosensors [18–22]. The selective reaction between an analyte and the corresponding antibody ensures the specificity of the immunosensor in biological fluid analysis. The specificities of immunosensors are determined in part by the affinity constants of the antibody–antigen interactions [23]. While a high affinity constant will give rise to high specificity, too high an affinity constant will result in an irreversible immunosensor, unless protein denaturing conditions are used.

The major processes involved in any biosensor system are shown in Fig. 5 [24]. There are a number of ways in which the binding event can be transduced into a measurable signal. Typical transducers include electrochemical, optical, acoustic and semiconductor devices.

7.1. Immunosensors based on electrochemical transducers

Electrochemical measurements fall into two broad classes, both of which have been applied for immunosensing applications. Amperometric devices involve the measurement of the current flowing between two electrodes in response to the

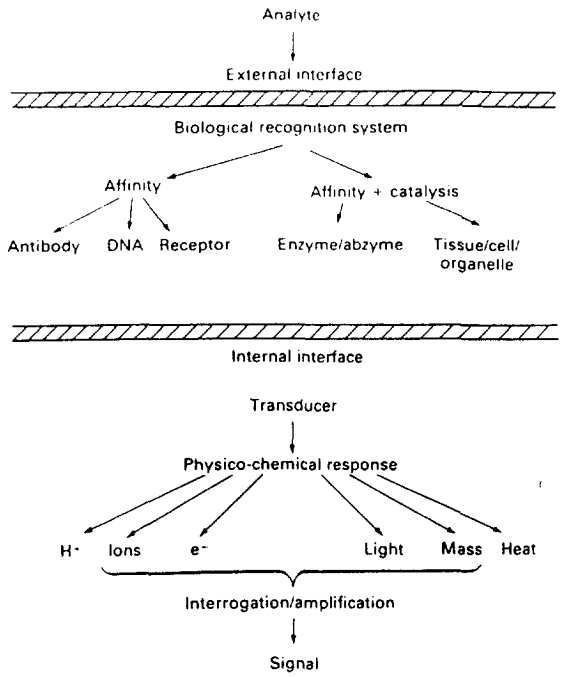

Fig. 5. Scheme representing the processes taking place in a biosensor system, analyte recognition, signal transduction and readout. Reproduced with permission from Ref. [24].

application of a defined voltage. In contrast, potentiometric devices (e.g. pH electrode) require the derived voltage to be determined with reference to a second electrode under conditions of zero current flow.

Deasy et al. [25] have recently reported on an immunosensor, based on a competitive amperometric immunoassay, for the quantitation of 7-hydroxycoumarin (umbelliferone). The antibody-based biosensor employed horseradish peroxidase-labelled anti-7-hydroxycoumarin, with the enzyme-catalysed reaction involving the reduction of hydrogen peroxide in the presence of a mediator (hydroquinone). Sensor preparation involved immobilisation of the thyroglobulin bound 7-hydroxycoumarin antigen within a Nafion film on a glassy carbon surface. The electrode was then incubated in a solution containing both the enzyme labelled anti-7-hydroxycoumarin and increasing concentrations of the free 7-hydroxycoumarin analyte. Competition between the free and immobilised forms of the antigen allows for the quantitation of free 7-hydroxycoumarin in solution. This competitive enzyme-linked immunosorbent assay is outlined in Fig. 6. Injection of the enzyme sub-

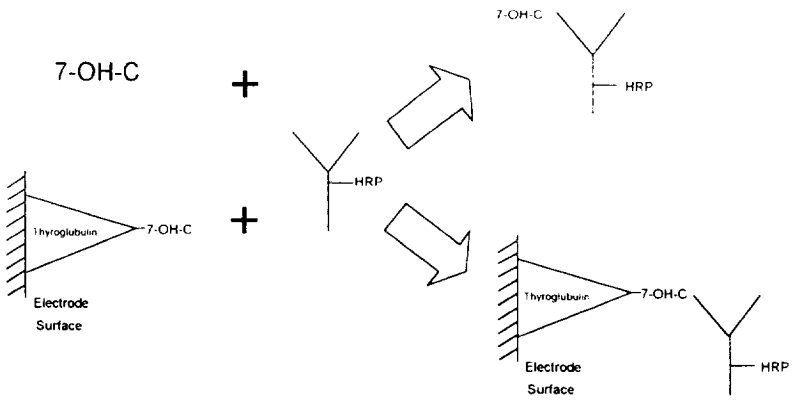

Fig. 6. Outline of the competitive enzyme linked immunosorbent assay used in the 7-hydroxycoumarin (7-OH-C) immunosensor. Reproduced with permission from Ref. [25].

strate, hydrogen peroxide, resulted in an increase in the steady-state cathodic current. This amperometric detection system is shown in Fig. 7.

A similar amperometric immunosensor was developed for the quantitation of Factor VIII, a glycoprotein that is measured in the diagnosis of haemophilia [26]. Some other recently developed amperometric immunosensors include a sensor for the determination of cholinesterase [27] and enzyme immunosensors based on electropolymerised polytyramine-modified electrodes [28]. Further to these, Sadik and Wallace have used pulsed amperometric detection in the determination of human serum albumin using antibody containing conducting polymer [29].

Blackburn et al. [30] have recently described a potentiometric biosensor employing catalytic antibodies as the molecular recognition element. In their prototype, a pH microelectrode was modified with a monoclonal antibody that catalysed the hydrolysis of phenyl acetate to produce protons which would then be sensed at the potentiometric electrode.

