DEVELOPMENT OF ANTIBODY-BASED ASSAYS FOR THE DETECTION OF AFLATOXINS AND ORGANOPHOSPHATES

A thesis submitted for the degree of Ph.D.

by

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October 2001

Based on research carried out at

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Under the supervision of Professor Richard O' Kennedy

This thesis is dedicated to my Mam, and to Dad who passed away while I was studying

"The reward of a thing well done is to have it done"
Ralph Waldo Emerson (1803-1882)

"At last I have my reward!"
Stephen J. Daly (2001)

Declaration

I hereby certify that the material, which I now submit for assessment on the programme of study leading to the award of Ph.D., is entirely my own work and has not been taken from the work of others save and to the extent that such work has been cited and acknowledged within the text.

Signed: _Stephen Daly_

Stephen J. Daly

Date: 18th Oct 01

Acknowledgements

This thesis would never have been started or completed without the help of my supervisor Professor Richard O' Kennedy. I am very indebted to him for all of his help, encouragement, pep-talks, as well as all of the other opportunities he has given me to travel and develop other skills throughout my time in DCU. It has been a pleasure to work for you Richard.

My family have been a great source of help and support to me throughout my life, and I am very grateful to them for keeping me going-both financially and mentally! So, to Eleanor and Robert, Catherine, Maura and John, Hugh and Mary-Kate, Caroline, Brian, and Cathal and Linda, thank you for always being there, giving me lifts everywhere, as well as regularly embursing me! Thanks also to Martin for many a lift back on a Sunday night.

I have really enjoyed working with the Applied Biochemistry group and have to thank all of my colleagues throughout the years, namely, Bernie, Gary, Ciaran, Deirdre, Mike, John, Brian, Jane, Lorna, Paul Leonard, Stephen, Aoife, Lynsey, and Joanne.

A special thanks to Tony who has been a true friend, and a great help and source of advice to me always. I couldn't forget my closest friend Paul, who I have studied with for the last 9 years! Thanks for some great times, and keeping me sane (as well as insane!) throughout my Ph.D. It has been a pleasure. Another good friend of mine is Keith, who I have also studied with for a number of years, and owe thanks to also. A very special thanks to my girlfriend Úna, who over the last year has been a great support and true friend to me.

Finally I would like to thank my parents. Unfortunately my Dad is no longer with us, but I am very indebted to him for all he did for me in life. My Mam is still great source of support to me, and I definitely would never have done this without her support and encouragement. Thanks for everything.

Table of contents:

Abbreviations

ACh	Acetylcholine
AChe	Acetylcholine esterase
AFB_1	Aflatoxin B_1
AFB_1-BSA	Aflatoxin B_1-bovine serum albumin
APC	Antigen presenting cell
BCA	Bicinchoninic acid assay
BIA	Biospecific interaction analysis
BSA	Bovine serum albumin
C	Constant
Ca	Circa
CBD	Cellulose binding domain
cDNA	Complementary deoxyribonucleic acid
CDR	Complementary determining region
CFU	Colony forming units
C_H	Constant heavy chain
C_L	Constant light chain
CM	Carboxy-methyl
CV	Coefficient of variation
D	Diversity
DEPC	Diethyl-pyrocarbonate
Dex	Dextran
DNA	Deoxyribonucleic acid
EDC	N-ethyl-N-(dimethyl-aminopropyl)carbodiimidehydrochloride
ELISA	Enzyme-linked immunosorbent assay
EST	Expressed sequence tag
EU	European Union
Fab	Antigen binding fragment
FTIR	Frustrated total internal reflection
Fv	Variable fragment

GC	Gas chromatography
H	Heavy chain
HAMA	Human anti-murine-antibody immune response
HAT	Hypoxanthine, Aminopterin, and Thymidine
HBS	Hepes buffered saline
Hcc	Hepatocellular carcinoma
HCFHrp	Human complement factor H-related protein
HGPRT	Hypoxanthine guanidine phosphoribosyl transferase
His	Histidine
HPLC	High performance liquid chromatography
HRP	Horse-radish peroxidase
IDA	Iminodiacetic acid
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
IMAC	Immobilised metal affinity chromatography
IPTG	Isopropyl-β-D-galactopyranoside
J	Joining
KLH	Keyhole limpet hemocyanin
L	Light chain
LC	Lethal concentration
LD	Lethal dose
LE	Low expression
LED	Light emitting diode
MALDI-TOF	Matrix-assisted laser desorption ionization-time of flight
MRL	Maximum residue level
MHC	Major Histocompatibility Complex
MIA	Molecularly imprinted sorbent assay
mRNA	Messenger ribonucleic acid
MS	Mass spectroscopy
MW	Molecular weight

NE	Non expression
NHS	N-hydroxysuccinimide
NMR	Nuclear magnetic resonance
NUI	National University of Ireland
OD	Optical density
OP	Organophosphate
δ-PD	δ-phenylenediamine
PBS	Phosphate buffered saline
PBST	Phosphate buffered saline-tween
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
PFMD	Pulse flame photometric detection
pH	Negative log of the hydrogen ion concentration
pI	Isoelectric point
PMSF	Phenyl methyl sulphonylfluoride
PVDF	Polyvinylidene fluoride
RIA	Radioimmunoassay
RT-PCR	Reverse transcription polymerase chain reaction
scFv	Single chain variable fragment
SD	Standard deviation
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SEC	Size Exclusion Chromatography
SELDI	Surface enhanced laser desorption/ionization
SIP	Selectively infective phage
SOC	Super optimal catabolite
SOE-PCR	Splice-by-overlap extension polymerase chain reaction
SPIA	Sol particle immunoassay
SPR	Surface plasmon resonance
StAb	Stabilised single chain antibody
TAE	Tris acetic acid-ethylene diamine tetraacetic acid
TBS	Tris buffered saline

Tc	Cytotoxic T-cell
TCP	3,5,6-trichloro-2-pyridinol
TED	Tris (carboxymethyl) ethylenediamine
Th	T-helper
TIR	Total internal reflection
TLC	Thin layer chromatography
TNF	Tumour necrosis Factor
TPB	Trans-4-phosphono-2-butenic acid
TY	Tryptone yeast extract
TYE	Tryptone yeast extract medium
UV	Ultra violet
V	Variable
V_H	Variable heavy chain
V_L	Variable light chain

Units

μg	microgram
(k)Da	(kilo) Daltons
μl	microlitre
μM	micromoles
°C	degrees Celsius
AU	arbitrary units
cm	centimetres
g	grams
h	hours
K	degrees Kelvin
kg	kilogram
l	litre
m	metre
M	molar
mg	milligram
min	minute
ml	millilitre
mm	millimetres
mM	millimolar
nM	nanomolar
mol	molar
pg	picogram
ppb	parts per billion
p.s.i.	pounds per square inch
rpm	revolutions per minute
kRU	(Kilo) response units
sec, s	seconds
U	Units
v/v	volume per unit volume
w/v	weight per unit volume

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Daly, S.J., Dillon, P.P., Dunne, L., Manning, B.M., and O' Kennedy. R. (2001). Comparison of single chain Fv and polyclonal antibodies for 'real-time' biosensor-based assays for aflatoxin-B_1. "Environ", Dublin City University, 27-28 Jan., p. 105.

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Abstract

The work described in this thesis involves the production and characterisation of antibodies and recombinant antibodies to aflatoxin B_1 (AFB_1), a potent mycotoxin and carcinogen. The antibodies produced were then applied for use in a surface plasmon resonance-based biosensor (BIAcore), which measures biomolecular interactions in 'real-time'. Production of hapten-protein conjugates of the organophosphate pesticides chlorpyrifos and pirimiphos, and characterisation of anti-chlorpyrifos antibodies were also successfully performed.

Rabbit polyclonal and single chain Fv (scFv) antibodies were produced to aflatoxin B_1. The scFv's were produced from an established phage display system, which incorporated a range of different plasmids for scFv expression. Both sets of antibodies were used in the development of competitive ELISA's and also for the development of BIAcore-based inhibition immunoassays. The antibodies were also used in the detection of AFB_1 in spiked grain samples by ELISA and BIAcore. Both the polyclonal and scFv antibodies were found to be suitable for the detection of AFB_1, and the assays were reproducible.

The production of an activated form of the organophosphate pirimiphos for the production of a pirimiphos-protein conjugate was attempted. However it was not possible to conjugate this pesticide by any suitable means. Chlorpyrifos-BSA conjugates were produced with different molar substitution ratios, and were used for the development of a competitive ELISA with polyclonal anti-chlorpyrifos antibodies.

Chapter 1

Introduction

1.1. The immune system

Throughout a lifetime, the mammalian vertebrate immune system encounters a large variety of infectious microbial agents such as viruses, bacteria, fungi, and parasites. These organisms can cause pathological damage, and if they are allowed to multiply unchecked, they will eventually kill their host. The immune system has evolved a powerful range of separate but inter-linked mechanisms for identifying and combating such pathological organisms. The two functional divisions of the immune system, which incorporate these protective mechanisms, are the innate system and the adaptive immune systems.

Innate immunity acts as the first line of defence, and consists of a series of physical and physiological barriers. The skin and mucous membranes are the body's first line of defence against infection. Physiological factors such as pH, temperature, and degradative substances such as lysozyme act in addition to this. If an organism penetrates the epithelial surface it encounters phagocytic cells, which come from monocytes/macrophages. These break down non-specifically by a process known as phagocytosis (Roitt *et al.*, 1989). This involves engulfing foreign particles, and breaking them down using potent intracellular enzymes and chemicals. The inflammation caused when tissue damage occurs also contributes to the innate immune system by facilitating the emigration of phagocytes and the lysis of pathogenic microorganisms.

The adaptive immune system differs from the innate system, as it is directed against specific molecules, and it possesses an 'immunological memory'. The system can be further divided into cell-mediated immunity and humoral immunity.

1.1.1. *Cell mediated immunity*

The key components of cell mediated immunity are T-lymphocytes, or T-cells, which complete their maturation in the thymus. These mature T-cells migrate from the thymus to the lymphoid organs such as the lymph nodes and the spleen. The two main types of T-cells are cytotoxic T-cells (Tc), and T-helper cells (Th). T-cells

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cannot normally be activated by free antigens present in body fluids. Instead they only respond to antigenic epitopes displayed on the surface of the body's own cells. These epitopes are known as the major histocompatibility complex (MHC), and they act like a red flag calling the T-cells into action against cells infected by the pathogen represented by that particular antigen (Davey, 1992). Th-cells recognise antigen in conjunction with MHC II proteins and are responsible for the release of a group of chemicals called cytokines. Cytokines play a crucial role in the regulation of the immune response, as their secretion regulates neighbouring cells. Th cells are made up of two distinct populations, which are differentiated by the cytokines they secrete- Th-1 and Th-2 cells. Th-1 cells are primarily responsible for the activation of Tc-cells by secreting the cytokines interleukin-2 (IL-2), interferon gamma (IFN-γ) and tumour necrosis factor beta (TNF-β) (Male *et al.*, 1996). However, they also play a role in the activation of B-lymphocytes. Th-2 cells are predominantly responsible for the activation of B-lymphocytes by secreting the cytokines IL-4 and IL-5 (Hannigan, 2000; Male *et al.*, 1996). Tc-cells recognise antigen in conjunction with MHC I proteins, which are found on all nucleated cells of the body. When it docks on the surface of an infected cell, it releases perforin, a protein that forms an open lesion on the cell membrane, thus leading to cell lysis. As well as destroying the host cell the Tc-cell also deprives the invader of a place to reproduce and exposes the pathogen to circulating antibodies.

1.1.2. *Humoral immunity*

The humoral immune response is mediated by B-cells in conjunction with Th-cells and their secretion of cytokines. After a macrophage engulfs a pathogen by phagocytosis it breaks it down into smaller pieces, and displays pieces of the digested antigen on its surface. In this context, the macrophage is known as an antigen-presenting cell (APC). B-cells and dendritic cells can also function as APC's (Male *et al.*, 1996). A feature of all APC's is that they express MHC II proteins on their surface (Lydyard *et al.*, 2000). Degraded antigen components bind to the MHC II molecule within the cell and are then exported to the cell surface. Th-cells then recognise this self/non-self complex of MHC II and antigen. This contact activates

the Th-cell, which proliferates and forms a clone of Th-cells keyed to the specific antigen. A co-stimulatory signal from the APC (secretion of the cytokine IL-1) also activates the Th-cells to secrete cytokines. It is these cytokines that are responsible for the stimulation of B-cells. IL-2 plays a key role in B-cell proliferation. Antigens are also processed by B-cells, although uptake is specific for a particular antigen. When antigen binds to antigen receptors on a B-cell, the cell takes in a few of these foreign molecules, and displays the antigen fragments bound to class II MHC markers on the cell surface. The Th-cells receptors recognise this and bind to the antigen-MHC-complex. This results in B-cells synthesising receptors for IL-4 (Th-2 response) which causes their proliferation, and in-turn production of IL-5. This cytokine is responsible for the differentiation of B-cells into memory and plasma cells. Figure 1.1 shows a schematic diagram of the sequence of events that lead to antibody production in the immune system. Plasma cells are responsible for the secretion of large amounts of specific antibody (2000/second), and have a lifespan of 4-5 days (Campbell, 1993). B-memory cells do not secrete antibodies, but instead, remain in the body for long periods of time with specific antibody cell-surface receptors primed for future exposure to the antigen. Memory T-cells also exist, and result from the initial encounter with antigen that generates an antigen specific T-cell response. Once a subsequent encounter occurs with the antigen, the memory cells are rapidly restimulated and proliferate to combat the antigen. One surface molecule found on T-cells is CD45. It has been found that this molecule changes, when a T-cell is activated by an encounter with an antigen. T-cells that have not yet encountered antigen are said to have the CD45RA molecule, while memory T-cells have the CD45RO molecule (Hannigan, 2000). However, it is not entirely certain that this distinction is completely reliable, as it is thought that cells can change CD45 formats through other event sequences.
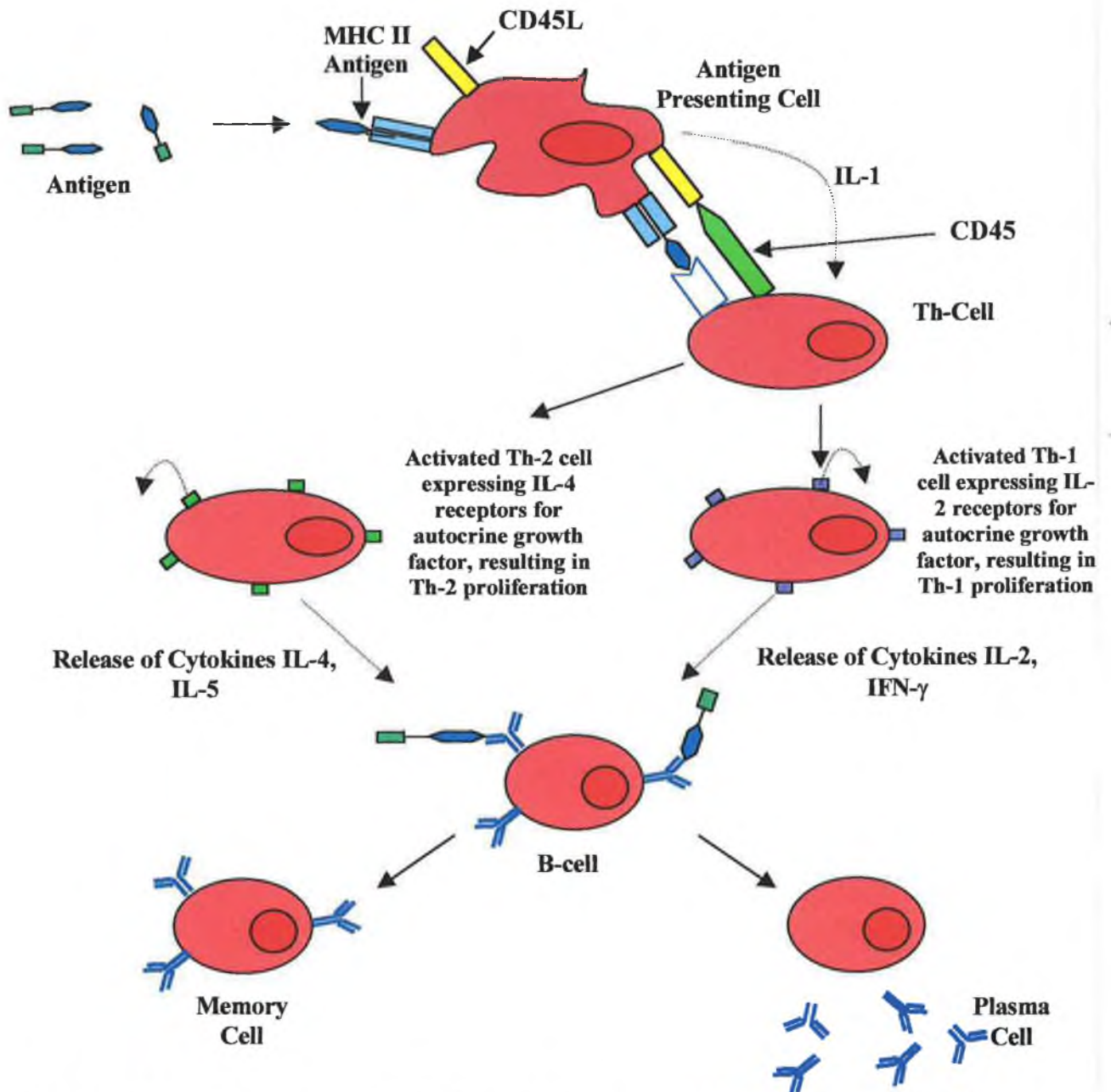
Figure 1.1. *The generation of the humoral immune response. Antigen is initially phagocytosed by the APC and presented on the surface as MHC II antigen-complex. Th-cells bind to the MHC II antigen-complex, and become activated to produce cytokines (dotted arrows) such as IL-1, which triggers T-cell differentiation and proliferation by autocrine growth factor. Th-1 and Th-2 cells secrete different cytokines that enhance B-cell proliferation. However, it is the Th-2 –derived cytokines that predominantly enhance B-cell proliferation. B-cells with specific antigen receptors on their surface bind to antigen in solution, and the proliferation of these B-cells is stimulated by the presence of cytokines. This results in differentiation into memory and plasma cells. The plasma cells are capable of producing large amounts of soluble antibody following affinity maturation in the germinal centres.*

1.1.3. *Immunogenicity*

Immunogenicity is the ability of a particular molecule to provoke an immune response. In order for a molecule to be immunogenic, it must possess three properties, namely: a degree of foreignness, a large molecular weight, and a degree of chemical complexity. The immune system must be able to recognise the antigen as foreign, and therefore, the greater the genetic distance between the source species of a molecule and the species of the host, the greater the immune response will be. Molecules with a molecular weight of greater than 1 kDa are generally capable of provoking an immune response (Davey, 1992). Smaller molecular weight compounds such as drugs or pesticides are not capable of provoking an immune response, and therefore, need to be conjugated to a larger carrier molecule in order to become immunogenic. The greater the chemical complexity of a substance, the more immunogenic it will be. Synthetic polymers of a single amino acid generally do not give rise to an immune response, whereas those containing a variety of amino acids are more effective immunogens.

Once an animal has been exposed to an antigen, either through immunisation or natural causes (e.g. injury) the immune system begins to work on combating it. The selective proliferation of lymphocytes to form clones of effector cells against an antigen constitutes the primary immune response. Between exposure to the antigen and the maximum production of effector cells, there is a lag period of 5-10 days. During this lag period, the lymphocytes selected against the antigen are differentiating into effector T-cells and antibody producing plasma cells. If the body is exposed to the same antigen at a later date, the response is faster and more prolonged. This is known as the secondary immune response, and is based on the immune systems ability to recognise previously encountered antigen i.e. immunological memory (Figure 1.2).

The effectiveness of the secondary immune response is dependent on the immunogenicity of the antigen used. Generally large insoluble molecules tend to be better immunogens than small insoluble molecules, as they can be rapidly

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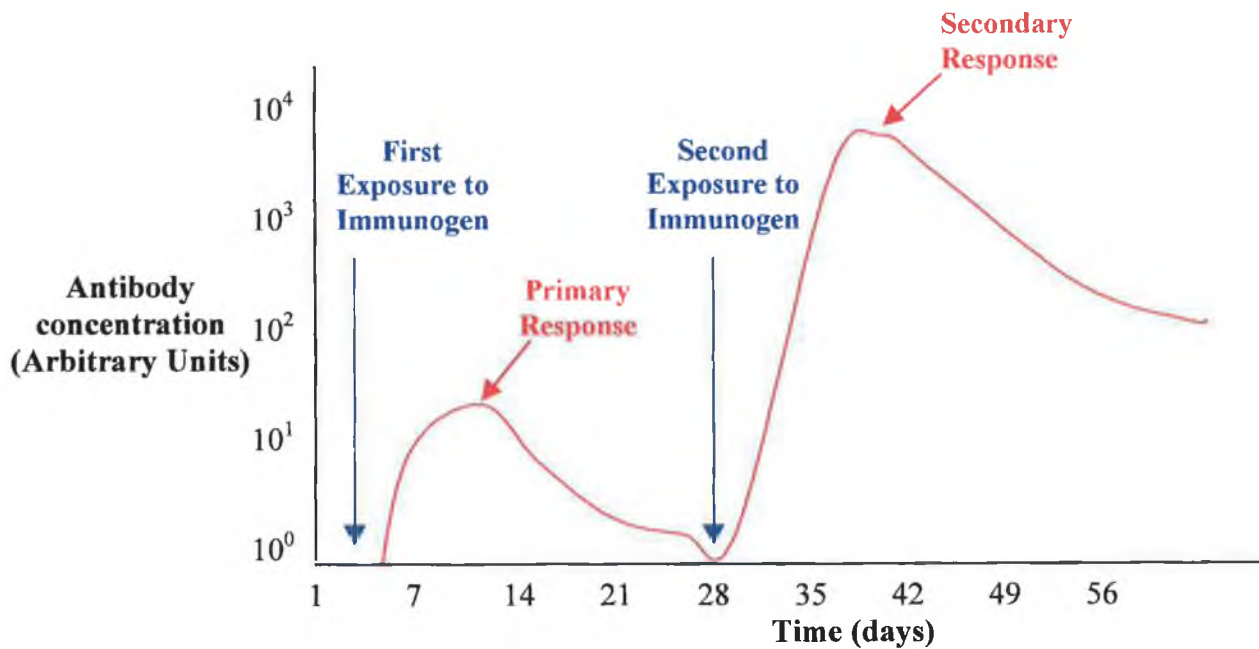
Figure 1.2. *Immunological memory. Initial exposure to the immunogen stimulates the primary immune response, with the eventual production of antibodies against the immunogen after a short time lag. A second exposure to the immunogen on day 28 produces a faster and greater secondary immune response, which is due to specific long-lived memory cells.*

phagocytosed, and effectively processed and presented by APC's. Administrating an adjuvant along with the immunogen can enhance the immune response significantly. An adjuvant is a substance that non-specifically stimulates the immune system. Adjuvants function in two main ways. Firstly, they protect the antigen by trapping it in a local deposit, and secondly, they attract macrophages and increase the local rate of phagocytosis. Freund's complete is one of the most widely used adjuvants in animals. It consists of mineral oil, emulsifying agent, and heat-killed *Mycobacterium tuberculosis* (Cooper, 1982). When the immunogen is mixed with the adjuvant and injected, the emulsifying agent causes the oil to form into small droplets, and results in the immunogen being released slowly from the injection site. The mycobacteria enhance macrophage activity and lead to the stimulation of Th cells. These factors provoke an inflammatory response, thus giving the immune system the 'kick-start' to

developing a large immune response to the immunogen. Freund's incomplete is the less pathogenic adjuvant, as it does not contain any mycobacteria. It is frequently used for second and subsequent immunisations- a process known as 'boosting'. Boosting is carried out in order to achieve the secondary immune response, and therefore, a greater amount of specific antibody in the serum.

Dosage and route of administration can also effect the immune response. Low doses of immunogen will not significantly activate the lymphocytes, while high doses can result in lymphocytes entering a non-responsive state. Different routes of administration can also be used, such as sub-cutaneous, intravenous, or intra-peritoneal. Sub-cutaneous injection will cause an administered substance to gather in the local lymph nodes, while intravenous injection carries the immunogen straight to the spleen. Intra-peritoneal injection is the most commonly used method for immunising mice with antigens. This is because of the large volume of the peritoneal cavity, thus allowing for larger volumes of immunogen to be administered. It is also a useful route for injecting particulate antigens such as heat-killed bacteria. These cannot be injected intraveneously as they may cause embolisms in the mouse.

1.2. Antibody structure and function

Antibodies constitute a class of glycoproteins called immunoglobulins (Igs) that are produced by the immune system against foreign antigens. Five distinct classes of Ig exist in most higher mammals. These are IgM, IgA, IgD, IgE, and IgG (Lydyard *et al.*, 2000). IgM's are pentameric in structure, and are the first circulating antibodies to appear in response to initial exposure of antigen. IgA antibodies are dimeric in structure, and are found in many body secretions, as they prevent the attachment of bacteria and viruses to epithelial surfaces. IgD antibodies are monomeric, and are mostly found on the external surfaces of B-cells functioning as antigen receptors required for initiating differentiation of B-cells that produce antibodies against the antigen. IgE antibodies are also monomeric in structure, and represent only a very small proportion of total antibodies in the blood. They are involved in the release of histamine, and other chemicals that cause an inflammatory reaction (Campbell,

1993). IgG's are the major immunoglobulin found in the blood system, as they constitute for 70-75 % of the total immunoglobulin pool (Roitt *et al.*,1989). The basic structure of the IgG molecule is shown in Figure 1.3. It consists of two identical heavy (H) chains and two identical light (L) chains stabilised and linked together by inter-and intra-chain disulphide bonds.

The L-chains weigh about 25 kDa, and the H-chains approximately 50 kDa giving the total molecular weight of approximately 150 kDa for the monomeric structure. The H and L-chains can be further divided into domains on the basis of the variability of their amino acid composition. Each L-chain has one variable (V_L) and one constant (C_L) domain, while the H-chain has one variable (V_H) and three constant domains (C_H1, C_H2, C_H3). The hinge region is a short amino acid sequence situated between the C_H1 and C_H2 regions of the antibody's heavy chains. It gives flexibility for the two arms of the molecule (Lydyard *et al.*, 2000).

The constant regions of the antibody are responsible for effector functions and complement binding, while the V_H and V_L domains are responsible for the specific recognition of antigen. These domains exhibit a high variability in amino acid sequence. This is a highly organised process within specific areas of the variable region, known as hypervariable regions. These form a region complementary in structure to the antigen epitope, and are thus called the complementary determining regions (CDRs). The CDR can be further divided into three sub-regions (CDR1, CDR2, and CDR3), as well containing more conserved regions known as framework residues. The CDR sequences are exposed on the variable region of the antibody and form a cleft that is the antigen-binding site. The combination of the CDR sequences results in a precise amino acid sequence and shape of the antigen-binding site, which confers an antibody's particular specificity (Roitt *et al.*, 1989).
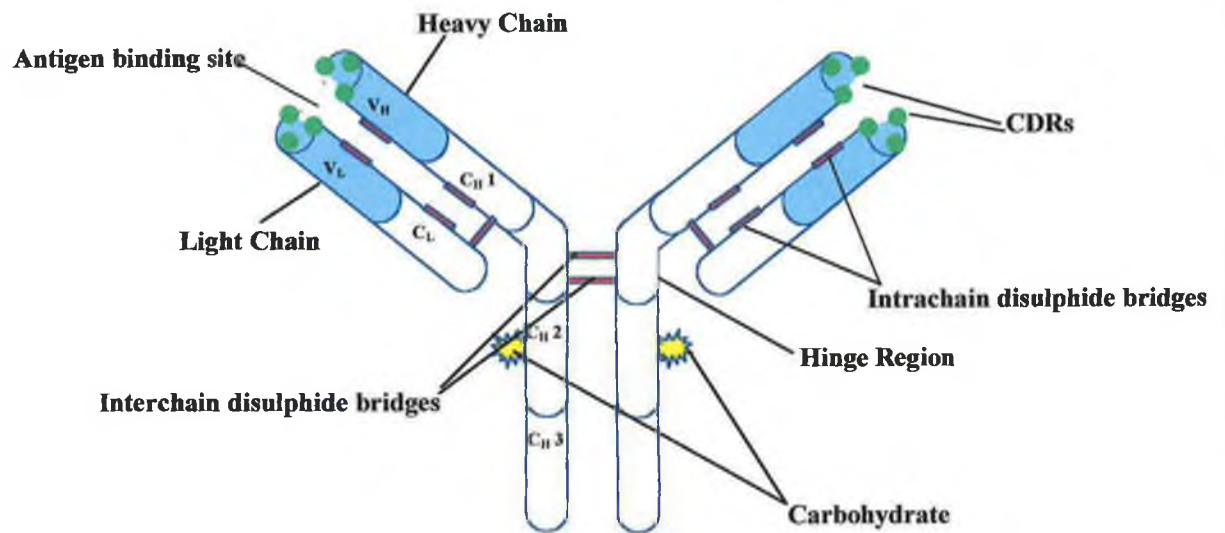
Figure 1.3. *Structure of the immunoglobulin IgG₁ molecule. The antibody is composed of two identical light (25 kDa) and heavy (50kDa) chains. The H chains comprise of five distinct domains: one variable (V_H), three constant (C_H1, C_H2, C_H3), and one hinge region. The hinge region provides the flexibility to allow for the individual antibody-binding sites to bind to antigen. The light chains comprise of a single variable (V_L) and a single constant (C_L) region. Interchain disulphide bonds connect the heavy and light chains, while intrachain disulphide bonds exist in the variable and constant regions giving the folded globular structure characteristic to antibodies. The complementary determining regions (CDRs) confer the specificity to the particular antibody-binding site, and have a high amino acid sequence variability.*

The IgG molecule can also be broken down into various fragments by means of enzymatic degradation, as well as being produced genetically. Figure 1.4 shows a structure of an IgG molecule and its various fragments. Treatment of the IgG with the enzyme pepsin cleaves the two heavy chains below the interchain disulphide bonds and forms a $F(ab')_2$ fragment. This consists of two light chains, along with two heavy chains and the hinge region. Papain will cleave the IgG molecule above the disulphide bridges, giving rise to two separate Fab fragments which bind antigen (Roitt *et al.,* 1989). Protein engineering has allowed for the development Fab fragments as well as scFv antibody fragments. The production of scFv fragments is becoming increasingly popular, as they can also offer high levels of sensitivity and specificity towards a given antigen compared to that of whole IgG's. A scFv antibody fragment consists of one variable heavy chain and one variable light chain stabilised together by a synthetic peptide linker (or in some cases the insertion of cysteine residues to form a disulphide bridge). This results in the production of a more stable antibody fragment compared to that of the Fv molecule. The genetic production of these antibody fragments will be discussed later in this chapter.

Antibodies do not destroy antigenic invaders directly. Instead, the binding of the antibody-antigen complex serves the basis of many effector functions. The simplest of these is neutralisation, which blocks key binding sites on a particular antigen, thus making it ineffective. Agglutination is another method it uses, where it exploits its two binding sites to cross-link adjacent antigens (Lydyard *et al.,* 2000). Both of these methods enhance phagocytosis, and antigen processing and presentation. Another important effector mechanism of antibodies and the humoral immune response is the activation of complement. Complement is a group of more than 20 plasma proteins that act with elements of the innate and adaptive immune system (Hannigan, 2000). Antibodies often combine with complement proteins, to activate complement and produce lesions in an invading cell's membrane, and thus lysing it.
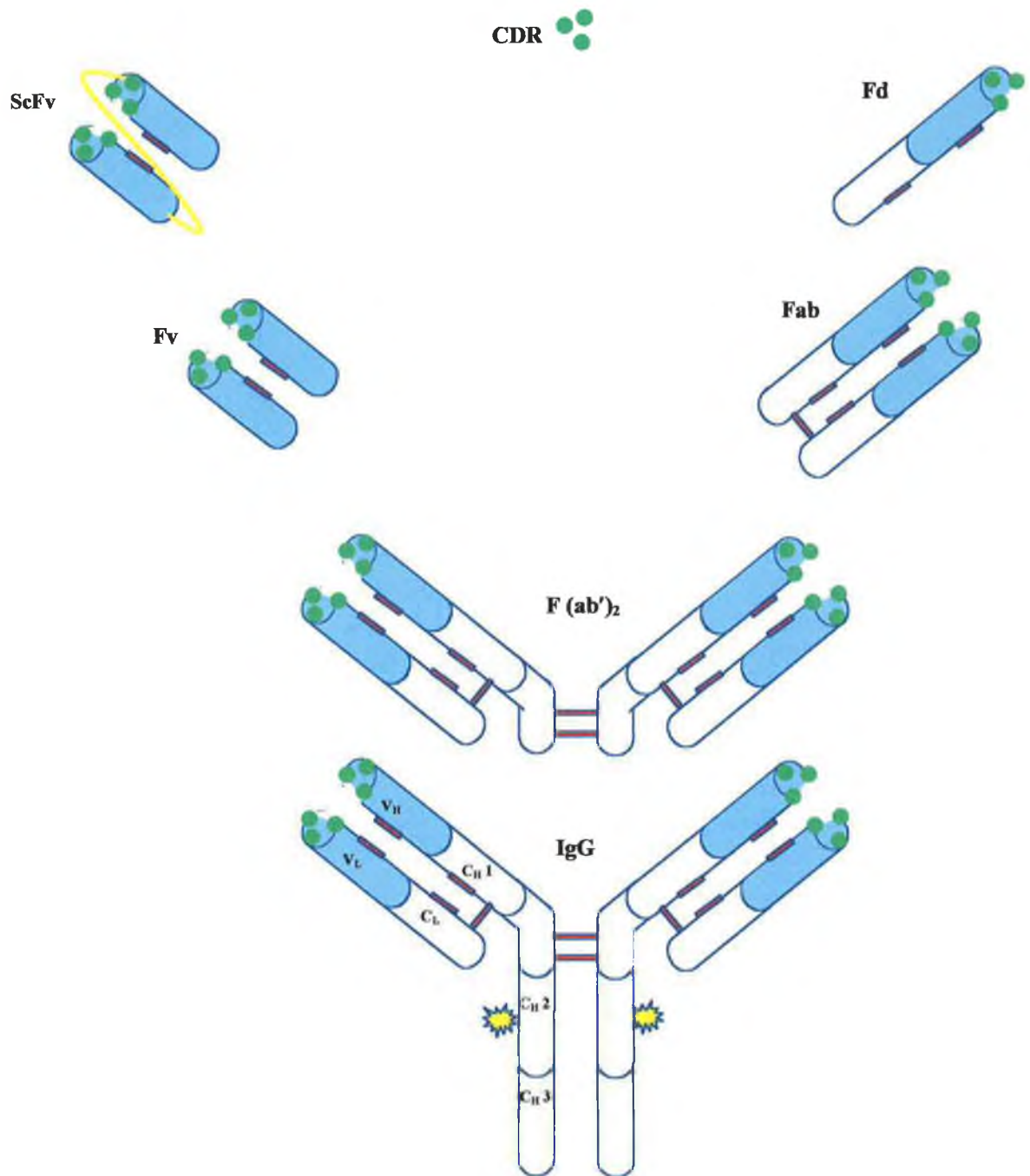
Figure 1.4. *Structure of the immunoglobulin IgG$_1$ molecule and its various fragments. The F (ab')$_2$ fragment consists of two antigen binding fragments linked by the hinge region, while the Fab fragment is only one antigen binding fragment. An Fv molecule consists of one variable heavy chain and one light variable chain. The scFv molecule is an Fv stabilised by a synthetic linker. The Fd fragment comprises of a variable heavy and one constant heavy chain molecule, while the complementary determining region (CDR) is the smallest antigen-binding fragment. These various fractions of the whole antibody molecule may be generated either through genetic or enzymatic and chemical manipulation.*

1.3. Antibody diversity and its genetic basis

The variable regions of the heavy and light chains of an antibody are what encode for the antibody's diversity. Heterogeneity within these regions ensure a diverse repertoire of antibodies that can combat any pathogen the immune system encounters. Four distinct processes have been identified for the generation of this somatic diversity (Tonegawa, 1983), and they are namely: combinatorial diversity, junctional site diversity, junctional insertion diversity, and somatic mutation.

In the germ-line cells, there are three sets of genes involved in immunoglobulin coding. One set codes for the heavy chain genes, and two sets code for the light chain genes, which are designated by the Greek letters kappa (κ) and lambda (λ). These differ significantly in the amount of amino acid sequence in their constant domains. The three sets of germ line genes (heavy chain, and κ and λ light chain genes) occur on three different chromosomes on both human and mouse cells. Figure 1.5 shows the three immunoglobulin gene sets in human germ line cells.

Variable light chain genes are encoded by the V and J regions, while the heavy chain genes are encoded by the V, D, and J regions. After B-cell maturation the various VDJ, and VJ sequences recombine to form a complete functional gene. During the recombination process, when a D region is brought adjacent to a V region, a procedure called 'looping out' occurs, where the intervening gene segments are deleted by recombinase enzyme (Figure 1.6). As a result, joining can occur with various combinations on the genes, and the number of possible permutations is the product of the number of V, D, and J sequences particular to each sequence.

Junctional site diversity arises at the V_L-J_L, V_H-D and D-J_H junctions, because the joining ends are imprecise. This leads to changes in amino acid sequences at the junction sites, thus altering the binding site specificity.
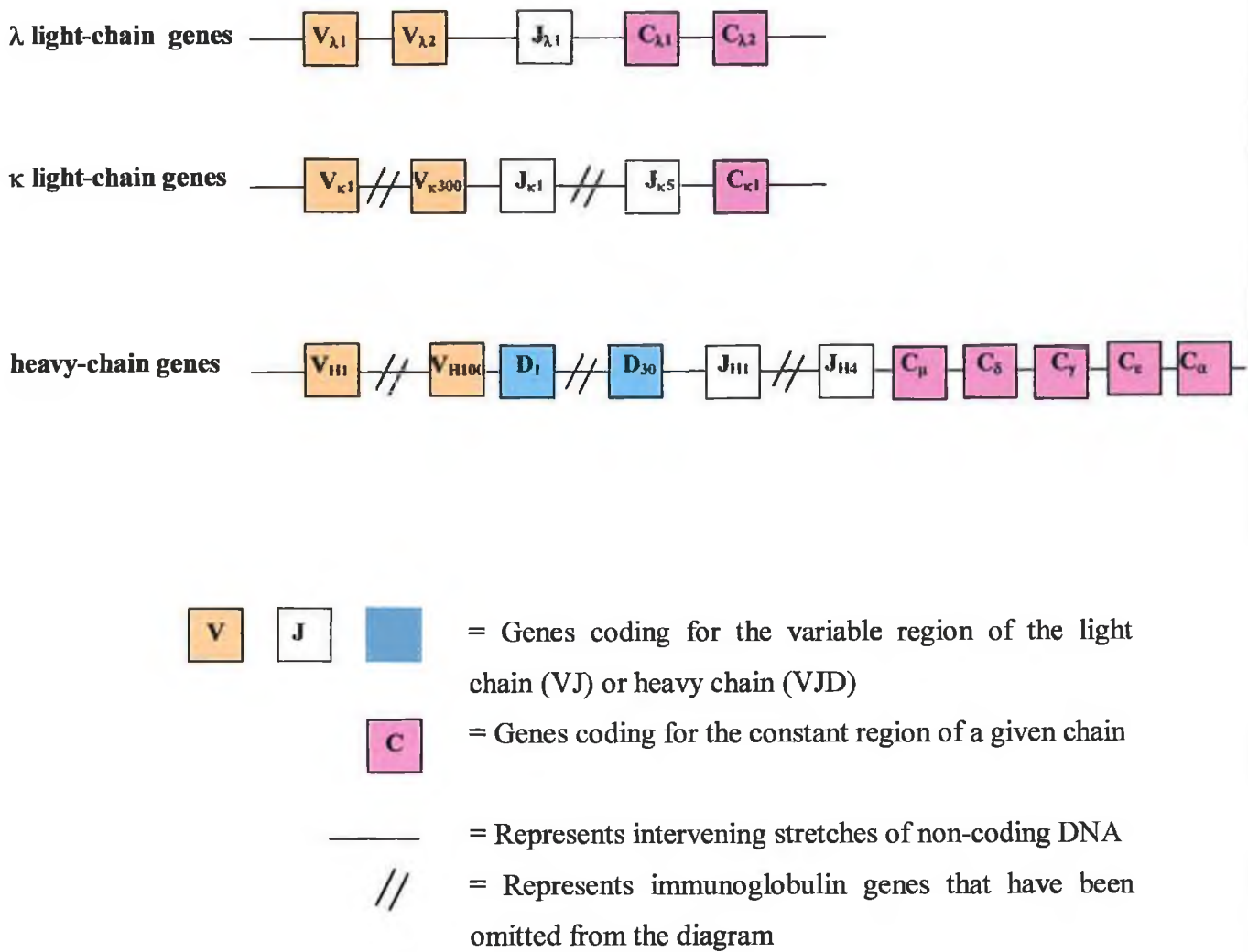
Figure 1.5. *The three immunoglobulin gene sets in human germ-line cells, prior to somatic recombination. These three gene sets are on different chromosomes in the human and mouse genome. Diagram taken from Davey (1992).*
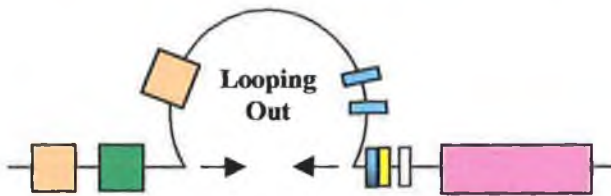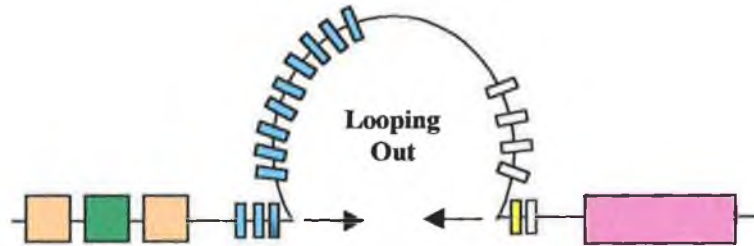
Figure 1.6. *The two separate processes involved in the recombination of a heavy chain gene. Heavy chain gene segments are composed of V (variable), D (diversity), and J (joining) segments. The constant regions encode for the antibody isotype. The first recombination step involves bringing the D and J segments together by the process of 'looping out' the intervening DNA by recombinase enzymes. These also introduce further variability at the point of cleavage (junctional site diversity). The second step also involves looping out of DNA genes, in order to bring the V region alongside the D J regions. This determines the specificity of the antibody and gives rise to a functional B-cell variable heavy chain gene.*

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Junctional insertion diversity only occurs in the V_H-D and D-J_H junctions, where one to several nucleotides are inserted in between the sequences.

More variation arises after contact with antigen. Single base changes occur in the DNA of activated B-cells, during the process of memory-cell formation. This is called somatic mutation, and the V, D and J genes are particularly susceptible to this type of mutation that can generate a small, but significant, variation in the antibody-antigen binding sites. One of the consequences of somatic mutation is that some of the binding sites produced by the mutated DNA have a better affinity for the antigen, and some have a lesser affinity. This is because the mutations occur randomly in the DNA coding for the variable regions, and therefore, sometimes improving the 'goodness of fit' between the antigen-binding site and the epitope- and also- sometimes reducing it. Somatic mutations mainly occur during memory B-cell formation, so the memory B-cell from a single clone ends up with binding-sites for the same antigen but with a range of different affinities. The memory cells with the higher affinity antibodies on their surface will be more likely to bind the antigen upon a second exposure, than the lower affinity antibodies. Thus, cells producing a higher-affinity antibody will be selected for activation during the secondary immune response. This process is known as affinity maturation (Davey, 1992).

Class or isotype switching occurs in mature B-cells as a result of antigenic stimulation. Figure 1.5 shows that constant H-regions are found downstream of the variable region genes, in the sequence μ, δ, γ, ε, α. The C_μ-gene is the first one and codes for the constant domains of the heavy chains of the IgM class. Transcription always takes place in the one direction (from 5' to 3'), and as a result, the primary mRNA transcript made from this DNA contains the transcribed version of C_μ, thus ensuring the resulting antibodies are IgM. These are the predominant class of antibody secreted by B-cells for first contact with antigen. However, as the primary immune response proceeds, some of the V, D, and J sequences of the B-cell undergo further DNA recombination with the C-genes (except for the δ gene, which has no

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switch region). The C_γ is next in the order and is brought to lie immediately downstream of the genes coding for the variable region of the heavy chain. This will be then transcribed into mRNA, and the cell will switch from synthesising IgM antibodies to IgG antibodies (Davey, 1992). Antigen and T-cell-derived cytokines regulate this mechanism, and allows for the production of antibodies with unique specificities and effector functions to a particular antigen.

1.4. Production of antibodies

1.4.1. *Hapten-carrier conjugate production*

A mentioned previously in section 1.1.3, hapten-molecules such as pesticides, food contaminants, and drugs usually have a molecular weight of less than 1 kDa. As a result of this, they generally do not elicit an immune response on their own, and need to be conjugated to a larger protein for immunisation purposes. The most frequently used carriers are highly immunogenic proteins, but lipid bilayers, polymers (e.g. dextran), and synthetic organic molecules have also been used (Hermanson, 1996). It is important for the carrier molecule to be highly immunogenic, non-toxic *in vivo*, as well as contain suitable functional groups for covalent linkage. Haptens may also have to be chemically modified, in order to conjugate them effectively to a protein. 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 moiety than the immunogen, so false positive results of observing non-specific binding of antibodies produced to the protein part of the conjugate are minimised. Danilova *et al.* (1994) recommended that, when dealing with very small haptens, the conjugate used for screening should possess a different carrier molecule and coupling chemistry than used for immunisation. However, in situations when it is not possible to synthesise a second protein conjugate for use in the screening stages, certain controls can be introduced into a particular assay to minimise false positives. These include incorporating the protein (that was conjugated to the hapten) into the antibody diluent buffer. This ensures that any of the non-specific antibodies to the protein are 'mopped up' before

they can bind to the protein conjugate on the plate, and thus detected in the assay. In order to ensure that there is sufficient protein in the diluent buffer to remove the non-specific interactions, another control must be ensured, where the antibodies are also assayed against the protein with the protein in the diluent buffer. One of the most widely used proteins for conjugation to haptens is bovine serum albumin (BSA). This is highly soluble with a molecular weight of 67 kDa. It also has a variety of functional groups available for conjugation including $-NH_2$, and $-COOH$ groups.

1.4.2. *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 increase. 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 (Davey, 1992). The animals in which polyclonal antiserum is 'raised' tend to be the larger species of domestic animal such as rabbits, sheep, 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 serum 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 described the average affinity value for the mixture of antibodies in a polyclonal antibody population (Davey, 1992).

While an individual antibody molecule may exhibit a high specificity of binding for a particular antigen-epitope, the presence of similar epitopes on different molecules (but structurally related) may result in the antibodies binding to these structurally related molecules. This effect is known as 'cross-reactivity' and is more prevalent in a polyclonal antibody population due to their heterogeneity. However, it may also be encountered when dealing with a homogenous antibody population.

One of the main advantages in the production of polyclonal antibodies is their relative inexpense, as well as the relatively short time-frame 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.

1.4.3. *Monoclonal antibodies*

In order to produce a homogenous antibody population i.e. a monoclonal antibody- it is necessary to isolate and propagate one individual B-cell clone. B-cells can only be cultured *in vitro* for a short period of time, after which they will die. Kohler and Milstein (Kohler & Milstein, 1975) reported the fusion of a B-cell to an immortalised myeloma cell resulting in an immortalised fusion product that could continuously produce monoclonal antibodies. This fusion product was called a hybridoma (hybrid-myeloma).

In order to produce a monoclonal antibody by somatic cell fusion, it is necessary to immunise a mouse or rat with the antigen of interest. After a period of time when a high enough antibody titre is obtained, the animal is given a final intravenous injection of the immunogen (without adjuvant). This results in the immunogen being directly carried to the spleen, where a large number of B-lymphocytes producing specific antibodies are present. The animal is usually sacrificed 3-4 days after this and the spleen removed.

A variety of myeloma cells are available as suitable fusion partners such as X63-Ag8.653 or Sp2/0-Ag14 cell lines. They are tumorigenic B-lymphocytes that were produced from a myeloma cell line developed after injection of mineral oil into the peritoneal cavity of mice. These myeloma cells are deficient in an enzyme called hypoxanthine guanidine phosphoribosyl transferase (HGPRT). As a result of this, they are unable to proliferate in the presence of HAT (Hypoxanthine, Aminopterin, and Thymidine) medium. This is because aminopterin blocks *de novo* synthesis purines and pyrimidines, which are required for DNA synthesis. HGRPT deficient

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cells are incapable of using the salvage pathways for DNA synthesis as they do not have the requisite enzymes systems to do so.

Splenocytes carry the functional enzyme HGRPT, but are not capable of living in culture over a long period of time, and will die off. Therefore, once a fusion has been carried out, only splenocyte-myeloma cells will survive as they have the functional requirements to survive in HAT medium i.e. HGRPT enzyme (from the splenocyte) and the property of immortality (from the myeloma).

In order for a fusion to be effectively carried out, attachment and joining of both cells needs to be enhanced. One of the most common methods used is fusion using polyethylene glycol (PEG). This is a polywax, that enhances cell fusion and the transfer of nuclei (Hurrell, 1985). Another method used for the generation of a hybridoma is electrofusion. This involves the generation of hybrid cells using high voltage electrical pulses to induce the fusion process. Dielectrophoresis is carried out first, which leads to cell-cell contact. This is followed by high voltage pulsing leading to the breakdown of the adjacent cell membranes (Zimmermann *et al.*, 1987). Fusion using PEG is generally more popular as it less stressful on the cells. However, electrofusion can increase the fusion efficiency 100-fold compared to that of PEG-based method.

After a number of weeks, only hybridomas will have survived, and the culture supernatants are assayed for the presence of specific antibody, and eventually only hybridomas derived from a single cell are produced. This is achieved by a process known as cloning by limiting dilution, in which the contents of positive wells are divided and sub-divided a number of times (Goding, 1996). A schematic diagram describing the generation of a monoclonal antibody is shown Figure 1.7.

The average amount of antibody in culture supernatant from conventional cell culture flasks is usually only between 10 and 100 μg/ml (Epstein & Epstein, 1986). In order to increase production of the quantity of antibodies, ascites fluid have been used.

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This entails the injection of the fused hybridoma cells into the peritoneal cavity of a mouse or rat, and allowing them to proliferate. The resulting ascites fluid can contain up to 1000 times as much specific antibody as spent cell culture medium (Galfre & Milstein, 1981). However, the disadvantage is that the resulting hybridomas can contain a high concentration of contaminating protein. More recently, there is also concern over the welfare of the animals using this procedure (Marx *et al.,* 1997).

Monoclonal antibodies can also be produced to antigens following *in vitro* immunisation (Borrebaeck *et al.,* 1983). The process involves harvesting B-lymphocytes from the spleen of a naïve mouse (i.e. not immunised) and incubating them in culture with the antigen of interest for 5-9 days. Somatic cell fusion is then carried out on the cells after this period of time. The technique is advantageous in-that it offers a shorter immunisation schedule, as well as that reduced amounts of antigen are required. However, the shortfall is that the antibody response raised is primary, and therefore it is predominantly an IgM response, with reduced quantities of IgG. A primary *in vivo* immunisation can also be incorporated into the procedure followed by a secondary *in vitro* boost. This strategy has been shown to produce greater quantities of IgG's with increased affinities (De Boer *et al.,* 1989).
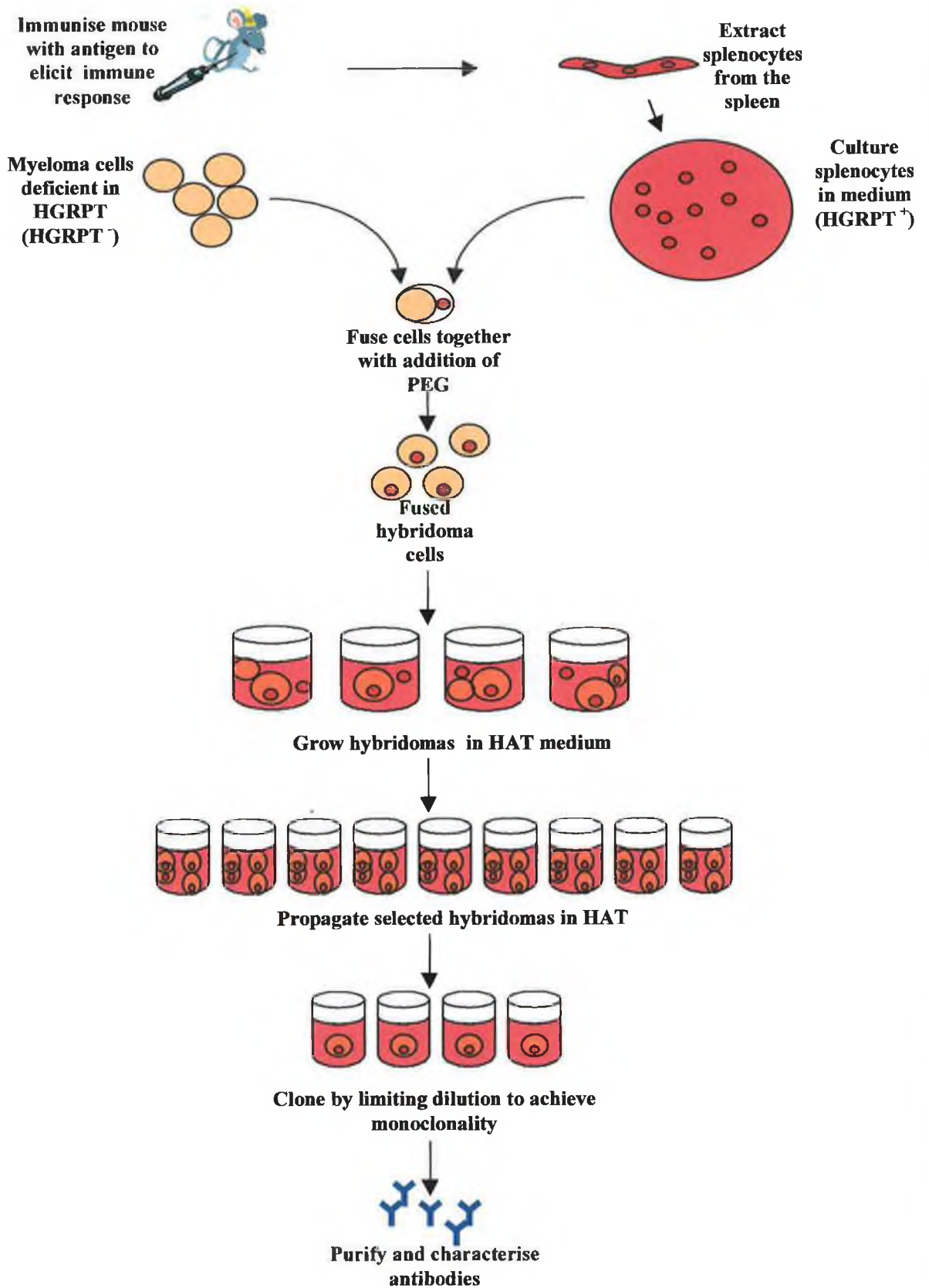
Figure 1.7. *Flow diagram showing the principle steps in the production of a murine monoclonal antibody.*

1.4.4. *Chimeric/Humanised antibodies*

One of the few disadvantages of hybridoma technology has been the inability to extend it as a general method for the generation of human monoclonal antibodies. Although murine-derived monoclonal antibodies have been widely applied in clinical diagnostics, they have had a limited amount of success in human therapy (Clark, 2000). Two major problems have been encountered with the use of murine antibodies for therapy. Firstly, although the murine antibodies can be highly specific toward their target antigens, they do not always activate the appropriate human effector functions such as complement and Fc receptors. Secondly, when the antibodies were used for human therapeutic purposes, the patients immune system would recognise them as foreign and thus reduce their effectiveness. This is known as the human anti-murine-antibody immune response (HAMA). HAMA can produce several undesirable side-effects such as allergic reactions, as well as an increase in the clearance rate of the administered antibody from the serum (Iwahashi *et al.*, 1999).

In order to overcome HAMA, murine monoclonal antibodies were transformed into chimeric antibodies (Boulianne *et al.*, 1984). A chimeric antibody combines the entire variable (V) domain of a mouse antibody with a human antibody constant (C) domain. Humanised antibodies have also been developed, and these consist of the mouse complementary determining regions being grafted onto human V-region framework regions and expressed with human C regions (Sung Co & Queen, 1991). The first fully humanised antibody, CAMPATH-1H, bound to an antigen on lymphocytes, and was used to treat patients with non-Hodgkin's lymphoma (Hale *et al.*, 1988). Other strategies to overcome the immunogenicity of therapeutic antibodies include: selecting human antibody V-regions from phage libraries by affinity selection on antigen (Winter *et al.*, 1994), as well as constructing transgenic mice that have had there own immunoglobulin genes replaced with human immunoglobulin genes. Thus, they can produce human antibodies upon immunisation (Mendez *et al.*, 1997).

Although the humanisation process has been relatively successful, it does have some problems such as reduction of the antibody affinity after humanisation (Riechmann *et al.*, 1988). It is rarely sufficient to combine the CDR's from a murine antibody with a completely human framework. This is because certain residues in the original murine framework make key contacts with the CDR's that help maintain their conformation. When the murine framework is replaced with the human one, the contacts are altered, therefore altering the shape of the CDR's and reducing the affinity of the antigen (Sung Co & Queen, 1991). However, the reduction in affinity can be minimised by careful selection of framework regions that are homologous to the starting antibody, or by reintroduction of important murine framework residues back into the engineered antibody. Sims *et al.* (1993) humanised a rat anti-human antibody that bound to CD 18, a cell surface glycoprotein that promotes the interaction of leukocytes with each other. They compared the rat V regions with human V region sequences, and the most homologous human frameworks were chosen. The comparison involved matching CDR length and composition, as well as framework identity. They found that the functional blocking characteristics of the rat antibody were also retained by the humanised antibody. Parajuli *et al.* (2001) demonstrated that a humanised anti-ganglioside GM2 antibody as well as its mouse-human chimeric counterpart could promote the lysis of lung cancer cells by blood mononuclear cells of lung cancer patients. Gangliosode GM2 is widely and substantially expressed in human cancer cells, and is therefore, a possible target for cancer therapy. This shows the potential use of humanised/chimeric antibodies for the treatment of cancer patients.

1.5. Applications of monoclonal antibodies

Antibodies and antibody-based immunoassays have been extensively used as diagnostic tools, and today they are still one of the fastest growing technologies for the analysis of biomolecules and drugs (Fitzpatrick *et al.*, 2000). Major trends in antibody-based diagnostics over the past few decades, have included increasing assay specificity, detection technologies and sensitivity (Borrebaeck, 2000). This has been

achieved by the development of a number of different assay formats based either on competitive or non-competitive principles.

Juric (2001), report the use of a mouse monoclonal antibody that could rapidly and specifically target-known sites of leukemia in patients. The antibody was produced against CD33, a cell surface glycoprotein found on most myeloid leukemias. The antibody was labelled with ^{131}Iodine (I)-M195. Subsequent trials showed elimination of large leukemic burdens after treatment with higher doses of the labelled antibody. A humanised version of the antibody was also produced, and was found to display rapid targeting of leukemia, and pharmacology similar to that of its murine counterpart without significant immunogenicity. They also found that treatment with supersaturating doses of the humanised antibody produced complete remissions in some patients with advanced myeloid leukemias.

Immunotoxins have also been applied for the treatment of cancer. An immunotoxin is a fusion protein consisting of a toxin connected to a monoclonal antibody or growth factor. Plant- and bacteria- derived toxins work in the same way: they bind to the cell surface through a monoclonal antibody, internalise into an endosome, translocate to the cytosol, and finally, inhibit protein synthesis that leads to cell death. Hematologic tumours are easier to target than solid tumours, as they are easier to access for the immunotoxin than for intravascular tumour cells. Anti-CD 22 and anti-CD19 immunotoxins have been used in the treatment of B-cell lymphomas and leukemias (Funaro *et al.*, 2000). Immunodrugs are similar to immunotoxins, as both of them use antibodies to carry the respective drug to the tumour target.

Presently, there are more than eight licensed monoclonal antibodies commercially available in Europe and the U.S. (Fitzpatrick *et al.*, 2000). They are used for the treatment of a range of diseases such as graft rejection, rheumatoid arthritis, non-Hodgkin's lymphoma, metatstatic breast cancer, and respiratory synctial virus infection. Some of the antibodies are either humanised or chimeric, while one of them, OKT3 (Orthoclone-Ortho Biotech) is a murine antibody.

Antibodies can be produced against protein markers on cancer cells and used for diagnostic purposes. Antibody kits for cancer detection are predominantly used for the management of cancer patients, as opposed to an initial diagnosis. The BTA test™, manufactured by Bion Diagnostic Sciences Inc., is used for recovering bladder cancer patients to monitor their risk of a relapse of cancer. The kit is lateral flow immunoassay that uses a monoclonal antibody specific to human complement factor H-related protein (hCFHrp). This is a tumour-related antigen that is secreted in urine by bladder cancer cells (Sarosdy et al.,1997; Kinders et al., 1998) . The test is used as an adjunct to cystoscopy for helping physicians assess patient progress after treatment.

Antibodies have been produced against a wide range of other molecules for diagnostic purposes including cytokines (Yun et al., 2000), polyamines (Schipper et al., 1991), viruses (Shum et al., 2001), and pesticides (Bruun et al., 2001). Future applications for antibodies include their use in proteome analysis, which will be discussed later in this chapter. A recent development of non-biological alternatives to antibodies are 'plastibodies', which may significantly improve immunoassay technology. Plastibodies are based on the concept of molecular imprinting. This entails mimicking the binding site of a natural antibody and moulding it directly into a polymer (Borrebaeck, 2000). The polymeric constructs have subsequently been used in so-called molecularly imprinted sorbent assays (MIA) against theophylline and diazepham. However, a general problem with the design of molecular alternatives to antibodies is that very low affinity variants are often obtained. Walshe et al. (1997) produced a plastibody to 7-hydroxycoumarin. The polymer was packed into a cartridge and used as a solid-phase extraction sorbent for 7-hydroxycoumarin from urine. The method was found to be linear for the extraction of 7-hydroxycoumarin between 10-50 μg/ml as well as being reproducible. It highlighted the potential of plastibodies as suitable alternatives to convention liquid-liquid extraction procedures.

1.6. Antibody engineering

Improvements in the efficacy and the relative ease of use of recombinant DNA technology has meant it is now possible to produce specific antibody using genetic engineering. Recombinant antibody display technology has been utilised for the generation of antibody fragments from several species including human, mouse, sheep and rabbit. This has resulted in the engineering of antibody fragments, which include the Fab and scFv fragments. Due to their small size, scFv fragments have many applications in therapeutics, as they allow for easy access through tissues. Antibody fragments smaller than scFv's can also be engineered, which have the ability to bind antigen; these include the Fd, and the complementary determining region (CDR), the smallest antibody fragment capable of binding to an antigen (Figure 1.4). Extensive reviews of antibody engineering are available elsewhere (Kontermann & Dubel, 2001; Borrebaeck, 1992).

The genetic material coding for an antibody fragment can be obtained from a variety of sources. These include immunised or non-immunised animals, such as mice (Caton & Koprowski, 1990) and sheep (Li et al., 2000), as well as humans (M[C]Call et al., 1998), or a repertoire of naïve, semi-synthetic or synthetic genes (Winter, 1998). The genes are isolated from cells of the immune system, which may be found in the spleen, peripheral blood, bone marrow or the lymph nodes. Classical hybridomas can also be used as the raw material for cloning the antibody variable gene segments. The genes for the antibody heavy and light chains can then be amplified up for insertion into a vector (Orlandi et al., 1989). A vector is used to transfer the genetic information into the host organism, which then produces the desired protein. Plasmids are naturally occurring closed circles of DNA that are commonly used as vectors in antibody engineering. Subsequently they may be used to transfect a range of different hosts for expression (Figure 1.8). These include bacterial, yeast, mammalian, and insect expression systems (Verma et al., 1998). Each has their individual advantages, potential applications and limitations. Bacteria cannot assemble whole glycosylated antibodies, but are useful for the production of antibody fragments. Complete antibodies can be expressed in yeast, but they can contain high-

mannose, multiple-branched oligosaccharides and have been shown to be defective in effector functions. Fully functional antibodies can also be expressed in mammalian cells, as it takes advantage of the sophisticated refolding machinery that is located in the endoplasmic reticulum of these cells. Insect expression systems are advantageous for large-scale production of proteins as they are cost effective and have a high affinity of expression.

Antibody engineering has allowed for the construction of large antibody libraries that can be used for the *in vitro* selection of many different molecules. In recent years the selection process has been made easier with the use of surface-display vectors for displaying antibody fragments on the surface of bacteriophage and bacteria. Both of these factors combined have enabled the development of more effective strategies for drug and target discovery.
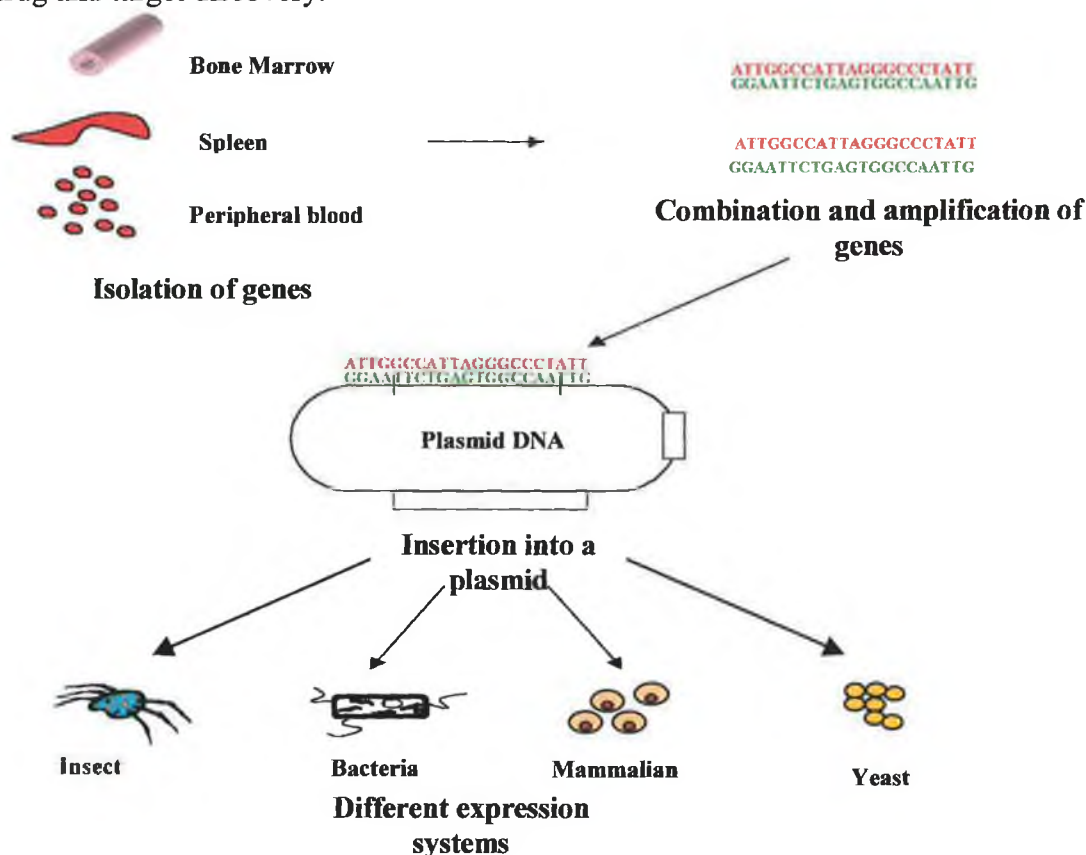
Figure 1.8. *Summary diagram showing the different sources for isolation of antibody genes and the different systems that can be used for the expression of scFv antibody fragments.*

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1.7. Phage display technology

Bacteriophages are viruses whose hosts are bacterial cells. Discovered over 80 years ago, they have played a key role in the development of modern biotechnology (Marks & Sharp, 2000). Bacterial phage display is by far the most important tool in the isolation and engineering of recombinant antibodies. Its principle entails the batch cloning of DNA encoding millions of variants of certain ligands (e.g. peptides, antibody fragments etc.) into the phage genome as a fusion to the gene encoding one of the phage coat proteins (pIII, pVI, or pVIII) (Hoogenboom *et al.*, 1998). When expressed, the coat protein fusion is incorporated into new phage particles that are assembled in the bacterium. This results in the ligand being presented on the phage surface, while its genetic material resides within the phage particle. The connection between ligand genotype and phenotype allows for the enrichment of specific phage using selection on immobilised target.

Phage display was developed in 1985 by Smith (Smith, 1985), when it was demonstrated that foreign DNA fragments could be inserted into filamentous phage gene III to create a fusion protein with a foreign sequence in the middle. The filamentous phage used was a member of the M13 family. These non-lytic phage infect strains of *E.coli* containing the F conjugative plasmid. Gene III of filamentous phage encodes a minor coat protein, pIII, which is located at one end of the virion. The amino terminal half of pIII binds to the F-pilus of *E.coli* during infection, while the carboxy-terminal half is buried in the virion and participates in morphogenesis. A foreign sequence can be inserted between the two domains without significantly affecting pIII function. Phage particles attach to the tip of the F-pilus that is encoded by genes on the plasmid, and the phage genome, a circular stranded DNA molecule is translocated into the cytoplasm. The genome can then replicate using phage and host-derived proteins, and is packaged by the infected cell into a rod-shaped particle that is released into the media. All virion proteins undergo transport to the cell periplasm prior to assembly and extrusion. Figure 1.9 shows what the typical structure of a filamentous phage.
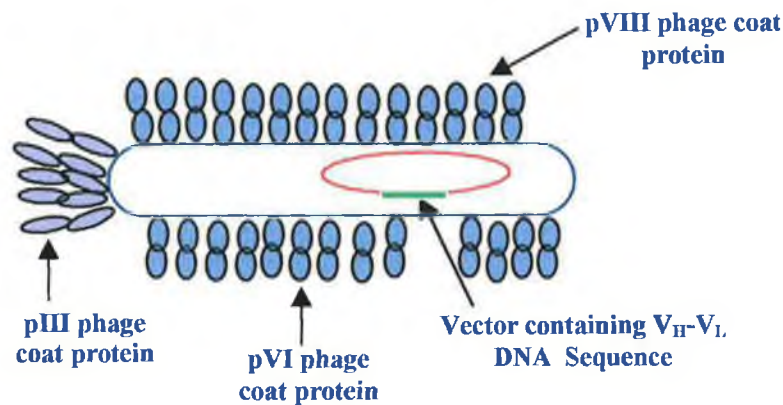
Figure 1.9. *Structure of filamentous Fd or M13 phage. ScFv's are displayed on the surface of the phage as a fusion with the pIII phage coat protein. The pVIII coat protein can also be used for display of antibody fragments on its surface. The plasmid DNA containing the genetic information coding for the antibody fragment is found within the phage particle. Production of phage particles displaying the scFv, within a bacterium, is induced by superinfection of a helper phage into the bacterium.*

Several filamentous phage have been used for the display of ligands. Attempts to display Fab' fragments fused to pVIII, the phage major coat protein have also been successful. (Gram *et al.,* 1992). However, although the pVIII site is popular for peptide phage display, it is not suitable for the efficient display of large polypeptides such as antibodies.

Early work also concentrated on the use of λ phage, as it was a well understood system, with a number of suitable vectors. Huse *et al.* (1989) produced a combinatorial Fab' antibody library using such a vector. However, it was soon noted that methods based on λ phage could only produce relatively small libraries (10^6), as well as the fact that screening procedures were poor.

M^CCafferty *et al.* (1990) were the first to clone and express antibody variable domains on the surface of Fd filamentous phage. The antibody was a murine anti-lysozyme antibody with the V_H and V_L fragments linked via the (Gly-Gly-Gly-Gly-Ser)$_3$ linker to the gene III of the Fd phage. This system also used a phage vector that carried all of the genetic information for the phage life cycle. However, presently, phagemids are the more commonly used vectors for display. Phagemids are small plasmid vectors that carry gene III with the appropriate cloning sites (for the insertion of cloned antibody genes) and the phage intergenic region (for rolling circle replication and packaging) (Benhar, 2001). The scFv may be fused to the phagemid at the N-terminus of an intact gene III, or at the N-terminus of a truncated gene III lacking the first two N-terminal domains. Phagemids have high transformation efficiencies, and therefore, are ideally suited for the production of very large library repertoires.

Most phagemids use a lacZ promoter to drive expression of the antibody gene III fusion product. In order to achieve display of the antibody-gene III product, glucose, acting in catabolite repression of the lac promoter is removed or depleted, leading to the upregulation of the lac promoter, and the expression of sufficient fusion product to generate monovalent phage particles. It is often necessary to regulate the system tightly, as expression mediated toxicity can be a problem with antibody fragments. The phagemid DNA encoding antibody-gene III fusions is preferentially packaged into phage particles using a helper phage such as such as M13K07 or VCS-M13. These supply all of the structural proteins necessary. The helper phage genome encodes wild-type pIII, and as a result, over 90 % of rescued phage display no antibody at all, and the majority of the rescued phage particles that do display the fusion product will only contain a single copy. Therefore, the use of a helper phage is required which supplies the necessary structural proteins needed to correctly package the phage particle is required. Figure 1.10 shows an example of a typical phagemid and the major components required for the production of functional scFv antibody fragments.

Restriction enzyme digestion sites

| LacZ | pelB | V$_H$.V$_L$ Fragments | H$_6$ tag | ✳ | geneIII |

Phagemid Vector

| Col E1 ori | Antibiotic Resistance | M 13 intergenic region |
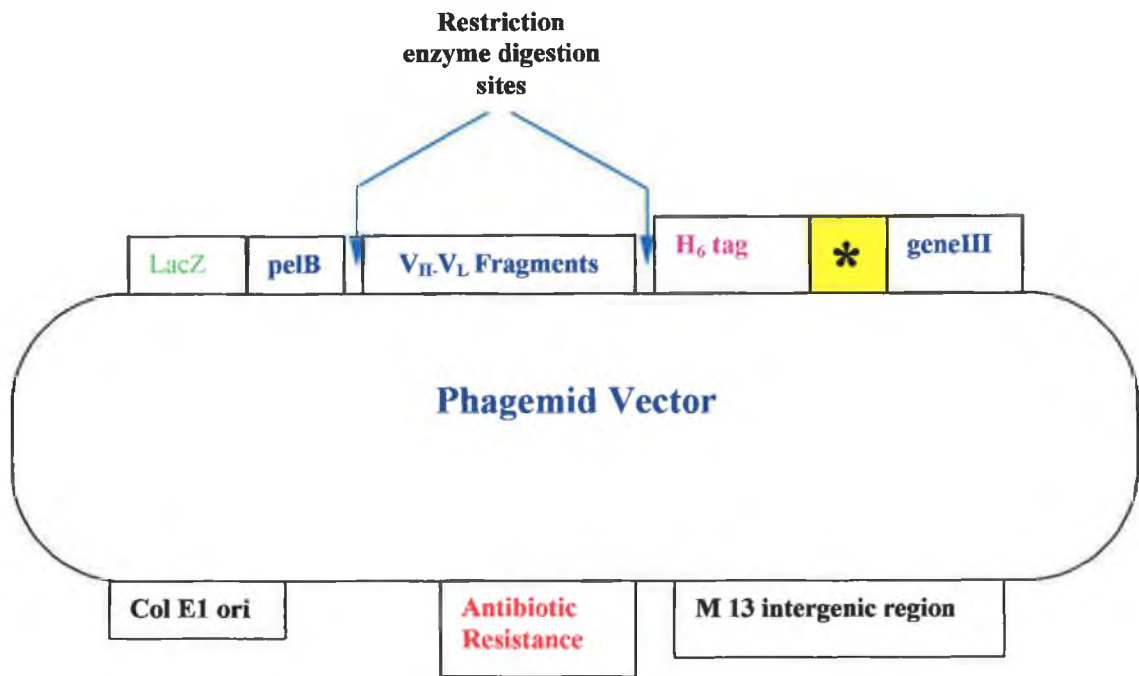
Figure 1.10. *Example of a (hypothetical) phagemid showing the major components required for phage display of a scFv antibody. ScFv fragments may be displayed on phage using a phagemid like this that expresses the V$_H$ and V$_L$ fragments fused to a coat protein gene, gene III, of filamentous phage Fd. The V$_H$ and V$_L$ fragments may be ligated separately into the phagemid, or alternatively, as is shown here, they may be assembled as one prior to ligation. Bacteria harbouring the phagemid are superinfected with helper phage to drive production of phage particles carrying the scFv fragment. The phagemid contains an antibiotic resistance gene (e.g. chloramphenicol, ampicillin) as a selective marker. Gene III codes for phage Fd, while the amber codon (*) allows for soluble expression of antibody in non-suppressor bacterial strains. The H$_6$ and tag region code for a histidine stretch, and peptide tag that can be used for purification and detection purposes of the antibody fragment. A pelB leader sequence directs expressed protein to the bacterial periplasm, while the lacZ promoter is responsible for control and expression of scFv-gene III fusion product.*

1.7.1. *Construction of an antibody phage display library*

Figure 1.11 outlines the main steps taken in the construction of an antibody phage display library. Firstly mRNA is extracted from either hybridomas, or spleen cells from a mouse that was either naïve or pre-immunised with antigen. The advantage of using spleen cells from a mouse pre-immunised with antigen means that there is a greater quantity of genetic material for specific antibodies produced to that antigen in the spleen. However, if one wanted to produce a phage display library for screening of a number of antigens, then a naïve library is more useful. If one uses the genetic material obtained from a hybridoma, it will result in cloning the antibody fragment of the parent monoclonal antibody. Reverse transcription-polymerase chain reaction (RT-PCR) is carried out to reverse transcribe the messenger RNA (mRNA) to its complementary DNA (cDNA) sequence. Primers are then used in the amplification of the heavy and light variable-genes by PCR, which are required for scFv production. Several sets of universal primers are available for the amplification of a large selection of V-genes (De Haard *et al.*, 1998). These primers can be engineered to contain certain restriction sites necessary for cloning. The restriction sites chosen are not contained within the scFv sequence. Commonly used restriction enzymes include SfiI and NotI, as they rarely cut within antibody genes, on the basis of recognition of eight base-pairs. The amplified DNA, which encodes millions of different variations of the scFv, is cloned as a fusion to the gene encoding one of the phage coat proteins (e.g. gene III), into a particular vector. The V-genes can either be cloned in as two separate pieces of DNA, or alternatively, they may be annealed together using splice by-overlap extension (SOE)-PCR (See Section 4.1), and then cloned into the phagemid vector as one piece. The ligated phagemid vector is then used to transform a suppressor strain of *E.coli*, such as Xl-1 blue. Expression of scFv displaying phage is then carried out, with the aid of a particular helper phage required to provide structural proteins necessary for correct packaging of the phage. This can then be used in the screening process for the detection of a 'positive' clone.

Genetic source for antibody fragment production
•Non-immunised/Immunised Mouse
•Hybridomas

Extract RNA

Reverse Transcription

V_H

V_L

PCR amplifying V_H and V_L regions

Plasmid

Restriction enzyme digest of V_H-V_L fragments

Ligation of amplified DNA into plasmid (either as 2 pieces or 1 annealed piece of DNA)

Superinfection with helper Phage

Phage display scFv antibody

Transform into *E.coli*

Figure 1.11. *Phage display library construction. Genetic material encoding sequences derived from mRNA is reverse transcribed, and amplified up using PCR. The DNA encoding the scFv is restriction enzyme digested and ligated to a particular vector either as one or two separate pieces of DNA. This is then transformed into a bacterium for the creation of an antibody phage display library. The scFv is displayed on the surface of the phage when fused to a coat protein, after super infection of the harbouring vector with a helper phage.*

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Library size is a particularly important factor, when producing a phage display library, as the number of clones in a library is a function of its diversity and the affinity of the antibodies selected from it. A typical library size would consist of 10^7 clones (Hoogenboom *et al.*, 1998). Sheets *et al.* (1998) produced a large naïve human phage-display library consisting of 6.7 x 10^9 clones. The library was then used to select for scFv's to fourteen different protein antigens. Analysis of these antibodies revealed high affinities for number of them.

As mentioned previously, the helper phage genome encodes wild-type pIII, and therefore, over 90 % of rescued phage will not display any antibodies on their surface. Rondot *et al.* (2001) developed a new method for increasing the number of phage particles displaying scFv fragments on their surface. They have developed a new helper phage known as hyperphage. Hyperphage have a wild type pIII phenotype and can therefore infect *E.coli* cells via their F-pilus with high efficiency. However, they lack a functional pIII gene, which means that the phagemid-encoded pIII antibody fusion is the sole source of pIII in phage assembly. This results in a considerable increase in the fraction of phage particles carrying antibody fragments on their surface. Results using hyperphage showed a 400-fold increase in antigen-binding activity. After two rounds of panning (against tetanus toxoid), it was found that more that 50 % of the phage were found to bind to antigen, compared to 3 % when conventional helper phage was used.

The majority of vectors used for antibody phage display carry a small peptide tag linked to the 3' end of the scFv permitting its detection by various immunological assays. However, these tags are not applied for the actual capture and purification of antibody-displaying phage particles. Usually, phage particles are isolated from culture supernatant by polyethylene-glycol (PEG) precipitation. A more recent alternative to PEG precipitation, is where a ligand-binding peptide is displayed in many copies on the phage surface as a fusion to the major phage coat protein pVIII. This peptide, which co-expresses with the scFv, antibody can then be used as an affinity tag for the selective concentration of displaying phage particles only.

Berdichevsky *et al.* (1999) expressed an scFv antibody as an in-frame fusion protein with a cellulose binding domain (CBD) derived from the *Clostridium thermocellum* cellulosome. Cellulosomes are multi-enzyme complexes devoted to the efficient degradation of cellulose and hemicellulose by cellulolytic microorganisms. The CBD domain serves as an affinity tag, allowing for the rapid capture of phage, and concentration from crude culture supernatants. CBD retains its cellulose binding properties when fused to a heterologous protein. Therefore, it binds to, and can be eluted from cellulose under mild conditions. Functional phage displaying scFv's expressing the tag can be separated from non-functional fragments by binding and elution with cellulose. The functional scFv's can then be used for screening against a particular antigen. They found the tag to be particularly useful in the selection for scFv fragments from naïve libraries. Comparison of panning for a scFv-specific to a human Alzheimers β-amyloid peptide using the CBD tag, as well as without it, was carried out. They found that using cellulose treatment throughout the panning allowed the isolation of specific scFv antibodies, while exhaustive panning without cellulose treatment failed to produce any antigen positive clones.

A third method for the functional selection of specific phage is the use of selectively infective phage (SIP) technology (Jung *et al.*, 1999). SIP is particularly advantageous, as there is direct coupling of the antibody-antigen interaction with phage infectivity and amplification. It works on the principle of displaying an antibody library on non-infective phage particles. This is done, by deleting part of the gene III protein on the phage (amino terminal domains), which are essential for phage infectivity but not phage morphogenesis. Infection is restored by binding a fusion protein consisting of the cognate antigen, and the amino terminal domains of pIII responsible for pilus binding and infection. The fusion product may be supplied either *in vitro*, or *in vivo*. For *in vitro* SIP, phage displaying the scFv and the fusion product need to be separately purified and combined together in defined amounts to yield infective phages. The fusion product is encoded on a plasmid, and the ligand can be either genetically fused to it, or in the case of a hapten chemically coupled to it.

The *in vivo* system entails encoding the antibody and antigen on the same genome, and producing them in the same cell. Therefore, in this case the ligand can only be a protein.

