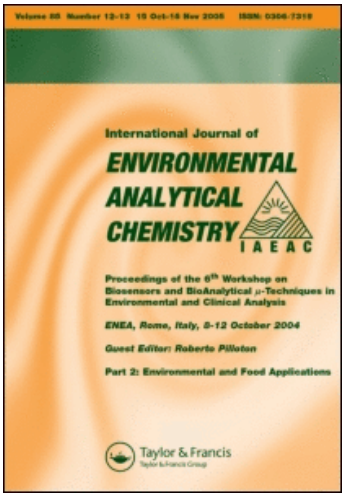

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Development and Use of Antibodies in Surface Plasmon Resonance-Based Immunosensors for Environmental Monitoring

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DEVELOPMENT AND USE OF ANTIBODIES IN SURFACE PLASMON RESONANCE-BASED IMMUNOSENSORS FOR ENVIRONMENTAL MONITORING

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The interaction between antibody and antigen is characterised by relatively high affinity and specificity, making this type of reaction a prime candidate for use as an analytical tool. The interaction may be combined with biosensors in the production of immunosensors for environmental monitoring. Polyclonal and monoclonal antibodies have had a significant impact in analytical detection systems over the past few decades with antibody fragments becoming important in recent years. Production of antibodies to small haptens requires the initial conjugation of hapten to a larger carrier molecule. Once hapten–carrier conjugates have been produced, polyclonal, monoclonal and various antibody fragments may be produced by differing protocols. A critical step in the production of antibody fragments is the development of efficient screening procedures to identify suitable antibody-producing clones and this has been reviewed in this article. Various antibody types may then be used in the generation of immunosensors for the monitoring of environmental pollutants. The selection of the appropriate sensor technology applicable for the determination of an antibody–antigen interaction is of prime importance for immunosensor development. One example of such an application is surface plasmon resonance-based biosensors, as they provide real-time analysis of interactions between the antibody and antigen of interest.

Keywords: Antibody; Immunosensor; BIAcore; Environmental monitoring; Inhibitive assay

INTRODUCTION

The use of immunochemical techniques for environmental monitoring has grown over the past few years. The most important antibody-based approaches currently used for environmental monitoring are immunoassays, immunosensors, immunochromatography and immunolabelling. This article will examine the current techniques for the production of antibodies and antibody fragments and their applications in immunosensors.

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For the production of antibodies, animals must first be immunised with a suitable antigen. Some antigens may be too small to elicit an immune response on their own and so require conjugation to a larger carrier molecule. The success of this process depends on the chemical groups available for conjugation on both molecules and can be problematic. Serum is collected for the purification of polyclonal antibodies, while the spleen cells are harvested for use in both monoclonal antibody production and the production of recombinant antibodies. Each antibody type possesses various characteristics listed in Table I.

The development of rapid analytical devices such as biosensors for the detection of compounds has grown enormously over the past few decades [1,2]. Biosensors are devices incorporating a biological sensing element coupled to a variety of transducing mechanisms (i.e. electrochemical, optical–electronic). When biological molecules interact specifically and reversibly, a change occurs in one or more physico-chemical parameters associated with the interaction. This change causes the production of ions, electrons, gases, heat, mass or light. These quantities are then converted by transducers into electrical signals and displayed in a suitable form. The transducer translates the response of the biological component into a readable electronic signal [2]. The use of biosensors has ranged from environmental monitoring [3], medical applications [4] to the detection of coumarins [5], mycotoxins [6] and pesticides [7].

The biological component of a biosensor should possess a high degree of specificity and stability, should not contaminate the sample and should retain biological activity when immobilised [8]. The specificity of the biosensor is completely dependent on the properties of the biological component as the biological component must recognise the analyte for there to be an interaction. Biosensors may be divided into two categories, the catalytic sensor and the affinity sensor. The catalytic sensor works on the principle that the molecules bind and alter the analyte in some way. The affinity-based sensors actually monitor the binding between the two molecules. The use of biomolecules and biological systems in biosensors was reviewed by McCormack *et al.* [9],

TABLE I Review of antibody characteristics

Characteristic	*Comment*
Ease of production	Monoclonal and polyclonal antibody (Ab) production is established technology while recombinant antibody technology is still under development
Specificity	High specificity for detection of individual molecules or class specific for group of compounds
Affinity	High affinity – 'once-off use' when dissociation is not required (ELISA) Lower affinity – facilitates re-use
Sensitivity	Sensitive assays – polyclonal and monoclonal Abs Possible to produce highly sensitive recombinant Abs
Association/dissociation	'On/off' constants important for assay development as association and dissociation of antibody decides the application of antibody to particular assay
Stability	High stability – monoclonal, polyclonal Abs are relatively stable compared with recombinant Abs; however, it is possible to improve stability in genetically engineered Abs High stability is a very useful property Stable antibodies may be stored long term
Cost of production	Low cost – polyclonal Higher cost – monoclonal and recombinant antibodies
Availability	Readily available – polyclonal and monoclonal Abs Not readily available at present – recombinant Abs
Capacity for improvement	Possible to improve Abs produced using recombinant technology

and may be divided into the following groups: enzymes, antibodies, whole cells, receptors, tissues, nucleic acids, lectins and antigens. Recently, several commercial biosensors have been developed using surface plasmon resonance (SPR) technology as their detection method. In particular, the BIAcore SPR-based biosensor has become a very important tool in the detection of a number of analytes using antibodies as the biological component.

ANTIBODY STRUCTURE

Antibodies or immunoglobulins are a group of glycoproteins present in the serum of all mammals. Rodney Porter [10] in 1966 proposed the basic four-polypeptide chain model for antibodies. The structure of antibodies varies depending on their isotype. However, antibody structure is generally represented by the IgG antibody as shown in Figure 1.

