

BIOSENSOR-BASED STUDIES ON COUMARINS

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by

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Declaration

I hereby certify that the material, which I now submit for assessment on the programme of study leading to the award of PhD, 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: _____

Gary J. Keating

Date: _____

Abstract

Polyclonal antibodies to 7-hydroxycoumarin - the main metabolite of coumarin, a plant constituent with many clinical applications - were produced, purified and characterised. This antibody preparation was used to develop a competitive immunoassay for 7-hydroxycoumarin which was carried out on the BIAcore, an optical biosensor based on the phenomenon of surface plasmon resonance (SPR). The immunoassay was optimised and internally validated.

Monoclonal antibodies were generated by somatic cell fusion using the spleens of mice immunised with a 7-hydroxycoumarin-protein conjugate. The screening of cell culture supernatants by BIAcore and ELISA was compared, and all of 13 clones isolated by limiting dilution were found to be reactive to the drug-protein conjugate, but not to free drug.

The panning of phage displayed antibodies from a naïve library using the BIAcore was investigated, and, although the amount of bound phage was insufficient to generate an SPR signal, subsequent analysis of eluate demonstrated that enrichment had taken place.

BIAcore was also used to affinity rank a panel of 3 genetically-produced single chain Fv antibodies against coumarin-BSA and to examine kinetic and affinity data for the interaction of one of these antibodies with immobilised drug-protein conjugate.

The interaction of two antibody preparations against the fungal toxin aflatoxin B₁ (AFB₁), a member of the coumarin family, was studied using both BIAcore and ELISA methods. High affinity constants rendered the monoclonal and polyclonal antibodies unsuitable for use in regenerable immunosensor formats. A range of competitive and sandwich ELISAs for the detection of AFB₁ were developed using both types of antibody. In addition, methods to facilitate better design of formats for use with BIAcore were established using ELISA to mimic regeneration conditions on the sensor chip surface, and to calculate equilibrium affinity constants.

Supercoiled plasmid DNA was also immobilised on the BIAcore sensor surface, and the inhibition of the enzyme topoisomerase II by the coumarin antibiotic novobiocin, as well as the direct binding of a range of coumarin-protein conjugates to nucleic acids, were investigated.

Abbreviations

Ab	antibody
Abs	absorbance
AFB ₁	aflatoxin B ₁
AFB ₁ -BSA	aflatoxin B ₁ bovine serum albumin conjugate
Ag	antigen
BCA	bicinchoninic acid
BIA	biospecific interaction analysis
BSA	bovine serum albumin
°C	degrees Celcius
CDR	complementarity determining region
CE	capillary electrophoresis
conc	concentration
CV	coefficient of variation
Da	daltons
DMEM	Dulbecco's modification of Eagle's medium
DMSO	dimethylsulphoxide
DNA	deoxyribonucleic acid
EDC	N-ethyl-N'-(dimethylaminopropyl)carbodiimide
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
Fab	antigen-binding portion of IgG molecule
Fc	effector portion of IgG molecule
Fv	variable portion of IgG molecule
g	gramme
h	hour
HAT	hypoxanthine, aminopterin, thymidine
HBS	HEPES buffered saline
HGPRT	hypoxanthine guanine phosphoribosyl transferase
HPLC	high performance liquid chromatography

HRP	horseradish peroxidase
HSA	human serum albumin
IgG	immunoglobulin class G
IgM	immunoglobulin class M
i.v.	intravenous
kDa	kilodaltons
KLH	keyhole limpet haemocyanin
l	litre
LED	light emitting diode
mA	milliamperes
mAb	monoclonal antibody
mg	milligramme
ml	millilitre
mm	millimetre
mM	millimole
mol	moles
mV	millivolts
µg	microgramme
µl	microlitre
µM	micromole
NADPH	nicotinamide adenine dinucleotide phosphate
NHS	N-hydroxysuccinimide ester
nm	nanometre
nM	nanomolar
7-OHC	7-hydroxycoumarin
7-OHCG	7-hydroxycoumarin glucuronide
7-OHC-BSA	7-hydroxycoumarin bovine serum albumin conjugate
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PBST	phosphate buffered saline containing 0.05% (v/v) Tween

PCR	polymerase chain reaction
PDA	photodiode array
PEG	polyethylene glycol
ppb	parts per billion
ppm	parts per million
r	regression coefficient
rpm	revolutions per minute
RU	response units
S.D.	standard deviation
SDS	sodium dodecyl sulphate
sec	seconds
TLC	thin layer chromatography
UV	ultraviolet
UDPGT	uridine diphosphate glucuronyl transferase
v/v	volume per unit volume
w/v	weight per unit volume

Publications and Presentations

Publications:

Killard, A.J., **Keating, G.J.** and O'Kennedy, R. (1998), Production and characterisation of anti-coumarin scFv antibodies, *Biochem. Soc. Trans.*, **26**, S33.

McCormack, T., **Keating, G.J.**, Killard, A.J., Manning B. and O'Kennedy, R. (1998), Biomaterials for Biosensors in *Principles of Chemical and Biological Sensors*, Wiley and Sons, Chichester, England, 133-194.

Keating, G.J. and O'Kennedy, R. (1997), Chemistry and Occurrence of Coumarins in *Coumarins – Biology, Applications and Mode of Action*, Wiley and Sons, Chichester, England, 23-66.

Bogan, D., **Keating, G.J.**, Reinartz, H., Duffy, C., Smyth, M. and O'Kennedy, R. (1997), Analysis of Coumarins in *Coumarins – Biology, Applications and Mode of Action*, Wiley and Sons, Chichester, England, 267-302.

Keating, G.J. and O'Kennedy, R. (In preparation), BIAcore immunoassay for quantitative determination of total 7-hydroxycoumarin in serum.

Presentations:

Keating, G.J., McCormack, T. and O'Kennedy, R. (1995), The use of BIAcore to study the effects of fluorophore-labelling on antibody binding, *European BIA symposium*, Stockholm, Sweden.

Keating, G.J. and O'Kennedy, R. (1995), The development of a biosensor-based quantitative assay for the determination of 7-hydroxycoumarin in biological samples, *2nd Annual ABI Workshop*, Tampere, Finland.

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CHAPTER 1

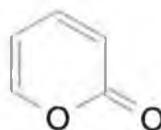
INTRODUCTION

1.0 Introduction

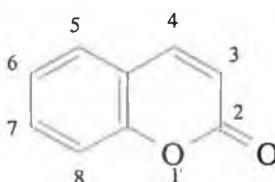
The work described in this thesis concerns the use of an optical immunosensor and other analytical techniques to study members of the coumarin family and their immunological interactions. The first part of this chapter describes the chemistry, biosynthesis, metabolism and clinical and analytical uses of coumarin compounds. The second half of the chapter deals with biosensor technology in general, and the use of optical immunosensors in particular.

1.1 Coumarins

The coumarins belong to a group of compounds known as the benzopyrones, all of which consist of a benzene ring joined to a pyrone - a six-membered heterocyclic ring containing one oxygen atom and five sp^2 -hybridised carbons. Both γ - and α -pyrones exist, with the prefixes referring to the position at which the oxygen atom is found. The structure of α -pyrone is shown below:



Coumarin (shown below) and the other members of the coumarin family are benzo- α -pyrones, while the other main members of the group - the flavonoids - contain the γ -pyrone group.



The coumarins display a characteristic fluorescence upon excitation with ultra-violet light, in addition to having a large range of physiological activity.

1.1.1 History of coumarins

The name coumarin is derived from *Coumarouna odorata*, the botanical name for the tonka tree – one of the many plant sources in which the compound is found. The molecule was

first isolated in 1820 by Vogel, who extracted coumarin both from the tonka bean and clover. It is the presence of coumarin in the latter which is responsible for the distinctive odour of new-mown hay. The structure of the compound was determined in 1868.

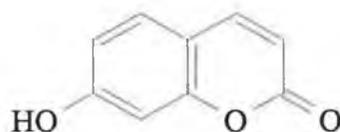
1.1.2 Chemistry of coumarins

Many coumarins are comprised of isoprenoid chains joined to one of the carbons of the nucleus, or to an oxygen atom, for example, a phenolic oxygen. The isoprenoid chains may consist of one, two or three units, and many variations of this type of structure are to be found among the coumarins. Interactions of the prenyl group with an *ortho*-phenolic group can lead to the formation of another heterocyclic ring. Oxidative interaction may also occur. As the vast majority of the coumarins are oxygenated at C-7, 7-hydroxycoumarin is frequently considered to be the parent compound for the more structurally complex members of the family. Coumarins may also be found in nature in combination with sugars, as glycosides. A variety of chromatographic and spectroscopic methods for the identification of coumarins exist. The coumarins can be roughly categorised as follows:

a) Simple

These members of the coumarin family are the hydroxylated, alkoxyated and alkylated derivatives of the parent compound, coumarin, along with their glycosides.

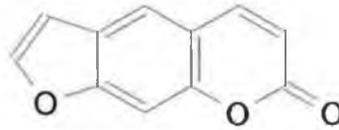
7-hydroxycoumarin, a member of this group, is shown here:



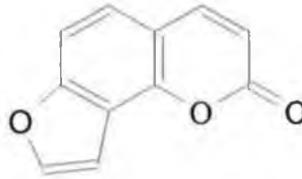
b) Furanocoumarins

These compounds consist of a five-membered furan ring attached to the coumarin nucleus. Most of the members of this group are derivatives of the linear furanocoumarin, psoralen, or its more stable angular isomer, angelicin. (The terms “linear” and “angular” refer to the orientation of the furan ring with respect to the coumarin nucleus.) The dihydrofuranocoumarins are also members of this group.

Psoralen:

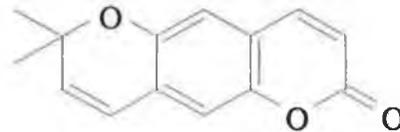


Angelicin:

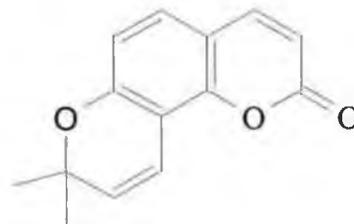


c) Pyranocoumarins

Members of this group are analogous to the furanocoumarins, and contain a six-membered ring. As with the furanocoumarins, linear and angular types exist, e.g.:



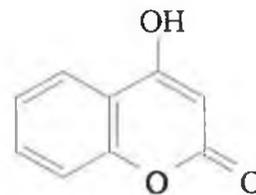
Xanthyletin:



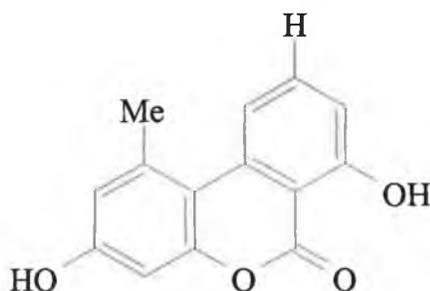
Seselin:

d) Coumarins substituted in the pyrone ring

Examples of this group include 4-hydroxycoumarin and the 3-methyl-4-hydroxycoumarins, such as autumnariol:



4-hydroxycoumarin:



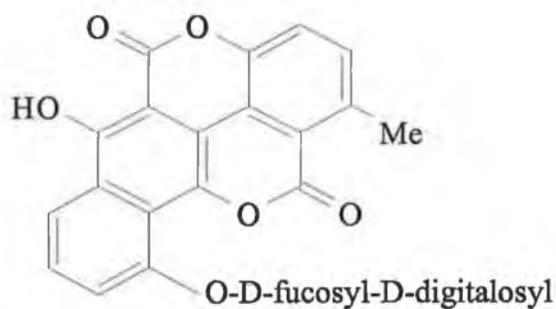
Autumnariol:

1.1.3 Nomenclature of coumarins

The nomenclature of coumarin compounds is frequently problematic. The parent compound, coumarin, is variously known as 2-oxo-1,2-benzo-pyran, 5,6-benzo- α -pyrone, 5,6-benzo-2-pyrone, 1,2-benzopyrone, 2*H*-1-benzopyran-2-one, *cis-o*-coumaric acid lactone, 2*H*-benzo[*b*]pyran-2-one, coumarinic anhydride and 2*H*-chromen-2-one. Members of the coumarin family have been isolated from hundreds of species of plants and microorganisms, giving rise to another problem in the naming of the compounds. Many of the coumarins that have been isolated are known by common names, which are generally derived from the Latin or colloquial name of the plant in which they have been discovered, or from the place where the plant grows, resulting in the same compound having several different trivial names. Frequently, the trivial name which has been assigned to such a compound gives little or no information as to its structural composition; and in some cases may even be misleading. For example, 7-hydroxycoumarin is also known as umbelliferone, (due to its occurrence in members of the Umbelliferae), skimmetin, hydrangin and Dichrin A.

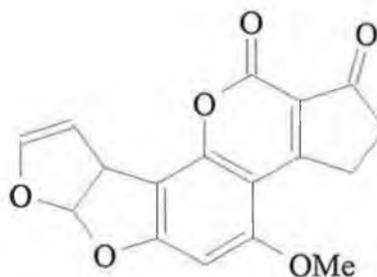
1.1.4 Occurrence

Although most of the natural coumarins in existence have been isolated from the higher plants, some members of the family have been discovered in microorganism and animal sources. Yeast of the genus *Streptomyces* produce antibiotics like novobiocin, chartreusin, (shown below) and coumermycin A₁:



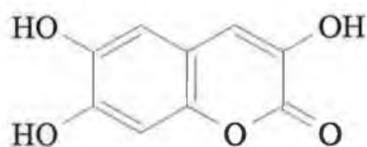
Chartreusin:

The aflatoxins are a group of highly toxic fungal metabolites, which occur in the fungus *Aspergillus* sp. The most commonly-occurring member of the group is aflatoxin B₁ (AFB₁), which is produced by *Aspergillus flavus* and *A. parasiticus*:

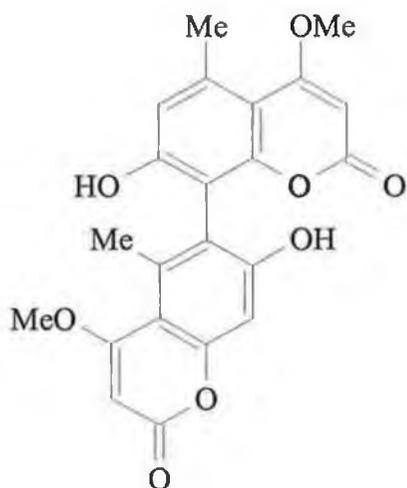


AFB₁:

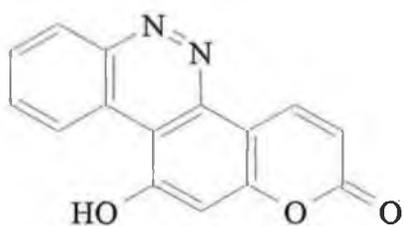
Dasycladus vermicularis - a member of the green algal family Dasycladaceae - has been shown to produce 3,6,7-trihydroxycoumarin (Menzel *et al.*, 1983):



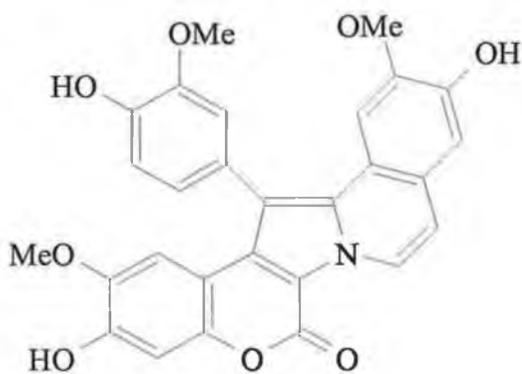
The mould *Emericella desertorum* produces desertorin A, B and C (Nozawa *et al.*, 1997), the first of which is shown below:



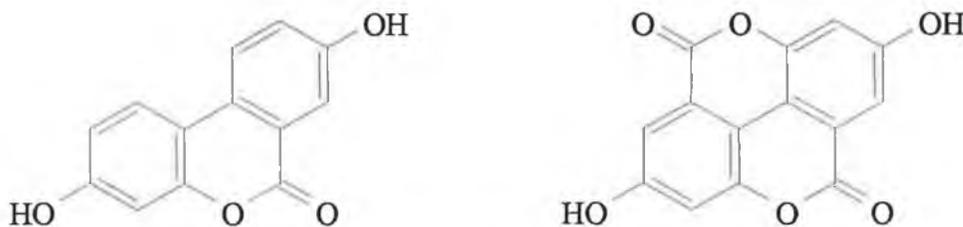
Necatorin (below) has been extracted from the wild mushroom *Lactarius necator* (Suortti and von Wright, 1983):



Lamillarin A, B, C and D are metabolites of the mollusc *Lamellaria* sp (Andersen *et al.*, 1985). The structure of lamillarin D is shown below:



The *Castoreum pigments* are also of animal origin, having been extracted from secretions of the beaver scent gland (Dean, 1963):

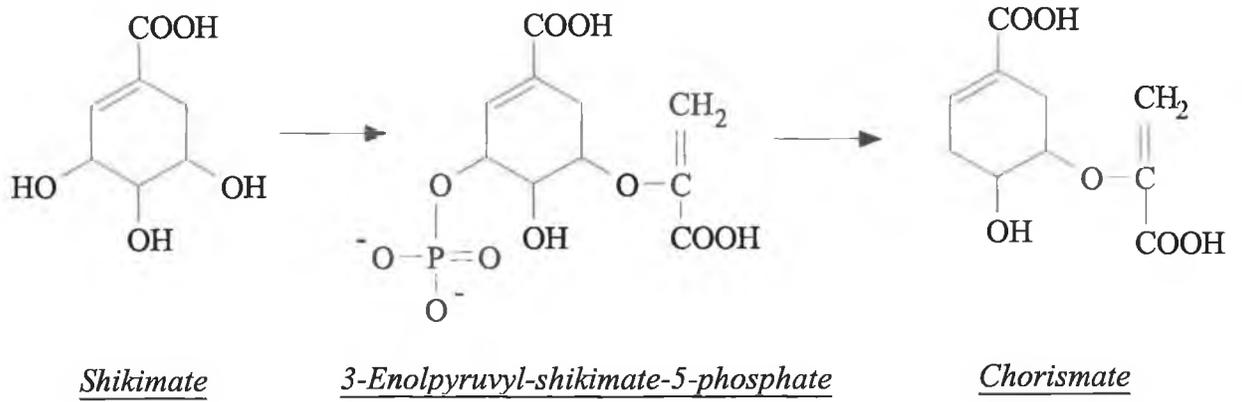


As stated above, though, most coumarins occur in the higher plants, with the richest sources being the Rutaceae and Umbelliferae, in which all parts of the plant have been discovered to contain coumarins. The coumarins belong to a group known as the secondary products of plant metabolism, the exact roles of which in plant physiology are not clearly understood. While being distributed throughout all parts of the plant, the coumarins occur at the highest levels in the fruits, followed - in order of decreasing occurrence - by the roots, stems and leaves. In addition, seasonal changes and environmental conditions may affect the occurrence in various parts of the plant.

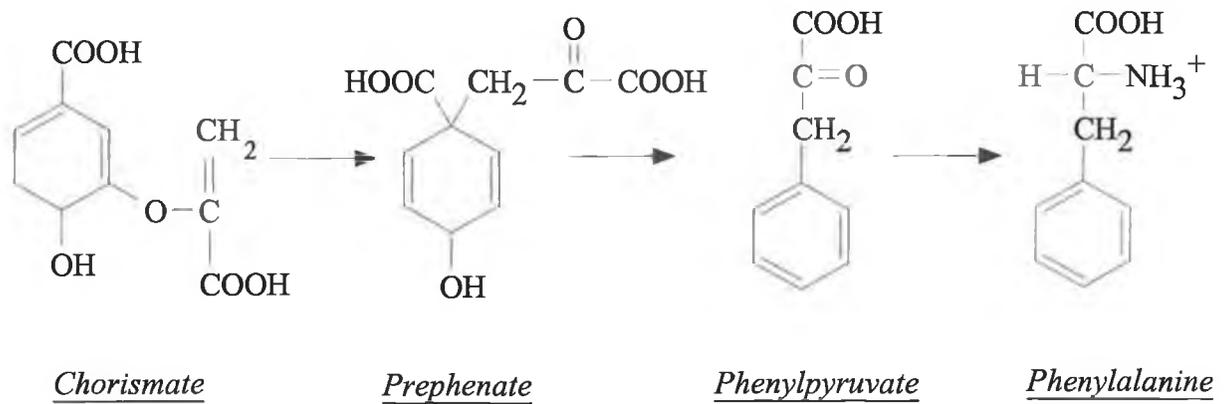
Murray *et al.* (1982) and Keating and O'Kennedy (1997) have given a comprehensive overview of the chemistry and occurrence of natural coumarins, including tables of the botanical sources of all of the coumarin compounds known at time of publication.

1.1.5 Biosynthesis of coumarins

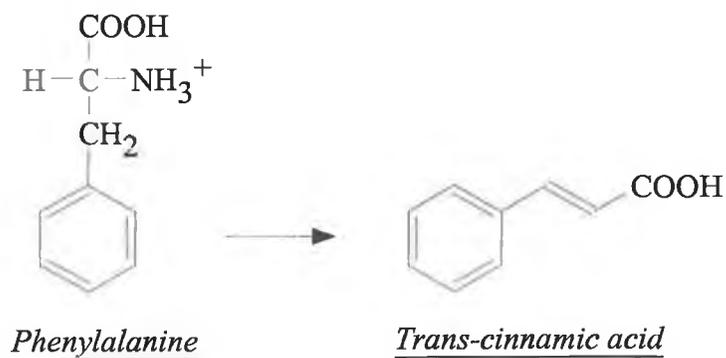
In the higher plants, coumarins are generally produced via the shikimate-chorismate biosynthetic pathway, as a derivative of cinnamic acid, which is also a precursor of many other natural products, such as methyl salicylate, cinnamic aldehyde and amygdalin. The shikimate-chorismate pathway is responsible for the formation of the aromatic amino acids. Phosphoryl group transfer from ATP followed by nucleophilic displacement results in the formation of 3-enolpyruvyl-shikimate-5-phosphate from shikimate, and the elimination of phosphate gives chorismate:



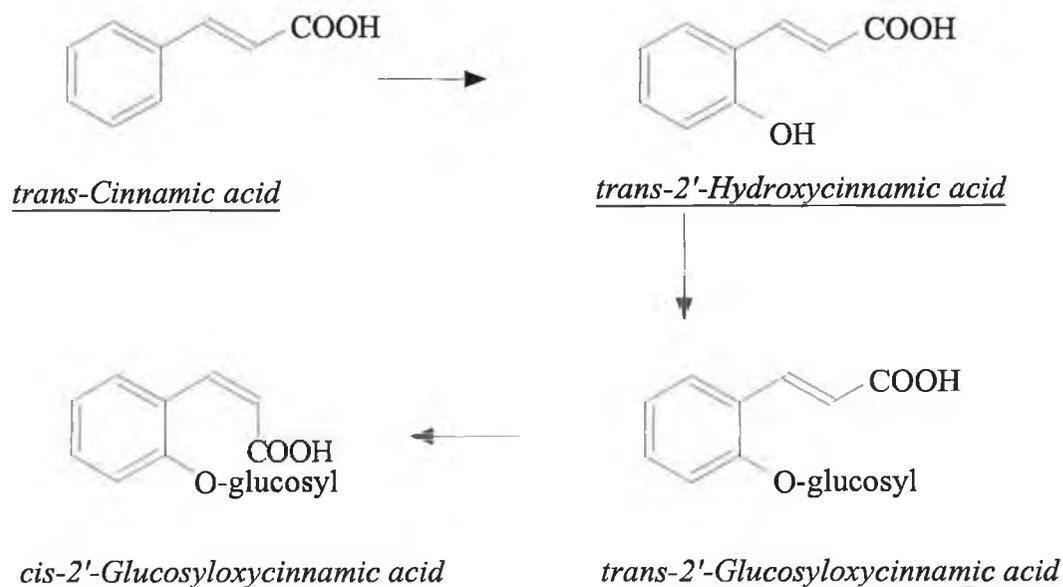
The enzyme chorismate mutase then catalyses the rearrangement of chorismate to prephenate, which is converted to phenylpyruvate by means of a 1,4 elimination. A glutamate-dependant transamination results in the formation of phenylalanine:



Phenylalanine is then converted to *trans*-cinnamic acid by the action of the enzyme phenylalanine ammonia-lyase, which eliminates NH_3^+ :

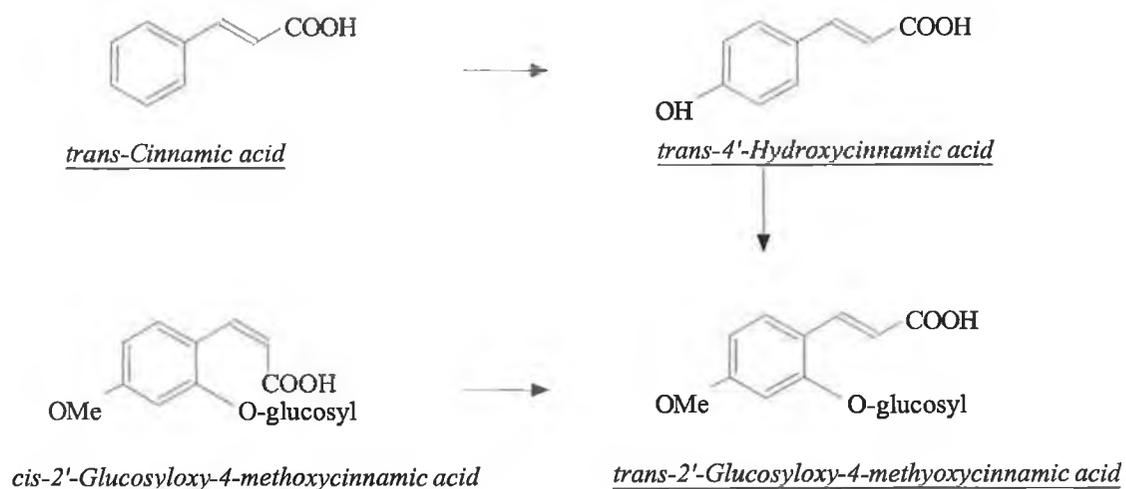


From this point, simple coumarins which are oxygenated at C-7 follow a biosynthetic route different to those which are not oxygenated at this position. In the latter case, the *trans*-cinnamic acid is first 2'-hydroxylated, then glucosylated to give *trans*-2'-glucosyloxycinnamic acid. This is then converted to the *cis*-isomer:

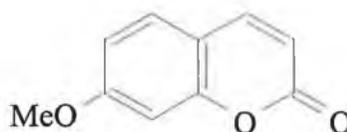


cis-2'-Glucosyloxycinnamic acid is also known as coumarinyl glucoside, and is the bound form in which coumarin exists in plants. The hydroxylation and glucosylation of the *trans*-cinnamic acid are enzyme-catalysed reactions, while the *trans*-*cis* isomerisation is mediated by UV light.

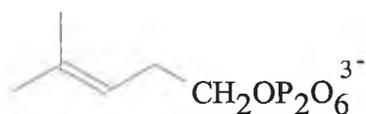
The 7-oxygenated simple coumarins arise as a result of *para*-hydroxylation, rather than the *ortho*-hydroxylation which is the first step in the formation of the non-oxygenated compounds. As in the previous reaction scheme, a glucosylation reaction is also involved, followed by UV-dependent *trans*-*cis* isomerisation. The scheme below shows the formation of *cis*-2'-glucosyl-4-methoxycinnamic acid, the bound form of herniarin:



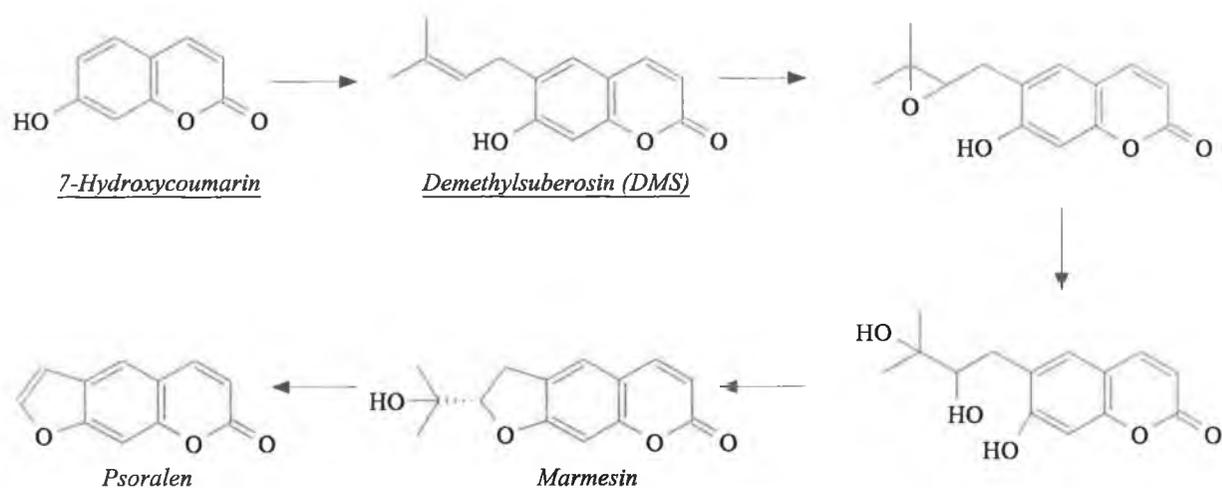
The structure of the unbound compound, herniarin, is shown below:



The furanocoumarins (both linear and angular) are produced using 7-hydroxycoumarin as the parent compound, with the other two carbons of the furan ring coming from malevonic acid. The malevonnate derivative dimethylallylpyrophosphate, below, is involved in the prenylation of position 6 of 7-hydroxycoumarin to form the furanocoumarin precursor demethylsuberosin (DMS).



It is thought that the double bond of the prenyl group in DMS undergoes epoxidation prior to cyclisation to a dihydrofuranocoumarin, which is then converted into a furanocoumarin, as in the synthesis of psoralen illustrated below:



The formation of angular furanocoumarins proceeds by an analogous series of reactions, with - for example - angelicin being formed from 7-hydroxycoumarin via colombianetin, following prenylation at position 8 to give osthenol. In the case of furanocoumarins which possess hydroxyl or methyl ether groups, *O*-methylation and hydroxylation appear to occur after psoralen or angelicin have been fully formed.

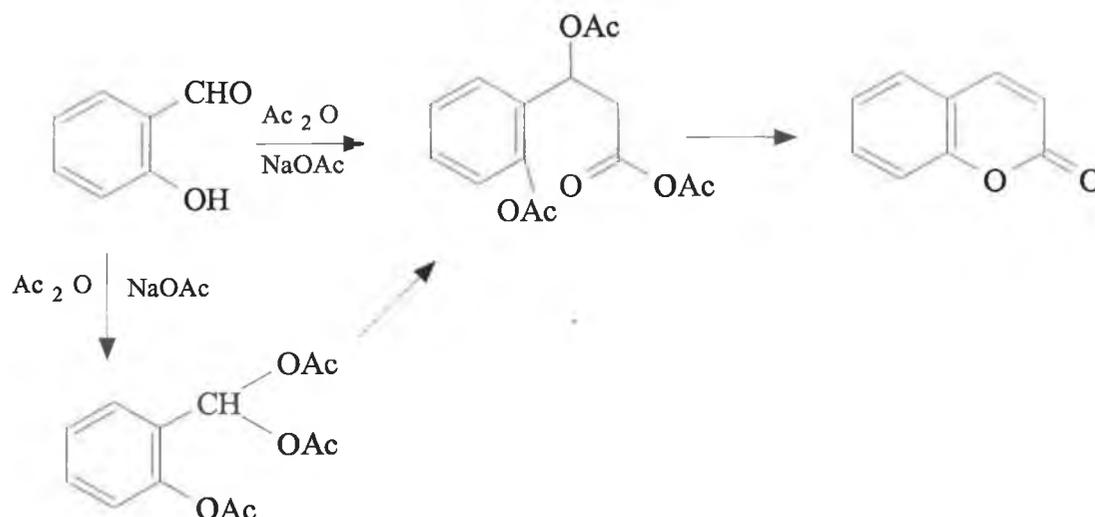
It seems most likely that the linear and angular pyranocoumarins are formed by a biosynthetic route similar to that of the furanocoumarins, differing only in the last cyclisation step.

Although the shikimate-chorismate pathway is the predominant route for the biosynthesis of coumarin compound, including the 4-hydroxycoumarins, exceptions do exist. The phenylcoumarins, such as coumestrol, have been found to originate partly from the shikimate-chorismate pathway and partly from the polyketide pathway, while the aflatoxins are synthesised entirely from acetate via polyketide intermediates like averufin and versicolorin A.

In plants, it seems that the main site of coumarin synthesis is in the leaves, although synthesis in other parts of the plant - notably the roots and aerial organs - also takes place.

1.1.6 Chemical synthesis

The classical reaction for the formation of coumarin - Perkin's reaction - involves heating *o*-hydroxybenzaldehyde with sodium acetate and acetic anhydride at 180°C:



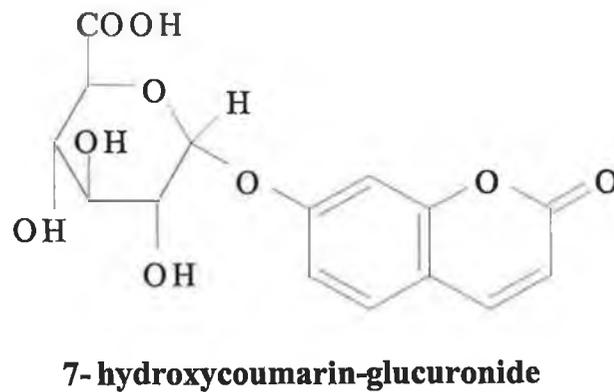
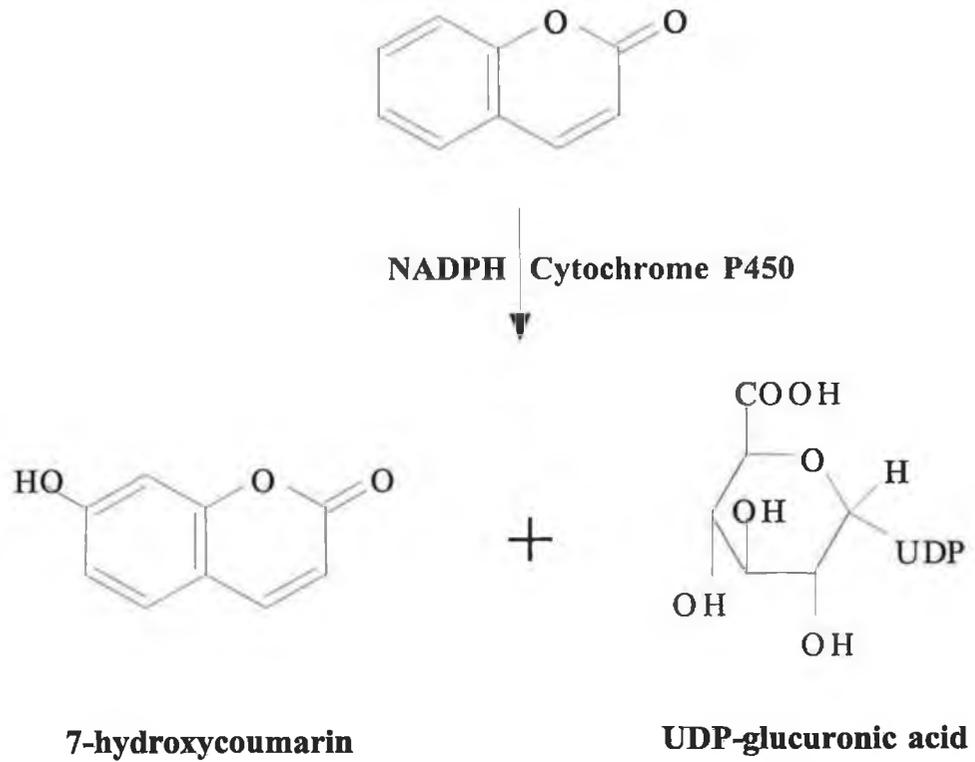
Since this reaction was first used for the preparation of coumarin in 1868, a wide range of the natural coumarins have been chemically synthesised. The synthesis of simple coumarins with methoxy or hydroxy groups is generally still carried out by means of the Perkin reaction, despite the fact that yields are frequently poor.

As the formation of the pyrone ring is the most important step in many of the chemical syntheses of coumarin, a variety of methods have been developed to introduce functional groups into this structure. One strategy is to prepare a phenol which contains the desired substituents of the coumarin before the pyrone ring is formed. Alternatively the coumarin nucleus may be synthesised first, and a process such as *C*- or *O*-alkylation or nuclear oxygenation employed to produce the required compound.

1.1.7 Coumarin metabolism

The main phase I metabolite of coumarin in man is 7-hydroxycoumarin, and the hydroxylation reaction is catalysed by a specific cytochrome P450 enzyme in the liver, called CYP2A6, which has an absolute requirement for nicotine adenine dinucleotide phosphate (NADPH). 7-hydroxycoumarin is then glucuronidated - a common metabolic fate for xenobiotics, which renders them more water-soluble and results in detoxification. The glucuronidation reaction is catalysed by uridine diphosphate glucuronyl transferase (UDPGT). The UDPGTs are a group of membrane-bound enzymes which conjugate glucuronic acid to compounds with oxygen-, nitrogen-, sulphur- and carbon-containing functional groups. The metabolic fate of coumarin in man is illustrated in Figure 1.1.

Figure 1.1 The metabolic fate of coumarin in man. Coumarin is rapidly hydroxylated to form 7-hydroxycoumarin by a cytochrome P450 enzyme with an absolute requirement for NADPH. 7-hydroxycoumarin is then conjugated to UDP-glucuronic acid by the enzyme UDP glucuronyl transferase (UGT) to form 7-hydroxycoumarin-glucuronide. Another enzyme, β -glucuronidase, can deconjugate this glucuronide.



Coumarin metabolism is subject to major interspecies differences, and hydroxylation at all available positions, as well as opening of the pyrone ring, has been documented. This metabolic diversity is mirrored by dissimilarities in the toxicity of coumarin in different species. In rats, for example, 3-hydroxylation followed by ring-opening appears to be the main metabolic route, and this pathway - relatively insignificant in man - seems to result in the highest degree of hepatotoxicity. The metabolism of coumarin has been reviewed recently by Pelkonen *et al.* (1997), and the primary mammalian metabolites are shown in Figure 1.2.

In all of the metabolic pathways investigated, a hydroxylation step is necessary for phase II conjugation to take place by means of UDP-glucuronosyl transferases and sulphotransferases.

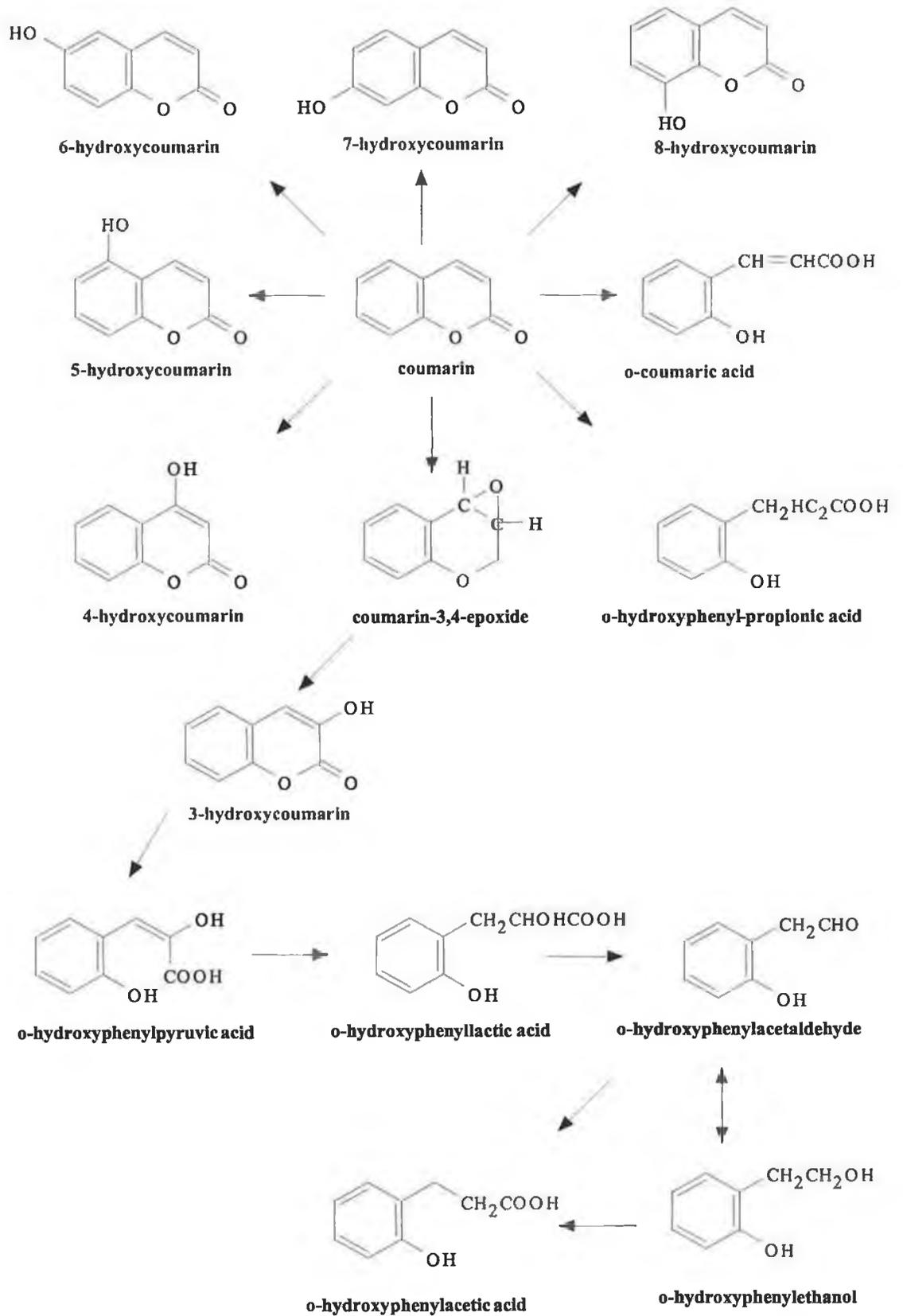
In man, as in all other species studied, metabolism of coumarin is rapid and almost complete, with the free drug having a half-life of only one hour after either oral or intravenous administration.

As coumarin is so quickly metabolised, it is thought to be a prodrug for 7-hydroxycoumarin, to which compound the clinical effects of coumarin are usually attributed. It has been shown (Sharifi *et al.*, 1993a) that both free and glucuronidated 7-hydroxycoumarin can be detected in circulation many days after administration, and a cyclic system of conjugation and deconjugation has been postulated for the action of the drug *in vivo* (Casely-Smith and Casely-Smith, 1986).

1.1.8 Clinical applications

Plants which naturally contain biologically-active coumarins seem to benefit from immunity from infections by fungal pathogens, and some plants also repel beetles and other insects. In addition, the strong absorption of ultra-violet light characteristic of coumarin and its derivatives shields young plants from damage by sunlight. Over the years, a variety of physiological effects in man have been attributed to the coumarins, ranging from bacteriostatic action to diuretic effects to narcotic properties. (It is interesting to note that many of the biologically-active coumarins contain a 7-hydroxy group.) These reports have led to many animal and clinical trials being undertaken with coumarins for the treatment of a large variety of disorders.

Fig 1.2 The metabolism of coumarin in mammals. Hydroxylation at all available positions, and the opening of the pyrone ring, have been documented. (Taken from Bogan, 1996)



Most of the studies have been carried out on the parent compound and its primary metabolite in man, 7-hydroxycoumarin, although the synthetic 4-hydroxycoumarin warfarin is currently in the most widespread use.

One of the most exciting potential uses of coumarin is in the treatment of cancer. Unlike many other chemotherapeutic agents, the use of coumarin is associated with relatively few side-effects. The major problem with oral administration of the drug has been nausea brought on by the aromatic nature of the parent compound, a problem which can be circumvented by the substitution of 7-hydroxycoumarin. The *in vivo* action of the drug in several types of cancer has been demonstrated, and - together with results from cell-culture experiments - these findings have pointed to a number of different possible mechanisms for the anti-tumour action of coumarins.

The programmed cell death necessary for normal growth, and for the removal of damaged and senescent cells, is known as apoptosis, and interference with this process - leading to an increase in the number of rapidly-dividing cells - is a characteristic of malignant cells. In experiments on tumour cells in culture, coumarin, 7-hydroxycoumarin and the derivative *o*-coumaric acid were seen to exhibit dose-dependant cytotoxic effects at high drug concentrations (100 -500 mg/ml). The dead cells were shown to display morphology consistent with apoptosis, such as shrinkage and compaction of chromatin (Marshall *et al.*, 1991a; Roskopf *et al.*, 1992; Kahn *et al.*, 1994).

In addition to these cytotoxic effects, coumarins have also been shown to possess a cytostatic activity, on both normal and cancerous cells. One example of this is the action of esculetin on muscle cells (Huang *et al.*, 1993), which is thought to be due to a decrease in tyrosine kinase activity. The synthetic derivative 8-nitro-7-hydroxycoumarin has been shown to possess both cytotoxic and cytostatic activity (Egan *et al.*, 1997).

The growth of normal cells is strictly controlled at various stages of the cell cycle such as cell division and DNA replication. Many tumour cells, though, display abnormalities in the cell cycle, allowing the reproduction of damaged DNA. In a model system, coumarin has been shown to modulate the cell cycle, decreasing the number of cells undergoing DNA replication, and leading to a reduction in cell proliferation (Tseng *et al.*, 1987).

A range of substances known as growth factors have been linked to the regulation of cell growth. The binding of these factors to specialised receptors control signalling pathways

within the cell. When both growth factor and receptor are produced by the same cell, the regulatory process is known as an "autocrine loop". Examples of growth factors are epidermal growth factor (EGF) and platelet-derived growth factor (PDGF). It is thought that aberrations of growth factors and their specific receptors play a part in some types of cancer. Glioma is a malignant condition of the glial cells. It has been demonstrated in *in vitro* studies that treatment of glioma cells with 7-hydroxycoumarin leads to an inhibition of cell growth, with concomitant reduction in levels of PDGF (Seliger and Pettersson, 1994). This suggests that 7-hydroxycoumarin controls glioma cell growth by inhibition of an autocrine loop.

Coumarins also exert an anti-tumour activity by affecting the body's immune system ("immunomodulatory effects"), causing defence mechanisms to be activated which can remove tumours and prevent recurrence of surgically-removed tumours (Zlabinger, 1997).

A variety of clinical trials of coumarins in the treatment of cancer, in which one or more of the above mechanisms were thought to be operative, have been carried out.

Marshall *et al.* (1991b) carried out a phase I trial on patients with advanced malignancies, in which coumarin was administered along with cimetidine - a substance with known immunomodulatory effects. Thirty-seven of the fifty-four patients treated were suffering from metastatic renal cell carcinoma, among which group there was a response rate of approximately 20%, with the best results occurring in patients with metastases in the lung, with low tumour burdens, and who had undergone prior nephrectomy. A subsequent study (Kokron *et al.*, 1991), carried out solely on patients with renal cell carcinoma, reported a comparable number of responders, with similar profiles.

A clinical trial on patients with metastatic prostate cancer (Mohler *et al.*, 1992), in which 3 g of coumarin was administered daily, gave a response rate of 8% (3 of 40 individuals). Favourable results have also been obtained in a trial of coumarin on patients with malignant melanoma (Thornes *et al.*, 1989).

The evidence from all of the clinical trials so far conducted is that coumarin is most effective in cancer treatment when used as a secondary (or "adjuvant") therapy in patients with good performance status and small tumour loads. In general, the drug appears to be of most use in the prevention of tumour recurrence following radical surgery.

One area in which coumarin, although not yet in routine use, has proved to be of very significant benefit, is in the treatment of high-protein oedema. An oedema is a medical condition characterised by the accumulation of plasma protein in tissue, leading to swelling of the affected tissue (as a result of osmosis) and pain. High-protein oedemas (defined as those in which protein concentrations in tissues are at or above 10 g/l) cause blood vessels to be damaged, and may result in secondary bacterial and fungal infections, and even death.

All of the coumarins tested have been shown to be effective at reducing high-protein oedemas. They do this by increasing the number and proteolytic activity of macrophages at the site of the oedema. It is thought that they may also exert an influence on other cell types involved in the response to injury. The excess proteins present are broken down, and the fluid returns to the blood-stream. Coumarin, in common with most other benzopyrones, binds to proteins *in vivo*. One study using radio-labelled coumarin administered to rats has demonstrated that 66% of the drug is bound to serum proteins (Piller and Schmitt, 1977). This protein binding allows coumarin to enter tissues easily, and greatly enhances its efficacy in the treatment of oedema.

In a clinical trial, administration of 400 mg per day of coumarin was reported to decrease the size of oedemas by 20% in patients with elephantiasis (Casely-Smith *et al.*, 1993).

Oedema is also associated with diseases which cause chronic inflammation, and disorders ranging from bedsores to brucellosis (Thornes, 1983) have been shown to respond to treatment with coumarin. In another study on animal tissue, coumarin, 7-hydroxycoumarin, and 4-hydroxycoumarin were found to possess an anti-inflammatory activity, as evinced by their inhibition of prostaglandin formation (Lee *et al.*, 1981).

In vivo, approximately 20-30% of administered 7-hydroxycoumarin will be bound to red blood cells, upon which it produces a haemmorheological effect - increasing the flexibility of erythrocytes and reducing aggregation. This property, along with its anti-oedema activity, is the basis for the use of coumarin in the treatment of venous disorders. Variocose veins and haemorrhoids have been treated using coumarin, and a combination of coumarin and troxerutin has been tested on patients with chronic venous insufficiency (CVI), with good results (Bosse *et al.*, 1985).

The action of coumarins on oedema, together with other effects such as those on the functions of the lymphatic system and glucose uptake by red blood cells, indicate that the

drugs - while not currently in widespread use - may also prove clinically important in the treatment of burns (Piller, 1997).

Coumarin and 7-hydroxycoumarin are also of interest for their ability to scavenge free radicals, which cause damage to blood vessels and cell membranes. This property may be of use in the treatment of AIDS and HIV infection, of which disorders such oxidative stress is symptomatic (Greenspan and Aruoma, 1994).

Despite the antimicrobial effects of novobiocin (and the other coumarin antibiotics), and the spasmolytic activity of hymecromen, neither have gained general clinical acceptance. The furanocoumarin 8-methoxypsoralen, however, is significant for its use in the treatment of dermatological diseases such as psoriasis.

As mentioned above, warfarin and its derivatives are currently the most clinically important of the coumarins. Warfarin (3-(α -acetylbenzyl)-4-hydroxycoumarin) was first synthesised by Ikawa *et al.* in 1944. The activity of warfarin and the other coumarin anti-coagulants is based on their inhibition of vitamin K-dependant clotting factors, culminating in the production of abnormal prothrombin, which is unable to bind the calcium ions necessary for the proper operation of the clotting mechanism. Warfarin is in widespread clinical use as an oral anticoagulant in the treatment of acute myocardial infarction, threatened stroke, and venous thrombosis. It has also been investigated for use in other disorders.

The effects of warfarin upon the cellular immune system were studied in a clinical test on patients with *Herpes simplex* infection (Berkarda, 1993). Patients receiving a low (non-anticoagulant) dose of the drug displayed a 4-fold lower instance of recurrence of cold sores than those in the control group.

IgA nephropathy is a disorder caused by accumulation of the immunoglobulin IgA in cells of the kidney, leading to renal failure. Macrophage-like cells in the kidney are instrumental in the progression of the disease, which has been shown to be arrested by administration of non-anticoagulant doses of warfarin, along with two other drugs - cyclophosphamide and dipyridamole (Woo *et al.*, 1987).

In a clinical trial, treatment with warfarin has been demonstrated to increase survival and tumour reduction in patients with small cell carcinoma of the lung (SSCL), a malignant condition associated with a coagulative effect (Zacharski *et al.*, 1981).

Coumarins are so-called "suicide substrates" for serine proteases, binding irreversibly to the enzyme. One such enzyme is the HIV-1 protease responsible for the spread of the causative organism of AIDS. Coumarins also block the production of the naturally-occurring tripeptide glutathione (GSH), which is implicated in the regulation of HIV replication. In an *in vitro* study (Bourinbaïar *et al.*, 1993a) low doses of warfarin (10^{-9} - 10^{-8} M) inhibited the infection of lymphocytes - by both adjacent infected cells and free virus - by 50%. These results were corroborated by a second study which also reported a similar activity for 7-hydroxycoumarin (Bourinbaïar *et al.*, 1993b). In a separate study, a structurally-related 4-hydroxycoumarin, phenprocoumon, was found to possess a greater inhibitory activity than warfarin (Thaisrivongs *et al.*, 1994). The data from all sets of experiments indicates that the antiviral effect of the coumarins tested was at least as specific as that of AZT. In addition, the coumarins exhibit greater oral availability and less rapid excretion than the peptidomimetic compounds previously studied as protease inhibitors.

The clinical applications of coumarins are summarised in Table 1.1.

1.1.9 Other uses of coumarin

In addition to their clinical applications, the coumarins are also widely used in analytical science as labelling and derivatising agents and as dyes in laser applications. Many of these applications are linked to the characteristic fluorescence of coumarins, and have recently been reviewed by Cooke *et al.* (1997). The compound 7-amino-4-methyl-coumarin-3-acetic acid (AMCA) is commonly used as a fluorescent label for proteins. The compound has a relatively large Stoke's shift of 100 nm, does not change the isoelectric point of labelled proteins, and is resistant to photobleaching. N-hydroxysuccinimide and sulphosuccinyl esters of AMCA are available, and have been used to label antibodies (Khalfan *et al.*, 1986), cell surface antigens (Aubry *et al.*, 1990) and nucleic acids (Wiegant *et al.*, 1993).

4-methyl-7-hydroxycoumarin (or 4-methylumbelliferone) and its derivatives are frequently used as fluorescent substrates for enzyme assays, due to the fact that conjugates of these compounds exhibit little or no fluorescence until the coumarin moiety is released by enzymic cleavage.

Table 1.1 A summary of the clinical applications of coumarins which have been investigated to date.

Compound	Clinical application	Reference
Coumarin	Cell cycle modulation	Tseng <i>et al.</i> , 1987
	Reduction of oedema	Casely-Smith <i>et al.</i> , 1993
	Haemorrhological effect	Bosse <i>et al.</i> , 1985
	Burn treatment	Piller, 1997
7-hydroxycoumarin	Induction of apoptosis	Marshall <i>et al.</i> , 1991
	Autocrine loop inhibition	Seliger and Petterssen, 1994
	Immunomodulation	Zlabinger, 1997
	Free radical scavenging	Greenspan and Aruoma ,1994
Esculetin	Cytostatic activity	Huang <i>et al.</i> , 1993
8-nitro-7-hydroxycoumarin	Cytostatic/cytotoxic action	Egan <i>et al.</i> , 1997.
4-hydroxycoumarin	Anti-inflammatory effect	Lee <i>et al.</i> , 1981
Phenprocoumon	Protease inhibition	Thaisrivongs <i>et al.</i> , 1994
Warfarin	Anticoagulant	Ikawa <i>et al.</i> , 1944
	Immunomodulation	Berkarda, 1993
	Macrophage reduction	Woo <i>et al.</i> , 1987
	Protease inhibition	Bourinabaiar <i>et al.</i> , 1993a

A variety of conjugates of 4-methylumbelliferone are currently available for use in assays for enzymes as diverse as glucuronidase (Mead *et al.*, 1955) and lysozyme (Yang and Hamaguchi, 1980). They are also of value in the identification of bacterial strains, such as coliforms (Park *et al.*, 1995).

Peptide derivatives of 7-amino-4-methylcoumarin (AMC) have been used for the measurement of protease activity (Kunugi *et al.*, 1985). Derivatives of 7-amino-4-trifluoromethyl coumarin (AFC) are both chromogenic and fluorogenic, and have also been used for the detection of proteases, such as those produced by Gram-negative bacteria (Agban *et al.*, 1990).

The fluorescence of coumarin compounds is sensitive to the presence of various ionic species - a phenomenon which has led to the use of molecules such as esculin and umbelliferone as pH indicators (Dement, 1995) and 3-acetyl-4-hydroxycoumarin for the detection of metal ions (Bhat and Jain, 1960). Coumarin-based compounds have also been used for the measurement of intracellular ion concentrations. One example of this is the use of fluorescent probes containing a coumarin moiety for the detection of intracellular calcium (Iatridou *et al.*, 1994).

Coumarins such as 4-bromo-7-methoxycoumarin (BrMmC) and 4-bromomethyl-7-acetoxycoumarin (BrMaC) can react with carboxylic acids or imidic nitrogens to form fluorescent compounds, and have been used for the detection of a range of chemically and biologically important substances - such as prostaglandins (Wintersteiger and Juan, 1984) - following initial derivatisation and separation by HPLC. Derivatisation with coumarins can also be used to detect biological compounds bearing hydroxy and thiol groups.

The degree of formation of 7-hydroxycoumarin from administered coumarin by the cytochrome P450 enzyme CYP2A6 can be measured fluorescently, and has been used clinically as a test of liver function (Sotaniemi *et al.*, 1995). The measurement of other compounds such as 7-ethoxycoumarin and 7-vinyloxy coumarin may also be used to monitor cytochrome P450 activity. Kobayashi *et al.* (1998) monitored the rate of production of 7-hydroxycoumarin from 7-alkoxycoumarins by mutant cytochrome p450 enzymes expressed by *E. coli*, in a study on the active site of the enzyme.

The broad absorption and fluorescent spectra of coumarin dyes has led to their use in the tuning of lasers to desired wavelengths. One example of this is the use of the coumarin 500

dye to set a laser to 488 nm for the fluorescence imaging of DNA samples undergoing capillary electrophoresis (Nilsson *et al.*, 1995). Coumarin 120 has been used as a dye for the matrix-assisted laser desorption ionisation (MALDI) mass spectrometric analysis of monosulphated oligosaccharides (Dai *et al.*, 1997).

1.2 Biosensors

Many different types of analyte have been studied using biosensor technology. The substances investigated have ranged from large proteins to smaller molecular weight analytes such as toxins and drugs. A biosensor is an analytical device consisting of a biological element integrated with a physico-chemical transducer to form a specific sensing system. The transducer translates the response of the biological component into a readable electrical signal. The first reported biosensor was the "enzyme electrode" described by Clark and Lyons in 1962. This sensor employed the enzyme glucose oxidase, which was immobilised at the surface of a platinum electrode. Glucose in samples was oxidised by the enzyme to form gluconic acid and hydrogen peroxide, and the concomitant decrease in O₂ concentration was detected at the electrode. Since then, a wide and varied selection of different biological components and transduction technologies have been combined to produce biosensors for the detection of a diverse range of analytes. These biosensors have been used in applications ranging from environmental monitoring (Dennison and Turner, 1995) to industrial process control (Ludi *et al.*, 1991) and medicine (Connolly, 1995).

1.2.1 The biological element

The biological element of a biosensor should possess a high degree of specificity and stability, should not contaminate the sample, and should retain biological activity when immobilised (Hall, 1990). The operation of the biological component in a biosensor is based on its specific recognition of analyte, and biosensors may be divided into two categories on the basis of this recognition. Catalytic biosensors employ molecules which bind to, and then alter, the analyte. The products of this catalytic reaction are then detected by the sensor. The other type of biosensor, the affinity sensor, detects the binding event itself.

The range of biological components which have been used in biosensors, and their immobilisation onto sensor surfaces, have been reviewed recently by McCormack *et al.* (1998). Enzymes, cells, tissues, antibodies, antigens, receptors and nucleic acids have all been employed.

The use of glucose oxidase in the first biosensor has already been mentioned. Enzymes are the most frequently used biological component of catalytic biosensors, and glucose biosensors have probably been the most successful (Schumann and Schmidt, 1992). The action of oxidoreductases, involves the transfer of electrons in oxidation/reduction reactions, making them especially well-suited for use in biosensors which detect electrochemical changes.

The inherent lack of stability exhibited by purified enzymes can be overcome by using complete cell or tissue sample as the biological element. Xuili *et al.* (1992) reported a tissue-based biosensor in which rabbit thymus tissue was used to detect adenosine, due to the action of adenosine deaminase. A novel tissue-based biosensor for the detection of the swellfish tetrodotoxin, employing an immobilised frog bladder membrane, was reported by Cheun *et al.* (1996). The membrane contains Na⁺ channels which control the passage of the ion. The blocking of these channels by tetrodotoxin is measured by an attached Na⁺ electrode. Svitel *et al.* (1998) immobilised intact *Gluconobacter oxydans*, *Saccharomyces cerevisiae* and *Kluyveromyces marxianus* cells at the surface of an oxygen electrode, to construct biosensors for the detection of glucose, sucrose and lactose, respectively.

Cell receptors have been used in the production of affinity biosensors. Molecular cell receptors are proteins which bind to specific substances such as hormones, and undergo conformational change. The interaction between receptors and their specific ligand can be detected by means of mass change. As with enzymes, however, purified receptors often display decreased stability. Rogers *et al.* (1991) described the use of the nicotinic acetylcholine receptor AChR to measure fluorescently-labelled neurotoxins. The binding of a range of bacterial toxins to glycolipid cell receptors was investigated by MacKenzie *et al.* (1997), using an optical sensing system.

The binding of complementary strands of DNA is known as hybridisation, and, as it is highly specific in nature, has been used as the basis for affinity biosensors. In this type of sensor, DNA with a particular sequence is immobilised at the sensor surface, and the

binding of DNA with a complementary sequence is monitored, generally by mass change. Fawcett *et al.* (1988) reported such a biosensor for the detection of *Salmonella typhimurium* in food samples. Bianchi *et al.* (1997) described a similar hybridisation biosensor method for the detection of HIV-1 sequences in PCR products. This method was simple, fast and reproducible, and -due to the amplification step – could detect low levels of analyte.

DNA-protein interactions have also been studied using biosensors. The binding of regulatory proteins to particular stretches of DNA controls the translation of various genes. Parsons *et al.* (1995) immobilised double-stranded DNA on a sensor surface to investigate the binding of lactose repressor protein to the *LAC*-operon.

Another class of biological affinity ligands which may be used in biosensors are the lectins. These are molecules which bind to carbohydrate groups with high affinity, and usually possess broad specificity, binding to several different carbohydrates. The use of an immobilised lectin, concanavalin A, in a biosensor to detect the binding of red blood cells has been described (Quinn *et al.*, 1997).

The main group of molecules used in affinity biosensors, however, are antibodies, and analytical devices which exploit the selectivity and sensitivity of these specialised recognition molecules are known as immunosensors.

1.2.2 Immunosensors

Immunosensors may employ either the antibody or its specific antigen as the immobilised component, and both indirect and direct types of immunosensor may be constructed. Indirect immunosensors utilize labels such as fluorophores and enzymes, and can generally be used only for end-point analysis. Direct immunosensors, however, detect the binding of antibody and antigen by measuring changes in parameters such as mass, heat and potential, and can be used for real-time monitoring (Morgan *et al.*, 1996). The ideal immunosensor should possess an intrinsic signal-generation property, a fast response time, and should exhibit reversible binding (Byfield and Abuknesha, 1994). Immunosensors using a variety of different transduction methods have been reported, and some examples these are shown in Table 1.2.

Table 1.2 Some examples of immunosensors based on different transduction technologies.

Transduction method	Analyte	Reference
Potentiometric		
Gas-sensing	Digoxin	Keating and Rechnitz, 1985
Ionophoric	Prostaglandin	Connell <i>et al.</i> , 1983
FET	Atrazine	Colapicchioni <i>et al.</i> , 1991
PSA	HSA	Wang <i>et al.</i> , 1998
Amperometric	Factor VIII	Manning <i>et al.</i> , 1994
Conductimetric	IgG	Sergeyeva <i>et al.</i> , 1998
Piezoelectric		
QCM	2,4-Dichlorophenylacetic acid	Skladal <i>et al.</i> , 1994
SAW	HSA	Weisch <i>et al.</i> , 1996
Calorimetric	Pro-insulin	Birnbaum <i>et al.</i> , 1986
Optical		
Chemiluminescent	HCG	Starodub <i>et al.</i> , 1994
TIR fluorescence	Cocaine	Ogert <i>et al.</i> , 1992
FCFD	Prostate-specific antigen	Daniels, 1995
Ellipsometry	γ -Interferon	Ruzgas <i>et al.</i> , 1992
Interferometry	HCG	Heideman <i>et al.</i> , 1993
IAsys	CLq	Gorgiani <i>et al.</i> , 1997
BIOS-1	Pesticides	Bier and Schmid, 1994
SPR fluorescence	hCG	Attridge <i>et al.</i> , 1991
BIAcore	Biotin	Haines <i>et al.</i> , 1995

1.2.2.1 Potentiometric immunosensors

Potentiometric transduction is based on the accumulation of potential across a membrane/sample interface. Many potentiometric biosensors employ ion-selective electrodes (ISEs), in which only particular ions can pass through the membrane, generating a potential which is proportional to the concentration of ions. The most common example of an ISE is a pH electrode, which is sensitive to hydrogen ions. Gas sensitive potentiometers can be constructed by coupling a pH electrode to a gas-permeable membrane, and measuring the change in pH caused by dissolved gas. A gas-sensing electrode of this type was used by Keating and Rechnitz (1985) to measure digoxin concentrations in an immunosensor utilising an immobilised decarboxylating enzyme in conjunction with a carbon dioxide electrode.

Antibody-sensitive potentiometric immunosensors have also been described, in which ionophoric antigen (or antigen conjugated to an ionophore) is immobilised at the surface of an ISE.

An ionophore is a neutral carrier ligand which can complex metal ions, and the binding of antibody to these antigens alters their ionophoric properties, causing a change in potential. An immunosensor of this type has been reported for the measurement of prostaglandin concentration (Connell *et al.*, 1983). An alternate configuration involves the immobilisation of monoclonal antibody at the electrode membrane (Bush and Rechnitz, 1987).

Field effect transistors (FETs) are basically miniaturised potentiometers in which a semiconductor such as silicon is used to measure charge accumulation. With the addition of an ion-selective electrode to form an ISFET, the background interference and high sample consumption of ISEs can be considerably reduced. The small size of ISFETs also makes them ideal for *in vivo* electrolyte monitoring. Colapicchioni *et al.* (1991) reported an ISFET-based immunosensor for the detection of human IgG and the herbicide atrazine, in which glucose oxidase-labelled antibodies were immobilised onto the ISFET. Binding of the analyte to antibody affected the enzymatic production of H⁺ ions, which were monitored by the ISFET.

Wang *et al.* (1998) have described a disposable immunosensor based on potentiometric stripping analysis (PSA) for the detection of HSA. Anti-HSA was immobilised at the sensor surface, and HSA in samples was allowed to compete with bismuth-labelled HSA for

binding sites. Following the removal of unbound labelled species, Bi^{3+} was released and detected by PSA.

In general, however, potentiometric-based immunosensors have been less than successful, due to their low signal-to-noise ratios and dependence upon environmental conditions, such as pH and ionic strength, which are caused by the low charge density of most biomolecules compared to background interferences (Morgan *et al.*, 1996).

1.2.2.2 Amperometric immunosensors

Amperometric transducers measure the flow of current through an electrochemical cell at constant voltage. The redox reaction of analyte at the sensing electrode generates a current directly proportional to the analyte concentration. Many molecules such as proteins, however, are not inherently electroactive, and must be labelled with oxidoreductase enzymes in order to generate electroactive species. The biological component of an amperometric immunosensor is generally immobilised on a selectively permeable electrode membrane, to prevent protein fouling of the electrode surface and control mass transport (Vagdama and Crump, 1992).

Alkaline phosphatase has been used to label antibodies in a range of immunoassays, including a method for the detection of α -acid glycoprotein with a reported limit of detection of 1 ng/ml (Doyle *et al.*, 1984).

An amperometric immunoassay for theophylline was reported by Athey *et al.* (1993). In this assay system, glucose-6-phosphate dehydrogenase-labelled theophylline was immobilised at the electrode surface, and competed with unlabelled theophylline in the sample for binding to anti-theophylline antibody. Binding of antibody to labelled enzyme blocked the production of NADH. NADH was detected electrochemically, and the current was directly proportional to the concentration of free theophylline in solution.

Manning *et al.* (1994) described an amperometric immunosensor for the determination of the glycoprotein factor VIII in which factor VIII was immobilised on the surface of a glassy carbon electrode. The immobilised protein and factor VIII in samples competed for binding sites on horseradish peroxidase-labelled anti-factor VIII antibody. Hydrogen peroxide and hydroquinone were then added, and horseradish peroxidase (HRP) catalysed the reaction

leading to the production of benzoquinone. Benzoquinone was then reduced electrochemically, and the current was measured.

Santandreu *et al.* (1997) have reported a novel amperometric immunosensor employing a biocomposite which contains graphite, rabbit IgG and resin. The biocomposite acts as both the reservoir for the biomolecule and the transducer. This immunosensor was used in a model system for the detection of rabbit IgG.

Rishpon and Ivnitski (1997) developed a disposable amperometric immunoassay format based on enzyme channelling, which was used in a model system for the detection of bacterial cells and viral antigens.

1.2.2.3 Conductimetric immunosensors

Conductimetric transducers measure the overall conductance of a solution - a property which is frequently altered by chemical reactions. Enzyme-based conductimetric biosensors can be constructed by immobilising an enzyme on a set of electrodes and measuring the conductance of a substrate-containing solution upon application of an electric field.

Although little work on the development of immunosensors based on this principle has been carried out, Sandberg (1993) described a polymer-based conductimetric immunosensor for pesticide detection. Recently, Sergeyeva *et al.* (1998) reported a new approach to conductimetric immunosensors which employed an iodine-selective thin film composed of phthalocyanine. This system was used to detect IgG in buffer and serum samples.