1.7.2. *Screening for scFv antibodies from a phage display library*

The feature of the display of ligands on the surface of phage is the linkage between phenotype (surface displayed ligand) and ligand genotype, encoded within the phage genome. This allows the enrichment for antigen-specific phage antibodies using immobilised label or antigen. Figure 1.12 outlines the screening procedure for the enrichment of specific phage antibodies. Phage that display a specific antibody are retained on a surface with coated antigen, while non-adherent phage are washed away. Bound phage can then be eluted from the surface and re-infected into bacteria for further growth and enrichment. Phage clones can be analysed for binding after each round of panning. A phage ELISA is the common method used for such analysis, where antigen is immobilised on the surface, and phage displaying a scFv antibody are added to the well. The phage can be detected using a secondary antibody specific to a tag encoded for on the phagemid vector (e.g. c-myc tag)

If a clone were found to be positive in a phage ELISA, the assay would be repeated in a competitive format. This is particularly important when selecting for scFv's specific to small haptens, as often, during panning, scFv's directed against the protein-conjugate molecule, or just the protein part of the conjugate are selected for, and amplified. Therefore, it is necessary to ensure the scFv recognises free hapten in solution. It is desirable to use more than one hapten-protein conjugate when panning for scFv's to such small molecules. This ensures non-specific phage that bind to the protein will not be continually selected and amplified, and therefore generating high-absorbance non-specific signals in phage ELISA.
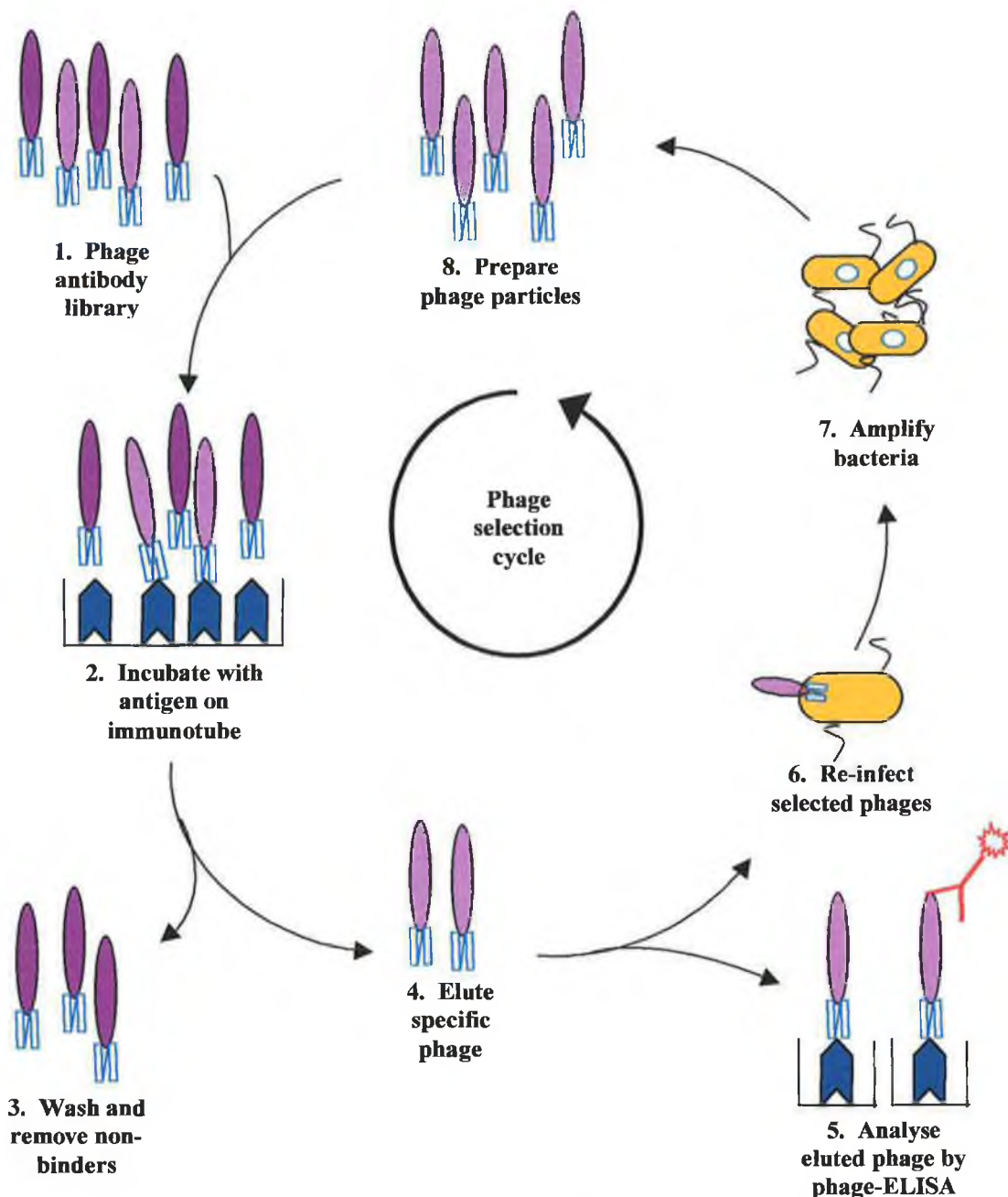
Figure 1.12. *Cycle for the selection of phage from an antibody phage-display library.* ***1.*** *Phage is produced from an antibody library.* ***2.*** *Antigen is coated on an immunotube, and the library is incubated with it in solution.* ***3.*** *Non-bound phage are washed away, while **(4)** specific phage are eluted from the antigen.* ***5.*** *A number of phage clones are selected for recognition to antigen (either in a competitive or non-competitive format).* ***6.*** *The phage is also re-infected into* <u>E.coli</u> *for* ***(7)*** *amplification and rescue.* ***8.*** *Phage particles from the amplified library are used to incubate with antigen in a subsequent round of panning. Clones are analysed by phage ELISA after each round of panning in order to select clones that bind specifically to the antigen. This process of enrichment and selection increases the probability of selecting for 'positive' clones after each round of panning.*

Any method that separates clones that bind to antigen, from clones that do not, can be used as a selection method for phage-display scFv's. Such methods include biopanning on immobilised antigen coated onto solid supports, columns, or BIAcore sensor chips. Selection can also be carried out using biotinylated antigen, panning on fixed prokaryotic cells, and on mammalian cells (Hoogenboom *et al.*, 1998). All the methods mentioned here are *in vitro*-based, and rely on a relatively large amount of antigen being available for the selection process. It is more difficult to select for phage antibodies with a limited amount of antigen. *In vivo*-based screening can be used for selection when one has a limited amount of antigen. Johns *et al.* (2000) used *in vivo* selection for the selection of scFv antibodies to vascular endothelium (found in the thymus). It is difficult to isolate fresh endothelial cells with intact surface molecules and unchanged phenotype from any tissue. The procedure entails producing phage from a library as normal, and injecting them intravenously into a mouse. After a two-hour incubation period, the thymus is removed, and teased out in PBS. Elution buffer is added, and the phage are eluted, and re-infected back into bacteria. They found the method to be successful for the isolation of a scFv to an endothelial protein. However, characterisation of the protein antigen also needs to be carried out, in order to determine the exact molecule to which the antibody generated is specific against.

1.8. Soluble expression of scFv antibodies

Once a phage-clone is found to specifically recognise the antigen of interest, the DNA encoding that particular scFv can be subcloned into an enhanced expression vector. This may be carried out for a number of reasons such as high expression of phage-displaying scFv's or soluble scFv's (Krebber *et al.*, 1997(a)), or expression of a soluble scFv attached to alkaline phosphatase for the direct detection of antigen (Muller *et al.*, 1999). These enhanced expression vectors may also contain a gene coding for a histidine tag to aid in scFv purification and detection. Soluble expression of scFv's is also enhanced by switching from a suppressor strain of *E.coli* (e.g. XL-1 blue) to a non-suppressor strain of *E.coli* such as JM83 (Krebber *et al.*, 1997). An amber codon is usually present on phagemid vectors between the antibody and the

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pIII gene. In suppressor strains, this allows direct expression of the scFv as a fusion partner with phage coat protein. In the case of non-suppressor strains, the amber serves as a stop codon, which enhances soluble scFv expression. ScFv expression is directed to the periplasm of the bacterium, which resembles the natural route of antibody production in the endoplasmic reticulum of the lymphocyte (de Haard *et al.*, 1998). This ensures the antibody folds correctly according to its natural conformation. Therefore, this production route, via the endoplasmic reticulum enhances the chances of obtaining a functional scFv. A proportion of periplasmic scFv may also leak out into the culture supernatant. ScFv antibodies can also be produced as cytoplasmic inclusion bodies (Huston *et al.*, 1988; Cho *et al.*, 2000) followed by re-folding *in vitro*. However, problems can arise with refolding, as the formation of incorrect intramolecular disulphide bridges can occur.

Kipriyanov *et al.* (1997) developed a system for high-level production of scFv antibodies in small scale *E.coli* cultures. They found that the addition of sucrose to the culture medium greatly increased (15-25 fold) the yield of soluble scFv's produced. They also found that scFv's could be made to accumulate in the periplasm or be secreted into the medium by simply changing the incubation conditions and the concentration of the inducer. Aggregation of recombinant proteins in the periplasm of *E.coli* could be reduced by growing the cells in the presence of certain sugars such as sucrose. These sugars are small enough to diffuse into the periplasmic space, but are not metabolised. The sugars cause an increase in osmotic pressure, and therefore, enlarge the periplasmic space, which allows more scFv's to be secreted into it.

Soluble scFv antibodies have also been expressed in mammalian cells (Ridder *et al.*, 1995), yeast (Freyre *et al.*, 2000), plants (Longstaff *et al.*, 1998), and insects (Kretzschmar *at al.*, 1996). These systems offer advantages such as better protein folding pathways as in the case of yeast and mammalian cells. Insect and plant-based systems do not require large fermenters with sterile culture media and, therefore, the secreted proteins are free from mammalian viruses and bacterial endotoxins. As a

result it could be possible to grow cells in serum-free media, that do not contain mammalian contaminants, thus simplifying purification.

A more novel expression system for the production of recombinant antibodies has been the use of transgenic milk (Pollock *et al.*, 1999). The principle involves linking the gene for the scFv antibody to mammary gland-specific regulatory elements on a vector. The resulting transgene is then introduced by pronuclear microinjection into embryos of the selected species (e.g. goat or sheep). The embryos are then transferred to the uterus of a surrogate mother and carried to term. The transgenic offspring are identified, and when mature are either bred or hormonally induced to lactate. The expression level of the target protein in the milk of the transgenic animals is determined and a suitable founder line is chosen for the generation of a production herd. Dairy goats are ideal for this application, as they provide relatively high yields of milk (300-800 liters/300-day lactation), and recombinant antibody concentrations are quite good with 1-5 g/l of protein being recorded (Pollock *et al.*, 1999). With these types of conditions, a herd of transgenic goats could yield up to 300 kg of purified product per year. The other advantage of using goats is that it only takes 16-18 months from the time of initiating microinjection to full lactation. A number of recombinant antibodies have been expressed in transgenic milk with good success rates (Pollock *et al.*, 1999).

1.9. Purification of scFv antibodies

Several methods are available for the purification of scFv antibodies. These include antigen affinity-chromatography (Owen *et al.*, 1992; Casey *et al.*, 1995; Cho *et al.*, 2000), protein A chromatography (Mahler *et al.*, 1997)), cation exchange and size exclusion chromatography (Kretzschmar *et al.*, 1996), human constant light chain tagging (Ridder *et al.*, 1995; Longstaff *et al.*, 1998), and immobilised metal affinity chromatography (IMAC) (Kipriyanov *et al.*, 1997; Freyre *et al.*, 2000).

IMAC is one of the most popular methods for scFv purification, as it can ensure high ligand stability, high protein loading, mild elution conditions, low cost and complete

recovery of ligand following regeneration (Arnold *et al.*, 1991). However, it has been noted in our laboratory that this method is not as effective as initially thought. This will be further discussed in Chapter 4. IMAC involves the insertion of the genes coding for a histidine (His) tag, containing 4-6 histidine residues, into the scFv sequence. Insertion at the N or C terminus ensures that the antigen binding site is not effected (Casey *et al.*, 1995). The column consists of transition metals such as Ni^{2+}, Co^{2+}, Zn^{2+} and Cu^{2+}, chelated by nitrotriacetic acid (NTA), iminodiacetic acid (IDA) or tris (carboxymethyl) ethylenediamine (TED) (Arnold *et al.*, 1991; Muller *et al.*, 1998). His-tagged proteins bind to the metal-chelate solid support, while impurities are eluted. Specifically bound protein is then eluted using low pH. This confers a positive charge on the histidine residues making them incapable of binding metal ions. Alternatively, imidazole can be used for elution, which competes with histidine for metal binding. The advantage of the imidazole-based elution system is that it does not denature the protein. Janknecht *et al.* (1991) used it to purify human serum response factor to greater than 95% purity. Casey *et al.* (1995) have utilised Cu^{2+}-IDA chelated IMAC to purify a His-tagged protein to 90% purity, with a higher yield than that of antigen affinity chromatography (10mg/l as opposed to 2.2mg/l). Kipriyanov *et al.* (1997) have shown that IMAC can also be used to successfully purify scFv's from *E. coli* periplasmic extracts.

1.10. Applications of scFv antibodies

The ability to produce a large number of recombinant antibodies in bacterial cells and to select for antibodies that bind to unique or non-dominant epitopes demonstrates the power of recombinant antibody technology. It has resulted in recombinant antibodies being produced to a large number of molecules and proteins, for use in assay development, antibody therapy and proteomics. A number of these applications are described in the following sections.

1.10.1. *Application of scFv's in residue analysis*

The detection of food residues and contaminants has become increasingly important in today's society, as there is a strong focus within the EU for food quality and safety

Many of the detection methods using recombinant antibody technology to date have been ELISA based.

Mycotoxins are a serious problem in the grain industry, as they are highly potent carcinogens. Yuan *et al.* (1997) produced a soluble scFv antibody to zearalenone from an anti-zearalenone hybridoma cell line. The antibody fragments could detect as little as 14 ng/ml for 50 % inhibition of binding to zearalenone. This was similar to that of the parent monoclonal antibody in a competitive indirect enzyme-linked immunosorbent assay (ELISA). Yuan *et al.* (2000) also expressed an anti-zearalenone scFv antibody in Arabidposis plants. This scFv 'plantibody' could detect as little as 11.2 ng/ml for 50 % inhibition of binding of zearalenone. The antibody was found to be as sensitive as its bacterially expressed scFv counterpart, as well as its parent monoclonal antibody. Moghaddam *et al.* (2001) produced scFv antibodies to aflatoxin B_1 from human lymphocyte, and semi synthetic antibody phage display libraries. The scFv antibodies produced could detect as little as 14 μM AFB_1 in solution.

ScFv's have also been produced to pesticides for detection purposes (Longstaff *et al.*, 1998; Strachan *et al.*, 2000). Stabilisation of the fragments has also been carried out in order to improve their tolerance in polar solvents. This is further discussed in section 6.1.

M^{C}Elhiney *et al.* (2000) isolated scFv fragments to the cyanobacterial hepatotoxin microcystin-LR from a naïve human phage display library. Cyanobacterial toxins are environmental pollutants commonly responsible for human and animal poisoning worldwide. In a competition ELISA, the most sensitive antibody clone selected from the library could detect as little as 4 μM of free microcystin-LR for 50 % inhibition of binding. Other recombinant antibodies such as Fab fragments have also been produced to environmental toxins including botulinum toxin complex (Emanuel *et al.*, 2000). This recombinant antibody was applied in a number of assay set-ups including ELISA, surface plasmon resonance (SPR), flow cytometry, and a hand-held

immunochromatographic assay. The antibody was found to be sensitive in all of assay formats assessed, indicating encouraging signs for future applications of recombinant antibodies as routine detection molecules.

1.10.2. *Medical applications of scFv antibodies*

Engineered antibody fragments such as scFv's are replacing monoclonal antibodies in many areas, especially in medical applications such as cancer treatment and other forms of potential *in vivo* therapy. Developments in the areas of chimerisation and humanisation, as well as other characteristics of scFv antibodies, such as their size, tissue penetration and pharmacokinetic characteristics have been of great benefit. A number of naïve human phage display libraries are available for the selection scFv's specific to a particular antigen. Krebs *et al.* (2001) report the production and of a human combinatorial antibody library with 2×10^9 members, optimised for high-throughput generation and targeted engineering of human antibodies for various applications. Although recombinant antibodies provide effective and highly specific *in vivo* targeting reagents for tumours, they are cleared and removed rapidly from the blood due to their small size and single binding site. Covalently linked dimers or non-covalent dimers of scFv's (also known as diabodies) have shown improved targeting and clearance properties due to their higher molecular weight (60-120 kDa) and increased avidity. Diabodies are constructed by two functional scFv's joined together by a short 5-residue polypeptide linker. Careful selection of the linker length dictates the size and the valency of the scFv multimer formed. The production of a monomeric scFv requires that the linker employed be at least 12 residues in length. If one selects a linker of 3-10 residues in length, the scFv cannot fold into a functional scFv, and thus associates with a second scFv to form a diabody (60 kDa) (Figure 1.13). Reducing the linker length below 3 residues forces the scFv to form triabodies (90 kDa) or tetramers (120 kDa) (Little *et al.*, 2000). Molecules of diabody and triabody are relatively flexible from the orientation of antibody binding sites, and show higher functional affinity with reduced kidney clearance rates. Recombinant antibodies and their fragments now represent approximately 30 % of all biological proteins undergoing clinical trials for FDA approval of engineered cancer therapeutic

antibody. Human antibodies derived from phage display have already entered phase II/III clinical trials for treatment of rheumatoid arthritis and ocular fibrosis (Ryu & Nam, 2000).

Other medical applications of recombinant antibodies include their potential role in diagnostics. Cho *et al.* (2000) produced a murine scFv antibody to human plasma apolipoprotein A-I. This is a major protein component of plasma high-density lipoproteins (HDL), which play an important role in the process of reverse cholesterol transport from peripheral tissues to the liver for excretion. Lower levels of apolipoprotein A-I and HDL cholesterol are significantly correlated with increased risk of cardiovascular disease. As a result, production of a recombinant antibody to such a protein, may be useful in a diagnostic application for prediction of cardiovascular disease.
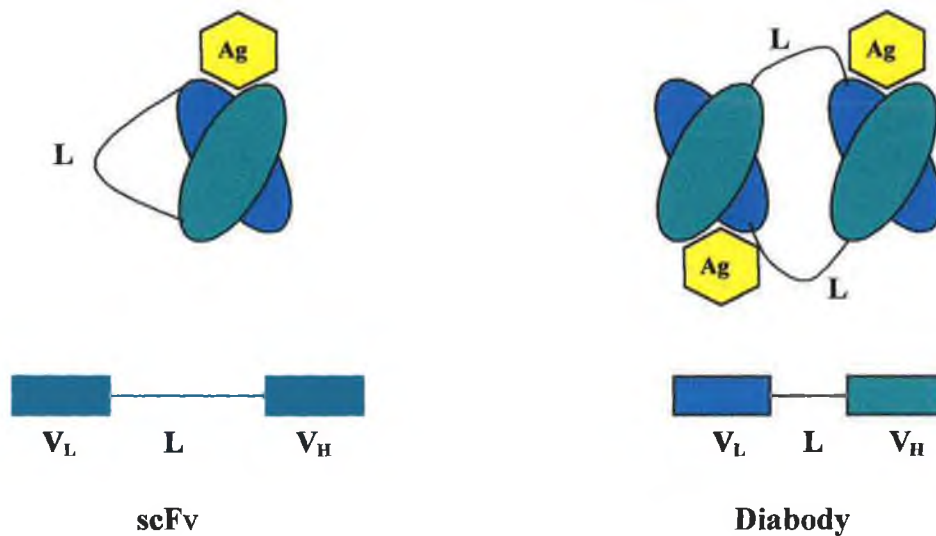
scFv

Diabody

Figure 1.13. *Schematic diagram of scFv antibody fragment and diabodies showing antigen-binding sites. When joined by a peptide linker (L) at least 12 residues long, scFv antibody fragments are monomeric (30 kDa). Diabodies (60 kDa) are formed when a linker between 3 and 12 residues is used. Reducing the linker length below 3 residues forces the scFv to fold into a triabody (90 kDa) or tetramer (120 kDa).*

1.10.3. *Application of scFv's to proteomics*

Proteomics can be defined as the study of protein properties (expression level, post-translational modification, interactions etc.) on a large scale to obtain a global, integrated view of disease processes, cellular processes and networks at protein level (Blackstock & Weir, 1999). Recombinant antibodies now play a significant role in proteomics, due to considerable advances made in the construction of large antibody phage display libraries, with high affinity antibodies being produced to target antigen. They can be produced quickly and cheaply, and can theoretically be produced against every human protein. Such a collection, in conjunction with screening technologies that use high density antibody arrays to identify these differentially expressed proteins, are being applied to the whole field of proteomics for selection of differentially expressed proteins.

There are different selection methods being adapted to apply antibody phage display technology to the proteomics field (Holt *et al.*, 2000). The three main areas are selection on complex antigen, conventional but highly parallel selection, and library versus library selection i.e. a library of antibodies selected against a library of antigens.

Selection on complex antigen entails the simultaneous selection of a library of antibodies against a complex antigen such as whole cells or cellular extracts. Although this can lead to a large panel of antibodies, phage selection generally biases antibodies recognising the most abundant protein present. As a result there is not a wide diversity of antibodies to all the targets in a given sample (Hoogenboom *et al.*, 1999). Instead, a number of antibodies are produced to different epitopes on the most abundant protein.

In order to overcome the problem of selecting large numbers of antibodies to the most abundant antigen a more strategic approach is required. This is known as conventional but highly parallel selection. Instead of selection against a complex antigen, selection of an individual protein, peptide or expressed cDNA can be

performed in parallel. Although this method has good potential for high-throughput it requires expensive robotics. It can also be problematic as the selected antibodies might only recognise the protein fragment used in the screening process and not the parental protein.

It is also possible to select a library of antibodies against a library of antigens, thereby allowing a large number of antibody-antigen combinations to be selected. An example of this system is selectively infective phage (SIP) (Krebber *et al.*, 1997(b)). As mentioned previously, SIP temporarily destroys the basic infectivity of the M13 filamentous phage, thereby enabling the direct coupling of a productive protein-ligand interaction to the phage. The infectivity can then be restored, allowing phage amplification and avoiding an elution step from a solid matrix. To date it has only been applied to model selections of antibody-antigen pairs, and has not yet been used to select antibody versus antigen libraries. The yeast two-hybrid system is another method of library versus library selection (Visintin *et al.,* 1999). It entails scFv antibodies being linked to a transcriptional transactivation domain within a eukaryotic cell. These linked scFv's can then interact with target antigen linked to a LexA-DNA binding domain, thereby activating a reporter gene. This method is also useful as is overcomes such problems as incorrect folding of antibodies in the cytoplasm. It has been used in a model selection where a single scFv was isolated from a mixture of half a million clones, indicating the procedure has the potential to capture antibodies of different specificities from complex mixtures.

Phage antibodies are also being applied to functional genomics using what is known as the ProAb™ approach (Pennington & Dunn, 2001). Presently there is a large amount of information currently available in databases of short DNA sequences called 'expressed sequence tags' or EST's, which represent most of the expressed human genome. These EST's can be translated into protein. Peptides are synthesised chemically to represent the protein sequence, and these peptides can then be used as antigens against which phage antibodies are selected. The antibodies can then be

used as detection reagents in immunocytochemistry, thereby revealing the distribution and abundance of the gene product in normal and diseased tissues.

Another screening method used in proteomics is the ProxiMol™ technique (Pennington & Dunn, 2001). Catalysed reporter deposition (Bobrow *et al.*, 1989; Bobrow *et al.*, 1992) was adapted to allow the isolation of phage antibodies binding in close proximity to a biotinylated guide molecule. When trying to screen antibodies to a cell surface antigen, a guide molecule (e.g. a biotinylated target ligand, or an HRP-conjugated antibody) is added to intact cells, together with phage from a $>10^{11}$ human antibody library. These diverse antibodies bind specifically to the ligand, to the target, and to all other cell surface markers. Biotin-tyramine reagent is added and a reactive biotin species is generated; phages binding within approximately 25nm of the guide ligand are covalently labelled with biotin. These phage antibodies, recognising the target and near neighbours, are recovered using streptavidin-coated magnetic beads.

Both of these methods can be successfully used in conjunction with each other, as antibodies selected using the ProAb™ strategy can be applied as guide molecules in ProxiMol™ method selections.

Immunoassay technology is limited to the analysis of a few thousand assays per day. When screening from an antibody phage display library the first round of phage selection can yield up to 10^7 clones. ELISA screening using 96-well plates only allows a small percentage of selected clones to be screened. The use of antibody arrays, however, can increase throughput up to several hundred times (Ekins *et al.*, 1990). Arraying antibodies and performing parallel screens using the same antibody array on different biological samples enables the identification of antibodies that bind to differentially expressed proteins. This form of application is useful, as antibodies selected against diseased samples could be screened in parallel with diseased and non-diseased samples. Unlike DNA arrays, antibodies cannot be synthesised on the surface of chips. Instead they have to be spotted on the chips in an array format and

coupled to a downstream high throughput detection system (Borrebaeck, 2000). Examples of detection systems would include fluorescent tags, nano-electrodes, and in the case of smaller arrays surface plasmon resonance and MALDI-TOF (matrix-assisted laser desorption ionization-time of flight) mass spectroscopy.

Antibody arrays take many forms from spatial patterning of just a few molecules on a solid support to high-density microarrays. Different supports can be used for the array including glass, nitrocellulose, and polyvinylidene fluoride (PVDF). Mendoza et al. (1999) produced a biochip that consisted of an optically flat, glass plate containing 96 wells formed by an enclosing hydrophobic Teflon™ mask. Each well contained four identical 36-element arrays comprising of eight different antigens and a marker protein. This 96 well microarray format was compatible with automated robotic systems and supported low cost, highly parallel assay format. Liu and Marks (2000) electroblotted the proteins ErbB2 and bovine serum albumin from SDS-PAGE onto nitrocellulose, and selected phage display antibodies to them. Antibodies were obtained with as little as 10^{-1} ng of antigen, and were used as reagents in both ELISA and Western blotting. De Wildt et al. (2000) developed a technique for high throughput screening of recombinant antibodies based on the creation of high-density antibody arrays. The method entailed the robotic picking and high-density gridding of bacteria containing antibody genes followed by filter-based ELISA screening to identify clones that express binding antibody fragments. This method was found to screen up to 18,342 clones at a time and simultaneously against 15 different antigens. Antibodies have also been used in the analysis of cellular changes occurring in cultured human cells, using a very small array and detected by SELDI (surface enhanced laser desorption/ionization) mass spectroscopy (Davies et al., 1999). The antibodies were used as the capture molecules on the chip array for retaining the target proteins from a biological sample. These proteins were then characterised using SELDI mass spectroscopy.

Information about gene expression at the protein level will make a great contribution to medicine and diagnostics in the twenty first century. Recombinant antibody arrays

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will have a wide range of applications in proteomics and will have a significant impact in the field.

1.11. Immunoassays

The first immunoassay was developed by Yalow and Berson in 1960 (Yalow & Berson, 1960), for the determination of insulin in blood. Since then, immunoassays have been commonly used in analytical biochemistry for the detection of a wide range of compounds such as hormones, drugs and viruses. Different assay formats are available, and these have been reviewed extensively elsewhere (Price & Newman, 1997).

Enzyme-Linked Immunosorbent Assay (ELISA) is one of the most common techniques used for measuring antibody-antigen interactions. ELISA's are heterogeneous assays because the antibodies or antigens are immobilised on a solid phase. Figure 1.14 shows a schematic diagram of a direct non-competitive ELISA. In this type of assay, the analyte such aflatoxin-B_1-BSA (AFB$_1$-BSA) conjugate is adsorbed onto the solid support matrix primarily through hydrophobic interactions. After a suitable incubation time (e.g. 1 hour at 37° C) the plate is washed using phosphate buffered saline (PBS) solution containing 0.05 % (v/v) tween, and phosphate buffered saline (3-times with each). Any remaining absorption sites on the plate are then blocked with casein protein dissolved in PBS, in order to reduce non-specific binding interactions. After another one-hour incubation and washing procedure, various dilutions of antibody are added to the coated wells of the plate, and allowed to react with the immobilised analyte. After incubation, unbound antibodies are then washed away, and an anti-species antibody molecule conjugated to an enzyme is added. Following further incubation and washing, the enzyme substrate is added, and a coloured product is produced. The intensity of the colour can then be read spectrophotometrically at the appropriate wavelength. This allows for the quantitative measurement of antibody binding to an antigen.

Another assay format commonly used is the indirect competitive ELISA. Figure 1.15 shows a schematic diagram of this. It is similar to the direct non-competitive ELISA, as analyte in the form of a hapten–protein conjugate (e.g. AFB_1-BSA) is adsorbed onto the wells of an immunoplate and blocked in the same manner. A solution containing a limiting constant amount of antibody (e.g. anti-AFB_1 antibody) and varying known concentrations of antigen (e.g. AFB_1) are then applied to the wells of the immunoplate and allowed to incubate. The washing step removes any of the antibody that has bound to the free antigen in solution, and the subsequent steps in the assay allow for the detection of the amount of antibody bound to the immobilised conjugate surface. In this assay format, as the concentration of free antigen (i.e. AFB_1) in solution increases, less-antibody is available for binding to the solid-phase immobilised antigen (i.e. AFB_1-BSA conjugate). As a result, an inverse relationship exists between intensity of the coloured solution formed, and the concentration of free antigen in solution. The assay format can be applied for the detection of unknown antigen concentrations in solution, by reference to a standard curve.
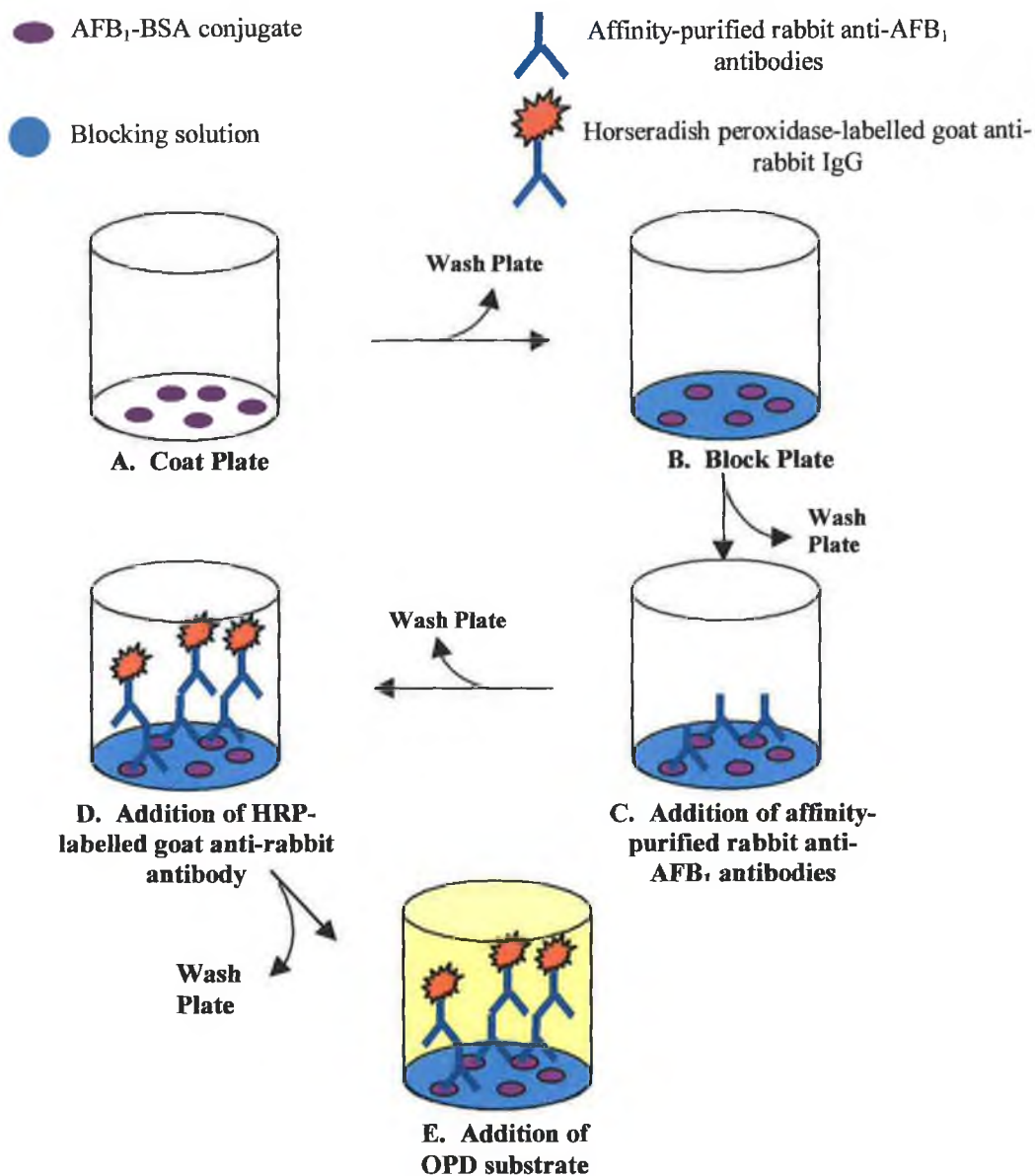
Figure 1.14. *Schematic diagram showing the principle of direct ELISA. Each step requires an incubation period and washing procedure to remove excess non-bound material. **A.** Aflatoxin B_1-BSA conjugate is used to coat wells of a 96-well immunoplate, and sticks to the plate primarily by hydrophobic interactions. **B.** The wells of the immunoplate are then coated with 2-4 % (w/v) of casein protein in PBS, to block any remaining absorption sites on the plate. **C.** The antibody samples are then added to the wells of the plate, and allowed to incubate for a suitable period of time followed by washing. **D.** A species-specific secondary enzyme-labelled antibody is added, and after suitable incubation and washing, substrate is added, which allows for colour development. The absorbance is then measured spectrophotometrically at the appropriate wavelength for the substrate.*
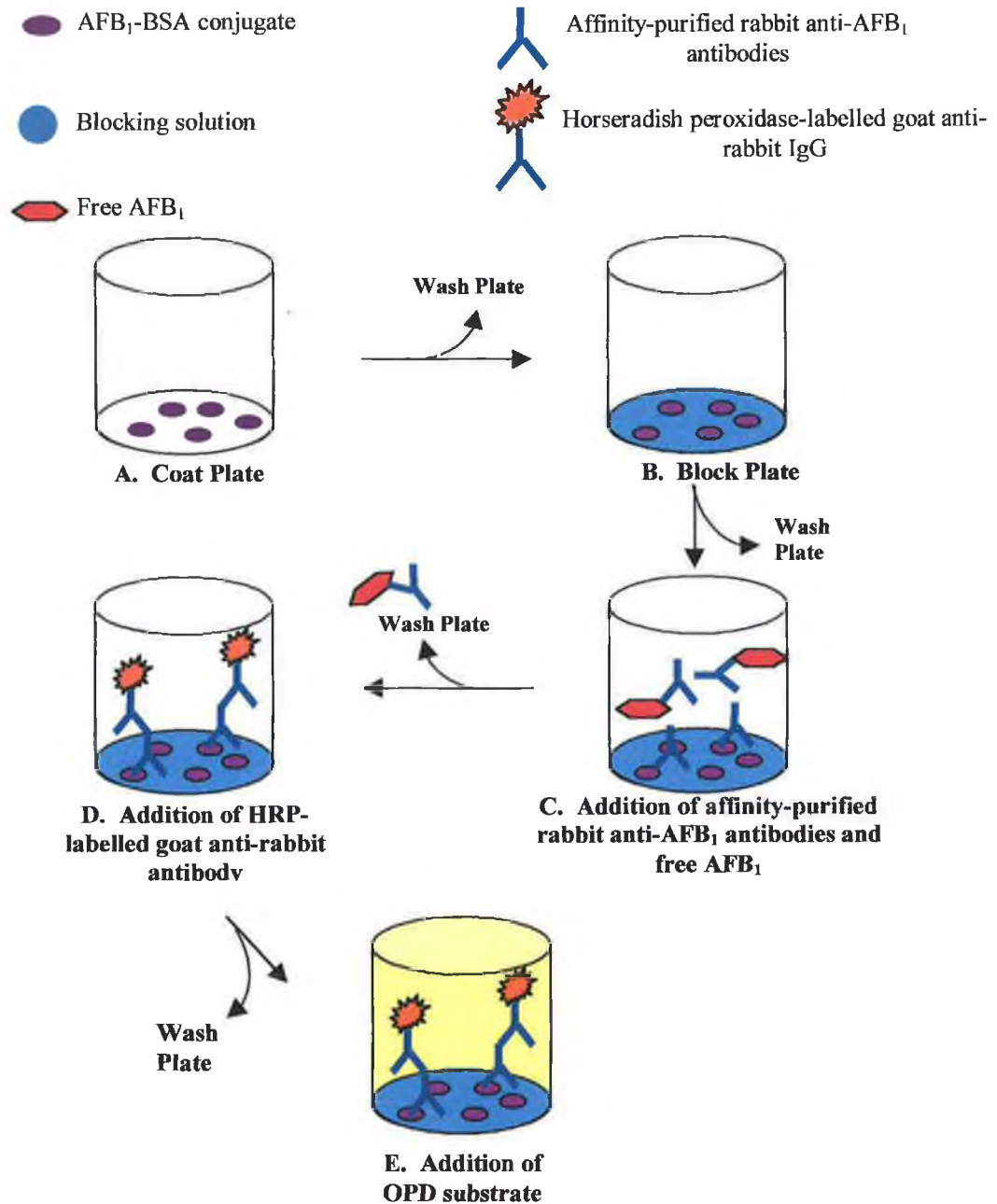
Figure 1.15. *Schematic diagram showing the principle of a competitive ELISA, used for the measurement of the range of detection of an antibody for a given antigen. The ELISA shown here is described in the context of measuring free AFB_1. The assay is similar to the direct ELISA, except that at step C, a solution of limiting constant amount of antibody and varying known concentrations of antigen are applied to wells of the immunoplate. Competition occurs between free antigen in solution and immobilised antigen on the immunoplate for binding to the antigen-specific antibody. The washing step removes any bound antibody – free AFB_1 complex. The secondary antibody then detects the amount of antibody bound to the conjugate on the plate surface, followed by the addition of substrate for colour development.*

1.12. Aims of research

The aim of this project was to produce antibodies and antibody fragments to aflatoxin B_1, utilising well established technologies, as well as exploiting combinatorial phage display technology.   It was also envisaged to develop different types of immunoassays using these antibodies for the detection of aflatoxin B_1. An additional aim of the project was to produce pesticide-protein conjugates and possibly generate antibodies using them, as well as utilise them in different immunoassay set-ups.

Chapter 3 describes the production of polyclonal antibodies to AFB_1, their purification and use in ELISA for the detection of AFB_1. It also includes cross reactivity studies and the application of one polyclonal antibody preparation for the detection of AFB_1 in spiked grain samples.

Chapter 4 describes the production of a combinatorial antibody phage display library derived from the RNA obtained from a mouse immunised with AFB_1-BSA conjugate. Single chain Fv antibodies were produced from the library, and were applied for use in an ELISA for the detection of AFB_1.

Chapter 5 describes the characterisation of polyclonal and scFv anti-AFB_1 antibodies, by applying them for the detection of AFB_1 on an SPR-based biosensor. Both antibodies were also applied for the detection of AFB_1 in spiked grain samples, and solution affinity analysis was carried out on the scFv antibody.

Chapter 6 describes the production of a pirimiphos-hapten molecule containing an amino group for conjugation to proteins.   It also includes the production of chlorpyrifos-BSA conjugates, their spectrophotometric characterisation, and application on BIAcore. A competition ELISA for the detection of chlorpyrifos was also developed using antibodies and conjugates obtained from collaborators (IFR Norwich, U.K.).   The development of an inhibition-based BIAcore assay for the detection of chlorpyrifos, using this antibody preparation was also attempted.

Chapter 2

Materials & Methods

2.1. Materials and equipment

All reagents used were of analytical grade, and with the exception of those tabulated below, and all reagents were purchased from Sigma Chemical Co., Poole, England. All commercial antibody preparations were also purchased from Sigma unless otherwise stated.

Reagent	Supplier
Acetic acid Hydrochloric acid Sodium chloride	Riedel de-Haen, Wunstrofer, Straβe 40, Apartado de Correos, D-3016, Seelze 1, Hannover, Germany.
Amino-dextran	Molecular Probes, Eugene, Oregon, U.S.A.
Bicinchoninic acid assay (BCA) kit	Pierce and Warriner (UK) Ltd., Chester, U.K.
C_{18} reverse phase cartridge filter	SEP-PAK®, Waters (A division of Millipore), Millipore Corporation, Milford, Massachusetts, U.S.A.
CM-5 Chips	Pharmacia Biosensor AB, Uppsala, Sweden.
CM-Dextran Chlorpyrifos O-carboxymethylhydroxylamine	Fluka Chemicals, Gillingham, Dorset, U.K.
DNA Ligase	Boreinnger Mannheim, Bell Lane, Lewes, East Sussex, U.K.
Epicurian coli Supercompetent Cells	StrataGene, San Diego, California, U.S.A.
HPLC-grade solvents	Lab-Scan, Stillorgan, Dublin, Ireland.
ProBond Resin PCR Optimiser Kit	In Vitrogen BV, 9704 CH Groningen, The Netherlands.
SfiI enzyme	New England Laboratories, 73 Knowl Place, Wilbury Way, Hitchin, Hertfordshire, U.K.
Tryptone Yeast Extract Bacto Agar	Oxoid Ltd., Wade Rd., Basingstoke , Hampshire, U.K.
Wizard Plus PCR-prep kit Wizard Plus SV Mini-prep kit Reverse Transcription system Low-melt Agarose 5-bromo-4-chloro-3'-indolyphosphate/ nitro blue tetrazolium chloride (BCIP/NBT) reagent	Promega Corporation, Madison, WI, U.S.A.

The equipment used and its suppliers are listed below.

Equipment	Supplier
3015 pH meter	Jenway Ltd., Essex, U.K.
Amicon 8050 Ultrafiltration Cell	Amicon Inc., Beverly, Massachusetts 01915, U.S.A.
Atto dual minislab AE-6450 Atto AE-6100	Atto Corp., Hongo 7-Chrome, Bunhyo-Kui, Tokyo, Japan.
BIAcore 1000 BIAcore 3000	Pharmacia Biosensor AB, Uppsala, Sweden.
Biometra PCR machine	Anachem Ltd., Anachem House, Charles St., Luton, Bedfordshire, U.K.
BioRad wet blotter	BioRad, BioRad House, Maylands Ave., Hemel Hempstead, Hertfordshire, U.K.
Eppendorf centrifuge (5810 R)	Eppendorf AG, 10 Signet Court, Swann Rd., Cambridge, U.K.
Eppendorf tubes	Sarstedt, Wexford, Ireland.
Grant Waterbath (Y6)	Grant Instruments (Cambridge) Ltd., 29 Station Rd., Shepreth, Royston, Hertfordshire, U.K.
Hermle Centrifuge (Z 200 M/H)	Hermle Labortechnik, 78564-Wehingen, Gosheimerstr. 56, Germany.
Image Master VDS	Pharmacia Biotech, San Francisco, California, U.S.A.
NUNC Maxisorb plates	NUNC, Kamstrup DK, Roskilde, Denmark.
Orbital incubator	Gallenkamp, Leicester, U.K.
RM6 Lauda waterbath Millipore filtration device	AGB Scientific Ltd., Glasnevin, Dublin, Ireland.
SB1 Blood tube rotator	Stuart Scientific, London, England.
Sorvall Refrigerated Centrifuge	Du Pont Instruments, Newtown, Connecticut, U.S.A.
Stuart Platform shaker (STR 6)	Lennox, Naas Rd., Dublin, Ireland.
Titretek Twinreader Plus Sterile universal containers	Medical Supply Company, Damastown, Mulhuddart, Dublin 15, Ireland.
Tomy Autoclave (SS 325)	Mason Technology, Greenville Hall, 228 South Circular Rd., Dublin 8, Ireland.
UV-160A spectrophotometer	Shimadzu Corp., Kyoto, Japan.
UVP ImageStore 7500 gel documentation system	Ultra Violet Products, Upland, California, U.S.A.

2.2. Culture media formulations

2 X Tryptone Yeast extract (TY) Medium	Tryptone	16 g/l
	Yeast Extract	10 g/l
	NaCl	5 g/l
Non-expression (NE) medium	2 X TY medium	
	1% (v/v) glucose	
	25 μg/ml chloramphenicol	
	30 μg/ml Tetracycline	
Low-expression (LE) medium	2 X TY medium	
	1% (v/v) glucose	
	25 ug/ml chloramphenicol	
	0.5 mM isopropyl-β-D-galactopyranoside (IPTG)	
Expression medium	2 X TY medium	
	25 ug/ml chloramphenicol	
Tryptone Yeast extract (TYE) medium	2 X TY medium	
	Bacteriological agar	15 g/l
Super Optimal Catabolite (SOC) medium	Tryptone	20 g/l
	Yeast Extract	5 g/l
	NaCl	0.5 g/l
	KCl	2.5 mM
	$MgCl_2$	20 mM
	Glucose	20 mM
	pH	7.0
	Glucose and $MgCl_2$ added separately following autoclaving	

2.3. Antibody Production

2.3.1. *Licensing*

All procedures involving animals were approved and licensed by the Department of Health and Children, and every care was taken to minimise the level of distress caused to the animals.

2.3.2. *Health Warning*

Aflatoxin B_1 is carcinogenic and should be handled with extreme care.

2.3.3. *Immunisation protocol for the production of rabbit anti-aflatoxin B_1 antisera*

Phosphate buffered saline, containing 0.15 M NaCl, 2.5 mM potassium chloride, 10 mM disodium hydrogen phosphate and 18 mM sodium dihydrogen phosphate, pH 7.4, was prepared. This buffer will be referred to throughout as PBS. A stock 5 mg/ml solution of aflatoxin B_1-BSA (AFB$_1$-BSA) conjugate was made up in PBS. This was diluted to a concentration of 200 µg/ml in PBS. An equal volume of Freund's Complete adjuvant was added to 200 µg/ml of the conjugate. The mixture was vortexed until it formed an emulsion. 1 ml of this mixture was injected sub-cutaneously at several sites into a New-Zealand white rabbit. This procedure was repeated 21 days later, using Freund's Incomplete adjuvant. The rabbit was bled from the marginal ear vein 14 days after this immunisation, and the cycle of reboosting and bleeding was continued until a specific antibody titre of greater than 1/100,000 was obtained. The rabbit was exsanguinated, and the serum was collected, allowed to clot for 2 h at room temperature and stored overnight at 4° C. It was then centrifuged at 4000 rpm for 20 minutes, and the supernatant collected and stored at –20° C until required for use.

2.3.4. *Immunisation protocol for BALB/c mice for the production of scFv anti-aflatoxin B_1 antibodies from splenomic RNA*

A stock 5 mg/ml solution of AFB$_1$-BSA conjugate was prepared in PBS. This was diluted to a concentration of 50 µg/ml in PBS. An equal volume of Freund's

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Complete adjuvant was added to 50 µg/ml of the conjugate. The mixture was vortexed until it formed an emulsion. 6-10 week old BALB/c mice were injected sub-cutaneously with 250 µl of the immunogen at several sites. On day 21, the mice were re-immunised intraperitoneally with the same dose of immunogen and Freund's Incomplete adjuvant. On day 28 a blood sample was taken from the tail, and intraperitoneal boosts, followed by blood sampling, were continued until a satisfactory titre of greater than or equal to 1/50,000 was obtained. 3-4 days prior to being sacrificed, the mice were injected with 250 µl of 50 µg/ml AFB_1-BSA conjugate in PBS. This was administered intravenously via the tail vein.

2.4. Preparation of serum

2.4.1. *Preparation of rabbit serum*
For estimation of antibody titre, a 2 ml blood sample was collected from the marginal ear vein in Sterilin universal containers, and allowed to clot for 2 h at room temperature. The clot tightened overnight at 4° C, before being centrifuged at 4,000 rpm for 20 min. The supernatant (serum) was then removed and stored at -20° C until required for further use.

2.4.2. *Preparation of mouse serum*
For the estimation of antibody titre in mouse blood, 10-20 µl of blood was taken from the tail, and allowed to clot at room temperature for 30 min. Following centrifugation at 13,000 rpm for 20 min the serum was removed and stored at -20° C until required for further use.

2.5. Polyclonal antibody purification
Rabbit serum was initially purified by saturated ammonium sulphate precipitation. It was then affinity-purified by protein G affinity-chromatography. Subtractive immunoaffinity chromatography was also carried out on the serum to remove all 'BSA-binding' antibodies.

2.5.1. *Saturated ammonium sulphate precipitation*

10 ml of rabbit serum was stirred on ice, and an equal volume of cold saturated - 100% (w/v) - ammonium sulphate was added, dropwise. The mixture was stirred on ice for 1 h. It was then centrifuged at 3000 rpm for 20 min. The supernatant was removed and the pellet was washed twice in 10 ml of 45% (v/v) ammonium sulphate. The final pellet was dissolved in 5 ml of PBS and dialysed overnight at 4°C against 5 l of PBS.

2.5.2. *Protein G affinity-chromatography*

The composition of the buffers used in this procedure are given in Table 2.1.

A Protein G column was prepared by pouring 2 ml of protein G immobilised on sephadex into a small column. It was allowed to settle and was equilibrated with 20 ml of Running buffer. 2 ml of the dialysed saturated ammonium sulphate-treated antibody solution (obtained as described in section 2.5.1.) was added to the column. The effluent from the column was collected and re-applied to the column three times. The column was then washed with 20 ml of wash buffer. The column was re-equilibrated with 20 ml of running buffer. Bound immunoglobulin was eluted from the column by the addition of elution buffer. This was allowed to run into the column, and remain in it for 5 min. Fifteen 0.5 ml fractions were collected to which 100 μl of neutralising buffer had already been added to each tube in order to prevent denaturation of the eluted antibody. The collected fractions were then analysed at 280 nm using quartz cuvettes. The fractions containing protein were pooled and dialysed in 5 l PBS overnight at 4°C. The protein G column was washed and stored in the recommended buffer (PBS with 20% (v/v) ethanol), at 4°C.

Table 2.1. Composition of running buffer, wash buffer, neutralising buffer, and elution buffer for protein G affinity-chromatography.

Running Buffer	PBS - 0.05% (v/v) Tween, 0.15 M NaCl
Wash buffer	PBS - 0.05% (v/v) Tween, 0.35 M NaCl
Neutralising buffer	2 M Tris/HCl, pH 8.6
Elution Buffer	0.1 M glycine/HCl, pH 2.7

2.5.3. *Subtractive immunoaffinity chromatography*

A BSA immobilised immunoaffinity column was obtained as a gift from Dr. Anthony Killard. Subtractive immunoaffinity chromatography was carried out on protein G-purified antibody to remove all BSA-binding antibodies from the antibody population. The method, as described in section 2.5.2, was used. The antibody solution was then sterile filtered through a 0.22 μm filter, and 0.05% (v/v) azide was added. It was aliquoted into 1 ml quantities, and stored at –20° C until required for use.

2.6. Bicinchoninic acid (BCA) protein assay

Standard protein solutions ranging in concentration from 0.01 – 1.5 mg/ml were prepared using purified immunoglobulin G (IgG). 10 μl of each standard or sample was added, in triplicate, to wells of a 96-well plate. 190 μl of BCA reagent was added to each sample. The plate was incubated for 30 min at 37° C. The absorbances of the wells were measured at 560 nm using a micro titre plate-reader (Titretek Twinreader Plus), and a standard curve was constructed from which the protein concentrations of the samples could be calculated.

2.7. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed to assess antibody purity. It was carried out using an Atto dual minislab AE-6450 gel electrophoresis system. The composition of gels, electrophoresis buffer and sample loading buffer are given in Table 2.2. Protein sample were diluted with sample loading buffer (4:1, sample:buffer) and boiled for 10 minutes. Approximately 10-15 µl of sample was loaded onto a gel. Molecular weight markers were also loaded onto the gel. When the gel was being used for Western blotting, it was necessary to use pre-stained molecular weight markers. The gel was electrophoresed at 20 mA per plate and was run until the dye had migrated to the bottom of the gel plates.

2.8. Coomassie blue staining

Gels were stained for 30 minutes in Coomassie blue staining solution (0.2% (w/v) Coomassie blue R250 in 30: 10: 60 (v/v/v) methanol: acetic acid: water), and destained overnight in destaining solution (10: 7: 53 (v/v/v) methanol: acetic acid: water).

Table 2.2. Composition of stacking gel, resolving gel, electrophoresis buffer and sample loading buffer for SDS-PAGE.

Stacking gel	5% (w/v) acrylamide
	0.13% (w/v) bis-acrylamide
	125 mM Tris
	0.1% (w/v) SDS
	0.15% (w/v) ammonium persulphate
	0.25% (v/v) TEMED
Resolving gel	10% (w/v) acrylamide
	0.27% (w/v) Bisacrylamide
	375 mM Tris
	0.1% (w/v) SDS
	0.08% (w/v) ammonium persulphate
	0.08% (v/v) TEMED
Electrophoresis buffer	25 mM Tris
	192 mM glycine
	0.1% (w/v) SDS
Sample loading buffer	60 mM Tris
	25% (v/v) glycerol
	2% (w/v) SDS
	14.4 mM 2-mercaptoethanol
	0.1% (w/v) bromophenol blue

2.9. Western blotting

Proteins were transferred from acrylamide gels (prepared in section 2.7) to nitrocellulose using a BioRad wet blotter in electrophoresis buffer containing 20% (v/v) methanol, for 90 minutes at 72 V. The membrane was blocked using 5% (w/v) non-fat milk powder, 0.01% (v/v) antifoam A, 0.02% (v/v) sodium azide in Tris Buffered Saline (TBS) (50 mM Tris-HCl, 150 mM NaCl, pH 7.4) for 2 h at room temperature or overnight at 4° C. Monoclonal anti-FLAG antibody was then added in blocking solution containing 1 mM calcium chloride ($CaCL_2$) for 1.5 h at room temperature. The nitrocellulose was washed three times in TBS containing 1 mM $CaCL_2$ for 10 min each time. Alkaline phosphatase-labelled anti-mouse IgG was added to the filter in TBS containing 5% (w/v) fat free milk powder and 1 mM calcium chloride for 1.5 h while shaking at room temperature. Final washing consisted of four 10 min washings in TBS, containing 1 mM $CaCl_2$, prior to development with 'one-step' 5-bromo-4-chloro-3'-indolyphosphate/ nitro blue tetrazolium chloride (BCIP/NBT) reagent (Promega). Colour development was stopped by the addition of 2 mM ethylene diamine tetra acetic acid (EDTA) in PBS.

2.10. Preparation of standard concentrations of hapten for use in competition ELISA

2.10.1. *Aflatoxin B₁ preparation*

A 2 mg/ml solution of free aflatoxin B_1 was prepared in methanol. Working standards were prepared in PBS containing 5% (v/v) methanol, at varying concentrations.

2.10.2. *Chlorpyrifos preparation*

A 50 mg/ml solution of free chlorpyrifos was prepared in methanol. Working standards were prepared in PBS containing 5% (v/v) methanol, at varying concentrations.

2.10.3. *Preparation of grain matrix samples*

AFB_1 samples in grain matrix were supplied as a gift from Mrs. Sue Patel, RHM Technology Ltd., The Lord Rank Centre, London Road, HP12 3QR, High Wycombe, U.K. A blank sample of grain was extracted according to the method described by Scudamore and Patel (2000), and then spiked with different concentrations of AFB_1 according to the official *AOAC* methods of analysis (Scott, 1995). The samples were then diluted to a final methanol concentration of 5% (v/v).

2.11. Enzyme-linked immunosorbent assay (ELISA)

A range of different ELISA methods were used for the detection of antibodies in rabbit and mouse serum, and phage supernatants, for the quantitative determination of aflatoxin B_1, and chlorpyrifos, and for the assessment of the cross-reactivity of purified antibody. In addition, ELISA was also used to study regeneration conditions for suitability of antibody use in an immunosensor. PBS containing 0.05% (v/v) Tween 20 was used to wash microtitre plates, and is referred to in all sections commencing from section 2.11 as PBST.

2.11.1. *Estimation of rabbit and mouse serum antibody titres to specific antigens*

The levels of specific antibody in serum were measured using ELISA. A 96-well microtitre plate was coated by adding 100 μl of aflatoxin B_1-BSA conjugate dissolved in PBS at a concentration of 50 μg/ml to each well. Due to the availability of only one protein-toxin conjugate, a second microtitre plate was coated with BSA at a concentration 50 μg/ml in order to estimate the antibody response to BSA. The plates were incubated overnight at 4° C. The plates were emptied and washed six times, three times with PBST and three with PBS only. The plates were then blocked by addition of 100 μl per well of PBS containing 2% (w/v) milk powder and incubated for 1 h at 37° C. (For both coating and blocking of plates, incubation steps could also be carried out at 37° C for 1 hour or 4° C overnight).

Serum from immunised rabbits or mice was then diluted (1/100 to 1/200,000 for rabbits; 1/300 to 1/61,400 for mice) in PBS containing 0.1% (w/v) BSA. This was carried out for two purposes. Firstly, to ensure that 0.1% (w/v) BSA was sufficient to remove the non-specific interactions, and secondly, to observe the specific antibody response to the AFB_1 part of the conjugate. The serum was also diluted in PBS containing no BSA, in order to observe the non-specific antibody response to BSA. The serum diluted in the PBS 0.1% (w/v) BSA was added to the AFB_1-BSA coated plate, as well as the BSA coated plate. The serum diluted in the PBS was added to the BSA coated plate only. All samples were added to the plate at 100 µl/well. This was incubated for 1 h at 37° C. The plate was washed three times in PBST and three times in PBS. After washing, 100 µl of horseradish peroxidase (HRP)-conjugated-anti-rabbit or anti-mouse secondary antibody, diluted in PBS (1/5,000 dilution), was added to each well and incubated for 1 h at 37° C.

After repeating the washing step, 100 µl of substrate (0.4 mg/ml δ-phenylenediamine (δ-PD), in 0.05 M phosphate citrate buffer, pH 5.0, and 0.4 mg/ml of urea hydrogen peroxide) was added into each well and incubated for 30 min at 37° C.

2.11.2. *Standard checkerboard ELISA for determination of optimal antibody dilution and conjugate concentration for use in a competition ELISA*

A 96-well microtitre plate was coated with different concentrations of the conjugate of interest (aflatoxin B_1-BSA conjugate, chlorpyrifos-BSA, chlorpyrifos-dextran dissolved in PBS) ranging from 0 to 50 ug/ml by adding 100 µl of conjugate to each well. The plates were incubated overnight at 4° C, after which they were emptied and washed six times, three times with PBST and three with PBS only. It was then blocked by addition of 100 µl per well of PBS containing 2% (w/v) milk powder and incubated for 1 h at 37° C. Serial dilutions of the antibody of interest were carried out. 100 µl per well of each antibody dilution was added to the wells (in at least triplicate). This resulted in a set of titre curves being produced for each different conjugate concentration. The plate was incubated for 1 h at 37° C. After washing, 100 µl of horseradish peroxidase (HRP)-conjugated-

anti-rabbit secondary antibody, diluted in PBS, was added to each well and incubated for 1 h at 37° C. Colour was developed as described in section 2.11.1. From the titre curves obtained, the antibody dilution that gave half the maximum absorbance, and the lowest conjugate concentration that provided sufficiently high absorbances was chosen for use in competition ELISA.

2.11.3. *Standard competitive ELISA using polyclonal antibodies*

Microtitre plates were coated by adding 100 µl of conjugate (aflatoxin B_1-BSA conjugate, chlorpyrifos-BSA, chlorpyrifos-dextran dissolved in PBS) to each well. The plates were incubated overnight at 4° C. The plates were emptied and washed six times, three times with PBST and three times with PBS. They were then blocked by adding 100 µl per well of PBS containing 2% (w/v) milk powder, and incubated for 1 h at 37° C. Stock solutions were prepared for each analyte at varying concentrations to produce a set of standard solutions (as described in sections 2.10.1-2.10.3). Washing was carried out as described before, and 50 µl of rabbit anti-hapten antibody was added into each well with 50 µl of hapten standards. The plate was incubated for 1 h at 37° C, and then washed three times in PBST and once with PBS. 100 µl/well of HRP labelled anti-rabbit IgG was then added, and the plate incubated for 1 h at 37° C. Colour was developed as described in section 2.11.1.

2.11.4. *Estimation of cross reactivity of purified polyclonal anti-AFB$_1$ antibodies*

Stock solutions of aflatoxin B_2, M_1, M_2, G_1, G_2, B_{2a}, and G_{2a} were prepared in methanol and diluted in PBS-5% (v/v) methanol with a range of concentrations from 31.25 to 500 ng/ml. The assay was carried out in the same way as that for the competition ELISA (2.11.3) except that standards of the other aflatoxins were added to the plate with the anti-AFB$_1$ antibody.

2.11.5. *Estimation of cross reactivity of purified polyclonal anti-chlorpyrifos antibodies*

Stock solutions of parathion, fenitrothion, carbophenothion, etrimfos, and dichlorvos were prepared in methanol and diluted in PBS-5% (v/v) methanol with a range of concentrations from 40 to 12,500 ng/ml. The assay was carried out in the same way as that for the competition ELISA (2.11.3), except that standards of the other pesticides were added to the plate with the anti-chlorpyrifos antibody.

2.11.6. *The use of ELISA to examine the efficiency and effects of regeneration on an immobilised AFB₁-BSA surface*

In an experiment to examine the efficiency of regenerating conjugate immobilised at a surface, 96-well microtitre plates were coated with 50 µg/ml AFB$_1$-BSA and blocked with PBS containing 2% (w/v) milk powder.

After washing, 100 µl/well of a 1/5,000 dilution of polyclonal anti-AFB$_1$ antibody in PBS was added to the plates, and was then incubated at 37° C for 1 h.

A set of solutions of 1M ethanolamine containing various percentage concentrations of acetonitrile was then prepared. Antibody solution was aspirated from the plates, which were washed three times in PBST and three times in PBS. 100 µl of each of the different ethanolamine-acetonitrile solutions was then added to wells, and the plates were incubated for 10 minutes at room temperature. Following incubation, the plates were washed three times in PBST and three times in PBS.

100 µl/well of HRP-labelled anti-rabbit antibody (1/5,000 in PBS) was then added, and the plates incubated at 37° C for 1 h. The colour was then developed as described in section 2.11.1. To examine the effect of the regeneration reagent upon the conjugate surface, the assay was repeated as before, except that the ethanolamine-acetonitrile solutions were added to the wells immediately after the blocking step. After washing the anti-AFB$_1$ antibody was added, incubated for 1 h at 37° C and the plate was washed as before. HRP-labelled anti-rabbit antibodies were then added, and the plates were washed and incubated as previously. Finally, colour development was carried out as described in section 2.11.1.

2.12. Agarose Gel Electrophoresis

DNA samples from 10 µl to 20 µl were prepared in 6X loading solution (with 0.25% (w/v) bromophenol blue and 40% (w/v) sucrose). These samples were loaded into wells of agarose gels, (typically 0.5-2% (w/v)). Agarose gels were prepared using 50X TAE (242 g/l Tris, 57.1 ml/l glacial acetic acid, 100 ml/l 0.5 M EDTA, pH 8.0) and type 1A agarose. Low melt agarose was used as the alternative when purifying specific DNA products. Ethidium bromide was added to the gels at 0.5 µg/ml. The samples were electrophoresed at 70V for 1 to 2 h.

2.13. Synthesis and characterisation of pesticide-protein conjugates

2.13.1. *Chemical derivatisation of pirimiphos*

A number of novel strategies were carried out in the attempt to derivatise an active form of pirimiphos for conjugation to proteins. These methods performed by Dr. Ollie Egan, and Dr. Kieran Nolan. Nuclear magnetic resonance (NMR), and spectroscopy analysis of the compounds were also performed by Dr. Egan and Dr. Nolan. Mass spectroscopy was carried out at the Department of Chemical Sciences, National University of Ireland (NUI), Cork.

2.13.2. *Conjugation of amino-pirimiphos to BSA*

10 mg of amino-pirimiphos was dissolved in 10 mM HCl and cooled on ice for 10 min. A chilled solution of 5 ml of 0.1 M $NaNO_2$ was added dropwise and allowed to mix for 30 min. This created an excess of nitrous acid, which was decomposed by the addition of 5 g of urea. 50 mg of BSA was dissolved in 0.2 M borate buffer, pH 9.0. This was added to the reaction mixture. This was allowed to mix for 2 h on ice, before being dialysed overnight in 5 l of PBS at 4° C.

2.13.3. *Chemical derivatisation and purification of chlorpyrifos for conjugation to a protein*

An activated form of chlorpyrifos was produced for conjugation to the protein BSA, by the method described by Manclus *et al.*, (1994). These methods are described in the following sections 2.13.3.1 to 2.13.3.3.

2.13.3.1. *Synthesis of O,O-Diethyl O-[3,5-Dichloro-6-[(2-carboxyethyl)thio]-2-pyridyl] Phosphorothioate*

3-mercapto-propanoic acid was dissolved in 3 ml of ethanol to a final molar concentration of 10 mM. Two equivalents of KOH were added to the solution and heated until dissolved. 10 mM chlorpyrifos, dissolved in 3 ml of ethanol, was added, and the reaction mixture was refluxed for 1 h. After this time the reaction mixture was filtered and the solvent was removed under reduced pressure. 5% (w/v) of $NaHCO_3$ was added to the residue in order to remove any residual water, followed by washing with hexane (2 X 5 ml). The aqueous layer was acidified to pH 4.0 and extracted with dichloromethane (3 X 5 ml). The extract was dried over Na_2SO_4 and concentrated by rotary evaporation.

2.13.3.2. *Purification of O,O-Diethyl O-[3,5-Dichloro-6-[(2-carboxyethyl)thio]-2-pyridyl] Phosphorothioate*

The activated chlorpyrifos compound was reconstituted in 4 ml of the solvent mixture hexane/tetrahydrofuoran (THF)/acetic acid (75: 25: 1). A C_{18} reverse phase cartridge filter was used to purify the compound. This was carried out by connecting the cartridge to a glass syringe, and washing it through with approximately 20 ml of the solvent mixture. The reconstituted chlorpyrifos product was injected through the column, and 500 μl fractions were collected. A further 3 ml of solvent was injected through the cartridge filter and the fractions collected. These fractions were then analysed by thin layer chromatography (TLC) using a silica plate. Fractions showing only one spot on the TLC plate (R_F 0.41, same solvent mixture used to dissolve the chlorpyrifos) were pooled and concentrated by rotary evaporation.

2.13.3.3. *Production of chlorpyrifos-BSA protein conjugates*

5 mg of activated chlorpyrifos was dissolved in 1 ml of ethanol and the pH was adjusted to 8.5 using 0.2 M borate buffer, pH 9.0. This was placed on a stirrer and 100 mM NHS (N-hydroxysuccinimide) and 400 mM EDC (N-ethyl-N- (dimethyl-aminopropyl) carbodiimide hydrochloride) were added to the reaction mixture. This was allowed to mix for 10 min. BSA was dissolved in 0.2 M borate buffer, pH 9.0, at varying concentrations depending on the molar excess of hapten to

protein required for each conjugation (50, 25, 10, and 5 molar excesses of hapten were used for conjugation). The BSA was then added dropwise to the reaction mixture, and the solution was left stirring at room temperature for 1 h. After this time the conjugate was dialysed in 5 l PBS overnight at 4° C. It was sterile filtered through a 0.22 μm filter, and 0.05% (v/v) of sodium azide was added for preservation. The conjugate was stored at -20° C until required for further use.

2.14. Characterisation of pesticide-protein conjugates

2.14.1. *Ultraviolet spectroscopy*
Pesticide-protein conjugates were analysed by UV spectral analysis from 200-400nm. UV spectra of similar concentrations of 'control' protein were also prepared and recorded. The UV spectra of the respective pirimiphos and chlorpyrifos molecules were also recorded over a similar wavelength range. Direct comparison of the three spectra gave putative confirmation of conjugation of the respective pesticide molecules to the protein molecules.

2.14.2. *ELISA*
ELISA was also used as a method for the characterisation of chlorpyrifos-BSA protein conjugates. This was carried in the same way as described in section 2.11.3, where the chlorpyrifos conjugate of interest was coated on the surface of a 96-well microtitre plate at a concentration of 50 μg/ml, and serial dilutions of a polyclonal anti-chlorpyrifos antibody was added to the plate (either in the presence, or without free chlorpyrifos). Colour development confirmed recognition of the antibody for the pesticide-protein conjugate.

2.15. Production of a single chain Fv antibody library to aflatoxin B_1
A pre-immunised scFv phage display library was produced according to the method described by Krebber *et al.* (1997(a)).

2.15.1 *Synthesis of AFB_1-dextran conjugate*
This procedure was performed according to Langone and Van Vunakis (1976) and involved the production of AFB_1 O-carboxymethylhydroxylamine, followed by

coupling of the activated aflatoxin molecule to amino-dextran using carbodiimide coupling chemistry.

2.15.1.1. *Preparation of AFB₁ O-carboxymethylhydroxylamine*

A solution of AFB_1 (15 mg: 0.054 mM) and O-carboxymethylhydroxylamine hydrochloride (123.2 mg: 0.64 mM) were mixed in 3.53 ml of ethanol, and 0.704 ml of aqueous NaOH (1.6 mM). This solution was refluxed for 3 h, and then allowed to sit overnight at room temperature. After this time the brown reaction mixture was concentrated to 1 ml using rotary evaporation. 5 ml of distilled water was added, and the pH adjusted to 9.5 with 1 M NaOH. This was then washed with two 10 ml portions of ethyl acetate. The aqueous layer was acidified to pH 2 with 6 M HCl and stored at $0°$ C overnight to yield a yellow oxime precipitate. This was collected by centrifugation, and vacuum dried over anhydrous calcium sulphate. The product was stored in 1 ml of dimethyl sulfoxide (DMSO), and frozen at $-20°$ C.

2.15.1.2. *Conjugation of AFB₁ O-carboxymethylhydroxylamine to amino-dextran*

10 mg of AFB_1 O-carboxymethylhydroxylamine in DMSO was taken, and the pH adjusted to 8.5 using 0.2 M borate buffer, pH 9.0. This was placed on a stirrer and 100 mM NHS and 400 mM EDC were added to the reaction mixture. This was allowed to mix for 10 min. 100 mg of amino-dextran was dissolved in 0.2 M borate buffer, pH 9.0, and was added dropwise to the reaction mixture. The solution was left stirring in the dark at room temperature for 1 h. After this time the conjugate was dialysed in 5 l PBS overnight at $4°$ C. It was stored at $4°$ C in the dark until required for further use.

2.15.1.3. *Evaluation of AFB₁-dextran conjugate using BIAcore*

Preconcentration of the AFB_1-dextran conjugate was carried out as described in section 2.20.1. Following this, the conjugate was immobilised on the CM-dextran gel surface as described in section 2.20.2. Monoclonal anti-AFB_1 antibody was injected over the immobilised conjugate surface in order to observe its binding

capacity. The surface was regenerated using 1 M ethanolamine containing 20% (v/v) acetonitrile, pH 12.0.

2.15.2. *Preparation of mouse spleen RNA*

Trizol reagent is a 'ready-to-use' reagent for the isolation of total RNA from cells and tissues, based on the procedure of Chomczynski and Sacchi (1987). The reagent, a mono-phasic solution, contains phenol and guanidine isothiocyanate. Trizol reagent maintains the integrity of the RNA, while disrupting cells and dissolving cell components.

All glassware and reagents were prepared using diethyl-pyrocarbonate (DEPC)-treated ultrapure water and autoclaved at 121°C, and 15 p.s.i. for 15 min. This was required to remove all contamination resulting from RNAse enzymes.

Following immunisation of mice, as described in section 2.3.4, mice were sacrificed by exsanguination and their spleens were removed using aseptic technique. The weight of the spleen was recorded. The spleen was homogenised with 1 ml of Trizol™ reagent and incubated at room temperature for 5 min. 200 µl of chloroform per ml of Trizol was added, and this was shaken for 15 seconds before being allowed to incubate for 2-3 min at room temperature. This was then centrifuged at 14,000 rpm for 15 min. The upper aqueous phase containing the RNA was carefully removed, and the pellet was discarded. The RNA was precipitated using 500 µl of isopropanol per ml of Trizol. The precipitate was incubated for 10 min at room temperature, and then centrifuged at 14,000 rpm for 10 min. The precipitate was removed, and the brown pellet remaining was washed with 1 ml of 75% (v/v) ethanol per ml of Trizol used. The eppendorf was inverted as opposed to vortexing, as vortexing would cause shearing and loss of the RNA. This was centrifuged at 10,000 rpm for 5 min. The ethanol was removed from the pellet, and the pellet was completely dried at 37° C. This was done by covering the eppendorf tube with parafilm, and making small perforations in it. This was then placed in a 37° C oven until the pellet was judged to be dry. The pellet was resuspended in 30 µl of DEPC-ultrapure water.

The optical density (O.D.)$_{260}$ and O.D.$_{280}$ of each extract were determined. This was used to determine the concentration of RNA from the standard that a solution

of RNA with an O.D.$_{260}$=1.0 contains 40 ug of RNA per ml. The purity of the RNA was also determined from the ratio of O.D.$_{260}$ / O.D.$_{280}$. This should be equal to 2.0. If this number was lower than 2.0 contamination of the of the extract with protein had occurred.

2.15.3. *Reverse transcription of mouse spleen RNA*

Reverse transcription was performed using the Promega polymerase chain reaction (PCR) – related Reverse Transcription System. The cDNA was synthesised using random hexamer primers (mix of 5 base fragments of varying sequence (d NTP)) which were obtained separately from the kit. The d NTP mix was prepared in sterile ultrapure water and mixed thoroughly before use. All buffers were allowed to completely thaw on ice and were mixed well before use. The components, and their concentrations for the reaction, are listed below.

Component	Stock concentration	Concentration in 20 µl reaction	X 1
MgCl$_2$	25 mM	5 mM	4 µl
10X buffer	10 X	1 X	2 µl
dNTP Mix	10 mM	1 mM	2 µl
RNAase Inhib	40 U/µl	1U/µl	0.5 µl
Random Primer	0.5 µg/µl	0.2 µg/reaction*	0.4 µl
AMV RT	25 U/µl	15 U/reaction*	0.6 µl
RNA		7-10 µg/reaction*	Varied

* Total concentration per reaction NOT per reaction volume as for other components.

X 1= Amount used per reaction

2.15.4. *Amplification of antibody light and heavy chain genes using polymerase chain reaction (PCR)*

2.15.4.1. *PCR Primers*

PCR primers were obtained from Sigma-Genosys Ltd., London Rd., Pampisford, Cambridge, CB2 4Ya, England. Primers used were as follows:-

Variable light chain back primers:-

LB1   5'gccatggcggactacaaa<u>GAY</u>ATCCAGCTGACTCAGCC3'

LB2   5'gccatggcggactacaaa<u>GAY</u>ATTGTTCTC<u>W</u>CCCAGTC3'

LB3   5'gccatggcggactacaaa<u>GAY</u>ATTGTG<u>MTM</u>ACTCAGTC3'

LB4   5'gccatggcggactacaaa<u>GAY</u>ATTGTGYTRACACAGTC3'

LB5   5'gccatggcggactacaaa<u>GAY</u>ATTGT<u>R</u>ATGAC<u>M</u>CAGTC3'

LB6   5'gccatggcggactacaaa<u>GAY</u>ATT<u>M</u>AGAT<u>RAM</u>CCAGTC3'

LB7   5'gccatggcggactacaaa<u>GAY</u>ATTCAGATGA<u>YD</u>CAGTC3'

LB8   5'gccatggcggactacaaa<u>GAY</u>AT<u>Y</u>CAGATGACACAGAC3'

LB9   5'gccatggcggactacaaa<u>GAY</u>ATTGTTCTCA<u>W</u>CCAGTC3'

LB10   5'gccatggcggactacaaa<u>GAY</u>ATTG<u>W</u>GCT<u>S</u>ACCCAATC3'

LB11   5'gccatggcggactacaaa<u>GAY</u>ATT<u>ST</u>RATGACCCA<u>R</u>TC3'

LB12   5'gccatggcggactacaaa<u>GAY</u>R<u>TTK</u>TGATGACCCA<u>R</u>AC3'

LB13   5'gccatggcggactacaaa<u>GAY</u>ATTGTGATGAC<u>B</u>CAG<u>K</u>C3'

LB14   5'gccatggcggactacaaa<u>GAY</u>ATTGTGATAAC<u>Y</u>CAGGA3'

LB15   5'gccatggcggactacaaa<u>GAY</u>ATTGTGATGACCCAG<u>W</u>T3'

LB16   5'gccatggcggactacaaa<u>GAY</u>ATTGTGATGACACAACC3'

LB17   5'gccatggcggactacaaa<u>GAY</u>ATTTTGCTGACTCAGTC3'

LBλ   5'gccatggcggactacaaa<u>GAT</u>GCTGTTGTGACTCAGGAATC3'

Variable light chain forward primers:-

LF1   5'ggagccgccgccgcc(agaaccaccaccacc)2ACGTTTGATTTCCAGCTTGG3'

LF2   5'ggagccgccgccgcc(agaaccaccaccacc)2ACGTTTTATTTCCAGCTTGG3'

LF4   5'ggagccgccgccgcc(agaaccaccaccacc)2ACGTTTTATTTCCAACTTTG3'

LF5   5'ggagccgccgccgcc(agaaccaccaccacc)2ACGTTTCAGCTCCAGCTTGG3'

LFλ   5′ggagccgccgccgcc(agaaccaccaccacc)2ACCTAGGACAGTCAGTTTGG3′

Variable heavy chain back primers:-

HB1   5′ggcggcggcggctccggtggtggtggatccGAKGTRMAGCTTCAGGAGTTC3′

HB2   5′ggcggcggcggctccggtggtggtggatccGAGGTBCAGCTBCAGCAGTC3′

HB3   5′ggcggcggcggctccggtggtggtggatccCAGGTGCAGCTGAAGSASTC3′

HB4   5′ggcggcggcggctccggtggtggtggatccGAGGTCCARCTGCAACARTC3′

HB5   5′ggcggcggcggctccggtggtggtggatccCAGGTYCAGCTBCAGCARTC3′

HB6   5′ggcggcggcggctccggtggtggtggatccCAGGTYCARCTGCAGCAGTC3′

HB7   5′ggcggcggcggctccggtggtggtggatccCAGGTCCACGTGAAGCAGTC3′

HB8   5′ggcggcggcggctccggtggtggtggatccGAGGTGAASSTGGTGGAATC3′

HB9   5′ggcggcggcggctccggtggtggtggatccGAVGTGAWGYTGGTGGAGTC3′

HB10   5′ggcggcggcggctccggtggtggtggatccGAGGTGCAGSKGGTGGAGTC3′

HB11   5′ggcggcggcggctccggtggtggtggatccGAKGTGCAMCTGGTGGAGTC3′

HB12   5′ggcggcggcggctccggtggtggtggatccGAGGTGAAGCTGATGGARTC3′

HB13   5′ggcggcggcggctccggtggtggtggatccGAGGTGCARCTTGTTGAGTC3′

HB14   5′ggcggcggcggctccggtggtggtggatccGARGTRAAGCTTCTCGAGTC3′

HB15   5′ggcggcggcggctccggtggtggtggatccGAAGTGAARSTTGAGGAGTC3′

HB16   5′ggcggcggcggctccggtggtggtggatccCAGGTTACTCTRAAAGWGTSTG3′

HB17   5′ggcggcggcggctccggtggtggtggatccCAGGTCCAACTVCAGCARCC3′

HB18   5′ggcggcggcggctccggtggtggtggatccGATGTGAACTTGGAAGTGTC3′

HB19   5′ggcggcggcggctccggtggtggtggatccGAGGTGAAGGTCATCGAGTC3′

Variable heavy chain for primers:-

HF1   5′ggaattcggccccccgaggcCGAGGAAACGGTGACCGTGGT3′

HF2   5′ggaattcggccccccgaggcCGAGGAGACTGTGAGAGTGGT3′

HF3   5′ggaattcggccccccgaggcCGCAGAGACAGTGACCAGAGT3′

HF4   5′ggaattcggccccccgaggcCGAGGAGACGGTGACTGAGGT3′

2.15.4.2. *PCR reaction*

Standard multiplex PCR reaction mixture was as follows:

Component	Stock concentration	Concentration in a 50 μl reaction	X 1
Forward Primers	Varied	0.1 nmol each primer /reaction	Varied
Reverse Primers	Varied	0.1 nmol each primer /reaction	Varied
10 X	10 X	1 X	5.0 μl
MgCl$_2$	25 mM	1.0 mM	2.0 μl
dNTP	20 mM	0.4 mM	1.0μl
cDNA	Varied		1.0 μl/ reaction
Taq Polymerase	5U/μl	5U/reaction	1.0 μl
Water			Make up reaction to 50 μl

X1 = Amount used per reaction

The PCR thermal cycling conditions were as follows:

94° C X 1 min

63° C X 30 sec

58° C X 50 sec

72° C X 1 min

repeat by 7 cycles

94° C X 1min

63° C X 30 sec

72° C X 1 min

repeat by 24 cycles

72° C X 10 min (optional)

All ramping rates were at 3°C/sec.

After the PCR was completed, the amplified DNA was electrophoresed on an agarose gel as described in section 2.12. A clean 400 base pair fragment was observed indicating that the required specific PCR product was generated.

2.15.5. *Purification of PCR reaction products*

PCR reaction products were gel-purified using a Wizard PCR-prep purification kit. The products were electrophoresed using an Atto AE-6110 gel electrophoresis system. Typically a 0.5% (w/v) low-melt agarose gel was prepared as described in section 2.12 The PCR products were loaded onto the gel, and this was electrophoresed at 70 V for 1-2 h. After this time the gel was observed through a protective shield under ultra violet (UV) light. The fragment of interest was excised from the gel and placed in a 1.5 ml eppendorf tube. The gel fragment was then incubated in a 70° C waterbath until it had fully melted. 1 ml of resin was immediately added and mixed thoroughly with the melted gel for 15-20 sec. A Wizard minicolumn was assembled with a 5 ml syringe barrel attached to it, and this was placed on a 1.5 ml eppendorf tube. The gel-resin mix was pipetted into the barrel, and was slowly plunged through the column. The syringe was removed and the barrel re-attached to the column. 2 ml of 80% (v/v) isopropanol was pipetted into the barrel, which was used to wash the column. The syringe was removed and the column inserted into a fresh 1.5 ml eppendorf tube. The column and eppendorf tube were centrifuged at 14,000 rpm for 2 min to remove any remaining isopropanol from the column. The minicolumn was once again transferred to a fresh 1.5 ml eppendorf tube, and 20 µl of autoclaved ultrapure water added to the column. This was allowed to incubate for 1 min at room temperature before being centrifuged at 14,000 rpm for 20 sec. The DNA eluted from the column was stored at -20° C until required for use.

After purification, the DNA fragments were re-electrophoresed with quantitative molecular weight marker. The concentration of DNA in the purified fragment (relative to the band of known concentration in the marker) was determined densiometrically using the Image Master VDS system. In the case of a Promega 100 base pair marker, the 500 base pair fragment was used as the reference for quantification, where 5 µl of marker contained 150 ng of DNA and 50 ng of the other fragments.

2.15.6. *Splice by overlap extension (SOE)–PCR amplification conditions*

The components for the SOE-PCR are as follows:

Component	Stock concentration	Concentration in 50 µl reaction	X 1
V_H	As determined	10 ng/ reaction	
V_L	As determined	10 ng/ reaction	
10 X	10 X	1 X	5 µl
$MgCl_2$	25 mM	1 mM	2 µl
dNTP	20 mM	0.4 mM	1 µl
Water			Make up to 50 µl
Sc Forward	Varied	0.05 nmol/reaction	Varied
Sc Back	Varied	0.05 nmol/reaction	Varied
Taq	5 U/µl	5 U/reaction	1 µl

X1 = Amount used per reaction

The PCR thermal cycling conditions were as follows:

92° C X 1 min

45° C X 50 min

72° C X 1 min

repeat X 5 cycles

pause

Add:

        0.05 mM Sc forward and Sc back primers

        5 U Taq DNA polymerase per reaction

92° C X 1 min

68° C X 30 sec

72° C X 1 min

repeat X 25 cycles

After the SOE-PCR was completed, the amplified DNA was electrophoresed on an agarose gel as described in section 2.12. A clean 800 base pair fragment was observed indicating that the required specific PCR product was generated.

2.15.7. *Plasmid purification of pAK 100 using Wizard Plus SV miniprep DNA purification system*

All plasmids were donated as a kind gift from Prof. Dr. Andreas Pluckthun, Biochemisches Institut der Universitat, Zurich, CH-8057 Zurich, Switzerland.

A single colony of *Escherichia coli* (*E. coli*) XL I-blue cells containing the plasmid pAK 100 was picked from an agar plate and grown in non-expression media overnight shaking in 37° C. The culture was then centrifuged at 7,000 rpm for 5 min at 4° C. The supernatant was removed and the tube was placed inverted on a piece of tissue in order to remove any remaining supernatant. The pellet was thoroughly resuspended in 250 μl of cell resuspension solution. This was transferred to a sterile 1.5 ml eppendorf tube, and 250 μl of cell lysis solution was added. The tube was carefully inverted four times causing the solution to clear. If the solution did not clear immediately it was necessary to wait up to five minutes in order for it to clear. After this, 10 μl of alkaline phosphatase was added and the tube carefully inverted four times, and incubated for five minutes. 350 μl of neutralisation solution was added and the tube was once again carefully inverted four times. The tube containing the cell lysate was then centrifuged at 14,000 rpm for 10 min. A spin column was inserted into a 2 ml collection tube and the cleared lysate was pipetted into the spin column. At this point it was important not to disturb the white precipitate remaining in the centrifuge tube. The spin column containing the lysate was centrifuged at 14,000 rpm for 1 min. The column was then removed from the tube and the liquid discarded. It was re-inserted into the tube and 750 μl of column wash solution was added. This was centrifuged again at 14,000 rpm for 1 min at room temperature. The wash step was repeated using 250 μl of wash solution and re-centrifuged at 14,000 rpm for 2 min. The column was then transferred to a sterile 1.5 ml eppendorf, and 50 μl of autoclaved ultra pure

water was added.   This was centrifuged at 14,000 rpm for 1 min in order to elute the DNA. The eluted DNA was stored at -20° C until required for further use.

2.15.8. *Restriction enzyme digestion of pAK 100, and the annealed V_H-V_L DNA*

The plasmids used in this recombinant phage display system are unique in that they only require one restriction enzyme digestion prior to ligation into the plasmid. The pAK vectors contain a tetracycline resistance gene bounded by SfiI sites while the SOE primers also incorporate an SfiI site into both ends of the fragment.

