The production of polyclonal, monoclonal and genetically-derived scFv antibody fragments for the detection of the β-Lactam antibiotic, cephalexin, in milk

A thesis submitted for the degree of Ph.D.

By

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"A thinker sees his own actions as experiments and questions, as attempts to find out something. Success and failure are for him answers above all"

Friedrich Nietzsche

Declaration

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

Signed 

Date: Oct 2006.

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Table of Contents:

Abbreviations

Ab	antibody
Abs	absorbance
ADAC	7-aminodesacetoxy-cephalosporanic acid
Ag	antigen
Ala	alanine
AP	alkaline phosphatase
APA	(+)-6-aminopenicillanic acid
APC	antigen-presenting cell
ARM	antibody, ribosome and mRNA
ATCC	American Type Culture Collection
AvAab	Aart van Amerongan antibody
BAD	biotin acceptor domain
BCA	bicinchoninic acid
B-cells	Bursa-equivalent-derived small lymphocytes
BIA	biomolecular interaction analysis
BSE	bovine spongiform encephalopathy
bp	base pairs
BSA	bovine serum albumin
Cam	chloramphenicol
CAT	Cambridge Antibody Technology
CBD	cellulose binding domain
cDNA	complimentary DNA
CDR	complementarity determining region
CE	capillary electrophoresis
Ceph	cephalexin
CephM1	anti-cephalexin monoclonal antibody
CephP1	anti-cephalexin polyclonal antibody
cfu	colony forming unit
$C_{H(1-3)}$	heavy chain constant domain
C_L	light chain constant domain
CM	carboxymethylated
conc (c)	concentration
CR	cross-reactivity
CSPI	Centre for Science in the Public Interest
CV	coefficient of variation

CVM	Centre for Veterinary Medicine
CVMP	Committee for Veterinary Medicinal Products
CWT	cell wall transamidase
DAF	Department of Agriculture and Food
DIC	directly immobilised cephalexin
DIP	delayed infectivity panning
DMEM	dulbecco's modified eagle's medium
DNA	deoxyribonucleic acid
dNTP	deoxynucleotidyl triphosphates
E. coli	*Escherichia coli*
EDC	N-ethyl-N'-(dimethylaminopropyl) carbodiimide
EDF	Environmental Defence Fund
EDTA	ethylenediaminetetra-acetic acid
EEC	European Economic Community
EF	evanescent field
ELISA	enzyme-linked immunosorbent assay
EMEA	European Medicines Evaluation Agency
EP-PCR	error-prone PCR
EU	European Union
EUMRL	EU maximum residue limit
Fab	antigen-binding region of an antibody
FACS	fluorescence activated cell sorting
FAO	Food and Agriculture Organisation
Fc	fragment crystallisable (constant region of antibody)
FCA	Freund's Complete Adjuvant
FCS	foetal calf serum
FDA	Food and Drug Administration
FSAI	Food Safety Authority of Ireland
Fv	fragment (variable)
GFI	guidance for industry
GFP	green fluorescence protein
Glu	glutamic acid
Gly	glycine
HAT	hypoxanthine aminopterin thymidine
HBS	Hepes buffered saline
HHMI	Howard Hughes Medical Institute
His	histidine

HT	hypoxanthine thymidine
HPLC	high performance liquid chromatography
HRP	horse radish peroxidase
HAS	human serum albumin
HuCal	human combinatorial antibody library
$IC_{(50, 90 \text{ or } 100)}$	inhibition concentration (at 50%, 90% or 100%)
IDF	International Dairy Federation
IgA	immunoglobulin class A
IgD	immunoglobulin class D
IgE	immunoglobulin class E
IgG	immunoglobulin class G
IgM	immunoglobulin class M
IMAC	immobilised metal affinity chromatography
IMS	immunomagnetic separation
IPTG	isopropyl-β-D-galactopyranoside
J	joining antibody germ-line gene cluster
JECFA	Joint Expert Committee on Food Additives
Kan	kanamycin
KLH	keyhole limpet Haemocyanin
L	leucine
LB	Luria Bertani media
LC	liquid chromatography
LE	low expression
LOD	limit of detection
Log	logarithmic
Lys	lysine
mAb	monoclonal antibody
MDC	minimal detectable concentration
MHC	major histocompatibity complex
mRNA	messenger RNA
MRL	maximum residue limit
MW	molecular weight
N	glutamine
ND	not determined
NAG	n-acetylglucosamine
NAM	n-acetylmuramic acid
NCTC	National Collection of Type Cultures

NHS	N-hydroxysuccinimide
NK	natural killer
NTA	nitrilotriacetic acid
OD	optical density
OPD	o-phenylenediamine dihydrochloride
PAGE	polyacrylamide gel electrophoresis
PASA	parallel affinity sensor array
PBP(s)	penicillin binding protein(s)
PBS	phosohate buffered saline
PBS-T	PBS containing 0.05% (v/v) Tween 20
PBS-TM	PBS-T containing 5% (w/v) powdered skimmed milk
PCR	polymerase chain reaction
PEG	polyethylene glycol
pH	log of the hydrogen ion concentration
pI	isoelectric point
ProG	protein G
Q	glutamine
RI	refractive index
R.T.	room temperature
RT-PCR	reverse transcriptase PCR
RU(s)	response unit(s)
S	serine
SAS	saturated ammonium sulphate
SCC	somatic cell count
scFv	single chain Fv antibody fragment
SD	standard deviation
SDS	sodium dodecyl sulphate
SPR	surface plasmon resonance
TAA	tumour associated antigen
TCC	thyroglobulin-conjugate chip
TES	testosterone
Tet	tetracycline
Thyro-Ceph	thyroglobulin-cephalexin
TMB	3,3′,5,5′-tetramethylbenzidine
upH_2O	ultra-pure H_2O
UV	ultraviolet
V	variable antibody germ-line gene cluster

V_H	variable region of heavy chain antibody fragment
V_L	variable region of light chain antibody fragment
WGSR	Working Group on the Safety of Residues
WHO	World Health Organisation
WT	wild type
XR	cross reactant

Units

µg	microgram
(k)Da	(kilo) Daltons
(k) bp	(kilo) base pair
µl	microlitre
µM	micromoles
°C	degrees Celsius
cm	cetimetres
g	grams
h	hours
kg	kilogram
l	litre
m	metre
M	molar
mg	milligram
min	minute
ml	millilitre
mm	millimetres
mol	moles
nM	nanomolar
ng	nanogram
nM	nanomolar
rpm	revolutions per minute
RU	response units
s	seconds
V	volt (s)
v/v	volume per unit volume
w/v	weight per unit volume

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Abstract

This thesis describes the production of antibodies for the detection of cephalexin and their application in ELISA and Biacore 'real-time' surface plasmon resonance (SPR)-based biosensor assays. The use of antibiotics in food-producing animals has many beneficial effects (e.g. treatment of mastitis in dairy cattle). However, their use and misuse has also been implicated in the increased incidence of resistant microbial strains with the associated public health risks and economic losses for food processors. Antibiotic residues can adversely affect bacterial starter cultures used in the production of milk-based products such as cheese and yoghurts. The β-lactam antibiotic cephalexin was chosen as the target for the production of antibodies due to its generic cephem-based structure, solubility, and, in particular its use of this antibiotic in treatment of udder infections (e.g. mastitis).

Cephalexin-protein conjugates were produced and used for the generation of polyclonal and monoclonal antibodies that were then purified and characterised by SDS-PAGE, Western blotting and ELISA. The specificity of these antibodies was determined and they were used in displacement immunoassays for the detection of the relevant antibiotic in 'spiked' samples of PBS and whole milk.

Recombinant single chain Fv (scFv) antibody fragments were produced using phage-display technology. Naïve and immune phage-display libraries were panned using cephalexin conjugates. In addition, a monoclonal antibody-secreting hybridoma cell line was used in the production of a phage-displayed recombinant scFv. The recombinant scFv (wild type) derived from the parental hybridoma cell was purified and characterised by SDS-PAGE, Western blotting and ELISA. Wild type scFv was subsequently used as a template for the production of a mutant scFv phage-displayed library using random mutagenesis and error-prone PCR. Mutant scFv antibodies were isolated, expressed and then purified using immobilised metal affinity chromatography (IMAC). Two mutant clones expressing scFv antibodies selected showed improved functional expression and assay performance when compared to wild type scFv. Comparative gene and amino-acid sequence analysis and protein modelling was carried out on wild type and selected mutant recombinant scFv antibodies and confirmed the presence of mutations in the framework and variable heavy chain complemetarity determining region amino acid sequences.

All antibodies were evaluated in ELISA and Biacore assay formats for the detection of cephalexin in milk samples. The polyclonal, monoclonal and recombinant scFv antibody-based assays demonstrated varying sensitivity and reproducibility but achieved cephalexin

detection below the legislated European Union maximum residue limit (EUMRL) in milk (100 μg/Kg).

Chapter 1

Introduction

1.1 Project Overview

The main aim of this work was the production of antibodies to cephalexin and their subsequent use in the development of assays for its specific detection in milk. This introductory chapter reviews aspects of the general project background, including detection of small molecule contaminants in foods, mastitis and antibiotic residues in milk, and the β-lactam antibiotics with a focus on cephalosporins. In addition, public health concerns with regard to the use of antimicrobials in food-producing animals, current detection methods, the immune system, antibody production and engineering techniques are also described. Introductions are presented at the beginning of each of the relevant chapters which describe the particular technologies relevant to that section.

1.2 Detection of contaminants in foods

Contaminants may be introduced into food by industrial or environmental pollution, during growth or at any stage in the processing leading to the final packaged product. For example reports in the media about Sudan Red, a known carcinogen detected in different foods and ingredients highlighted the overall amplified awareness of such contaminants in public, industrial and legislative domains. Food contamination arising from antibiotics used in veterinary medicine is an increasingly important public health issue. Antibiotic use in food-producing animals is associated with potential direct (i.e. allergic reactions) and indirect hazards (i.e. increased occurrence of antibiotic resistance pathogenic bacteria). Food ingestion is considered to be a major route of exposure to many of these contaminants. Small molecules such as antibiotics with molecular weight $\leq$ 5000 Daltons are called haptens. They are molecules that are too small to elicit a direct immune response without being first conjugated to a larger carrier (e.g. protein). Many publications describe the production of antibodies that are capable of detecting very small quantities of hapten contaminants in an appropriate immunoassay format (Stanker *et al.*, 1989; Daly *et al.*, 2001; Dillon *et al.*, 2003; Miura *et al.*, 2003; Baeumner, 2003; Oi *et al.*, 2005).

The European Union has significant legislation with regard to small molecule food contaminants. The legislation covers a broad range including, for example, veterinary medicines (e.g. antibiotic residues in milk), food additives and environmental or industrial pollutants, for example, plant mycotoxins, which may include aflatoxins (potent carcinogens) in grain and nut products that are poorly stored (Anadón *et al.*, 1999; Stolker *et al.*, 2005). The use of most pharmacologically active substances in food-producing animals (i.e. veterinary medicines) is governed by the EU Council Regulation 2377/90/EC. This regulation

describes the procedure for the establishment of maximum residue limits (MRL's) for veterinary medicinal product residues in foodstuffs of animal origin. Veterinary medicine residue problems are addressed in reports from the Joint FAO/WHO Expert Committee on Food Additives (JECFA), the Working Group on the Safety of Residues (WGSR) of the Committee for Veterinary Medicinal Products (CVMP) of the European Medicines Evaluation Agency (EMEA). The CVMP are responsible for the establishment of maximum residue limits (MRL's). EU council regulation 2377/90/EC contains four Annexes that classify each potential contaminant relative to its potential exposure risk. Annex I includes substances for which final MRL's (e.g. cephalexin, appendix 1) have been established. Annex II includes those substances where the establishment of MRL values for the protection of public health it is not considered necessary. The use of this group of substances is permitted in certain food-producing animals under defined conditions. Annex III includes compounds for which provisional MRL's have been set out, yet not all requirements for the establishment of the MRL have been met. Finally, Annex IV lists substances that have no MRL established, for the simple reason that their presence in foods at whatever limit constitutes a hazard to public health. Included in the legislation are other substances (i.e. originating from sources other than veterinary medicine) that are required to be monitored for public health reasons (i.e. aflatoxins) and other plant-associated toxins (Than *et al.,* 2005).

Overall, there is a myriad of potential hapten contaminants that may be present in a vast number of different foodstuffs. For each contaminant with a listed MRL, a value was established for a particular foodstuff. For example, β-Lactam antibiotics, include many different compounds and have MRL's in kidney tissue ranging from 25-300 μg/kg. However, in milk the MRL's range from 4-100 μg/kg. This highlights the need for an extremely flexible, sensitive and rapid analytical methodology capable of multi-analyte detection with high-throughput. Such systems should be capable of detecting residues of the parent compound and its metabolites in diverse sample matrices (e.g. milk, tissue, plant material or honey).

The sample matrix is usually determined by the point in production at which testing is performed, i.e. from the final end food product (e.g. milk) to the actual food-producing animal (e.g. animal tissue, feed, water or urine). Analytical techniques must be capable of specifically detecting a multitude of different compounds (Table 1.1). Antibody-based analytical techniques meet such requirements, being highly specific, available in many different flexible assay formats and are very robust.

Current techniques employed for the detection of such contaminants almost always involves LC-MS-based techniques. This usually involves extensive sample pre-treatment and extraction processes. Due to the bulky nature of the equipment, relatively low throughput and the expertise required to carry out such analyses, they are not suitable for 'in the field' or 'user-friendly' measurement by untrained persons. Such techniques are carried out as confirmatory tests, with certain rapid detection technologies available for particular compound types. However, within the EU there is no obligation to use standardised methods in the residue control of food-producing animals. Techniques are required to satisfy performance characteristics, limits and other criteria which encourage the development of new analytical approaches (e.g. antibody micro-arrays) (Christiansen, 2002).

Table 1.1. Examples of some potential contaminants, the respective MRL ranges for that foodstuff and the foods in which they may be found.

Contaminant	Specific MRL (µg/kg)	Foodstuff
Antibiotics		
- Aminoglycosides (e.g. gentamycin)	50-750	Meat & milk
- β-Lactams (e.g. cephalexin)	4-100	Milk
- Macrolides	40-2000	Meat, Milk & Eggs
- Sulphonamides	100	Milk & Eggs
- Tetracyclines	100-600	Meat
- Quinolones	30-600	Fish
- Chloramphenicol	0.3	Meat & Seafood
Toxins		
- Aflatoxins	1-8	Nuts & Grains

1.3 Mastitis and antibiotic residues in milk

Antimicrobial agents, generally referred to as antibiotics, are used in food-producing animals for three main reasons. Firstly, in the treatment of diseases, secondly as a prophylactic to prevent disease and finally as feed additives to promote beneficial effects such as increased body mass. In dairy cattle worldwide, one of the most prevalent and costly diseases is mastitis. This is an inflammation of the mammary gland caused by micro-organisms, usually

bacteria, that invade the cows' udder, proliferate, and produce toxins that are harmful to surrounding tissues. Mastitis may be sub-clinical, i.e. no visible signs of disease, or clinical displaying mild or severe signs of infection which include clots present in the milk, swelling of infected udder quarters, fever and the potential death of infected animal. Mastitis may be caused by a multitude of micro-organisms classified as environmental or contagious. The latter resulting from infection by various *Streptococci* (e.g. *S. agalactiae*) and *Staphylococci* (e.g. *S. aureus*) species, environmental causative micro-organisms include various coliforms (e.g. *E. coli*) and environmental *Streptococci* (e.g. *S. dysgalactiae*).

1.3.1 *Diagnosis of mastitis*

Cows already infected with contagious bacteria are the main source of infection within herds and hence early detection and treatment is of the utmost importance. Clinical mastitis is easy to detect for veterinarians and trained dairy personnel, but detection of sub-clinical cases of mastitis can be a challenge. Diagnostic methods are focused on detection of inflammatory products, somatic cell count (SCC), and bacterial growth. The International Dairy Federation (International Dairy Federation, 1971), have decided that milk SCC and bacteriological examination are the main parameters that define whether or not an animal should be regarded as infected. Hamann (2002) suggested that a SCC of 100,000 cells/ml in raw milk represents a 'normal' physiological limit in an udder quarter. A SCC of approximately >200,000 cells/ml may be taken as an indicative (i.e. 250,000 to 1,000,000 cells/ml) of udder quarter infection. The specific identification of the causative agent is critical to the subsequent treatment of infected animal udder quarter, as this dictates if the use of particular anti-microbial treatment (e.g. antibiotics) is necessary.

1.3.2 *Effects on Milk Production, Composition and Quality*

Mastitis reduces yield and alters milk composition resulting in a very negative economic impact on producers. Mastitis-causing pathogens produce toxins that can directly damage milk-producing tissue of the mammary gland and the presence of bacteria initiates inflammation within the mammary tissue in an attempt to eliminate the invading micro-organisms (see innate immune system, section 1.9.1). The inflammatory immune response contributes to the decreased production and altered compositional changes observed in milk from infected animals. In sub-clinical mastitis there are significant changes in the types of proteins present. The major milk protein is casein. Casein has a high nutritional value and contains most of the milk calcium within its structure. It is also very important in cheese manufacturing. The casein content of milk from mastitic cows is reduced, while the content of

relatively lower quality (in terms of food production) whey proteins or blood serum proteins, such as serum albumin and immunoglobulins, increases in the milk. Increased concentration of certain enzymes in mastitic milk released as a result of mammary tissue damage can also negatively affect milk quality in terms of flavour. For example, an increase in the enzyme lipase results in an elevated level of free fatty acids that can produce 'off-flavours' in milk. This can result in significant economic loss to the producer, from direct milk production and the production of value-added milk products (e.g. cheese and yoghurt). The prevalence of mastitis in dairy cattle worldwide and the bacterial nature (in most cases) of the disease is used as a major justification for the legal application of anti-microbials in milk-producing animals.

1.3.3 *Mastitis treatment*

Mastitis may be best prevented using hygienic farming/milking procedures. Treatment of mastitis when it does occur invariably involves using antibiotics at various stages of the disease (i.e. sub-clinical or clinical mastitis). Antibiotics should ideally only be administered during 'dry' as opposed to lactation periods, where the potential for contamination of milk is removed. Sub-therapeutic treatment of lactating cows can result in milk containing antibiotic residues unless the animal is removed from milking schedules. Cows with mammary bacterial infections are usually treated with intra-mammary antibiotic infusions.

There are a host of potential antibiotics available for treatment. Infusions usually contain high levels of penicillins, cephalosporins and cloxacillins and some others found to be the most effective therapeutic agents. In general, the beta (β)-lactam antibiotics (i.e. penicillins, cephalosporins etc.) are the most widely used antibiotics in both human and animal medicine. There are a range of cephalexin monohydrate suspensions marketed for the treatment of mastitis in bovines, ovines and caprines, for example, Cefa-Milk ® (www.engormix.com). Antibiotic preparations are usually in the form of slow release bases, allowing them to remain in the udder for extended periods of time. Preparations are also available as combination therapies, for example, Cefa-milk ® Forte, a suspension of cephalexin monohydrate and gentomycin sulphate.

Efflux of the antibiotic is directly affected by milking frequency and may vary from cow-to-cow, so animals receiving treatment outside recommended dry periods should remain off milking for an extended period post-treatment. If this is not adhered to or the antibiotic persists within a particular cow potential contamination of resulting milk with residues is greatly enhanced. Milking of cows while undergoing treatment is also counterproductive to

antibiotic therapy as effectively the antibiotic will be removed from infected udder quarters which may result in development of chronic mastitis.

1.4 The beta (β)-lactam antibiotics and mechanism of antimicrobial activity

β-lactam antibiotics are a very large family of antimicrobial drugs containing reactive beta lactam ring systems. The ring systems contained within this class of antibiotics represent the bio-active components. Encompassed in their chemical structure are ring systems found in five distinct conformations or sub-groups (figure 1.1). The sub-groups are termed penam (i.e. penicillins), cephem (i.e. cephalosporins), penem, carbapenem and monobactam depending on their respective ring structures. In general, beta lactam bacteriocidal activity is attributed to their ability to bind a group of bacterial proteins, loosely referred to as penicillin binding proteins (PBP). PBPs are enzymatic proteins that function in bacterial cell wall synthesis. Irreversible-binding of such PBPs is facilitated by the highly strained and reactive nature of β-lactam ring structures with variety of stable leaving groups. PBPs bind irreversibly to β-lactams, as their respective ring structures spatial arrangements closely resemble the conformation of certain PBP natural peptidoglycan substrates (e.g. D-alanine-D-alanine). A generalised binding mechanism of natural substrate and generic beta-lactam is shown in Figure 1.2. This results in reduced or inhibited cross-linking and disruption of bacterial wall synthesis, which is followed by inhibition of cell division or cell lysis and death.

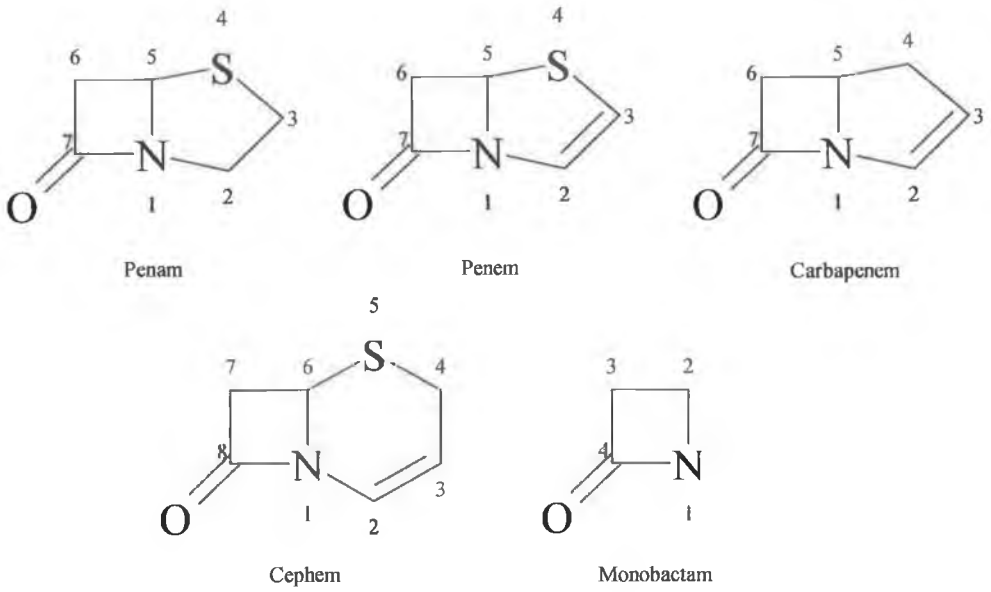
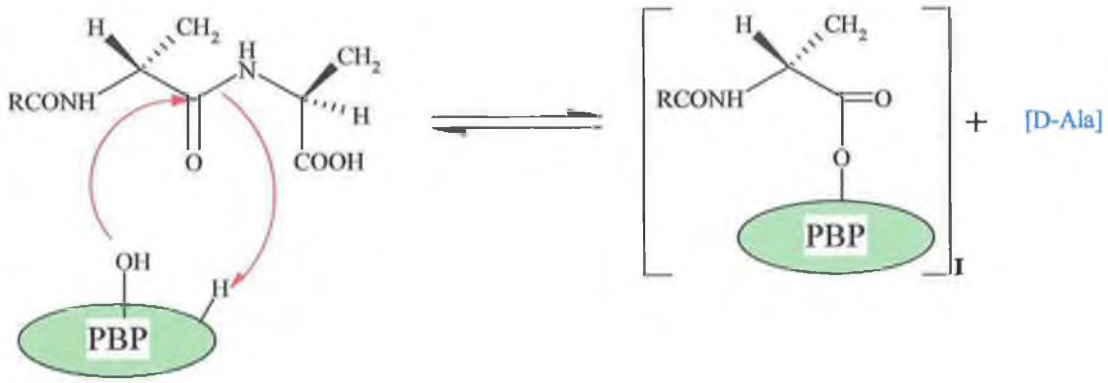


Figure 1.1. The core β-lactam ring structures for each of the five beta-lactam antibiotic sub-groups, penam, penem, carbapenem, cephem and monobactam are shown. Atoms are numbered with respect to nitrogen at position one.

The biochemical makeup of the bacterial cell wall is a distinguishing feature. Cells can be visually classified by cell wall make up, as Gram (after the Danish biologist Hans Christian Gram) positive or negative using established staining methods. Cell walls have provided a target for the development of specific antimicrobial agents to combat various infections that bacteria cause. Overall, cell walls serve three main purposes within bacteria. They provide (i) a semi-permeable barrier through which only certain substances may pass (ii) a barrier against osmotic stress, and (iii) prevent digestion by host enzymes.

There are various PBPs implicated in cell wall synthesis although their functions are not fully understood. Various β-lactam antibiotics bind in a dissimilar manner to different PBPs producing a variety of effects. Binding to PBP-1 (transpeptidase or transamidase) produces cell lysis. Binding to PBP-2 (transpeptidase) leads to irregular oval cells that are incapable of cell division, while binding to PBP-4-6 (carboxypeptidases) have no lethal effects.

PBP binding natural substrate (D-Ala-D-Ala)



PBP irreversible binding beta-lactam substrate

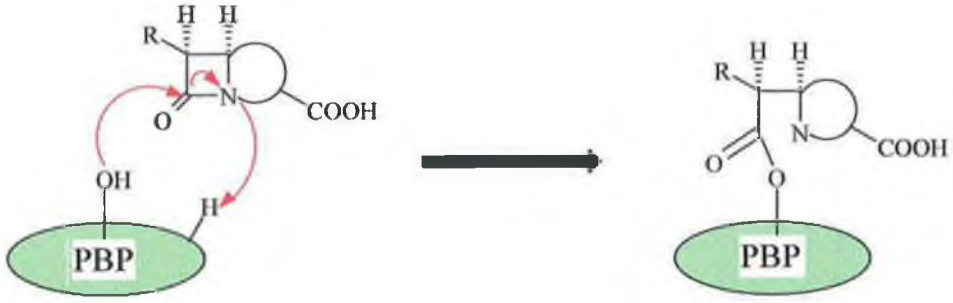


Figure 1.2. Overview of a penicillin-binding protein (PBP) binding to its natural substrate (top of figure) and to a generic beta-lactam antibiotic (bottom of figure). Binding of PBP to the D-alanine-D-alanine (D-Ala) portion of peptidoglycan moieties (R, represents the remaining molecule) which is the natural substrate, is reversible and results in formation of a reactive intermediate complex (**I**), shown in square brackets. This intermediate complex subsequently undergoes further reaction, as dictated by the particular PBP, during bacterial cell wall synthesis. In contrast, binding of PBP to the beta-lactam ring on the antibiotic (generic antibiotic structure shown, where R, represents functional group) is irreversible and results in formation of a stable PBP-beta-lactam conjugate, thus inhibiting PBP function in bacterial cell wall synthesis. Red arrows represent the movement of electrons in bond formation from nucleophilic to corresponding electrophilic participants.