1.2.2.4 Piezoelectric immunosensors

Piezoelectric immunosensors measure the mass change that results from antibody-antigen binding, and are based on a natural property of asymmetric crystals. Piezoelectricity occurs when anisotropic crystals are subjected to mechanical stress, causing the generation of electrical charge. If an AC voltage at the resonance frequency is applied to the crystals, oscillations are induced, and 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 oscillating frequency (Δf) is defined by the Sauerbrey equation:

Equation 1.2.2.4.1

$$\Delta f = \frac{-2f_o^2 \Delta m}{A\sqrt{\mu_q \rho_q}}$$

Where f_o is the resonance frequency of the unloaded crystal, A is its surface area, μ_q is the shear modulus, and ρ_q the density. This phenomenon has led to the use of piezoelectric crystals for the transduction of mass change in immunosensors. Although a variety of naturally abundant crystals, and some man-made polymers and ceramics, possess piezoelectric properties, quartz is the most frequently used. For immunosensor applications, the crystal, situated between two electrodes, is coated with either antigen or antibody, and AC voltage is applied, causing the crystal to oscillate at its normal frequency. Specific binding of the other interactant decreases the frequency of oscillation, which can be related to analyte concentration. This type of immunosensor is known as a quartz crystal microbalance (QCM). The main drawback of QCM-based immunosensors is that the crystals are prone to interference from solvent adsorption and viscous drag when used in liquid phases (Walton *et al.*, 1991). Non-specific binding to the surface is also frequently problematic (Morgan *et al.*, 1996). In order to overcome the problems of liquid phase measurement, the resonance frequency of the crystal can be determined in air prior to the addition of sample. The crystal may then be dried, and any change in resonance frequency due to biospecific binding can be calculated. This so-called "dip and dry" method has been used for antibody-based QCM determination of analytes such as pesticides (Guilbault *et al.*, 1992) and Herpes virus (König and Gratzel, 1994).

Skladal *et al.* (1994) reported a piezoelectric crystal-based method for the direct real-time investigation of the binding of monoclonal antibodies to the herbicide 2,4-dichlorophenoxyacetic acid in solution. Harteveld *et al.* (1997) developed a piezoelectric competitive immunosensor for the detection of the biological warfare agent Staphylococcal enterotoxin B (SEB) in solution, which had a limit of detection of 0.1 $\mu\text{g/ml}$.

Surface acoustic wave - or SAW - transduction also involves the use of piezoelectric crystals. In this system, the oscillation of the crystal is at a higher frequency. By applying a voltage across a group of interlaced metal electrodes known as an interdigital transducer (IDT), an acoustic wave can be generated, and is detected by another IDT situated millimeters away. Any adsorbed substance on the crystal surface, such as a biomolecule,

slows the acoustic wave, and this change in velocity is proportional to the concentration of adsorbed material. The type of SAW transducer most commonly used in biosensors is known as a shear horizontal acoustic plate mode device (SH-APM). SAW biosensors are still at a developmental stage, although Weisch *et al.* (1996) monitored the binding of anti-human serum albumin (HSA) to HAS. Gizeli *et al.* (1997) also reported a SAW-based model immunosensor to detect binding and rate constants for rabbit anti-goat IgG.

1.2.2.5 Calorimetric immunosensors

Calorimetric transduction is based on the measurement of heat generated by a biological reaction, using a thermistor. The biological component can either be directly attached to the thermistor, or bound to a column in which the thermistor is embedded. Although of most use in biosensors based on enzymes, due to their associated heat production, the advent of miniaturised thin-film thermistors lead to the construction of several calorimetric immunosensors. One such device was reported by Mattiasson *et al.* (1977) for the detection of gentamicin. Birnbaum *et al.* (1986) reported a similar method for the measurement of human pro-insulin, which had a limit of detection of 100 ng/ml.

1.2.3 Optical immunosensors

Possibly the largest group of transducers used in immunosensor technology has been the optical transducers. Optical immunosensors do not require the use of a reference cell, do not consume analyte and possess the potential for multi-analyte sensing (Byfield and Abuknesha, 1994). A variety of different types of optical immunosensor format have been reported.

As with the other transduction technologies discussed above, both direct and indirect optical immunosensors can be designed. Enzyme labels which generate fluorescent or luminescent products can be used to produce indirect optical immunosensors. The production of luminescent species removes the need for a light source, and chemiluminescence-based immunosensors for the detection of human chorionic gonadotropin (hCG) and IgG have been described by Starodub *et al.* (1994).

In a direct optical immunosensor, light is directed towards the sensing surface, and then reflected out again. Information about the physical events occurring at the surface can be

obtained from the reflected light. Most sensors of this type, and all commercially available immunosensors, are based on total internal reflection and the generation of an evanescent wave.

1.2.3.1 The evanescent wave

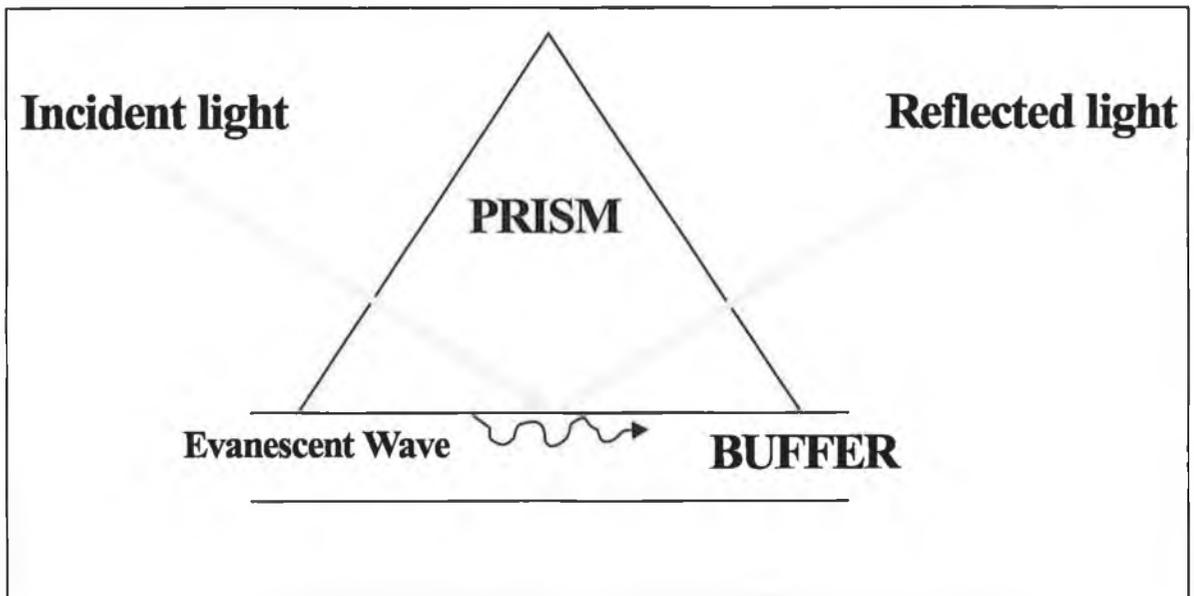
The refractive index of a material is a measure of its ability to bend light, and is the ratio of the speed of light in that material compared to the speed of light in a vacuum. When light is directed through a medium of high refractive index at an angle greater than or equal to the critical angle, total internal reflection (TIR) takes place - in other words, all of the incident light is reflected out of the high refractive index medium, and no refraction occurs. If an interface exists between the high refractive index material (for example, the glass in a prism) and a medium of lower refractive index, then a high-frequency electromagnetic field, known as the evanescent wave, is generated parallel to the interface (Sutherland and Dahne, 1987). The evanescent wave is a fraction of the wavelength of the incident light, and penetrates a short distance (in the order of one wavelength) into the medium of lower refractive index. 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 1.3.

1.2.3.2 Fibre-optic immunosensors

Many of the optical immunosensors which have been described make use of optical fibre technology. Fibre-optic waveguides consist of a high refractive index cylindrical core, composed of glass, quartz or polymer, surrounded by a cladding. If the cladding is removed, and the core of the fibre immersed in sample solution, an evanescent wave can be propagated at the core/sample interface. Optical fibres are inexpensive, flexible, do not experience electrical interference, and can carry several wavelengths of light simultaneously (McCormack, 1995).

In the optical immunosensor format known as TIR fluorescence, the evanescent wave excites fluorescent molecules close to the sensor surface, and the resultant fluorescence is coupled back into the waveguide and measured by a photodetector. This allows fluoroimmunoassays, performed on the fibre surface, to be monitored in real-time, and removes the need for a separation step.

Figure 1.3 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 and a lower refractive index buffer layer, an electromagnetic field component of the light, the evanescent wave, is propagated parallel to the interface.



Ogert *et al.* (1992) reported a flow immunosensor for cocaine based on TIR fluorescence. Antibody to the drug was immobilised on the surface of an optical fibre, and fluorescently-labelled antigen was allowed to bind. Sample was then passed over the surface, and any cocaine present displaced the labelled drug from antibody binding sites. The fluorescent signal obtained was thus inversely proportional to the amount of cocaine in the sample.

A fibre-optic immunosensor for the detection of the pesticide atrazine was described by Oroszlan *et al.* (1993). In this sensor, anti-atrazine antibodies were immobilised on the fibre core, and atrazine in samples competed with pesticide labelled with fluorescein isothiocyanate (FITC) for antibody binding sites. This assay had a measuring range of 0.5 - 200 nM.

Hanbury *et al.* (1997) developed a fibre-optic immunosensor for the detection of myoglobin. In this system, fluorophore-labelled anti-myoglobin was entrapped within a polyacrylamide gel at the distal end of an optical fibre. Binding of myoglobin to antibody resulted in quenching of fluorescence due to energy transfer between the fluorophore and the haem group of myoglobin.

1.2.3.3 Fluorescence capillary flow device (FCFD)

Another type of fluorescent immunosensor is the fluorescence capillary flow device (FCFD), first described by Badley *et al.* (1987). In this device, two parallel glass plates are held 0.1 mm apart. The lower plate bears immobilised antibody of the required specificity and fluorescently-labelled antigen is dosed onto the upper plate in such a way that it dissolves in the presence of sample. When sample is added, free and fluorophore-labelled analyte compete for antibody binding sites.

A light source is focussed onto the lower plate, and when light is directed at the plate, antibody-bound fluorophore becomes excited by the evanescent wave. An aperture in front of the photodetector ensures that only fluorescence from antibody-bound molecules is detected. The observed fluorescence is inversely proportional to the amount of analyte in the sample. This system was used by Daniels (1995) for the measurement of prostate-specific antigen in blood samples, with a limit of detection of 3.3×10^{-12} M.

1.2.3.4 Attenuated total internal reflection

The technique of attenuated total internal reflection is based on the measurement of the reduced intensity of reflected light due to the absorption of energy by the evanescent wave. An optical film absorbs reflected incident light, and the attenuated light intensity is calculated as a fraction of the incident wavelength. A reflectance method of this type has been reported for monitoring immunological reactions (Arwin and Lundstrom, 1985), but the system is not in widespread use.

1.2.3.5 Diffraction grating

Tsay *et al.* (1991) described a direct optical immunosensor based on the action of a diffraction grating. Antibody or antigen was immobilised on a silicon surface, onto which light was directed. Interaction with the other binding partner resulted in the formation of a grating on the surface, which caused the diffraction of light.

1.2.3.6 Ellipsometry

The optical technique of ellipsometry measures the change of phase of polarisation state of light as a result of reflection from a planar surface, and can be used to study the binding of proteins. Light is directed onto a mirrored surface, and any changes in phase and amplitude of reflected light are related to the amount of absorbed protein on the surface. This method has been used in immunosensors for the determination of gamma-interferon and human serum albumin (Ruzgas *et al.*, 1992).

1.2.3.7 Integrated optics (IO) immunosensors

Planar optical waveguides are an alternative to optical fibres, and have been used in the construction of optical immunosensors. These waveguides consist of a high refractive index dielectric film sandwiched between two dielectric materials of lower refractive index. (A dielectric medium is a solid which contains no free charges.) Materials currently employed in waveguides include glass, silica and lithium niobate. The use of miniaturised waveguides in biosensors is known as integrated optics (IO), and several IO-based immunosensors have been reported.

1.2.3.7.1 Interferometry

The optical technique of interferometry is based on characteristic interference of light beams, and, if combined with a flow cell, can be used to measure biomolecular interactions. A single polarised light wave is composed of two partial waves: an electric field (TE) and a magnetic field (TM). A ligand is immobilised on the surface of a waveguide and a polarised laser is directed into the waveguide, causing the excitation of TE and TM fields. The evanescent wave generated within the waveguide interacts with immobilised ligand, and binding of analyte causes a change in the effective refractive indices of TE and TM fields. These are reflected backwards and forwards between mirrored surfaces in the device, and each time light is reflected, some couples out. TE and TM waves which are coupled out of the waveguide interfere with each other, and changes in refractive index at the sensor surface can be measured by continuously monitoring changes in the relative phase between TE and TM. This method was used by Heideman *et al.* (1993) to study the binding of immobilised anti-hCG antibody to its specific antigen. Brecht and Gauglitz (1995) used interferometry to measure the concentration of atrazine in drinking water, in a competitive immunosensor with a limit of detection of 0.1 ppb.

1.2.3.7.2 IAsys biosensor

A waveguide-based biosensor which combines the action of the evanescent wave with interferometry has been described by Cush *et al.* (1993), and is marketed as IAsys by Fisons Applied Sensor Technology. The sensor consists of a glass prism coated with a thin layer of silica and a high refractive index resonant layer (usually titanium), which is in contact with the sample solution. A laser beam is directed into the prism, where it undergoes total internal reflection. At a specific angle - the resonance angle - a fraction of this light is directed towards the resonant layer. An evanescent wave is generated at the interface between sample and resonant layer, and travels along this interface for a short distance (approximately 1 mm), before coupling back into the prism. The angle of incident light at which this occurs can be detected by monitoring the phase of reflected TE and TM modes. These two modes are in phase before resonance is reached, but the coupling of light into the resonant layer produces a delay in the reflected light, and causes TE and TM to shift out of

phase. The binding of biomolecules at the sensing surface changes the resonance angle at which this occurs, and can be measured in real-time.

The IAsys biosensor utilises a disposable micro-cuvette sample cell consisting of the prism, silica coupling layer and resonant layer coated with dextran for covalent attachment of biomolecules (Fortune, 1993). The micro-cuvette is stirred constantly, which reduces any mass transport limitation of analyte to the sensor surface, and the use of a well-based system also minimises sample consumption.

Hall and Winzor (1997) immobilised a monoclonal antibody to apocarboxypeptidase A on the surface of the IAsys sensor, and used a stepwise titration procedure to thermodynamically characterise the interaction between antibody and apocarboxypeptidase A. The equilibrium association constant for the interaction was calculated.

Gorgiani *et al.* (1997) used the IAsys instrument to derive affinity constants for the interaction between the complement component CLq and human and rabbit IgG

1.2.3.7.3 Grating coupler biosensor

A grating coupler is a set of fine corrugations which is etched into the surface of a planar waveguide film, causing light to couple in and out of the waveguide. An IO biosensor employing a grating coupler has been described by Tiefenthaler (1993), and is marketed by Artificial Biosensing systems as the BIOS-1. In this sensor the grating coupler is located between the glass substrate and the waveguiding film. The sample layer is situated on the other side of the film. A laser directs polarised light into the waveguide, and the grating causes it to couple in and out, creating an evanescent wave. The coupling angle of the input laser beam is proportional to the amount of binding at the sample/waveguide interface. This "incoupling angle" is continuously monitored by rotating the sensor surface against a fixed-angle beam of light, affording real-time analysis of biomolecular binding events. A BIOS-1 based immunosensor for the detection of nanomolar amounts of pesticides has been reported by Bier and Schmid (1994).

1.2.3.8 Surface plasmon resonance (SPR)

Several immunosensors based on the phenomenon of surface plasmon resonance (SPR) have been reported. SPR is a physical phenomenon related to the evanescent wave. If the

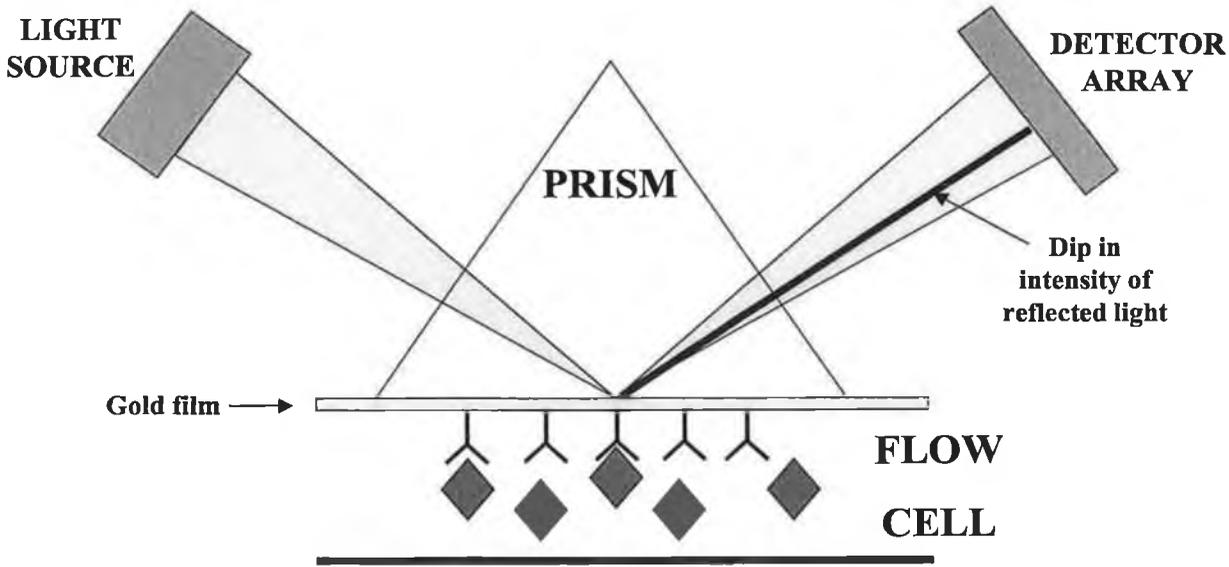
interface between the medium of higher and lower refractive indices is coated with a thin layer of metal, then the propagation of the evanescent wave will interact with the electrons of the metal layer. Metals contain electron clouds at their surface which can couple with incident light at certain angles. These electrons are called plasmons, and the passage of the evanescent wave through the metal layer causes the plasmons to resonate, forming a quantum mechanical wave known as a surface plasmon. This surface plasmon wave takes up some of the energy of the incident light, and this can be observed as a "dip" in the intensity of reflected light at a certain angle (Panayotou *et al.*, 1993). The angle at which this dip occurs is called SPR angle, and is dependent upon the refractive indices of the prism, metal and sample layers, as well as the wavelength and polarisation state of the incident light. If all the other factors are constant, however, the SPR angle measures the refractive index at the metal film side of the interface. The refractive index is directly proportional to concentration, and it is this property which is exploited to monitor biomolecular interactions.

Attridge *et al.* (1991) combined a fluorescent label with an SPR detector to create an SPR fluoroimmunosensor for the determination of hCG in serum. Severs and Schasfoort (1993) reported an enhanced SPR-based immunoassay which they called the Enhanced SPR Inhibition Test (ESPRIT). In this system, a competition is set up at the SPR sensor surface for antibody binding sites. Binding of antibody to the sensor surface is thus inversely proportional to the amount of free analyte in solution. In a second enhancement step, latex particles coated with antigen are added. These particles bind to the antibody at the sensor surface, and increase the SPR signal.

1.2.3.8.1 BIAcore

The most popular SPR-based biosensor in current use is the BIAcore, manufactured by Pharmacia (Jönsson, 1991; Fägerstam and O'Shannessy, 1993). This is a fully automated system which combines continuous flow and SPR detection to perform label-free real-time biospecific interaction analysis (BIA). The operation of SPR in the BIAcore instrument is shown in Figure 1.4.

Figure 1.4 The operation of surface plasmon resonance (SPR) in BIAcore. Light from a high-intensity LED is directed into a prism, and the evanescent wave which is generated excites surface electrons (“plasmons”) in a gold layer, leading to a dip in the intensity of reflected light at a particular angle known as the SPR angle. This SPR angle is sensitive to the refractive index at the gold film side of the interface, and any biospecific interaction, for example, the binding of antibody to antigen, changes the SPR angle.



The basic disposable sensor chip used with BIAcore is illustrated in Figure 1.5, and consists of a glass slide covered in a 50 nm thick gold film, onto which a carboxymethylated dextran matrix is attached by a hydroxyalkyl thiol linker layer. (Gold is very suited for use in SPR, as it is chemically inert and generates sharp reflectance minima.) The carboxymethylated dextran (CM-dextran) layer is about 100 nm thick, and facilitates the covalent attachment of biomolecules by a variety of different immobilisation chemistries.

This sensor chip docks with the optical system of the instrument via an opto-interface layer of silicone polymer. A wedge-shaped beam of light from a near-infrared high-intensity light-emitting diode (LED) passes through the prism, generating SPR at the gold film surface, and a 2-dimensional photodiode array at the other side of the prism measures the intensity of reflected light at all angles. The change in SPR angle caused by biomolecular interaction at the sensor surface is shown in Figure 1.6. In the BIAcore, this change is monitored continuously and displayed on a PC in the form of a sensorgram, in which resonance units (RUs) are plotted against time. All proteins, regardless of amino acid sequence, give the same refractive index change, and a signal of 1,000 RU corresponds to a 0.1° shift in the SPR angle, which is equivalent to a protein concentration of 1 ng/mm^2 at the sensor surface. The dynamic range of the instrument is between 1 and 30,000 RU.

A typical sensorgram for binding of a biomolecule to immobilised ligand is shown in Figure 1.7. The initial level baseline is due to running buffer flowing over the sensor surface. Injection of sample over this surface causes an initial sharp rise in signal. This is due to a difference in bulk refractive index between the running buffer and the sample plug. The binding of the two interactants can be seen as a steady increase in signal over the course of the injection. A sharp decrease in signal at the end of the injection indicates that running buffer of lower refractive index is again passing over the surface. The difference in response units (RU) between the signal after the injection and the initial baseline corresponds to the amount of sample which remains bound to the surface.

The BIAcore possesses several advantages over other optical biosensor systems based on evanescent wave or SPR sensing. The use of the novel CM-dextran hydrogel provides a convenient attachment for biomolecules and acts as a physical barrier to protect the gold film from the non-specific adsorption of protein to which previously-reported SPR sensors were prone (Cullen and Lowe, 1990).

Figure 1.5 Diagrammatic representation of a BIACore sensor chip. The SPR signal is generated at a 50 nm gold layer on a glass slide. A hydroxyalkyl thiol linker layer connects this gold film to a carboxymethylated dextran gel, onto which biomolecules may be covalently immobilised.

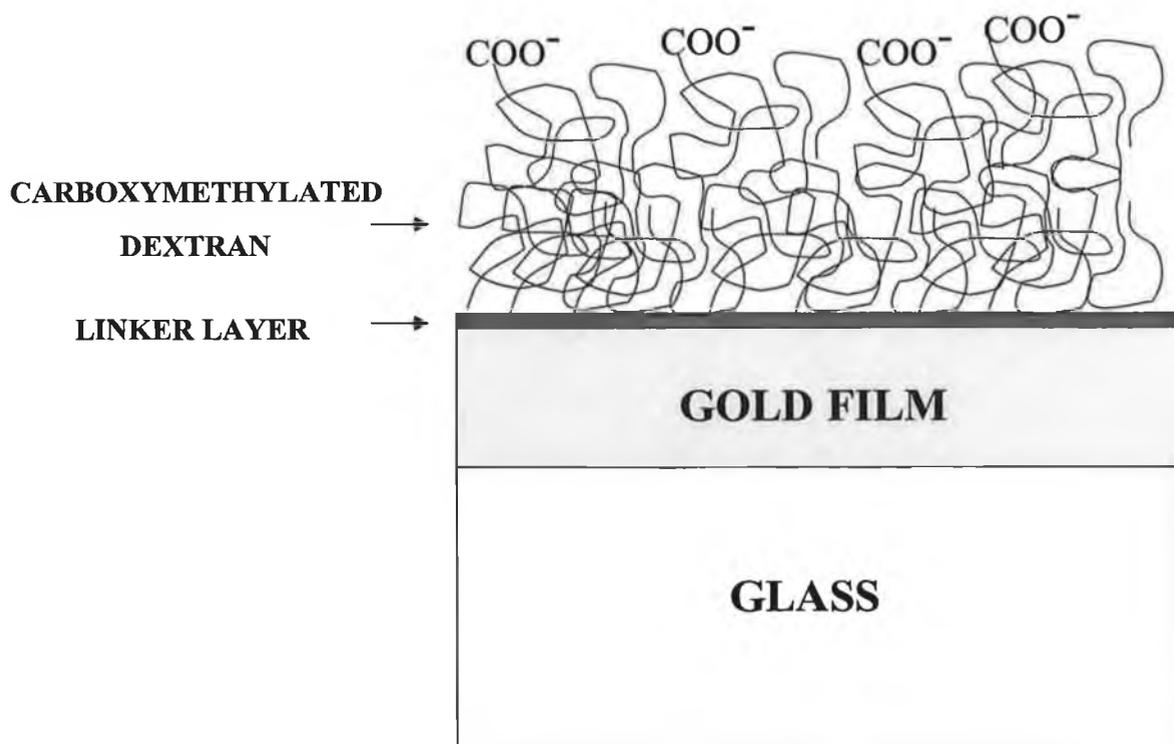


Figure 1.6 The SPR angle can be seen as a dip in the intensity of reflected light. In the BIAcore, light reflected through the prism is analysed by a 2-dimensional photodiode array. Biomolecular interaction at the sensor chip surface causes a mass change, and an increase in the SPR angle. The change in SPR angle is monitored continuously and displayed as a sensorgram.

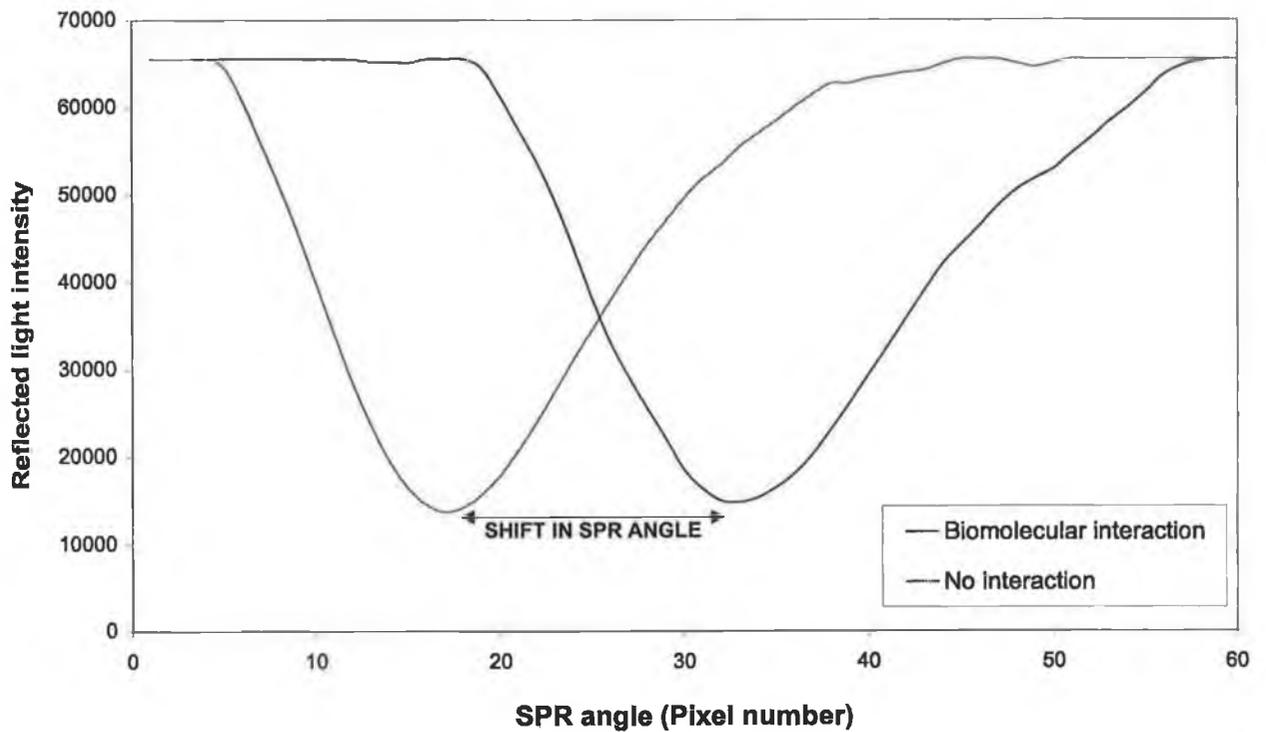
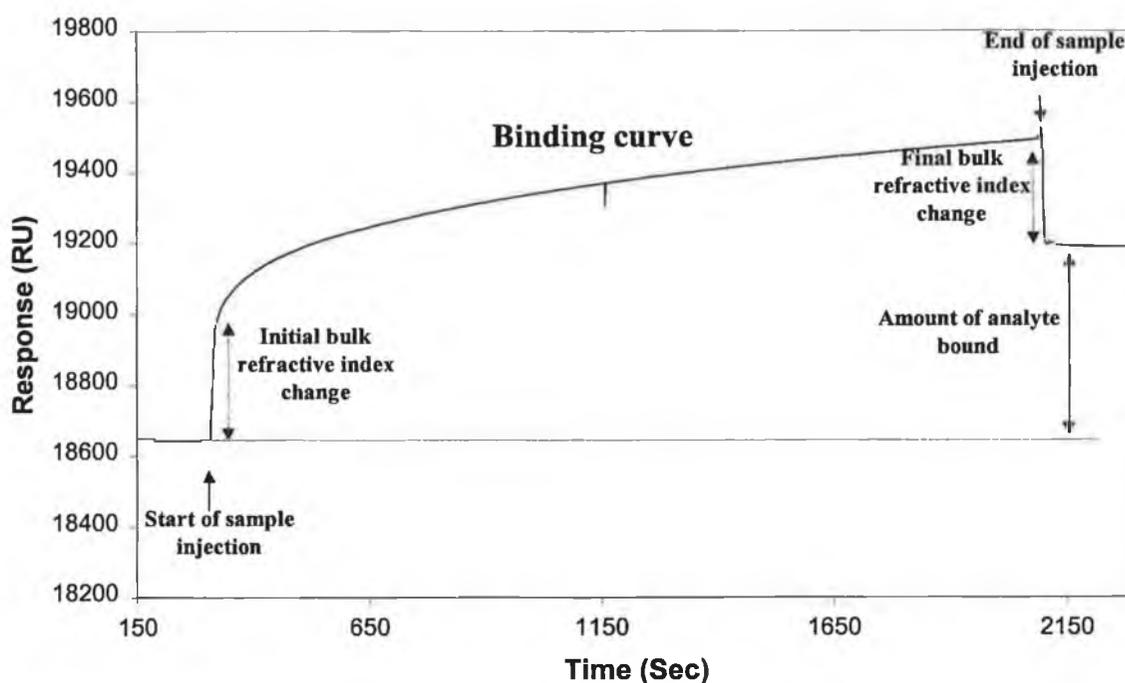


Figure 1.7 A typical BIAcore sensorgram for binding of analyte to immobilised ligand. The initial steady baseline is due to flow of running buffer over the immobilised ligand surface. Injection of sample causes an initial sharp signal increase due to differing bulk refractive index. The binding of the two interactants can then be seen as a gradual increase in signal. The signal falls sharply at the end of the sample injection, as lower refractive index buffer again flows over the surface. The difference in RUs between the initial baseline and the signal after the sample injection represents the amount of analyte bound.



On average, only 40% of the carboxy groups on the dextran matrix are derivatised in a typical immobilisation procedure, and the electrostatic repulsion caused by the remaining COO⁻ groups confers 3-dimensional mobility upon the covalently-bound biomolecules. The movement of immobilised molecules thus approximates their behaviour in solution.

The delivery of sample to the sensor surface is carried out by an autosampler, together with an integrated micro-fluidics system (IFC), which optimises sample transport while minimising consumption. A sample recovery feature reduces sample consumption still further. The IFC consists of a series of precision-cast channels in a hard silicon polymer, with small diameter sample loops and a low dead volume (approximately 0.4 µl) which is controlled by a set of pneumatically operated diaphragm valves, enabling the accurate delivery of samples between 1 and 50 µl in volume (Sjolander and Urbaniczky, 1991). Larger sample volumes (up to 750 µl) can be injected directly from the autosampler needle. Upon docking with the sensor chip, the IFC forms four parallel flow cells - each of which is 50 µm thick with a volume of 60 nl - allowing four different interactants to be immobilised and studied separately. The flow of running buffer and sample through the system is controlled by precision pumps, and flow rates of 1 - 100 µl can be used. The programmable autosampling facility allows for extensive unattended operation, and can accommodate large sample numbers and replicates.

Data collection is handled by the controlling PC, and sensorgrams are displayed instantaneously. Dedicated software is available for the determination of concentration, kinetic and affinity data. The instrument has a large dynamic measuring range and concentrations from 10⁻¹¹ to 10⁻³ M, affinity constants from 10⁵ to 10¹⁰ M⁻¹, and rate constants from 10³ to 10⁶ have been determined (Griffiths and Hall, 1993).

The BIAcore system does have some inherent drawbacks. Due to the nature of SPR detection, only molecules with a relatively high molecular mass (> 2,000 Da) can be directly sensed. The interaction of smaller molecules must be detected by indirect methods such as competitive assays. Binding events involving large structures like cells may also be difficult to detect due to the limited penetration depth of the evanescent wave (approximately 1 µm). In addition to these intrinsic problems, the high cost of BIAcore sensor chips must be considered.

The BIAcore instrument described here was the first to be made commercially available. Several other related products have since been launched on the market. The BIAlite system is a miniaturised BIAcore, which lacks autosampling and programming facilities. BIAcore X, a similar instrument, has a wider dynamic range and lower detection limit in terms of analyte molecular weight (approximately 200 Da). The most recent product, BIAcore 2000, combines these features with an increased signal-to-noise ratio and multi-channel operation. BIAcore has been used to study a wide range of biomolecular interactions including the specific binding of antibody to antigen, enzyme to substrate, receptor to ligand and the interaction between complementary sequences of DNA. Perhaps the most interesting aspect of the system is its ability to detect biospecific binding in real-time, allowing the determination of association and dissociation rates.

The use of the technique in immunosensor systems for concentration determination, kinetic and affinity analyses, and antibody production - as well as nucleic acid studies - is described in the following chapters.

Aims

The aim of this project was to investigate the use of optical biosensor technology for the study of the coumarin family of compounds. The performance of BIAcore, an optical biosensor based on the phenomenon of surface plasmon resonance (SPR), was compared to more established methods of analysis.

Chapter 3 describes the production of polyclonal antibodies to 7-hydroxycoumarin, their purification and use in a competitive ELISA. Hybridomas generated by somatic cell fusion, using animals immunised with 7-hydroxycoumarin-BSA, were tested for specific antibody production using ELISA and BIAcore. A genetically generated antibody fragment to a joint coumarin-BSA epitope was studied using BIAcore, and kinetic and affinity constants were derived. The possibility of using BIAcore for the panning of naïve libraries of phage display antibodies was also examined.

In Chapter 4, the polyclonal antibodies to 7-hydroxycoumarin were used in the development of a BIAcore-based competitive immunosensor for the detection of free drug in serum samples. The method displayed good reproducibility and linearity.

Chapter 5 concerns comparative BIAcore and ELISA studies on two commercial antibody preparations against the coumarin aflatoxin B₁. Competitive and direct immunoassay formats, using both direct and indirect immobilisation, were investigated. The importance of antibody affinity, and the use of ELISA methods for initial optimisation of immunosensor performance, are discussed.

In Chapter 6 the action of the coumarin antibiotic on the DNA-modifying enzyme topoisomerase II was investigated. Supercoiled plasmid DNA was labelled with biotin and immobilised on the surface of the BIAcore sensor chip and the inhibition of topoisomerase-DNA binding was visualised in real-time. Direct interactions between coumarin-protein conjugates and immobilised DNA were also studied.

CHAPTER 2

MATERIALS AND METHODS

2.1 Materials and equipment

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

Reagent	Supplier
Acetic acid Hydrochloric acid Sodium chloride Tween 20	Riedel de-Haen, Hannover, Germany
Bicinchoninic acid assay (BCA) kit	Pierce and Warriner (UK) Ltd., Chester, England
Biotin-Chem-Link kit	Boehringer-Mannheim Ltd., Lewes, East Sussex, England
BriClone Normal Rat Kidney (NRK) cells	BioResearch Ireland, Dublin City University, Ireland
CM-Dextran	Fluka Chemicals, Gillingham, Dorset, England
HPLC-grade solvents	Lab-Scan, Stillorgan, Dublin, Ireland
Hydrogen peroxide	BDH Chemicals Ltd., Poole, Dorset, England
Topoisomerase II	United States Biochemical Corp., Cleveland, Ohio, USA.
Wizard Plus Mini-prep kit	Promega, Madison, WI, USA

The equipment used and its suppliers are listed below.

Equipment	Supplier
Beckman L8-70M Ultracentrifuge	Beckman Instruments Ltd., Fullerton, CA, USA
BIAcore 1000	Pharmacia Biosensor AB, Uppsala, Sweden
Diaphot Inverted Microscope	Nikon, Tokyo, Japan
Eppendorf tubes Sterile universal containers	Sarstedt, Wexford, Ireland
Heraeus Labofuge GL Titretek Twinreader Plus	Medlabs, Dublin, Ireland
HB 2448K Laminar flow cabinet	Holten, Allerod, Denmark
NUNC Maxisorb plates NUNC Covalink plates	NUNC, Kamstrup DK, Roskilde, Denmark
PREM PCR machine	Wessex Instrumentation Ltd., Hampshire, England
3015 pH meter	Jenway Ltd., Essex, England
Orbital incubator	Gallenkamp, Leicester, England
RM6 Lauda waterbath	AGB Scientific Ltd., Glasnevin, Dublin, Ireland
SB1 Blood tube rotator	Stuart Scientific, London, England.
Sterile cell culture lab-wear	Brownes, Foxrock, Dublin, Ireland
UV-160A spectrophotometer	Shimadzu Corp., Kyoto, Japan
UVP ImageStore 7500 gel documentation system	Ultra Violet Products, Upland, CA, USA.

Methods

2.2 Antibody production

2.2.1 Licensing

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

2.2.2 Immunisation protocol for the production of rabbit anti-7-hydroxycoumarin 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 1.5 mg/ml solution of 7-hydroxycoumarin-thyroglobulin in PBS was prepared. An equal volume of Freund's Complete Adjuvant was added, and the mixture was vortexed until it formed an emulsion. 1 ml of this preparation was then injected subcutaneously at several sites into a New Zealand White rabbit. This procedure was repeated 21 days later, with Freund's Incomplete Adjuvant being used. The animal was bled from the marginal ear vein 11-13 days after this immunisation, and the cycle of reboosting and bleeding was continued until a sufficiently high specific antibody titre in the serum was obtained.

2.2.3 Immunisation protocol for BALB/c mice for the production of monoclonal antibodies

A 2 mg/ml solution of 7-hydroxycoumarin-ovalbumin conjugate in PBS was prepared. An equal volume of Freund's Complete Adjuvant was added, and the mixture was vortexed until it formed an emulsion. 6-10 week old BALB/c mice were anaesthetised with Halothane, and 250 µl of the immunogen was injected subcutaneously at several sites at volumes of approximately 250 µl. On day 21, a similar dose of immunogen in Freund's Incomplete Adjuvant was administered intraperitoneally, and on day 28 a blood sample was taken from the tail. Intraperitoneal boosts followed by blood sampling were continued until a satisfactory titre was obtained.

3-4 days prior to being sacrificed, the mice were injected with 250 μ l of 2 mg/ml 7-hydroxycoumarin protein conjugate in PBS. This was administered intravenously via the tail vein.

2.3 Preparation of serum

2.3.1 Preparation of rabbit serum

For estimation of titre, 5 ml blood samples from the marginal ear vein, collected in Sterilin universal containers, were allowed to clot for 2 h at room temperature, and the clot allowed to tighten overnight at 4°C, before being centrifuged at 4,000 rpm for 20 minutes. The supernatant was then removed and stored at -20°C. For purification of polyclonal antisera, the animal was anaesthetised and sacrificed by cardiac puncture, and the collected whole blood treated as described above.

2.3.2 Preparation of mouse serum

For the estimation of specific antibody titre in mouse blood, 10-20 μ l of blood was taken from the tail, and allowed to clot at room temperature for 30 minutes. Following centrifugation at 13,000 rpm for 20 minutes the supernatant was removed and stored at -20°C.

2.4 Polyclonal antibody purification

Rabbit serum was first partially purified by saturated ammonium sulphate precipitation, and then affinity-purified by column chromatography using protein G.

2.4.1 Saturated ammonium sulphate precipitation

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

2.4.2 Protein G chromatography

A protein G column was prepared by pouring 1 ml of protein G immobilised on sephadex into a 5 ml syringe. The column was equilibrated with 20 ml PBS. 2.5 ml of the dialysate from ammonium sulphate precipitation was added to the column. 1 ml PBS was added to the eluate, and the whole amount was then re-applied to the column. 4 ml PBS was added to this eluate before reapplication. All the collected eluate was then applied to the column again. The column was then washed with 25 ml of PBS. Bound immunoglobulin was then eluted by addition of 0.1 M glycine/HCl, pH 2.5, to the column. Twelve 1 ml fractions were collected in eppendorf tubes to which 100 μ l of 1.5 M Tris/HCl, pH 8.7, had been added in order to neutralise the pH and prevent denaturation of antibody.

The presence of protein in fractions was determined by monitoring their absorbance at 280 nm, and those fractions containing protein were pooled and dialysed overnight at 4°C against 5 l PBS, with two changes of buffer. The protein G column was stored for re-use at 4°C in PBS containing 20% (v/v) ethanol.

2.5 Bicinchoninic acid (BCA) protein assay

Standard protein solutions from 0.1 - 1 mg/ml in PBS were prepared using either bovine serum albumin (BSA) or - if determining immunoglobulin concentrations - IgG. To 10 μ l of standard or sample in wells of 96-well microtitre plates, 190 μ l of BCA working reagent was added. After gentle mixing, the plate was incubated at 37°C for 30 minutes. The absorbance of the wells at 560 nm was then measured using a microtitre plate-reader, and a standard curve was constructed from which the protein concentrations of samples were calculated.

2.6 Polyacrylamide gel electrophoresis

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed to assess antibody purity.

The composition of gels, electrophoresis buffer and sample loading buffer are given in Table 2.1. Samples were diluted with sample loading buffer (4:1, sample : buffer) and boiled for 10 minutes. 10 μ l of samples were loaded onto the gel.

Table 2.1 Composition of stacking gel, resolving gel, electrophoresis buffer and sample loading buffer for SDS-PAGE.

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

Pre-stained molecular weight markers containing carbonic anhydrase (29 kDa), ovalbumin (45 kDa), BSA (66 kDa), phosphorylase B (97.4 kDa), β -galactosidase (116 kDa) and myosin (205 kDa) were also loaded. The gel was electrophoresed at 200mV until the dye had migrated to the bottom. Gels were stained for 20 minutes in 0.1% (w/v) Coomassie Brilliant Blue in acetic acid : methanol : water (2:9:9 v/v/v), and destained overnight in acetic acid : methanol : water (1:1:8 v/v/v). Destained gels were visualised using a UVP ImageStore 7500 gel documentation system .

2.7 Enzyme-linked immunosorbent assay (ELISA)

A range of different ELISA methods were used for the detection of antibodies in rabbit and mouse serum and hybridoma supernatants, the quantitative determination of 7-hydroxycoumarin and aflatoxin B₁, and assessment of the cross-reactivity of purified antibody. In addition, ELISAs were used to study regeneration conditions for an immunosensor, and to derive an equilibrium affinity constant for a monoclonal antibody. Both sandwich and competitive ELISA formats were employed. PBS containing 0.05% (v/v) Tween 20 was used to wash microtitre plates, and is referred to throughout as PBST.

2.7.1 Estimation of rabbit antibody titres

Levels of specific antibody in serum from immunised rabbits were measured using ELISA. 7-hydroxycoumarin-BSA (10 μ g/ml) was added to wells of a 96-well microtitre plate (NUNC Maxisorb) at 100 μ l/well, and incubated at 37°C for 1 h. The plate was then washed three times in PBST and once in PBS. 150 μ l/well of 1% (w/v) BSA in PBS (blocking solution) was then added to the plate, which was again incubated for 1 h at 37°C. The plate was then washed three times in PBST and once in PBS. (For both coating and blocking of plates, incubation steps could also be carried out at 4°C overnight.)

Serum from both immunised and control (unimmunised) rabbits was then diluted from 1/200 to 1/50000 in PBS and added to appropriate wells at 100 μ l/well. The plate was incubated for 1 h at 37°C. The plate was washed three times in PBS and once in PBST. Commercial anti-rabbit IgG antibody labelled with horseradish peroxidase (HRP) at a dilution of 1/10,000 in PBS was then added to the plate at 100 μ l/well. The plate was incubated for 1 h at 37°C.

After washing three times in PBST and once in PBS, chromogenic substrate for HRP (10 mg o-phenylenediamine (OPD) in 25 ml of citrate/phosphate buffer, pH 5.0, with 5 µl of 30% (v/v) H₂O₂) was added to the plate at 100 µl/well. The plate was incubated at 37°C for 15-30 minutes, or until the colour produced was sufficiently strong. The absorbance of the wells at 405 nm was then measured using a microtitre plate-reader.

2.7.2 Determination of the working dilution of purified rabbit antibody in a competitive ELISA for 7-hydroxycoumarin.

A 96-well microtitre plate (NUNC Maxisorb) was coated with 10 µg/ml of 7-hydroxycoumarin-BSA and blocked with 1% (w/v) BSA as described in section 2.7.1. A 1 mg/ml stock solution of 7-hydroxycoumarin in a mixture of 30% (v/v) ethanol and 70% (v/v) PBS was prepared by dissolving 10 mg of the drug in 3 ml of absolute ethanol, and slowly adding 7 ml PBS. This stock solution was diluted in PBS to prepare standard solutions of 100, 80, 40, 20, 10, and 5 µg/ml. Dilutions of purified rabbit anti-7-hydroxycoumarin in PBS with 1% (w/v) BSA from 1/50 to 1/2,000 were prepared.

A set of standard curves at each different antibody dilution was produced by adding 50 µl of appropriate standard and 50 µl of diluted antibody to each well, mixing gently, and incubating for 1 h at 37°C. After washing three times with PBST and once with PBS, 100 µl/well of a 1/10,000 dilution of commercial HRP-labelled anti-rabbit antibody were added, and colour was developed and read as in section 2.7.1.

2.7.3 Covalent coupling of 7-hydroxycoumarin-4-acetic acid to Covalink plates for estimation of antibody cross-reactivity

EDC-mediated N-hydroxysuccinimide (NHS) esterification was used to directly link 7-hydroxycoumarin to amine groups on derivatised 96-well microtitre plates. To solutions of 7-hydroxycoumarin-4-acetic acid (1-5 mg/ml) in absolute ethanol, equimolar amounts of NHS and 3-ethyl-3-(3-diethylaminopropyl)carbodiimide (EDC) were added. The reaction mixtures were transferred to 10 ml blood tubes and mixed at room temperature on a blood tube rotator for 30 minutes 150 µl of the mixtures was then added to wells of a NUNC Covalink plate, and incubated for 1 h at 37°C (or overnight at room temperature). The wells were washed three times in PBST and once in PBS.

150 μ l of blocking solution was then added to each well, and the plate was incubated for 1 h at 37°C - or overnight at room temperature. The plate was then washed three times in PBST and once in PBS.

2.7.3.1 Estimation of cross-reactivity of purified polyclonal anti-7-hydroxycoumarin

NUNC Covalink plates were coated with 7-hydroxycoumarin and blocked as described in section 2.7.3. Stock solutions of coumarin, 4-hydroxycoumarin, 7-hydroxycoumarin, 7-hydroxycoumarin-4-acetic acid, warfarin and 7-hydroxywarfarin were prepared in methanol, and diluted in PBS to a range of concentrations - 50, 25, 12.5, 6.25 and 3.125 μ g/ml. 50 μ l of each solution was added to appropriate wells of the microtitre plate along with 50 μ l of purified polyclonal anti-7-hydroxycoumarin (1/200 in PBS). The plates were incubated for 1 h at 37°C and then washed three times in PBST and once in PBS. 100 μ l/well of HRP-labelled anti-rabbit IgG was then added, and the plate incubated for 1 h at 37°C. Colour was developed and read as in section 2.7.1.

2.7.4 Estimation of mouse antibody titres

Levels of specific antibody in serum from immunised mice was estimated by ELISA. 96-well microtitre plates were coated with 100 μ l/well of ovalbumin, BSA, and two different 7-hydroxycoumarin-BSA conjugates (diazo- and NHS/EDC-coupled) at 50 μ g/ml and incubated for 1 h at 37°C. The plates were blocked with 5% (v/v) FCS as in section 2.7.3. Serum from both immunised and control (unimmunised) mice was then diluted from 1/200 to 1/12,800 in PBS containing 1% (w/v) BSA and 5% (v/v) FCS and added to appropriate wells of the above plates, and to wells of a Nunc Covalink plate which had been coated as in section 2.7.3, using a 5 mg/ml solution of 7-hydroxycoumarin-4-acetic acid. In addition, similar dilutions of polyclonal anti-7-hydroxycoumarin antibody, purified as in section 2.4, were added. The plates were then incubated at 37°C for 1 h. After washing three times with PBST and once with PBS, 100 μ l/well of either HRP-labelled anti-mouse or anti-rabbit IgG diluted 1/5,000 with PBS containing 1% (w/v) BSA and 5% (v/v) FCS was added to each plate. Colour was developed and read as described in section 2.7.1.

2.7.5 Screening of hybridoma supernatants

Cell culture supernatants from hybridomas produced by cell fusion were screened for specific antibody production by ELISA. 96-well microtitre plates were coated with 100 μl /well of 7-hydroxycoumarin-protein conjugate or native protein at 50 $\mu\text{g}/\text{ml}$ and blocked with 5% (v/v) FCS as in section 2.7.3.

Neat hybridoma supernatant and positive control (1/200 polyclonal anti-7-hydroxycoumarin antibody) were added to both plates at 100 μl /well, followed by incubation for 1 h at 37°C. After washing three times in PBST and once in PBS, HRP-labelled antibodies were added and colour developed and read as in section 2.7.1.

2.7.6 Competitive ELISA for the quantitative determination of aflatoxin B₁.

Commercially available monoclonal and polyclonal antibodies were used in a competitive ELISA format for the detection of aflatoxin B₁ (AFB₁). 96-well microtitre plates were coated with 300 μl /well of 0.5 $\mu\text{g}/\text{ml}$ of aflatoxin B₁ - BSA conjugate in PBS, and incubated for 1 h at 37°C.

The plates were then washed three times with PBST and once with PBS. 350 μl /well of 5% (v/v) FCS in PBS (blocking solution) was then added to the plates, which was again incubated for 1 h at 37°C. The plates were then washed three times in PBST and once in PBS. (For both coating and blocking of plates, incubation steps could also be carried out at 4°C overnight.)

Stock AFB₁ solution was prepared at a concentration of 2 mg/ml in methanol, and this was diluted in PBS containing 5% (v/v) FCS to produce a set of standard solutions ranging in concentration from 1 $\mu\text{g}/\text{ml}$ to 10 pg/ml. 100 μl /well of each standard was then added to the coated plates, together with 100 μl /well of either monoclonal or polyclonal anti-AFB₁ antibody. Following gentle mixing, the plates were incubated at 37°C for 1 hour. After washing three times in PBST and once in PBST, 100 μl /well of HRP-labelled secondary antibody (either anti-mouse or anti-rabbit) was added, and the plates again incubated for 1 h at 37°C. After another washing step, HRP substrate was added, and the absorbance at 405 nm determined, as in section 2.7.1.

2.7.7 Direct sandwich ELISA for AFB₁ determination.

A sandwich assay for AFB₁ was performed with either polyclonal or monoclonal anti-AFB₁ directly coated onto the ELISA plate. A 1/5,000 dilution of anti-AFB₁ in PBS was coated onto wells of a 96-well microtitre plate at 100 µl/well and incubated for 1 h at 37°C. The plate was washed and blocked with 5% (v/v) FCS as in section 2.7.6.

Stock AFB₁-BSA solution was prepared at 1 mg/ml in PBS, and this was diluted in PBS containing 5% (v/v) FCS to produce a set of standard solutions ranging in concentration from 10 pg/ml to 1 µg/ml. 100 µl of each standard was added to appropriate wells of the plate, which was then incubated for 1 h at 37°C. After washing three times with PBST and once with PBS, 100 µl/well of a 1/5000 dilution of complementary anti-AFB₁ was added, and the plate again incubated for 1 h at 37°C. Washing, addition of labelled anti-species antibody, and development of colour were performed as in section 2.7.1.

2.7.8 Sandwich ELISAs for AFB₁ using protein A capture

Protein A was used to capture polyclonal and monoclonal anti-AFB₁ for use in a sandwich assay. A 96-well microtitre plate was coated with 100 µl/well of 0.5 µg/ml protein A, and the plate blocked with 5% (v/v) FCS as in section 2.7.6. 100 µl/well of a 1/5,000 dilution of either polyclonal or monoclonal anti-AFB₁ was then added, and the plates incubated for 1 h at 37°C. The sandwich assays were then performed as in section 2.7.7.

2.7.9 Sandwich AFB₁ ELISA using an anti-mouse antibody for capture

A sandwich ELISA for AFB₁ was performed in which a commercially available anti-mouse IgG antibody was employed to capture monoclonal anti-AFB₁. A 96-well microtitre plate was coated with 100 µl/well of a 1/500 dilution of anti-mouse IgG (Fc specific) in PBS, and incubated for 1 h at 37°C. The plate was washed and blocked with 5% (v/v) FCS as in section 2.7.6. After a further washing step, 100 µl/well of a 1/5000 dilution of monoclonal anti-AFB₁ in PBS containing 5% (v/v) FCS was then added, and the plate was again incubated for 1 h at 37°C, and then washed three times with PBST and once with PBS. Standard solutions of AFB₁-BSA were then prepared, and from this point on the assay was performed as in section 2.7.7.

2.7.10 ELISA to examine the efficiency and effects of regeneration on immobilised antibody surfaces

In an experiment to examine the efficiency of regenerating antibody immobilised at a biosensor surface, two 96-well microtitre plates were coated with 100 μ l/well of a 1/5,000 dilution of either mouse monoclonal or rabbit polyclonal anti-AFB₁, and incubated for 1 h at 37°C.

The plates were then washed three times in PBST and once in PBS, and then blocked with 5% (v/v) FCS as in section 2.7.6. After washing, 100 μ l/well of 0.5 μ g/ml AFB₁-BSA in PBS was added to the plates, which were then incubated at 37°C for 1 h.

A set of solutions of NaOH was then prepared, ranging in concentration from 5 to 1000 mM. AFB₁-BSA solution was aspirated from the plates, and they were washed three times in PBST and once in PBS. 100 μ l of each of the NaOH solutions were then added to appropriate wells, and the plates were incubated for 10 minutes at room temperature. Following incubation, the plates were washed three times in PBST and once in PBS.

100 μ l/well of complementary anti-AFB₁ antibody - 1/5,000 in PBS containing 5% (v/v) FCS - was then added. Monoclonal antibody was added to the plate coated with polyclonal antibody, and polyclonal antibody to the plate coated with monoclonal antibody.

The plates were then incubated at 37°C for 1 h. After washing three times in PBST and once in PBS, 100 μ l of appropriate HRP-labelled anti-species antibody diluted 1/5000 in PBS containing 5% (v/v) FCS was added. The plates were washed again, and colour was developed and read as in section 2.7.1.

To examine the effect of the regeneration reagent upon the antibody surfaces, the assay was re-run as before, except that the NaOH solutions were added to the wells immediately after the blocking step. Following washing, the AFB₁-BSA solution was added, and then the complementary anti-AFB₁ antibody.

2.7.11 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 biosensor surface, two 96-well microtitre plates were coated with AFB₁-BSA and blocked with 5% (v/v) FCS as described in section 2.7.6.

After washing, 100 μl /well of a 1/5000 dilution of either monoclonal or polyclonal anti-AFB₁ in PBS containing 5% (v/v) FCS was added to the plates, and they were then incubated at 37°C for 1 hour.

A set of solutions of NaOH was then prepared, ranging in concentration from 2 to 1000 mM. Antibody solution was aspirated from the plates, and they were washed three times in PBST and once in PBS. 100 μl of each of the NaOH solutions were then added to appropriate wells, and the plates were incubated for 10 minutes at room temperature. Following incubation, the plates were washed three times in PBST and once in PBS.

100 μl /well of appropriate HRP-labelled anti-species antibody - 1/5,000 in PBS containing 5% (v/v) FCS - was then added, and the plates incubated at 37°C for 1 hour. After washing three times in PBST and once in PBS, substrate was added, and the absorbance of wells at 405 nm read.

To examine the effect of the regeneration reagent upon the conjugate surface, the assay was re-run as before, except that the NaOH solutions were added to the wells immediately after the blocking step. Following washing, the monoclonal and polyclonal antibodies were added, and then HRP-labelled anti-species antibodies.

2.7.12 Measurement of the equilibrium affinity constant for monoclonal anti-AFB₁

Determination of the affinity constant for the monoclonal anti-AFB₁ antibody was carried out by a variation of the method of Friguet *et al.* (1985).

A 96-well microtitre plate was coated with 200 μl /well of AFB₁-BSA conjugate in PBS, and blocked with 250 μl /well of 5% (v/v) FCS, as in section 2.7.6. A range of standards of free aflatoxin B₁ were prepared by diluting stock AFB₁ (2 mg/ml in methanol) in PBS. Standards ranged in concentration from 7.82×10^{-7} M to 8×10^{-4} M. 100 μl of each standard was added to 100 μl of a 1/10,000 dilution of monoclonal anti-AFB₁, and the mixture was incubated overnight at room temperature. 100 μl of each reaction mixture was then added to the conjugate-coated wells. A series of dilutions of monoclonal anti-AFB₁ in PBS, from 1/10,000 to 1/20,000,000 were also prepared, and added at 100 μl /well to a separate series of wells on the same plate.

The plate was incubated for 1 h at 37°C, and then washed three times in PBST and once in PBS. 100 µl/well of HRP-labelled anti-mouse IgG was then added, and the plate again incubated at 37°C for 1 hour. Substrate was added, and colour developed and read, as in section 2.7.1.

The linear portion of the antibody dilution curve was used to calculate the amount of unbound antibody in the equilibrium solutions, and a Klotz plot was constructed (see section 5.5).

2.7.13 Measurement of antibody concentration in hybridoma supernatant

A microtitre plate was coated with 100 µl/well of anti-mouse IgG (Fc specific) and blocked with 5% (v/v) FCS as described in section 2.7.3. Culture supernatant from hybridomas was spiked with mouse IgG at concentrations of 0.625, 1.25, 2.5, 5 and 10 µg/ml and these solutions were added to the plate at 100 µl/well and incubated for 1 h at 37°C. The wells were washed three times in PBST and once in PBS, and then 100 µl/well of HRP-labelled anti-mouse IgG was added. After a further 1 h incubation at 37°C, colour was developed and read as in section 2.7.1. A set of standard curves were constructed, and the concentration of monoclonal antibody in the original supernatants was calculated from the y-axis intercept.

2.8 Cell culture techniques

All cells were cultured aseptically in a laminar flow cabinet, and incubated at 37°C in a humid 5% (v/v) CO₂ atmosphere.

2.8.1 Culture of SP2 cells

Semi-adherent SP2 cells were cultured in Dulbecco's Modification of Eagle's Medium (DMEM) containing 10% (v/v) FCS, 2 mM L-glutamine, 10 mM HEPES buffer, and 25 µg/ml gentamicin (referred to as S₁₀). 1 ml of frozen cell suspension was thawed in a waterbath at 37°C and diluted slowly with 9 ml of medium, before being centrifuged for 10 minutes at 2000 rpm. The cell pellet was then resuspended in 5 ml of medium and incubated in a 25 cm³ tissue culture flask (T25).

For subsequent passages, cells were taken into suspension by gently washing the surface of the flask with medium, using a sterile pasteur pipette.

2.8.2 Culture of adherent cell lines

Adherent normal rat kidney (NRK) cells were cultured in S_{10} medium. When cells had grown to confluency in culture flasks, the medium was poured off, and the flask rinsed three times with 10 ml of sterile PBS. 2 ml of trypsin solution containing 0.25% (w/v) trypsin and 0.02% (w/v) EDTA was added to the cells, which were incubated at 37°C.

The cells were inspected under the microscope, and when none remained adhering to the surface, 10 ml of S_{10} medium was added. The cell suspension was then centrifuged, and resuspended in S_{10} medium.

2.8.3 Cell counting

Cells were counted by mixing an equal volume of cell suspension and Trypan Blue stain. After 1-2 minutes the mixture was examined on an Improved Neubauer Haemocytometer, and the number of viable cells (those which had not taken up the dye) was recorded.

2.8.4 Mycoplasma testing

Mouse myeloma cell lines were tested for the presence of mycoplasma before being used for cell fusion procedures. The Hoechst stain was used for visualisation of mycoplasma. NRK cells were grown up for at least three passages in antibiotic-free medium.

Glass coverslips were prepared by washing in detergent, rinsing in deionised water and methanol, drying with a lint-free cloth and sterilising in a hot air oven at 160°C for 1 h. After trypsinisation, NRK cells were counted and diluted to 1×10^4 , and 1 ml aliquots were placed onto sterile coverslips in petri-dishes, and incubated overnight at 37°C. The following day, 2 ml of conditioned medium from the cell lines to be tested were added to the NRK cells growing on coverslips, and cultured for a further 2 days. The coverslips were then removed and gently washed 3 times in PBS. The cells were then fixed by incubating for 6 minutes in cold Carnoy's fixative (glacial acetic acid : methanol, 1:3 v/v), and the coverslips were again washed 3 times in PBS.

Hoechst stain 33258 was prepared at 50 ng/ml in sterile PBS and the coverslips were incubated in this solution for 10 minutes, before being washed and mounted onto slides and examined under an oil immersion lens at a magnification of 100x, using a fluorescence microscope. Uncontaminated cells show strong fluorescence in the nuclei only, whereas cells contaminated with mycoplasma also exhibit cytoplasmic staining.

2.8.5 Freezing of cell lines

Cells which had reached 50% confluency (covered 50% of the surface) in 75 cm³ tissue culture flasks (T75s) were suitable for freezing. The cell suspension was centrifuged at 2,000 rpm for 10 minutes.

The resulting pellet was resuspended in 1 ml of FCS with 10% (v/v) dimethylsulfoxide (DMSO) and transferred to a sterile cryotube, which was initially lowered gradually into the gas phase and then the liquid phase of liquid nitrogen in a cryocontainer.

2.8.6 Production of antibodies by somatic cell fusion

Two different types of media were used in the course of this procedure. Serum-free medium - DMEM containing 2 mM L-glutamine and 10 mM HEPES - was used for all steps up to and including addition of PEG. For subsequent steps, DMEM containing 10% (v/v) FCS, 2 mM L-glutamine, 5% (v/v) BriClone, 25 µg/ml gentamicin, 100 nM hypoxanthine, 400 µM aminopterin, and 16 nM thymidine - referred to as S₁₀-HAT - was used.

500 µl/well of S₁₀-HAT was added to eight 48-well cell culture plates, which were then placed in the incubator. SP2 cells in the log phase of growth were counted and a volume of cell suspension containing 1x10⁷ cells was centrifuged for 10 minutes at 2,000 rpm. The cell pellet was then washed twice in serum-free medium, and the final pellet resuspended in this medium and placed in the incubator.

A BALB/c mouse, immunised as in section 2.2.3, was sacrificed by cervical dislocation. The spleen was removed aseptically, and transferred to a petri dish containing 5 ml of serum-free medium in a laminar flow cabinet. Using a needle bent at mid-point and a 5 ml syringe, holes were pricked in the spleen, and medium was drawn up and washed through it to remove splenocytes. This was repeated until the spleen capsule appeared pale.

The splenocyte suspension was then centrifuged for 10 minutes at 2,000 rpm, and a cell count was performed. Typically, a spleen will yield in the region of 1×10^8 cells. The spleen cell pellet was then resuspended in 5 ml of serum-free medium, and mixed with the previously prepared SP2 suspension to give a spleen cell to SP2 ratio of approximately 10:1.

The cell mixture was centrifuged at 2,000 rpm for 10 minutes and then washed twice in serum-free medium.

After the final washing step, all supernatant was removed from the pellet using a sterile pasteur pipette. 1 ml of 50% (w/v) PEG (molecular weight 1540 Da), which had been pre-heated to 37°C, was added slowly to the pellet using a 1 ml pipette, and a stopwatch was started:

For the *first 30 seconds* the mixture was taken up and aspirated.

30 seconds after addition of PEG the pipette was removed.

95 seconds after addition of PEG 0.5 ml of S₁₀-HAT was added slowly.

2 minutes after addition of PEG 1 ml of S₁₀-HAT was added slowly.

3 minutes after addition of PEG 1 ml of S₁₀-HAT was added slowly.

4 minutes after addition of PEG 1 ml of S₁₀-HAT was added slowly.

5 minutes after addition of PEG 5 ml of S₁₀-HAT were added slowly.

The mixture was then centrifuged at 2,000 rpm for 10 minutes, resuspended gently in 10 ml of S₁₀-HAT, and incubated for 15 minutes at room temperature. A further 5 ml of S₁₀-HAT was then added, and one drop from a 10 ml pipette was added to each well of seven of the previously-prepared 48-well cell culture plates.

As a negative control, 5 ml of SP2 cell suspension was prepared in S₁₀-HAT and added, dropwise, to the remaining 48-well cell culture plate.

The plates were left undisturbed in the incubator for 12 days, after which time they were inspected under the microscope. By this stage, hybridoma colonies could be seen growing in some wells of the plates, while SP2 cells in the control plate were dead. The cells were then fed by gently removing half of the supernatant and adding a similar volume of S₁₀-HAT containing 10 mM HEPES. Supernatants from wells containing hybridomas were screened for antibody production as described in section 2.7.5.

Antibody-secreting hybridomas were scaled up from 48-well plates into 24-well plates, and, after subsequent rounds of screening, into 12-well and 6-well plates. After 2-3 weeks, aminopterin was removed from the medium. When cells had been scaled up into 12-well plates, hypoxanthine, thymidine and BriClone were removed from the medium.

2.8.7 Cloning of hybridomas by limiting dilution

Monoclonality of hybridomas was ensured by means of cloning by limiting dilution. Positive hybridomas were cultured to 50 - 70% confluence in 6-well plates. A cell count was performed, and 100 μ l of hybridoma suspension in S₁₀-BriClone was added to each well of two 96-well cell culture plates at a concentration of 10 cells per well.

When cells had reached confluence in the 96-well plates, supernatants were screened for specific antibody production, and positive hybridomas were scaled up. When growth had reached 50-70% confluence in 6-well plates and cells had been weaned off BriClone, the hybridomas were plated out in S₁₀-BriClone at 1 cell per well into 96-well plates. Wells seen to contain single clones were then screened for monoclonal antibody production and scaled up.

2.9 BIAcore studies

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

2.9.1 Preconcentration studies

For all investigations, proteins were immobilised on the sensor surface by means of N-hydroxysuccinimide esterification. The standard conditions used lead to activation of 30-40% of the carboxyl groups on the dextran, and allow covalent attachment of biomolecules via primary amine groups. In order for the coupling reaction to proceed satisfactorily, it is necessary for an initial "preconcentration" step, resulting from electrostatic binding of protonated amine groups on the biological component to negatively-charged carboxyl groups on the chip surface, to take place.

For the native form of a protein, preconcentration can be facilitated by adjusting the pH below the isoelectric point (pI). However, modification of proteins by, for example, conjugation to drug 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 in 10 mM sodium acetate at a range of different pHs, passing these solutions over an underderivatised chip surface, and monitoring the degree of electrostatic binding. The highest pH at which satisfactory preconcentration was observed was chosen as the pH for immobilisation.

2.9.2 Immobilisation of interactants

The carboxymethylated dextran surface of the sensor chip was first derivatised by injection of a 1:1 mixture of 3-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) and N-hydroxysuccinimide (NHS), at concentrations of 400 mM and 100 mM, respectively. The interactant to be immobilised was diluted in 10 mM sodium acetate at the appropriate pH, and at a typical concentration of 200-1,000 µg/ml. This solution was then injected over the derivatised chip surface for 20 minutes. Unreacted NHS groups were capped, and non-covalently bound protein removed, by injection of 1 M ethanolamine hydrochloride, pH 8.5, for 7 minutes.

2.9.3 BIAcore inhibitive 7-hydroxycoumarin immunoassay

An indirect inhibition assay system was used for the detection of 7-hydroxycoumarin in human serum samples. All additions of reagents and incubation steps were automated. A flow rate of 10 µl/min was used throughout.

10,000 RU of diazo-coupled 7-hydroxycoumarin-BSA conjugate was immobilised on sensor chips as in section 2.9.1.

Human serum was centrifuged at 13,000 rpm and filtered (0.22 µm) to remove any precipitate formed by freeze-thawing. Standard solutions in the range 0.5 to 80 µg/ml were prepared by spiking serum with a stock solution of 1 mg/ml 7-hydroxycoumarin in a mixture of 30% (v/v) ethanol : 70% (v/v) PBS. Samples were prepared by spiking serum with a stock solution of 7-hydroxycoumarin-glucuronide (1 mg/ml in a mixture of 30% (v/v) methanol : 70% (v/v) ultrapure water).

The majority of 7-hydroxycoumarin in serum is present as a glucuronide conjugate, which is not recognised by the polyclonal antiserum and must be deconjugated prior to analysis. The enzyme used for deconjugation of 7-hydroxycoumarin-glucuronide was β -glucuronidase, which was at a concentration of 5,000 units/ml in 10 mM sodium acetate, pH 5.0. 180 μ l of enzyme solution was added to an equal volume of sample or standard, mixed, and incubated at 37°C for 2 hours. BIAcore sample blocks were brought up to a temperature of 37°C by means of an attached waterbath.

Purified polyclonal anti-7-hydroxycoumarin antibody (see section 2.4) was diluted to a protein concentration of 0.8 mg/ml in PBS containing 4% BSA, 4 mg/ml CM-dextran and 0.2% Tween 20. 45 μ l of this antibody solution was added to 45 μ l of the enzyme-sample mixture, mixed, and incubated for 10 minutes. 20 μ l of this mixture was then injected over the immobilised 7-hydroxycoumarin-BSA surface, and the change in response recorded.

After binding of excess antibody, the surface was regenerated by injecting a 1 minute pulse of 5 mM NaOH, one of 20 mM HCl and another pulse of 5 mM NaOH.