The antibody is composed of four polypeptide chains, with two identical heavy chains (H) and two identical light chains (L) (called κ (kappa) or λ (lambda)) [11]. The light chain has a molecular weight of approximately 25 kDa, whereas the heavy chain has a molecular weight of approximately 50 kDa. The two heavy chains are held together by interchain disulphide bonds, while various antibody chains are held together by intrachain disulphide bonds. The amino terminal end of the antibody is characterised by variability (V) of the sequences in both the heavy (H) and light (L) chains, which are known as the V_H and V_L regions. The remainder of the molecule is constructed of constant regions (C_H and C_L). These constant regions are broken up into three distinct regions: C_H1, C_H2 and C_H3. A hinge region is also present, which is found between the C_H1 and C_H2 domains, allowing conformational changes to

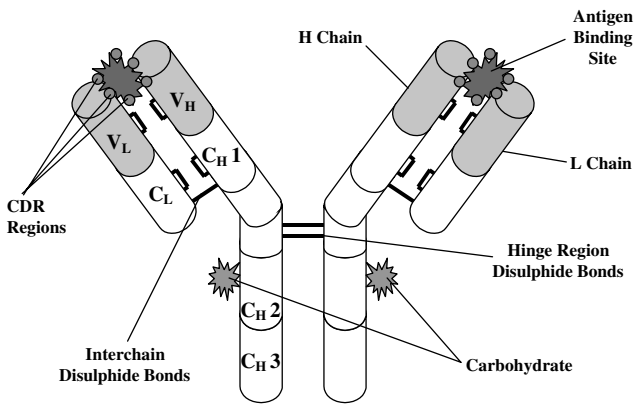

FIGURE 1 Diagrammatic representation of an immunoglobulin molecule. The amino terminal end of the antibody is characterised by variability (V) of the sequences in both the heavy (H) and light (L) chains, which are known as the V_H and V_L regions, respectively. The remainder of the molecule is constructed of constant regions (C_H and C_L). These constant regions are broken up into three distinct regions: C_H1, C_H2 and C_H3. There is also a hinge region which is found between the C_H1 and C_H2 domains which allows conformational changes to occur in the antibody resulting in the flexibility of between 60 and 180°. The CDR regions confer the specificity of the antibody to a particular antigen and this is known as the antigen-binding site. The CDR regions in both light and heavy chain V regions are sub-divided into three CDRs (CDR1, CDR2, CDR3). The two heavy chains are held together by interchain disulphide bonds while various antibody chains are held together by intrachain disulphide bonds.

occur in the antibody resulting in flexibility of between 60 and 180°. This flexibility allows the binding sites on each arm of the antibody to work independently from one another.

The binding site of an antibody to an antigen is located within the variable region of the antibody. The properties of antigenic recognition and specificity are comprised of approximately 110 amino acid residues located in the variable region. These are referred to as hypervariable regions forming a region complementary in structure to the antigen epitope (defined as a single antigenic determinant: functionally it is the portion of an antigen which combines with the antibody paratope). Hypervariable regions are also referred to as complementarity determining regions (CDRs) and the intervening peptide segments, which are approximately 10 amino acid residues long, are known as framework regions (FR). The CDR regions in both light and heavy chain V regions are sub-divided into three CDRs (CDR1, CDR2, CDR3). The light and heavy chain CDRs form the six hypervariable loop structures producing a three-dimensional structure, which acts as the antigen-binding pocket.

ANTIBODY CHARACTERISTICS

Antibody Affinity

The interaction formed when an antibody comes into contact with its specific antigen is determined by a combination of different physical forces, hydrophobic interactions, hydrogen bonds, electrostatic binding and van der Waals' forces [12].

Hydrophobic interactions involve the association of non-polar residues in aqueous environments. Electrostatic binding is the result of attraction between oppositely charged groups on the two interactants, the effects of which may be greatly enhanced due to the removal of water molecules as a result of hydrophobic interactions. Hydrogen bonds are formed between hydrophilic groups such as OH, NH_2, and COOH and are formed when hydrogen atoms are shared between two other atoms. Van der Waals' forces are due to the interactions between the electron clouds of adjacent non-polar groups on the neighbouring molecules. This results in the induction of oscillating dipoles between the two molecules producing a net attractive force. The strength of these forces is only effective over a small distance, resulting in the epitope on the antigen and the binding site on the antibody becoming the defining factor for the strength of the interaction overall.

The term affinity is defined as the sum of the attractive and repulsive forces for a single antibody–antigen interaction and may be described in terms of reaction kinetics. At equilibrium, the formation of an antibody–antigen complex may be expressed as:

$$Ab + Ag \underset{k_d}{\overset{k_a}{\longleftrightarrow}} Ab.Ag, \tag{1}$$

where Ab is antibody and Ag is antigen and $Ab.Ag$ is the antibody–antigen complex and k_a and k_d are the association and dissociation rate constants, respectively.

$$K_A = \frac{k_a}{k_d} = \frac{[Ab.Ag]}{[Ab].[Ag]}. \tag{2}$$

The affinity of the antibody for the antigen is given by the equilibrium association constant K_A.

$$K_D = \frac{1}{K_A} = \frac{k_d}{k_a} = \frac{[Ab].[Ag]}{[Ab.Ag]}. \quad (3)$$

The affinity of the antibody–antigen interaction may also be stated as the equilibrium dissociation constant, K_D, which is a reciprocal of the K_A value.

Antibody Specificity

Antibody specificity depends on its ability to discriminate between different haptens (small molecules which can act as an epitope but which are incapable by themselves of eliciting an antibody response) with structural similarities to the haptens to which the antibody was raised. Since the strength of the binding of antibody to hapten depends on the affinity of the antibody for the hapten, specificity of the antibody can be related to its relative affinity for the hapten. Antibodies may display relative affinities for more than one hapten with varying degrees of affinity for each. This may be referred to as cross-reactivity and is designated as relative affinity of the antibody rather than absolute affinity for a particular hapten. Cross-reactivity of an antibody with a number of haptens is usually present when the hapten contains similar or identical epitopes. Generally, both polyclonal and monoclonal antibodies can possess high levels of cross-reactivity; however, cross-reactivity can be a greater problem with monoclonals as there is only one population of antibody present.

Although antibody specificity is an advantageous characteristic for low-level detection of trace amounts of individual haptens, there are applications in which broad-specificity antibodies with the ability to recognise groups of haptens would be desirable. Such antibodies may be applied in cost-effective screening programmes and for assays with the purpose of providing a positive or negative result for the presence of specific analytes. Alcocer *et al.* [7] produced broad-specificity antibodies against a group of organophosphate pesticides by using phosphonic acid (TPB) as a generic hapten. Results showed that detection of a number of pesticides was possible using this method.