The components for restriction enzyme digestion of the V_H -V_L **insert** are as follows:

Component	Volume/ Reaction
NEB buffer 2	1.5 µl
BSA	0.2 µl
SfiI	0.5 µl
V_H -V_L fragment	10 µl
Water	3.5 µl

The components for restriction enzyme digestion of the **pAK vector** are as follows:

Component	Volume/ Reaction
NEB buffer 2	5 µl
BSA	0.5 µl
SfiI	1 µl
PAK vector	10 µl
Water	34µl

Both sets of components for both reactions were incubated in a 50° C waterbath overnight.

After this time the digested vectors and digested V_H-V_L inserts were electrophoresed on a 1% (w/v) low melt agarose gel as described in section 2.12. In the case of the pAK vector there was a clear distinction between the cut vector (4,300 base pairs) and the tetracycline gene cut (2,100 base pairs). However, in the case of the V_H-V_L fragment the distinction was not as apparent, with only a small number of base pairs being cut from the 800 base pair DNA fragment. In order to observe the cut more clearly in the case of the V_H-V_L fragment, it was electrophoresed on a 2-2.5 % (w/v), agarose gel to aid separation.

The cut fragments were purified as described in section 2.15.5 and following purification were electrophoresed on an agarose gel in order to determine their concentrations densiometrically as described in section 2.15.5.

2.15.9. *Insertion of V_H-V_L fragment into pAK 100 plasmid vector*

Gel purified SOE-V_H-V_L fragment was ligated into the pAK 100 plasmid vector as follows:

Component	Stock concentration	Concentration in a 20 µl reaction
pAK 100	As determined	1.5 X V_H-V_L fragment*
V_H-V_L fragment	As determined	1 X*
Ligase	As supplied	10 U/reaction
5X Ligase buffer	5X	1X

All reactions were made up to a final volume of 10 µl.

* Using the concentrations of V_H-V_L and vector digests, a sufficient volume was added to ensure a ratio of vector to insert of 1.5:1

Incubation was overnight at 16° C. It was preferable to use the ligated construct immediately, but, if necessary, it was possible to store it at –20° C.

2.15.10. *Transformation of E.coli XL1-blue with pAK 100 plasmid vector containing SOE-V_H-V_L fragment*

The supercompetent *E.coli* cells were completely thawed on ice, before being mixed gently by hand. 100 μl of the cells were added into each of two pre-chilled 25 ml tubes. (One tube was for the experimental transformation and one tube was for the control transformation). 1.7 μl of β-mercaptoethanol provided with the kit was added to each of the two Sterilin 25 ml tubes containing the bacteria. The tubes were gently swirled, and then incubated on ice for 10 min, with gentle swirling every 2 min. 50 ng of the ligated DNA vector was added to one of the aliquots and gently swirled. As a control, 1 μl of the pUC 18 control plasmid was added to the other aliquot of cells and swirled gently. Both tubes were incubated on ice for 30 min. The tubes were then heat-pulsed in a 42° C water bath for 45 seconds. This length of time of the heat pulse was critical for obtaining the highest efficiency, as there is a defined 'window' of the highest efficiency resulting from the heat pulse. The defined 'window' for these particular cells was between 40-50 seconds, after which time transformation efficiencies are greatly reduced. The tubes were further incubated on ice for another 2 min. After this time 0.9 ml of preheated (42° C) SOC medium was added, and the tubes incubated at 37° C for 1 hour with shaking at 225-250 rpm. The tubes were then centrifuged at 2000 rpm for 12 min and resuspended in 400 μl each of 2 X TY. Serial dilutions (diluted to 10^{-10}) were carried out on both transformed samples, and were plated out on selective TYE medium (TYE containing 25 μg/ml of chloramphenicol, and 1% (v/v), glucose for the experimental transformation; TYE containing 50 μg/ml ampicillin, and 1% (v/v), glucose for the control transformation). The remaining experimental transformed sample was plated out on selective medium plates for production of library stocks. The plates were incubated for 24 h at 37° C.

2.15.11. *Production of stocks of the antibody phage display library*

After the transformed bacteria were allowed to grow overnight, the diversity of the library was determined from the experimental transformant dilution plates, while the transformation efficiency was determined from the control transformant

dilution plates. The bacteria on the stock experimental plates were aseptically scraped off with 2 X TY (using a glass spreader). The bacteria were then centrifuged at 4000 rpm in a bench centrifuge for 10 min. The supernatant was removed and the bacterial pellet containing the transformed antibody phage display library was resuspended in 3 ml of 2 X TY containing 15% (v/v) sterile glycerol. The cultures were aliquoted and "flash-frozen" using liquid nitrogen. They were then stored at -80° C until required for further use.

2.16. Screening for scFv phage display antibodies from a pre-immunised anti-AFB$_1$ phage display library

All culture media formulations used in the production of scFv displaying phages, and soluble scFv antibodies are described in section 2.2.

2.16.1. *Production of scFv displaying phages*

50 ml of non-expression (NE) medium was inoculated with 100 µl of approx 10^5 cells from the glycerol stock library. The culture was shaken at 37° C until O.D. $_{550}$=0.5. The culture was then allowed to sit for 10 min at 37°C. 10^{11} colony forming units (c.f.u.) of helper phage (2 µl -VCSM13 (StrataGene)) and 25 µl of 1 M IPTG solution was added. After 15 min incubation at 37° C the culture was diluted in 100 ml of low-expression (LE) medium. The culture was then shaken for 10 h at 26° C for phage production. Two hours after infection 30 µg/ml of kanamycin was added. Phage particles were purified and concentrated 100-fold by two PEG/NaCl precipitations.

It was necessary to inoculate 4 ml of 2 X TY containing 30 µg/ml tetracycline with a single colony of XL-1-blue when it was intended to carry out a phage titre as well as PEG/NaCl precipitation the following day. This was carried out in duplicate (as well as a control) and incubated overnight at 37° C in a shaking incubator.

2.16.2. *PEG/NaCl precipitation*

150 ml of the *E.coli* XL-1-blue-library was transferred to a sterile 300 ml Sorvall tube and centrifuged at 10,000 rpm for 10 min at 4° C. One fifth of the volume of

PEG/NaCl was added to the supernatant. This was mixed and incubated at 4° C (on ice) for at least one hour. The mixture was then centrifuged in a Sorvall refrigerated centrifuge at 10,000 rpm for 30 min at 4° C. The pellet was re-suspended in 40 ml of ultrapure water and 8 ml of PEG/NaCl. This was mixed and incubated at 4° C for 20 min. After this time, the mix was centrifuged at 10,000 rpm for 20 min at 4° C. The supernatant was removed and the pellet re-centrifuged at 7,000 rpm for 10 min at 4° C. Any remaining supernatant was removed and the pellet was re-suspended in 2 ml of sterile-filtered PBS. The phage was then micro-centrifuged at 4000 rpm for 10 min at 4° C, to remove any bacterial debris. The phage precipitate was retained and stored at 4° C (short-term) or at −80° C in a 15% (v/v), sterile glycerol solution (long-term).

2.16.3. *Phage titre*

4 ml of 2 X TY containing 50 μg/ml tetracycline was inoculated with a single colony of XL-1-blue overnight at 37° C. The *E.coli* XL-1-blue culture was then diluted 1/100 in 5 ml of 2 X TY containing 50 ug/ml tetracycline. This was grown shaking at 37° C for approximately 2 h until $O.D._{550} = 0.5$-0.6. The culture was then allowed to sit at 37° C for 15 min. After this time serial dilutions $(10^{-1}-10^{-11})$ of the phage were prepared using the bacterial culture. This was then incubated for 30 min at 37° C without shaking. 100 μl of each dilution was spread on TYE plates containing 1% (v/v) glucose and 25 ug/ml chloramphenicol. The plates were allowed to grow overnight at 37° C. The colony forming units were counted in order to calculate the titre of the stock phage.

2.16.4. *Selection of antigen binders by panning*

For selection, an immunotube was coated overnight at 4° C with AFB_1-BSA or AFB_1-dextran (AFB_1-Dex) diluted in PBS (50-100 ug/ml). The tube was washed five times with PBST and five times with PBS. This was then blocked with 4% (w/v) milk in PBS and allowed to incubate for 1 h at 37° C. After this time the tube was again washed, and 1 ml of phagemid particles was added to 3 ml of PBS which in total contained 2% (w/v), milk. This was allowed to incubate in the immunotube for 2 h at room temperature, gently shaking. After washing (as before) bound phage was eluted from the tube by adding 800 μl 0.1 M

glycine/HCl, pH 2.2 for 10 minutes. This was then neutralised using 48 µl of 2 M Tris. The phage was then used for titre, as described previously.

2.16.5. *Preparation of Library Stocks*

Once phage was eluted from an immunotube (as described above), it was re-infected into an *E.coli* XL-1-blue culture. 4 ml of 2 X TY containing 50 µg/ml tetracycline was inoculated with a single colony of XL-1-blue overnight at 37° C. The *E.coli* XL-1-blue culture was then diluted 1/100 in 5 ml of 2 X TY containing 50 ug/ml tetracycline. This was grown shaking at 37° C for approximately 2 h until O.D.$_{550}$ = 0.5-0.6. The culture was then allowed to sit at 37° C for 15 min. Approximately 1.5-2.0 ml of the eluted phage was added to a 5 ml culture and was allowed to incubate for 15 min at 37° C. After this time the culture was centrifuged in a bench centrifuge at 4000 rpm for 10 min. The supernatant was discarded and the pellet resuspended in 400-500 µl of 2 X TY. The resuspended culture was then spread on TYE plates containing 1% (v/v) glucose and 25 ug/ml chloramphenicol (100 µl of culture per plate). The plates were allowed to grow overnight at 37° C. After this time the bacteria were scraped off the plates with 2 X TY (using a glass spreader). This was then centrifuged at 4000 rpm for 10 min. The supernatant was removed and the pellet was resuspended in 3 ml of 2 X TY containing 15% (v/v), sterile glycerol. The cultures were aliquoted and "flash-frozen" using liquid nitrogen. They were then stored at −80° C until required for further use.

2.16.6. *Preparation of clones for non-competitive phage ELISA*

Individual wells of a 96 well culture plate containing 200 µl/well of 2 X TY, 1% (v/v) glucose, 25 ug/ml chloramphenicol, and 30 µg/ml tetracycline were inoculated with single colonies from plates growing bacteria from a round of panning. The plate was shaken (150 rpm) overnight at 37° C and used as the primary 'master' plate to inoculate an identical plate. 30 µl of 60% (v/v) sterile glycerol was added to each well of the master plate and stored at -20° C. The second plate was grown (shaken at 150 rpm) for 6-8 h at 37° C. The plate was allowed to stand for 15 min at 37° C. 25 µl of 2 X TY containing 1% (v/v)

glucose, 25 ug/ml chloramphenicol, 1.5 mM IPTG, and 5×10^9 VCSM13 helper phage per ml was then added to each well, and allowed to stand at 37° C for 15-30 min followed by shaking (150 rpm) for 1-2 h at 26° C. The plate was centrifuged in a bench centrifuge at 4000 rpm for 10 min. The supernatant was discarded and replaced with 200 µl 2 X TY containing 1% (v/v), glucose 25 ug/ml chloramphenicol, 1.5 mM IPTG, 30 ug/ml kanamycin, and grown at 150 rpm overnight at 26° C. After the overnight incubation the plate was centrifuged in a bench centrifuge at 4000 rpm for 10 min. The supernatant was analysed by phage ELISA.

2.16.7. *Non-competitive phage ELISA for the detection of positive clones to* AFB_1

A 96-well microtitre plate was coated by adding 100 µl of aflatoxin B_1-BSA conjugate or AFB_1-Dex conjugate dissolved in PBS at a concentration of 50 ug/ml to each well. The plate was incubated overnight at 4° C. The plate was emptied and washed six times, three times with PBST and three with PBS only. The plate was then blocked by addition of 100 µl per well of PBS containing 2% (w/v), milk powder and incubated for 1 h at 37° C. (For both coating and blocking of plates, incubation steps could also be carried out at 37° C for 1 h or 4° C overnight). 75 µl of supernatant and 25 µl of 4% (w/v), milk in PBS were added to each well for 1 h at 37° C. Plates were emptied and washed as described before, and incubated for 1 h at 37° C with 1/1,000 dilution of anti-M13 antibody (anti-helper phage antibody). This was followed by addition of a 1/5,000 dilution of HRP-labelled anti-rabbit antibody. This was incubated for 1 h at 37° C followed by colour development as described in section 2.11.1 The enzymatic reaction was stopped by the addition of 25 µl 4 M H_2SO_4 to each well. The absorbance of the wells at 492 nm was subsequently measured using a microtitre plate-reader.

2.16.8. *Preparation of clones for competitive phage ELISA*

Positive clones recognising either AFB_1-BSA or AFB_1-Dex in the non-competitive format were grown from the master plate (prepared in section 2.16.6) in 2 ml of NE medium at 37° C. After reaching O.D.$_{550}$= 0.3, 1 ml of NE medium

complemented with 5×10^9 cfu VCSM13 and 1.5 mM IPTG was added. The cultures were allowed to produce scFv-displaying phages overnight at 24° C in a shaking incubator. The cultures were then centrifuged at 4,000 rpm for 10 min and the supernatant containing the phage displayed scFv's was removed and analysed for recognition of free AFB_1.

2.16.9. *Competitive phage ELISA for the detection of clones recognising free* AFB_1

A competitive phage ELISA was carried out on clones that recognised either AFB_1-BSA or AFB_1-Dex on a non-competitive format. The ELISA was carried using the same method as described in section 2.16.7 except that standards of free AFB_1 were added to the ELISA plate at same time of addition of the phage clones.

2.17. Isolation and soluble expression of scFv anti-AFB_1 antibodies

2.17.1. *Purification and isolation of 'positive'* V_H-V_L *fragment from pAK 100*

Plasmid purification of pAK 100 containing DNA of scFv antibody recognising free AFB_1 was carried out using the Wizard Plus SV miniprep DNA purification system as described in section 2.15.7. The plasmid was then restriction enzyme-digested from the pAK 100 vector as described in section 2.15.8 and electrophoresed on a low-melt agarose gel as described in section 2.12. The fragment excised from the gel (800 base pair fragment), was then purified using the Wizard PCR-prep purification kit as described in section 2.15.5 prior to being ligated into the high expression vector pAK 400.

2.17.2. *Restriction enzyme digestion of pAK 400*

Restriction enzyme digestion of the soluble high expression vector pAK 400 was carried out in the same way as described in section 2.15.8.

2.17.3 *Insertion of* V_H-V_L *fragment into pAK 400 plasmid vector*

Ligation of the positive V_H-V_L fragment into the vector pAK 400 was carried out in the same way as described in section 2.15.9.

2.17.4. *Preparation of fresh competent E.coli JM-83 using calcium chloride*

A single colony of the non-suppressor *E.coli* bacteria JM 83 was used to inoculate 5 ml of 2 X TY containing 50 μg/ml of streptomycin sulphate. This was grown overnight while shaking at 37° C. The *E.coli* culture was then diluted 1/100 in 100 ml of 2 X TY containing 50 μg/ml of streptomycin sulphate. This culture was grown until the $O.D._{550}= 0.3\text{-}0.4$. The cells were cooled on ice for 10-15 min before being collected by centrifugation at 4° C (2,000 rpm for 20 min in a bench centrifuge). The cells were maintained at 4° C for the rest of the procedure. The supernatant was decanted off and the cells were resuspended in 20 ml of 100 mM $MgCl_2$. This was re-centrifuged at 2000 rpm for 20 min, and the cells resuspended in 20 ml of 50 mM $CaCl_2$. The cells were left on ice for 30 min before being centrifuged again at 4° C (2000 rpm for 20 min in a bench centrifuge). The supernatant was decanted off and the cell pellet was resuspended in 2 ml of 50 mM $CaCl_2$ per 100 ml culture. The washed bacterial cells were then ready for transformation. If necessary, it was possible to "flash-freeze" the cells using liquid nitrogen and store them at –80° C in 15% (v/v) sterile glycerol.

2.17.5. *Transformation of E.coli JM-83 with pAK 400 plasmid vector containing 'positive' V_H-V_L fragment*

3 μl of the ligated DNA vector was added to a 200 μl aliquot of fresh $CaCl_2$ - competent *E.coli* JM-83 cells. As a control, 3 μl of 50 mM $CaCl_2$ was also added to a 200 μl aliquot of the competent cells. The transformations were left on ice for 60 min. After this, they were placed in a 42° C waterbath for exactly 2 min, and then immediately back on ice. The contents of the tubes were then added to 700 μl of 2 X TY, and incubated for 1 h at 37° C with gentle shaking. Serial dilutions (diluted to 10^{-8}) were carried out on both transformed samples. These were plated out on selective TYE medium (TYE containing 25 μg/ml of chloramphenicol, and 1% (v/v), glucose). The remaining experimental-transformed sample was plated out on selective medium plates containing 25 μg/ml of chloramphenicol, and 1% (v/v), glucose, in order to grow stocks of the library. The plates were incubated for 24 h at 37° C. The production stocks of the bacteria containing the ligated pAK 400 were made in the same way as described in section 2.15.11.

2.17.6. *Soluble expression of scFv fragments in pAK 400*

In order to obtain soluble expression of scFv antibodies, 20-200 ml of expression medium was inoculated with 200 µl - 2 ml of preculture (JM 83 harbouring the pAK 400 expression plasmid for the anti-AFB$_1$ scFv antibody). This was grown while shaking at 37° C until O.D.$_{550}$= 0.5. Expression was then induced by the addition of 1 mM IPTG and allowed to proceed for 4 h at 24° C. The culture was then centrifuged at 4,000 rpm in a bench centrifuge for 15 min. The supernatant was removed and analysed for soluble expression of scFv antibodies .

2.17.7 *Analysis of the cellular distribution of scFv antibodies*

Cell pellets were analysed for periplasmic expression of scFv by resuspending in 100 µl – 1 ml of PBS containing 0.1% (w/v) phenyl methyl sulphonylfluoride (PMSF) and 1.5% (w/v) streptomycin sulphate. They were then subjected to three freeze-thaw cycles and centrifuged in a bench centrifuge at 3,000 rpm in a bench centrifuge for 10 min. Lysates were analysed for soluble expression of scFv antibodies by ELISA.

2.18. Purification and concentration of soluble scFv antibody fragments

ProBond™ resin (Invitrogen) was used for the isolation of the soluble scFv antibodies from bacterial culture supernatant. ProBond™ resin allows for single column purification of expressed proteins that contain polyhistidine tags (Hemdan *et al.*, 1989).

2.18.1. *Purification of soluble scFv fragments by immobilised metal affinity chromatography (IMAC)*

5 ml of ProBond™ resin was poured into a column and allowed to settle. The column was then calibrated with 20 ml of wash buffer (20 mM phosphate buffer with 500 mM NaCl, pH 7.8). 20 ml of the bacterial cell culture supernatant was added to the column and re-applied a number of times, in order to allow optimal binding of the scFv's to the column. The column was then washed with 40 ml of wash buffer. 20 ml of elution buffer (wash buffer containing 300 mM imidazole) was added and allowed to sit in the column for 10 min, before fractions were

collected. All of the fractions were monitored for protein content at O.D.$_{280}$, and elution continued until there was no more protein being eluted from the column. The fractions containing protein were pooled and dialysed overnight at 4° C against 5 l PBS.

2.18.1.1. *Stripping and recharging of ProBond™ resin*

The column was washed twice with 8 ml of 50 mM EDTA to strip away chelated nickel ions. This was followed by washing once with 16 ml of 0.5 M NaOH in order to denature or elute any non-specific proteins remaining on the resin. It was then washed with 16 ml of sterile distilled water. The column was recharged with 16 ml of a 5 mg/ml solution of nickel chloride (NiCl$_2$) followed by one more washing with 16 ml of distilled water. The charged column was stored in water containing 20% (v/v) ethanol, until required for use. The resin was always used for the same recombinant antibody, and only used 3-4 times before disposal.

2.18.2. *Concentration of purified scFv antibody*

Soluble scFv antibodies, in PBS, were concentrated five-fold using a stirred ultrafiltration cell with a 76 mm diaflo ultrafiltration membrane, with a molecular weight cut-off of 10,000 daltons. The concentrate was sterile filtered, and 0.05% (v/v) sodium azide was added for preservation. It was stored at 4° C until required for use.

2.19. ELISA analysis of soluble scFv antibodies

2.19.1. *Checkerboard ELISA for determination of optimal scFv antibody dilution and conjugate concentration for use in a competition ELISA*

A 96-well microtitre plate was coated as described in section 2.11.2. Serial dilutions of the scFv antibody were carried out in PBS. 100 μl per well of each dilution of scFv antibody was added to the wells (at least in triplicate) in the absence of free hapten. This resulted in a set of titre curves being produced for each different conjugate concentration. The plate was incubated for 1 h at 37° C, followed by washing, and the addition of a 1/400 dilution of anti-FLAG monoclonal antibody, which recognises the histidine tag on the scFv antibody.

After incubation and washing, 100 µl of horseradish peroxidase (HRP) - conjugated - anti-mouse secondary antibody, diluted in PBS, was added to each well and incubated for 1 h at 37° C. Colour was developed as described in section 2.11.1. From the titre curves obtained, the antibody dilution that gave half the maximum absorbance, and the lowest conjugate concentration that provided sufficiently high absorbances was chosen.

2.19.2. *Competitive ELISA using soluble scFv antibodies for the detection free AFB₁*

A competitive ELISA was carried using the same method as described in section 2.19.1 except that the ELISA plate was coated with one concentration of conjugate, and standards of free AFB₁ were at same time of addition of the soluble scFv antibody.

2.19.3 *Estimation of cross reactivity of soluble anti-AFB₁ scFv antibodies*

Cross reactivity studies were carried out in the same way as described in section 2.11.4 for the preparation of aflatoxin standards, and section 2.19.2 for the competition assay.

2.20. BIAcore studies

CM5 research grade sensor chips were used in all cases. Running buffer for all BIAcore experiments was Hepes buffered saline (HBS) buffer, pH 7.4, containing 10 mM HEPES, 150 mM NaCl, 3.4 M EDTA, and 0.005% (v/v) Tween 20. All solutions were filtered (0.22 µm) and running buffer was degassed by sonication prior to use.

2.20.1. *Preconcentration studies*

Proteins and protein-conjugates were immobilised on the sensor surface by means of N-hydroxysuccinimide esterification. This allows covalent attachment of biomolecules via primary amine groups. In order for the coupling reaction to be carried out, an initial "preconcentration" step is required. At pH values greater than 7, the CM-dextran surface has a net negative charge (negatively charged carboxyl groups), while proteins at pH values below their isoelectric point (pI) will

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have a net positive charge (protonated amine groups), and will, therefore, be electrostatically attracted to the chip surface.

For the native form of a protein, preconcentration can be facilitated by adjusting the pH below the isolectric point (pI). However, modification of proteins by, for example, conjugation to hapten molecules often radically alters the pI. Therefore, when dealing with modified proteins, the correct pH for preconcentration had to be determined experimentally. This was done by preparing protein solutions (usually at a concentration of 50 ug/ml) in 10 mM sodium acetate at a range of different pH's from 3.9 to 5.0. These solutions were then injected over an underivatised chip surface, and the degree of electrostatic binding was monitored. The highest pH at which satisfactory preconcentration was observed was chosen as the pH for immobilisation.

2.20.2. *Coupling reaction of proteins and hapten-protein conjugates to cm-dextran gel*

The carboxymethylated dextran (CM-dextran) matrix was activated by mixing equal volumes of 100 mM NHS (N-hydroxysuccinimide) and 400 mM EDC (N-ethyl-N-(dimethyl-aminopropyl) carbodiimide hydrochloride) and injecting the mixture over the sensor chip surface for 7 min at a flowrate of 5μl/min. The interactant to be immobilised (concentration: 50 - 200 μg/ml) was dissolved in 10 mM acetate buffer (at the required pH), and injected over the surface for 20 min at a flowrate of 2 μl/min. The unreacted sites on the sensor chip surface were then capped by injection of 1 M ethanolamine, pH 8.5, for 7 minutes.

2.20.3. *Removal of non-specific interactions*

In certain situations non-specific interactions may occur with an inhibition assay format, where the antibody being injected over the immobilised conjugate surface may non-specifically bind to the CM-dextran surface, as well as the protein part of the hapten-protein conjugate. This is particularly common with a polyclonal antibody population due to its heterogeneous nature. The problem is overcome by 'spiking' the antibody diluent buffer, when necessary, with CM-Dextran (~100μg/ml) or the protein the hapten is conjugated to (~1mg/ml).

2.20.4. *General surface plasmon resonance-based inhibition immunoassay*

An indirect inhibition assay system was used for the detection of aflatoxin B_1 in PBS and spiked grain samples. All additions of reagents and incubation steps were automated.

Approximately 4,000-8,000 response units (RU) of aflatoxin B_1-BSA conjugate was immobilised on the chip surface for each assay.

The antibodies were diluted in HBS running buffer, and depending on the non-specific interactions, CM-dextran and/or BSA were also added. All buffers and solutions used were prepared using ultrapure water de-gassed and sterile filtered.

A stock solution of AFB_1 was made, as described in section 2.10.1, and standard solutions within the desired range were prepared in PBS containing 5% (v/v) methanol from this stock.

Each sample was incubated with an equal volume of the anti-aflatoxin antibody or scFv for 10 min when the system was automated, or alternatively the samples could be incubated for 1 h at 37° C and then passed over the surface of the chip. This was carried out three times for each concentration. Regeneration of the surface of the chip was carried out using, 10-100 mM HCl/NaOH or 1 M ethanolamine with 20%(v/v) acteonitrile, pH 12.0.

A calibration curve was constructed from these results by plotting the change in response (in RU) for each standard against the log of concentration. The intra-day variability of the assay was investigated by running a set of three standards across the linear range three times in one day, and determining the coefficient of variation (C.V.) between the calculated AFB_1 standards for each set of three.

The inter-day variability of the assay was assessed by running three sets of standards across the linear range on three different days, and determining the C.V. between the calculated AFB_1 concentrations for the standards from each of the three standard curves.

2.20.5. *Solution affinity analysis of scFv antibodies using BIAcore*

Approximately 5,000 RU of AFB_1-BSA conjugate was immobilised as described in section 2.20.2. Serial dilutions of purified anti-AFB_1 scFv of known concentration were passed over the immobilised surface, and a calibration curve was constructed of mass bound measured in terms of response units, versus scFv concentration

(nM). A known concentration of scFv was then incubated with varying concentrations of AFB_1 (nM), and allowed to reach equilibrium for 2 h at 37° C. These samples were then sequentially injected over the immobilised surface and the binding response calculated. The response values measured were used to calculate the amount of 'free scFv' in the equilibrium mixtures, from the constructed calibration curve. A graph was then constructed of toxin concentration (nM) versus 'free scFv concentration' (nM), and using the solution phase interaction models in BIAevaluation 3.1 software, the overall constant could be determined.

Chapter 3

Production, and Applications of Polyclonal Antibodies to Aflatoxin B_1

3.1. Introduction

Aflatoxins were discovered in 1960 following the deaths of several thousand turkey poults throughout England, due to consumption of contaminated Brazilian groundnut meal (Murray *et al.*, 1982). They are a group of highly toxic fungal secondary metabolites that occur in *Aspergillus* species (O'Kennedy *et al.*, 1997). The fungus contaminates foodstuffs and feeds, as well as crops such as maize, cottonseed, peanuts, and tree nuts during growth but particularly while in storage. Contamination is most common in tropical and sub-tropical countries where humidity is high, and, therefore, allows favourable conditions for the fungus to grow. Aflatoxins are members of the coumarin family and the most significantly occurring compound is aflatoxin B_1 (AFB$_1$) (Figure 3.1), which is produced by certain strains of *Aspergillus flavus (A. flavus)* and *Aspergillus parasiticus (A. parasiticus)*. Other aflatoxins designated B_2, G_1, and G_2 are also produced, but AFB$_1$ is generally present in the largest quantity, and is the most toxic. Aflatoxin M_1 (AFM$_1$-or 4-hydroxy-AFB$_1$) is a hydroxylated metabolite of AFB$_1$, which is excreted in the milk of dairy cattle after they consume aflatoxin-contaminated food. AFQ$_1$ and AFP$_1$ are metabolites of AFB$_1$ in the mouse and rhesus monkey (Dalezios *et al.*, 1971).

3.1.1. *Aflatoxin production conditions and biosynthesis*

The *Aspergillus* species that produce aflatoxins are widely distributed saprobic moulds. They grow naturally on many substances ranging from rubber to foodstuffs depending on the climatic conditions. *A. flavus* species generally infect nuts as the fungus is airborne, while *A. parasiticus* is most commonly found in the soil, so contamination of cereals is mainly due to this subspecies. For aflatoxin production, relative humidity (RH) levels need to be between 83-88 %, with levels increasing as the RH increases up to 99% (Gourama and Bullerman, 1995). Although *A. flavus* requires optimum growth temperatures between 36-38° C, it was found that AFB$_1$ is produced between 24-35° C (Gqaleni *et al.*, 1997). Carbon dioxide levels and pH may also have effects on aflatoxin production. Optimum pH levels from 4 to 6 are particularly favourable, while increasing concentrations of carbon dioxide from 20 to 100 % gradually inhibits aflatoxin production.
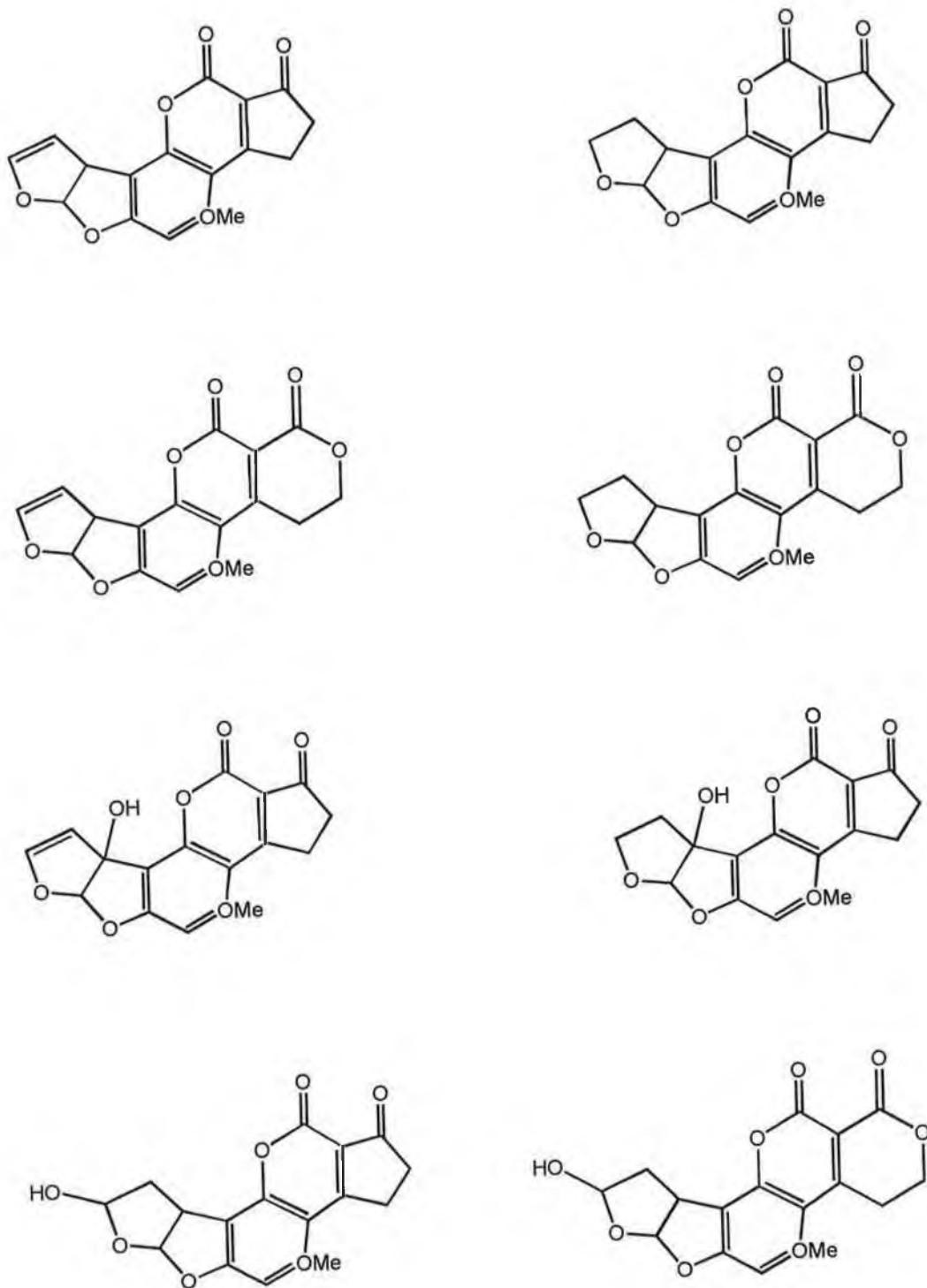
Figure 3.1. Chemical structure of aflatoxin B_1 (AFB$_1$) and its structural analogues.

Aflatoxins produced by the *Aspergillus* species are secondary metabolites, and are therefore not essential for growth of the fungi. Their functional role in the producing fungus is not known. Acetate polyketides are the main precursors of aflatoxins, and their biosynthesis is long and complex. It is characterised by diverse oxidative rearrangement processes (Minto and Townsend, 1997). The pathway outlined in Figure 3.2 is for the biosynthesis of AFB_1 and AFB_2 (Trail *et al.*, 1995).

3.1.2. *Aflatoxin biotransformation*

The most serious toxic actions associated with AFB_1 exposure are mutagenicity and carcinogenicity, which result from metabolic activation. This involves oxidative activation in the liver and kidneys by cytochrome P 450 and its enzymes (Minto and Townsend, 1997). Cytochrome P 450 is associated with extra-mitochondrial electron transport in the liver, and is involved with drug detoxification, and mixed function oxidation reactions. It is the principle enzymatic system involved in the biotransformation of AFB_1 to AFB_1-8-9-epoxide, referred to also as AFB_1-2-3-epoxide, which is highly toxic. P 450 enzymes capable of activating AFB_1 include members of the 1A, 2B, 2C and 3A subfamilies (Pelkonen *et al.*, 1997). Activation is essential for aflatoxin to produce its carcinogenic, mutagenic, and DNA-binding actions. AFB_1 can be activated to form either an endo-epoxide or an exo-epoxide (Massey *et al.*, 1995) (Figure 3.3). Only the exo-epoxide binds to DNA. It does this by electrophilically attacking the N^7 atom of Guanine, with the reaction producing a DNA lesion known as 2,3-dihydro-2-(N^7-guanyl)-3-hydroxyaflatoxin B_1 (AF-N^7-Gua). This is studied as an indicator compound of genotoxic damage (Groopman *et al.*, 1984). It is also thought to be the major cause of lesions on the liver that account for the carcinogenic and mutagenic nature of AFB_1. The exo-epoxide has also been shown to cause G-T transversions at codon 249 of the p 53 tumour suppressor gene, the altered sequence of which is associated with human cancers (Woloshuk and Preito, 1998). Hydration of the exo-epoxide can also occur, forming the dihydrodiol of AFB_1. This dihydrodiol inhibits protein synthesis *in vitro* and this may explain the cause of necrosis of the liver, which causes death (Gourama and Bullerman, 1995).

Polyketide

↓

Norsolorinic Acid

↓

Averantin

↓

Averufani

↓

Averufin

↓

Versiconal hemiacetal

↓

Versiconal

↓

Versicolorin B

↙   ↘

Versicolorin A	Versicolorin B
↓	↓
Sterigmatocystin	Dihyrosterigmatocystin
↓	↓
O-Methylsterigmatocystin	Dihydro O-methylsterigmatocystin
↓	↓
Aflatoxin B_1	Aflatoxin B_2
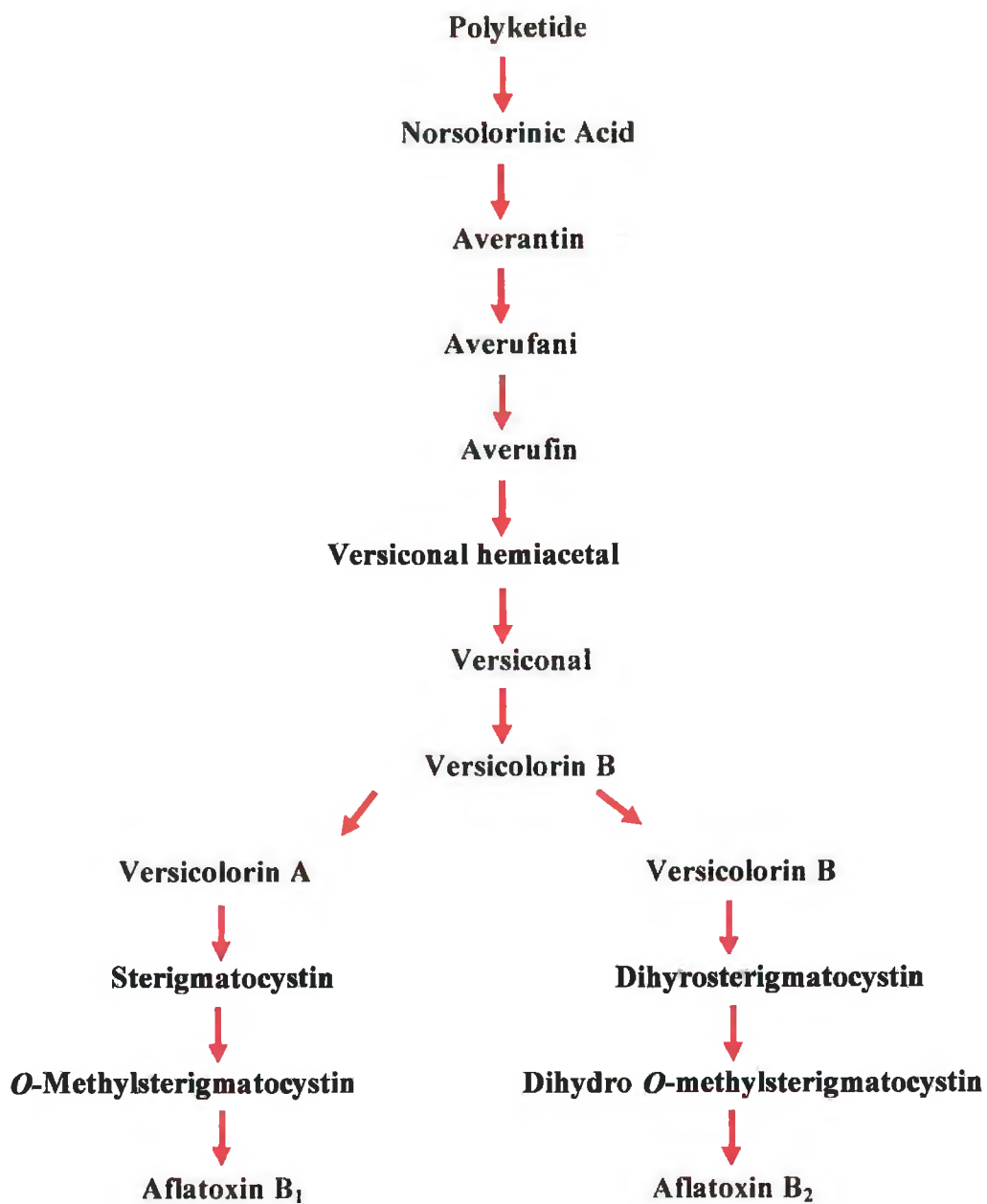
Figure 3.2. *Biosynthetic pathway of* AFB_1 *and* AFB_2.

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Biotransformation via cytochrome P 450

AFB₁

AFB₁ exo-epoxide

AFB₁ endo-epoxide

Binding to DNA
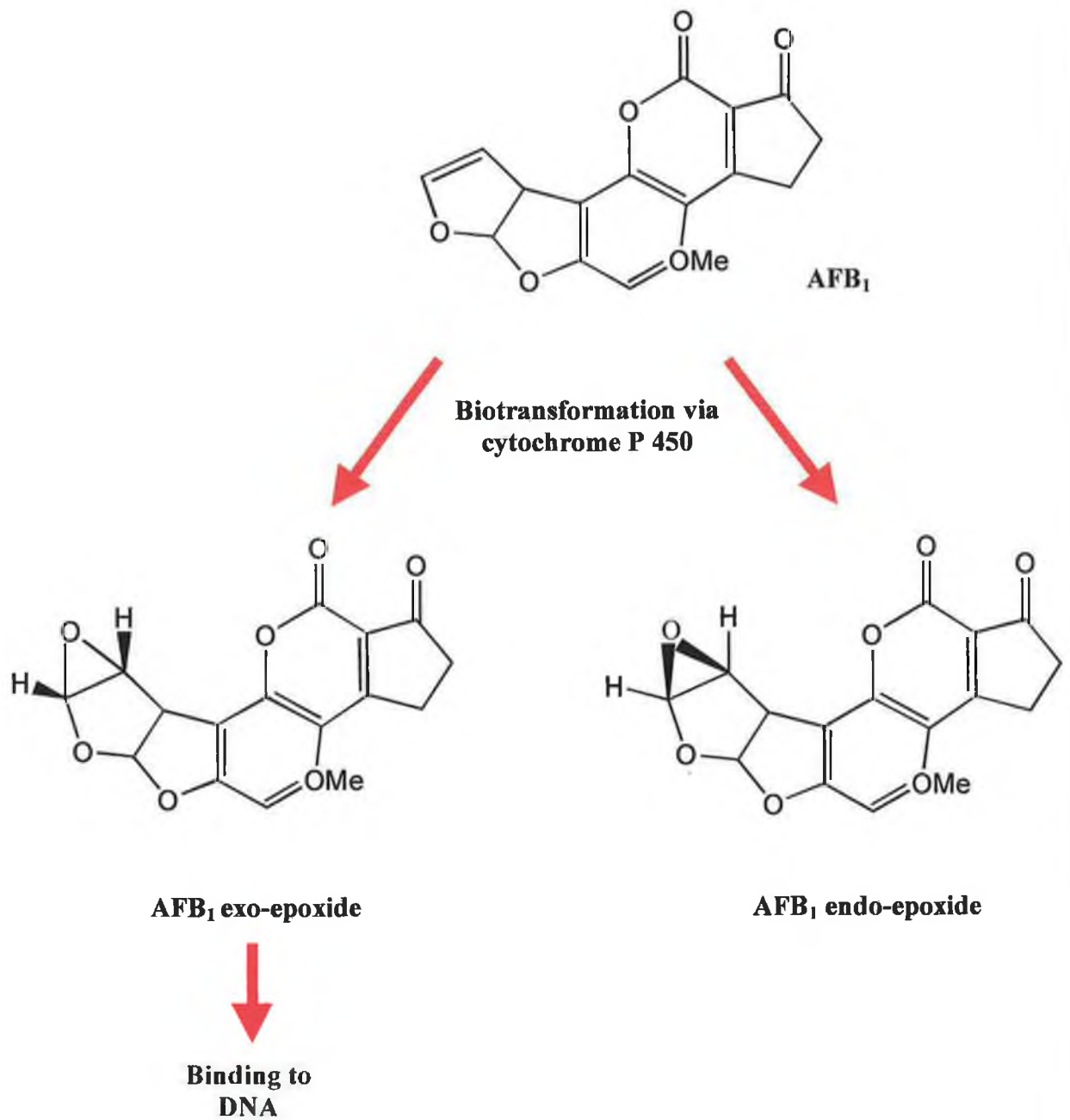
Figure 3.3. *Formation of exo-epoxide and endo-epoxide of AFB₁.*

3.1.3. *Carcinogenicity and toxicity*

AFB_1 is a very potent carcinogen and has been linked to human hepatocellular carcinoma. The international agency for research on cancer regards it as a human carcinogen (Ward *et al.,* 1990). Aflatoxicosis is a very rare disease and only a few instances of it have been reported. Symptoms of aflatoxicosis in animals and humans have been categorised in two general forms.

A. Acute aflatoxicosis is produced when moderate to high levels of aflatoxins are consumed. Specific, acute episodes of disease ensue and may include haemorrhage, acute liver damage, oedema, alteration in digestion, and possibly death.

B. Chronic aflatoxicosis results from ingestion of low to moderate levels of aflatoxins. The effects are usually subclinical and difficult to recognise. Some of the common symptoms are impaired or slower food conversion rates (U.S. Food and Drug administration. 'Foodborne pathogenic micro-organisms and natural toxins handbook'. http://vm.cfsan.fda.gov/~mow/chap41.html).

Although a wide range of LD_{50}'s have been reported for different species, most are in the region of 0.5 – 10 mg/kg of body weight. Animal studies indicate that aflatoxins can cause cancer in the liver, kidney and lungs, with the liver being the most sensitive and principle target organ (Nawaz *et al.,* 1995). However, it is often difficult to prove that aflatoxins are a cause of liver cancer, because, in tropical climates where aflatoxins occur most frequently, hepatitis B is very common (Chen *et al.,* 1996). Immunotoxicological effects, caused by AFB_1 have also been reported (Silvotti *et al.,* 1997). In this study, piglets were fed with AFB_1, and a number of effects including impairment of macrophage and granulocyte function were reported.

3.1.4. *Occurrences of aflatoxin poisoning*

Links between dietary exposure to aflatoxins and increased risks of primary hepatocellular carcinoma has been reported in several ethnic groups (Gerbes & Caselmann, 1993; Harrison *et al.,* 1993). For example, in Taiwan, an ecological correlation study was carried out that showed an association between urinary

aflatoxin level and hepatocellular carcinoma (hcc). Residents in an area called Penghu Islets, Taiwan, were reported to have the highest mortality rate caused by liver cancer in the country. Peanuts, the major agricultural product in this area were heavily contaminated by aflatoxins (Chen *et al.*, 1996). The study found that the onset of hcc among residents in the Penghu Islets was 10 years earlier than in residents of Taiwan island, and this was at least partly attributable to aflatoxin exposure.

One of the most significant accounts of aflatoxicosis in humans, occurred in northwest India in 1974. More than 150 villages in adjacent districts of two neighbouring states in were affected by the outbreak. It was found that 397 persons were affected, of whom 108 died. In this outbreak, contaminated corn was the major dietary constituent, and aflatoxin levels ranging from 0.25 to 15 mg/kg were found. The daily aflatoxin B_1 intake was estimated to have been at least 55 ug/kg body weight for an undetermined number of days. The patients experienced high fever, rapid progressive jaundice, oedema of the limbs, pain, vomiting, and swollen livers. Histopathological examination showed extensive bile duct proliferation and periportal fibrosis of the liver together with gastrointestinal haemorrhages. A 10-year follow-up of the Indian outbreak found the survivors fully recovered with no apparent ill effects from the experience.

A second outbreak of aflatoxicosis was reported from Kenya in 1982. There were 20 hospital admissions with a 60% mortality rate. Daily aflatoxin intake was estimated to be at least 38 ug/kg body weight for an undetermined number of days.

In a deliberate suicide attempt, a laboratory worker ingested 12 ug/kg body weight of aflatoxin B_1 per day over a 2-day period, and 6 months later, 11 ug/kg body weight per day over a 14-day period. There were no ill effects, except for transient rash, nausea and headache. Hence these levels may serve as possible no-effect levels for aflatoxin B_1 in humans. In a 14-year follow-up, a physical examination and blood chemistry analysis, which included tests for liver function proved to be normal (U.S.

Food and Drug administration. 'Foodborne pathogenic micro-organisms and natural toxins handbook'. http://vm.cfsan.fda.gov/~mow/chap41.html).

3.1.5. *Analysis and detection of aflatoxins*

Due to the widespread occurrence of the toxin-producing fungi in cereals, major efforts have been made to develop rapid and sensitive methods for detection of aflatoxins. Thin-layer chromatography and high-pressure liquid chromatography have been traditional methods used (Nawaz *et al.*, 1995), but can lack sensitivity and consistency due to the presence of many components in food and biological matrices that can interfere with physiochemical analysis. Selection of representative samples is a major problem, as the content of aflatoxin can vary from grain sample to sample due to individual 'hot spots' of mycotoxin production in an individual grain or nut consignment. Gourama and Bullerman (1995) gave an example, where in a peanut lot, one peanut kernel alone was reported to contain several hundred micrograms of AFB_1. This kernel could consequently contaminate 1,000 other kernels with a high level of aflatoxin. This emphasises the importance of adequate sampling of grains and nuts. Scudamore and Patel (2000) describe in detail a survey for aflatoxin and other mycotoxins in maize imported to the United Kingdom. It entailed sampling from all the major ports as well as from maize mills. This was carried out by taking incremental samples each of 0.5 kg from consignments on route to mills over a 2 h period. In the case of sampling at the mills, 0.5 kg incremental samples were also taken using an automatic sampling probe. This ensured the ability to take a sample from the 'centre' of a load and not just on the surface. In order to achieve a good degree of uniformity within samples for accurate analysis, it is strongly recommended to finely grind the grain as this will aid in more equal distribution of the mycotoxin within the sample. Extraction can be carried out with polar solvents such as methanol, acetone or chloroform. However, chloroform is generally the solvent of choice (Patel *et al.*, 1996). Once the aflatoxin has been extracted it can be further purified in one of two ways.

A. Using a column clean-up step: This entails passing the extracted sample through a silica column pre-washed with a solvent such as toluene. The sample is then passed

through the column, which is washed again with toluene to remove any impurities. The aflatoxin is then eluted from the column using chloroform and methanol mixed together. The solvent can then be removed from the sample by rotary evaporation, and the sample dissolved in mobile phase buffer (if the sample is going to be analysed by HPLC).

B. Immunoaffinity clean-up can also be utilised for aflatoxin sample purification. Immunoaffinity columns are commercially available, and contain a monoclonal antibody specific for aflatoxin B_1 (or another mycotoxin if required). An extracted sample is passed through the column and the aflatoxin binds to the monoclonal antibody immobilised on the column. The column is washed with PBS and the aflatoxin is then eluted from the column under gravity, using acetonitrile. The acetonitrile is then evaporated, and the aflatoxin sample re-dissolved in mobile phase buffer.

Both of these methods are effective in their application, but also require stringent internal controls. This entails spiking 'non-contaminated' grain sample with a known concentration of aflatoxin, and then extracting it the same way as the unknown grain samples, which monitors the effectiveness of the extraction procedure. Immunoaffintiy columns are used for a higher degree of sample cleanup (Patel *et al.* 1996) i.e. those containing high levels of co-extractives, which could not be removed using the silica column. The silica column method is also effective in sample cleanup and also offers recoveries of between 70-110 % throughout a given study (Scudamore *et al.*, 1999).

Other methods include extracting the sample as described already, evaporating off the solvent without a clean-up step, resuspending it in buffer and analysing it. Alternatively, the extracted sample could be directly diluted in buffer and analysed (Linskens *et al.*, 1992). Both of these methods are more applicable for immunoassay analysis, which normally does not require as many sample clean-up steps. Immunoassays can offer high levels of sensitivity, specificity, and are capable of high sample throughput (Linskens *et al.*, 1992). In order to produce antibodies to

aflatoxins and most mycotoxins they must be covalently linked to an immunogenic carrier molecule such as a protein (e.g. Bovine serum albumin-BSA), which will elicit a strong immune response following immunisation. This is because aflatoxins have a low molecular weight (i.e. less than 1,000 daltons). The protein conjugate is also used at the screening and characterisation stages of antibody production. Monoclonal and polyclonal antibodies have been produced using an aflatoxin B_1-BSA (AFB$_1$-BSA) conjugate (Ward *et al.*, 1990). Immunoassays (and ELISA in particular) have found widespread use in aflatoxin analysis. Langone and Van Vunakis (1976) were one of the first to develop an immunoassay to AFB$_1$ in serum, urine, and crude extracts of corn and peanut butter supplemented with aflatoxin. Antibodies were produced in New Zealand white rabbits immunised with AFB$_1$-poly-l-lysine conjugate. Results showed that as little of 1μg/kg of sample could be measured. The radioimmunoassay for AFB$_1$ was found to be at least as sensitive as the chromatographic and spectral techniques available.

ELISA is more widely used in present day, as radioisotopes are more expensive and hazardous to use. Aldao *et al.* (1995) developed an indirect competitive ELISA for the quantification of AFB$_1$ in peanuts. It was found that the assay had a limit of detection of 0.25 μg/kg (ppb), which was more than satisfactory to meet international compulsory limits (~5 ppb) for commercial preparations of the particular food. Aflatoxin test kits have also been produced employing ELISA. Abouzied (1998) developed a highly sensitive competitive ELISA using polyclonal antibodies to AFB$_1$. The assay had a limit of detection of 0.05 ng/ml (0.25 ppb), and was validated against high-performance liquid chromatography using corn samples. The assay was found to be reproducible, and the kit was stable for at least nine months when stored at 4° C. There are also commercially available kits for the detection of AFB$_1$. An Oxford-based company called Digen Ltd., develop and sell a kit called "Ridascreen®, Aflatoxin TOTAL". This is a competitive enzyme immunoassay for the quantitative analysis of aflatoxin in cereals, fruits nuts, spices, coffee and feeds. The assay only takes one and a-half-hours from start to finish. It can detect as little as 1.75 ppb, with a recovery rate from extraction of 85% ± 15 %.

Dipstick assays employing antibodies for the detection of mycotoxins have also been developed. Schneider *et al.* (1995) produced a membrane-based visual dipstick enzyme immunoassay for the simultaneous detection of up to five mycotoxins including AFB_1. The visual detection limit for AFB_1 was 2 ng/ml in buffer solution, while in extracted sample it was 30 ng/g. Niessen *et al.* (1998) also developed a dipstick assay for the detection of AFB_1. This was a lateral flow one-step sol particle immunoassay (SPIA), with signals generated by colloidal carbon particles onto which monoclonal antibody specific for AFB_1 was coupled. A diagram of the SPIA is outlined in Figure 3.4. The capture ligand (aflatoxin B_1-BSA (AFB_1-BSA) conjugate) was spotted onto plastic backed nitrocellulose strips in a line format. In the same way a control line was spotted above the test line, using secondary antibodies directed against the AFB_1 specific antibodies (used to test assay performance). In a research set-up, the strips (1) were mounted in a vertical position on a perspex bridge (2). A piece of filter paper was placed on the top end of the strip (3) in order to serve as a fluid drain. The bottom end of the strip was positioned to take a droplet (4) pipetted onto a piece of parafilm. The droplet contained a detection ligand (anti-AFB_1 antibody) coupled to colloidal carbon particles. Upon passing the immobilised capture ligand the detection ligand on the colloidal conjugate specifically bound, resulting in the formation of a visual signal, i.e. a black line. This assay was used for sample analysis in a competitive format. The droplet of fluid also contains the target molecule of interest (AFB_1 sample). During the run the detection ligand would bind the target molecule, which would result in less binding of the detection ligand conjugate to the capture ligand and, as a consequence, loss of signal intensity. Figure 3.5 shows an example of a possible format of a SPIA dipstick for the detection of AFB_1. A detection limit of 0.1 ppb (ng/ml) AFB_1 could be achieved in buffer, while a detection limit of 10 ppb was achieved in extracts of an uncontaminated blank sample spiked with AFB_1.
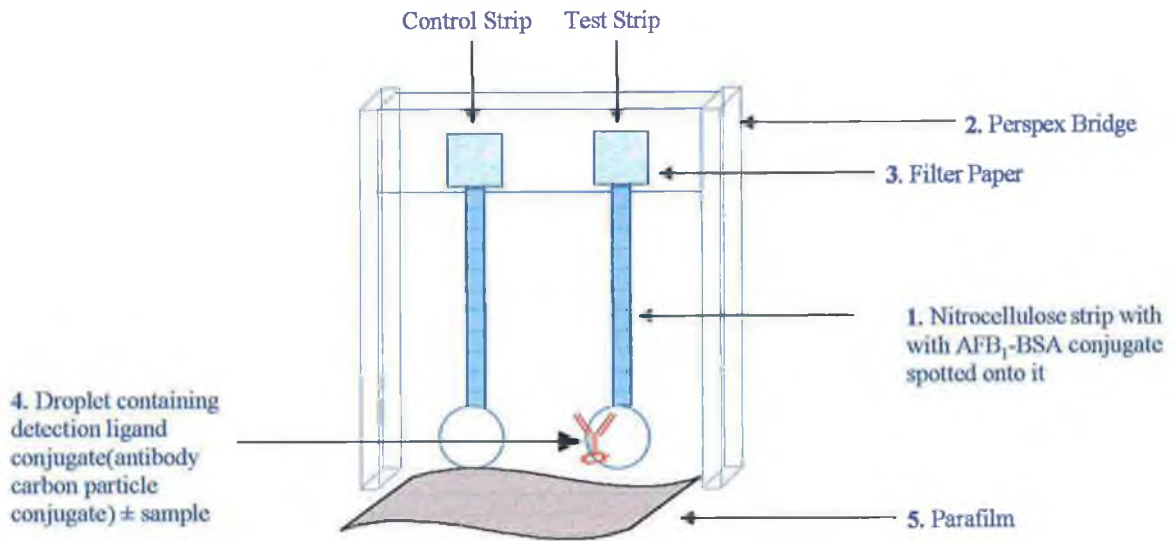
Figure 3.4. Schematic diagram for SPIA.
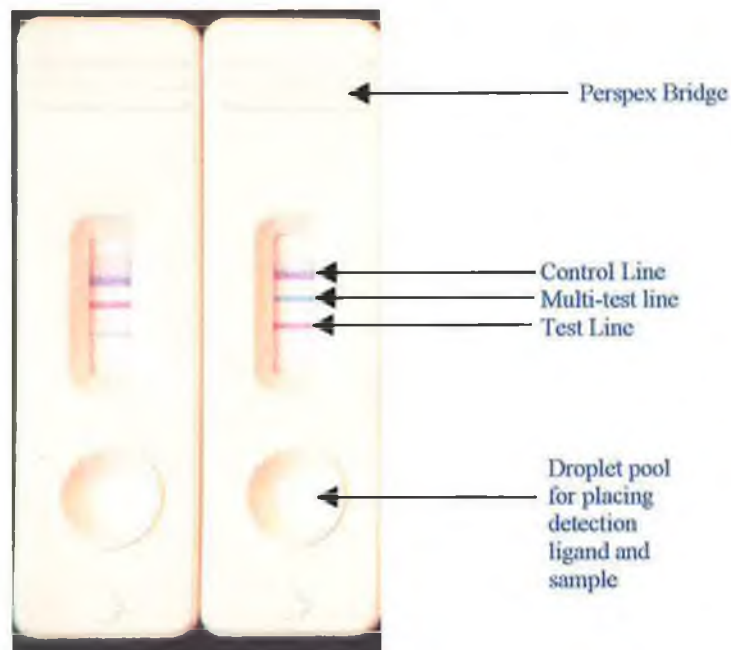
Figure 3.5. Visualisation of an SPIA for the detection AFB$_1$. Three lines are visible on the strip. One of the lines is a control line, while a second one is the test line. The third line is a multi-analyte line for the detection of different ligands. In the case of AFB$_1$, this could be applied for the detection of a number of aflatoxins, providing the antibody was cross-reactive to various other aflatoxins.

3.1.6. *Control of aflatoxins*

Following the discovery of aflatoxins in 1960, several countries have developed legislation to regulate and control mycotoxins (mainly AFB_1) in food and feed. The Food and Drug Administration (FDA) in the United States have established regulatory working guidelines on acceptable levels of aflatoxin in food and feeds. The action level for food is 20 ppb (ng/g) total aflatoxin, while the action level for AFM_1 in milk is 0.5 ppb. This low level for milk was set due to the fact that milk-containing AFM_1 may present a risk to infants (Gourama & Bullerman, 1995). Within the EU, the maximum residue level (MRL) for AFB_1 has been set at 2 ppb, while the U.K. Ministry of Agriculture, Fisheries and Food have proposed that nuts and nut products destined for human consumption should not contain more than 4 ppb of total AFB_1 (Niessen *et al.,* 1998).

From this, it is clear the aflatoxins pose a serious problem to farmers and food processors throughout the world, and as a result there is a great emphasis put on detection methods for such contaminants. Additionally there is also emphasis put on inhibition of *Aspergillus* growth in grain and nuts thus eliminating aflatoxin production.

3.1.7. *Chapter outline*

This chapter describes the production of polyclonal antibodies to AFB_1, their purification and use in ELISA for the detection of AFB_1. It also includes cross reactivities and the application of one polyclonal antibody population for the detection of AFB_1 in spiked grain samples.

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3.2. Results

3.2.1. *Production and characterisation of polyclonal anti-AFB₁ antibodies*

Polyclonal antibodies were produced in two rabbits (rabbits 1 & 2, respectively) against AFB_1 using a commercially available AFB_1-BSA conjugate. They were purified by saturated ammonium sulphate precipitation and protein G affinity-chromatography, as well as by subtractive immunoaffinity chromatography on a 'BSA-immobilised' column. The purity of the antibodies was investigated by SDS-PAGE, and the working dilution for the antibodies in competitive ELISA for AFB_1 was assessed. A linear range of detection was optimised for both antibodies using a competition ELISA and an assessment of the cross reactivity of the antibodies was also made. One of the antibody populations was then applied for the detection of AFB_1 in spiked grain samples using competitive ELISA.

3.2.2. *Estimation of rabbit titres*

New Zealand white rabbits were immunised with a commercially available AFB_1-BSA conjugate as described in section 2.3.3. The rabbits received a sub-cutaneous injection of the conjugate together with an equal volume of Freund's Complete adjuvant. Blood was drawn periodically to estimate the specific antibody titre, which was assessed by ELISA as described in section 2.11.1. One half of a 96-well microtitre plate was coated with AFB_1-BSA conjugate, and the other half of the microtitre plate was coated with BSA. The plate was blocked with 2 % (w/v) casein protein. Serum was then diluted from 1/100 to 1/200,000 in PBS containing 0.1% (w/v) BSA. The serum was also diluted in PBS containing no BSA. The serum diluted in the PBS 0.1% (w/v) BSA was added to the AFB_1-BSA-coated wells, as well as the BSA-coated wells, while the serum diluted in the PBS was added to the BSA-coated wells only. The antibody responses were then detected using a horse-radish peroxidase-labelled anti-rabbit antibody followed by the addition of a chromogenic substrate. The titre was taken to be the highest dilution of immunised serum which gave a signal greater than the corresponding dilution of control serum, i.e. the serum diluted in PBS containing 0.1% (w/v) BSA and added to the BSA-

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coated plate. Background levels (i.e. PBS containing 0.1% (w/v) BSA-added to the BSA-coated plate) were also considered when determining the highest antibody titre. However, these were minimal. The final titre for serum for rabbit 1 was 1/204,800, while the final titre for serum for rabbit 2 was 1/102,400. Figures 3.6 and 3.7, respectively show these results, including the antibody responses to BSA, with or without BSA in the diluent buffer. The figures also demonstrate that there was a greater antibody response to AFB_1 than to BSA, as well as that 0.1 % (w/v) BSA was sufficient to remove non-specific binding of the serum to the BSA part of the AFB_1-BSA conjugate.
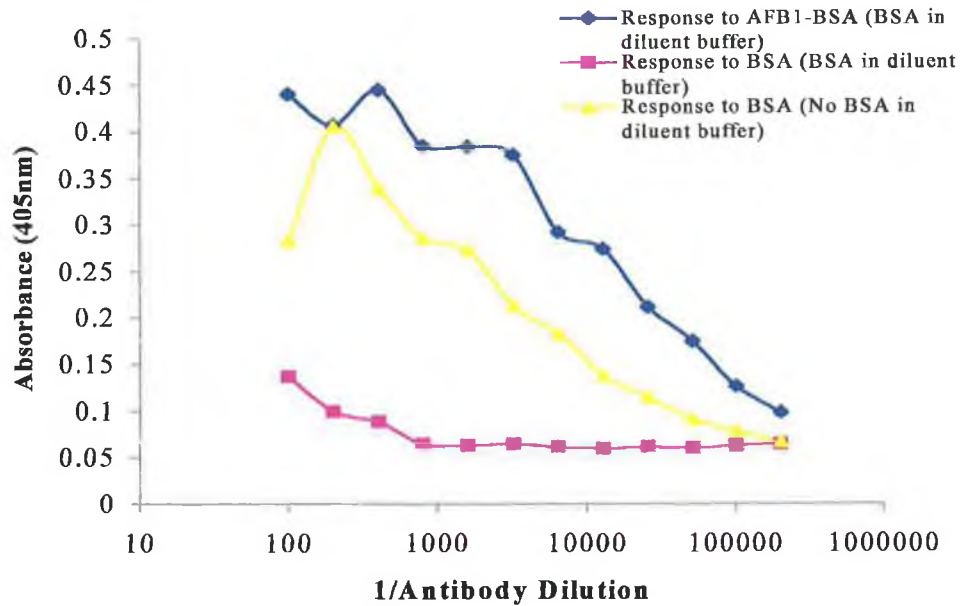
Figure 3.6. *Final titre of serum obtained from rabbit 1. The serum was diluted in PBS as well as PBS containing 0.1% (w/v) BSA, and added to wells of a microtitre plate coated with AFB₁-BSA conjugate. Diluted serum was also added to wells of a microtitre plate coated with BSA. The final titre recorded was in excess of 1/204,800.*
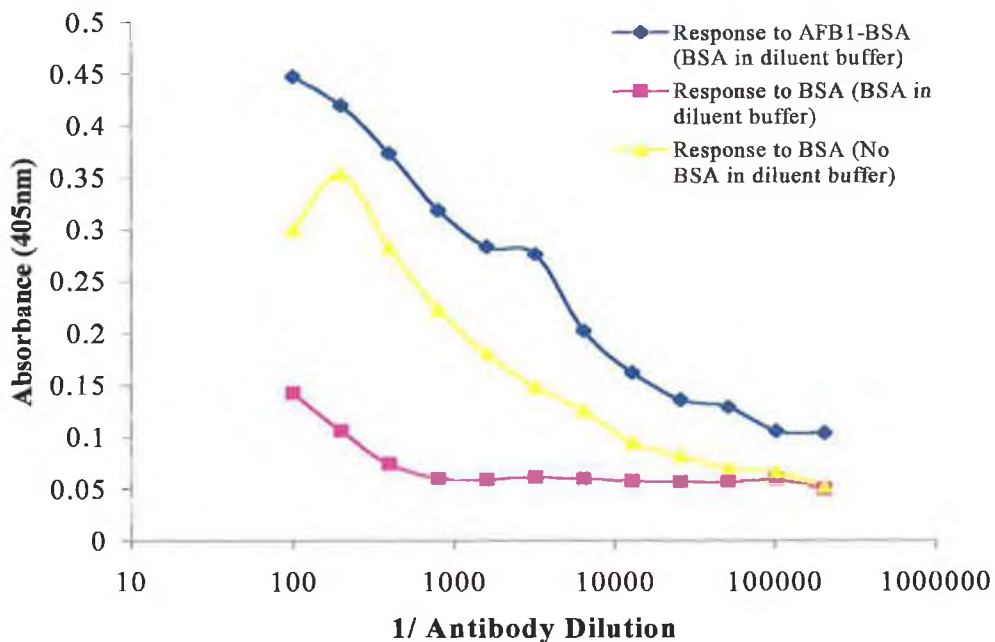
Figure 3.7. *Final titre of serum obtained from rabbit 2. The serum was diluted in PBS as well as PBS containing 0.1% (w/v) BSA, and added to wells of a microtitre plate coated with AFB₁-BSA conjugate. Diluted serum was also added to wells of a microtitre plate coated with BSA. The final titre recorded was 1/102,400.*

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3.2.3. *Purification of polyclonal antiserum*

The serum was initially purified by saturated ammonium sulphate precipitation as described in section 2.5.1. It was further purified by protein G affinity-chromatography as described in section 2.5.2. IgG bound to the protein G column, and were eluted after washing, by addition of glycine buffer. The presence of antibody in the collected fractions was monitored by absorbance at 280 nm. Typical elution profiles for serum obtained from rabbits 1 & 2 are shown in Figure 3.8. The profiles show that a higher protein concentration of antibody was eluted from the serum obtained from rabbit 1, as well as that the majority of the protein eluted in fewer fractions compared to that of the serum of rabbit 2. These fractions were pooled and dialysed. Both antibody populations were further purified by subtractive immunoaffinity chromatography. Figure 3.9 shows titre curves for both antibody populations after subtractive immunoaffinity chromatography was carried out. The purification was successful as it removed all of the BSA binding antibodies while not affecting the specific antibody titre to AFB_1.

3.2.4. *Characterisation of purified antibodies by SDS-PAGE*

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) is a technique which separates proteins on the basis of their molecular weights. The purity of both polyclonal antibody populations from rabbits 1 & 2, respectively, was assessed by SDS-PAGE as described in section 2.7. The antibody was electrophoresed under reducing conditions, along with a molecular weight marker containing proteins of different weights. Purity was assessed as a comparison between serum, ammonium sulphate-precipitated antibody, and the affinity-purified antibody. Figures 3.10 and 3.11, respectively, show the gels obtained for both antibodies. Boiling the antibody samples together with mercaptoethanol causes the polypeptide chains of the immunoglobulins to separate, resulting in two main sample bands being observed. One band, with a molecular weight of *ca.* 50 kDa shows the presence of the heavy chains. A second band is barely visible at *ca.* 25 kDa and indicates the presence of the antibody light chains. The figures also demonstrate that

the purification steps have worked effectively in the isolations of pure immunoglobulins from serum.
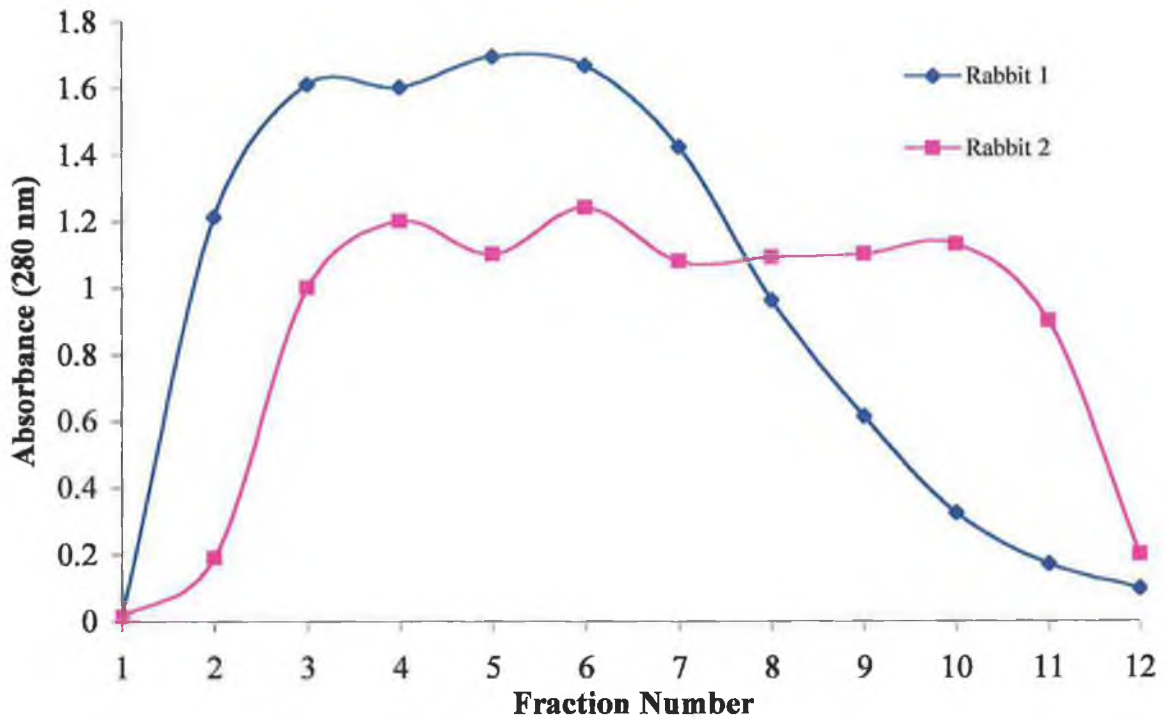
Figure 3.8. *Typical elution profiles for polyclonal anti-AFB₁ antibodies from a 1 ml protein G affinity column. Saturated ammonium sulphate precipitated samples were passed through the column, followed by 20 column volumes of wash buffer. Bound antibody was then eluted using 0.1 M glycine, pH 2.7. 0.5 ml fractions were collected, and the protein content was measured by absorbance at 280 nm. In the case of rabbit 1, fractions 2-7 were collected and pooled, while for rabbit 2, fractions 3-11 were used.*
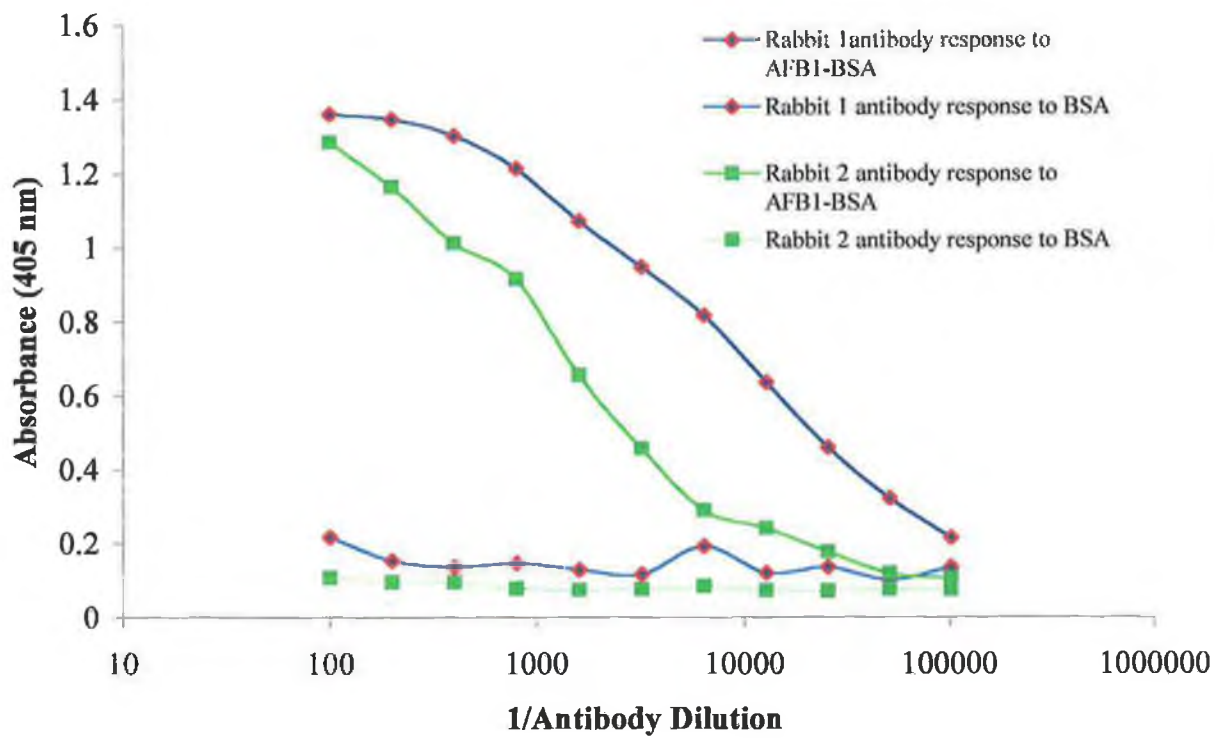
Figure 3.9. *Titres of serum obtained for rabbits 1 & 2, which were purified by saturated ammonium sulphate precipitation, protein G affinity-chromatography and subtractive immunoaffinity chromatography. The curves indicate that subtractive immunoaffinity chromatography was successful in removing all of the BSA-binding antibodies, while not reducing the specific antibody response to AFB_1-BSA.*
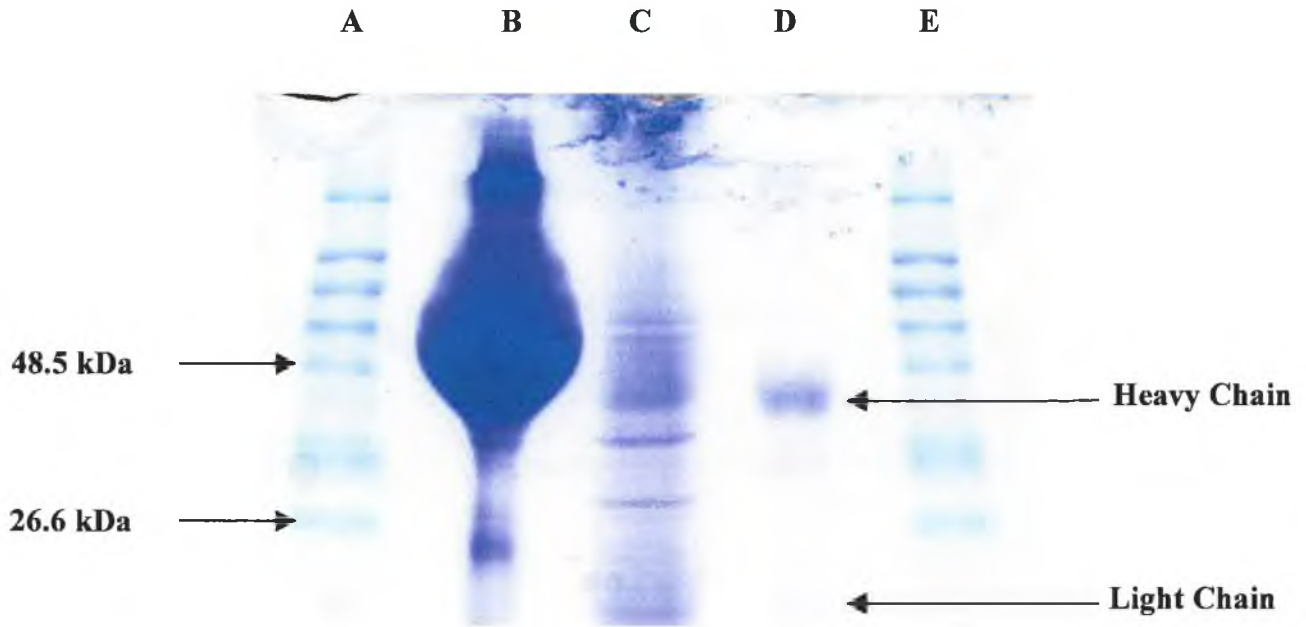
Figure 3.10. *SDS-PAGE showing serum (B), ammonium sulphate precipitate (C), and affinity-purified polyclonal anti-AFB₁ antibodies (D) from rabbit 1. These were electrophoresed under reducing conditions. Lanes A and E contained markers consisting of α_2 macroglobulin (180 kDa), β galactosidase (116 kDa), fructose-6-phosphate kinase (84 kDa), pyruvate kinase (58 kDa), fumarase (48.5 kDa), lactate dehydrogenase (36.5 kDa), and triosephosphate isomerase (26.6 kDa). The purified sample in lane D has one band below the 48.5 kDa markers, and one less distinct one below that for the 26.6 kDa marker consistent with the presence of the heavy chain and the light chain of IgG. The less intense light chain may be due to the fact that the staining method used was not sensitive enough to detect it.*
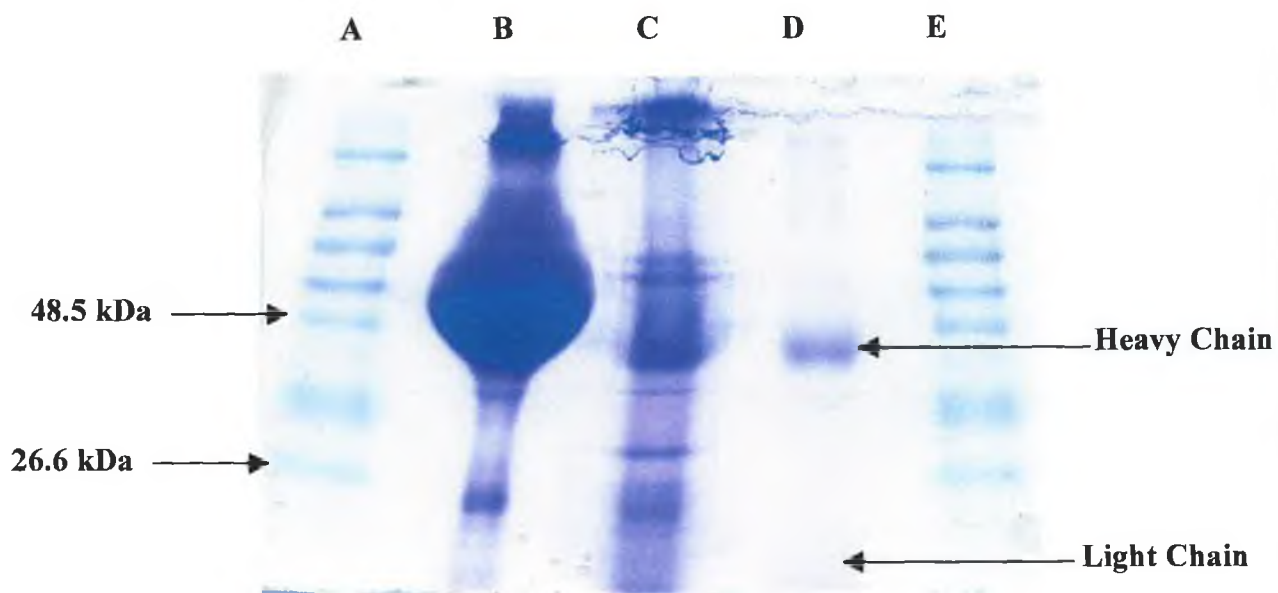
Figure 3.11. *SDS-PAGE showing serum (B), saturated ammonium sulphate precipitate (C), and affinity-purified polyclonal anti-AFB₁ antibodies (D) from rabbit 2. These were electrophoresed under reducing conditions. Lanes A and E contained markers consisting of the same proteins as mentioned previously. The purified sample in lane D has one band below the 48.5 kDa markers, and one less intense band below that for the 26.6 kDa marker consistent with the presence of the 50 kDa heavy chain and the 25 kDa light chain of IgG. The less intense light chain may be due to the fact that the staining method used was not sensitive enough to detect it.*

3.2.5. Determination of working dilution of antibodies in competitive ELISA for AFB$_1$

A checkerboard ELISA was carried out as described in section 2.11.2 in order to determine the optimal antibody dilution and conjugate concentration for use in competitive ELISA. Rows of wells of a 96-well microtitre plate were coated with concentrations of AFB$_1$-BSA ranging from 0 to 50 µg/ml, and blocked with 2 % (w/v) casein protein. Dilutions of the purified polyclonal anti-AFB$_1$ antibodies from 1/100 to 1/100,000 were prepared and added to the plate. After incubation, enzyme-labelled anti-rabbit antibody was added followed by chromogenic substrate. Figures 3.12 and 3.13 show the overlaid titre curves obtained from the antibodies isolated from rabbit's 1 & 2, respectively. The antibody dilution that gave half the maximum absorbance was chosen, and the lowest conjugate concentration that provided sufficiently high absorbances was chosen. For rabbit 1, a 1/10,000 dilution of antibody was used in the competition ELISA, with a conjugate concentration of 20 µg/ml. For rabbit 2, the antibody dilution used was 1/20,000 with a conjugate concentration used of 50 µg/ml.

3.2.6. *Competitive ELISA for the detection of AFB$_1$*

A series of standards of AFB$_1$ ranging in concentration from 12-25,000 ng/ml for rabbit 1 and 24-1,563 ng/ml for rabbit 2 were prepared as described in section 2.10.1. 50 µl of each of these standards were then added to an AFB$_1$-BSA-coated plate (see section 2.11.3). 50 µl/well of each of the affinity-purified anti-AFB$_1$ antibodies obtained from rabbits 1 & 2, respectively, were prepared at the appropriate dilution and added to the plates. After incubation and washing, enzyme-labelled anti-rabbit antibody was added followed by chromogenic substrate. The linear range of detection for the antibodies obtained from rabbit 1 was 12 to 25,000 ng/ml, while for rabbit 2 it was 24 to 1,563 ng/ml. For intra-day assay variation, each concentration was assayed five times on the one day and the mean absorbance of bound antibody for each antigen concentration was plotted versus antigen concentration. Figures 3.14 and 3.15 show the intra-day assay linear ranges of detection of AFB$_1$ for rabbit's 1 & 2, respectively. Tables 3.1 and 3.2 show the coefficients of variation (C.V.'s) for

both rabbits. Intra-day percentage C.V.'s for the calibration plots of both the assays were below 9 %.