The cell walls of the Gram negative organisms are the more complex than Gram positive and both types are sensitive to beta-lactams due to presence of PBPs. The Gram positive cell wall serves as a good generic model for explanatory purposes. Gram positive cell walls consist of a characteristic set of carbohydrates and proteins on the external surface (e.g. antigenic determinants) and inside this is a layer comprised of repeating units of N-acetylglucosamine (NAG) and N-acetylmuramic acid (NAM) peptidoglycans arranged in polymeric chain form. This layer of the cell wall is highly cross-linked lending greater intrinsic stability to the barrier. Cross-linking is catalysed by a cell wall transamidase (CWT) also known as PBP-1. Each NAM unit is covalently linked to an oligopeptide chain that contains both L and D-amino acids (i.e. alanine [Ala], lysine [Lys], glycine [Gly] and glutamic acid [Glu]). CWT transiently binds a single oligopeptide on strand one through a surface serine residue and this bond is subsequently attacked by an amino terminus of a free glycine residue on strand two oligopeptide (Figure 1.3).

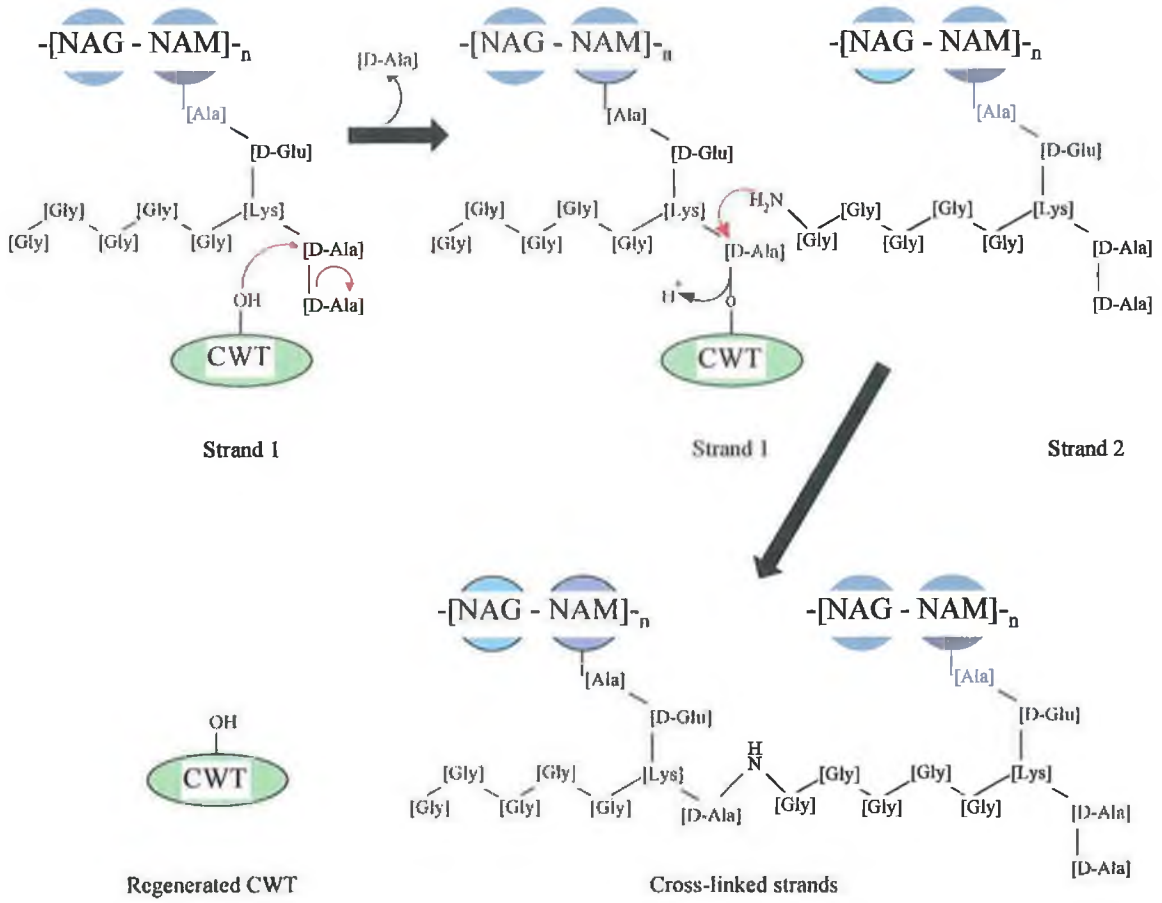


Figure 1.3. Cell wall transamidase (CWT) or PBP-1-catalysed cross-linking of N-acetylglucosamine (NAG) and N-acetylmuramic acid (NAM) polymeric (denoted by small n above) peptidoglycans. Each NAM unit is covalently linked to an oligopeptide (R-L-ala-D-glu-L-lys-D-ala-D-ala) chain containing both L- and D-amino acids. CWT transiently binds to a single NAG-NAM polymeric unit (strand 1) via R-D-Ala-D-Ala oligopeptide (red arrows depict the movement of electrons from nucleophilic to electrophilic moiety). Binding occurs through a CWT surface serine residue (-OH) and this intermediate bond is subsequently attacked by a free glycine residue amino terminus (red arrow) of an adjacent strand oligopeptide (strand 2). This results in covalently cross-linked strands with the release of D-alanine and a proton. CWT is regenerated and is ready for a new catalytic cycle.

1.5 Introduction to Cephalexin and Cephalosporins

Cephalexin is a member of the cephalosporin sub-family of β-lactam antibiotics. It contains a cephem β-lactam ring system that is characteristic of these antibiotics and once intact confers their antimicrobial activity. Four generations of cephalosporin-based antimicrobials have evolved since their discovery by Guiseppe Brotzu, in the 1940's. The various cephalosporins contain the core cephem β-lactam ring structure substituted with different side chain groups that affects their antimicrobial activity and resistance to hydrolysis mediated by β-lactamase enzymes. This classification may be further elaborated by their relative activity against various pathogenic microbes into limited-, intermediate-, and broad-spectrum cephalosporins. Cephalexin may be described as a semi-synthetic first-generation cephalosporin with a limited-spectrum of activity. Cephalexin hydrate (Figure 1.4) was chosen as the test compound for the production of antibodies in this work. This was due to its relative stability, availability and it also serves as a generic model for the cephalosporin (cephem) sub-group. The target analyte is unmodified cephalexin, i.e. no secondary metabolite is produced. The current MRL for cephalexin is 100 μg/kg of milk (Appendix 1).

A few first- and second-generation β-lactams including cephalexin have strictly regulated approval for use worldwide for the treatment of mastitis infections in dairy cattle. Consequently, this group of antimicrobials are most frequently analysed in milk quality assurance programs globally. FEDESA, the European Animal Health Association, a body that actively promotes the responsible use of animal health products to ensure the health and welfare of European animal populations, estimated that the usage of antibiotics in animals, as opposed to humans, in the EU accounted for 48% of the overall total usage. This 48% figure is further broken down into therapeutic use (33%) and the remaining 15% as feed additive (FEDESA Antibiotics for Animals, 1999). The efficiency of antibiotics in general as growth promoters is reflected by their use as animal food additives.

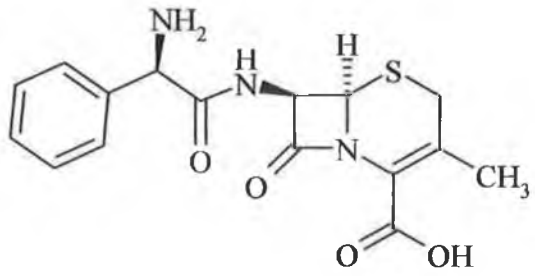


Figure 1.4. The chemical structure of cephalexin (Cephalexin hydrate contains an associated water molecule).

1.6 Public Health issues regarding use of antibiotics in animals

The use of antibiotics in food-producing animals has had many beneficial effects (e.g. longer life expectancy, increase body mass, economic gain from healthier livestock). However, their use and misuse has also been implicated in the increased incidence of resistant microbial strains. The generally accepted mechanism by which antibiotic resistance is acquired involves the antibiotic itself exerting a selective pressure upon microorganisms. During the 1990's several scientists raised concerns regarding fluoroquinolone-resistant food borne pathogens. These concerns were compounded by the temporal association between the approval of fluoroquinolone for therapeutic use in poultry in Europe and the emergence of fluoroquinolone-resistant *Campylobacter spp.* in humans (US Food and Drug Administration, FDA, URL: www.fda.gov/cvm A and B). In December 1998 EU health ministers voted to ban four specific antimicrobials that were widely used at sub-therapeutic levels (i.e. as prophylactics or to promote animal growth). Although none of these antimicrobials were from the β-lactam family they were widely used in the treatment of human disease. This highlights the general lack of data and understanding regarding the potential risks associated with the use of antimicrobials in food-producing animals.

Similar action was sought in the United States whereby a citizen's petition was made to the FDA's Centre for Veterinary Medicine (CVM) on behalf of the Centre for Science in the Public Interest (CSPI), the Environmental Defence Fund, the Food Animal Concerns Trust, the Public Citizens Health Research Group, and the Union of Concerned Scientists. The petition requested the withdrawal of approvals for the sub-therapeutic use of medically important antibiotics in livestock feeds. The CSPI specified a priority list, seven antibiotics were requested to be withdrawn, penicillin included. However, although no specific action was taken with regard to the petition, the FDA released updated guidelines for industry regarding the use of anti-microbial drugs for food animals. The guidelines set out a framework for the qualitative risk assessment to be carried out by prospective drug sponsors seeking approval for their drug for use in food-producing animals.

Within these guidelines, anti-microbial drugs are classified into three categories based on their perceived risk. Category 1 (high risk estimate) are for strictly limited use and include third generation cephalosporins (e.g. cefataxime) among others. Category 2 (medium risk estimate) require intermediate restriction on use and include fourth-generation cephalosporins. Finally, category 3 (low risk estimate) have the least restricted use, which includes the fist and second-generation cephalosporins (e.g. cephalexin) (FDA, Guidance for Industry (GFI) #152 ("Evaluating the Safety of Antimicrobial New Animal Drugs with

Regard to their Microbiological Effects on Bacteria of Human Health Concern") October 2003). All of the categories (1-3) are further classified with regard to their relative importance in human medicine. High-risk estimate drugs are considered critically important, medium risks are highly important, and low risk is considered important.

The limitations placed on the usage of certain antibiotics by the US and EU regulatory bodies and general guidelines regarding the responsible use of such drugs are based largely on the assumption that selective pressure is the only mechanism by which resistance is gained. However, recent work (Beaber *et al.*, 2004) carried out by researchers at the Howard Hughes Medical Institute (HHMI) and Tufts University School of Medicine suggests a more direct involvement by the antibiotic itself. The work focused on a set of antibiotic resistance genes designated SXT. This is a 100-kb self-transmissible, conjugative, chromosomally integrating element, which confers resistance to four different antibiotics (i.e. chloramphenicol, sulphamethoxazole, trimethoprim, and streptomycin).

The SXT genetic element was derived from *Vibrio cholerae* in 1993 and is found in almost all clinical isolates of the strain in Asia. When they sequenced this region of the bacterial chromosome they discovered it contained a gene (*setR*) encoding a repressor protein (SetR). This protein was similar to one found in lambdoid phage viruses. It is responsible for the maintenance of the viral genes in an inactive state. Damage to the bacterial chromosome activates an "SOS response". Hence, the repressor is in effect 'turned off', triggering the virus to replicate and spread from the damaged bacteria. During the course of their work they found that the SetR protein did act as a repressor to two other genes, *setC* and *setD*, which are transcriptional activators of excision and transfer processes of the SXT genes. They also found that another protein, RecA, renders SetR inactive via proteolysis which gives rise to the 'SOS response'. The 'SOS response' may be activated by different stimuli, e.g. UV light. However, more importantly the stimuli also include antibiotics. They found that the 'SOS response' was activated by the fluoroquinolone antibiotic ciprfloxacin (Beaber *et al.*, 2003). This work and other such studies (Jerman *et al.*, 2005; Maiques *et al.*, 2006) demonstrate an alternative means by which dissemination of antibiotic resistance can occur and highlights the need for more stringent controls on the general use of antimicrobials especially with regard to their use in food-producing animals.

Risk assessment reviews on penicillin hypersensitivity revealed a very small number of previously sensitised individuals from whom there is reasonable clinical and documentary evidence that penicillin residues in milk triggered an allergic reaction, usually a rash. Although these cases are very rare (less than 10 cases reported in the last 25 years), this

illustrates the continuing need to control antibiotic residues vigilantly (Dewdney *et al.*, 1991). Anaphylactic reactions to mainly penicillin-based β-lactams are sometimes reported. However, in cephalosporins such cases are very rare indeed occurring in approximately 2 cases per 10,000. In these instances, the dosage required to cause reactions is of a therapeutic order, yet, for the development of hypersensitivity the levels may be much lower.

1.7 Public and Industrial concerns about food safety and its implications

There are also economic concerns, along with the health risks associated with the presence of antibiotic residues in the food chain. Antibiotic residues can affect bacterial starter cultures used in the production of milk-based products such as cheese and yoghurts. This can cost considerable amounts of money in lost, reduced or spoiled production, which is of concern to large and small-scale producers alike.

Public concern with regard to food safety (i.e. the presence of veterinary medicine residues, such as antibiotics in foods) has become a major factor in the establishment of testing programmes. A recent study by the Food Safety Authority of Ireland (FSAI) reported that from a list of unrelated issues that worry consumers, food safety ranked fourth with 53% of the people surveyed. The specific worries that they tended to have were primarily with regard to the presence of pesticide and herbicide residues (70%) and Bovine Spongiform Encephalopathy (BSE) (67%). However, 63% of the people who took part in the survey report having concerns regarding antibiotic residues in foodstuffs. These results were based on a list of prompted responses (FSAI, Consumer Attitudes to Food Safety in Ireland, 2003). A similar study also carried out by the FSAI on industry attitudes to food safety in Ireland placed antibiotic residues sixth on a list of prompted concerns (FSAI, Industry Attitudes to Food Safety in Ireland, 2003).

Consumers are now increasingly aware of potential contaminants in food (e.g. antibiotic residues, pesticides, hormones etc.) and, as a consequence, some of the larger supermarket chains now include a record of the animal origin and statements like "Antibiotic free" on the packaging in the case of certain meat products.

1.8 Current testing techniques for the detection of antibiotics in milk

Within the EU countries, national testing and control programs are compulsory in order to ensure that all foods from animal origin remain free from veterinary drug residues. The EU reference method for the determination of antibiotic and sulphonamide residues in raw and heat-treated milk is the International Dairy Federation (IDF) microbial inhibition test method. This method uses *Bacillus stearothermophilus* var. *calidolactis*, ATCC 10149 as the test organism due to its relatively high sensitivity to inhibitory substances (IDF 1991). The test involves a colour change that is dependant on the growth of *B. stearothermophilus*. However, it is quite complex and time-consuming. Current test kits based on the IDF microbial inhibition test with improved performance characteristics (e.g. Delvotest® SP (Gist-brocades/DSM), Charm Farm Test (Charm Sciences)) are routinely used that utilise *B. stearothermophilus*. The Delvotest® SP test is a broad spectrum test i.e. non-specific detection of a range of β-lactams, tetracyclines, sulphonamides, macrolides and aminoglycosides to name but a few. This test is presently the standard method required by the Department of Agriculture and Food (DAF) in Ireland. The Delvo® SP test is capable of detecting a range of antimicrobial drugs in milk at or below their respective MRL, for example manufacturers declare a sensitivity range of 40-60 µg/kg for cephalexin (FSAI, 2002).

In addition to the above assay kits, so called 'rapid' test methods are also available. In contrast to microbial inhibition tests that are relatively lengthy and used to test for a broad spectrum of antimicrobials, rapid test methods generally require less time and detect specific families of antibiotics. At pasteurising plants rapid antibiotic screening tests are often used prior to the standard Delvo® SP test to avoid delays. Rapid screening methods are based on a variety of assay techniques for example, immunoassays (IDEXX Parallux™ Antibiotic test kit, IDEXX Inc.). This test kit is a solid-phase fluorescent immunoassay (Kumar *et al.*, 1997) for determination of β-lactams, sulphonamides and tetracyclines in milk. It is a laboratory-based test that requires trained staff and expensive equipment (e.g. Parallux Processor). Different families of antimicrobial residues are detected using a variety of available cartridges. There is no sensitivity quoted by the manufacturer for cephalexin. This method is employed as a tanker release test throughout the UK and Northern Ireland (FSAI, 2002) and by a number of companies in the Republic of Ireland. IDEXX also produce the SNAP® Beta-lactam test, which detects a range of beta-lactam antibiotics in raw bovine milk. The kit is more conducive to 'on-site' or farm testing as it is more user friendly than the Parallux™ kit. The SNAP® Beta-lactam test is an enzyme-linked receptor-binding assay, as yet the manufactures state sensitivity to cephalexin is to be confirmed.

Immunoassay-based detection methods for β-lactams have been reported in the literature (Meyer *et al.*, 1999; Gaudin *et* al., 2001) along with other methods describing various methods for determining penicillin and cephalosporin sub-families. These include UV-vis-spectroscopy (Martinez *et al.*, 2002), High Performance Liquid Chromatography (HPLC) (Samanidou *et al.*, 2003), matrix solid phase dispersion (MSPD) (O' Keeffe, 1999), Liquid Chromatography (LC) (Suhren and Knappstein, 2003) and Capillary Electrophoresis (CE) (Hernandez *et al.*, 2003). Such techniques require extensive sample preparation or extraction steps (Fedeniuk and Shand, 1998). Some researchers have also developed novel biosensors in order to detect various β-Lactams. In such cases the antibiotic is detected using an immobilised β-Lactamase enzyme that converts β-Lactams (e.g. penicillin) in the sample to the requisite acid (i.e. penicilloic acid) thus increasing the pH, which can be detected using a glass-pH electrode *in situ* (Patel, 2002).

Overall, immunoassay techniques for the detection of beta-lactams are more conducive to the production of a portable 'user-friendly' device and offer many advantages including, increased specificity, sensitivity and flexibility, in terms of potential target analyte by the incorporation of different specific antibodies for each analyte.

1.9 The Immune system

1.9.1 *Innate immunity*

The innate immune system is characterised by its 'non-specific' nature and consists of two main components. Firstly, the physical barriers including, the skin, self-cleaning barriers (e.g. mucus membranes), stomach acid and non-pathogenic bacterial flora. These represent the first line of external defence. Secondly, the internal innate immune system via the inflammatory response which results in increased blood flow and vascular permeability to affected areas, followed by an localised accumulation of leukocytes (e.g. monocytes and neutrophils) and certain soluble factors. Neutrophils, monocytes and natural killer (NK) cells migrate to affected areas guided by chemotactic factors. Monocytes that exit the circulatory system and enter tissues mature to become macrophages. Neutrophils and macrophages are the functional phagocytes of the body (Paape *et al.*, 2002), whereas NK-cells recognise cells that fail to express major histocompatibility complex (MHC) class I molecules, and lyse these cells through various killing mechanisms.

Phagocytosis is the ingestion of large bodies (e.g. micro-organisms, cellular debris) by formation of endocytic vesicles termed phagosomes. In order for phagocytosis to occur the body must first be bound to the phagocyte surface via specialised cell-surface receptors. In effect this triggers the phagocyte to begin engulfing the body using its plasma membrane, thereby enclosing it in a phagosome. The phagosome then fuses with a lysosome (intra-cellular body containing digestive enzymes) upon which the ingested particle is broken down. Material that can be utilised (e.g. antigenic determinants) is transported initially to the cytosol and indigestible material remains in the lysosome forming residual bodies. Macrophages carry out phagocytosis and play an important role as antigen-presenting cells (APC) by presenting processed antigenic material in association with MHC class II molecule on the cells plasma membrane surface. APC's, including B-lymphocytes and dendritic cells, present surface bound antigen to T-lymphocytes which in turn trigger further cascades via various cytokines. Macrophages also ingest damaged cells, connective tissue matrix and neutrophils that have become apoptotic from phagocytosis.

1.9.2 *Acquired immunity*

If an invading micro-organism survives the external and internal innate immune barriers the adapted or acquired immune response comes into play. The main distinguishing feature of acquired immunity with regard to innate immunity is its high specificity. Acquired immunity can be sub-divided into two discriminate types, humoral and cell-mediated responses. The cell-mediated response, involves only T-lymphocytes, where the active agent is the T-cells. T-cells can be further distinguished into at least four distinct varieties i.e. CD4+ helper (T_{H1} and T_{H2}), CD8+ cytotoxic (T_C), Natural Killer (T_{NK}) and regulatory (T_R) cells.

The humoral or antibody response involves the concerted action of both B- and T-lymphocytes resulting in the production of soluble immunoglobulins (Ig) or antibodies into the blood stream. B-lymphocytes mature in the bone marrow, whereas, T-lymphocytes mature in the thymus gland. Following maturation both cell types migrate to secondary lymphoid organs (e.g. spleen and lymph nodes). Lymphocytes may be naïve or 'primed' (i.e. exposed to specific antigen). Naïve and 'primed' lymphocytes are partly differentiated or fully differentiated, respectively, as triggered by specific antigen binding. Lymphocytes continuously re-circulate in the body 'patrolling' for their respective antigen. Primed lymphocytes home back to their predilection organ, which is the tissue or the draining lymph node of that tissue, where they first encountered their specific antigen (Mackay, 1992). Naïve lymphocytes only traffic the route from blood to peripheral lymph nodes and back to blood via efferent lymph. Lymphocyte trafficking is directed by homing receptors, *i.e.* cell-surface

molecules that selectively interact with molecules on endothelial cells, and these receptors may be locally up-regulated during inflammation (Dailey, 1998).

Upon binding with their specific epitope (i.e. minimum portion of antigen to which the antibody binds), B-cells with membrane bound antibody will multiply and differentiate with the aid of CD4+ T_H-cells. The humoral response requires various similar cell-to-cell interactions to select ideal complementary B-cell clones (with respect to optimum antigen binding). This is mediated by MHC class II. MHC class II molecules are glycoproteins that are exclusively expressed by APC's. They function in the presentation of antigen on plasma membranes of APC including B-cells to the CD4+ T_H cells. This triggers a signalling cascade within T_H-cells resulting in the release of various cytokines which initiate rapid cell proliferation and differentiation (Figure 1.5). B-cells may differentiate into two main variants i.e. 'plasma' and 'memory'. 'Plasma' B-cells are short-lived, characterised by a large endoplasmic reticulum and continuously secret specific soluble antibody. Memory clones persist and confer protection from a particular antigen (i.e. pathogenic bacteria) for extended periods of time. A main function of Igs is to facilitate phagocytosis by acting as opsonins. Moreover, they can activate complement and mediate inflammatory reactions, prevent bacterial colonisation, neutralize toxins, and trigger NK-cells to kill antibody-coated cells (Leibson, 1997; Sanchez-Mejorada and Rosales, 1998; Perussia, 2000; Tizard, 2000; Vivier *et al.*, 2004).

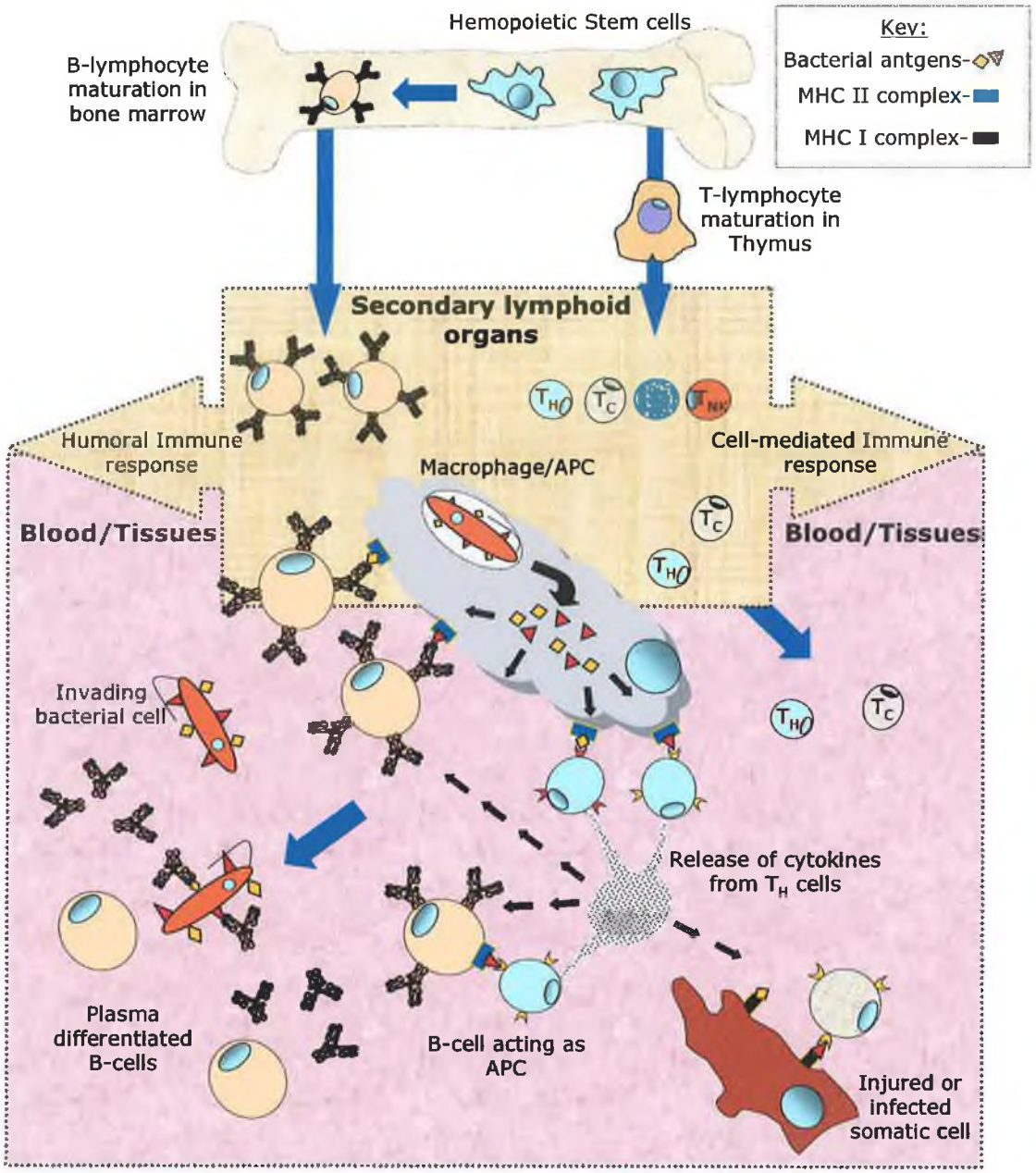


Figure 1.5. A schematic diagram representing a generic overview of the adaptive immune response. Lymphocytes are produced in the bone marrow, where B-cells mature and T-cells migrate to the thymus gland prior to maturation and formation of four distinct cell types (T-Helper $CD4^+$ (T_{H1+H2}), T-Cytotoxic $CD8^+$ (T_C), Natural killer-T (T_{NK}) and T-Regulator (T_R)). Invading bacteria are identified as foreign and are engulfed by phagocytosis (e.g. by a macrophage) where they are systematically dismantled. Macrophages then display processed bacterial antigens (represented as red triangles and yellow squares in above key) in association with MHC class II complex (blue rectangles, above key) on their outer surface for binding of complementary Ig-containing B- or T-cells. B-cells may also act as APC. B-cells containing complementary Ig and T_H $CD4^+$ cells bind to MHC class II antigen and stimulate the generation of a signalling cascade of cytokines. Cytokines direct the T-cell activation and

B-cell differentiation. T_C $CD8^+$ cells may also degrade host cells that display foreign antigens on their surface in complex with MHC class I molecules (represented as black rectangles, as shown in the key).