PRODUCTION OF ANTIBODIES

Hapten–Carrier Conjugate Generation

Haptens or molecules with a molecular weight of less than 1 kDa are unable to elicit an immune response. This characteristic produces the need for conjugation of these haptens to larger carrier molecules resulting in hapten–carrier conjugates capable of producing an immune response. In order for some haptens to be chemically conjugated to larger carrier molecules, modification of the structure of the hapten may be required to produce the functional groups needed for the conjugation chemistries to the carrier molecule. The most frequently used carriers are highly immunogenic proteins (i.e. BSA, bovine serum albumin; OVA, ovalbumin; THY, thyroglobulin; etc.), but lipid bilayers, polymers (e.g. dextran), and synthetic organic molecules have also been used [13]. It is important for the carrier molecule to be highly immunogenic, non-toxic *in vivo*,

as well as containing suitable functional groups for conjugation to the hapten of interest.

The coupling chemistry used to prepare such immunogenic hapten–carrier protein molecules is an important consideration for the production of antibodies with the correct specificity. Many methodologies for conjugation exist and vary depending on the functional groups that are available on both the antigen and the carrier molecule. These range from carbodiimide-mediated hapten–carrier conjugation (forming amide bonds), NHS ester-mediated hapten–carrier conjugation (forming amide bonds), NHS ester-maleimide heterobifunctional cross-linker-mediated hapten–carrier conjugation (forming thioether bonds) to diazonium conjugation (forming diazo linkages) and the Mannich condensation (forming alkylamine linkages).

To produce efficient conjugation reactions, one objective is to optimise the number of haptens conjugated to each carrier molecule. This is carried out to direct the production of antibodies to the hapten part of the conjugate rather than the carrier molecule in order to reduce the production of non-specific antibodies. However, this strategy also produces its own problems. Conjugates which possess a high hapten to carrier ratio may become insoluble if the hapten is hydrophobic. This would result in the conjugate becoming unusable for the development of assay and selection strategies.

Production of conjugates should also take into consideration the orientation of the hapten to produce optimal presentation to the immune system as without the correct orientation and presentation, specific antibodies to the hapten may not be produced. To achieve optimal presentation to the immune system the main epitope of the hapten of interest should be placed as far away from the carrier as possible.

Hapten–protein conjugates are also used in the screening stages of antibody production. When screening for the detection of specific antibody, it is desirable to use a conjugate containing a different protein carrier moiety than the immunogen, so that false positive results due to binding of antibodies against the protein part of the conjugate are minimised. Danilova [14] recommended that, when dealing with very small haptens, the conjugate used for screening should possess a different carrier molecule and coupling chemistry than that used for immunisation. One of the most widely used proteins for conjugation to haptens is BSA. This is highly soluble with a molecular weight of 67 kDa, and contains a variety of functional groups available for conjugation.

Although phage display and affinity selection techniques have been found to be widely applicable to the isolation of high-affinity antibody fragments, these selection techniques can prove ineffective in some instances. Attempts to isolate high-affinity antibody clones to small, hydrophobic hapten molecules have proven difficult [15]. Coumarin and 7-hydroxycoumarin are two such molecules, and standard affinity ligand selection processes appear to be ineffective with such molecules. Affinity selection is based on the principle that high-affinity interactions are selected out at the expense of lower affinity ones. In most hapten–protein conjugates, the hapten is large and complex enough to provide a significant number of interactive forces for high affinity binding. In this instance it is then easy to remove unwanted affinity interactions to the carrier protein and the linker molecule. However, in the case of coumarin and 7-hydroxycoumarin, few affinity interactions are available, which makes affinity-dominant interactions with the carrier and the linker far more likely to contribute to interactions with the antigen-binding site when high affinity interactions are being selected. This has been shown to result in clones with only minimal affinity for the hapten and a large reliance on the carrier. Great care must be taken

to prevent this. Using different carrier proteins is not always a guarantee of success as identical coupling chemistries to different carrier proteins normally results in linkages to the same amino acids that form the binding dominant interaction. Further steps must be taken, such as modifying the coupling chemistry and the orientation of the hapten to the carrier. Although the interactions being selected for may still remain of relatively lower affinity, they will not become dominated by stronger, but unwanted interactions.

Polyclonal Antisera

When an animal is injected with a multivalent antigen, an immune response is elicited, and the titre of antibody specific for the epitopes on that antigen increases. If several injections of the antigen are given over a period of weeks or months the specific antibody response will increase. The blood of the host animal will contain a heterogeneous mixture of antibodies directed against different epitopes on the immunogen, and binding with a variety of affinities. This is known as polyclonal antiserum [16]. The animals in which polyclonal antiserum is 'raised' tend to be the larger species of domestic animal, such as rabbits, sheep, chickens, donkeys and goats. This is because of their relative ease of handling, as well as the fact that it is possible to obtain large quantities of antibody without harming the animal.

The concept of antibody affinity cannot be applied to polyclonal antisera due to the heterogeneous nature of the population. Instead the term 'avidity' is used to describe the average affinity value for the mixture of antibodies in a polyclonal antibody population [16].

One of the main advantages in the production of polyclonal antibodies is their relative inexpense, as well as the relatively short timeframe in which they can be obtained. A possible disadvantage is that consistency between batches cannot be guaranteed and therefore, there may be a greater degree in variation in assays using polyclonal antibodies.

Monoclonal Antibody Technology

The development of monoclonal antibody technology was first reported by Köhler and Milstein [17]. Before this, all antibodies produced were polyclonal in nature. Although polyclonal antibodies were useful in many areas, their heterogeneous nature also limited their application. However, the homologous nature of monoclonal antibodies allows the generation of antibodies with differing characteristics such as higher specificity and affinity by selecting for such characteristics.