The inter-day assay variation was calculated by performing the assay over five separate days, and a separate calibration curve plotted for each normalised absorbance (absorbance/absorbance 0 (A/Ao)) value versus the respective AFB_1 concentration for each assay carried out on each day. The normalised mean values for these assays were calculated and plotted as one assay. Figures 3.16 and 3.17 show the inter-day variability assays for each of the antibody populations obtained from rabbits 1 & 2. The percentage C.V.'s for each of the inter-day assays are shown in Tables 3.3 and 3.4, respectively. All of the percentage C.V.'s for both assays were no greater than 9 % except for one concentration shown on Table 3.3 for the assay developed using rabbit 1 antibodies.
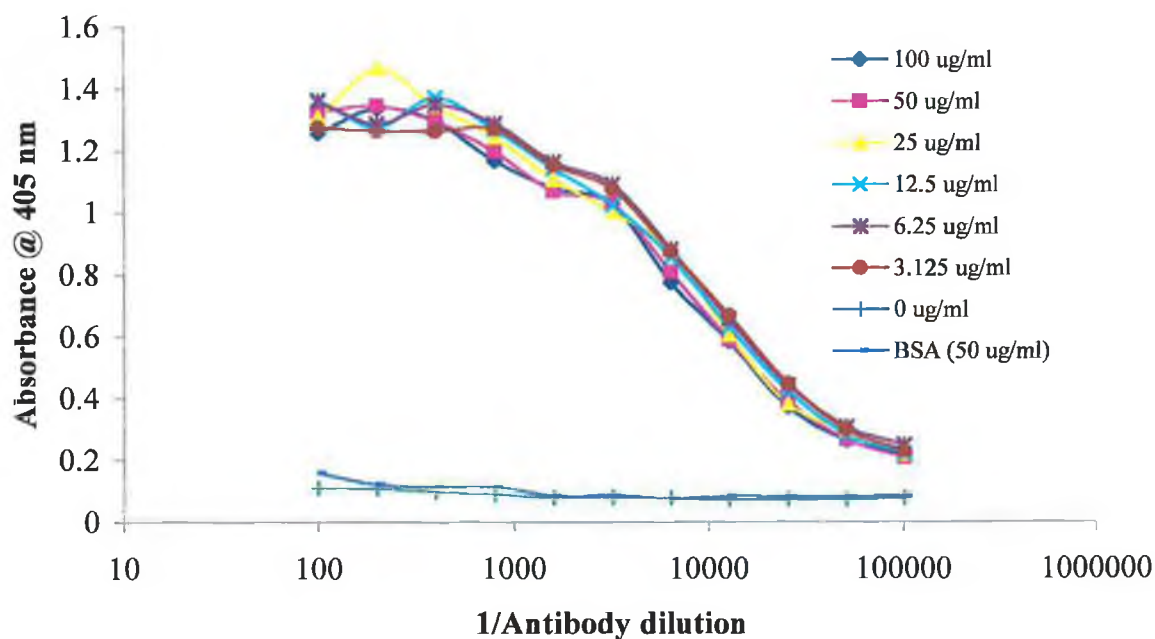
Figure 3.12. *Determination of optimal conjugate loading density and antibody dilution.*
Wells of a microtitre plate were coated with AFB₁-BSA conjugate at a range of
concentrations between 0 and 100 μg/ml. One row on the plate was coated with 50 μg/ml of
BSA as a control. Serial doubling dilutions of the affinity-purified anti-aflatoxin antibody
(Rabbit 1) were carried out and then added to the plate as described in section 2.11.3. The
optimal conjugate concentration chosen was 20 μg/ml, and the optimal antibody dilution (i.e.
the one that gave half the maximum absorbance) was 1/10,000.
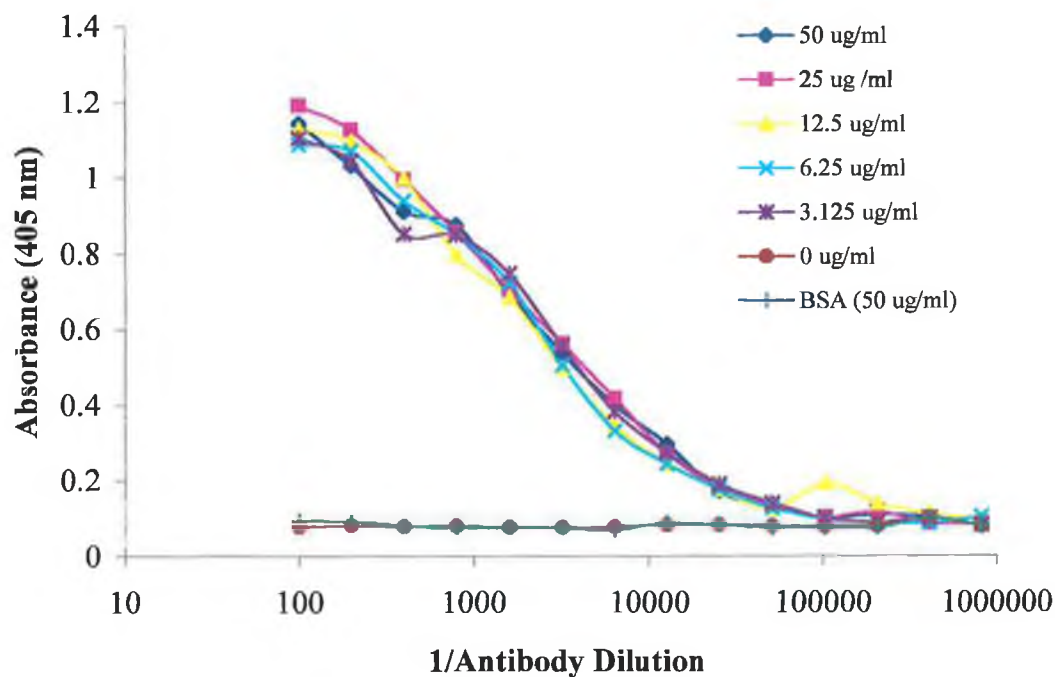
Figure 3.13. Determination of optimal conjugate loading density and antibody dilution. Wells of a microtitre plate were coated with AFB_1-BSA conjugate at a range of concentrations between 0 and 50 $\mu g/ml$. One row on the plate was coated with 50 $\mu g/ml$ of BSA as a control. Serial doubling dilutions of the affinity-purified anti-aflatoxin antibody (Rabbit 2) were carried out and then added to the plate as described in section 2.11.3. The optimal conjugate concentration chosen was 50 $\mu g/ml$, and the optimal antibody dilution (i.e. the one that gave half the maximum absorbance) was 1/20,000.
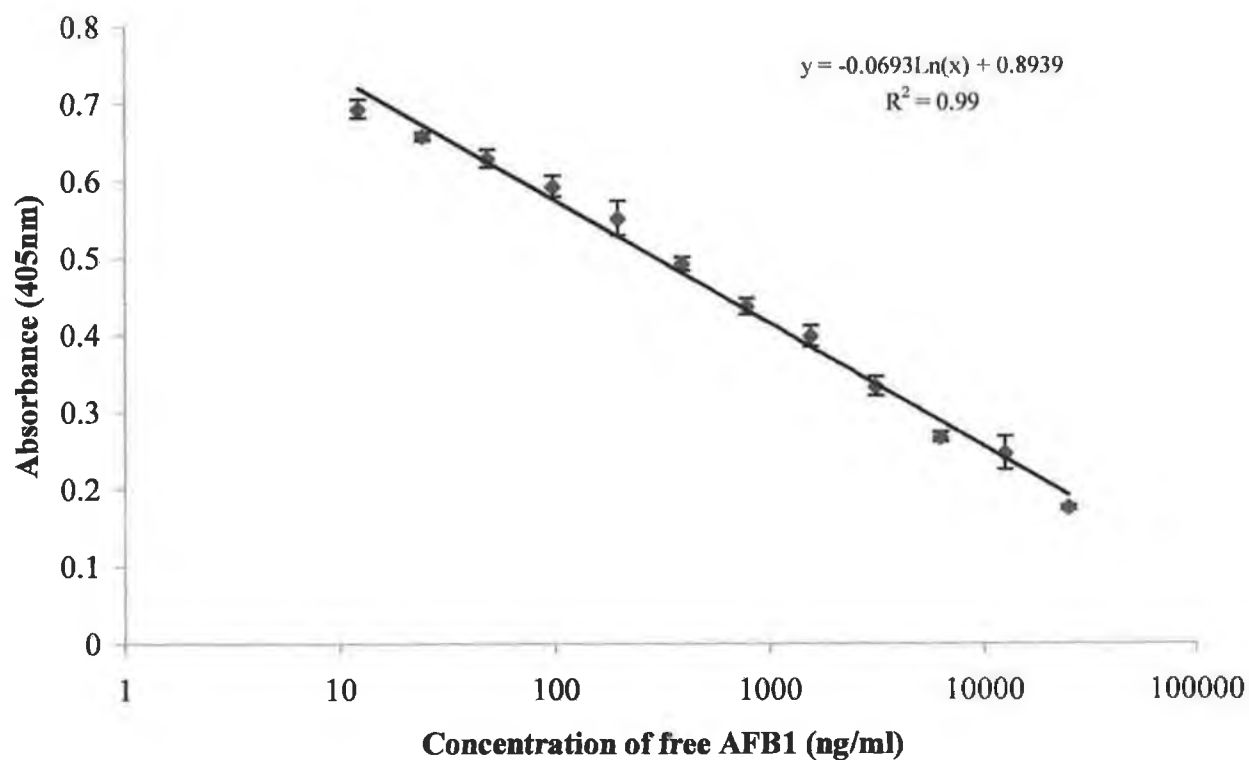
Figure 3.14. *Intra-day assay variation for competitive ELISA for AFB₁ using affinity-purified anti-AFB₁ antibodies obtained from rabbit 1. The linear range of detection was 12.0 to 25,000 ng/mL. The error bars on each calibration point indicate the standard deviation of the mean of five measurements.*

Table 3.1. Intra-day assay coefficients of variation for the determination of free AFB_1 using affinity-purified antibody obtained from rabbit 1. Five sets of twelve standards were assayed the same day, and the coefficient of variation (C.V.'s) were calculated as the standard deviation (S.D.) expressed as a percentage of the mean values for each standard.

Aflatoxin B_1 concentration (ng/ml)	Calculated mean ± S.D.	Coefficient of variation (%)
25,000	0.176 ± 0.003	1.73
12,500	0.246 ± 0.021	8.65
6,250	0.267 ± 0.006	2.32
3,125	0.333 ± 0.013	3.78
1,563	0.398 ± 0.014	3.42
781	0.437 ± 0.010	2.29
391	0.499 ± 0.009	1.79
195	0.551 ± 0.023	4.09
98	0.593 ± 0.014	2.34
49	0.629 ± 0.011	1.79
24	0.658 ± 0.005	0.79
12	0.694 ± 0.012	1.75

Table 3.2. *Intra-day assay coefficients of variation for the determination of free AFB$_1$ using affinity-purified antibody obtained from rabbit 2. Five sets of seven standards were assayed the same day, and the C.V.'s were calculated as the standard deviation (S.D.) expressed as a percentage of the mean values for each standard.*

Aflatoxin B$_1$ concentration (ng/ml)	Calculated mean ± S.D.	Coefficient of variation (%)
1,563	0.312 ± 0.005	1.64
781	0.344 ± 0.004	1.27
391	0.374 ± 0.009	2.43
195	0.399 ± 0.004	1.01
98	0.429 ± 0.004	0.97
49	0.456 ± 0.008	1.66
24	0.471 ± 0.006	1.32

$$y = -0.0698Ln(x) + 1.0803$$
$$R^2 = 0.99$$
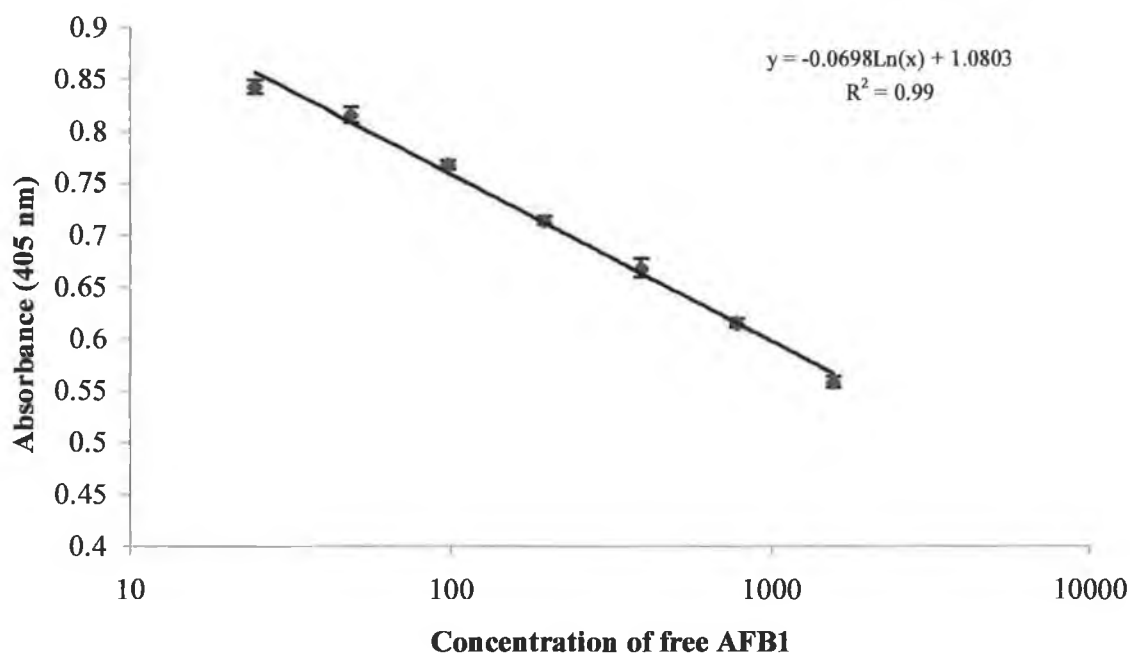
Figure 3.15. *Intra-day assay variation for competitive ELISA for AFB$_1$ using affinity-purified anti-AFB$_1$ antibodies obtained from rabbit 2. The linear range of detection was 24.0 to 1,563 ng/ml. The error bars on each calibration point indicate the standard deviation of the mean of five measurements.*
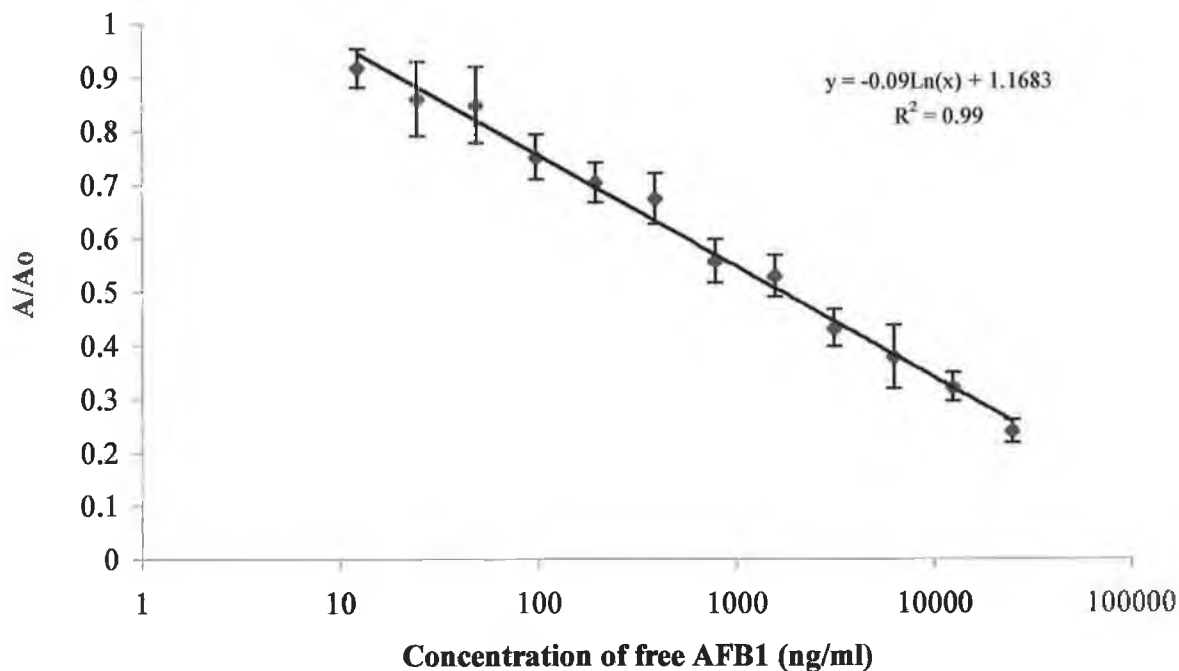
Figure 3.16. *Inter-day assay variation for competitive ELISA for AFB_1 using affinity-purified anti-AFB_1 antibodies obtained from rabbit 1. The linear range of detection was 12.0 to 25,000 ng/mL. The error bars on each calibration point indicate the standard deviation of the mean of five measurements. The results were normalised by dividing the absorbance values (A) by the absorbance value obtained for zero concentration of antigen (Ao) in the assay.*

Table 3.3. *Inter-day assay coefficients of variation for the determination of free AFB_1 using affinity-purified antibody obtained from rabbit 1. Five sets of twelve standards were assayed on five different days, and the C.V.'s were calculated as the standard deviation expressed as a percentage of the mean normalised values for each standard.*

Aflatoxin B_1 concentration (ng/ml)	Calculated mean ± S.D.	Coefficient of variation (%)
25,000	0.238 ± 0.022	9.08
12,500	0.321 ± 0.027	8.46
6,250	0.377 ± 0.059	15.59
3,125	0.431 ± 0.035	8.02
1,563	0.528 ± 0.039	7.36
781	0.556 ± 0.040	7.24
391	0.672 ± 0.047	6.93
195	0.703 ± 0.037	5.20
98	0.750 ± 0.041	5.49
49	0.848 ± 0.070	8.29
24	0.859 ± 0.070	8.07
12	0.917 ± 0.036	3.88

Table 3.4. *Inter-day assay coefficients of variation for the determination of free AFB$_1$ using affinity-purified antibody obtained from rabbit 2. Five sets of seven standards were assayed on five different days, and the C.V.'s were calculated as the standard deviation expressed as a percentage of the mean normalised values for each standard.*

| Aflatoxin B$_1$ concentration (ng/ml)	Calculated mean ± S.D.	Coefficient of variation (%)
1,563	0.520 ± 0.036	6.92
781	0.603 ± 0.043	7.17
391	0.669 ± 0.013	1.97
195	0.720 ± 0.016	2.16
98	0.764 ± 0.007	0.95
49	0.832 ± 0.018	2.21
24	0.859 ± 0.026	2.99

$$y = -0.0807Ln(x) + 1.1355$$
$$R^2 = 0.9853$$

Figure 3.17. *Inter-day assay variation for competitive ELISA for AFB$_1$ using affinity-purified anti-AFB$_1$ antibodies obtained from rabbit 2. The linear range of detection was 24.0 to 1,563 ng/ml. The error bars on each calibration point indicate the standard deviatio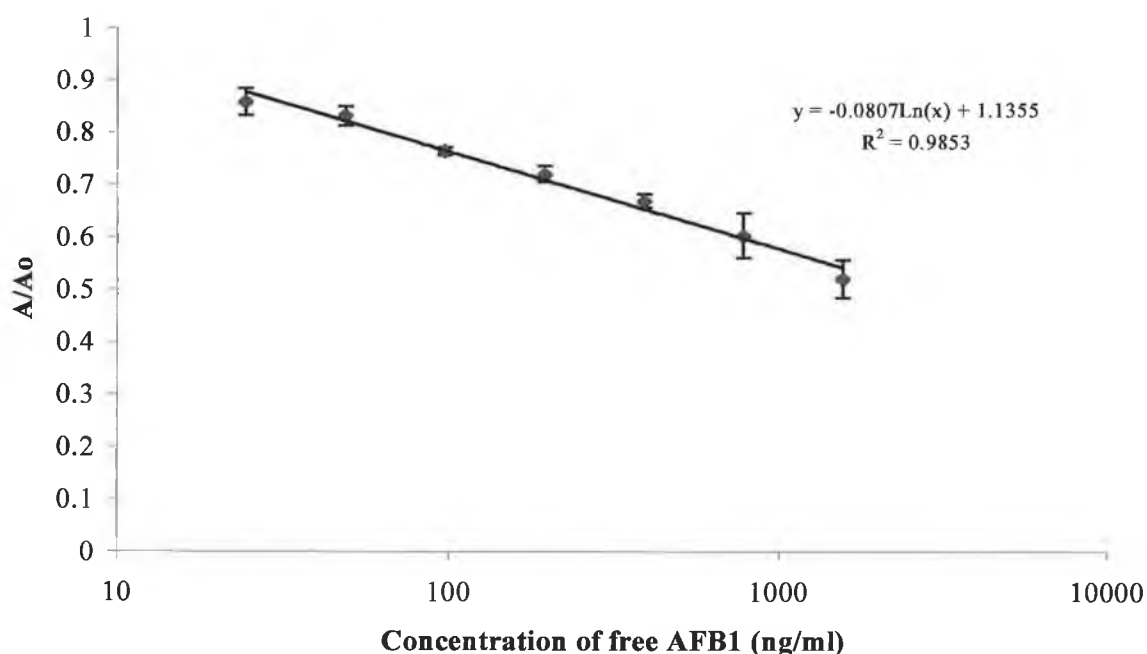n of the mean of five measurements. The results were normalised by dividing the absorbance values (A) by the absorbance value obtained for zero concentration of antigen (Ao) in the assay.*

3.2.7. *Cross-reactivity studies*

Cross reactivity studies were carried out on the antibodies obtained from rabbits 1 & 2 to seven other structurally related aflatoxins, as described in section 2.11.4. Standard curves for each of the aflatoxins were produced as described in previously in 3.2.6. The results were normalised and plotted. The slope of the line of the standard curve for each toxin was expressed as a percentage of the slope of the line for binding to AFB_1. Table 3.5 shows the cross-reactions of the affinity-purified antibodies obtained from rabbits 1 & 2. Both antibody populations showed a high level of cross-reaction to the structurally related aflatoxins.

Table 3.5. *Cross-reactions of affinity-purified anti-AFB₁ antibodies obtained from rabbits 1 & 2 observed to seven other aflatoxins. Standard curves were produced for each of the aflatoxins in the same way as that for AFB₁. Each concentration for each point on the standard curve was assayed in triplicate and the means plotted and normalised in order to construct standard curves.*

Aflatoxin	Rabbit 1 % Cross Reactivity	Rabbit 2 % Cross Reactivity
B_1	100	100
B_2	67.5	47.9
G_1	65.5	65.0
G_2	50.1	29.9
M_1	35.0	34.0
M_2	1.7	<1
B_{2a}	33.4	37.3
G_{2a}	15.5	<1

3.2.8. *Optimisation of a competitive ELISA for the detection of AFB₁ in spiked grain samples*

The affinity-purified antibodies obtained from rabbit 1 were chosen for use in the detection of AFB_1 in spiked grain samples. Spiked grain samples were prepared as described in section 2.10.3, and were applied in a competitive ELISA as described in section 2.11.3. The linear range of detection for the detection of AFB_1 in spiked grain samples was 98 to 12,500 ng/ml. Figure 3.18 shows this result as the intra-day assay. This was carried out by running five sets of each concentration on the one day, and calculating the mean absorbance, which was plotted against concentration of free toxin. Table 3.6 shows the percentage C.V.'s for the assay, which were all lower than 7 %. Inter-day assays were also carried out by running five sets of each standard on five different days. The results were normalised and plotted as mean of all five sets of results. Figure 3.19 shows the inter-day graph plotted along with percentage C.V.'s in Table 3.7, which were no greater than 12 %.

Table 3.6. Intra-day assay coefficients of variation in grain samples spiked with AFB_1 using affinity-purified antibody obtained from rabbit 1. Five sets of eight standards were assayed the same day, and the C.V.'s were calculated as the standard deviation (S.D.) expressed as a percentage of the mean values for each standard.

Aflatoxin B_1 concentration (ng/ml)	Calculated mean ± S.D.	Coefficient of variation (%)
12,500	0.191 ± 0.050	2.82
6,250	0.208 ± 0.005	2.17
3,125	0.269 ± 0.009	3.16
1,563	0.330 ± 0.017	5.01
781	0.377 ± 0.019	5.08
391	0.426 ± 0.022	5.17
195	0.477 ± 0.031	6.55
98	0.531 ± 0.020	3.80
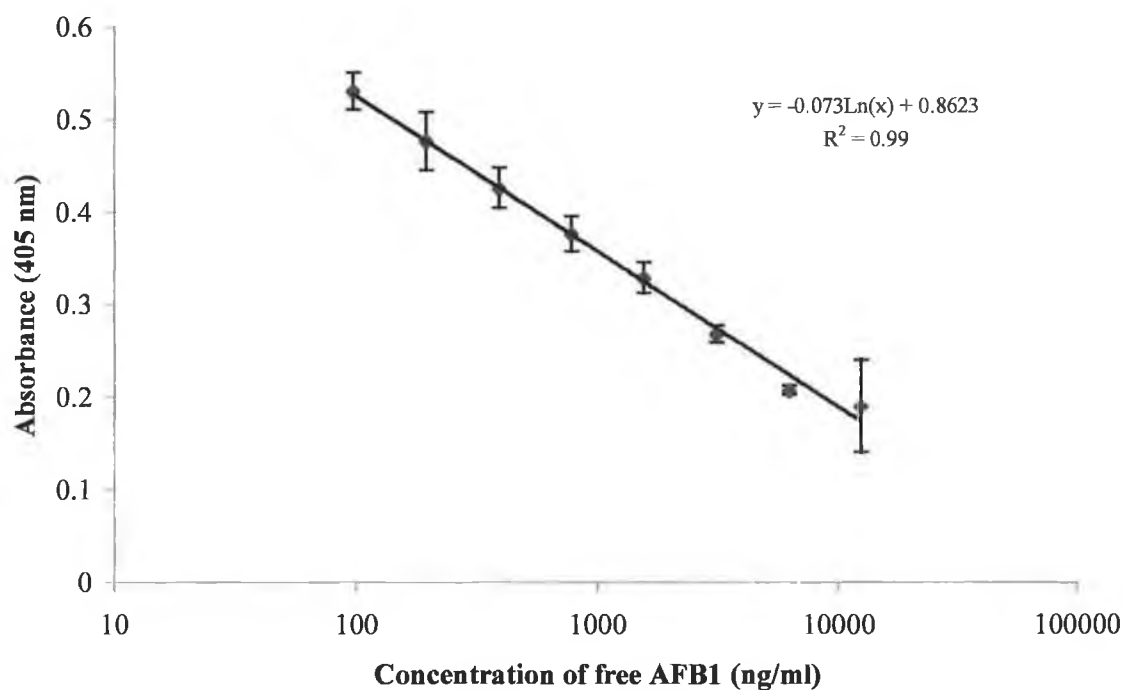
Figure 3.18. Intra-day assay variation for competitive ELISA in grain samples spiked with AFB_1, using affinity-purified anti-AFB_1 antibodies obtained from rabbit 1. The linear range of detection was 98 to 12,500 ng/mL. The error bars on each calibration point indicate the standard deviation of the mean of five measurements.

Table 3.7. *Inter-day assay coefficients of variation in grain samples spiked with AFB₁ using affinity-purified antibody obtained from rabbit 1. Five sets of eight standards were assayed on five different days, and the C.V.'s were calculated as the standard deviation expressed as a percentage of the mean normalised values for each standard.*

Aflatoxin B₁ Concentration (ng/ml)	Calculated mean ± S.D.	Coefficient of variation (%)
12,500	0.300 ± 0.036	12.10
6,250	0.381 ± 0.009	2.55
3,125	0.451 ± 0.031	6.96
1,563	0.552 ± 0.036	6.52
781	0.611 ± 0.054	8.76
391	0.708 ± 0.055	7.80
195	0.771 ± 0.071	9.28
98	0.830 ± 0.094	11.28

$y = -0.1116 \text{Ln}(x) + 1.3574$
$R^2 = 0.99$
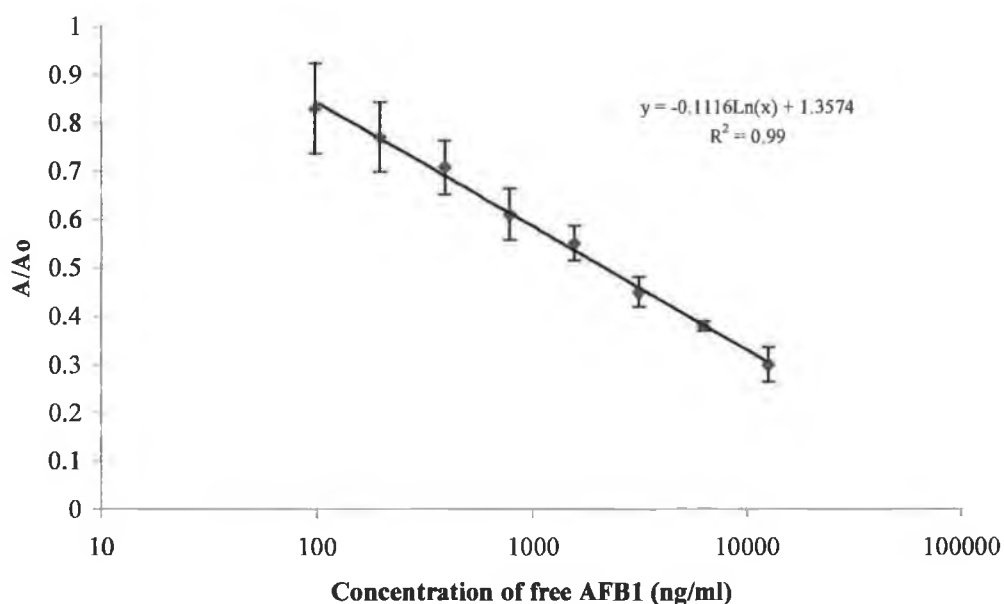
Figure 3.19. *Inter-day assay variation for competitive ELISA in grain samples spiked with AFB₁, using affinity-purified anti-AFB₁ antibodies obtained from rabbit 1. The linear range of detection was 98 to 12,500 ng/ml. The error bars on each calibration point indicate the standard deviation of the mean of five measurements. The results were normalised by dividing the absorbance values (A) by the absorbance value obtained for zero concentration of antigen (Ao) in the assay.*

3.3. Discussion

The work presented in this chapter is concerned with the production of polyclonal antibodies to aflatoxin B_1, and their characterisation and application in ELISA. Aflatoxin B_1 is a small hapten with a molecular weight of 320 Da. Due to its small size, the toxin needed to be conjugated to a larger protein such as BSA in order to elicit an immune response in an animal following immunisation. This conjugate was commercially available, and according to the manufacturers, consisted of 7-8 AFB_1 molecules for every BSA molecule present. Two New Zealand white rabbits were immunised with this protein conjugate with the aim to produce antibodies. Both rabbits gave high antibody titres, with rabbit 1 giving a slightly higher titre than rabbit 2.

Only one protein conjugate was commercially available, and because of the hazards involved with handling free AFB_1, it was decided to use this conjugate for both the immunisation and analytical steps. As a result, when carrying out antibody titres using this conjugate, a number of controls also needed to be included. Serial dilutions of the serum were carried out in both PBS, and PBS containing 0.1 % (w/v) BSA. The serum diluted in the PBS-BSA was added to AFB_1-BSA-coated wells of a microtitre plate, in order to ensure specific antibody binding to AFB_1 was observed (and not to BSA). The PBS-BSA-diluted serum was also added to BSA-coated wells of a microtitre plate, in order to ensure that 0.1 % (w/v) BSA was sufficient to remove the non-specific binding of the antibody population to BSA. The serum diluted in PBS only was added to BSA-coated wells, which demonstrated the non-specific immune response to BSA. These controls worked effectively, as 0.1 % (w/v) BSA was sufficient to remove the non-specific interactions, as well as showing that there was a greater immune response to the AFB_1 part, as opposed to the BSA part of the conjugate. This indicates that AFB_1 is highly immunogenic, which may be due to the manner in which AFB_1 was conjugated to the protein. Holtzapple *et al.* (1996) state that when designing an immunogen, it is preferable to introduce new functional groups (for attachment of the hapten to the carrier) at positions originally unfunctionalised compared to derivatising determinant groups that are already present

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on the molecule. They also state that antibodies generally recognise the part of the molecule most-distal to the point of attachment of the carrier protein than other regions. In the case of AFB_1, an O-carboxymethylhydroxylamine linker was added on the carbonyl group on the cyclopentanone ring of the molecule. Holtzapple *et al.* (1996) have carried out molecular modelling studies on the structurally similar AFM_1 molecule which was linked in the same was as AFB_1. They found that generation of an oxime at the carbonyl group on the cyclopentanone ring did not cause major structural damage in any of the other rings. As a result, it is possible that a similar situation has occurred with AFB_1. O-carboxymethylhydroxylamine is also a relatively short linker, and as a result the hapten would be kept in close proximity to the BSA molecule. The molecule was not structurally changed in any significant manner during conjugation, and it was also held in close proximity to BSA. Both of these findings, coupled with the fact that there is a relatively high substitution ratio of hapten: protein, suggest an explanation for such a high and specific antibody titre for AFB_1 in both rabbits.

Purification of the serum was carried out by ammonium sulphate precipitation, and protein G affinity-chromatography. This worked effectively, with all of the bound immunoglobulin eluting easily from the protein G column. The low pH of the glycine buffer did not affect the integrity of the eluted immunoglobulins, as each fraction tube contained 2 M Tris, which neutralised the pH of the eluted antibodies. Subtractive immunoaffinity chromatography was also carried out on the antibody populations, in order to remove all of the 'BSA-binding antibodies'. This was highly effective with only background binding observed when the purified antibodies were titered against BSA (Figure 3.9). Subtractive immunoaffinity chromatography has been used elsewhere for the isolation of anti-BSA antibodies from an anti-AFB_1 antibody population (Aldao *et al.*, 1995). They found the technique to be successful in the depletion of anti-BSA antibodies from the population. SDS-PAGE was carried out on both antibody populations in order to determine their purity. Both antibody populations showed high degrees of purity, compared to that of serum or the ammonium sulphate precipitate. However, in both electropherograms (Figures 3.10

and 3.11) the light chain band at 25 KDa is not very apparent. This may be due to its lower molecular weight as well as its lower affinity for the dye. It may also have migrated on the gel at a different rate to the serum and ammonium sulphate sample, due to both samples being in matrices of different protein concentrations.

Antibodies achieve their highest degrees of sensitivity in a competitive ELISA when the primary antibody concentration approaches zero i.e. when the antibody response in decreasing antigen concentrations is approaching the absorbance achieved for primary antibody binding to immobilised conjugate in the absence of free antigen on the microtitre plate. As a result of this, limits of detection of antibodies in a competitive ELISA are a function of varying antibody affinities (in the case of polyclonal antibodies), and the equilibrium between both free and immobilised forms of the antigen. Consequently, it is important to optimise conjugate loading density for application in a competitive ELISA, as using too much conjugate on the microtitre plate surface will shift the binding equilibrium in favour of binding to the ELISA plate, and cause reduced sensitivity to free antigen in solution (i.e. the desired detectable antigen). It is also necessary to optimise the antibody concentration for use in the assay, as too high an antibody concentration will result in an excess of free antibody in solution, and therefore will only be suitable to detect high concentrations of free antigen in solution. It is essential therefore, to optimise the concentration of hapten-conjugate and antibody for use in a competitive ELISA. Two separate checkerboard ELISA's were carried out for both antibody populations obtained from rabbits 1 & 2, respectively, as described in section 2.11.2. The antibody dilution for rabbit 1 was 1/10,000 with a conjugate loading density of 20 μg/ml for the assay. In the case of rabbit 2 the antibody dilution was 1/20,000 with a conjugate loading density of 50 μg/ml.

Competition ELISA's were optimised for both antibody populations using these parameters. Both assays showed good levels of sensitivity. Antibodies obtained from rabbit 1 gave a linear range of detection of between 12 and 25,000 ng/ml (Figure 3.14), while antibodies obtained from rabbit 2 had a linear range of detection

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of between 24 and 1,563 ng/ml (Figure 3.15). Inter-day assay variation studies were carried out by analysing five sets of standards across the respective linear ranges on five different days and calculating the C.V.'s as the standard deviation expressed as a percentage of the mean normalised values for each standard.

Both assays were reproducible over five different days and the C.V.'s for them are shown in Tables 3.3 and 3.4, respectively. The C.V.'s for both assays are well within the acceptance limits, as they are all less than 10 %. One point in the rabbit 1 assay was above 10 %, which was possibly due to a pipetting error when the assay was being carried out on one of the given five days. Both assays are quite sensitive, with the assay optimised with rabbit 1 antibodies being the more sensitive. It had a limit of detection of 12 ng/ml while the assay optimised with rabbit 2 assay had a limit of 24 ng/ml. The assays also demonstrate good precision profiles (i.e. the quantitative measure of the variation between measurements, usually the C.V.'s versus the nominal concentration of analyte in the sample). However other assays have been produced that are more sensitive than these ELISA's developed. Aldao *et al.* (1995) developed a competition ELISA with a detection limit of 25 pg/ml, while Ward *et al.* (1990) have also developed competitive assays with picogram limits of detection. Both of these assays were also developed using polyclonal antibody populations, and the same assay format was used as described here. Neither publication mentions the final titres obtained from the immunised rabbits. As a result, it may be that higher antibody titres were obtained from the rabbits and hence more sensitive antibodies for the detection of AFB_1. Notwithstanding these factors, both assays described here are quite sensitive and highly reproducible and, therefore, can be used for the detection of AFB_1 in grain samples, when applying certain maximum residue levels i.e. U.S. maximum residue levels (M.R.L.'s)-20 ppb.

Cross-reactivity studies were also carried out on both antibody populations. Cross-reactivity may be defined as the measure of antibody response to structurally related molecules, as a result of shared epitopes. The procedure is described as in section 2.11.4 and Table 3.5 shows the results of the studies. Both antibody populations

showed high degrees of cross reactivity, especially to AFB_2 and AFG_1. Holtzapple *et al.* (1996) carried out molecular modelling studies on all the main aflatoxins, in an attempt to characterise the cross-reactivity of monoclonal anti-AFM_1 antibodies. They found that the antibody showed similar levels of reactivity towards AFB_1 and AFG_1. This may be due to the fact that the conjugated protein predominantly masked the cyclopentanone ring where it is linked to the hapten. AFB_1 and AFG_1 only differ in structure on this particular ring, and the rest of the molecule is identical. From these findings, it is possible to extrapolate that both antibody populations showed a high degree of cross-reactivity to AFG_1, as the only position in which they differ structurally was shielded by the conjugated protein. It is also thought that the methoxyl group on all AFB structures is immunodominant, i.e. plays a large role in what epitopes the antibody recognises antigen (Langone & Van Vunakis, 1976). This may give a reason why both antibody populations showed levels of cross-reactivity to all of the structurally related aflatoxins. Differing ranges of cross-reactivity of anti-AFB_1 antibodies has also been found elsewhere. Groopman *et al.* (1984) described a monoclonal antibody with equal specificity for aflatoxins B_1, B_2, and M_1, while Aldao *et al.* (1995) produced polyclonal antibodies with a high level of specificity for AFG_1. Although it is generally favourable to produce specific antibodies for a particular hapten, it can be advantageous from a regulatory viewpoint to produce a more 'broad-specificity antibody'. This could be particularly useful for the detection of aflatoxins, as the total amount of aflatoxin in a sample is regulated, and thus the antibody reacting to the major forms of the toxin would be appropriate.

The antibody population obtained from rabbit 1 was deemed to be the more sensitive for use in competition ELISA. As a result of this, it was decided to use these antibodies for the detection of AFB_1 in spiked grain samples. Spiked grain samples were prepared as described in section 2.10.3, and used as samples for a competitive ELISA with the same format as described in section 2.11.3. The linear range of detection of AFB_1 in the spiked grain samples was 98 to 12,500 ng/ml. Figures 3.18 and 3.19 show the intra-day and inter-day graphs for both assays while Tables 3.6 and 3.7 show the percentage C.V.'s for both assays. The precision profiles for the intra-

day assay were excellent with C.V.'s no higher than 5 %. The inter-day C.V.'s showed slightly higher C.V.'s of up to 12 % for certain concentrations on the curve. The assays were carried out in the same methanol concentration as that for the assays optimised in PBS. However, it is apparent that the grain matrix has affected the robustness and sensitivity of the competition ELISA. When the assay was performed in PBS the limit of detection was 12 ng/ml, and when it was carried out in spiked grain matrix the limit of detection was 98 ng/ml. These results indicate that although a reproducible assay was developed for the detection of AFB_1 in grain samples, further work needs to be carried out to make it more sensitive. Different international agencies have set maximum residue levels between 3 and 20 ppb (ng/g), and as a result it would be desirable to optimise the assay for the detection of such quantities of AFB_1. Different extraction techniques could be employed which would entail the removal of all solvent from the samples, and re-suspend them in PBS. This may reduce the effects the matrix might have on the assay.

3.4. Conclusions

This chapter highlights the serious problem mycotoxins pose to the food industry, and the important role antibodies can play in their detection. AFB_1-BSA conjugate was effective in the production of antibodies to AFB_1 in rabbits. Immunoaffinity chromatography proved to be a simple and effective method for the isolation of the polyclonal antibodies from extraneous protein found in serum. The polyclonal antibodies proved to be relatively sensitive and specific to AFB_1 as well as a number of structurally related aflatoxins. The assays optimised were highly reproducible, and demonstrated good precision. One of the antibody populations was used in the detection of AFB_1 in spiked grain samples. It showed that the grain matrix affected the antibody's performance in the assay, as it was not as sensitive as the assay optimised in PBS. Further work in the extraction of AFB_1 from grain samples needs to be carried out in order to optimise conditions for a more sensitive assay for the detection of AFB_1. This may have a significant impact on the future performance of the assay, as well as routine analysis of aflatoxins in food.

Chapter 4

Production and Characterisation of Murine Single Chain Fv Antibodies to Aflatoxin B_1 derived from a Pre-Immunised Antibody Phage Display Library system

4.1. Introduction

In recent years, phage-display technology has become increasingly powerful, and has transformed the way in which we produce binding-ligands, such as antibodies or peptides for a given target. The technology has enabled the tailor-made design of repertoires of ligands *ab initio*, and their expression on the surface of filamentous phage for the selection of a ligand with the desired properties (Hoogenboom *et al.*, 1998). Phage-display has proven to be particularly successful for the isolation of monoclonal single chain Fv (scFv) antibodies, either from a hybridoma, naïve spleen cells, or spleen cells that were pre-immunised with antigen *in vivo*. The enrichment technique of affinity selection (panning) is particularly advantageous as it allows for the selective amplification of phage particles carrying antibody specificities of interest from the rest of the population. This chapter focuses on the 'Krebber'-based system (Krebber *et al.*, 1997), which utilises phage-display technology for the production of soluble scFv antibodies. Mice were immunised with AFB_1-BSA conjugate, and the RNA from their spleens was used for the construction of an antibody phage-display library, which was used to produce soluble scFv antibodies specific for AFB_1.

4.1.1. *The Krebber-based phage display system for the production of scFv antibodies*

One of the main advantages of phage-display technology is the direct link of DNA sequence to protein function. As a result, single clones can be rapidly screened for antigen binding, as well as being selected from pools in the same experimental set-up. Phage-display has some drawbacks, where aberrant chains are often expressed on the surface of phage, but are non-toxic to the bacterial cell. As a result, cells expressing functional scFv-gene III fusions have a growth disadvantage. This is because they are competing with the non-functional scFv-gene III fusions for growth nutrients, as well non-specifically binding in the phage ELISA assay. The scFv-gene III fusion protein can cause vector instability, and create deletions in the antibody fusion genes. These shortfalls of phage-display technology prompted the development of a regulatable vector system, which allowed tight product suppression during all

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propagation steps as well as controlled expression of low amounts of scFv-gene III fusion protein for phage display. This re-designed system has a number of significantly improved features.

With previous antibody phage-display systems the primer sets used were too restricted to amplify either particular light or heavy chains. As a result, the mouse primers used in this system (section 2.15.4.) have been extended and optimised, as they incorporate all mouse V_H, V_λ, and V_κ sequences collected in the Kabat data base (Kabat *et al.*, 1991). It also combines the extended primer sets described by Kettleborough *et al.* (1993), Ørum *et al.* (1993) and Zhou *et al.* (1994). In addition, the V_L back primer set encodes a shortened version of the FLAG peptide, which introduces only three additional amino acids at the N-terminus of V_L. This allows easy detection of the scFv using a commercially available monoclonal antibody with an appropriate label.

The amplified V_H and V_L fragments are assembled by splicing-by-overlap extension (SOE)-PCR (Horton *et al.*, 1989). This simplifies the restriction enzyme digestion step, as only one piece of DNA on the plasmid needs to be cut. In addition, ligation of the scFv-DNA fragment into the vector is also a simplified one-step procedure. In order to avoid incorrect overlaps during the SOE-PCR, the four (Gly$_4$Ser) repeats in the single chain linker region are encoded by different codons, while reduction of dimerization of the scFv is ensured with the linker between the V_H and V_L being 20 amino acids in length rather than the usual 15 amino acid length.

Figure 4.1 shows a diagram of two of the phagemid vectors used in this system, pAK 100, and pAK 400. The vector pAK 100 has a tetracycline resistance cassette (*tetA* and *tetR*; 2101kb) inserted between two different restriction enzyme (SfiI) sites which is used as the recipient of the scFv-DNA. Digestion with SfiI results in a vector that can be easily separated from uncut vector by gel-electrophoresis. The vector also has a strong upstream t$_{HP}$ terminator sequence between the *lacI* gene and the *lac* promoter gene. This factor, along with glucose repression of the *lac* promoter, completely

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abolishes background expression before induction. Hence, selection against toxic scFv-gIII fusion proteins is avoided during the propagation steps, and plasmid stability is significantly improved. The *lac* repressor is encoded on the phagemid, which ensures strain-independent *lac* promoter repression. There is also a synthetic Shine-Dalgarno sequence combined with a *pelB* sequence, which gives only moderate levels of translation, and therefore only low levels of scFv-gIII expression upon induction by IPTG.
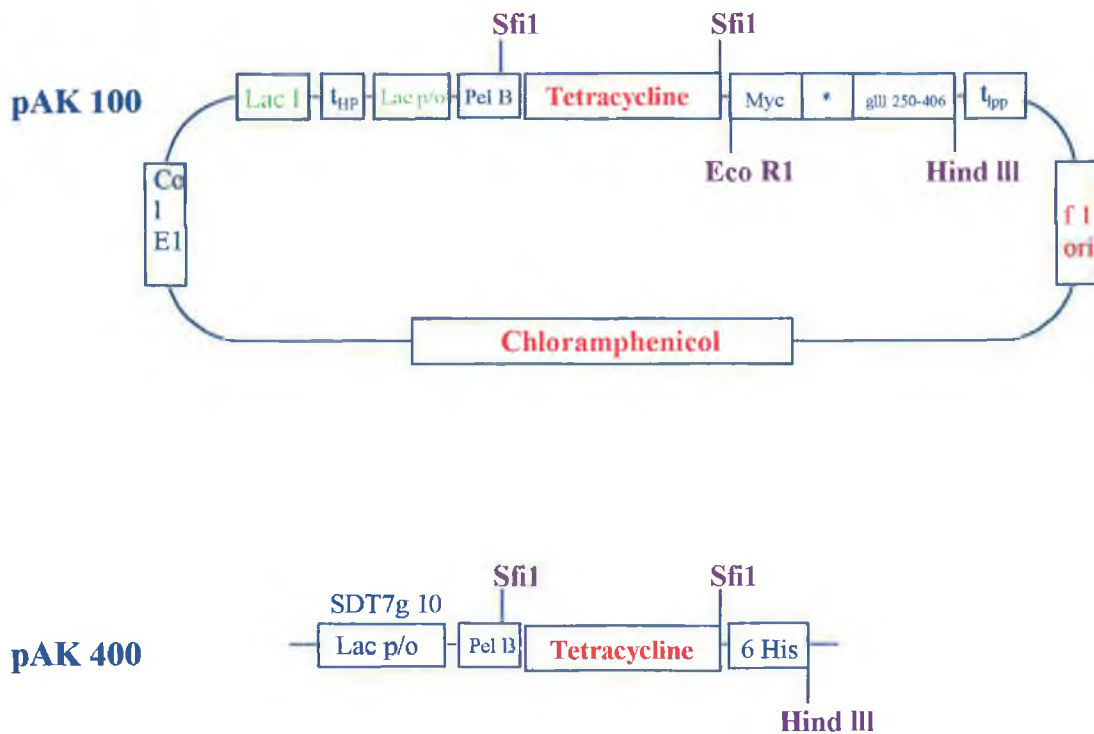
Figure 4.1. *Phage-display vector pAK 100, and related vector, pAK 400. Both vectors contain a tet resistance cassette (tet A and tet R; 2101 bp) to facilitate the monitoring of the SfiI restriction-enzyme cutting. The in-frame fusion to gene 111 250-406 in pAK 100 leads firstly into a c-myc-tag which is used as a detector handle, followed by an amber codon (asterisk). It is possible to switch to soluble expression of scFvs with pAK 400 (i.e. using a non-suppressor strain such as JM 83) depending on the bacterial strain used. C-terminal his-tag fusions can be used for purification by immobilised metal-affinity chromatography (IMAC) as well as for detection by an anti-tag antibody. The expression strength of the antibody can be enhanced by replacing the original Shine Dalgarno (SD 2) sequence by the stronger SDT7g10, as in the case of pAK 400.*

PAK 100 is engineered to achieve low levels of expression, and is therefore not useful for large-scale production of soluble antibody fragments. Thus, a compatible high-expression vector has also been engineered. PAK 400 has a stronger Shine-Dalgarno sequence (SDT7$_g$10), and as a result the expression strength is greatly enhanced. Removal of the geneIII and the absence of helper phage allows it to propagate as a plasmid, and soluble production of scFvs is induced in the same way as pAK 100 by up-regulation of the *lacZ* promoter. It also has a C-terminal his-tag fusion, which can be used for IMAC purification and detection using a labelled anti-his-tag antibody. Both vectors use chloramphenicol as the resistance marker, as it allowed more stringent selection than ampicillin. This is because the resistance protein does not leak into the medium. It was also found that chloramphenicol is more advantageous than kanamycin or tetracycline as it does not reduce phage titres as much (Johansen *et al.*, 1995).

The restriction enzyme digestion used in this system is unique, as SfiI is the only enzyme used for directional cloning of scFv fragments into pAK 100. SfiI is a particularly useful restriction enzyme as it recognises eight bases, interrupted by five non-recognised nucleotides (5'-GGCCNNNN/NGGCC-3': where "N" is any base and "/" the point of cleavage). SfiI restriction sites are, therefore, very rare in antibody sequences, which reduces the possibility of internal digestion of sequences. The enzyme is also unique as it always cuts two sites at once and therefore single-cut plasmids or inserts do not occur as intermediates. Two different sticky ends were designed to allow cloning of the scFv fragment in a directional manner. In contrast to the palindromic sticky ends 3 base pair overhangs derived from SfiI sites render it impossible to self dimerise by either insert or vector.
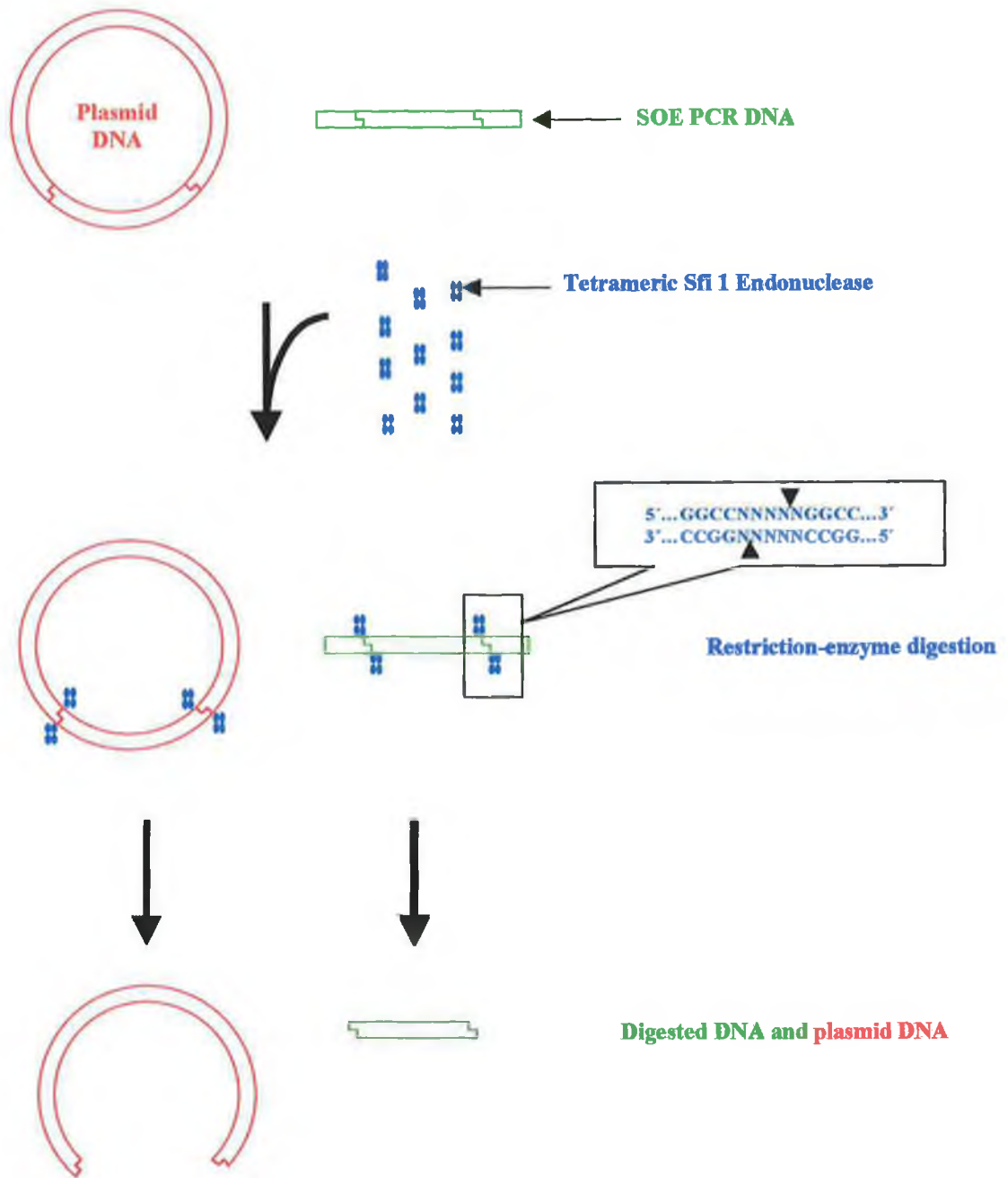
Figure 4.2. *Schematic diagram showing the restriction-enzyme digestion of plasmid DNA and SOE-PCR product using SfiI.*

Figure 4.3 (a) shows a diagrammatic representation of how an antibody phage display library may be produced using the Krebber-based system. The genetic material (mRNA) can originate from an immunised mouse if one were to produce a pre-immunised phage display library, or alternatively a naïve mouse for production of a naïve library. It is also possible to obtain the genetic material from a hybridoma, and clone the scFv fragment of the parent monoclonal antibody. The mRNA is then reverse-transcribed to produce DNA. The V_H and V_L fragments are amplified by PCR using the designated primers and the DNA. SOE-PCR is used to anneal the V_H-V_L fragments together as one. Restriction enzyme digestion is carried out on the SOE product as well as the vector (pAK 100), which enables ligation of the scFv fragment into pAK 100. This is transformed into *E. coli,* and results in an antibody phage display library. Once the bacteria are harbouring the phagemid vector, they can be superinfected with helper phage to drive production of phage particles carrying the scFv fragment, as a fusion product with the phage coat protein pIII on the surface and genes encoding the scFv inside the particle. Expression of the fusion product and its subsequent incorporation into the mature phage coat results in the ligand being presented on the phage surface, while its genetic material resides within the phage particle. This connection between ligand genotype and phenotype allows the enrichment of specific phage using selection on an immobilised target.

Figure 4.3 (b) shows an outline of the panning procedure, where phage-expressing scFv's are exposed to (for example) hapten-protein conjugate. After a short incubation period, the non-specific phage are washed away, and bound phage are then eluted from the conjugate. The specific phage may be amplified and re-selected against conjugate, or alternatively they may be assayed for recognition to free hapten. Once a phage clone recognising free hapten has been selected, the phagemid DNA can be purified and the specific scFv fragment cut from the vector. The scFv fragment can then be re-ligated into a high expression vector such a pAK 400 for soluble expression of scFv fragments.

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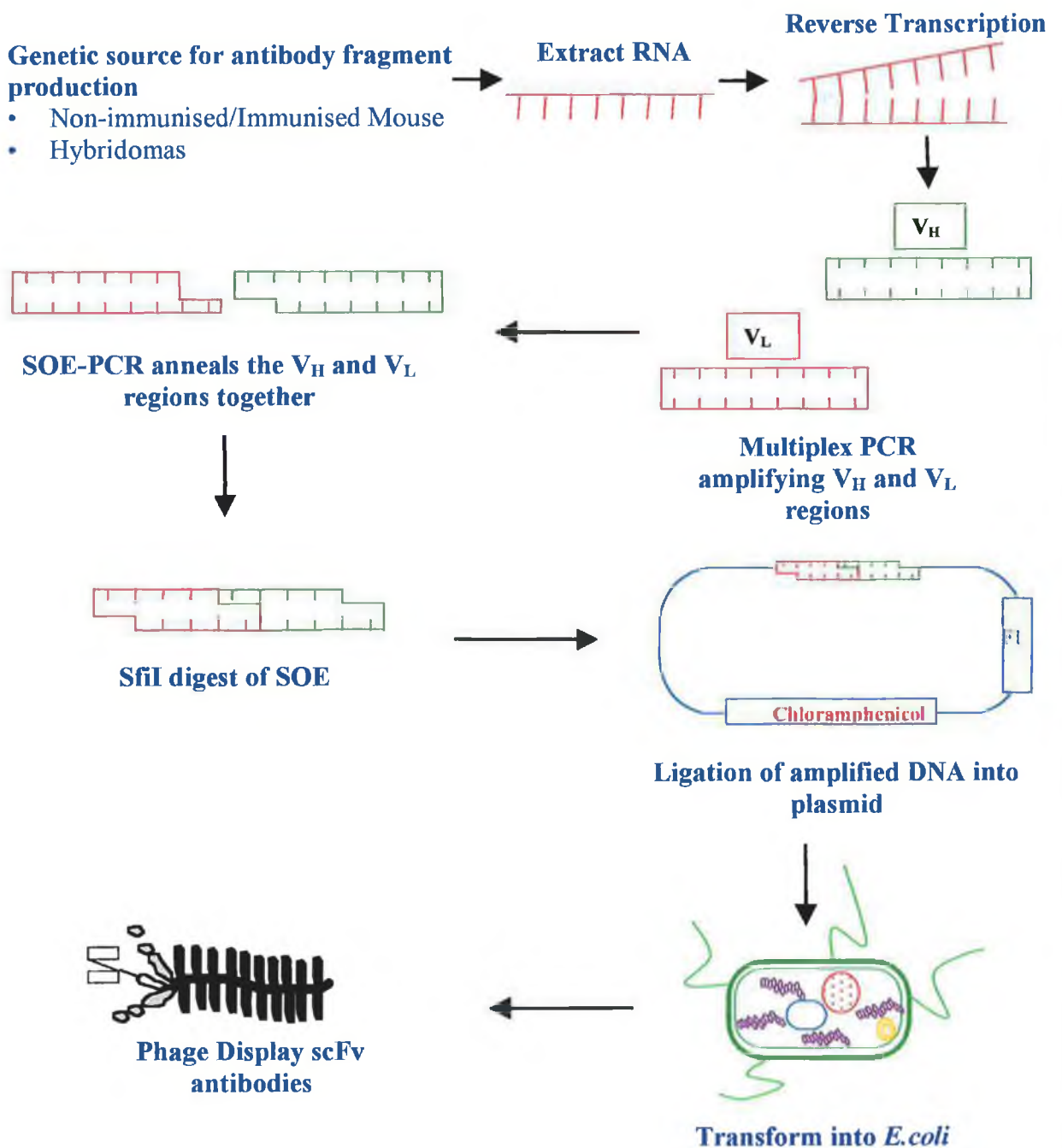
Figure 4.3 (a). *The Krebber-based system for phage display library construction. Genetic material from spleens or hybridomas encoding sequences derived from mRNA is reverse transcribed and amplified up by PCR. SOE-PCR is used to assemble the V_H and V_L chains as one. The DNA encoding the scFv is restriction enzyme-digested and ligated to a particular vector i.e. pAK 100. This is then transformed into E. coli. The scFv is displayed on the surface of the phage when fused to a coat protein, after superinfection of the harbouring vector with a helper phage.*

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Phage ELISA for selection of phage-display scFv antibodies recognising hapten-protein conjugate

Wash-step for removal of non-specific phage

Elution of specific phage

SDT7g 10

Lac p/o Pel B Tetracyclin 6 His

Sfi1 *Sfi1*

Hind III

Purify and cut DNA coding for specific scFv antibody and ligate into pAK 400

Phage ELISA for selection of phage-display scFv antibodies to free hapten

Soluble scFv expression
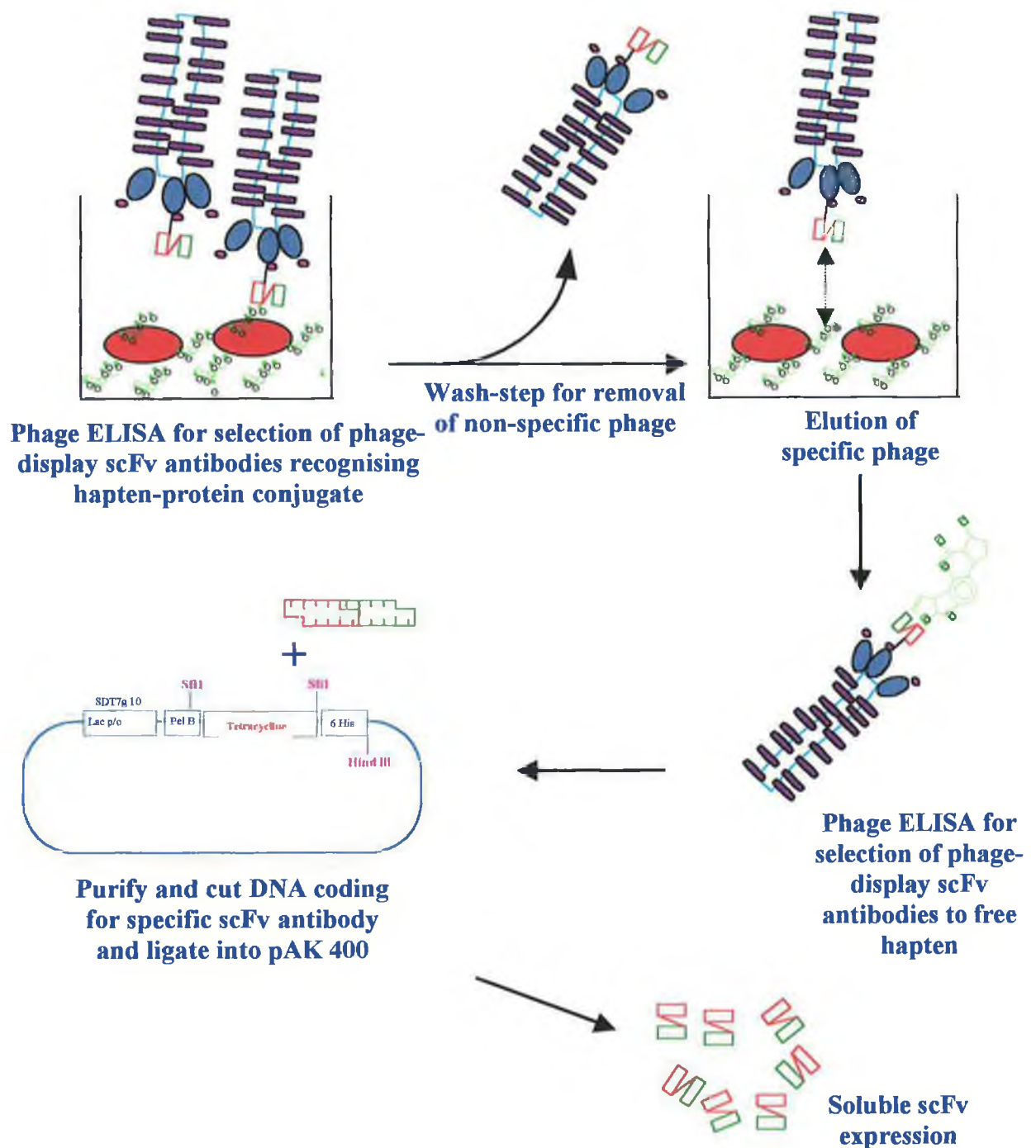
Figure 4.3(b). Schematic diagram of panning procedure for the selection of phage-displaying scFv antibodies recognising free hapten, and soluble expression of scFv antibodies. Phage ELISA is used to detect and amplify specific phage to hapten-protein conjugate. These phage colonies are then analysed for recognition of free hapten. Plasmid DNA is purified from 'positive' clones, restriction enzyme digested, and re-ligated into the high-expression vector pAK 400 for soluble expression of scFv antibodies.

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4.2. Results

4.2.1. *Estimation of mouse titres*

Two Balb/C mice were immunised with a commercially available AFB_1-BSA conjugate as described in section 2.3.4. The mice received an intra-peritoneal injection of the conjugate together with an equal volume of Freund's Complete adjuvant. Blood was drawn periodically to estimate the specific antibody titre, which was assessed by ELISA as described in section 2.11.1. One half of a 96-well microtitre plate was coated with AFB_1-BSA conjugate, and the other half of the microtitre plate was coated with BSA. The plate was blocked with 2 % (w/v) milk. The mouse serum was then diluted from 1/300 to 1/614,400 in PBS containing 0.1% (w/v) BSA. The serum was also diluted in PBS containing no BSA. The serum diluted in the PBS 0.1% (w/v) BSA was added to the AFB_1-BSA-coated wells, as well as the BSA-coated wells, while the serum diluted in the PBS was added to the BSA-coated wells only. The antibody responses were then detected using a horseradish peroxidase-labelled anti-mouse antibody followed by chromogenic substrate. The titre was taken as highest dilution of immunised serum which gave a signal greater than the corresponding dilution of control serum, i.e. the serum diluted in PBS containing 0.1% (w/v) BSA and added to the BSA-coated wells. The final titre for serum from mouse 1 was 1/153,600, while the final titre for serum from mouse 2 was 1/76,800. Figures 4.4 and 4.5, respectively, show these results, including the antibody responses to BSA, with or without BSA in the diluent buffer. The figures also show that there was a greater antibody response to AFB_1 than to BSA, as well as that 0.1 % (w/v) BSA was sufficient to remove non-specific binding of the serum to the BSA part of the AFB_1-BSA conjugate. The serum titrations were deemed high enough responses to indicate that splenomic RNA would be utilised following a final intravenous boost.
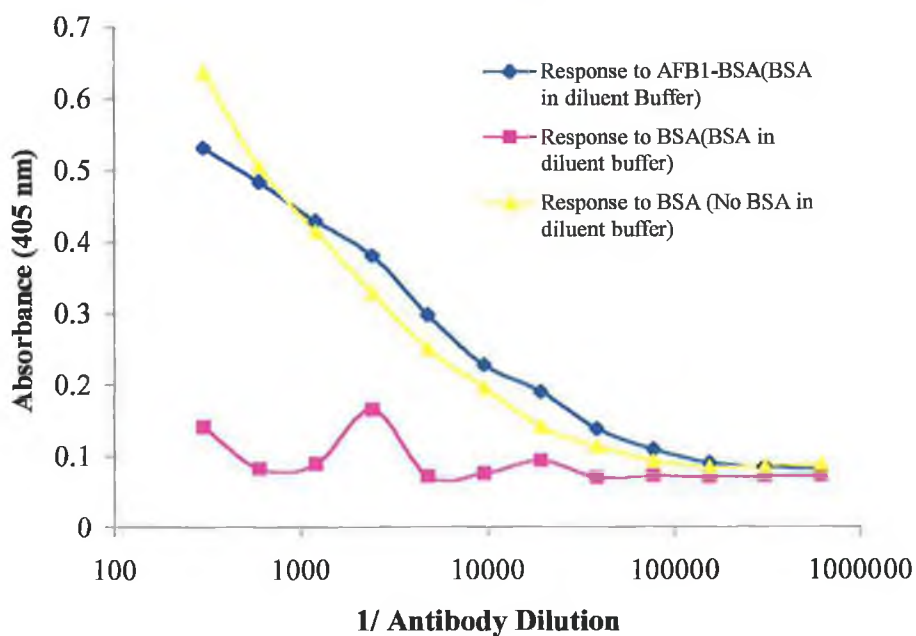
Figure 4.4. *Final titre of serum obtained from mouse 1. The serum was diluted in PBS as well as PBS containing 0.1% (w/v) BSA, and added to wells of a microtitre plate coated with AFB₁-BSA conjugate. Diluted serum was also added to wells of a microtitre plate coated with BSA. The final titre recorded was in excess of 1/153,600.*
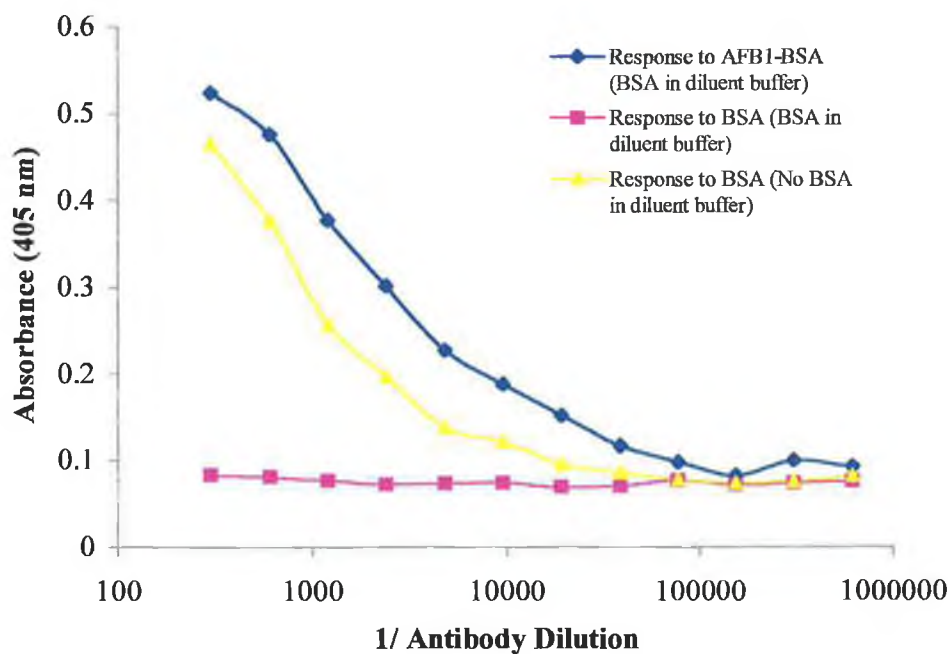
Figure 4.5. *Final titre of serum obtained from mouse 2. The serum was diluted in PBS as well as PBS containing 0.1% (w/v) BSA, and added to wells of a microtitre plate coated with AFB₁-BSA conjugate. Diluted serum was also added to wells of a microtitre plate coated with BSA. The final titre recorded was in excess of 1/76,800.*

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4.2.2. *Preparation of splenomic RNA*

Splenomic RNA was isolated from the spleens of the two mice (1 & 2) as described in section 2.15.2. Table 4.1 shows the yields of RNA obtained from the two mice, where the spleen from mouse 1 yielded 11.74 $\mu g/\mu l$, and the spleen from mouse 2 yielded 9.68 $\mu g/\mu l$.

Table 4.1. *U.V.-visible readings for determination of total RNA concentration from extracted spleen cells. The absorbance at 260 nm indicated the amount of RNA in the sample, while the absorbance at 280 nm indicated the amount of background protein in the sample. The concentrations of RNA were calculated on the basis that $\lambda 260 = 1.0$ is equivalent to 40 μg of RNA per μl.*

	λ 260	λ 280	Concentration of RNA (μg per μl)
Mouse 1	1.174	0.672	11.74
Mouse 2	0.968	0.546	9.68

4.2.3. *Amplification of mouse heavy and light chains using PCR*

The V_L back primer mix (LB 1-17 and LBλ, representing a total of 131 variants) paired with five V_L forward primers (LF 1, 2, 4, 5 and LFλ) were used to amplify the V_L domains. The V_H back mix (HB 1-19, representing a total of 94 variants) coupled with four V_H forward primers (HF 1-4) were used to amplify the V_H domains. The domains were amplified from a mixture of cDNA obtained from mice 1 & 2, respectively. Using the standard multiplex PCR protocol (section 2.15.4.2), single amplified bands ranging from 375-402 bp for the V_L, and 386-440 bp for V_H were achieved. Figure 4.6 shows typical bands obtained from the multiplex PCR reaction,

where two batches of heavy chains were amplified, and one batch of light chain was amplified.

Figure 4.7 shows an SOE-PCR where the heavy and light chains were annealed together to form an 800 bp fragment. In order to optimise this reaction properly, the heavy and light chains were analysed densiometrically to determine the concentration of DNA in respective fragments. The bands shown in Figure 4.6 were analysed densiometrically, and the results are given in Table 4.2. 10 ng of each of the V_H and V_L fragments was used to carry out the SOE-PCR.
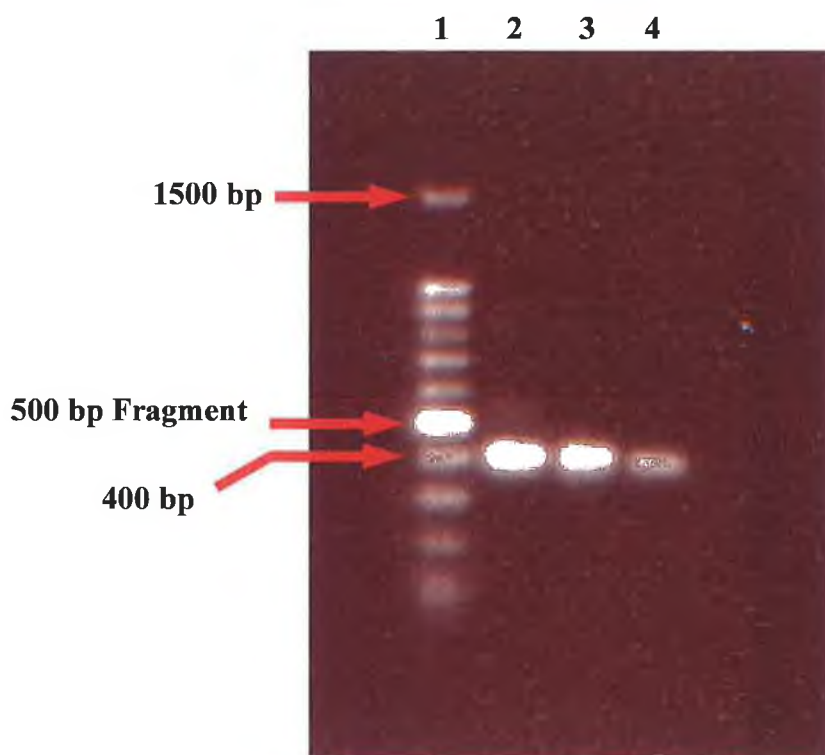
Figure 4.6. *Amplification of murine heavy (V_H) and light chains (V_L) from a mixture of DNA obtained from two mice immunised with AFB$_1$-BSA conjugate. Lane 1: 100 base pair (bp) ladder. Lanes 2-3: Amplified heavy chains. Lane 4: Amplified light chains. In the case of the heavy chains, a sharp band with the predicted size 375-402 bp was obtained, while for the light chain, a sharp band with the predicted size 386-440 bp was obtained. These bands were observed as expected just below the 500-bp fragment on the gel picture.*

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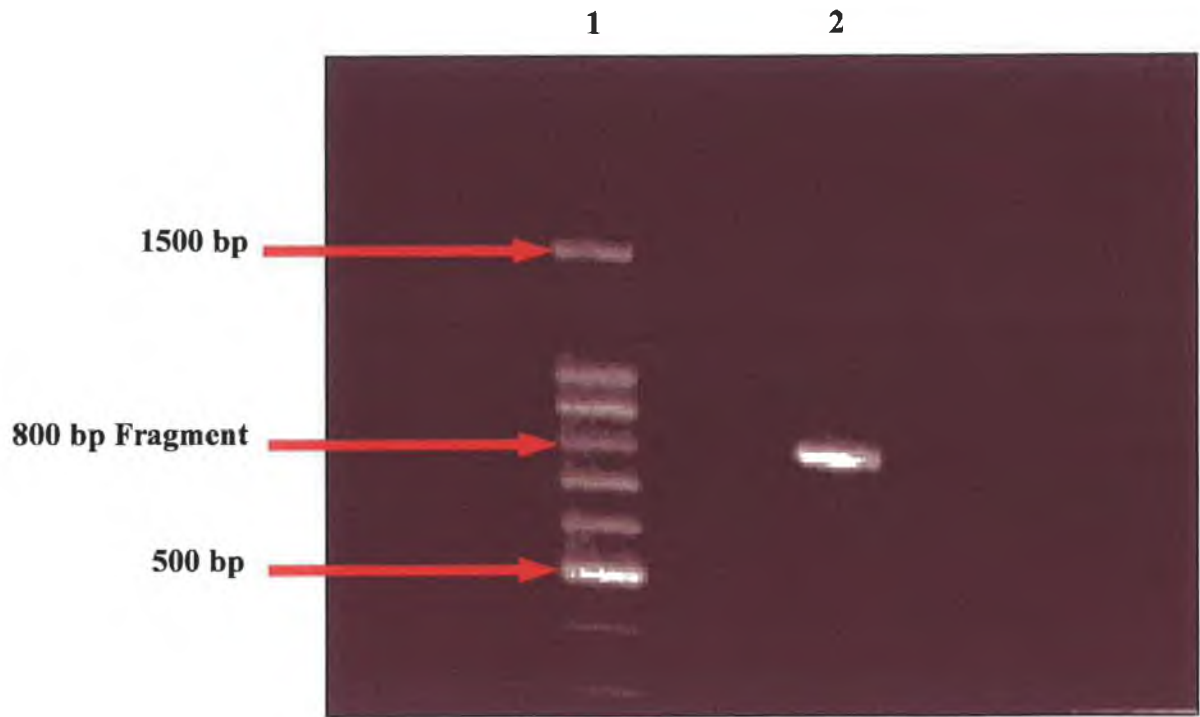
Figure 4.7. *SOE-PCR annealing the V_H and V_L genes. Lane 1: 100 bp ladder. Lane 2: SOE-PCR. A specific band is obtained for the SOE product just below the 800 bp fragment on the ladder, which is as expected, both the heavy and light chains annealed together.*

4.2.4. *Preparation of SOE-PCR product and pAK 100 for ligation reaction*

The SOE-PCR product was digested with the restriction enzyme SfiI as described in section 2.15.8. Figure 4.8 shows a gel photograph of the digestion of the PCR product. Only a small change in weight is observed between undigested and digested product. However, there is an apparent difference between the two fragments, which is highlighted by the presence of the yellow line placed across the bands. This indicated the digestion was successful. Densiometric analysis was also carried out on digested SOE-PCR product, and Table 4.2 shows that 9.8 ng/μl was present in the pooled samples of Lanes 3 & 4 on Figure 4.8.

Figure 4.9 shows a gel photograph of the restriction enzyme digestion of pAK 100. It is easy to differentiate between the cut vector (at approximately 4144 bp) and the tetracycline gene (2101 bp) which was cut from the vector. The photograph also shows that a small amount of the vector also remained uncut. However, there was sufficient vector digested to gel-purify and use for the ligation reaction. Densiometric analysis was also carried out on pAK 100, and Table 4.2 shows that 14.8 ng/μl of plasmid DNA was present in the sample.
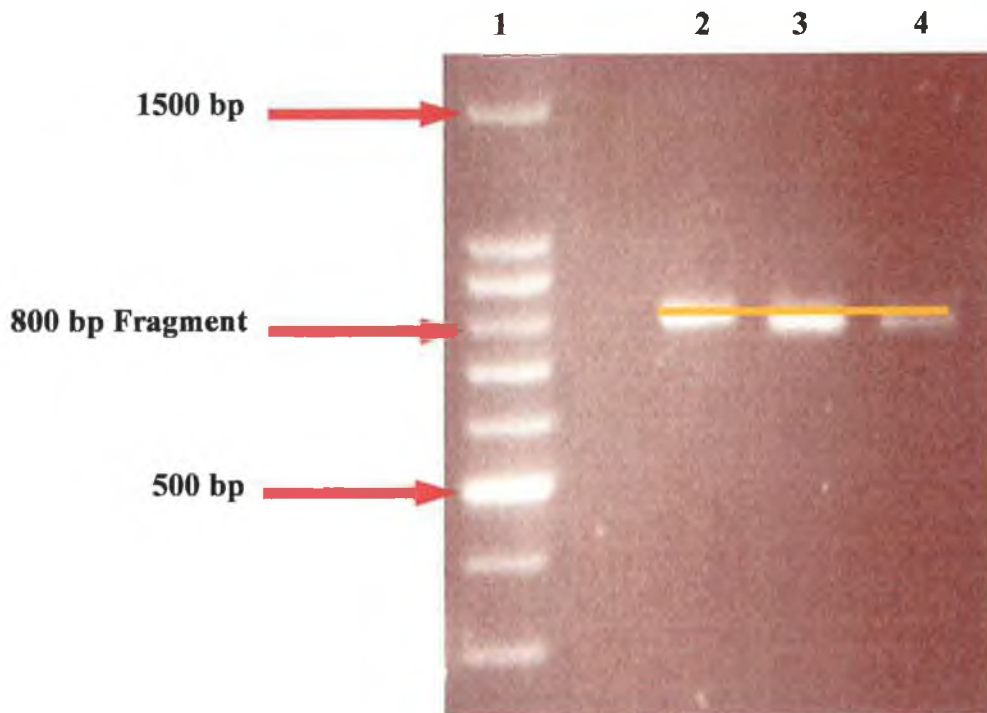
Figure 4.8. *Restriction enzyme digest of SOE-PCR product using SfiI. Lane 1: 100 bp ladder. Lane 2: Undigested SOE product. Lane 3: Digested PCR product. Lane 4: Digested PCR product. A yellow line has been placed through the 800 bp fragment in order to observe the change between the bands. Only a minor change is observed between the digested and undigested bands, as only a small number of base pairs are removed from the DNA fragment, and hence a small change in separation.*
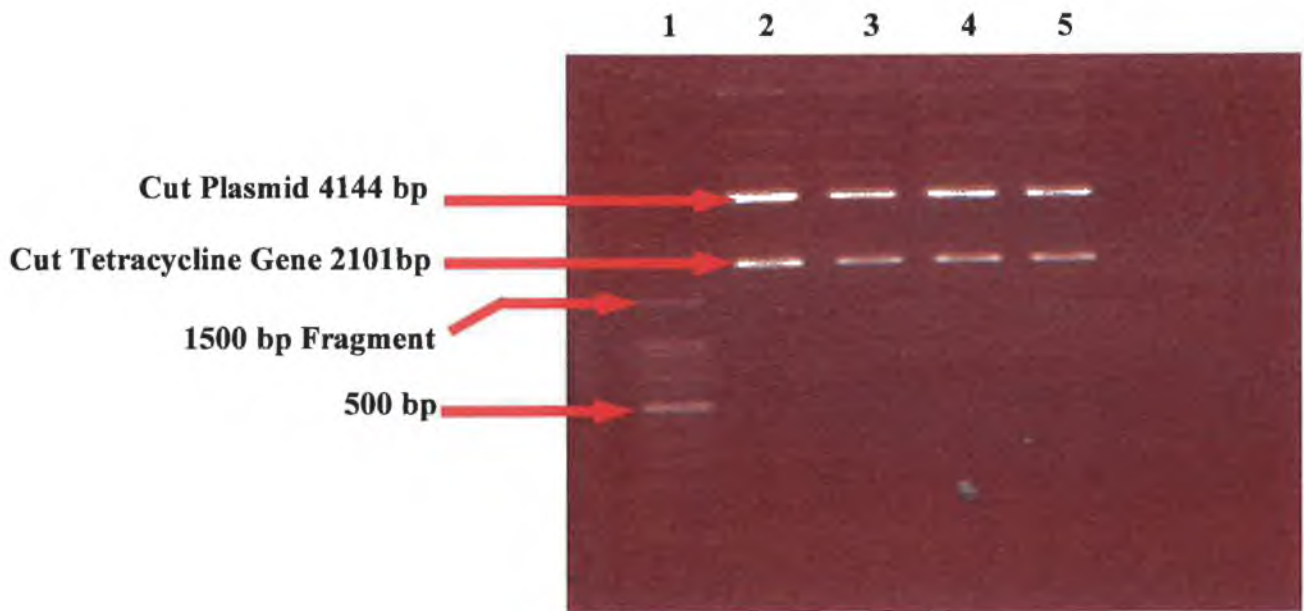
Figure 4.9. *Restriction enzyme digest of pAK 100 using SfiI. Lane 1: 100 bp ladder. Lanes 2-5: Digested pAK 100. Both the cut tetracycline gene, and the digested plasmid can be observed on the gel photo. Three weak-intensity bands are also visible above cut plasmid. These may represent some remaining uncut plasmid as well as partially cut products.*

Table 4.2. *Concentrations (ng/μl) of all of the various DNA products required for production of the antibody phage display library. All of the concentrations were densiometrically calculated from the standard ladder, where 5 μl of the 100 bp ladder was equivalent to 150 ng of DNA.*

DNA Product	Concentration (ng/μl)
100 bp Ladder	150
V_H Fragment (batch 1)	29.44
V_H Fragment (batch 2)	26.08
V_L Fragment	17.32
Digested SOE product	9.8
pAK 100 Vector	14.8

4.2.5. *Transformation of pAK 100 containing V_H- V_L genes into E. coli*

Supercompetent *E. coli* cells with a transformation efficiency of 1 x 10^9 cfu/μg of plasmid DNA were heat-pulse transformed with the ligated plasmid DNA as described in section 2.15.10. This resulted in the production of a phage display library consisting of 5 x 10^3 clones. This library was then used for subsequent panning procedures.