1.9.3 *Antigens and antibody structure*

Antigens may be of various different chemical classes ranging from biological macromolecules (e.g. peptides, carbohydrates, and nucleotides) to non-biological molecules. The relative molecular mass of the antigen plays an important role in the subsequent immune response, if it has a relatively low (<5 kDa) molecular mass (e.g. illicit drugs, pesticides, antibiotics) it may not be recognised by the organism's immune system. Such compounds are known as haptens. In the production of antibodies this obstacle is overcome by coupling such haptens to larger protein carrier. Antibodies will be generated that are specific to the desired hapten and also the carrier protein. Bovine serum albumin (BSA), ovalbumin (OVA), thyroglobulin (THY), and non-protein polymers e.g. dextran, are generally used for coupling to haptens to elicit an immune response. Antibodies are produced and circulated within the blood of the organism as already stated; such antibody populations are heterogeneous in nature and are termed as polyclonal (i.e. mixture of antibodies reactive to many different epitopes produced by a variety of parental cells). Monoclonal or monospecific antibodies (i.e. specific to a single epitope) can be produced by somatic cell fusion, isolating B-cells and fusing them with a myeloma cell line to produce hybridomas.

Antibodies are globular glycoproteins that are generally referred to as immunoglobulins (Ig). In humans there are five different classes of Ig or isotypes. These can be distinguished biochemically (i.e. each contains its own particular type of heavy chain) and functionally as IgM, IgA, IgD, IgG and IgE. IgG represents a good generic model to use to describe antibody structure since it is most common in serum (70-75% of the total Ig population) and most frequently utilised in immunoassay and biosensor applications (Fitzpatrick *et al.*, 2000). It is stable during isolation, purification and chemical coupling. The IgG class consists of 4 polypeptide chains i.e. two 50 kDa heavy (H) chains each approximately 440 amino acids long and two 25 kDa light (L) chains about 220 amino acids long. There are repeating domains, each with about 110 amino acid residues within both of these chains (Zubay, 1993). Each heavy chain is covalently linked to the corresponding light chain via disulphide bonds. The two heavy chains also linked via disulphide bonds in close proximity to the hinge axis (Figure 1.6).

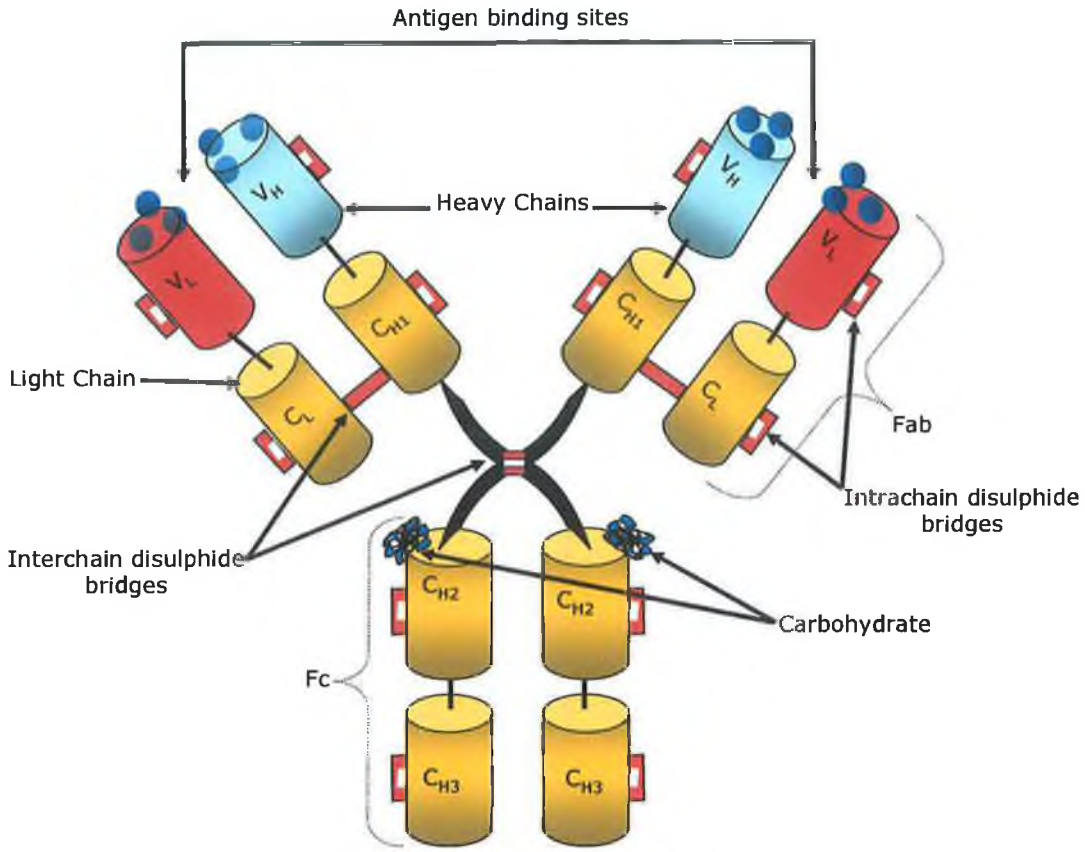


Figure 1.6. The basic IgG structure is shown. The IgG class consists of 4 polypeptide chains i.e. two heavy (H) and two light (L). On the IgG molecule the H and L chains are sub-divided into constant (C) (represented in gold) and variable (V) domains relative to their amino acid sequence. The L chain has both a variable (V_L) (represented in red) and a constant (C_L) and the H chain has a single variable domain (V_H) (represented in blue) and three constant domains (C_{H1}, C_{H2}, C_{H3}). The antigen-binding region containing the complementarity-determining (CDR) loop regions (indicated by 6 blue circles (3X V_H and 3X V_L) within antigen binding site). The antibody specificity and relative affinity (binding strength) to its complementary antigen is determined by the CDRs.

The basic IgG structure may be cleaved using proteases. Papain digestion yields two separate univalent Fab fragments as it cuts the IgG above the hinge axis. The Fc portion of the molecule is so called as it readily crystallises. It carries out important effector functions within the humoral immune response (e.g. triggering phagocytosis of pathogens). Proteolytic digests using the enzyme pepsin have yielded divalent $F(ab')_2$ fragments indicating the cut occurs below the disulphide bonds proximal to the hinge region.

In the IgG molecule the H and L chains are sub-divided into constant (C) and variable (V) domains determined by their amino acid sequences. The L chain has a variable (V_L) domain at the N-terminus end and a constant (C_L) domain at the C-terminal end. The H chain has a single variable domain (V_H) and three constant domains (C_H1, C_H2, C_H3), at N- and C-terminals, respectively. V_H and V_L domains each contain three localised areas of high-sequence variability (hypervariable regions) within the three-dimensional structure form the antigen-binding site. These areas are termed the complementarity-determining (CDR) regions and determine the specificity and relative affinity (binding strength) of the antibody to its complementary antigen. The remaining amino acid sequences of each variable domain are relatively conserved and are referred to as framework regions.

Heavy chains are classified according to amino acid sequence and confer different functional and effector capabilities to resulting immunoglobulins. The four different isotypes of Ig in humans are IgA, IgD, IgE, IgG and IgM. Each contains its own type of heavy chain; designated α, δ, ε, γ, and μ, respectively. The IgG class is further divided into sub-classes IgG1, IgG2a, IgG2b, IgG3 in mice and IgG1, IgG2, IgG3, IgG4 in humans, depending on the number and distribution of disulphide bonds. Antibodies also contain one of two possible light chain forms, designated κ or λ, which differ appreciably in the constant region sequence.

1.9.4 *Antibody synthesis and diversity*

The mammalian humoral immune response has vast potential antibody diversity, expressing as many as 10^{11} distinct antibody binding capacities. The antibody repertoire or binding-diversity is so vast as to make an individual antibody germ-line hypothesis highly implausible. In fact no complete antibody genes exist in germ-line cells; antibody diversity is generated via the two distinct processes of somatic recombination and somatic point hyper-mutation. All antibody polypeptide chains are derived from split genes encoded in separate sections. Antibody genes are assembled during B-cell maturation by somatic recombination, whereby the genes are constructed from various different basal genetic elements or clusters. In humans the chromosomes 2, 22, and 14 encode gene clusters associated with the κ light chains, the λ light chains and the heavy chains, respectively (Zubay, 1993). Elegant studies (Tonegwa, 1983) have demonstrated that antibody gene construction requires the splicing of non-sequential germ-line clusters including variable (V), joining (J) and constant (C) exons to form compete heavy (i.e. V_H, C_{H1}, C_{H2} etc.) and light chain (i.e. V_L and V_H) genes. Heavy chain gene synthesis also involves a diversity (D) element located between (J) and (C) exons. During initial B-cell differentiation, light chain genes are assembled by somatic recombination of germ-line V_L (encoding approximately 95 amino acids on the V_L

polypeptide domain) and J (encoding the remaining V_L domain amino acids) exons. This process is referred to as V-J joining, and occurs by random joining of a single V_L exon (from approximately 300 distinct variations in murine germ-line cells) with one J exon (of a possible 5). Heavy chain genes are constructed in an analogous manner. However, further diversity is introduced into heavy chains via the D genetic element. During the somatic recombination event, heavy chain gene synthesis proceeds by D-J joining followed by V-DJ joining (Figure 1.7). Diversity is initially generated by one of a potential 15 active D segments recombining with one of four possible J segments, followed by recombination with any one of potentially 200-1000 V_H segments. During V-J, D-J and V-DJ gene recombination events, sequence diversity is also promoted by modifications to nucleic acid sequence within these regions termed 'junctional diversity'.

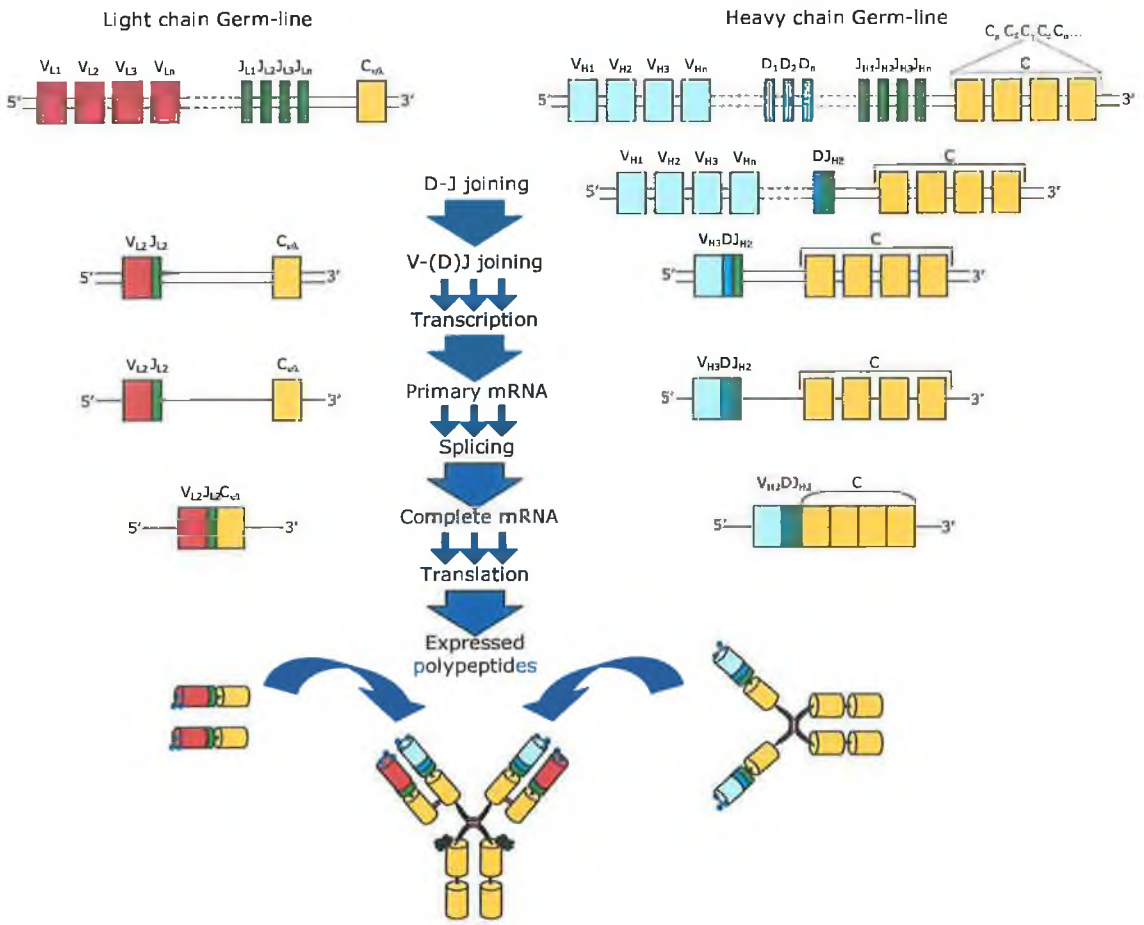


Figure 1.7. This is a representation of the variable light and heavy chain gene construction from germ-line gene segments by somatic recombination. Each respective exon is represented by a coloured box and introns are represented by lines. Light chain variable region genes are constructed by VJ joining, from two sets of distinct segments, variable (V) and joining (J), as shown above on the left. Each constant region is encoded by a spatially separated exon, that is joined by post-translational mRNA splicing to remove introns, in the process forming a complete light chain gene. Heavy chain variable genes are constructed by initial D-J joining followed by V-DJ joining, from three segment groups (V), diversity (D) and (J), as shown above (right). Regions encoding alternative heavy chain constant domains are spatially separated from variable regions by large introns and are joined to variable exons by processing of primary RNA transcript. Light and heavy chain genes are translated into large polypeptide chains that associate to form a complete antibody (IgG depicted above). The diagram presented was adapted from Janeway *et al.* (1999).

The second process mediating generation of antibody-binding diversity is somatic point hyper-mutation. The mechanism of somatic point hyper-mutation of Ig genes is still not fully understood. A variety of models have been put forward that suggest the involvement of DNA polymerase(s), as reviewed by Jacobs and Bross (2001). The hyper-mutation process also requires various other factors as suggested by Storb *et al.* (1998). They proposed the involvement of a mutator factor that loads onto an initiating RNA polymerase. They later report (Longacre *et al.*, 2003) that this mutator factor is possibly a recently discovered activation-induced deaminase (Revy *et al.*, 2000; Muramatsu *et al.*, 2000). Following the hyper-mutation process the repair phase may involve naturally error-prone DNA polymerases (Storb and Stavnezer, 2002; Longacre *et al.*, 2003). Various comparative studies of germ-line and somatic antibody genes have demonstrated that V_L-J_L and V_H-D_H-J_H segments were subject to high frequency mutations during somatic differentiation. The density of observed mutations is about three fold higher within the CDR encoded regions. During the humoral response beneficial mutations within these gene regions are selected for by the presence of antigen. In effect somatic hyper-mutation and junctional diversity gives the immune system the ability to produce antibodies that are progressively 'engineered' to have higher affinity to their respective antigen.

1.10 Monoclonal antibody production by somatic cell fusion

Kohler and Milstein in 1975 (Kohler and Milstein, 1975) first reported the Nobel Prize-winning method of somatic cell fusion between a myeloma and an immune lymphoblast. Each individual resultant hybridoma (*hybrid*-myel*oma*) combines the immortality of the myeloma with the antibody production capabilities of the lymphocyte, thus serving as a stable source of mono-specific antibody. The technique has been refined and many alternative protocols developed. However, all are based on the same core elements.

The technique involves the extraction of lymphocytes from the spleen or lymph nodes of an immunised animal (e.g. mouse) subsequent to their fusion with relevant myeloma cells (non-Ig secretory immortalised cells). The myeloma-lymphocyte fusion product contains two different nuclei, termed heterokaryons, which in due course form a cell containing a single fused nucleus (hybridoma) with genetic information from both parent cells. Hybridomas continue to grow and divide indefinitely compared to lymphocytes, which only grow and divide for a short period of time *in vitro*. The cell fusion process is facilitated using for example, a polywax such as polyethylene glycol (PEG) that enhances the cell fusion process and the transfer of nuclei. Originally the somatic cell fusion was facilitated using Sendai virus, but this was problematic (Sikora and Smedley, 1984). Hybridoma cells secrete a single

antibody type into the growth media or supernatant at a concentration range of 1 to 100 μg/ml (Falkenberg, 1998). Specific antibody activity is identified using a suitable assay. Positive fractions may then be further separated by limited dilution until the expressed antibody is monospecific, or monoclonal, derived from a single parent hybridoma (Figure 1.8). The technique makes it possible to produce and maintain indefinitely homogenous populations of monoclonal antibodies with defined specificities and affinities. Monoclonal antibodies are finding ever-diversifying applications including environmental and food immunodiagnostics and as clinical therapeutics (Fitzpatrick *et al.*, 2000; Jessup *et al.*, 2000).

Increasingly monoclonal antibody production techniques have been coupled with or superseded by recombinant phage-display technologies (Siegel *et al.*, 1997; Burioni *et al.*, 1998; Yoo *et al.*, 2002).

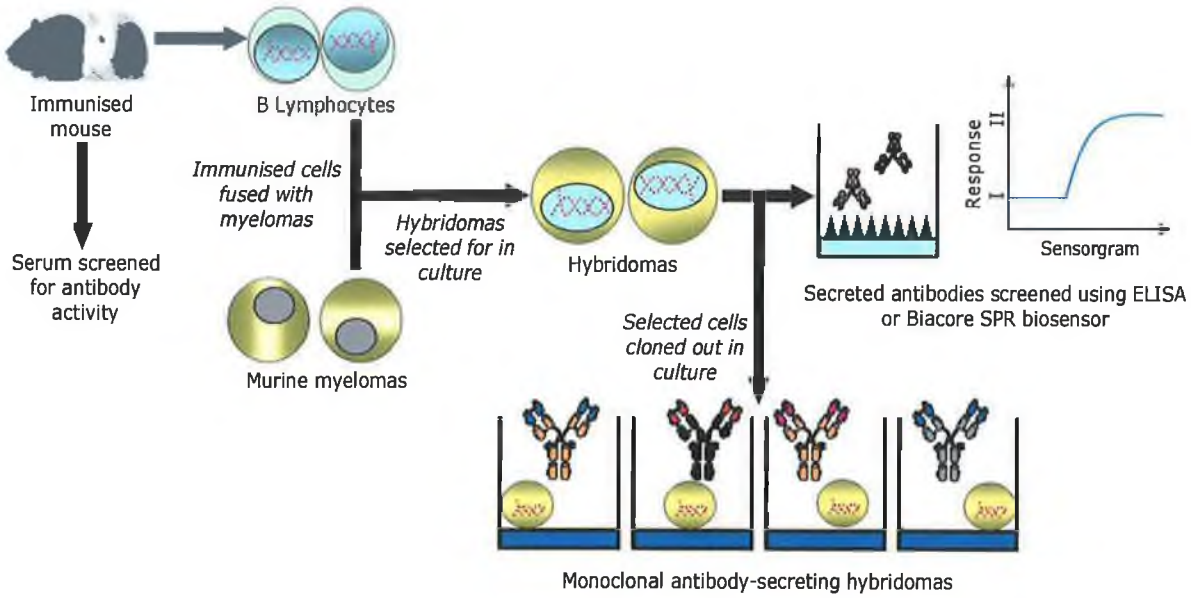


Figure 1.8. Schematic overview of key steps involved in the production of monoclonal antibody-secreting murine hybridoma cells by somatic cell fusion. Initially, immunised mice are screened for specific antibody activity using an appropriate assay technique. Once sufficient antibody titre is obtained lymphocytes are extracted from the animal's spleen or lymph nodes. Lymphocytes are then fused with myeloma cells to produce hybridoma cells that are subsequently screened and cloned out to isolate a single cell type in culture secreting a single antibody idiotype.

1.11 Recombinant Antibody production

1.11.1 *Introduction*

Monoclonal antibody fragments i.e. single chain Fv (scFv) and Fab are now routinely produced using combinatorial library-display technologies. Protein-display technologies including phage-, ribosomal-, and cell surface-display-based methods have revolutionised affinity protein research in general. Such methodologies have accelerated the production, screening and selection of high-affinity monoclonal antibodies to a myriad of different targets. Various antibody libraries have been produced by isolating genetic material encoding antibodies obtained from a variety sources, including hybridoma cell lines, spleenocytes or lymph tissues isolated from pre-immunised (immune) or naïve sources (non-immune). In addition, antibody libraries have been constructed from fully synthetic antibody or antibody 'like' genes yielding novel antigen-binding proteins or 'affibodies' (Fernández, 2004; Nygren and Skerra, 2004). Synthetic antibody libraries consist of stable protein structural framework regions based on Ig domains with an affinity function grafted onto it, thus, yielding novel synthetic binding proteins.

The key factors of display systems are the vast library sizes obtained (i.e. binding function diversity) and the direct link between phenotype (binding function) and genotype (encoding gene), that facilitates the selection and amplification of antibodies with desired characteristics. This technology permits relatively rapid production, identification and isolation of monoclonal antibody fragments with diversity of antigen binding function, novel binding affinities and specificities from vast library repertoires and subsequent engineering to further improve desired characteristics.

The main advantage of recombinant antibody production is its inherent capacity for molecular biology-based manipulations. Using established recombinant techniques antibodies are now routinely produced incorporating affinity tags (e.g. polyhistidine, strepavidin) to aid subsequent purification and detection or immobilisation (i.e. on a biosensor chip). In addition, antibody genes can be expressed in a range of plasmid vectors that enable expression as fusion proteins in bacterial hosts yielding bifunctional or bispecific antibodies. Bifunctional antibodies contain a single antigen-binding capacity covalently linked to a specific biological function, for example an enzyme (e.g. alkaline phosphatase). Bispecific antibodies contain two distinctly different antigen-binding capacities. Recombinant protein-display technologies also facilitate engineering or evolution of proteins. This has enabled enhanced antibody expression, stability and binding characteristics by selection. In effect it is possible to 'tailor-

make' and screen vast numbers of monoclonal antibodies to obtain the optimum characteristics for use as diagnostic or therapeutic agents. Combinatorial antibody library display and selection processes are also conducive to automation that permits the development of high-throughput production and isolation systems.

1.11.2 *Protein display technology*

Overall, protein display technologies can be sub-divided into three main categories, bacteriophage (phage), ribosomal (i.e. mRNA-protein complex) and cell surface (i.e. bacterial) based. All three methods permit the simultaneous selection of high-affinity antibodies and the genetic material that encodes it. Phage-display is by far the most widely used *in vitro* technique for the display of large antibody fragment repertoires and selection of high-affinity clones against many different types of targets. This method will be discussed in greater detail in this thesis. A brief description of ribosomal and cell-surface display techniques will be given. All three techniques are used to produce scFv and Fab antibody fragments as opposed to whole IgG molecules (Figure 1.6).

Ribosomal display methods exploit the cellular role of ribosomes in the synthesis of proteins and are based on the formation of stable antibody, ribosome and mRNA complexes (ARM complex) *in vitro*. This system maintains the link between antibody (phenotype) and mRNA (genotype) together with the ribosome (basal protein-production machinery). A diagrammatic overview of the technique is presented in figure 1.9. One of the main advantages of ribosomal over other display technologies is that it is cell-free thus it permits the production of potentially very large antibody repertoires ($\leq 10^{14}$) (Hudson and Souriau, 2003). Vast repertoires are facilitated as the technique is not limited by microbial cell transformations, does not require any cloning and mRNA products of selection are utilised directly as templates for reverse-transcription polymerase chain reaction (RT-PCR). Since it is a cell-free method it also facilitates the production of antibodies that may be toxic to cellular expression hosts or prone to degradation by intra-cellular proteolytic enzymes. Ribosomal display is limited in that it requires ribonuclease-free environments and optimisation of buffer composition to allow formation of stable ARM complexes with correctly folded antibody. The technique has been extensively reviewed (He and Taussig, 2002).

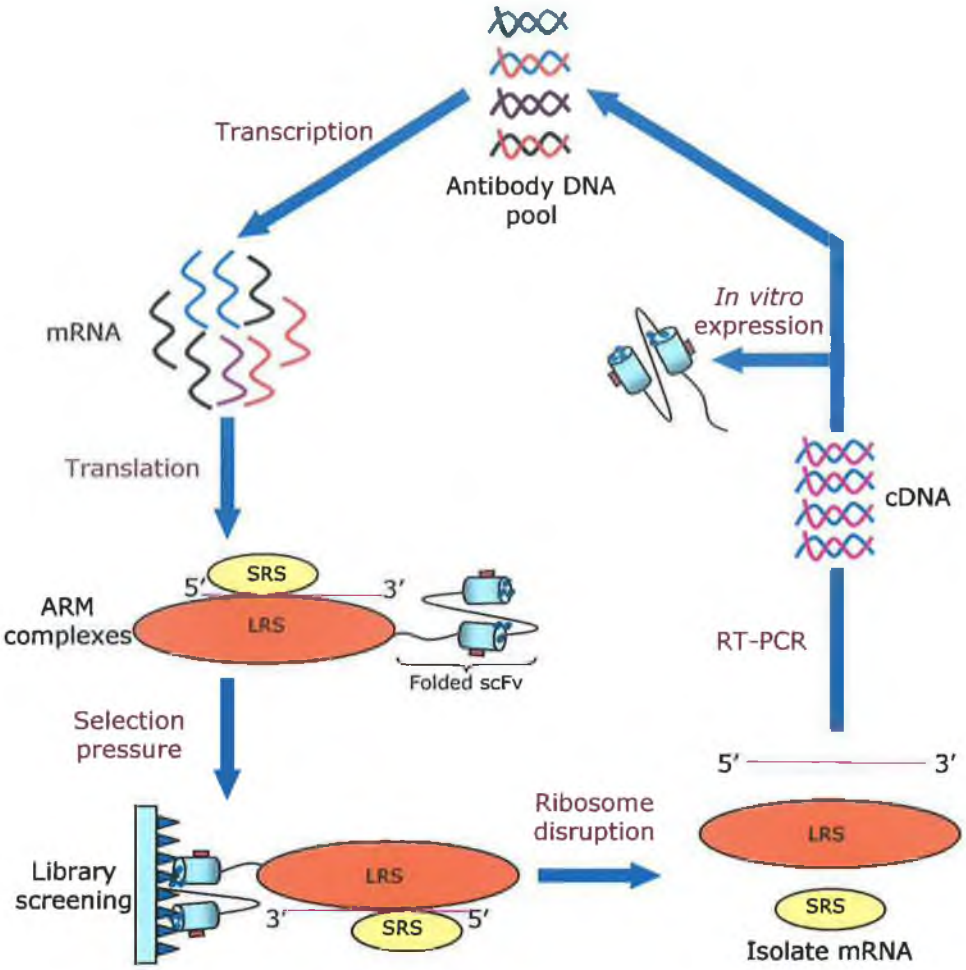


Figure 1.9. Diagrammatic representation of the main steps involved in ribosome-display library production and panning (blue coloured arrows). Firstly, it involves the transcription of antibody genetic diversity yielding mRNA followed by translation mRNA sequences into properly folded antibody by formation of stable ARM complex with large (LRS) and small (SRS) ribosomal subunits under optimised buffer conditions. Panning is carried out on immobilised antigen to select for specific scFv-ARM complexes. Subsequently, the ARM complexes are disrupted and mRNA used as the template for RT-PCR. The resulting cDNA can be subjected to further rounds of selection by repeating the process or used for *in vitro* expression of antibody.