For the production of monoclonal antibodies (Fig. 2) by somatic cell fusion, an initial immunisation of the host animal with the antigen is required. This primes the animal for the production of specific antibodies to the antigen of interest. Production of antibody to the specific antigen is monitored closely until the titre is sufficient to perform a fusion. The spleen is removed from the animal and the splenocytes harvested in order to perform the procedure. The other fusion partner in this procedure is a mouse myeloma cell line (e.g. X63-Ag8.653 and Sp2/0-ag14). Myeloma cells are tumorigenic B-lymphocytes, which may be cultured *in vitro*. These cells are found to lack the enzyme hypoxanthine guanidine phosphoribosyl transferase (HGPRT) and are unable to proliferate in the presence of HAT (Hypoxanthine, Aminopterin and

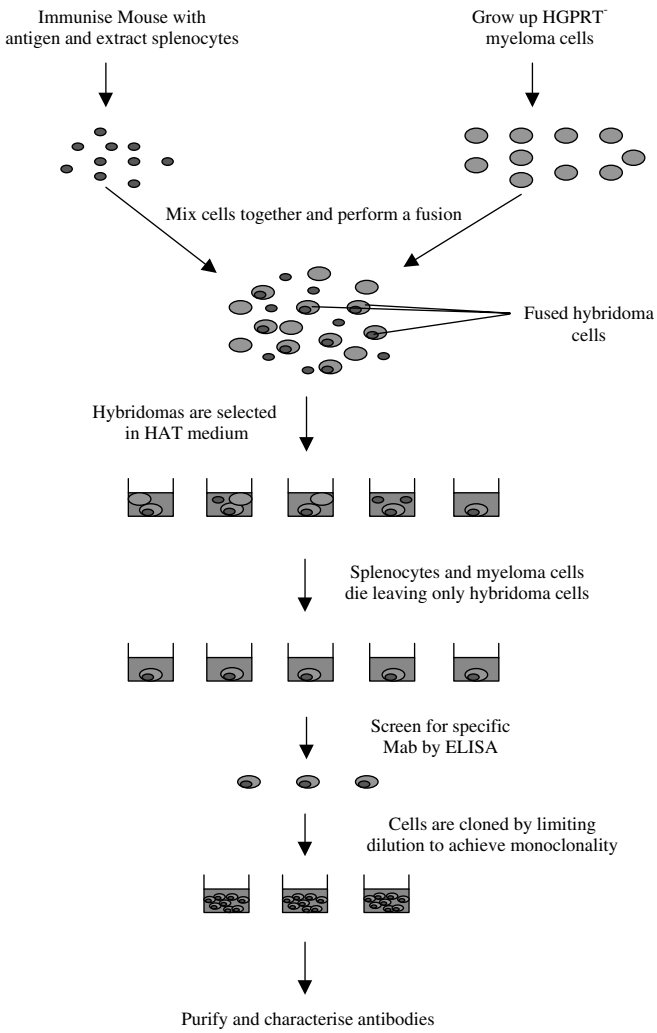

FIGURE 2 Principle of monoclonal antibody production.

Thymidine) medium. Aminopterin blocks the *de novo* biosynthesis of purines and pyrimidines needed for the production of DNA. One of the characteristics of $HGPRT^-$ cells is that they cannot utilise the salvage pathways for DNA synthesis as they do not possess the required enzyme systems. However, splenocytes carry the required enzymes to produce DNA through the salvage pathway but are unable to proliferate outside the body and die off naturally. Fused cells, however, will proliferate in HAT medium as they have acquired the ability to use the salvage pathway to produce DNA from the spleen cell and the characteristic to grow continuously from the myeloma cell, resulting in a hybridoma cell capable of antibody production.

Splenocytes from the immunised animal are fused with myeloma cells by the addition of polyethylene glycol (PEG), which promotes membrane bridging, cell fusion and transfer of nuclei [18]. Once hybridomas have been selected, using HAT medium, the culture supernatants are screened for hybridomas producing specific antibodies to

the antigen. Eventually, hybridomas from a single cell are produced using procedures such as cloning by limiting dilution where cultures are recloned until they eventually derive from a single cell [19].

Monoclonal antibodies may also be produced by *in vitro* immunisation [20]. For the production of monoclonal antibodies using this method, spleen cells are harvested from naïve mice and incubated with the antigen of interest for a period of 5–9 days, before the somatic cell fusion procedure. Antibodies produced using *in vitro* immunisation are as a result of a primary response and are generally IgM antibody molecules with low quantities of IgGs. The production of IgM antibodies is a disadvantage of this system as, in general, these antibodies have a lower affinity than IgG antibodies. However, this system reduces both the immunisation time and the amount of antigen required for the procedure compared to *in vivo* immunisation. Bonwick *et al.* [21] have demonstrated the ability of this method to produce antibodies by generating antibodies to flucofluron and sulcofuron. However, the lack of papers in the literature suggests that there has been relatively little success in the development of assays using antibodies produced by *in vitro* immunisation.

Engineering of Antibody Fragments

Antibody engineering can be defined as the construction of genetically engineered designer antibodies. Over the last fifteen years, genetic and molecular techniques have been used as a means of generating monoclonal antibodies and antibody fragments [22]. Recombinant antibody display technology is utilised in the generation of antibody fragments from several species including human, rabbit, mouse and sheep. This has resulted in the engineering of antibody fragments (Fig. 3), which include the antigen-binding (Fab) and single chain variable fragments (scFv). This truncated Fab fragment consists of one V_L domain and one V_H domain. The Fv fragment can be very unstable for use in therapeutics or immunoanalysis because it lacks the interchain disulphide bond, which is present in the Fab fragment [23]. Protein engineering has facilitated the development of a more stable Fv fragment. A synthetic peptide linker (or in some cases the insertion of cysteine residues to form a disulphide bridge) can be incorporated into the Fv fragment to produce a more stable single chain Fv (scFv) fragment. A single chain Fv fragment is composed of a heavy chain variable region joined to a light chain variable region by a flexible 15 amino-acid linker. Smaller antibody fragments may also be engineered capable of binding to antigen. These include the Fd region which consists of one V_H, one C_H1 domain from the heavy chain, and the complementary determining region (CDR), which is the smallest antibody fragment with the ability to bind to antigen.

The DNA with the genes required to code for antibody fragments is available from a number of sources, which include the genetic material from non-immunised or immunised animals or humans and genes from a repertoire of naïve, semi-synthetic or synthetic genes [24]. Messenger RNA may also be attained from hybridomas expressing antibodies to a specific antigen and used to provide the raw material for cloning the antibody variable gene segments [25].

Due to the advent of antibody phage display technology, isolation of antibodies with higher affinities has become possible while simultaneously reducing the size of the antibody [26]. This is possible with the use of different selection strategies capable of isolating antibodies with the required affinities.