A calibration curve was constructed by plotting the change in response (in RU) for each standard against the log of concentration, and sample concentrations were determined from this curve.

The intra-day variability of the assay was investigated by running a set of five standards across the linear range five times in one day, and determining the coefficient of variation (CV) between the calculated 7-hydroxycoumarin concentrations for each set of five.

The inter-day variability of the assay was assessed by running five sets of standards across the linear range on five different days, and determining the CV between the calculated 7-hydroxycoumarin concentrations for the standards from each of the five standard curves.

2.9.4 Aflatoxin studies

Stock solutions of AFB₁ and AFB₁-BSA were prepared as in sections 2.7.6 and 2.7.7. Monoclonal and polyclonal antibodies to AFB₁, as well as protein A and Fc-specific anti-mouse IgG, were immobilised as in section 2.9.1.

AFB₁-BSA was separated from any free toxin by means of size exclusion chromatography. A PD-10 column was first equilibrated with 25 ml of PBS. 1 ml of AFB₁-BSA was then made up to 2.5 ml with PBS, and this was applied to the column.

3.5 ml of PBS was then added to the column to elute AFB₁-BSA. The column was stored in PBS containing 0.02% (w/v) sodium azide. Flow rates throughout all aflatoxin BIAcore studies were 5 µl/min.

2.9.5 DNA studies

Streptavidin was immobilised on the sensor chip at a concentration of 50 µg/ml in 10 mM sodium acetate, pH 4.8, as described in section 2.9.1. This surface was used to capture biotin-labelled plasmid DNA. Flow rates throughout all BIAcore DNA studies were 2 µl/min.

2.10 Phage counts

E. coli TG1 cells were cultured in 2x TY medium - 1.6% (w/v) tryptone, 1% (w/v) yeast extract and 0.5% (w/v) sodium chloride - containing 1% (w/v) glucose. The cells were grown to late log phase at 37°C with shaking. Samples of phage which had been eluted from the BIAcore were serially diluted in 2x TY medium, and 50 µl of each dilution was added to 50 µl of *E. coli* TG1 and incubated at 37°C for 30 minutes. The infected *E. coli* samples were plated onto solid medium containing 1% (w/v) glucose and 0.1% (w/v) carbenicillin, and incubated overnight at 37°C with shaking. The number of plaques on the agar plates was then counted, and the number of phage present in the original samples was calculated and reported as titre-forming units per millilitre (TFU/ml).

2.11 Extraction of supercoiled plasmid DNA

E. coli TG1(pHEN1) cells were cultured in 2x TY medium containing 1% (w/v) glucose and 0.01% (w/v) glucose, and incubated overnight at 37°C with shaking. The culture was then centrifuged at 4000 rpm for 10 minutes, and the supernatant was aspirated. A Wizard Mini-prep kit was used to extract plasmid DNA. The cell pellet was resuspended in 400 µl of cell resuspension solution (50 mM Tris, pH 7.5, 10 mM EDTA, 100 µg/ml RNase A) and transferred to a sterile eppendorf tube. 400 µl of cell lysis solution (0.2 M NaOH, 1% (w/v) SDS) were added, and the eppendorf tube was inverted 4 times. 800 µl of neutralisation solution (1.32 M potassium acetate) were added, and the mixture was incubated for 10 minutes at room temperature and then centrifuged for 10 minutes at 13,000 rpm.

The supernatant was added to the barrel of a sterile 5 ml syringe, along with 1 ml of DNA purification resin. The mixture was then passed through an attached Minicolumn, followed by 2 ml of 4.2 M guanidine hydrochloride in a mixture of 40% (v/v) isopropanol and 60% (v/v) ultrapure water. 2 ml of column wash solution (80 mM potassium acetate, 8.3 M Tris-HCl, pH 7.5, 40 μ M EDTA and 55% (v/v) ethanol) were then passed through the column, which was then centrifuged in a sterile eppendorf tube for 2 minutes at 13,000 rpm, to remove any remaining wash solution. DNA was eluted by adding 50 μ l of ultrapure water to the column and centrifuging at 13,000 rpm for 2 minutes in a sterile eppendorf tube.

The concentration of the extracted DNA was calculated by measuring the absorbance at 260 nm. An absorbance reading of 1.00 indicated a DNA concentration of 50 μ g/ml.

The extracted plasmid DNA was kept at -20°C for long-term storage, and at 4°C for short-term storage.

2.12 Biotin labelling of extracted DNA

Extracted DNA was labelled with biotin using a Boehringer-Mannheim Biotin-Chem-Link kit. 50 μ g of DNA were added to 50 μ l of Biotin-Chem-Link Reagent (a *cis*-platinum-biotin complex) in a sterile eppendorf and incubated for 30 minutes at 85°C on a PCR machine. The eppendorf was centrifuged briefly to collect condensate, and 5 μ l of Stop Reagent was added.

Labelled DNA was separated from remaining free biotin by precipitation. To 50 μ l of labelled DNA, 5 μ l of 3 M sodium acetate, pH 5.2, and 100 μ l of absolute ethanol were added. The mixture was incubated for 1 h at -20°C, and was then centrifuged for 10 minutes at 13,000 rpm.

The liquid was carefully drawn off using a pipette and 200 μ l of 70% (v/v) ethanol were added and aspirated to wash the pellet. The precipitated DNA was resuspended in 200 μ l of ultrapure water.

2.13 Agarose gel eletrophoresis

The purity of extracted DNA and the action of topoisomerase II and novobiocin were assessed by agarose gel electrophoresis.

Agarose was dissolved, with heating, at a concentration of 1% (w/v) in 1x TAE buffer, containing 40 mM Tris, 20 mM acetic acid and 1 mM EDTA. Ethidium bromide was added a final concentration of 0.5 µg/ml. The solution was poured into a mini-gel plate, with the comb inserted, and allowed to solidify. 5 µl of sample DNA was added to 13 µl of ultrapure water and 2 µl of gel loading buffer (containing 0.25% (w/v) bromophenol blue and 40% (w/v) sucrose). The gel was covered with 1x TAE buffer, and 20 µl of each sample was loaded, together with 20 µl of pre-stained lambda phage DNA markers. The gel was electrophoresed at a voltage of 80 mV, and photographed under UV light.

2.14 Southern blots

Biotin-labelled DNA was detected by Southern blotting after transfer of nucleic acids from agarose gels to nitrocellulose membranes by capillary action.

The agarose gel was removed from the plate and the DNA was denatured by soaking in 1.5 M NaCl / 0.5 M NaOH, for 45 minutes. This step, and all subsequent incubation and washing steps, were carried out at room temperature on a rotary platform. The gel was rinsed in ultrapure water, and then neutralised by soaking in 1 M Tris, pH 7.4, containing 1.5 M NaCl, for 30 minutes. A basin was filled with 20x SSC (3 M NaCl, 0.3 M sodium citrate, pH 7.0), and a strip of filter paper was draped across a glass plate suspended above the basin, with each end of the paper immersed in the buffer. The gel was then inverted and placed on top of the filter paper. A piece of nitrocellulose was cut to the size of the gel, and dipped in ultrapure water. The nitrocellulose was then soaked in 20x SSC for 5 minutes. The gel was surrounded with Parafilm, and the wet nitrocellulose membrane was placed on top. Two pieces of filter paper the same size as the gel were soaked in 20x SSC and placed on top of the nitrocellulose membrane. A stack of filter papers about 8 cm high was placed on top of this and weighed down with a 500 g weight. The capillary transfer of DNA was allowed to proceed overnight.

The nitrocellulose membrane was then removed and incubated for 1 h in a 5% (w/v) solution of dried milk powder in PBS. It was then washed 3 times (10 minutes each) in phosphate-free wash buffer (0.15 M NaCl, 0.05 M Tris, pH 7.5). A 1/4,000 dilution of avidin-alkaline phosphatase conjugate was prepared in wash buffer containing 5% (w/v) milk powder.

The nitrocellulose membrane was incubated in 20 ml of conjugate solution for 1 hour, and then washed 3 times in wash buffer. Substrate buffer was prepared, containing 100 mM Tris/HCl, pH 9.5, 100 mM NaCl and 5 mM magnesium chloride. 66 μ l of BCIP (5-bromo-4-chloro-3-indoyl phosphate) and 33 μ l of NBT (nitro blue tetrazolium) were added to 10 ml of substrate buffer, and the nitrocellulose membrane was incubated in this solution at 37°C until colour developed.

CHAPTER 3

PRODUCTION AND CHARACTERISATION OF ANTIBODIES TO COUMARIN COMPOUNDS

3.1 Introduction

This chapter describes the production and characterisation of a variety of different types of antibody using protein conjugates of coumarin compounds. Polyclonal antiserum to 7-hydroxycoumarin was raised in rabbits and purified by means of ammonium sulphate precipitation and protein G affinity chromatography. The purity of the antibody was tested by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). The working dilution of this antibody in a competitive ELISA for 7-hydroxycoumarin was determined, and the degree of cross-reactivity of the antibody for a variety of coumarin compounds was assessed.

Somatic cell fusion was used to generate monoclonal antibodies, using spleen cells from mice immunised with 7-hydroxycoumarin-BSA. Cell culture supernatants were screened by ELISA, and, after cloning by limiting dilution, the antibodies produced were found to bind to the drug-protein conjugate, but not to free 7-hydroxycoumarin. A panel of 9 monoclonal antibodies to 7-hydroxycoumarin-BSA were affinity ranked using the BIAcore.

Three genetically-engineered single chain Fv (scFv) fragments against coumarin-BSA were affinity ranked using the BIAcore, and an observed affinity constant for the interaction of one of the antibodies with the conjugate was derived using BIAcore kinetic software.

BIAcore was also used to pan a library of phage-displayed antibodies for anti-7-hydroxycoumarin antibodies. Although no binding signals could be obtained during the panning process, phage counts carried out on the eluate showed that specific elution had taken place.

3.2 The immune system

The vertebrate immune system consists of a range of separate but inter-related cells and molecules, all concerned with protecting the parent organism against infectious agents (Roitt, 1994; Kuby, 1997). Both innate and acquired immunity exist.

The innate immune system consists of a series of physical and physiological barriers. The skin and mucous membranes provide the body's first line of defence against infection. In addition, physiological factors such as pH, temperature and the presence of degradative substances such as lysozyme also act to protect the body from pathogens. Foreign material is also broken down non-specifically by two processes known as endocytosis and

phagocytosis. Endocytosis involves the breakdown of extracellular macromolecules, whereas particulate matter is broken down by phagocytosis. In both cases, the foreign matter is internalised by cells, and digested by enzymes prior to elimination. Endocytosis can be performed by almost any cell type, but only certain cells, such as monocytes and macrophages, are capable of phagocytosis. The inflammation generated in response to tissue damage also contributes to innate immunity, facilitating the emigration of phagocytes and the lysis of pathogenic microorganisms.

Unlike the processes involved in innate immunity, the acquired immune system is directed against specific molecules, and possesses immunological memory. Two different types of response, cellular and humoral, are generated when components of the acquired immune system encounter a foreign (or "non-self") substance.

The humoral immune response is mediated by antibodies, the specialised binding proteins produced by B lymphocytes. Each B lymphocyte expresses approximately 10^5 antibody molecules on its surface. The substance to which an antibody binds is known as an antigen. When a B cell expressing a particular antibody is activated by the binding of antigen, it proliferates, leading to the production of large amounts of specific antibody. This process is known as clonal selection. The selected B cells also differentiate into two cell types. Effector B cells (known as "plasma cells"), secrete large amounts of soluble antibody, and have a half-life of only a few days. Memory B cells, on the other hand, express membrane-bound antibody and have a longer life-span. Memory B cells are responsible for the rapid and strong antibody response to secondary infections, and are involved in the use of vaccines.

Antibodies can trigger the destruction of large infectious agents by binding to discrete sites on their surface. Smaller pathogens such as viruses and toxins can also be neutralised by the binding of antibody. The distinct chemical group on the surface of an antigen which is recognised by an antibody is called an epitope, and the immune system is capable of producing several million individual antibodies, each of which binds to a different epitope. One of the main characteristics of antibodies is their high degree of specificity. Antibodies can distinguish between proteins which differ in composition by only a single amino acid residue.

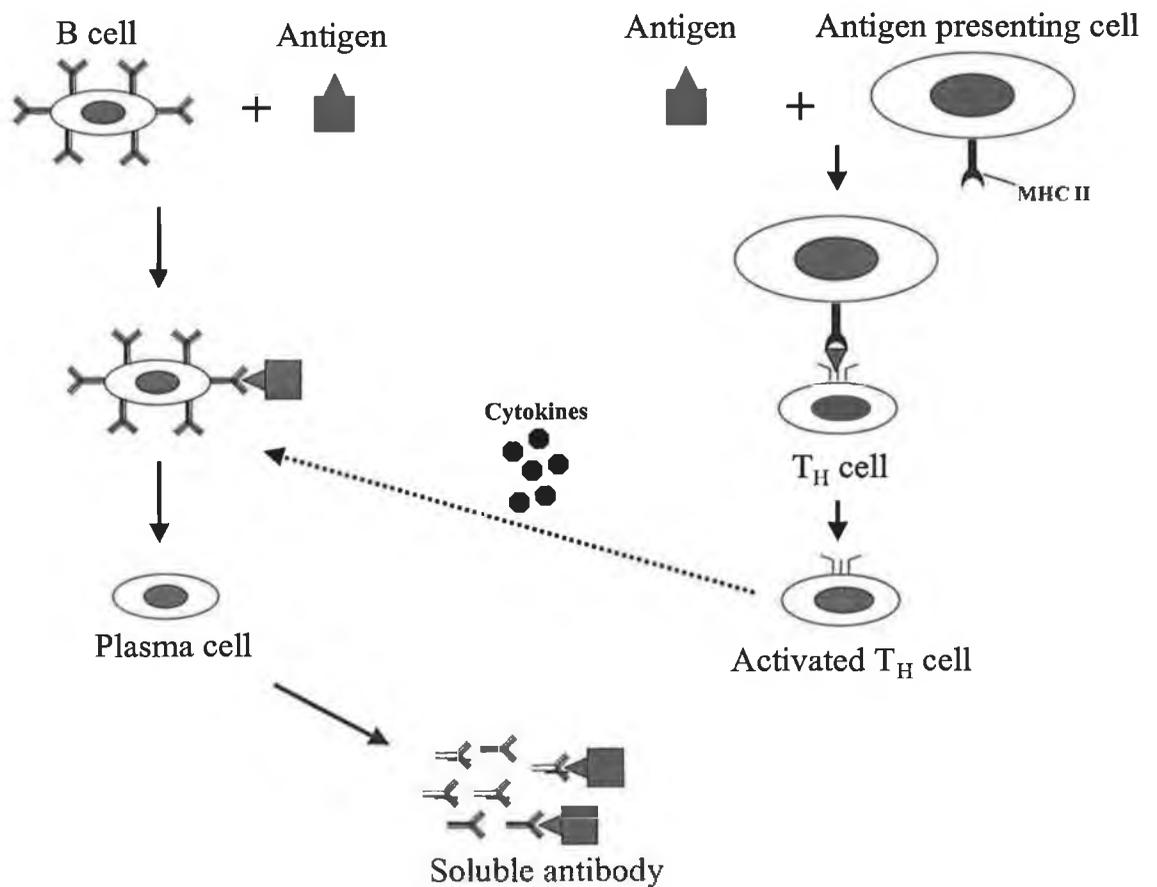
The cellular immune response involves the production of T lymphocytes. Like B lymphocytes, T cells also have membrane-bound antigen-recognition molecules. While B cells can recognise antigen in solution, however, T cells can identify antigen only if it is associated with a cell membrane protein of the major histocompatibility complex (MHC) group. When a T lymphocyte encounters specific antigen associated with an MHC molecule on the surface of another cell, it also undergoes clonal selection, and differentiates into memory and effector cells.

There are two categories of T lymphocyte, T helper (T_H) and T cytotoxic (T_C) cells. T_H cells recognise antigen associated with MHC II proteins, and are activated to produce cytokines. These are growth factors which are necessary for the production of B cells, T cells and macrophages. There are two subsets of T_H cells, which are differentiated by the particular cytokines they produce. T_H1 cells, among whose secretory products are interferon gamma ($IFN-\gamma$) and tumour necrosis factor beta ($TNF-\beta$), are responsible for cell-mediated functions, such as the activation of cytotoxic T lymphocytes. T_H2 cells secrete mainly interleukins, and are involved in B-cell activation.

T_C cells bind to antigen associated with MHC I molecules, which causes activation to cytotoxic T lymphocytes (CTLs). These cells exert a cytotoxic effect on cells of the body which display antigen on their surface, such as virally-infected or tumour cells.

The generation of the humoral immune response, then, is dependent upon the production of cytokines, which is in turn dependent upon the activation of T_H cells. A type of cell known as an antigen presenting cell (APC) is responsible for this process. These cells must express class II MHC proteins on their surface, as well as being able to deliver a signal which stimulates the activation of T_H cells. B cells, macrophages and dendritic cells can all function as APCs. APCs internalise antigen by phagocytosis or endocytosis. Degraded antigen components then bind to MHC II molecules within the cell, and are exported to the cell surface. T_H cells bind to the joint antigen fragment-MHC II complex, and a co-stimulatory signal from the APC then activates the T_H cell, causing cytokines to be produced. The sequence of events leading to the production of soluble antibody is illustrated in Figure 3.1.

Figure 3.1 The generation of the humoral immune response. Antigen presenting cells internalise and degrade antigen. Antigen fragments are then presented on the surface of cells together with class II major histocompatibility complex (MHC) molecules. T helper (T_H) cells bind to the antigen-MHC II complex, and become activated to produce cytokines. 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 causes differentiation into plasma cells, and the production of specific soluble antibody.



3.2.1 Immunogenicity

The ability of a particular molecule to provoke an immune response is known as immunogenicity, and is dependent upon a variety of factors such as foreignness, molecular weight and chemical composition.

During the development of an embryo, any B cells expressing antibody against self molecules are killed off, and molecules recognised by the remaining antibody population are considered to be foreign. The greater the genetic distance between the source species of a molecule and the species of the host, the greater will be the immune response it generates. For example, bovine serum albumin (BSA) does not result in antibody production in cows, but is strongly immunogenic in sheep.

Substances with a molecular weight of approximately 100,000 Da are the best immunogens, while molecules with a molecular mass of less than 5,000 Da usually do not cause antibody production. Small molecules of this type are known as haptens. Haptens – including very low molecular weight substances such as drugs and toxins - while themselves non-immunogenic, are able to combine with antibodies. By coupling haptens to a large carrier molecule and immunising with the hapten-carrier conjugate, specific anti-hapten antibodies can be produced. Carrier molecules are generally highly immunogenic proteins.

The greater the chemical complexity of a substance, the more immunogenic it will be. Synthetic polymers consisting of a single amino acid generally do not give rise to an immune response, whereas those containing a variety of amino acids are much more effective immunogens. The inclusion of amino acids with aromatic side-chains increases the immunogenicity still more (Kuby, 1997).

For proteinaceous immunogens, the different types of structural organisation also play a role in immunogenicity. Proteins possess four different levels of structure. The linear amino acid sequence of the protein is referred to as the primary structure. This polypeptide chain is folded into regular arrangements such as α -helices and β -pleated sheets, giving rise to secondary structure. Tertiary structure is the overall conformation of the protein, caused by folding of the areas between secondary structures, and resulting in the presence of individual domains with separate functional properties. Proteins which are polymeric in nature are said to possess quaternary structure. All four levels of structural arrangement contribute to a protein's immunogenicity.

The ease with which a molecule can be processed and presented by APCs is of critical importance to its immunogenicity. Large insoluble molecules, which are rapidly phagocytosed, tend to be better immunogens than small insoluble molecules.

The immune responsiveness of the host animal is also of importance. Immune responsiveness is genetically controlled by genes coding for the MHC molecules. The use of inbred animal strains, which are produced by inbreeding of brother and sister littermates, and are genetically identical, removes any variations in immune responsiveness between animals. One example of an inbred strain is the BALB/c mouse.

The dosage and route of immunisation also affect the immune response. Very low doses of immunogen will fail to activate sufficient numbers of lymphocytes, while high doses result in lymphocytes entering an unresponsive state.

Immunogens can be administered by a variety of routes, the choice of which can alter the type of immune response given. For example, subcutaneous injection causes the administered substance to gather in local lymph nodes, whereas intravenous injection carries the immunogen straight to the spleen.

The response to an immunogen can be enhanced by administering it along with an adjuvant. Adjuvants are substances which non-specifically stimulate the immune system. One of the most widely-used of these agents is Freund's Complete Adjuvant, which consists of mineral oil, emulsifying agent and heat-killed *Mycobacterium tuberculosis*. When the immunogen is mixed with the adjuvant and injected, the emulsifying agent causes the oil to form into small droplets, which results in the immunogen being released slowly from the injection site. The presence of mycobacteria activates macrophages, leading to the stimulation of T_H cells, and the adjuvant also provokes an inflammatory response. The less pathogenic Freund's Incomplete Adjuvant, which does not contain mycobacteria, is frequently used for second and subsequent injections - a process known as "boosting". The amount of specific antibody in the serum of an immunised animal is known as the titre, and boosting is repeated until a sufficiently high titre is reached.

3.2.2 Antibody structure

There are a range of different types of antibody, with varying functions *in vivo*. Those most commonly produced and used for analytical applications have been either entire or partial

antibodies of the subclass immunoglobulin G (IgG). The structure of a typical IgG molecule is shown in Figure 3.2. The antibody is composed of four polypeptide chains - two heavy chains (H) and two light chains (L) - held together by a number of inter- and intra-chain disulphide bonds. The light chains weigh about 25 kDa, and the heavy chains approximately 50 kDa, giving the entire IgG molecule a relative molecular mass of 150-160 kDa. The H and L chains are further subdivided into domains on the basis of the variability of their amino acid composition. Each L chain contains one variable (V_L) and one constant (C_L) domain, while the H chain consists of a single variable domain (V_H) and three constant domains (C_{H1} , C_{H2} and C_{H3}). As shown in the figure, the variable regions of the light and heavy chains associate with each other, as do the C_L and C_{H1} regions. The C_{H2} and C_{H3} domains of each heavy chain also pair with those on the other heavy chain. Between each C_{H1} and C_{H2} domain lies the hinge region, which introduces a degree of flexibility for the two "arms" of the molecule.

While the constant regions of the molecule are responsible for effector functions and complement binding, it is the V_H and V_L domains of the antibody which are involved in the specific recognition of antigen. Within each of these domains, which exhibit high variability in amino acid sequence from one molecule to the next, are three discrete "hypervariable" regions. The combined hypervariable loops from a pair of V_H and V_L domains make up the six complementarity determining regions (CDRs), which are situated at the end of each antibody arm and form the antigen binding site. The CDRs comprise 15-20% of the variable domain (Kuby, 1997), and the remaining areas - which exhibit much lower variability - are known as "framework regions". The variable regions of heavy and light chains are coded for by several hundred genes, which genetic diversity, together with somatic mutation and the combining of different light and heavy chains, allows for the production by the immune system of more than 10^8 different antibody specificities.

The IgG molecule can be broken down into various fragments by means of enzymatic degradation. These fragments, together with those produced by means of genetic engineering, are shown in Figure 3.3. Treatment with pepsin cleaves the two heavy chains below the intrachain disulphide bonds, resulting in the formation of an $F(ab')_2$ fragment, which consists of the two light chains joined together by the hinge region.

Figure 3.2 The structure of an immunoglobulin G molecule. The molecule consists of two heavy and two light chains, which are held together by both inter- and intra-chain disulphide bonds. The antigen binding sites are located at the end of the "arms" of the structure, and molecular recognition involves both the complementarity determining regions (CDRs) and the framework regions (FRs).

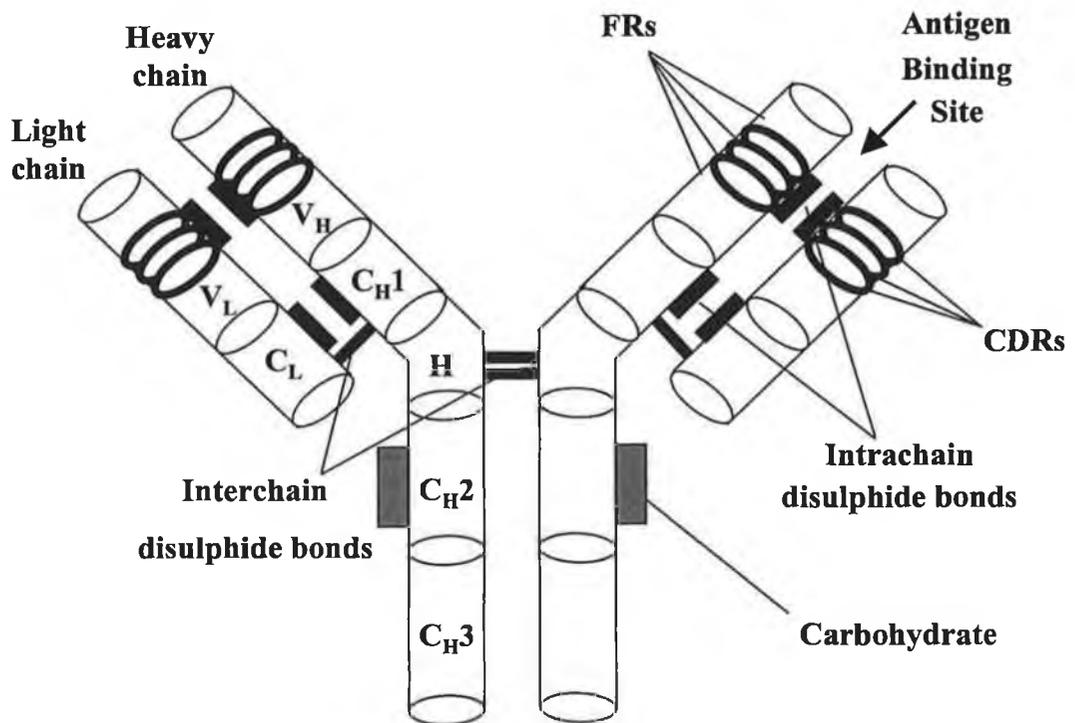
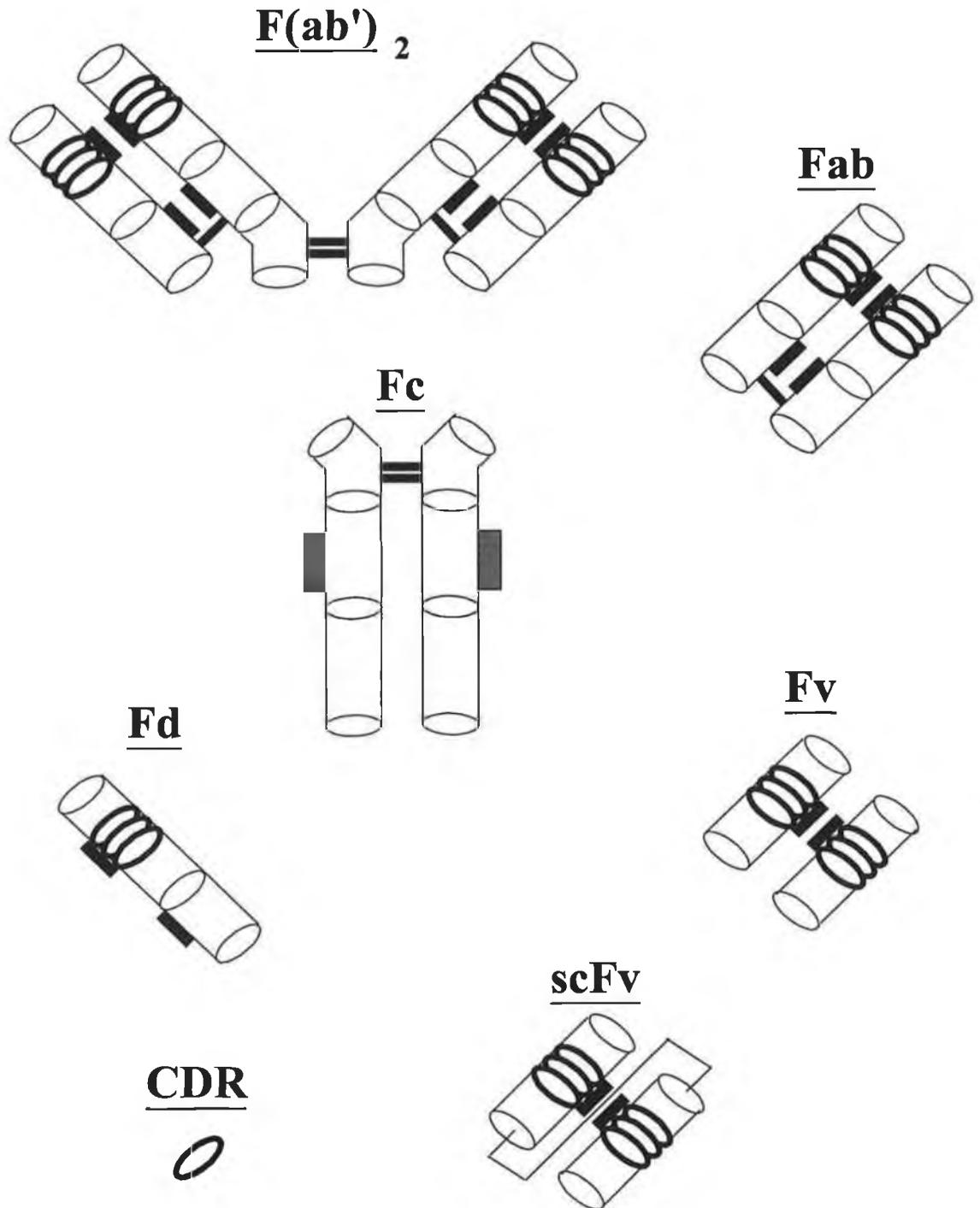


Figure 3.3 Antibody fragments. $F(ab')_2$ and F_c fragments are obtained from papain digestion of IgG, and Fab fragments are generated by treating with pepsin. Other fragments have been produced by antibody engineering. These include the antigen-binding fragment (Fab); Fd fragment – consisting of the heavy-or light-chain moiety of a single Fab; variable fragment (Fv); single chain variable fragments (scFv); and individual complementarity determining region (CDR).



The action of papain cleaves the molecule above the disulphide bridge, giving the two Fab fragments, which bind antigen, and the Fc fragment, which has effector functions.

While IgG is the predominant type of antibody in circulation, there are four other classes of immunoglobulin, all of which basically consist of two identical light and heavy chains joined by disulphide bridges, although some are polymeric in structure. IgM is a pentameric protein, and is produced in response to primary infections. IgA may be present as a monomer or a dimer, and is the predominant antibody in salivary and mucous secretions. IgD is a membrane component of many B lymphocytes, and is thought to be involved with lymphocyte differentiation. IgE is present in only trace amounts in serum, and plays a role in immunity against parasites, in addition to being implicated in many hypersensitivity conditions such as hay-fever.

The IgG class of immunoglobulins is further subdivided into four subclasses or isotypes - called IgG₁, IgG₂, IgG₃, IgG₄ - based on the type of heavy chain which is present.

3.3 Antibody-antigen binding

As stated above, the variable regions of the antibody molecule are involved in the specific recognition of antigen, and antibody-antigen binding occurs as a result of contacts between the surfaces of both interactants. X-ray crystallographic analysis of antibody molecules has made possible the visualisation of the shape and size of antigen-binding sites. The antigen-binding sites of antibodies against small molecules, including haptens, appear to form deep concave pockets (Garcia *et al.*, 1992). Protein antigens, on the other hand, bind to specific antibody via flat undulating areas on the antibody surface (Amit *et al.*, 1986), and the two surfaces in this type of interaction have matching depressions and protrusions. Hanin *et al.* (1997) demonstrated complementarity of the hydrophobic or hydrophilic nature of the individual amino acid residues making contact between CDRs on a monoclonal antibody and a neuropeptide antigen, and used BIAcore to show that modification of this "hydropathic profile" resulted in dramatic reduction in binding.

Wilson and Stanfield (1995) studied the binding of Fab fragments to a range of different antigens, and reported that for small antigens such as haptens, approximately 60% of the contact area is "buried" within the antibody binding site, whereas a maximum of 15% of the contact area for large protein antigens was buried. In the same study, it was reported that at

least 4 of the 6 CDRs make contact with the epitope, and that more heavy chain than light chain residues are involved. Laune *et al.* (1997) demonstrated that residues from the framework regions also play a role in antigen binding. The individual contributions of the heavy and light chain variable regions to binding were investigated by Noel *et al.* (1996) using BIAcore. They reported that the heavy chain of a murine antibody to human thyroglobulin was able to bind antigen in the absence of the light chain, but that the reverse was not the case.

With protein antigens, only those amino acid residues on the surface of the protein are accessible to the antibody. Due to the nature of the biological environment, these amino acids are predominantly hydrophilic in nature. In the average interaction between an antibody and a protein antigen, between 15 to 22 amino acids on the antigen make contact with the antibody (Amit *et al.*, 1986). The amino acids which make up the epitope may be either contiguous - i.e. run in sequence along the polypeptide chain - or non-sequential. Non-sequential epitopes consist of amino acids which, although far apart in terms of primary structure, are brought together by the tertiary structure of the antigen. In addition, protein epitopes are generally situated on the most mobile regions of the structure, allowing greater flexibility for combining with binding sites on antibodies. Holmes *et al.* (1998) demonstrated that CDRs could exhibit a similar flexibility, in that binding of a synthetic antibody to its antigen could reverse adverse conformations introduced by genetic engineering.

3.3.1 Antibody affinity

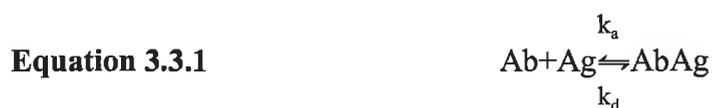
The strength of the bond formed by an antibody and its specific antigen is determined by a combination of different physical forces, the same type of weak non-covalent forces which govern other basic biochemical interactions such as DNA replication and enzyme-substrate binding. These forces are hydrophobic interactions, electrostatic binding, van der Waals forces, and hydrogen bonds (Tijssen, 1985).

Hydrophobic interactions occur when the conjunction of non-polar groups in a binding site repulses water molecules from a binding site. Electrostatic binding occurs between oppositely charged groups on the two interactants, and its effect is greatly enhanced by the removal of water molecules as a result of hydrophobic interactions. Van der Waals forces

are a result of attraction between the electron clouds of adjacent non-polar groups on the binding partners. Hydrogen bonds are formed when hydrogen atoms are shared between two other atoms, and are stronger than van der Waals forces.

The individual bond energies of these weak non-covalent interactions are negligible – especially in an aqueous environment - and their effect is significant only when they occur in large numbers. In addition, non-covalent interactions are only effective over a small distance, making the goodness of the fit between the epitope and the binding site on the antibody the defining factor for 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 terms of reaction kinetics. For an antibody (Ab) and an antigen (Ag) at equilibrium, the formation of antibody-antigen complex (Ab-AG) can be expressed as:



where k_a and k_d are the association and dissociation rate constants, respectively. The affinity of the antibody for the antigen is given by the equilibrium association constant (K):

Equation 3.3.2

$$K = \frac{k_a}{k_d} = \frac{[\text{AbAg}]}{[\text{Ab}][\text{Ag}]}$$

The affinity of an antibody may also be quoted as the equilibrium dissociation constant, K_D , which is the reciprocal of the K value.

3.3.2 Implications of antibody affinity for immunosensor design

Optimisation of regeneration conditions is a necessary, but frequently problematic and time-consuming, aspect of immunosensor assay development. Some knowledge of the nature of the binding forces between the antibody and the antigen can considerably speed up the process, although the reverse is usually the case, with insight into the type of attractive

forces which predominate being gained as the optimal regeneration conditions are discovered.

Antibody-antigen bonds which are significantly electrostatic in nature are generally easily broken by the use of extremes of pH or ionic strength. Chaotropic reagents disrupt the steric complementarity of the binding sites, while the use of organic solvents interferes with hydrophobic interactions. Van der Waals forces can be affected by contact with low surface tension organic acids. For interactions in which the main attractive forces are exothermic hydrogen bonds (“cold antibodies”), increased temperatures may be used for regeneration. Zeder-Lutz *et al.* (1997) reported that the affinity of a Fab fragment for lysozyme, as measured with BIAcore, decreased with increasing temperature.

3.4 Antibody production

3.4.1 Production of hapten-carrier conjugates

As discussed in section 3.2.1, haptens are not themselves capable of generating an immune response, and must first be linked to a carrier molecule. 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). Carriers must be inherently immunogenic, possess suitable functional groups for covalent linkage, and be non-toxic *in vivo*. Haptens may be coupled to carriers via existing reactive groups, while some molecules may require initial derivatisation to introduce such groups. The use of spacer molecules to distance the hapten from the surface of the protein may be beneficial (Erlanger, 1980).

Hapten-protein conjugates are also used at the screening stage of antibody production. When screening for the presence of specific antibody it is necessary to use a conjugate containing a different protein moiety than the immunogen, to minimise false positive results. Danilova (1994) recommended that, when dealing with very small haptens, the conjugate used for screening should possess a different carrier molecule and coupling chemistry than that used for immunisation. Carriers for use in screening must have good solubility, even when derivatised. This is not a necessity for immunogens, however. As discussed in section 3.2.1, precipitated molecules often produce good immune responses.

For the work presented in this chapter, three different proteins - BSA, ovalbumin and thyroglobulin - were conjugated to drugs, both for screening and immunisation purposes. Conjugation was carried out by either diazonium conjugation and EDC-mediated NHS-esterification.

Bovine serum albumin (BSA) is a highly soluble protein with a molecular weight of 67 kDa, which has a variety of available functional groups for derivitisation. Ovalbumin is the major protein present in hen egg whites, and has a molecular weight of 43 kDa. Thyroglobulin is a multisubunit prohormone protein produced by the thyroid gland, with a molecular weight of 670 kDa.

Many aromatic ring systems contain active hydrogens which can easily be displaced by attacking electrophilic groups. Diazonium groups are particularly reactive with these active hydrogens, and are a useful means of attachment for molecules with few available functional groups (Hermanson, 1996). Amino groups on phenolic compounds can be converted to diazonium groups by reaction with sodium nitrite in acidic conditions. The diazonium group reacts rapidly with the aromatic ring of tyrosine and histidine residues on protein molecules. Egan (1993) and Bogan (1996) used this procedure to convert 3-amino-7-hydroxycoumarin to the diazonium derivative before conjugation to protein, as shown in Figure 3.4. The conjugates produced were used in immunisation and screening steps.

The formation of amide bonds between amino and carboxyl groups can be effected by the combined use of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide - EDC - and N-hydroxysuccinimide, or NHS. EDC reacts with carboxyl groups to form highly reactive *O*-acylisourea intermediates, and the addition of NHS leads to the production of more stable esters. Reaction with amine groups on the other compound to be conjugated results in the formation of amide bonds (Staros *et al.*, 1986). The reaction scheme for the production of protein conjugates of 7-hydroxycoumarin, with initial derivatisation of 7-hydroxycoumarin-4-acetic acid (John Quinn, DCU, Personal communication), is given in Figure 3.5.

Coupling occurs predominantly at the ϵ -amine group of lysine residues on the protein.

Other types of coupling chemistry for hapten-carrier conjugates are also widely used. These include the use of heterobifunctional crosslinkers, such as NHS ester-maleimide and the homobifunctional crosslinker glutaraldehyde, which reacts with primary amine groups (Hermanson, 1996).

Figure 3.4 Reaction scheme for the production of a diazonium-coupled 7-hydroxycoumarin-protein conjugate. 3-amino-7-hydroxycoumarin is first treated with sodium nitrate to form the diazonium derivative, which is then reacted with protein. Conjugation occurs mainly at tyrosine and histidine residues.

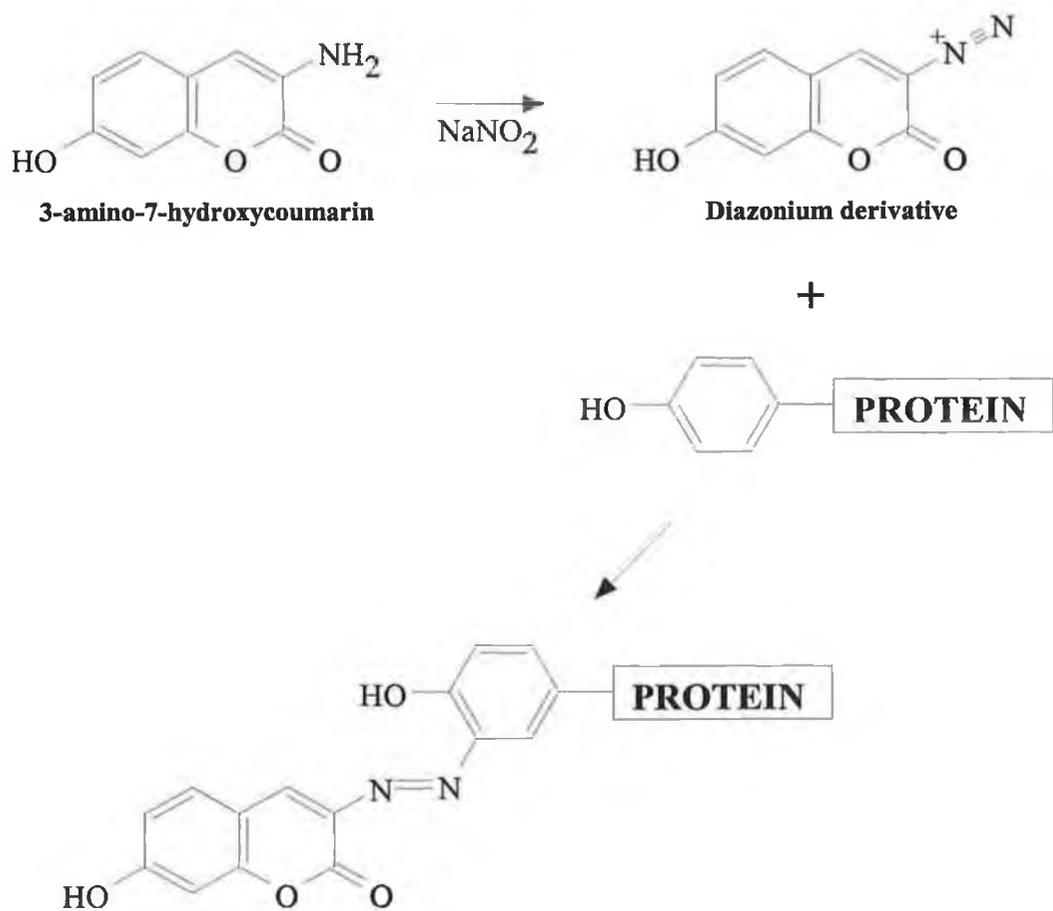
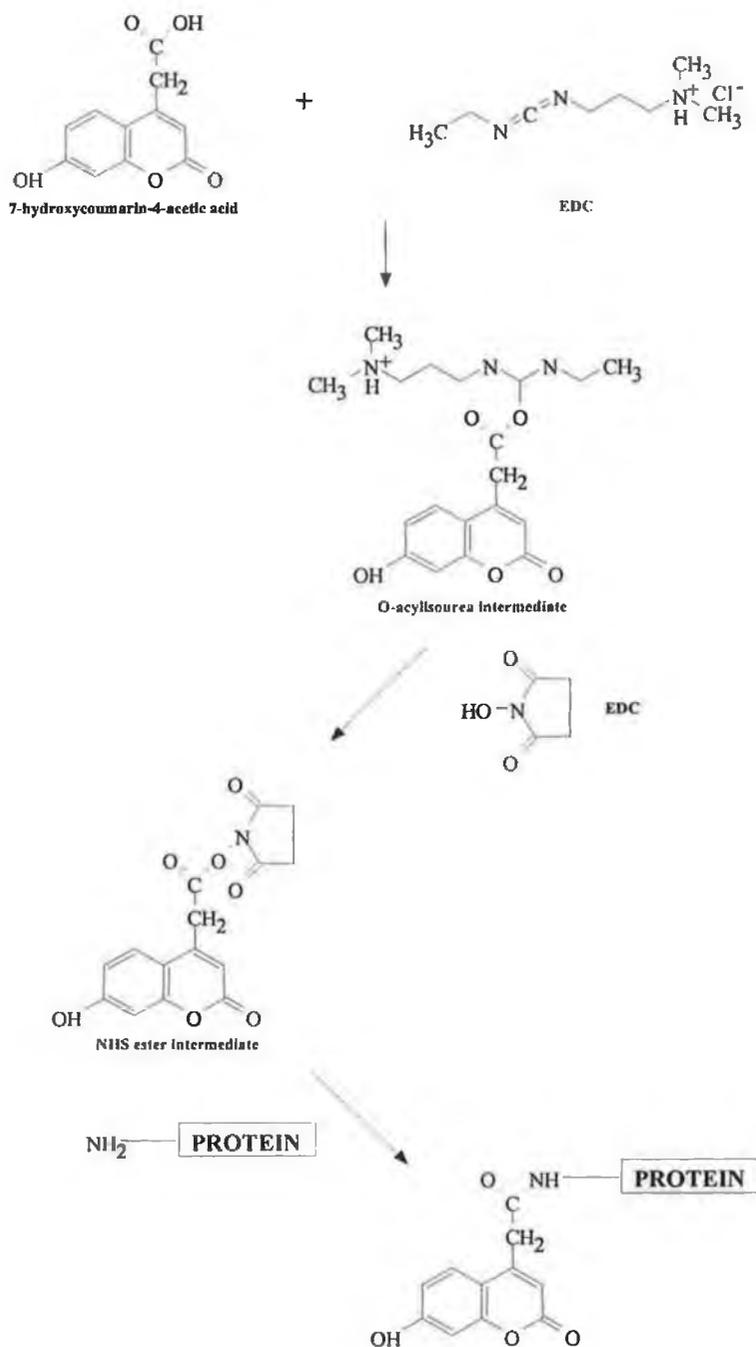


Figure 3.5 Conjugation of 7-hydroxycoumarin to proteins by EDC-mediated NHS esterification. Treatment of 7-hydroxycoumarin-4-acetic acid with EDC results in derivatisation at the carbonyl group and formation of an active o-acylisourea intermediate. Reaction with NHS causes NHS esterification, and addition of protein leads to the formation of amide bonds. The ϵ -amino groups of lysine are the predominant site of conjugation.



3.4.2 Polyclonal antisera

The serum produced by any host animal in response to immunisation is known as a polyclonal antiserum. 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. When producing a polyclonal antiserum, the host animal is generally chosen on the basis of ease of handling and the quantity of serum obtainable. Rabbits, sheep and goats are among the species most commonly used.

The concept of antibody affinity cannot be applied to polyclonal antisera, due to the heterogeneity of their composition. Instead, the term "avidity" is used to describe an average affinity value for the mixture of antibodies in a polyclonal antiserum. The binding of polyclonal antibodies to an antigen with several epitopes is often many times stronger than that of a single antibody to a monovalent antigen, as a result of the formation of multiple antibody-antigen bridges between molecules.

While individual antibody molecules exhibit high specificity of binding, the presence of similar epitopes on different (frequently related) molecules may result in antibodies binding to antigens other than those against which they were raised. Due to their mixed population, this effect - known as "cross-reactivity" - is more prevalent among polyclonal antibodies; it may also, however, be encountered when dealing with homogeneous antibody preparations. In this and subsequent chapters, the term cross-reactivity will be used to denote binding of antibodies to shared or similar antigenic determinants on different molecules. The related phrase of "non-specific binding" is taken to refer to any other extraneous interaction which takes place (usually between proteins) in a biological matrix, generally as a result of electrostatic attraction.

From a practical aspect, the production of polyclonal antibodies is a relatively inexpensive and rapid process. Consistency between batches of antisera is impossible to guarantee, however, and even with large molecular weight immunogens, a maximum of 30% of the antibodies obtained will be of the desired specificity (Catty, 1988).

3.4.3 Monoclonal antibodies

In order to produce a homogeneous antibody preparation - or monoclonal antibody - it is necessary to isolate and propagate one individual B cell clone. B lymphocytes, however,

can be cultured *in vitro* for only a short time. The production of monoclonal antibodies was pioneered by Kohler and Milstein (1975), when they immortalised antibody-producing cells by fusing them with a continuously growing cell line.

Monoclonal antibody production by somatic cell fusion requires initial immunisation of a host animal with the molecule of interest. When the titre of antibodies in the blood has reached a satisfactory level, the animal - usually a mouse or rat - is given a final intravenous injection of the immunogen (without adjuvant). This results in the presence in the spleen of a large number of specific B lymphocytes when the animal is sacrificed three or four days subsequently (Campbell, 1986).

The other fusion partner in the process is a mouse myeloma cell line. Myeloma cells are tumorigenic B lymphocytes which can be readily cultivated *in vitro*, and those used for monoclonal antibody production should not themselves secrete any antibody. They are also deficient in an enzyme called hypoxanthine guanine phosphoribosyl transferase (HGPRT). The final number of fused cells will be low, which requires that selective media be used to promote their growth. HGPRT⁻ cells cannot utilise the salvage pathway of nucleic acid synthesis, and when cultured in medium containing hypoxanthine, aminopterin and thymidine (HAT medium) - which blocks the *de novo* synthesis of nucleic acid production - only fused cells will proliferate.

Splenocytes from the immunised animal are fused with myeloma cells by the addition of polyethylene glycol (PEG), which promotes membrane bridging and communication. Nuclear fusion will occur in a certain proportion of the fused cells, and a percentage of these cells will undergo mitosis and chromosome mixing (Freshney, 1983). The resulting cells - which possess the immortality of the myeloma line and the antibody production capability of the splenocytes - are called "hybridomas".

The cell mixture, which will contain splenocytes, myelomas and hybridomas, is divided into aliquots and cultured in selective HAT medium in multi-well cell culture clusters. After a number of weeks, by which time only the hybridomas should survive. The culture supernatants are assayed for the presence of specific antibody and eventually hybridomas derived from a single cell are produced.. This can be achieved by a process known as cloning by limiting dilution, in which the contents of positive wells are divided and subdivided a number of times (Goding, 1996). The predominant protein in the culture

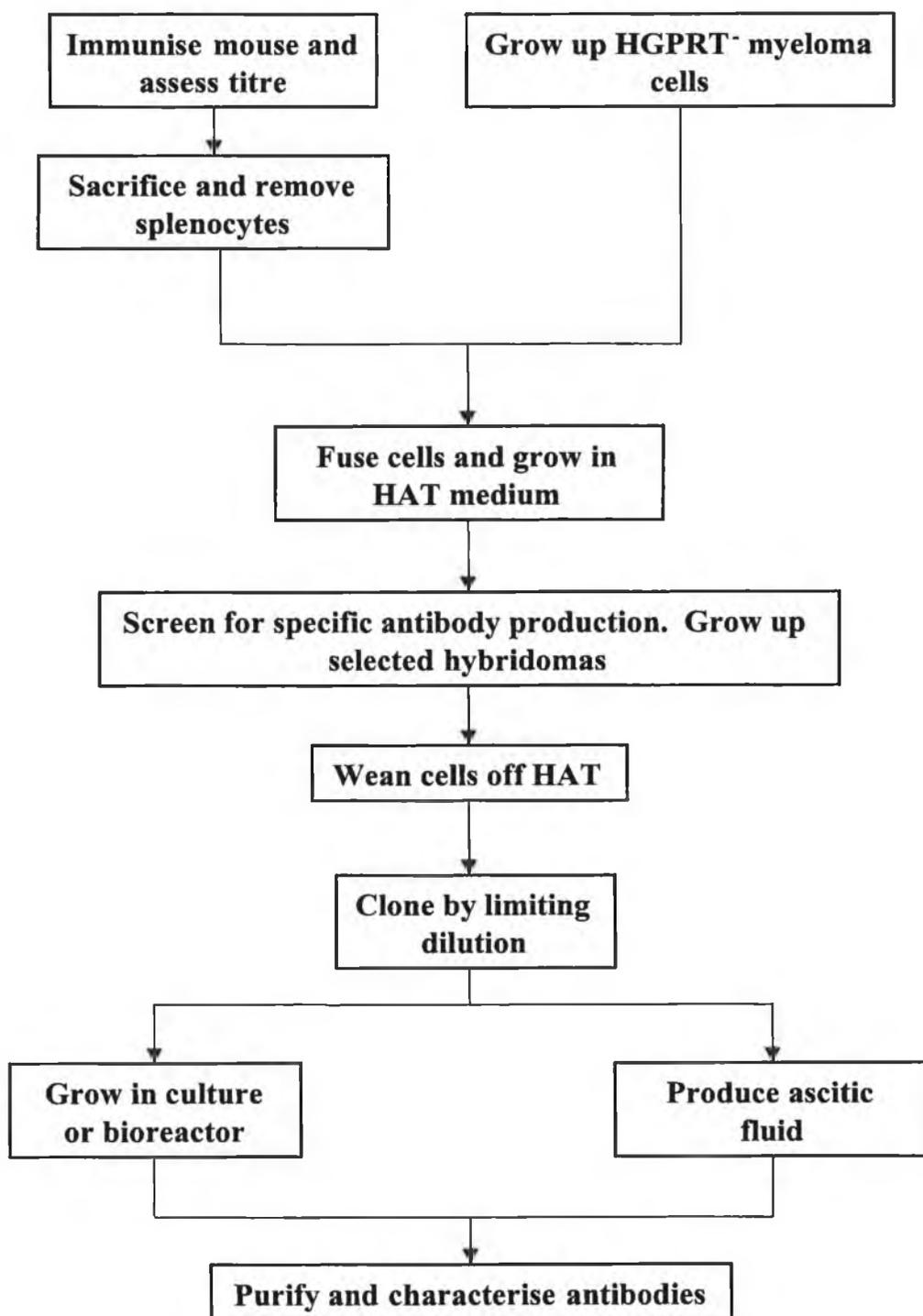
supernatant from these cells will be monoclonal antibody of the required specificity. As the average amount of antibody in supernatant from conventional cell culture flasks is only between 10 and 100 mg/ml (Epstein and Epstein, 1986), the generation of ascites fluid has traditionally been used to produce large amounts of antibody. When hybridoma cells are injected into the peritoneal cavity of a mouse or rat they rapidly proliferate, and the resulting ascites fluid which forms can contain up to 1000 times as much specific antibody as spent cell culture medium (Galfré and Milstein, 1981). The high yields from this method, however, are accompanied by the presence of considerable amounts of contaminating protein. Concern has also been widely expressed for the welfare of animals used to produce ascites fluid (Marx *et al.*, 1997). These factors have led to the development of high cell density bioreactors which allow the *in vitro* production of large quantities of high purity antibody (Evans and Miller, 1988).

Although the description above deals with mouse and rat antibodies, it is possible for human monoclonal antibodies to be produced in a similar fashion. Routine immunisation of humans is not, of course, possible. Nor is the use of the spleen as the source of lymphocytes; instead, B cells are generally taken from the tonsils or other lymphoid tissues. An alternative approach is to use the technique of *in vitro* immunisation. With this procedure, the antigen of interest is added to a mixture containing unsensitised B lymphocytes, together with macrophages, T helper cells, and other stimulatory and growth factors required to carry out the immune response (Carroll *et al.*, 1989).

Whatever the method used to generate specific B cells, the lack of availability of non-secreting human myeloma cell lines means that human lymphocytes are fused with mouse myelomas, resulting in the production of hybridomas which are frequently unstable due to chromosome loss.

A schematic representation of the steps involved in producing a murine monoclonal antibody is given in Figure 3.6. The inherent specificity of monoclonal antibodies can enhance the sensitivity of many immunoassays. However, the high affinities of these antibodies may render them unsuitable for use in regenerable immunosensor formats (see Chapter 5). The generation of monoclonal antibodies is also a much more expensive and labour-intensive process than that required to produce polyclonal antisera.

Figure 3.6 Flow diagram showing the main steps in the production of a murine monoclonal antibody. Spleen cells from an immunised mouse are fused with non-secreting myeloma cells and cultured in selective HAT medium. Cells producing specific antibody are cloned to ensure homogeneity, and monoclonal antibody is purified from ascitic fluid or culture medium.



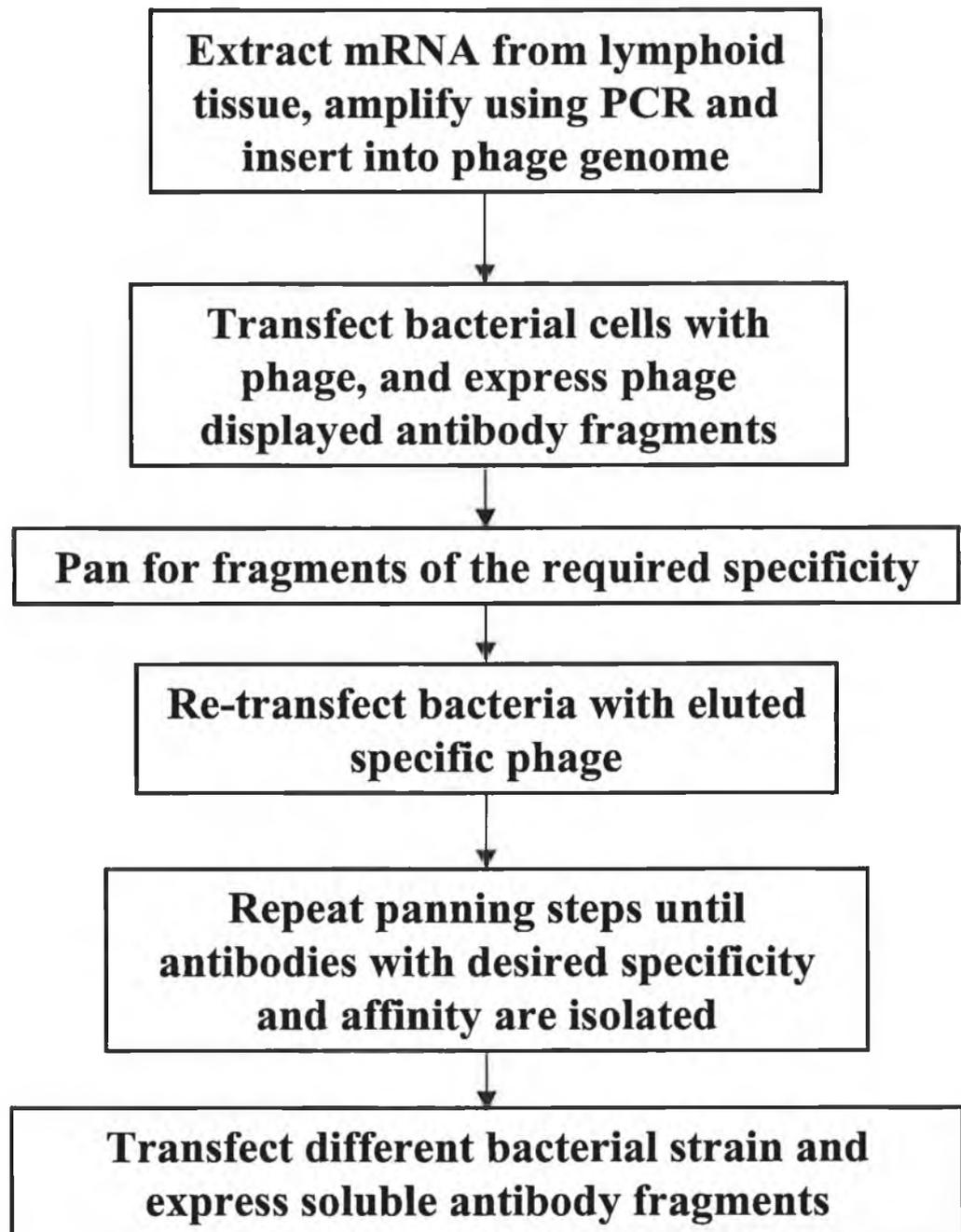
3.4.4 Antibody engineering

The continuing improvement in both the efficiency and the ease of use of recombinant DNA technology has meant that it is now possible to produce specific antibody by means of genetic engineering. The genes for antibody heavy and light chains can be isolated from cells of the immune system and inserted into a vector. A vector is a means of transferring genetic information into a host organism ("transfecting"), which then produces the desired protein. It is generally unpractical to incorporate entire antibody genes into a vector, and usually only those fragments necessary for antigen binding are used.

Plasmids are naturally-occurring closed circles of DNA which have been widely used as vectors in the field of antibody engineering. They may be used to transfect a range of different hosts. Better *et al.* (1988) utilised a plasmid vector to produce a Fab fragment against human carcinoma which was secreted by *E. coli*. Yeast was used by Horwitz *et al.* (1988) as the host organism for the production of a Fab fragment, and functional antibodies were expressed in plants by Hiatt *et al.* (1989).

A more recent development in the genetic engineering of antibodies is that of phage display technology. A bacteriophage, or phage, is a virus which infects bacteria, and can thus be used as a vector. However, by coupling inserted antibody genes to one of two phage coat protein genes, the antibody fragment can be displayed on the surface of the phage particle (McCafferty *et al.*, 1990). This means that phage bearing specific fragments can be selected on the basis of affinity, and transfecting into bacterial cells. This selection is achieved by adding a mixture of phage to antigen immobilised on a solid support. Phage particles expressing unspecific antibody fragments, or fragments with low affinity, are washed away, and binders are eluted. This process is known as "panning". Panning can be repeated several times, and each step should result in an enrichment of the culture supernatant. When the required specificity and affinity have been obtained, the phage particles are transfecting into a different strain of *E. coli* which causes the antibody fragment to be expressed as a soluble protein, and secreted into either the cytoplasm or the growth medium. A flow chart showing the main steps involved in producing antibody fragments from a phage display library is given in Figure 3.7.

Figure 3.7 Flow diagram showing the main steps in the production of antibody fragments from a combinatorial phage display library.



The entire repertoire of antibodies expressed on the surface of all the phage particles prior to panning is known as a combinatorial phage display library. One way of producing such a library is to immunise an animal, remove lymphoid cells, extract DNA coding for antibody and insert this into phage. In such a system, large amounts of the antibody being expressed should be of the desired specificity. A procedure of this type was employed by Clackson *et al.* (1991) to generate antibody fragments directed against the hapten phenylloxazolone from immunised mice. Amersdorfer *et al.* (1997) also used the spleens of immunised mice to generate antibodies to botulinum neurotoxin type A, which may have therapeutic applications for neutralisation of the toxin.

However, the necessity for immunisation means that this approach comes no closer to solving the problems involved with human monoclonal antibody production. An alternative technique is to use lymphoid tissue from an unimmunised individual to construct what is known as a "naïve" library. Due to the genetic hypervariability discussed in section 3.2, such a library will contain antibodies to all possible antigens, and fragments of the required specificity can be isolated by repeated panning and enrichment steps. Marks *et al.* (1991) used a naïve phage display library to produce antibody fragments specific to turkey egg-white lysozyme. Dorsam *et al.* (1997) produced IgM fragments against a range of steroids including digoxin and progesterone from a naïve human library.

Antibody engineering has made possible the production of antibody fragments smaller than those produced by enzymatic degradation. These are shown in Figure 3.1. The variable fragment, Fv, is composed of the V_H and V_L domains. The lack of a disulphide linkage makes the Fv fragment inherently unstable, however, and methods to covalently join the two chains together, forming a single chain Fv (scFv), have been developed. An scFv directed against a human tumour marker has been produced by Savage *et al.* (1993).

An Fd fragment - consisting of either the V_L and C_L or V_H and C_H1 domains joined together - has also been generated, as has a single CDR region. The production of CDR regions is of great importance in the so-called "humanisation" of antibodies, and Riechmann *et al.* (1988) have created humanized antibodies in which only the CDR regions are of mouse origin.

Hybrid molecules such as bifunctional antibodies have also been produced by antibody engineering. Bifunctional antibodies are bivalent antigen-binding molecules, in which the two arms of the antibody are specific for two different antigens. Their production and

applications have been reviewed by Nolan and O'Kennedy (1992). Bifunctional antibodies were first produced for use in cancer chemotherapy by targeting tumour cells and providing close contact with the chemotherapeutic agent (Raso and Griffin, 1981). They may also be used to obviate the need for antibody labelling in immunoassays (Reinartz *et al.*, 1996). The chemical means used to conjugate labels such as enzymes and fluorophores may damage the biological efficiency of the immunological component. If the label is bound by one arm of the bifunctional antibody, however, there is no necessity for chemical modification of the structure. Both biological and chemical methods for the production of bifunctional antibodies have been developed. Biological production is based on fusing two hybridomas of different specificities (Reading, 1981), or fusing a hybridoma specific for one antigen with splenocytes from an animal immunised with a second antigen (Milstein and Cuello, 1984). Chemical methods of bifunctional antibody production basically involve cleaving two antibodies and joining Fab fragments of differing specificities, or preparing heteroconjugates, which consist of two separate monoclonal antibodies that have been chemically crosslinked.

Holliger *et al.* (1993) engineered a hybrid molecule consisting of the V_H and V_L domains of one antibody covalently linked to the V_H and V_L domains of another. This novel bifunctional antibody fragment was named a "diabody". Atwell *et al.* (1996) generated a stable bispecific scFv dimer with reactivity for glycophorin and N9 neuraminidase. This dimeric protein was formed by non-covalent association of the two variable domains, and was designated a bisFv. Using a different approach to the production of a bifunctional antibody fragments, Pearce *et al.* (1997) created an scFv-streptavidin fusion, with specificity for neuraminidase, by genetic methods. This was linked to a biotinylated anti-ferritin Fab' to form a bispecific molecule. Iliades *et al.* (1997) reported that scFv fragments which were produced by directly linking V_H and V_L domains formed into stable trimers, called "triabodies".

3.5 Antibody purification

The purification of antibodies from serum, culture supernatant, bacterial cell lysate or ascites fluid is often a necessity. The extent of purification required depends upon the final

application, and ranges from the removal of any non-immunoglobulin material to the isolation of specific antibody to a particular antigen.

Ammonium sulphate precipitation is frequently employed as a preliminary crude purification step. This procedure separates proteins on the basis of their solubility. Addition of saturated ammonium sulphate to an antibody-containing solution increases the number of hydrophobic interactions between the proteins, and results in precipitation of the immunoglobulin fraction, among other proteins. Approximately 50% of the extraneous protein can be removed by this treatment.

Further purification of the IgG fraction of the sample can be achieved by chromatographic methods. Ion-exchange chromatography may be used to purify antibodies. As antibodies are more basic than other serum proteins, they can be separated by using anion exchangers such as DEAE-cellulose (English, 1994).

Column chromatography with protein A or protein G may also be used to isolate IgG. Protein A is a 42 kDa polypeptide produced by *Staphylococcus aureus*. The protein has 4 binding sites for the Fc region of IgG, two of which may be occupied at any time. The binding sites for protein A are located on the C_H1 and C_H2 domains. Protein G is a 30 kDa protein which is found in the cell walls of β -haemolytic streptococci. This protein also binds strongly to mouse and rabbit IgG, with a higher affinity for some isotypes (most notably IgG₁) than protein A. Proteins A and G can be immobilised on a solid matrix, such as Sepharose gel, and poured into a column (Surolia *et al.*, 1982). When the sample is added, any IgG present will bind to the column, and can subsequently be eluted by changes of pH or ionic strength (Hudson and Hay, 1980). Antibodies can also be purified on the basis of size, using HPLC (Carty and O'Kennedy, 1988).

Specific antibodies may also be isolated by affinity column chromatography, where the antigen is immobilised on a solid support. One such method has been described for the purification of specific anti-7-hydroxycoumarin antibodies from rabbit serum (H. Reinartz, Personal communication). In this procedure, 7-hydroxycoumarin-4-acetic acid was coupled to EAH sepharose by EDC/NHS esterification and poured into a column. Serum from an immunised rabbit was passed down the column, and specific antibodies were eluted with 50 mM phosphate buffer containing 0.5 M NaCl, pH 11.0. A yield of 4% for specific anti-7-hydroxycoumarin was reported.

3.6 The role of BIAcore in the analysis of antibody production

In recent years, BIA technology has become a widely-used tool in the production and characterisation of antibodies. Screening for specific monoclonal antibodies in cell culture supernatants is traditionally carried out by ELISA. In addition to being a laborious and time-consuming procedure, ELISA is an equilibrium system, and binders with high dissociation constants (i.e. low to moderate affinity) may be lost. The ease of automation, ability to analyse crude samples, and high sample throughput of BIA instrumentation would seem to make it ideally suited for such a task.

BIAcore has been utilised to screen for antibody fragments produced by genetic means. Soluble Fab fragments produced using a recombinatorial phage display library (established from *in vitro* immunised lymphocytes) were screened on BIAcore by Duenas *et al.* (1996). Binders were selected on the basis of their rates of dissociation from the immobilised antigen.

The use of BIAcore to pan an entire phage display library has been investigated by Malmberg and Borrebaeck (1995). They devised a model system in which a mixture containing 10% specific and 90% non-specific phage-displayed antibody fragments were injected over an immobilised antigen. Bound phage particles were eluted from the chip, and the eluate was collected and re-analysed, and exhibited a 5-fold increase in specific phage. Duenas *et al.* (1996) also used BIAcore to screen a phage display library generated from immunised lymphocytes, and demonstrated that soluble Fab fragments derived from phage collected during the later stages of elution had lower dissociation rates than those which were more readily eluted.

BIAcore can also be used to characterise antibodies, with respect to their specificity, subclass and concentration. One valuable application is that of pairwise epitope mapping of a panel of antibodies. In this procedure, a capture molecule is immobilised at the sensor surface and the first antibody is bound. The antigen is then added, followed by a second antibody. If the two antibodies are specific for the same epitope, then the second antibody will not bind. By analysing all the possible pairs of antibodies in this way, an epitope map of the antigen can be constructed. Fägerstam *et al.* (1990) used this procedure to study the binding of 29 murine monoclonal antibodies to recombinant HIV-1 proteins. Allauzen *et al.*

(1995) also investigated the binding of a panel of 7 monoclonal antibodies to insulin by this method.

Yu *et al.* (1998) utilised BIAcore to characterise the binding behaviour of hybrid biomolecules consisting of the Fab' fragment of a rat monoclonal antibody linked to the cardiac protein Troponin I.

Johne *et al.* (1993) used BIAcore to isotype antibodies to human heart myoglobin, and found the results to be comparable to those obtained from commercial isotyping kits.

By ensuring that conditions of mass transport limitation prevail (see section 3.7), the concentration of scFv, Fab, diabody and whole antibody in crude samples can be determined (Abraham *et al.*, 1995; Kazemier *et al.*, 1996). Schier and Marks (1996) employed mass transport limited analysis of this type to measure the percentage of specific phage present in bacterial cell lysate, and used the results to determine the antigen concentration for the next round of panning.