The third recombinant-based selection platform is cell surface display. In this technique proteins or antibodies are displayed on the surface of intact cells, including yeast (e.g. *Saccharomyces cerevisiae*), bacteria (e.g. *Escherichia coli*) and even some mammalian cell types. This method is more analogous to phage-display in that it is a cell-based technique and requires cloning of antibody gene repertoires into cell-based expression systems. Cell-surface display has been found to be less applicable to the generation of large antibody libraries (in the order of $<10^8$ clones). A yeast-display-based method used in conjunction with random mutagenesis techniques to diversify variable heavy (V_H) and light (V_L) domain genes has yielded a very high affinity (48 fM) antibody (Boder *et al.*, 2000).

1.11.3 *Combinatorial phage-display libraries*

A combinatorial phage display library consists of a heterogeneous mix of phage clones each displaying a different functional antibody, typically of the order of 10^9 clones. In the reported literature, phage displayed antibody libraries almost always consist of either single chain Fv (scFv) or Fab fragments. Since the discovery of the technique as a means of displaying peptides on the surface of a bacteriophage (Smith *et al.*, 1980 and Smith, 1985), it has proven to be a robust and powerful means of selecting high-quality antibody fragments. Phage-display was successfully applied to existing hybridoma cell lines, as a means of cloning and expressing their respective antibodies in a recombinant bacterial host, and in the production of combinatorial antibody libraries. Phages (Bacteriophage) are viruses that infect bacterial cells, introducing their genetic material in the process. This permits the introduction of foreign DNA (i.e. natural or synthetic antibody genes) into host bacterial cells (e.g. *E. coli*) and the expression and replication of both protein and genetic material encoding it, as shown in Figure 1.10.

Bacteriophage fd and M13 are filamentous non-lytic bacteriophage, which are the most commonly used phages for antibody phage-display (Hoogenboom, 2005). Phage-display DNA vector systems are usually truncated phage plasmids (termed phagemids) with phage and *E.coli* origins of replication and contain the inserted DNA (i.e. antibody gene) adjacent to a phage coat protein gene (e.g. gIIIp). When expressed within bacterial hosts it produces a hybrid antibody-gIIIp fusion protein. Phagemid vectors do not contain all the requisite genes encoding proteins for complete phage replication and, therefore, helper phage are required in order to produce and package complete virions displaying antibody fragments as fusion partners with coat proteins.

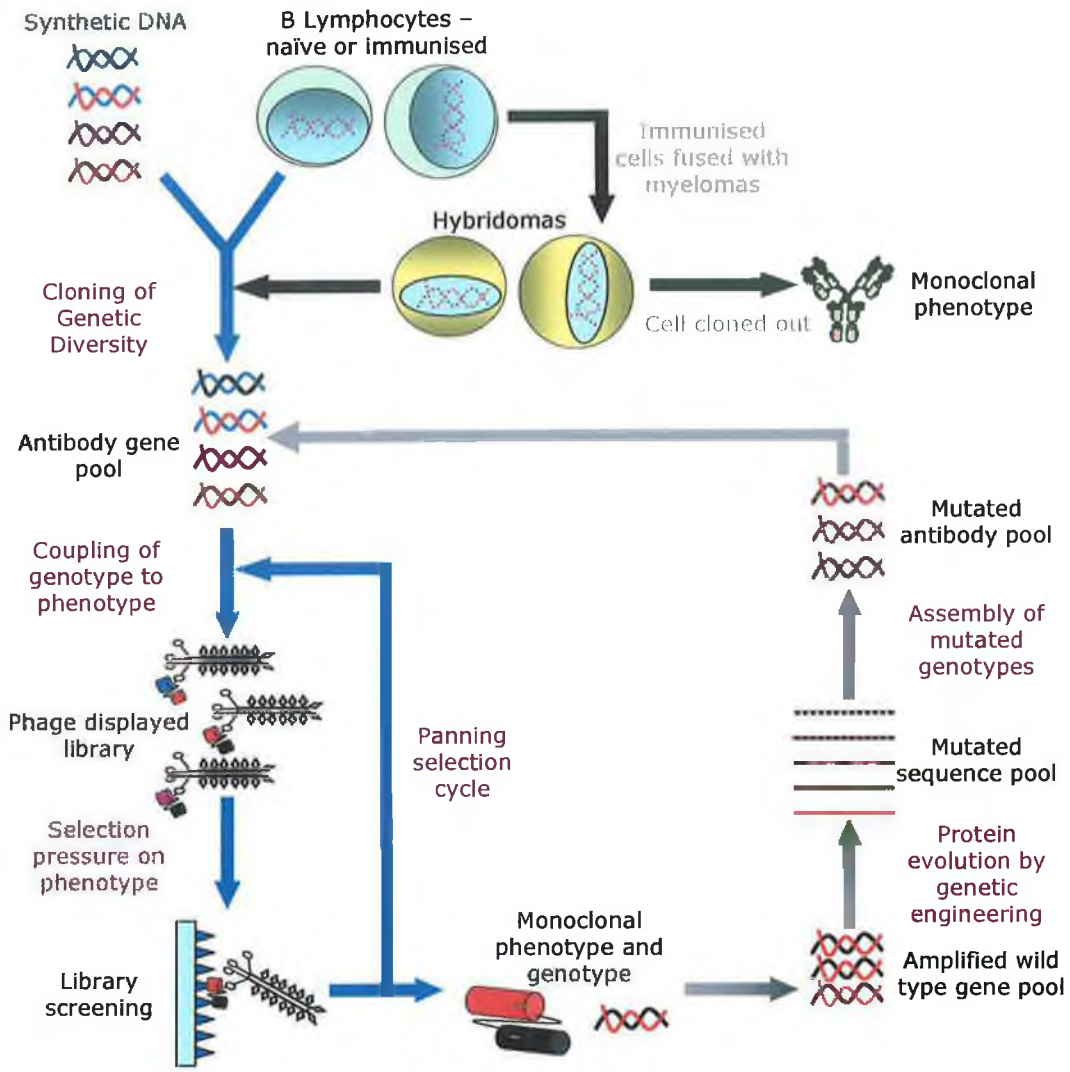


Figure 1.10. Schematic overview of major steps involved in production of phage-display libraries (blue coloured arrows), from initial cloning of genetic diversity and production of antibody gene pool by extraction of mRNA from populations of hybridomas (black arrows) or from B-cells taken from the spleen of a naive / pre-immunised animal. Antibody (i.e. Fab or scFv) cDNA gene constructs are then ligated into a phagemid vector and transfected into competent bacterial cells (e.g. *E. coli* XL1 blue). The antibody fragment is co-expressed with phage coat protein and preferentially packaged into phage particles using a helper phage (e.g. M13-K007), in effect coupling genotype and phenotype. Phage-displayed antibodies are then subjected to a panning selection cycle. Subsequently, the required antibody is obtained by isolating infected single *E. coli* colonies on selective agar. The basic steps involved in the production of mutant libraries are also shown (grey arrows).

Successful phage-display-based methods require very stringent control over the expression of scFv-coat protein due to two main reasons. Firstly, the antibody coat protein fusion is potentially very toxic to the host cell and secondly, as a consequence of the need for subsequent infection with helper phage (superinfection). Superinfection is inhibited by the intrinsic expression of phage coat proteins. Expression is invariably controlled by transcriptional regulation via the *lac* promoter. Alternatively, other regulatory systems, for example the *tet*A promoter (Rau *et al.*, 2002), have been used. Upon infection with helper phage hybrid antibody coat protein fusions are incorporated into the phage particles as they are assembled in the hosts' periplasmic space and then released from the cell. As helper phage contains its own copies of wild type coat proteins (e.g. gIIIp) they will compete with antibody-coat protein fusion for incorporation into the newly forming phage particles. This has been overcome by researchers in various ways, for example, by producing helper phage that are deficient in gIIIp gene and preferentially package insert-containing phagemid into infectious virions (Rondot *et al.*, 2001 and Soltes *et al.*, 2003).

The key advantage of this technique is the link between phenotype (i.e. scFv or Fab displayed on phage surface) and genotype (i.e. the genetic information encoding scFv or Fab contained within the infectious phage virions). Once the phage displaying the antibody infects a new bacterial host, it produces multiple progeny each an identical copy containing the displayed antibody on the surface and genes encoding it within. Heterogeneous polyclonal library populations of phage-displayed antibodies can be 'enriched' for desired binding characteristics by affinity selection or 'panning'. Library panning can be carried out in several ways, as will be discussed in section 1.11.3.2 and can play a major role in obtaining high quality antibodies. Panning strategies are usually user defined and dependant on two main criteria, the pan stringency and desired yield. Increased panning stringency will result in a lower yield of phage; however, increased stringency will yield more optimum clones (i.e. of higher affinity to target antigen). Therefore, a balance between these two factors must be found in the development of a successful panning strategy.

1.11.3.1 *Phage-display library production*

Over the past ten years many different methods and approaches have been described for the production of phage display libraries. Libraries have been produced containing antibody gene repertoires (of the order of 10^{11} individual clones) from immune, non-immune (naïve) and semi- or fully synthetic origins (i.e. based on synthetic PCR products). A number of synthetic scFv and Fab libraries have been reported in the literature (Azriel-Rosenfeld *et al.*, 2004; Lee

et al., 2004; Sidhu *et al.*, 2004) Library quality is generally evaluated in terms of the size (i.e. the number of clones) usually presented as the transformation efficiency and, more importantly, the quantity of different functional clones (i.e. true library diversity).

Although phage-display technology has to a degree negated the need for animals in the production of antibodies, display libraries are commonly produced from immunised organisms. Organisms used as genetic donors in phage library production include mice, human and recently chickens (Finlay *et al.*, 2005). Each organism should be taken on their individual merits (e.g. number of primers required to amplify all potential antibody variants from germline sequences) with regard to what is required by the user. Generally the underlying principles of library synthesis remain the same. Methodologies mainly differ in:

(i) The number and types of primers used for V_H and V_L gene amplification (dependant on consensus antibody germline families).
(ii) DNA vectors (e.g. phagemid or phage) may contain different affinity tags and sites for binding restriction enzymes, used to introduce antibody genes into vectors in the correct orientation.
(iii) Phage coat protein used for fusion partner to antibody.
(iv) Helper phage used for library rescue (e.g. selectively infective as opposed to non-selective helper phage).

Overall antibody gene constructs are assembled using PCR. The V_H and V_L domain genes may be intersected by encoded linker sequence of various lengths (scFv format) or contain terminal cysteine residues for disulphide bridge formation (dsFv format). Fab antibody fragments are produced by the inclusion of a constant domain gene into the construct. The majority of libraries produced utilise the scFv format. However, many Fab libraries are also reported. Phage-display systems also incorporate various polypeptide tags into the expressed antibody to aid in downstream purification and detection. A comprehensive evaluation of affinity tags is presented by Lichty *et al.* (2005). Antibodies are almost invariably screened by ELISA using commercial labelled secondary antibodies specific to M13 phage or FLAG polypeptide tag for example. The appropriate system is defined by the specific users' requirements.

A good representative example system for phage-display library production is described by Krebber *et al.* (1997). This work describes the production of a murine single chain Fv (scFv) antibody library from initial RNA extraction and cDNA synthesis to scFv gene construction by a splicing by overlap extension (SOE) PCR. The technique was used to develop

recombinant scFv antibodies from hybridomas and immunised mice spleenocytes. The system lists a full set of murine antibody gene primers (designed to cover all known potential V_H and V_L genes) based on consensus sequence analysis. Primers also incorporated the necessary restriction sites required for the incorporation into compatible DNA vectors. The protocol utilised *Sfi* 1, a single rare cutting restriction enzyme. The scFv gene constructs consist of V_H and V_L domains joined by a region encoding a flexible 20 amino acid glycine serine repeat linker.

In initial library production antibody gene constructs are cloned in frame with a gIIIp gene, for expression as fusions with gIIIp minor phage coat protein using the pAK100 phagemid vector. Expression is tightly controlled using the *lac* promoter system. Transcription may be inhibited by culturing transformed *E. coli* cells in growth media supplemented with glucose. Alternatively, transcription may be induced by removal of glucose and addition of isopropylthiogalactoside (IPTG). Once suitable monoclonal scFv has been identified the system allows scFv expression as a single entity (i.e. not as a fusion with gIIIp) by transferring into an *E. coli* strain that recognises amber stop codons. The amber stop codon is present between scFv and gIIIp genes on the pAK100 phagemid. Alternatively enhanced soluble expression is facilitated using the compatible vector series. The scFv construct can be excised using *Sfi* 1 restriction sites and can be sub-cloned into the pAK400 vector. This vector contains no gIIIp gene downstream of inserted scFv and soluble expression is enhanced by incorporation of a Shine-Dalgarno translational initiation sequence. The authors also expressed scFv in dimeric form using the pAK500 vector that introduces a protein helix (leucine zipper) into the scFv structure that promotes dimerisation by association. The pAK600 vector permits the production of bifunctional scFv by expression of a scFv-alkaline phosphatase fusion hybrid. Purification and detection of scFv is facilitated by incorporation of FLAG, six histidine residues and myc polypeptide tags.

In addition to generating combinatorial libraries from immunised source organisms, so called 'single-pot', universal or naïve phage-display libraries have been produced and offer alternative vast antibody repertoires for panning with potential target antigens. There are a range of universal naïve antibody libraries currently available including n-CoDeR™, a Fab library from Bioinvent (www.bioinvent.com), BMV, a scFv library from Cambridge Antibody Technology (www.cambridgeantibody.com), HuMade™, a scFv library from Crucell (www.crucell.com) and the HuCAL*Gold Fab library from MorphoSys (www.morphosys.com). Due to their commercial nature little information regarding their exact production methods is made public.

The Human Combinatorial Antibody Library (HuCal) (Knappik *et al.*, 2000) is a fully synthetic library. Library production was performed by analyzing the human antibody repertoire in terms of structure, amino acid sequence diversity and germline usage. Knappik and co-workers found that 95% of human antibody diversity was represented by seven variable heavy (V_H) and seven variable light (V_L) framework germline gene families. Framework consensus sequences for each family were derived and optimised for expression in *E. coli*. Synthetic genes were modular by construction and mutually compatible by design which facilitated introduction of different complementarity determining regions (CDRs). Hence the library binding diversity was increased using CDR gene regions diversified by randomising CDR3 regions of both V_H and V_L domain master genes. The randomised CDR3 regions were generated from mixed trinucleotides and introduced into the modular framework genes using optimised restriction sites. The modular nature of this library facilitated rapid evolution of binders by selection and modification with pre-built CDR cassette libraries. This work was applied to scFv and Fab antibody fragment formats.

1.11.3.2 *Library panning strategies*

Phage-display technology is tailored for the selection of antibodies with the optimum expression and affinity from vast antibody repertoires. Selection of the highest affinity antibodies is largely determined by the panning process. The simplest method of panning a library is based on ELISA, where immobilised antigen is incubated with prepared polyclonal phages (Figure 1.11). Non-specifically bound phages are removed by multiple washing steps and specific scFv-phage eluted using a gentle acidic or basic solutions. Alternatively, phage antibodies may be eluted using excess antigen in solution or by successively lowering soluble antigen concentration after each round of panning thereby increasing the selective pressure toward high affinity clones. Following this, retained phages are used to infect *E.coli* in logarithmic growth phase, in effect multiplying up the specific binding population. Polyclonal phage-antibody populations may be subjected to successive rounds of panning to greatly enrich the specific binding population. Typically, three or four rounds of panning are carried out on library populations depending on the library quality. This is necessary due to non-specific binding limiting the enrichment of optimum antibody binders.

Monoclonal antibodies (i.e. scFv or Fab) can be obtained simply by isolating single phage infected *E. coli* colonies on selective agar after successive rounds of the panning process. On the whole, the panning strategy should take into account the potential function of the desired antibody. For example, if the antibody is going to be used to detect a protein target in aqueous

solution, it should be selected for under those conditions, as solid phase immobilisation or adsorption conditions may alter the true epitope conformation.

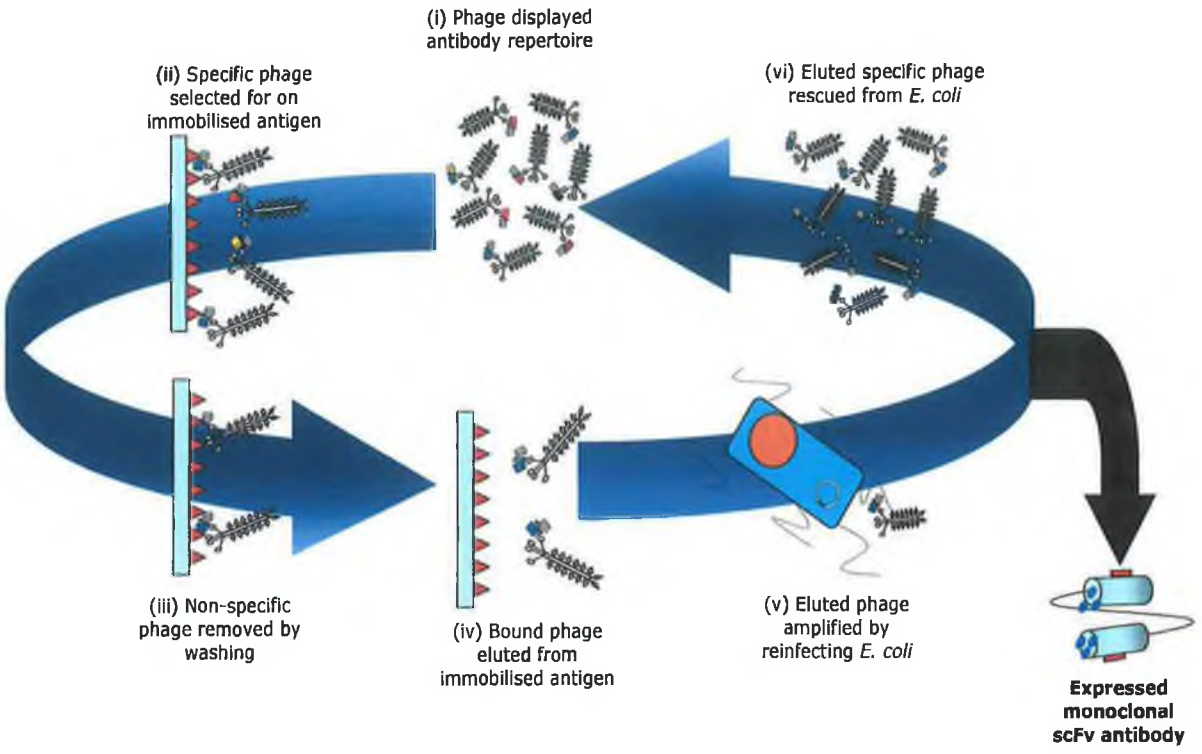


Figure 1.11. Diagrammatic representation of typical steps involved in basic panning of combinatorial phage-displayed libraries. (i) The polyclonal phage-displayed antibodies are packaged and isolated from *E. coli.* (ii) Phages are the subjected to selective pressure on immobilised antigen. (iii) Non-specific phages are removed by repetitive washing using appropriate buffer. (iv) Specific bound phages are eluted from immobilised antigen using specific conditions (i.e. acidic or basic solutions) (v) Exponential growth phase *E. coli* containing F' plasmid are infected by eluted phages. Following infection monoclonal phage-antibodies can be obtained by isolating single infected colonies on selective agar (represented by black block arrow). (vi) Panned phages amplified in *E. coli* are purified from bacteria and subjected to subsequent rounds of panning.

If, however, target antigen is a hapten i.e. small molecules (<5 kDa) higher affinity clones can be selected for by successively limiting both the concentration of free antigen over subsequent panning rounds and the contact time between phages antibodies and target. Panning with complex antigen preparations (e.g. whole cells, cell extracts or hapten-protein conjugates) can utilise subtractive panning. This involves removing potential non-specific binding phage antibodies from the library population by panning with the non-target components of the

mixture and discarding the panned phage. This reduces the non-specific phage scFvs in the population. Novel panning strategies have included combining cell display and phage display libraries, as reported by (Benhar *et al.*, 2000). The "delayed infectivity panning" or DIP technology described used bacterial cells to display the target antigen on their surface. These cells were used for capture of specific-phage and as subsequent hosts for scFv-displaying phages. A reverse variation of the DIP method was also applied to an epitope mapping technique (Mazor *et al.*, 2005), by displaying a characterised scFv on a bacterial cell surface and using this to capture phage-displayed polypeptide variants of the epitope region.

In the first instance phage-displayed antibodies are generally screened for potential antigen binding by phage ELISA in microtitre immunoplates. Subsequent to expression and purification, soluble antibody fragments (i.e. scFv or Fab) may be further characterised in displacement or affinity assays using biosensors (e.g. Biacore™ series of surface plasmon resonance (SPR)-based biosensors). Novel library screening systems have been developed based on engineered display systems that facilitate arraying. The phage antibodies are expressed as cellulose-binding domain (CBD) fusions with immobilisation onto cellulose-based filters followed by probing with appropriately labelled antigen (Azriel-Rosenfeld *et al.*, 2004).

1.11.3.3 *Antibody engineering*

The potential application of antibodies as analytical and therapeutic tools has been greatly enhanced as a direct consequence of recombinant display technology and protein engineering techniques. Using established molecular biology-based methods it is now possible to improve or alter antibody characteristics in terms of binding affinity or avidity, stability or cross-reactivity and intrinsic immunogenic properties (i.e. 'humanised' antibodies). Antibodies can be manipulated by linking them *in vivo* to moieties that allow enhanced signal transduction or improved immobilisation strategies to facilitate application in novel assay formats or bio-sensing devices (Killard *et al.*, 1995; Darmanin Sheehan *et al.*, 2003; O'Kennedy *et al.*, 2005).

There are two basic ways in which antibodies can be engineered using recombinant methods (Figure 1.12). Firstly, antibodies may be produced in multimeric form, as bispecific entities or as fusion proteins with biologically active moieties. Antibody functional affinity (avidity) may be enhanced by expressing scFv monomers as a 'diabody' (dimeric scFv with divalent binding capacity), 'triabody' (trivalent) or 'tetrabody' (tetravalent). Multiple binding domains and dual specificities (bispecific antibodies (bsAbs)) have been readily combined to yield

novel binding constructs (Lu *et al.*, 2003). Antibody gene sequences may be also mutated to beneficially alter binding characteristics, expression profile and stability. Antibody engineering, in combination with phage-display, removes the requirement for extensive theoretical-based binding site design approaches, as nascent antibodies can be quickly identified and enriched by panning. This combined technology has been successfully applied to 'humanise' murine antibodies (Mazor *et al.*, 2005) by grafting murine heavy and light chain CDRs into human framework regions. Antibody engineering will have profound implications on the use of antibodies as therapeutic agents and the potential applications of immunodiagnostic devices.

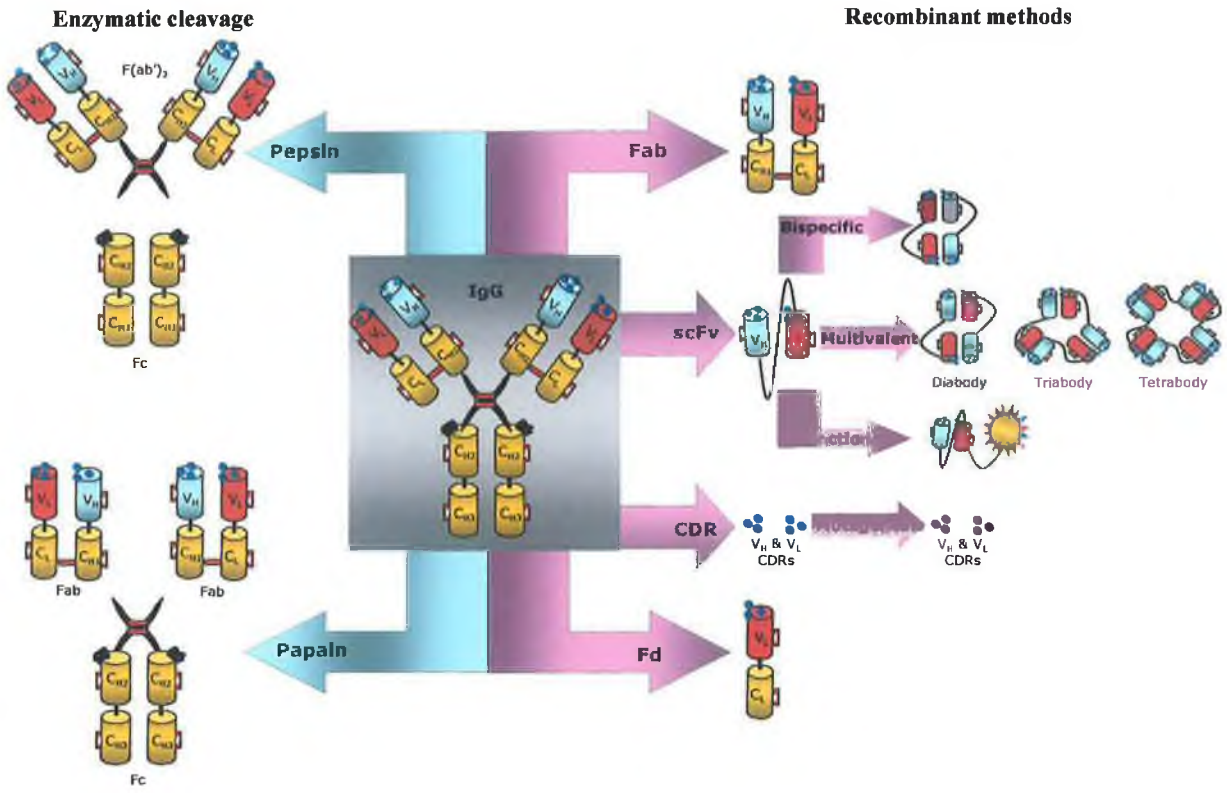


Figure 1.12. Illustration of potential antibody fragments produced by enzymatic cleavage and recombinant methods. Chemical and enzymatic cleavage methods have yielded several antibody-derived fragments including the Fab monomer (antigen-binding) by digestion using papain and $F(ab)_2$ dimer using pepsin, and readily crystallisable Fc fragments. Recombinant production techniques have yielded Fv fragments (V_H and V_L with the CDR region), Fab and single chain Fv (scFv) fragments consisting of an Fv with a linker peptide. Antibody engineering has led to the production of bispecific and bifunctional antibodies. Multimeric scFvs have been produced by altering linker length. Diabodies, triabodies and tetrabodies, consist of two, three and four associated scFv monomers, respectively. Molecular-biology techniques also permit mutation of individual CDR regions followed by incorporation into stable framework regions. The multiple domain structure of antibodies facilitates chemical, enzymatic and genetic manipulations to yield novel antibody derivatives or fragments that are stable and retain strong affinity towards the relative antigen.