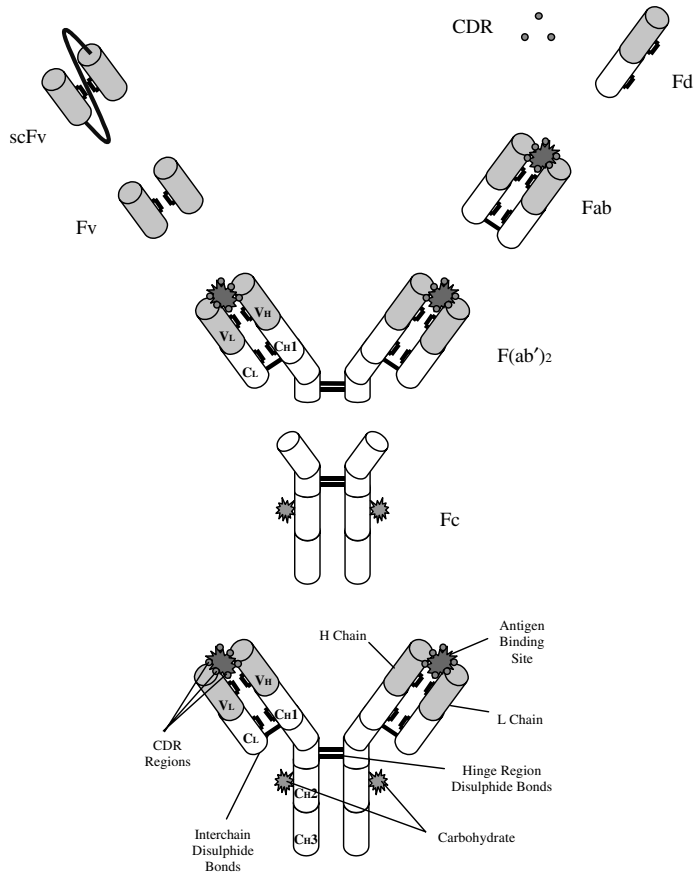

FIGURE 3 Illustration of an antibody and antibody fragments. The fragments illustrated above may be produced by genetic or enzymatic and chemical manipulation. The whole antibody may be initially broken up into either Fab (antigen-binding fragment) or $F(ab')_2$ (two antigen-binding fragments linked) and Fc (crystalline fragments) regions. Fab fragments may be further broken up into Fv (variable fragments), scFv (single chain Fv) and individual CDR regions which are all capable of antigen binding.

Construction of Combinatorial Phage Display Libraries

Recombinant antibody libraries are defined as repertoires which contain high numbers of clones, each capable of producing a different antibody. These libraries may be used in the selection of clones producing hapten-specific antibodies. The first step in the production of a recombinant antibody library (Fig. 4) using phage display is the isolation of mRNA from sources such as peripheral blood, spleen cells or bone marrow. The isolated mRNA should contain the full repertoire of antigen specificities and may be used in the production of complementary DNA (cDNA) using reverse transcription. The technology behind the use of phage display as a tool to produce antibodies is the use of this cDNA in the polymerase chain reaction (PCR). Utilising suitable primers, PCR may be applied for the amplification of heavy and light chain genes for antibody production. These amplified gene fragments are then joined together and cloned into a suitable phage expression vector. Once this occurs the antibody may be displayed on the surface of the phage allowing the antibody on the surface

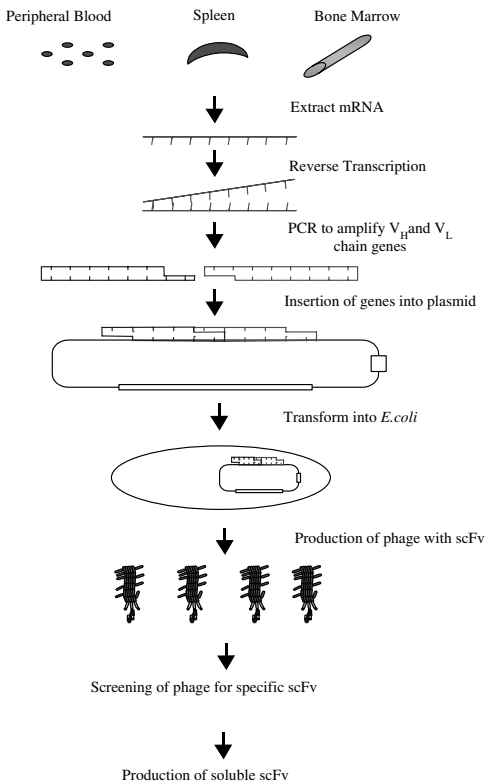

FIGURE 4 Diagram showing a generic model for the production of a combinatorial antibody phage display library. Messenger RNA was initially isolated from a suitable source as listed above. The mRNA undergoes reverse transcription and PCR amplification of antibody heavy and light chain genes using families of variable 5′ primers and constant 3′ primers. Light and heavy chain genes are joined together and ligated into a plasmid followed by transformation into *E. coli*. Phage expression is brought about by infection with helper phage, and affinity selection takes place against the desired antigen immobilised on a solid support. When specific clones have been isolated, soluble antibody may be expressed by religating specific light and heavy chain genes into a vector for soluble expression.

to be treated exactly like an antibody for selection and affinity studies [27]. Other characteristics encoded on the phage vector include an antibiotic resistance marker, the phage coat protein, an origin of replication, a promoter, and a tag to aid in scFv purification.

The *lacZ* promoter is present to produce strain-dependent *lac* promoter expression. Under control of the *lac*Z promoter expression of the vector can be induced using isopropyl-β-D-galactopyranoside (IPTG) and suppressed in the presence of glucose. Helper phage is used for the packaging of phagemid DNA into a virion. This allows the expression of recombinant antibodies on the surface of the phage. Initially specific antibodies are produced by propagation of *Escherichia coli* resulting in the excretion of virions into the supernatant followed by screening the supernatant for hapten-specific antibodies. These antibody fragments are usually linked to one of two phage coat proteins (pIII or pVIII) on the surface of phage particles (Fig. 5). Following the isolation of the specific antibodies required, the DNA from these clones are sub-cloned into plasmids with the characteristic for soluble expression of antibody.