3.7 BIAcore for measuring antibody affinities

The concept of antibody affinity has been discussed in section 3.3. The real-time aspect of biospecific interaction analysis makes it a useful tool for the estimation of affinity constants and association and dissociation rates, based on the assumption that the antibody-antigen interaction obeys pseudo-first order reaction kinetics (Karlsson *et al.*, 1991).

From equation 3.3.2, the rate of production of analyte-ligand complex can be expressed as:

$$\text{Equation 3.7.1} \quad \frac{d[AB]}{dt} = k_a[A][B] - k_d[AB]$$

where [A], [B] and [AB] are the concentrations of analyte, ligand and analyte-ligand complex, respectively, k_a is the association rate constant, and k_d is the dissociation rate constant. In BIAcore investigations, the ligand is immobilised on the surface of the sensor chip, so the concentration of AB is identical to the concentration of bound analyte, which is proportional to the observed response, R:

$$\text{Equation 3.7.2} \quad \frac{dR}{dt} = k_a[A][B] - k_dR$$

The concentration of free ligand - [B] - is the difference between total and bound ligand. The total amount of ligand on the chip surface is determined indirectly as it becomes saturated with analyte. In other words, the maximum response due to antibody binding - R_{\max} - will be proportional to the total ligand concentration, and $(R_{\max} - R)$ will be proportional to the free ligand concentration:

$$\text{Equation 3.7.3} \quad \frac{dR}{dt} = k_a [A] (R_{\max} - R) - k_d R$$

As the analyte is supplied to the surface in continuous flow, the concentration of free analyte - [A] - can be considered to be constant, and identical to that of the original free analyte concentration. The rate equation can therefore be rewritten as:

$$\text{Equation 3.7.4} \quad \frac{dR}{dt} = k_a C (R_{\max} - R) - k_d R$$

Where C is the concentration of injected analyte in moles. The equation can be rearranged as follows:

$$\text{Equation 3.7.5} \quad \frac{dR}{dt} = k_a C R_{\max} - (k_a C + k_d) R$$

This implies a linear relationship between the derivative of the binding curve, and the observed response. In theory, then, the rate constant can be obtained by plotting dR/dt against R.

In order to calculate the rate constants in this way, however, it is necessary to know the value of R_{\max} - the response obtained when the immobilised ligand is saturated with analyte.

This practical terms, this requires very high levels of analyte: injecting 10 times the amount needed to give 50% saturation will result in only 91% saturation. In addition the time taken to reach equilibrium may be very long. The necessity of determining the R_{\max} value experimentally can be avoided if the slopes of plots of dR/dt against R (i.e. $k_a C + k_d$) over a range of concentrations are plotted against concentration. This results in a line with the equation:

Equation 3.7.6 $\text{slope (dR/dt vs R)} = k_a C + k_d$

which has a slope of k_a and an intercept on the abscissa of k_d .

Once the sample plug has passed over the chip surface and is replaced by running buffer, the change in response is due solely to the dissociation constant, provided that no significant re-binding of dissociated analyte to the surface occurs:

Equation 3.7.7 $dR/dt = -k_d R$

Analyte re-binding can be minimised by the use of faster flow rates. Integrating equation 3.7.7 with respect to time gives:

Equation 3.7.8 $\ln \frac{R_{t_1}}{R_{t_n}} = k_d (t_n - t_1)$

where R_{t_1} is the response at an arbitrarily chosen time 1, and R_{t_n} is the response at later time n . If the log of decrease in response is plotted against time, the slope of this line will be k_d .

Since the value of dR/dt at equilibrium is zero, by introducing the affinity constant ($K = k_a/k_d$) the equation can be rearranged as follows:

Equation 3.7.9 $R/C = KR_{\max} - KR$

Therefore, by plotting R/C against R for a range of concentrations at equilibrium, a line with a slope of K and an intercept on the abscissa of KR_{\max} can be obtained.

Some practical considerations must be taken into account in order that BIAcore kinetic and affinity experiments produce accurate data. The fundamental factor governing the association and dissociation of antibody and antigen is the mass transport of analyte across a stationary layer of solution at the surface of a flow cell. Diffusion across this layer is

dependent upon the concentration gradient and the thickness of the layer, the latter depending upon the flow rate. In order for correct calculation of kinetic parameters, any mass transport limitation must be overcome, so that the rate of binding is controlled only by the association and dissociation constants. The flow rate used in kinetic determinations should be high enough to remove mass transport effects, but low enough to reduce sample wastage.

Perhaps the most important point to address in experimental design is which of the two interactants to immobilise. In general, this should be the component which is more stable to regeneration. Ideally the more precious sample should be immobilised in order to minimise its consumption. When performing kinetic analyses it is necessary to use a range of analyte concentrations. In an antibody-antigen system, the absolute concentration of antigen is often much easier to determine than that of the antibody preparation, making it preferable to immobilise antibody. This approach is not, however, practicable where the antigen in question is of low molecular weight.

The amount of ligand immobilised should be sufficiently high to allow measurement of a reasonable range of responses, while remaining low enough to avoid steric hindrance and mass transport limitation. Generally speaking, an R_{\max} value of 300-2000 RU will satisfy these requirements. The amount of ligand to be immobilised in order to obtain an R_{\max} value in this range can be calculated using the following equation:

Equation 3.7.10
$$R_L = (300 - 2000) \times \frac{1}{s} \times \frac{MW_L}{MW_A}$$

where R_L is the number of response units of immobilised ligand, s is the number of analyte molecules which can bind each ligand molecule, MW_L is the molecular weight of the ligand, and MW_A is the molecular weight of the analyte.

The concentration of analyte to be used should be sufficiently high to avoid mass transport limitations, yet low enough to give a signal in a satisfactory time for derivative readings to be taken.

Recently, some researchers have reported significant deviations from the pseudo first-order kinetic behaviour assumed for the interactants in the above model. O'Shanessy and Winzor

(1996) concluded that these differences were due to the heterogeneity of the immobilised ligand sites, and suggested that the net observed rate constant might be used to calculate more accurate k_a and k_d values. Nieba *et al.* (1996) reported the use of a competitive method for measuring affinity constants which exhibited considerable differences from values calculated using binding kinetics.

Results

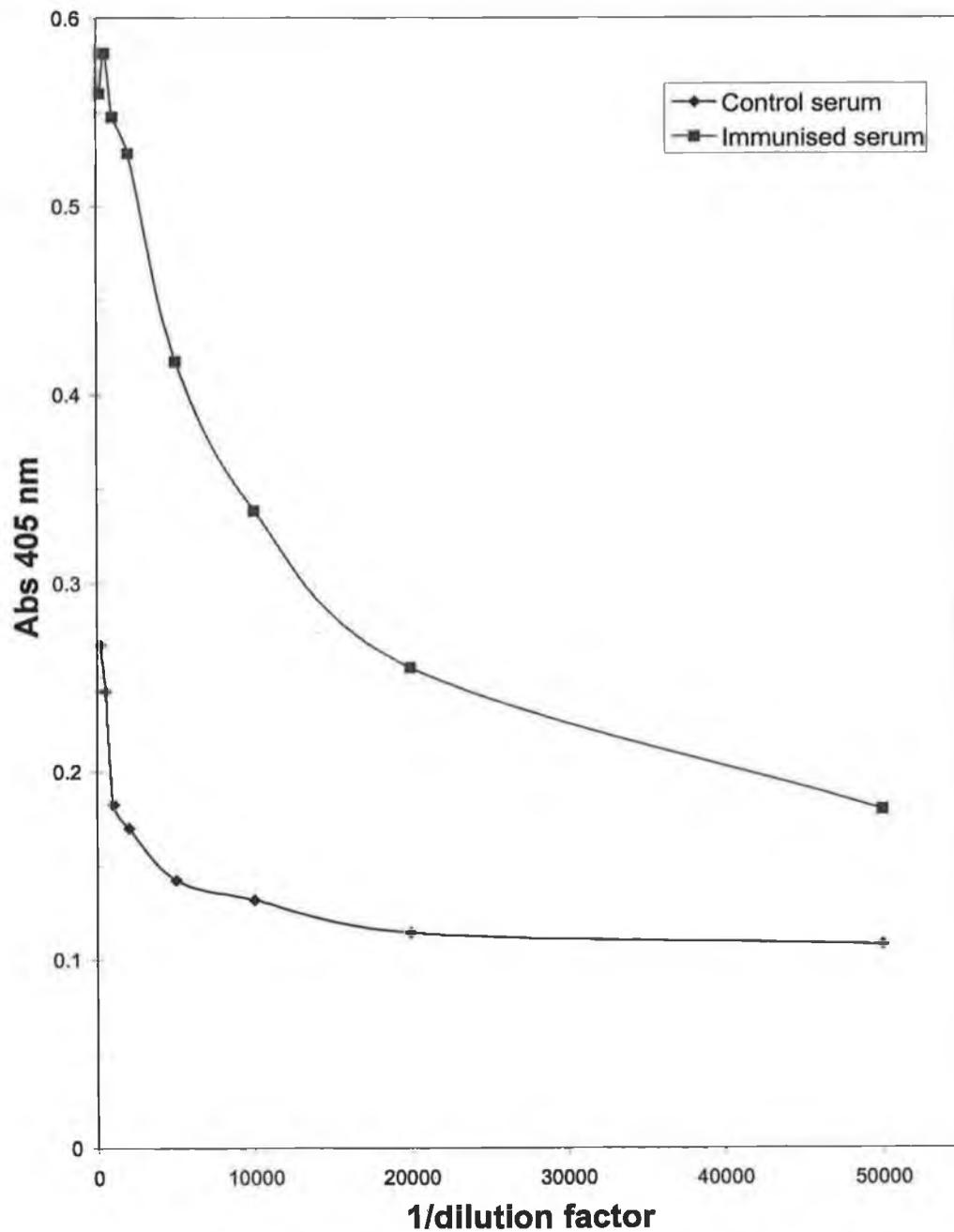
3.8 Production and characterisation of polyclonal anti-7-hydroxycoumarin

Polyclonal antibodies to 7-hydroxycoumarin were produced, purified by saturated ammonium sulphate precipitation and protein G affinity chromatography. The purity of the antibody was investigated by SDS-PAGE, and the working dilution of antibody in a competitive ELISA for 7-hydroxycoumarin was assessed. The coating and blocking of chemically-activated microtitre plates with 7-hydroxycoumarin was optimised, and these plates were used in an enzyme-linked immunosorbent assay (ELISA) to examine the degree of cross-reactivity of the antibody preparation with a range of related coumarin derivatives.

3.8.1 Estimation of titre

New Zealand White rabbits were immunised with a diazo-coupled thyroglobulin conjugate of 7-hydroxycoumarin as described in section 2.2.2. The rabbits were injected subcutaneously with a solution of the conjugate in Freund's adjuvant. Blood was drawn periodically to estimate the titre of specific antibody. The titre was assessed by ELISA, as outlined in section 2.7.1. 96-well microtitre plates were coated with 7-hydroxycoumarin-BSA and blocked with 1% (w/v) BSA. Dilutions of rabbit serum (see section 2.3.1) from 1/200 to 1/50000 were prepared. Control serum from an unimmunised rabbit was diluted in the same way, and both sets of samples were added to the plate, followed by labelled anti-rabbit antibody and 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. The final titre of serum was greater than 1/50000, and an overlay plot of the results for immunised and control serum is shown in Figure 3.8.

Figure 3.8 Overlay plot showing the titres for immunised and non-immunised (control) rabbit serum. Serial dilutions of both were added to wells coated with 7-hydroxycoumarin. At a dilution of 1/50000, serum from the immunised animal still gives a higher reponse than the control serum.



3.8.2 Purification of polyclonal antiserum

The serum was initially partially purified by saturated ammonium sulphate precipitation, as described in section 2.4.1. The precipitate was then further purified by protein G affinity chromatography as in section 2.4.2. IgG in the sample bound to the protein G on the column, and was then eluted, after washing, by addition of glycine buffer. The presence of antibody in collected fractions was determined by monitoring the absorbance at 280nm. A typical elution profile for anti-7-hydroxycoumarin from the protein G column is shown in Figure 3.9.

Fractions containing antibody were pooled and dialysed, and the concentration of IgG was determined by BCA assay as described in section 2.5. The average protein concentration of pooled fractions was 4-5 mg/ml.

3.8.3 Characterisation of purified antibody by SDS-PAGE

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) is a technique which separates proteins on the basis of molecular weight. The purity of the polyclonal antibody preparation was assessed by SDS-PAGE as described in section 2.6. The antibody was electrophoresed under reducing conditions, along with a series of molecular weight markers, on a gel containing 5% (w/v) acrylamide. The results are shown in Figure 3.10. Boiling together with mercaptoethanol causes the polypeptide chains of the immunoglobulin to separate, and the two main sample bands on the electropherogram - one between the 45 kD and 66 kD markers, and the other below the 29 kD marker - correspond to the 50 kD heavy chain and the 25 kD light chain of IgG. The "spreading" seen for the second band is probably due to a combination of high protein concentration and excessive voltage.

3.8.4 Working dilution of antibody in a competitive ELISA for 7-hydroxycoumarin

Egan and O'Kennedy (1993b) described the use of polyclonal anti-7-hydroxycoumarin in a competitive ELISA for the free drug (see Chapter 4). The working dilution of this antibody in such an immunoassay format was assessed as described in section 2.7.2.

Figure 3.9 A typical elution profile for polyclonal anti-7-hydroxycoumarin from a 1 ml protein G affinity column. Crude sample was passed down the column, followed by 20 column volumes of wash buffer. Bound antibody was then eluted with 0.1 M glycine, pH 2.5, 1 ml fractions were collected, and the protein content was measured by absorbance at 280 nm. The majority of IgG is eluted in the first 3 fractions.

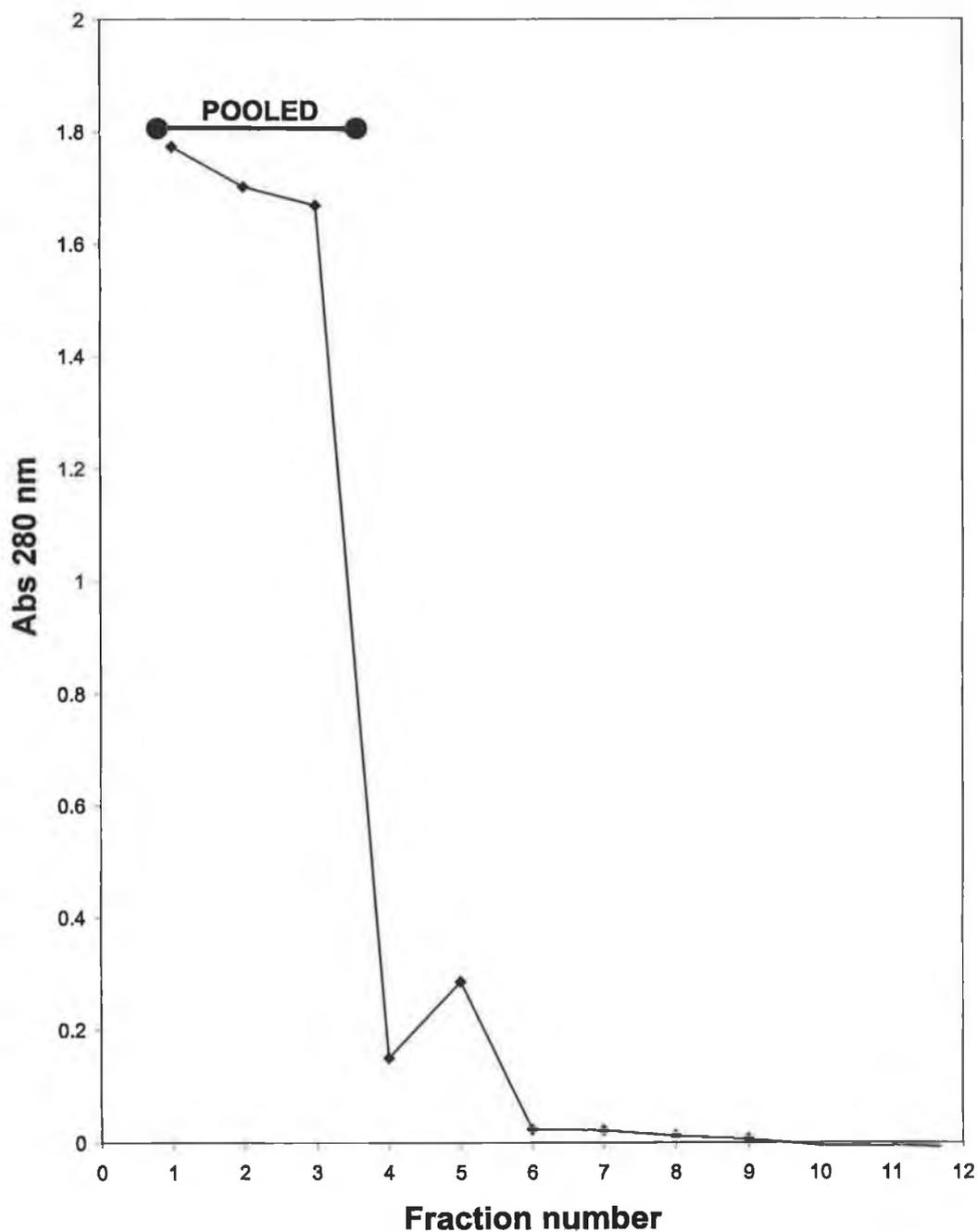
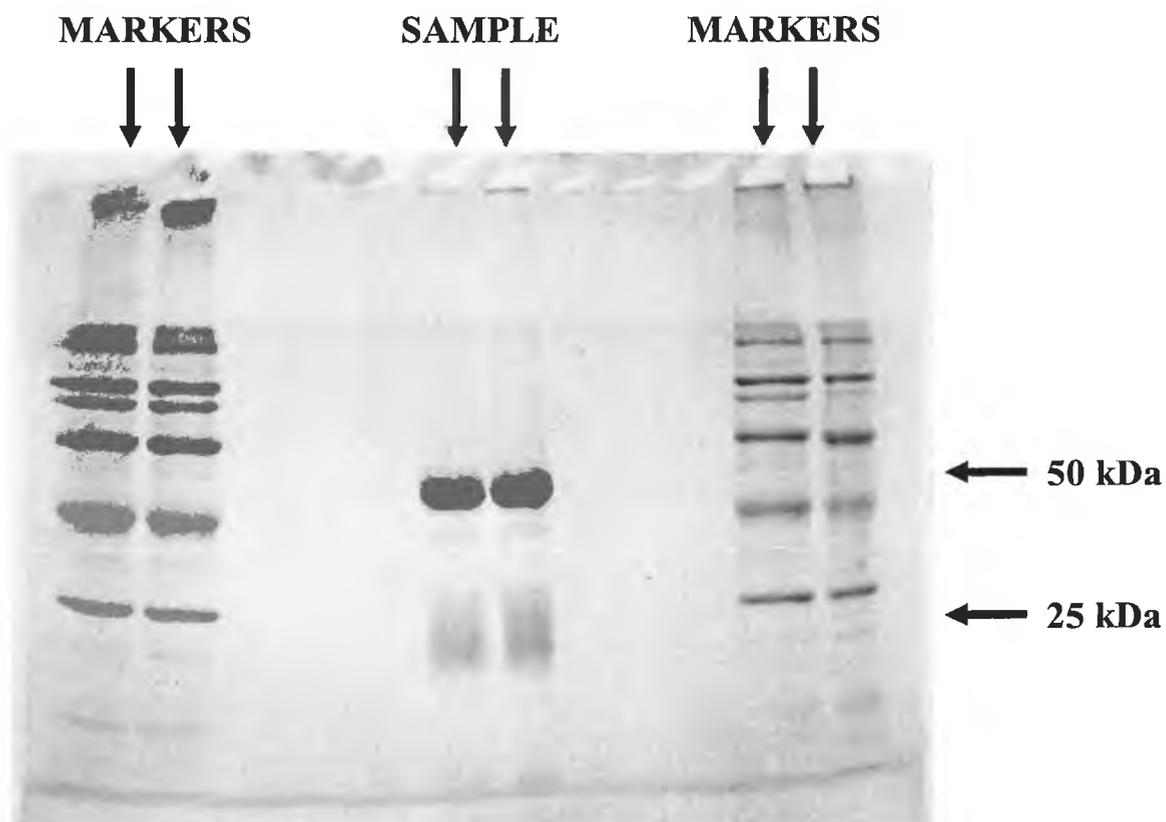


Figure 3.10 SDS-PAGE of purified polyclonal anti-7-hydroxycoumarin. Rabbit antiserum which had been purified by saturated ammonium sulphate precipitation and protein G affinity chromatography was electrophoresed under reducing conditions. Markers containing carbonic anhydrase (29 kDa), ovalbumin (45 kDa), BSA (66 kDa), phosphorylase B (97.4 kDa), β -galactosidase (116 kDa) and myosin (205 kDa) were also run. The sample has one band between the 45 kDa and 66 kDa markers, and one below that for the 29 kDa marker, consistent with the presence of the 50 kDa heavy chain and 25 kDa light chain of IgG. The “spreading” seen for the 25 kDa sample band is probably due to a combination of high protein concentration and high voltage.



Wells of a microtitre plate were coated with 7-hydroxycoumarin-BSA, and blocked with 1% (w/v) BSA. Dilutions of purified rabbit anti-7-hydroxycoumarin from 1/50 to 1/2000 were prepared, as well as a range of standard solutions of 7-hydroxycoumarin. Equal volumes of appropriate standard and antibody solution were added to the wells. After incubation, enzyme-labelled anti-rabbit IgG was added, followed by a chromogenic substrate. An overlay plot of the results for the different antibody dilutions is shown in Figure 3.11. An antibody dilution of 1/200 performs well in the assay, giving a standard curve with an r^2 value of 0.98 and a measuring range of 2.5 - 40 $\mu\text{g/ml}$ of 7-hydroxycoumarin, as shown in Figure 3.12.

3.8.5 Cross-reactivity of polyclonal anti-7-hydroxycoumarin

The coating and blocking of Covalink plates (NUNC) with 7-hydroxycoumarin-4-acetic acid was first optimised. These microtitre plates were then used to assess the cross-reactivity of purified polyclonal antibody with a range of coumarin derivatives.

3.8.5.1 Optimisation of coating and blocking for Covalink plates

The polystyrene surface of Covalink microtitre plates is derivatised with amino groups, allowing the direct covalent attachment of molecules via a range of chemistries. NHS esterification was used to link 7-hydroxycoumarin-4-acetic acid to the plates as described in section 2.7.3. Plates were coated with a 1 mg/ml solution of 7-hydroxycoumarin-4-acetic acid and blocked with a range of different reagents: 1% (w/v) gelatin, 5% (w/v) milk powder, 2% (w/v) ovalbumin, and 5% (v/v) foetal calf serum (FCS). Antibody was added to these wells, and to uncoated wells which had been blocked using the same reagents. Labelled anti-rabbit antibody and chromogenic substrate were then added, and the absorbance of wells at 280 nm was measured. 5% (v/v) FCS was found to give the best reduction in background reading.

Wells of covalink plates were then coated with a range of different concentrations of 7-hydroxycoumarin-4-acetic acid: 1, 2, 3, 4, and 5 mg/ml. These wells were then blocked with 5% (v/v) FCS. A range of dilutions of purified anti-7-hydroxycoumarin from 1/50 to 1/1600 were prepared, and 100 μl /well of appropriate dilutions were added to the plate.

Figure 3.11 An overlay plot for the assessment of the working dilution of purified anti-7-hydroxycoumarin in a competitive ELISA for 7-hydroxycoumarin. Antibody at various dilutions was mixed with standard 7-hydroxycoumarin solutions in the wells of microtitre plates coated with 7-hydroxycoumarin. The absorbance of wells at 405 nm is inversely proportional to the amount of drug present. Results are the averages of duplicate analyses.

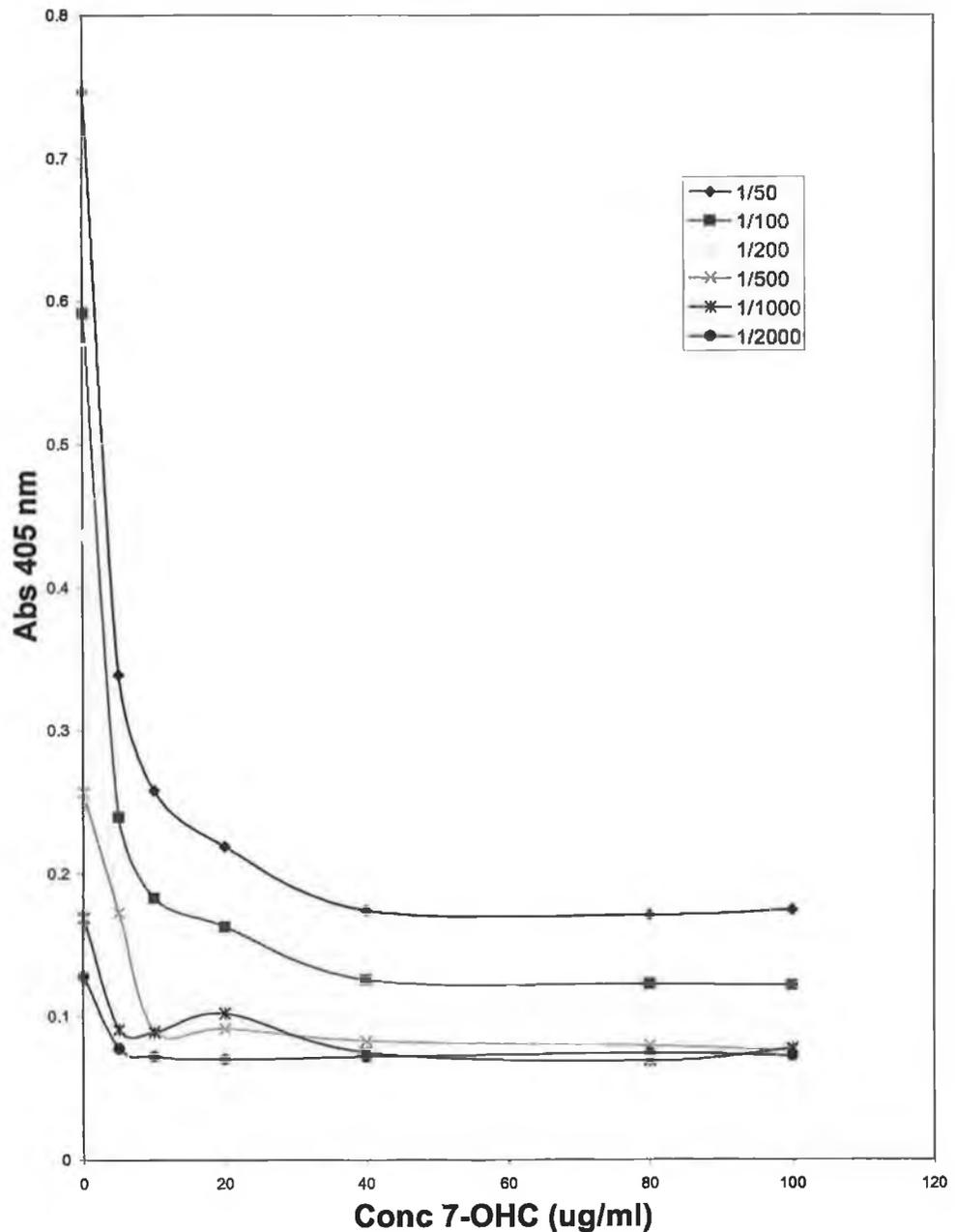
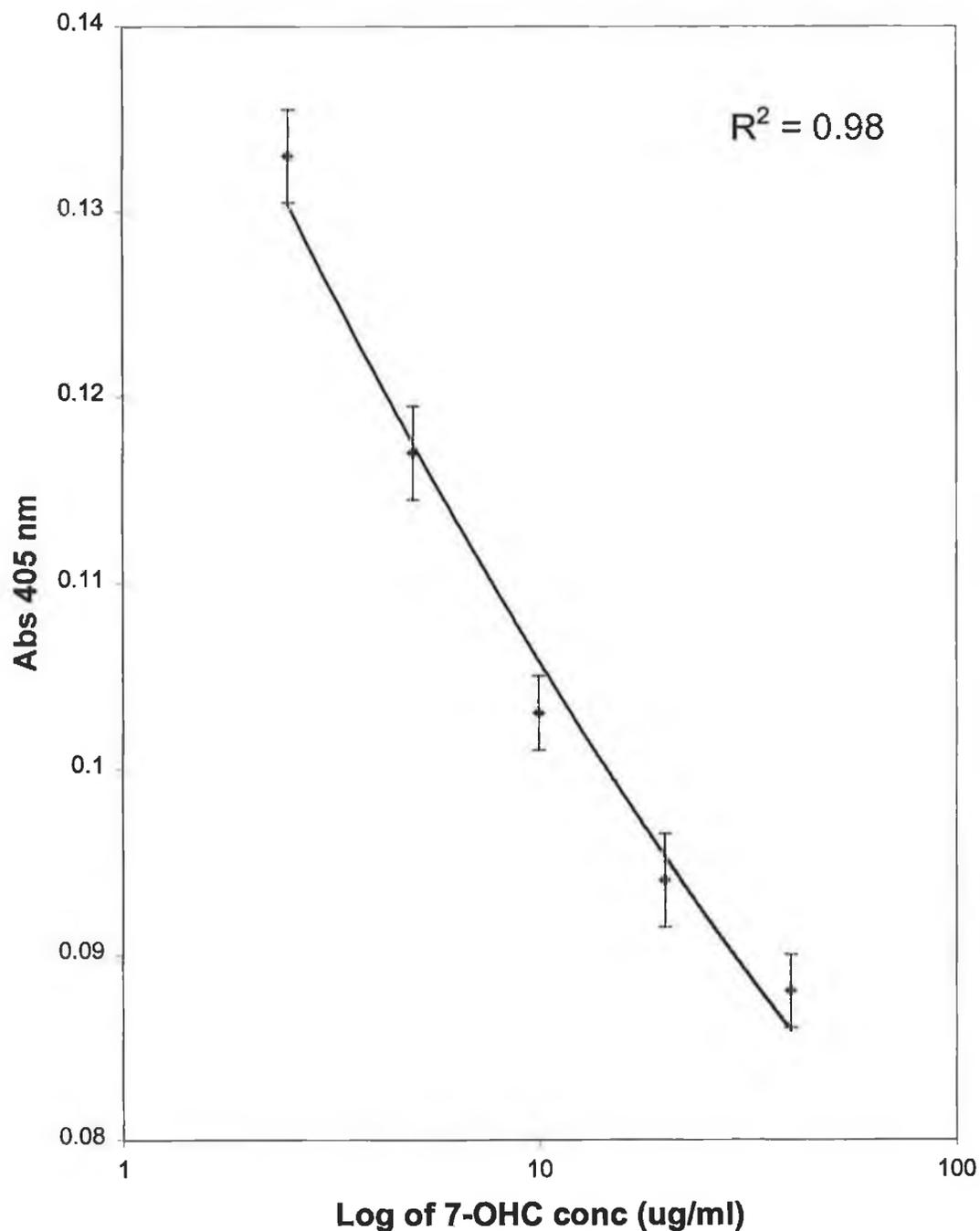


Figure 3.12 Standard curve for 7-hydroxycoumarin in a competitive ELISA format using a 1/200 dilution of purified polyclonal antibody. The absorbance at 405 nm is inversely proportional to the amount of 7-hydroxycoumarin present. The results shown are the average of five replicate analyses, and all coefficients of variation were below 2.5%. The assay has a measuring range of 2.5-40 $\mu\text{g/ml}$ and an r^2 value of 0.98.



Following addition of labelled anti-species antibody and chromogenic substrate, the absorbance of wells at 280 nm was measured. The results of this experiment are shown in Figure 3.13. It can be seen that the response at all antibody dilutions increases up to a coating concentration of 5 mg/ml.

At higher concentrations, 7-hydroxycoumarin-4-acetic acid is insoluble. Coating with 5 mg/ml of 7-hydroxycoumarin-4-acetic acid and blocking with 5% (v/v) FCS were thus taken to be the optimal conditions.

3.8.5.2 Assessment of antibody cross-reactivity by ELISA

The degree of cross-reactivity of purified polyclonal anti-7-hydroxycoumarin was determined by competitive ELISA as described in section 2.7.3.1. Wells of a Covalink microtitre plate were coated with 7-hydroxycoumarin-4-acetic acid and blocked with FCS. Solutions containing from 0 - 50 µg/ml of 7-hydroxycoumarin, warfarin, coumarin, 4-hydroxycoumarin, 7-hydroxywarfarin, and 7-hydroxycoumarin-4-acetic acid were prepared. 50 µl of appropriate solutions were added to wells of the microtitre plate, together with an equal volume of polyclonal antibody.

The absorbance reading for the wells containing 50 µg/ml of 7-hydroxycoumarin represented 100% inhibition of antibody binding. The degree of cross-reactivity of the antibody was calculated from the percentage inhibition produced by 50 µg/ml of each of the compounds tested, and is shown in Table 3.1. It can be seen that the antibody exhibits low cross-reactivity with warfarin and coumarin, moderate cross-reactivity with 4-hydroxycoumarin and 7-hydroxywarfarin, and is highly cross-reactive with free 7-hydroxycoumarin-4-acetic acid, 50 µg/ml of which results in 86% inhibition.

3.9 Production and characterisation of monoclonal antibodies

BALB/c mice were immunised with a 7-hydroxycoumarin-protein conjugate and the titre of antibodies in serum was estimated by ELISA, using a range of immobilised substances. Spleen cells from immunised mice were fused with a myeloma cell line, and culture supernatants from the resultant hybridomas were screened for antibody production by BIAcore and ELISA, with an immobilised drug-protein conjugate.

Figure 3.13 The effect of covalently linking increasing amounts of 7-hydroxycoumarin-4-acetic acid to Covalink microtitre plates. 1,2,3,4 and 5 mg/ml solutions of the compound were used to coat plates, and doubling dilutions of purified polyclonal antibody were added to wells. The binding increases up to a coating concentration of 5 mg/ml.

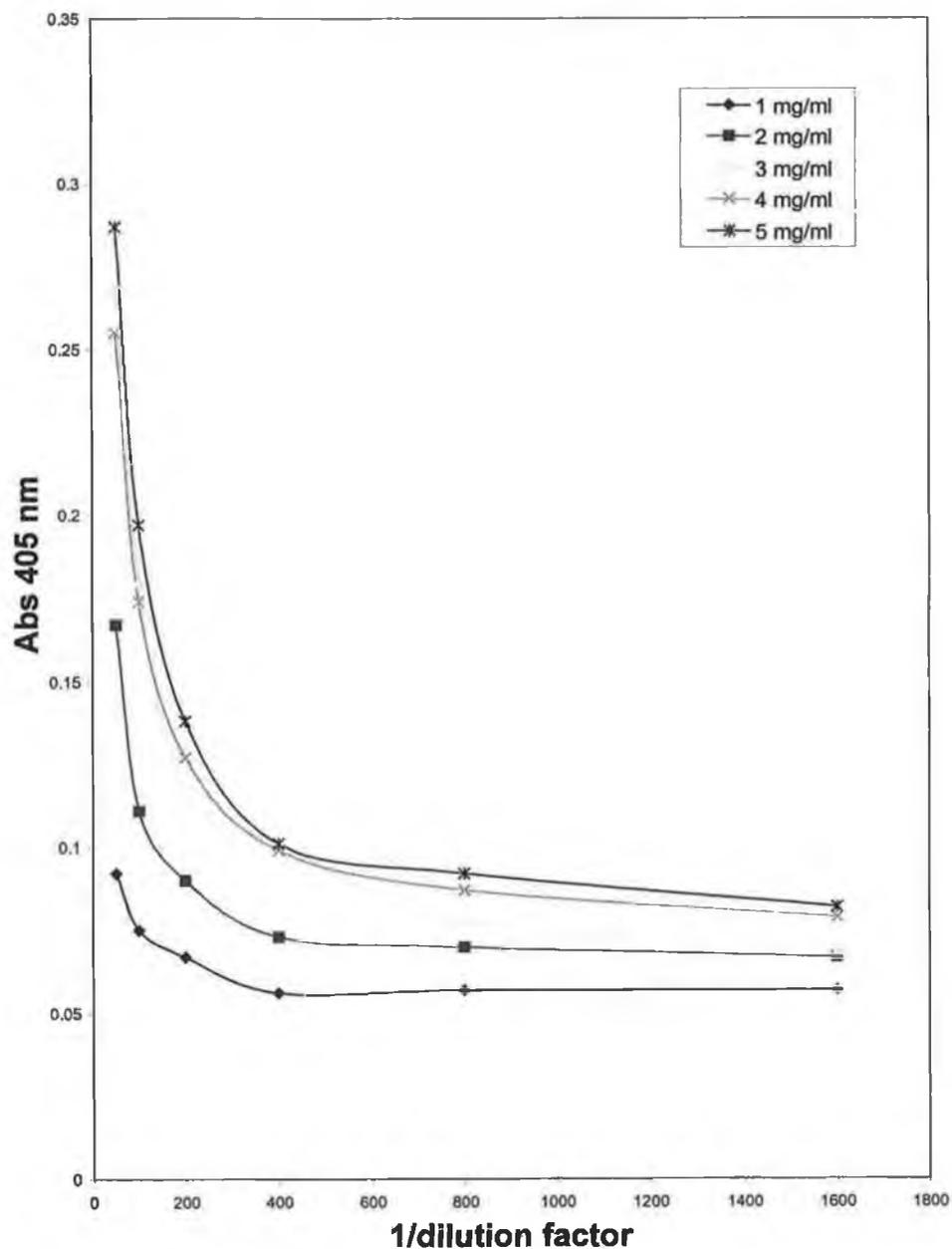


Table 3.1 Cross-reactivity of purified polyclonal anti-7-hydroxycoumarin. The antibody was tested in a competitive ELISA format. 7-hydroxycoumarin-4-acetic acid was covalently linked to Covalink plates, and antibody was added along with various concentrations of coumarin compounds. The percentage inhibition caused by 50 µg/ml of each compound was compared to the 100% inhibition of binding caused by 50 µg/ml of 7-hydroxycoumarin.

Compound	Percentage inhibition at 50 µg/ml
7-hydroxycoumarin	100%
7-hydroxycoumarin-4-acetic acid	86%
7-hydroxywarfarin	40%
4-hydroxycoumarin	41%
Warfarin	13%
Coumarin	13%

Hybridomas from positive wells were scaled up and rescreened against both drug-protein conjugate and native protein. 3 surviving hybridoma populations which exhibited significantly greater binding to drug-protein conjugate than to unconjugated BSA were cloned by limiting dilution. 13 positive clones were isolated which, when tested in a competitive ELISA format, were found not to bind to free drug. The affinities of the panel of antibodies for drug-protein conjugate were ranked using BIAcore.

3.9.1 Estimation of titre

BALB/c mice were immunised with an EDC/NHS-coupled 7-hydroxycoumarin-ovalbumin conjugate as described in section 2.2.3. The mice were injected subcutaneously and intraperitoneally with a solution of the conjugate in Freund's adjuvant. Blood was taken and the titre of specific antibodies was estimated by ELISA as in section 2.7.4. 96-well microtitre plates were coated with BSA, ovalbumin, diazo-coupled 7-hydroxycoumarin-ovalbumin and EDC/NHS-coupled 7-hydroxycoumarin-ovalbumin. In addition, Covalink plates were coated using 7-hydroxycoumarin-4-acetic acid.

Dilutions of mouse serum (see section 2.3.2) from 1/200 to 1/12800 were prepared. Serum from an unimmunised mouse and purified polyclonal anti-7-hydroxycoumarin were also prepared at the same dilutions, for use as negative and positive controls, respectively. Dilutions of immunised serum, negative and positive controls were added to appropriate wells, followed by labelled anti-species antibody and chromogenic substrate. The test serum gave a high response on the ovalbumin-coated plate, but did not bind to the BSA surface. Both immunised and control serum gave a similarly low response on the Covalink plate. Figure 3.14 shows that neither immunised nor control serum gave a significant response to the diazo-coupled conjugate, whereas the positive control (purified polyclonal antibody) bound strongly. On wells coated with EDC/NHS-coupled drug-protein conjugate, however, serum from the immunised mouse gave an even higher response than the positive control, and had a titre of greater than 1/12800. This result is shown in Figure 3.15. The mouse was then injected intravenously 3/4 days before being sacrificed.

Figure 3.14 Overlay plot showing the lack of binding of mouse serum to wells coated with a diazonium-coupled 7-hydroxycoumarin-BSA conjugate. Serum from an immunised mouse (Mouse 1) and pre-immune serum (Neg control) gave similarly low responses, while positive control (polyclonal anti-7-hydroxycoumarin) bound strongly to the immobilised conjugate.

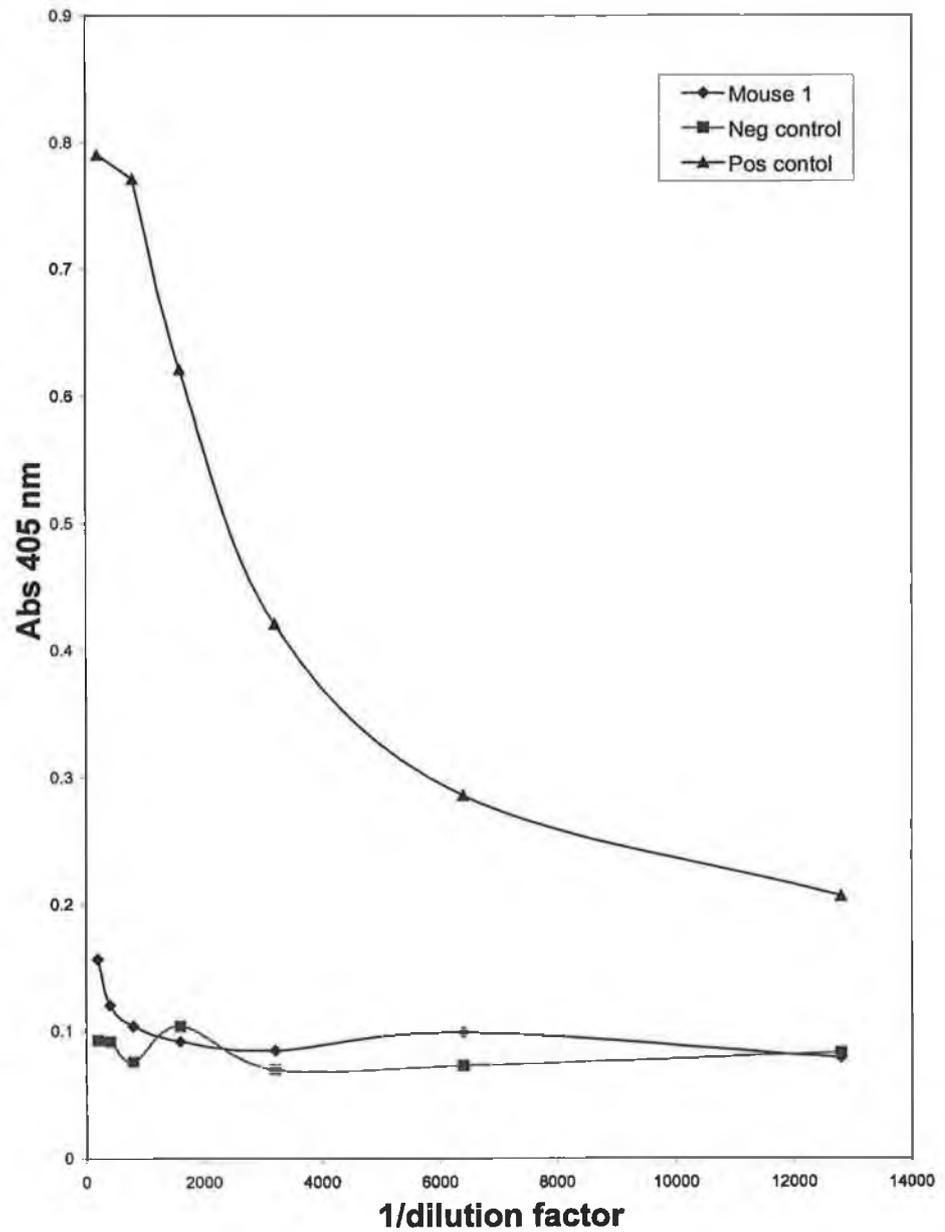
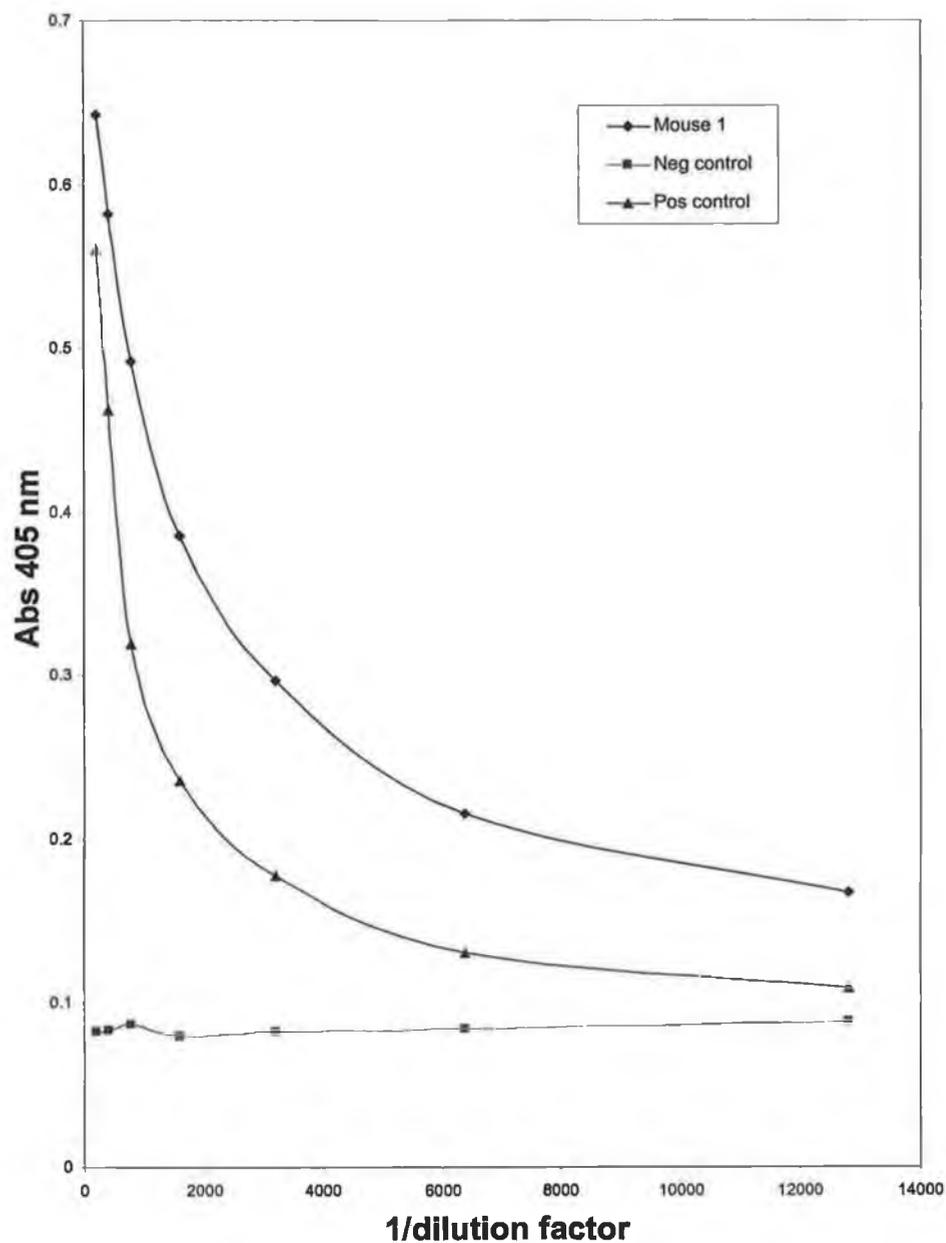


Figure 3.15 Overlay plot showing the titre of immunised mouse serum on wells coated with EDC/NHS-coupled 7-hydroxycoumarin-BSA conjugate. Serum from an immunised mouse (Mouse 1) gave a higher reponse than the positive control (polyclonal anti-7-hydroxycoumarin). The titre of the serum was greater than 1/12800.



3.9.2 Somatic cell fusion

Spleen cells from the immunised BALB/c mouse were fused with SP2 myeloma cells as described in section 2.8.6. The SP2 cell line was first tested for the presence of mycoplasma (see section 2.8.4). Splenocytes were mixed with SP2s in a 10:1 ratio, and then fused by the addition of PEG. The fused cells were distributed into eight 48-well cell culture clusters containing HAT medium (including BriClone). The plates were visually inspected 12 days later, and approximately 85% of wells were found to contain colonies of hybridoma cells, while SP2 cells which had been cultured in HAT medium in a set of control wells were dead. The cells were fed by removal of supernatant and addition of a similar volume of HAT medium.

The removed supernatant was assayed for the production of specific antibody as described below. The hybridomas were monitored daily, and those which were producing antibody and had attained 50 - 70% confluence (covered 50 - 70% of the surface) were scaled up into cell clusters with larger surface areas as described in section 2.8.6. The cells were gradually weaned off hypoxanthine, thymidine, aminopterin, and BriClone.

3.9.3 Screening of cell culture supernatants

Hybridoma supernatants from 48-well masterplates were screened for specific antibody production as soon as 50% confluence had been reached. Initial screening by ELISA is described in section 2.7.5. Neat culture supernatants were added to wells of plates coated with 7-hydroxycoumarin-BSA, followed by labelled anti-mouse antibody and chromogenic substrate. Purified polyclonal anti-7-hydroxycoumarin was included in all assays as a positive control.

Of the 336 hybridoma-containing wells which were screened, 27 produced a positive result when screened by ELISA against 7-hydroxycoumarin-BSA. The same supernatants were assayed by BIAcore. 7-hydroxycoumarin-BSA was immobilised on the sensor chip as in section 2.9.2, and neat culture supernatants were injected over this surface. No binding signal was obtained for any of the supernatants tested.

Those 27 hybridomas which had given positive screening results with ELISA were scaled up to 24-well cell culture plates. Supernatants from all hybridomas were then rescreened by ELISA. Wells of microtitre plates were coated with 7-hydroxycoumarin-BSA and native

BSA, and a sample of each culture supernatant was added to both wells. 12 of the positive hybridoma populations had ceased antibody secretion after scaling up, and the results for the other 15 supernatants from this round of screening are shown in Figure 3.16. It can be seen that of the 15 hybridomas tested, only 5 bound significantly more strongly (at least 2-fold greater) to 7-hydroxycoumarin-BSA than to BSA. Supernatants from the remaining wells were subsequently found also to bind to native thyroglobulin.

The 5 hybridomas which bound significantly to drug-protein conjugate were further scaled up to 12-well clusters, and supernatants were again screened by ELISA. At this stage, 2 of the hybridomas had ceased to secrete antibody. A summary of the results from ELISA screening of original hybridomas is given in Table 3.2.

3.9.4 Cloning by limiting dilution

The remaining three positive hybridomas were cloned by limiting dilution as outlined in section 2.8.7. Hybridomas were grown up in culture flasks and then added to wells of 96-well microtitre plates at 10 cells/well. When sufficient growth had occurred, the supernatants were again screened by ELISA against 7-hydroxycoumarin-BSA. 2 positive wells were identified, and the contents of these wells were gradually scaled up to 6-well plates, and then again cloned out into 96-well plates at 1 cell/well. Screening of these plates by ELISA yielded 13 positive clones, which were again scaled up.

3.9.5 Assessing reactivity of clones to free 7-hydroxycoumarin

The 13 clones isolated were assayed by ELISA for reactivity to free 7-hydroxycoumarin. Microtitre plate wells were coated with 7-hydroxycoumarin-BSA as for screening. Culture supernatants from the cloned hybridomas were added to wells together with 7-hydroxycoumarin at a final concentration in the wells of 50 µg/ml. Controls in which supernatant was diluted by the same factor with PBS were also set up. The assay was then performed as normal. The results are given in Figure 3.17, and clearly show that the presence of free 7-hydroxycoumarin in the wells does not inhibit binding to immobilised 7-hydroxycoumarin-BSA. 50 µg/ml is one of the most concentrated standards in both the ELISA and BIAcore competitive assays for 7-hydroxycoumarin, in which purified polyclonal antibody is used.

Figure 3.16 Supernatants from 15 hybridoma colonies screened against both 7-hydroxycoumarin-BSA and native BSA. Most of the antibodies produced reacted strongly with the unconjugated protein, but supernatants 2, 3, 8, 11 and 12 exhibited at least 2-fold higher binding to the drug-protein conjugate. 1/200 purified polyclonal anti-7-hydroxycoumarin was used as the positive control ("POS").

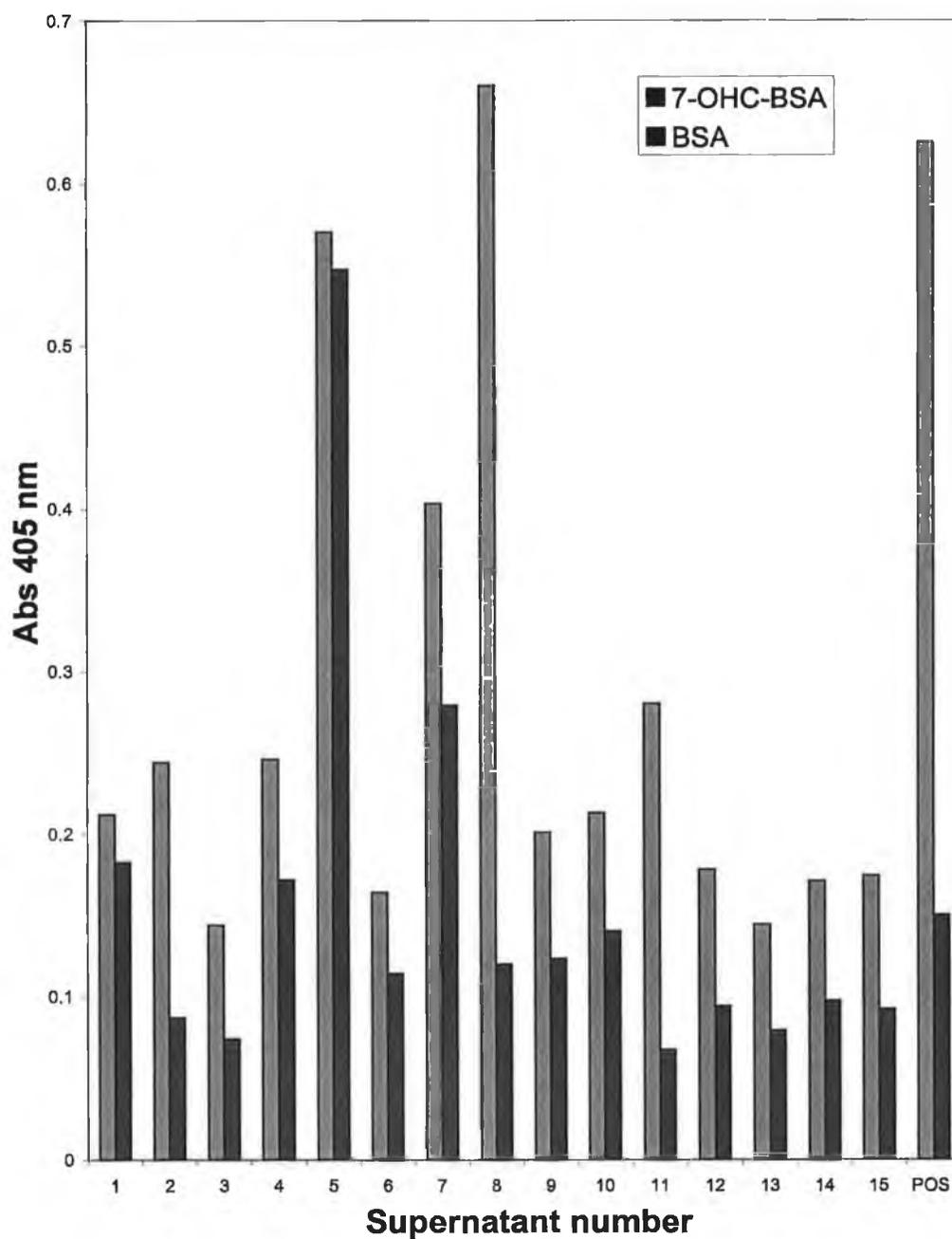
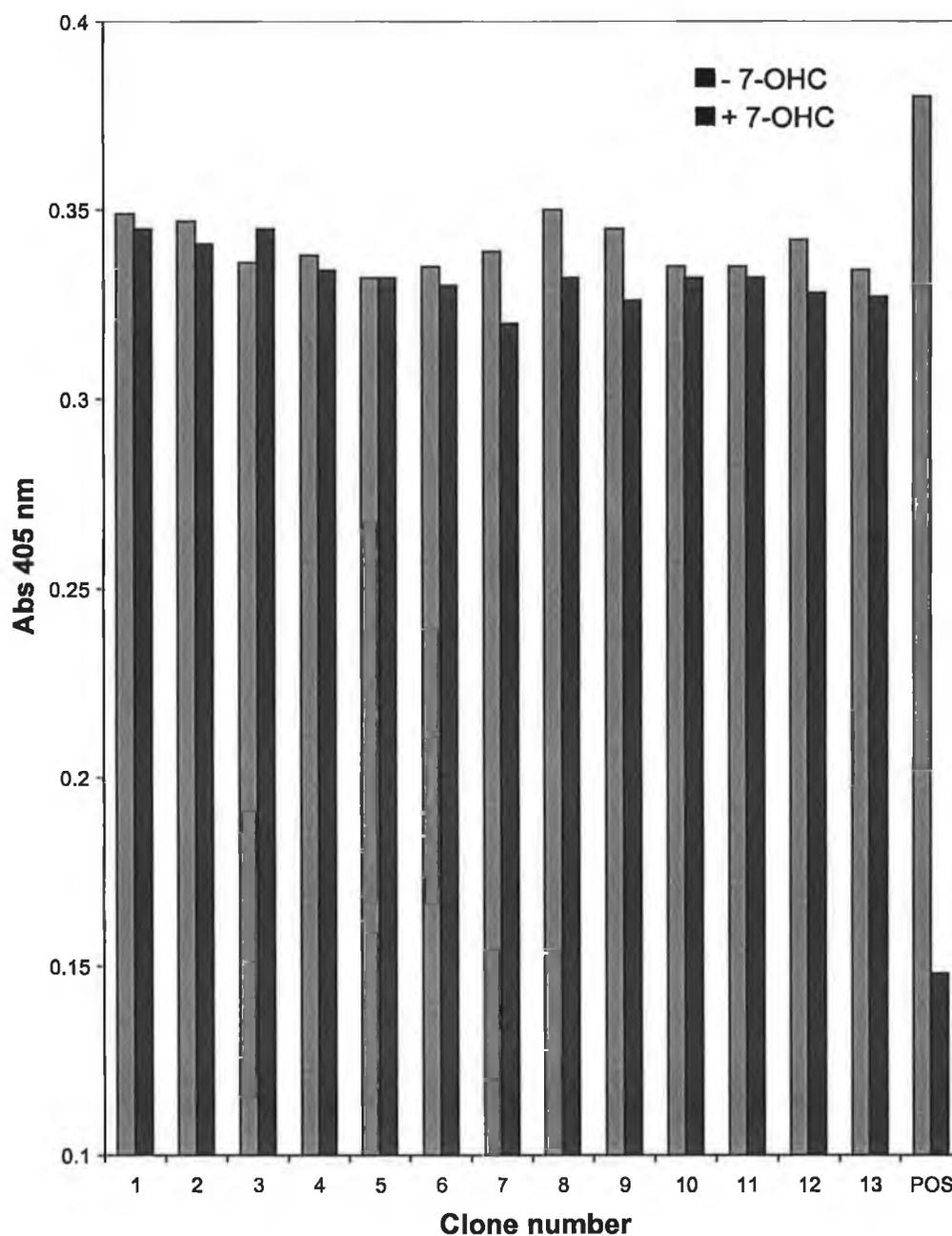


Table 3.2 Summary of results from the screening of hybridoma supernatants. Supernatants were tested against drug-protein conjugates and unconjugated protein.

Percentage of wells with hybridoma colonies	87.5%
Number of hybridoma supernatants screened	336
Number of supernatants positive for 7-hydroxycoumarin-BSA	27
Number of supernatants with significantly higher binding for drug-protein conjugate than native BSA	5
Number of positives cloned by limiting dilution	3

Figure 3.17 13 monoclonal antibodies tested in a competitive ELISA format. Wells of a microtitre plate were coated with 7-hydroxycoumarin-BSA, and antibody was added, with and without free 7-hydroxycoumarin. 1/200 purified polyclonal anti-7-hydroxycoumarin was used as the positive control ("POS"). The presence of free drug in wells containing antibody does not cause any appreciable inhibition. As the original stocks were shown not to bind significantly to native BSA, these results indicate that the antibodies produced are directed against a joint drug-protein epitope.



The microgramme quantities of antibody present in culture supernatant would be saturated by the addition of such a high amount of 7-hydroxycoumarin. This demonstrates that the monoclonal antibodies produced do not recognise free drug.

3.9.6 Affinity ranking of monoclonal antibodies

Of the 13 clones which were produced, 4 did not survive the scaling process. The affinities of the remaining 9 monoclonal antibodies for 7-hydroxycoumarin-BSA were ranked using BIAcore. Neat supernatants were injected over an immobilised 7-hydroxycoumarin-BSA surface (see section 2.9.2), and the response generated by the binding of each antibody was recorded. The concentration of mouse IgG in each culture supernatant was calculated by the method of standard addition, as described in section 2.7.13.

The relative affinities of the antibodies for the antigen - expressed as the BIAcore response divided by the calculated immunoglobulin concentration - are illustrated in Figure 3.18. The results suggest that the clones numbered 3,4,5 and 6 might be identical. The same may be true for numbers 8,11 and 12.

An example of a sensorgram obtained for the one of the hybridoma supernatants - clone number 3 - is given in Figure 3.19. This sensorgram is typical of those obtained for all 11 clones. It can be seen that the bulk refractive index change due to the composition of the cell culture supernatant is considerable, and appears to mask an initial high rate of antibody binding, as no large increase in signal during the rest of the injection phase is present to account for the binding signal of 670 RU. The shape of this sensorgram, with its rapid association and dissociation phases, suggests that the antibodies may have only a weak overall affinity for 7-hydroxycoumarin-BSA.

3.10 BIAcore-based kinetic and affinity studies on scFv fragments against a coumarin-BSA conjugate

A human semi-synthetic phage display library (Nissim *et al.*, 1994) - a naïve library which had been subjected to random mutagenesis in order to increase genetic diversity - underwent three rounds of panning on microtitre plates against a coumarin-BSA conjugate. Three clones which displayed reactivity to coumarin-BSA and produced soluble antibody were isolated, and expressed from large-scale culture.

Figure 3.18 Affinity ranking of a panel of 9 monoclonal antibodies on BIAcore. Neat hybridoma supernatants were injected over an immobilised 7-hydroxycoumarin-BSA surface, and the resultant binding signals were divided by the concentration of antibody in each supernatant.

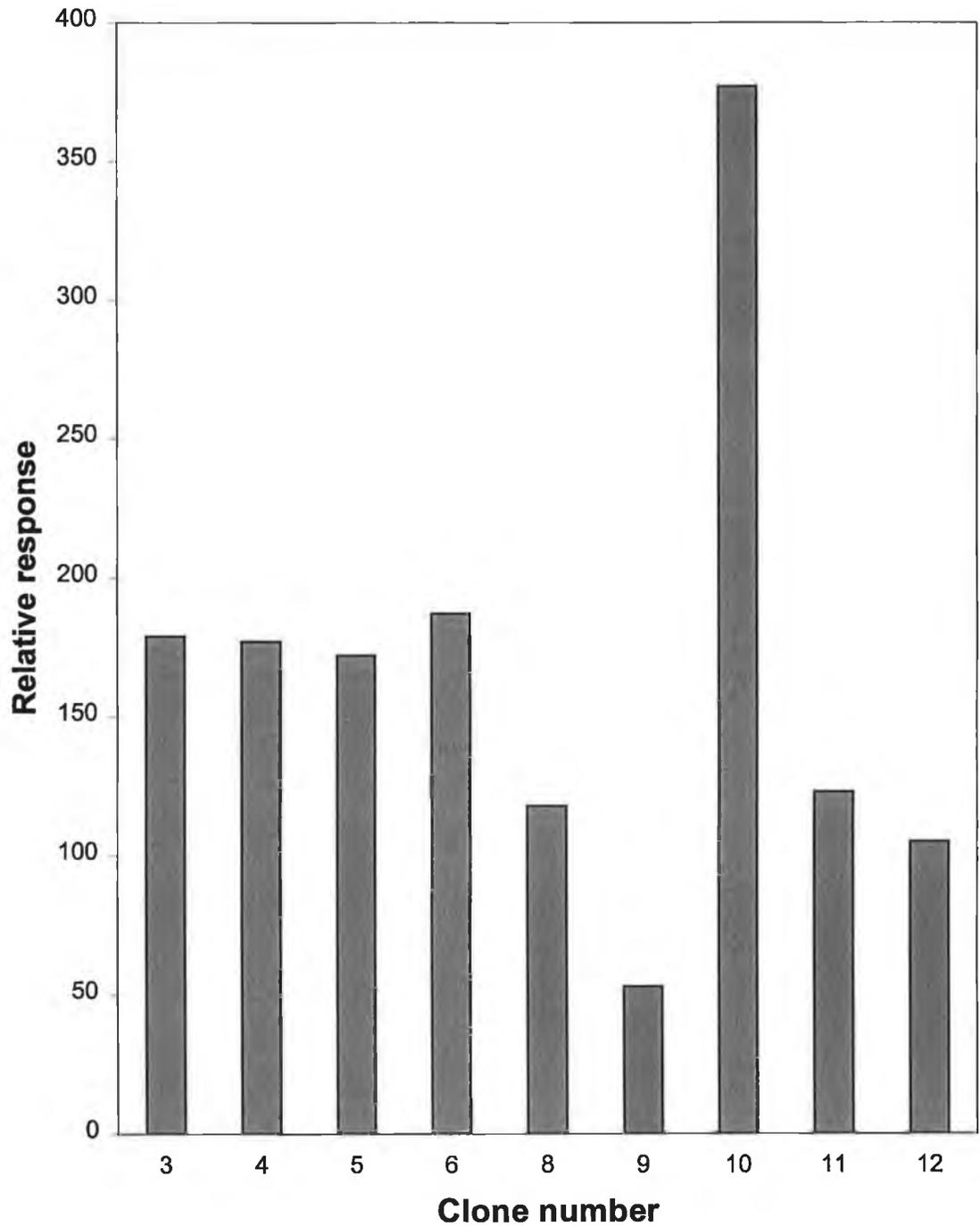
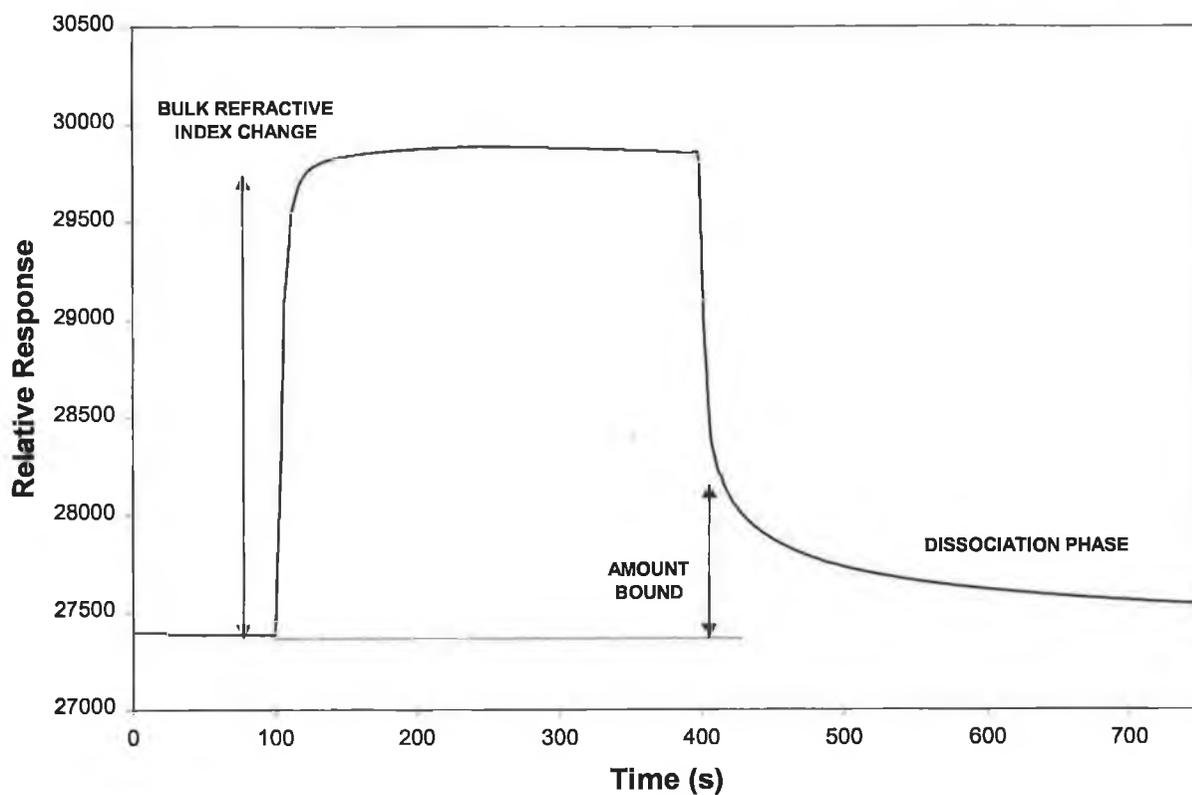


Figure 3.19 Sensorgram obtained for the binding of monoclonal antibody number 3 to immobilised 7-hydroxycoumarin-BSA. The initial bulk refractive index change appears to mask a fast association phase, which together with the rapid dissociation suggest an overall weak affinity constant for the antibody. The difference between the signal after injection and the initial baseline represents the amount of antibody bound.



None of the three antibodies selected exhibited binding to free coumarin. Bacterial periplasmic lysates from all three were purified by column chromatography with immobilised coumarin-BSA. The three clones were designated α -C5, α -C13 and α -C14 (“ α -C” stands for “anti-coumarin”) The production and purification of antibodies was carried out by Anthony Killard, DCU. BIAcore was then used to affinity rank the purified scFvs and to calculate apparent affinity and rate constants for one of the antibody fragments.