Various methods described permit two antibody specificities to be combined in single molecules in the process forming 'bispecific' antibodies (Lu et al., 2003 & 2004). Work in this field has mainly focused on the development of dual targets for tumour associated antigens (TAA) and immune effector cell surface receptors. The potential of bispecific antibodies as cancer therapeutics is extremely promising with clinical trials for cancer treatment being carried out on a number of bsAbs. Traditional means of producing bsAbs used complex chemistries (chemically cross-linking two F (ab') fragments at hinge region) or using hybrid-hybridoma or quadroma production using tissue culture techniques (Nolan and O'Kennedy, 1993; Segal *et al.*, 2001). These methods invariably resulted in poor yields of heterogeneous product, as well as being problematic and expensive.

Bifunctional antibodies have been produced by expressing antibodies as chimeric fusion proteins with reporter moieties or affinity ligands *in vivo,* using phage-display techniques. Hence, scFvs can be expressed as a fusion partner with biologically significant domains while retaining biological function (Berdichevsky *et al.*, 1999; Cloutier *et al.*, 2000; Brennan *et al.*, 2003; Santala and Lamminmäki, 2004). Cloutier and co-workers described the production and application of an expression vector that permits *in vivo* biotinylation of scFv antibodies by subsequent expression in *E. coli* of scFv-BAD (biotin acceptor domain) fusion. The BAD domain is a 15 amino acid long peptide that is biotinylated in the presence of BirA enzyme. Thus, they produced *in vivo* biotinylated-scFv that was then purified using affinity chromatography. Biotinylated-scFv subsequently was tetramerised on strepavidin molecules to create what they termed a StAb or 'streptabody'. Competitive assays using StAbs showed an avidity improvement of one log in comparison to the relevant monomer-scFv.

A cellulose binding domain (CBD) is another example of high-affinity tag that was successfully expressed as a scFv fusion partner. Berdichevsky and colleagues (1999) reported a phage-display method that utilises the high affinity of CBD to cellulose as a novel means of phage capture and elution, demonstrating in the process that scFv-CBD fusion was expressed as monomer and retained both antibody and cellulose binding functions. This system offers an alternative enrichment step during the library panning process that reduces the potential capture of 'insert loss' virions. It may prove advantageous over more commonly used affinity tags as cellulose is cheap and readily available in pure form.

Other workers have developed expression systems for producing *in vivo* fluorescently labelled scFvs in prokaryotic (Griep *et al.*, 1999; Morino *et al.*, 2001) and eukaryotic cells. One report (Piepp *et al.*, 2004) describes the production of two sets of eukaryotic expression vectors that permit the expression of scFv fusions with mutant form of green fluorescent protein (GFP+)

and red fluorescent protein (DsRed) in insect and mammalian cells. The scFv-fluorescent protein fusions were applied in fluorescence activated cell sorting (FACS). The advantages of such *in vivo* methods for covalently linking individual components over more traditional chemistry-based ones are numerous. They typically yield more homogenous product and remove the need for laborious optimisation of chemical approaches and separation techniques.

The second main approach to antibody engineering is carried out by random or site-directed mutagenesis, or variable chain 'shuffling' techniques. These techniques have been used on scFv or Fab gene sequences to beneficially alter binding characteristics, expression profile or intrinsic stability. Researchers have taken many different approaches to what is in essence the 'evolution' of antibodies to obtain desired characteristics. Recombinant display technologies facilitate antibody evolution and selection, by mimicking, to an extent, the natural acquired immune system. Random or point mutations can be introduced into various antibody genes or particular gene regions followed by mutant antibody phage library construction and panning. Work in this area has focused on a variety of obstacles to ideal antibody design. This includes, for example, overcoming problems associated with transfer from eukaryotic (i.e. hybridomas) to prokaryotic (i.e. *Escherichia coli*) expression systems, detrimental immune responses to potential immunotherapeutics and generally improving the overall antibody affinity or specificity characteristics.

In vitro affinity maturation has been successfully achieved by numerous researchers yielding antibodies with increased affinity in comparison to the original or 'wild type' antibody. One such study (Schier *et al.*, 1996) describes the mutation of V_H and V_L CDR3 regions found in a human anti-c-erbB-2 tumour antigen scFv, previously isolated from a naïve phage-display library. Mutations were introduced stepwise, firstly into wild type V_L CDR3 gene sequences using a designed oligonucleotide which partially randomised 9 amino acid residues located in this region. A phage-display library of the resulting mutant clones was constructed and subsequently panned for c-erbB-2-specific scFv. The highest affinity clone obtained was found to have a 16-fold lower dissociation rate constant (K_d) with respect to wild type. Following this V_H CDR3 mutant libraries were constructed from the new mutant clone. Panning of these libraries yielded clones with a further 9-fold decrease in K_d. They obtained a scFv with 1230-fold overall increase in affinity compared to original wild type by combining the mutations isolated from the individual V_H CDR3 libraries.

Alternative methods of producing mutated phage-display antibody libraries have yielded different antibody improvements. Random mutagenesis of wild-type antibody genes

combined with cleverly designed panning strategies have yielded antibodies with improved affinity and cross-reactive characteristics (Zhang *et al.*, 2003). Other studies utilised random mutation of the entire scFv gene by error-prone PCR to produce antibodies with increased or broader specificity ranges (Korpimäki *et al.*, 2002). In this work a phage-displayed scFv was produced from monoclonal antibody-secreting hybridoma cells specific to sulphonamide antimicrobials and used to construct a mutant scFv library. Mutant scFv gene sequences were produced using wild type scFv gene as template for error-prone PCR (Fromant *et al.*, 1995, Cadwell and Joyce, 1998). Mutant scFv obtained was capable of detecting a broader range of sulphonamide compounds in comparison to the wild type antibody. The authors of this work later reported further improvement of the scFv obtained in terms of its sulphonamide specificity range, by using a site-directed mutagenesis approach (Korpimäki *et al.*, 2004).

Antibodies with increased as opposed to broader specificity, have been successfully obtained using variations of this technique. Saviranta *et al.*, (1998), used an anti-17β-estradiol Fab that was previously isolated and characterised, to fabricate a mutant phage-display library. Mutant Fab genes were produced using error-prone PCR to amplify V_H domains and subsequently used to construct Fab genes. The library was selected for decreased testosterone (TES) cross-reactivity by the addition of a large excess TES as a competitor to the panning reaction. Resulting mutant Fab clones retained their anti-17β-estradiol activity and displayed a 20-fold improvement over original wild type Fab with regard to their potential to cross-react with TES. Random point mutation and variable chain shuffling (discussed later in this section) techniques were combined to alter antibody specificity and assay sensitivity to similar antigens. The original scFv antibody was s-triazine specific and following manipulations clones were isolated with a 22-fold lower IC50 to the related pesticide atrazine (Rau *et al.*, 2002).

Random mutations may also be introduced *in vivo* by transforming mutator strains of *E. coli*. Such cells, introduce point mutations at a substantially higher rate in comparison to normal strains following transformation. An example protocol (Coia *et al.*, 2001) was reported that utilises various mutator strains of *E. coli* to facilitate the introduction of a random point mutation per kilobase of target DNA sequences, by adjustment of bacterial growth conditions. The authors have used this method in parallel with phage-display and selection to obtain scFv antibodies with enhanced affinities and increased expression levels.

Another observable benefit of antibody engineering, in conjunction with recombinant display technologies is the selection of clones with improved expression levels. Mutant antibodies may gain advantage from engineering by, for example, point mutations contained within

framework regions that have little or no effect on affinity and permit elevated expression in prokaryotic cells. Enhanced expression results in greater antibody yields and is directly selected for during the panning process.

In contrast to methods based on random or site-directed mutagenesis within variable heavy and light chains some workers have focused on exploring the potential of chain shuffling. This involves cross-pairing V_H and V_L chains isolated from different antibodies. One report (Collet *et al.*, 1992) describes using a binary system of replicon-compatible plasmids to test potential promiscuous recombination of V_H and V_L chains within sets of human Fab fragments isolated from combinatorial antibody libraries. The results of this work found that V_H chain could recombine with multiple V_L chains while retaining antigen binding. Although crosses showed similar apparent binding constants, they mainly differed in amount of active Fab expressed in the bacterial host. This work implicated V_H chain dominance in antigen-binding by exploring several further direct chain crosses using V_H and V_L chains derived from antigen-specific and nonrelated Fab clones. They found that only crosses containing V_H derived from antigen-specific clones retained binding activity. This work illustrates a method for potentially improving the stability of antibody obtained from bacterial cultures and implicates the V_H chain as an optimum site for mutagenesis with regard to engineering binding characteristics. Antibody stability is a key factor in their performance as potential biopharmaceuticals and analytical tools. Stability of scFv antibody fragments depends largely on the intrinsic stability of its constituent V_H and V_L domains as well as the stability of the interface between each domain (V_H and V_L). These are properties dependant on particular individual sequences. Wörn and Plückthun (2001) have extensively reviewed scFv stability engineering. A range of engineering techniques have been successfully applied to overcome poor stability. These include, for example, antigen-specific CDR grafting onto stable framework regions and variable light-chain shuffling (Rojas *et al.*, 2004).

1.12 Thesis outline

The aim of the work presented in this thesis was the production of polyclonal, monoclonal and genetically-derived scFv antibody fragments for the detection of the beta-lactam antibiotic residue, cephalexin, in milk. The work describes the production and characterisation of polyclonal (Chapter 3) and monoclonal antibodies, using hybridoma (Chapter 4) and recombinant phage-display technologies (Chapter 5). Antibodies were used in the development of ELISA and biosensor-based immunoassays capable of detection of cephalexin below its listed maximum residue limit (MRL) in milk.

A polyclonal antibody was generated from cephalexin-bovine serum albumin (BSA) conjugate, cephalexin conjugates were produced by covalently binding it to various proteins for use as immunogens and analytical reagents. A panel of monoclonal antibodies was produced by somatic cell fusion procedures.

Recombinant antibody production techniques included the production of phage-display libraries from pre-immunised mice, the phage-display of scFv constructed from hybridoma cells and panning of a naïve human combinatorial library (BMV library). A mutant phage-display library was generated using positive scFv-secreting clone using random mutagenesis and error-prone PCR. Phage-display libraries were panned using cephalexin conjugates and bound phages were retained by competitive cephalexin elution and with mild acid shock elution strategies. Soluble scFv antibodies were isolated, expressed and purified from mutant libraries. Selected clones were sequenced and point mutations were identified in CDR and structural framework regions.

All antibodies were purified and characterised by ELISA, SDS-PAGE and Western blotting. Subsequently, antibodies were evaluated in the development of inhibition immunoassays for the detection of cephalexin 'spiked' samples of PBS and whole milk (pasteurised and homogenised). Assays were initially carried out by ELISA and transferred to the SPR-based BIAcore 3000™ 'real-time' biosensor. The research encompassed the development of ELISA, and BIAcore-based displacement assays for the detection of cephalexin in milk. Working assays with desired performance characteristics (i.e. sensitivity and reproducibility) were validated. A range of semi-quantitative ELISA and Biacore-based assays were developed for the specific detection of cephalexin in milk. Antibodies produced were generally capable of detecting cephalexin at and below the MRL in milk, in ELISA and BIAcore-based assays.

Chapter 2

Materials & Methods

2.1 General Formulations

2.1.1 *Reagents and chemicals*

All standard laboratory reagents were purchased from Sigma Chemical Co., Poole, Dorset, England and were research grade unless stated otherwise.

Table 2.1. Reagents used and suppliers.

Reagent	Supplier
Acetic acid Hydrochloric acid	Riedel de-Haen AG, Wunstorfer, Strabe 40, D-30926, Hannover, Germany.
Blue Ranger® pre-stained molecular weight markers BCA protein assay kit	Pierce, 3747 North Meridian Rd., PO box 117, Rockford, IL 61105, USA.
Amine coupling kit	BIAcore AB, 2 Meadway Court, Meadway Technology Park, Stevenage, Herts SG1 2EF, UK.
DNA ligase	Boehringer-Mannheim Ltd., East Sussex, UK.
Briclone	Archport Ireland Ltd., Dublin City University, Glasnevin, Dublin 9, Ireland.
Bacteriological Agar Tryptone Yeast Extract Phosphate buffered saline tablets	Oxoid, Basingstoke, Hampshire, RG24 8PW, UK.
Trizol Foetal calf serum L-Glutamine (100x) (200mM) Non-essential amino acids (100x) Sodium pyruvate (100mM)	Gibro BRL, Renfew Rd., Paisley PA49RF, Scotland.
PCR Optimizer kit	Invitrogen, 9704-CH-Groningen, Netherlands.
PCR Primers	MWG-Biotech Ltd, Milton Keynes, MK12 5RD, UK.
Perfectprep® gel cleanup kit	Eppendorf AG, 22331, Hamburg, Germany.
Wizard® Plus Mini-prep kit PCR prep DNA purification kit Reverse-Transcription kit RQ1 RNase-Free DNase 1 and buffer	Promega Corporation, 2800 Wood Hollow Rd., Madison, WI 53711-5399, USA.

Restriction enzymes and relevant buffers	New England Biolabs, Hitchin, Herts., England.
VCSM13 Bacteriophage	Stratagene, North Torrey Pines Rd. La Jolla, USA.

2.1.2 *Consumables*

Table 2.2. Consumables used and suppliers.

Consumable	Supplier
1.5 ml and 0.5 ml micro-centrifuge tubes, 50 ml (falcon) polypropylene tubes, pipette tips, Sterile 96-well plates	Sarstedt Ltd., Sinnottstown lane, Drinagh, Wexford, Ireland.
Sterile 20 ml universals	Medical Supply Company, Damastown, Mulhuddart, Dublin 15, Ireland.
Nunc Maxisorp™ 96-well plates Tissue culture plastic-ware	Nunc, Kamstrup, DK, Roskilde, Denmark.
Nitrocellulose membrane	Schleicher and Schuell Bioscience GmbH, Hahnestrasse 3, D-37586, Dassel, Germany.
Acrodisc® 0.2 µm syringe filters Omega™ 10 and 50 kDa membrane filters	PALL Corp., Newquay, Cornwall, UK. (Distributed by VWR International Ltd., Rath Business Park, Ashbourne, Co. Meath, Ireland.)
Durapore® 0.2 µm and 0.45 µm membrane filters	Millipore, 290 Concord Rd., Billerica, MA 01821, USA. (Distributed by AGB Scientific Ltd., Dublin Industrial Estate, Dublin 11, Ireland.)
BIAcore CM5 sensor chip	BIAcore AB, 2 Meadway Court, Meadway Technology Park, Stevenage, Herts SG1 2EF, UK.

2.1.3 *Equipment*

Table 2.3. Equipment used and suppliers.

Equipment	Supplier
BIAcore 1000™ BIAcore 3000™	BIAcore AB, 2 Meadway Court, Meadway Technology Park, Stevenage, Herts SG1 2EF, UK.
SB1 Blood tube rotator	Stuart Scientific, Holmthorpe Industrial Estate, Redhill, Surrey, UK.
Heraeus Christ Labofuge 6000 Biofuge A Microcentrifuge	Heraeus Instruments Inc, 111-A Corporate Boulevard, South Plainfield, New Jersey, USA.
Beckman J2-J21 centrifuge	Beckman-Coulter Inc., 4300N Harbour Boulevard, South Plainfield, New Jersey, 07080, USA.
Sorvall RC-5B refrigerated centrifuge	Du Pont instruments, Newtown, Connecticut, USA.
Titretek Twinreader Plus	Medical Supply Company, Damastown, Mulhuddart, Dublin 12, Ireland.
3015 pH meter	Jenway Ltd., Essex, England.
Orbital incubator	Sanyo Gallenkamp PLC., Monarch Way, Belton Park, Loughborough, Leicester, UK.
RM6 Lauda waterbath T-Gradient BIOMETRA-PCR Millipore Filtration Apparatus (250 ml)	AGB Scientific Ltd., Dublin Industrial Estate, Glasnevin, Dublin 9, Ireland.
Stirred Ultrafiltration Cell 8400	Amicon Inc., Beverly, Massachusetts 01915, USA.
Stuart Platform Shaker STR6	Lennox, P.O. Box 212A, John F. Kennedy Drive, Naas Rd., Dublin 12, Ireland.
UV-160A spectrophotometer	Shimadzu Corp., 1 Nishinokyo-Kuwabaracho, Nakagyo-ku, Kyoto 604, Japan.
UVP ImageStore 7500 gel documentation system	Ultra Violet Products, Upland, CA., USA.
CO_2 Tissue culture incubator Thermoforma series II 3110 FASTER BH2000 Laminar air flow unit	Biosciences, 3 Charlemont Terrace, Crofton Rd., Dun Laoghaire, Dublin, Ireland.
Laminar Flow Unit Holten 2448K	Holten Laminar, A/S Gydevang 17, DK 3450 Allerod, Denmark.

2.1.4 *Microbiological culture media formulations*

2.1.4.1 *Bacterial culture medium*

Table 2.4. Bacterial growth media used and components with amounts used. (Solid medium was made by adding 15 g/l bacteriological agar to the media below).

Media Type	Components and amounts	
2 x Tryptone and yeast extract (TY) medium	Tryptone	16 g/l
	Yeast Extract	10 g/l
	NaCl	5 g/l
Luria Bertani broth (LB) medium	Tryptone	10 g/l
	Yeast Extract	5 g/l
	NaCl	5 g/l
Non-expression medium (NE)	Tryptone	16 g/l
	Yeast Extract	10 g/l
	NaCl	5 g/l
	Glucose	1%, (v/v)
	Chloramphenicol	25 µg/ml
Low expression medium (LE)	Tryptone	16 g/l
	Yeast Extract	10 g/l
	NaCl	5 g/l
	Glucose	1%, (v/v)
	Chloramphenicol	25 µg/ml
	IPTG	0.5 mM
SOB medium	Tryptone	20 g/l
	Yeast Extract	5 g/l
	NaCl	0.5 g/l
	KCl	2.5 mM
	$MgCl_2$	20 mM
Super optimal catabolites (SOC) medium	Tryptone	20 g/l
	Yeast Extract	5 g/l
	NaCl	0.5 g/l
	KCl	2.5 mM
	$MgCl_2$	20 mM
	Glucose	20 mM
	pH	7.0

2.1.4.2 *Mammalian cell culture medium*

All mammalian cell lines were routinely cultured in DMEM (Dulbecco's modification of Eagle's medium) supplemented with 10 % (v/v) foetal calf serum (FCS), 2 mM L-glutamine, and 25 μg/ml gentamycin (referred to as DMEM). Subsequent variations of DMEM were used and are described in section 2.5.2.1.

2.1.5 *Bacterial strains used*

Liquid cultures were stored at –80°C with 15-20% (v/v) glycerol and at 4 ° C as streak agar plates (for no longer than one month).

Bacterial strain	Source	Comments
E. coli XL-1 Blue	Stratagene	Cultured in 2xTY broth
E. coli JM83	Stratagene	Cultured in 2xTY broth
E. coli TG1	Stratagene	Cultured in 2xTY broth
E.coli TOP10F'	Stratagene	Cultured in 2xTY and SB broth

2.1.6 *General buffer formulations*

2.1.6.1 *Phosphate buffered saline (PBS) wash buffer*

One tablet (DA, Oxoid) was dissolved per 100 mls of distilled water according to the manufacturer's instructions. When dissolved, prepared PBS contained 0.15 M NaCl, 2.5 mM potassium chloride, 10 mM disodium hydrogen phosphate and 18 mM sodium dihydrogen phosphate, and the pH was 7.4. This buffer will be referred to throughout as PBS.

2.1.6.2 *PBS-Tween (PBS-T) wash buffer*

Tween 20 surfactant (Sigma) was added to PBS to a final concentration of 0.05% (v/v) and mixed.

2.1.6.3 *PBS-TM dilution buffer*

PBS-T was supplemented with 2 % (w/v) dried skimmed milk powder, and used for preparing antibody dilutions.

2.1.6.4 *Blocking solution / buffer*

Dried skimmed milk powder was dissolved in PBS-T to give 5% (w/v) solution. This was used to block remaining absorption sites.

2.1.6.5 *Tris-acetic acid-EDTA buffer (TAE)*

A 50 X stock solution of TAE buffer was prepared in a final volume of 1 litre by dissolving 242 g Tris, and addition of 57.1 ml glacial acetic acid followed by 100 ml of 0.5 M EDTA, with a pH of 8.0. All agarose gels were run in 1 X TAE by diluting the stock solution 1/50 with ultra pure water.

2.1.6.6 *Hepes Buffered Saline (HBS)*

Hepes buffered saline (BIAcore running buffer) containing 50 mM NaCl, 10 mM HEPES, 3.4 mM EDTA and 0.05% (v/v) Tween-20 was prepared by dissolving 8.76 g of NaCl, 2.56 g of HEPES, 1.27 g of EDTA and 500 µl of Tween 20 in 800 ml of distilled water. The pH of the solution was then adjusted to pH 7.4, by the addition of NaOH. The final volume was then made up to 1,000 ml. The solution was filtered through a 0.2 µm filter and degassed prior to use.

2.1.6.7 *MES buffer*

2-[N-Morpholino]-ethansulfonic acid (MES) buffer was prepared to a concentration of 5 mM, by dissolving 0.019g of MES in 15 ml of distilled water. The pH of the solution was adjusted to 5.8 by addition of NaOH. (For the production of KLH conjugates, this was supplemented with 0.9 M NaCl to aid solubility).

2.2 Production of polyclonal antibodies to cephalexin

2.2.1 *Antigen preparation*

Coupling of the cephalexin to carrier protein was achieved using established EDC (1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride) / NHS (N-Hydroxysuccinimide) chemistry. EDC reacts with carboxylic acids to from highly active o-acylisourea intermediates that subsequently react with primary amines; NHS is used to stabilise the active intermediate produced by this reaction. Initially it was decided to activate the carboxyl group on the cephalexin molecule with subsequent addition of protein carrier (Activated Ester Coupling).

However, the carboxyl groups on the proteins were also activated followed by the addition of the cephalexin as a source of amine (Reverse Activated Ester Coupling). BSA (Bovine Serum Albumen), OVA (Ovalbumen), Thy (Thyroglobulin), KLH (Keyhole Limpet Haemocyanin), Biotin-Avidin, and Dextran conjugates with cephalexin were synthesised using the general coupling schemes that follow.

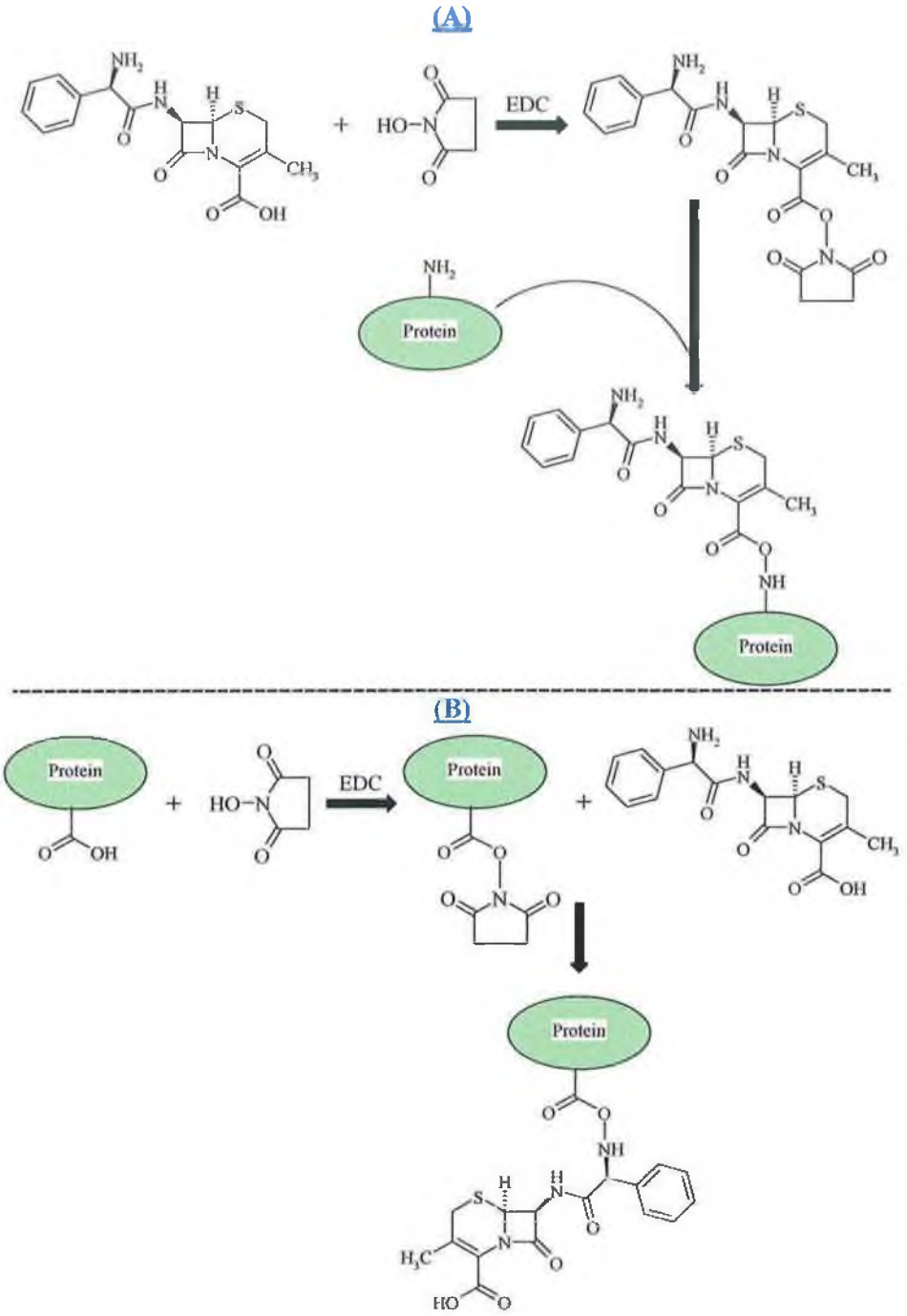


Figure 2.1. Diagrammatic representation of generic reaction schemes used in the production of cephalexin conjugates. (**A**) The scheme in A is designated as cephalexin-activated coupling, where the carboxylic acid (-COOH) functional groups on the cephalexin molecule are activated using NHS / EDC and subsequently reacted with free amine ($-NH_2$) groups present on the relevant protein (e.g. BSA) surface. **B**, (above) Shows the opposite process to that shown in (A), designated as protein-activated coupling, whereby carboxylic acid groups on the relevant protein surface are activated using NHS / EDC and subsequently reacted with free amines provided by cephalexin.

2.2.1.2 *Preparation Cephalexin Conjugates (Cephalexin-activated ester coupling).*

A 20 mg/ml solution of cephalexin was made up to a final volume of 4 ml with 0.2 M borate buffer, pH 8.5. Concentrations of EDC (N-ethyl-N-(dimethyl-aminopropyl) carbodiimide hydrochloride) and NHS (N-hydroxysuccinimide) were then added to give a final molarity of 0.4 and 0.1 M, respectively, in the solution and allowed to react at room temperature without agitation for 10 min. Protein (BSA, OVA, THY) was prepared at a molar ratio of 1:100 to Cephalexin in 4 ml of 0.2 M borate buffer, pH 8.5, and added drop wise to the Cephalexin solution. This solution was allowed to react at room temperature for 2 h with mild stirring. The solution was dialysed against 50 volumes of PBS overnight at 4°C and further dialysed against 50 volumes of PBS at 4°C for 4 h.