4.2.6. *Screening for phage-display scFv antibodies to AFB₁*

4.2.6.1. *Production of AFB₁-dextran conjugate*

An AFB_1-dextran conjugate was synthesised for use in the panning procedure, as it is desirable to use more than one hapten-carrier conjugate when selecting for scFv antibodies to small molecules by phage display. The conjugate was produced as described in sections 2.15.1.1 and 2.15.1.2, and its performance was assessed by BIAcore analysis as described in section 2.15.1.3. An introduction to BIAcore and how to interpret the SPR signal is given in section 5.1. Figure 4.10 shows a sensorgram of a preconcentration study of the conjugate onto a CM-dextran chip surface. The conjugate (concentration used-50 μg/ml) was prepared in 10 mM acetate buffer at a range of incremental pH's from 3.8 to 5.0. Each conjugate concentration was passed sequentially over an underivatised chip surface at 5 μl/min for 2 minutes as described in section 2.20.1. The differing degree of preconcentration was measured by the response for each conjugate solution at the various pH values, prior to the end of the injection. Following injection, the flow of the running buffer over the chip surface was sufficient to dissociate the electrostatic attraction between the protein and the CM-dextran gel surface. Figure 4.10 shows that an increase in preconcentration occurs at pH values above 4.5. The studies showed the optimal pH for immobilisation of AFB_1-dextran conjugate was 5.0 in 10 mM acetate buffer, and was thus used for immobilisation.
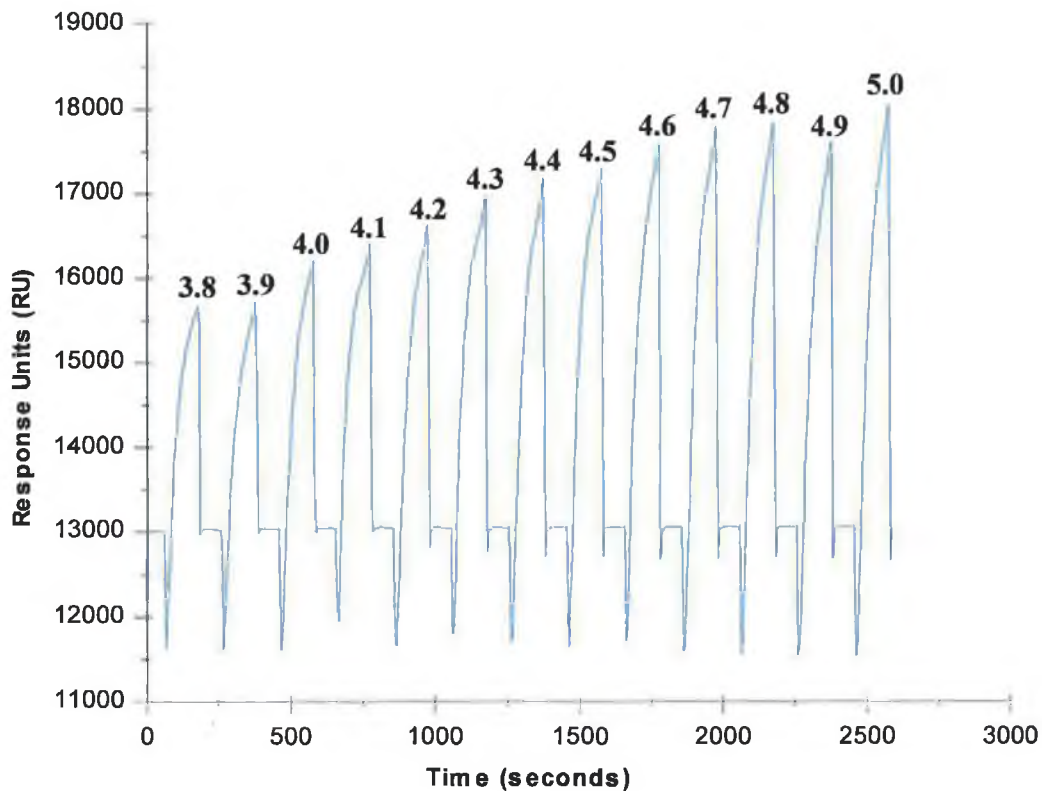
Figure 4.10. *Preconcentration of AFB₁-dextran conjugate onto a CM-dextran chip surface. Solutions of AFB₁-dextran at 50 µg/ml in 10 mM acetate buffer at pH increments of 0.1 were passed over an unactivated CM-dextran surface at 5 µl/min for 2 minutes. The low ionic strength of the buffer used favours the electrostatic attraction between the negatively charged dextran layer and the positively charged amino-dextran conjugate (i.e. below isoelectric point). The degree of 'preconcentration' was measured from the response prior to the end of each sample injection. The ionic strength of running buffer (150 mM NaCl) was sufficient to remove the electrostatically attracted conjugate from the sensor chip surface. The optimal pH for immobilisation of AFB₁-dextran onto the CM-dextran chip surface was calculated to be pH 5.0, and subsequently all immobilisations of AFB₁-BSA were carried out in 10 mM acetate buffer, pH 5.0.*

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EDC/NHS coupling chemistry was used for activation of the CM-dextran gel surface for the immobilisation of AFB_1-dextran conjugate, as described in section 2.20.2. Figure 4.11 shows an immobilisation sensorgram where 2,773 RU of AFB_1-dextran conjugate was immobilised onto the sensor surface after capping with 1M-ethanolamine hydrochloride, pH 8.5. This deactivated any remaining NHS-esters, and eluted any non-covalently bound material.

Affinity-purified polyclonal anti-AFB_1 antibodies (obtained from rabbits 1 & 2 (See Chapter 3)) were diluted in HBS buffer and injected over the immobilised conjugate surface. Figure 4.12 shows a sensorgram of this, which resulted in 67.3 RU of a 1/100 dilution of antibody (obtained from rabbit 1) binding to the surface. Regeneration of the surface was carried out using 1 M ethanolamine containing 20 % (v/v) acetonitrile, pH 12.0, which removed all of the bound antibody from the surface. A 1/50 dilution of antibody (obtained from rabbit 2) was then injected over the surface, which resulted in 95 RU binding to the immobilised conjugate. The surface was once again regenerated using the same regeneration solution. These studies indicated that conjugation of AFB_1 to amino-dextran was a successful procedure, although the antibody binding response was low, indicating a low substitution ratio of the hapten to the amino-dextran.
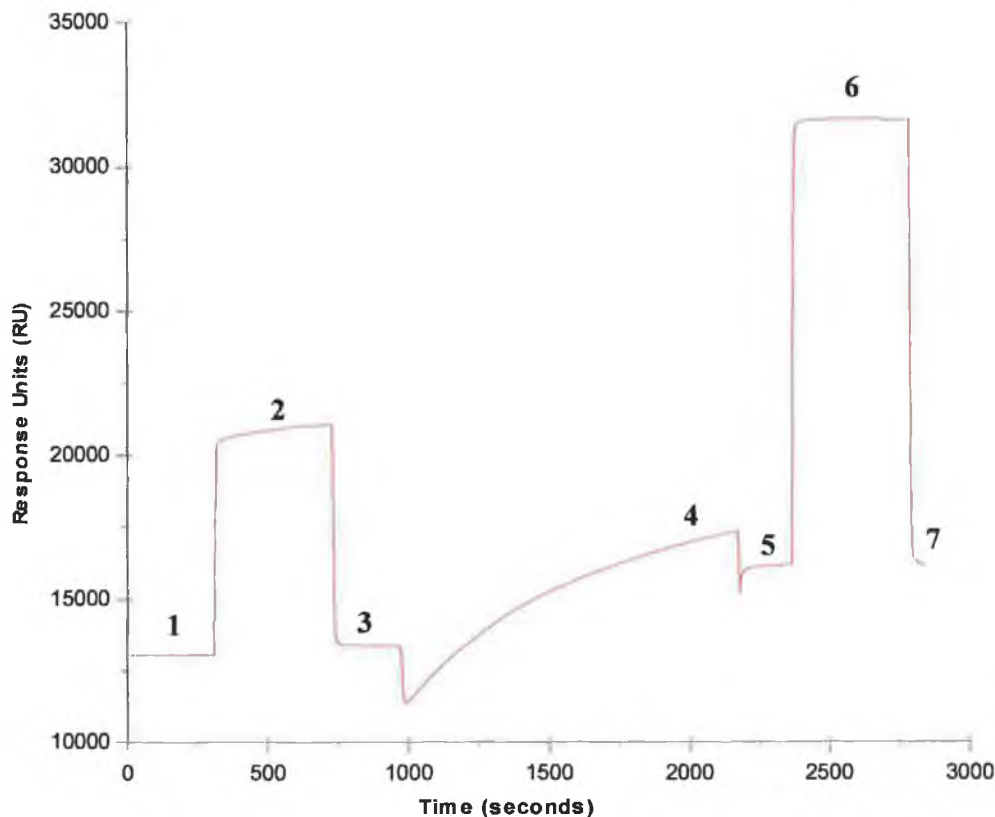
Figure 4.11. *Immobilisation of AFB$_1$-dextran conjugate onto a CM-dextran chip surface:*

1. *HBS running buffer is passed over the chip surface and a baseline measurement is recorded.*

2. *After mixing, a solution containing 0.05 M NHS and 0.2 M EDC is injected over the chip surface to activate it. The large increase in SPR signal is mainly due to a bulk refractive index change.*

3. *An increase of approximately 200 RU is recorded following activation of the chip surface.*

4. *A solution containing 50 µg/ml of AFB$_1$-dextran conjugate in 10 mM acetate buffer (pH 5.0) is passed over the chip surface for 20 minutes.*

5. *The amount of bound conjugate adsorbed is recorded whilst most of the non-covalently bound protein has been eluted.*

6. *Any remaining surface NHS-esters are deactivated by the addition of 1 M ethanolamine hydrochloride, pH 8.5. This also elutes any non-covalently bound conjugate.*

7. *2,773 RU of AFB$_1$-dextran conjugate bound to the surface following the immobilisation procedure.*

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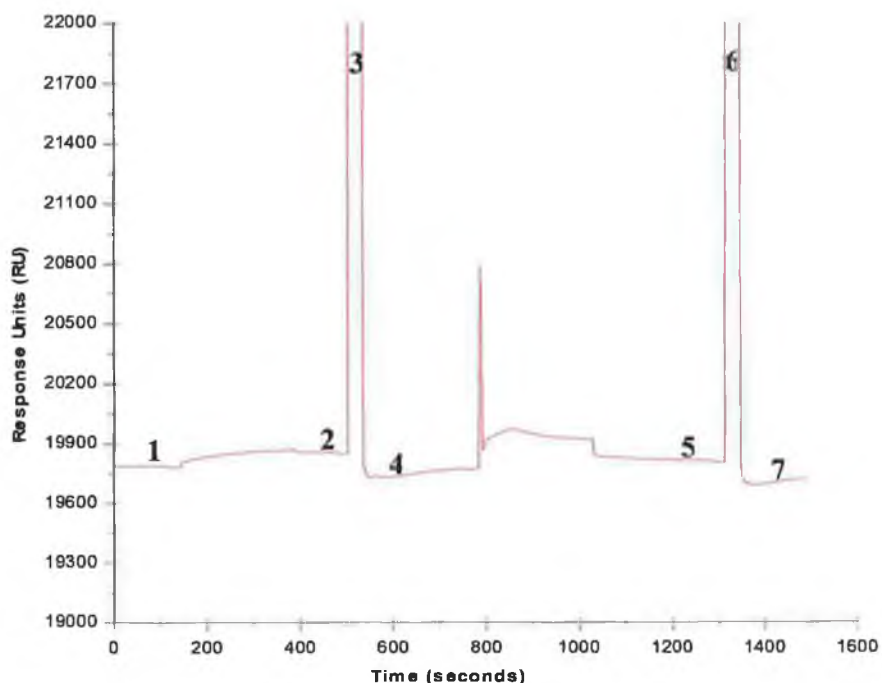
Figure 4.12. *Binding curve of polyclonal antibodies binding to an immobilised AFB$_1$-dextran conjugate.*

1. *HBS running buffer is passed over the chip surface and a baseline measurement is recorded.*

2. *A 1/100 dilution of affinity-purified polyclonal anti-AFB$_1$ antibody (obtained from rabbit 1) was injected over the immobilised chip surface resulting in 67.3 RU binding to the AFB$_1$-dextran surface.*

3. *Regeneration of the bound antibody from the conjugate surface was carried out by injecting a 2-minute pulse of 1M-ethanolamine containing 20%(v/v) acetonitrile, pH 12.0. The large increase in SPR signal is mainly due to a bulk refractive index change.*

4. *The surface was completely regenerated removing an additional 50 RU from the immobilised conjugate surface.*

5. *A 1/50 dilution of affinity-purified polyclonal anti-AFB$_1$ antibody (obtained from rabbit 2) was injected over the immobilised chip surface resulting in 95.0 RU binding to the AFB$_1$-dextran surface.*

6. *Regeneration of the bound antibody from the conjugate surface was carried out by injecting a 2-minute pulse of 1M-ethanolamine containing 20%(v/v) acetonitrile, pH 12.0. The large increase in SPR signal is mainly due to a bulk refractive index change.*

7. *The surface was completely regenerated removing an additional 28 RU from the immobilised conjugate surface.*

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4.2.6.2. *Panning procedure for the detection of scFv phage display antibodies*

Table 4.6 gives a summary of all of the phage titres obtained from the three rounds of panning that were carried out, including the number of clones assayed after each round, and the number that recognised free toxin.

After the library was constructed, phage was produced from it (as described in section 2.16.1) giving a titre of 2.5 x 10^9 cfu/ml of phage. The phage were then panned against an AFB_1-BSA conjugate (concentration used was 50 µg/ml). Stocks of the panned library were prepared as described in section 2.16.5, and a phage titration was also carried out (section 2.16.3) which showed that 3 x 10^5 cfu/ml of phage was produced after one round of panning. 96 random bacterial clones from round 1 of panning were selected for analysis, and phage was produced from them. These phage displaying scFv antibodies were analysed by phage ELISA for recognition of AFB_1-BSA conjugate. Eight clones were found to significantly recognise the conjugate. Table 4.4 shows the absorbances obtained from the phage ELISA, while Figure 4.13 shows the results in the form of a 3-D bar graph. The three clones with the highest absorbances were analysed for recognition to free AFB_1, by competitive phage ELISA. However, when analysed none of the three recognised free AFB_1. Figure 4.14 shows the competitive phage ELISA where no competition was observed.

A second round of panning was then carried out. This entailed producing phage from the panned library stocks from round 1 of panning. AFB_1-dextran was used for this, where 200 µg/ml was coated onto an immunotube. A phage titre of 2.8 x 10^7 cfu/ml was obtained, and once again 96 random clones were analysed for recognition to AFB_1-BSA conjugate. Table 4.5 shows the absorbances obtained from the phage ELISA, while Figure 4.15 shows the data depicted as a 3-D bar graph. Ten positives recognising the conjugate were obtained, and the clones with the three highest absorbances were analysed for recognition to free AFB_1. However, the phage ELISA was unsuccessful, with none of the three clones recognising free AFB_1 (Figure 4.16).

Table 4.3. *Data obtained from three panning rounds of an antibody phage-display library produced for the detection of a scFv fragment to aflatoxin B_1. The library was produced from RNA obtained from a mouse that was pre-immunised with the AFB_1-BSA conjugate prior to using the RNA.*

	Conjugate used for panning	Phage Titre cfu/ml	No. of clones assayed	No. of positive clones recognising AFB_1-BSA conjugate	No. of clones recognising free toxin
Phage Production	-	2.5×10^9	96	0	0
Round 1 of panning	AFB_1-BSA 50μg/ml	3.0×10^5	96	8	0
Round 2 of panning	AFB_1-Dextran 200μg/ml	2.8×10^7	96	10	0
Round 3 of panning	AFB_1-BSA 50μg/ml	2.4×10^7	96	7	6

Table 4.4. *Phage ELISA data of 96 randomly selected clones from the pre-immunised anti-AFB₁ phage display library after one round of panning. Eight positives were detected, with the numbers in bold being the clones that were chosen to be assayed for recognition free AFB₁.*

	1	2	3	4	5	6	7	8	9	10	11	12
A	1.112	1.043	1.215	1.077	1.018	1.073	0.976	0.905	1.206	0.977	0.85	0.269
B	0.958	1.04	1.117	1.075	1.015	0.913	1.058	1.218	0.771	0.900	1.131	1.093
C	1.09	1.18	1.07	1.043	1.143	1.072	1.045	1.117	1.072	1.157	1.378	1.115
D	1.066	1.038	1.043	1.007	1.069	1.104	1.001	0.894	0.737	1.100	1.154	1.099
E	1.092	1.077	0.864	1.138	1.073	0.94	0.899	0.913	1.096	1.123	1.16	0.949
F	1.142	1.156	0.991	1.167	1.142	1.016	1.089	0.936	1.108	1.145	1.135	1.259
G	0.893	1.02	1.024	0.75	1.261	0.857	0.894	1.008	1.135	1.297	1.137	0.834
H	0.884	1.194	0.918	0.906	1.173	0.809	0.941	0.963	1.213	1.023	1.177	1.143
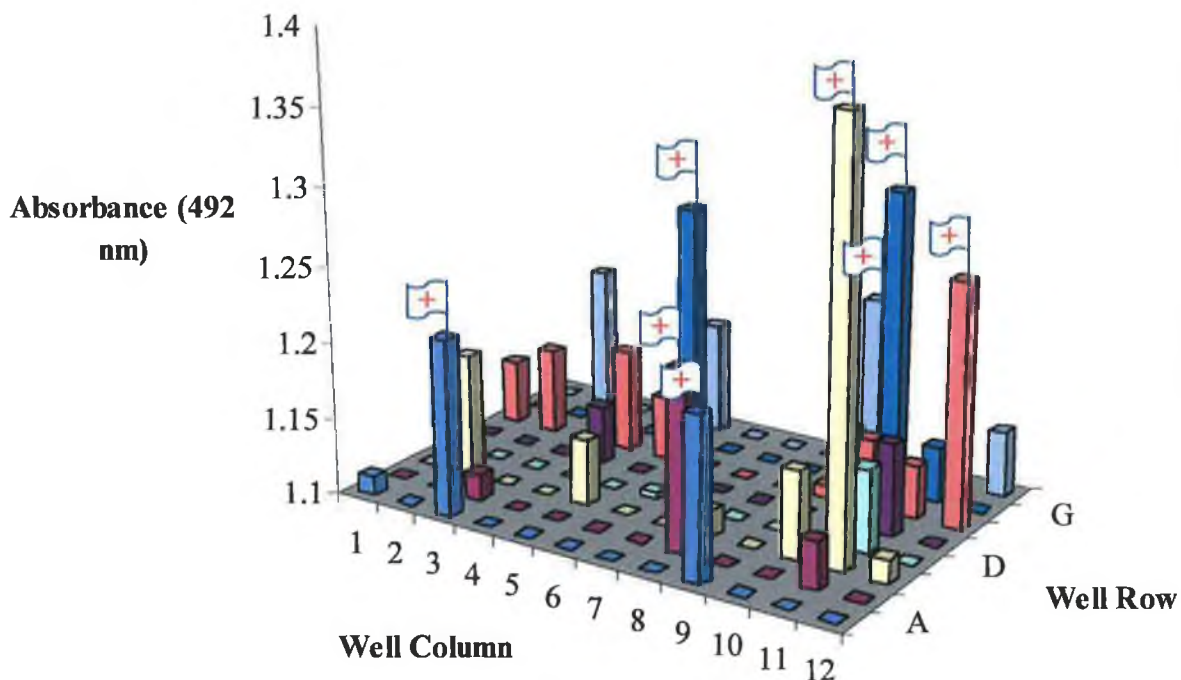
Figure 4.13. *Bar graph of phage ELISA analysis from the pre-immunised anti-AFB₁ library. The bars with flags represent the eight positive clones that correlate with the clones shown in Table 4.4.*
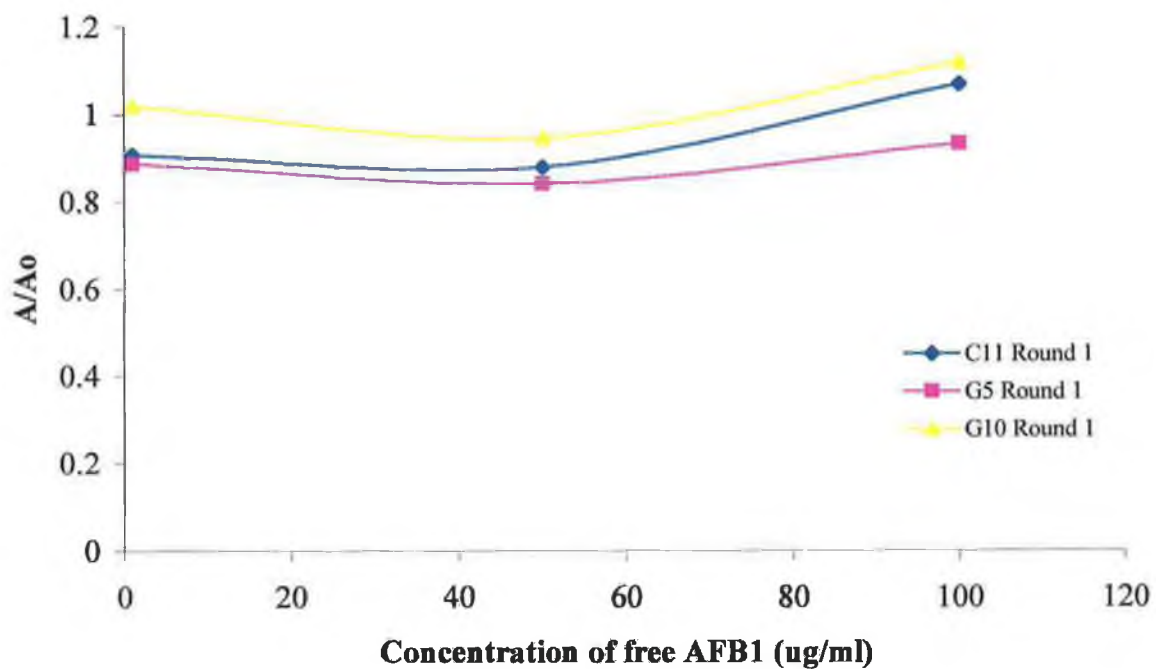
Figure 4.14. *Competition phage-ELISA of three clones selected after one round of panning for recognition to free AFB₁. The three clones selected, gave the highest absorbances in the non-competitive phage-ELISA, and were therefore selected for further analysis. None of the clones showed recognition for the free toxin.*

Table 4.5. *Phage ELISA data of 96 randomly selected clones from the pre-immunised anti-AFB₁ phage-display library after two rounds of panning. Ten positives were detected, with the numbers in bold being the clones that were chosen to be assayed for recognition free AFB₁.*

	1	2	3	4	5	6	7	8	9	10	11	12
A	0.748	0.728	0.661	1.007	0.922	0.623	0.573	0.669	0.724	0.623	0.632	0.813
B	0.842	0.682	0.655	0.673	0.576	0.537	1.122	0.673	0.645	0.498	0.989	**1.136**
C	0.726	0.795	0.771	0.144	0.749	0.584	0.658	0.888	0.570	0.498	0.678	0.896
D	**1.196**	0.947	0.720	0.684	0.952	0.615	0.617	0.592	0.800	0.497	0.69	0.882
E	0.913	0.881	**1.262**	0.595	0.714	0.980	0.951	0.889	0.543	0.603	0.617	0.941
F	0.948	1.039	0.731	0.997	0.602	0.667	0.891	0.726	0.566	0.699	0.593	0.625
G	0.73	0.646	0.558	0.722	0.613	1.056	0.611	0.697	0.524	0.568	0.536	1.004
H	0.891	0.681	0.741	0.640	0.466	0.619	0.567	0.519	0.758	0.880	0.639	0.566
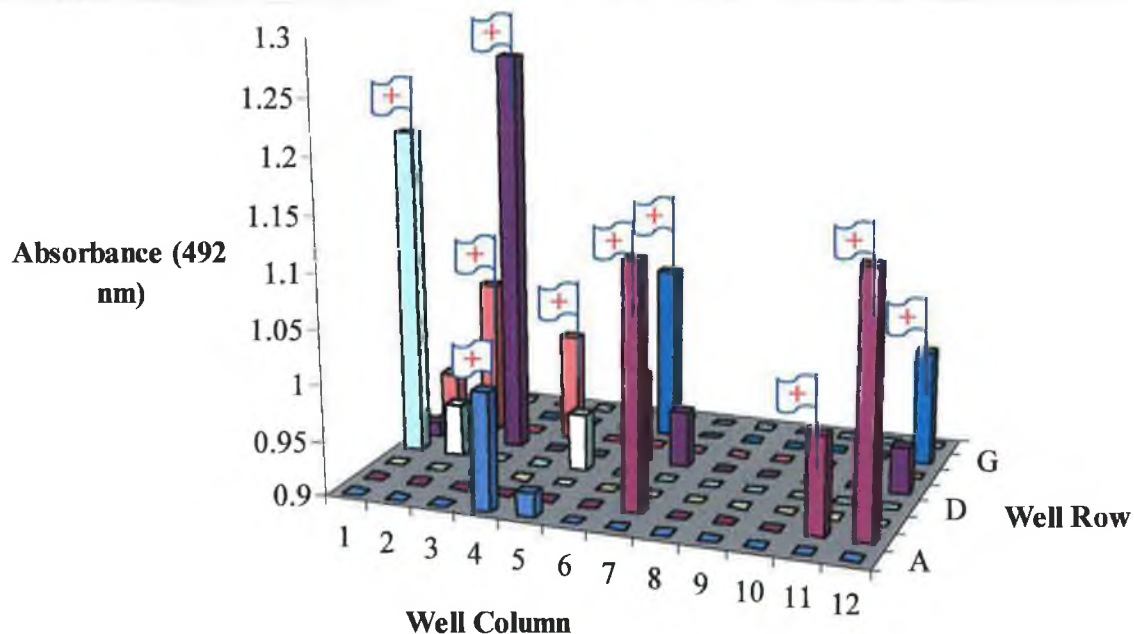
Figure 4.15. *Bar graph of phage ELISA analysis from the pre-immunised anti-AFB₁ library. The bars with flags represent the ten positive clones that correlate with the clones shown in Table 4.5.*

Figure 4.16. *Competition phage-ELISA of three clones selected after two rounds of panning for recognition to free AFB₁. The three clones selected, gave the highest absorbances in the non-competitive phage-ELISA, and were therefore selected for further analysis. None of the clones showed recognition for the free toxin.*

A third round of panning was subsequently carried out, as the previous two rounds yielded no clones recognising free AFB_1. This was carried out using the stocks produced after round two of panning. This pan was carried out using an AFB_1-BSA conjugate (50 $\mu g/ml$) and yielded a phage titre of 2.4×10^7 cfu/ml of phage. 96 clones selected from this round were grown, and phage was produced from them. These phage-displaying clones were then tested for activity to AFB_1-dextran and AFB_1-BSA. The clones displayed very low levels of activity towards the AFB_1-dextran (data not shown). However, eight positives were obtained from the assay using the BSA-conjugate. Table 4.6 shows the absorbances obtained from the phage ELISA, while Figure 4.17 shows the results depicted as a 3-D bar graph. Seven of the clones were re-grown on a 5 ml scale and tested for activity to free aflatoxin B_1. This was carried out using three different concentrations of aflatoxin B_1, 100, 50, and 1 $\mu g/ml$ respectively. Six of the clones showed recognition for free aflatoxin B_1. Figure 4.18 shows this result. Non-specific interactions were minimal, as the clones did not recognise BSA, or milk-coated wells (data not shown).

Table 4.6. *Phage ELISA data of 96 randomly selected clones from the pre-immunised anti-AFB₁ phage-display library after three rounds of panning. Six positives were detected, with the numbers in bold being the clones that were chosen to be assayed for recognition free AFB₁.*

	1	2	3	4	5	6	7	8	9	10	11	12
A	0.196	0.245	0.461	0.305	0.421	0.304	0.282	0.317	0.365	**0.524**	0.33	0.321
B	0.220	0.192	**0.851**	0.374	0.235	0.228	0.187	0.258	0.25	0.199	0.246	0.240
C	0.263	0.288	0.293	**0.956**	0.253	0.242	0.206	0.221	0.239	0.201	0.197	0.271
D	**0.473**	0.265	0.245	0.234	0.225	0.258	0.202	0.252	**0.544**	0.232	0.209	0.232
E	0.286	0.295	0.214	0.244	0.202	0.233	0.195	0.206	0.216	0.458	0.236	0.173
F	0.219	0.25	0.239	**0.706**	0.282	0.306	**0.574**	0.209	0.202	0.222	0.206	0.202
G	0.223	0.24	0.217	0.294	0.236	0.227	0.237	0.207	0.187	0.205	0.208	**0.601**
H	0.216	0.242	0.224	0.25	0.211	0.222	0.225	0.224	0.194	0.203	0.282	0.257

Figure 4.17. *Bar graph of phage ELISA analysis from the pre-immunised anti-AFB₁ library. The bars with flags represent the seven positive clones that correlate with the clones shown in Table 4.6.*
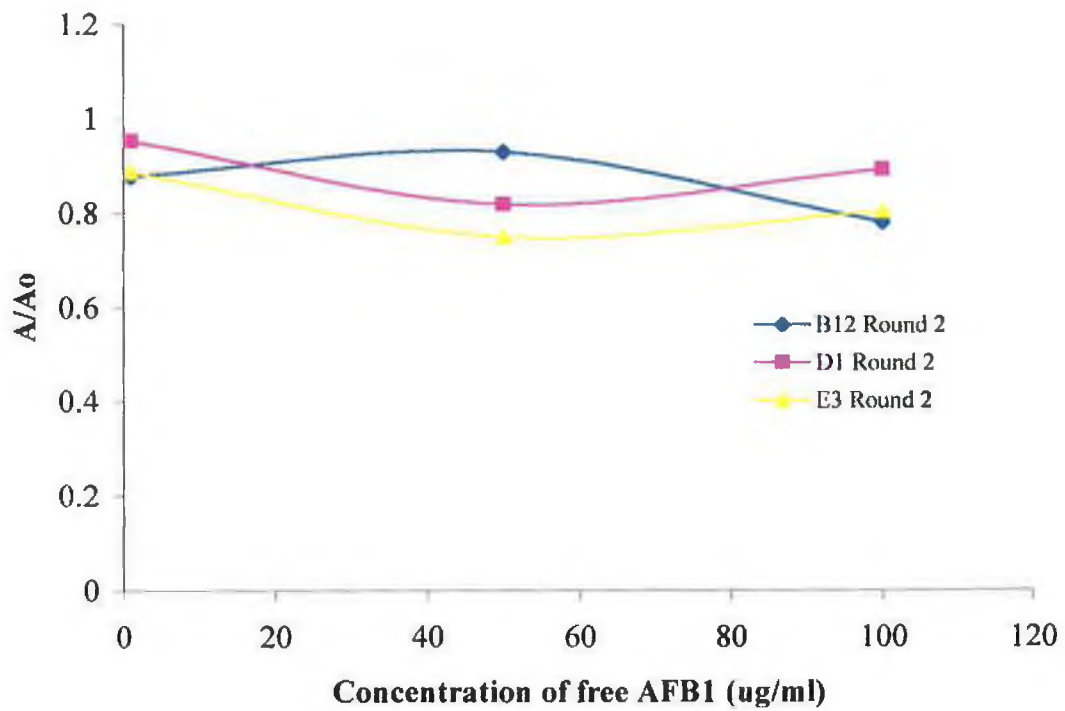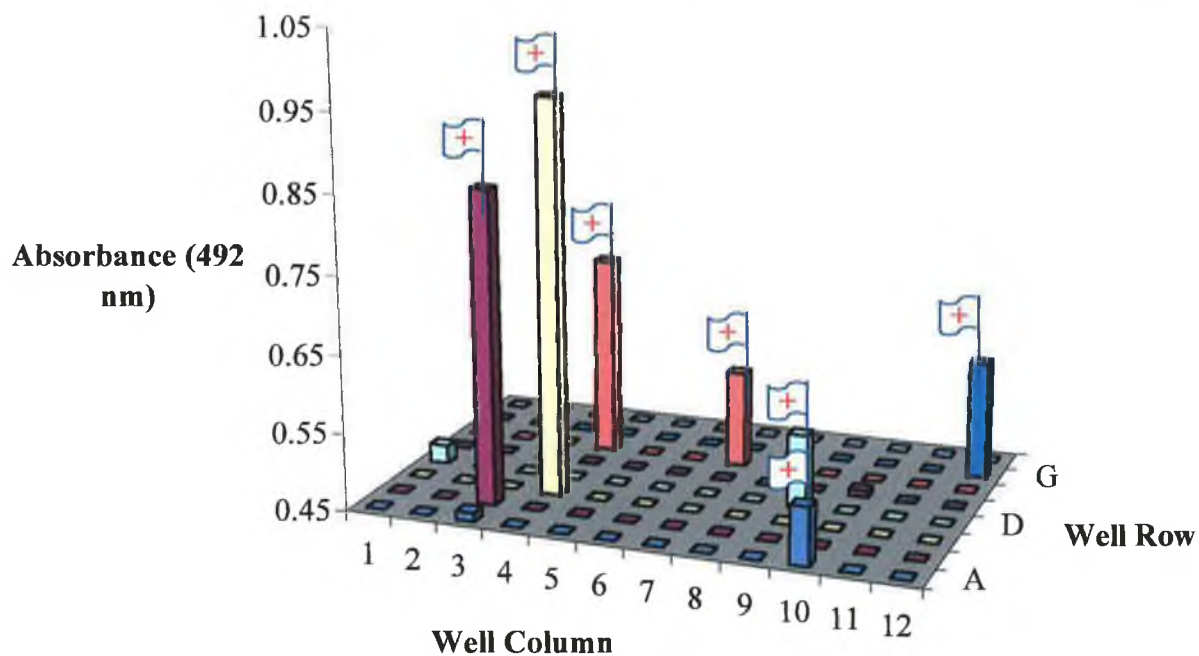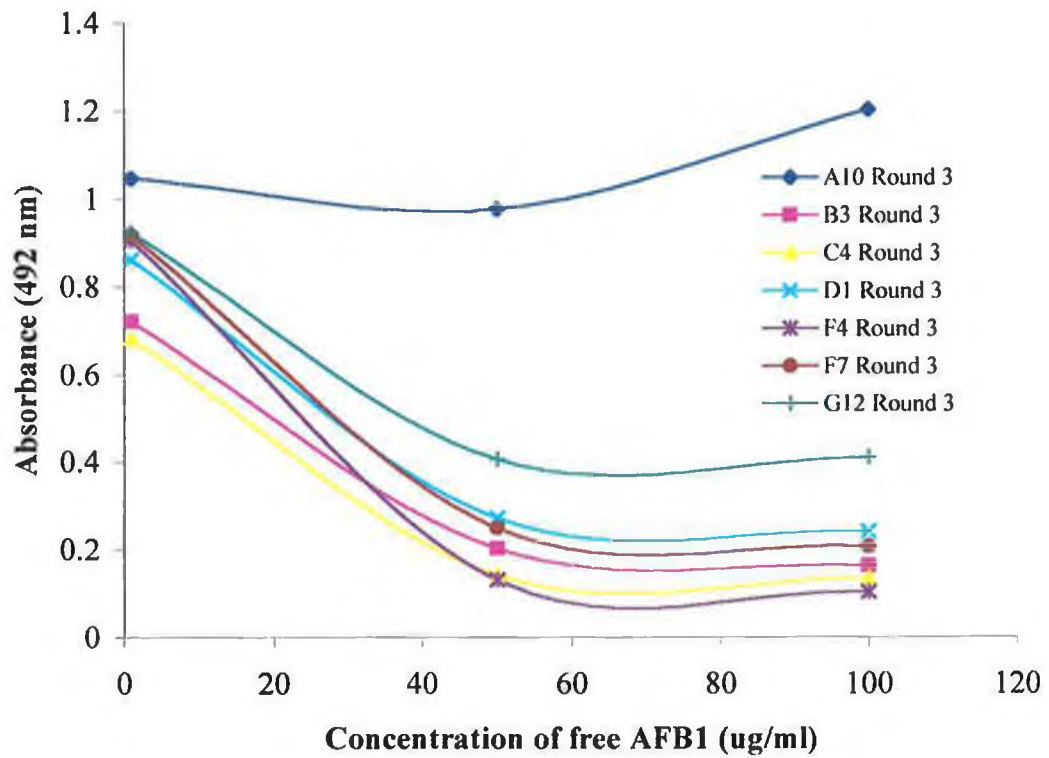
Figure 4.18. *Competition phage-ELISA of seven clones selected after three rounds of panning for recognition to free AFB₁. All seven of the positives were analysed for recognition to free AFB₁ by phage ELISA. Six of the clones selected showed recognition for the free toxin over the range 1-100 ug/ml.*

4.2.7. *Production of soluble scFv antibodies*

4.2.7.1. *Restriction enzyme digestion of plasmid DNA and pAK 400*

Once it was confirmed that the positive clones recognised free AFB_1, three of them were chosen (B3, C4, & F4) for production as soluble scFv antibodies. In order to do this, the bacteria containing the 'positive' plasmid DNA was grown on 5 ml scale and the DNA was purified from the bacteria as described in section 2.17.1. The plasmid DNA was restriction enzyme digested using SfiI, and electrophoresed on a low-melt agarose gel. The cut 800 bp fragment separated on the gel, and was purified as described in section 2.15.5. Figure 4.19 shows a gel photograph of the restriction enzyme digestion of the three clones B3, C4, and F4, from pAK 100. Some of the vector remained uncut, and this was redigested overnight in order to obtain the maximum yield of positive DNA.

Figure 4.20 shows a gel photograph of the restriction enzyme digestion on pAK 400. The cut vector (at approximately 4144 bp) and the tetracycline gene (2101 bp) which was cut from the vector can be easily differentiated on the photograph. A small amount of the vector also remained uncut, however, there was sufficient vector digested to gel-purify and use for the ligation reaction.

4.2.7.2. *Ligation and transformation of pAK 400 into JM 83 E. coli cells*

The 'positive' scFv DNA was ligated into pAK 400 as described in section 2.17.3, and was successfully transformed into the non-suppressor *E.coli* strain JM 83.

Stocks of the transformed bacteria were made, and used to inoculate media for induction of scFv production as described in section 2.17.6.
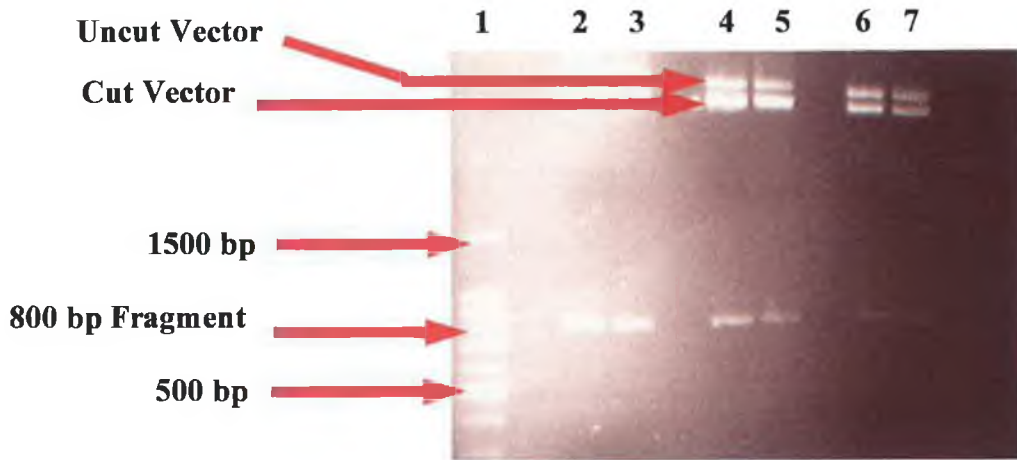
Figure 4.19. Restriction enzyme digest of purified pAK 100 (using SfiI) containing the DNA of three 'positive' clones that recognised free AFB₁. Lane 1: 100 bp ladder. Lanes 2-3: Clone B3. Lanes 4-5: Clone C4. Lanes 6-7: Clone F4. Due to the poor quality of the gel photograph (over-exposure of light) the cut DNA for clone F4 is not very visible, as is also the case with the uncut and cut vector from clone B3. However these were visible to the naked eye when the gel was visualised under UV light. It is possible to see that there was still some uncut vector in the samples, which was re-cut overnight.
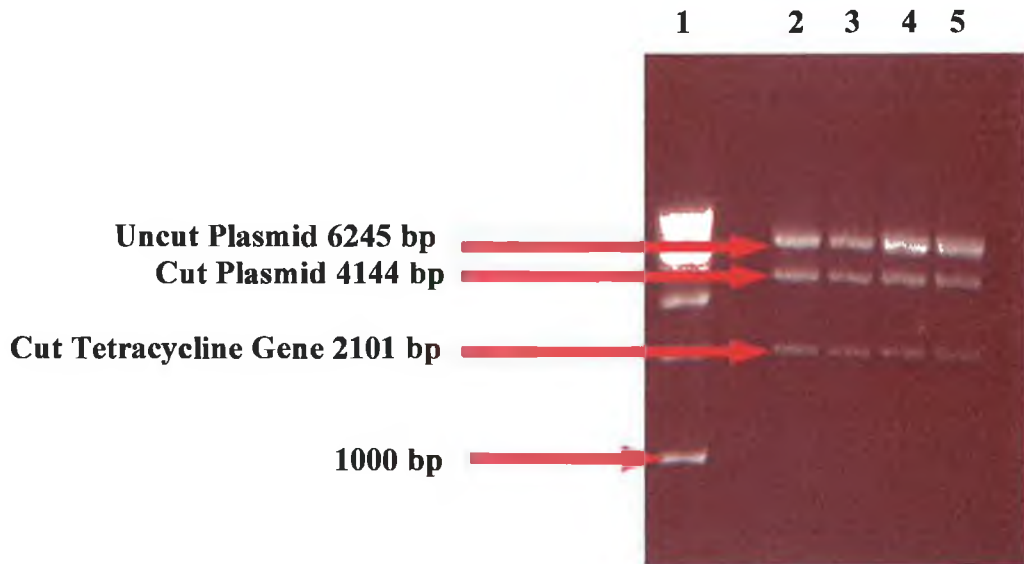
Figure 4.20. Restriction enzyme digest of pAK 400 using SfiI. Lane 1: 1 KB ladder. Lanes 2-5: pAK 400. It is clear from the gel photograph that partial digestion of the plasmid occurred with some uncut plasmid visible. However, there was sufficient cut plasmid to isolate and purify.

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4.2.7.3. *Induction of soluble scFv expression and analysis of cellular distribution*

Soluble scFv expression was induced on the transformed JM 83 cells as described in section 2.17.6. Culture supernatants and periplasmic lysates of the three clones were analysed for soluble expression of the scFv fragments. Figure 4.21 shows the results of an ELISA, which analysed the culture supernatants and the periplasmic lysates from the three clones B3, C4, and F4. From the graph it can be seen that there is minimal secretion of scFv clone-B3 into culture supernatant or periplasmic lysate. The scFv clone-C4 showed a high level of secretion into the supernatant with a moderate level of secretion into the periplasm. Scfv clone-F4 showed an equal amount of secretion of antibody into both culture supernatant and the periplasm. It was decided to work with only one clone from this stage, and, therefore, scFv clone-C4 was chosen for production of soluble scFv antibodies on a large scale and to purify for further analysis.
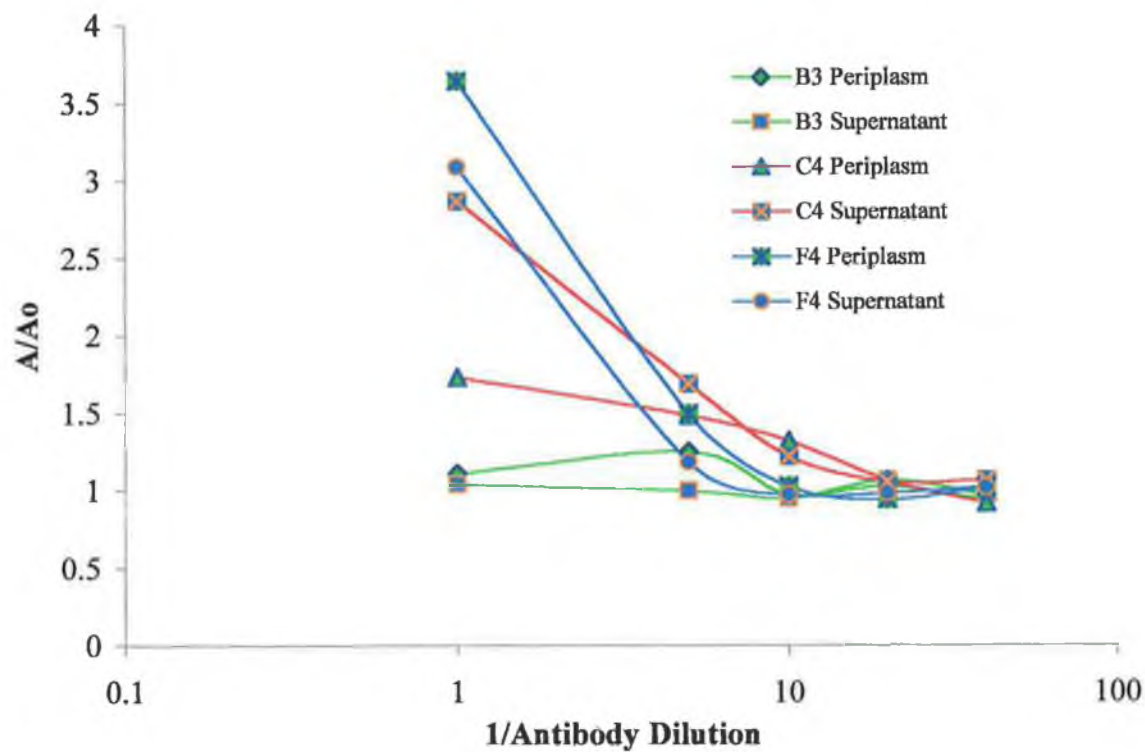
Figure 4.21. *ELISA analysis of periplasmic lysates and culture supernatants of three soluble-expressed scFv anti-AFB₁ clones. The periplasmic lysate data is represented as green markers surrounded by a blue line, while the culture supernatant data is represented as the blue and orange markers. The data shows that clone B3 neither secretes soluble scFv's into the periplasm or the supernatant. Clone C4 secretes into both the periplasm and the supernatant, with a higher degree of secretion into the supernatant. Clone F4 also secretes into the periplasm and the supernatant.*

4.2.7.4. *Purification of soluble scFv fragments by IMAC*

Culture supernatant containing scFv antibodies (clone C4) was concentrated five-fold as described in section 2.18.2. The concentrated supernatant was then passed through the IMAC column for purification (section 2.18.1). The purified scFv antibodies were dialysed into PBS and also concentrated five-fold. Figure 4.22 shows a SDS-PAGE of purified scFv antibody versus culture supernatant. It is apparent from the gel photograph that the purification procedure was not as effective as anticipated in the removal of non-specific proteins from the sample when compared with the culture supernatant. In order to confirm the presence of scFv antibody in the purified samples, a Western blot was carried out. Detection of the scFv was achieved using an anti-FLAG antibody. Figure 4.23 shows a picture of the Western blot. It can be seen that the 32 KDa fragment is quite clear and specific in the purified sample. However, it cannot be seen in the culture supernatant sample, as it may not be concentrated sufficiently.
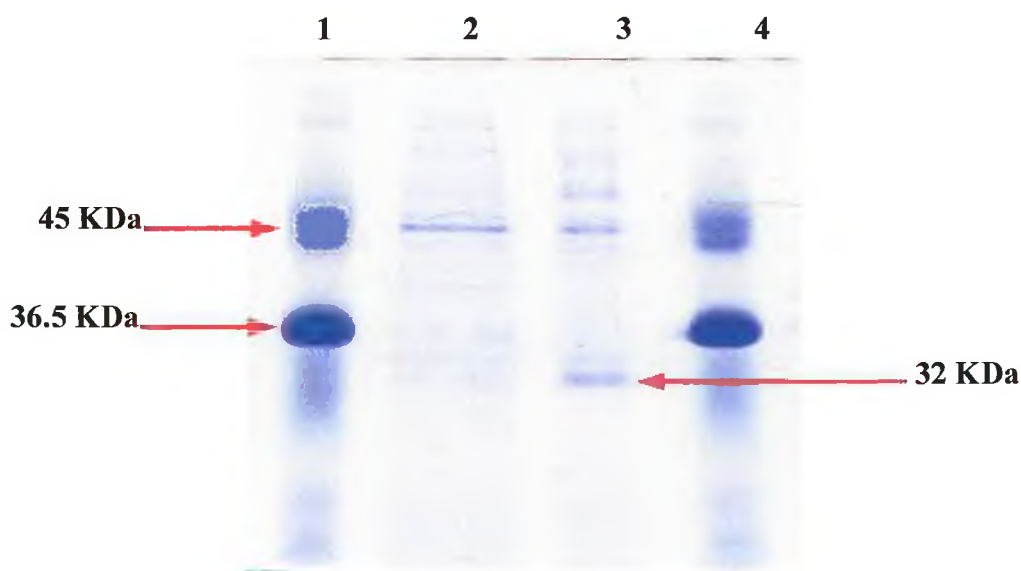
Figure 4.22. *SDS-PAGE analysis of IMAC-purification of C4 clone. Lanes 1 & 4: Molecular weight markers ranging from 6.5 KDa to 45 KDa. Lane 2: Supernatant. Lane 3: IMAC-purified supernatant. The 32 KDa protein fragment is more apparent in the purified fraction than the supernatant, mainly because the purified fractions were concentrated five-fold after the purification procedure. The concentration of non-specific protein in the purified fractions is also quite high indicating the procedure was not as successful as anticipated.*
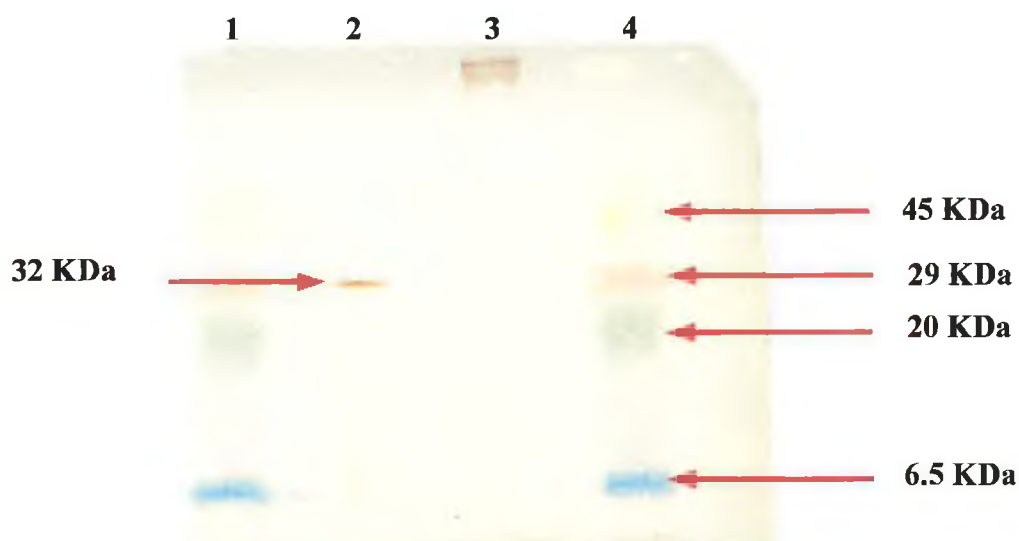
Figure 4.23. *Western blot analysis of IMAC-purification of C4 clone. Lanes 1 & 4: Coloured molecular weight markers. Lane 2: IMAC-purified supernatant. Lane 3: Supernatant. The 32 KDa protein fragment is more apparent in the purified fraction than the supernatant, mainly because the purified fractions were concentrated five-fold after the purification procedure.*

4.2.8. *ELISA analysis of soluble scFv antibodies*

4.2.8.1. *Determination of working dilution of scFv antibody in competitive ELISA for AFB₁*

A checkerboard ELISA was carried out as described in section 2.19.1 in order to determine the optimal antibody dilution and conjugate concentration for use in competitive ELISA. Rows of wells of a 96-well microtitre plate were coated with concentrations of AFB_1-BSA ranging from 0 to 50 µg/ml, and blocked with 2 % (w/v) milk powder. Dilutions of the purified scFv anti-AFB_1 antibody from 1/2 to 1/32 were prepared and added to the plate. After incubation, anti-FLAG antibody was added followed by another incubation step. Enzyme-labelled anti-mouse antibody was then added followed by chromogenic substrate. Figure 4.24 shows the overlaid titre curves obtained for the scFv antibody. The antibody dilution that gave

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half the maximum absorbance was chosen, and the lowest conjugate concentration that provided sufficiently high absorbances was chosen. A 1/4 dilution of antibody was used in the competition ELISA, with a conjugate concentration of 12.5 μg/ml.
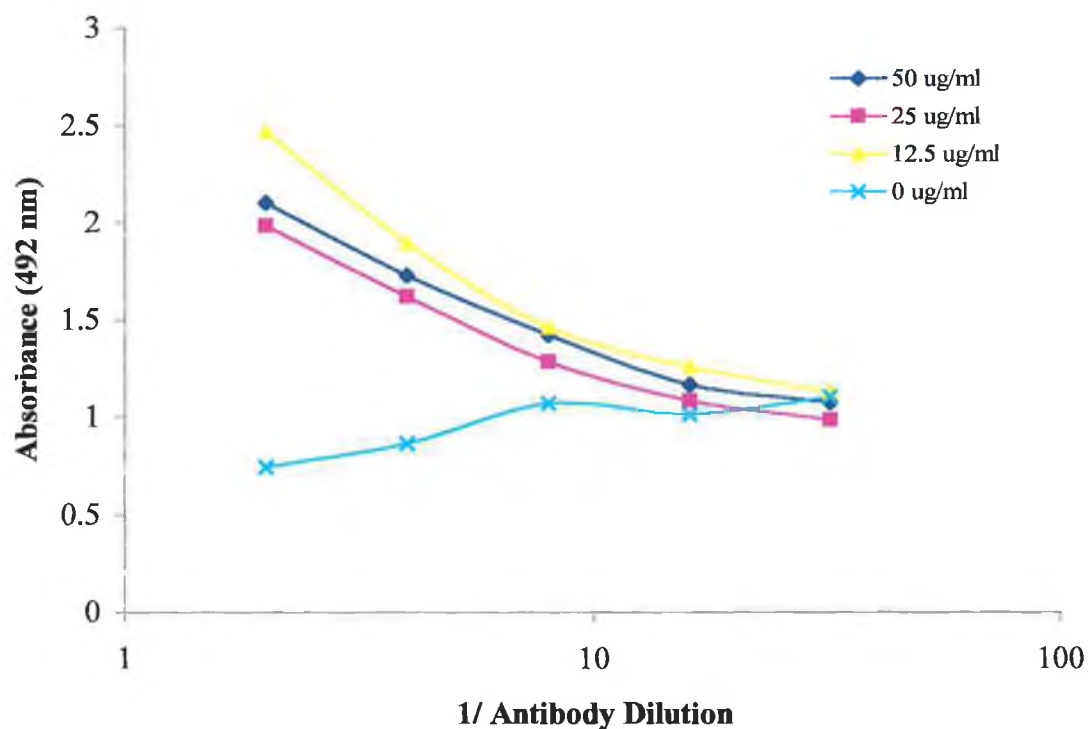
Figure 4.24. *Determination of optimal conjugate loading density and antibody dilution. Wells of a microtitre plate were coated with AFB_1-BSA conjugate at a range of concentrations between 0 and 50 μg/ml. Serial doubling dilutions of the IMAC-purified anti-aflatoxin scFv antibody (C4) were carried out and then added to the plate as described in section 2.19.1. The optimal conjugate concentration chosen was 12.5 μg/ml, and the optimal scFv antibody dilution (i.e. the one that gave half the maximum absorbance) was 1/4.*

4.2.8.2. *Competition ELISA for the detection of AFB₁ using scFv antibody*

A series of standards of AFB_1 ranging in concentration from 98-1,560 ng/ml were prepared as described in section 2.10.1. 50 μl of each of these standards were then added to an AFB_1-BSA coated plate (section 2.19.2). 50 μl/well of the scFv anti-AFB_1 antibody was prepared at a 1/2 (1/4 final dilution) dilution and added to the plate. After incubation and washing, anti-FLAG antibody and enzyme-labelled anti-mouse antibody were added followed by chromogenic substrate. The linear range of detection for the scFv antibody was 98 to 1,560 ng/ml. For intra-day assay variation, each concentration was assayed three times on the one day and the mean absorbance of bound antibody for each antigen concentration was plotted versus antigen concentration. Figure 4.25 shows the intra-day assay linear range of detection of AFB_1 for the scFv antibody, while Table 4.7 shows the coefficients of variation (C.V.'s) for the assay. Intra-day percentage C.V.'s for the assay were below 4.6 %.

The inter-day assay variation was calculated by performing the assay over three separate days, and a separate calibration curve plotted for each normalised absorbance (absorbance/absorbance 0 (A/Ao)) value versus the respective AFB_1 concentration for each assay carried out on each day. The normalised mean values for the assay were calculated and plotted. Figure 4.26 shows the inter-day variability assay for the scFv antibody, while the percentage C.V.'s for the inter-day assay are shown in Tables 4.8. The percentage C.V.'s for the assay were less than 12 %.

Table 4.7. Intra-day assay coefficients of variation for the determination of free AFB_1 using scFv anti-AFB_1 antibody (C4). Three sets of five standards were assayed the same day, and the C.V.'s were calculated as the standard deviation (S.D.) expressed as a percentage of the mean values for each standard.

Aflatoxin B_1 concentration (ng/ml)	Calculated mean ± S.D.	Coefficient of variation (%)
1,560	0.852 ± 0.039	4.59
780	1.081 ± 0.027	2.54
390	1.435 ± 0.022	1.57
195	1.665 ± 0.056	3.39
98	2.044 ± 0.068	3.36
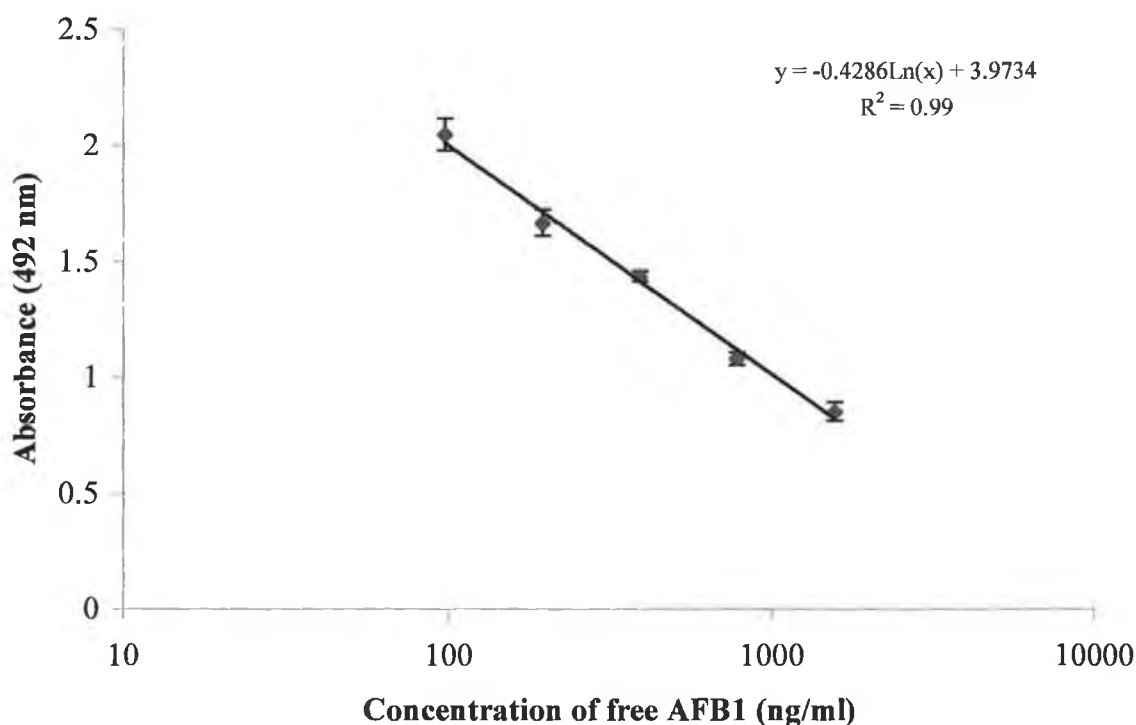
Figure 4.25. Intra-day assay variation for competitive ELISA for AFB_1 using scFv anti-AFB_1 antibody (C4). The linear range of detection was 98 to 1,560 ng/ml. The error bars on each calibration point indicate the standard deviation of the mean of three measurements.

Table 4.8. *Inter-day assay coefficients of variation for the determination of free AFB$_1$ using scFv anti-AFB$_1$ antibody (C4). Three sets of five standards were assayed on three different days, and C.V.'s were calculated as the standard deviation expressed as a percentage of the mean normalised values for each standard.*

Aflatoxin B$_1$ concentration (ng/ml)	Calculated mean ± S.D. A/Ao	Coefficient of variation (%)
1,560	0.404 ± 0.039	9.71
780	0.490 ± 0.058	11.90
390	0.643 ± 0.056	8.74
195	0.753 ± 0.060	8.07
98	0.858 ± 0.022	2.67

$$y = -0.1691Ln(x) + 1.6392$$
$$R^2 = 0.99$$
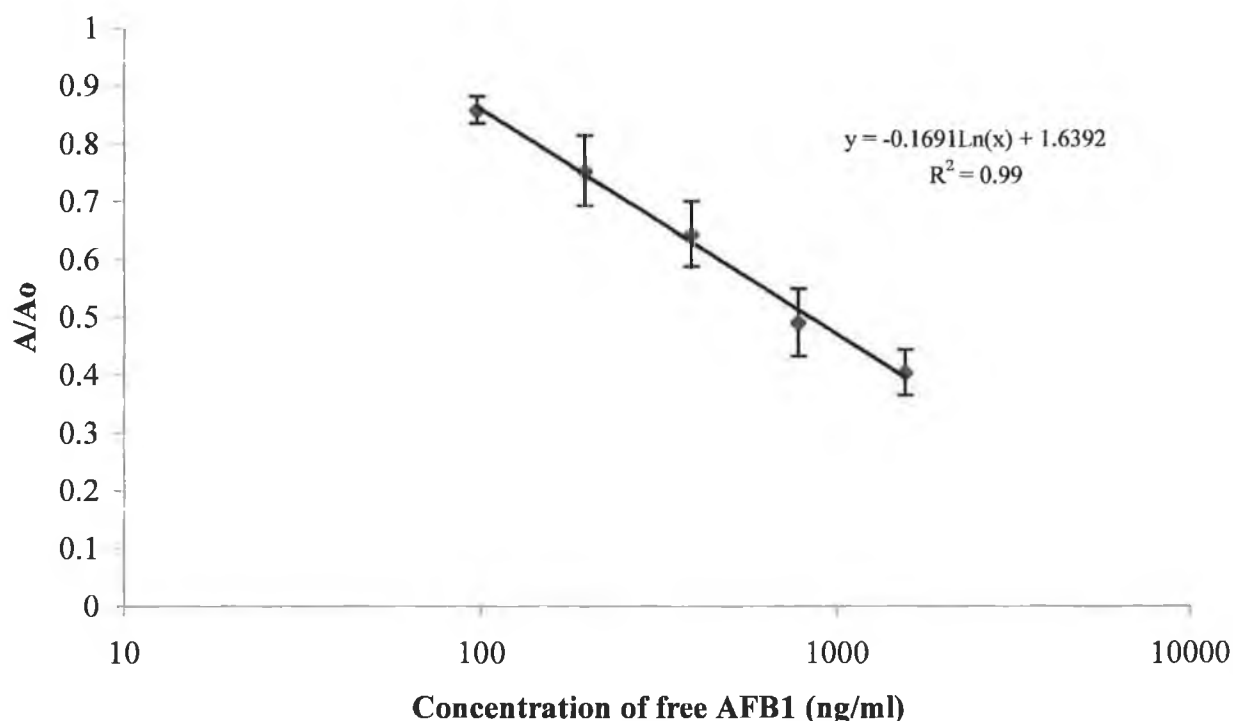
Figure 4.26. *Inter-day assay variation for competitive ELISA for AFB$_1$ using scFv anti-AFB$_1$ antibody (C4). The linear range of detection was 98 to 1,560 ng/ml. The error bars on each calibration point indicate the standard deviation of the mean of three measurements.*

4.2.8.3. *Cross-reactivity studies*

Cross reactivity studies were carried out on the scFv antibody to seven other structurally related aflatoxins, as described in section 2.19.3. Standard curves for each of the aflatoxins were produced as described previously in 4.2.8.3. The results were normalised and plotted. The slope of the line of the standard curve for each toxin was expressed as a percentage of the slope of the line for binding to AFB_1. Table 4.9 shows the cross-reactions of the scFv antibody, which showed a high level of cross-reaction to the structurally related aflatoxins.

Table 4.9. *Cross-reactions of scFv anti-AFB_1 antibody (C4) observed to seven other aflatoxins. Standard curves were produced for each of the aflatoxins in the same way as that for AFB_1. Each concentration for each point on the standard curve was assayed in triplicate and the means plotted and normalised in order to construct standard curves.*

Aflatoxin	scFv % Cross Reactivity
B_1	100
B_2	53.1
G_1	75.6
G_2	67.9
M_1	50.4
M_2	22.8
B_{2a}	17.8
G_{2a}	21.7

4.3. Discussion

The work presented in this chapter showed the use of a pre-immunised antibody phage display library in order to produce soluble scFv antibodies, and their characterisation by ELISA. In order to produce the phage display library, Balb/c mice were immunised with an AFB_1-BSA conjugate. Only one protein conjugate was commercially available, and was therefore used for both the immunisation and analytical steps. When carrying out antibody titres using this conjugate, the same controls as mentioned and explained in chapter 3.3 were included. These controls worked effectively, and it was apparent from them that there was a greater immune response to the AFB_1 portion, as opposed to the BSA part of the conjugate. The high antibody titre to the toxin-protein conjugate was indicative of large quantities of mRNA translating for specific AFB_1-BSA antibodies. The spleen is rich in B-cells, and therefore rich in mRNA coding for these specific B-cells. As a result, once a sufficiently high enough specific antibody titre was obtained, the spleen was removed from the mice, and the RNA extracted from it.

Extraction of the RNA was successful, and good yields were obtained from the spleen. There was also a certain amount of protein (as expected) in the samples. However, the quantity of protein was not greater than the amount of RNA present. A complementary strand of DNA (cDNA) was synthesised from the RNA by reverse transcription, and this was used for amplification of the heavy and light chains by PCR.

Amplification of the heavy and light chains was carried out using the extended primer mix (Chapter 2.15.4.), which incorporated all of the mouse V_H, V_λ, and V_κ sequences collected in the Kabat data base (Kabat *et al.*, 1991). It also combined the extended primer sets described by Kettleborough *et al.* (1993), Ørum *et al.* (1993), and Zhou *et al.* (1994). This ensured that a larger repertoire of DNA coding for the mouse heavy and light chains was effectively and specifically amplified, and as a result a larger population of antibodies available for selection during the screening process. It can

be seen (Figure 4.6) that the heavy and light chains were specifically amplified, using an annealing temperature of 63° C.

Once the V_H and V_L fragments were amplified, they were densiometrically analysed, to determine the concentration of DNA for optimisation of the SOE-PCR. A greater quantity of heavy chain DNA was amplified compared to the light chain. However, there was sufficient light chain to proceed with the SOE-PCR. Approximately 10 ng of each of the V_H and V_L's was used, and this was adequate for the reaction to work successfully (Figure 4.7). SOE-PCR produced an 800 bp fragment, as the heavy and light chains are approximately 400 bp long each.

Both the SOE-PCR product and the vector pAK 100 had to be restriction enzyme-digested prior to the ligation step. SfiI was the restriction enzyme used, and, as described in section 4.1, it is unique as it cuts two sites at once on a piece of DNA. It is also useful, as the sites at which it cuts at are quite rare in antibody sequences, and therefore it eliminates the possibility of internal digestion, and incorrect sequence lengths. Digestion of the SOE product results in a very small change in weight of the piece of DNA. It is therefore quite difficult to differentiate between cut and uncut DNA. In order to aid the visual differentiation, the samples were electrophoresed on a 2-2.5 % agarose gel, which improved the separation of the fragments. Figure 4.8 shows an agarose gel of digested and undigested fragment, and it is just possible to differentiate between the two fragments on the gel, indicating the reaction was successful. Digestion of the pAK 100 vector is visually easier to differentiate, as the cut tetracycline cassette is 2101 bp in length. This is easily visualised on the gel (Figure 4.9). It can also be seen on the gel that a certain amount of vector remained uncut. Possibly there was not enough SfiI to digest all the vector present in the sample. However, it is more favourable to have 'too-little' enzyme rather than 'too-much', especially in the case of SfiI. In reactions, with the enzyme in excess of DNA sites, each site becomes loaded with SfiI tetramer, and thus preventing the protein from binding the two sites necessary for full activity. Hence, it is easier to re-digest a sample to obtain complete digestion, rather than add too much initially, and not achieve any or sufficient digestion.

Once both the vector and the SOE product were digested, they were ligated and transformed into supercompetent *E.coli* XL-1 blue cells. An antibody phage display library consisting of 5 x 10^3 clones was produced. This library would be considered as very small in size, as it is possible to generate libraries consisting of 10^7 or 10^8 clones. However, given the fact that the library was produced from RNA that originated from a pre-immunised mouse, it was decided to attempt panning from the library, rather-than repeating the transformation procedure to produce a larger library.

In order to select for small haptens from a phage display library it optimal to use more than one hapten-protein conjugate. Therefore a second AFB_1 conjugate needed to be synthesised. Amino-dextran was chosen as the carrier molecule, as previously, it has been shown to yield low background values in ELISA (Xiao *et al.*, 1995). This feature of amino-dextran is optimal for use in the panning procedure, as it is necessary to keep background signals to a minimum to ensure a non-biased selection for positive clones recognising free hapten. AFB_1 was activated producing the compound AFB_1 O-carboxymethylhydroxylamine, which contained a COOH group on the cyclopentanone ring. The COOH group was then used for coupling to amino groups on the dextran using conventional carbodiimide coupling chemistry. Due to laboratory safety, it was unsuitable to analyse the conjugate by ultra-violet spectroscopy. BIAcore™ (1000) was therefore used to analyse the conjugate, as it was a rapid and safer method to use. The conjugate was preconcentrated onto a blank dextran surface in order to optimise the pH in acetate buffer for immobilisation. The pH used was 5.0, which was relatively high, indicating a low substitution ratio of toxin on the dextran molecule. Immobilisation of the conjugate onto the activated dextran gel surface resulted in 2,773 RU (1,000 RU = 1 ng/mm^2) being immobilised. The two polyclonal antibodies described in chapter 3 were then injected over the surface. Both showed low levels of binding to the conjugate surface. The antibodies obtained from rabbit 1 gave 67 RU of binding, while the antibodies obtained from rabbit 2 resulted in 95 RU binding. The antibodies obtained from rabbit 1 showed a significantly better binding response to an AFB_1-BSA conjugate (~350 RU) (Chapter

5.2) at the same dilution (1/100). This also confirmed a relatively low substitution ratio on the dextran conjugate.

After producing phage from the library initially, a phage titre of 2.5×10^9 cfu/ml of phage was produced. Round 1 of panning was carried out using an AFB_1-BSA conjugate coated onto an immunotube at 50 µg/ml. This yielded a phage titre of 3.0×10^5 cfu/ml of phage. A lower phage titre after the first round was expected, as it selected out a large amount of non-specific phage display antibodies. 96 random clones were picked and grown to produce phage from them. They were tested for recognition to AFB_1-BSA conjugate. The background signal was quite high, and it was difficult to completely differentiate between positive clones and background. 8 of the clones shown a certain level of recognition for the conjugate i.e. those that gave the highest absorbances. Three of these were then assayed for recognition of free AFB_1, but none of them were positive. Therefore, a further round of panning needed to be carried out to select for phage display antibodies recognising AFB_1.

After the first round of panning, it is desirable to switch the carrier molecule on the conjugate for the second round. This ensures that non-specific phage recognising the protein (from the round 1 conjugate) are not re-selected and amplified. Instead, only phage generally recognising the hapten will be selected for, and thus increasing the chances of selecting and amplifying a specific phage-antibody to the hapten. As a result of this, AFB_1-dextran was used for round 2 of panning. A high concentration of 200 µg/ml was used to coat the immunotube, due to the low substitution ratio on the conjugate. This yielded a phage titre of 2.8×10^7 cfu/ml, which was higher than the titre obtained from round 1. 96 random clones were then selected and assayed for recognition to AFB_1-BSA conjugate, and ten clones were positive. In the case of this assay the backgrounds were significantly lower compared to the ELISA after round 1 (Tables 4.4 and 4.5). Three of these positive clones were then assayed for recognition to free AFB_1. However, none of them were positive, indicating there was still a large number of non-specific phage in the population. This prompted a third

round of panning to be carried out, in order to make the selection conditions more stringent for the detection of positive clones.

AFB_1-BSA conjugate was used for round 3, as the previous round ensured that non-specific 'BSA-binding' phage antibodies were removed from the selection process. As the number of rounds of panning increase, the selection for a specific phage-antibody becomes more stringent, as each round (providing different conjugates are used) ensures a reduction in the amount of non-specific phage, and greater amplification of specific phage. Round 3 gave a phage titre of 2.4×10^7 cfu/ml, which was slightly less than that obtained from round 2. 96 random clones were picked and assayed for selection to AFB_1-BSA conjugate. Seven clones showed good recognition for the conjugate, with significantly lower background levels compared to the round 2 ELISA (Table 4.6). The seven clones were then assayed for recognition to free AFB_1, in which six of them showed recognition over the range 1-50 μg/ml. The panning process was highly successful, as selection of phage antibodies to small haptens is generally quite difficult.

It has been mentioned elsewhere (Moghaddam *et al.*, 2001) that selection of a large number of antibodies to low molecular weight haptens conjugated to BSA or biotinylated can often be unsuccessful for the detection of antibodies that bind the non-derivatised form of the hapten. Moghaddam *et al.* (2001) screened for phage display scFv antibodies to AFB_1 from non-immunised human phage libraries, as well as semi-synthetic phage libraries. It was found that a large percentage of the antibodies isolated from the non-immunised (human) library specifically bound AFB_1-BSA conjugate but did not bind free AFB_1, and if so, had at least 100-times less affinity than for the BSA conjugated form. However, they did find that a percentage of the scFv antibodies that bound to both free toxin and conjugate dramatically increased, when free AFB_1 was used for elution of phage from AFB_1-BSA during the elution step of the panning. With respect to the semi-synthetic library, after three rounds of panning using the same conjugate, antibodies with high affinities to free AFB_1 were detected. This is a similar finding to what is presented in

this chapter, except that two different conjugates were used. However, they used AFB_1 for elution of the phage from the immunotube, which may have increased the stringency of the procedure.

Three of the clones (B3, C4, and F4) that recognised free AFB_1 were then selected for expression as soluble scFv antibodies. Restriction enzyme digestion of the pAK 100 plasmid containing the 'positive' scFv fragment was successfully carried out, as was digestion of the high expression vector pAK 400, which contains the enhanced Shine-Dalgarno sequence. Ligation of the scFv fragments into the plasmid was carried out and these were then transformed into the non-suppressor *E.coli* strain JM 83, which enhances soluble scFv expression. Analysis of the periplasmic lysates and the culture supernatants was carried out by ELISA. One of the clones (F4) did not show significant levels of secretion of soluble scFv into the periplasm or the culture supernatant, indicating that the scFv antibodies were not secreting correctly from the intracellular compartment of *E.coli*. Due to the arduous task in obtaining intracellular scFv antibodies, it was decided not to pursue work with this particular clone. Clone B3 showed equal levels of antibody secretion both into the periplasm, as well as the culture supernatant. Clone C4, however, demonstrated a larger degree of secretion into the supernatant rather than the periplasm. It was therefore decided to concentrate on working with clone C4, as there was a predominant form of expression and, therefore, a quicker route to obtaining and purifying the scFv's.

After scFv antibodies were produced in culture supernatant, they were concentrated five-fold and then purified using ProBond™ resin, which is an IMAC-based column (Janknecht *et al.*, 1989). This procedure did not work as effectively as anticipated. Figure 4.22 shows a SDS-PAGE of purified scFv antibody versus culture supernatant. With respect to purity, there is very little difference between the two samples as they both have a number of bands common to each other. IMAC was only useful in isolating the antibodies from the culture supernatant, but not isolating them from the proteins in the supernatant. It may be possible to further optimise the procedure by dialysing the culture supernatant into column running buffer prior to passing it

through the column. This would remove some of the non-specific proteins below the molecular weight cut-off of 10 KDa, and therefore improving antibody purity. Muller *et al.* (1998) state that if the protein of interest such as an scFv antibody is only present as a small fraction in the supernatant, several contaminating proteins can remain. These can bind to the IMAC column under certain purification conditions and co-elute. Certain proteins from *E.coli* have been identified which may bind to IMAC columns during purification. These include superoxide dismutase (8 histidines (his)), chloramphenicol acetyltransferase (12 his), cAMP receptor protein (6 his), heat shock protein (14 his), host factor-1 protein (5 his), and wondrous histidine-rich protein (18 his). Some of these proteins, therefore, may account for the impurities observed on the SDS-PAGE gel. The IMAC column used in this procedure used iminodiacetate (IDA) as the metal-chelating compound and nickel (Ni^{2+}) as the binding-metal. Arnold (1991) states that due to the higher affinity of imidazole (elution compound used in IMAC) for copper (Cu^{2+}), histidine containing proteins are more strongly bound to Cu (II)-IDA, than to IDA chelates of other metals. It may be therefore more favourable to use copper as the chelating metal, rather than nickel which was being used. Alternative methods of purification could also be implemented such as production of an affinity column using an anti-FLAG antibody. However, given the quality of the antibody available, and the expense, it was not feasible to produce such a column. It was also possible to produce an AFB_1 affinity column for purification of the scFv antibodies, but this was not feasible given the hazards associated with AFB_1. Both of these affinity columns use low pH buffers for elution, and this may affect antibody activity. Hence, it was more favourable to use IMAC, as low pH elution was not required, with an accompanying greater chance of maintaining the integrity of the antibody.

Western blot analysis was also carried out to identify the scFv antibodies. Figure 4.23 shows the Western blot, where a single band can be observed for purified sample indicating the presence of scFv antibodies. No significant band was observed in culture supernatant. This is probably because the sample was not sufficiently concentrated for the anti-FLAG antibody to detect it in the blot.

It was important to initially optimise the concentration of AFB_1-conjugate and scFv antibody for use in a competitive ELISA. A checkerboard ELISA was carried out for the scFv antibody as described in section 2.19.1. The antibody dilution used was 1/4 with a conjugate loading density of 12.5 μg/ml for the assay.

A competition ELISA was optimised for the scFv antibody using these parameters. The assay was relatively sensitive, with a limit of detection of 98 ng/ml. Inter-day assay variation studies were carried out by analysing three sets of standards across the respective linear ranges on three different days and calculating the C.V.'s as the standard deviation expressed as a percentage of the mean normalised values for each standard.

The assay was reproducible over three different days with the C.V.'s below 11.9 % (Table 4.8). Yuan *et al.* (1997) produced scFv antibodies to the mycotoxin zearalenone, and found that the soluble antibodies could detect as little as 14 ng/ml for 50 % inhibition of binding. The scFv antibodies produced by Moghaddam *et al.* (2001) could detect as little as 14 μM AFB_1 in solution. It is clear that these antibodies are more sensitive than the scFv produced here. The polyclonal antibodies produced to AFB_1 discussed in Chapter 3 are also significantly more sensitive (limits of detection 12.2 & 24 ng/ml, respectively) than the anti-AFB_1 scFv antibody. However, it is thought that the assay could be further optimised if a more sensitive anti-FLAG antibody could be obtained. Enzyme-labelling the anti-FLAG antibody could also improve the sensitivity of the assay, as it would reduce the number of incubation steps, as well as reduce the probability of the enzyme-labelled anti-species antibody reducing the sensitivity.

Cross-reactivity studies were also carried out on the scFv antibody. The procedure is described as in section 2.19.3 and Table 4.9 shows the results of the studies. The scFv antibody showed high degrees of cross reactivity, especially to AFG_1> AFG_2> AFB_2> M_1 (in decreasing order). These are similar findings to the polyclonal antibodies discussed in chapter 3, where they were also cross-reactive to AFG_1 as the

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major functional group which both compounds differ from is masked by the protein which is attached on the cyclopentanone ring. Moghaddam *et al.* (2001) analysed their scFv antibodies for cross reactivity using BIAcore. They found that the antibody was able to bind as effectively to AFG_1 as AFB_1. This is a similar finding to what was observed with the scFv antibody reported here. However, the scFv did not show any recognition for AFM_1 or AFM_2. It is favourable to produce specific antibodies for a particular hapten, but it could be advantageous from a regulatory viewpoint to apply this scFv antibody for the detection of total aflatoxin concentration in a sample.

4.4. Conclusions

The production of an antigen-specific antibody phage display library from the RNA obtained from a mouse pre-immunised with AFB_1-BSA conjugate was successful. The stringency of the panning procedures using two toxin-carrier conjugates sequentially reduced the number of non-specific phage after each round of panning. It resulted in the highly successful isolation of seven phage display clones, six of them recognising free AFB_1 in solution. Soluble production of scFv antibodies (clone C4) was induced using the high expression vector pAK 400. After purification by IMAC, a competition ELISA was optimised for the scFv antibody. The assay was relatively sensitive, but it was thought it could be further optimised using a more sensitive anti-FLAG antibody. The scFv showed a high level of cross-reactivity to structurally similar aflatoxins. The phage display system used was found to be highly robust, and generally straightforward for the production of scFv antibodies. SOE-PCR was particularly useful, as it reduced the number of ligation steps, coupled with the ease of restriction enzyme digestion using SfiI. The use of a number of expression vectors eliminated the problems associated with expression levels, and enabled the production of highly stable scFv antibodies.

Chapter 5

Development and Application of Surface Plasmon Resonance-Based Immunoassays for Aflatoxin B_1 using Polyclonal and Single Chain Fv antibodies

5.1. Introduction

Biosensor development over the last few decades has grown enormously, and now, biosensors are an established and reliable analytical tool. A biosensor may be defined as any probe or transducer that incorporates a biological component as the key functional element in the overall transducer sequence (Vadgama & Crump, 1992). Both the transducer and biological element can then work together to relate the concentration of analyte to a measurable electronic signal. The first biosensor reported (Clark & Lyons, 1962) incorporated an enzyme as the bio-component operating in combination with an electrochemical transducer for the detection of glucose in samples. Since then, interest in the biosensor area has grown substantially, and their development has been extensively reviewed elsewhere (Pancrazio *et al.*, 1999; Rogers, 2000). Trends in biosensor development today show miniaturised, chip-based, microarrays capable of multi-analyte detection (Ekins, 1998; de Wildt *et al.*, 2000).

5.1.1. *Components of a biosensor*

In the design of any biosensor, the biological component is an essential part and should possess a high degree of specificity and stability. It is also important that it should not contaminate the sample, as well as retain its biological activity when immobilised. The biologically sensitive components can be an enzyme, organelle, membrane component, a bacterial cell or whole cell, or an antibody or antigen (Sethi, 1994). These materials are responsible for the recognition of the test species in the mixture and provide the sensitivity and specificity for the entire device. When the biological molecules interact specifically and reversibly, a change in one or more physio-chemical parameters occurs. These changes may produce ions, electrons, gases, heat, mass or light, which are then converted into electrical signals by the transducers, that process and display the information in a suitable form. A number of different transducer elements are available for use in biosensors (Sethi, 1994). These include electrochemical, which measure conductance, piezo-electric, which measure mass change, and optoelectronic, which measure optical changes. Biosensors-based on optoelectronics often incorporate the phenomenon of surface plasmon resonance

(SPR) as a method of detection. SPR-based biosensors are important tools in the biotechnology industry, as they have the ability to generate large amounts of qualitative and quantitative data simultaneously. A number of SPR-based biosensors are commercially available which include; the BIAcore range of instruments (BIAcore AB), the IAsys instrument (Affinity Sensors), the Texas Instruments SPR device (TI-SPR), and BIOS-1 system (Windsor Scientific Ltd). This chapter will focus on surface plasmon resonance-based biosensors and their applications in the detection of analytes.