Coumarin BSA conjugate was immobilised on a BIAcore sensor chip as in section 2.9.2. Native BSA was also immobilised on a separate flow cell. The three antibodies were passed over each surface at a concentration of 100 nM. 5 minute injections at a flow rate of 20 μ l/min were used. The non-specific interaction of α -C5 with the BSA surface is shown in Figure 3.20, and is characteristic of the response seen for the other two clones.

An overlay plot of the binding curves for the three clones to immobilised coumarin-BSA is given in Figure 3.21, and shows the affinity ranking of the three scFvs to be in the order α -C13 > α -C5 > α -C14.

For the kinetic analysis of α -C5, purified scFv at concentrations of 10, 25, 50, 75 and 100 nM were injected over the immobilised coumarin-BSA surface. BIAevaluation software was used to derive the rate constants for the interaction, as described in section 3.7. The linear portion of the dissociation phases at all antibody concentrations was selected from a plot of $\ln(t1/t0)$ against time, and these data were fitted to a simple dissociation model ($AB \rightarrow A+B$). The k_d value obtained from this plot was used to calculate k_a values, following selection of linear portions from the association phase of the sensorgrams. Average calculated k_a and k_d values were 1×10^5 and 5×10^{-4} , respectively, giving an overall affinity constant, for the interaction of 1×10^{-9} M. However, as shown in Figure 3.22, there is a significant divergence between the calculated dissociation curve and the observed BIAcore data. This indicates that the data do not fit the simple dissociation model, and that the interaction deviates from pseudo-first order reaction kinetics.

3.11 BIAcore panning of a phage display library

A naïve phage display library (Nissim *et al.*, 1994) was panned using BIAcore. 7-hydroxycoumarin-BSA was immobilised on the sensor chip as described in section 2.9.2.

Figure 3.20 Overlay plot showing the degree of non-specific binding of monoclonal scFv antibody α -C5 to an immobilised BSA surface as determined by BIAcore studies. 100 μ l of scFv antibody at a concentration of 100mM was passed over both BSA and coumarin-BSA surfaces. 40 RU of non-specific binding to BSA occurred, and similar results were obtained for the other two scFvs tested.

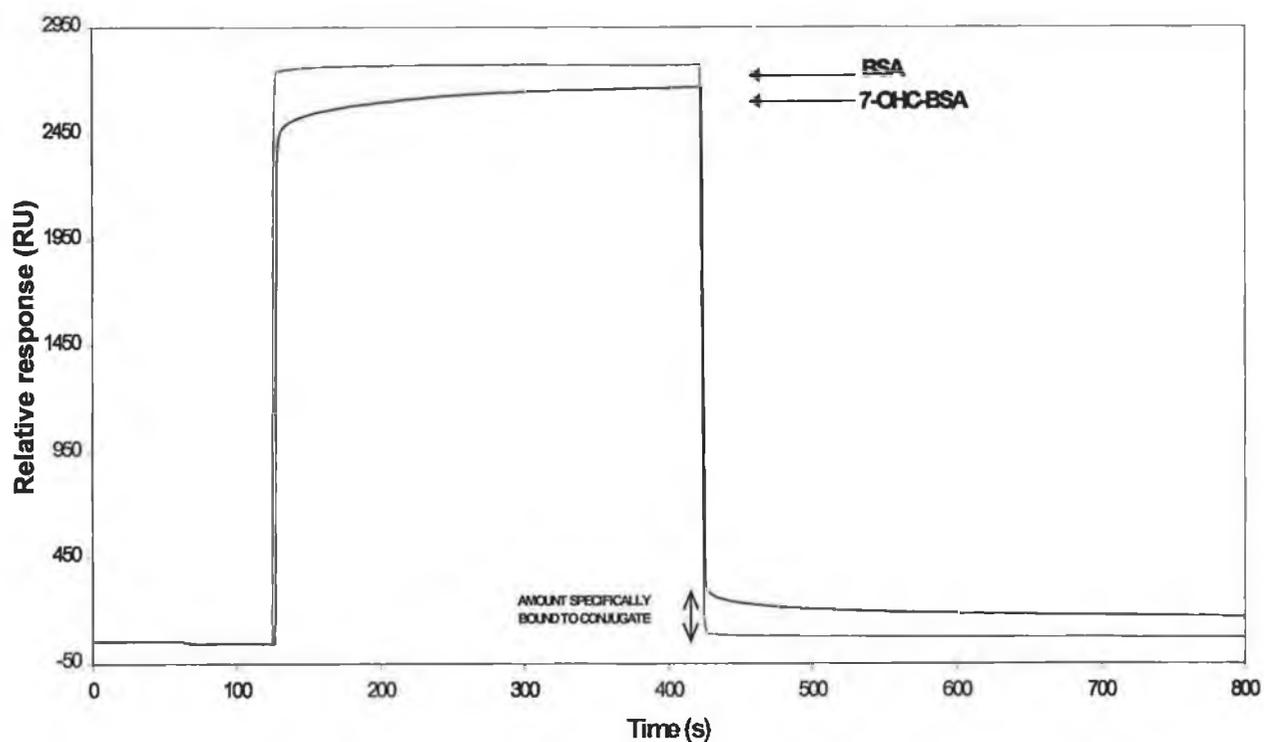


Figure 3.21 Affinity ranking of three scFv fragments against coumarin-BSA. Coumarin-BSA was immobilised on the BIAcore and purified scFvs at a concentration of 100mM were passed over this surface. The relative affinities of the antibodies are in the order α -C13 > α -C5 > α -C14.

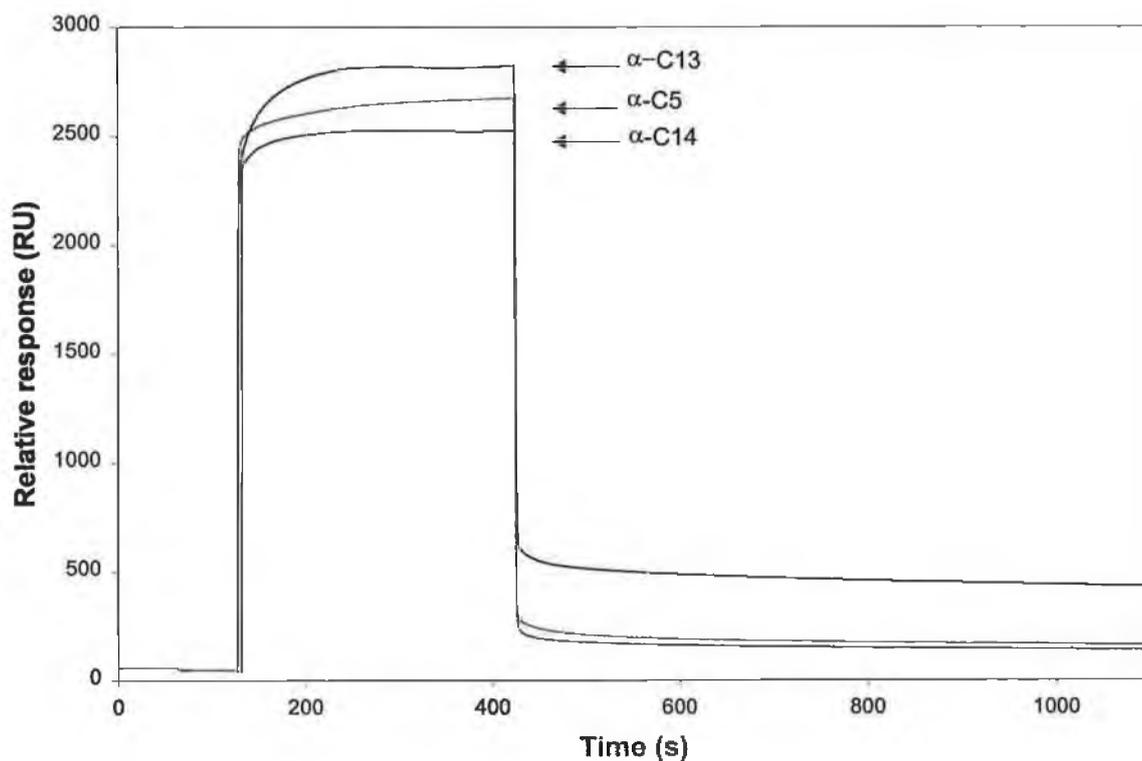
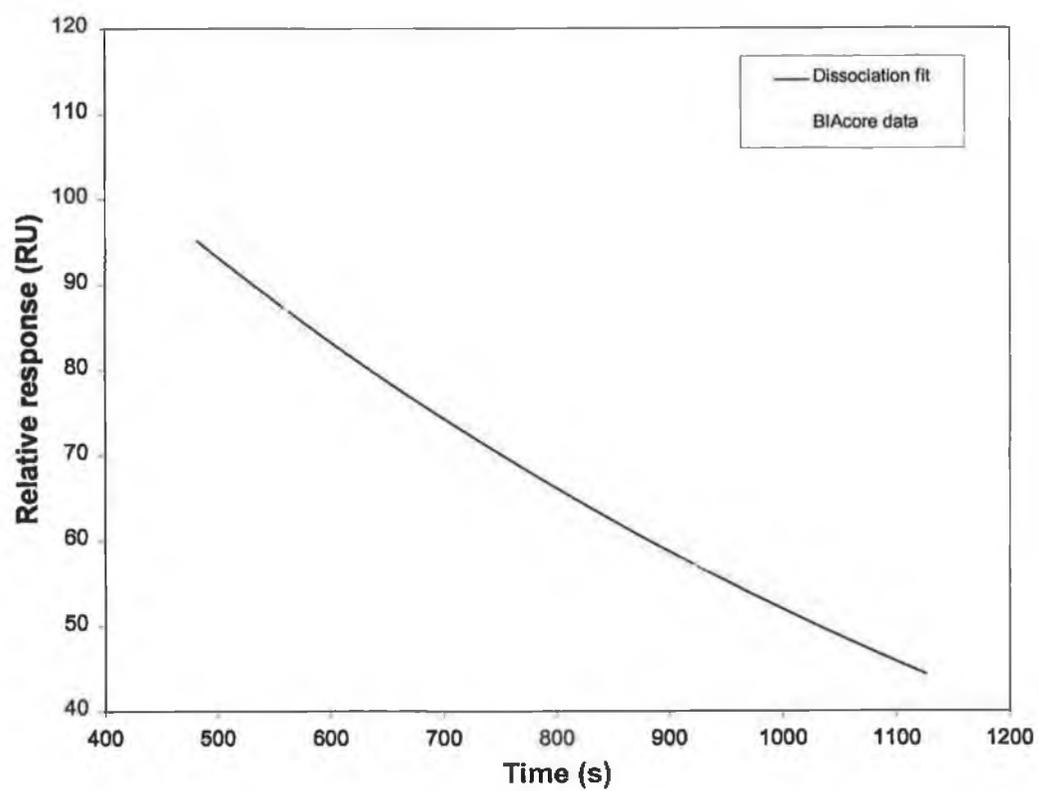


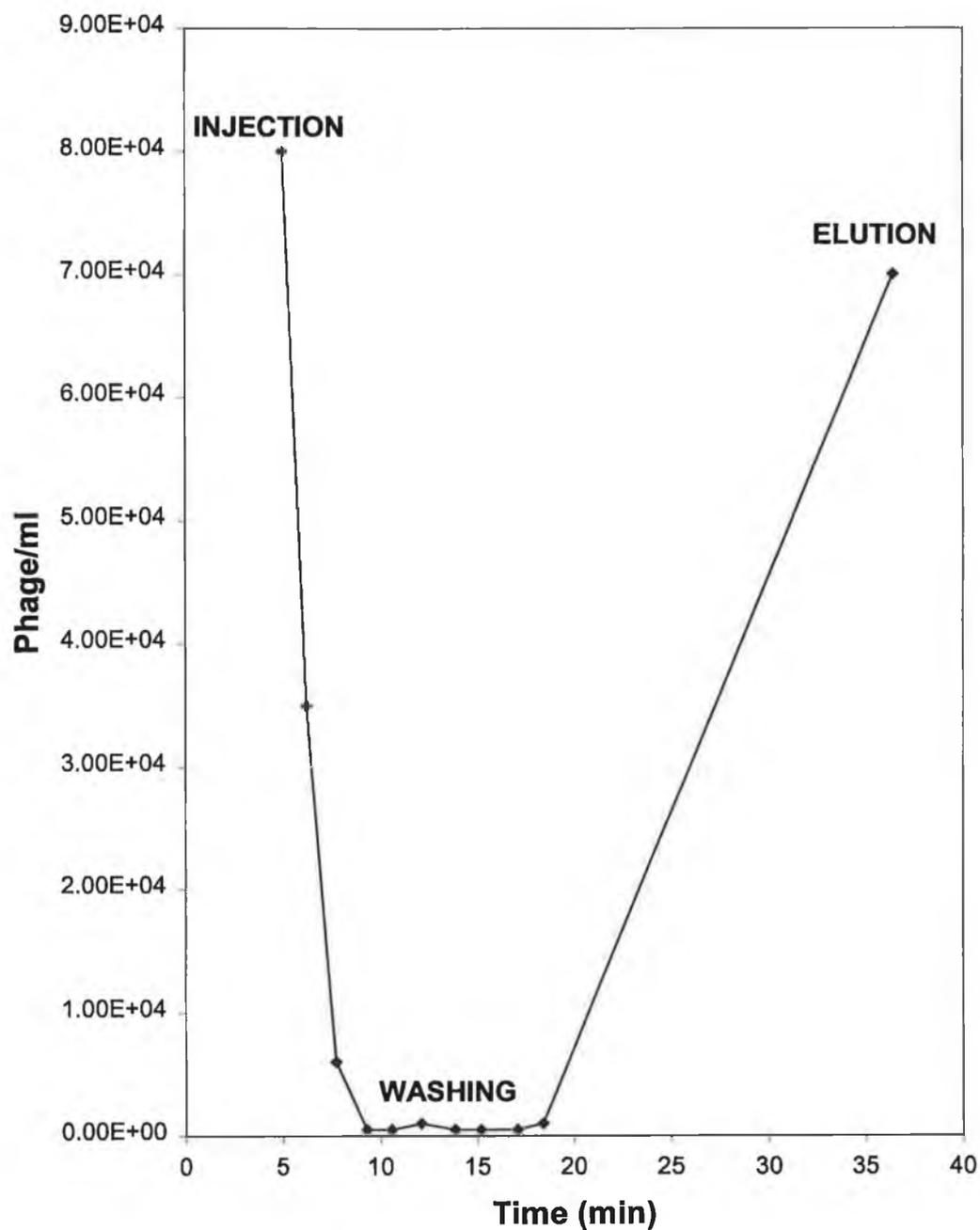
Figure 3.22 Dissociation plot for scFv antibody α -C5 from BIAevaluation software. The bad fit between the calculated dissociation curve and the BIAcore data indicates a deviation from pseudo-first order reaction kinetics



Neat bacterial cell lysate containing 5.5×10^9 phage particles/ml was injected over the sensor surface for 5 minutes at a flow rate of $10 \mu\text{l}/\text{min}$. No binding signal was generated as a result of this injection. The flow rate was increased to $100 \mu\text{l}/\text{min}$ for 15 minutes, to wash the surface. A 20 minute injection of glycine buffer, pH 2.2, was then passed over the surface to remove any specifically bound phage. Samples of the eluate from the BIAcore flow cell were taken regularly throughout the experiment, and these samples were assayed for the presence of phage as described in section 2.10. *E. coli* cells were infected with the eluted samples, and plated out onto selective medium containing carbenicillin. Only infected cells grow on the medium, as the presence of phage confers carbenicillin resistance, and the amount of phage in the original sample is calculated from the number of colonies visible on the agar plates.

The results are shown in Figure 3.23, and demonstrate that, although the amount of specific phage in the cell lysate was not sufficiently large to generate a change in SPR angle, the sample collected after treatment with glycine buffer contained 100-fold more phage than those collected during the washing phase. This indicates that specific elution of phage displayed antibodies was achieved. The final number of phage specifically eluted was 1.4×10^4 . This is 20,000-fold less than the original amount of 2.75×10^8 phage injected over the surface. As all of the phage in the eluted sample are specific for the immobilised conjugate surface, this represents a 20,000-fold enrichment step.

Figure 3.23 Phage counts for samples taken during BIAcore analysis of bacterial cell lysates. Phage from a naïve library were injected over an immobilised 7-hydroxycoumarin-BSA surface at 5.5×10^9 phage particles per ml. Samples were taken during the injection, washing and elution phases, and analysed for the presence of phage. The eluted sample contained 7×10^4 specific phage per ml. When compared to the counts for samples taken during the washing phase, this shows that specific elution took place.



3.12 Discussion

The work presented in this chapter is concerned with antibodies produced - by immunisation, somatic cell fusion and genetic engineering - to small haptens. Together with reports from the literature, and other results generated in our laboratory, these findings highlight the problems encountered when dealing with small haptens and their protein conjugates. The limitations of solid-phase selection strategies - both microtitre plate and BIAcore-based - for antibodies to such molecules are discussed here. The importance of the choice of coupling chemistry and carrier protein for the production of drug-protein conjugates is also emphasised.

Polyclonal antibodies to 7-hydroxycoumarin were produced by immunising New Zealand White rabbits with a diazonium-coupled 7-hydroxycoumarin-thyroglobulin conjugate. Serum from immunised rabbits gave a good titre compared to control serum (Figure 3.8), and, following purification by ammonium sulphate precipitation and protein G affinity chromatography, the antibody preparation was shown to have a high degree of purity by SDS-PAGE (Figure 3.10).

The working dilution of this antibody in a competitive immunoassay for free 7-hydroxycoumarin was estimated. A dilution of 1/200 was found to perform with good reproducibility and linearity (Figure 3.12), and this value compares favourably to that quoted by Egan (1993) for another polyclonal antibody against 7-hydroxycoumarin in the same assay system.

The cross-reactivity of the polyclonal antibody was assessed by ELISA. Microtitre plates with active amine groups were used in order to minimise non-specific binding of antibodies to the carrier protein in drug-protein conjugates. The coating (by EDC/NHS coupling of 7-hydroxycoumarin-4-acetic acid) and blocking of these plates was first optimised, and the inhibition of antibody binding by a range of coumarin compounds was then investigated by competitive ELISA (Table 3.1). Little cross-reactivity with coumarin or warfarin was evident, but the antibody displayed moderate cross-reactivity to 4-hydroxycoumarin and 7-hydroxywarfarin, and high cross-reactivity to 7-hydroxycoumarin-4-acetic acid. The presence of a hydroxy group, whether at the 4- or 7- position, appears to be an absolute requirement for antibody binding. As 7-hydroxylation is the predominant metabolic fate of coumarin in man (Pelkonen *et al.*, 1997) cross-reactivity with other simple hydroxylated forms of coumarin should not affect the use of this antibody preparation in analytical applications. The antibody was subsequently successfully employed in the development of

a BIAcore-based immunoassay for free 7-hydroxycoumarin in human serum samples (see Chapter 4).

Prior to the production of monoclonal antibodies, BALB/c mice were injected with an EDC/NHS-coupled 7-hydroxycoumarin-ovalbumin conjugate. After several immunisations, the titre of specific antibodies in the mouse serum was assessed by ELISA, with a range of different immobilised interactants. Figure 3.15 shows that, on a microtitre plate coated with EDC/NHS-coupled 7-hydroxycoumarin-BSA, serum from the immunised mouse gives a higher response than that given by the positive control (purified polyclonal antibody), and had a titre of greater than 1/12800. The degree of similarity between ovalbumin and BSA can lead to misleading results if antibodies immunised with a conjugate of one protein are screened with a conjugate containing the other (Hermanson, 1996). On control wells coated with native BSA, however, the serum from the immunised animal gave no significant response. This indicated that the response given by the serum in the 7-hydroxycoumarin-BSA wells was not simply due to cross-reactivity between anti-ovalbumin antibodies and the BSA component of the immobilised conjugate.

While a good titre was obtained for the mouse serum using plates coated with EDC/NHS-coupled 7-hydroxycoumarin-BSA, no significant binding of serum components to wells coated with a diazonium-coupled BSA conjugate of 7-hydroxycoumarin was observed, although the positive control gave a good response (Figure 3.14). Similarly, control and immunised serum gave almost identically low responses on a plate to which 7-hydroxycoumarin had been covalently coupled. The final implications of these findings will be discussed in detail below, but at the screening stage it seemed probable that these differences in response could have been the result of proximity effects, differing orientation of drug molecules, or steric hindrance.

Splenocytes from the immunised animal were then fused with myeloma cells using PEG, and 87.5% of the wells of 48-well cell culture clusters were found to contain hybridomas, while unfused cells died in HAT medium. 336 cell supernatants were assayed by ELISA for antibody production, using microtitre plates coated with EDC/NHS-coupled 7-hydroxycoumarin-BSA. 27 positive results were obtained, but when the supernatants were subsequently assayed against both conjugated and unconjugated BSA, 22 of the antibodies had either ceased secretion or bound strongly to the native form of the protein (Figure 3.16). Further investigation of these supernatants showed that they also bound to thyroglobulin. The isolation of antibodies which bind non-specifically to protein, probably as a result of

electrostatic interactions, is an inevitable consequence of screening with a hapten-protein conjugate. Breen (1987) found large numbers of such "sticky" immunoglobulins when screening for antibodies against human plasma apolipoproteins. The logistics of testing initial fusion supernatants, involving large numbers and small volumes of samples, mean that comparative screening against both conjugated and unconjugated protein is difficult to carry out until the apparent "positives" have been scaled up. Killard (1998) found that more complex screening strategies, such as competitive methods, were difficult to incorporate at earlier stages in the process, as optimisation of the assay conditions was not possible.

Of the five hybridoma colonies which produced antibodies that bound significantly more strongly to 7-hydroxycoumarin-BSA than to native BSA, two ceased secretion of antibody before they could be cloned. Such loss of antibody production is a frequently encountered problem, and can occur because of overgrowth of the antibody producing hybridomas by other cells in the well, or as a result of chromosome loss (McCullough and Spier, 1990).

The three hybridoma populations which were eventually cloned by limiting dilution grew slowly in the original 48-well plates, and were detected as positive two weeks after the start of the screening process. It is probable that hybridomas which are producing antibody naturally divide more slowly than non-secreting cells, because of their added metabolic burden (Reuveny and Lazar, 1989). In addition, the kinetics of antibody production may complicate the screening process. Most mouse monoclonal cell lines produce antibody at the beginning of the exponential growth phase and during the stationary and decline phases. However, some secrete antibody at the start of the exponential phase and during the decline phase only; while others only produce antibody during the exponential phase of growth (Merten *et al.*, 1987). These differences in production kinetics, exacerbated by the presence in culture supernatants of proteases which can cleave antibody molecules (Schlaeger *et al.*, 1986), can make the timing of screening steps an important consideration.

Cloning of the three hybridoma populations by limiting dilution resulted in the production of a panel of eleven monoclonal antibodies. As these were derived from the contents of two wells initially subcloned at 10 cells/well it is unlikely, although not impossible, that they represented eleven different antibodies. The results from affinity ranking on BIAcore suggest that only 4 different antibodies are present.

When these antibodies were tested in a competitive ELISA format, none of them displayed any significant binding to free 7-hydroxycoumarin (see Figure 3.17). The results from previous rounds of screening show that no binding occurs to unconjugated BSA, indicating

that the antibodies produced are interacting with a combined epitope on the drug-protein conjugate. In the light of these findings, the results from the initial titres of mouse serum take on a greater importance.

The serum from the immunised mouse was found to bind strongly to EDC/NHS-coupled 7-hydroxycoumarin-protein conjugate, but not to the same two components joined via a diazonium linkage. The drug molecule in the diazonium conjugate is joined closely to the protein (Figure 3.4), whereas the EDC/NHS conjugate contains a two-atom "spacer" between drug and protein, due to the use of 7-hydroxycoumarin-4-acetic acid (Figure 3.5). As there was no response for mouse serum on a Covalink plate onto which 7-hydroxycoumarin-4-acetic acid had been linked via EDC/NHS esterification, the antibodies produced by immunisation with an EDC/NHS conjugate appear to be binding to a joint epitope consisting of the drug molecule, the "bridge" region, and the protein. Although the animal was immunised with an ovalbumin conjugate and antibodies were screened with a BSA conjugate, the amino acid residues involved in conjugation are the same for both proteins.

Similar findings with regard to the screening of monoclonal antibodies against haptens were reported by Danilova (1994). In this study, a range of haptens were coupled to proteins for immunisation, and the effect of the coupling chemistry and carrier molecule used to screen by ELISA were investigated. In one experiment, the hormone thyroxine was joined to keyhole limpet haemocyanin (KLH) by glutaraldehyde, and mice were immunised with this conjugate. Hybridoma supernatants were screened by ELISA with an immobilised EDC-coupled thyroxine-BSA conjugate. Of six monoclonal antibodies produced from one fusion, three did not bind to free thyroxine, and one had only a weak affinity for the unconjugated hormone. Although the two conjugates used in this study contained different proteins and used different coupling chemistries, in both cases the hormone was linked to the protein via lysine groups.

Delcros *et al.* (1995) also investigated the screening of antibodies against small molecules using ligands immobilised by two different chemistries. The polyamines putrescine, spermidine and spermine are low molecular weight cations which are necessary for cell differentiation and growth. Wells of microtitre plates were coated with gelatine, and polyamines were immobilised covalently by either reaction with EDC/NHS or glutaraldehyde cross-linking. A monoclonal antibody raised against an EDC/NHS-coupled spermine-thyroglobulin conjugate was assessed for binding to immobilised polyamines. In

wells to which the polyamines had been linked via EDC/NHS esterification, antibody bound to spermine and spermidine to a similar degree, but very little binding to putrescine was observed. These findings were in agreement with the results of equilibrium analysis. In wells to which ligands had been coupled using glutaraldehyde, however, the antibody exhibited similar levels of binding to all three immobilised polyamines. These results were repeated when immunised mouse serum was tested on the same surfaces. The authors concluded that the affinities of antibodies for free and immobilised polyamines could not be compared, due to structural differences caused by the different immobilisation chemistries used. Such structural differences would be much less significant for larger antigens.

Fasciglione *et al.* (1996) studied the generation of monoclonal antibodies to the hydrophobic haptens theophylline, heme, pyrrolequinoline quinone (PQQ) and the pentapeptide Leu-Trp-Gly-Phe-Ala. These haptens were coupled to gelatin, BSA, KLH and recombinant hepatitis B core antigen using EDC, and the resultant conjugates were used to immunise BALB/c mice. All of the monoclonal antibodies produced bound either to the hapten-carrier conjugate or to chemically-modified lysine groups on any carrier molecule. None of the antibodies bound to free hapten. The haptens were then coupled to highly hydrophilic carriers such as Sepharose and chemically modified poly-L-lysine. When these conjugates were used as immunogens, monoclonal antibodies to free hapten could be isolated. The authors postulated that the covalent binding of small hydrophobic haptens resulted in alterations to the tertiary structure of carrier proteins, causing the haptens to be “hidden” inside the protein, and limiting their interaction with the immune system. They suggested that this could be avoided by the use of highly hydrophilic carriers for hydrophobic haptens.

In order to produce the scFv fragments used in section 3.10, Killard (1998) used a diazonium-coupled coumarin-BSA conjugate to pan a naive phage display library, and selected three clones. These three antibodies were found not to bind to free coumarin, and the binding of the antibodies to drug-protein conjugates appeared to be independent of the particular protein used.

As discussed in section 3.4.6, the panning of phage display libraries using microtitre plates coated with protein conjugates is in many respects similar to the screening of hybridoma supernatants by ELISA. Phage are added to wells of the plate, and those expressing specific antibody are allowed to bind. The plate is then washed with a solution containing detergent and NaCl, and phage which remain bound are eluted and used to transfect

bacteria, leading to an enrichment in the number of specific phage. The inability to generate antibodies to free coumarin in this fashion, together with the failure to produce antibodies to free 7-hydroxycoumarin by somatic cell fusion, may indicate an inherent problem with microtitre plate-based screening and selection strategies for antibodies to small haptens.

Danilova (1994) recommended that for low molecular weight haptens the conjugate used for screening should differ from the immunogen in terms of carrier protein, coupling chemistry and the amino acid residue at which conjugation occurs. Even such precautions, however, may not be sufficient to overcome some of the fundamental difficulties encountered when dealing with small haptens.

A procedure which used a 7-hydroxycoumarin affinity column to purify antibodies specific for 7-hydroxycoumarin from a polyclonal antiserum, resulted in a yield of approximately 4% (H. Reinartz, Personal communication). This indicates that the 7-hydroxycoumarin molecule, even when conjugated to a carrier protein, is only weakly immunogenic - probably due to its low molecular weight and lack of numerous functional groups. Along with the results from antibody screening, this suggests that polyclonal antisera to small, weakly immunogenic molecules such as coumarin and 7-hydroxycoumarin function on the basis of avidity, and that individual antibodies are likely to have only weak affinities. If this assumption is correct, it is probable that the washing steps involved in both panning phage libraries and screening hybridoma supernatants actually remove those low affinity antibodies directed solely against the drug molecules. This type of solid phase selection strategy may thus be biased towards the selection of antibodies to combined hapten/bridge region/protein epitopes, which will have a higher affinity.

The use of an optical biosensor such as BIAcore has the potential to obviate the problems associated with microtitre-based screening procedures. In addition to the ability to detect the binding of low to moderate affinity antibodies in real-time, the use of automated BIAcore methods can reduce the time-consuming nature of screening multiple hybridoma supernatants by ELISA. However, as stated in section 3.9.3, the injection of neat culture supernatants from 48-well plates over an immobilised 7-hydroxycoumarin-BSA surface did not give any binding signals, although the same supernatants yielded some positive results when assayed by ELISA against the same conjugate. Equilibrium methods such as ELISA are more sensitive than BIAcore, and the low number and affinity of antibodies to 7-hydroxycoumarin present at the initial screening stage appear to be below the limit of

detection. Not until cells had reached confluency in 12-well plates could a binding signal be obtained for the supernatant. There are no reports in the literature of BIAcore being used to screen neat hybridoma supernatants. Bynum *et al.* (1995) used immunoprecipitation as the primary selection method for antibodies against human milk fat globule (HMFG). Positive hybridomas were scaled up to 24-well plates and supernatants were then analysed by BIAcore. The average signal for the positive supernatants was only 180 RU. Unfortunately, then, BIAcore cannot be used - in this application - at the two stages of the screening process for which its sample throughput and automation seem ideally suited: initial screening and the screening of clones.

The sensorgram obtained for the binding of clone number 3 (Figure 3.19) suggests that even the antibodies generated against the combined conjugate epitope may have a weak overall affinity. The initial bulk refractive index change appears to mask a rapid association phase, and swift dissociation is evident after the end of the sample injection.

As the concentrations of the three purified scFv antibodies to coumarin were known, they could be directly affinity ranked using BIAcore (Figure 3.21). BIAcore kinetic software was also used to derive observed k_a , k_d and K_A values for one of the antibody fragments. However, the bad fit between the BIAcore data and the dissociation curve based on the simple dissociation model (Figure 3.22) indicates a deviation from pseudo-first-order kinetics. This could be ascribed to the significant interaction of the scFv with BSA (Figure 3.18), but it is probable that the nature of the combined drug-protein epitope and the number of coumarin molecules on each conjugate molecule (approximately 12 moles of coumarin per mole of BSA) contribute to the complexity of the binding events. Such heterogeneity of immobilised ligand sites has been reported as the major cause of deviation from pseudo-first-order kinetics (O'Shannessy and Winzor, 1996). Bowles *et al.* (1997) found that the binding of monoclonal anti-paraquat antibodies to a paraquat analogue covalently immobilised on the BIAcore chip surface displayed marked deviations from pseudo-first-order kinetics.

The use of BIAcore to pan a naïve phage display library was also investigated. Bacterial cell lysate containing 2.75×10^8 phage particles was injected over an immobilised 7-hydroxycoumarin surface. As with the screening of hybridoma supernatants, the sensitivity of the instrument was not sufficient to detect binding of specific phage. The flow rate was increased, and running buffer passed over the surface, to remove any non-specifically bound phage. Glycine buffer was then injected to elute specific phage. Phage counts

carried out on samples taken during injection, washing and elution phases, show that specific elution did take place, with a 100-fold increase in the amount of phage compared to samples taken during the washing step. This compares favourably with the 5-fold increase in specific phage reported by Malmberg and Borrebaeck (1995) for their model system.

A total of 1.4×10^4 phage were recovered during the elution phase. As these were all specific for the immobilised conjugate, and the initial amount of phage injected was 20,000-fold larger, this represents a 20,000-fold specific enrichment. The lack of an SPR signal during the injection is not surprising, considering the small number of specific phage present in a naïve library. Malmberg *et al.* (1996) reported that the binding of 10^{10} specific phage particles was necessary in order to cause a change in SPR angle. A sample from a naïve library containing such an amount of specific phage would be viscous in the extreme, and impossible to run on the BIAcore. However, by reinfecting bacteria with the eluted phage, and continuing this process for several rounds of panning, it seems possible that sufficient enrichment to produce a binding signal would eventually occur. Individual clones with low to moderate affinity could then be isolated.

3.13 Summary

A polyclonal antibody to 7-hydroxycoumarin was produced by immunising a rabbit with a protein (BSA) conjugate of the drug. The antibody was purified and found to possess suitable specificity for use in ELISA techniques. The use of this antibody in a biosensor-based immunoassay for 7-hydroxycoumarin will be described in Chapter 4.

BALB/c mice were immunised with another 7-hydroxycoumarin-protein conjugate, and found to have a high specific antibody titre by ELISA. Somatic cell fusion using the spleen of an immunised mouse, however, failed to generate an antibody against the free drug. These results highlighted the limited immunogenicity of small haptens, and accentuated some of the problems with screening by ELISA. Although BIAcore was used successfully to affinity rank both whole antibodies and scFv fragments for joint drug-protein epitopes, the limit of sensitivity of the biosensor rendered it unsuitable for use in the early, labour-intensive, stages of screening. Affinity and kinetic constants for the interaction of scFv fragments with a drug-protein conjugate was derived using BIAcore, but the heterogeneity of the immobilised ligand caused significant deviation from pseudo-first-order kinetics.

The panning of phage display libraries using BIAcore was also investigated. Specific elution of phage from a naïve library was demonstrated, and the method may warrant further study.

CHAPTER 4

THE DEVELOPMENT OF A BIAcore-BASED INHIBITIVE IMMUNOASSAY FOR QUANTITATIVE DETERMINATION OF TOTAL 7-HYDROXYCOUMARIN IN SERUM

4.1 Introduction

This chapter describes the production of a quantitative method for the detection of free 7-hydroxycoumarin in human serum samples. Initially, a model immunoassay system for drug measurement in phosphate buffered saline (PBS) was set up using an immobilised drug-protein conjugate and polyclonal anti-7-hydroxycoumarin antibodies. Standard curves using human serum spiked with 7-hydroxycoumarin were then produced, and the molarity of enzyme buffer, incubation time of samples with enzyme, and methods for the removal of non-specific binding were optimised. The precision, accuracy, percentage recoveries and efficiency of regeneration of the assay were assessed, and found to be within suggested limits for BIAcore immunoassays.

4.2 Analysis of 7-hydroxycoumarin

Currently, a range of sophisticated techniques, ranging from chromatography and spectroscopy to immunoassay and electrochemical detection, exist for the quantitative detection of 7-hydroxycoumarin. Some of these methods are suitable for the measurement of total drug concentration in complex matrices, although deconjugation of the glucuronide conjugate and extensive sample clean-up in the form of solvent extraction are a prerequisite for many procedures.

Early methods of 7-hydroxycoumarin analysis included paper chromatography (Feigl *et al.*, 1955) and colorimetry (Ensminger, 1952), and were crude and generally qualitative in nature.

Thin layer chromatography (TLC) is an analytical technique typically involving a silica stationary phase onto which sample is applied, and an organic mobile phase which facilitates separation of sample components as they migrate along the solid support. The method of Indahl and Scheline (1971) allowed the qualitative analysis 7-hydroxycoumarin, which was detected by observing fluorescence at 254 nm. The use of TLC to quantitatively detect 7-hydroxycoumarin in urine samples was reported by Cholerton *et al.* (1992). In this procedure, urine samples were treated with β -glucuronidase and extracted into chloroform, and drug levels were measured by fluorescence densitometry. The limit of detection for this method was 1 ng/ml.

High-performance liquid chromatography (HPLC) is a separative technique based upon differences in polarity of the analytes. For 7-hydroxycoumarin analysis, reverse phase HPLC may be used. In this method, the stationary phase is generally a column containing a silica surface with hydrophobic chains of 8 or 18 carbon atoms (termed C₈ or C₁₈). The analyte is applied to the column in a more polar mobile phase, and binds to the column via hydrophobic interactions. Sample components are eluted in order of decreasing polarity, and quantitative measurement is usually carried out by measuring absorbance at a defined wavelength.

Moran *et al.* (1987) described a reverse-phase HPLC method for 7- hydroxycoumarin in urine. β -glucuronidase was added to urine samples, which were then freeze-dried and reconstituted prior to separation. The limit of quantification for this method was 500 ng/ml. Egan and O'Kennedy (1992) improved upon the above method by reducing the incubation times and amount of enzyme added. Instead of freeze-drying, samples were extracted into diethyl ether. The limit of detection of this method was also 500 ng/ml.

Sharifi *et al.* (1993b) used an extracted 7-hydroxycoumarin glucuronide from rabbit urine as a standard, removing the necessity for deconjugation, and allowing measurement of free and glucuronidated drug in urine and plasma samples, which were diluted with water and methanol prior to analysis. The limit of detection of this method was 0.3 ng/ml, for both metabolites.

Bogan and O'Kennedy (1996) reported a similar method for determination of 7-hydroxycoumarin and its glucuronide in serum, plasma and urine. No deconjugation step was necessary, and samples were treated with trichloroacetic acid before being applied to the column. The limit of detection of this method was 50 ng/ml for 7-hydroxycoumarin, and 200 ng/ml for its glucuronide.

In a study on 7-ethoxycoumarin *O*-deethylase activity, Evans and Relling (1992) measured 7-hydroxycoumarin in reaction mixtures after extraction into chloroform and reconstitution with methanol-water. After elution, the drug was detected by fluorescence at 470 nm. A limit of quantification of 70 ng/ml was reported.

The *in vitro* metabolism of 7-hydroxycoumarin to 7-hydroxycoumarin glucuronide by uridine diphosphate glucuronyl transferase (UDPGT) was followed using reverse-phase HPLC by Killard *et al.* (1996). TCA was added to samples to precipitate large proteins, and

a gradient elution method was used. The limit of detection of 7-hydroxycoumarin was 500 ng/ml.

Gamache *et al.* (1993) used HPLC with coloumetric array detection to measure 7-hydroxycoumarin (and other derivatives) in juice beverages. They reported a limit of detection in the order of 1 ng/ml.

Capillary electrophoresis (CE) is a separative technique based on the movement of charged species in an applied electric field. Samples migrate through an electrolyte buffer in a narrow bore (20 -100 μm in diameter) fused silica capillary by a combination of electrophoretic mobility and electroosmotic flow (the characteristic movement of liquid in an electric field). The separated sample components are detected by means of UV spectroscopy.

Bogan *et al.* (1995) described a CE method for the quantitative determination of free and total 7-hydroxycoumarin in serum and urine samples. Samples were treated with β -glucuronidase to deconjugate 7-hydroxycoumarin glucuronide, although this step was found to interfere with the separation of internal standard (warfarin). Samples were prepared by extracting into diethyl ether and reconstituting with electrolyte buffer. 7-Hydroxycoumarin was detected at 210 nm and this method had a limit of detection of 1 $\mu\text{g}/\text{ml}$.

The CE method of Bogan *et al.* (1996a) allowed the determination of both 7-hydroxycoumarin and its glucuronide in urine without the need for deconjugation or extraction steps. Detection was at 320 nm, and the reported limit of quantification for 7-hydroxycoumarin was 2 $\mu\text{g}/\text{ml}$.

In comparative studies on the CYP2A6 activity of liver microsomal preparations, levels of 7-hydroxycoumarin in neat unextracted enzyme reaction mixtures were measured by CE (Deasy *et al.*, 1995; Bogan *et al.*, 1996b). Absorbance was monitored at 214 nm, and the limit of detection was 1 $\mu\text{g}/\text{ml}$.

Direct spectroscopic analyses, which exploit the characteristic absorbance and fluorescence of the compound, have also been reported for the detection of 7-hydroxycoumarin.

Tan *et al.* (1976) reported a spectrofluorimetric method for the determination of 7-hydroxycoumarin in whole blood. Samples were extracted first into ether and then into glycine buffer, and the fluorescence was measured at excitation and emission wavelengths of, respectively, 370 and 450 nm. In a further study (Ritschel *et al.*, 1977), samples were

treated with β -glucuronidase prior to estimation of total 7-hydroxycoumarin concentrations. The limit of detection in the above methods was 1 ng/ml. Direct fluorimetry using the above wavelengths has also been used to investigate the binding of 7-hydroxycoumarin to serum proteins and red blood cells (Ritschel *et al.*, 1981). In a study on liver microsome preparations of eight different species, fluorimetry was used - following TCA precipitation - to determine coumarin 7-hydroxylase activity and its inhibition by antibodies specific for cytochrome P450 (Kaipainen *et al.*, 1985).

Conway *et al.* (1984) described a novel method for monitoring the β -glucuronidation of 7-hydroxycoumarin, in which a fluorimeter was linked to microlight guides on the surface of liver samples by optical fibres. The decrease in content of 7-hydroxycoumarin was followed using excitation and emission wavelengths of 366 nm and 450 nm, respectively.

Rautio *et al.* (1992) and Iscan *et al.* (1994) both reported the use of a spectrofluorimetric assay for detection of 7-hydroxycoumarin in urine. Samples were treated with β -glucuronidase and extracted into chloroform prior to analysis, and the method had a limit of detection of 5 nM. In a separate study, a similar method was used to investigate the effect of grapefruit juice on coumarin 7-hydroxylase activity (Merkel *et al.*, 1994).

Egan and O'Kennedy (1993a) described a spectrofluorimetric microassay for measuring 7-hydroxycoumarin levels in urine and plasma. Both neat samples and samples extracted into diethyl ether were used, with similar results. The assay was performed in 96-well microtitre plates, and the limit of detection was 0.5 μ g/ml.

In addition to spectrographic and separative techniques, immunoanalytical methods have also been used for the determination of 7-hydroxycoumarin.

Egan and O'Kennedy (1993b) reported an antigen-inhibition enzyme-linked immunosorbent assay (ELISA) for 7-hydroxycoumarin in urine. A protein conjugate of 7-hydroxycoumarin is coated onto microtitre plates, and urine samples are added to wells, along with polyclonal antibodies to 7-hydroxycoumarin. Free and immobilised drug compete for antibody binding sites, and, following the addition of a secondary labelled anti-species antibody, and colour development with a chromogenic substrate (*ortho*-phenylenediamine), the increase in absorbance is inversely proportional to the concentration of 7-hydroxycoumarin. The immunoassay had a limit of detection of 0.5 μ g/ml.

Reinartz *et al.* (1996) described a one-step antigen-inhibition ELISA for 7-hydroxycoumarin urine samples. A bispecific antibody, specific for both 7-hydroxycoumarin and alkaline phosphatase, was produced chemically. The dual activity of the antibody obviated the need for a secondary enzyme-labelled antibody. Microtitre plate wells were coated with 7-hydroxycoumarin-BSA, and urine samples or standards, bispecific antibody, and alkaline phosphatase were added. A chromogenic substrate for alkaline phosphatase (*para*-nitrophenol phosphate) was added, and absorbance at 414 nm was measured. The limit of detection of this method was 20 ng/ml for free 7-hydroxycoumarin. For determination of total drug, a deconjugation step was necessary. β -glucuronidase was added to samples, which were then diluted before analysis. For total 7-hydroxycoumarin detection, the limit of quantification was 30 ng/ml. When samples were extracted into diethyl ether and reconstituted with PBS, concentrations as low as 6 ng/ml could be detected.

Dempsey *et al.* (1993a) developed an electrochemical immunosensor for 7-hydroxycoumarin measurement. Polyclonal antibodies to the drug were immobilised on the surface of a glassy carbon electrode, behind a permeable cellulose dialysis membrane. Binding of drug to antibody results in a decrease in electroactivity, as detected by DC amperometry. The limit of detection of the sensor system was 10 μ M.

Deasy *et al.* (1994) described an enzyme-linked competitive electrochemical immunosensor for detection of 7-hydroxycoumarin. 7-hydroxycoumarin-thyroglobulin was immobilised at the surface of a glassy carbon electrode. Antibodies to 7-hydroxycoumarin labelled with horseradish peroxidase (HRP) were mixed with standard solutions of the drug, and the electrode was added. Excess labelled antibody bound to the immobilised conjugate and, after washing and addition of substrate (hydrogen peroxide), the electroactivity of the HRP at the electrode surface was inversely proportional to the amount of 7-hydroxycoumarin in the original sample, and was determined by DC voltammetry. The limit of quantification for the sensor was 24 μ M.

Lu *et al.* (1996) reported the use of a regenerable enzyme immunosensor for 7-hydroxycoumarin. The assay format was similar to that described by Deasy *et al.* (1994), except that potassium ferrocyanide (a mediator in the enzyme reaction) was present, and the sensor surface could be regenerated by washing with 10 mM HCl.

Direct electroanalysis has also been utilised for the detection of 7-hydroxycoumarin. Dempsey *et al.* (1993b) used differential pulse voltammetry to measure total drug levels in urine, following treatment with β -glucuronidase, extraction, and reconstitution into methanol. Carrazon *et al.* (1989) used a range of voltammetric techniques to detect 7-hydroxycoumarin in micelles and emulsions. Using differential pulse voltammetry with a rotating glassy carbon electrode they achieved a limit of detection of 0.28 μM .

To summarise, a wide variety of analytical approaches have been reported for the detection of 7-hydroxycoumarin. Chromatographic, spectroscopic, immunological, biosensor and electroanalytical techniques have all been used, and sample matrices have ranged from urine and plasma to fruit juices and foodstuffs. Table 4.1 lists the different methods of detection, and indicates whether deconjugation and extraction methods were used.

4.3 BIAcore and the measurement of low molecular weight analytes

For molecules of low molecular weight (less than 2000), the mass change caused by binding to the BIAcore sensor surface is too small to result in a change in SPR signal. When developing BIAcore-based assays for the quantitative determination of low molecular weight analytes such as drug molecules, then, it is necessary to use an indirect sensing method, of which there are two main types: inhibition and competitive.

Inhibition formats are analagous to the antigen-inhibition ELISA methods described in section 4.2. The analyte (or an analogue or protein conjugate) is immobilised on the sensor chip, and samples containing analyte are mixed with a high molecular weight interactant (for example, specific antibody). When the mixture is passed over the sensor chip, excess antibody binds to the immobilised surface, giving a change in relative response inversely proportional to the amount of analyte free in solution.

In competitive techniques the antibody (or other interactant) is immobilised on the sensor chip. Sample is mixed with a high molecular weight analogue, and the mixture passed over the antibody surface. The increase in SPR signal is due to the binding of the high molecular weight component, and, as with the inhibitive method, is inversely proportional to the amount of analyte in the sample.

Table 4.1 Summary of reported methods of 7-hydroxycoumarin analysis.

Method	Sample Matrix	Enzyme Added	Clean-up	LOD	Reference
TLC	Urine	Yes	Extraction	1 ng/ml	Cholerton <i>et al.</i> , 1992
HPLC	Urine	Yes	Freeze-drying	0.5 µg/ml	Moran <i>et al.</i> , 1987
	Urine	Yes	Extraction	0.5 µg/ml	Egan and O'Kennedy, 1992
	Urine	No	Dilution	0.3 ng/ml	Sharifi <i>et al.</i> , 1993 b
	Urine	No	TCA	50 ng/ml	Bogan and O'Kennedy, 1996
	Enzyme Reaction Mixture	-	Extraction	70 ng/ml	Evans and Relling, 1992
	Enzyme Reaction Mixture	-	TCA	0.5 µg/ml	Killard <i>et al.</i> , 1996
	Juice Drinks	-	None	1 ng/ml	Garnache <i>et al.</i> , 1993
CE	Serum , Urine	Yes	Extraction	1 µg/ml	Bogan <i>et al.</i> , 1995
	Urine	No	None	2 µg/ml	Bogan <i>et al.</i> , 1993a
	Enzyme Reaction Mixture	-	None	1 µg/ml	Deasy <i>et al.</i> , 1995
	Enzyme Reaction Mixture	-	None	1 µg/ml	Bogan <i>et al.</i> , 1993b
Fluorimetry	Whole Blood	-	Extraction	1 ng/ml	Tan <i>et al.</i> , 1976
	Whole Blood	Yes	Extraction	1 ng/ml	Ritschel <i>et al.</i> , 1977
	Microsome Preparation	-	TCA	-	Kaipainen <i>et al.</i> , 1985
	Liver Samples	-	None	-	Conway <i>et al.</i> , 1984
	Urine	Yes	Extraction	5 nM	Rautio <i>et al.</i> , 1992
	Urine	Yes	Extraction	5 nM	Iscan <i>et al.</i> , 1994
	Urine	Yes	Extraction	-	Merkel <i>et al.</i> , 1994
	Urine, Plasma	-	None	.5 µg/ml	Egan and O'Kennedy, 1993a
ELISA	Urine	-	None	.5 µg/ml	Egan and O'Kennedy, 1993b
	Urine	Yes	Dilution	30 ng/ml	Reinartz <i>et al.</i> , 1996
Immunosensor	Buffer	-	-	10 µM	Dempsey <i>et al.</i> , 1993a
	Buffer	-	-	24 µM	Deasy <i>et al.</i> , 1994
	Buffer	-	-	-	Lu <i>et al.</i> , 1996
Voltammetry	Urine	Yes	Extraction	-	Dempsey <i>et al.</i> , 1993b
	Micellar Preps	-	None	0.28 µM	Carrazon <i>et al.</i> , 1989

Wagner *et al.* (1995) described the use of an inhibition technique to detect the pesticide 2,4-dichlorophenoxyacetic acid in tobacco extracts using the BIAlite instrument. A protein conjugate of the pesticide was immobilised on the sensor chip, and extracted tobacco samples were mixed with antibody and passed over this surface. Recoveries from spiked samples in the order of 90 - 95% were reported, with detection limits in the low ppm range using polyclonal antiserum, which were reduced to the ppb range with the use of monoclonal antibodies.

A BIAcore inhibition immunoassay for the determination of the antibiotics sulphametazine and enrofloxacin has been reported by Sternesjo *et al.* (1995), with limits of detection in the low ppb range.

Minunni and Mascini (1993) developed an indirect inhibition assay for the analysis of the pesticide atrazine in drinking water, which employed a monoclonal antibody for primary detection, and a polyclonal antibody for signal enhancement.

Vitamin levels in food were studied by Haines *et al.* (1995). They measured the concentration of biotin and folic acid in homogenized and clarified food extracts using monoclonal antibodies in an inhibition immunoassay format, and their reported results compared well to those obtained with standard microbiological assays.

Haines and Patel (1995) reported a BIAcore inhibition assay for the detection of the food borne pathogens *Salmonella* and *Listeria*. Polyclonal antibodies to bacterial cell wall antigens were added to cultures of food samples. The culture was filtered at 0.22 μm to remove all bacteria, and the filtrate passed over a BIAcore sensor chip bearing an immobilised anti-Fab antibody. Free antibody bound to the surface, giving a signal inversely proportional to the amount of bacteria in the original culture sample.

In a paper reporting a BIAcore method for the determination of antibody concentration, Wong *et al.* (1997) noted that few guidelines for the validation of BIAcore assays existed, although the system has been in use since 1990. They recommended the values of a range of parameters for the validation of quantitative BIAcore assays, including percentage recoveries, inter- and intra-day coefficients of variation, and baseline shift and rebinding after regeneration.

In light of the number of BIAcore inhibition immunoassays reported in the literature, this assay format was chosen for the development of a quantitative method for detection of 7-

hydroxycoumarin in complex biological samples. A schematic diagram of the proposed system, with immobilised drug-protein conjugate and free polyclonal antibody, is given in Figure 4.1.

Results

4.4 Model system for 7-hydroxycoumarin measurement in PBS

To establish the basis for a method to quantitatively detect levels of 7-hydroxycoumarin in serum, a model system for the measurement of the drug in PBS was set up. The running buffer for all the following experiments was HBS, pH 7.4, as described in section 2.9, and a flow rate of 10 $\mu\text{l}/\text{min}$ was used throughout.

The first step in the development of the assay was the immobilisation of a diazo-coupled 7-hydroxycoumarin-BSA conjugate. The immobilisation chemistry used was EDC-mediated N-hydroxysuccinimide esterification. The reaction scheme is shown in Figure 4.2. As discussed in section 2.9.1, this coupling method involves binding to the carboxymethylated surface via amine groups, and requires preconcentration of protein to the dextran layer by lowering the pH of solution to below the isoelectric point. A preconcentration study of 7-hydroxycoumarin-BSA was carried out as in section 2.9.1, with conjugate solutions in 10 mM sodium acetate at varying pHs being injected over an underivatized chip. From the results (Figure 4.3), it can be seen that the optimum pH for immobilisation is 4.0. This is the lowest recommended pH for immobilisation on BIAcore sensor chips, as below this value protonation of carboxy groups causes collapse of the gel matrix and decreased mobility of attached biomolecules. A 2 mg/ml solution of 7-hydroxycoumarin-BSA in 10 mM sodium acetate, pH 4.0, was immobilised as in section 2.9.2.

Figure 4.1 Schematic representation of the BIAcore inhibitive immunoassay format for quantitative determination of 7-hydroxycoumarin. Standards and samples containing 7-hydroxycoumarin are premixed with antibody and injected over an immobilised 7-hydroxycoumarin-BSA surface. Excess antibody binds to the sensor surface, and the signals are recorded and a standard curve constructed.

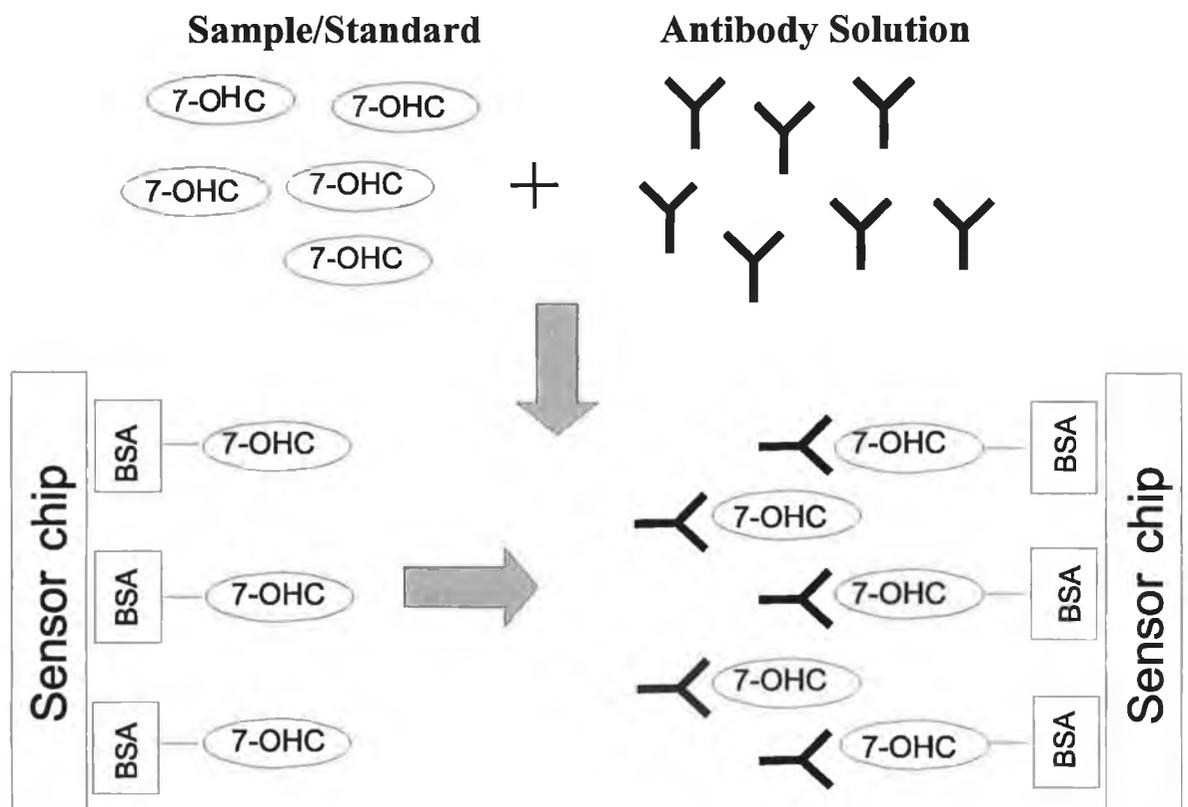


Figure 4.2 NHS/EDC coupling of proteins to the BIAcore sensor chip surface. Protein at a pH lower than its isoelectric point is injected over the surface following EDC-mediated NHS derivatisation. Unreacted sites are capped with ethanolamine.

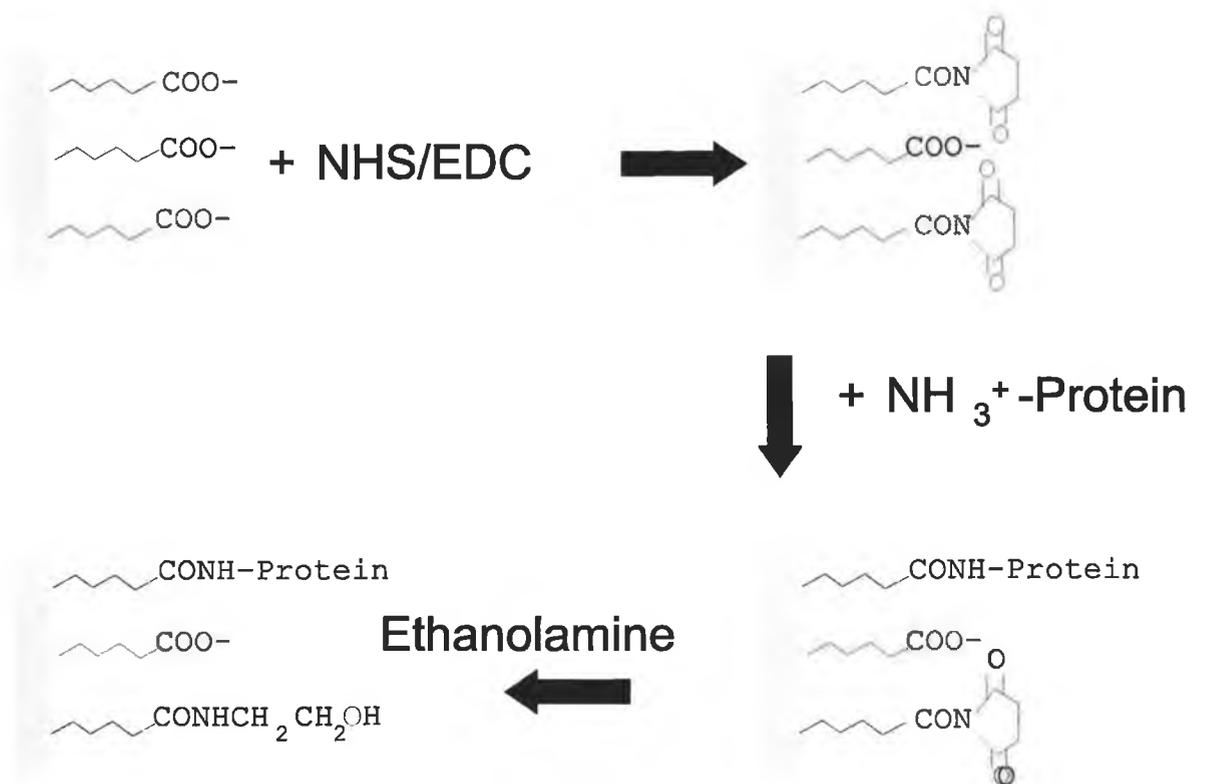
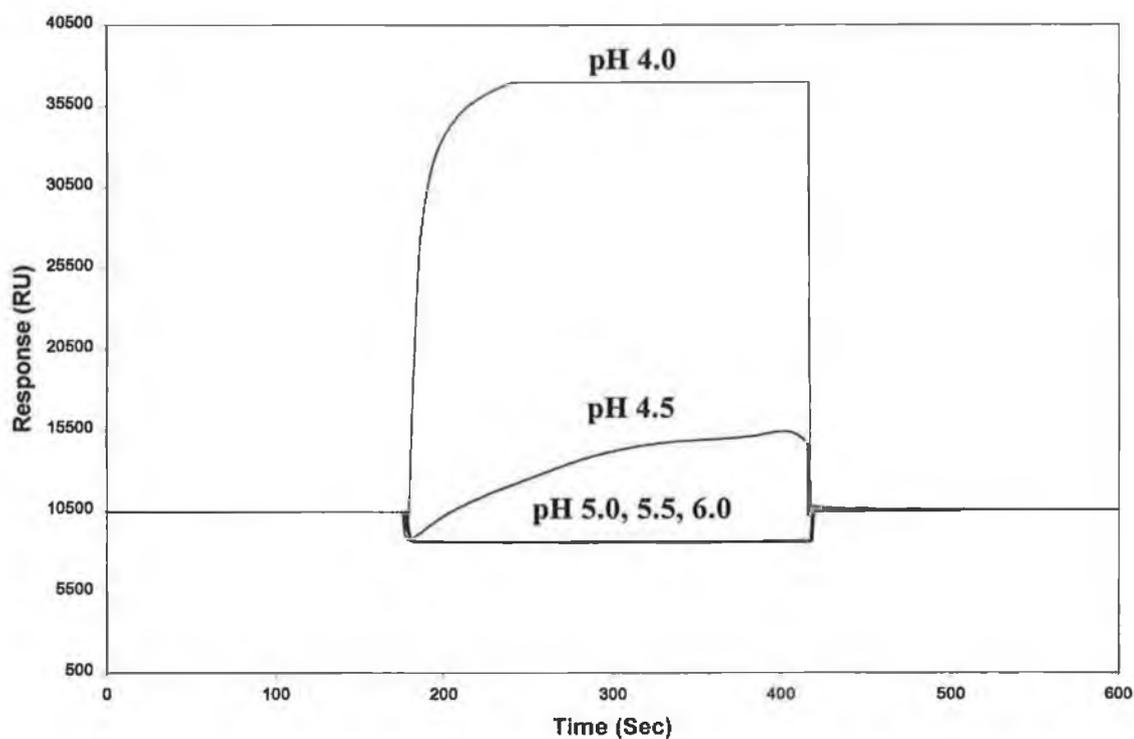


Figure 4.3 Overlay plot showing the preconcentration profile for 7-hydroxycoumarin-BSA. Conjugate at 100 $\mu\text{g/ml}$ in 10 mM sodium acetate at pH 6.0, 5.5, 5.0, 4.5, and 4.0 was injected over an underivatized sensor chip surface, and the degree of electrostatic binding was monitored. The optimal pH for preconcentration is 4.0.



The chip surface was activated with a mixture of EDC and NHS, and the conjugate solution was injected over the activated surface - unreacted groups were then capped by injection of 1M ethanolamine/HCl, pH 8.5. A typical sensorgram for immobilisation of 7-hydroxycoumarin-BSA is shown in Figure 4.4.

A number of standards of 7-hydroxycoumarin, ranging from 0.1 to 50 µg/ml, were prepared by diluting a stock solution of the drug (1 mg/ml in a mixture of 30% (v/v) ethanol : 70% (v/v) PBS) in PBS. Purified polyclonal antibody to 7-hydroxycoumarin (see Chapter 3), at a concentration of 0.7 mg/ml in PBS, was then mixed with an equal volume of standard. The mixture was incubated for 5 minutes at room temperature and then a 20 µl aliquot was injected over a chip surface on which approximately 10,000 RU of 7-hydroxycoumarin-BSA had been immobilised. The surface was regenerated by injection of 10 µl of the following sequence of reagents: 5 mM NaOH, 20 mM HCl, and 5 mM NaOH.

After regeneration, the next standard-antibody mixture was injected. A typical sensorgram showing binding and regeneration for a single standard is shown in Figure 4.5. A calibration curve was produced by plotting the change in response for each standard (caused by the binding of excess antibody in each sample to the immobilised conjugate) against the concentration of 7-hydroxycoumarin (Figure 4.6). The standard curve for this model system is linear in the range 0.1 - 50 µg/ml, with an r^2 value of 0.99.

4.5 Development of serum-based assay

Having constructed a standard curve for 7-hydroxycoumarin in PBS and demonstrated the principle of the inhibitive immunoassay system, an analytical method for the determination of drug levels in human serum, incorporating a deconjugation step for 7-hydroxycoumarin glucuronide in samples, was optimised and validated.

4.5.1 Non-specific binding of polyclonal antibody to BSA

Binding of the polyclonal antibody to the BSA of the conjugate immobilised on the sensor chip would result in high background signals and have a damaging effect on the sensitivity of the assay. The extent of non-specific binding was assessed by immobilising similar amounts of 7-hydroxycoumarin-BSA and unconjugated BSA (10,000 and 13,000 RU respectively) on separate flow cells, as in section 2.9.3.

Figure 4.4 Typical sensorgram from immobilisation of 7-hydroxycoumarin-BSA. (A) Sensor chip surface is derivatised with a mixture of EDC and NHS. (B) Conjugate at 2 mg/ml in 10mM sodium acetate, pH 4.0, is injected over this surface for 20 minutes (C) Unreacted sites are capped by injection of ethanolamine.

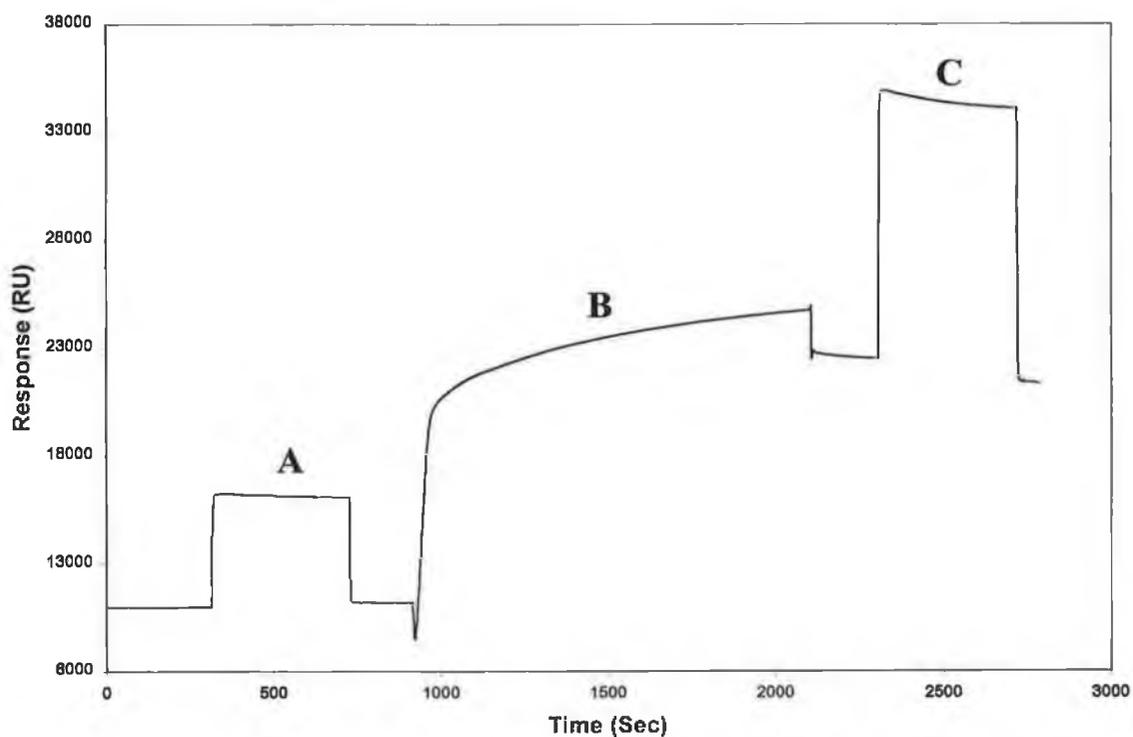


Figure 4.5 A typical sensorgram for one standard from the BIAcore 7-hydroxycoumarin immunoassay. (A) Standard containing free drug is mixed with antibody and injected over a 7-hydroxycoumarin-BSA surface; the binding signal is inversely proportional to the amount of drug in the sample mixture. The surface is then regenerated by one minute pulses of 5mM NaOH (B), 20mM HCl (C), and 5mM NaOH (D).