2.2.1.3 *Preparation of Cephalexin Conjugates (Protein-activated coupling).*

A 10 mg/ml solution of protein was made up to a final volume of 4 ml with 5 mM MES buffer, pH 6. Concentrations of EDC and NHS were then added to give a final molarity of 0.4 and 0.1 M, respectively, in the solution and allowed to react at room temperature without agitation for 10 min. Protein (BSA, OVA, THY) was prepared at a molar ratio of 1:100 to Cephalexin in 4 ml of MES buffer, pH 6, and added drop wise to the Cephalexin solution. This solution was allowed to react at room temperature for 2 h with mild stirring. The solution was dialysed against 50 volumes of PBS overnight at 4°C and further dialysed against 50 volumes of PBS at 4°C for 4 h.

2.2.1.4 *Preparation of Cephalexin-KLH Conjugate.*

Succinylated Keyhole Limpet Heamocyanin (KLH) (Sigma, research grade) was dissolved in 5 mM MES buffer (0.9 M NaCl), pH 6, to give a 10-mg/ml solution of protein in a final volume of 1 ml. A stock solution of 10-mg/ml Cephalexin was made up in MES. 150 µl of this was added to the protein solution. From a 10-mg/ml stock solution of EDC a 100µl portion was added to the solution containing the protein and cephalexin. The reaction mixture was vortexed and allowed to react at room temperature for 2 h with mild stirring. The solution was dialysed against 50 volumes of PBS overnight at 4°C and further dialysed against 50 volumes of PBS at 4°C for 4 h.

2.2.1.5 *Preparation of Cephalexin-Biotin-Avidin Conjugate.*

EZ-Link™ NHS-Biotin (N-Hydroxysuccinimidobiotin) purchased from Pierce was dissolved in 1 ml DMSO to give a 1-mg/ml solution; 100µl of this was reacted with a 20 molar equivalent of cephalexin dissolved in PBS, pH 7.4. The reaction mixture was allowed to react overnight at 4°C. The following day the reaction was further reacted with 1/10 molar equivalent avidin for 2 h at room temperature. The solution was dialysed against 50 volumes of PBS overnight at 4°C and further dialysed against 50 volumes of PBS at 4°C for 4 h.

2.2.2 ***Immunogen preparation and immunisation***

All processes involving animals were licensed by the Department of Health and Children and care was taken to minimise the stress to the animals involved. Immunogen for the production of polyclonal antibodies consisted of a 1 mg/ml solution of Cephalexin-Bovine Serum Albumin (Ceph-BSA) conjugate in PBS and an equal volume of Freund's complete adjuvant. This solution was vortexed until a stable emulsion was formed. New Zealand White female rabbits were injected subcutaneously at a number of sites with 1 ml of the emulsion. The immunisation was repeated 21 days later with Freund's Incomplete adjuvant. Bleeds were taken from the marginal ear vein of the animals between 10 and 15 days after immunisation. Re-boosting and bleeding procedures were repeated until adequate antibody titre was obtained. Once sufficient titre was obtained the animals were sacrificed and the serum removed.

2.2.3 ***Purification of polyclonal antibody from whole serum***

Purification of polyclonal antibody from rabbit serum was carried out by precipitation with saturated ammonium sulphate, followed by affinity purification using a protein G column.

2.2.3.1 *Saturated ammonium sulphate precipitation*

The ammonium sulphate precipitation of antibody from rabbit serum was carried out according to the method of (Hudson and Hay, 1989). Saturated ammonium sulphate (SAS) was prepared by dissolving 500 g of ammonium sulphate in 500 ml distilled water at 50°C, leaving the solution overnight at 4°C and adjusting the pH to 7.2. 10 ml of cold saturated ammonium sulphate (100%, w/v) was added drop-wise to an equal volume of rabbit serum on ice with gentle stirring. The mixture was allowed to stir at room temperature for 30 minutes,

followed by centrifugation at 3,000 rpm for 20 minutes. The supernatant was discarded and the precipitate washed twice in 10 ml of 45% (w/v) ammonium sulphate. The washed pellet was dissolved in 5 ml of PBS and dialysed in 5 L of PBS overnight at 4°C to remove residual ammonium sulphate.

2.2.3.2 *Protein G affinity chromatography*

A 2 ml protein G-sepharose column was poured and equilibrated with 50 ml of PBS, pH 7.4, containing 0.3 M NaCl (running buffer). 2 ml of the dialysed ammonium sulphate rabbit immunoglobulin fraction was applied to the column and the flow rate adjusted to 1 ml/min. The sample was then re-applied to the column (twice) followed by washing the column with 20 ml of PBS, pH 7.4, containing 0.5 M NaCl and 0.05% (v/v) Tween (wash buffer). Affinity captured-antibody was eluted with 0.1 M glycine/HCl, pH 2.2. 1 ml fractions were collected and immediately neutralised to pH 7.0 by addition of 100 µl of 1.5 M Tris/HCl, pH 8.7, to each fraction, in order to prevent denaturation of antibody. The optical density of each fraction was recorded at 280 nm, and those fractions containing protein were pooled and dialysed overnight in PBS at 4°C, and the preservative agent sodium azide added (0.05% (v/v) solution). The sample was aliquoted (0.5 ml fractions) and stored at -20°C.

2.3 Antibody characterisation

In general, antibodies were characterised using enzyme-linked immunosorbent solid-phase immunoassays, Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE), Western blotting and Bicinchoninic acid (BCA) protein assays.

2.3.1 *Solid-phase immunoassays*

2.3.1.1 *Enzyme-Linked Immunosorbent Assay (ELISA) for determination of antibody titre*

Nunc 96 well ELISA plates were coated with antigen (10-20 µg/ml of cephalexin-protein conjugate) for 1 hour at 37°C or overnight at 4°C. The plates were incubated at 37°C for 1 hour, or at 4°C overnight and washed 3 times with PBS. The plates were then blocked with 200 µl of a PBS-T 5% (w/v) blocking solution and incubated at 37°C for 1 hour and washed as before. Dilutions of antibody were prepared in dilution buffer (PBS-T) containing 1% (w/v) dried milk powder and 100 µl of each dilution added to the plates in triplicate. The plates were then incubated at 37°C for 1 hour, followed by 3 washes with PBS and 3 washes

with PBS-T. A 1/2,000 dilution of secondary antibody (horse radish peroxidase (HRP)-labelled goat anti-rabbit antibody (polyclonal work) or HRP-labelled goat anti-mouse (monoclonal work)) was prepared in PBS-TM and 100 µl of this solution added to the plates and incubated at 37^{0}C for 1 hour. The plates were washed 3 times with PBS and 3 times with PBST, as before, and 100 µl of freshly prepared o-phenylenediamine dihydrochloride (OPD) substrate added to each well. The absorbance was determined at 450 nm after 30 minutes using a Titertek Plate Reader Plus®. Antibody titres were taken as the lowest absorbance value that can be distinguished from background plus twice the standard deviation (SD).

2.3.1.2 *Checkerboard ELISA for determination of optimal concentrations of immobilised conjugates*

96-well plates were coated with varying antigen concentrations with each antigen concentration added to a different row on the ELISA plate. Plates were blocked, as described in section 2.3.1.1, with PBS containing 5% (w/v) dried skimmed milk and 100 µl samples of antiserum were serially diluted in PBS-Tween containing 2% (w/v) milk (PBS-TM) added to each well and detected as in section 2.3.1.1.

2.3.1.3 *Inhibition ELISA for antibody characterisation*

Nunc ELISA plates were coated with antigen and blocked as described in section 2.2.3. Dilutions of free antigen (e.g. cephalexin or cross reactants) were made in PBS or whole milk and mixed with an equal volume of antibody. This mixture was incubated for 30 minutes at 37^{0}C. The plates were washed as before and 100 µl of each sample added to the plate in triplicate and incubated at room temperature for 1.5 hours with gentle mixing. Plates were washed three times with PBS and 3 times with PBS-Tween and 100 µl of 1/2000 dilution of HRP-conjugated goat anti-rabbit antibody in PBS-Tween containing 1% (w/v) Marvel added to each well. After incubation for 1 hour at 37^{0}C, 100 µl of OPD substrate was added to each well and the plates incubated for 30 minutes to 1 hour at 37^{0}C. Absorbance was determined at 450 nm. The results were plotted as a ratio (A/A_0), where the absorbance for a given antibody and cephalexin standard concentration (A) was divided by the measured absorbance for the antibody with no free cephalexin (A_0).

2.3.2 *Denaturing Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE)*

Polyacrylamide gel electrophoresis was carried out, as described by Laemmli (1970), in the presence of SDS. The experimental apparatus (Atto dual minislab system) was set up according to the manufacturers' guidelines. 12% (w/v) separating gels and 5% (w/v) stacking gels were normally used and prepared using following stock solutions as outlined in table 2.5:

(A) 30% (w/v) acrylamide containing 0.8% (w/v) bis-acrylamide.

Acrylamide	30 g
Bis-acrylamide	0.8 g

Dissolved in 100 ml dH_2O, mixed thoroughly and stored in the dark at 4°C.

(B) Separating gel buffer (4x) -1.5 M Tris-HCl, pH 8.8 containing 0.4% (w/v) SDS.

2 M Tris-HCl (pH 8.8)	75 ml
10% (w/v) SDS	4 ml
dH_2O	21 ml

Mixed thoroughly and stored at 4°C.

(C) Stacking gel buffer (4x) - 0.5 M Tris-HCl, pH 6.8 containing 0.4% (w/v) SDS.

1 M Tris-HCl (pH 6.8)	50 ml
10% (w/v) SDS	4 ml
dH_2O	46 ml

Mixed thoroughly and stored at 4°C.

(D) 10% (w/v) ammonium persulphate.

Ammonium persulphate	0.05 g

Dissolved in 500 µl of dH_2O and mixed thoroughly and made fresh as required.

The apparatus glass plates were washed thoroughly and wiped with acetone. Polymerisation of the separating gel acrylamide was catalysed by addition of ammonium persulphate solution (D) and the accelerator TEMED (N, N, N′, N′-tetramethyl-ethylenediamine) and the gel added to the space between the plates and covered with a layer of ethanol. Once the gel had polymerised, the ethanol was removed and the stacking gel was placed directly onto the

separating gel. A plastic comb was placed in this gel to create the wells for sample application. Once the gel had fully polymerised, the plates were then placed in the electrophoresis apparatus, the comb was removed and the chamber and wells filled with 1x electrophoresis buffer (25 mM Tris, 130 mM Glycine (electrophoresis grade), pH 8.3 and 0.1% (w/v) SDS).

5x Electrophoresis buffer (1L).

Tris-base (25 mM)	15 g
Glycine (130 mM)	72 g
SDS (0.1% (w/v))	5 g
pH	8.3

It was mixed thoroughly and volume adjusted to 1 L in a volumetric flask and stored at R.T.

Table 2.5. Quantities of stock solutions used for the preparation of separating and stacking gels. (A) 30% (w/v) Acrylamide solution containing 29.2% (w/v) acrylamide and 0.8% (w/v) Bis-acrylamide; (B) 4X separating gel buffer consisting of Tris-HCl, pH 8.8 (1.5M) and SDS (0.4% w/v) diluted in distilled water; (C) 4X stacking gel buffer consisting of Tris-HCl, pH 8.8, (0.5M) and SDS (0.4%, w/v) diluted in distilled water and (D) 100 mg/ml ammonium persulphate diluted in water.

Solution	Resolving Gel	Stacking Gel
Acrylamide (A)	5.0 ml	837 µl
Distilled Water	2.5 ml	2.87 ml
Separating Gel Buffer (B)	2.5 ml	-
Stacking Gel Buffer (C)	-	1.25 ml
Ammonium Persulphate (D)	100 µl	50 µl
TEMED	10 µl	10 µl

Excess sample buffer is essential to maintain reduction of protein sulfhydryls and to prevent intra-molecular disulfide bond formation through oxidised cysteines. A 5x stock solution of sample buffer was prepared to give the following final concentrations: 60 mM Tris-HCl (pH 6.8), 25% (w/v) glycerol, 2% (w/v) SDS, 14.4 mM 2-mercaptoethanol and 0.1% (w/v) bromophenol blue.

1 M Tris-HCl (pH 6.8)	0.6 ml
50% (v/v) Glycerol	5 ml
10% (w/v) SDS	2 ml
2- Mercaptoethanol	0.5 ml
1% (w/v) Bromophenol blue	1 ml
dH_2O	0.9 ml

Components were mixed thoroughly and stored in aliquots at -20°C.

Samples were mixed 5:1 with sample buffer and heated for 4-5 minutes at 100°C. Standard protein molecular weight markers, as outlined in table 2.6, were run in parallel with samples. A total sample volume of 20 µl was added to the respective wells on the gel. The power unit was run at 15 mA per gel until the sample had reached the bottom of the gel.

Gels were stained with Coomassie blue staining solution for 1-2 hours followed by destaining in 30% (v/v) methanol:10% (v/v) acetic acid:60% (v/v) distilled water for 2-3 hours. Coomassie blue staining solution was prepared as follows:

Coomassie blue R-250 (Sigma)	1.25 g
Methanol	227 ml
dH_2O	227 ml
Glacial acetic acid	46 ml

The staining solution was mixed thoroughly and filtered through Whatman filter paper (grade 1) and stored in a dark bottle at R.T.

Destaining solution was stored at R.T. and typically prepared as follows:

Methanol	300 ml
dH_2O	600 ml
Glacial acetic acid	100 ml

Destaining solution was mixed thoroughly and stored at R.T.

Table 2.6. Molecular weights of the protein standards used for SDS-PAGE and Western Blot analysis. The molecular weights of the Sigma wide range and Pierce Blue Ranger™ pre-stained markers indicate the apparent molecular weights of each protein after staining.

Protein standard	Sigma wide range marker	Pierce blue ranger marker
Aprotinin	6.5 kDa	-
α-Lactalbumin	14.2 kDa	-
Lysozyme	-	18 kDa
Trypsin Inhibitor	20 kDa	26 kDa
Trypsinogen	24 kDa	-
Carbonic Anhydrase	29 kDa	32 kDa
Glyceraldehyde-3-phosphate dehydrogenase	36 kDa	-
Ovalbumin	45 kDa	48 kDa
Glutamic Dehydrogenase	55 kDa	-
Albumin	66 kDa	85 kDa
Fructose-6-phosphate Kinase	84 kDa	-
Phosphorylase B	97 kDa	112 kDa
Galactosidase	116 kDa	-
Myosin	205 kDa	208 kDa

2.3.3 *Western blot analysis*

Following electrophoresis, the polyacrylamide gel was placed on top of three sheets of Whatman filter paper pre-soaked in transfer buffer. A sheet of nitrocellulose membrane, also pre-soaked in transfer buffer, was carefully placed on top of the gel and any air bubbles removed by rolling a clean 10 ml pipette over the sheets. Three more sheets of pre-soaked filter paper were added on top of the gel and air bubbles removed. The proteins were transferred from the gel to the membrane using a Bio-Rad Semi-dry Transfer system for 20 min at 20V at R.T.

After transfer, the blotted membrane was washed 3 times with PBS (0.15 M NaCl, 2.5 mM potassium chloride, 10 mM disodium hydrogen phosphate and 18 mM sodium dihydrogen phosphate, pH 7.4), prior to overnight incubation in blocking buffer (PBS containing 5% (w/v) powdered skimmed milk). Following blocking, the blot was washed 3 times in PBS buffer and then incubated with primary antibody (at the appropriate dilution in dilution buffer i.e. PBS-T containing 2% (w/v) skimmed milk powder) for 1.5 hours at room temperature while rocking. The blot was then washed 3 times in PBS and 3 times in PBS-T prior to incubation with enzyme-labelled secondary antibody (e.g. HRP-labelled goat anti-rabbit) diluted in PBS-T containing 2% (w/v) skimmed milk for 1.5 hours at R.T. The blot was washed 3 times in PBS-T then PBS. Following this the membrane was developed using appropriate substrate solution (i.e. 3,3′,5,5′-Tetramethyl-Benzidine (TMB) for HRP-labelled and 5-bromo-4-chloro-3'-indolyphosphate / nitro blue tetrazolium chloride (BCIP/NBT) for alkaline phosphatase-labelled secondary antibodies). Colour development was allowed to proceed at room temperature until the desired bands were apparent. The reaction was then stopped by washing extensively with distilled water or by rinsing with 100mM EDTA.

2.3.4 *Bicinchoninic acid (BCA) assay for estimation of protein concentration*

BCA reagents were provided as 'made-up' components in the BCA protein assay kit (Pierce). Solution 'A' comprised a mixture of sodium carbonate, sodium bicarbonate, Bicinchoninic acid and sodium tartrate in 0.1 M sodium hydroxide. Solution 'B' contained 4% (w/v) cupric sulphate. Working reagent (WR) was prepared by mixing solutions 'A' and 'B' in the ratio of 50:1, respectively. Appropriate protein standards were prepared in an analogous buffer to unknown samples (e.g. PBS) at concentrations of 0, 25, 125, 250, 500, 750, 1000, 1500 and 2000 µg/ml. The assay was carried out by pipetting 25 µl of each standard and unknown sample in triplicate into the wells of a 96-well plate. A 200 µl aliquot of working reagent was added to each of the wells. The plate was covered with tin foil and incubated at 37°C for 30 minutes. Following colour development the absorbance was read at 590 nm. Values obtained for each standard solution were used to construct a calibration curve by subtracting the blank (0 µg/ml) measurement. That was subsequently used to estimate the total protein content of the unknown samples.

2.4 Production of monoclonal antibodies to cephalexin

All mammalian cell cultures and reagents were manipulated aseptically in laminar air flow units and grown in a humidified 5 % (v/v) CO_2 atmosphere, at 37°C. Somatic cell fusion was employed as a means of producing 'immortal' *hybrid* cell lines secreting specific antibody from pre-immunised murine B-lymphocyte and myel*oma* cells.

2.4.1 *Immunogen preparation and Immunisation protocol for the production of monoclonal antibodies to Cephalexin*

All processes involving animals were approved and licensed by the Department of Health and Children and care was taken to minimise the stress to the animals involved. Balb/C mice of age 5-8 weeks old were used for the production of monoclonal antibodies by immunisation with immunogens that were prepared as described previously (section 2.2.2). Three separate immunogens containing Thyro-Ceph., KLH-Ceph., and BSA-Ceph conjugates at a concentration of 1 mg/ml were used in the generic immunisation schedule described below:

Day 1: A 1-mg/ml solution of immunogen was prepared in PBS and mixed with an equal volume of Freund's Complete Adjuvant (FCA). This solution was then vortexed to form an emulsion, and 150-200 µl of the emulsion used to immunise mice by intra-peritoneal (i.p.) injection.

Day 21: Mice were re-immunised in the i.p. cavity (with a similar dose of the immunogen, above in Freund's Incomplete Adjuvant (FICA) or PBS).

Day 28: Mice were bled from the tail vein, and the specific antibody titre determined.

Post-Day 28: Mice were boosted through the i.p. cavity with a similar mix of antigen and FICA or PBS as per day 21.

Post-Day 28: The above immunisation and blood sampling sequence was continued until a satisfactory antibody titre was recorded. Once the antibody titre was sufficiently high, the mouse was boosted by i.p. cavity injection with the respective immunogen.

The mouse was then sacrificed by cervical dislocation; the spleen was removed and used for subsequent monoclonal antibody fusion procedures.

2.4.2 *Mammalian cell culture*

2.4.2.1 *Preparation of mammalian cell culture media*

All cell lines were grown in DMEM (Dulbecco's modification of Eagle's medium) supplemented with 10 % (v/v) foetal calf serum (FCS), 2 mM L-glutamine, and 25 μg/ml gentamycin (referred to as DMEM).

Three additional media preparations were prepared for somatic cell fusion procedures. (i) DMEM lacking foetal calf serum was prepared for use during somatic cell fusion procedures. (ii) HAT (Hypoxanthine, Aminopterin and Thymidine) medium consisted of DMEM supplemented with 1 mM sodium pyruvate, non-essential amino acids (NEAA; 1 %, v/v), 100 μM hypoxanthine, 400 nM aminopterin, and 16 μM thymidine. This particular culture medium allowed for the selective growth of hybridoma cells post-fusion in the presence of Sp2/0 cells, which are unable to proliferate in HAT medium because they lack the requisite enzyme systems. (iii) DMEM supplemented with 1 mM sodium pyruvate, non-essential amino acids (1 %, v/v), 100 μM hypoxanthine and 16 μM thymidine was also prepared (referred to as HT medium).

2.4.2.2 *Recovery of frozen cells*

Cells were recovered from liquid nitrogen by rapidly thawing at 37°C, and transferring to a sterile universal containing 5 ml DMEM. The cells were then centrifuged at 2,000 r.p.m. for 10 minutes, resuspended in fresh culture medium, transferred to a tissue culture flask and incubated at 37°C in a humidified 5 % (v/v) CO_2 incubator. Prior to incubation, a 100 μl sample of the resuspended pellet was tested for cell viability using Trypan blue (section 2.5.2.3). Only cell preparations with > 90 % viability on recovery from frozen stocks were cultured further.

2.4.2.3 *Cell counts and viability testing*

All cell counts were made using an improved Neubaüer counting chamber. Viable cell counts were obtained by mixing 100 μl of cell suspension with 20 μl of a commercial 0.25 %, (w/v) isotonic Trypan blue solution (Sigma). The viable cell count was carried out within 5 minutes of the addition of Trypan blue as the solution is cytotoxic. A sample of this mixture was then used to perform the cell and viability count. Viable cells excluded the dye and remained

white, whereas dead cells were stained blue. Cells were visualised under phase contrast on an inverted microscope at 40 X magnifications. All cells were pelleted from growth media by centrifugation at 2,000 r.p.m. for 5 minutes unless otherwise stated.

2.4.2.4 *Growth of suspension cell lines*

The Sp2/0 (ATCC CRL 1581) cell line was cultured in DMEM. The cells were sub-cultured, using a split ratio of 1:4, at approximately 70 % confluency. For sub-culturing, the cells were flushed off the surface of the flask using a Pasteur pipette, collected and centrifuged at 2,000 r.p.m. The pellet was then resuspended in 4 ml of fresh culture medium. 1 ml of the resuspended pellet was then transferred to T-75 flasks containing 14 ml of fresh pre-warmed (37°C) DMEM.

2.4.2.5 *Storage of cell lines*

Cells were harvested from the surface of a confluent T-75 flask by flushing the surface of the flask with a Pasteur pipette, and pelleted by centrifugation at 2,000 r.p.m. Pellets were resuspended in 3 ml of a 10 % (v/v) solution of DMSO (Dimethyl sulphoxide) in FCS and 1 ml aliquots were transferred into 3 cryovials. A freezing-tray was used to gradually cool the cells, over a 3 hr. period, in the vapour-phase of liquid nitrogen. They were then immersed in the liquid-phase for long-term storage.

2.4.2.6 *Mycoplasma screening*

Cell lines were screened for Mycoplasma contamination in conjunction with the National Cell and Tissue Culture Centre (NCTCC), Dublin City University. Mouse myeloma cell lines used for somatic cell fusion procedures and selected hybridoma cell lines were cultured for 2 weeks prior to screening in antibiotic-free media. This was to ensure that any intracellular antibiotic was removed. Cells were split at approximately 70% confluence and seeded at a low density to prolong contact with growth media. Conditioned medium was decanted and stored at -20°C for subsequent screening. Mycoplasma screening was carried out using Hoechst 33258 DNA-intercalating fluorescent dye. Uncontaminated cells show relatively strong fluorescence in the nuclei only, whereas cells that are contaminated are detected by cytoplasmic staining of the mycoplasmic DNA.

2.4.3. *Hybridoma production and isolation*

2.4.3.1. *Somatic cell fusion*

Sp2/O (ATCC CRL 1581) cells were grown for at least two weeks, prior to fusion. Cells were not grown above 50 % confluency, and were sub-cultured at a split ratio of 1:2 the day before fusion. On the day of fusion, the medium was poured off the Sp2/0 cells, and fresh DMEM lacking FCS (section 2.4.2.1) was added. Cells were resuspended and counted. The cells were stored at room temperature until required.

Lymphocytes, from an immunised mouse were harvested from the extracted spleen, resuspended in DMEM lacking FCS, and counted. The lymphocytes and Sp2/O cells were mixed to give a cell ratio of approximately 5-10 lymphocytes per Sp2/0 cell. This cell mixture was pelleted by centrifugation at 1,250 r.p.m. and washed 4 times with 5 ml of DMEM lacking FCS.

All of the supernatant, from the final wash, was removed, except for 50-100 µl (simply pouring off the supernatant sufficed). The cells were resuspended by tapping the outside of the centrifuge tube until the cell pellet was in suspension. The cell suspension was placed in an ice-water bath, and 1.5 ml of 50 % (v/v) PEG (polyethylene glycol, molecular weight 1540 Da) previously cooled to 4°C, was added over a 1 minute period, with constant mixing. Mixing was continued for another 1.5 minutes. The centrifuge tube was removed from the ice-water bath, and enclosed in the palm of the hand, while adding 20 ml of pre-warmed (37°C) DMEM lacking FCS at a constant rate over a 5 minute period with continual slow swirling. The resulting suspension was placed at 37°C in a water bath for 15-20 minutes. Following this the suspension was centrifuged, at 1,000 r.p.m. for 10 minutes, and the cells resuspended at a cell density of 1.2×10^6 cells/ml, in HAT medium supplemented with 5 % (v/v) Briclone (section 2.4.2.1). The suspension was plated (termed master plates), in 96 well plates, at 0.1 ml per well, and incubated for 7 days. On day 7, 75 µl of fresh HAT medium supplemented with 5 % (v/v) Briclone was added to each well. Wells were then fed as required.

2.4.3.2 *Screening for specific monoclonal antibody production*

100 µl of 'spent' hybridoma supernatant was removed from each of the master plate wells, and replaced with 100 µl fresh DMEM supplemented with 5 % (v/v) Briclone. The 'spent' hybridoma medium was then screened for specific antibody production by conventional ELISA, as described in section 2.3.1.1. Positive wells from the initial screening were then transferred to 24-well plates, 12-well and subsequently to 6-well plates for cloning by limiting dilution. Positive populations were continuously monitored in displacement inhibition assays, as described in section 2.3.1.3.

2.4.3.3 *Cloning of hybridomas secreting cephalexin-specific antibodies*

Post-fusion cells from wells that tested positive in the screening ELISA were expanded in HAT medium, from 96-well plates, to 24-well plates, 6-well plates and finally to T25 flasks. Cells were cloned by limiting dilution when they had reached 50 % confluency. Briefly, cells were resuspended, and diluted to a concentration of 100 cells/ml in HT supplemented with 5 % (v/v) Briclone. 100 µl aliquots were added to wells in fresh 96-well plates yielding approximate cell densities of 10 cells per well. After 7 days, the plates were examined microscopically and wells containing single colonies were noted. On days 10-12 screening for positive wells proceeded, using an ELISA, as per section 2.3.1.1, with neat medium from the wells, as the test sample. Only cells that demonstrated inhibition in the presence of free cephalexin in ELISA assay formats were expanded further. Positive wells were expanded, as already described, and re-cloned at 1 cell/well seeding densities. Finally, another round of cloning was carried out in DMEM containing 10 % (v/v) FCS, NEAA (1 %, v/v), and 1 mM sodium pyruvate i.e. lacking HT and Briclone supplements, at a 0.5 cell/well seeding density. Positive single colonies were considered to be monoclonal at this stage.