5.1.2. *Surface plasmon resonance*

In order to explain the principle of SPR, it will be described in the context of BIAcore instruments, which incorporate the SPR phenomenon to monitor biomolecular interactions in 'real-time'. At an interface between two transparent media of different refractive index such as glass and water, light coming from the side of higher refractive index is partly reflected and partly refracted. Above a certain critical angle of incidence no light is refracted across the interface and total internal reflection (TIR) occurs at the metal film-liquid interface. This is where light travels through an optically dense medium such as glass, and is reflected back through that medium at the interface with a less optically dense medium such as buffer. Although the incident light is totally reflected, the electromagnetic field component, termed the evanescent wave, penetrates a distance on the order of one wavelength into the less optically dense medium, in this case buffer. The evanescent wave generated at the interface between a glass prism (high refractive index) and a layer of buffer (lower refractive index) is illustrated in Figure 5.1. If the interface between the media of higher and lower refractive indices is coated with a thin metal film (a fraction of the light wavelength), then the propagation of the evanescent wave will interact with the electrons on the metal layer. Metals contain electron clouds at their surface, which can couple with incident light at certain angles. These electrons are also known as plasmons, and the passage of the evanescent wave through the metal layer causes the plasmons to resonate, forming a quantum mechanical wave known as surface plasmon. Therefore, when surface plasmon resonance occurs, energy from the
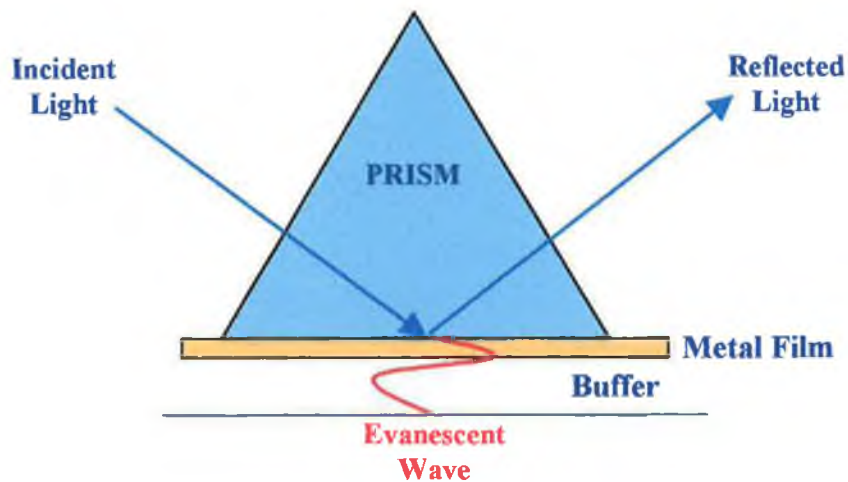
Figure 5.1. *The evanescent wave. Light directed into a glass prism at an angle greater than the critical angle undergoes total internal reflection. At the interface between the prism (high refractive index) and the buffer (lower refractive index), an electromagnetic field component of the light, the evanescent wave propagates into the medium of lower refractive index on the non-illuminated side.*

incident light is lost to the metal film resulting in a decrease in the reflected light intensity. The resonance phenomenon only occurs at an acutely defined angle of the incident light. This angle is dependent on the refractive index of the medium close to the metal-film surface. Changes in the refractive index of the buffer solution (e.g. an increase in surface concentration of solutes), to a distance of about 300 nm from the metal film surface will therefore alter the resonance angle. Continuous monitoring of this resonance angle allows the quantitation of changes in refractive index of the buffer solution close to the metal-film surface. This is illustrated in Figures 5.2 to 5.4 where binding of the analyte shifts the SPR angle from position 1 to position 2.

The reflected light angle at which SPR occurs is determined by three main parameters, which include the properties of the metal film (optical constants, thickness and uniformity etc.), the wavelength of the incident light, and the refractive index of the media on either side of the metal film.
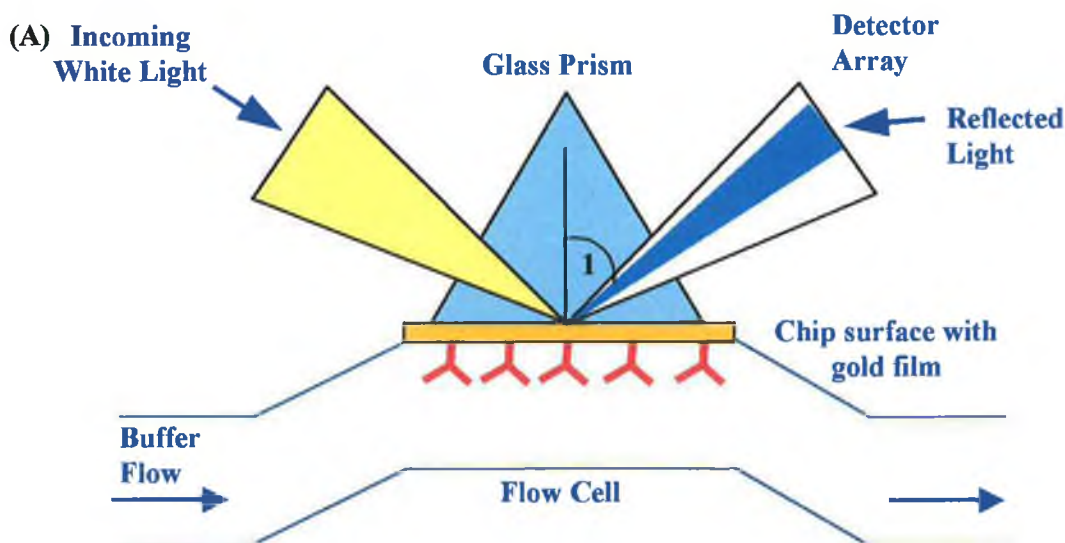
Figure 5.2. *Schematic basis of SPR technology with the BIAcore instrument.*

(A) Antibody (Y) is immobilised on the gold surface using conventional coupling chemistry such as EDC/NHS. Plan polarised light is emitted from a high efficiency emitting diode, and is focused on the gold chip surface in the shape of a wedge-shaped beam using a glass prism, under conditions of total internal reflection. The reflected light is then measured by a two-dimensional photo-diode array. The evanescent wave penetrates into the medium of lower refractive index at the metal-coated interface under conditions of total internal reflection. The angle at which this occurs is known as the resonant angle (i.e. SPR angle = angle 1), and a sharp shadow or dip occurs in the reflected light at angle 1), designated by the blue line in the reflected light. The angle at which SPR occurs is dependent on a number of factors including the refractive index of the layer adjacent to the metal film. SPR is therefore used to probe and monitor the interactions occurring at the chip-surface in 'real-time'.
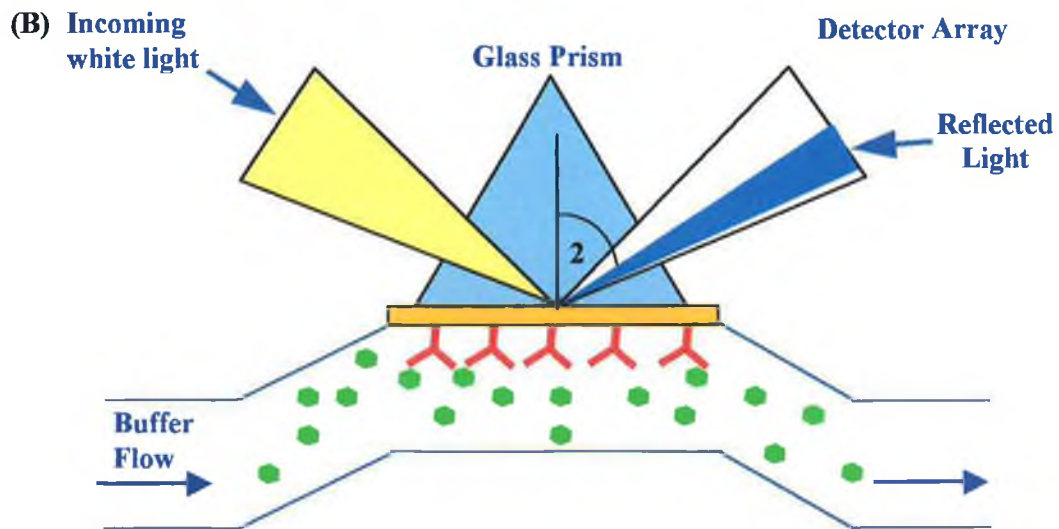
Figure 5.3. (B) *Antigen (●) is then injected over the immobilised-antibody chip surface. Binding of the antigen to the immobilised antibody causes an increase in the mass bound at the sensor chip surface and a subsequent change in the refractive index at the sensor chip surface, causing a shift in the resonant angle of the reflected light (i.e. from angle 1 (Figure 5.2) to angle 2). The shift in the resonant angle is proportional to the change in the mass of analyte bound at the sensor chip surface.*

(C)
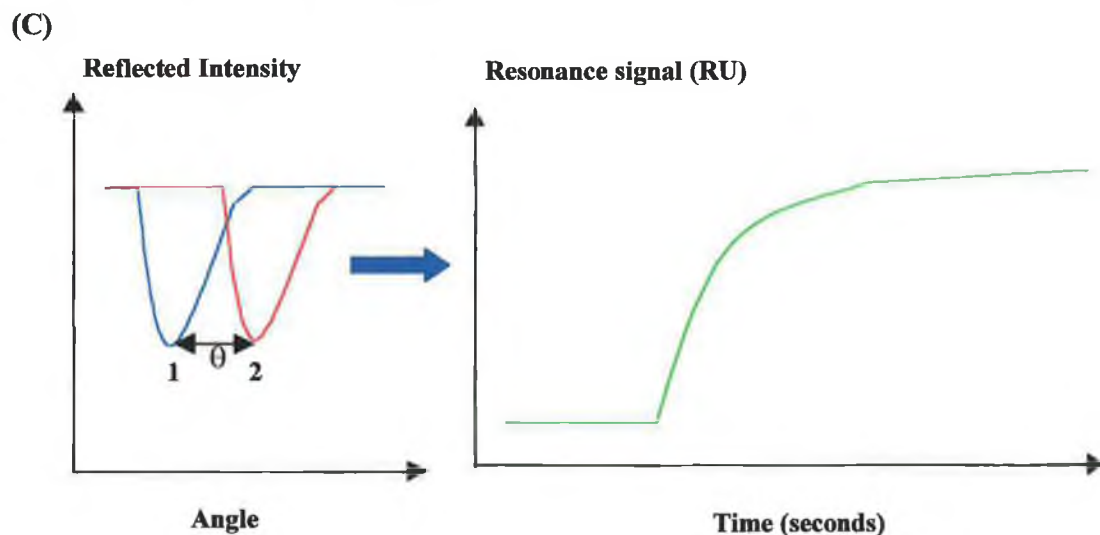
Figure 5.4. (C) *Plot of the change in the resonant angle (θ) measured in 'real-time' following antibody: antigen binding versus time. This is interpolated by the instrument software to generate the characteristic SPR response curve, where the change in the SPR angle is converted to arbitrary response units. The response signal measured is proportional to the concentration of bound analyte.*

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In real-time BIA, the metal film properties, wavelength, and refractive index of the glass (denser medium) are all kept constant, and as a result SPR can be used to monitor the refractive index of the aqueous layer immediately adjacent to the metal (gold) layer. As a result, any changes in mass in the buffer will cause a change in SPR angle, which is monitored in 'real-time' and quantified as a sensorgram. A mass change of approximately 1 kRU (1,000 RU) corresponds to a mass change in surface protein concentration of 1 ng/mm^2, or about 6 mg/ml in bulk protein concentration. Typical responses for surface binding of proteins in BIA are of the order of 0.1-20 kRU. A more extensive review of SPR has been carried out by Liedberg & Lundstrom (1993).

5.1.2.1. *The sensor chip*

The sensor chip incorporated in the BIAcore system is depicted in Figure 5.5. It consists of a glass substrate onto which a 50 nm-thick gold film is deposited. This gold film is derivatised with a monolayer of a long chain hydroxalkyl thiol, which serves a barrier to proteins and other ligands from coming into direct contact with the metal surface. A carboxy-methylated dextran layer is covalently deposited onto the hydroxalkyl thiol layer. This is approximately 100 nm thick, and serves to produce a hydrophilic layer suitable for macromolecular interaction studies as well as containing functional groups (carboxyl groups) available for ligand immobilisation chemistries. Covalent attachment of biomolecules to the flexible dextran layer preserves a hydrophilic environment around the immobilised molecules and gives a high degree of accessibility to the molecular surfaces. Immobilisation of proteins on the sensor-chip surface results in 30-40 % of the carboxyl groups in the matrix being activated under typical immobilisation conditions using EDC/NHS coupling chemistry. The dextran matrix is chemically stable in most buffers used in biomolecular interaction studies and can be exposed to extremes of pH for short periods without deterioration. Direct immobilisation of proteins onto the gold surface itself can result in significant denaturation of the immobilised protein. Carboxymethylation of the dextran layer also places a net negative charge on the dextran, which is completely charged above pH 7. This allows for positively charged

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proteins (i.e. below their pI) to become electrostatically attracted to the bound dextran layer, and effectively 'pre-concentrates' the protein to the chip for subsequent immobilisation procedures.
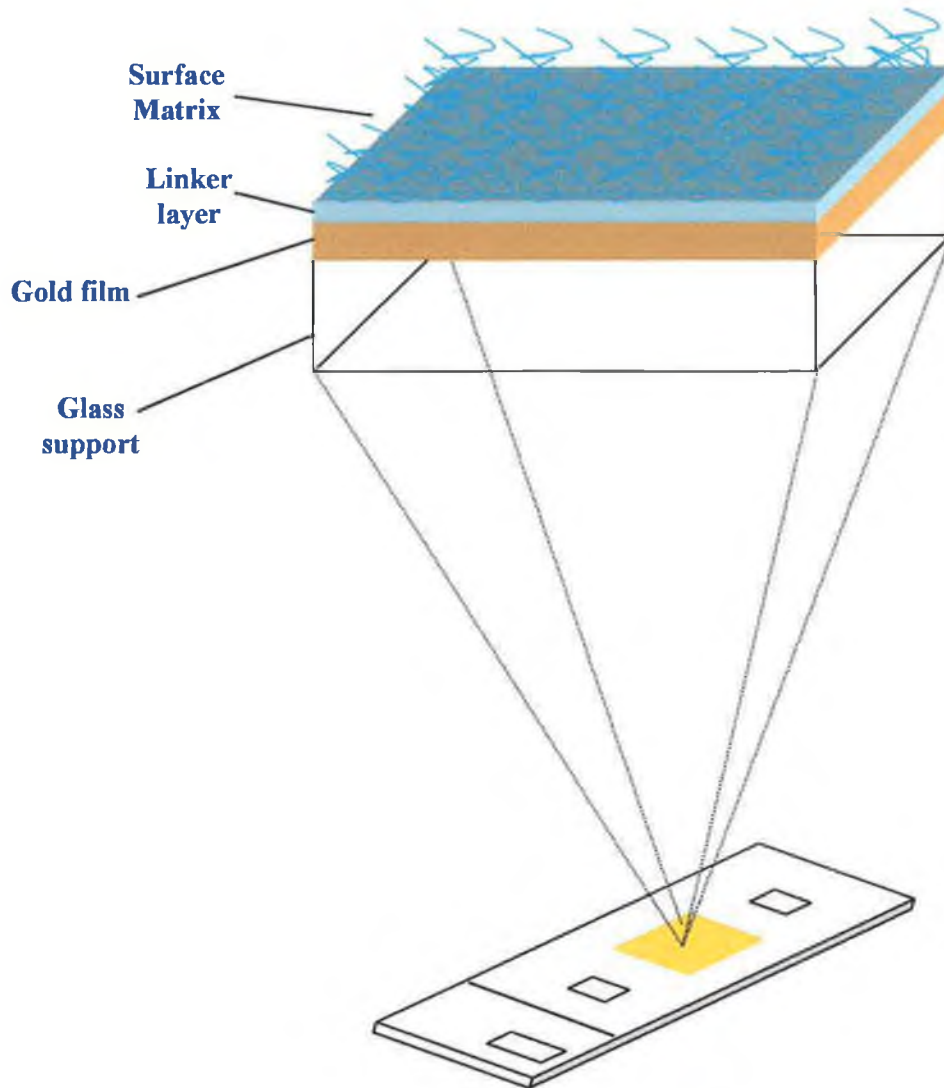
Figure 5.5. Surface of a CM 5 sensor chip consisting of three layers: glass, a thin gold film, and a matrix layer. The matrix is bonded to the gold film through an inert linker layer.

5.1.2.2. *Interpreting the SPR signal*

The SPR signal originates from changes in the refractive index of a surface layer consisting of both bulk solution and the surface matrix with adsorbed biomolecules. A typical sensorgram for binding of a bimolecule to immobilised ligand is shown in Figure 5.6.
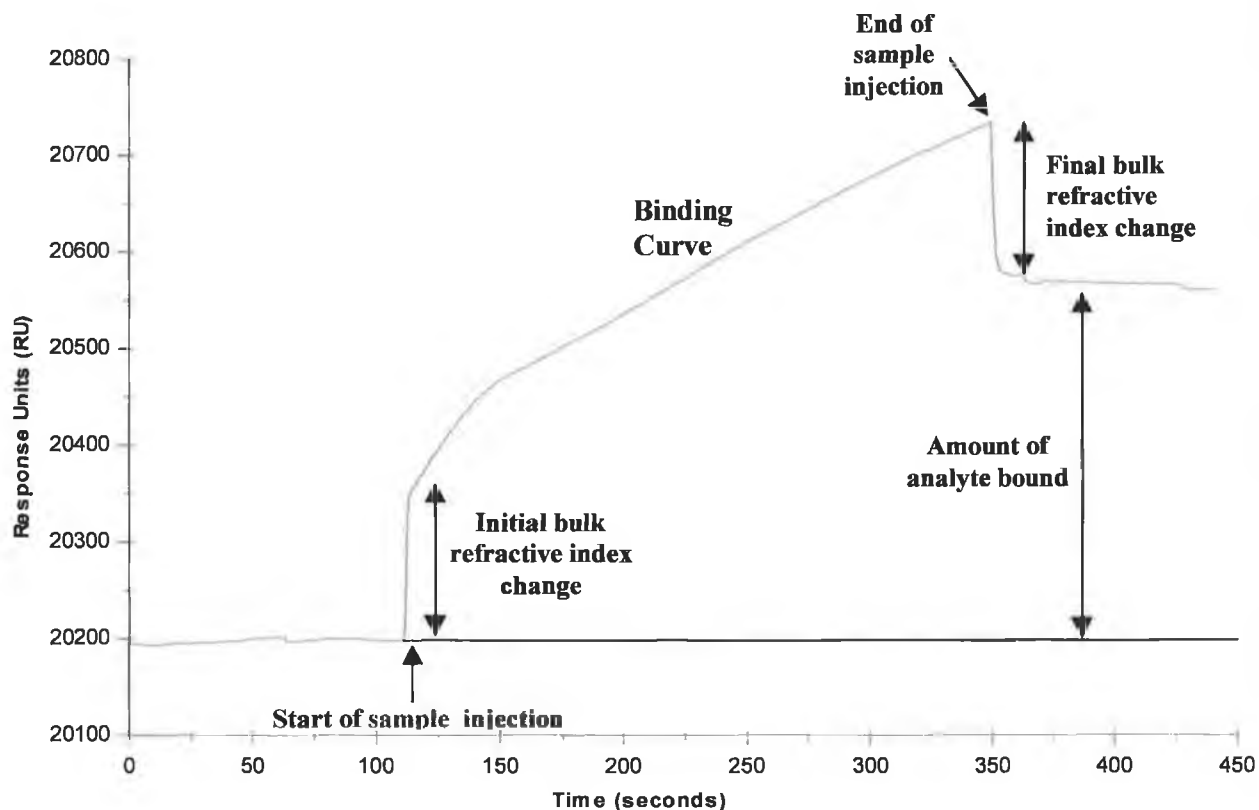
Figure 5.6. *A typical BIAcore sensorgram for binding of analyte to immobilised ligand. The initial steady baseline is due to running buffer flowing over the immobilised ligand surface. Injection of sample causes an initial sharp signal increase due to the difference in bulk refractive index. The binding of two interactants can then be seen as a gradual increase in signal. The signal falls sharply at the end of the sample injection, as the lower refractive index buffer flows over the surface. The difference in response units (RU) between the initial baseline and the signal after the sample injection represents the amount of analyte bound.*

5.1.3. *Resonant mirror-based devices*

An example of a resonant mirror-based device is the Iasys system. It is also similar to the BIAcore device in that polarised light is totally internally reflected at the underside of the sensor surface. In this case, the sensor surface has the metal layer replaced by a dielectric resonant layer (Cush *et al.*, 1993). It is approximately 100 nm thick, and made of titanium which is of high refractive index. This is separarated from the prism by a low index layer made of silica. Figure 5.7 shows a schematic diagram of the system. Incident light of focused on the prism-silica interface which results in frustrated total internal reflection (FTIR). This evanescently couples light into the high index or wave guiding layer. Within this waveguiding layer, total internal reflection (TIR) occurs generating an evanescent field at the biointerface. Efficient coupling occurs only for certain incident angles where phase matching between the incident beam and the resonant modes of the high index layers is achieved. At the resonant point, light couples into the high index layer, and propagates along the sensing surface before coupling back into the prism. During resonance, a phase shift of the order of 2π in reflected light occurs and causes constructive interferance that produces sharp peaks of light at resonance. These changes in angular position are monitored in real-time as they are directly related to changes in refractive index at the biointerface surface. The IAsys system has also utilised a cuvette-based design as opposed to a flow cell configuration, which continually stirs to reduce mass transport effects and has a much larger surface area than the BIAcore chip.
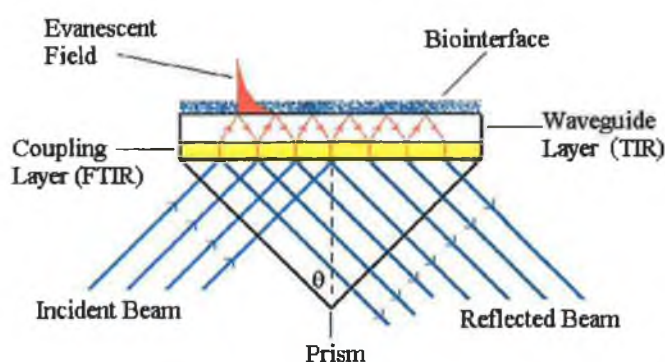
Figure 5.7. *Structure of resonant mirror sensing device. Incident light is directed through a prism, and frustrated total internal reflection occurs at the boundary with the low refractive index layer. An evanescent field is generated which couples light into the high refractive index waveguiding layer. The guided mode gives rise to an evanescent field at the biointerface. The incident angle at which resonance occurs is dependent on the refractive index at the biointerface surface. Binding interactions at this surface cause a shift in angle of reflected light, which are related to changes in mass at the sensor chip surface.*

5.1.4. *Minature Texas Instruments-SPR device*

In 1996 Texas Instruments Incorporated developed and released a fully integrated and minature surface plasmon resonance sensor. The TISPR-1 contains a light emitting diode (LED), polarizer, thermistor (to allow for correction due to temperature fluctuations) and two 128 silicon photo diode arrays housed in an epoxy resin moulding which was designed in the form of the Kretschmann geometry prism (Figure 5.8(a)). The device was developed for use as a non-destructive, low-cost, and small-sized smart sensor. The wedge shaped beam reflects off the SPR sensing layer onto the PDA array by means of a mirror. The SPR minimum is then measured in 'real-time' following dedicated signal processing (Kukanskis *et al.*, 1999). The system offers the capability for 'remote-sensing' and is available in a hand-held pipette-style format called the Spreeta™ device. A schematic of the device in illustrated in Figure 5.8(b) with the attached flowcell.

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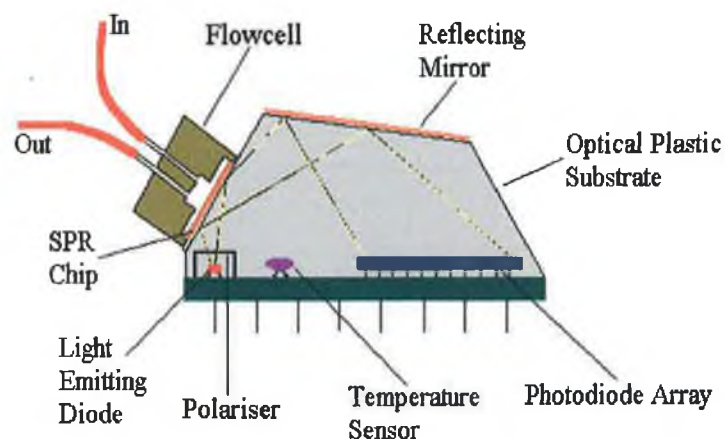
Figure 5.8(a). Cross-section of miniature TI-SPR instrument. Mass changes are related to changes in the resonant angle and position of the reflectance minima, which are detected in 'real-time'.
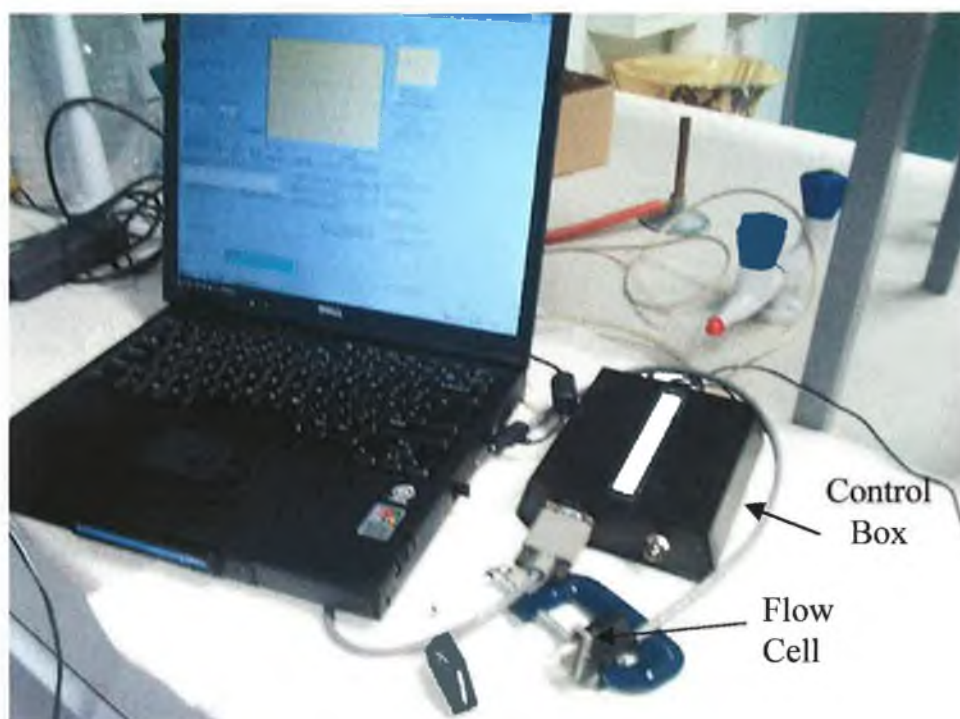
Figure 5.8(b). TI-SPR device fitted with flow-cell. The control box interfaces with the device, which allows it to be installed onto a PC. This, together with dedicated software (SPR-MINI MS-Windows software) allows control of data acquisition, processing and display as a virtual control panel as seen on the laptop.

5.1.5. *Applications of SPR-based biosensors*

Antibodies have been extensively applied to SPR-based biosensors, as they are molecules that are highly sensitive and specific for their antigens. A biosensor which monitors antibody-antigen interactions may also be known as an immunosensor. An extensive range of analytes have been detected and measured by immunosensors, and more specifically SPR-based immunosensors. These include drugs, proteins, hormones, as well as environmental contaminants such as pesticides and food contaminants. One of the main advantages of SPR-based immunosensors is the absence of labelling requirements and the ability to investigate the kinetics of the antibody-antigen interaction. Another advantage is the re-usability of the sensor-chip surface, due to a regeneration step which can remove bound antibody from immobilised antigen on the chip surface, or vice-versa. Some of the more recent applications of SPR-technology incorporating antibodies as a detection method are reviewed in the following sections.

5.1.5.1. *Detection of small molecules using BIAcore*

In certain cases, molecules of low molecular weight (less than 2,000 daltons), may not show a mass change when binding to the sensor surface, as they are too small to give a significant change in refractive index, and hence change in SPR signal. As a result, when developing a quantitative method for detection of low molecular weight compounds, it is necessary to use an indirect sensing method. This can either be **inhibitive** - where sample is incubated together with antibody, and the mixture passed over an immobilised conjugate surface for binding of remaining free antibody. It gives a change in relative response inversely proportional to the amount of analyte free in solution. Alternatively a **competitive** format can be used - in which standard samples and high molecular weight hapten-conjugate compete for binding sites on an immobilised antibody surface. The increase in SPR signal is due to binding of the high molecular weight hapten-conjugate, and, similar to the inhibitive method, is inversely proportional to the amount of analyte in the sample. Figure 5.9 shows a diagrammatic representation of both assay formats.
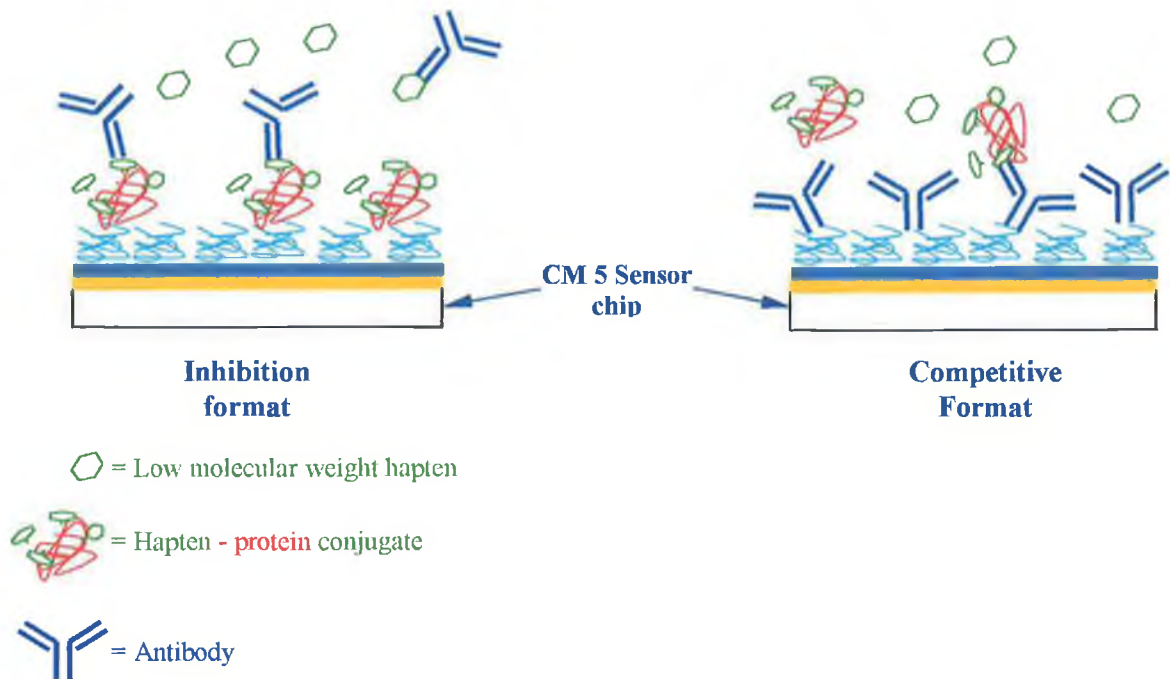
*Figure 5.9. Diagrammatic representation of **inhibition** and **competitive** BIAcore-based immunoassays for the detection of low-molecular weight haptens. Inhibition assay involves sample and antibody mixed together, and the mixture passed over an immobilised conjugate surface for binding of remaining free antibody. A competitive format consists of standard samples and high molecular weight hapten - conjugate competing for binding sites on an immobilised antibody surface.*

Effective regeneration of the sensor surface is necessary for both of these formats if they are to be used routinely. The antibody-antigen interaction can often be very strong, and therefore very difficult to dissociate, despite using strong chaotrophic reagents. When selecting an antibody for use in a regenerable immunosensor, it is necessary to choose one with a moderate affinity to allow easy regeneration of the sensor surface. If the choice of antibody is limited, alternative immobilisation strategies can be used (Quinn *et al.,* 1999).

Inhibition immunoassays have been used for the detection of organophosphate pesticides by Alcocer *et al.* (2000(a)). Polyclonal antibodies were produced to phosphonic acid (trans-4-phosphono-2-butenic acid (TPB), with the aim of producing an antibody preparation that recognised a number of organophosphates. Parathion-BSA conjugate was immobilised on the sensor-chip surface, and antibody and free parathion were injected over the chip surface, which resulted in the free antibody in solution binding to the conjugate surface. Regeneration of the bound antibody from the immobilised conjugate surface was carried out using two one-minute pulses of 20 mM HCl and NaOH. The antibody population had a linear range of detection of 1.562 to 50 μg/ml, and the assay was highly reproducible. Another example, where hapten-protein conjugates were immobilised on the chip surface for use in inhibition assays was carried out by Keating *et al.* (1999). They used a 7-hydroxycoumarin-BSA conjugate, for immobilisation, and found the assay had a measuring range of 0.5 – 80 μg/ml for the detection of free 7-hydroxycoumarin. The BIAcore-based immunoassay was easily regenerable, reproducible, entailed no sample clean-up, and compared well to established methods of analysis.

Direct immobilisation of hapten onto the CM-5 sensor-chip surface can also be used for an inhibition assay. Fitzpatrick (2001) employed the direct immobilisation method for warfarin. 140 consecutive regeneration cycles with a monoclonal anti-warfarin antibody were carried out on a 4'-aminowarfarin immobilised surface. The surface was regenerated with two 30-second pulses of 30 mM HCl. The binding responses demonstrated coefficients of variation of 0.82 % over the course of 140 cycles, and no decrease in the measured binding response over the course of the regeneration study. An inhibition assay was optimised using this sensor-chip surface. The linear range of detection for the assay was 0.49 to 500 ng/ml, with inter-day coefficients of variation no greater than 9.5 %.

Crooks *et al.* (1998) directly immobilised the sulphonamide drugs-sulfamethazine and sulfadiazine onto the surface of a CM-5 sensor-chip surface using conventional EDC/NHS coupling chemistry. The assay was used for the detection of these

respective drugs in pig-bile samples. Analysis of 2,081 samples for both sulphonamide residues over an 8-month period showed the method to be highly reproducible and reliable. Van der Gaag *et al.* (1999) have also used the direct immobilisation of hapten method for the detection of aflatoxin B_1. It was found that the assay was sensitive enough for detection of aflatoxins in food and feed.

Direct immobilisation of haptens to a CM-dextran gel surface is only possible where an amino group (NH_2) is present on the hapten, as it can bind to the carbodiimide activated COO $^-$ groups on the CM-dextran gel surface. Alternative methods of direct immobilisation have been attempted (unpublished observations/studies), where ethylenediamine is immobilised onto the dextran gel surface, and a hapten containing a COO $^-$ group is EDC/NHS activated remote from the chip, and then injected over the ethylenediamine-immobilised surface. Although this method partially works, it also results in some of the EDC/NHS present in the activated hapten solution reacting with unreacted COO $^-$ groups on the sensor chip surface. This causes the ethylenediamine to 'cross-link' to the activated COO $^-$ groups, and hence the activated hapten cannot effectively bind to the dextran gel surface.

Analysis of other small molecular weight compounds such as hormones (Mani *et al.*, 1997), and also enzyme inhibitors has been carried out. Markgren *et al.* (1998) immobilised the enzyme HIV-1 proteinase directly onto the sensor-chip surface. They then injected a variety of inhibitors and non-interacting drugs over the sensor surface, and were able to select and rank the inhibitors based on their association and dissociation rates. Myszka & Rich (2000) used immobilised antibody surfaces for the direct detection of the hormone progesterone. They immobilised three flow-cell surfaces with three monoclonal antibodies (anti-progesterone, anti-testosterone, and anti-mouse Fc) within a BIAcore 2000. The fourth flow cell remained blank as a control surface to correct control bulk refractive index changes, as well as observe non-specific interactions. Progesterone (M.W.= 315 daltons) was then injected over the respective surfaces, and binding was observed between the progesterone and the anti-progesterone antibodies. A lower degree of binding was observed with the anti-

testosterone antibodies while no binding was observed towards the anti-mouse Fc antibody. This example illustrates how multiple surfaces can be used to compare simultaneously a compound's selectivity for different target ligands. This type of biosensor data can also be use for the estimation of kinetics by fitting the data to simple interaction models.

5.1.5.2. *Applications of BIAcore in antibody engineering*

Analysis of recombinant antibodies and phage displayed antibodies can be easily and effectively carried out using BIAcore. The biosensor was used in a number of applications including the screening process, kinetic selection of phage-displayed antibodies, as well as the characterisation and epitope mapping of fragments.

When antibody fragments are produced from a phage-display library, thousands of different specificities are available for selection. These different phage–displaying antibodies are usually selected against antigen using ELISA. ELISA is an equilibrium method, and therefore candidates with high off rates may be lost. BIAcore, however, can be used to evaluate an entire binding pattern of a selected panning round. Bacterial culture supernatants can be directly injected over the the sensor surface, without further purification (Marks *et al.*, 1992).

The phage selection proccess can also be aided by BIAcore. Malmborg and Borrebaeck (1995) selected for phage-displaying Fab fragments specific for lysozyme by injecting the phage population over an immobilised lysozyme surface. Phage particles specifically bound to the immobilised lysozyme, and were then eluted, and re-injected over the surface for a second round of selection.

BIAcore is also a useful tool for the determination of kinetics of respective antibodies and their respective fragments (Malmborg *et al.*, 1996). Active antibody concentrations can also be determined using BIAcore (Abraham *et al.*,1995; Kazeimer *et al.*, 1996) under the conditions of mass transport limited binding.

Recombinant antibody quality can sometimes be a problem in a given preparation from *E.coli*. A simple two-site BIA assay was developed to measure the

concentration of assembled Fab' in the presence of unassembled antibody chains and fragments (Lawson *et al.*, 1997). The assay involved the initial capture of the Fab' antibody from a sample using an immobilised anti-heavy chain antibody (or anti-light chain antibody). This was followed by binding of a complementary antibody (anti-light or heavy chain antibody) in a second step. The initial capture step detects heavy or light chains in the sample (depending on what antibody was first immobilised), as well as unassembled chains or fragments in the mixture. However the second step using the complementary antibody only measures intact Fab' antibodies, since light chains will not be identified by anti-heavy chains and vice-versa. By replacing one of the antibodies in the two-site assay with antigen, the same assay format can be used to determine the integrity and activity of recombinant antibodies and constructs such as scFv-hinge-Fc. The assay can be applied with confidence to culture supernatant or crude extract, allowing the optimal harvest time for the culture to be determined.

5.1.5.3. *Proteomic applications*

At present, proteome analysis is carried out using a combination of 2-D gel electrophoresis and matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI/TOF-MS). Both of these methods combined are widely accepted and powerful tools in proteome analysis. However, after these initial proteomic analyses, more questions arise about their interactions. Do the proteins in question interact with each other? What other kind of molecules could bind to proteins that are specifically expressed during certain biological events or diseases? BIAcore can be used to monitor these types of interactions, as well as possessing the advantage of being capable of high-throughput analysis. Alternatively, MS analysis can be carried out on protein samples after they have be analysed by BIAcore-provided the analysed species is retained after analysis. Krone *et al.* (1997) combined BIAcore and MALDI/TOF-MS for the analyis of the peptide myotoxin *a*. Three flow-cells of a CM-5 sensor chip were immobilised with polyclonal anti-myotoxin *a* antibodies, while the fourth flow-cell was used as a control. Samples of myotoxin *a* were then injected over the surface for analysis. The sensor chip was then removed from the machine, and prepared for MS analysis, where the proteins

could be analysed. Femtomole quantities of the peptide were detected by direct MALDI analysis of the sensor chips used during BIAcore analysis. Epitope tagging of peptides has also been carried out prior to BIA/MS analysis (Nelson *et al.*, 2000). Tags were fused or inserted into nominally unknown genes for the purpose of tracking proteins throughout expression. Once the proteins were expressed, they were digested with trypsin, resulting in epitope tagged peptides. BIAcore was used to selectively isolate (using immobilised antibodies), detect and quantify the peptides from the digested media. The sensor chips were then analysed by MALDI-TOF in order to characterise the tagged peptides, and identify the protein resulting from the gene. This method was capable of detecting low-to sub-femtomole quantities of peptide, and shows that BIA/MS is a rapid method to isolate, detect and identify epitope-tagged proteins from complex biological mixtures.

5.1.6. *Determination of antibody affinity constants*

The strength of the bond formed between an antibody and its specific antigen, is determined by a combination of different physical forces such as hydrophobic interactions, electrostatic binding, van der Waals forces, and hydrogen bonds (Tijssen, 1985).

Hydrophobic interactions occur when conjugation of non-polar groups in a binding site repel water molecules away from the binding site. Electrostatic binding occurs between oppositely charged groups on the antibody and antigen, and its effect is greatly enhanced by the removal of water molecules from the binding site, as a result of hydrophobic interactions. Van der Waals forces are as a result of the attraction of electron clouds on adjacent non-polar groups of the binding partners to each other. Hydrogen bonds are formed when hydrogen atoms are shared between two separate atoms, and are stronger than van der Waals forces.

The individual bond energies of these weak non-covalent interactions are minimal, especially in aqueous environments, and their effects are only significant in large numbers. Non-covalent interactions are also only effective over a small distance, making the 'goodness of fit' between the epitope and the binding site on the antibody the significant factor in determining the strength of the bond.

The sum of the attractive and repulsive forces for a single antibody-antigen bond is known as the antibody affinity, and may be described in the following expressions:

$$[Ab] + [Ag] \underset{Kd}{\overset{Ka}{\longleftrightarrow}} [Ab:Ag] \qquad (1)$$
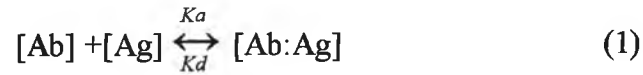
[Ab] = Antibody

[Ab] = Antigen

[Ab:Ag] = Antibody-antigen complex

Ka = Association rate constant

Kd = Dissociation rate constant

The overall affinity of the antibody for an antigen is given by the equilibrium association rate constant.

$$K_A = \frac{[Ab:Ag]}{[Ab]:[Ag]} = \frac{Ka}{Kd} = \frac{1}{K_D} \qquad (2)$$

The affinity of an antibody may also be referred to as the equilibrium dissociation rate constant, K_D, which is the reciprocal of the K_A value.

Two antibodies with similar affinity constants (K_A) may possess completely different kinetic rate constants, and consequently vary considerably in their time taken to reach equilibrium, which can be a significant factor when choosing an antibody for a specific use, e.g. for use in a regenerable immunosensor, or ELISA. When choosing an antibody for use in a regenerable immunosensor such as BIAcore, it is desirable to have an antibody with a high association rate constant (Ka) and a moderate-to-high dissociation rate constant (Kd). However, when choosing an antibody for use in ELISA, it is desirable to choose on with a high association rate constant (Ka) and a low dissociation rate constant (Kd).

Various techniques are available for the determination of antibody affinity constants, which include ELISA-based methods (Friguet *et al.*, 1985). This method relies on the concentration measurement of free antibody in solution after equilibrium with antigen has been reached. BIAcore can also be used to determine equilibrium dissociation

rate constants. The method employed utilises the same method as that employed in ELISA (Friguet *et al.*, 1985), but has several advantages over this technique. The short contact time of the antibody-antigen mixture in the flow cell (compared to incubation step in ELISA) and continual flow of fresh equilibrium mixture over the antigen-coated sensor surface limits the possibility of re-equilibration of the antibody-antigen mixture, and subsequent under estimation of the affinity constant. The method also does not require additional labelling of either reagent, or the addition of secondary reagents, which may affect the intrinsic thermodynamic binding constants.

5.1.7. *Chapter outline*

This chapter describes the characterisation of polyclonal and scFv anti-AFB_1 antibodies, by applying them for the detection of AFB_1 on a SPR-based biosensor. BIAcore 1000™ was used in the analysis of the polyclonal antibody, while BIAcore 3000™ was used for the analysis of the scFv antibody. Both antibodies were also applied for the detection of AFB_1 in spiked grain samples, and solution affinity analysis was carried out on the scFv antibody.

5.2. Results

5.2.1. *Preconcentration of AFB$_1$-BSA conjugate*

10 mM acetate buffer was prepared at a range of incremental pH's from 3.9 to 4.9. A solution of 50 µg/ml of AFB$_1$-BSA was prepared in acetate buffer at each pH. Each conjugate concentration was passed sequentially over an underivatised chip surface at 5 µl/min for 2 minutes as described in section 2.20.1. The differing degree of preconcentration was measured by the response prior to the end of the injection for each conjugate solution at the various pH values. Following injection, the flow of the running buffer over the chip surface was sufficient to dissociate the electrostatic attraction between the protein and the CM-dextran gel surface. Figure 5.10 shows that an increase in preconcentration occurs at pH values below 4.1. The preconcentration studies showed the optimal pH for immobilisation of AFB$_1$-BSA conjugate was 3.9 in 10 mM acetate buffer. This pH was thus used for all subsequent immobilisations.

5.2.2. *Immobilisation of AFB$_1$-BSA conjugate*

EDC/NHS coupling chemistry was used for activation of the CM-dextran gel surface for the immobilisation of AFB$_1$-BSA conjugate, as described in section 2.20.2. Figure 5.11 shows a typical immobilisation sensorgram where 6,766 RU of AFB$_1$-BSA conjugate was immobilised onto the sensor surface after capping with 1M-ethanolamine hydrochloride, pH 8.5. This deactivated any remaining NHS-esters, and eluted any non-covalently bound material.

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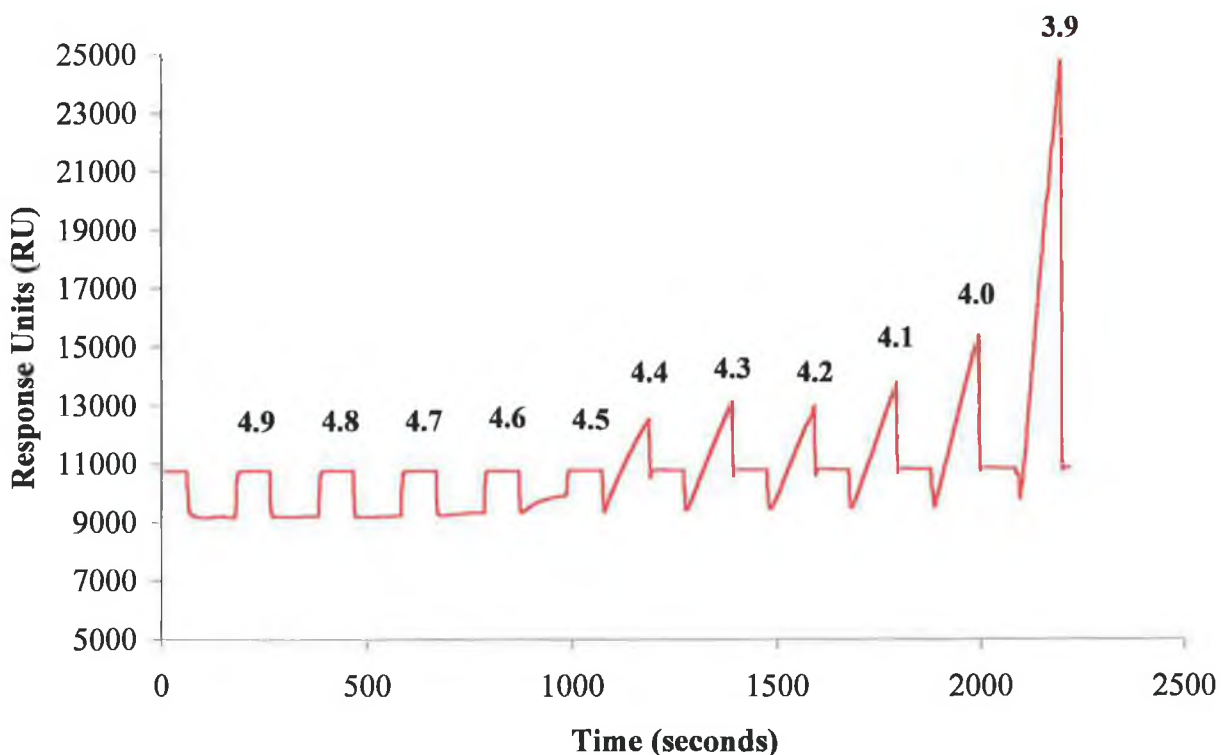
Figure 5.10. *Preconcentration of AFB₁-BSA conjugate onto a CM-dextran chip surface. Solutions of AFB₁-BSA at 50 μg/ml in 10 mM acetate buffer at pH increments between 3.9 and 4.9 were passed over an unactivated CM-dextran surface at 5 μl/min for 2 minutes. The low ionic strength of the buffer used favours the electrostatic attraction between the negatively charged dextran layer and the positively charged protein (i.e. below the isoelectric point). The degree of 'preconcentration' was measured from the response prior to the end of each sample injection. The ionic strength of running buffer (150 mM NaCl) was sufficient to remove the electrostatically attracted conjugate from the sensor chip surface. The optimal pH for immobilisation of AFB₁-BSA onto the CM-dextran chip surface was calculated to be pH 3.9, and subsequently all immobilisations of AFB₁-BSA were carried out in 10 mM acetate buffer, pH3.9.*
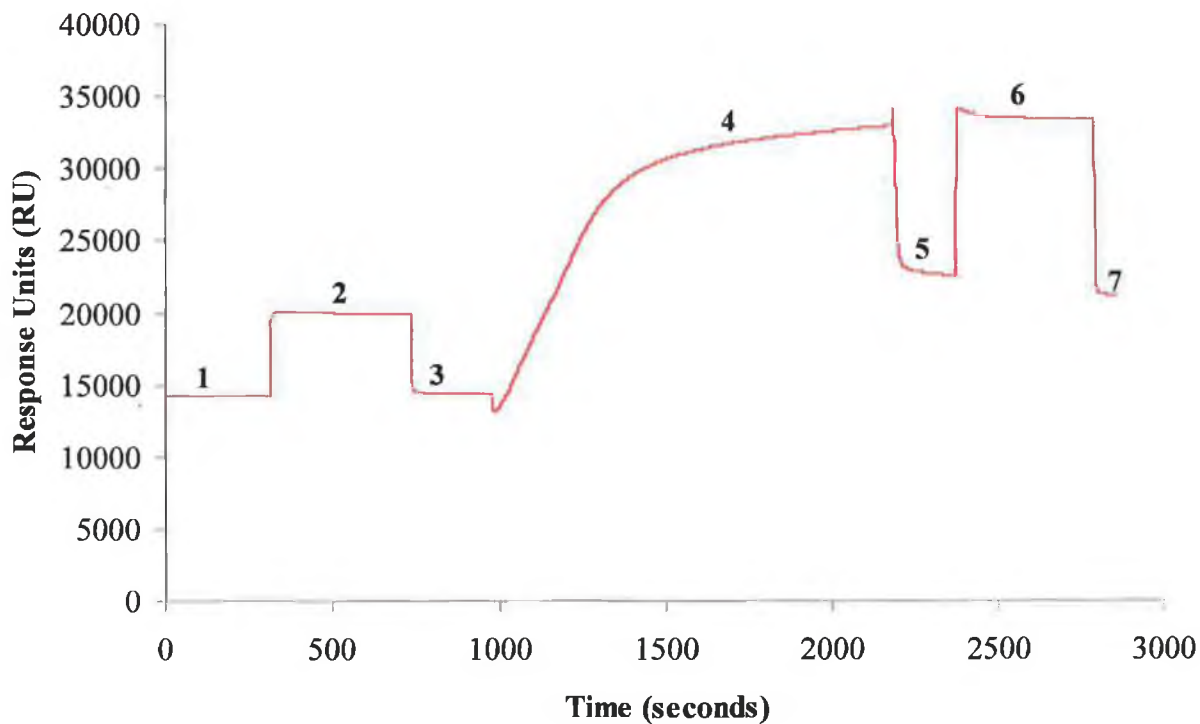
Figure 5.11. *Immobilisation of AFB$_1$-BSA conjugate onto a CM-dextran chip surface:*

1. *HBS running buffer is passed over the chip surface and a baseline measurement is recorded.*

2. *After mixing, a solution containing 0.05 M NHS and 0.2 M EDC is injected over the chip surface to activate it. The large increase in SPR signal is mainly due to a bulk refractive index change.*

3. *An increase of approximately 200 RU is recorded following activation of the chip surface.*

4. *A solution containing 50 μg/ml of AFB$_1$-BSA conjugate in 10 mM acetate buffer (pH 3.9) is passed over the chip surface for 20 minutes.*

5. *The amount of bound conjugate adsorbed is recorded whilst most of the non-covalently bound protein is eluted.*

6. *Any remaining surface NHS-esters are deactivated by the addition of 1 M ethanolamine hydrochloride pH 8.5. This also elutes any non-covalently bound conjugate.*

7. *6,766 RU of AFB$_1$-BSA conjugate bound to the surface following the immobilisation procedure.*

5.2.3. *Development of a SPR-based inhibition immunoassay for AFB₁*

A number of parameters had to be optimised for the development of an inhibition assay for AFB$_1$. These included removal of non-specific interactions, optimisation of antibody dilution for the assay, as well as effective regeneration conditions.

5.2.3.1. *Regeneration conditions*

Affinity-purified polyclonal anti-AFB$_1$ antibody was diluted to 1/100 in HBS running buffer and injected over the immobilised AFB$_1$-BSA immobilised surface. Initial studies showed that the antibody bound to the toxin-protein conjugate with a high avidity, and very little dissociation of the antibody from the conjugate surface was observed. It was also found that regeneration of the antibody from the AFB$_1$-BSA conjugate surface was not possible using HCl or NaOH solutions. As a result of this, a regeneration solution consisting of 1M ethanolamine with 20% (v/v) acetonitrile, pH 12.0, was used. An ELISA was initially carried out in order to determine the suitability of this regeneration solution on an AFB$_1$-BSA surface.

AFB$_1$-BSA conjugate was coated onto wells of a microtitre plate, which were then blocked with 2% (w/v) casein protein. 100 µl/well of the affinity-purified polyclonal anti-AFB$_1$ antibody was then added to the wells, and incubated for 1 hour at 37° C. After washing, 100 µl of a range of 1M ethanolamine solutions-containing varying percentages (v/v) of acetonitrile was added to appropriate wells, incubated for 10 minutes at room temperature, and then aspirated. The affinity-purified polyclonal antibody was then added, followed by HRP-labelled anti-rabbit antibody and chromogenic substrate. The assay was then repeated with the regeneration solutions added to the plate after coating and before incubation with antibody. It could be seen that addition of increasing percentage-concentrations of acetonitrile (in the 1M ethanolamine solutions) resulted in increased regeneration, while pre-treatment of the conjugate surface did not significantly decrease the binding of polyclonal antibody at higher concentrations (Figure 5.12.).
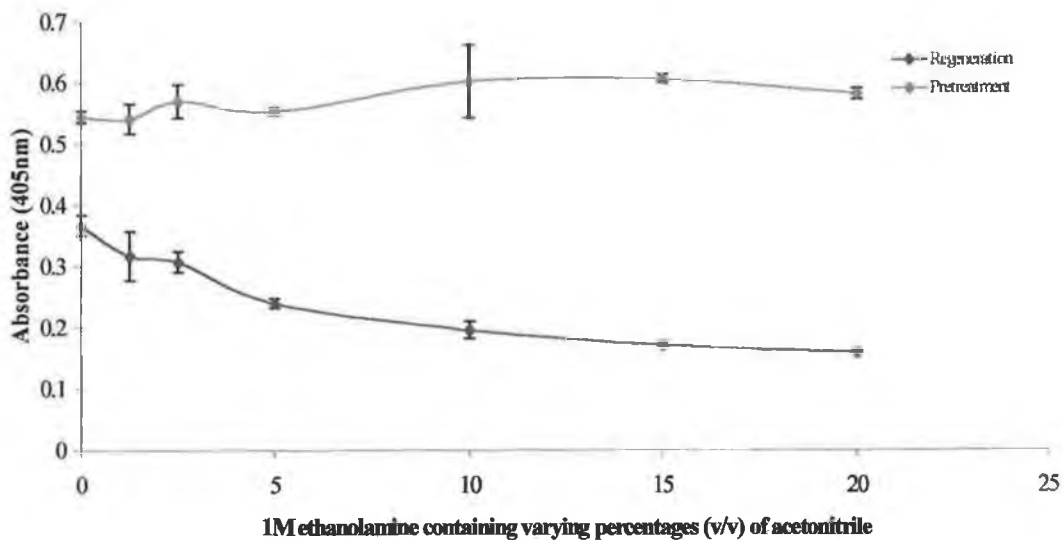
Figure 5.12. The effects of regeneration with 1M ethanolamine, pH 12.0, containing varying percentages (v/v) of acetonitrile on AFB$_1$-BSA coated onto wells of a 96-well microtitre plate. Increasing concentrations of the regeneration solution were added to coated wells before ('Pretreatment') and after ('Regeneration') incubation with affinity-purified anti-AFB$_1$ antibody. The amount of bound polyclonal antibody was measured by ELISA, with addition of HRP-labelled anti-rabbit IgG. The results shown are the averages of five analyses.

From the ELISA experiment, it was decided to use 1M ethanolamine with 20% (v/v) acetonitrile, pH 12.0, as the regeneration solution for removal of bound polyclonal antibody from the AFB_1-BSA immobilised surface. A 2-minute pulse of the solution at a flowrate of 10 µl/min was used. Although it was successful for regeneration of the surface, it did not remove all the bound antibody each time. However, the accumulation of this bound antibody did not significantly affect binding over a large number of injections. The efficiency of the regeneration process was evaluated by performing multiple (i.e. 25) binding-regeneration cycles over the AFB_1-BSA-coated surfaces (Figure 5.13.). The surface binding capacity of the antibody oscillated slightly over the twenty-five cycles, but it did not significantly affect the performance of the assay.

Initial studies, using a 1/8 dilution in HBS of scFv anti-AFB_1 antibody showed that it was easily regenerated from the AFB_1-BSA immobilised surface using a 1-minute pulse of 10 mM NaOH. This completely removed all bound scFv antibody after each binding-regeneration pulse. As a result, it was not necessary to carry out an investigative ELISA to examine the efficiency of regeneration using this regeneration solution. Figure 5.14 shows a graph of the reproducibility of regeneration for the scFv antibody over fifty regeneration cycles. Binding of the scFv antibody to the conjugate surface was highly reproducible, with approximately 250 RU of antibody binding to the surface each time. The regeneration solution (10 mM NaOH) did not affect the binding throughout the regeneration study.
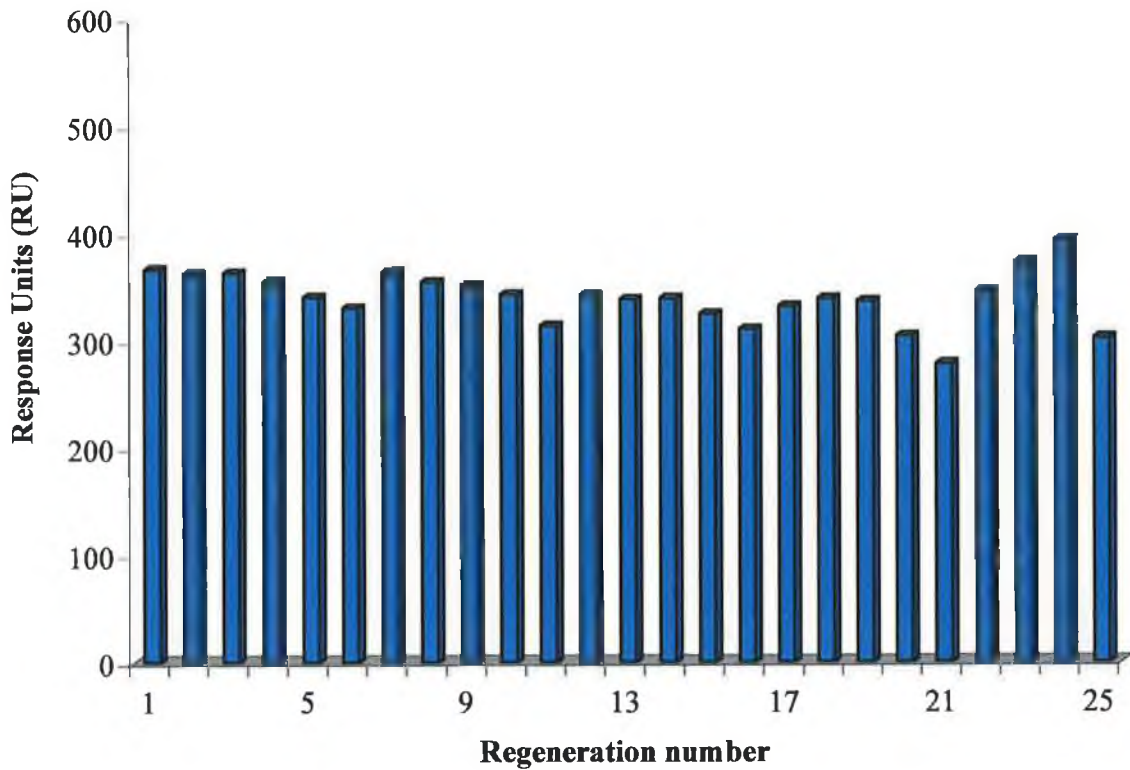
Figure 5.13. *Graph showing the reproducibility of regeneration of a sensor chip immobilised with AFB₁-BSA on the surface. Twenty-five consecutive regeneration cycles of a 4-minute binding pulse of polyclonal anti-AFB₁ were carried out. This was followed by a 2-minute injection of 1M-ethanolamine containing 20%(v/v) acetonitrile pH 12.0, as the regeneration solution. Approximately 15% of the antibody bound remained on the surface after each regeneration cycle. This did not seem to significantly affect the reproducibility of the binding.*
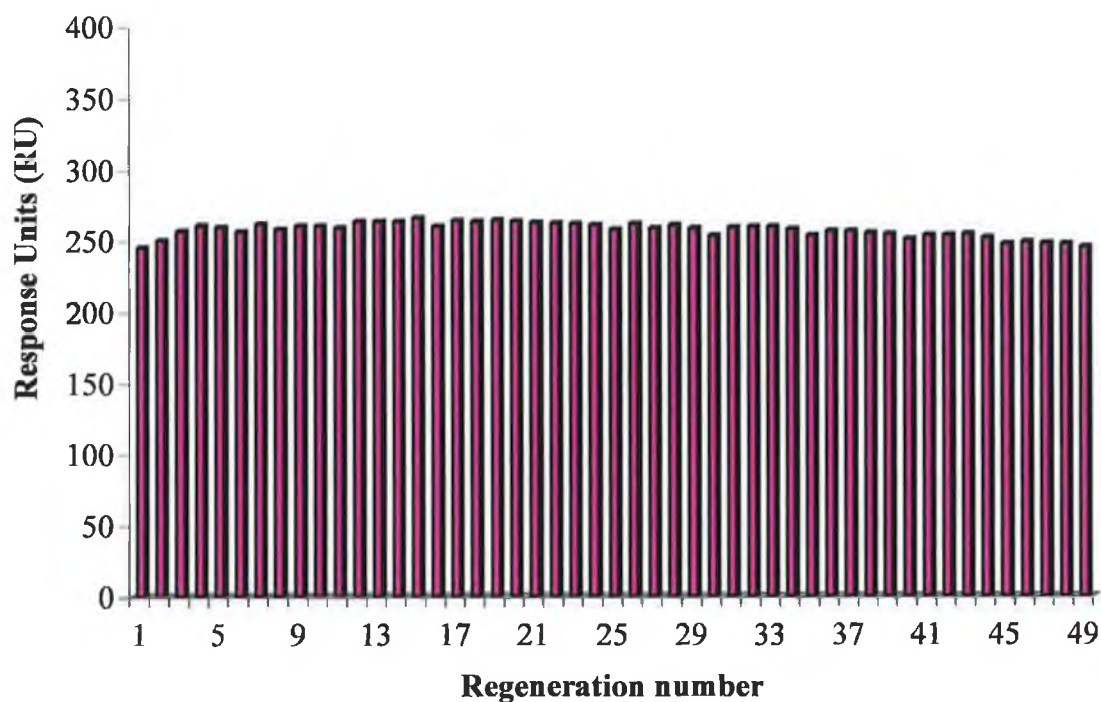
Figure 5.14. *Graph showing the reproducibility of regeneration of a sensor chip immobilised with AFB₁-BSA on the surface. Fifty consecutive regeneration cycles of a 3-minute binding pulse of scFv anti-AFB₁ were carried out. This was followed by a 1-minute injection of 10 mM NaOH as the regeneration solution. This completely removed all of the bound antibody after each binding cycle, which resulted in highly reproducible binding cycles, as no significant decrease in the measured binding response over the course of the regeneration study was observed.*

5.2.3.2. *Assessment of non-specific binding*

The degree of non-specific binding of the polyclonal and scFv antibodies to both the dextran layer, and the immobilised BSA portion of the toxin-protein conjugate, was assessed. This was carried out, by passing the respective antibody at the requisite dilution (1/100 for the polyclonal antibody, and 1/8 for the scFv antibody) over a blank CM-dextran surface, as well as an immobilised BSA surface as described in section 2.20.3. Figure 5.15 shows overlaid sensorgrams of the injection of polyclonal anti-AFB_1 antibody over an AFB_1-BSA immobilised surface, a BSA immobilised surface, as well as a CM-dextran layer. There was negligible non-specific binding of the antibody to the BSA protein ($< 5RU$). The antibody solution did not have to be pre-incubated with BSA, as all of the BSA binding antibodies were removed by subtractive-immunoaffinity chromatography. It was found that pre-incubation of the sample with 100 µg/ml of carboxy-methylated dextran (CM-dextran) removed all non-specific interactions to the CM-dextran surface. This was subsequently used in all antibody dilution preparations.

Figure 5.16 shows overlaid sensorgrams of the injection of scFv anti-AFB_1 antibody over an AFB_1-BSA immobilised surface, a BSA immobilised surface, as well as a CM-dextran layer. A negligible amount of non-specific binding (<5 RU) was observed to the BSA and CM-dextran surfaces, and as a result the antibody solution did not have to be pre-incubated with either BSA or CM-dextran.
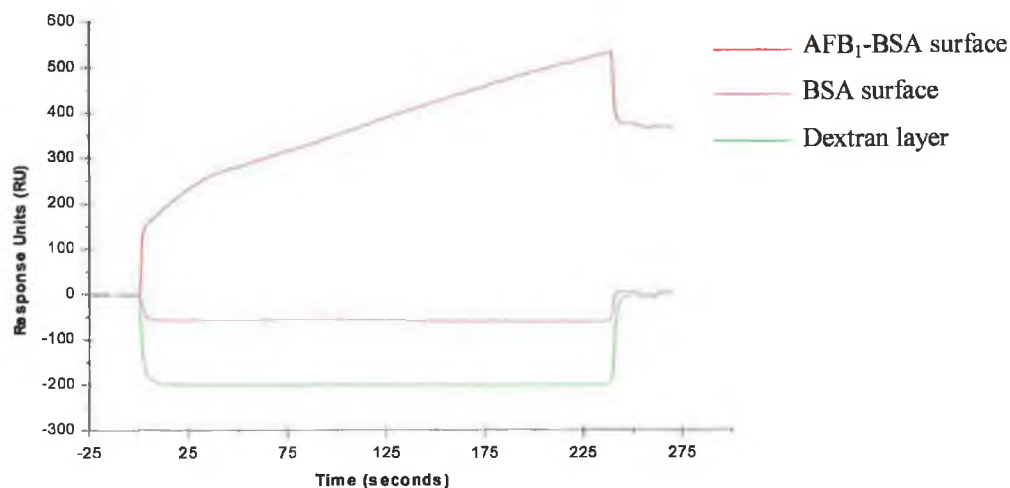
Figure 5.15. *Injection of a 1/100 dilution of affinity-purified polyclonal anti-AFB₁ antibody over immobilised AFB₁-BSA conjugate, BSA, as well as a CM-dextran gel layer at 10 μl/min for 4 minutes. A negligible amount of binding of the antibody to the BSA and dextran surfaces (< 5 RU) was observed using 100 μg/ml of CM-dextran in the diluent buffer, while 368 RU of antibody bound to the immobilised AFB₁-BSA conjugate surface. The differences in SPR signal are due to changes in the bulk refractive index of the antibody solution.*
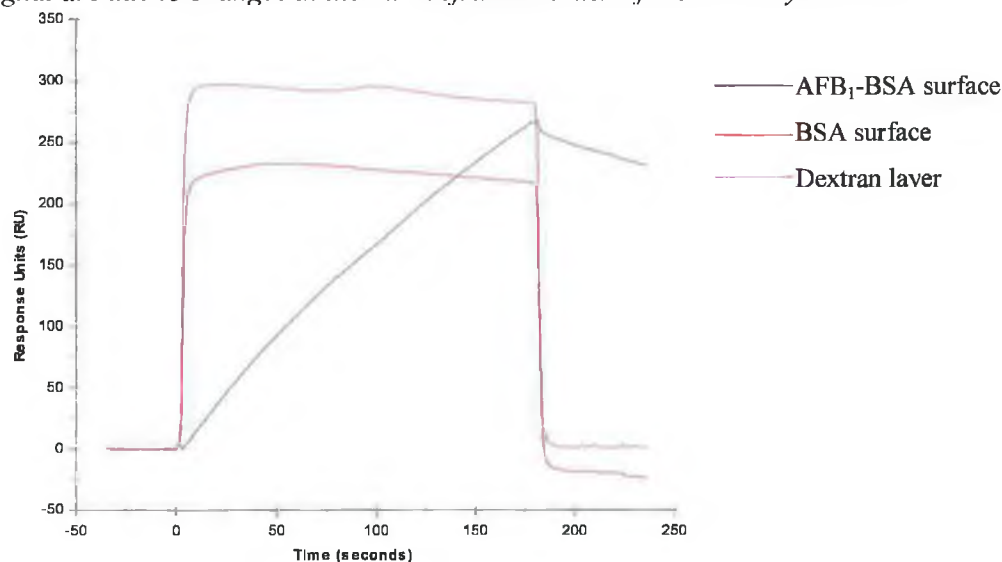
Figure 5.16. *Overlaid sensorgrams showing binding of a 1/8 dilution of anti-AFB₁ scFv antibody to an AFB₁-BSA surface, BSA surface, and a CM-dextran gel layer, at 10 μl/min for 3 minutes. A negligible amount of binding of the scFv antibody to the BSA and dextran surfaces (< 5 RU) was observed, while 245 RU of antibody bound to the immobilised AFB₁-BSA conjugate surface. The differences in SPR signal are due to changes in the bulk refractive index of the antibody solution.*

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5.2.3.3. *The determination of working range of model inhibition assay in PBS*

For determination of the working range of the inhibition immunoassays for both the polyclonal and scFv antibodies, dilutions of AFB_1 were firstly prepared as described in section 2.10.1. Dilutions of AFB_1 were prepared in PBS-5 % (v/v) methanol ranging from 0.75 to 98 ng/ml for the polyclonal antibody inhibition assays, and 3 to 195 ng/ml for the scFv inhibition assays. The polyclonal affinity-purified antibodies were mixed with the corresponding dilution of AFB_1 using the autosampler on the BIAcore, and allowed to equilibrate for 10 minutes at room temperature. In the case of the scFv antibodies, they were manually pre-mixed with the respective concentrations of AFB_1 and allowed to incubate for 1 hour at 37° C, before being injected over the conjugate-immobilised surface. This was carried out, as it was a more economical method of using scFv antibody. The samples for both antibodies were passed over an immobilised AFB_1-BSA surface in random order, and regenerated using the appropriate pre-determined regeneration solution.

A typical antibody binding response curve for the polyclonal anti-AFB_1 antibody is illustrated in Figure 5.17. The response was measured following the binding of unbound antibody from the equilibrated antibody: antigen mixtures. A standard curve was constructed by plotting concentration of free AFB_1 versus amount of unliganded antibody bound in response units (RU). Figure 5.18 shows the intra-day assay plot for the polyclonal affinity-purified anti-AFB_1 antibody. The assay had a range of detection between 0.75 and 98 ng/ml. Table 5.1 shows the intra-day C.V.'s, which are the standard deviation expressed as a percentage of the mean. These varied between 3.5 and 13.6 %. In order to determine the inter-day coefficients of variation the assay was carried out on three different days, and the binding response of each antigen concentration was divided by the antibody response determined in the presence of zero antigen concentration (R/Ro). The mean of these normalised results for each of three assays were plotted, and the C.V's between them were calculated. Figure 5.19 shows the inter-day assay plot, with a range of detection between 0.75 and 98 ng/ml. Table 5.2 shows the inter-day coefficients of variation for the assay, which range between 2.5 and 17 %.
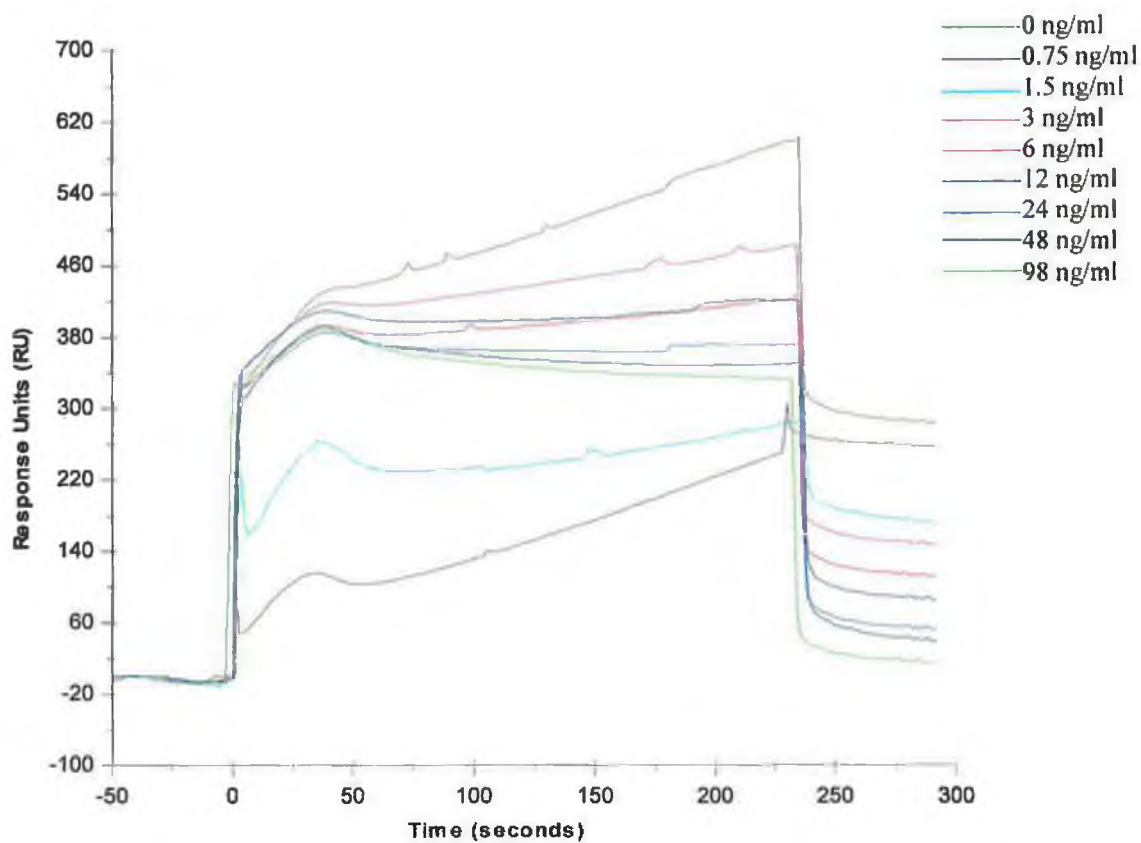
Figure 5.17. *Overlaid interaction curves for various polyclonal antibody-AFB₁ concentrations equilibrated in PBS. The measured binding responses for each concentration were used to construct a calibration curve. The antibody bound showed minimal dissociation from the sensor chip, and was regenerated using 1 M ethanolamine containing 20 % (v/v) acetonitrile, pH 12.0.*

Table 5.1. *Intra-day coefficients of variation for polyclonal anti-AFB$_1$ antibody. Three sets of eight standards were run on the same day and the coefficient of variation (C.V.'s) were calculated as the standard deviation (S.D.) expressed as a percentage of the mean values for each standard.*

Aflatoxin B$_1$ concentration (ng/ml)	Calculated mean ± S.D. (RU)	Coefficient of variation (%)
98.0	43.70 ± 2.50	5.73
48.0	62.60 ± 7.18	11.48
24.0	84.03 ± 7.64	9.09
12.0	110.66 ± 3.96	3.58
6.0	134.90 ± 6.23	4.62
3.0	168.46 ± 15.23	9.04
1.5	198.10 ± 21.07	10.64
0.75	214.03 ± 29.04	13.57
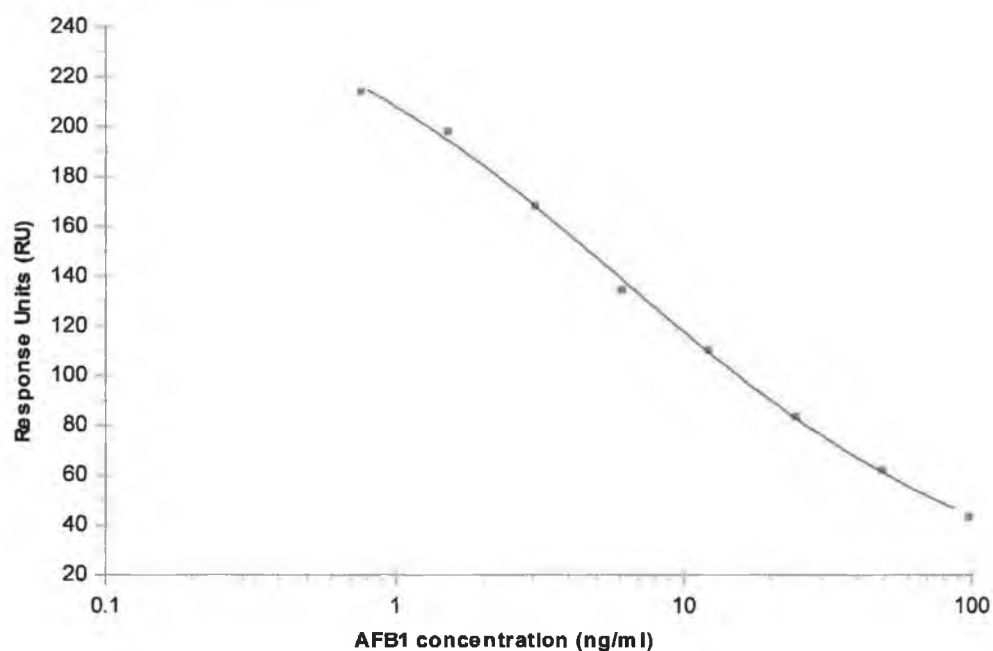
Figure 5.18. *Intra-day assay curve for AFB$_1$ in PBS using polyclonal anti-AFB$_1$ antibody. The range of detection for the antibody was between 0.75 and 98 ng/ml. The calibration plot was constructed using BIAevaluation 3.1 software package. The results shown are the average of triplicate measurements.*

Table 5.2. *Inter-day coefficients of variation for polyclonal anti-AFB$_1$ antibody. Three sets of eight standards were run on three different days, and the measured binding responses were used to calculate the normalised binding response values. From these, the C.V.'s were calculated as the standard deviation expressed as a percentage of the mean normalised values for each standard.*

Aflatoxin B$_1$ concentration (ng/ml)	Calculated mean ± S.D. (R/Ro)	Coefficient of variation (%)
98.0	0.18 ± 0.03	17.02
48.0	0.25 ± 0.02	9.56
24.0	0.34 ± 0.01	5.26
12.0	0.44 ± 0.02	5.61
6.0	0.56 ± 0.00	1.43
3.0	0.72 ± 0.01	2.50
1.5	0.82 ± 0.03	3.63
0.75	0.91 ± 0.03	3.61

Figure 5.19. *Inter-day assay curve for AFB$_1$ in PBS using polyclonal anti-AFB$_1$ antibody. The range of detection for the antibody was between 0.75 and 98 ng/ml. The calibration plot was constructed using BIAevaluation 3.1 software package. The results shown are the average of triplicate measurements.*

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A typical antibody binding response curve for the scFv anti-AFB$_1$ antibody is illustrated in Figure 5.20. A standard curve was constructed by plotting concentration of free AFB$_1$ versus the amount of unliganded antibody bound in response units (RU). Figure 5.21 shows the intra-day assay plot for the scFv anti-AFB$_1$ antibody. The assay had a range of detection between 3 and 195 ng/ml. Table 5.3 shows the intra-day C.V.'s, which varied between 0.16 and 1.0 %. In order to determine the inter-day coefficients of variation, the assay was carried out on three different days and the binding response of each antigen concentration was normalised, plotted, and the C.V.'s between them were calculated. Figure 5.22 shows the inter-day assay plot, with a range of detection between 3 and 195 ng/ml. Table 5.4 shows the inter-day coefficients of variation for the assay, which ranged between 3 and 20 %.

Figure 5.20. *Overlaid interaction curves for various equilibrated scFv antibody-AFB$_1$ concentrations in PBS. The measured binding responses for each concentration were used to construct a calibration curve. The antibody bound began to dissociate from the sensor chip immediately after the injection was completed. Regeneration of surface was carried out using 10 mM NaOH.*

Table 5.3. *Intra-day coefficients of variation for scFv anti-AFB₁ antibody. Three sets of eight standards were run on the same day and the C.V.'s were calculated as the standard deviation expressed as a percentage of the mean values for each standard.*

Aflatoxin B_1 concentration (ng/ml)	Calculated mean ± S.D. (RU)	Coefficient of variation (%)
195	31.06 ± 0.32	1.03
98	42.46 ± 0.40	0.95
48	64.26 ± 0.32	0.50
24	125.40 ± 0.43	0.35
12	210.86 ± 0.30	0.14
6	288.73 ± 0.81	0.28
3	312.43 ± 0.50	0.16
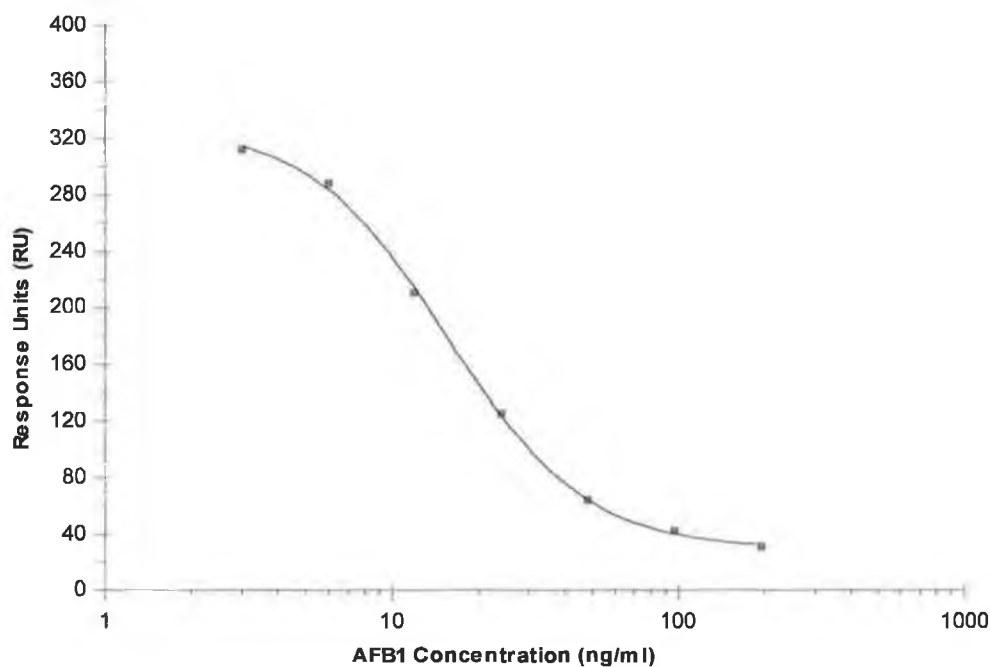
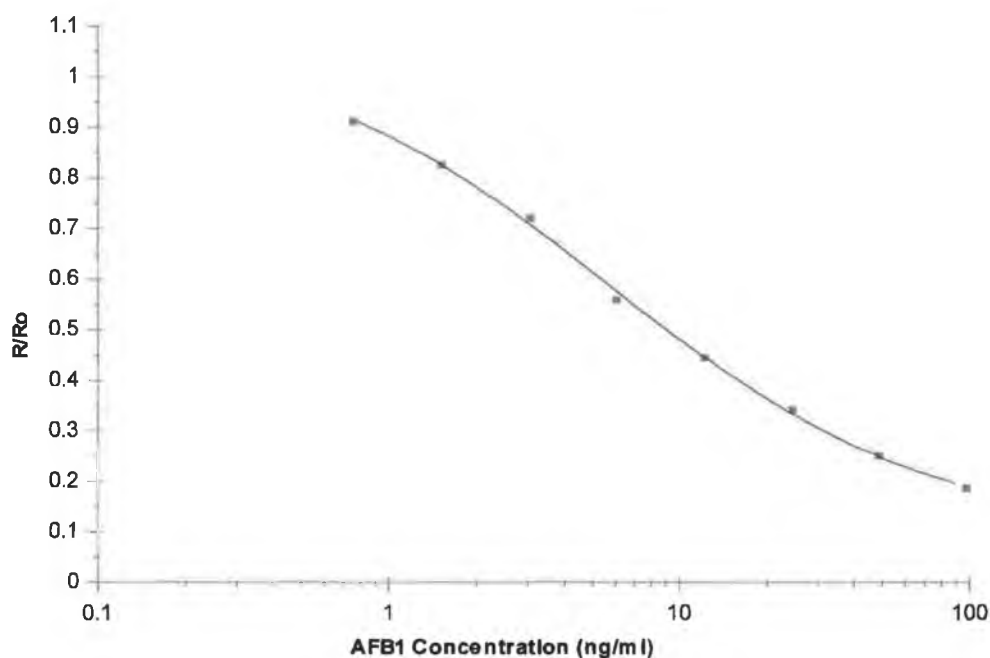***Figure 5.21.*** *Intra-day assay curve for AFB₁ in PBS using scFv anti-AFB₁ antibody. The range of detection for the antibody was between 3 and 195 ng/ml. The calibration plot was constructed using BIAevaluation 3.1 software package. The results shown are the average of triplicate results.*
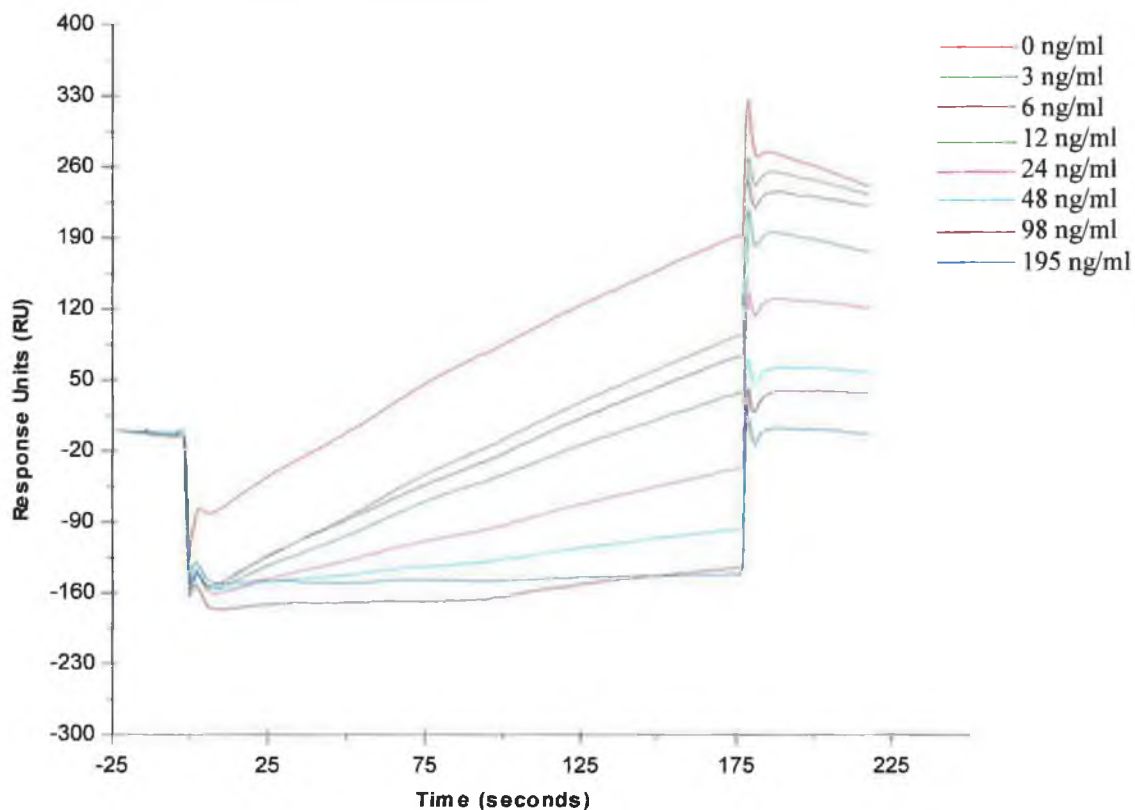
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Table 5.4. Inter-day coefficients of variation for scFv anti-AFB$_1$ antibody. Three sets of eight standards were run on three different days, and the measured binding responses were used to calculate the normalised binding response values. From these the C.V.'s were calculated as the standard deviation expressed as a percentage of the mean normalised values for each standard.