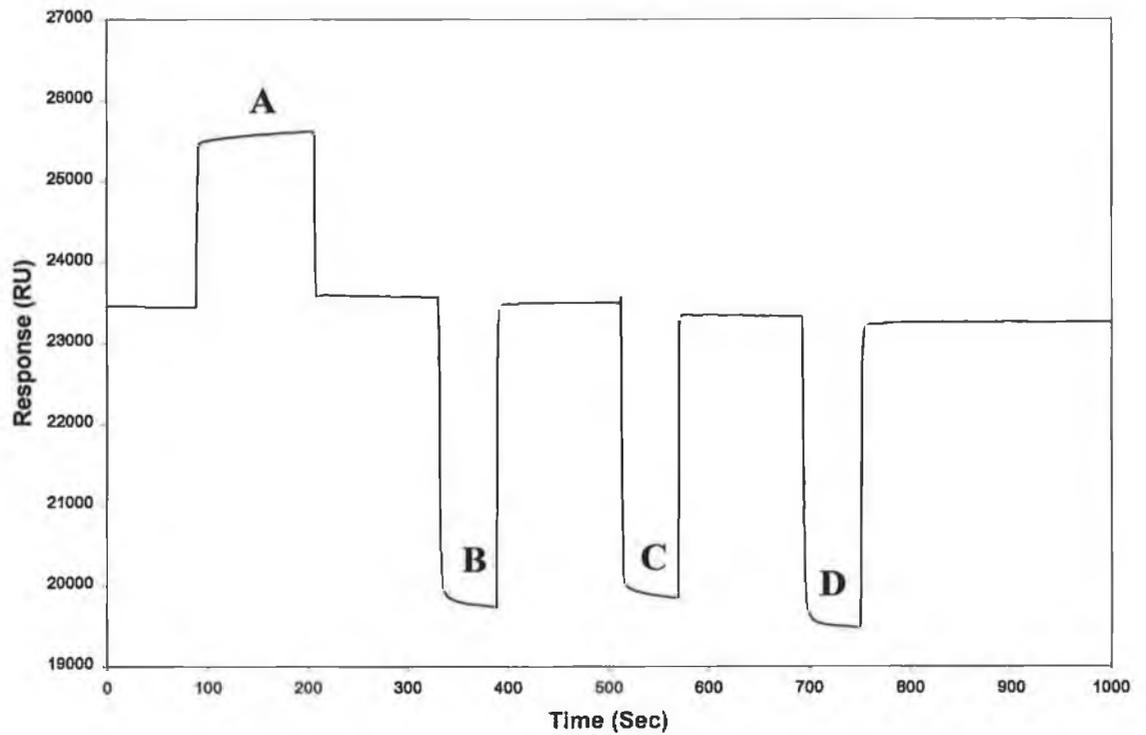
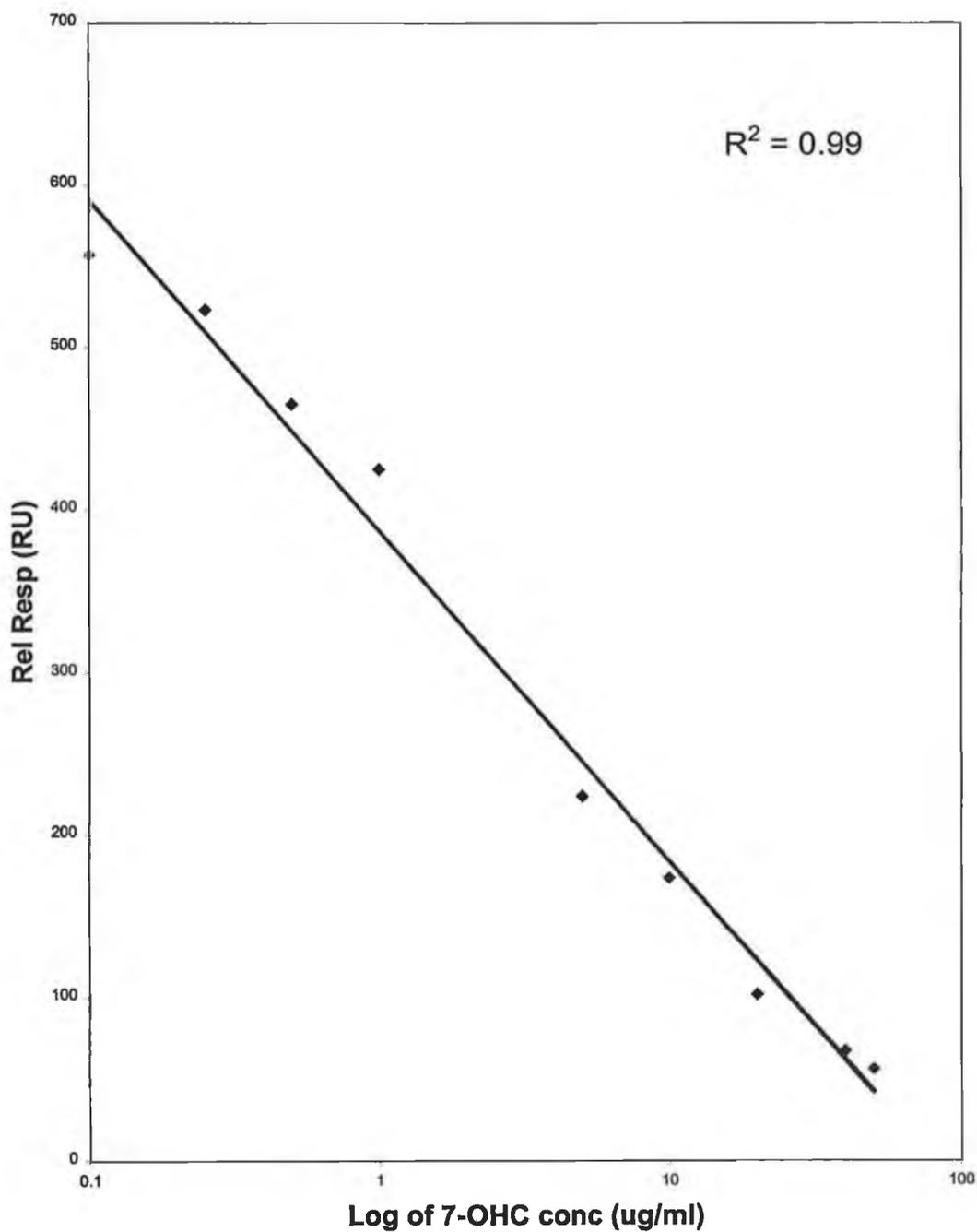


Figure 4.6 Standard curve for 7-hydroxycoumarin from BIAcore immunoassay model system. A range of 7-hydroxycoumarin standards was prepared in PBS, mixed with polyclonal antibody, and injected over a 7-hydroxycoumarin-BSA surface. The standard curve is linear over the range 0.1 – 50 $\mu\text{g/ml}$.



Polyclonal anti-7-hydroxycoumarin at a concentration of 0.25 mg/ml in PBS (approximating to the working dilution after mixing with sample) was passed over both surfaces (Figure 4.7). Binding of antibody to native BSA was shown to be negligible - 4 RU compared to 235 RU for the conjugate surface - under these assay conditions.

4.5.2 Cross-reactivity of polyclonal antibody to 7-hydroxycoumarin glucuronide

The specificity of polyclonal antibody is of critical importance for the performance of the immunoassay. Antibody (0.7 mg/ml in PBS) was mixed with 7-hydroxycoumarin at 50 µg/ml in human serum, 7-hydroxycoumarin glucuronide at 40 µg/ml in human serum, and control serum. These three samples were then injected over a 7-hydroxycoumarin-BSA surface. An overlay plot of the sensorgrams for the free drug and glucuronide samples is shown in Figure 4.8.

The binding of the sample containing free drug is much lower than that of the glucuronide sample, which gives the same response as the control, in which no 7-hydroxycoumarin was present. This demonstrates that - within the parameters of the BIAcore assay - the antibody exhibits no cross-reactivity to the glucuronidated form of 7-hydroxycoumarin.

4.5.3 Optimisation of enzyme buffer molarity

The enzyme β -glucuronidase can be used to deconjugate 7-hydroxycoumarin glucuronide, producing free 7-hydroxycoumarin and glucuronic acid. The enzyme has an optimum pH of 5.0, and in previously reported HPLC and CE assays (Egan and O'Kennedy, 1992; Bogan *et al.*, 1995) has been used at a concentration of 5000 units/ml in 1M sodium acetate, pH 5.0. In an immunologically-based assay, however, a buffer of such high ionic strength might reasonably be expected to interfere with antibody activity. To investigate the optimal molarity of enzyme buffer, 7-hydroxycoumarin glucuronide solutions at a range of concentrations (50, 40 and 20 µg/ml) were added to equal volumes of enzyme solution in differing molarities of buffer (5,000 U/ml β -glucuronidase in 10 mM, 100 mM and 1 M sodium acetate, pH 5.0). These samples were incubated for 2 hours at 37°C, then mixed with antibody and injected over a 7-hydroxycoumarin-BSA surface (Figure 4.9).

Figure 4.7 An overlay plot showing the interaction of polyclonal serum with a 7-hydroxycoumarin-BSA surface (10000 RU immobilised), and a native BSA surface (13000 RU immobilised). The degree of non-specific binding of antibody to BSA (4 RU) is not significant when compared to the response on the conjugate surface (235 RU).

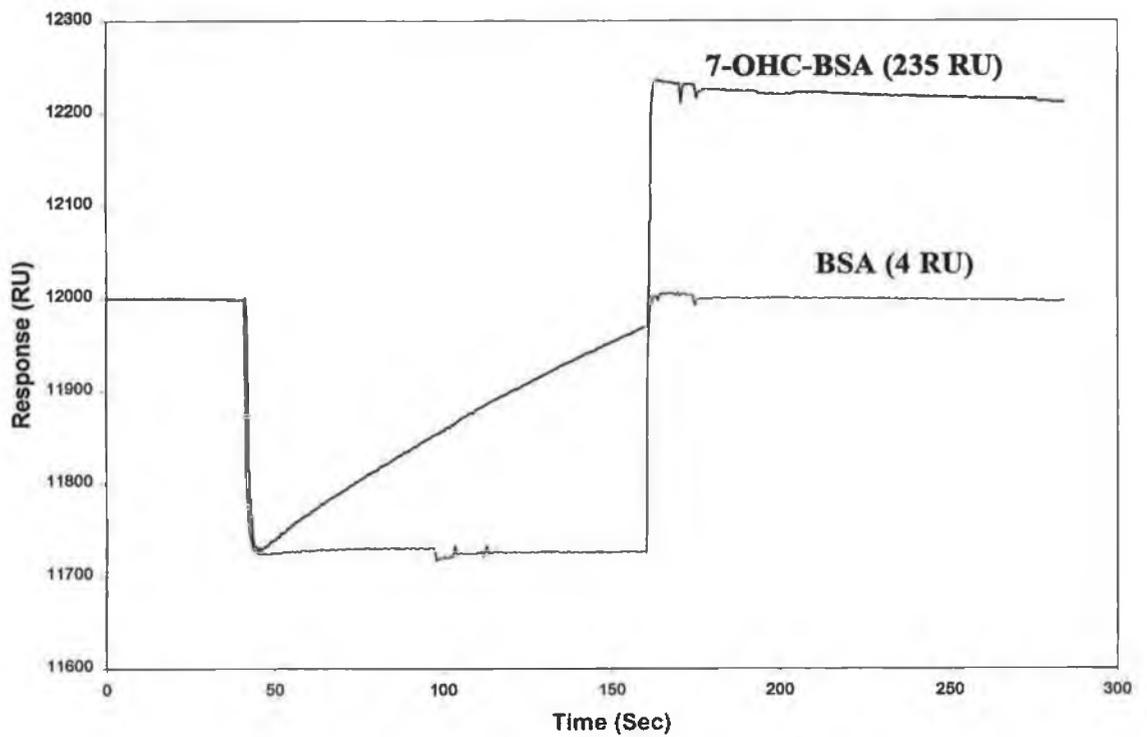


Figure 4.8 Overlay plot demonstrating the degree of cross reactivity of polyclonal antiserum with 7-hydroxycoumarin glucuronide. Antibody was mixed with both free and glucuronidated forms of 7-hydroxycoumarin and injected over an immobilised 7-hydroxycoumarin-BSA surface. No unbound antibody remains in the free drug sample (-6 RU), and the signal from the glucuronide sample (323 RU) indicates that the polyclonal antibody does not display any significant cross-reactivity for 7-hydroxycoumarin. The small negative signal for the 7-hydroxycoumarin sample represents some removal of non-covalently bound conjugate from the sensor surface.

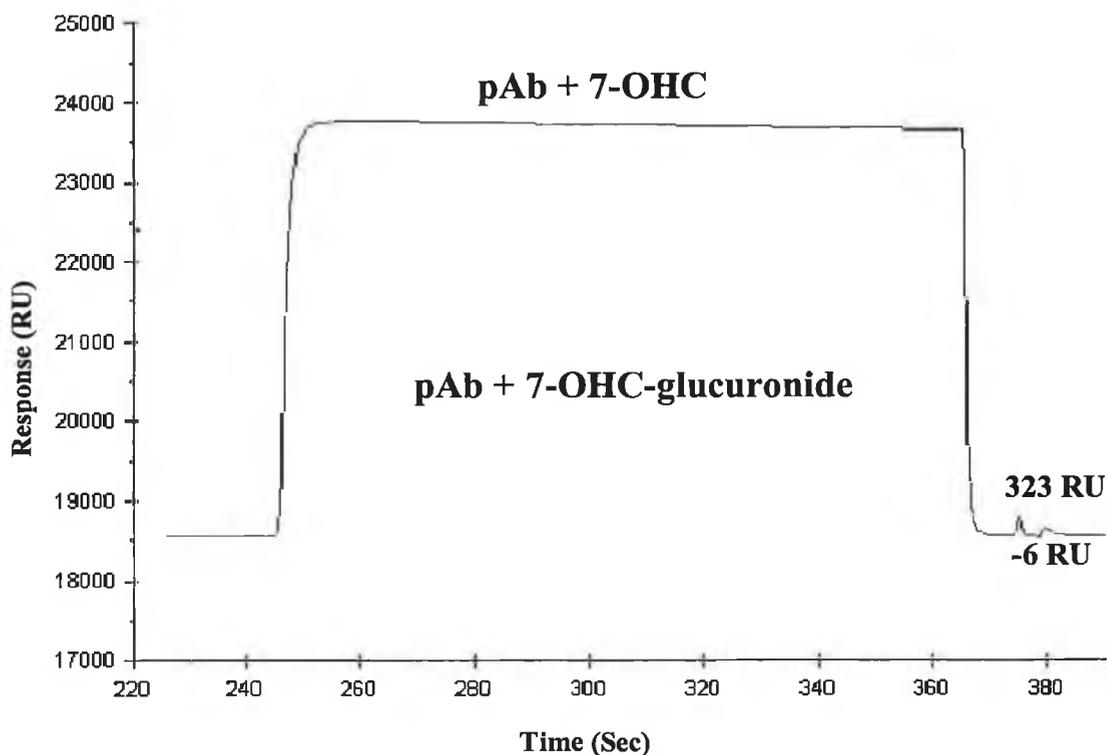
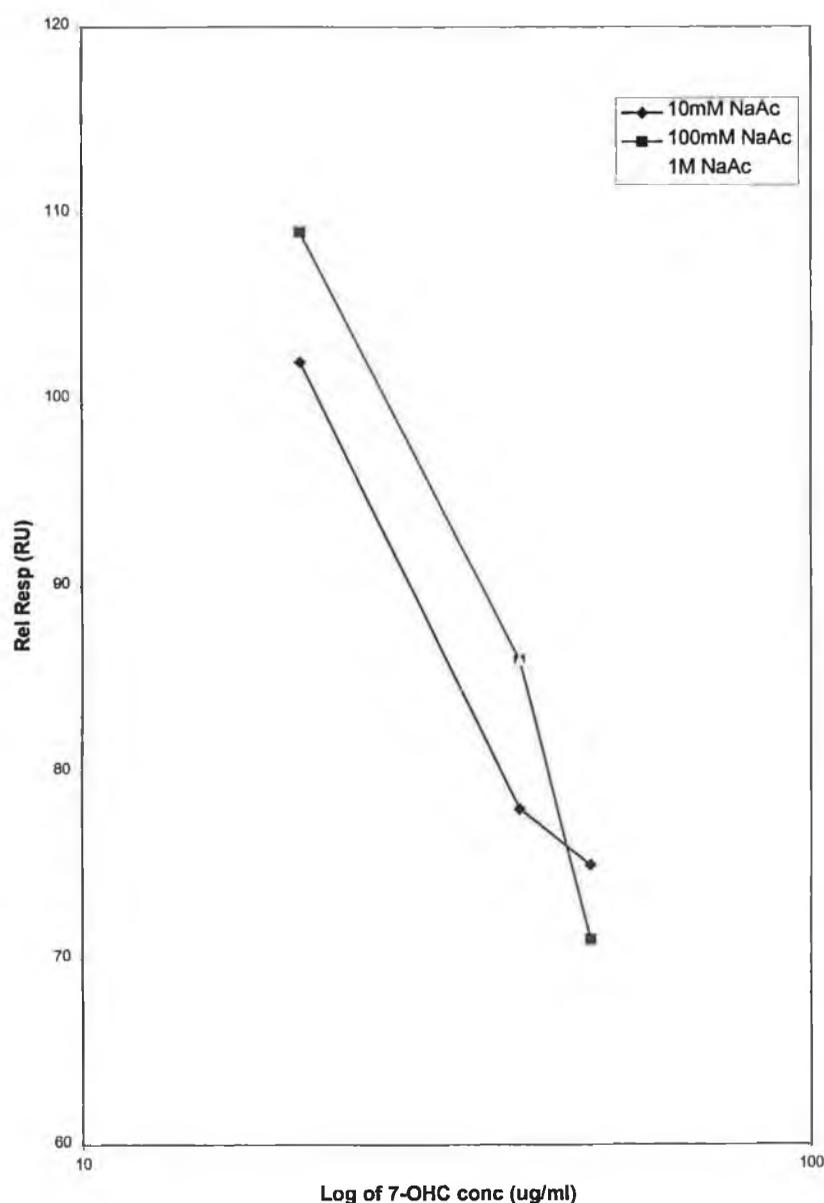


Figure 4.9 Optimisation of enzyme buffer molarity. Human serum was spiked with 50, 40 and 20 $\mu\text{g/ml}$ of 7-hydroxycoumarin glucuronide and these samples were mixed with 5000 units/ml of β -glucuronidase in 10mM, 100mM and 1M sodium acetate, pH 5.0. Antibody was then added to the samples, and they were injected over a 7-hydroxycoumarin-BSA surface. The samples in 100mM sodium acetate show good differences in response for the different concentrations, whereas 10mM acetate seems insufficient to buffer the enzyme, and the ionic strength of 1M sodium acetate appears to disrupt antibody-antigen binding.



The results show that enzyme in 10 mM sodium acetate is not deconjugating the glucuronide at higher concentrations, presumably due to insufficient buffering power. The three samples in 1 M acetate, however, give virtually the same response, suggesting that the ionic strength of the enzyme solution is disrupting antibody-antigen interactions. 100 mM sodium acetate was chosen as the enzyme buffer for the assay, as the response for this set of samples clearly increases with increased concentration of glucuronide conjugate. An incubation time for samples with enzyme solution of 2 hours at 37°C was chosen to ensure that the enzyme reaction had run to completion.

4.5.4 Removal of serum non-specific binding

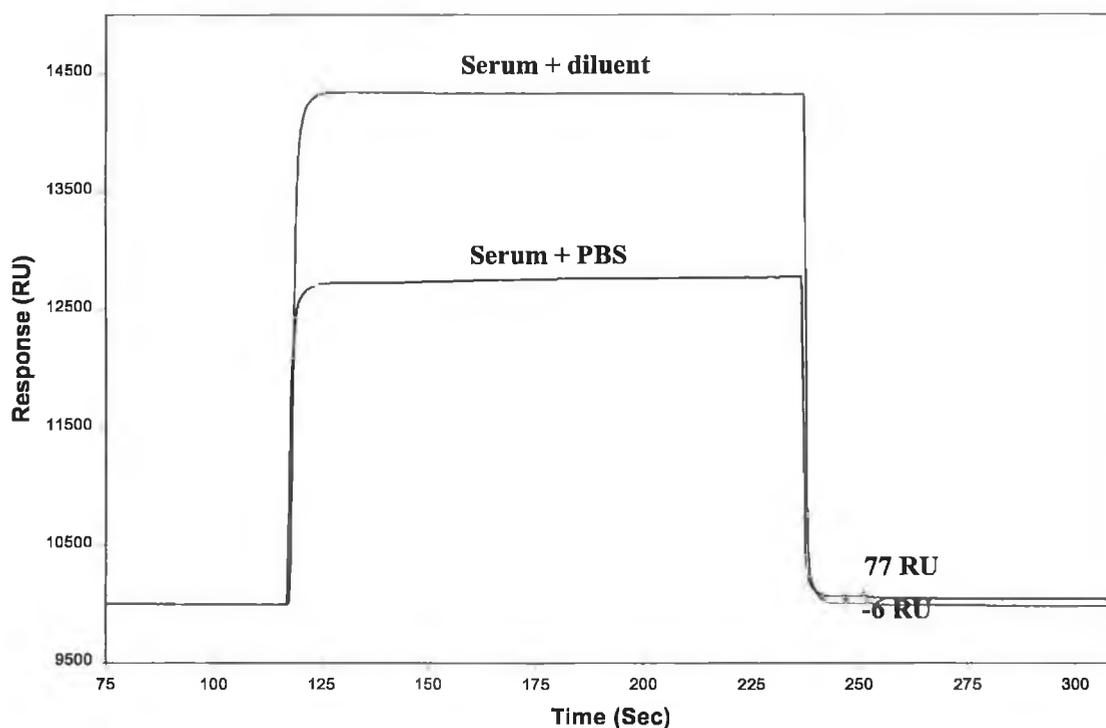
The use of a complex matrix such as human serum in the assay introduced problems with non-specific binding. Injections of neat serum over BIAcore sensor chips, both underivatized and with immobilized conjugate, resulted in high background responses due to binding of components in the serum to the protein moiety of 7-hydroxycoumarin-BSA and to the dextran layer on the chips.

In order to reduce these interactions, which would be damaging to assay performance, a diluent buffer containing BSA, carboxymethylated dextran and Tween 20 was prepared. Human serum was mixed and incubated for 10 minutes at room temperature with equal volumes of both PBS and diluent buffer (PBS containing 4% (w/v) BSA, 4 mg/ml carboxymethylated dextran and 0.2% (v/v) Tween 20), and both solutions were injected over an immobilized 7-hydroxycoumarin-BSA surface (Figure 4.10). Preincubation with the mixture of protein, CM-dextran and detergent effectively removed the non-specific binding of the serum sample. To avoid additional dilution and incubation steps in the assay, this mixture was used as the antibody diluent buffer, and the incubation time of samples with antibody was extended from 5 minutes to 10 minutes.

4.5.5 Efficiency of regeneration

One minute pulses of 5 mM NaOH, 20 mM HCl and 5 mM NaOH in sequence were injected over the sensor chip surface after each standard and sample injection to remove bound antibody.

Figure 4.10 Removal of non-specific serum binding by diluent buffer. Human serum was mixed 1:1 with both PBS and diluent buffer (PBS containing 4% (w/v) BSA, 4 mg/ml CM-dextran and 0.02% (v/v) Tween 20), and passed over an immobilised 7-hydroxycoumarin surface. Pre-mixing of sample with the diluent buffer effectively removes non-specific binding of serum components to the sensor surface. Although the sample containing diluent buffer produces a high initial bulk refractive index change (due to its protein content), no significant change in relative response is seen after the injection. The small negative signal (-6 RU) is probably due to the removal of non-covalently bound conjugate from the surface by the action of the detergent.



To assess the efficiency of this regeneration process, antibody solution (1/200 in PBS) was injected over this surface and regenerated, and this cycle was repeated for 65 cycles. The absolute response after regeneration decreased by only 2% over the 65 cycles, and Figure 4.11 shows that the signal produced by binding of antibody stayed within 20% of the initial response for 64 cycles. This indicates that the surface is stable over 64 regeneration cycles.

4.5.6 Linear range

The final optimised immunoassay format for 7-hydroxycoumarin is as shown in Figure 4.12, and is described in section 2.9.3. An overlay plot of the binding curves for a typical set of standards is shown in Figure 4.13, and a standard curve of relative response against concentration of 7-hydroxycoumarin in serum is given in Figure 4.14. The assay is linear over the range 0.5 - 80 µg/ml, with typical r^2 values of 0.99.

4.5.7 Recoveries of 7-hydroxycoumarin from spiked samples

The percentage recoveries of 7-hydroxycoumarin from samples spiked with 7-hydroxycoumarin glucuronide were calculated as described in section 2.9.3. Known amounts of 7-hydroxycoumarin glucuronide were added to human serum and these spiked samples, along with standards containing unconjugated 7-hydroxycoumarin, were mixed with β -glucuronidase and assayed for free drug. Their concentrations were read from the standard curve, and the percentage recoveries were calculated from the ratio of the molecular weights of both metabolites (Table 4.2). The calculated recoveries for a set of three replicates of five spiked samples were in the range 95.4 – 103.2%.

Figure 4.11 Regeneration profile for an immobilised 7-hydroxycoumarin-BSA surface. A 1/200 dilution of purified polyclonal anti-7-hydroxycoumarin was injected over the surface, and regenerated with 5 mM NaOH and 20 mM HCl. After 64 cycles of binding and regeneration, the response after injection of antibody (130.5 RU) was within 20% of the original signal (156.8 RU).

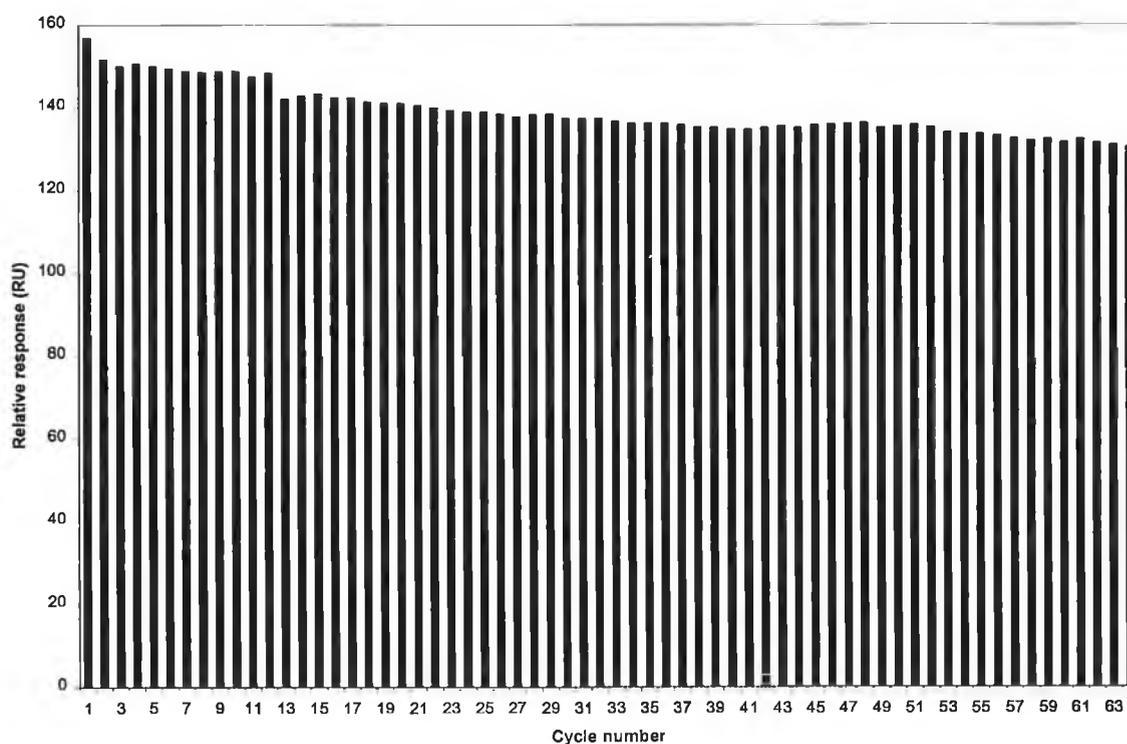


Figure 4.12 Flow diagram of BIAcore 7-hydroxycoumarin immunoassay.

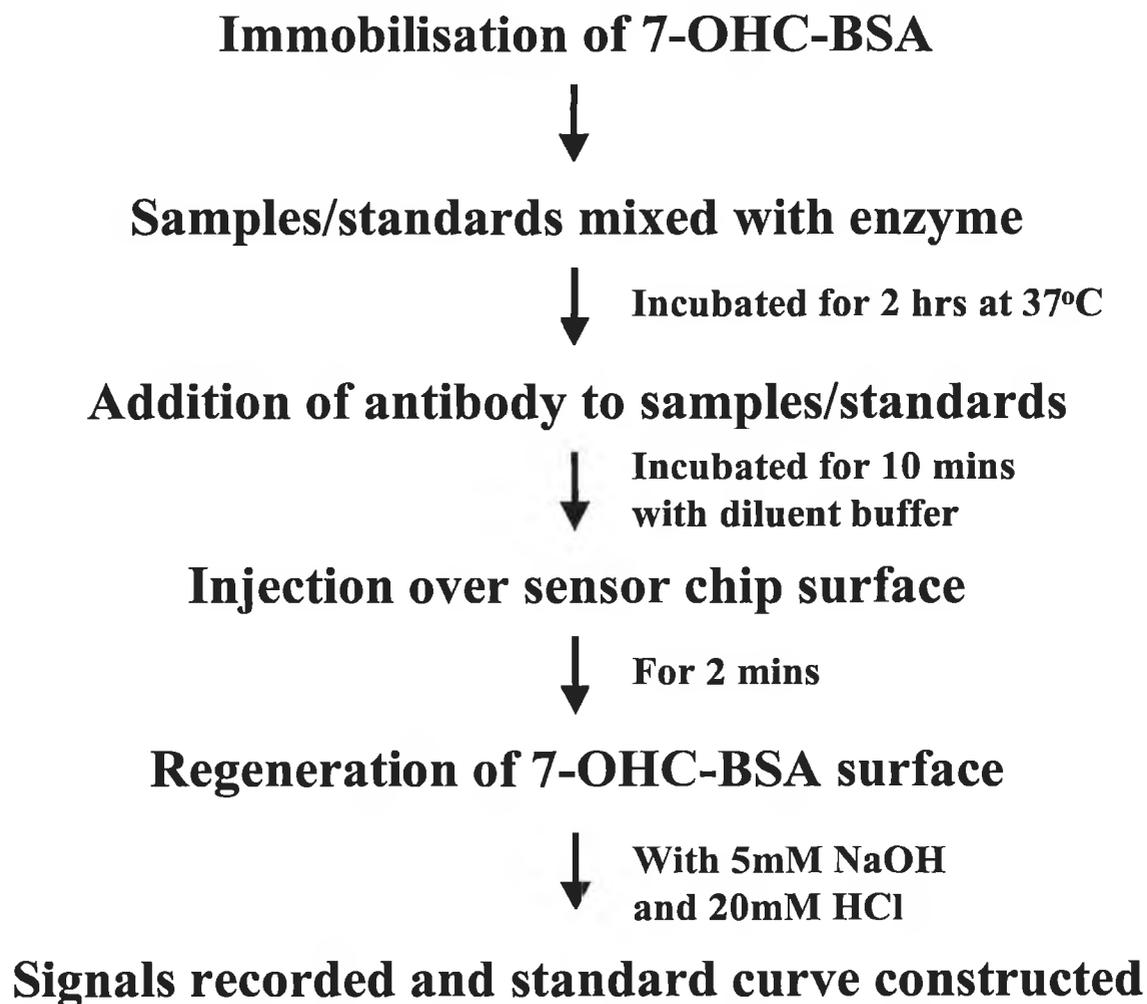


Figure 4.13 An overlay plot for a typical set of binding curves. 7-hydroxycoumarin standards containing 0.5 - 80 ug/ml of free drug were mixed with antibody and injected over a 7-hydroxycoumarin-BSA surface.

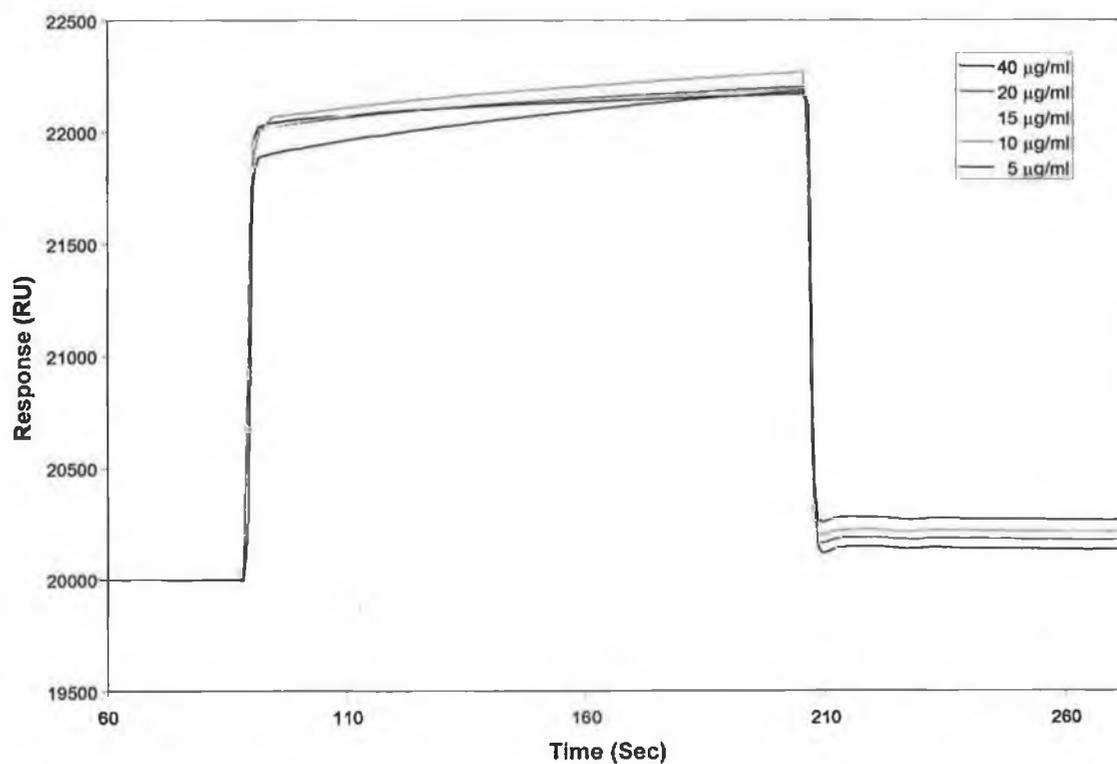


Figure 4.14 Linear range of the BIAcore 7-hydroxycoumarin assay in serum. Standards from 0.5 - 80 $\mu\text{g/ml}$ were prepared, mixed with β -glucuronidase and antibody and injected over the sensor surface. BIA Concentration Evaluation software was used to fit the data to a smoothed spline model.

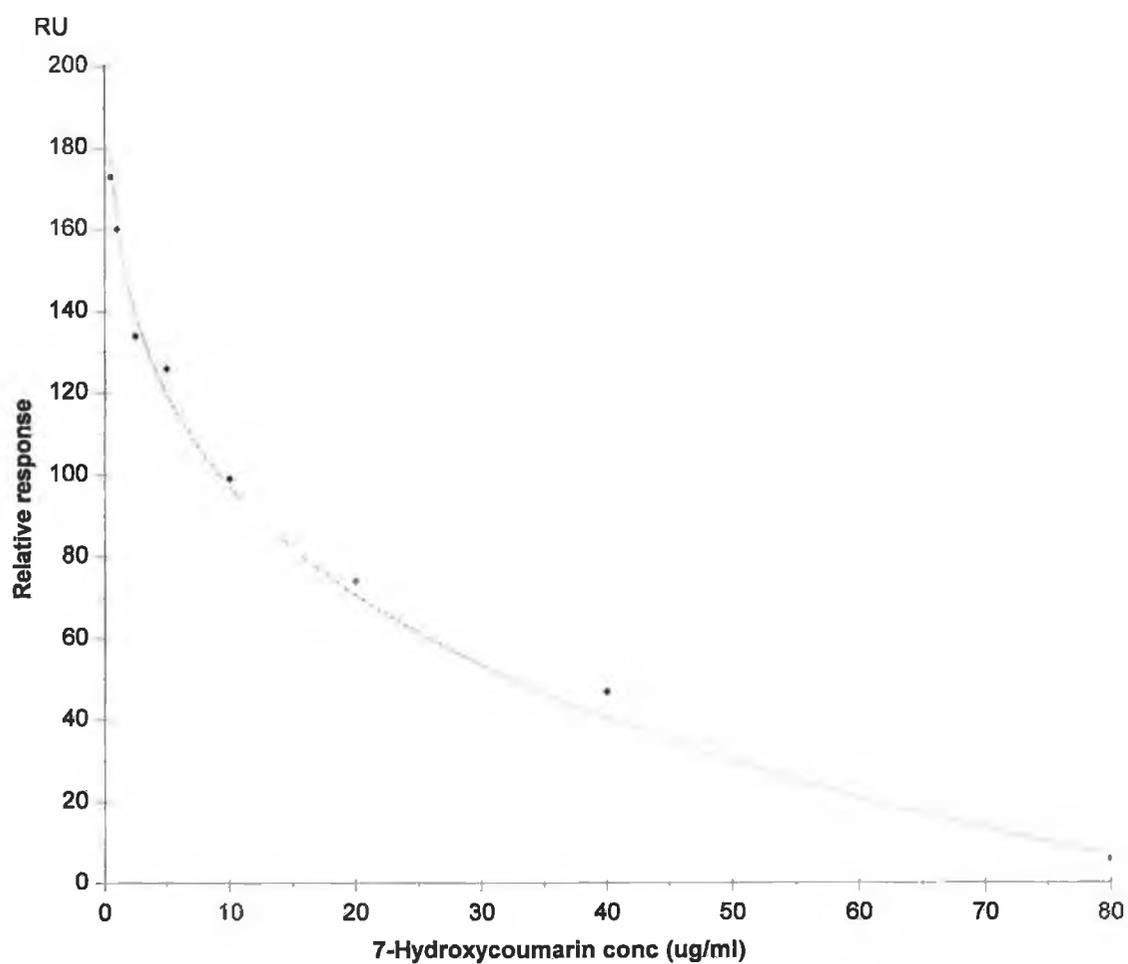


Table 4.2 Percentage recoveries for the BIAcore 7-hydroxycoumarin assay. Human serum was spiked with varying concentrations of 7-hydroxycoumarin glucuronide, β -glucuronide was then added, and the samples assayed for deconjugated 7-hydroxycoumarin. Percentage recoveries were determined on the basis of the ratio between the molecular weights of the two metabolites. Standard variations (S.D.s) were calculated for three replicates of each sample.

7-OHC concentration ($\mu\text{g/ml}$)	% Recovery \pm S.D. (n=3)	Coefficient of variation (%)
37.08	97.8 \pm 4.7	4.84
18.54	95.4 \pm 8.4	8.85
13.91	99.8 \pm 5.8	5.83
9.27	103.2 \pm 6.0	5.81
4.64	101.9 \pm 0.0	0.0

4.5.8 Intra-day variability

The intra-day variability of the assay was investigated by running a set of five standards across the linear range five times in one day, and determining the coefficient of variation (CV) between the calculated 7-hydroxycoumarin concentrations for each set of five (Table 4.3). Intra-day CVs ranged from 1.93% to 8.2%.

4.5.9 Inter-day variability

The inter-day variability of the assay was investigated by running five sets of standards across the linear range five times on five different days, and determining the CV between the calculated 7-hydroxycoumarin concentrations for the standards from each of the five curves (Table 4.4). Intra-day CVs ranged from 5.02% to 8.76%.

4.6 Urine analysis

The quantitative determination of 7-hydroxycoumarin in urine samples was also investigated. Using a method otherwise identical to that for analysis in serum, standards and samples were prepared in control urine and assayed for the presence of free drug, following treatment with β -glucuronidase. Although a standard curve with an r^2 value of 0.99 and a linear range of 0.5 - 50 $\mu\text{g/ml}$ (Figure 4.15) could be constructed, and recoveries in the range 95 - 105% were demonstrated, analysis in urine displayed very poor reproducibility.

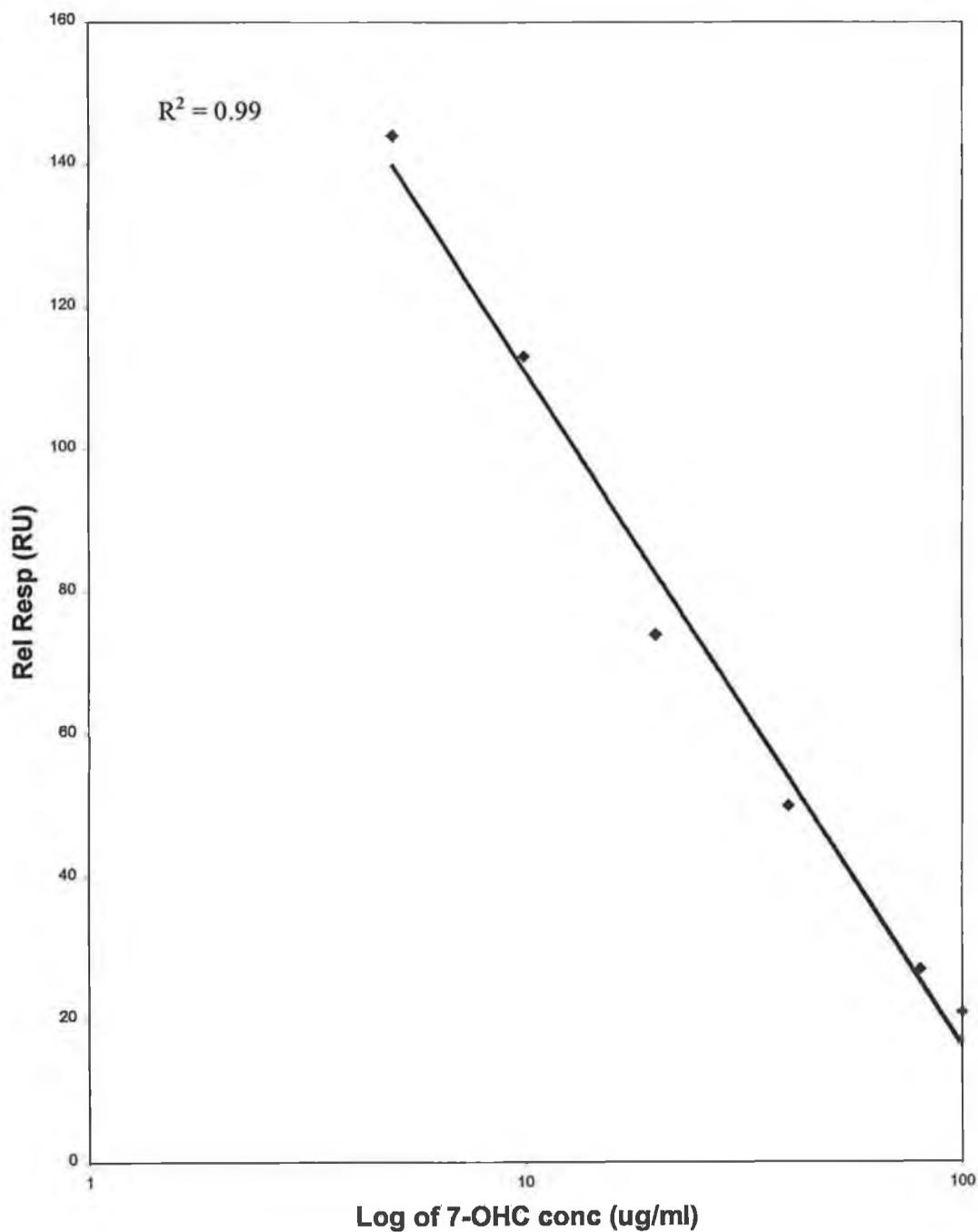
Table 4.3 Intra-day coefficients of variation for the BIAcore 7-hydroxycoumarin assay. Five sets of five standards were run on the same day and the coefficients of variation (CVs) between the calculated concentrations from the standard curve were determined.

7-OHC concentration ($\mu\text{g/ml}$)	Calculated mean \pm S.D. ($\mu\text{g/ml}$)	Coefficient of variation (%)
5	5.36 ± 0.39	7.26
10	9.95 ± 0.15	1.93
15	14.29 ± 0.54	3.78
20	19.19 ± 0.68	3.44
40	41.11 ± 3.34	8.20

Table 4.4 Inter-day coefficients of variation for the BIAcore 7-hydroxycoumarin assay. Five sets of five standards were run on five different days, and the CVs between the calculated values from each standard curve were calculated.

7-OHC concentration ($\mu\text{g/ml}$)	Calculated mean \pm S.D. ($\mu\text{g/ml}$)	Coefficient of variation (%)
5	4.62 ± 0.29	6.31
10	11.22 ± 0.56	5.02
15	15.99 ± 1.40	8.76
20	18.66 ± 1.00	5.02
40	39.16 ± 2.23	6.31

Figure 4.15 Standard curve for BIAcore 7-hydroxycoumarin immunoassay in urine. Free drug was spiked into neat control urine, and the samples were assayed without an extraction step. The linear range is 5- 100 $\mu\text{g/ml}$, although the method is not very reproducible, probably as a result of batch-to-batch differences in pH and ionic strength of control urine.



4.7 Discussion

A model system for 7-hydroxycoumarin detection in PBS was first established. The first step in setting up the immunoassay system was the immobilisation of 7-hydroxycoumarin BSA. The results of the preconcentration study (Figure 4.3) show that electrostatic binding of the drug-protein conjugate is not evident until the pH of solution is lowered to 4.5. Comparing this to the pI for native BSA (5.1) demonstrates the effect of chemically modifying the protein, underlining the need for preliminary optimisation of preconcentration in order to conserve sample and save time. By using the RECOVERY facility on the BIAcore during these investigations, the quantity of conjugate used can be kept to a minimum.

Immobilisation of 7-hydroxycoumarin was carried out by means of a defined chemistry, and was highly reproducible, resulting in approximately 10,000 RU of conjugate being bound to the chip. This responds to a protein concentration at the sensor surface of 10 ng/mm².

A model inhibition immunoassay was then developed, in which 7-hydroxycoumarin standards in PBS were mixed with polyclonal antiserum to the drug and injected over the immobilised surface. The high concentration of antibody used (700 µg/ml) is due to the fact that the antibody preparation was only partially purified by protein G chromatography, and also reflects the small number of specific antibodies present in antisera raised against a drug-carrier immunogen (Heiko Reinartz, Personal communication). The assay had a linear range of 100 ng/ml to 50 µg/ml, and displayed excellent linearity.

Having shown that the polyclonal antibody was suitable for use in an assay of this type, and that the antibody-antigen binding was regenerable, the aim was to develop a method for detection of total 7-hydroxycoumarin in complex biological matrices.

The main limiting factor in the sensitivity of any immunoassay is the affinity and specificity of the antibody used. Figure 4.7 shows that there is no binding of polyclonal antibody to immobilised BSA. It is interesting to note, however, that in the course of screening for monoclonal antibody production by ELISA (see Chapter Three) - an assay format involving long incubation steps - the same antibody was shown to bind significantly to control wells coated with BSA. The short contact time between sample and sensor surface in the BIAcore system appears to minimise this non-specific interaction.

The antibody was also shown not to bind significantly to 7-hydroxycoumarin glucuronide (Figure 4.8). This finding (not surprising, in view of the fact that glucuronic acid is attached at the 7-position) meant that, as with all of the other immunologically-based detection methods discussed above, deconjugation of the glucuronide conjugate by β -glucuronidase would be necessary for determination of total 7-hydroxycoumarin content of samples. Reinartz *et al.* (1996) used 5000 units/ml of enzyme in 1 M sodium acetate, pH 5.0, in an antigen-inhibition ELISA for estimation of total 7-hydroxycoumarin, but performed a 1:10 dilution of sample prior to addition of sample to microtitre plate wells. Several molarities of enzyme buffer were investigated for use in the BIAcore assay (Figure 4.9), in an attempt to find an concentration of sodium acetate at which both the enzyme reaction and the antibody-antigen interaction could occur, thus removing the need for dilution or other pretreatment of sample. 100 mM sodium acetate was found to be suitable.

The majority of the previously reported methods of 7-hydroxycoumarin analysis have involved sample clean-up, in the form of extraction and reconstitution, dilution, freeze-drying, or precipitation of proteins with TCA. If such a step were to be avoided for the BIAcore immunoassay, it was clear that the background binding of serum components to the sensor surface would have to be reduced. Human serum gave a significant binding signal when injected over both underivatized flow cells and 7-hydroxycoumarin-BSA surfaces. It was thought that the addition of carboxylated dextran and BSA to serum samples might absorb out substances which were binding to these components, and Figure 4.10 shows that incubation with CM-dextran and BSA in a diluent buffer, together with the detergent effects of Tween 20, effectively remove non-specific binding of serum. This mixture was then used as the antibody diluent.

When an inhibitive immunoassay incorporating deconjugation and removal of non-specific binding in neat serum samples was performed, it had a linear range of 500 ng/ml to 80 μ g/ml. The limit of detection is thus 2.5 times higher than that for the model system (taking into account the extra dilution step with enzyme). In addition, by comparing Figures 4.6 and 4.4, it can be seen that the relative responses for the standards in the model system range from 100 - 600 RU, whereas those for the assay in serum are between 10 and 170 RU. However, by the time the more complex sample is injected over the chip surface, it contains serum, 7-hydroxycoumarin, β -glucuronidase, sodium acetate, PBS, CM-dextran, BSA,

Tween 20 and polyclonal antibody, and immunoanalytical detection in such a complex matrix is obviously only achieved at the expense of sensitivity.

The regeneration of immunosensors is of critical importance for their performance. The antibody-antigen interaction at the sensor surface in this assay was disrupted by brief (1 minute) pulses of alternating low molarity base and acid. The change in pH between the two solutions is sufficient to regenerate the surface. Wong *et al.* (1997) recommended that the two criteria of baseline shift and binding capacity should be used for evaluating regeneration. The absolute response of immobilised 7-hydroxycoumarin-BSA after 65 regeneration cycles decreases by only 2%, while Figure 4.11 shows that the rebinding of antibody to this surface remains within 20% of the initial response for 64 cycles. Wong *et al.* considered an immobilised surface to be stable while the rebinding signal was within this 20% value.

When samples containing 7-hydroxycoumarin glucuronide were assayed along with a range of 7-hydroxycoumarin standards, the recoveries of free drug were all close to 100%, over 3 replicates (Table 4.2). This indicates that the deconjugation process is highly efficient. Inter-day and intra-day coefficients of variation for the method are all below 8.76% for 5 replicates (Tables 4.3 and 4.4). Both the percentage recoveries and coefficients are within the limits recommended by Wong *et al.* (1997).

Overall, the BIAcore inhibitive immunoassay for quantitative determination of total 7-hydroxycoumarin in serum compares well with the analytical methods listed in Table 4.1. Although some of the other techniques do have lower limits of detection, all of these (with the exception of the method of Gamache *et al.*, 1993) involve some type of sample clean-up. In particular, the method described here has a lower limit of detection than the other three immunosensors discussed. Of all the antibody-based methods reported, only that of Reinartz *et al.* (1996) has a lower limit of quantification. This may, as mentioned above, be due to the dilution step involved, or it may reflect the greater sensitivity of ELISA compared to BIAcore. Wagner *et al.* (1995) reported increased sensitivity in their inhibitive BIAcore immunoassay when they used monoclonal antibodies in place of polyclonal antiserum. The use of monoclonal antibodies in this assay might result in enhanced sensitivity, although the greater affinity of these antibodies may create problems with regeneration (see Chapter 5).

Although the BIAcore method was used to construct a standard curve in neat urine with a linear range of 5 - 100 $\mu\text{g/ml}$ and an r^2 value of 0.99, it proved difficult to reproduce this assay using different batches of urine. It seems that batch-to-batch variations in the pH and ionic strength of urine interfere with the antibody-antigen interaction. Although it would be possible to adjust the pH of control urine used in the preparation of standards, the smaller volumes of patient samples might be problematic, and it appears that non-immunological methods of detection, such as HPLC and CE, are inherently more suitable for the analysis of neat urine samples.

One of the most noteworthy attributes of the BIAcore immunoassay is its degree of automation. Standards, samples, enzyme, antibody and regeneration solutions are prepared and placed in the BIAcore sample racks. An automated method then controls addition of, and incubation with, both enzyme and antibody, injection of sample, and regeneration of the sensor surface. The temperature of the enzyme/sample mixtures is maintained at 37°C by means of an attached waterbath. These factors make this method of analysis easier to perform than any of the others discussed previously, and allow less opportunity for operator error.

4.8 Summary

A model system for the detection of 7-hydroxycoumarin using BIAcore was established. 7-hydroxycoumarin-BSA was immobilised on the sensor chip and 7-hydroxycoumarin was premixed with a polyclonal anti-7-hydroxycoumarin antibody and injected over the surface. Excess antibody bound to the immobilised conjugate, generating an SPR signal inversely proportional to the amount of 7-hydroxycoumarin in the sample.

An inhibition immunoassay for the determination of total 7-hydroxycoumarin in human serum samples was then developed. A diluent buffer to minimise the non-specific binding of serum components to the sensor surface was included in the assay. The regeneration of the sensor surface was investigated, and the baseline shift and rebinding capacity were found to be acceptable over 64 cycles. Recoveries of free drug from samples spiked with 7-hydroxycoumarin-glucuronide were close to 100%, and inter- and intra-day coefficients of variation were less than 8.76% for 5 replicates. The assay had a measuring range of 0.5 – 80 µg/ml.

In conclusion, then, the BIAcore-based immunoassay for 7-hydroxycoumarin described was easily regenerable, reproducible, entailed no sample clean-up, and compared well to established methods of analysis.

CHAPTER 5

COMPARATIVE BIAcore AND ELISA STUDIES ON ANTI-AFLATOXIN ANTIBODIES

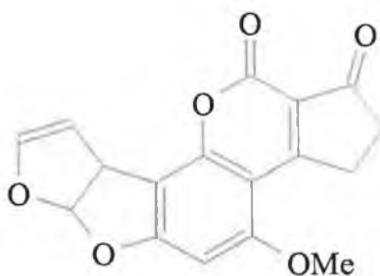
5.1 Introduction

In this chapter, a variety of immunoassay formats were investigated on the BIAcore, using commercially available polyclonal and monoclonal antibodies to aflatoxin B₁ - a mycotoxin and member of the coumarin family. The high affinity of both antibody preparations for the antigen presented severe difficulties in the regeneration of the sensor surface, and no meaningful quantitative or kinetic data could be obtained. In a series of comparative studies with established ELISA techniques, both antibodies were used to construct standard curves for free and conjugated toxin, using both sandwich and competitive assay formats. The results presented indicate the lack of suitability of the antibodies investigated for regenerable biosensor applications.

The antibodies were used to develop an ELISA-based model system for the initial optimisation of BIAcore regeneration conditions. The regeneration of antibody and toxin-protein conjugate surfaces was mirrored on 96-well microtitre plates, and a competitive ELISA system was employed to calculate the equilibrium dissociation constant for the monoclonal anti-aflatoxin antibody. Preliminary investigations of this type can be used to considerably reduce the expense and time of BIAcore experiments.

5.2 Chemistry, occurrence and toxicity of aflatoxins

The aflatoxins are a group of highly toxic secondary fungal metabolites that occur in *Aspergillus* species. They are members of the coumarin family, and the most commonly occurring compound is aflatoxin B₁ (AFB₁), which is produced by certain strains of *A. flavus* and *A. parasiticus*. The structure of AFB₁ is shown below:



Other aflatoxins - designated B₂, G₁ and G₂ - are also produced, but AFB₁ is generally present in the largest quantity, and is definitely the most toxic.

A range of metabolites of AFB₁ have been isolated. AFM₁ (or 4-hydroxy-AFB₁) is a major metabolite of AFB₁ in rats and cows, among other species (de Iongh *et al.*, 1964). AFQ₁ and AFP₁ have been shown to be major metabolites of AFB₁ in the mouse and Rhesus monkey (Dalezios *et al.*, 1971; Hsieh *et al.*, 1974). Other metabolites of the toxin include aflatoxicol, deshydroaflatoxin D₁, AFB_{2a} and AFG_{2a}.

Under high humidity and temperature conditions, *A. flavus* and *A. parasiticus* can grow on certain foodstuffs, predominantly peanuts, tree nuts (e.g. Brazil nuts and pistachio nuts), and oilseeds, such as corn. The consequent contamination of these foods with aflatoxins is of major concern, particularly for the health of livestock. Aflatoxins have also been considered for use as biological weapons (Paddle, 1996).

At the molecular level, aflatoxins interfere with the replication and transcription of nucleic acids, protein and lipid metabolism, and enzyme activity. The effects of aflatoxin poisoning in animals include cirrhosis, acute necrosis, and carcinoma of the liver. In addition, Silvotti *et al.* (1997), in a study on piglets fed with aflatoxins, discovered a range of immunotoxicological effects, including impairment of macrophage and granulocyte function.

The first recorded outbreak of aflatoxicosis occurred in 1960, when 100,000 turkeys in England died as a result of consuming contaminated groundnut meal (Austwick, 1978). Although a wide range of LD₅₀s has been reported for different species, most are in the region of 0.5 - 10 mg/kg body weight.

AFB₁ has been shown to be a potent carcinogen in fish, birds, rodents and non-human primates (Newborne and Butler, 1969). The carcinogenic effects of the toxin are known to require metabolic activation. This probably occurs by conversion of AFB₁ into its 2,3-epoxide, which binds strongly to nucleic acids (Martin and Garner, 1977), although a more recent study has demonstrated 8,9-epoxidation by lipoxygenase, pointing to another possible route for hepatocarcinogenesis (Roy and Kulkarni, 1997).

As aflatoxin contamination of foodstuffs for human consumption rarely reaches very high levels, little is known of the effects of acute aflatoxin poisoning in man. Several outbreaks of aflatoxicosis have occurred, however, the most notable taking place in India in 1974. In

some 150 villages in neighbouring districts in the northwest of the country 108 of the 397 individuals affected died. The symptoms of the poisoning included fever, jaundice, oedema, vomiting and inflammation of the liver. The cause of the outbreak was contaminated corn, and it was estimated that the average daily dietary intake of AFB₁ was at least 55 µg/kg body weight, for an undetermined number of days.

Although the main cause of hepatocarcinoma in man is known to be the hepatitis B virus, it is thought that, in areas of where high exposure is common, aflatoxin exposure interacts with the virus to increase the incidence of the disease (Peers *et al.*, 1987). In a large-scale study in Shanghai, Ross *et al.* (1992) found a definite correlation between urinary levels of aflatoxin B₁, its metabolites AFP₁ and AFM₁, and their DNA-adducts, and liver cancer.

5.3 Detection of aflatoxins

Many of the detection systems employed for the analysis of aflatoxins have been chromatographic in nature. Column chromatography followed by TLC was the method of choice for early aflatoxin studies. Silica plates were developed in chloroform:acetone (9:1), and quantitation was achieved by fluorodensitometry and use of internal standards.

More recently, ease of use and automation have led to HPLC being widely used for the measurement of aflatoxins. Although absorbance at 360-365 nm can be used for the determination of AFB₁ and AFB₂, the greater sensitivity of detection of fluorescence has made it a more popular alternative. Pre- or post-column derivatisation has frequently been used to increase the fluorescence signal.

Roch *et al.* (1995) found that pre-column derivatisation of AFB₁ and AFB₂ resulted in a sensitive HPLC assay, with a limit of quantification for AFB₁ of 1.1 µg/kg of groundnut meal. Samples were extracted into acetone:water (85:15) before analysis.

HPLC separation with precolumn derivatisation using trifluoroacetic acid and detection of fluorescence was used by Torres Espinosa *et al.* (1995) to quantify AFB₁ levels in extracted corn samples, with a limit of detection of 5 ng/g.

Cepeda *et al.* (1996) used HPLC separation with a methanol:water mobile phase, together with fluorescent detection (following post-column addition of cyclodextrins to enhance fluorescence) to measure aflatoxin levels in extracted food samples, with a limit of detection of 4 mg/l.

Niedwetzki *et al.* (1994) used an immunoaffinity column for sample clean-up prior to HPLC analysis, with post-column derivatisation and fluorescent detection. This assay was carried out on filtered nut and fruit extracts, using an automated workstation.

Kussak *et al.* (1995a) also employed an immunoaffinity column for pre-column clean-up of urine samples prior to HPLC analysis with post-column bromine derivatisation and fluorescence detection. Recoveries of 103% and a limit of quantification for AFB₁ of 6.8 pg/ml were reported with this method. In a separate study, Kussak *et al.* (1995b) also used an immunoaffinity column for clean-up of dust and urine samples before HPLC separation and detection by electrospray ionisation tandem mass spectrometry, with a detection limit of AFB₁ of 4 pg.

The use of antibodies in aflatoxin analysis has not been confined to sample clean-up for HPLC. As discussed in Chapter 4, immunoanalytical techniques, if correctly designed, can facilitate analyte detection in complex matrices without laborious extraction procedures. A variety of immunoassays for the determination of aflatoxins have been reported. Many of these, however, are semi-quantitative methods of analysis, with "cut-off" concentrations corresponding to hazardous toxin levels in particular foodstuffs. The average legal limit for AFB₁ in food products is 10 µg/kg, with some variations between countries (Van Egmond, 1989).

A semi-quantitative enzyme-linked immunosorbent assay for aflatoxins has been reported by Trucksess and Stack (1994). Corn samples were extracted into methanol:water (80:20) and the extracts, together with HRP-labelled aflatoxin, were added to polyclonal antisera immobilised on a filter membrane. After washing with water, a chromogenic substrate (containing hydrogen peroxide and tetramethylbenzidine) was added. Test samples with an AFB₁ content of greater than 20 ng/g failed to produce any colour change after a 1 minute incubation.

Another semi-quantitative immunoassay system was described by Abouzied and Pestka (1994). In this assay, monoclonal anti-aflatoxin antibodies were immobilised on nitrocellulose membranes, and a competition between AFB₁ in extracted corn samples and HRP-labelled AFB₁ was set up. The colour intensity upon addition of chromogenic substrate was inversely proportional to the amount of mycotoxin present. The limit of detection of this method was 0.5 ng/ml.

Schneider *et al.* (1995) reported a "dipstick" type enzyme immunoassay for AFB₁. This semi-quantitative assay involves immobilising antibodies to AFB₁ (among other mycotoxins) onto a dipstick membrane. The dipstick is then immersed in a test-tube containing extracted sample and labelled toxin, followed by a second incubation with substrate. Complete suppression of colour formation indicated a positive detection of aflatoxin. The detection limit of the assay was 30 ng/g in wheat samples. A similar dip-strip assay format was described by Sashidar (1993), with a limit of quantification for AFB₁ of 10 ppb.

Ram *et al.* (1986) reported a competitive ELISA for quantitative determination of aflatoxin B₁ in corn and cottonseed extract, which utilised polyclonal antibodies and had a limit of detection of 1 mg/ml.

Ward *et al.* (1990) described the production of polyclonal and monoclonal antibodies to AFB₁, and their use in a microtitre plate-based quantitative ELISA. They reported limits of detection for their model system of 1 pg/ml with both types of antibody.

Ramakrishna *et al.* (1990) used monoclonal antibodies to measure aflatoxin in extracted barley samples. They reported a limit of detection for aflatoxin B₁ of 100 pg/ml/.

Bacigalupo *et al.* (1994) developed two different immunoassay formats to detect AFB₁ in extracts of soya seeds, dried figs and raisins. Using polyclonal antibodies in a time-resolved fluoroimmunoassay, they reported a limit of quantification of 0.5 µg/kg, while a limit of detection of 0.2 µg/kg was achieved with an immunoenzymatic assay.

5.4 Enzyme-linked immunosorbent assay (ELISA)

The production and characterisation of polyclonal and monoclonal antibodies have been discussed in Chapter 3. Enzyme-linked immunosorbent assay (ELISA) - an analytical technique which exploits the sensitivity and specificity of the antibody-antigen interaction - has been reviewed by O'Kennedy (1989) and Gosling (1990). Basically, either antibody or its complementary antigen is labelled with an enzyme, and the interaction is allowed to proceed. A chromogenic substrate is then added, which generates colour upon reaction with enzyme. The amount of coloured product formed is then determined by measuring the absorbance at a particular wavelength, and is directly proportional to the concentration of the labelled component.

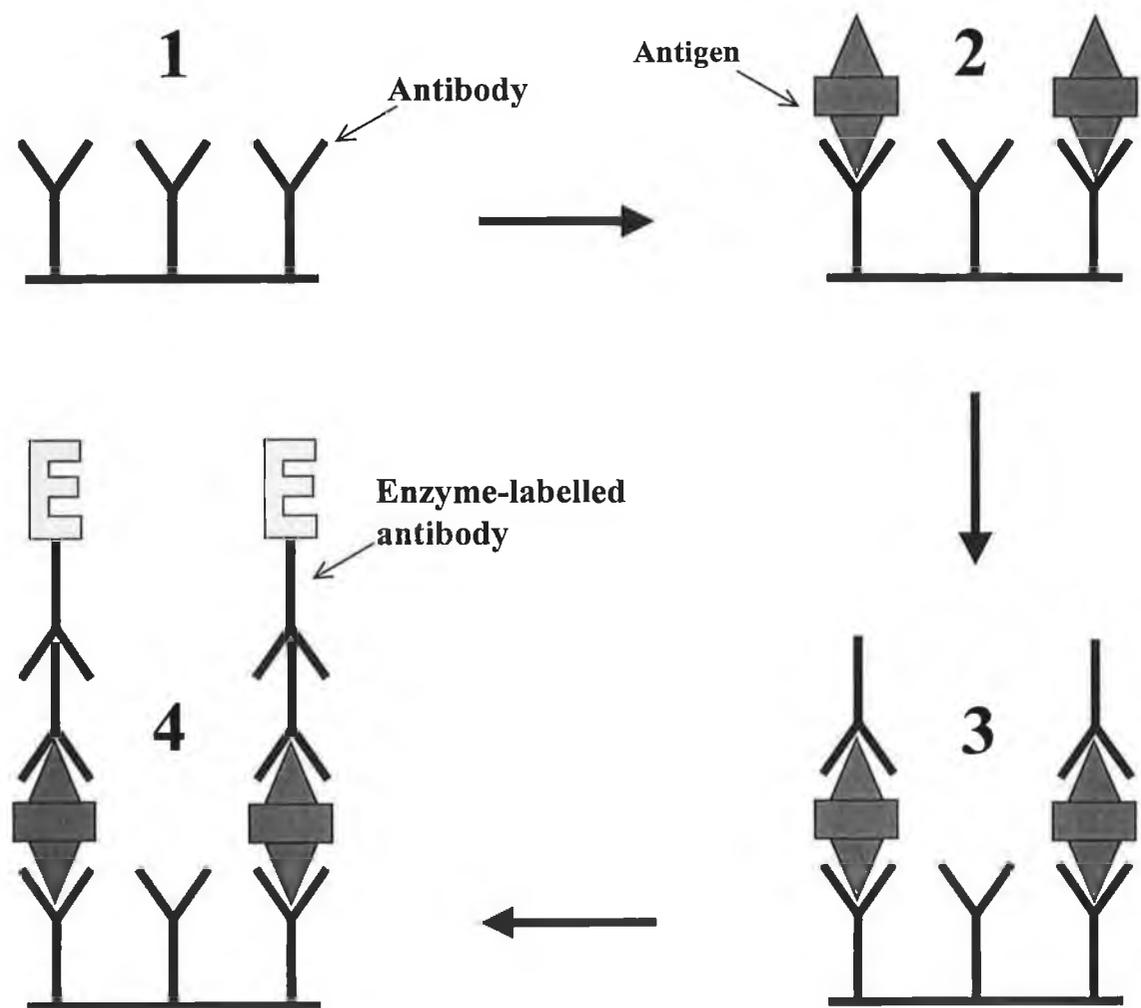
In this chapter, a variety of ELISA formats are used, both for the quantitative determination of AFB₁, and to calculate an equilibrium affinity constant for the interaction between monoclonal anti-AFB₁ and free toxin. The ELISAs described here are heterogeneous assays, in which one component is immobilised by passive adsorption onto the wells of a 96-well polystyrene microtitre plate, and subsequent washing steps are needed to separate free from surface-bound interactants. ELISA systems can be divided into two categories: non-competitive ("sandwich") and competitive assays.

A typical sandwich assay format is depicted in Figure 5.1. Antibody is first immobilised on the wells of a microtitre plate. The remaining surface of the plate is then "blocked" by addition of a high molecular weight protein or mixture of proteins - typically bovine serum albumin (BSA) or foetal calf serum (FCS). Sample containing antigen is then added to the wells. A second antibody against the analyte (which interacts with a different epitope) is then allowed to bind. Finally, an anti-species antibody to the secondary antibody, labelled with an enzyme, is added, and, following incubation with chromogenic substrate, the absorbance of each well is measured. In between each of these steps, the wells of the plate are washed with a detergent-containing solution to remove any unbound antibody or antigen.

Utilising a labelled secondary antibody can reduce the number of steps in a sandwich assay; but the ready availability of commercial labelled anti-species antibodies, together with the inherent risks to activity posed by enzyme conjugation, make the use of anti-species antibodies a more acceptable option, affording greater sensitivity.

Ideally, the addition of secondary antibody should introduce an amplification effect. For optimum assay performance, an immobilised monoclonal antibody should be used for capture of analyte, and a polyclonal antiserum (or heterogeneous mixture of monoclonal antibodies) for secondary binding. The interaction of the polyclonal antibody at multiple epitopes will then increase the response. If the situation is reversed - i.e. coating with polyclonal antibody and secondary addition of monoclonal antibody - then the number of sites available for monoclonal antibody binding may be reduced.

Figure 5.1 Schematic diagram of a typical sandwich ELISA format. (1) Wells of an ELISA plate are coated with antibody. (2) Antigen is added and allowed to bind, after which a secondary antibody is added (3). An enzyme-labelled (E) anti-species antibody is then allowed to bind to the secondary antibody, and the colour generated upon addition of chromogenic substrate is directly proportional to the amount of antigen present (4). The wells are washed between every step to remove unbound components.



The absorption of antibodies onto the walls of a microtitre plate well is an essentially random process, and the conformation of many of the immunoglobulins may prevent the binding of antigens to their active sites. This type of conformational problem may be overcome by using an initial "capture" molecule to correctly orientate the antibodies.

The ability of protein A to bind specifically to IgG has been discussed in section 3.5. By coating plates with protein A or an Fc-specific anti-IgG antibody and then adding the primary antibody, the correct orientation of the Fab portion can be assured.

Figure 5.2 shows the type of competitive ELISA format used in this chapter. Antigen is immobilised on the surface of a microtitre plate, which is then blocked as before. Solutions containing antigen are mixed with an excess of antibody, and this mixture is added to the wells. Unbound antibody is allowed to bind to immobilised antigen, and then an enzyme-labelled secondary antibody is added. The colour produced upon addition of chromogenic substrate is inversely proportional to the concentration of antigen in the original solution. As in the sandwich assay, the plates are washed between each step to remove unbound components. This type of assay is more properly known as an antigen inhibition ELISA, to distinguish it from a competitive ELISA format in which labelled and unlabelled antigen compete for binding sites on an immobilised antibody surface.