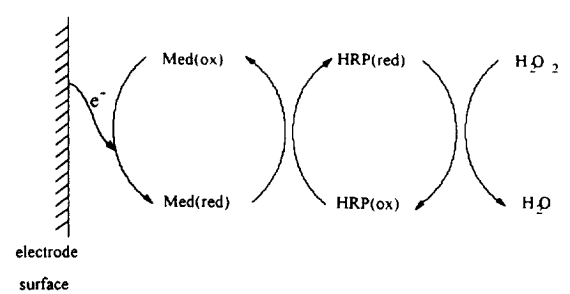

Fig. 7. Schematic diagram showing the amperometric detection system used in the 7-OH-C immunosensor.

7.2. Immunosensors based on optical devices

A surface plasmon resonance (SPR) immunosensor is an SPR device modified with an antibody layer. When the antigen binds, minute changes in the refractive index of the SPR device are detected as a shift in the angle of total absorption of light incident on a metal layer carrying the antibodies. The SPR device can consist of a prism or a glass slide carrying the thin metal layer. Recently, Morgan and Taylor [31] have developed a surface plasmon resonance immunosensor based on the streptavidin–biotin complex. Fig. 8 shows the principle and the apparatus used for this sensor. Nice et al. [32] have published work on the mapping of the antibody and receptor-binding domains of granulocyte colony-stimulating factor using an optical biosensor. The data was obtained using a BIAcore biosensor, with surface plasmon resonance detection.

Optical immunosensors can also be based on internal reflections in a light guide with antibodies immobilised on the surface of the guide which is in contact with the analyte. Use is then made of the evanescent wave penetrating a fraction of a wavelength into the optically rarer medium, when light coming from a denser medium is incident on the interface with an angle above the critical angle. Fluorescent techniques can be used to good advantage with these devices. Bright [33] has reported on probing biosensor interfaces, by multi-frequency phase and modulation total-internal reflection fluorescence.

There are quite a number of immunosensors in the recent literature using the fiber optic approach (e.g. [34,35]). Bier et al. [36] have for instance

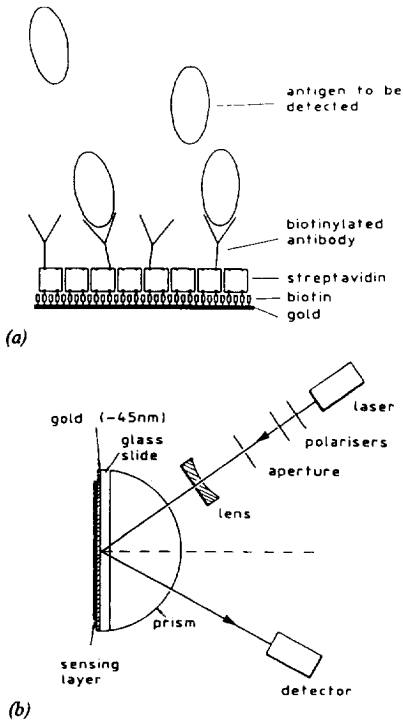

Fig. 8. (a) Cross section through the sensing element showing its structure. (b) Diagram of the SPR apparatus. Reproduced with permission from Ref. [31].

made use of a fiber optic immunosensor for the detection of pesticides. Aminohexylatriazine was immobilised on one end of a silica fiber and the fiber was inserted into a flow-through glass cell. Triazine antibodies (conjugated with fluorescein isothiocyanate) were detected after binding to the fiber surface based on the decrease in the fluorescence signal caused by the presence of triazines due to the inhibition of antibody binding to the fiber.

7.3. Immunosensors based on surface acoustic wave devices

Surface acoustic wave (SAW) devices, which can be sensitive to changes in the elasticity, density or electrical conductivity of a surface on which a surface acoustic wave propagates, can also be used as immunosensors. The device consists of a piezoelectric crystal, such as quartz carrying thin-film interdigital electrode arrays. Radiofrequency excitation of the electrode pair creates a synchronous mechanical surface wave, which is propagated on the surface of the piezoelectric substrate and received by another electrode pair. Koenig and Graetzel [37] have used a piezoelectric immunosensor for the detection of human T-lymphocytes. The frequency difference between the coated crystal (coated with antibody) and a reference crystal was measured, then the coated biosensor was placed in a solution of human T-lymphocytes and a second frequency difference was measured.

Prusak-Sochaczewski and Luong [38] have also used a piezoelectric crystal immunosensor for the determination of human serum albumin. The sensor was prepared by coating the crystal with protein A and reacting this with anti-human serum albumin antibody. This surface could then be used in the determination of human serum albumin.

7.4. Immunosensors based on semiconductor devices

In immuno field effect transistors (ImmunoFETs), the gate of an ion selective field effect transistor is covered with an antibody-bearing membrane. They can be constructed for the detection of the minute potential changes associated with the formation of the antibody–antigen complex. The advantages of the FET devices, i.e. fast response, good signal to noise ratio and small size, are well suited to flow injection analysis. An example of an immunoFET was developed by Schasfoort et al. [39], who reported on an immunosensor to monitor the immunological reaction between human serum albumin and its antibody.

8. Conclusions

Immunosensors are designed by a combination of immobilised antibody (or antigen) with an electronic device. A high selectivity may be attained as a result of the specific affinity of antigen for the corresponding antibody. However, the immunochemical reaction of matrix-bound antibody (or antigen) can be influenced by non-specific adsorption of coexisting substances onto the matrix. This problem of interference from coexisting substances (proteins or small molecules) is still a problem in the design of immunosensors and needs further work in the future.

Monoclonal antibody technology has been a primary source of antibodies for some time, but alternative genetic methods are replacing it, removing the need for difficult cell culture techniques. Also, new chemical, biological and genetic methods for

the production of bifunctional antibodies and antibody fragments also show great promise in generating novel antibody species for use in sensor and other applications. On the sensor side, the trend seems to be towards disposable sensors, which are mass produced, cheap and easy to use. An example of this is the use of screen printed electrodes in immunosensor development.

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