Aflatoxin B$_1$ concentration (ng/ml)	Calculated mean ± S.D. (R/Ro)	Coefficient of variation (%)
195	0.09 ± 0.01	11.06
98	0.14 ± 0.01	12.39
48	0.27 ± 0.05	19.95
24	0.53 ± 0.02	4.01
12	0.79 ± 0.02	3.5
6	0.89 ± 0.03	3.95
3	0.96 ± 0.03	3.35

Figure 5.22. Inter-day assay curve for AFB$_1$ in PBS using scFv anti-AFB$_1$ antibody. The range of detection for the antibody was between 3 and 195 ng/ml. The calibration plot was constructed using BIAevaluation 3.1 software package. The results shown are the average of triplicate results.

5.2.3.4. *Detection of AFB₁ in spiked grain samples*

Spiked grain samples were prepared as described in section 2.10.3, and were applied in inhibition assays for both the affinity-purified polyclonal anti-AFB$_1$ antibody, and the scFv anti-AFB$_1$ antibody, as described in section 2.20.4.

The affinity-purified polyclonal antibodies were mixed with concentrations of spiked AFB$_1$ between 0 and 98 ng/ml using the autosampler on the BIAcore, and allowed to equilibrate for 10 minutes at room temperature. They were then were passed over an immobilised AFB$_1$-BSA surface in random order, and regenerated using the appropriate pre-determined regeneration solution. Figure 5.23 shows a typical binding response curve for the affinity-purified polyclonal anti-AFB$_1$ antibody, where the amount of unliganded antibody bound to the conjugate surface was measured. The range of detection of the polyclonal antibody for the detection of AFB$_1$ in the spiked grain samples was between 0.75 and 98 ng/ml. Figure 5.24 shows a graph of the intra-day assay variation, while Table 5.5 shows the percentage C.V.'s for the intra-day assay. These varied between 0.9 and 5.7 %. The assay was also reproducible on three separate days, and Figure 5.25 shows the inter-day assay variation, which also had a linear range of detection between 0.75 and 98 ng/ml. The inter-day C.V.'s were between 0.7 and 11.4 %, and these are shown in Table 5.6.
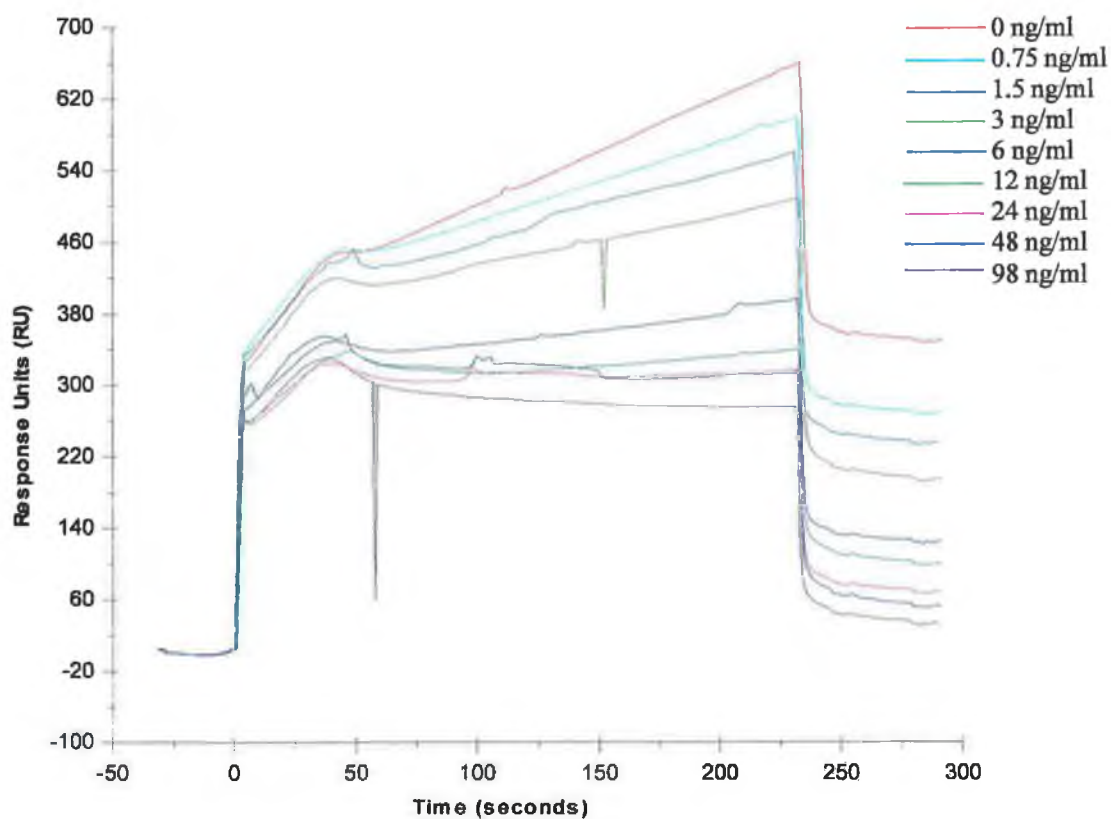
Figure 5.23. *Overlaid interaction curves for various equilibrated polyclonal antibody-AFB₁ concentrations in spiked grain samples. The measured binding responses for each concentration were used to construct a calibration curve. The antibody bound showed minimal dissociation from the sensor chip, and was regenerated using 1 M ethanolamine containing 20 % (v/v) acetonitrile, pH 12.0.*

Table 5.5. Intra-day assay coefficients of variation in grain samples spiked with AFB_1 using affinity-purified polyclonal anti-AFB_1 antibody. Three sets of eight standards were run on the same day and the C.V.'s were calculated as the standard deviation expressed as a percentage of the mean values for each standard
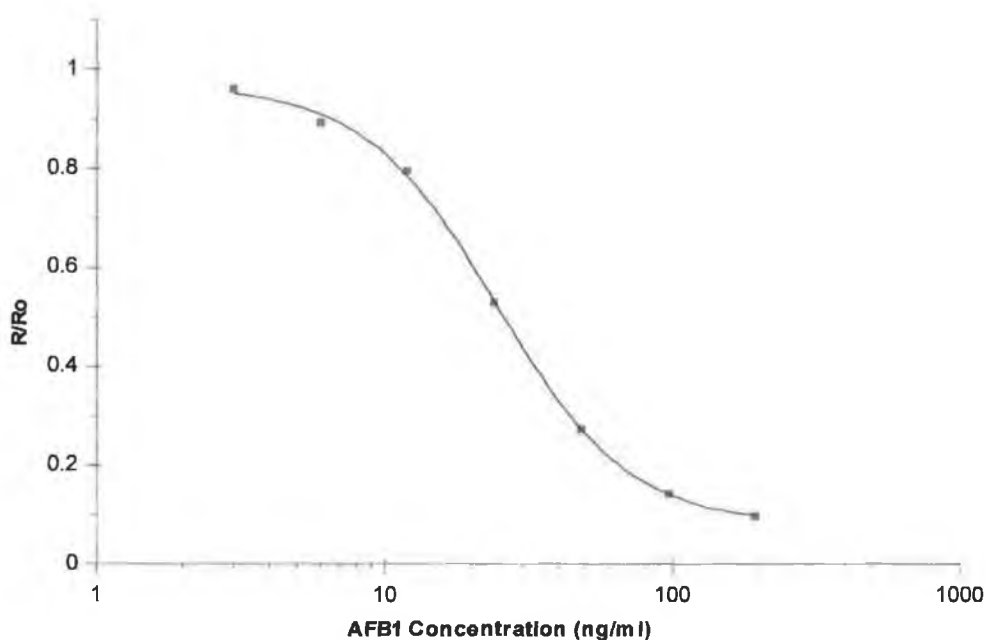
Aflatoxin B_1 concentration (ng/ml)	Calculated mean ± S.D. (RU)	Coefficient of variation (%)
98.0	49.16 ± 2.45	5.00
48.0	74.96 ± 4.29	5.73
24.0	89.40 ± 2.95	3.30
12.0	112.50 ± 3.22	2.86
6.0	136.80 ± 3.93	2.88
3.0	166.93 ± 1.55	0.93
1.5	224.20 ± 3.86	1.72
0.75	264.63 ± 5.99	2.26
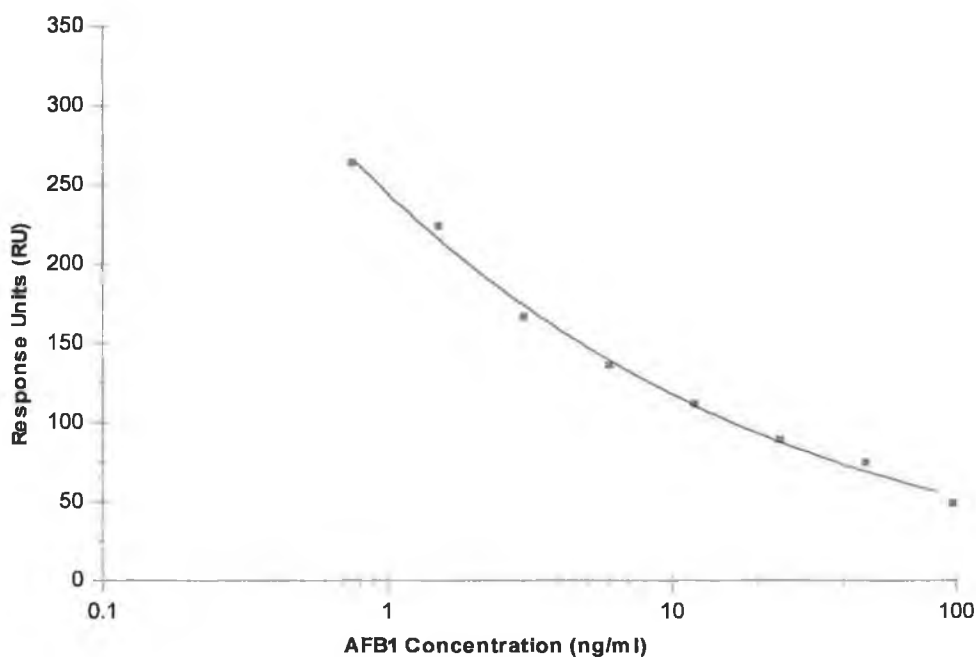
Figure 5.24. Intra-day assay curve for AFB_1 in grain samples spiked with AFB_1 using affinity-purified polyclonal anti-AFB_1 antibody. The range of detection for the antibody was between 0.75 and 98 ng/ml. The calibration plot was constructed using BIAevaluation 3.1 software package. The results shown are the average of triplicate results.

Table 5.6. Inter-day coefficients of variation in grain samples spiked with AFB_1 using affinity-purified polyclonal anti-AFB_1 antibody. Three sets of eight standards were run on three different days, and the measured binding responses were used to calculate the normalised binding response values. From these the coefficient of variation (C.V.'s) were calculated as the standard deviation expressed as a percentage of the mean normalised values for each standard.

Aflatoxin B_1 concentration (ng/ml)	Calculated mean ± S.D. (R/Ro)	Coefficient of variation (%)
98.0	0.15 ± 0.009	6.28
48.0	0.24 ± 0.02	10.36
24.0	0.31 ± 0.01	4.48
12.0	0.42 ± 0.02	6.20
6.0	0.49 ± 0.02	4.29
3.0	0.60 ± 0.06	11.44
1.5	0.77 ± 0.006	0.77
0.75	0.94 ± 0.02	2.40

Figure 5.25. Inter-day assay curve for AFB_1 in grain samples spiked with AFB_1 using affinity-purified polyclonal anti-AFB_1 antibody. The range of detection for the antibody was between 0.75 and 98 ng/ml. The calibration plot was constructed using BIAevaluation 3.1 software package. The results shown are the average of triplicate results.

The scFv anti-AFB$_1$ antibodies were manually pre-mixed with the respective concentrations of spiked AFB$_1$ and allowed to incubate for 1 hour at 37° C, before being randomly injected over the conjugate immobilised surface. Regeneration was carried out using the appropriate pre-determined regeneration solution. Figure 5.26 shows overlaid interaction curves for various equilibrated scFv antibody-AFB$_1$ concentrations in spiked grain samples. The amount of unliganded antibody bound to the immobilised conjugate surface was measured, and this was used to construct a standard curve. Figure 5.27 shows the intra-day assay curve for the range detection for the scFv antibody. This was between 0.75 and 48 ng/ml, with percentage C.V.'s between 0.1 and 16.5 %. Inter-day variation studies were also carried out by running the assay on three separate days, normalising the results, and calculating the percentage C.V.'s between the assays. Figure 5.28 shows the inter-day assay curve with a range of detection for the scFv antibody between 0.75 and 48 ng/ml. Table 5.8 shows the percentage C.V.'s which varied between 3.7 and 11.8 %.
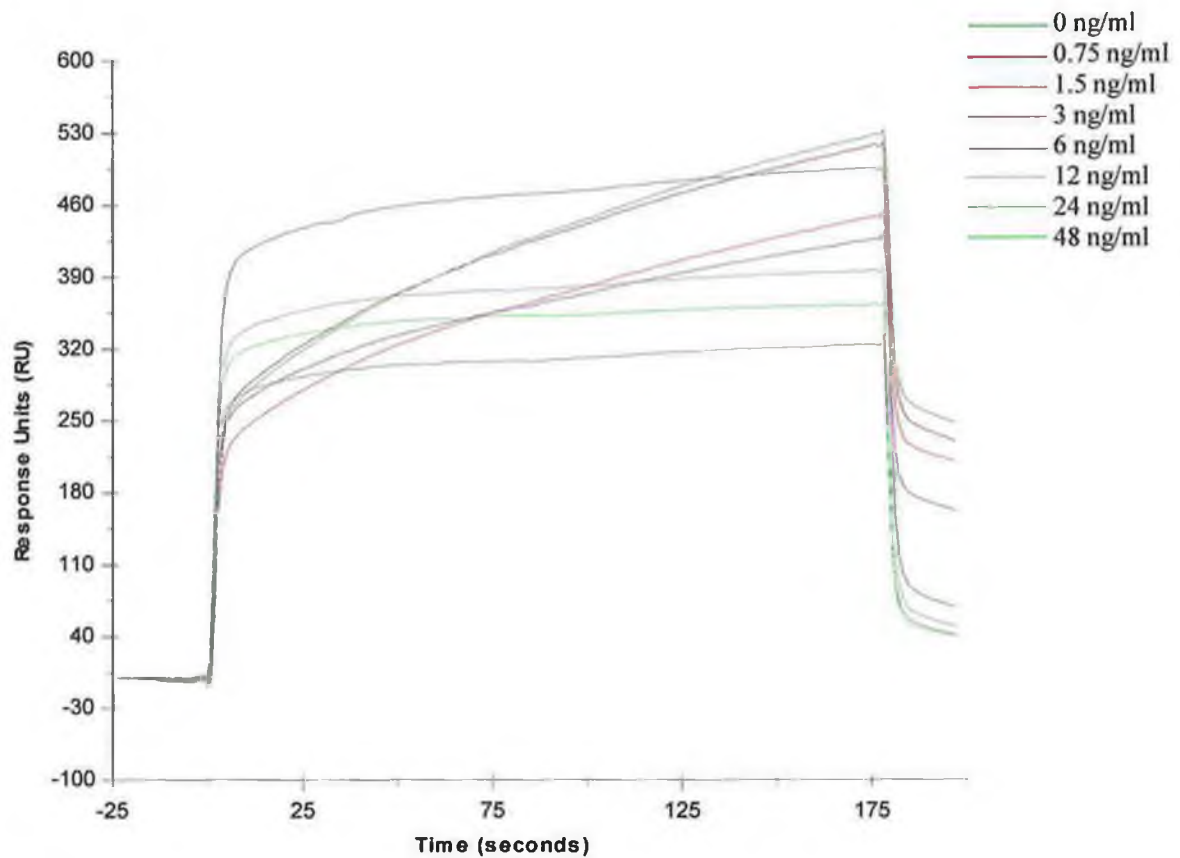
Figure 5.26. *Overlaid interaction curves for various equilibrated scFv antibody-AFB₁ concentrations in spiked grain samples. The measured binding responses for each concentration were used to construct a calibration curve. The antibody bound began to dissociate from the sensor chip immediately after the injection was completed. Regeneration of surface was carried out using 10 mM NaOH.*

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Table 5.7. Intra-day assay coefficients of variation in grain samples spiked with AFB_1 using scFv anti-AFB_1 antibody. Three sets of eight standards were run on the same day and the C.V.'s were calculated as the standard deviation expressed as a percentage of the mean values for each standard.
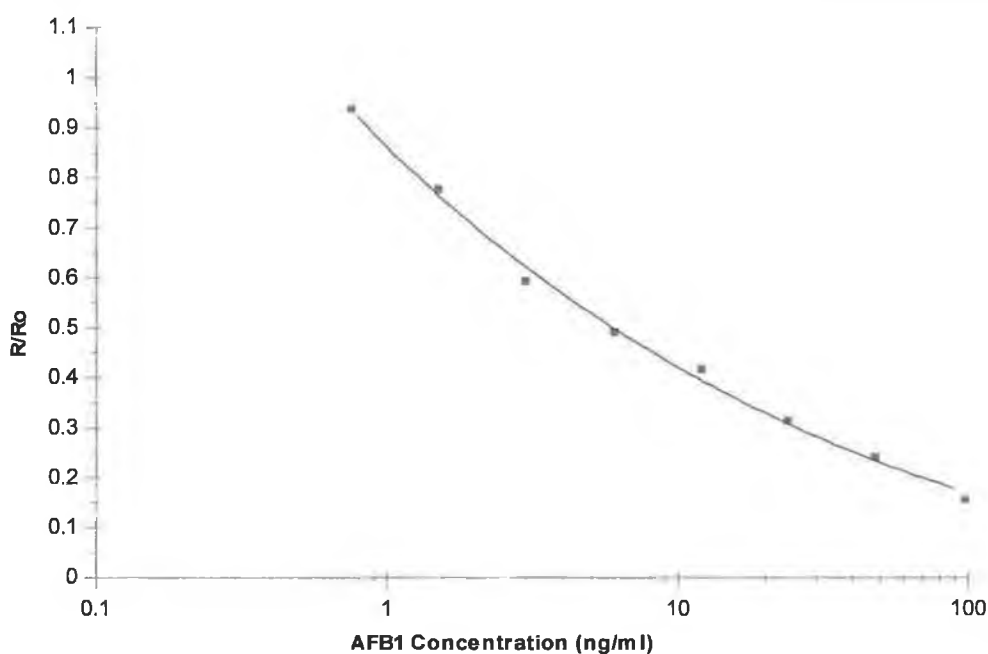
Aflatoxin B_1 concentration (ng/ml)	Calculated mean ± S.D. (RU)	Coefficient of variation (%)
48.0	39.93 ± 2.57	6.44
24.0	46.36 ± 7.66	16.53
12.0	61.03 ± 2.54	4.16
6.0	90.03 ± 4.81	5.35
3.0	132.96 ± 0.23	0.17
1.5	168.43 ± 10.73	6.3
0.75	194.43 ± 12.29	6.32
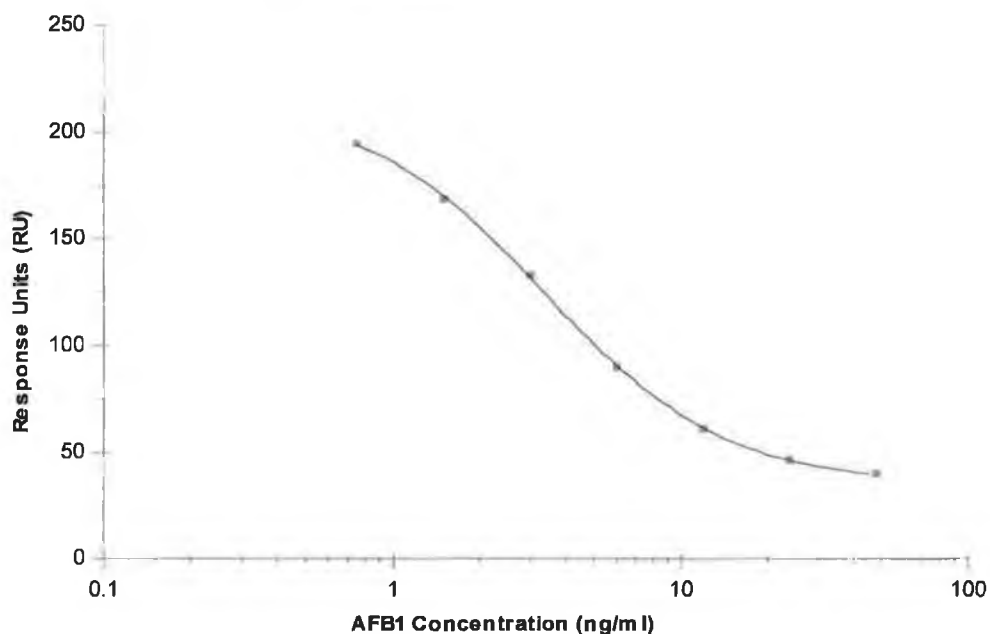
Figure 5.27. Intra-day assay curve for AFB_1 in grain samples spiked with AFB_1 using scFv anti-AFB_1 antibody. The range of detection for the antibody was between 0.75 and 48 ng/ml. The calibration plot was constructed using BIAevaluation 3.1 software package. The results shown are the average of triplicate results.

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Table 5.8. Inter-day coefficients of variation in grain samples spiked with AFB₁ using scFv anti-AFB₁ antibody. Three sets of eight standards were run on three different days, and the measured binding responses were used to calculate the normalised binding response values. From these the C.V.'s were calculated as the standard deviation expressed as a percentage of the mean normalised values for each standard.

| Aflatoxin B₁ concentration (ng/ml)	Calculated mean ± S.D. (R/Ro)	Coefficient of variation (%)
48.0	0.19 ± 0.007	4.13
24.0	0.24 ± 0.01	7.47
12.0	0.33 ± 0.04	11.83
6.0	0.44 ± 0.01	4.06
3.0	0.67 ± 0.02	3.58
1.5	0.84 ± 0.03	4.63
0.75	0.93 ± 0.03	3.75
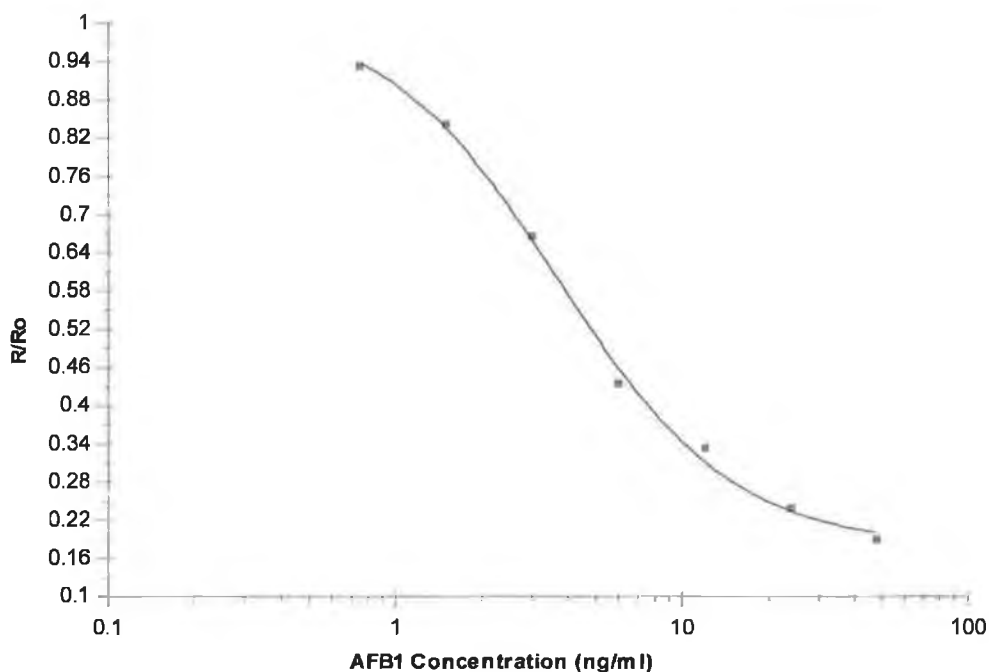
Figure 5.28. Inter-day assay curve for AFB₁ in grain samples spiked with AFB₁ using scFv anti-AFB₁ antibody. The range of detection for the antibody was between 0.75 and 48 ng/ml. The calibration plot was constructed using BIAevaluation 3.1 software package. The results shown are the average of triplicate results.

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5.2.4. *Solution phase steady state affinity determinations*

BIAcore was also used to determine the dissociation rate constant of the scFv anti-AFB_1 antibody. A known concentration of scFv anti-AFB_1 antibodies (2.09×10^{-8} M) were serially doubly diluted in HBS buffer, and passed sequentially over an immobilised AFB_1-BSA conjugate surface. The binding responses obtained were used to construct a standard curve of antibody concentration (bound to the immobilised toxin-protein conjugate) versus response units, as described in section 2.20.5. This is shown in Figure 5.29. A known concentration of scFv antibody (1/8 dilution = 2.6125×10^{-9} M) was then mixed with serial doubling dilutions of AFB_1 ranging in concentration from 1.5 to 390 ng/ml (9.62×10^{-12} to 6.25×10^{-7} M). These were allowed to attain equilibrium. Each equilibrium mixture was then injected over an immobilised AFB_1-BSA conjugate surface, for the detection of 'unligated' scFv anti-AFB_1 antibodies. The concentration of free scFv antibody at equilibrium was determined by reference to the standard curve. The model described below was used to calculate the solution equilibrium dissociation constant

$$B_{free} = \frac{B - A - K_D}{2} + \sqrt{\frac{(A + B + K_D)^2}{4} - AB} \tag{3}$$

Where B_{free} = Free concentration of scFv anti-AFB_1 antibody

A = Total concentration of AFB_1

B = Total scFv anti-AFB_1 antibody concentration

K_D = Equilibrium dissociation constant

The equilibrium dissociation constant was calculated by constructing a plot of free scFv anti-AFB_1 antibodies versus AFB_1 concentration as shown in Figure 5.30. Equation (3), which describes the model for solution phase affinity assumes that the antibodies are monovalently bound, which is the case with an scFv antibody. The dissociation rate constant for this monovalent model was found to be, $K_D = 6.91 \times 10^{-8}$ M.

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$$y = 4E+10x + 6.3598$$
$$R^2 = 0.99$$
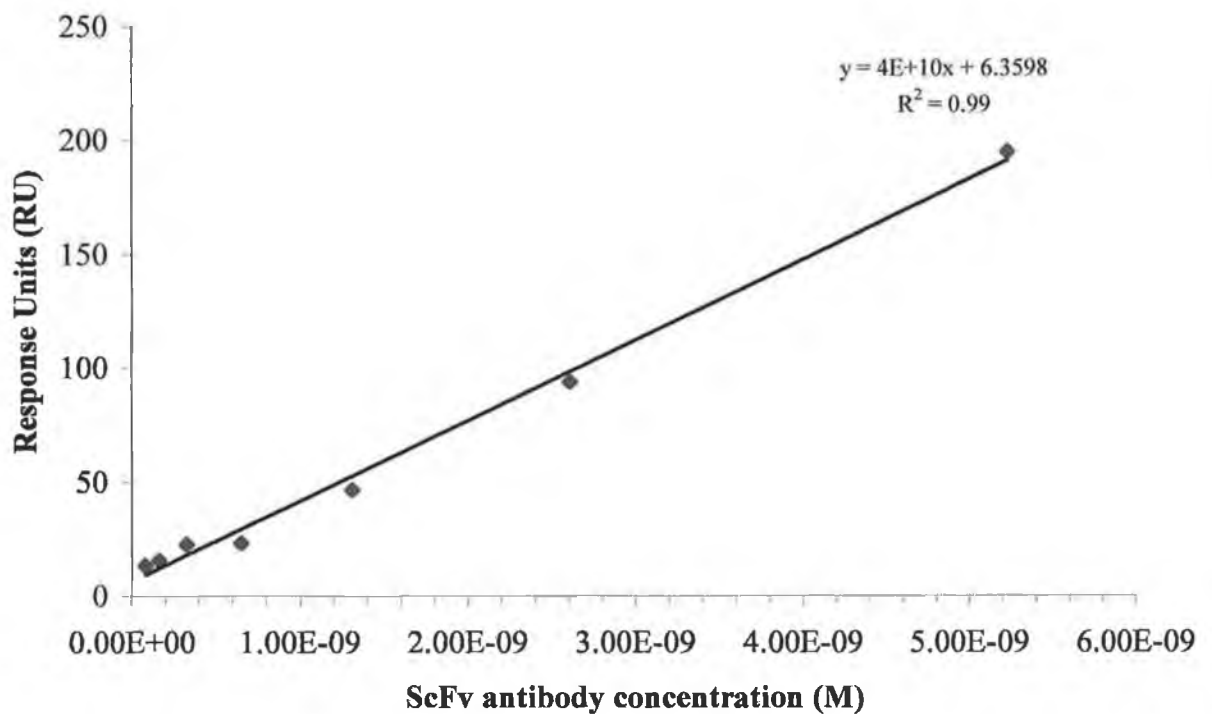
Figure 5.29. *Serial doubling dilutions of scFv anti-AFB$_1$ antibodies of known concentration (M) were passed sequentially over an AFB$_1$-BSA-coated sensor chip surface. A calibration plot was constructed of scFv anti-AFB$_1$ antibodies (M) versus response measured (RU). The calibration plot was then used to calculate the concentration of free scFv antibody at equilibrium. The results shown are singlet measurements.*
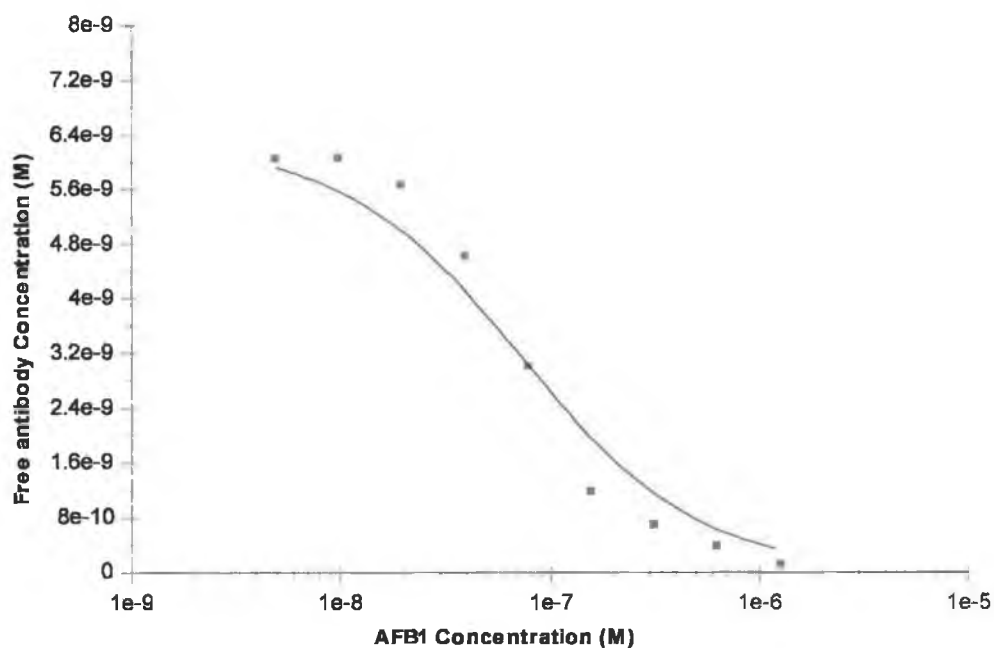
Figure 5.30. *Determination of overall solution equilibrium affinity constant between scFv anti-AFB₁ antibody and AFB₁ on an AFB₁-BSA-coated chip surface. AFB₁ concentrations were plotted against free scFv anti-AFB₁ antibody concentration, determined by reference to a calibration plot of scFv anti-AFB₁ antibodies. A 1:1 interaction model was used to describe the interaction, and fitted to the data set using BIAevaluation software, deriving an equilibrium dissociation constant of $K_D = 6.91 \times 10^{-8}$ M, for the interaction between the scFv antibody and AFB₁. The model described in equation 3 fitted to the data does not describe the interaction particularly well as can be seen from the plot.*

5.2.5. *Results summary*

Table 5.9 gives a summary of the results found in this chapter for both the polyclonal and scFv antibodies.

Table 5.9. *Summary of the results obtained from the polyclonal and scFv anti-AFB$_1$ antibodies in the different BIAcore-based inhibition assay set-ups. It was not possible to calculate an equilibrium dissociation rate constant for the polyclonal antibody population because of its heterogeneous nature.*

Inhibition assay-type	Affinity-purified polyclonal anti-AFB$_1$ antibody	ScFv anti-AFB$_1$ antibody
Range of detection in PBS (ng/ml)	0.75 - 98	3 - 195
Range of detection in spiked grain matrix (ng/ml)	0.75 - 98	0.75 - 48
Equilibrium dissociation rate constant (K$_D$) (M)	N/A	6.91×10^{-8}

5.3. Discussion

In order to achieve a successful immobilisation of toxin-protein conjugate onto a CM-dextran gel surface, it is essential to maximise the interaction between the CM-dextran and the conjugate (AFB$_1$-BSA). When the pH is greater than 7, the CM-dextran surface has a net negative charge (negatively charged carboxyl groups), and proteins at pH values below their isoelectric point (pI) will have a net positive charge (protonated amine groups). As a result, the positively charged protein will be electrostatically attracted to the negatively charged CM-dextran layer. Low ionic strength buffers such as 10 mM sodium acetate are employed for protein immobilisation procedures as they favour such electrostatic interactions. As a result, a 'preconcentration' step has to be carried out in order to maximise this interaction for subsequent immobilisation procedures. For the native form of a protein such as BSA, preconcentration can be facilitated by adjusting the pH below the isolectric point (BSA pI = 4.4). However, modification of proteins by, for example, conjugation to hapten molecules often radically alters the pI. Therefore, when dealing with modified proteins, the correct pH for preconcentration needs to be determined experimentally. The AFB$_1$-BSA conjugate used had a relatively high substitution ratio of 7-10 molecules of AFB$_1$ for every BSA molecule. This resulted in the pI of the conjugated BSA molecule being reduced to less than 4.4, as there were fewer positively charged amines available on the protein for preconcentration onto the negatively charged dextran layer. Hence, the pH for optimal pre-concentration of the toxin-protein conjugate was lower. The pH at which the toxin-protein conjugate preconcentrated onto the dextran gel surface was 3.9. This was indicative of a protein containing a high substitution ratio of hapten.

EDC/NHS coupling chemistry was used to immobilise AFB$_1$-BSA conjugate onto the CM-dextran surface. Water-soluble carbodiimide EDC transforms surface carboxyl groups into active ester groups in the presence of NHS. The surface NHS esters are then available for reaction with amine groups on the hapten-protein conjugate. The reaction works best between pH 6 and 9, and its efficiency decreases rapidly below pH 4.5. Immobilisation of AFB$_1$-BSA conjugate needed to be carried out in acetate buffer

pH 3.9, and, as a result, the immobilisation procedure was not optimal. However, on average, a sufficient amount of conjugate (between 4,000 – 8,000 RU) could be immobilised onto the sensor surface to allow for efficient analytical studies.

When designing SPR-based immunoassays, an important factor in their design is the regenerability of the sensor-chip surface in order to carry out multiple analyses. Ideally it should be possible to carry out fifty or more regeneration cycles on a given immobilised surface, and ligand-binding capacity should be maintained within 20 % of positive control values (Wong *et al.*, 1997). Regeneration studies were carried out on both the polyclonal and scFv anti-AFB_1 antibodies for binding to an immobilised AFB_1-BSA conjugate surface.

Initial studies with the affinity-purified polyclonal anti-AFB_1 antibody indicated that it was difficult to remove, after binding to the AFB_1-BSA conjugate surface using HCl and NaOH. It was not possible to completely regenerate the surface without affecting the binding capacity of the immobilised conjugate. ELISA was used as a method to investigate the effects of regeneration on immobilised conjugate. The use of a novel regeneration solution, consisting of 1M ethanolamine, 20% (v/v) acetonitrile, pH 12.0, was assessed in ELISA. This solution combined high ionic strength and extreme pH as well as chaotrophic properties. It was found to be previously successful for use in surface regeneration of high affinity molecules (Quinn *et al.*, 1999). The studies showed that this regeneration solution did not significantly affect the integrity of the conjugate surface, as well as that increasing concentrations of acetonitrile in the 1M-ethanolamine solution removed increasing amounts of antibody from the surface.

The combined properties of this solution enabled successful regeneration, and facilitated consistent binding of the polyclonal antibody to the AFB_1-BSA surface each time (Figure 5.13.). The regeneration solution did not seem to completely remove all of the bound antibody, although there was consistent binding of the antibody after each injection. This was probably due to the 'higher-affinity' antibodies in the heterogeneous solution of polyclonal antibody that were more difficult to regenerate. The use of high salt concentrations and organic solvents as regeneration solutions could

also have resulted in false signals, as contraction of the dextran gel can sometimes occur, giving rise to inappropriate detector responses. It is significant for future biosensor applications that compatibility of the immobilised-chip surface (even if limited) for organic solvents was demonstrated. Consequently, the possibilities for using small proportions of such solvents to aid solubility of analytes will extend the range of contaminants that could be covered.

Regeneration studies involving the scFv antibody showed that it was easily regenerated from the AFB_1-BSA immobilised surface using a 1-minute pulse of 10 mM NaOH. The binding responses were highly consistent throughout the regeneration study with a variation of binding of approximately 8 %. There was no decrease in measured binding response throughout the study (i.e. cycle 1= 244.7, cycle 49= 244.3). The was probably due to pre-treatment of the conjugate surface with twenty 1-minute pulses of 10 mM NaOH, which would have removed any loosely bound conjugate from the immobilised surface. As a result, when scFv antibody was injected over the conjugate surface, no leeching of scFv antibody-conjugate complex from the surface was observed. Therefore, a decrease in binding signal was not observed throughout the regeneration study, and the binding-regeneration pulses were true to the signal observed.

Non-specific interactions between the polyclonal antibody and the BSA portion of the protein conjugate were minimal, as subtractive immunoaffinity chromatography (using a BSA column) was carried out on the antibody solution. This removed all BSA-binding antibodies. As a result BSA did not have to be incorporated into the diluent buffer. The addition of CM-dextran to the diluent buffer ensured that there were no non-specific interactions to the CM-dextran surface. The scFv antibody displayed no non-specific binding to BSA or the CM-dextran gel surface. This is probably due to the fact that it is monoclonal in nature, and therefore recognises only one epitope, which is present of the AFB_1 molecule.

After optimising the binding capacity and the degree of non-specific binding of both the polyclonal and scFv antibodies, the working range of detection of the BIAcore-

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based assays were then evaluated. A standard curve for the polyclonal antibody of the relative response against the aflatoxin B_1 concentration is shown in Figure 5.19. The limit of quantification of the assay was 0.75 ng/ml, and the assay had a detection range between 0.75-98.0 ng/ml. Each sample was analysed in triplicate and in random during all analyses. Intra-day variability of the assay was assessed by running three sets of standards on one day, and determining the coefficients of variation between the calculated concentrations for each set of three. The C.V. values ranged from 3.58 to 13.57%, and are shown in Table 5.1. Inter-day variability tests were also carried out, in which three sets of standards across the linear range were assayed on three different days. The C.V.-values for this are shown in Table 5.2, and ranged from 2.5 to 17.02%. The limit of quantification for the assay using the scFv antibody was 3 ng/ml, with a range of detection between 3 and 195 ng/ml (Figure 5.21). Intra-day and inter-day variability studies were carried out in the same manner as that for the scFv antibody. The intra-day C.V.'s were between 0.16 and 1.03 % (Table 5.3), while the inter-day C.V.'s were between 3.35 and 19.95 % (Table 5.4).

Both antibodies showed a high level of sensitivity for the detection of AFB_1, with the polyclonal antibody being slightly more sensitive (Limit of detection = 0.75 ng/ml) than the scFv antibody (Limit of detection = 3 ng/ml). The assays are also reproducible with good levels of precision, and reproducibility. However, with both assays some of the C.V.'s are above 10 %. In the case of the polyclonal antibody the C.V.'s may have been higher due to the use of such a harsh regeneration solution. As mentioned previously, contraction of the dextran gel can sometimes occur with the use of such harsh regeneration solutions, and as a result may have given rise to more variable detector responses. However, the assay was reproducible over three days. The scFv showed very high intra-day degrees of precision with only the highest C.V. at 1.03 %. This was probably a result of stringent pre-treatment of the immobilised surface prior to assay analysis, as well as that it was a very mild regeneration solution. This in turn gave rise to accurate binding responses during a given assay. The C.V.'s for the scFv inter-assay were slightly higher at certain points, indicating only a moderate degree of reproducibility. Findlay *et al.* (2000) say that since the basis of

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immunoassays is the antibody-antigen interaction, they may be less precise than chromatographic techniques, and as a result, existing methods with respect to degrees of assay precision are too stringent. They recommend minimal acceptance limits of 20 % (25 % at the limits of quantification) for precision profiles. Wong *et al.* (1997) describe the validation of an assay using BIAcore 2000 for the detection and quantification of humanised monoclonal antibody in mouse serum. The assay was also designed to detect the presence of antibodies against the humanised antibody in the serum samples. They examined degrees of precision, accuracy, specificity, sensitivity, linearity, and ligand stability within the assay. They state that their acceptance criteria for validation is applicable to all biosensor-based immunoassays used to determine the concentration of an analyte, or to determine the presence of antibodies capable of binding to that agent. In addition, they stated that results should be quantitated within 20 % C.V. as a measure of precision. When taking these requirements into consideration, both assays shown in this chapter are within the required acceptance limits, and can be deemed reproducible.

The polyclonal and the scFv antibody were also used for the detection of AFB_1 in spiked grain samples, using BIAcore. The polyclonal antibody showed the same range of detection in spiked grain as that for PBS (0.75-98 ng/ml) with good degrees of precision (Intra-day C.V.'s: 0.93 –5.73 %) and also a high degree of reproducibility (Inter-day C.V.'s: 0.77-11.44 %). The scFv antibody was more sensitive for the detection of AFB_1 in grain samples compared to the model PBS assay, with a detection range between 0.75 and 48 ng/ml. The assay also showed good levels of precision (Intra-day C.V.'s: 0.17-16.53 %) and reproducibility (Inter-day C.V.'s: 3.75-11.83 %). Linskens *et al.* (1992) state that low concentrations of methanol extraction solvent can enhance the performance of some assays, and hence this may give a possible reason for the scFv antibody being more sensitive for the detection of AFB_1 in grain samples. Van der Gaag *et al.* (1999) developed a BIAcore-based inhibition assay for the detection of AFB_1 in spiked grain samples. They used a commercially available monoclonal antibody, and directly immobilised AFB_1 onto the sensor-chip surface. The assay could detect levels as low as 0.2 ppb (ng/ml). The assays shown in this

chapter are not as sensitive as this, but are capable of detecting as low as the EU maximum residue level set for AFB_1 (3 ppb (ng/g)) in grain samples.

BIAcore was also used to determine the solution phase affinity constant of the scFv anti-AFB_1 antibody. The assay was based on the detection of free unliganded scFv antibody after incubation with free drug concentrations of AFB_1. The model describing the solution phase affinity assumed the antibodies were monovalently bound, which was the case with scFv antibodies. The equilibrium dissociation constant for the scFv antibody was $K_D = 6.91 \times 10^{-8}$ M indicating the scFv antibody has a relatively high affinity constant.

5.4. Conclusions

Both of the polyclonal and scFv antibodies examined bound specifically to aflatoxin B_1-BSA with a high avidity. The use of organic solvents combined with high ionic strength was also assessed. This solution did not damage the conjugate surface, but it could not regenerate all of the different polyclonal antibodies in the given population. It was possible to develop an SPR-based inhibition immunoassay for both the polyclonal and scFv antibodies. These assays were then applied for the detection of AFB_1 in spiked grain samples. Both assay types showed a high level of sensitivity, being capable of detecting AFB_1 below the maximum residue levels designated by the EU. Solution-phase equilibrium analysis was also carried out on the scFv antibody demonstrating it had a high dissociation rate constant. The availability of an 'on-line' antibody-based biosensors should have a significant impact on routine surveillance and analysis of agri-food materials.

Chapter 6

Synthesis of Organophosphate-Protein Conjugates and their Applications in Immunoassays

6.1. Introduction

A pesticide is any substance used to control, repel, or kill any pest. Organophosphate (OP) pesticides were developed in the early nineteenth century, and since then they have been extensively used for the treatment of cotton, corn, wheat, and a large variety of other agricultural crops. Some of them are also administered orally to cattle for the control of insect parasites. Others are used externally on cattle for the control of external parasites (e.g. Warble fly). OP's are the most commonly used pesticides primarily because of their low mammalian toxicity, they penetrate grain to a limited extent, and they decompose slowly in storage (Skerritt *et al.*, 1992). However, a consequence of their widespread use in the agricultural industry is that OP's are commonly detected as residues in a large number of food products, and therefore, there are growing concerns over possible adverse effects of long term, low-level exposure to these compounds.

6.1.1. *Organophosphate chemistry and structure*

OP's are distinguished by a central phosphorous atom and numerous side chains. A generalised structure is shown in the Figure 6.1 (a). The X on the structure is generally a leaving group of variable structure. Most of the pesticides are dimethoxy and diethoxy compounds. OP's are usually categorised based on the atoms immediately surrounding the central phosphorous atom. Phosphates are the prototype for the entire class and are those compounds where all four atoms surrounding the phosphorous are oxygen (e.g. dichlorvos). A large number of the OP's have sulphur double bonded to the phosphorous in the place of the oxygen. This is illustrated in Figure 6.1 (b). The X denotes the presence of an aromatic structure. Figure 6.1 (c) shows the structure of pirimiphos, while Figure 6.1 (d) shows the structure of chlorpyrifos. Both of these OP's are commonly used on grain materials while in storage for processing into various products, and will be discussed in greater detail later in the chapter.

R—O   O
   \\ ‖
    P
   / \\
R—O   X

(a)

R—O   S
   \\ ‖
    P
   / \\
R—O   X

(b)

H3C—O—P(=S)—O—[pyrimidine ring with CH3, N, N, N(CH2CH3)2]

(c)

H3C–H2C—O—P(=S)—O—[pyridine ring with Cl, Cl, Cl]

(d)
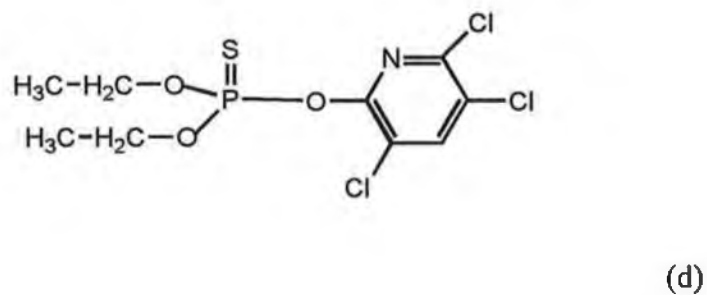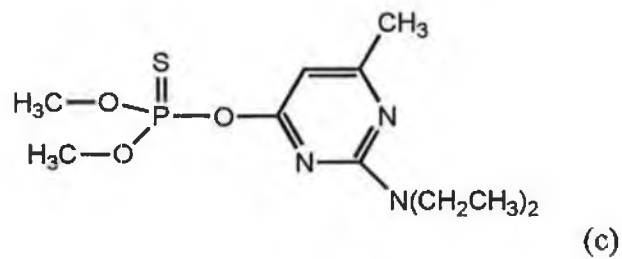
Figure 6.1. *(a) Generic organophosphate structure. (b) Organophosphate structure showing sulphur bonded to the phosphorous. (c) Structure of the pirimiphos-methyl, which has an aromatic structure in place of the X. (d) Structure of chlorpyrifos, which also has an aromatic ring structure. (X= side chain specific for individual OP's).*

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6.1.2. *Mode of action and toxicology*

OP's are metabolised in a similar way for insects as for mammals. Acetylcholine (ACh) is produced in nerve cells and allows the transfer of nerve impulses from one nerve cell to a receptor cell e.g. from a muscle cell to another nerve cell. When the nerve transmission is completed, an enzyme known as acetylcholinesterase (AChE) speeds up the breakdown of acetylcholine in order to stop the nerve transmission. OP's render acetylcholinesterase inactive, and as a result the nerve transmission continues indefinitely. This causes a wide variety of symptoms in mammals such as weakness or paralysis of the muscle, which will eventually result in death. This is a particularly rapid process for insects. The inhibition of acetylcholinesterase results in over-stimulation of the parts of the nervous system that contain acetylcholine. This mainly affects the para-sympathetic nervous system, as well as the central nervous system (O' Malley, 1997).

In humans OP's can be absorbed through the skin, inhaled, or digested. Skin absorption is a very slow process, and, therefore, significant absorption only occurs after prolonged exposure to the pesticide (Kamrin, 1997). However, if the skin is inflamed (e.g. if one had dermatitis), absorption can happen significantly faster, as it is more susceptible to uptake of the pesticide.

OP's vary considerably in their toxicity potency. The major determinant of potency is the binding affinity of the parent compound and/or its metabolic products for AChE. The binding affinity in-turn is determined by structural characteristics such as lability of the P-X bond and overall hydrophobicity and steric characteristics of the molecule (Storm *et al.*, 2000). P=O compounds are generally more potent then P=S compounds. This is because of the greater electronegativity of the oxygen, which weakens the P-X bond, thereby enhancing binding to AchE.

OP's are highly toxic by all routes of exposure. Upon inhalation, the first effects are usually respiratory. The responses are quite allergic in nature, and therefore, common symptoms may include bloody or runny nose, coughing, chest discomfort,

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difficulty in breathing, and wheezing due to constriction or excess fluid in the bronchial tubes. Severe toxicity affects the central nervous system and the peripheral nervous system (Kamrin, 1997). Usually signs of acute toxicity become noticeable when the normal activity of acetyocholinesterase is reduced by one half. Death may occur if enzyme activity falls to between 10-20% of normal functioning levels and may be caused by respiratory failure or cardiac arrest (Kamrin, 1997). Although hazardous, the majority of OP's, with the exception of dichlorvos are not thought to be carcinogenic.

6.1.3. *Environmental and ecological effects*

The toxicity of OP's for birds varies from slight to highly toxic. However, the majority, such as dichlorvos and parathion are highly toxic to wild birds such as mallard ducks and pheasants. Fish also fall victim to the toxicity of OP's. The 96-hour lethal concentration 50 (LC_{50}) of a number of OP's in water is between 0.003-0.9 mg/l, showing that very low concentrations are required for dangerous levels to be reached.

The pesticides have a relatively short half-life under many field conditions, and therefore pose little threat to organisms found in soil or groundwater over a long-term period. Lakes and streams may be susceptible to pesticide 'run-off' if application occurs prior to rainfall. OP breakdown in water is only significantly achieved with high pH levels (Kamrin, 1997).

6.1.4. *Pirimiphos and chlorpyrifos*

Pirimiphos-methyl is generally used to treat grains post-harvest for pests such as rice weevil (*Sitophilus orzyae*), and red flour beetle (*Tribolium castaneum*) (Desmarchelier *et al.*, 1981). It is used to different extents in Australia, Europe, Asia, and North America, predominantly because of the different biological effectiveness against particular insects and mite species. It is one of the more persistent OP's that remains during storage and cooking, as its mammalian toxicity is somewhat lower than other OP's. Its LD_{50} in rats is 2000 mg/kg (Cremlyn, 1991). Pirimiphos is particularly effective against insects that have become resistant to organochlorine

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insecticides and malathion. It is fast acting and has both contact and fumigant action. The ethyl ester, pirimiphos-ethyl, is used as an insecticide in mushroom farms and greenhouses (Cremlyn, 1991).

Chlorpyrifos is a broad-spectrum OP, widely used for the control of various crop pests in soil and on foliage, household insects and animal parasites (Manclus *et al.*, 1994). These include cutworms, corn rootworms, cockroaches, and lice. It is also used on grain, cotton, field fruit, nuts and vegetable crops. The pesticide is poisonous both through skin contact and ingestion (Kamrin, 1997). It is moderately toxic to mammalian species but extremely toxic to a wide range of non-target aquatic organisms. Its toxicity is class II (moderately toxic), with a significantly lower LD_{50} of 95-270 mg/kg compared to pirimiphos (Kamrin, 1997).

6.1.5. *Detection of organophosphates*

OP's such as pirimiphos and chlorpyrifos are commonly found as residues in a large number of food products due to their wide use in the agricultural industry as insecticides and herbicides. This poses potential health hazards, and as a result, there is a requirement for monitoring and surveillance programs for these residues. Maximum residue limits for each pesticide in different products have been defined by the Codex Alimentarius Commission. However, individual countries and most companies usually prescribe 'nil' residue limits. This is usually understood to be <0.1 ppm (Skerritt *et al.*, 1992).

6.1.5.1. *Chromatographic techniques for the detection of organophosphates*

Chromatographic techniques have been the most common and reliable systems used for the detection of OP's in recent times. Although preparation time and sample clean-up procedures are time-consuming, they still offer a high level of reliability and sensitivity for detection. Bottomly and Baker (1984) developed a multi-residue method for the determination of a number of pesticides including pirimiphos in grain samples. The pesticides were extracted with acetone-methanol, and were separated from co-extractives by a partition process with dichloromethane and chromatography on an acidic aluminium oxide column. Quantitative determinations were made by a

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packed column gas-liquid chromatography using a flame photometric detector. The method gave 84% recovery of pirimiphos from fortified grain samples containing 0.1mg kg^{-1} of the pesticide.

Grob & Kalin (1991) developed a modified gas chromatography (GC) method for the detection of pesticides. It incorporated on-line Size Exclusion Chromatography–Gas Chromatography (SEC-GC). The method permitted automated integration of sample preparation into the GC analysis and eliminated the corresponding manual work. The method was suitable for analysing many strongly differing components at the same time, and because high molecular weight materials were removed, it allowed an on-column introduction into GC without contaminating the column inlet. This resulted in the development of a technique that was sufficiently sensitive for the analysis of pesticides.

The GC detectors used to analyse the organophosphates, such as flame photometric detectors and nitrogen/phosphorous detectors, are sensitive to phosphorous but are not specific to phosphorous. As a result they are prone to interferences from phosphorous containing compounds. Residues in cole crops such as cabbage and broccoli are particularly difficult to analyse (Stan & Kellner, 1989). This is because of numerous volatile co-extractives containing nitrogen and sulphur that are found in the *Brassica* genus and are therefore sensed by these detectors. [31]Phosphorous Nuclear Magnetic Resonance (^{31}PNMR) can be used for the analysis of organophosphate pesticide residues (Mortimer & Dawson, 1991). The technique is useful, as extraction of large samples and the prolonged use of NMR acquisition times are not required. Minimal clean-up of extracts was required, and samples only took 30 min for analysis. However, problems were observed with the influence of solvents on chemical shifts. This limited the number of organophosphates that could be analysed using $CDCl_3$.

More recent chromatographic methods for the detection of OP's include gas chromatography-pulse flame photometric detection-mass spectroscopy (GC-PFPD-

MS). The PFMD indicates the exact chromatographic time of suspected peaks for their MS identification and then provides elemental information (Dagan, 2000). Automatic GC-MS analysis is another method used for the detection of OP's. This entailed analysis of a sample by GC, and if a suspect peak was observed, MS analysis was carried out to identify the peaks. However, it was found that GC-PFPD-MS was significantly more sensitive for the detection of OP's compared to GC-MS (Dagan, 2000).

GC is also useful for multi-residue analysis of pesticides in wine (Soleas *et al.*, 2000). The method was capable of simultaneous quantitation of 17 pesticides including chlorpyrifos. The widespread use of pesticides during grape production has led to a greater presence of pesticide residues in wines, which are sold on the international markets. The method was capable of detecting pesticide levels as low as 2 µg/l, with recovery from spiked samples greater than 80 %.

6.1.5.2. *Immunoassay-based techniques for the detection of organophosphates*
The detection methods mentioned in section 6.1.5.1 usually have time-consuming clean-up procedures, with multiphase columns and several solvent manipulations. This has enhanced the need to develop more rapid and sensitive techniques for OP detection. Immunoassay technology offers an important tool for the development of such detection systems. It is possible to apply immunoassays directly to test portions, and hence quantitative data can be obtained significantly faster. They are relatively cheap to develop and can give very low degrees of sensitivity.

Solvent selection is one of the limiting factors in the development of immunoassays for pesticide detection, as antibodies generally work best in an aqueous environment. Most pesticides are only soluble in organic phases, or are only sparingly soluble in water. For the extraction of pesticides from solid foods, a variety of solvents have been tested, such as acetone, ether, petroleum ether, methanol, acetonitrile or hexane. Direct analysis of extracts by immunoassay requires the use of solvents that are miscible in water at low concentrations, as well as being non-denaturing to the

antibody. Immunoassays and antibodies tolerate solvents to different levels, and therefore, individual assays must be assessed for tolerance of varying concentrations of a given solvent (Dankwardt, 2000). Interference levels of solvents must also be assessed by running appropriate controls and blanks in a given assay. Clean-up steps can also be introduced to reduce interference by separating the analyte of interest from the matrix. This can be carried out by C_{18} columns or immunoaffinity chromatography (Aga *et al.*, 1994; Marx *et al.*, 1995). Strachan *et al.* (1999) developed a more novel way for antibodies to tolerate solvents. They produced scFv antibodies to the pesticide atrazine. They also found that stabilisation of the antibody fragment using an inter-domain disulphide bond significantly greatly improved its tolerance for solvent (i.e. methanol). This stabilised single chain antibody was referred to as a stAb. They found that the stAb's could tolerate as much as 50 % (v/v) methanol in an assay, and up to 19 % (v/v) DMSO.

Radioimmunoassays (RIA's) were some of the earliest immuno-based assays used for the detection of OP's and pesticides in general. Ercegovich *et al.* (1981) developed a RIA for the detection of parathion, using polyclonal antibodies being raised to a parathion-BSA conjugate in rabbits. The assay had a lower limit of detection of 4 ng of parathion in sample-free solutions, and between 10-120 ng in blood plasma and lettuce without any clean-up of the sample extract.

ELISA's have also been developed for the detection of OP's. Coupling chemistries of the pesticide hapten to the carrier protein can influence the specificity and sensitivity of an assay (McAdam *et al.*, 1992). Monoclonal and polyclonal antibodies were produced to the OP fenitrothion. It was coupled to the carriers through its phosphate group, which generated the most specific antibodies with the highest affinities (both monoclonal and polyclonal antibodies). The pesticide was also coupled via its aromatic nitro group following its reduction and amide formation with an adipic spacer arm. This produced monoclonal and polyclonal antibodies of moderate affinity. This has led to the proposal that antibodies of greater specificity and higher affinity were produced by conjugation of the pesticide through the thiophosphate

group, as it exposed the functional groups unique to fenitrothion in the immune system. The antibodies generated could also detect fenitrothion in grain samples with a limit of detection of 3 ng (Hill *et al.*, 1992).

M^C Adam & Skerritt (1993) developed a generic method for the production of a number of organophosphate conjugates (i.e. fenitrothion, chlorpyrifos-methyl and ethyl, and pirimiphos-methyl). It entailed the synthesis of a bifunctional reagent that allowed coupling to carrier molecules through a derivatised phosphate ester. Immunoassays were successfully developed for these pesticides using the conjugates produced. The assays were highly sensitive with 3 ppb being detected for fenitrothion, 0.02 ppb for chlorpyrifos-methyl, and 0.2 ppb for pirimiphos (Skerritt *et al.*, 1992). The antibodies used in these assays were also tested for reaction to other pesticides, as a variety of pesticides can be used to treat stored grain samples, in order to control different insects. They showed low levels of cross reactivity to other OP's, indicating that the conjugation strategy used was successful for the development of highly specific antibodies.

Manclus *et al.* (1994) have also synthesised chlorpyrifos conjugates for the production of monoclonal antibodies to chlorpyrifos. The conjugation strategy used here is simpler to that described by M^C Adam & Skerritt (1993). It entailed synthesis of a spacer arm on the aromatic ring by chlorine substitution. The assay developed was sensitive (limit of detection = 1.3 ng/ml) and specific for the detection of chlorpyrifos, with low levels of cross reactivity to other OP's.

Manclus and Montoya (1996) developed another assay for chlorpyrifos and for 3,5,6-trichloro-2-pyridinol (TCP). This is the major metabolite of chlorpyrifos in water, as it undergoes chemical hydrolysis. TCP is charged at neutral pH, and, therefore, more leachable into groundwater and surface water than the parent molecule. Conjugate synthesis of TCP was carried out in the same way as described by Manclus *et al.* (1994), except that once the activated chlorpyrifos hapten was synthesised, chemical hydrolysis was carried out to convert it to TCP (containing the activated spacer arm)

(Figure 6.2). Monoclonal antibodies were produced to both haptens, with limits of detection in ELISA of 6 ng/ml for chlorpyrifos, and 0.034 ng/ml for TCP. The TCP assay was particularly useful, as no extraction steps or clean-up procedures were required for the analysis of the environmental water samples.

ScFv antibodies have also been produced to chlorpyrifos (Alcocer *et al.*, 2000(b)). Two chlorpyrifos hybridoma cell lines were used as the source of immunoglobulin genes for the production of the recombinant scFv antibodies in *E. coli*. The cloning system was the same as that as described in Chapter 4. Functional scFv antibodies were detected without any prior selection, as the genes came from functional anti-chlorpyrifos hybridomas. The extended primer mix used (Krebber *et al.*, 1997) was also a factor in the success of the screening procedure. The scFv's produced showed limits of detection as low as their parental monoclonal antibodies (7 ng/ml).
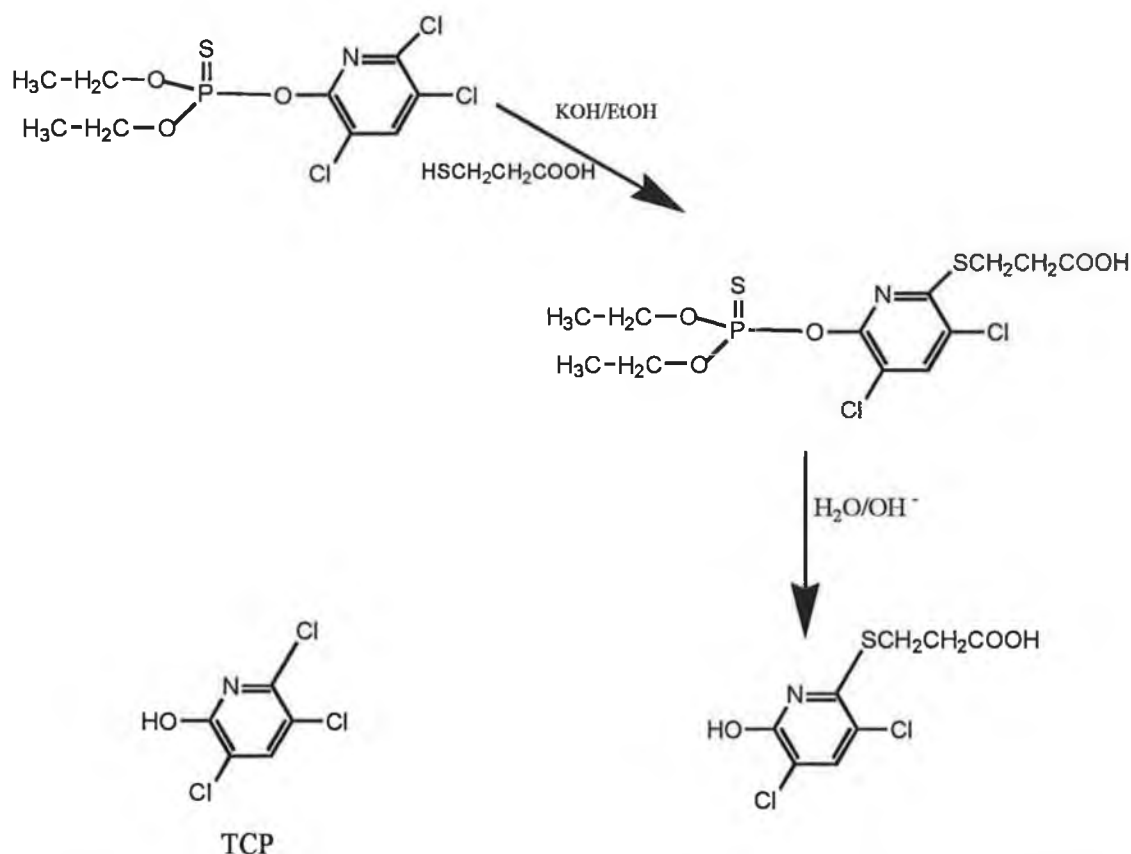
Figure 6.2. Reaction scheme for the synthesis of chlorpyrifos hapten for the production of chlorpyrifos conjugates, as well as the reaction scheme for the synthesis of 3,5,6-trichloro-2-pyridinol (TCP) hapten, the main transformation product of chlorpyrifos.

Garrett *et al.* (1997) produced scFv antibodies to the OP parathion. This was also produced from the genetic material of a hybridoma cell line secreting anti-parathion monoclonal antibodies. The scFv and the parent monoclonal antibody showed similar characteristics when analysed in ELISA. The limits of detection for the monoclonal antibody and scFv-based ELISA's were 1.6 and 2.3 ng/well, respectively. Both antibodies were also assessed by ELISA for stability in methanol extracted samples. The scFv antibody was stable in methanol concentrations up to 5 % (v/v), but this deteriorated in concentrations over 10 % (v/v). Similar results were found for its parent monoclonal antibody, indicating that scFv antibodies can be as useful as monoclonal (and polyclonal) antibodies for the detection of food contaminants in samples.

6.1.5.3. *Biosensor-based techniques for the detection of organophosphates*

Piezoelectric-based biosensors have been used for the detection of the pesticide parathion (Ngwainbi *et al.*, 1986; Horaceck *et al.*, 1998). Piezoelectric biosensors measure mass changes that result from antibody-antigen binding interactions. Piezoelectricity occurs when anistropic crystals are subjected to mechanical stress, causing the generation of an electric charge. If an AC voltage is applied to the resonance crystals, it results in oscillations. The optimal resonance frequency at which this takes place is dependent upon the mass at the crystal surface. The relationship between the change of mass at the crystal surface (Δm) and the change in the oscillating frequency (Δf) is defined by the Sauerbrey equation.

$$\Delta f = -K\Delta m$$

Ngwainbi *et al.* (1986) coated piezoelectric crystals with anti-parathion antibodies, which were used for detection of parathion in the gas phase. This caused a mass change on the crystals, and generated a frequency shift proportional to the concentration of the pesticide. The antibodies could detect as little as 36 ppb of parathion in this assay format.

Regeneration of these sensor surfaces is central to the development of biosensor-based assays, as it ensures the re-usability of a given surface. Horaceck *et al.* (1998) immobilised parathion to piezoelectric crystals using albumin and dextran as two different spacer/carrier molecules. However it was not possible to regenerate bound scFv from BSA-parathion-coated crystals. The dextran-parathion-coated crystals were easily regenerated using proteinase K. This is a useful regeneration solution, when not working with proteins on a sensor surface. In this case, the piezoelectric biosensor was actually used as an antibody characterisation tool, as opposed to a tool used for pesticide detection.

SPR-based biosensors have also been applied as a detection method for OP's (Alcocer *et al.*, 2000(a)). Broad specificity polyclonal antibodies were produced against a group of organophosphates using the hapten – phosphonic acid (trans-4-phosphono-2-butenic acid; TPB) conjugated to keyhole limpet hemocyanin (KLH). The antibodies were used in a BIAcore-based inhibition assay for the detection of parathion using a parathion-BSA conjugate. The limit of detection for the antibody in this assay format was 1.5 ng/ml, with good levels of reproducibility.

Namera *et al.* (2000) developed a direct colourmetric method for the detection of OP's in urine. Many people die through suicidal ingestion of OP's each year, and therefore rapid measurement of OP's is important for the prompt medical management of suspected patients exposed to these compounds. The assay was based on detecting colour complexes, which resulted from reactions of OP's and the chromogenic reagent 4-(4-nitrobenzyl)pyridine (NBP) in urine. NPB reacted with the thiophosphate group on the pesticide to form a purplish-blue colour. The coloured complex could then be determined spectrophotometrically. The detection limit for the assay in urine was 0.1-10 µg/ml in urine. Approximately 27 OP's were detected in urine samples using this method, including chlorpyrifos, which had a limit of detection of 0.3 µg/ml.

6.1.6. *Chapter outline*

This chapter describes the production of a pirimiphos hapten containing an amino group for conjugation to proteins. It also describes the production of chlorpyrifos-BSA conjugates, their spectrophotometric characterisation, and application on BIAcore. A polyclonal anti-chlorpyrifos antibody preparation and a chlorpyrifos-dextran conjugate were obtained from collaborators at the Institute of Food Research, Norwich Research Park, Colney, Norwich, NR 4 7UA, U.K. These were used in a competitive ELISA for the detection of chlorpyrifos. An investigation of the development of an inhibition-based BIAcore assay for the detection of chlorpyrifos, using this antibody population was also undertaken.

6.2. Results

6.2.1. *Pirimiphos hapten synthesis and characterisation for conjugate production*

6.2.1.1. *Synthesis of amino-pirimiphos*

Amino-pirimiphos was synthesised for use in the production of pirimiphos-protein conjugates. Figure 6.3 shows the scheme of the reaction used. The two starting compounds for the reaction, 2-amino-4-hydroxy-6-methyl pyrimidin and dimethyl chlorothiophosphate, were purchased commercially. Sodium anhydride was used to treat 2-amino-4-hydroxy-6-methyl pyrimidin in order to deprotonate the H from the OH group. This de-protonated hydrogen then formed hydrogen gas with the hydrogen from sodium anhydride. The deprotonated compound reacted with dimethyl chlorothiophosphate to form 'amino-pirimiphos'. Chlorine was a by-product from the reaction and formed a salt with the sodium from sodium anhydride.

6.2.1.2. *Nuclear magnetic resonance (NMR), and mass spectral analysis (MS) of amino-pirimiphos*

NMR analysis was carried out on the compound in order to confirm that the desired structure was synthesised. Figure 6.4 (a) shows the Hydrogen (H) NMR spectra of amino-pirimiphos, while Figure 6.4 (b) shows a structure of amino pirimiphos with the hydrogen atoms labelled A-E corresponding to the peaks in Figure 6.4 (a). All of the peaks on the spectra can be correctly accounted for on the structure.

Carbon (C) NMR was also carried out on the compound to account for the correct number of carbon atoms on the structure. Figure 6.5 (a) shows the C NMR spectra of amino-pirimiphos, and Figure 6.5 (b) shows the carbon atoms labelled on an amino-pirimiphos structures, which correspond to the peaks on Figure 6.5 (a). Once again all of the peaks are correctly identified, indicating the desired synthetic reaction had occurred to produce amino-pirimiphos.

1. NaH $\longrightarrow$ Na$^+$ +H$^-$

2.

2-amino-4-hydroxy-6-methylpyrimidin

3.

Dimethylchlorothiophosphate

+ Cl$^-$

Na$^+$ (1) + Cl$^-$ (3) $\longrightarrow$ Na$^+$ + Cl$^-$
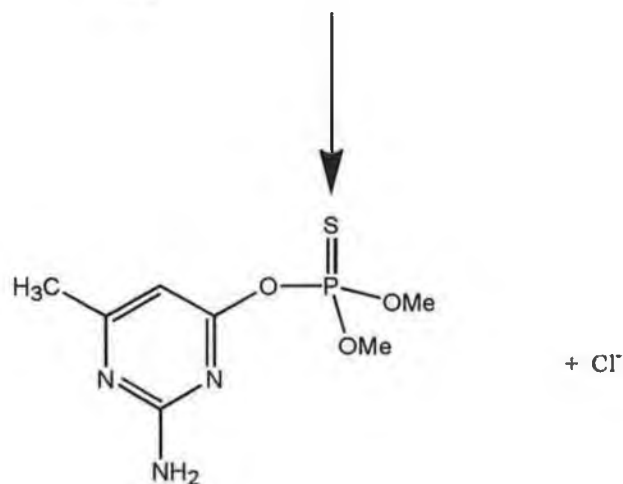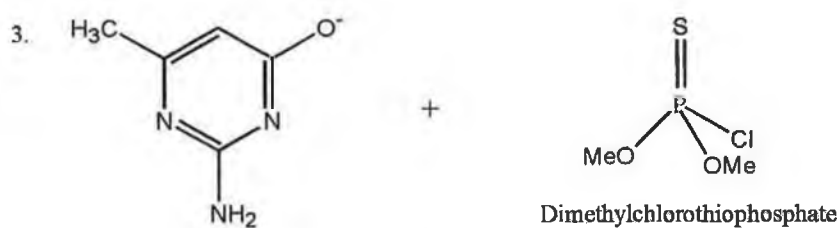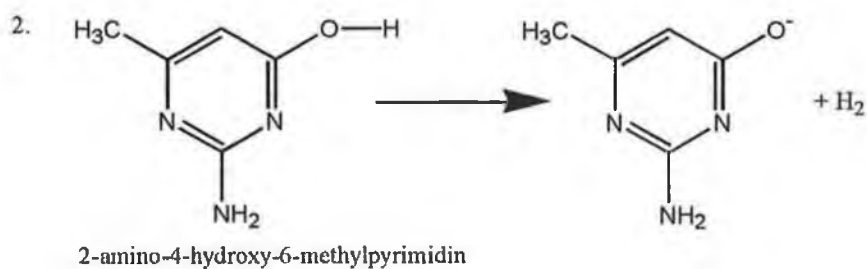
Figure 6.3. *Reaction scheme showing the synthesis of amino–pirimiphos. Sodium anhydride reacted with-2-amino-4-hydroxy-6-methylpyrimidin to de-protonate the hydrogen, which formed hydrogen gas with the H$^-$ of NaH. The deprotonated compound reacted with dimethyl chlorothiophosphate to form amino-pirimiphos with chlorine as a by-product. This formed a salt with the Na of NaH.*

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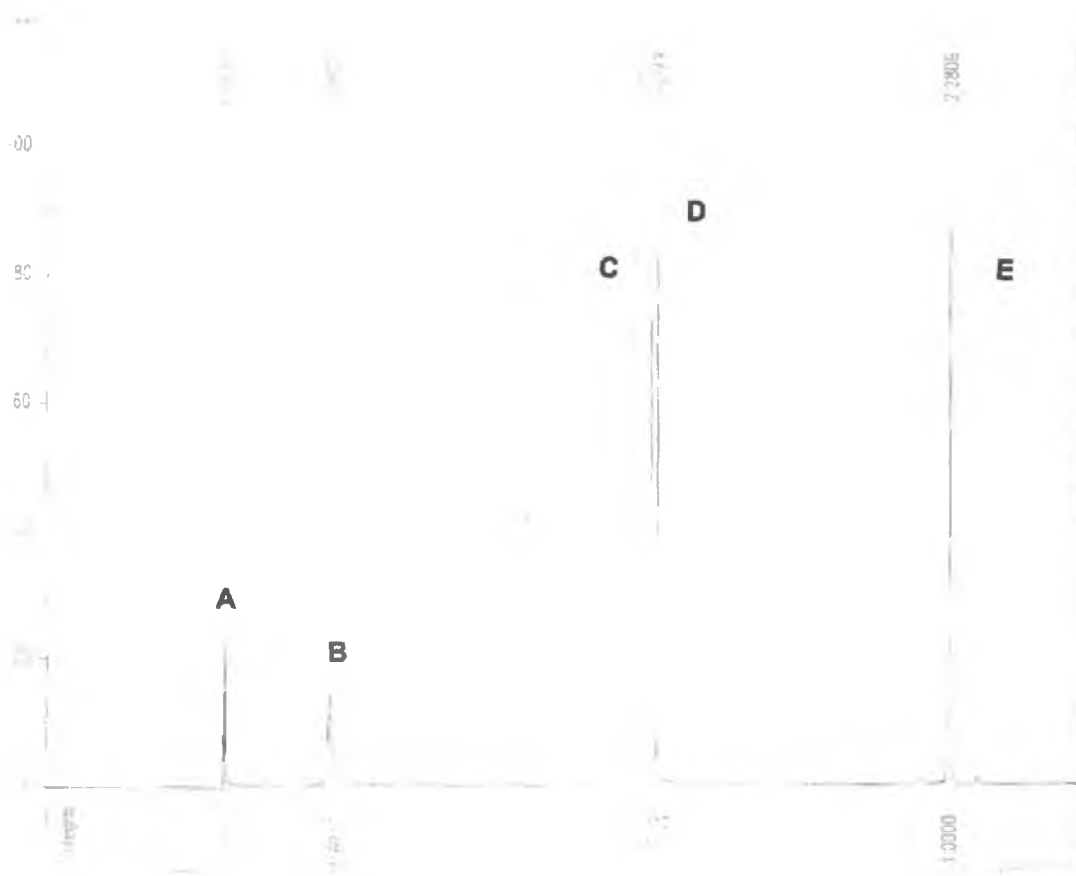
Figure 6.4. (a) H NMR of amino-pirimiphos. All of the relevant peaks are labelled A-E, and correspond to the atoms labelled on the amino-pirimiphos molecule in Figure 6.4. (b).
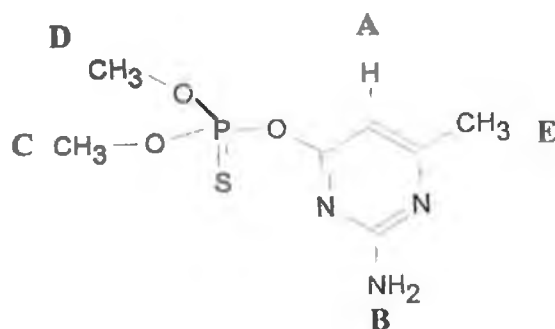
Figure 6.4. (b) Structure of amino-pirimiphos showing the corresponding hydrogen atoms on the H NMR in Figure 6.4 (a).
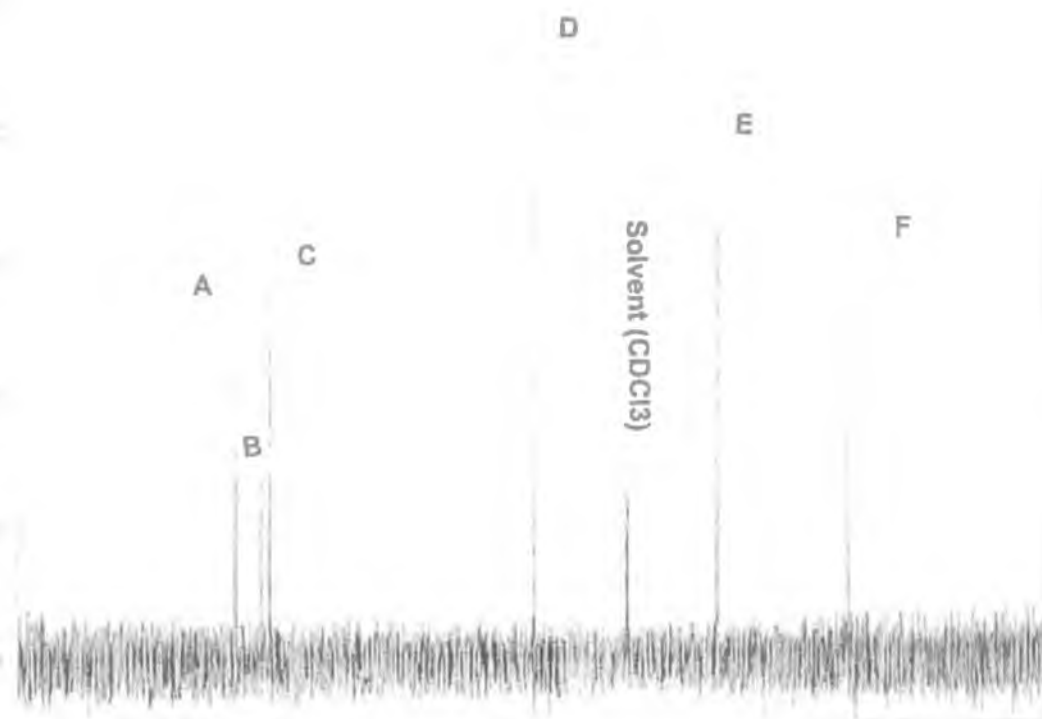
Figure 6.5. *(a)* C NMR of amino-pirimiphos. All of the relevant peaks are labelled A-F, and correspond to the atoms labelled on the amino-pirimiphos molecule in Figure 6.5. (b).
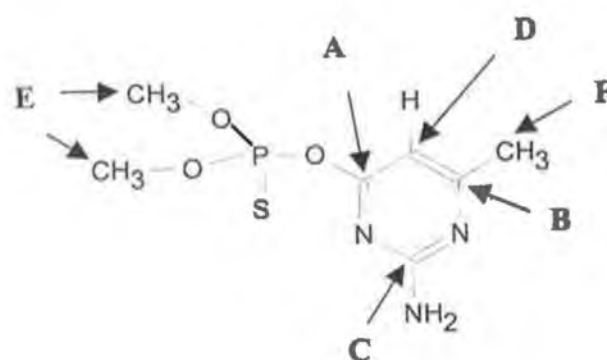
Figure 6.5. *(b)* Structure of amino-pirimiphos showing the corresponding carbon atoms on the C NMR in Figure 6.5 (a)

MS analysis was carried out to confirm the correct molecular weight of the compound, as well as to analyse it for the presence of other partially synthesised products. Figure 6.6 shows the MS analysis of amino-pirimiphos. The two main molecular weight peaks identified are that for 249 Da, and 279 Da. The 249 Da peak corresponds to amino-pirimiphos, while the 279 Da peak indicates the presence of amino-pirimiphos intermediate compound.