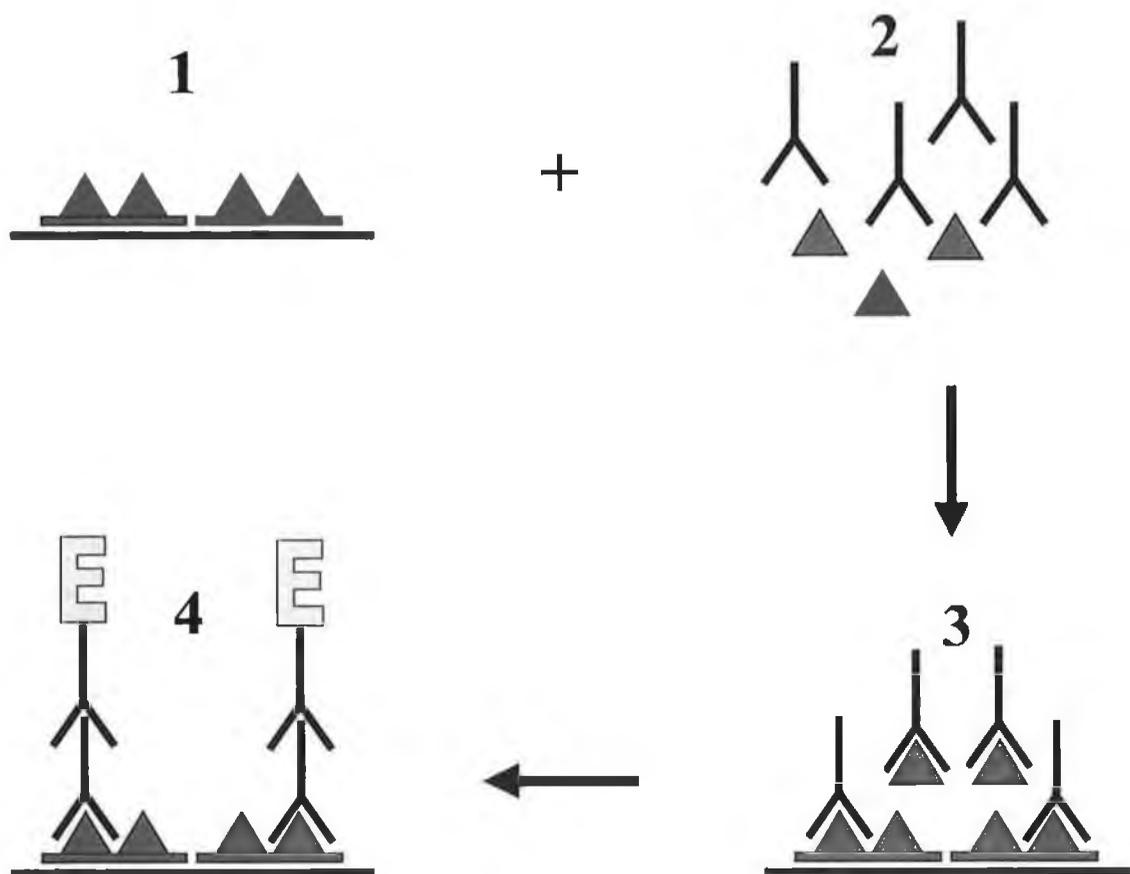
In general, competitive ELISA is the method of preference for detection of low molecular weight analytes such as drugs and toxins, as the small size of these molecules precludes the multiple-epitope binding necessary in a sandwich assay.

5.5 Measurement of antibody affinity by ELISA

The concept and theory of antibody affinity have been discussed in section 3.3. A variety of methodologies, including equilibrium dialysis, fluoroimmunoassay and radio-immunoassay, may be used to determine the affinity of an antibody for its specific antigen. The use of BIAcore to calculate kinetic and affinity data was described in section 3.7. In this chapter, an ELISA method is used to calculate this value for a commercial monoclonal anti-AFB₁ antibody.

Van Heyningen *et al.* (1983) outlined a simple competitive ELISA procedure for ranking antibody affinities, which gave an approximate K_D value.

Figure 5.2 Schematic diagram of a typical competitive ELISA format. (1) A protein conjugate of a low molecular weight analyte is coated onto wells of an ELISA plate. Antibody and analyte are mixed (2) and added to the wells, where immobilised and free antigen compete for antibody binding sites (3). An enzyme-labelled anti-species antibody is then added, and the colour generated upon addition of chromogenic substrate is inversely proportional to the amount of analyte present (4). The wells are washed between every step to remove unbound components.



This method, however, relies upon interaction of antibody with immobilised antigen, and, as a result, is unsuitable for the calculation of true affinity constants. Friguet *et al.* (1985) described an improved ELISA method, which allows determination of the equilibrium dissociation constant by measuring the amount of free antibody in solution after equilibrium with antigen has been reached. In addition, this procedure can be used to calculate the dissociation constant even if the absolute concentration of antibody is not known.

The assay is based upon the assumption that monoclonal antibody at constant concentration will reach equilibrium if incubated with differing concentrations of antigen for a sufficient period of time. The amount of remaining unbound antibody in each equilibrium solution is then measured by ELISA.

Antigen is coated onto a wells of a microtitre plate, and aliquots of the antibody-antigen mixtures are added. Following incubation and washing, an enzyme-labelled secondary antibody is allowed to bind, chromogenic substrate is added, and the absorbance is recorded. In preliminary studies, Friguet *et al.* (1985) found a linear relationship between the amount of antibody added to wells and the final absorbance reading. This allowed them to state that if antibody at a total concentration i_0 is incubated with antigen at a given concentration, then i , the free antibody concentration, will be equal to the absorbance A , as measured by ELISA, divided by A_0 - the absorbance for antibody in the absence of antigen:

Equation 5.5.1
$$\frac{i}{i_0} = \frac{A}{A_0}$$

This assumes that the equilibrium in the liquid phase is not disrupted in the course of the ELISA.

Using the mass conservation equations, the concentrations of bound antibody (x) and free antigen (a) at equilibrium can be calculated:

Equation 5.5.2
$$x = i_0 - i$$

Equation 5.5.3
$$a = a_0 - x$$

where a_0 is the total concentration of antigen. The three values x , a and i_0 can be related to K_D , the dissociation constant, by the Klotz equation (Klotz, 1953):

$$\text{Equation 5.5.4} \quad \frac{1}{x} = \frac{1}{i_0} - \frac{K_D}{a \times i_0}$$

From equation 5.5.1, x and a can be related to the absorbance readings from ELISA:

$$\text{Equation 5.5.5} \quad x = i_0 \times A_0 - \frac{A}{A_0}$$

$$\text{Equation 5.5.6} \quad a = a_0 - i_0 \times A_0 - \frac{A}{A_0}$$

Hence, equation 5.5.4 can be rewritten as:

$$\text{Equation 5.5.7} \quad A_0 / A_0 - A = 1 + K_D / (a_0 - i_0 \times A_0 - A / A_0)$$

If the concentration of the antigen is much larger than that of the antibody, then a can be approximated by a_0 , and the previous equation becomes:

$$\text{Equation 5.5.8} \quad A_0 / A_0 - A = 1 + K_D / a_0$$

Therefore, by preincubating a constant concentration of antibody with at least a ten-fold molar excess of antigen, measuring the amount of remaining unbound antibody by ELISA, and plotting the reciprocal of the fraction of free antibody (A_0/A_0-A) against the reciprocal of the antigen concentration, a line with an intercept on the y-axis of 1, and a slope of K_D should be produced.

Results

5.6 BIAcore studies

The interactions between polyclonal and monoclonal anti-AFB₁ antibodies and a protein conjugate of the toxin were investigated in a range of experimental formats which included direct immobilisation of both antibodies, use of protein A and anti-species antibody capture layers, and immobilisation of AFB₁-BSA. Running buffer and flow rate throughout were as described in section 2.9.4.

5.6.1 Direct immobilisation of monoclonal anti-AFB₁

Monoclonal anti-AFB₁ was immobilised on the sensor chip surface as outlined in section 2.9.2. In order to minimise any reduction in antibody activity, the pH of immobilisation buffer (10 mM sodium acetate/HCl) was adjusted to 5.3, and antibody was diluted immediately prior to injection over the derivatised chip surface.

As the molecular weight of aflatoxin (312.3) is too small for direct sensing, a BSA conjugate of the toxin was used in these studies. The degree of non-specific binding of the native form of the protein was first assessed by injecting a 4 minute pulse of 1 mg/ml BSA over the immobilised monoclonal antibody surface (Figure 5.3). No non-specific interaction was observed.

The binding of AFB₁-BSA to the immobilised antibody was then investigated. Figure 5.4 shows the sensorgram obtained when a solution containing 7 µg/ml of AFB₁-BSA was injected over an immobilised antibody surface (~9000 RU) for 20 minutes. Approximately 550 RU of conjugate is bound to the monoclonal antibody, and very little decrease in response is evident in the dissociation phase of the sensorgram. This indicates that there is a very low dissociation constant for the interaction.

Low molarity acid and base had previously been used to disrupt antibody-antigen binding in a BIAcore-based immunoassay (see Chapter 4). The effects of regeneration with sodium hydroxide are shown in Figure 5.5. Monoclonal antibody was immobilised (~14000 RU), and a 4 minute injection of AFB₁-BSA produces a binding signal of 342 RU. Injecting a 1 minute pulse of 10 mM NaOH brings the signal down to 20 RU above baseline. Subsequent injection of another 20 µl of toxin-protein conjugate, however, results in only 36 RU of binding.

Figure 5.3 Typical sensorgram to estimate the degree of non-specific binding of monoclonal anti-AFB₁ to BSA. Approximately 4,500 RU of antibody were immobilised, and a five minute pulse of 1 mg/ml BSA was injected over the surface. No increase in signal relative to baseline is seen after the sample pulse has passed over the surface.

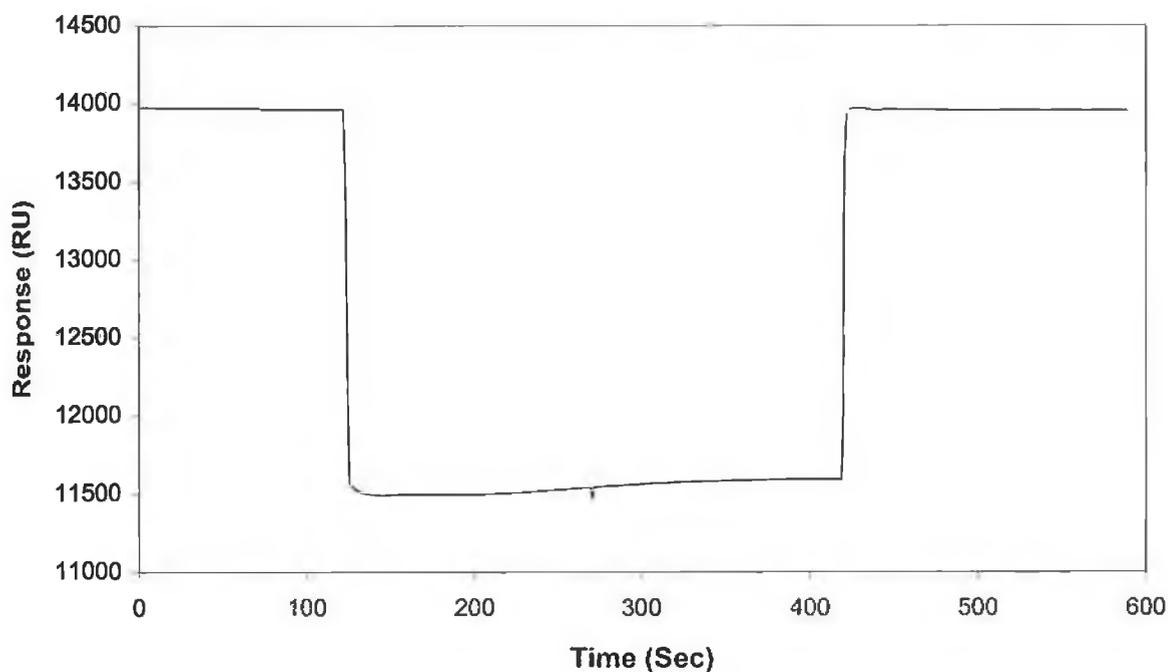


Figure 5.4 Sensorgram showing the binding of AFB₁-BSA to immobilised monoclonal anti-AFB₁. A 20 minute injection of a 7 µg/ml solution of conjugate over this surface results in an increase in response of 550 RU. The changes in signal at the beginning and end of the injection are due to differences in bulk refractive index between running buffer and sample. No significant decrease in signal is evident in the dissociation phase.

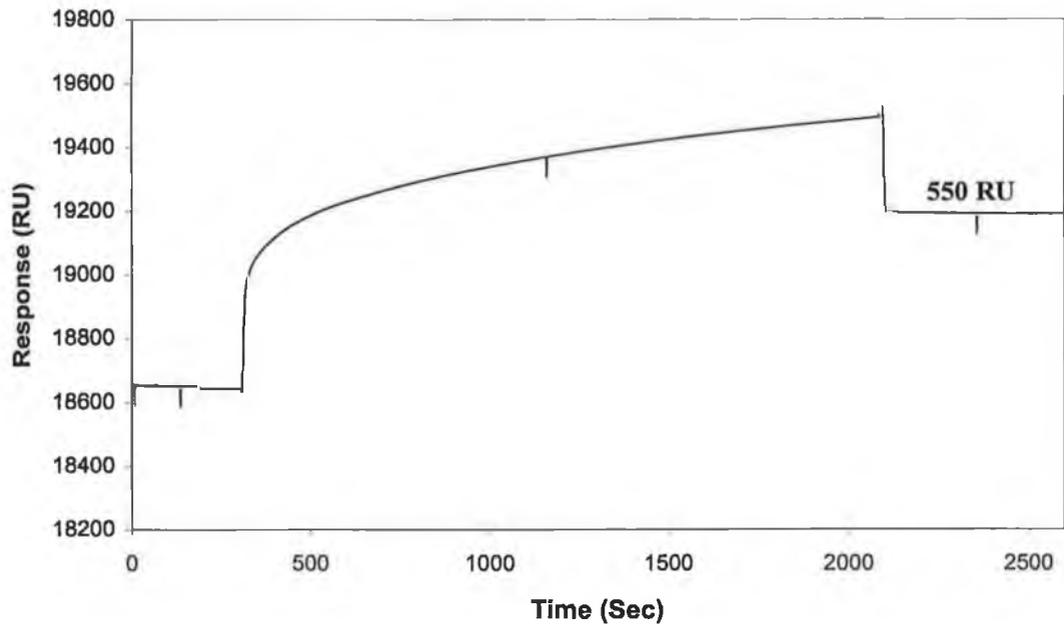
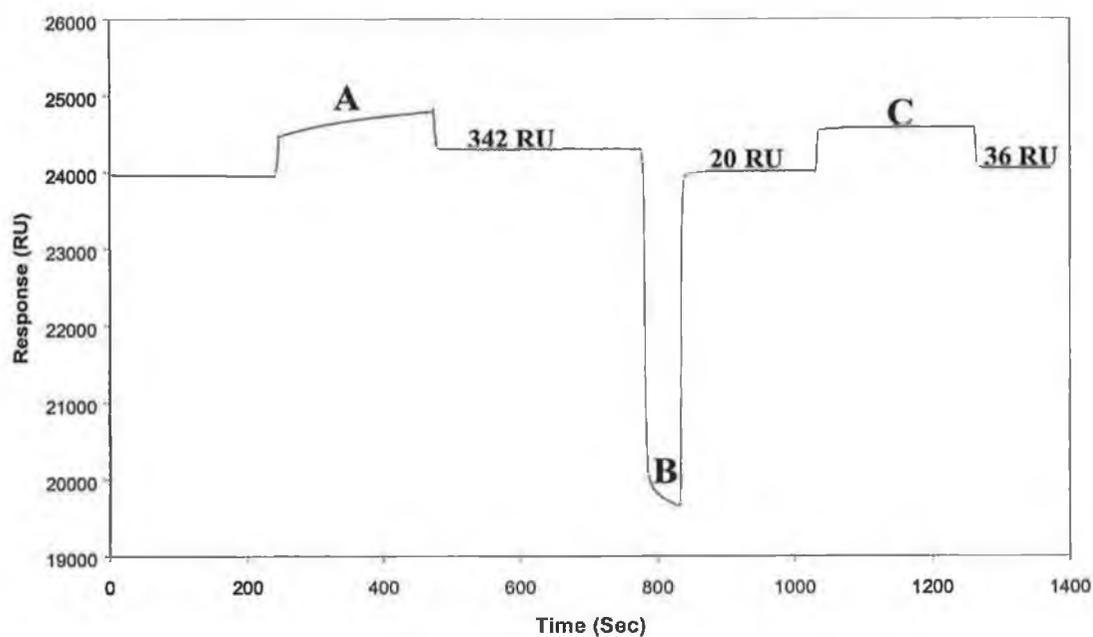


Figure 5.5 Sensorgram illustrating the effects of regeneration with sodium hydroxide on an immobilised monoclonal anti-AFB₁ surface. (A) A 4 minute injection of 7 µg/ml AFB₁-BSA produces a binding signal of 342 RU. (B) Injection of a 1-minute pulse of 10mM NaOH removes 322 RU of bound conjugate, but a further injection of toxin-protein conjugate (C) results in a response of only 36 RU.



This suggests that although injection of 10 mM NaOH is sufficient to almost completely regenerate the antibody surface, antibody activity is detrimentally affected by treatment with the base, to the extent that binding of conjugate after regeneration produces only 10% of the initial response.

The ability of a range of different solutions to regenerate the monoclonal antibody surface - on the basis of extremes of pH, hydrophobicity, and ionic strength - was investigated. The results are given in Table 5.1. Of the solutions tested, only 10 mM sodium hydroxide proved to be an effective regeneration reagent. It appears that satisfactory regeneration of the immobilised monoclonal antibody surface can only be achieved at the expense of antibody activity.

5.6.2 Indirect immobilisation of monoclonal anti-AFB₁ via protein A

As regeneration of the monoclonal antibody surface proved problematic, the use of a capture molecule to indirectly immobilise the antibody, thus removing the necessity for regeneration, was investigated.

The use of protein A to capture mouse and rabbit IgG in ELISA formats has been discussed in section 5.4. Protein A was immobilised on the sensor chip surface as in section 2.9.2, giving a signal of 5500 RU. 25 μ l of a 1/100 dilution of monoclonal antibody in PBS was injected over this surface, giving a response of 1460 RU. A subsequent injection of a 7 μ g/ml conjugate solution over the captured antibody, however, does not result in any binding signal (Figure 5.6). It can be seen from the sensorgram that the bound monoclonal antibody is dissociating from the protein A surface too rapidly to allow any binding of conjugate to be observed. This commercial antibody preparation is of the sub-class IgG₁, a type of mouse immunoglobulin for which protein A is known to have only a weak affinity.

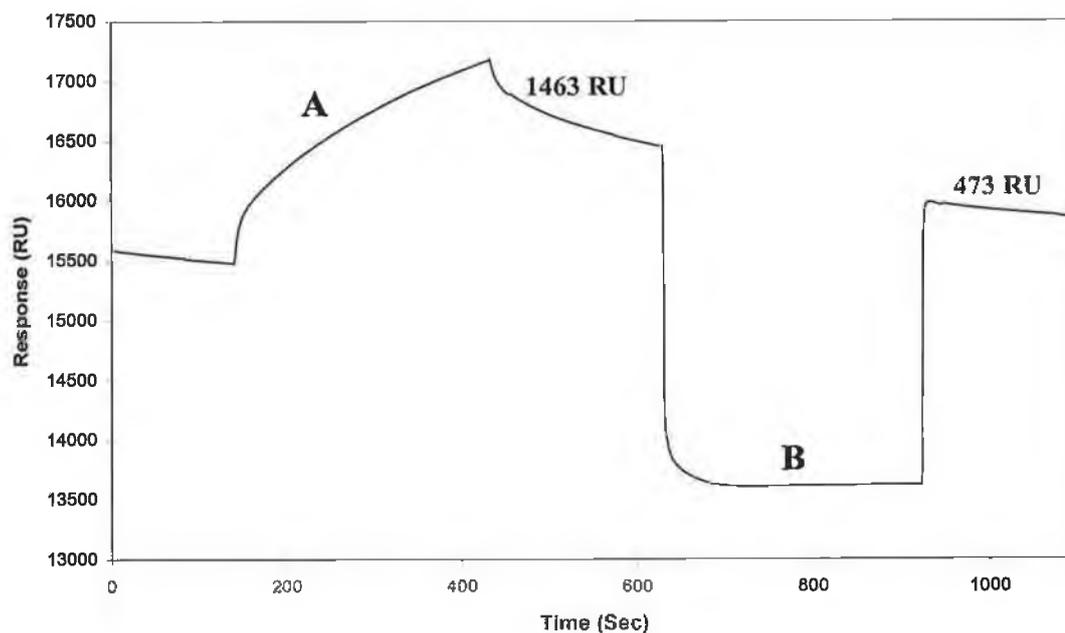
5.6.3 Indirect immobilisation of monoclonal anti-AFB₁ via an anti-mouse IgG antibody

A 1/200 dilution of Fc-specific anti-mouse IgG antibody in 10 mM sodium acetate, pH 5.45, was immobilised on the chip surface as described in section 2.9.2, giving a signal of ~9000 RU. Injection of a 1/50 dilution of monoclonal anti-AFB₁ for 10 minutes resulted in a binding signal of 1200 RU.

Table 5.1 Regeneration reagents tested on a monoclonal anti-AFB₁ surface. AFB₁-BSA was passed over immobilised antibody surfaces, and the ability of various reagents to disrupt antibody-antigen binding was assessed. The amount of regeneration is reported in terms of the percentage of the initial binding signal removed or added by each injection.

Regeneration Reagent	Contact Time	Percentage Change in Reponse
10mM HCl	2 min	+ 0.6%
10mM NaOH	1 min	- 93%
25% Ethanol	1 min	- 3.7%
20% Acetonitrile	1 min	- 8.6%
10mM NaCl	1 min	+ 6.3%
20mM NaCl	1 min	- 4.5%
50mM NaCl	1 min	- 1.1%

Figure 5.6 Sensorgram showing the dissociation of captured monoclonal anti-AFB₁ from an immobilised protein A surface. (A) Monoclonal antibody was injected over a protein A surface, with a binding signal of 1463 RU. However, significant dissociation is visible, and following injection of a 7 µg/ml solution of AFB₁-BSA (B), the response has decreased to 990 RU above the initial baseline.



When 7, 70 and 1000 $\mu\text{g/ml}$ solutions of AFB₁-BSA conjugate were passed over this captured antibody, however, no significant binding was observed (Figure 5.7). Further injections of increasing concentrations of anti-AFB₁ over immobilised anti-mouse IgG surfaces showed that a signal of 1200 RU was the saturation response for monoclonal antibody capture.

5.6.4 Direct immobilisation of polyclonal anti-AFB₁

Polyclonal anti-AFB₁ was immobilised at a 1/200 dilution as in section 2.9.2. The immobilisation buffer used was 10 mM sodium acetate, pH 5.8, and approximately 10000 RU of antibody was covalently bound to the sensor chip. Injections of AFB₁-BSA (at concentrations of 7, 70 and 1000 $\mu\text{g/ml}$) over this surface, however, resulted in no significant change in response.

The pH of immobilisation buffer was increased to 6.8, and antibody diluted in buffer immediately prior to injection over the derivatised chip surface. Again, 10000 RU of antibody were immobilised, but no signal was obtained when conjugate was passed over the IgG surface. Omission of the capping step - treatment of the immobilised surface with 1M ethanolamine, pH 8.5, to remove non-covalently bound material - did not result in any increase in antibody activity.

The AFB₁-BSA conjugate used was freshly reconstituted from lyophilisate, dialysed overnight at 4°C to remove any free toxin, and diluted in PBS containing 400 mM sodium chloride, in order to counteract any electrostatic repulsion between the highly-substituted conjugate and the carboxyl groups on the chip surface.

5.6.5 Indirect immobilisation of polyclonal anti-AFB₁ via protein A

As in section 5.6.2, protein A was immobilised on the sensor chip. A 5 minute injection of a 1/100 dilution of polyclonal anti-AFB₁ in PBS over this surface gave a response of approximately 2000 RU, but further injections of 7 and 70 $\mu\text{g/ml}$ of AFB₁-BSA over the captured antibody did not result in any binding (Figure 5.8).

Figure 5.7 (A) Monoclonal anti-AFB₁ is injected over an immobilised Fc-specific anti-mouse IgG surface, giving a response of 1216 RU. 5 minute injections of 7, 70 and 100 µg/ml of AFB₁-BSA (B, C and D, respectively) over this captured antibody do not result in any binding. Differences in shape of signal between B, C and D are due to differing bulk refractive indices.

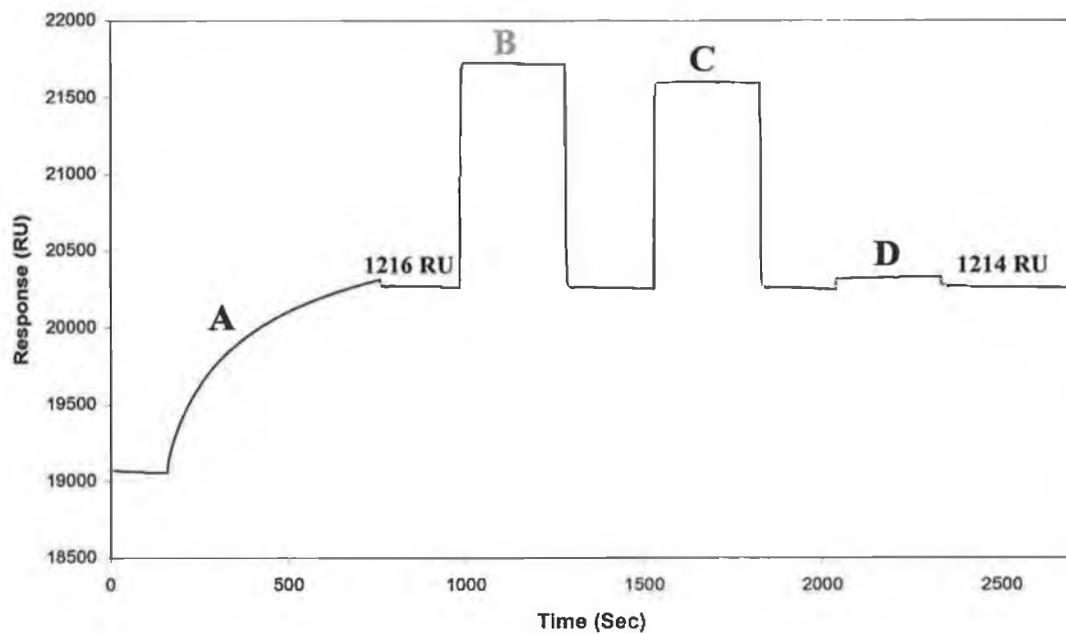
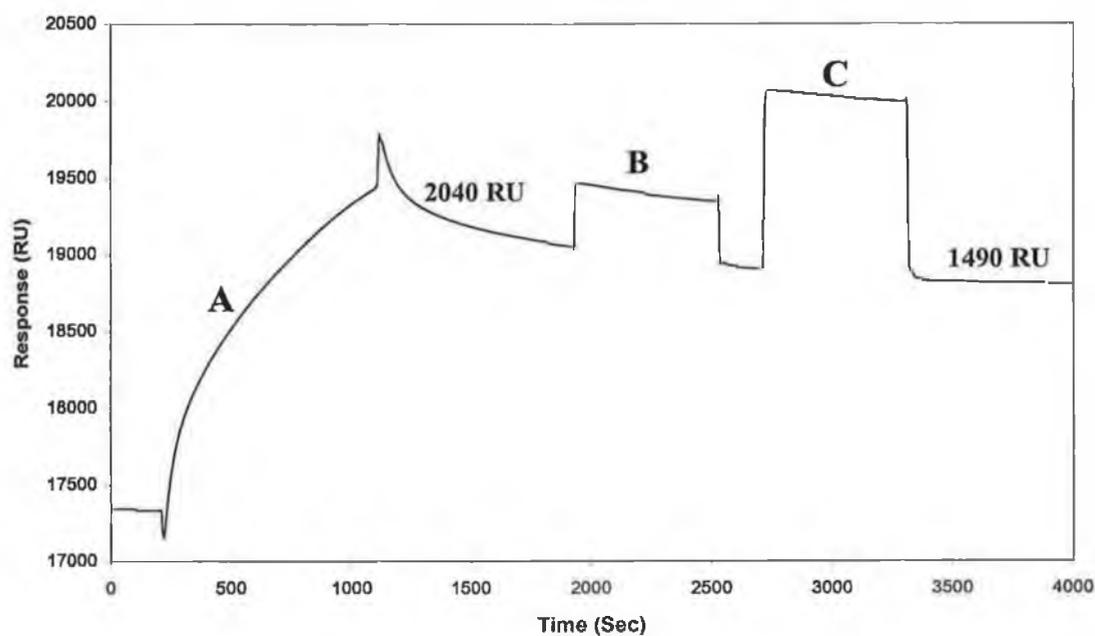


Figure 5.8 Sensorgram showing indirect immobilisation of polyclonal anti-AFB₁ on a protein A surface. (A) A 5 minute injection of antibody over immobilised protein A results in a signal of approximately 2,000 RU. However, dissociation of antibody from this surface is significant, and can be seen as a rapid decrease in signal as soon as the sample injection has finished. A steady decrease in signal continues during the injection of 7 and 70 $\mu\text{g/ml}$ solutions of AFB₁-BSA over the chip (B and C, respectively), after which the signal has decreased by 550 RU.



It can be seen from the sensorgram that - as with the attempt to utilise protein A to indirectly immobilise monoclonal antibody - there is significant dissociation of IgG from the protein A surface, and that this dissociation is too rapid to allow any binding of antigen to antibody to be discerned.

5.6.6 Direct immobilisation of AFB₁-BSA conjugate

As regeneration of immobilised antibody surfaces appears to be difficult to achieve while maintaining activity, investigation of the antibody-antigen interaction by directly immobilising AFB₁-BSA was examined.

A 200 µg/ml solution of AFB₁-BSA in 10 mM sodium acetate, pH 3.9, was immobilised as in section 2.9.2. A 5 minute injection of a 1/800 dilution of monoclonal anti-AFB₁ over this surface gave a binding signal of approximately 310 RU. From the data in Table 5.1, basic solutions appear to be the most effective at disrupting the bonds between monoclonal antibody and AFB₁-BSA. A 1 minute pulse of 75 mM NaOH, however, only removed about 90 RU of antibody binding (Figure 5.9).

When repeated 1 minute pulses of 100 and 200 mM NaOH had eventually brought the signal back to baseline level, another injection of 1/800 polyclonal antibody gave a decreased signal of 216 RU - indicating that the harsh regeneration conditions used had damaged the immobilised conjugate.

The binding of polyclonal antibodies to a conjugate surface was then examined. NaOH was again chosen as the regeneration agent. On a separate flow cell, approximately 2000 RU of AFB₁-BSA was immobilised.

The removal of bound polyclonal anti-AFB₁ from this surface using increasing molarities of NaOH was investigated, and only 100 mM NaOH was found to give complete regeneration. Figure 5.10 shows repeated binding/regeneration cycles, using a 1/100 dilution of polyclonal anti-AFB₁ and 100 mM NaOH, with varying contact times. Regeneration with a 10 minute injection of 100 mM NaOH (the flow rate here was reduced to 2 µl/min, in order to minimise sample consumption) seems to be the most effective.

In an attempt to develop a quantitative method for estimation of free AFB₁ analogous to that for 7-hydroxycoumarin described in Chapter 4, a fresh AFB₁-BSA conjugate surface was immobilised, and a range of standards of free toxin (0 - 40 µg/ml in PBS) prepared.

Figure 5.9 Sensorgram showing interaction of monoclonal anti-AFB₁ with immobilised AFB₁-BSA. **(A)** A 5 minute injection of antibody over the immobilised conjugate surface results in 309 RU of binding. **(B)** A 1 minute pulse of 75 mM NaOH over the chip removes only 90 RU of bound IgG. The differences in shape between the two injection signals are due to the differing bulk refractive indices of the two solutions.

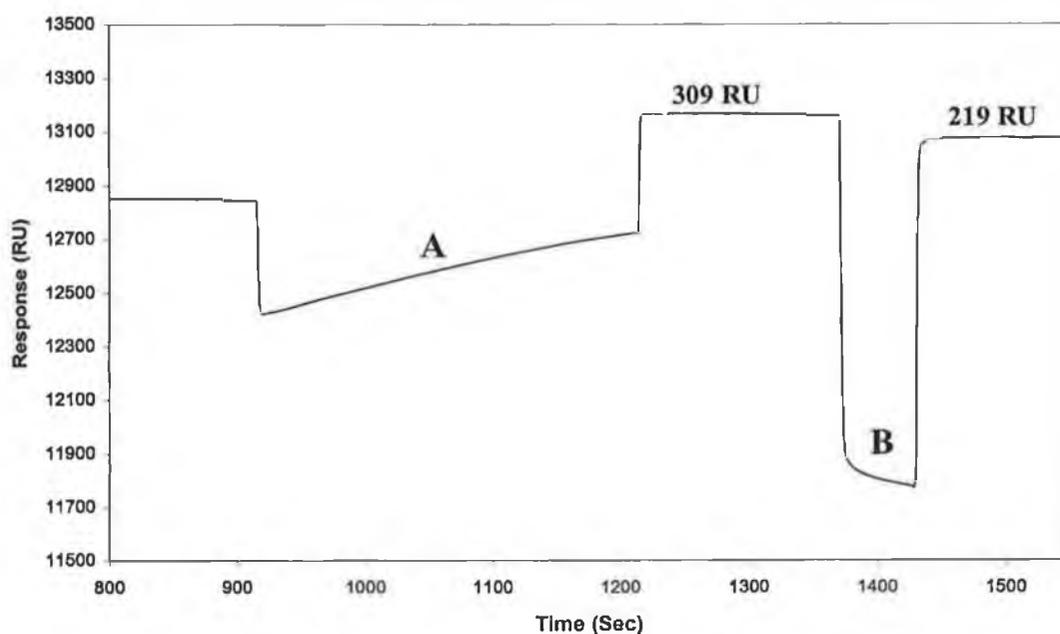
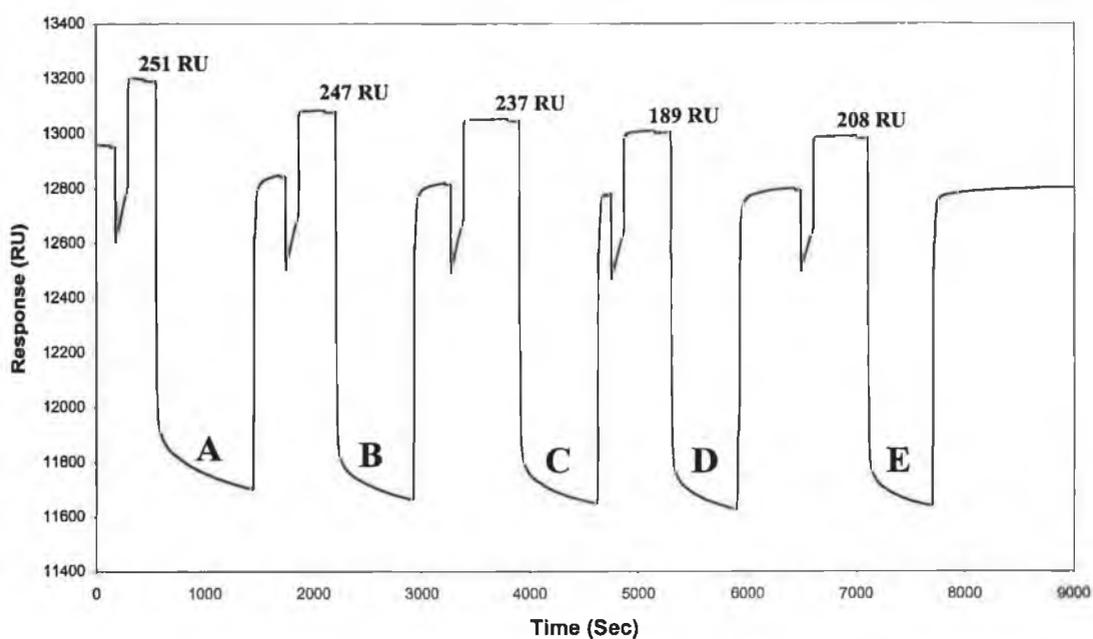


Figure 5.10 Sensorgram showing binding/regeneration cycles for polyclonal anti-AFB₁ bound to immobilised AFB₁-BSA. Antibody at a 1/100 dilution was injected over a conjugate surface, and 10mM NaOH was then passed over the chip. The contact times with regeneration reagent were (A) 15 minutes, (B, C) 12 minutes, and (D, E) 10 minutes. The antibody-binding signal decreases after A, B and C, but the response after D increases slightly, indicating that a contact time of 10 minutes is optimal for this concentration of base.



45 µl of each standard were then mixed and incubated with an equal volume of polyclonal anti-AFB₁ (1/100 in PBS). After a 10 minute incubation period, 2 µl of this mixture was passed over the chip. A 10 minute injection of 100 mM NaOH was then injected. The flow rate throughout was 2 µl/min. The change in response for each standard, and the signals after regeneration are given in Table 5.2. It can be seen that no standard curve can be constructed using these results, although the method seems to have worked in principle, with the 40 µg/ml standard giving a response of 3 RU, and the 5 µg/ml standard a signal of 192 RU. The regeneration of the surface appears to be responsible for the poor assay performance. The negative relative response values after treatment with 100 mM NaOH show that this regeneration strategy, which was optimised for the removal of 200-300 RU of bound antibody, is damaging the immobilised surface when lesser amounts of IgG are bound.

5.7 ELISA studies

The BIAcore results above demonstrate that regeneration of the two antibodies is problematic. To examine the efficiency of these antibody preparations in non-regenerable assay formats, a range of microtitre plate-based ELISA formats were employed. Both monoclonal and polyclonal antibodies were used in ELISAs for the quantitative determination of both free aflatoxin and the toxin-BSA conjugate. In addition, the regeneration of immobilised antibody and conjugate, and the effects of the regeneration reagents on these surface were investigated.

An indirect ELISA method was also used to calculate the equilibrium dissociation constant of the monoclonal antibody. Unless otherwise stated, two replicate analyses were performed for all of the ELISA studies reported in this chapter.

5.7.1 Competitive ELISA for AFB₁

Competitive ELISAs for the determination of free AFB₁, using both polyclonal and monoclonal antibodies, were carried out as described in section 2.7.6.

96-well microtitre plates were coated with AFB₁-BSA and blocked with 5% (v/v) FCS. A range of standard solutions of AFB₁ - containing from 1 µg/ml to 10 pg/ml - were then prepared in PBS.

Table 5.2 Results from attempted quantitative assay for free AFB₁, with immobilised AFB₁-BSA and premixing of toxin standards with polyclonal anti-AFB₁. The responses after injection of the standard-antibody mixture over the conjugate surface and regeneration (10 minute injection of 100mM NaOH) are shown for each standard.

AFB₁ Concentration (µg/ml)	Standard Response (RU)	Regeneration Reponse (RU)
40	3	- 188.6
20	1.1	- 130.2
10	27.3	- 111.6
5	192.2	- 73.6
2.5	126.3	- 76.4
0	69.4	- 72.5

100 μ l of each standard, together with 100 μ l of a 1/10,000 dilution of polyclonal anti-AFB₁, were added to each well and free and immobilised toxin allowed to compete for antibody binding sites.

HRP-labelled anti-rabbit antibody was then added, followed by a chromogenic substrate, and the absorbance of wells was measured. Figure 5.11 shows the standard curve obtained for the assay, which had a linear range of 10 pg/ml to 50 ng/ml, and an r^2 value of 0.93.

A similar assay was developed using the monoclonal anti-AFB₁ antibody, which was incubated together with standards at a dilution of 1/40,000, and HRP-labelled anti-mouse antibody. The standard curve for this assay is shown in Figure 5.12, and has an r^2 value of 0.99, with a linear range of 1 - 800 ng/ml.

5.7.2 Direct sandwich ELISA for AFB₁-BSA

Sandwich ELISAs for the determination of AFB₁-BSA, with direct coating of both polyclonal and monoclonal antibodies, were performed as described in section 2.7.7.

A 96-well microtitre plate was coated with 100 μ l/well of a 1/5000 dilution of polyclonal anti-AFB₁, and blocked with 5% (v/v) FCS. A range of standards of AFB₁-BSA (1 μ g/ml to 10 pg/ml) were prepared in PBS, and 100 μ l of each standard was added to appropriate wells, and incubated. 100 μ l/well of monoclonal anti-AFB₁ was then added, followed by HRP-labelled anti-mouse IgG, and a chromogenic substrate. The absorbance of wells at 405 nm was then measured. Figure 5.13 shows the standard curve obtained from this assay, which has a linear range of 1 - 100 ng/ml and an r^2 value of 0.99.

In a similar assay, 100 μ l/well of monoclonal anti-AFB₁ was coated onto a microtitre plate. Standards were added as before, followed by 100 μ l/well of polyclonal anti-AFB₁. HRP-labelled anti-rabbit was then added, and colour was developed and measured. This assay configuration gave a standard curve with a measuring range of 1 - 400 ng/ml, and an r^2 value of 0.97, as shown in Figure 5.14.

5.7.3 Sandwich ELISA for AFB₁-BSA using protein A capture

Sandwich ELISAs for AFB₁-BSA in which immobilised protein A was used to capture both polyclonal and monoclonal antibodies were carried out as in section 2.7.8.

Figure 5.11 Standard curve from competitive ELISA for AFB₁ using a 1/10,000 dilution of polyclonal antibody. The absorbance of wells at 405 nm is inversely proportional to the amount of free toxin present. Results shown are the averages of duplicate analyses. The method has a linear range of 10 pg/ml to 50 ng/ml.

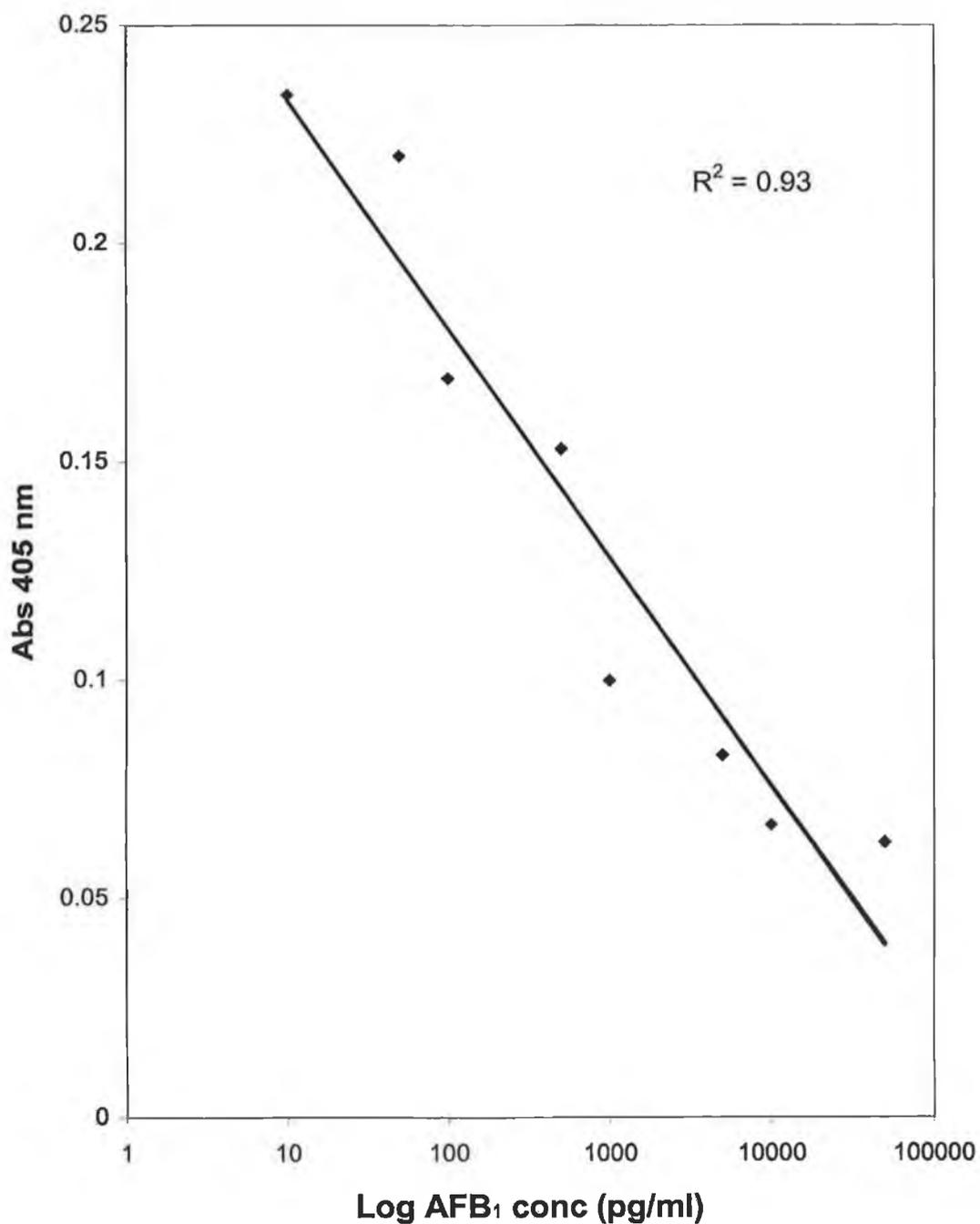


Figure 5.12 Standard curve from competitive ELISA for AFB₁ using a 1/4,000 dilution of monoclonal antibody. The absorbance of wells at 405 nm is inversely proportional to the amount of free toxin present. Results shown are the averages of duplicate analyses. The method has a linear range of 1 to 800 ng/ml.

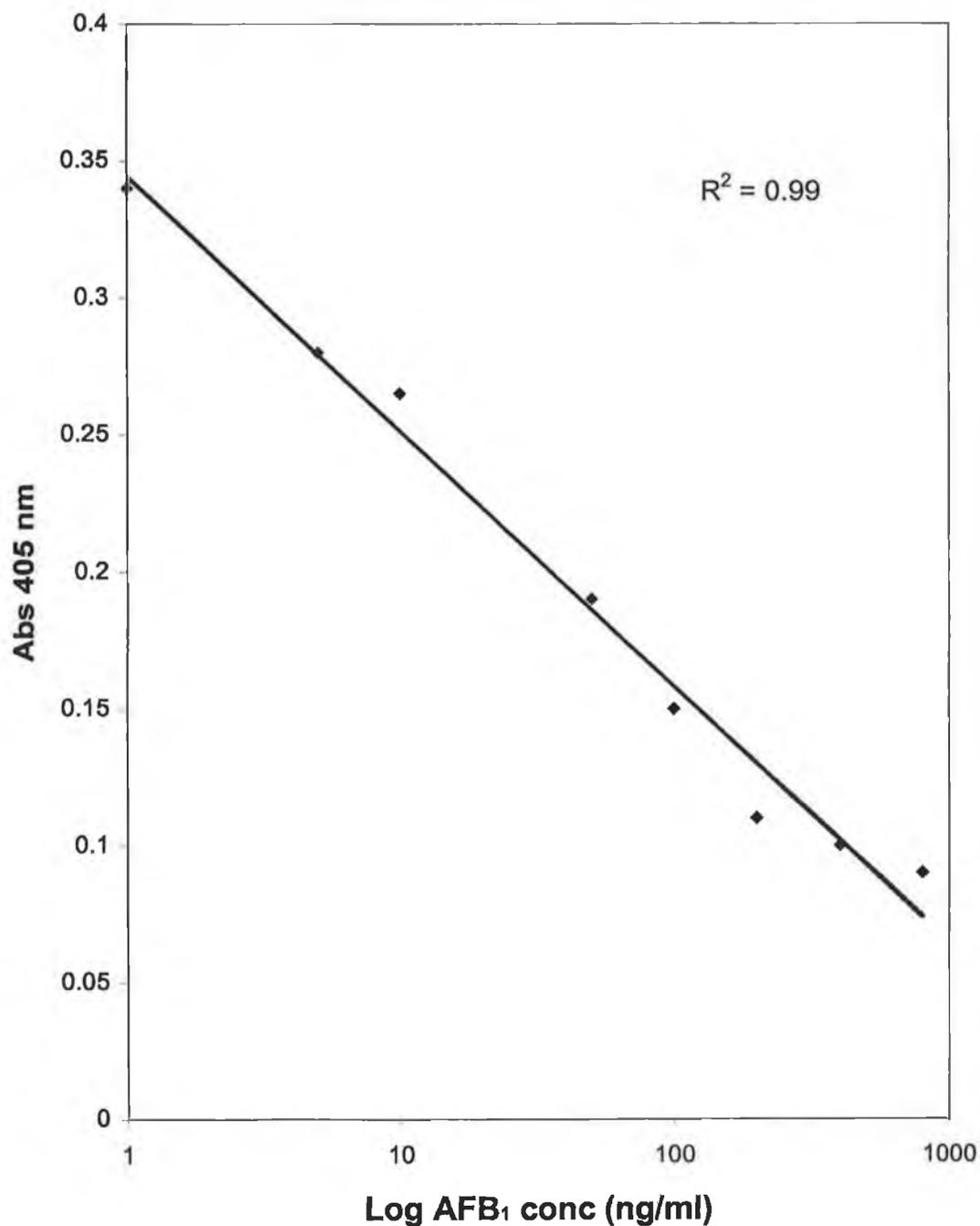


Figure 5.13 Standard curve from sandwich ELISA for AFB₁-BSA with direct coating of polyclonal antibody. The absorbance of wells at 405 nm is directly proportional to the amount of toxin-protein conjugate present. Results shown are the averages of duplicate analyses.

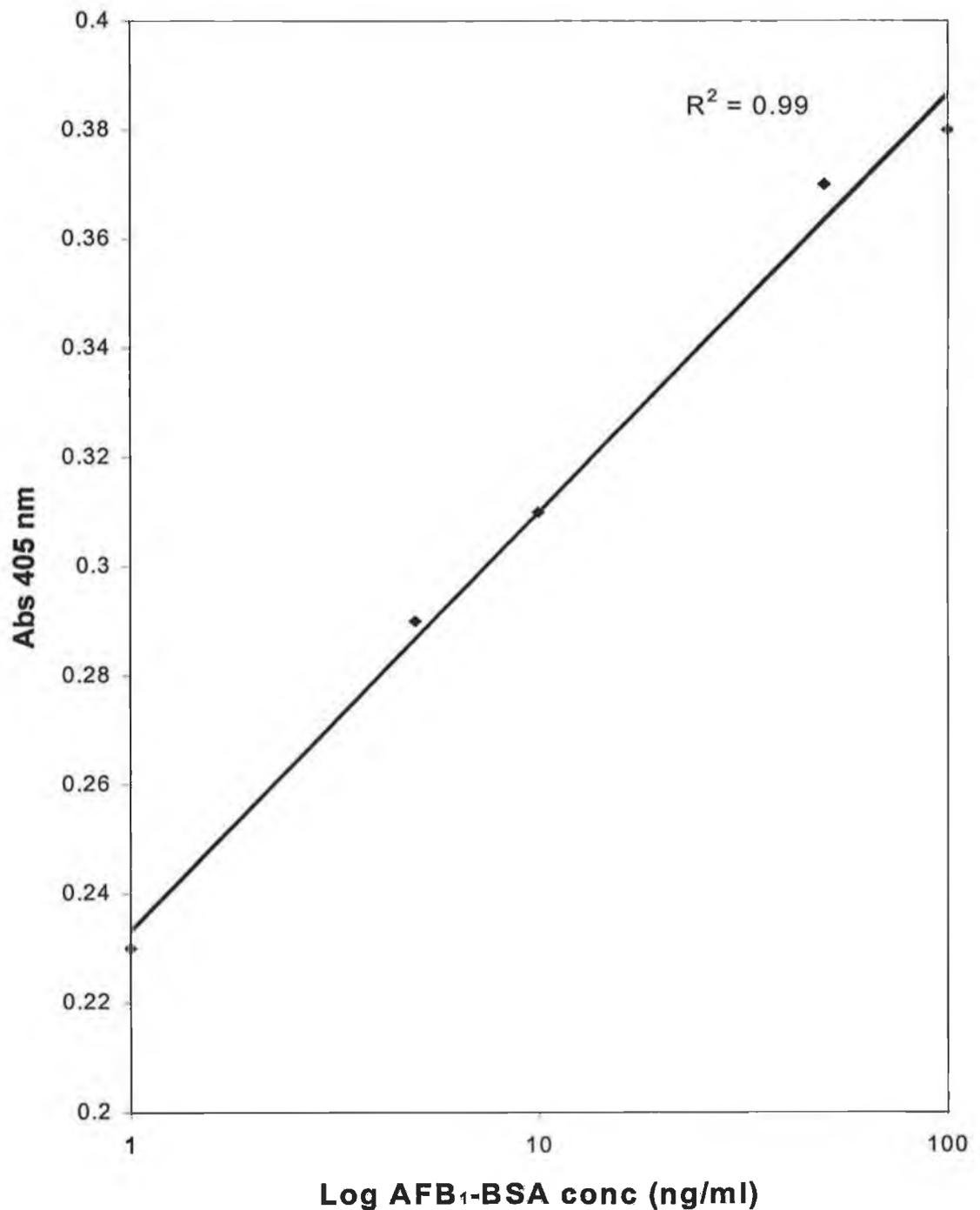
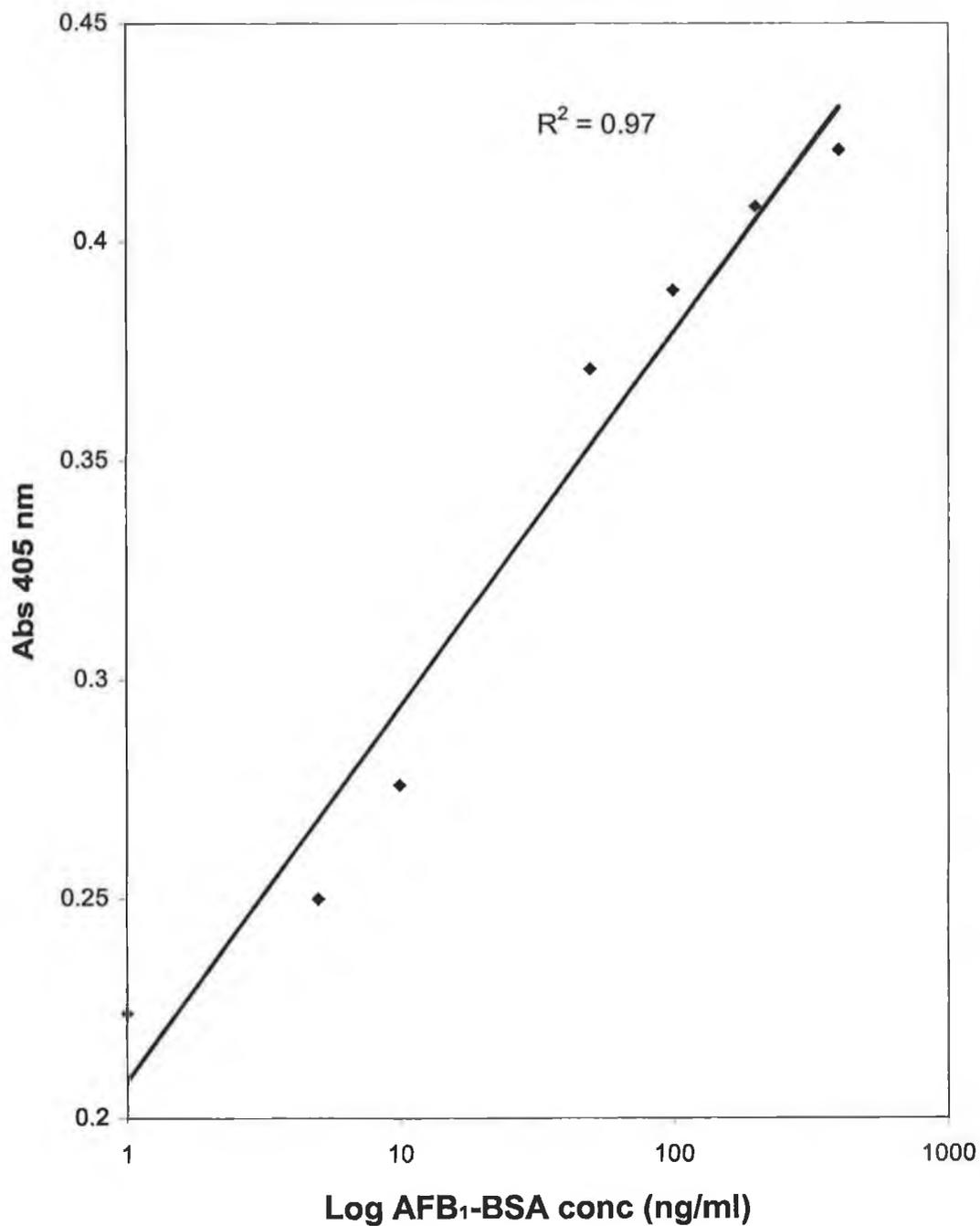


Figure 5.14 Standard curve from sandwich ELISA for AFB₁-BSA with direct coating of monoclonal antibody. The absorbance of wells at 405 nm is directly proportional to the amount of toxin-protein conjugate present. Results shown are the averages of duplicate analyses. The assay has a linear range of 1 – 400 ng/ml.



Wells of a 96-well microtitre plate were coated with protein A and blocked with FCS. 100 μ l/well of polyclonal anti-AFB₁ was then added. A range of standards of AFB₁-BSA, containing from 10 pg/ml to 1 μ g/ml, were then prepared, and 100 μ l of each solution was added to appropriate wells. 100 μ l/well of a mouse monoclonal anti-AFB₁ was then added, followed by an HRP-labelled anti-mouse antibody, and chromogenic substrate. Although there was a slight increase in response with increasing conjugate concentration, the maximum absorbance reading at 405 nm was 0.1, and it was not possible to construct a standard curve using the data obtained.

The capture of monoclonal antibody was also examined. Wells of a microtitre plate were coated with protein A, and 100 μ l/well of monoclonal anti-AFB₁ was added. After incubation with a range of AFB₁-BSA standards, polyclonal anti-AFB₁ was added, followed by an HRP-labelled anti-rabbit antibody, and chromogenic substrate.

There was no significant change in absorbance for the wells to which different concentrations of conjugate had been added, and no standard curve could be constructed.

5.7.4 Sandwich ELISA for AFB₁-BSA using an anti-mouse antibody for capture

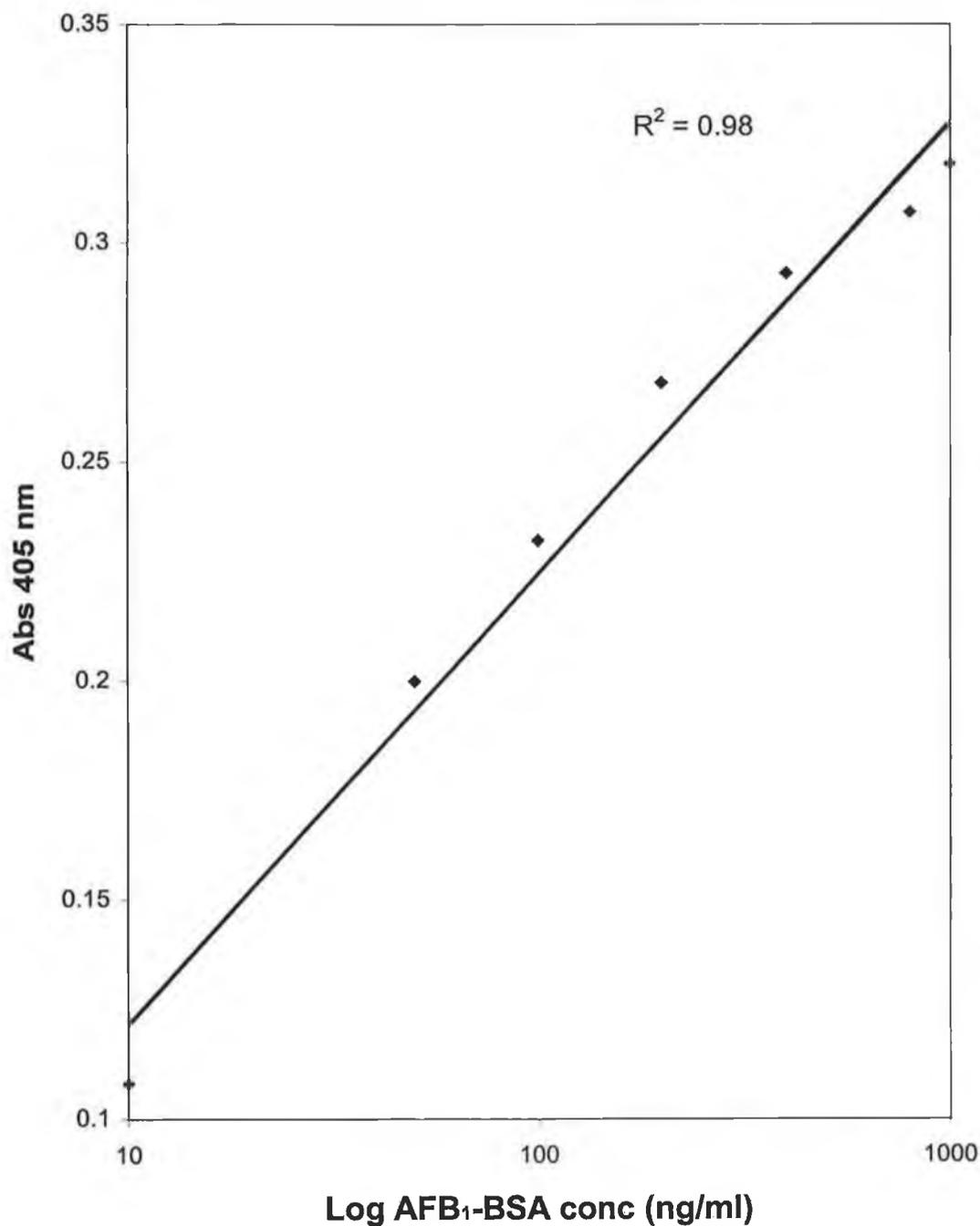
A sandwich ELISA for AFB₁-BSA in which an immobilised anti-mouse antibody was used to capture monoclonal anti-AFB₁ was performed as in section 2.7.9.

A 96-well ELISA plate was coated with 100 μ l/well of a 1/5000 dilution of Fc-specific anti-mouse IgG antibody. After blocking with 5% FCS, 100 μ l/well of a 1/5,000 dilution of mouse monoclonal anti-AFB₁ in PBS was added.

A range of standards of AFB₁-BSA containing from 10 pg/ml to 1 μ g/ml were then prepared, and 100 μ l of each solution was added to appropriate wells. 100 μ l/well of polyclonal anti-AFB₁ was then added, followed by an HRP-labelled anti-rabbit antibody.

After addition of, and incubation with, chromogenic substrate, the absorbance of the wells at 405 nm was measured. Figure 5.15 shows the standard curve for AFB₁-BSA constructed with these data, which has a linear range of 10 ng/ml to 1 μ g/ml, and an r^2 value of 0.98.

Figure 5.15 Standard curve from sandwich ELISA for AFB₁-BSA with initial coating of wells with Fc-specific anti-mouse IgG, and subsequent capture of monoclonal anti-AFB₁. The absorbance of wells at 405 nm is directly proportional to the amount of toxin-protein conjugate present. Results shown are the averages of duplicate analyses. The assay has a linear range of 10 ng/ml to 1 μg/ml.



5.7.5 Investigation of the efficiency and effects of regeneration by ELISA

BIAcore studies demonstrated that regeneration of antibody-antigen binding with sodium hydroxide affected the activity of the immobilised interactant. To corroborate these findings, ELISA methods were used to examine the efficiency of regeneration and effects of pretreatment with sodium hydroxide on both immobilised antibody and conjugate surfaces. Sandwich ELISAs were performed as described in section 2.7.10, to investigate the regeneration of immobilised polyclonal and monoclonal antibody surfaces. A 96-well microtitre plate was coated with polyclonal anti-AFB₁. 100 µl/well of a 0.5 µg/ml solution of AFB₁-BSA was added to the wells, and the plate was incubated at 37°C for 1 hour. A range of solutions containing from 5 mM to 1000 mM NaOH were prepared, and 100 µl/well of each of the solutions were added to appropriate wells for 10 minutes, before being aspirated. After washing, monoclonal anti-AFB₁ was added to the wells, followed by HRP-labelled anti-mouse antibody and chromogenic substrate. This assay demonstrates the ability of the differing concentrations of NaOH to disrupt the antibody-antigen interaction. The procedure was repeated with the NaOH solutions added to the plates after blocking, incubated for 10 minutes, and aspirated prior to addition of conjugate, in order to assess the effect of contact with base upon the ability of the immobilised antibody to bind antigen. An overlay plot of the absorbance at 405 nm against concentration of NaOH for these two assays is given in Figure 5.16, and shows that increasing concentrations of NaOH up to 400 mM result in increased levels of regeneration, while pretreatment of immobilised antibody with increasing molarities of base does result in some reduction of its antigen-binding capacity, especially at the higher concentrations at which regeneration is most effective. A similar assay format was used to investigate the effects of NaOH on immobilised monoclonal antibody. Monoclonal anti-AFB₁ was coated onto wells of a microtitre plate, and toxin-protein conjugate was then added. After treatment with base, polyclonal anti-AFB₁ was added, followed by HRP-labelled anti-rabbit antibody and chromogenic substrate. The assay was then repeated with the NaOH solutions added to the plate after blocking and before addition of conjugate. Figure 5.17 shows an overlay plot for the results of the two assays.

Figure 5.16 The effects of regeneration with sodium hydroxide on polyclonal anti-AFB₁ coated onto wells of a 96-well microtitre plate. Increasing concentrations of NaOH were added to coated wells before (“Pretreatment”) and after (“Regeneration”) incubation with AFB₁-BSA. The amount of bound conjugate was measured by sandwich ELISA, with addition of monoclonal anti-AFB₁ and HRP-labelled anti-mouse antibody. The results shown are the averages of duplicate analyses.

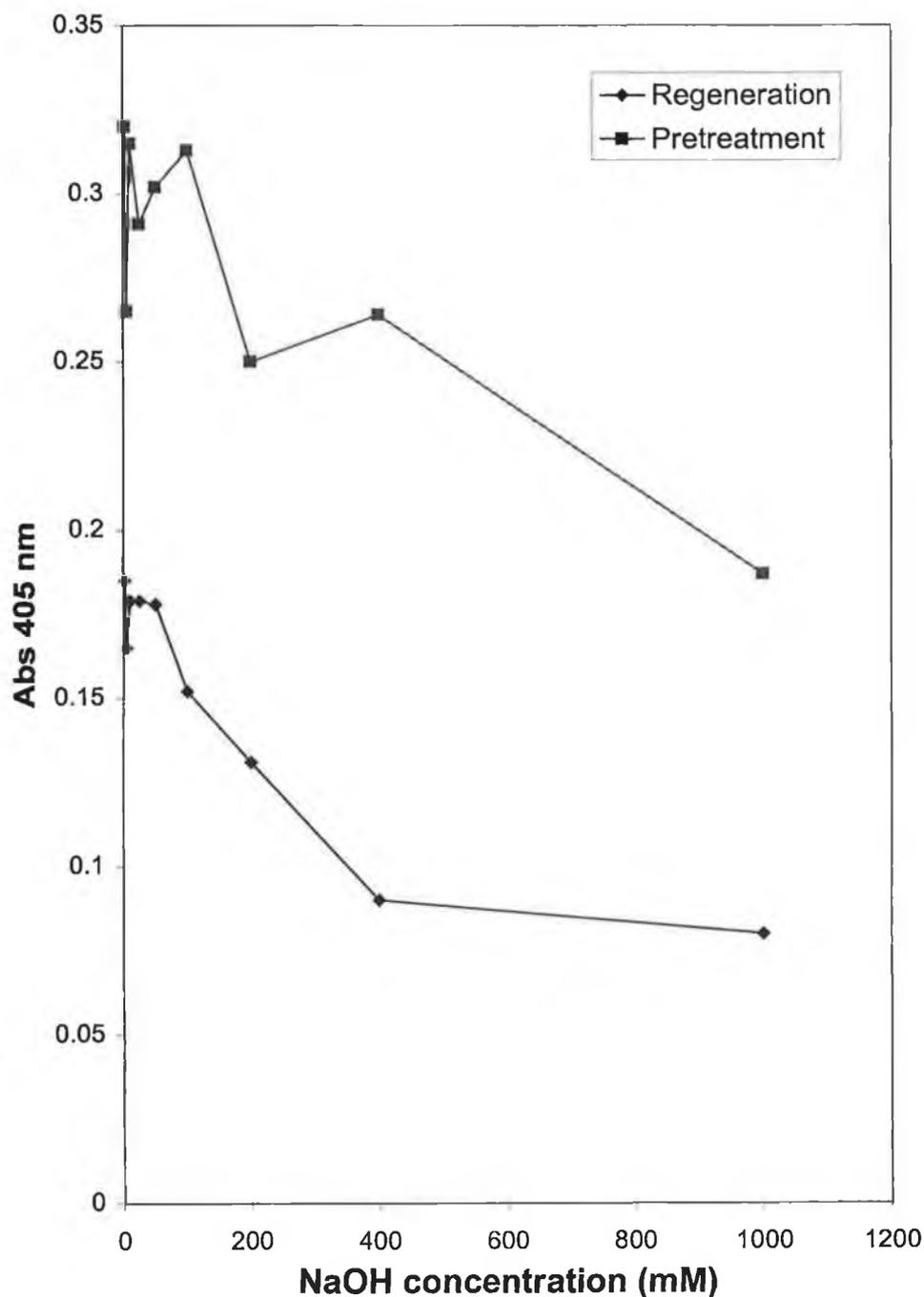
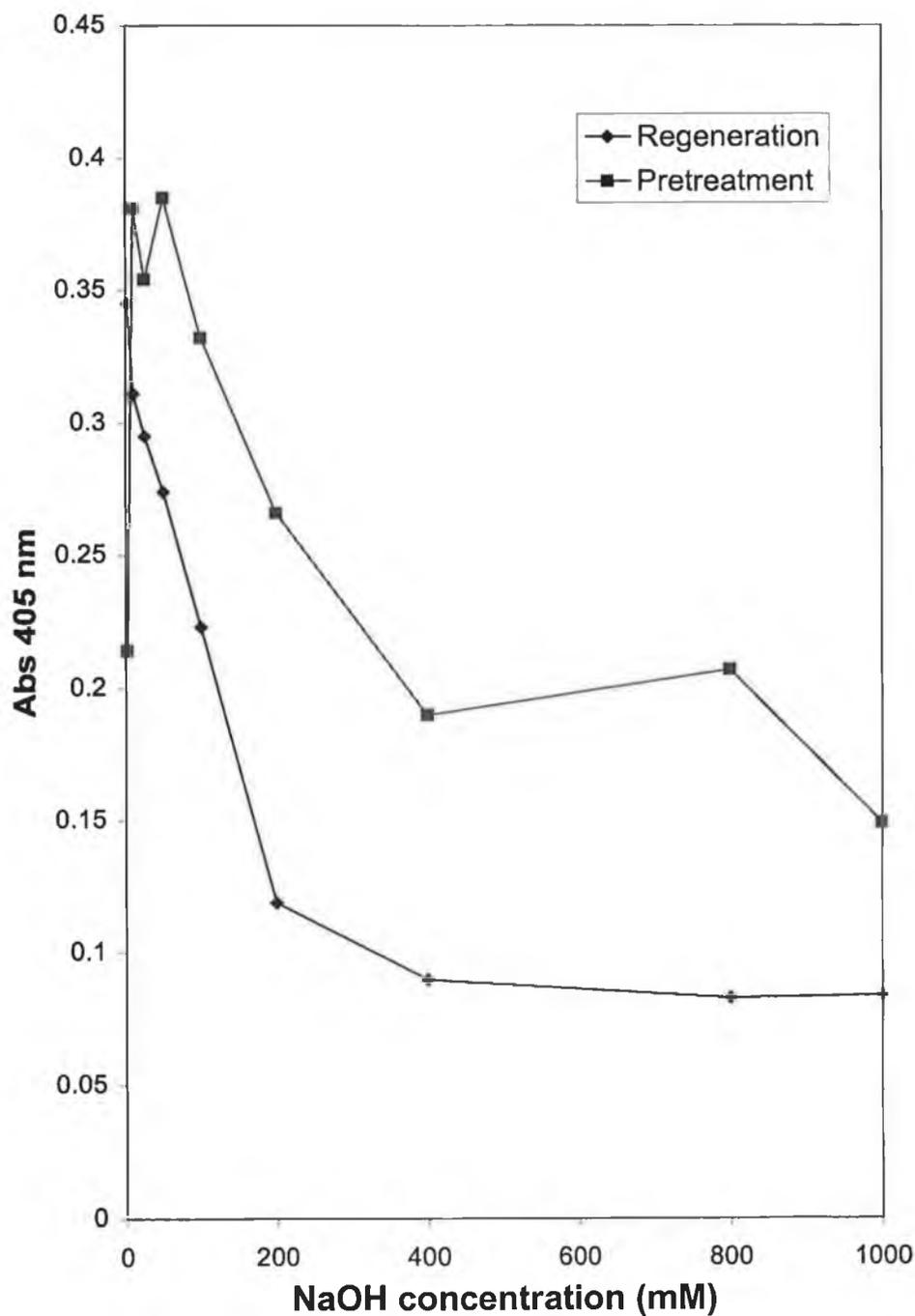


Figure 5.17 The effects of regeneration with sodium hydroxide on monoclonal anti-AFB₁ coated onto wells of a 96-well microtitre plate. Increasing concentrations of NaOH were added to coated wells before (“Pretreatment”) and after (“Regeneration”) incubation with AFB₁-BSA. The amount of bound conjugate was measured by sandwich ELISA, with addition of polyclonal anti-AFB₁ and HRP-labelled anti-rabbit antibody. The results shown are the averages of duplicate analyses.