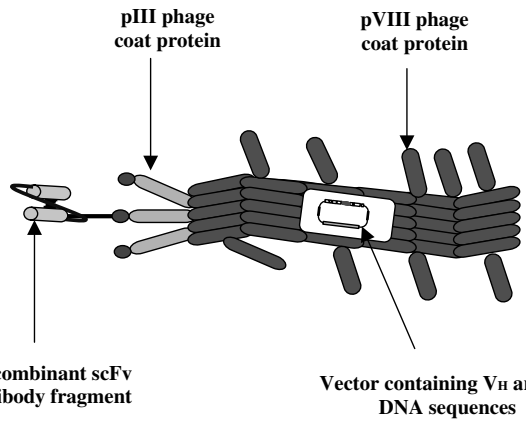

FIGURE 5 Diagram of filamentous phage expressing scFv on the surface of the phage as a fusion with the pIII phage coat protein. Light and heavy chain genes are present in the vector contained within the phage and the phage is ready for infection into *E. coli*.

Selection and Screening of Specific Phage Antibodies

Selection processes that isolate clones producing specific antibodies are of vital importance in obtaining antibodies that are required. The most popular selection methods include affinity selection (also known as biopanning) of phage displaying antibodies by use of immobilising antigen onto solid supports and columns or, alternatively, the use of the BIAcore biosensor for analysis of antibody binding to antigen immobilised onto the surface of a chip followed by selection of the specific antibodies.

The biopanning process (Fig. 6) for isolation of phage-expressing specific antibodies from combinatorial phage display libraries may be achieved by multiple rounds of phage binding to antigen, followed by washing to remove non-specific phage and elution to retrieve specific phage. This may be achieved with the use of immunoassay plates or immunotubes to which hapten–protein conjugates are adsorbed onto the surface or use of the BIAcore biosensor with conjugate immobilised onto a chip [28–31]. The higher the ratio of hapten bound to the protein, the higher the chance of phage antibodies binding to the hapten rather than the conjugate. However, as many haptens are hydrophobic, the more haptens present on the protein will result in insoluble complexes, which will inhibit the adsorption of the conjugate onto the surface of the immunoplate or immunotube.

Haptens covalently immobilised on the surface of immunoplates may also be used in the selection process for specific antibodies. This will decrease the possibility of selection of non-specific antibodies as the carrier–protein is no longer present in the selection process. However, immobilisation may depend on the availability of the functional groups on the hapten and is not suitable for all haptens.

Following production of recombinant antibodies from *E. coli*, it is necessary to screen large numbers of clones for antigen-specific antibodies with the required characteristics. This is achieved with the use of a simple ELISA [29] with coated antigen on the surface of an immunoplate. The polyclonal phage mixture is added to the wells of the plate and the binding allowed to come to equilibrium, followed by the use of enzyme-labelled secondary antibodies and chromogenic substrate for detection of these bound antibodies.

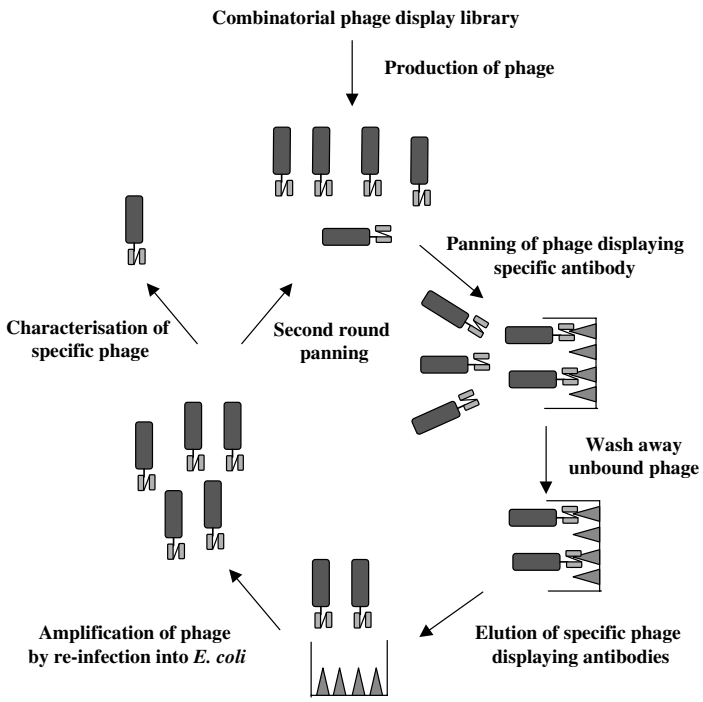

FIGURE 6 Illustration of affinity-selection panning process for the isolation of specific phage displaying antibodies. Phage libraries are subjected to several rounds of panning whereby phage displaying scFvs is captured by binding to specific antigen on a solid support. Phage is then eluted from the antigen and re-infection into *E. coli* for screening of further rounds of panning.

One problem associated with using a selection system such as the biopanning process and the screening assays are that these systems are required to reach a state of equilibrium. The use of these procedures results in the selection of high-affinity antibodies over lower-affinity antibodies [32], which may not be desirable in some assay configurations such as the BIAcore biosensor. The BIAcore has the ability to perform 'real-time' analysis of biomolecular interactions but requires antibodies with relatively high dissociation rates for the repeated regeneration of the sensor chip surface. However, due to the nature of the ELISA screening technique, in some cases the vast majority of antibodies isolated have low dissociation rates and would require more severe regeneration conditions to dissociate the antibody–antigen complex. This problem may be solved using the BIAcore biosensor for selection of positive antibody displaying phage with different affinities [31]. This development has become very important as the use of antibody and antibody fragments on biosensors is becoming more prevalent [33]. Antibodies selected using the BIAcore biosensor possess the characteristics for use on this sensor and so may be applied to the development of BIAcore biosensor assays.

By using the BIAcore biosensor for screening, it is possible to monitor the entire binding pattern of an antibody mixture. Unpurified antibody mixtures such as supernatant may be directly injected over a surface with the antigen of interest immobilised and the antibody binding patterns may be monitored in 'real-time' and screened on the basis of their dissociation rate constants as described by Marks *et al*. [34]. In this article,

antibody fragments were screened using the BIAcore biosensor and results showed the selected antibodies exhibited affinities improved by 300 times compared to conventional selection techniques.