2.5 Production of recombinant single chain Fv (scFv) antibodies to cephalexin

2.5.1 *Production of murine scFv antibody libraries*

Recombinant single chain variable fragment (scFv) production provides an alternative source of high affinity cephalexin-specific antibodies. Recombinant antibodies can be expressed in large quantities in *E. coli* and easily purified via engineered affinity tags, such as the polyhistidine tag or the Flag peptide tag. Antibody phage display has the advantage of a direct link between DNA sequence and protein function allowing modifications in the primary antibody sequence, (Hoogenboom *et al.*, 1998). This makes it possible to manipulate the genetic sequence (genotype) of antibodies and, hence, their characteristics (e.g. affinity and specificity). The link between the antibody genotype and phenotype (i.e. the expressed antibody protein) facilitates the enrichment of desired antibodies by panning with appropriate antigen.

2.5.2 *General molecular biology-based techniques*

2.5.2.1 *Agarose gel electrophoresis for DNA characterisation*

DNA was analysed by running on agarose gels (containing 0.5 μg/ml ethidium bromide for staining of DNA) in a horizontal gel apparatus. Gels were prepared by dissolving agarose (typically ranging between 0.7-1.2% (w/v)) in 1x TAE buffer by heating and 0.5 μg/ml ethidium bromide added to the cooled gel prior to setting. Following this gels were placed in the electrophoresis apparatus and 1 x TAE added as the running buffer. A 5X loading dye incorporated into each sample (1 μl dye + 5 μl sample) to facilitate loading and monitoring of samples unless red *Taq* (Sigma) PCR products were run. Maxi gels were frequently run at 100 volts for 1 hour or until the loading dye reached the end of the gel while mini gels were frequently run at 40-80 volts. Gels were then visualised on a UV transilluminator and photographed using a UV image analyser.

2.5.2.2 *Purification of PCR reaction products from agarose gels*

DNA (i.e. PCR product) was purified from agarose gels using the Wizard® PCR prep DNA purification kit (Promega). PCR products were typically run on a 1% (w/v) low melt agarose gel containing 0.5 μg/ml ethidium bromide (section 2.5.2.1). The desired base pair band fragments (e.g. approximately 400 bp for variable heavy and light chain antibody genes) were excised from the gel with a sterile scalpel and the DNA purified from the low melt agarose

according to manufacturers' guidelines. The excised gel samples were incubated at 70°C in a water bath/heating block causing the agarose to melt within 5 minutes. 1 ml of resin was immediately added and mixed thoroughly by pipetting for 15 seconds. For each PCR preparation a plunger from a 5 ml syringe was attached to a Wizard Mini-column and the resin was slowly pushed through. The column was then washed with 2 ml of 80% (v/v) isopropanol. The column was centrifuged for 2 minutes at 14,000 rpm to remove any residual buffers. The mini-column was placed in a fresh 1.5 ml micro-centrifuge tube and 20 µl of molecular grade ultra pure water (upH_2O) was applied to the mini-column and allowed to incubate for 1 minute at R.T. The column was centrifuged at 14,000 rpm for 20 seconds to elute the DNA, samples were and stored at –20°C until required.

2.5.2.3 *Purification of plasmid DNA*

Plasmid DNA purification was performed using the Wizard® Plus SV miniprep DNA purification kit (Promega). A single *E. coli* colony containing plasmid (e.g. *E.coli* XL1-Blue containing pAK100 phagemid vector, kindly donated by Andreas Pluckthun, University of Zurich, Switzerland) was picked from a stock agar streak plate and used to inoculate 5 ml of 2 x TY supplemented with 30 µg/ml tetracycline. The culture was grown overnight at 37°C with vigorous shaking. The cells were then pelleted by centrifugation for 5 min at 4000 rpm. The supernatant was carefully decanted and the pellet thoroughly resuspended in 250 µl resuspension buffer (50 mM Tris-HCl, pH 7.5, 10 mM EDTA and 10 µg/ml RNase A) and transferred to a sterile 1.5 ml microcentrifuge tube. Cell lysis solution (250 µl of 0.2 M NaOH and 1% (w/v) SDS) was added to the resuspended cells and mixed by inverting 4 times. The resulting mixture was incubated at R.T. for 5 minutes prior to the addition of 10 µl of alkaline protease solution and further mixing by inverting the tube 4 times and incubation for 5 minutes. Alkaline protease was added in order to inactivate endonucleases and other proteins released during cell lysis that can adversely affect the quality of DNA. 350 µl of neutralisation solution (Buffer containing 4.09 M guanidine hydrochloride, 0.759 M potassium acetate and 2.12 M glacial acetic acid, pH 4.2) was added and again mixed by inverting the tube 4 times. The resulting mixture was centrifuged at 14,000 rpm for 10 minutes at R.T. The cleared lysate (supernatant) was carefully decanted into a spin column in a fresh 1.5 ml tube and centrifuged at 14,000 rpm for 1 min at R.T. The column 'flow-through' was discarded from the 1.5 ml tube and 750 µl of wash solution (162.8 mM potassium acetate, 22.6 mM Tris-HCl, pH 7.5, and 0.109 M EDTA) pre-diluted with 95% (v/v) ethanol as per manufacturers' guidelines, was added. The spin column was re-inserted in the microcentrifuge tube and centrifuged at 14,000 rpm for 1 min at R.T. and the 'flow-through' discarded again. The wash procedure

was repeated using 250 µl of wash solution and centrifuged at 14,000 rpm for 2 minutes The 'flow-through' was again discarded. The spin column was transferred to a fresh sterile 1.5 ml microcentrifuge tube and the plasmid DNA finally eluted by adding 100 µl of ultra-pure molecular grade water to the column, followed by centrifugation at 14,000 rpm for 1 min at R.T. The volume collected in the 1.5 ml tube was kept for further analysis and the spin column discarded. DNA was stored at -20°C until required.

2.5.2.4 *Ethanol precipitation of DNA*

DNA was routinely precipitated by ethanol precipitation as a means of concentrating samples and crude rapid purification. Typically, $1/10^{th}$ the sample volume of 3 M sodium acetate buffer, pH 5.2, was added (i.e. 5 µl sodium acetate for a 50 µl PCR reaction product). PCR product was transferred to a 1.5 ml microcentrifuge tube and mixed with the appropriate volume of sodium acetate buffer. This was followed by the addition of 2x the original sample volume of cold (stored at -20°C) 100% (v/v) ethanol (i.e. 100 µl ethanol for a 50 µl PCR reaction product). The mixture was placed at -20°C for at least 2 hours (mostly overnight) and centrifuged at 14,000 rpm for 15 minutes at 4°C. The supernatant was carefully removed using a pipette and the remaining ethanol evaporated by incubation in a 37°C water bath. The resulting pellet was resuspended using the desired volume of molecular grade upH_2O and stored at -20°C until required.

2.5.2.5 *Quantification of DNA*

DNA quantification was carried out on an agarose gel with a quantitative molecular weight markers (Promega, 100 bp Molecular Weight Markers, Bioline, Hyperladder quantification markers), The Promega 100 bp markers 500 bp fragment was used as a reference for densiometric quantitation, as 5 µl contains 150 ng of the 500 bp fragment and 50 ng of the other molecular weight fragments. Supplementary means of estimating the concentration of DNA samples was carried out using UV spectroscopic analysis. Samples were typically diluted (1/50), transferred to a dedicated quartz cuvette and the absorbance measured at 260 nm. The concentration (µg/ml) of DNA was calculated using the following formula:

$$[\text{DNA}] = \frac{\text{Dilution factor (e.g. 50)} \times 50 \times \text{Absorbance}}{1000}$$

2.5.2.6 *Preparation of chemically competent E. coli cells*

Chemically competent cells were prepared as previously described by Inoue *et al.* (1990). Stocks of *E. coli* JM83 and XL1 blue were streaked on 2xTY agar and incubated at 37°C overnight. Approximately 10-12 large colonies were removed from the plate and inoculated into 250 ml of SOB medium in a 2L baffled flask. The culture was grown at 18°C with vigorous shaking (200-250 rpm) until an OD_{600} of 0.6 was reached. The culture was then placed on ice for 10 minutes before being transferred to two pre-cooled 250 ml centrifuge tubes and spun at 4,000 rpm at 4°C for 5 minutes in a Beckman J2-21 centrifuge. The pellets were resuspended in 40 ml of ice-cold TB buffer (10 mM Pipes, 15 mM $CaCl_2$, 150 mM KCl, pH adjusted to 6.7 with KOH and 55 mM $MnCl_2$, sterile filtered and stored at 4°C), placed on ice for 10 minutes, pooled and centrifuged as before. The cell pellet was gently resuspended in 20 ml of ice-cold TB buffer and DMSO added slowly with gentle swirling to a final concentration of 7% (v/v). After incubation in an ice bath for 10 minutes the cell suspension was dispensed in 1 ml aliquots into microcentrifuge tubes. The cells were then flash frozen in liquid nitrogen and stored at –80°C.

2.6.2.7 *Preparation of high efficiency electrocompetent E. coli cells*

Electrocompetent TOP10F' *E. coli* were prepared using a modified version of the protocol described by Barbas *et al.* (2001). In order for cells to be electrocompetent, *E. coli* must have a high resistance i.e. low ionic strength. This was achieved by repeatedly washing mid-log phase *E. coli* in low-salt buffers and eventually resuspending them in the same buffer supplemented with 10% (v/v) glycerol prior to flash freezing in liquid nitrogen at -80°C. TOP10F' cells were streaked on LB agar plates supplemented with 30 μg/ml tetracycline (Tet.) and incubated inverted at 37°C overnight. Single colonies were picked and used to inoculate 15 ml of pre-warmed (37°C) SB containing 30 μg/ml Tet. in polypropylene tubes (50 ml falcon tubes). Cultures were grown at 37°C overnight with shaking (250 rpm). 2.5 ml of overnight culture was used to inoculate two 2 L baffled culture flasks containing 500 ml SB broth supplemented with 0.4% (v/v) glucose, 10 mM $MgCl_2$ and 30 μg/ml Tet. The 2 L flasks containing cultures were grown at 37°C with shaking (250 rpm) until OD of 0.8 at 600nm was achieved. The cultures were then placed on ice in a 4°C environment for 15 min. All further steps were carried out as rapidly as possible and manipulated with pre-chilled pipettes in a cold room environment (4°C). The chilled cultures were decanted into two pre-chilled 500 ml centrifuge tubes and centrifuged at 4000 rpm for 10 min at 4°C. Supernatant was decanted and the pellets were resuspended in 25 ml of pre-chilled 10% (v/v) glycerol solution. After resuspending, both cultures were combined and added to 500 ml centrifuge

tube bringing the volume up to approximately 500 ml with glycerol solution followed by centrifugation as before. Pellets were resuspended in 500 ml 10% (v/v) glycerol solution and centrifuged a further two times. The pellet was then thoroughly resuspended in 25 ml glycerol solution and transferred to a pre-chilled 50 ml falcon tube and centrifuged at 3500 rpm for 15 min at 4°C. Most of the supernatant was carefully poured off until the pellet began to slide out of the tube. The remaining wet pellet was resuspended thoroughly and approximately 300 µl aliquots were placed in pre-chilled 1.5 ml sterile microcentrifuge tubes and immediately flash frozen in liquid nitrogen. Cell preparations were stored at -80°C until required (for no longer than 6 months).

2.5.3 *Isolation of murine antibody variable heavy (V_H) and light (V_L) chain genes from immunised mice*

2.5.3.1 *Immunisation procedure of BALB/c mice for the production of single chain Fv (scFv) antibody fragments to cephalexin.*

Solutions of 1 mg/ml cephalexin conjugates were prepared in PBS and added to an equal volume of Freunds' Complete adjuvant (FCA). This solution was vortexed until a stable emulsion was formed. 5-10 week old BALB/c mice were immunised subcutaneously with a total volume of 250 µl over several sites, as described in section 2.4.1. Further intra-peritoneal cavity immunisations were carried out after 21 days using Freunds' Incomplete adjuvant (FIA). Tail bleeds from immunised mice were taken 7 days later and the antibody titre against cephalexin determined as per section 2.3.1.1. Mice were re-boosted and the bleeding procedure repeated until an adequate serum antibody titre was obtained. The animals were then re-immunised 7-10 days prior to sacrifice. The mice were sacrificed by cervical dislocation and the spleens removed.

2.5.3.2 *Total RNA extraction from the spleens of BALB/c mice*

The spleen from an immunised mouse (immunised with various cephalexin conjugates) was removed aseptically and placed into a pre-weighed sterile universal. The spleen was weighed and 1 ml of Trizol reagent per 50 mg of spleen added. It was then homogenized, using a sterile homogeniser (Ultra-Turrax ®, model) for the mechanical disruption of tissues and cells. The apparatus was previously sterilised and desiccated, at 180°C overnight. The homogenized sample was dispensed into 1 ml aliquots and placed in sterile 1.5 ml centrifuge tubes. These were then left for 5 minutes at room temperature before the addition of 200 µl of

chloroform to each tube. The tubes were shaken for 15 seconds and incubated again at room temperature for 2-3 minutes. After incubation the tubes were centrifuged at 14,000 rpm for 15 minutes at 4°C. Three layers were observed, a lower red phenol/chloroform phase, a protein inter-phase and a colourless liquid upper phase containing the RNA. Transferring the aqueous phase to a fresh tube and adding 0.5 ml of isopropyl alcohol per tube precipitated the RNA. Samples were incubated at room temperature for 10 minutes and then centrifuged at 14,000 rpm for 10 minutes at 4°C. Small brown pellets were observed at the side of each tube. The supernatant was removed and the pellet washed by re-dissolving in 1 ml of 75% (v/v) ethanol per tube by gentle pipetting and inversion. Each tube was then centrifuged at 10,000 rpm for 5 minutes at 4°C, the ethanol decanted and the pellet dried at 37°C for 10 minutes. Each pellet was redissolved in 50 µl molecular grade (i.e. 'nuclease-free') ultra pure H_2O and pooled together in a fresh sterile 1.5 ml microcentrifuge tube.

2.5.3.3 *Reverse transcription of mouse spleen mRNA*

PCR can be performed directly from RNA but it is more successful to synthesize complementary DNA (cDNA) from total RNA for use in PCR. Complementary DNA was synthesized using a Promega cDNA synthesis kit containing random hexamer primers and oligo dT primers. Both primer sets i.e. random and hexamer were used as it is possible that the mRNA poly A tail could be sheared during the extraction process. Thus the use of the oligo dT primer could potentially reduce the amount of viable cDNA synthesized. The PCR reaction was set up as follows:

Component	Stock conc.	Conc. in 20µl	X1▼	X6
$MgCl_2$	25mM	5mM	4 µl	24 µl
10X Buffer	10X	1X	2 µl	12 µl
dNTP mix	10mM	1mM	2 µl	12 µl
RNase Inhib.	40U/µl	1U/µl	0.5 µl	3 µl
Primer	0.5µg/µl	0.2 µg/rxn*	0.4 µl	2.4 µl
AMV RT	25U/µl	15U/rxn*	0.6 µl	3.6 µl
Sterile H_2O	Made up to 20 µl including RNA		7.5 µl	45 µl
RNA	Varied	7-10 µg RNA/rxn*	3 µl	18 µl

▼ Volume for one reaction (X1) or six reactions (X6)

* total concentration per reaction

All reaction components, except enzymes, were placed on ice and left to thaw slowly. Enzymes were only removed when required and immediately kept at –20°C after use, in order to preserve activity. A 6X master mix was made up and the required volume of enzymes added before pipetting the mixture in to pre-labelled eppendorf tubes. Finally, the required volume of total RNA was added and mixed by pipetting.

2.5.4 *Amplification of antibody light and heavy chain genes*

2.5.4.1 *PCR Primers*

The listed PCR primers were obtained from Sigma-Genosys Ltd and designed to be compatible with the pAK vector system described by Krebber *et al.* (1997).

Variable light chain back primers

LB1 5′gccatggcggactacaaaGAYATCCAGCTGACTCAGCC3′

LB2 5′gccatggcggactacaaaGAYATTGTTCTCWCCCAGTC3′

LB3 5′gccatggcggactacaaaGAYATTGTGMTMACTCAGTC3′

LB4 5′gccatggcggactacaaaGAYATTGTGYTRACACAGTC3′

LB5 5′gccatggcggactacaaaGAYATTGTRATGACMCAGTC3′

LB6 5′gccatggcggactacaaaGAYATTMAGATRAMCCAGTC3′

LB7 5′gccatggcggactacaaaGAYATTCAGATGAYDCAGTC3′

LB8 5′gccatggcggactacaaaGAYATYCAGATGACACAGAC3′

LB9 5′gccatggcggactacaaaGAYATTGTTCTCAWCCAGTC3′

LB10 5′gccatggcggactacaaaGAYATTGWGCTSACCCAATC3′

LB11 5′gccatggcggactacaaaGAYATTSTRATGACCCARTC3′

LB12 5′gccatggcggactacaaaGAYRTTKTGATGACCCARAC3′

LB13 5′gccatggcggactacaaaGAYATTGTGATGACBCAGKC3′

LB14 5′gccatggcggactacaaaGAYATTGTGATAACYCAGGA3′

LB15 5′gccatggcggactacaaaGAYATTGTGATGACCCAGWT3′

LB16 5′gccatggcggactacaaaGAYATTGTGATGACACAACC3′

LB17 5′gccatggcggactacaaaGAYATTTTGCTGACTCAGTC3′

LBλ 5′gccatggcggactacaaaGATGCTGTTGTGACTCAGGAATC3′

Variable light chain forward primers

LF1 5′ggagccgccgccgcc(agaaccaccaccacc)$_2$ACGTTTGATTTCCAGCTTGG3′

LF2 5′ggagccgccgccgcc(agaaccaccaccacc)$_2$ACGTTTTATTTCCAGCTTGG3′

LF4 5′ggagccgccgccgcc(agaaccaccaccacc)$_2$ACGTTTTATTTCCAACTTTG3′

LF5 5′ggagccgccgccgcc(agaaccaccaccacc)$_2$ACGTTTCAGCTCCAGCTTGG3′

LFλ 5′ggagccgccgccgcc(agaaccaccaccacc)$_2$ACCTAGGACAGTCAGTTTGG3′

Variable heavy chain back primers

HB1 5′ggcggcggcggctccggtggtggtggatccGAKGTRMAGCTTCAGGAGTTC3′

HB2 5′ggcggcggcggctccggtggtggtggatccGAGGTBCAGCTBCAGCAGTC3′

HB3 5′ggcggcggcggctccggtggtggtggatccCAGGTGCAGCTGAAGSASTC3′

HB4 5′ggcggcggcggctccggtggtggtggatccGAGGTCCARCTGCAACARTC3′

HB5 5′ggcggcggcggctccggtggtggtggatccCAGGTYCAGCTBCAGCARTC3′

HB6 5′ggcggcggcggctccggtggtggtggatccCAGGTYCARCTGCAGCAGTC3′

HB7 5′ggcggcggcggctccggtggtggtggatccCAGGTCCACGTGAAGCAGTC3′

HB8 5′ggcggcggcggctccggtggtggtggatccGAGGTGAASSTGGTGGAATC3′

HB9 5′ggcggcggcggctccggtggtggtggatccGAVGTGAWGYTGGTGGAGTC3′

HB10 5′ggcggcggcggctccggtggtggtggatccGAGGTGCAGSKGGTGGAGTC3′

HB11 5′ggcggcggcggctccggtggtggtggatccGAKGTGCAMCTGGTGGAGTC3′

HB12 5′ggcggcggcggctccggtggtggtggatccGAGGTGAAGCTGATGGARTC3′

HB13 5′ggcggcggcggctccggtggtggtggatccGAGGTGCARCTTGTTGAGTC3′

HB14 5′ggcggcggcggctccggtggtggtggatccGARGTRAAGCTTCTCGAGTC3′

HB15 5′ggcggcggcggctccggtggtggtggatccGAAGTGAARSTTGAGGAGTC3′

HB16 5′ggcggcggcggctccggtggtggtggatccCAGGTTACTCTRAAAGWGTSTG3′

HB17 5′ggcggcggcggctccggtggtggtggatccCAGGTCCAACTVCAGCARCC3′

HB18 5′ggcggcggcggctccggtggtggtggatccGATGTGAACTTGGAAGTGTC3′

HB19 5′ggcggcggcggctccggtggtggtggatccGAGGTGAAGGTCATCGAGTC3′

Variable heavy chain forward primers

HF1 5′ggaattcggcccccgaggcCGAGGAAACGGTGACCGTGGT3′

HF2 5′ggaattcggcccccgaggcCGAGGAGACTGTGAGAGTGGT3′

HF3 5′ggaattcggcccccgaggcCGCAGAGACAGTGACCAGAGT3′

HF4 5′ggaattcggcccccgaggcCGAGGAGACGGTGACTGAGGT3′

2.5.4.2 *Components of PCR reaction for amplification of variable region light (V_L) and heavy (V_H) chain antibody genes*

The concentration of stock primers varied. However, 0.1 nmol concentrations of the forward and reverse primers were used per reaction.

Component	Stock concentration	Concentration in 50 µl
Forward Primers	varied	0.1 nmol of each primer/reaction
Reverse Primers	varied	0.1 nmol of each primer/reaction
10X Buffer	10 X	1 X
$MgCl_2$	25 mM	1.0 mM
dNTP	20 mM	0.4 mM
Ultrapure water		to 50 µl including *Taq* and DNA
cDNA	varied	6-8 µg of cDNA/50 µl reaction
Taq polymerase	5 U/µl	5 U/reaction (multiplex)

2.5.4.3 *PCR conditions for amplification of antibody light and heavy chain genes*

Typical thermal cycling conditions were as follows:

94°C X 5 min 'Hot-Start'
94°C X 1 min
63°C X 30 sec
58°C X 50 sec
72°C X 1 min
Repeat X 7 cycles

94°C X 1 min
63°C X 30 sec
72°C X 1 min
Repeat X 24 cycles
72°C X 10 min

All ramping rates were at 4°C/sec.

2.5.4.4 *Components of Splice by overlap extension (SOE)-PCR*

Agarose gel-purified (section 2.5.2.2) light and heavy chain genes (each approximately 400 bp) were spliced together and amplified to produce 800 bp fragments by SOE PCR.

SOE Primers

Single chain back (sc_{back}): 5′ttactcgcggcccagccggccatggcggactaccccg3′

Single chain forward (sc_{for}): 5′ggaattcggcccccgag3′

SOE-PCR reaction components for amplification and splicing of antibody light and heavy chain genes

Component	Stock concentration	Concentration in 50 µl
10 X buffer	10 X	1 X
$MgCl_2$	25 mM	1.0 mM
dNTP	20 mM	0.4 mM
Ultrapure water	to 50 µl including *Taq*, DNA and scfor and scback	
V_H	varied	10 ng/reaction
V_L	varied	10 ng/reaction
sc_{for}	varied	0.05 nmol /reaction
sc_{back}	varied	0.05 nmol/reaction
Taq polymerase	5 U/µl	5 U/reaction

SOE-PCR buffer compositions: buffer A (7.5 mM $MgCl_2$, pH 8.5); buffer B (10 mM $MgCl_2$, pH 8.5); buffer C (12.5 mM $MgCl_2$, pH 8.5); buffer D (17.5 mM $MgCl_2$, pH 8.5); buffer E (7.5 mM $MgCl_2$, pH 9.0); buffer F (10 mM $MgCl_2$, pH 9.0); buffer G (12.5 mM $MgCl_2$, pH 9.0); buffer H (17.5 mM $MgCl_2$, pH 9.0); buffer I (7.5 mM $MgCl_2$, pH 9.5) and buffer J (10 mM $MgCl_2$, pH 9.5).

SOE-PCR conditions for amplification of joined antibody light and heavy chain genes

92°C X 1 min
45°C X 50 min
72°C X 1 min
Repeat X 5 cycles
Add 0.05 mM sc_{for} and sc_{back} primers:
5 U *Taq* Polymerase/rxn

92°C X 1 min
68°C X 30 sec
72°C X 1 min
Repeat X 25 cycles

All ramping rates were at 4°C/sec.

2.5.4.5 *Preparation and purification of pAK100 vector using Wizard miniprep system (Promega).*

A single colony of *E.coli* XL1-Blue containing pAK100 phagemid vector (all vectors were kindly donated by Andreas Pluckthun, University of Zurich, Switzerland) was picked from a stock agar streak plate and used to inoculate 5 ml of 2 x TY supplemented with 30 μg/ml tetracycline. The culture was grown overnight at 37°C with vigorous shaking. Purification of the pAK100 phagemid vector was carried out using the Wizard miniprep system, as described in section 2.5.2.3.

2.5.4.6 *Digestion of pAK100 and antibody light and heavy chain genes SOE PCR products*

Digestion of pAK100 and SOE products was performed using *Sfi1* restriction enzyme. The pAK100 phagemid vector requires digestion with *Sfi1* restriction enzyme prior to ligation with the combined light and heavy chain gene SOE-PCR products. Antibody light and heavy chain gene SOE products were also digested with *SfiI*, for subsequent ligation into pAK100. Two units of *SfiI* enzyme was used to cut 30-50 ng/reaction SOE product or 50-100 ng/reaction pAK100 vector at 50°C overnight in a reaction volume of 10 μl.

2.5.4.7 *Ligation of antibody light and heavy chain genes into the pAK100 vector*

Agarose gel-purified SOE products (section 2.5.2.2) were ligated into the *Sfi1* digested pAK100 plasmid vector using a reaction mixture containing DNA ligase (Promega), reaction buffer, SOE V_L/V_H gene constructs and pAK100 digests, at a ratio of 1.5:1, and molecular grade upH_2O, to give a final volume of 10 µl. The reaction mixtures were incubated at 15°C overnight and DNA purified using ethanol precipitation (section 2.5.2.4). Following this the DNA was used to transform *E.coli* XL1-Blue chemically competent cells.

2.5.4.8 *Preparation of high efficiency chemically competent cells*

High efficiency chemically competent *E. coli* XL-1 Blue and JM83 cells were prepared as previously described in section 2.5.2.6.

2.5.4.9 *Transformation of E.coli XL1-Blue chemically competent cells with pAK100 vector containing light and heavy chain genes and measurement of transformation efficiencies*

High efficiency competent *E. coli* XL1-Blue cells were thawed on ice. Cells were mixed by gentle tapping and transferred into two 15 ml polypropylene tubes (one tube was for the experimental transformation and the other tube was for the positive control transformation). The contents of the tubes were stirred gently and incubated on ice for 10 min, swirling gently at 2 min intervals. 0.1-50 ng of pAK100 phagemid vector, containing light and heavy chain SOE-PCR product as insert was added to one of the tubes and swirled gently. The commercial (Invitrogen) control vector (1 µl of pUC18; 10 µg/µl) was added to the second tube and also swirled. Both tubes were then incubated on ice for 30 min. Cells were heat-pulsed for 30 sec in a water bath at 42°C, followed by incubation on ice for 2 min. 0.8 ml of LB medium, pre-warmed at 37°C for 10 min, was then added and the tubes incubated at 37°C for 1 h with shaking at 225-250 rpm. pAk100 transformants were plated out on LB agar supplemented with 25 µg/ml chloramphenicol and 1% (v/v) glucose. Control transformants were plated out on LB agar supplemented with 20 µg/ml of ampicillin. Both sets of plates were allowed to grow overnight inverted at 37°C. Transformed colonies were washed and scraped off the agar plates using sterile LB broth and used as library stocks. Concentrated library stocks were suspended in LB medium supplemented with 15% (v/v) glycerol and flash frozen in liquid nitrogen and stored at –80°C.