From the graph it can be seen that regeneration occurs with lower concentrations of NaOH than those needed for the polyclonal antibody, but that the effect of treatment with base on the activity of the antibody is also more pronounced.

ELISAs to investigate the effects of regeneration on an immobilised AFB₁-BSA surface were performed as described in section 2.7.11. Conjugate was coated onto wells of a microtitre plate, which were then blocked with 5% FCS. 100 µl/well of polyclonal anti-AFB₁ was then added to the wells, and incubated for 1 hour at 37°C. After washing, 100 µl of a range of NaOH solutions (5 - 400 mM) was added to appropriate wells, incubated for 10 minutes at room temperature, and then aspirated. Monoclonal anti-AFB₁ was then added, followed by HRP-labelled anti-mouse antibody and chromogenic substrate. The assay was then repeated with the NaOH solutions added to the plate after coating and before incubation with antibody. An overlay plot for the two assays is shown in Figure 5.18, and it can be seen that addition of increasing concentrations of NaOH results in increased regeneration, while pretreatment of the conjugate surface with base slightly decreases the binding of polyclonal antibody at higher concentrations.

This procedure was repeated using monoclonal anti-AFB₁ as the primary antibody, and the results are shown in Figure 5.19. While the regeneration of binding is again evident, pretreatment of the conjugate surface with increasingly concentrated solutions of NaOH does not seem to significantly affect interaction with the monoclonal antibody.

5.7.6 Determination of the affinity constant of monoclonal anti-AFB₁ by ELISA

The equilibrium dissociation constant of monoclonal anti-AFB₁ was determined by a variation of the method of Friguet *et al.* (1985), as described in section 2.7.12. The theory of this method of determining the affinity of an antibody when its absolute concentration is unknown is outlined in section 5.5. In their study, Friguet *et al.* found a linear relationship between the amount of antibody added to wells and the absorbance values. Preliminary studies with the monoclonal anti-AFB₁ antibody, however, showed that a log linear plot gave the best straight line, necessitating a modification of the experimental procedure.

Figure 5.18 The effects of regeneration with sodium hydroxide on AFB₁-BSA coated onto wells of a 96-well microtitre plate. Increasing concentrations of NaOH were added to coated wells before ("Pretreatment") and after ("Regeneration") incubation with polyclonal anti-AFB₁. The amount of bound antibody was measured by ELISA, with addition of HRP-labelled anti-rabbit IgG. The results shown are the averages of duplicate analyses.

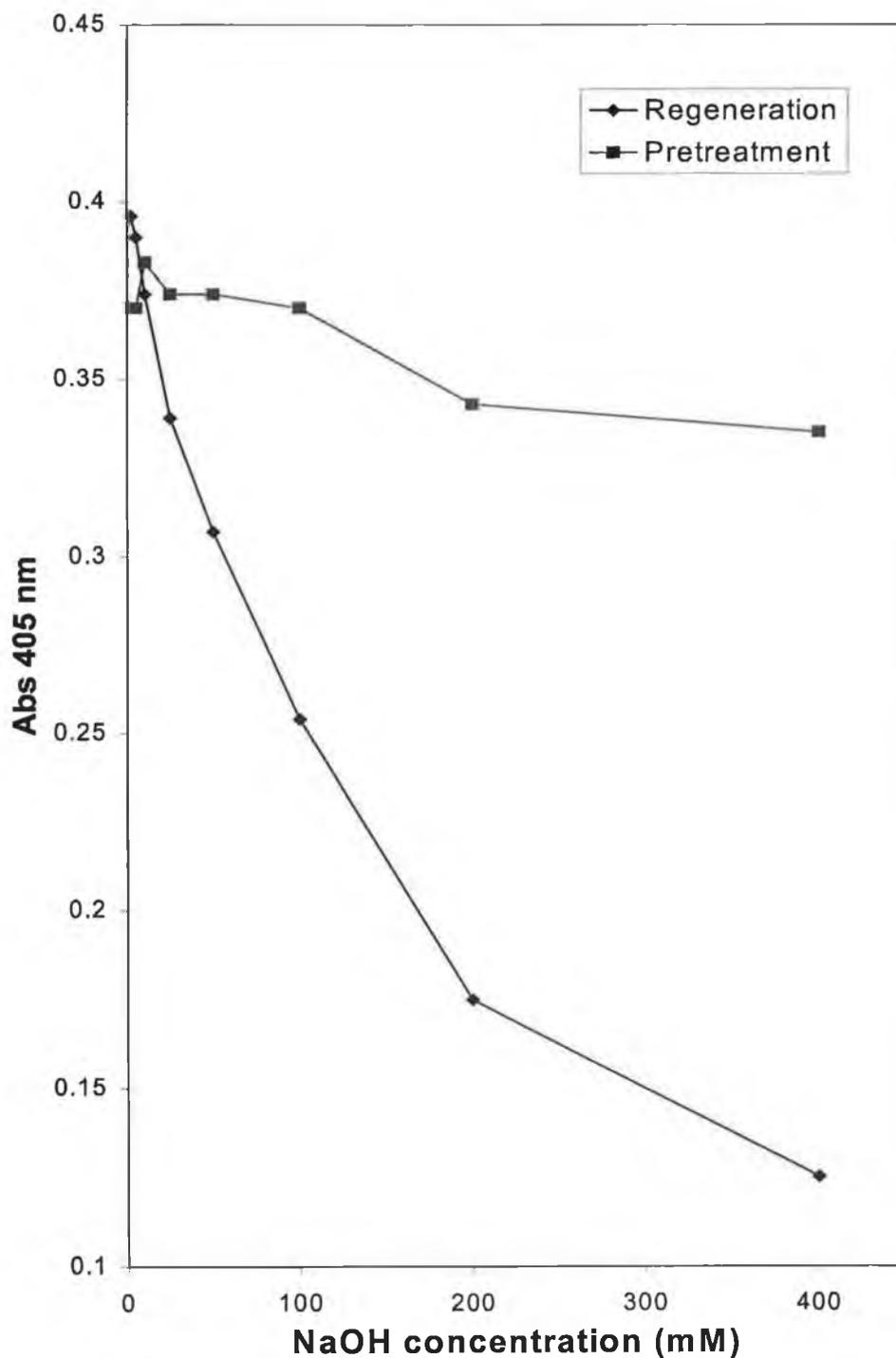
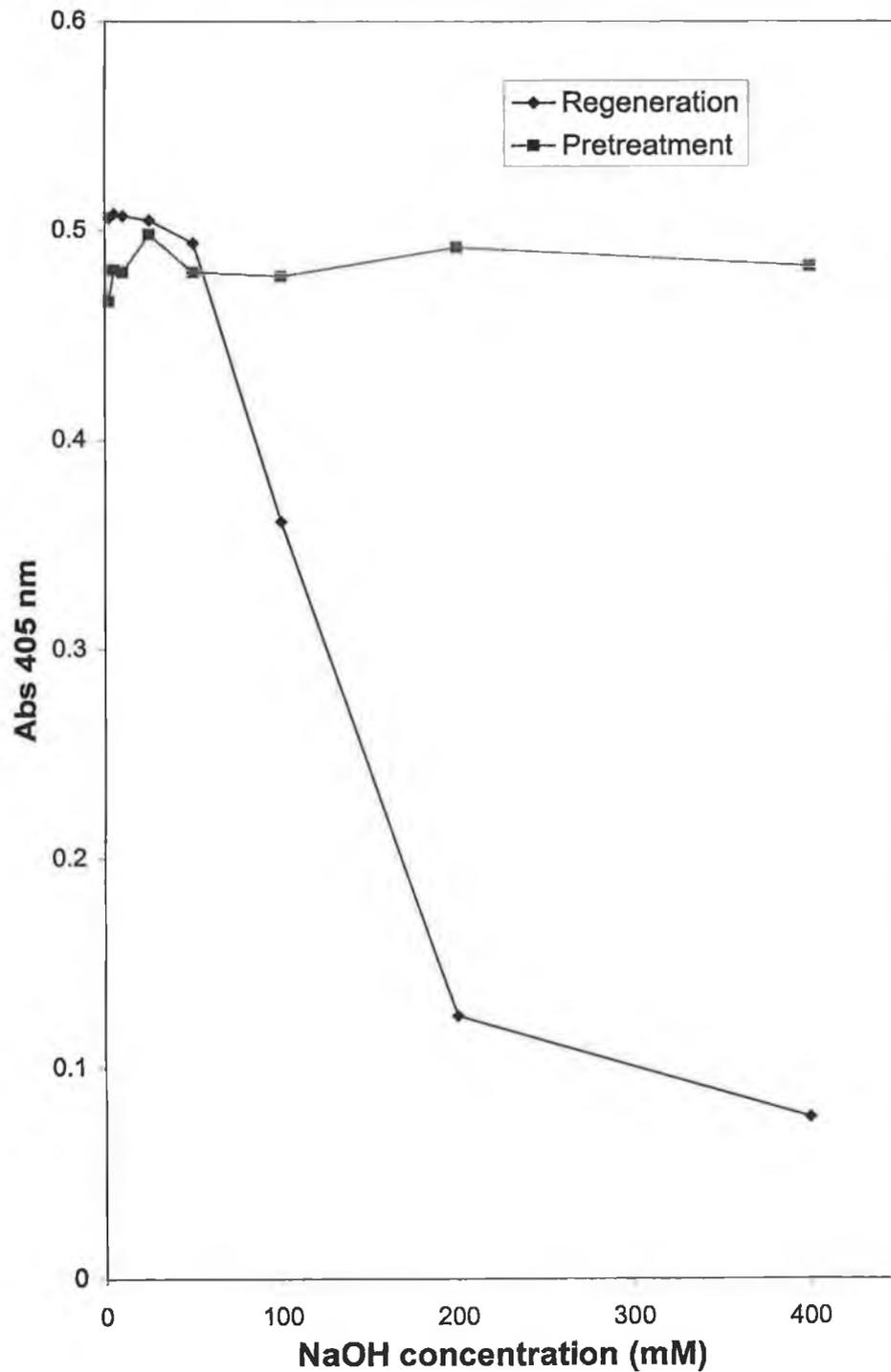
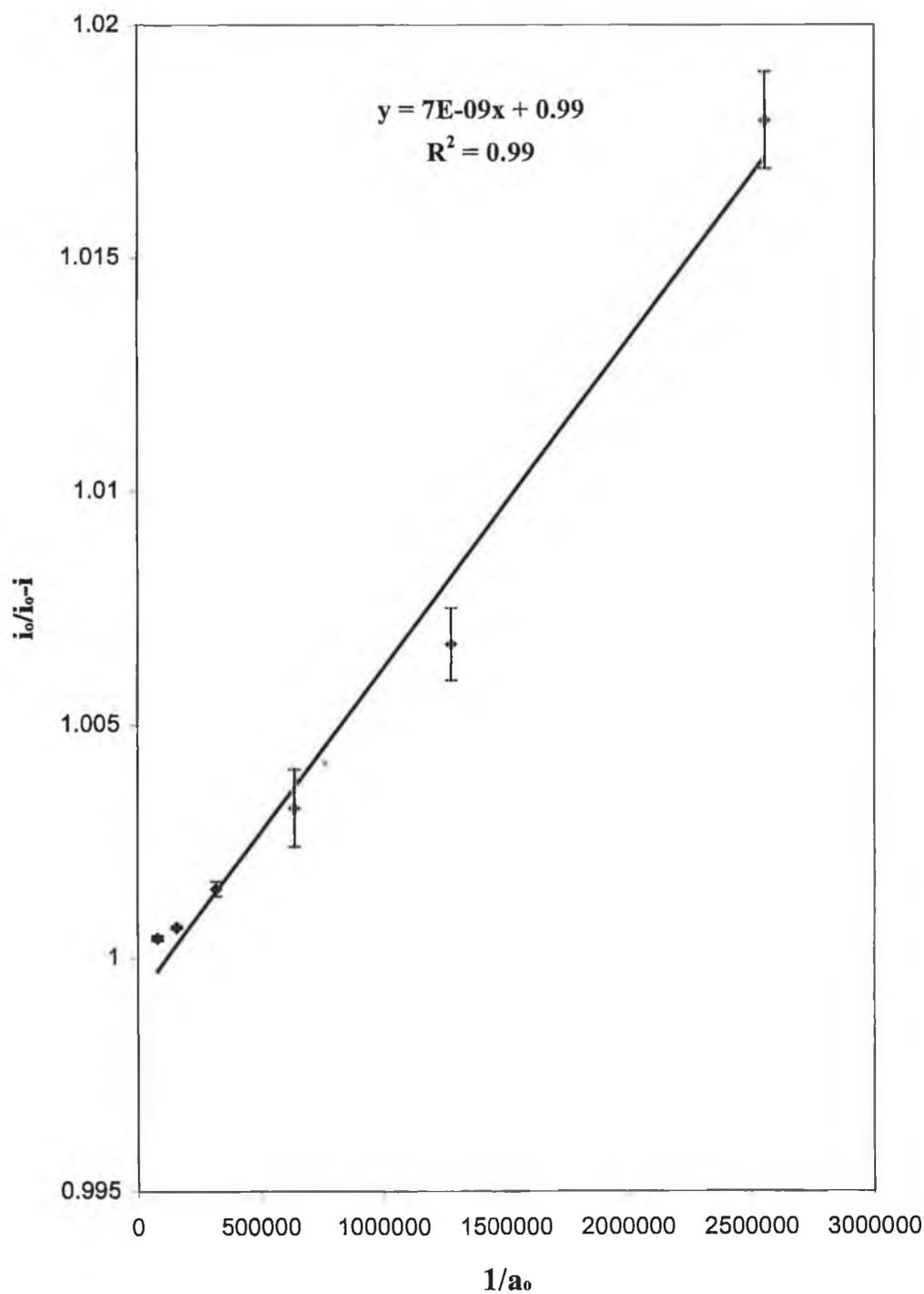


Figure 5.19 The effects of regeneration with sodium hydroxide on AFB₁-BSA coated onto wells of a 96-well microtitre plate. Increasing concentrations of NaOH were added to coated wells before ("Pretreatment") and after ("Regeneration") incubation with monoclonal anti-AFB₁. The amount of bound antibody was measured by ELISA, with addition of HRP-labelled anti-mouse IgG. The results shown are the averages of duplicate analyses.



A 96-well microtitre plate was coated with AFB₁-BSA. A range of standard solutions, containing from 3.9×10^{-7} to 4×10^{-4} M of free AFB₁, was prepared, and 100 μ l aliquots of each standard were incubated overnight at room temperature with a 1/10,000 dilution of monoclonal antibody, to allow equilibrium to be reached. These reaction mixtures were then added to the conjugate-coated wells. A range of dilutions of monoclonal antibody were also prepared, and added to separate individual wells. After incubation, HRP-labelled anti-mouse antibody and enzyme substrate were added, and the absorbance of wells at 405 nm was measured. Four replicates of both antibody dilutions and equilibrium mixtures were assayed, and coefficients of variation were below 4%. The absorbances of wells containing different antibody concentrations were used to construct a standard curve, from which the amounts of free antibody in the equilibrium solutions were calculated. The reciprocal of the fraction of free antibody in these solutions (i_0/i_0-i) was then plotted against the reciprocal of the antigen concentration ($1/a_0$). This Klotz plot is shown in Figure 5.20, and has an r^2 value of 0.99, and a y-axis intercept of 0.99. The equilibrium dissociation constant (K_D) for the monoclonal anti-AFB₁ antibody was calculated as 7×10^{-9} M.

Figure 5.20 Klotz plot for monoclonal anti-AFB₁. Antibody at constant concentration was incubated with a range of antigen standards. The amount of free antibody remaining in solution was calculated by indirect ELISA, from a separate antibody standard curve. The reciprocal of this value was plotted against the reciprocal of antigen concentration, and a value of 7×10^{-9} M was obtained for the K_D . The points shown are the averages of 4 replicate analyses.



5.8 Discussion

The results presented in this chapter clearly indicate the lack of suitability of the particular antibodies studied for use in a regenerable immunosensor, and highlight some general considerations to be taken into account when selecting the immunological component for such applications.

Monoclonal antibody to AFB₁ was immobilised on the BIAcore sensor chip, displayed no significant non-specific interaction, and injection of a protein conjugate of the toxin over this surface resulted in a strong binding signal (Figure 5.4). The lack of a visible dissociation phase in this sensorgram, however, indicated a high affinity constant for the interaction, and after treatment with a variety of regeneration reagents, the binding proved to be reversible only at the expense of antibody activity. This high affinity did not affect the use of antibody in a non-regenerable immunoassay format, however, and a sandwich ELISA in which monoclonal antibody was directly coated onto wells of a microtitre plate (Figure 5.14) displayed good linearity and sensitivity, with a measuring range of 1 - 400 ng/ml for AFB₁-BSA.

In an attempt to circumvent the problems of regenerating a directly immobilised monoclonal antibody surface, two different capture molecules were used. Although initial binding of antibody to protein A is high (Figure 5.6), the overall affinity of the molecule for the isotype of IgG used here is low, and the subsequent rapid dissociation makes binding of toxin-protein conjugate impossible to visualise. Hence, attempts to perform a sandwich ELISA using immobilised protein A to capture monoclonal antibody were also unsuccessful. The low affinity of the ligand for mouse IgG₁ is such that when polyclonal antibody is added in a subsequent step, it displaces any remaining bound primary antibody, resulting in high background signals, and no change in absorbance between standards.

Although no such dissociation of antibody captured with an Fc-specific anti-mouse IgG antibody is evident (Figure 5.7), binding of conjugate cannot be demonstrated. This appears to be due to the fact that a maximum of 1200 RU of monoclonal IgG can be captured by the immobilised anti-mouse antibody. This limits the amount of antigen which can bind, dramatically decreasing the effective binding capacity of the surface. Comparison of the standard curves from the sandwich ELISAs carried out using direct coating of monoclonal

anti-AFB₁ and capture by anti-mouse antibody (Figures 5.14 and 5.15, respectively) show that the direct immobilisation format is 10-fold more sensitive.

When polyclonal anti-AFB₁ is immobilised on the BIAcore sensor chip, no binding of toxin-protein conjugate to the antibody surface can be demonstrated. The pH of immobilisation buffer was increased to 6.8, and the capping of unreacted NHS esters with ethanolamine was omitted, to avoid inactivation of the antibody by contact with harsh reagents, but neither of these alterations promoted binding of conjugate, although 10000 RU of polyclonal antibody was immobilised. A sandwich ELISA in which polyclonal antibody was coated onto microtitre plates by passive adsorption, however, had a linear range of 1 - 100 ng/ml, with an r^2 value of 0.99. These results suggest that the NHS/EDC coupling of polyclonal anti-AFB₁ to the chip surface is affecting the activity of the antibody.

In a study on antibody fragments, Kortt *et al.* (1997) immobilised a mouse anti-idiotypic scFv fragment by this type of non-specific amine coupling, and found that it displayed a significantly reduced affinity for its antigen, an anti-viral neuraminidase antibody designated NC41, compared to fragments which had been immobilised in a site-directed fashion via a C-terminal thiol residue. They also immobilised the NC41 antibody by EDC/NHS coupling, and found that this had no effect upon its affinity for the scFv fragment. Upon investigating the amino acid sequence of the two antibodies, they concluded that the activity of the scFv was reduced as a result of immobilisation through lysine residues within the CDR region. Similar findings with regard to the effect of EDC/NHS immobilisation on antibody binding have been reported by Catimel *et al.* (1997).

The lack of activity of immobilised polyclonal anti-AFB₁, then, may be due to the fact that amine groups at the binding site of the antibody have been coupled to the carboxylated dextran of the BIAcore chip by the non-specific nature of the EDC/NHS immobilisation chemistry. Monoclonal anti-AFB₁, which is not inactivated by EDC/NHS immobilisation, may possess different functional groups in the antigen-binding site.

This problem could be avoided by using a capture molecule, but when indirect immobilisation of polyclonal anti-AFB₁ via protein A was investigated (Figure 5.8), the dissociation of the rabbit IgG was significant, and no subsequent binding of conjugate could be observed.

As the immobilisation of antibodies had been shown to pose difficulties in terms of regeneration and inactivation, and as strategies involving the use of capture molecules had proved unsuccessful, immunosensor formats based on the immobilisation of the AFB₁-BSA conjugate were investigated. Binding of monoclonal antibody to immobilised conjugate was demonstrated (Figure 5.9), but harsh regeneration conditions were necessary to disrupt this interaction, after which treatment the signals from further injections of monoclonal antibody decreased. This indicated that the binding capacity of the immobilised conjugate surface had been damaged by contact with the 75 - 100 mM sodium hydroxide. In a competitive ELISA in which AFB₁-BSA was coated onto microtitre plate wells and monoclonal antibody was mixed with free toxin, a linear measuring range of 1 - 800 ng/ml and an r^2 value of 0.99 were obtained (Figure 5.12).

The binding of polyclonal antibody to an immobilised AFB₁-BSA surface was also examined. Although good binding signals were obtained (Figure 5.10), regeneration could not be achieved without affecting the antibody-binding capacity of the conjugate surface. Regeneration conditions which were optimised for the removal of moderate levels of antibody were found to be too harsh when only small amounts of polyclonal antiserum were bound, preventing the development of an inhibitive BIAcore immunoassay for AFB₁. In a competitive ELISA format, however, the polyclonal antibody gave a standard curve with adequate linearity and a measuring range of 10 - 50000 pg/ml. This hundred-fold increase in sensitivity when using polyclonal rather than monoclonal antibodies is in agreement with the findings of Ward *et al* (1990), who reported that use of rabbit polyclonal antiserum in an ELISA for AFB₁ resulted in greater sensitivity than rat monoclonal antibody.

Apart from a general indication of a high affinity constant in the case of monoclonal anti-AFB₁, then, no useful information could be gained from the BIAcore studies of these two commercial antibody preparations, and no quantitative or kinetic experiments could be designed. Notwithstanding the limitations of the various capture systems - i.e. rapid dissociation and low levels of indirect immobilisation - the main problem was that of regeneration. One inherent difficulty with optical immunosensor systems is that of assessing whether actual regeneration of antibody-antigen binding is taking place. Non-covalently bound material which remains at the sensor surface after immobilisation can be removed by reagents used for regeneration, and the resulting decrease in detector response

may be mistaken for the reversal of antibody-antigen binding. It is usually possible to correct for this effect by pre-treatment of the immobilised surface with the regeneration reagents to be used, but this is not feasible where a novel interaction, for which the regeneration conditions are unknown, is being studied. In addition, the covalent bonds linking the immobilised component to the dextran gel may be disrupted by harsh chemical treatment, again resulting in a reduced signal. For example, the binding of 208 RU of polyclonal anti-AFB₁ to immobilised toxin-protein conjugate was almost completely regenerated by treating the surface with a 10 minute injection of 100mM NaOH, but when only 27 RU of antibody were bound to the surface, the same regeneration conditions brought the signal down to 111 RU below the baseline (Table 5.2), suggesting that some immobilised conjugate had been removed. The use of high salt concentrations and organic solvents sometimes result in contraction of the dextran gel, which can also give rise to confusing detector responses.

A series of ELISAs were performed to investigate the effects of regeneration on immobilised antibody and conjugate. Comparison of Figures 5.16 and 5.17 shows that when both polyclonal and monoclonal antibodies are coated onto microtitre plates, treatment with increasing concentrations of base results in increasing regeneration. However, the ability of both antibodies to bind to antigen is also clearly affected by contact with NaOH, the more pronounced effect on the monoclonal preparation perhaps being due to its homogeneity. When the AFB₁-BSA conjugate was coated onto wells (Figures 5.18 and 5.19), regeneration of both monoclonal and polyclonal antibody binding was evident, but pretreatment of the surface with base failed to significantly affect subsequent binding of antibody. This result seems to belie the BIAcore findings, but it should be remembered that two different methods of immobilisation were involved. It appears that the covalent bonds formed by NHS esterification are more susceptible to treatment with base than are conjugate molecules passively adsorbed onto the polystyrene of microtitre plates.

One practical consideration that emerged from the BIAcore investigations undertaken here was that of the stability of bio-conjugates. As discussed in section 3.4.1.1, these conjugates are of integral importance in the study of low molecular weight analytes, but coupling chemistries can be unstable, and the breakdown of these molecules can lead to misleading results. While AFB₁-BSA was initially found to bind strongly to immobilised monoclonal

anti-AFB₁, the binding signal proved difficult to reproduce after the conjugate had been stored at 4°C for some weeks. When an aliquot of the same batch of conjugate was immobilised, injections of antibody over this surface gave strong binding signals. It was thought that breakdown of the conjugate might result in the presence of free aflatoxin, which could be preferentially binding to immobilised antibody. Such binding would not cause a change in SPR angle. Free toxin, however, would be washed away from immobilised conjugate by continuous buffer flow. This hypothesis was proved when stock AFB₁-BSA, purified by size exclusion chromatography on a PD-10 column (section 2.9.4), was found to bind to immobilised antibody. These results highlight the importance of choosing a stable coupling chemistry.

The Klotz plot shown in Figure 5.20 gives a K_D value of 7×10^{-9} M for monoclonal anti-AFB₁. This high affinity value is at the theoretical upper limit for diffusion-limited reactions - which has been shown to govern the association rate of some hapten-antibody interactions (Kuby, 1997) - and confirms the inferences from BIAcore sensorgrams, as well as explaining the difficulty of regeneration. The much-lauded advantages of specificity and sensitivity conferred by monoclonal antibodies must be balanced against the necessity for reversible binding which is a prerequisite for regenerable biosensor applications. Although this antibody preparation performed well in various ELISA formats, it is inherently unsuitable for BIAcore use. As discussed in Chapter 3, screening procedures for antibodies tend to select on the basis of high affinity, but for antibodies which are to be used in biosensors, moderate affinity is preferable, and screening should, if possible, be carried out with this in mind.

Given the high cost of sensor chips, preliminary ELISA studies on the interactants to be investigated in BIAcore experiments can provide an economical source of essential data. The ELISA method of Friguet *et al.* (1985) for the calculation of antibody affinity constants should be employed initially to determine the suitability of the proposed antibodies for use in BIAcore experiments. Antibodies with high affinity constants (in the range 10^8 to 10^9) are likely to be inherently unusable. The ability of this method to measure antibody affinities in crude samples, such as hybridoma supernatant or ascitic fluid, can be used to select only antibodies with moderate affinity from a panel of monoclonal antibodies for BIAcore investigations.

Establishing optimal regeneration conditions on the BIAcore is generally a time-consuming task, involving the use of a number of sensor chips. The results presented here show that microtitre plate-based ELISAs can be used to approximate the optimal regeneration conditions for immobilised antibody and antigen surfaces. The effects of these regeneration solutions on the activity of immobilised biomolecules can also be assessed. While it is difficult to precisely relate the two formats in terms of contact time with the sensor surface, initial ELISA studies of this type can be used to identify the most effective type and concentration of regeneration reagent for the interaction.

5.9 Summary

A commercial monoclonal anti-aflatoxin B₁ preparation was immobilised on BIAcore sensor chips and injection of AFB₁-BSA resulted in a binding signal. The antibody-antigen interaction proved too strong to regenerate, however, and the use of protein A as a capture agent was unsuccessful, due to high rates of dissociation. Anti-mouse IgG was also used to capture monoclonal anti-AFB₁, but the amount of antibody which could bind to this surface was not sufficient to allow antibody-antigen interactions to be observed.

Commercial polyclonal anti-AFB₁ was also immobilised, but the activity of the antibody was reduced by the use of non-specific amine coupling to the dextran layer of the sensor chip. Attempts to capture polyclonal antibody via anti-rabbit IgG antibodies were unsuccessful due to high dissociation rates.

AFB₁-BSA was then immobilised on the sensor chip. Although binding of monoclonal antibody to this surface could be demonstrated, the harsh regeneration conditions which were necessary before re-using the chip considerably reduced the binding capacity of the surface. Similar results were obtained for binding of the polyclonal antibody to an AFB₁-BSA-coated surface.

Although not suitable for use with BIAcore, both polyclonal and monoclonal antibody preparations were successfully employed in quantitative ELISA techniques, in both competitive and sandwich assay formats.

ELISA methods were used to construct a model system for the optimisation of BIAcore regeneration conditions. The regeneration of immobilised antibody and conjugate BIAcore surfaces with NaOH and HCl was mimicked on microtitre plates, and the method of Friguet *et al.* (1985) was used to calculate the affinity constant of a monoclonal antibody against AFB₁. This type of ELISA study is proposed as an inexpensive preliminary investigative step for all BIAcore studies on antibody-antigen binding, which can help to minimise the use of costly sensor chips.

CHAPTER 6

BIAcore STUDIES ON THE INTERACTION OF COUMARIN COMPOUNDS WITH DNA AND TOPOISOMERASE II

6.1 Introduction

This chapter describes the use of BIAcore to examine interactions between coumarins and DNA. Plasmid DNA was extracted from bacterial cells and labelled with biotin. The biotin-labelled nucleic acid was bound to the sensor chip via an immobilised streptavidin surface. The effect of novobiocin upon the action of topoisomerase II – an enzyme which catalyses the relaxation of supercoiled DNA – was investigated. The unwinding of extracted plasmid DNA by topoisomerase II was demonstrated by agarose gel electrophoresis and Southern blotting. BIAcore was used to show the inhibition of enzyme activity by the coumarin antibiotic novobiocin, although the binding of enzyme to nucleic acid was transient, and no data on the kinetics of binding could be obtained.

The direct binding of protein conjugates of various coumarins to immobilised DNA was also investigated. No binding was observed upon injection of BSA conjugates of warfarin, coumarin, 7-hydroxycoumarin and aflatoxin B₁ over the surface.

6.2 Interactions of coumarins with DNA

Coumarin has been reported to cause DNA and chromosome damage in plant cells (Grigg, 1978), although antimutagenic activity has also been claimed for the drug in *E. coli*. (Ohta *et al.*, 1983). In a range of optical tests including spectrophotometric titration and circular dichromism, however, no binding of coumarin or 7-hydroxycoumarin to DNA in water-salt solutions could be detected (Y. Yevdokimov, Personal communication). The binding of furanocoumarins to DNA has been reported (Murray *et al.*, 1982).

The photobinding of furanocoumarins to DNA in the presence of UV light has been widely documented (Murray *et al.*, 1982). 5,7-dimethoxycoumarin has also been shown to possess photobinding activity (Jung *et al.*, 1983), as have the pyrrolocoumarins (Gia *et al.*, 1988)

As discussed in Chapter 5, the toxic activity of aflatoxin B₁ (AFB₁) has been ascribed to the binding of its epoxide metabolites to nucleic acids (Martin and Garner, 1977, Roy and Kulkarni, 1997). The identification and quantification of AFB₁-DNA adducts is of importance as an indication of the risk of cancer. Although the toxicity of AFB₁ is due to its epoxide, the binding of underivatised AFB₁ to DNA has been investigated. In a range of studies using spectrophotometric methods (Clifford and Rees, 1966), equilibrium dialysis (Black and Jirgensons, 1967) and fluorescence techniques (Neeley *et al.*, 1970), AFB₁ was found to bind weakly to double- and single-stranded nucleic acids, without intercalating or affecting the melting temperature (T_m) of DNA.

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6.3 Coumarin antibiotics and topoisomerase inhibition

The three coumarin antibiotics, novobiocin, coumermycin A, and chlorobiocin, are structurally related compounds which are produced by *Streptomyces* species. The structure of these three drugs is shown in Figure 6.1. The drugs are generally more active against Gram-positive than Gram-negative bacteria, as a result of differences in permeability (Maxwell, 1993), and inhibit DNA replication by binding to topoisomerases, specialised enzymes responsible for relaxing and supercoiling DNA.

In vivo, DNA usually has a closed structure, with no free ends. Supercoiling occurs when the double helix is twisted about its own axis, and may be either or negative (in the opposite direction to the right-handed helix) or positive (in the same direction). Only negative supercoiling occurs *in vivo*, and it is caused by unwinding a number of turns of the double helix and joining the ends. In addition to producing a more compact form of DNA, this action facilitates the separation of the two strands of the DNA molecule. This strand separation is of critical importance for the replication, transcription and recombination of DNA. As a result, the presence of topoisomerases in cells has been shown to be necessary for their viability. The enzymes operate by making a break in a stretch of DNA, stabilising it, and passing another strand of DNA through this break prior to rejoining it. This action is thought to be non-specific, with the enzyme recognising any two regions of double-stranded DNA which cross over each other (Lewin, 1990). Type I topoisomerases introduce transient breaks in one strand of the DNA duplex, while type II topoisomerases make double-strand breaks. The action of many topoisomerases is ATP-dependant.

The rapidly-dividing nature of cancer cells, involving high levels of DNA replication, has made topoisomerases a target for anti-tumour strategies, while the prokaryotic equivalent – DNA gyrase – is being studied as a target for therapeutic anti-bacterial agents (Maxwell, 1997).

Bacterial DNA gyrase is an unusual member of the type II topoisomerases, in that it has the ability to perform the energetically unfavourable task of introducing negative supercoils into relaxed closed circular DNA. One molecule of the enzyme can introduce about 100 supercoils per minute into a molecule of DNA.

The supercoiling ability of DNA gyrase is dependent upon the hydrolysis of ATP; although the enzyme can still relax supercoiled DNA in its absence, and this reaction is not affected by the coumarin antibiotics.

DNA gyrase is made up of two subunits, and the active form of the enzyme is a tetramer. Coumarin antibiotics bind to the B subunit, and the novobiocin-binding site has been pinpointed to a 24 kDa sub-domain of the N-terminal fragment of the enzyme (Gormley *et al.*, 1996). The novobiocin molecule is composed of three parts: a benzoic acid derivative, a coumarin residue, and a sugar (novobiose). Studies have shown that the coumarin portion (bearing the correct substituents) is the necessary component for binding DNA gyrase B. The mechanism of action of the coumarin antibiotics on DNA gyrase has been widely studied, and they have been found to function by competitively inhibiting the hydrolysis of ATP.

The inhibition of vaccinia topoisomerase, a type I eukaryotic enzyme, by coumarin antibiotics, occurs in a different manner. Novobiocin and coumermycin have been shown to interact with the enzyme in such a way as to block the binding of DNA (Sekiguchi *et al.*, 1996).

6.4 The use of BIAcore to study nucleic acid interactions

BIAcore has been used to study a variety of DNA interactions, including hybridisation, binding of receptors and anti-DNA antibodies, and the action of enzymes. In most of the reported experiments, DNA or oligonucleotides have been biotinylated and bound to the sensor chip via immobilised avidin or streptavidin.

Biotin, also known as vitamin H, is a small molecule which acts as a coenzyme for transcarboxylase and ATP-dependent carboxylases. Avidin is a tetrameric glycoprotein which is found in eggs, and has four high affinity binding sites for biotin. Streptavidin is a related molecule which exhibits lower non-specific binding. Both molecules have been extensively used in biosensors for the immobilisation of biotinylated biomolecules (Ebersole *et al.*, 1990; Reháč *et al.*, 1994).

Wood (1993) first reported the use of BIAcore to visualise nucleic acid hybridisation. In this study, biotinylated oligonucleotides were captured on the sensor surface and the binding of complementary strands was observed within 7 minutes. Gotoh *et al.* (1995) described a method to determine the kinetic parameters in DNA hybridisation, which was sensitive to the presence of mismatches, and could detect even a single base-pair

mismatch. In a study on the detection of HIV-1 genomic sequences, Bianchi *et al.* (1997) used an immobilised biotinylated oligonucleotide probe to produce a fast, reproducible method for the detection of HIV-1 complementary sequences in PCR products.

The presence in the body of high affinity autoantibodies specific for native double-stranded DNA is diagnostic of the disease systemic lupus erythematosus (SLE). Barbas *et al.* (1995) generated human Fabs to DNA from combinatorial phage display libraries, and used BIAcore to derive kinetic data for their binding to immobilised oligonucleotides. Sibille *et al.* (1997) used a similar strategy to study murine monoclonal antibodies derived from lupus-prone mice.

The interaction between MutS – a mismatch repair molecule from *E. coli* – and a range of oligonucleotides immobilised on BIAcore was investigated by Babic *et al.* (1996). Cheskis *et al.* (1997) studied the binding of the ligand inducible transcription factor, oestrogen receptor (ER), to DNA immobilised on the BIAcore in the presence and absence of a variety of ligands.

Nilsson *et al.* (1995) used biotin-avidin capture of oligonucleotides to study a range of different types of DNA manipulation. The assembly of a 69 base-pair fragment from 6 smaller fragments was demonstrated. The action of DNA ligase to join together separate oligonucleotides was visualised in “real-time”, as were strand separation and hybridisation. Two types of DNA polymerase, T7 polymerase and Klenow fragment, were examined on BIAcore, and injection of the enzymes, together with dNTPs, over a single-stranded DNA surface, lead to an increase in signal, corresponding to the synthesis of a complementary strand of DNA. In addition, the cleavage of DNA by the restriction endonuclease *XhoI* was demonstrated. A mini-sequencing experiment was also described, in which Klenow fragment and one of four ddNTPs were injected over immobilised single-stranded DNA bearing a double-stranded overhang. This was carried out on four surfaces for each of the four ddNTPs, and subsequent injection of polymerase along with all four dNTPs resulted in one sensorgram where no signals for DNA synthesis could be seen. The ddNTP injected in this flow cell was found to be complementary to the first base in sequence.

Uracil-DNA glycosylase is a DNA repair enzyme which removes uracil bases from DNA. Panayotou *et al.* (1998) immobilised biotinylated DNA, with and without uracil, and injected mutated uracil-DNA glycosylase from herpes simplex virus, and reported that the enzyme recognised uracil in the DNA, and not DNA itself.

Pond *et al.* (1997) used BIAcore to investigate the binding of human topoisomerase I to DNA. Three different types of biotinylated double-stranded DNA were immobilised on a streptavidin surface, and the effect of the base content and concentration of Mg^{2+} upon the binding of topoisomerase I was examined. The association and dissociation of the enzyme from DNA were found not to fit to simple models. The dissociation phase appeared to be biphasic in nature, and it was suggested that this was due to an initial high rate of dissociation of topoisomerase I from sites on DNA where it could not introduce single-strand breaks.

In the studies described below, supercoiled plasmid DNA was extracted from bacterial cells, labelled with biotin and bound to the BIAcore via immobilised streptavidin. The interaction of topoisomerase II, novobiocin and coumarin-protein conjugates with this surface was investigated.

Results

6.5 Extraction, purification and biotinylation of supercoiled plasmid DNA

Supercoiled plasmid DNA was extracted from bacterial cells as described in section 2.11. *E. coli* cells of the strain TG1 were used. These cells contained the plasmid pHEN1, a 3,000 base-pair phage display vector which carries a gene for carbenicillin resistance. After culturing cells overnight, the plasmid DNA was extracted using a commercial mini-column kit based on specific absorption of DNA onto a solid phase. The DNA was eluted from the mini-column with ultrapure water. The absorbance of the eluted material at 260 nm and 280 nm was measured, and the average A_{260}/A_{280} ratio was found to be 1.75, indicating good purity of DNA. 10 ml of bacterial culture were purified at a time, and the average yield of DNA was 15 μ g.

The extracted DNA was labelled with biotin using a commercial chemical labelling kit as outlined in section 2.12. A *cis*-platinum compound of biotin with an easily cleavable nitrate ligand was added in a 1:1 ratio to purified DNA in aqueous solution. This results in the cleavage of the nitrate group, and coordinative binding to guanosine and adenosine bases. Theoretically, one in every four bases can be labelled by this method, although the actual labelling density is generally one in ten. The labelled DNA was separated from any remaining free biotin by precipitation.

6.6 Investigation of topoisomerase II and novobiocin activity

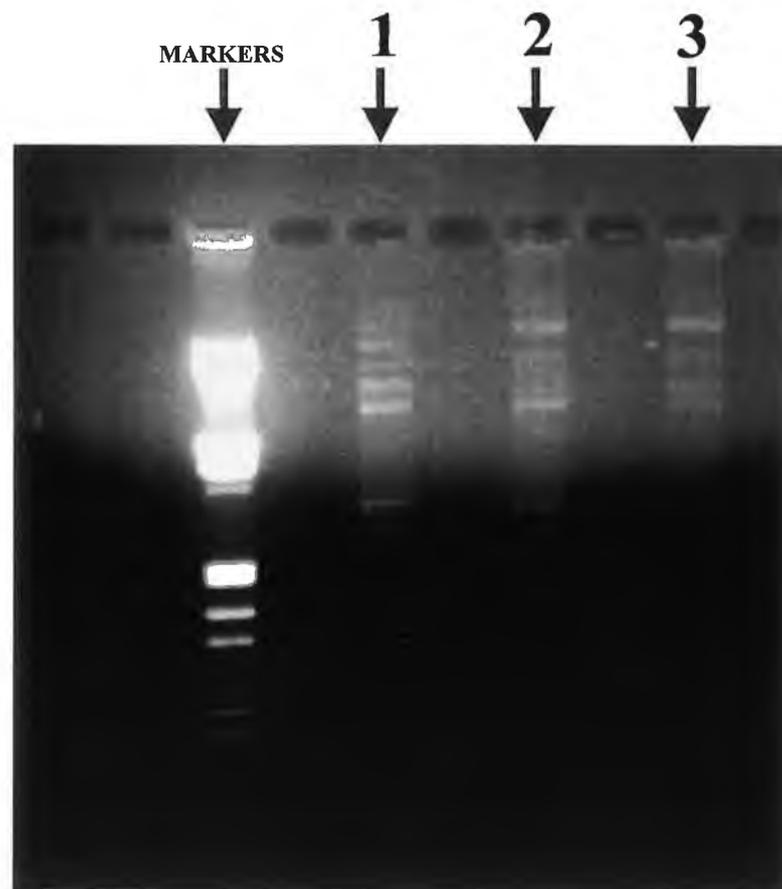
Agarose gel electrophoresis and Southern blotting were used to demonstrate the relaxation of supercoiled DNA by topoisomerase II. Biotinylated DNA was then captured by streptavidin immobilised on the BIAcore sensor chip, and enzyme solutions, with and without novobiocin, were injected over the surface to examine the effect of the coumarin antibiotic on the binding of enzyme.

6.6.1 Agarose gel electrophoresis

The action of the enzyme was demonstrated using agarose gel electrophoresis as described in section 2.13. A commercial preparation of topoisomerase II from *Drosophila melanogaster* was used. This is a dimeric protein with a molecular weight of 166 kDa, and an absolute requirement for divalent cation and ATP. One unit of the enzyme is defined as the amount needed to fully relax 0.3 µg of supercoiled pBR322 plasmid DNA in 15 minutes at 30°C (Osheroff *et al.*, 1983).

3 µg of biotinylated DNA was added to 40 units of topoisomerase II together with enzyme reaction buffer (containing a final concentration of 10 mM Tris-HCl, pH 7.9, 50 mM NaCl, 50 mM KCl, 5 mM MgCl₂, 100 µM EDTA, 15 µg/ml BSA and 1 mM ATP). This solution was incubated for 45 minutes at 30°C. A sample of the reaction mixture was added to loading buffer and loaded onto a 1% (w/v) agarose gel containing 0.5 µg/ml of ethidium bromide, along with a sample of the original DNA extract, and biotinylated DNA. All samples were loaded at a concentration of 1.1 µg/ml. Markers containing λ phage DNA digested with *EcoRI* and *HinD3* restriction enzymes were also run. The gel was electrophoresed at 80 mV, and photographed under ultraviolet light. The results are shown in Figure 6.2. It can be seen that the original extracted DNA and the biotinylated sample give almost identical bands, whereas the enzyme-treated sample lacks the lowest band. The different bands seen for the samples correspond to a variety of topological forms of DNA. Due to its topography, supercoiled DNA can move through the gel faster than the relaxed form, indicating that the topoisomerase II in sample 3 has relaxed the supercoiled plasmid DNA.

Figure 6.2 Agarose gel electrophoresis of extracted DNA samples. Extracted pHEN1 plasmid DNA (1), biotinylated plasmid DNA (2) and sample treated with topoisomerase II (3) were run along with markers (digested λ phage DNA). Extracted and biotinylated samples give similar bands, corresponding to differing topological forms of DNA. The more supercoiled the DNA is, the faster it moves through the gel. The absence of the lowest band in sample 3 demonstrates that the enzyme has relaxed supercoiled DNA.



6.6.2 Southern blotting

The biotinylation of the plasmid DNA was assessed by Southern blotting. This was carried out as described in section 2.14. DNA from the agarose gel was transferred onto a nitrocellulose membrane by means of capillary transfer. The nitrocellulose was then blocked by incubation with 5% (w/v) milk powder. After washing, the membrane was incubated together with an avidin-alkaline phosphate conjugate, and colour was developed after addition of an insoluble alkaline phosphate substrate.

A photograph of the nitrocellulose blot is shown in Figure 6.3. The biotinylated DNA bound to the avidin conjugate, and although faint, a pattern of bands mirroring those on the agarose gel can be seen. The lowest band – denoting supercoiled DNA – is more distinct for the biotinylated sample than the enzyme treated one. The “smearing” of colour running down both lanes may be due to biotin dissociating from the labelled DNA, or the presence of free biotin as a result of inefficient precipitation.

6.6.3 BIAcore-based investigation of the effect of novobiocin on topoisomerase II

DNA was captured on the sensor chip as outlined in section 2.9.5. Streptavidin was immobilised at a concentration of 50 µg/ml, giving a signal of approximately 15,000 RU. Biotinylated DNA at a concentration of 35 µg/ml in ultrapure water was injected over this surface for 25 minutes, resulting in the binding of 980 RU of DNA. The biotin-streptavidin interaction is very rapid, and no binding curve is seen (Figure 6.4).

5 nM topoisomerase II in reaction buffer (see section 6.6.1) was injected over this surface with and without 500 µg/ml novobiocin. A negative control containing 500 µg/ml novobiocin in PBS was also injected. The results are shown in Figure 6.5. The overlaid sensorgrams clearly show the effect of novobiocin on the topoisomerase-DNA interaction. The protein concentration in the two samples is the same, yet the initial bulk refractive index change for the sample without novobiocin is greater than that for the sample containing antibiotic. Binding of topoisomerase to the immobilised DNA is evident in the sample without antibiotic, and by just before the end of the injection, the signal for the sample containing novobiocin is 555 RU less than that for the antibiotic-free sample. The binding of topoisomerase II to DNA appears to be transient, however, as no increase in signal relative to baseline is seen after the sample pulse has passed over the surface.

Figure 6.3 Southern blotting of biotin-labelled DNA. Biotinylated DNA, with and without topoisomerase II, was transferred from agarose gel onto nitrocellulose, which was then probed with an alkaline phosphatase conjugate of avidin. The results confirm the biotinylation of plasmid DNA, and the pattern of bands obtained mirrors those on the gel. The lowest band, which represents supercoiled DNA, is fainter for the enzyme-treated sample than the untreated one, demonstrating the relaxation of supercoiled DNA by topoisomerase II.

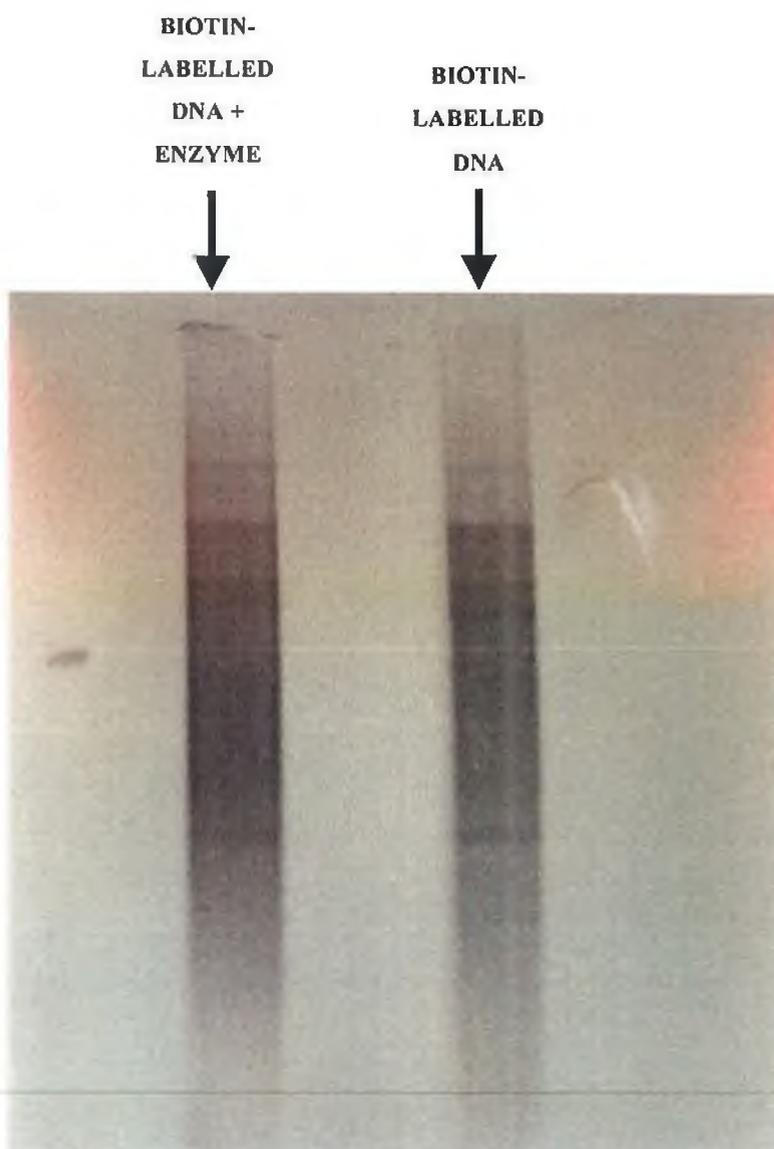


Figure 6.4 Capture of biotinylated DNA on a streptavidin surface. Approximately 15,000 RU of streptavidin were immobilised. A 25 minute injection of 35 $\mu\text{g/ml}$ biotinylated DNA was then passed over this surface. The binding of the biotin and avidin is very rapid, as no increase in signal can be seen during the sample injection. The binding is masked by the initial bulk refractive index change, reflecting the high affinity of the interaction. 980 RU of DNA were captured

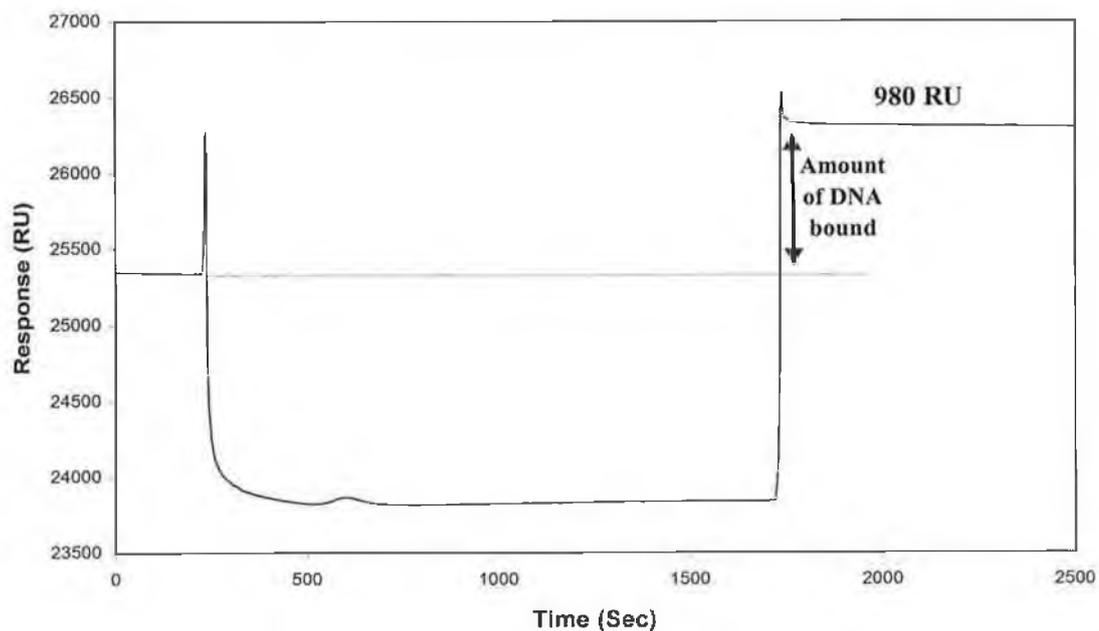
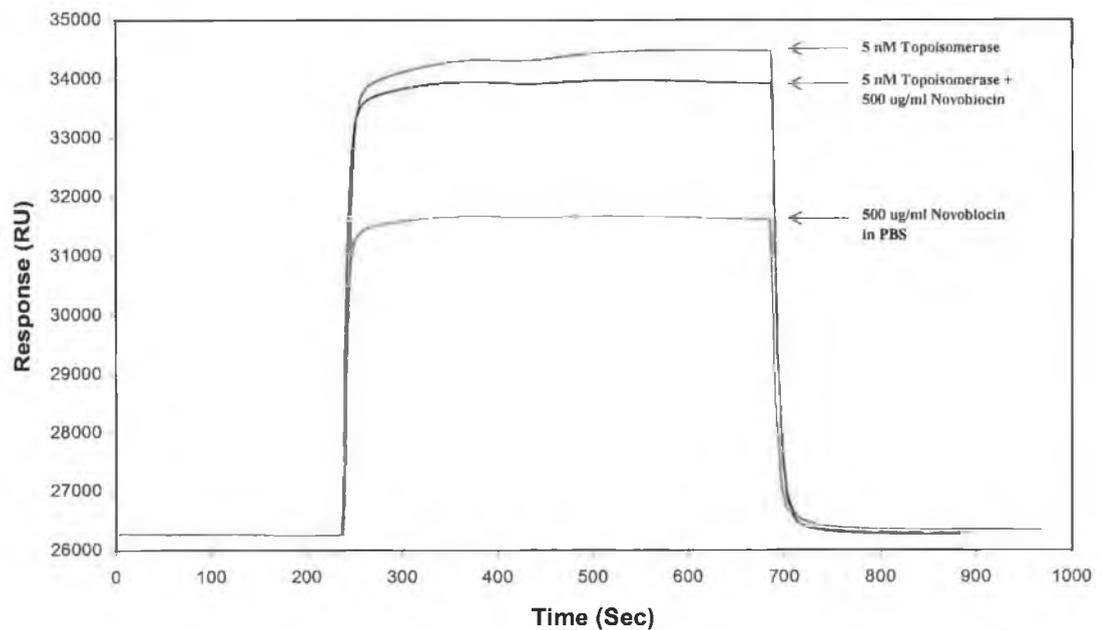


Figure 6.5 Overlay plot showing the effect of novobiocin on the action of topoisomerase II. Biotinylated DNA was immobilised on the sensor chip and 5 nM solutions of enzyme, with and without 500 $\mu\text{g/ml}$ novobiocin, were injected. A negative control containing 500 $\mu\text{g/ml}$ novobiocin in PBS was also passed over the surface. The inhibition of the topoisomerase-DNA interaction by novobiocin is demonstrated by the differences in binding signals. Although the protein concentration of the two samples is the same, the initial bulk refractive change is greater for the sample without novobiocin. A binding signal can be seen during for this sample, and, by the end of the injection, the response is 555 RU greater than that for the antibiotic-containing sample.

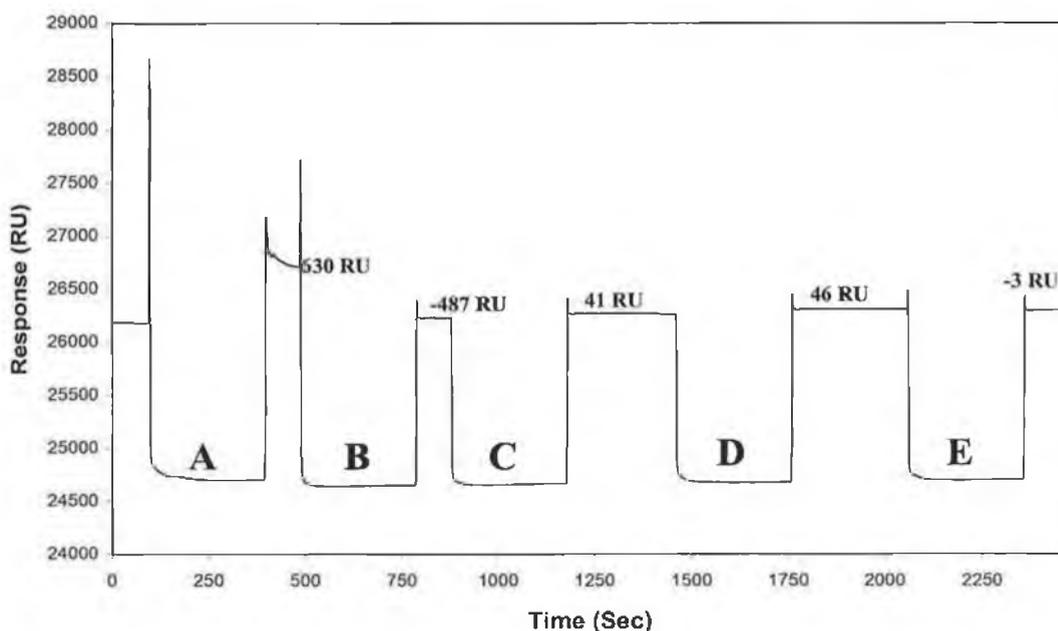


6.7 Direct interactions of coumarin-protein conjugates with BSA

The binding of a range of BSA conjugates to immobilised DNA was assessed on the BIAcore. Approximately 15,000 RU of biotinylated DNA was captured with streptavidin as in section 6.6.3. Sequential injections of 100 µg/ml of BSA, 7-hydroxycoumarin-BSA, coumarin-BSA, aflatoxin B₁-BSA and warfarin BSA were passed over the surface. The results are shown in Figure 6.6. Native BSA gave a binding signal of 630 RU, and the high level of dissociation observed immediately after the sample pulse has passed over the surface suggests that this is due to low affinity, non-specific, binding.

A large negative response (-487 RU) is seen after the injection of 7-hydroxycoumarin-BSA. A subsequent injection of 7-hydroxycoumarin-BSA over the DNA surface (data not shown), did not result in any such signal, indicating that the initial response was due to washing off of non-specifically bound native BSA from the first injection. No significant binding of any of the coumarin conjugates was observed.

Figure 6.6 Interaction of coumarin-protein conjugates with immobilised DNA. DNA was captured by immobilised streptavidin and 100 $\mu\text{g/ml}$ solutions of (A) BSA, (B) 7-hydroxycoumarin-BSA, (C) coumarin-BSA, (D) aflatoxin B1-BSA and (E) warfarin-BSA were injected over the surface. The non-specific binding of native BSA gives a response of 630 RU. The large negative signal after injection of 7-hydroxycoumarin-BSA (B) is due to the dissociation of native BSA, as a subsequent injection of 7-hydroxycoumarin-BSA (not shown) did not give any change in signal. None of the other coumarin conjugates gave any significant binding signals.



6.8 Discussion

The results presented here demonstrate the ease of immobilisation of biotin-labelled DNA on the BIAcore, and its use for the investigation of the properties of coumarin compounds. Commercial kits were used to extract and label plamid DNA, with good yields and purity.

The action of a topoisomerase II from the fruit fly *Drosophila melanogaster* was investigated using agarose gel electrophoresis (Figure 6.2). The enzyme was shown to effectively relax the biotinylated DNA. A Southern blot using an avidin-alkaline phosphatase probe showed that the biotinylation of DNA had been successfully achieved (Figure 6.3). The smearing seen in the photograph could be due to the dissociation of biotin from the DNA or incomplete separation of bound from free biotin by ethanol precipitation.

DNA was then bound to the sensor chip surface. Streptavidin was first immobilised and biotinylated DNA passed over this surface. The binding of streptavidin and biotin is very rapid, and no binding curve is seen during the sample pulse (Figure 6.4). This type of interaction is characteristic of these two binding partners, and similar sensorgrams were observed by Nilsson *et al.* (1995) when passing biotinylated DNA over immobilised streptavidin.

Topoisomerase and topoisomerase/novobiocin solutions were then injected over this surface (Figure 6.5). The sensorgrams show a definite, if transient, interaction between topoisomerase II and the supercoiled DNA. The addition of 500 µg/ml of novobiocin to the enzyme results in a sensorgram with lower initial bulk refractive index change, and no apparent binding of the enzyme. Although a binding curve for topoisomerase II is obtained, no enzyme remains bound once the sample pulse has passed over the surface.

In contrast, Pond *et al.* (1997) reported binding signals of 500-600 RY for human topoisomerase binding to double stranded DNA. In this study, DNA from *Micrococcus lysodeikticus*, *Clostridium perfringens* and salmon testis were immobilised, and topoisomerase I from human placenta was injected at 50-800 nM. This molar concentration of enzyme was at least ten-fold greater than that of topoisomerase II in the current study. However, the sensorgrams of topoisomerase II injections clearly show a binding event, and it must be concluded that the transient nature of the binding is a property of this enzyme, and not a concentration-dependent effect.

Although hardly conclusive, the results observed appear to indicate that the action of novobiocin upon eukaryotic topoisomerase II involves the blocking of DNA binding - as reported for vaccinia topoisomerase I by Sekiguchi *et al.* (1996) - rather than the inhibition of ATPase activity described for bacterial DNA gyrase (Maxwell, 1993). Vaccinia topoisomerase I was found to have one strong binding site and several weak binding sites for novobiocin, and these were thought to coincide with or overlap the DNA binding site. Unfortunately, the transient nature of the interaction observed for topoisomerase II makes the derivation of affinity data based on dissociation rates impossible.

The direct interaction between coumarin conjugates and immobilised DNA was then examined. BSA conjugates of coumarin, 7-hydroxycoumarin, warfarin and aflatoxin B₁ were injected over the surface and no binding was observed. The lack of binding of coumarin and 7-hydroxycoumarin agrees with the results of optical experiments on the binding of the drugs to DNA in water-salt solutions (Y. Yevdokimov, Personal communication). However, as discussed in section 6.2, the binding of aflatoxin B₁ to DNA has been well documented. The low molecular weight of the coumarins is such that they must be conjugated to proteins in order to generate an SPR signal, and it seems likely that this conjugation prevents the normal interaction between coumarins and nucleic acid.

6.9 Summary

Agarose gel electrophoresis and Southern blotting were used to demonstrate the relaxation of biotinylated DNA by topoisomerase II. This biotinylated DNA was then immobilised on the BIAcore surface, and the transient binding of topoisomerase to this surface, and its inhibition by novobiocin, were demonstrated. The direct binding of a range of BSA-coumarin conjugates to immobilised nucleic acid was also examined, with no interaction being observed.

A mechanism of action of novobiocin on topoisomerase II is suggested, which could benefit from further examination. The use of coumarin-BSA conjugates with long spacer arms to separate drug and protein could reduce steric hindrance and facilitate study of direct interactions between coumarins and DNA.

CHAPTER 7

CONCLUSIONS

The work described in this thesis is mainly concerned with the production and applications of antibodies to members of the coumarin family. Chapter 3 describes the production and characterisation of some anti-coumarin antibodies. Polyclonal antiserum to 7-hydroxycoumarin was produced by immunisation of rabbits, and displayed a good degree of purity and adequate working dilution in a competitive ELISA. Attempts to generate monoclonal antibodies to the drug by somatic cell fusion, however, were unsuccessful. Hybridomas were obtained and cloned by limiting dilution, but the antibodies obtained bound only to drug-protein conjugate, and not to free drug. These findings agreed with other results generated in our laboratory, and with reports in the literature. It appears that for small, weakly immunogenic haptens such as the simple coumarins, the nature of the immunogen used, both in terms of carrier protein and coupling chemistry, and the solid-phase screening procedure employed are of critical importance. The detection limit of the BIAcore biosensor meant that it could not be used to replace traditional ELISA screening methods.

Also in chapter 3, the BIAcore was used to derive kinetic and affinity data for the interaction between a genetically-generated scFv antibody to a joint epitope on a coumarin-protein conjugate. The interaction between the two was found not to fit to the simple mathematical model for antibody-antigen binding, indicating that the binding of antibodies to combined epitopes such as these may prove difficult to characterise.

Chapter 3 also contained a study on the possibility of using BIAcore for the panning of naïve phage display libraries. Bacterial culture supernatant was passed over an immobilised coumarin-protein surface, and then a low pH buffer was injected to remove any specifically-bound phage. Due to the low numbers of specific phage present in a naïve library, no signal was observed upon passing the supernatant over the surface. However, phage counts on the eluate from BIAcore indicated that specific elution had taken place. It seems possible that the use of BIAcore over successive rounds of panning and enrichment might result in the isolation of specific phage with the desired specificity.

Chapter 4 outlined the development of a novel method for the quantitative determination of 7-hydroxycoumarin in serum samples. The purified rabbit polyclonal anti-7-hydroxycoumarin antiserum produced in chapter 3 was used in an inhibitive BIAcore immunoassay for free drug. A model system in buffer was first established, and then the detection of drug in unextracted serum samples was optimised. The non-specific binding of serum components to the sensor chip surface was removed by use of a diluent buffer,

and samples were pre-incubated with the enzyme β -glucuronidase to deconjugate 7-hydroxycoumarin-glucuronide prior to analysis. All steps, including the addition of, and incubation with, enzyme and antibody were carried out on the BIAcore. The method was reproducible, with good inter- and intra-day coefficients of variation, and excellent recovery of drug from spiked samples.

Chapter 5 described comparative BIAcore and ELISA studies on commercial antibody preparations against aflatoxin B₁ a member of the coumarin family. A variety of assay formats were investigated on the BIAcore using monoclonal and polyclonal antibodies to the toxin. These formats included direct immobilisation of antibodies and antigen, as well as the use of anti-species antibodies and protein A. No regenerable immunosensor could be constructed, however, due to the difficulty of regenerating the bound antibodies. A series of ELISA studies were then carried out in which the same antibodies were successfully used in sandwich and competitive assays for the determination of free and protein-conjugated drug. These results highlighted the inherent unsuitability of high affinity antibodies for use in regenerable biosensors. An ELISA-based system for initial optimisation of BIAcore regeneration conditions was proposed, which – together with the use of an indirect ELISA to determine the affinity of monoclonal antibodies – could considerably reduce the time and expense of BIAcore experiments.

In chapter 6, some preliminary results dealing with BIAcore studies on the binding of coumarins to nucleic acids were presented. Plasmid DNA was extracted from bacterial cells, biotinylated and bound to the sensor surface via immobilised streptavidin. The binding of topoisomerase to this DNA was seen to be inhibited by the action of the coumarin antibiotic novobiocin, indicating a possible mechanism of action for the drug. The direct binding of a range of coumarin-protein conjugates to immobilised DNA was assessed, and none exhibited any significant interaction.

To summarise, then, some of the strengths and weaknesses of the BIAcore optical biosensor were highlighted. The instrument was used to perform an accurate and precise fully automated immunoassay for 7-hydroxycoumarin, which compared well with reported methods of detection. However, the sample throughput and automation of the BIAcore could not be used to speed up the screening of monoclonal antibodies from neat hybridoma supernatants, due to the low concentrations of antibody present. The system was also shown not to be compatible with the use of high affinity antibodies, due to difficulties with surface regeneration. This highlights the advisability of producing

antibodies with moderate affinity for use in biosensor applications where regeneration is required.

There are inherent problems with the production of monoclonal antibodies to low molecular weight, weakly immunogenic haptens, and the results presented here suggest that biosensor-based screening and careful attention to choice of carrier protein and coupling chemistry are necessary to generate and select antibodies to free hapten.

CHAPTER 8

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