BIOSENSORS

Surface Plasmon Resonance (SPR) Biosensors

Several biosensors have been developed based on the phenomenon of surface plasmon resonance. This phenomenon allows the detection of biomolecular interactions in 'real-time'. The principle behind SPR is described in the context of BIAcore for convenience. At an interface between two media of different refractive index (e.g. glass and water), light coming from the side of the higher refractive index is partly reflected and refracted. Above a certain critical angle of incidence, the light is totally internally reflected and no light is refracted across the interface between the two surfaces of different refractive index.

Under conditions of total internal reflection (TIR), an electromagnetic field component called the evanescent wave penetrates into the medium of lower refractive index a short distance in the order of one wavelength. As the evanescent wave moves further away from the interface into the lower dense medium, the wave decays exponentially. If the interface between the media is coated with a thin layer of metal (in the case of BIAcore, this metal layer is gold), containing electron clouds at the surface and the passage of the evanescent wave through this metal layer causes the plasmons to resonate which results in a quantum mechanical wave known as a surface plasmon. Some of the energy of the reflected light (incident light) is taken up by the surface plasmon wave, resulting in a dip in the intensity of reflected light at a certain angle being observed [35]. The incident light angle at which this dip is observed is known as the SPR angle. The SPR angle is dependent on a number of factors. These factors include the properties of the metal film (e.g. thickness, uniformity and composition), the wavelength and polarisation of incident light and the refractive index of the media on either side of the metal film. In real-time BIA, the properties of the metal film, the wavelength and refractive index of the denser medium are kept constant. The SPR signal can be used to monitor the refractive index of the aqueous layer immediately adjacent to the gold metal layer. The light source in the BIAcore instrument is a high-efficient light-emitting diode with a wavelength in the near infrared λ-region. This light is focused on an interface consisting of glass and gold on the sensor chip in a wedge-shaped beam, producing a fixed range of incident angles. A two-dimensional diode array is used to monitor the reflected light. Changes in the refractive index are a direct result of changes in the mass or concentration on the surface of the chip and this characteristic of SPR has been used to monitor biological interactions [36]. The change in reflected light is interpolated as a sensorgram.

At the heart of the BIAcore instrument, as with the majority of SPR-based commercially available instruments, is the sensor chip as shown in Fig. 7. The BIAcore sensor chip consists of a glass slide with a thin layer of gold deposited on one side. Gold was chosen because it possesses the characteristics of chemical inertness and good SPR response. This gold layer was in turn covered with a covalently bound carboxymethylated dextran matrix attached by a hydroxyalkyl thiol linker layer.

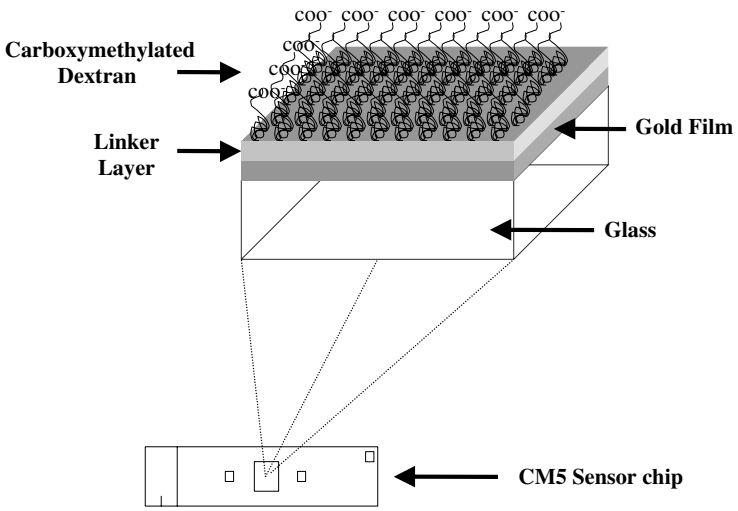

FIGURE 7 Diagram representing the surface of the BIAcore carboxymethylated 5 (CM5) sensor chip. The surface of the chip consists of: glass, a thin gold film and a carboxymethylated dextran layer. The carboxymethylated dextran matrix is bonded to the gold film through an inert linker layer. This matrix allows the covalent immobilisation of analytes onto the surface of the chip.

The matrix allows the covalent immobilisation of analytes onto the surface of the chip and increases sensitivity by increasing the binding capacity of the surface.

The BIAcore biosensor is also used in the development of inhibitive immunoassays to various metabolites (Fig. 8). With this system, either hapten or antibody may be immobilised onto the surface of the chip. Immobilisation of hapten directly may prove difficult depending on the functional groups available. This is why many assays developed immobilise protein–hapten conjugates onto the surface. Standards of free hapten are prepared and premixed with antibody and after a suitable incubation time, passed over the surface of the chip with protein–hapten conjugate immobilised. The hapten in solution inhibits the binding of antibody to the surface and the amount of antibody that binds to the surface is inversely proportional to the amount of free hapten in solution. The results of this immunoassay may be used in the construction of a standard curve and unknown concentrations determined from such a curve. This biosensor may be used in environmental monitoring as described by Daly *et al.* [6]. In this article an inhibition assay was developed for the detection of aflatoxin B_1 using carrier proteins (BSA) as the ligand for immobilisation onto the surface of the chip. Polyclonal antibodies were produced to aflatoxin B_1 and found to be regenerable using an organic solution consisting of 1 M ethanolamine with 20% (v/v) acetonitrile, pH 12.0. The polyclonal antibodies were added to standards of free aflatoxin and passed over the surface immobilised with aflatoxin B_1-BSA. The remaining free antibody bound to the surface of the chip, providing a signal inversely proportional to the amount of free aflatoxin in solution and a detection range of between 3 and 98 ng/mL was achieved.