7174579 ADC
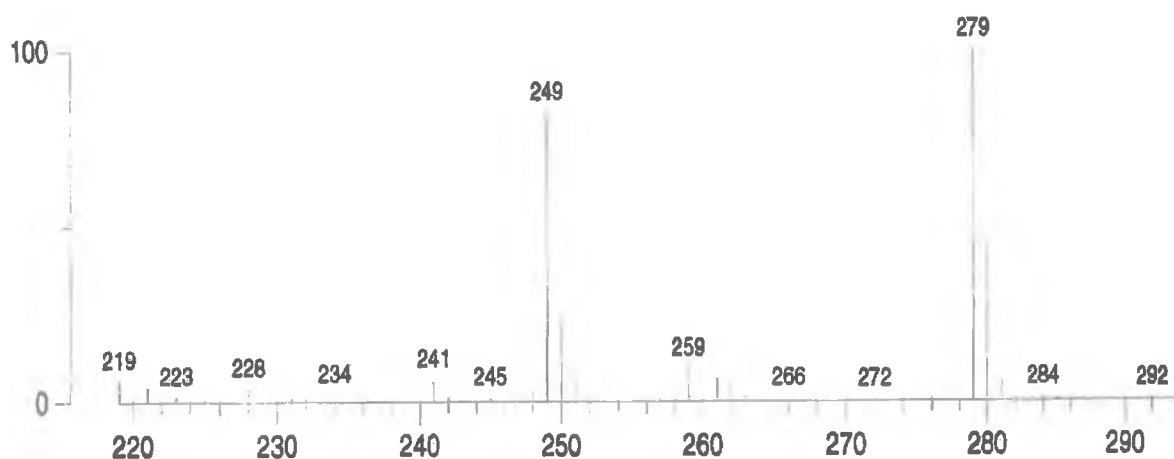
Figure 6.6. *MS analysis of amino pirimiphos molecule. The peak shown at 249 indicates the production of the amino-pirimiphos molecule. The peak at 279 indicates the presence of a higher molecular weight intermediate in the production of amino-pirimiphos.*

6.2.1.3. *Diazonium coupling of amino-pirimiphos to BSA*

Diazonium coupling of amino-pirimiphos to BSA was carried out as described in section 2.13.1, and the reaction scheme is shown in Figure 6.7. Briefly, amino-pirimiphos was activated in an acidified sodium nitrite solution to yield highly reactive diazonium ions. The activated diazonium ions then reacted with free amino groups on the protein to form a diazonium-coupled pesticide-protein conjugate. Conjugates containing diazonium bonds yield highly coloured complexes, and this is generally a good indication of whether the reaction was successful or not. In the case of amino-pirimiphos no change in the colour of the reaction mixture occurred. Figure 6.8 shows spectrophotometric analysis of BSA, diazo-coupled amino-pirimiphos and pirimiphos. The scan of the diazo-coupled amino-pirimiphos shows only one shoulder at 280 nm similar to that of the BSA scan. This indicated the reaction was definitely unsuccessful, as a second shoulder would have been observed at approximately 310 nm for pirimiphos.
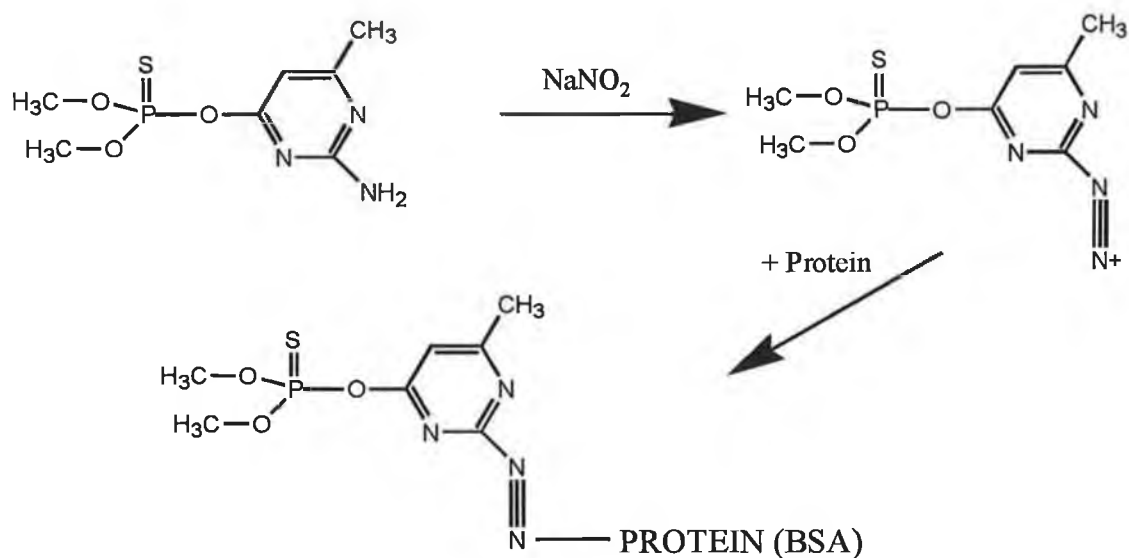
Figure 6.7. Reaction scheme for the production of diazo coupled amino-pirimiphos-BSA conjugate as described in section 2.13.2.

(A)

(B)

(C)
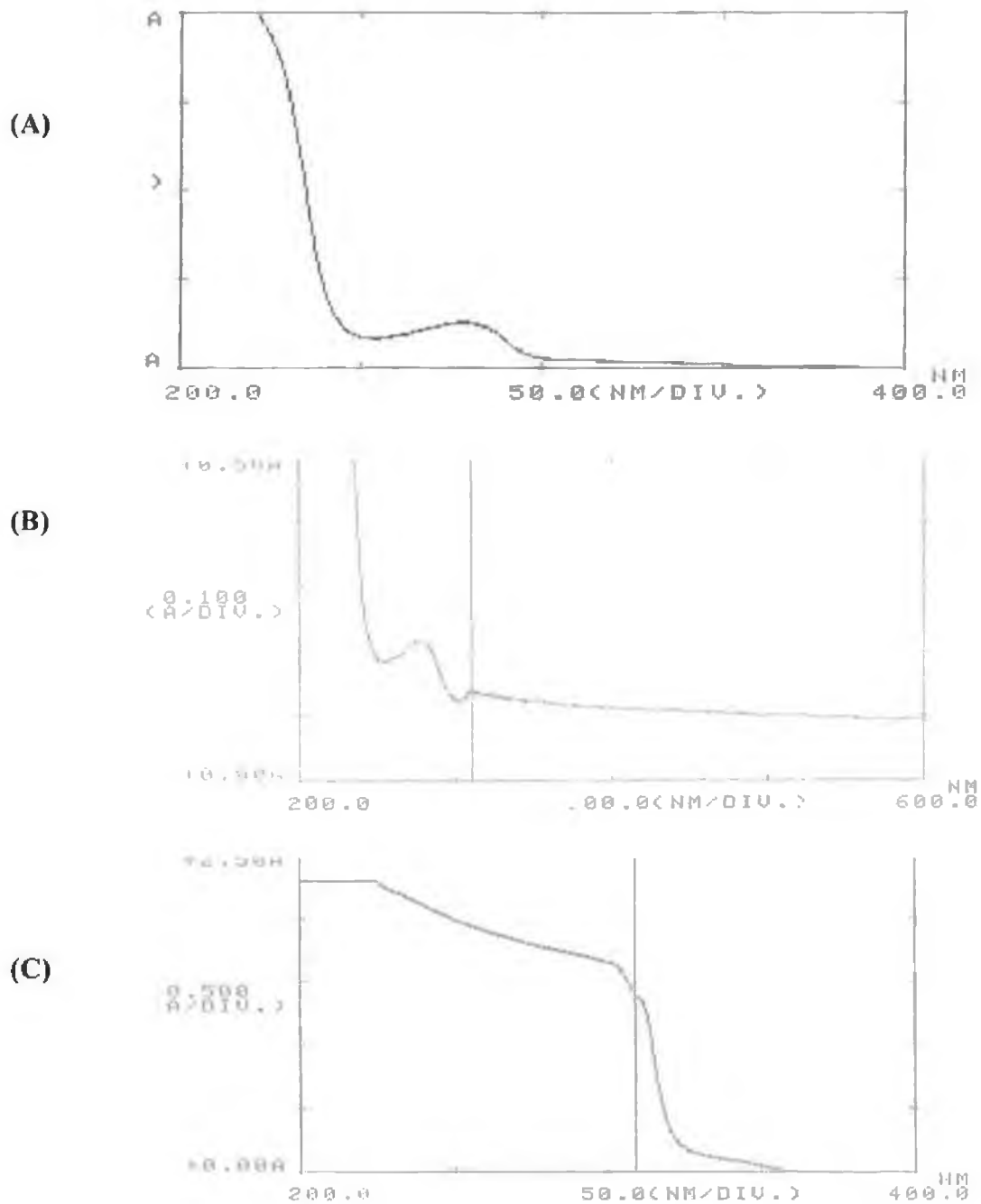
Figure 6.8. Spectrophotometric scan of (A) BSA, (B) Diazo coupled amino pirimiphos-BSA, (C) Pirimiphos. Comparison of amino-pirimiphos-BSA with BSA shows similar scans. The line on scan (B) is at 310 nm i.e. the optical density of pirimiphos (C). This indicates the conjugation was unsuccessful, as a distinctly larger shoulder should be present on the conjugated scan beside the protein peak at 280 nm, which would indicate linkage of amino-pirimiphos to BSA.

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6.2.2. *Development of an enzyme-linked immunosorbent assay for chlorpyrifos*

Polyclonal anti-chlorpyrifos anti-sera and a chlorpyrifos-dextran conjugate were obtained from collaborators at the Institute of Food Research, Norwich, U.K. The anti-sera was purified and used for the development of a competitive ELISA for the detection of chlorpyrifos. An investigation of the development of an inhibition-based BIAcore assay for the detection of chlorpyrifos, using this antibody population was also undertaken.

6.2.2.1. *Purification and characterisation of polyclonal anti-chlorpyrifos antibodies from serum*

The serum was initially purified by saturated ammonium sulphate precipitation as described in section 2.5.1. It was further purified by protein G affinity-chromatography as described in section 2.5.2. IgG bound to the protein G column and was eluted after washing, by addition of glycine buffer. The presence of antibody in the collected fractions was monitored by determining absorbance at 280 nm. A typical elution profile for the serum is shown in Figure 6.9. Fractions 2-13 contained protein with absorbances above 0.37 nm, and these were deemed acceptable for collection. The samples were then pooled and dialysed overnight in order to remove any remaining ammonium sulphate, as well as adjust the pH to 7.0.

The purity of the polyclonal antibody preparation was assessed by SDS-PAGE as described in section 2.7. The antibody preparation was electrophoresed under reducing conditions, with appropriate molecular weight markers. The presence of two single bands representing the heavy and light chains on the gel were used to assess the purity of the antibody sample. Figure 6.10 shows the gel. One band is observed below the 48.5 kDa markers, indicating the presence of the heavy chain. However, the light chain is not visible on the sample, as it would be expected to be visible just below the 26.6 kDa marker. The figure does demonstrate, however, purification steps have worked effectively.
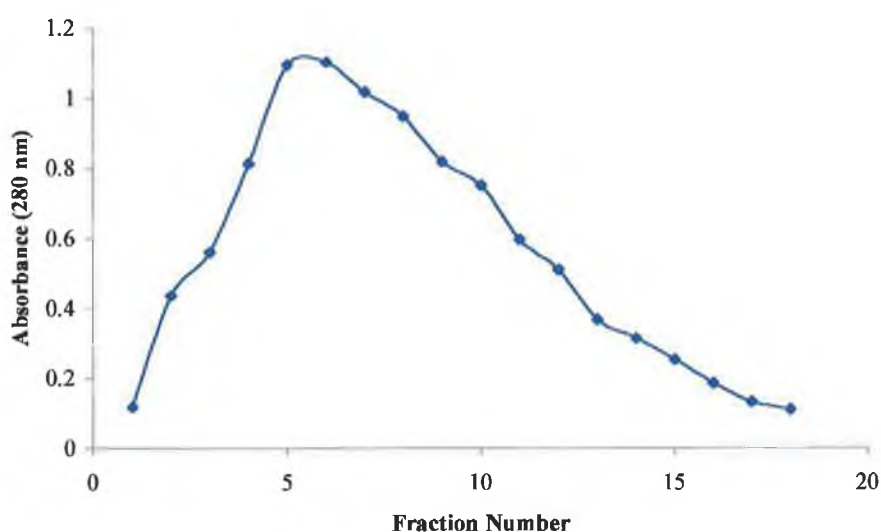
Figure 6.9. *Typical elution profile for polyclonal anti-chlorpyrifos antibody preparation from a 1 ml protein G affinity column. Saturated ammonium sulphate precipitated sample was passed through the column, followed by 20 column volumes of wash buffer. Bound antibody was then eluted using 0.1 M glycine, pH 2.7. 0.5 ml fractions were collected, and the protein content was measured by absorbance at 280 nm. Fractions 2-13, inclusive, were pooled as they contained the greatest proportion of anti-chlorpyrifos antibodies.*
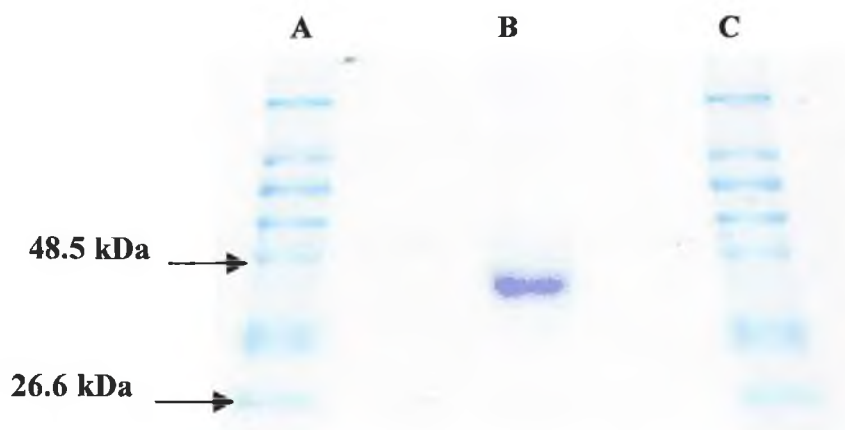
Figure 6.10. *SDS-PAGE showing (B) affinity-purified polyclonal anti-chlorpyrifos antibodies. The sample was electrophoresed under reducing conditions. Lanes A and C contained markers consisting of α_2 macroglobulin (180 kDa), β galactosidase (116 kDa), fructose-6-phosphate kinase (84 kDa), pyruvate kinase (58 kDa), fumarase (48.5 kDa), lactic dehydrogenase (36.5 kDa), and triosephosphate isomerase (26.6 kDa). The purified sample in lane B has one band below the 48.5 kDa markers, consistent with the presence of the heavy chain. The light chain at 25 kDa, however, is not visible on the gel.*

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6.2.2.2. *Determination of working dilution of antibodies in a competitive ELISA for chlorpyrifos*

A checkerboard ELISA was carried out as described in section 2.11.2 in order to determine the optimal antibody dilution and conjugate concentration for use in competitive ELISA. Rows of wells of a 96-well microtitre plate were coated with concentrations of chlorpyrifos-dextran ranging from 0 to 100 μg/ml, and blocked with 2 % (w/v) milk powder. Dilutions of the purified polyclonal anti-chlorpyrifos antibodies from 1/100 to 1/100,000 were prepared and added to the plate. After incubation, enzyme-labelled anti-rabbit antibody was added followed by chromogenic substrate. Figure 6.11 shows the overlaid titre curves obtained from the anti-chlorpyrifos antibodies. The antibody dilution that gave half the maximum absorbance was chosen, and the lowest conjugate concentration that provided sufficiently high absorbances was chosen. Therefore, 50 μg/ml of chlorpyrifos-dextran conjugate, and 1/600 dilution of antibody were chosen as the parameters for use in the competitive ELISA.

6.2.2.3. *Methanol concentration optimisation*

Chlorpyrifos is a very hydrophobic molecule. Therefore, it was essential to incorporate methanol into the free pesticide standards. The limit of detection of the antibody for chlorpyrifos can depend on the maximum amount of methanol the antibody and assay will tolerate. For this reason a competitive assay for chlorpyrifos was optimised using different methanol concentrations of 5, 10, 20, and 30 % (v/v). A competitive assay was carried out using chlorpyrifos standards ranging from 20 to 25,000 ng/ml with the four different methanol concentrations. Three of the concentrations-10, 20, and 30 % (v/v) methanol appeared to reduce the inhibitory effects of the free chlorpyrifos in the assay (Figure 6.12), while 5 % (v/v) of methanol did not seem to have as much of an effect. Therefore, 5% methanol was used for subsequent work.
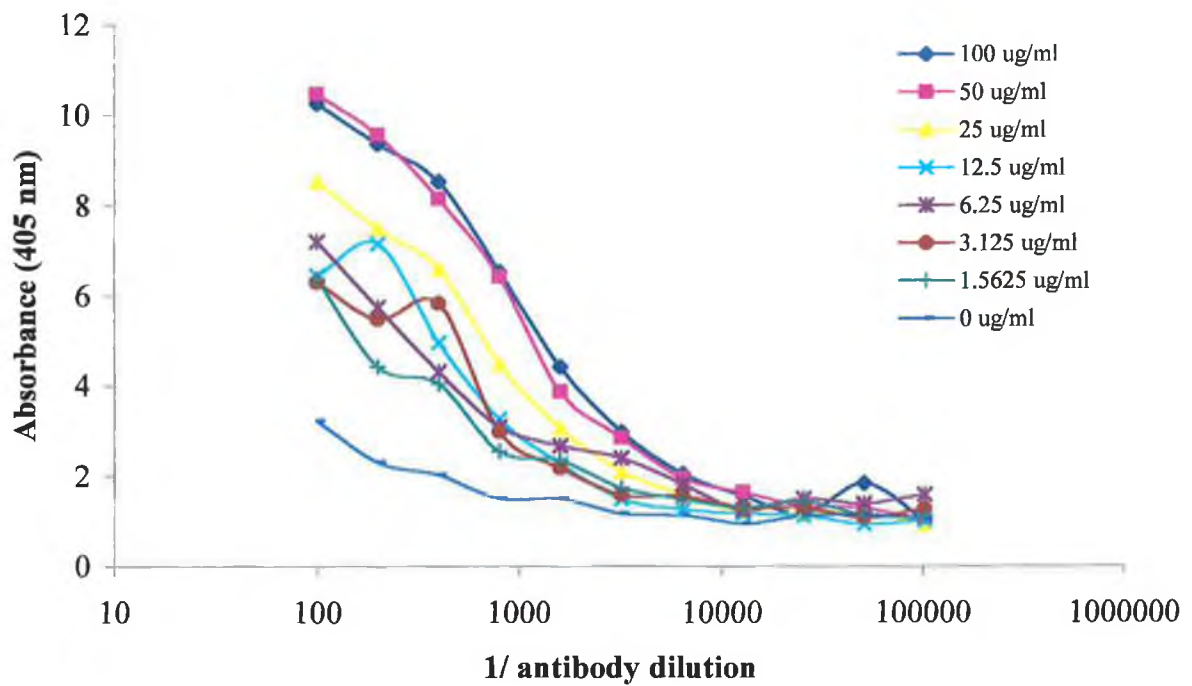
Figure 6.11. Determination of optimal conjugate loading density and antibody dilution. Wells of a microtitre plate were coated with chlorpyrifos-dextran conjugate at a range of concentrations between 0 and 100 μg/ml. Serial doubling dilutions of the affinity-purified anti-chlorpyrifos antibody were carried out and then added to the plate as described in section 2.11.3. The optimal conjugate concentration chosen was 50 μg/ml, and the optimal antibody dilution (i.e. the one that gave half the maximum absorbance) was 1/600.
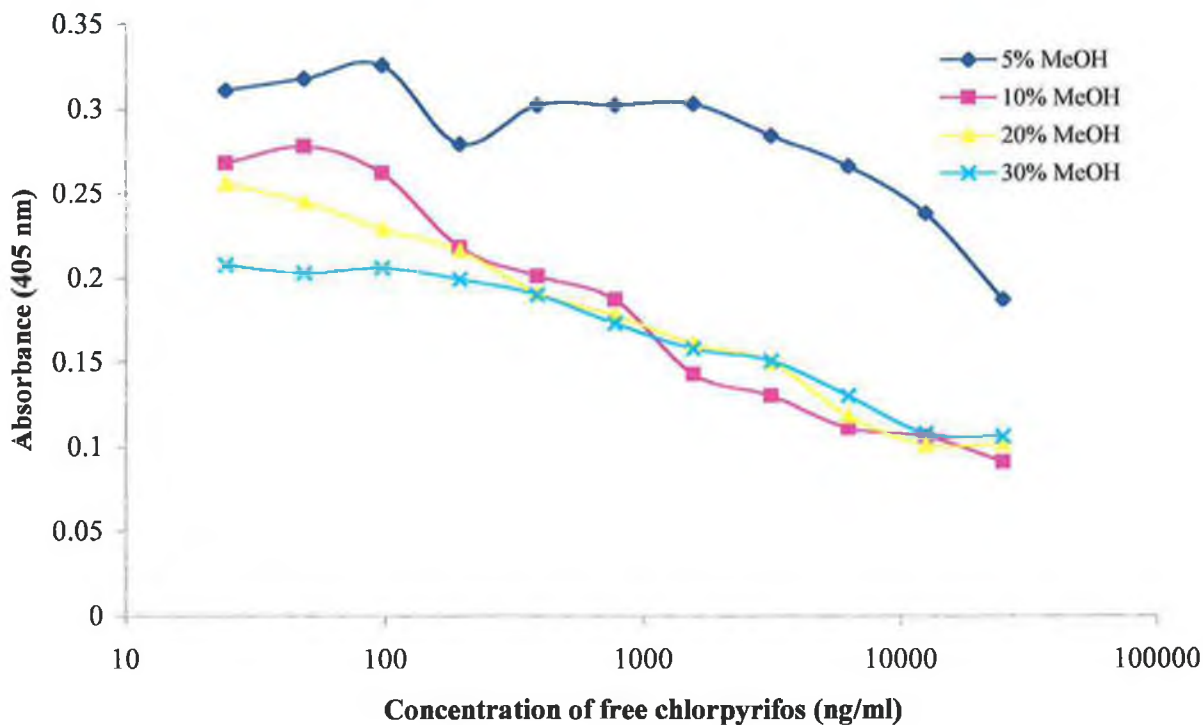
Figure 6.12. Competition ELISA for chlorpyrifos to determine the optimum methanol concentration for use in ELISA. Chlorpyrifos standards ranging from 20 to 25,000 ng/ml were prepared in methanol concentrations of 5, 10, 20 and 30 % (v/v) in PBS. 5 % (v/v) methanol appeared to have the least effect on the assay, although it was not suitably sufficient to maintain pesticide solubility above 1000 ng/ml. This can be seen from the graph, where the absorbances drop after this concentration.

6.2.2.4. *Competitive ELISA for the detection of chlorpyrifos*

A series of standards of chlorpyrifos ranging in concentration from 49-25,000 ng/ml were prepared as described in section 2.10.2. 50 μl of each of these standards were then added to a chlorpyrifos-dextran-coated plate (section 2.11.3). 50 μl/well of the affinity-purified anti-chlorpyrifos antibodies, were prepared at a 1/300 dilution (final dilution of 1/600) and added to the plate. After incubation and washing, enzyme-labelled anti-rabbit antibody was added followed by chromogenic substrate. The linear range of detection for the antibody population was 49 to 25,000 ng/ml. For intra-day assay variation, each concentration was assayed five times on the one day and the mean absorbance of bound antibody for each antigen concentration was plotted versus antigen concentration. Figure 6.13 shows the intra-day assay linear range of detection for chlorpyrifos. Table 6.1 shows the C.V.'s for the assay. Intra-day percentage C.V.'s for the calibration plot of the assay were all below 7.3 %.

The inter-day assay variation was calculated by performing the assay over five separate days, and a separate calibration curve plotted for each normalised absorbance (absorbance/zero absorbance (A/Ao)) value versus the respective chlorpyrifos concentration for each assay carried out on each day. The normalised mean values for the assay were calculated and plotted as one assay. Figure 6.14 shows the inter-day variability assay for the affinity-purified anti-chlorpyrifos antibody preparation. The percentage C.V.'s for the assay are shown in Table 6.2, and are all less than or equal to 10 %.

Table 6.1. *Intra-day assay coefficients of variation for the determination of free chlorpyrifos using affinity-purified anti-chlorpyrifos antibody. Five sets of ten standards were assayed the same day, and the C.V.'s were calculated as the standard deviation (S.D.) expressed as a percentage of the mean values for each standard.*

Chlorpyrifos concentration (ng/ml)	Calculated mean ± S.D.	Coefficient of variation (%)
25,000	0.200 ± 0.014	7.26
12,500	0.218 ± 0.007	3.21
6,250	0.266 ±0.007	2.87
3,125	0.332 ± 0.020	6.15
1,563	0.403 ± 0.015	3.76
781	0.488 ± 0.031	6.44
391	0.559 ± 0.027	4.92
195	0.651 ± 0.014	2.26
98	0.674 ± 0.001	0.23
49	0.742 ± 0.035	4.75
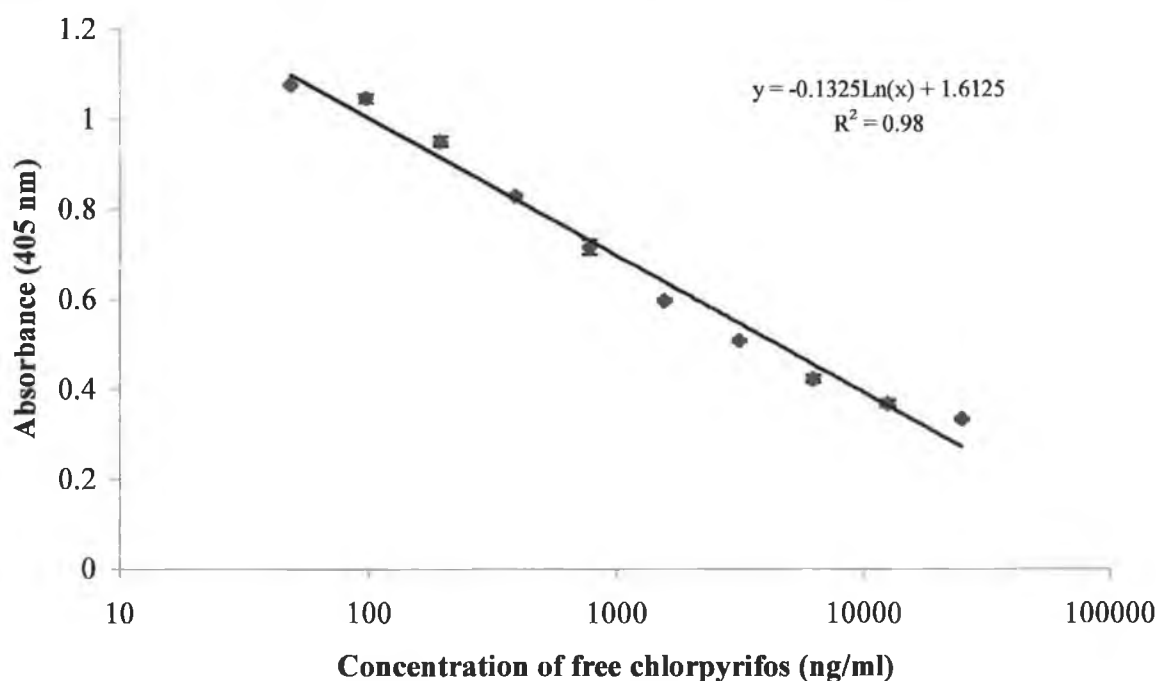
Figure 6.13. *Intra-day assay variation for competitive ELISA for chlorpyrifos using affinity-purified anti-chlorpyrifos antibodies. The linear range of detection was 49.0 to 25,000 ng/ml. The error bars on each calibration point indicate the standard deviation of the mean of five measurements.*

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Table 6.2. *Inter-day assay coefficients of variation for the determination of free chlorpyrifos using affinity-purified anti-chlorpyrifos antibody. Five sets of twelve standards were assayed on five different days, and the C.V.'s were calculated as the standard deviation expressed as a percentage of the mean normalised values for each standard.*

Chlorpyrifos concentration (ng/ml)	Calculated mean ± S.D.	Coefficient of variation (%)
25,000	0.316 ± 0.010	3.42
12,500	0.361 ± 0.036	10.07
6,250	0.400 ± 0.021	5.35
3,125	0.482 ± 0.024	5.10
1,563	0.594 ± 0.028	4.80
781	0.702 ± 0.037	5.38
391	0.814 ± 0.021	2.63
195	0.927 ± 0.035	3.79
98	0.981 ± 0.038	3.94
49	1.079 ± 0.027	2.57

$y = -0.1137Ln(x) + 1.3765$
$R^2 = 0.99$
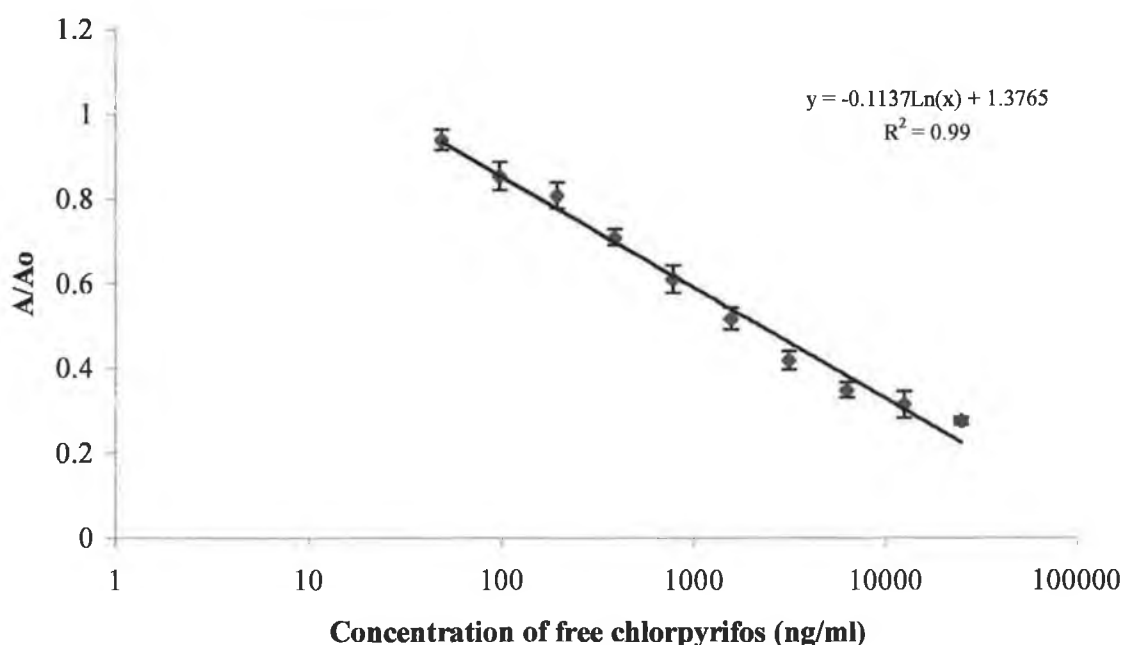
Figure 6.14. *Inter-day assay variation for competitive ELISA for chlorpyrifos using affinity-purified anti-chlorpyrifos antibodies. The linear range of detection was 49.0 to 25,000 ng/ml. The error bars on each calibration point indicate the standard deviation of the mean of five measurements.*

6.2.2.5. *Cross reactivity studies*

Cross reactivity studies were carried out on the antibody population to six other OP's, as described in section 2.11.5. Standard curves for each of the pesticides were produced as described previously in 6.2.2.6. The results were normalised and plotted. The slope of the line of the standard curve for each OP was expressed as a percentage of the slope of the line for binding to chlorpyrifos. Table 6.3 shows the cross reactivity of the affinity-purified anti-chlorpyrifos antibody population. The antibody was very cross-reactive to parathion and fenitrothion, and less reactive to mevinphos. However, it showed negligible recognition for the OP's, carbophenethion, and etrimfos.

Table 6.3. *Cross-reactivity of affinity-purified anti-chlorpyrifos antibody observed to six other organophosphates. Standard curves were produced for each of the organophosphates in the same way as that for chlorpyrifos. Each concentration for each point on the standard curve was assayed in triplicate and the means plotted and normalised in order to construct standard curves.*

Pesticide	% Cross reactivity
Chlorpyrifos-methyl	100
Parathion	50
Fenitrothion	50
Carbophenethion	0.5
Mevinphos	25
Etrimfos	1
Dichlorvos	5

6.2.3. *Production and characterisation of chlorpyrifos-BSA conjugates*

The structure of chlorpyrifos was modified in order to place a spacer arm on it for conjugation to proteins such as BSA. Figure 6.2 shows the reaction scheme for the synthesis of this modified chlorpyrifos hapten. Two chlorpyrifos-BSA conjugates were produced, one with a 25 molar (M) excess of pesticide per molecule of BSA and one with a 50 M excess of pesticide per molecule of BSA. Figure 6.15 shows the spectrophotometric scans of both of the pesticide-protein conjugates. Conjugation was successful in both cases, with shoulders at 280 nm (representing the protein-BSA) and at 310 nm representing chlorpyrifos. The presence of both of these shoulders indicated the conjugation had worked. The conjugate concentrations, and the substitution ratios of pesticide molecules: protein molecules was calculated spectrophotometrically, and these are shown in Table 6.4. The 50 M excess conjugate was calculated to have 27 pesticide molecules per BSA molecule, while the 25 M excess conjugate had 6 pesticide molecules per BSA molecule.

Both conjugates were also analysed by competitive ELISA, using a conjugate coating concentration of 50 µg/ml, and a 1/600 dilution of the affinity-purified anti-chlorpyrifos antibody. The free chlorpyrifos standards ranged from 49 to 25,000 ng/ml. Figure 6.16 shows a graph of the competition ELISA's using both conjugates. No competition was observed in the assay using the 25 M excess conjugate. However, competition was observed in the assay using the 50 M excess of conjugate.
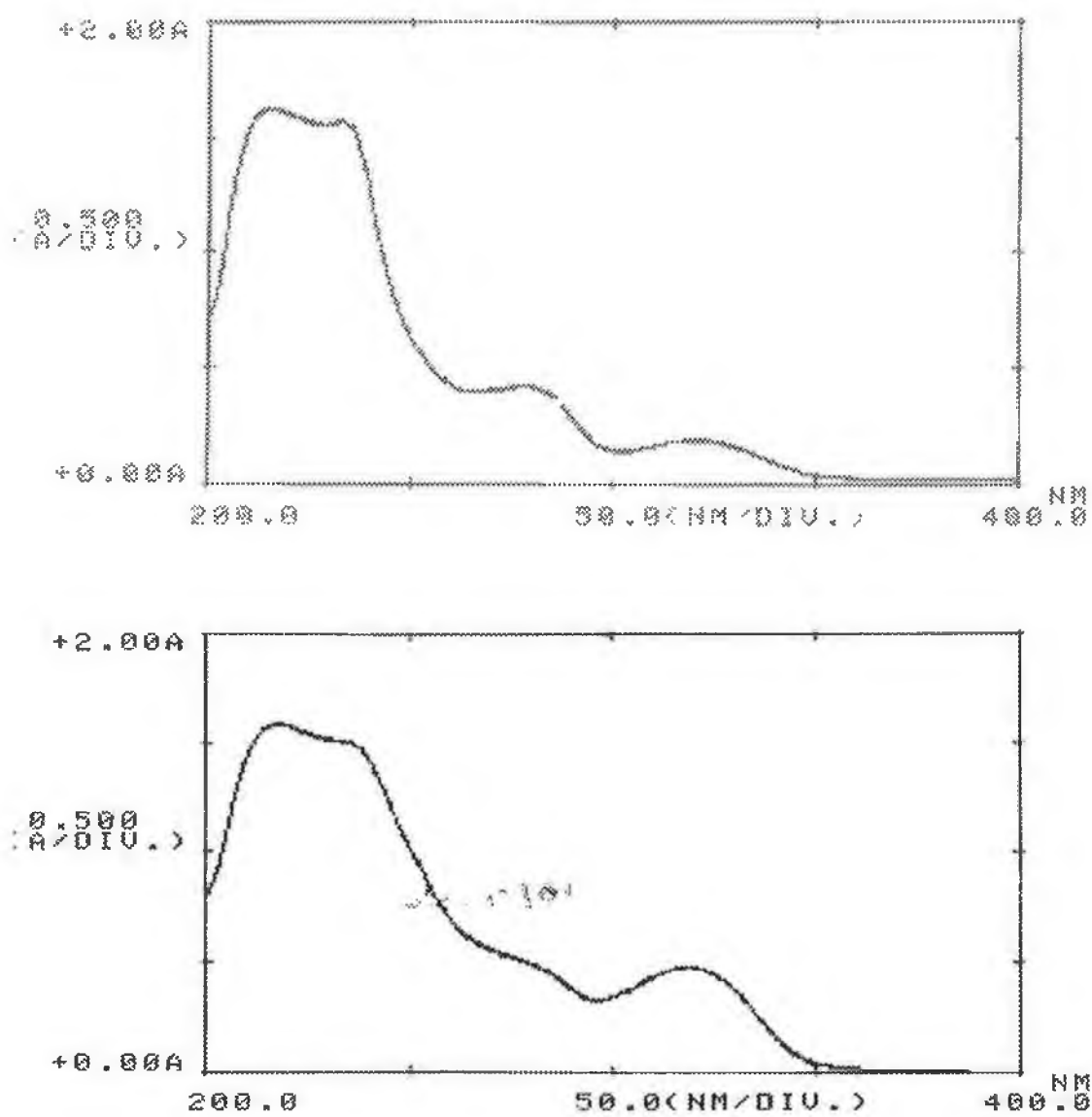
Figure 6.15. UV spectra of (A) chlorpyrifos-BSA conjugate containing a 50 M excess of pesticide, (B) chlorpyrifos-BSA conjugate containing a 25 M excess of pesticide. The absorbance at 320nm was read and used to calculate the chlorpyrifos concentration of the conjugates.

Table 6.4. *Characterisation of two chlorpyrifos-BSA conjugates, with 50 and 25 molar excess of pesticide to BSA. This table shows the conjugate concentration and the coupling ratio of pesticide-protein. These results were calculated in duplicate.*

Conjugate	Conjugate concentration mg/ml	Degree of conjugation BSA: Chlorpyrifos
Chlorpyrifos-BSA 25 M excess	7.4	1: 6
Chlorpyrifos-BSA 50 M excess	2.13	1: 27
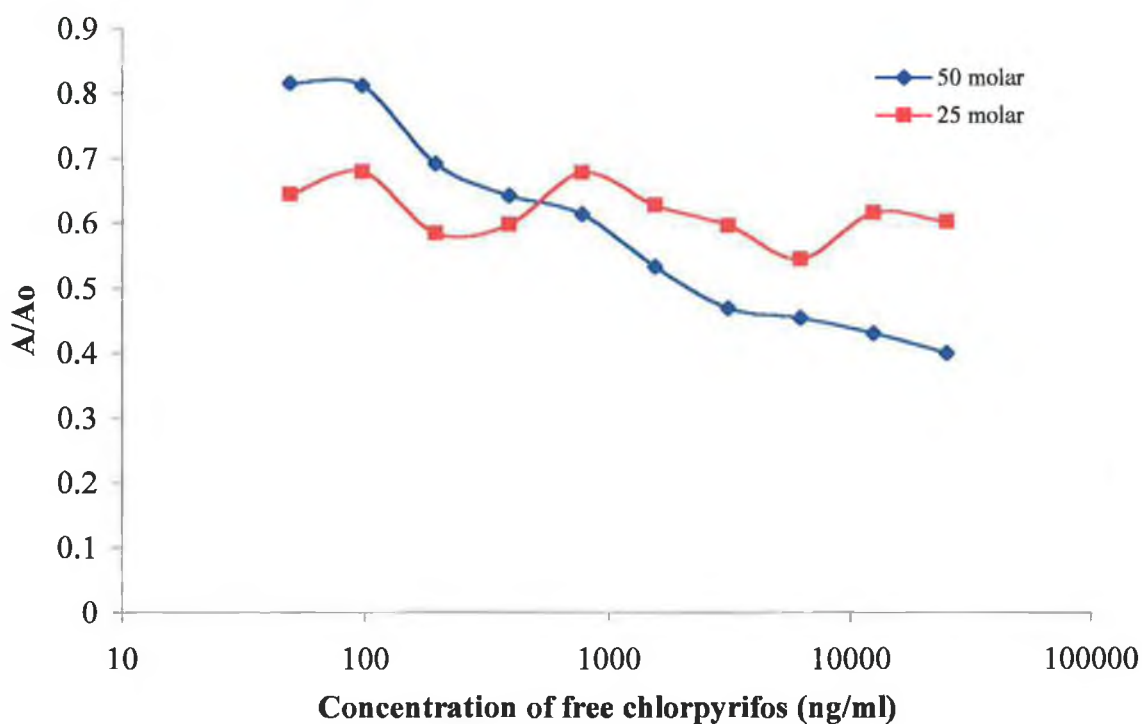
Figure 6.16. *Competitive ELISA's carried out using the two chlorpyrifos-BSA conjugates. No competition is observed with the 25 molar excess conjugate. However, competition was observed with the 50 molar excess conjugate.*

6.2.4. *Development of an inhibition BIAcore-based immunoassay for the detection of chlorpyrifos*

6.2.4.1. *Preconcentration studies of chlorpyrifos-BSA conjugates*

10 mM acetate buffer was prepared at a range of incremental pH's from 3.9 to 4.5. A solution of 50 μg/ml of the 25 and 50 M excess chlorpyrifos-BSA conjugates was prepared in acetate buffer at each pH. Each conjugate concentration was passed sequentially over an underivatised chip surface at 5 μl/min for 2 minutes as described in section 2.20.1. The differing degree of preconcentration was measured by the response prior to the end of the injection for each conjugate solution at the various pH values. Following injection, the flow of the running buffer over the chip surface was sufficient to dissociate the electrostatic attraction between the protein and the CM-dextran gel surface. Table 6.5 shows the degree of preconcentration in response units for each pesticide-protein conjugate. The studies showed the optimal pH for immobilisation of 25 M excess chlorpyrifos-BSA conjugate was 4.5, and the optimal pH for immobilisation of 50 M excess chlorpyrifos-BSA conjugate was 3.9 (both in 10 mM acetate buffer). These pH's were thus used for all subsequent immobilisations.

6.2.4.2. *Immobilisation of AFB_1-BSA conjugates*

EDC/NHS coupling chemistry was used for activation of the CM-dextran gel surface for the immobilisation of chlorpyrifos-BSA conjugates, as described in section 2.20.2. Table 6.6 shows the number of response units of each respective conjugate immobilised after capping with 1M-ethanolamine hydrochloride, pH 8.5. For the 25 M excess conjugate, 5,283.8 RU of conjugate were immobilised, while for the 50 M excess conjugate 9,826.9 RU were immobilised.

Table 6.5. *Pre-concentration conditions for both conjugates. The highlighted response represents the optimum pre-concentration and coupling pH for the corresponding conjugate. For subsequent immobilisations the conjugate was immobilised onto the sensor surface in acetate buffer using the corresponding pH.*

pH	25 molar excess (RU)	50 molar excess (RU)
4.5	3728.9	1249.8
4.4	2465.4	514.1
4.3	2619.7	1007.2
4.2	1680.2	750.1
4.1	2988.0	983.9
4.0	2714.9	1317.4
3.9	801.3	2673.7
3.8	326.8	2343.4

Table 6.6. *Table showing the number of RU immobilised for each conjugate. Both conjugates were immobilised onto individual flow cells on the sensor chip using conventional carbodiimide coupling chemistry as described in section 2.20.2.*

Conjugate	Immobilised conjugate Response Units (RU)
25 molar excess	5283.8
50 molar excess	9826.9

6.2.4.3. *Regeneration conditions*

Both chlorpyrifos-BSA conjugates were assessed for binding of the affinity-purified anti-chlorpyrifos antibodies on BIAcore. Figure 6.17 shows a diagram of the overlaid binding curves that were obtained after injecting the antibody (1/2 dilution) over both conjugate surfaces for 15 minutes at 5 µl/min. Binding to the 25 M excess conjugate resulted in 376.4 RU of antibody bound, while binding to the 50 M excess conjugate resulted in 183.6 RU binding. It was therefore decided to further use the 25 M excess conjugate for the optimisation of regeneration conditions, and development of an SPR inhibition-based immunoassay for chlorpyrifos.

Regeneration studies, using a 1/2 dilution in HBS of affinity-purified anti-chlorpyrifos antibody showed that it was easily removed from the chlorpyrifos-BSA (25 M excess) immobilised surface using a 1-minute pulse of 30 mM HCl. This completely removed all bound antibody after each binding-regeneration pulse. Figure 6.18 shows a graph of the reproducibility of regeneration for the antibody over forty regeneration cycles. Binding of the polyclonal antibody to the conjugate surface was highly reproducible, with approximately 150 RU of antibody binding to the surface each time. The regeneration solution (30 mM HCl) did not affect the binding throughout the regeneration study.

6.2.4.4. *Assessment of non-specific binding*

The degree of non-specific binding of the polyclonal antibody to both the dextran layer, and the immobilised BSA portion of the pesticide-protein conjugate, was assessed. This was carried out, by passing a 1/2 dilution of the polyclonal antibody over a blank CM-dextran surface, as well as an immobilised BSA surface as described in section 2.20.3. Figure 6.19 shows overlaid sensorgrams of the injection of polyclonal anti-chlorpyrifos antibody over a BSA immobilised surface, and a CM-dextran layer. There was negligible non-specific binding of the antibody to the BSA protein or the CM-dextran surface (< 5RU). The antibody solution did not have to be pre-incubated with BSA, as the antibodies were produced using a chlorpyrifos-KLH conjugate. As a result the antibody solution did not have to be pre-incubated with either BSA or CM-dextran.
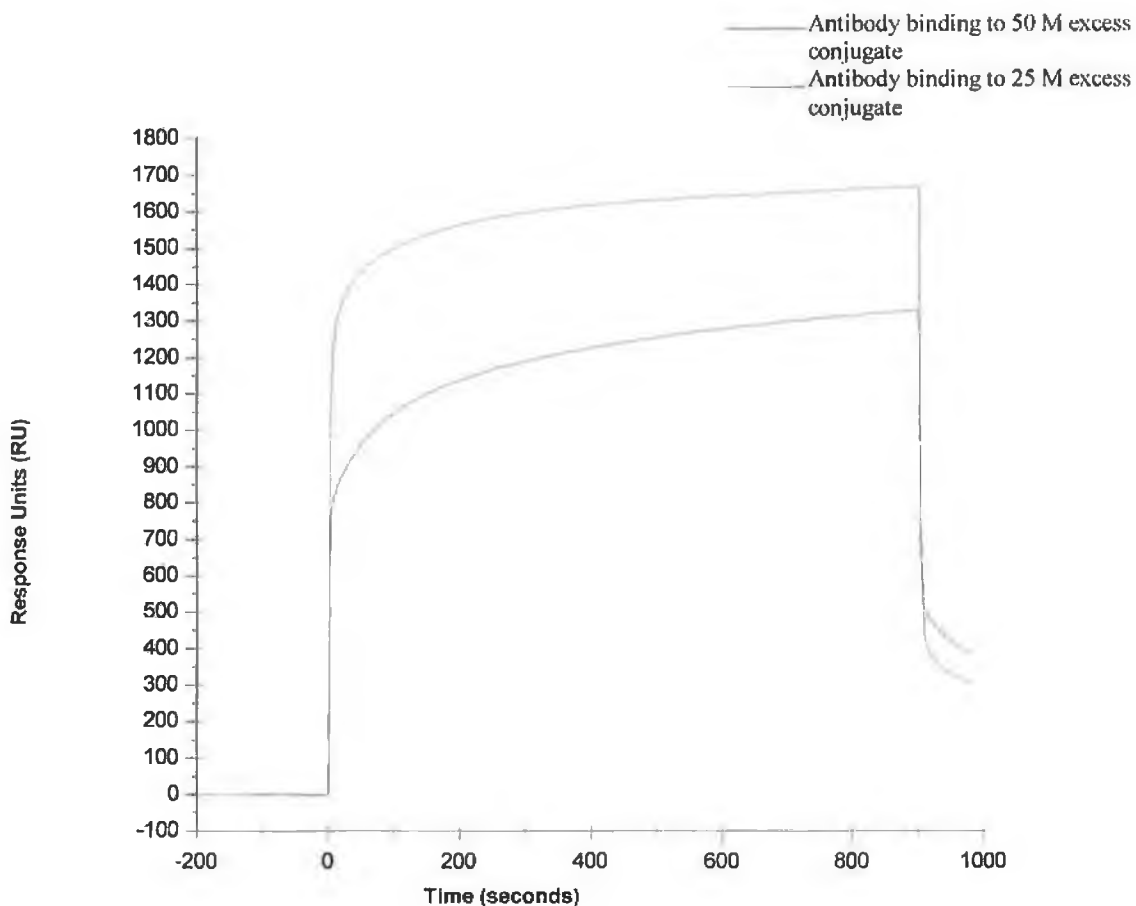
Figure 6.17. *Overlaid binding curves for affinity-purified anti-chlorpyrifos antibody (1/2 dilution) binding to a chlorpyrifos-BSA conjugate with a 25 M excess of pesticide, and a 50 M excess of pesticide. Binding to the 25 M excess conjugate resulted in 376.4 RU of antibody bound, while binding to the 50 M excess conjugate resulted in 183.6 RU binding. Both injections were carried out at a flowrate of 5 μl/min for 15 min. The differences in SPR signal are due to changes in bulk refractive index of the antibody solution.*
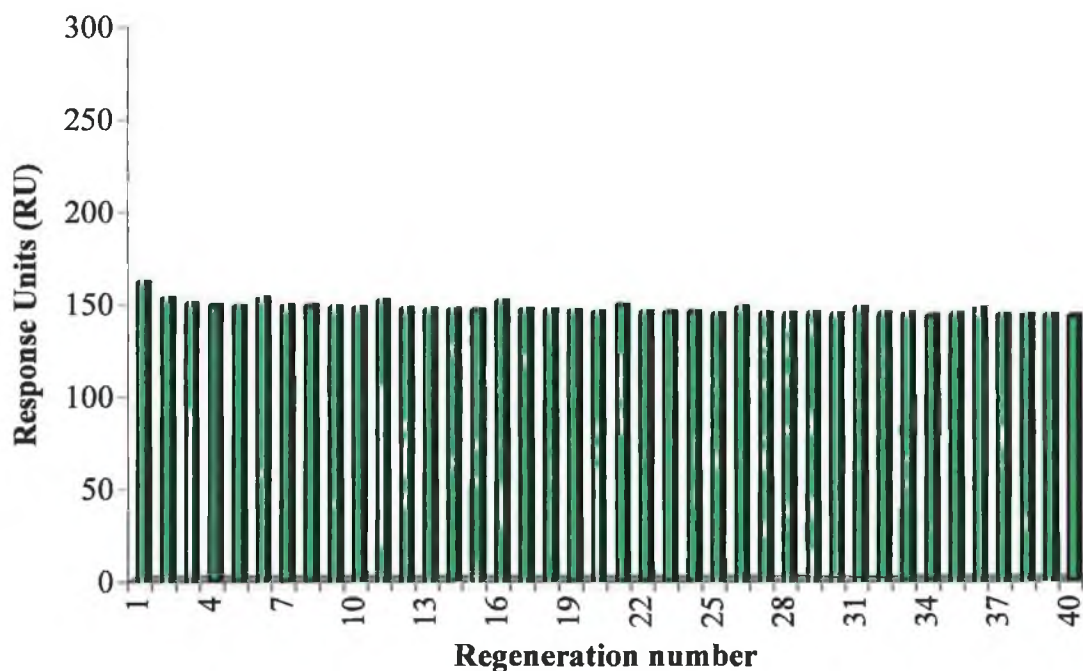
Figure 6.18. *Graph showing the reproducibility of regeneration of a sensor chip immobilised with chlorpyrifos-BSA (25 M excess) on the surface. Forty consecutive regeneration cycles of a 4-minute binding pulse of polyclonal anti-chlorpyrifos antibody were carried out. This was followed by a 1-minute injection of 30 mM HCl as the regeneration solution. This completely removed all of the bound antibody after each binding cycle, which resulted in highly reproducible binding cycles, as no significant decrease in the measured binding response over the course of the regeneration study was observed.*
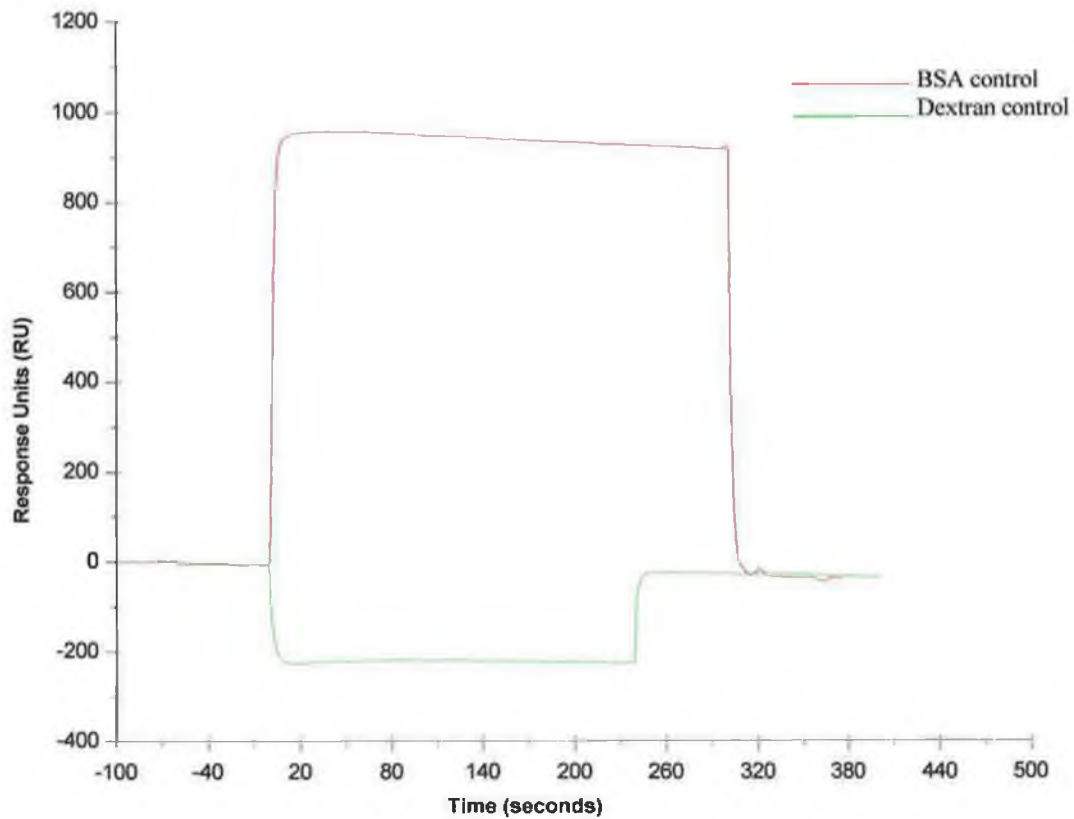
Figure 6.19. *Overlaid sensorgrams showing binding of a 1/2 dilution of affinity-purified anti-chlorpyrifos antibody to a BSA surface, and a CM-dextran gel layer, at 5 μl/min for 4 minutes (for the dextran control) and 5 μl.min for the BSA control. A negligible amount of binding of the antibody was observed to the BSA and dextran surfaces (< 5 RU). The differences in SPR signal are due to changes in bulk refractive index of the antibody solution.*

6.2.4.5. *Determination of working range of inhibition assay in PBS*

For determination of the working range of the inhibition immunoassay for the affinity-purified anti-chlorpyrifos antibodies, dilutions of chlorpyrifos were firstly prepared as described in section 2.10.2. Dilutions of chlorpyrifos were prepared in PBS-5 % (v/v) methanol ranging from 49 to 25,000 ng/ml. The antibodies were manually pre-mixed with the respective concentrations of chlorpyrifos and allowed to incubate for 1 hour at 37° C, before being injected over the conjugate-immobilised surface. This was carried out as it was a more economical method of using the antibodies. The samples were passed over the immobilised chlorpyrifos-BSA surface in random order, and the surface was regenerated using the 30 mM HCl. The response was measured following the binding of unliganded antibody from the equilibrated antibody: antigen mixtures. A standard curve was constructed by plotting concentration of free chlorpyrifos versus amount of unliganded antibody bound in response units (RU). Figure 6.20 shows the curves obtained from the inhibition assays using both chlorpyrifos-BSA conjugate i.e. the 25 and 50 M excess conjugates. It is apparent from these curves that no inhibition was observed in either case. It was not possible to pursue this work any further due to the limited supply of polyclonal anti-serum available from our collaborators.
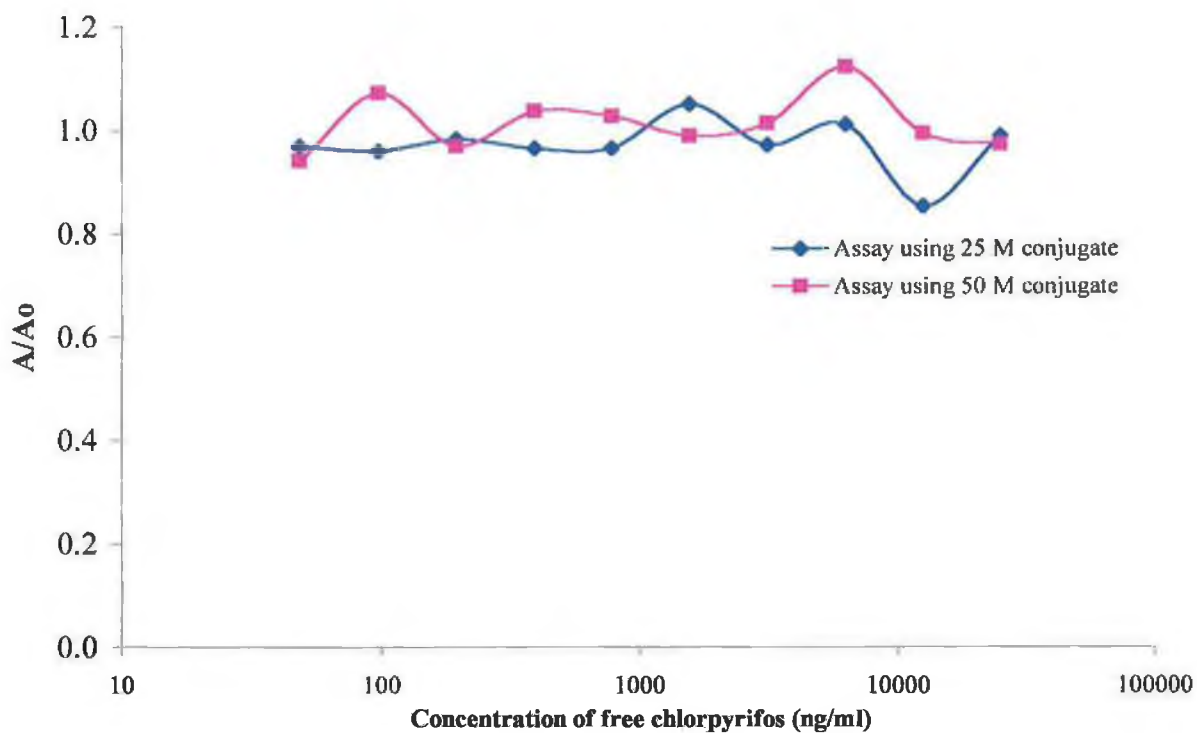
Figure 6.20. *Inhibition BIAcore-based assays for the detection of chlorpyrifos using affinity-purified anti-chlorpyrifos antibodies and chlorpyrifos-BSA conjugates with 25 and 50 M excesses of pesticide. No range of inhibition was observed for the antibody using either chlorpyrifos-BSA conjugate. The assays were repeated to ensure the result was correct.*

6.3. Discussion

It was intended to produce both polyclonal and scFv antibodies to the OP pirimiphos. Therefore, it was necessary to conjugate the pesticide to a carrier molecule such as BSA in order to elicit an immune response in an animal. Pirimiphos contains no suitable functional groups for conjugation to a protein or carrier molecule. As a result, a synthetic strategy for the production of a pirimiphos hapten containing a suitable functional group for conjugation was developed. An optimised method for the production of such a hapten was already developed (M^C Adam & Skerritt, 1993). It involved the synthesis of a bifunctional reagent that couples through the thiophosphate group of pirimiphos. This compound contained the characteristic phosphorous and sulphur atoms of organophosphates. However, the method for synthesising this compound was extremely time-consuming, and required the full-time commitment and expertise of an organic chemist to carry out the work. Therefore an alternative, less time-consuming procedure needed to be developed. This was done with the help of two organic chemists (section 2.13.1). Therefore, the compound amino-pirimiphos was produced, by reacting the two compounds 2-amino-4-hydroxy-6-methyl-pyrimidin, and dimethyl-chlorothiophosphate together (Figure 6.3). The resulting compound- amino-pirimiphos contained an amino group (NH$_2$) in the place of the amino-diethyl group (N(CH$_2$CH$_3$)$_2$) on the two position of the pyridin ring. The reaction was successful, and C & H NMR analysis was carried out to confirm its structure (Figures 6.4-6.5). Mass spectral analysis was also performed to confirm the correct molecular weight of the compound. This provided additional evidence that the correct structure was synthesised. Figure 6.6 shows the MS of the compound, and it can be seen that two peaks of relatively equal intensity are present; one at MW = 249, and one at MW = 279. The 249 peak is that of amino-pirimiphos, while the 279 peak is a non-specific by-product of the reaction. Figure 6.21 shows the possible structure of this compound. One of the methoxy groups on the thiophosphate group may have cleaved off and reacted with the amino group to form an amino-methoxy group at that position. The intensity of the peak also indicates that this compound is equally abundant as amino-pirimiphos, if not greater.
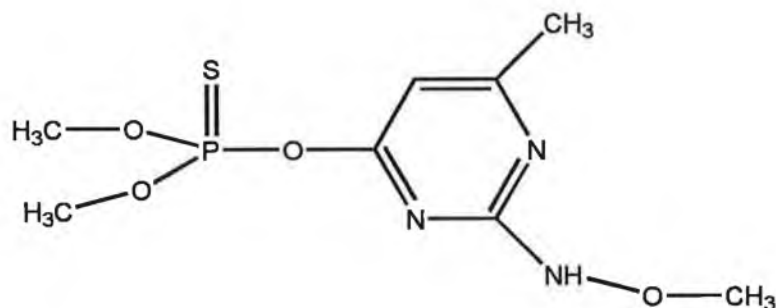
Figure 6.21. *Structure of compound that may have been a non-specific by-product of the reaction (MW= 279) for the synthesis of amino-pirimiphos.*

It was hoped, to couple the pesticide to the protein at the amino position by diazonium coupling. This reaction is outlined in Figure 6.7. The method involved the diazotization of amino-pirimiphos in acid with sodium nitrate. This approach was not successful as is evident from Figure 6.8. Initially it was thought solubility of the compound in acid was a problem, and therefore the acid concentration was increased significantly. However, the test reactions carried out revealed that harsh conditions were required to achieve successful conversion to the diazonium salt (data not shown). Addition of protein to such a solution would denature it, and might prevent its conjugation to the pesticide. One option was to isolate the diazonium salt prior to addition of the protein, and dissolve it in a less harsh solution. However, diazonium salts are highly reactive and dangerous when isolated. It is also thought that the presence of the two nitrogens on the ring structure, and the adjoining phosphorous, oxygen, and sulphur atoms had an electronegative effect on the amino group. As a result the amino group was not available for conjugation. Another reason why the diazonium-coupling reaction may not have worked was the predominance of the by – product shown in Figure 6.21. In this form the amino group was already substituted, and therefore not reactive for diazonium coupling.

Alternative approaches to activate the amino-pirimiphos compound were also carried out, such as nucleophilic attack of the amino group using electrophilic compounds

such as ethyl chloroacetate, and trifluoroacetic acid. It was thought these methods might work, as the 'electron rich' amino group would nucleophilically attack the 'electron deficient' compounds like ethyl chloroacetate, and trifluoroacetic acid, forming an amide group (Figure 6.22), and HCl as a by-product. However, attempts using both of these compounds failed to work. Once again it is thought that the presence of the two nitrogens on the ring structure, and the adjoining phosphorous, oxygen, and sulphur atoms had electronegative effect on the amino group.
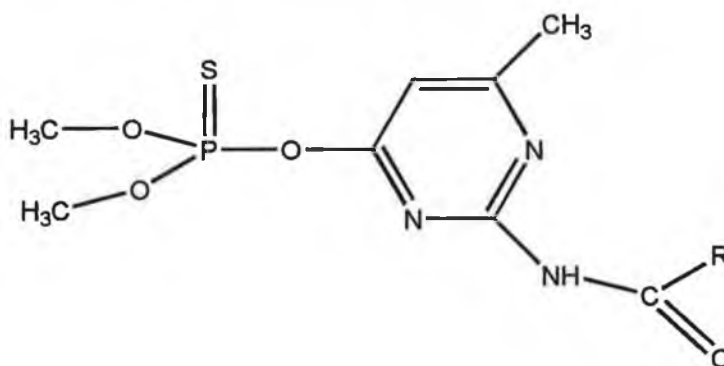
Figure 6.22. Structure of pirimiphos hapten with an amide group.

After all of these attempts to synthesise a conjugable form of the pesticide failed, it was then decided to terminate the project, given the amount of time and energy that had already been spent on it. Instead, analysis of anti-chlorpyrifos polyclonal serum was carried out. This serum was obtained from collaborators at the Institute of Food Research, Norwich, U.K., (Section 6.1.6).

Purification of the anti-chlorpyrifos serum was carried out by ammonium sulphate precipitation, and protein G affinity-chromatography. This worked effectively, with all of the bound immunoglobulin eluting easily from the protein G column. The low pH of the glycine buffer did not affect the integrity of the eluted immunoglobulins, as each fraction tube contained 2 M Tris, which neutralised the pH of the eluted antibodies. It was not necessary to carry out subtractive immunoaffinity chromatography as the rabbit was immunised with a chlorpyrifos-KLH protein conjugate. As a result, the antibody population did not recognise dextran or BSA

conjugated to chlorpyrifos, and were thus used for analysis of the antibodies. SDS-PAGE was also carried out on the antibody population in order to determine its purity. It showed a high degree of purity, as indicated by the presence of only one band on the gel. However, the electropherogram (Figure 6.10) shows that the light chain band at 25 KDa is not visible. This may be due to the high voltage denaturing the sample. The sensitivity of the staining also may not have been sufficiently sensitive to stain the light-chain band.

The conjugate loading density of chlorpyrifos-dextran was optimised for application in a competitive ELISA. It was necessary to optimise the antibody concentration for use in the assay, as too high an concentration would result in an excess of free antibody in solution, and therefore, would be only suitable for the detection of high concentrations of free antigen in solution. A checkerboard ELISA was carried out for the antibody as described in section 2.11.2. The antibody dilution used was 1/600 with a conjugate loading density of 50 µg/ml for the assay.

Due to the hydrophobicity of chlorpyrifos it was also essential to optimise the methanol concentration in the free chlorpyrifos standards. This was important in order to obtain a balance between pesticide solubility and assay tolerance of the solvent. 5% (v/v) methanol was chosen as the optimum concentration. Concentrations above 5% (v/v) inhibited the competition assay and concentrations below 5% (v/v) would have been unsuitable to prepare the free chlorpyrifos standards.

A competition ELISA was optimised for the antibody population using these parameters. The antibody was relatively sensitive with a range of detection between 49 and 25,000 ng/ml. The limit of detection for the antibody was 49 ng/ml. Inter-day assay variation studies were carried out by analysing five sets of standards across the linear range on five different days and calculating the C.V.'s as the standard deviation expressed as a percentage of the mean normalised values for each standard.

The assay was reproducible over five different days, as the C.V.'s were within the acceptance limits of less than or equal to 10 %. The assay also demonstrated good precision profiles (i.e. the quantitative measure of the variation between measurements, usually the C.V.'s versus the nominal concentration of analyte in the sample). M^C Adam & Skerritt (1993) developed an immunoassay for chlorpyrifos using a conjugate synthesised as described in section 6.1.5.2. Monoclonal anti-chlorpyrifos antibodies were produced after immunisation with the conjugate. The antibodies produced had a limit of detection of 0.2 ppb (ng/ml) in ELISA. Manclus *et al.* (1994) developed an immunoassay for chlorpyrifos using monoclonal anti-chlorpyrifos antibodies. The limit of detection for the assay was 1.3 ng/ml. Both of these assays are significantly more sensitive than the assay developed here. However, the antibodies used were monoclonal, and therefore a homogenous population. Although monoclonal antibodies are not always more sensitive than polyclonal antibodies, they often can be, due to their high avidity for one particular epitope on a hapten. Skerritt *et al.* (1992) state that individual countries commonly prescribe nil residue limits i.e. <0.1 ppb of pesticide in grain samples. The sensitivity of this polyclonal antibody population therefore needs to be optimised further in an ELISA set-up, if it were to be used for the detection of chlorpyrifos in contaminated grain samples.

Cross-reactivity studies were also carried out on the antibody population as described in section 2.11.5. Table 6.3 shows the results of the studies. The antibody showed high degrees of cross reactivity to parathion and fenitrothion, with some recognition for mevinphos. However, it showed negligible recognition for the two remaining OP's, carbophenethion and etrimfos. The monoclonal antibodies produced by Skerritt *et al.* (1992) were highly specific, and did not detect many other organophosphates or pesticides from other chemical groups. The antibodies produced by Manclus *et al.* (1994) were also highly specific showing minimal specificity for the other OP's. Once again, the reason for such specific antibodies was probably due to the fact that they were monoclonal, and therefore specific for only one epitope on the chlorpyrifos molecule. This epitope may be exclusive to the chlorpyrifos

molecule, and does not share any similarity on the other OP's. The polyclonal antibodies mentioned here were produced using a chlorpyrifos-KLH conjugate, which was conjugated in the same as described in Figure 6.2. This conjugation resulted in the thiophosphate part of the pesticide being exposed, which was not chemically modified in the reaction. This part of the molecule is common to all OP's and therefore antibodies produced using this conjugate may recognise this part of the hapten, and therefore other OP's. This was the case found here.

In order to develop an inhibition BIAcore-based assay for the detection of chlorpyrifos using the polyclonal antibodies, conjugates needed to be produced, as there was only a limited supply of the chlorpyrifos-dextran conjugate. BSA was chosen as the carrier molecule. Chlorpyrifos was chemically modified for conjugate production as described by Manclus *et al.* (1994) as shown in Figure 6.2. Two conjugates were produced, one with a 25 M excess of pesticide, and a second one with a 50 M excess of pesticide. The objective of synthesising two conjugates was to ensure the development of a sensitive SPR-based immunoassay, as sometimes over-substitution of a conjugate radically alters its pI below 4.0. If this occurs, it can be difficult to immobilise the conjugate using EDC/NHS coupling chemistry. Both conjugations were successful, and the molar substitution ratio of both conjugates was calculated. The 25 M excess conjugate had 6 pesticide molecules per BSA molecule, while the 50 M excess conjugate had 27 pesticide molecules per BSA molecule. These ratios were estimated spectrophotometrically, and therefore are not completely accurate for the calculation of actual ratios. However, they give good indications as to the substitution ratios, and it can be seen that limiting the molar ratio of pesticide did alter substitution. The conjugates were also analysed in a competition ELISA using the same parameters optimised for the competition ELISA using the chlorpyrifos-dextran conjugate. No competition was observed using the 25 M excess conjugate, while competition was observed using the 50 M excess conjugate. It may have been necessary to further optimise the assay using the 25 M excess conjugate in order to observe competition. However, it was apparent from the A/Ao values of the

assay that the antibodies did recognise the conjugate, thus showing the conjugate was suitable for antibody-binding interaction studies.

Preconcentration studies were carried out on the conjugates, in order to determine the optimal pH for immobilisation to the CM-dextran gel surface. The 25 M excess conjugate preconcentrated onto the surface at pH 4.5. This was expected due to the relatively low substitution ratio of the conjugate, and thus a larger number of free amines were available for preconcentration onto the dextran gel surface. As a result the pI of the protein was not radically changed. The 50 M excess conjugate preconcentrated on the dextran gel surface at pH 3.9. This was also as expected due to the high molar substitution ratio of pesticide on the protein, and, therefore, fewer free amines available for preconcentration onto the dextran gel surface. This high substitution reduced the pI of the protein, and hence pH 3.9 was required for immobilisation. Both conjugates were sufficiently immobilised onto the dextran gel surface, with 5,283.8 RU of the 25 M excess being immobilised, and 9,826.9 RU of the 50 M excess being immobilised.

In order to determine which pesticide-protein conjugate was the most suitable for use in the development of an SPR-based immunoassay, an antibody-binding saturation curve analysis was carried out on both conjugates using the affinity-purified anti-chlorpyrifos antibody. Figure 6.17 shows the overlaid binding curves obtained. Binding to the 25 M excess conjugate resulted in 376.4 RU of antibody bound, while binding to the 50 M excess conjugate resulted in 183.6 RU binding. As a result, the 25 M excess conjugate was subsequently used for the development of the assay.

Regeneration studies involving the affinity-purified anti-chlorpyrifos antibody showed that it was easily regenerated from the chlorpyrifos-BSA immobilised surface using a 1-minute pulse of 30 mM HCl. The binding responses were consistent throughout the regeneration study (approximately 150 RU binding each time) with a variation of binding well within the 20 % limit as mentioned in Chapter 5. Pre-treatment of the conjugate surface with ten 1-minute pulses of 30 mM HCl, removed

any loosely bound conjugate from the immobilised surface and therefore reduced leeching of antibody: conjugate complex from the surface giving regeneration. A significant decrease in binding signal was therefore not observed throughout the regeneration study. Non-specific interactions between the polyclonal antibody and the BSA part of the protein conjugate, and the CM-dextran gel surface were minimal, and there was no need to incorporate BSA or CM-dextran into the diluent buffer.

After optimising the regeneration conditions, and non-specific interactions of the polyclonal antibody binding to the conjugate surface, an attempt was made to develop an inhibition SPR-based assay. The same range of detection was chosen for the antibody as that optimised in the ELISA. However, as can be seen from Figure 6.20, no inhibition was observed. A lower range of detection for the antibody was also attempted (data not shown), but this too resulted in no inhibition being observed. The assay was also carried out using the 50 M excess conjugate. However no inhibition was observed in this situation either. A number of reasons may explain this result. Firstly, the range of detection of the antibody for the detection of chlorpyrifos may have increased in the BIAcore-based assay, thus causing problems with pesticide solubility. Due to the hydrophobicity of chlorpyrifos, it is only soluble in methanol at high concentrations. However, the antibodies used in this assay cannot tolerate high methanol concentrations (Figure 6.12). As a result of this problem, it was not possible to determine a range of detection for the antibodies at the higher concentrations, because the population would not be able to tolerate the high concentration of methanol.

Another reason this assay may not have worked was that the antibody had a high dissociation rate. Figure 6.17 shows the antibody saturation curves carried out, and it can be observed that after the injection the antibody did begin to rapidly dissociate from the immobilised conjugate surface. In the inhibition set-up, the antibodies may have bound to the free pesticide in solution, but, because of the high antibody dissociation rate, they dissociated from the free pesticide and, instead, bound-again to the conjugate surface. The conjugate surface contained a large amount of pesticide, and therefore the antibody population favoured binding to this instead of the free

pesticide. A possible solution to this problem was to make a conjugate with an even lower molar substitution ratio of pesticide, in order to shift the equilibrium of the assay in favour of the free pesticide and not the conjugate. This was attempted by producing conjugates (data not shown) with 5 and 10 M excesses of pesticide. However, when they were immobilised onto the sensor surface, there was not enough of antibody binding to them in order to develop an inhibition-based assay.

Although it was demonstrated that this antibody detected free pesticide in ELISA, it must be noted that ELISA is a different system to that of BIAcore. ELISA is equilibrium-based, and therefore the interaction between the antibody, the pesticide and the conjugate were allowed to reach a level of equilibrium before the detection step. In the BIAcore set-up, only the antibody and the pesticide were allowed to reach equilibrium before being injected over the conjugate surface, and, as a result, when injected over the surface, the antibodies 'recognise' this large quantity of pesticide-conjugate immobilised. Thus inhibition is prevented.

The reason any of these problems were encountered was because the antibodies produced probably had a low affinity for the pesticide. Chlorpyrifos is very hydrophobic, and this can cause problems in eliciting an immune response in an animal. Fasciglione *et al.* (1996) have found that carrier proteins can specifically modulate the antibody titre and the specificity of an immune response. This in turn may affect the affinity of the antibody. They do not recommend conjugating a hydrophobic hapten to a hydrophobic carrier (such as KLH, which was used in the production of this polyclonal antibody population). They have found that after conjugating such molecules together, the haptens hide inside the protein carrier itself by interactions with hydrophobic domains. The result of this interaction is the lack of any productive interaction/stimulation with specific B-cells. Therefore, it is more favourable to conjugate a hydrophobic hapten to a more hydrophilic carrier protein. This will reduce the number of domains that the hapten can shield in, and make it more available for presentation to the immune system. This will result in more specific antibodies with higher affinities.

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6.3. Conclusions

Conjugation of pirimiphos to a protein for use in the production of specific antibodies was unsuccessful. It was not possible to conjugate the modified form of the pesticide-amino-pirimiphos due to the presence of the two nitrogens on the ring structure, as well as the presence of the phosphorous, sulphur and oxygen atoms. These had an electronegative effect on the molecule, and made it impossible to activate the amino group.

The development of a competition ELISA for the detection of chlorpyrifos using affinity-purified polyclonal anti-chlorpyrifos antibodies was successful. The antibody was found to be relatively sensitive in the assay with good levels of reproducibility. Production of chlorpyrifos-BSA conjugates with different molar substitution ratios was also successful. These were used for the development of an inhibition BIAcore-based assay for the detection of free chlorpyrifos. However, this was unsuccessful, with the antibodies showing no level of inhibition in the SPR-based assay set-up using either conjugate. It is thought that the range of detection for the antibody may have changed, or its dissociation rate was too high to allow for inhibition to occur in the SPR-based immunoassay.

Chapter 7

Overall Conclusions

7.1. Overall conclusions

The research described in this thesis is mainly concerned with the production of antibodies and antibody fragments to the mycotoxin, aflatoxin B_1. Following their characterisation, the antibodies were used for the development of surface plasmon resonance-based immunoassays, for detection of aflatoxin B_1 in spiked grain samples. Pesticide-protein conjugates were also produced, characterised and evaluated for use in different immunoassay formats.

The work described in Chapter 3 entailed the production of polyclonal antibodies to aflatoxin B_1, their subsequent purification and characterisation. Protein G affinity-chromatography and immunoaffinity chromatography were used to isolate the antibodies following ammonium sulphate precipitation of relevant crude antiserum. Immunoaffinity chromatography was particularly successful in the removal of 'BSA-specific' antibodies, thus ensuring lower background signals when using the same toxin-protein conjugate for both immunisation and characterisation procedures. The polyclonal antibodies were relatively sensitive and specific to AFB_1. The optimised assays for the detection of aflatoxin B_1 were highly reproducible, and demonstrated good precision. Antisera was used for the detection of AFB_1 in spiked grain samples. However, the grain matrix affected the antibody's performance in the assay, thus reducing its sensitivity.

In Chapter 4 the production of single chain Fv antibodies to aflatoxin B_1 from an antibody phage display library was examined in detail. The library was produced using RNA obtained from the spleen of a mouse immunised with aflatoxin B_1-BSA conjugate. This was used for reverse transcription of RNA to cDNA, which was subsequently used for the amplification of the genes encoding the heavy and light chains of an antibody. SOE-PCR proved to be an efficient method for annealing the heavy and light chains, and, coupled with the ease of restriction enzyme digestion using SfiI, it aided in simplifying ligation of the DNA into the plasmid pAK 100. A small combinatorial library was produced. However, given the fact that the library was produced from RNA obtained from a mouse that was pre-immunised with

antigen, it was feasible to pursue screening from it. After three rounds of panning using two different toxin-protein conjugates, seven clones (six of which recognised free AFB_1) were successfully isolated. Soluble production of one of the scFv antibody clones was induced using the high expression vector pAK 400. After purification of the scFv by IMAC, a competition ELISA was optimised. The assay was relatively sensitive (limit of detection-98 ng/ml), but it was thought it could be further optimised using a more sensitive anti-FLAG antibody. The phage display system used was found to be highly robust with the use of a number of vectors for expression, and generally straightforward for the production of scFv antibodies.

The polyclonal and scFv antibodies produced were then applied for use in SPR-based immunoassays (Chapter 5). Regeneration of bound polyclonal antibody from the immobilised aflatoxin B_1-BSA conjugate surface was difficult, and required the use of organic solvents combined with high ionic strength. Although this solution did not regenerate all of the antibody from the surface, it removed a sufficient amount to allow for the development of an assay. The scFv antibody was easily removed from the immobilised conjugate surface, and demonstrated a high level of reproducibility for binding. SPR-based inhibition immunoassays were developed for both antibodies, which were sensitive and reproducible. These assays were then applied for the detection of AFB_1 in spiked grain samples. Both assays were highly sensitive and were capable of detecting AFB_1 below the maximum residue levels designated by the EU. Solution-phase equilibrium analysis was also carried out on the scFv antibody demonstrating it had a high dissociation rate constant.

Chapter 6 described the attempted production of a pirimiphos derivative for conjugation to a protein. This was unsuccessful, due to the presence of atoms on the pesticide ring structure having an electronegative effect on the molecule, thus making it impossible to activate for conjugation.
A competition ELISA was developed for the detection of chlorpyrifos, using affinity-purified polyclonal anti-chlorpyrifos antibodies (obtained from collaborators at IFR Norwich, U.K.). The antibody was found to be relatively sensitive in the assay with

good levels of reproducibility. Chlorpyrifos-BSA conjugates with different molar substitution ratios were also synthesised and used for the development of an inhibition BIAcore-based assay for the detection of free chlorpyrifos. However, this was unsuccessful, with the antibodies showing no level of inhibition in the SPR-based set-up using any of the conjugates.

In summary, this work highlights the potential use of immunoassays for the detection of food contaminants in samples. It shows that antibodies can be produced with relative ease to aflatoxin B_1, although, in the case of polyclonal antisera not all populations within a given antiserum are as sensitive as each other. The production of recombinant antibody fragments shows great promise, especially when derived from a mouse that was pre-immunised with antigen. It can also be seen that large library sizes are not necessarily essential when using pre-immunised libraries. Single chain Fv antibodies have been shown to be as sensitive as polyclonal antibodies for the detection of the aflatoxin in SPR-based assays. They also showed good solvent tolerance levels. This is significant for future biosensor applications that compatibility (even if limited) for organic solvents was demonstrated.

Organophosphate pesticides have proven to be difficult molecules to produce antibodies against. This is due mainly because they are difficult to conjugate to proteins, as they do not always contain a suitable functional group. They are also very hydrophobic in nature, thus making it difficult to achieve optimal conditions for the pesticide and protein to react without compromising their respective solubilities.

Future work could entail optimisation of aflatoxin extraction from grain samples using immunoaffinity columns, for the development of more sensitive and reproducible assays. These could then be used for reliable and routine analysis of food samples. It may also be possible to apply the antibodies for use in a 'on-site' dipstick assay and this will be examined shortly.

Chapter 8

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Appendix 1A

Glossary of terms and definitions commonly employed in Bioanalytical Validation procedures

The terms listed below were referred to for validation purposes of certain procedures carried out in this thesis. The criteria which they can be defined under have been reviewed elsewhere (Findlay *et al.*, 2000).

Mean

Describes the average of replicate *(x)* measurements. (i.e. $n_1+n_2+.........n_x/x)$

Precision

Is defined as the closeness of agreement, or variance between independent test results of multiple measurements of the same sample obtained under a set of specified analytical test conditions. It is normally expressed in terms of the relative standard deviation (% R.S.D.), or the coefficient of variation (% C.V.) of the determined concentration of a replicate number of assays. The degree of precision assessed between replicates (i.e. % C.V.) performed during a single assay batch, is commonly referred to as the intra-assay variation (also referred to as repeatability). The term inter-assay variation (also referred to as reproducibility) is used to describe the precision between assays when related to multiple batches.

Limit of Detection (L.O.D.)

Defines the lowest analyte concentration that the analytical technique can differentiate from background signals and is usually determined as background noise ± 3 standard deviations.

Robustness:

Is a term used to describe the ability of an analytical technique to withstand fluctuations in the described analytical test conditions. For immunoassay procedures the term could be used to describe changes in the ionic strength of the matrix, as well as pH and temperature changes.

Standard Curve:

This describes the relationship between the measured analyte response (i.e. absorbance, response units) and the analyte concentration.

Non-specific binding:

This describes matrix effects which affect the degree of binding of the antibody:antigen interaction, and can occur as a result of increased protein concentration and sample viscosity in the sample matrix (e.g. grain matrix), and also as result of altered ionic composition (e.g. urine) of the sample matrix.

Coefficient of Variation (% C.V.)

A quantitative measure of the precision of an analytical measurement expressed as a percent function of the mean value, also referred to as the Relative Standard Deviation (% R.S.D.).

$$\% \text{ C.V.} = [\text{S.D./Mean value}] \times 100$$

Precision profile:

A quantitative measure of the variation between measurements, usually the coefficient of variation versus the nominal concentration of analyte in the sample.

Normalised Response Values:

The response recorded in response units (RU) at each particular antigen concentration divided by the response recorded in the presence of zero antigen.
i.e.

Normalised Response = $\dfrac{\text{Response measured at particular antigen concentration}}{\text{Response measured at zero antigen concentration}}$

Normalised Absorbance Values:

The absorbance recorded (AU) at each particular antigen concentration divided by the absorbance recorded in the presence of zero antigen.
i.e.

Normalised Absorbance = $\dfrac{\text{Absorbance measured at particular antigen concentration}}{\text{Absorbance measured at zero antigen concentration}}$