The BIAcore biosensor has also been used in the detection of pesticides such as parathion [7] using a broad specificity antibody produced against a group of organophosphate pesticides. This antibody was found to be easily regenerable using mild solutions of NaOH and HCl. A parathion–BSA conjugate was immobilised onto the

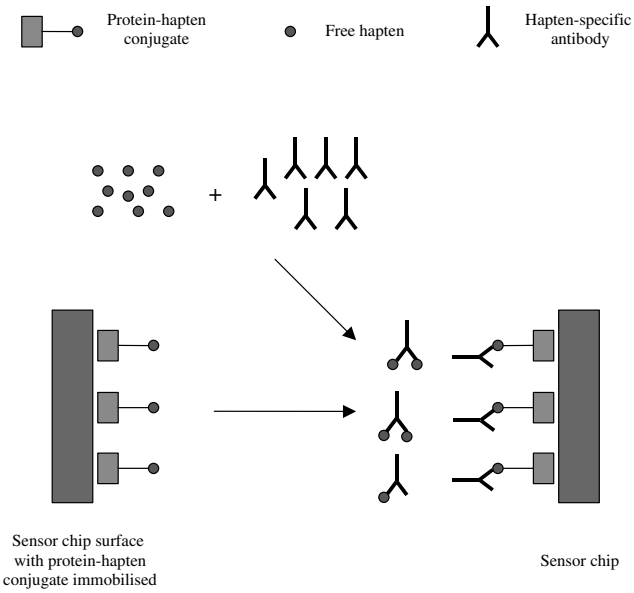

FIGURE 8 Schematic representation of the BIAcore inhibitive immunoassay format for quantitative determination of hapten. Standards of free hapten were prepared and premixed with antibody, and after a suitable incubation time, passed over the surface of a chip with protein–hapten conjugate immobilised on its surface. The excess antibody in the mixtures binds to the hapten immobilised on the sensor chip surface. The amount of antibody which binds is inversely proportional to the amount of free hapten in solution.

surface of a sensor chip and a mixture of antibody and varying concentrations of parathion were passed over the surface. The assay had a range of detection for parathion of between 3 and 100 μg/mL.

Keating *et al.* [5] also developed an inhibition assay for the determination of total-7-hydroxycoumarin (7-OHC) in human serum samples using the BIAcore biosensor. A 7-OHC–BSA conjugate was produced and immobilised onto the chip surface. Standards of free 7-OHC were premixed with a polyclonal anti-7-OHC antibody and injected over the surface. As in the previous assay excess antibody bound to the immobilised conjugate, generating a binding response that was inversely proportional to the amount of 7-OHC in solution. The assay had a range of detection for 7-OHC of between 0.5 and 80 μg/mL.

Further surface plasmon resonance-based assays have concentrated on the production of assays to β-lactam antibiotics [37], the antibiotics gentamicin [38] and chloramphenicol [39] and many more compounds. Results from these assays confirm the growing applications of immunosensors for environmental monitoring.

Miniature TI-SPR Sensor

The miniature TI-SPR device was first released in 1996 by Texas Instruments, and consists of a light-emitting diode (LED), polariser, thermistor allowing correction due to temperature changes and two 128 silicon photodiode arrays (Fig. 9). These components are mounted on a single platform using conventional semi-conductor-based opto-electronic manufacturing techniques [40]. The platform is encapsulated in an epoxy resin moulding structure in the form of the Kretchmann geometry prism.

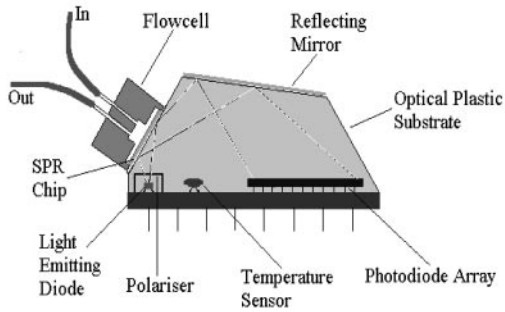

FIGURE 9 Cross-section of miniature TI-SPR instrument. The instrument consists of an electro-optical component mounted on a single platform encapsulated in an optically transparent plastic. Changes in mass are related to changes in the resonant angle and this change is measured by the photodiode array.

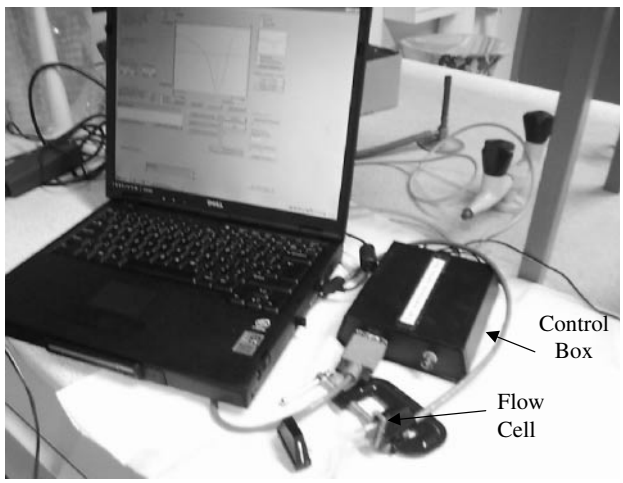

FIGURE 10 Schematic of TI-SPR device fitted with flow cell. A control box interfaces with the device and this may be installed on a personal computer. SPR-MINI MS-Windows software can be used for data acquisition and processing to produce a display via a virtual control panel as shown on the laptop.

The width of light produced by the LED is controlled by the polariser and reduces the emission of transverse electric radiation. Under conditions of total internal reflection the wedge-shaped beam is directed onto a linear photo diode array by a mirror. An SPR-induced minimum is determined by processing the signal from the photodiode array in 'real-time' using dedicated signal software [41]. Temperature fluctuations can also be corrected during analysis as there is a built in temperature sensor in the device. This system is available in a hand-held format and is known as the Spreeta^{TM} device, which possesses a control box housing the components of the instrument, which may be attached to a laptop to produce a portable device (Fig. 10). Such a portable system would offer many advantages for use in environmental monitoring, particularly where ''on site'' analysis is required.

CONCLUSION

Over the past few years, the application of antibodies for use in immunosensors for environmental monitoring has grown. However, the initial production of specific antibodies to small haptens depends on their ability to be joined chemically to larger immunogenic molecules and in the case of recombinant antibodies, the selection strategies used for isolation of positive clones is of significant importance. Immunosensors are produced by combining antibodies with a biosensor device with either the antigen or antibody immobilised. Antibody specificity and sensitivity are important in the detection of individual molecules using immunosensors. Low levels of detection are dependent on the affinity of the antibody for the antigen. Recombinant antibody technology has also been investigated indicating the applicability of such antibodies for use in immunosensors along with their monoclonal and polyclonal counterparts. However, further development of recombinant antibody technology is required before these antibodies overtake existing technologies.

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