2.5.5 *Isolation of scFv antibodies to cephalexin*

2.5.5.1 *Rescue of scFv displaying phage*

50 ml of non-expression (NE) medium (2xTY broth containing 1% (w/v) glucose and 25 μg/ml chloramphenicol) was inoculated with 100 μl of cells from a stock library antibody library. The culture was shaken at 37°C until the $O.D._{550} = 0.5$, at which point it was incubated for 10 minutes at 37°C without shaking. 10^{11} pfu of helper phage (VCSM13) and 25 μl of 1M IPTG solutions were added to the culture, followed by incubation for 15 minutes at 37°C without shaking. The culture was then diluted in 100 ml of low-expression (LE) medium (NE medium containing 0.5 mM IPTG) and then shaken for 2 hours at 26°C to allow phage production. After 2 hours 30 μg/ml kanamycin was added to the culture followed by incubation at 26°C with shaking (250 rpm) for a further 8 hours. Phage particles were purified and concentrated using PEG/NaCl precipitation and a phage titre carried out.

2.5.5.2 *PEG/NaCl Precipitation*

150 ml of the *E. coli* XL-1 blue/library was transferred to a sterile Sorvall 250 ml centrifuge tube and centrifuged at 10,000 rpm for 4°C for 10 minutes. Approximately 30 ml of autoclaved PEG/NaCl (36.52g NaCl and 50g PEG 10,000 in 250 ml H_20) solution was added to the supernatant, mixed and incubated at 4°C (on ice) for at least one hour. The mixture was then centrifuged at 10,000 rpm at 4°C for 30 minutes. The pellet was re-suspended in 40 ml of sterile ultra-pure H_2O and 8 ml of PEG/NaCl solution. The solution was then mixed and incubated at 4°C for 20 minutes. The mixture was then centrifuged at 10,000 rpm at 4°C for 20 minutes. The supernatant was removed and the pellet was re-centrifuged at 7,000 rpm at 4°C for a further 10 minutes. When all remaining supernatant had been removed, the pellet was re-suspended in 2 ml of sterile filtered PBS. The phage were then centrifuged at 4,000 rpm for 4°C for 10 minutes to remove any bacterial debris (pellet) and supernatant decanted into a fresh 1.5 ml tube. The concentrated phage was retained and stored at 4°C (short-term) or at –80°C (long-term) in a 15% (v/v) glycerol solution.

2.5.5.3 *Phage titre*

5 ml of 2xTY containing 30 μg/ml tetracycline was inoculated with a single colony of XL-1-blue overnight at 37°C. The culture was then diluted 1/100 in 5 ml of 2xTY with 30 μg/ml tetracycline and left to grow shaking at 37°C until $O.D._{550} = 0.5\text{-}0.6$. It was then allowed stand

at 37°C for 15 minutes without shaking. Serial dilutions (ranging from 10^{-1} to 10^{-11}) of phage were then prepared using the bacterial culture (180 µl *E.coli* XL-1 blue + 20 µl phage) and incubated for 30 minutes at 37°C without shaking. 100 µl of each dilution was spread on 2xTY agar plates containing 1% (w/v) glucose and 25 µg/ml chloramphenicol. The plates were allowed to grow overnight at 37°C. The colonies were counted in order to calculate the titre of the stock phage.

2.5.5.4 *Selection of antigen binders by bio-panning using Nunc immunotubes with immobilised cephalexin conjugate*

To facilitate specific scFv-displaying phage selection, immunotubes were coated overnight at 4°C with various cephalexin conjugates (e.g. thyroglobulin, bovine serum albumin, ovalbumin). This was then blocked with blocking solution i.e. PBS with 5% (w/v) dried skimmed milk powder and incubated for 1 hour at 37°C. Tubes were then washed five times with PBS-Tween and five times with PBS. 1 ml of library phage (10^{11} phage/ml) particles were added to 3 ml of PBS-T, containing 2% (w/v) powder milk. This was allowed to incubate, while gently shaking, in the immunotube for 2 hours at room temperature. After washing 10x PBS-T and 10x PBS, bound phage were eluted from the tube by adding 800 µl 0.1 M glycine/HCl, pH 2.2, for 10 minutes. This was then neutralised using 48 µl of 2 M Tris. A phage titre was then carried out as previously described in section 2.5.5.3, to determine the amount of phage recovered from each panning round. The phage titre agar plates were stored at 4°C for use in the phage ELISA.

2.5.5.5 *Re-infection of phage-displaying scFv*

5 ml of 2xTY containing 30 µg/ml tetracycline was inoculated with 50 µl of *E. coli* XL-1 blue stock were grown overnight at 37°C. The culture was then diluted 1/100 in 5 ml of 2xTY containing 30 µg/ml tetracycline and grown while shaking until the $O.D._{550}$ = 0.4-0.5. It was then allowed to stand for 10 minutes at 37°C. 700 µl of eluted phage, from the panning step, was added to the culture and allowed stand at 37°C for 30 minutes. This culture was then shaken at 37°C for 1 hour before being plated out on 2xTY agar plates containing 25 µg/ml chloramphenicol and 30 µg/ml tetracycline. It was then grown overnight at 37°C.

2.5.5.6 *Screening of individual phage-displaying scFv clones by ELISA*

96 well culture plates were filled with 200 µl/well of NE medium containing 30 µg/ml tetracycline and 25 µg/ml chloramphenicol. Single colonies were picked from the phage titre plates and transferred into each well, leaving 2 wells free as controls. The clones were allowed to grow, while shaking at 150 rpm overnight at 37°C. These were designated master plates and frozen with in media supplemented with 15% (v/v) glycerol. A second copy set of plates was prepared with 180 µl/well of NE medium. 20 µl from each well on a master plate were transferred to the corresponding well on the second plate and incubated at 37°C while shaking for approximately 6-8 hours. 25 µl of solution X (5.75 ml 2xTY, with 1% (w/v) glucose, 25 µg/ml chloramphenicol, 1.5 mM IPTG and 5 x 10^9 phage/ml) were added to each well after 5 hours. The culture was then allowed to stand for 30 minutes at 37°C followed by shaking at 150 rpm for 1 hour. The plate was then centrifuged at 4000 rpm for 10 minutes. The supernatants were removed by inversion and the media was replaced with 200 µl of solution Y (20 ml 2xTY, 25 µg/ml chloramphenicol, 30 µg/ml kanamycin, 1% (w/v) glucose and 1.5 mM IPTG). The plate was then grown overnight shaking at 150 rpm at 26°C. An ELISA plate was coated with the appropriate cephalexin conjugate overnight at 4°C. The following day the culture plates were centrifuged at 4000 rpm for 10 minutes and 75 µl of the supernatant was used for the phage ELISA. The coated ELISA plates were blocked using 5% (w/v) milk marvel in PBS. The plates were then washed 3 times with PBS and 75 µl of supernatant from the culture plates were added to the corresponding wells on the ELISA plates. 25 µl of 5% (w/v) milk marvel-PBS was then added in on top of each well and mixed gently. The phage were left to bind for 2 hours at room temperature and then washed with PBS-Tween. 100 µl/well of a 1/1000 dilution of rabbit anti-fd bacteriophage antibody was added and the plate was incubated for 1 hour at 37°C followed by HRP-labelled goat anti-rabbit IgG (1/2000 dilution). A non-inoculated well and non-transformed bacterial culture inoculated well i.e. containing no phagemid-scFv constructs, were used as negative controls. This was incubated for 30 min at 37°C and OPD substrate was used. The plates were read at 450 nm after 30 minutes.

2.5.5.7 *Preparation of bacterial glycerol stocks*

Bacteria cells were grown in 2xTY media containing the appropriate antibiotic at 37°C until the $O.D_{600}$ was 0.5-0.8. The cultures were then spun at 4,000 rpm for 10 minutes and the pellet resuspended in 2xTY containing 20% (v/v) glycerol to a volume of 1/4 the original

culture volume. 0.5 ml aliquots were transferred to 1.5 ml sterile microcentrifuge tubes and flash frozen with liquid nitrogen. Cell stocks were stored at –80°C.

2.5.5.8 *Expression of soluble scFv antibodies from E. coli harbouring the pAK400 vector with scFv gene inserts*

Enhanced soluble expression pAK400 vector was digested using *Sfi1* restriction enzyme as described in section 2.5.4.6. SOE V_L/V_H gene constructs, also digested using *Sfi1*, were ligated with linear pAK400 vector as outlined in section 2.5.4.7. Ligation reaction product was used to transform chemically competent *E. coli* JM83 cells. Transformed cells were grown in 2xTY media containing 25 µg/ml chloramphenicol and 30 µg/ml tetracycline at 37°C until the $O.D._{600}$ = 0.5-0.8. Expression of the scFv gene product was induced by the addition of 1 mM IPTG and incubated for a further 4-5 hours at 30°C with constant agitation. The cultures were later centrifuged at 4,000 rpm for 15 minutes and supernatant analysed as described in section 2.5.5.9. The remaining cell pellets were resuspended in PBS (0.15 M NaCl, 2.5 mM potassium chloride, 10 mM disodium hydrogen phosphate and 18 mM sodium dihydrogen phosphate, pH 7.4) to a volume of $1/10^{th}$ that of the original culture volume and sonicated for 45 seconds at an amplitude of 40 using a microtip Vibra Cell™ sonicator to lyse the bacterial cell walls. The cell debris was removed by centrifugation at 4,000 rpm for 5 minutes, followed by further centrifugation at 14,000 rpm for 10 minutes. The supernatant (cell lysate) was then analysed by ELISA, as described in section 2.5.5.9.

2.5.5.9 *ELISA for the determination of soluble scFv*

A screening ELISA was carried out with scFv antibody growth medium supernatants and cell lysates as described in section 2.3.1.1. In brief, dilutions of scFv antibody media or cell lysates were added to the wells of cephalexin conjugate-coated immunoplates and sequentially detected using two different secondary antibodies. A rabbit anti-histidine polyclonal was used followed by HRP-labelled goat anti-rabbit for the detection of bound scFv antibodies. In addition a mouse anti-FLAG monoclonal antibody was used and subsequently detected by HRP-labelled goat anti-mouse antibody. Colour was developed by the addition of OPD substrate solution.

2.5.6 *Production of scFv antibody from hybridoma cell line secreting monoclonal cephalexin-specific antibody*

2.5.6.1 *Preparation of V_H and V_L genes from hybridoma cells*

Cephalexin-specific antibody secreting hybridoma cell lines were produced by mammalian somatic cell fusion as described in section 2.4.3.1. Monoclonality was achieved using successive rounds of cloning by limiting dilution. Cell clone (CTh5) secreting specific anti-cephalexin antibody was used in the development of a single chain variable fragment (scFv) antibody fragment. CTh5 hybridoma cells were recovered from liquid nitrogen frozen stocks (section 2.4.2.2), transferred to a T25 culture vessel and incubated at 37°C in a humidified 5 % (v/v) CO_2 incubator. Prior to incubation, a 100 µl sample of the resuspended cells were tested for cell viability using Trypan blue staining (section 2.4.2.3). Only cells with > 90 % viability on recovery from frozen stocks were cultured further. Cells were allowed to grow until 70% confluent in T25 flasks, pelleted by centrifugation at 2,000 rpm for 10 min and resuspended in 2 ml fresh DMEM. 1 ml portions were transferred to two T75 culture flasks containing 14 ml DMEM. Flasks were incubated as before until they reached confluence and subsequently the cells were harvested. The cell pellets were pooled and centrifuged at 2,000 rpm for 10 min, and total RNA extracted using 1 ml Trizol reagent, as described in section 2.5.3.2.

2.5.6.2 *Amplification of V_H and V_L chain genes*

Amplification of variable H and L chain genes was carried using a modified version of the protocol described by Krebber *et al.* (1997). Heavy and light chain sequence primer mixes omitting the lambda light chain primers (CTh5 antibody was found to be of IgG2a isotype containing a kappa light chain), were used in separate PCR reactions. The reactions were carried out using, components as listed in section 2.5.4.2 and reaction conditions (section 2.5.4.3).

2.5.6.3 *Components of Splice by overlap extension (SOE)-PCR*

Heavy and light chain genes each approximately 400 bp were run on agarose gel and purified (section 2.5.2.2). Purified fragments were spliced together and amplified to produce 800 bp fragments by SOE-PCR. Reactions were set up using the components and conditions as described in section 2.5.4.4.

2.5.6.4 *Preparation of pAK100 vector containing SOE gene insert*

The pAK100 phagemid vector was purified (section 2.5.4.5) and both vector and SOE-PCR purified products were digested using *Sfi1* restriction enzyme digests as outlined in section 2.5.4.6. The agarose gel-purified (section 2.5.2.2) SOE and linear pAK100 vector products were ligated using DNA ligase (Promega) as described previously (section 2.5.4.7). The ligation DNA was purified and concentrated using ethanol precipitation (section 2.5.2.4). The resulting product was used to transform *E.coli* TOP10F' electrocompetent cells that were prepared as outlined in section 2.5.2.7.

2.5.6.5 *Transformation of E.coli TOP10F' electrocompetent cells with pAK100 vector containing CTh5 light and heavy chain genes and measurement of transformation efficiencies*

Electro-transformations were carried out using a gene pulser apparatus (Bio Rad) set up at 25 μF, 2.5 kV and 200 Ω (Barbas *et al.*, 2001). Electrocompetent *E. coli* TOP10F' cells were thawed on ice. A 5 μl aliquot of purified ligation product in a 1.5 ml microcentrifuge tube and 0.2 cm electroporation cuvette were placed on ice for 10 min. 50 μl of thawed cells was added to the ligation product mixed by pipetting, transferred to the pre-chilled cuvette and held on ice for 1 min. The cuvette was placed in the gene pulser apparatus and electroporated using the above conditions. It was then was flushed with 1 ml and then 2 ml portions of SOC medium at R.T. The combined 3 ml SOC medium wash was transferred to a 15 ml polypropylene tube and incubated at 37°C for 1 hour with shaking at 250 rpm. After this incubation period, 7 ml of SB medium was added. 1, 10, and 100 μl volumes of the transformants were plated on LB agar supplemented with 25 μg/ml chloramphenicol and 1% (v/v) glucose to ascertain the transformation efficiency. A Positive control (pUC18) transformation carried out using 1 μl of pUC18 (10 μg/μl, Invitrogen) vector. Control transformants were plated out on LB agar supplemented with 20 μg/ml of ampicillin. Both sets of plates were allowed to grow overnight inverted at 37°C. Transformed colonies were harvested from the agar plates and used as stocks. These stocks were suspended in 15% (v/v) glycerol, flash frozen in liquid nitrogen and stored at –80°C.

2.5.6.6 *Isolation of CTh5 scFv antibody to cephalexin*

A total of 400 individual colonies were picked from transformation efficiency streak LB agar plates (prepared as per section 2.5.6.5) and used to inoculate the wells of sterile microtitre plates containing 200 μl/well of NE medium containing 30 μg/ml tetracycline and 25 μg/ml

chloramphenicol. The plates were incubated overnight at 37°C, while shaking at 150 rpm. These were designated master plates and stored frozen (-80°C) in growth media supplemented with 15% (v/v) glycerol after a copy set of plates was prepared with 180 µl/well of NE medium. The NE medium was inoculated with 20 µl from each corresponding well on the relevant master plate. The copy plates were incubated with shaking at 37°C for approximately 6-8 hours. 25 µl of solution X (5.75 ml 2xTY, with 1% (w/v) glucose, 25 µg/ml chloramphenicol, 1.5 mM IPTG and 5 x 10^9 helper phage/ml) was added to each well after 5 hours. The culture was then allowed to stand for 30 minutes at 37°C followed by shaking at 150 rpm for 1 hour. The plate was then centrifuged at 4000 rpm for 10 minutes. The supernatants were removed by inversion and the media was replaced with 200 µl of solution Y (20 ml 2xTY, 25 µg/ml chloramphenicol, 30 µg/ml kanamycin, 1% (w/v) glucose and 1.5 mM IPTG). The plate was then grown overnight shaking at 150 rpm at 26°C. An ELISA plate was coated with the appropriate cephalexin conjugate overnight at 4°C. The following day the culture plates were centrifuged at 4000 rpm for 10 minutes and 75 µl of the supernatant was used for the phage ELISA. The coated ELISA plates were blocked using PBS-T (0.15 M NaCl, 2.5 mM potassium chloride, 10 mM disodium hydrogen phosphate and 18 mM sodium dihydrogen phosphate, 0.05% (v/v) Tween 20, pH 7.4) containing 5% (w/v) dried skimmed milk powder for 1 hour at 37°C and washed 3 times with PBS. To each corresponding ELISA screening plate well was added 75 µl of supernatant from the culture plates. 25 µl of 5% (w/v) milk marvel-PBS was then added in to each well and mixed gently. Phage supernatants were left to bind for 2 hours at room temperature with gentle rocking followed by 3x washes with PBS-T. 100 µl/well of a 1/1000 dilution of rabbit anti-fd bacteriophage antibody was added to each well and plates incubated for 1 hour at 37°C. The plates were washed 3x PBS-T and 3x PBS, followed by addition of HRP-labelled goat anti-rabbit IgG (1/2000 dilution) and incubation for 1 hour at 37°C. Plates were washed as before and the OPD substrate added. The absorbance of each well was read at 450 nm after 30 minutes.

2.5.6.7 *Soluble expression of CTh5 scFv*

Soluble scFv was expressed as outlined in section 2.5.5.8.

2.5.6.8 *Purification of soluble His-tagged scFv by immobilised metal affinity chromatography (IMAC)*

A 1 ml aliquot of Ni^{+}-NTA agarose resin (QIAgen) was added to a 20 ml universal tube and equilibrated with 20 ml of running buffer (PBS-T with 1% (v/v) Tween-20, 500 mM NaCl and 10 mM β-mercaptoethanol). The solution was centrifuged at 4000 rpm for 10 minutes and the supernatant (running buffer) removed. Sonicated cytoplasmic extracts (2-3 ml) containing His-tagged recombinant protein were added to the pellet (Ni^{+}-NTA resin) and mixed for 1-2 hours at room temperature on a rocking platform. The mixture was then centrifuged at 4000 rpm for 10 minutes and the supernatant (unbound material) removed. The pellet (resin with bound protein) was washed 3 times with 20 ml of running buffer. The proteins were eluted with 2-3 ml of sodium acetate buffer, pH 4.5, and neutralised with 2 M Tris buffer, pH 8.8. Purified proteins were stored at –20°C. This represents a generic protocol for IMAC-based scFv purification.

2.6 Production of mutant phage-display libraries

Following isolation and characterisation the wild type (WT) scFv gene construct was excised and used as template for random intra-gene digestion with RQ1 RNase-Free DNase 1 endonuclease (Promega) and subsequent error-prone PCR, in order to produce mutant WT phage-display libraries. WT gene fragments were used as 'self primers' in a PCR for re-amplification of the gene construct. The product of this PCR (fragments ranging from 600-800 bp) was amplified by SOE-PCR, as previously described, using with sc_{for} and sc_{back} primers. Multiple 50 µl volume SOE-PCR reactions were carried out to amplify a large amount of WT gene, yielding an 800 bp (approximately) band. The reaction product was run on agarose gel subsequent to purification. Each purified product was pooled and concentrated using ethanol precipitation so as to produce a concentrated working stock.

2.6.1 *DNase 1 endonuclease digests optimisation*

The DNase 1 concentration (U/rxn), WT DNA concentration (µg/µl) and digest time were optimised for the reaction so as to yield 50 bp fragments. The final digestion reaction consisted of 1 µl (1 U/µl) DNase, 2 µl buffer (400 mM Tris-HCl, pH 8, 100 mM $MgSO_4$ and 10 mM $CaCl_2$). The total reaction volume was made up to 20 µl with molecular grade H_20 and initiated by adding 5 µg/rxn WT SOE-PCR product. The reaction was allowed to proceed at R.T. for 5 minutes and stopped using 1 µ EDTA (20 mM, pH 8) followed by DNase inactivation by placing in a water bath at 65°C for 10 minutes. The digest products was

analysed on a 1% (w/v) agarose gel and fragments were purified and concentrated using ethanol precipitation (section 2.5.2.4).

2.6.2 *PCR of DNase 1 digestion products*

Purified DNase 1 digest product (fragments approximately 50 bp) was used as template for PCR reactions to re-construct scFv genes. PCR reaction components and conditions were as follows:

Component	**Stock concentration**	**Concentration in 50 µl**
Digested fragments	varied	300 ng
10 X buffer	10 X	1 X
$MgCl_2$	25 mM	1.0 mM
dNTP	20 mM	0.4 mM
Taq polymerase	5 U/µl	5 U/reaction
H_2O	to 50 µl including *Taq*, DNA, sc_{for} and sc_{back} per rxn.	

PCR conditions for amplification of digested light and heavy chain genes

95°C X 2 min 'Hot-start'
Add 5 U *Taq* Polymerase/rxn
95°C X 30 min
50°C X 1 min
72°C X 1 min
Repeat X 35 cycles
72°C X 6 min

All ramping rates were at 4°C/sec.

The PCR product was analysed on a 1% (w/v) agarose gel, purified and concentrated using ethanol precipitation (section 2.5.2.4).

2.6.3 *Error-prone PCR of reconstructed fragments*

Two separate reactions were set up using reconstructed scFv genes with sc_{for} and sc_{back} primers, designated R1* and R2*. The reaction components were as follows:

R1*

Component	Stock concentration	Concentration in 50 µl
Reconstructed fragment	varied	300 ng
5 X buffer A	5 X	1 X
dNTP	20 mM	0.4 mM
sc_{for}	varied	0.05 nmol /reaction
sc_{back}	varied	0.05 nmol/reaction
Taq polymerase	5 U/µl	5 U/reaction
H_2O	to 50 µl final volume including *Taq*, DNA , sc_{for} and sc_{back}	

PCR buffer A (7.5 mM $MgCl_2$, pH 8.5).

R2*

Component	Stock concentration	Concentration in 50 µl
Reconstructed fragment	varied	300 ng
5 X buffer A	5 X	1 X
dNTP	20 mM	0.4 mM
MnCl2	25 mM	0.5 mM
sc_{for}	varied	0.05 nmol /reaction
sc_{back}	varied	0.05 nmol/reaction
Taq polymerase	5 U/µl	5 U/reaction
H_2O	to 50 µl final volume including *Taq*, DNA , sc_{for} and sc_{back}	

PCR buffer A (7.5 mM $MgCl_2$, pH 8.5).

2.6.4 *Preparation of pAK100 vector containing SOE R1* and R2* gene constructs*

The pAK100 phagemid vector was purified (section 2.5.4.5) and both vector and R1* and R2* SOE-PCR ethanol-purified products were digested using *Sfi1* restriction enzyme digests, as outlined in section 2.5.4.6. The agarose gel-purified (section 2.5.2.2) R1*, R2* fragments and linear pAK100 vector were ligated using DNA ligase (Promega) (section 2.5.4.7). The ligation mixes were purified and concentrated using ethanol precipitation (section 2.5.2.4). The resulting products were used to transform *E.coli* TOP10F' electrocompetent cells (section 2.5.2.7), and to construct mutant R1* and R2* phage-display mutant library stocks.

2.6.5 *Selection of cephalexin binders by panning of mutant R1* and R2* phage libraries*

Both mutant phage-scFv libraries (i.e. R1* and R2*) were subjected to three rounds of panning with immobilised conjugates, as described in section 2.5.5.4, with additional phage elution using a 100 ng/ml solution of free cephalexin in PBS-T containing 2% (w/v) dried skimmed milk powder. A 1 ml solution of free cephalexin was added to each immunotube and incubated for 30 min at 37°C. This solution was then decanted and used to infect *E. coli* in the exponential growth phase (section 2.5.5.5). After eluting phages with cephalexin, any remaining bound phages were eluted using glycine solution, as per section 2.5.5.4. Subsequently, eluted phage fractions were used to infect *E. coli* XL1 blue. Phage-scFv clones were then screened as before (2.5.5.6).

2.7 Development of SPR-based immunoassay using a BIAcore 3000TM biosensor

Analysis was carried out on a BIAcore 3000™ instrument using a carboxymethylated dextran (CM5) sensor chips. The running buffer for all BIAcore experiments was HBS buffer, pH 7.4, containing 10 mM HEPES, 150 mM NaCl, 3.4 M EDTA, and 0.05% (v/v) Tween 20. The running buffer was filtered (pore size of 0.45 μm) and degassed using a filtration apparatus (Millipore sintered glass filtration unit) immediately before use.

2.7.1 *Preconcentration studies*

In order to optimise the conditions needed for the immobilisation of desired proteins on the sensor chip surface, 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, was performed. Preconcentration is in essence an optimisation of the buffer pH so that it falls below the isolectric point (pI) of the protein (i.e. cephalexin-protein conjugate)

and facilitates the maximum binding of the protein through electrostatic interactions. Therefore, protein solutions were prepared in 10 mM sodium acetate at a range of different pHs and these solutions passed over an underivatised chip surface, with the degree of electrostatic binding monitored. The pH at which the highest preconcentration of protein onto the underivatised surface was observed was chosen as the pH for immobilisation.

2.7.2 *General cephalexin-protein conjugates immobilisation conditions*

The carboxymethylated dextran matrix was activated by mixing equal volumes of 100 mM NHS (N-hydroxysuccinimide) and 400 mM EDC (N-ethyl-N-(dimethyl-aminopropyl) carbodiimide hydrochloride), and injecting the mixture over the sensor chip surface for 7 minutes at a flow rate of 5 µl/minute. The desired protein to be immobilised was diluted in 10 mM sodium acetate at the optimised pH with a typical concentration of 50-100 µg/ml. This solution was then injected over the derivatised chip surface for 60 minutes at 2 µl/minute. Un-reacted NHS groups were capped, and non-covalently bound protein removed by injection of 1 M ethanolamine hydrochloride, pH 8.5, for 5 min.

2.7.3 *Direct cephalexin immobilisation conditions*

The carboxymethylated dextran matrix was activated by mixing equal volumes of 100 mM NHS (N-hydroxysuccinimide) and 400 mM EDC (N-ethyl-N-(dimethyl-aminopropyl) carbodiimide hydrochloride), and injecting the mixture over the CM5 sensor chip surface for 16 minutes at a flow rate of 5 µl/minute. The surface was converted to amine functionality by injecting ethylene diamine (1M pH 8.5 with HCl) for 20 minutes at a flow rate of 5 µl/min. Immediately afterwards a 200 µg/ml cephalexin solution dissolved in equal volumes of 100 mM NHS (N-hydroxysuccinimide) and 400 mM EDC (BIAcore amine coupling kit) was injected over activated surface for 40 minutes at a flow rate of 5 µl/min. Un-reacted sites on the chip surface were capped, and non-covalently bound material removed by two sequential injections of 1 M ethanolamine hydrochloride, pH 8.5, for 5 min at a flow rate of 10 µl/min.

2.7.4 *Regeneration studies*

To assess the stability of immobilised cephalexin and conjugate surfaces, a known concentration of antibody was passed over the chip surface, and the surface regenerated by passing over various concentrations of NaOH and HCl ranging in concentration from 1-30 mM. This cycle of regeneration was usually completed for greater than 50 cycles, and the

binding signal measured to assess the stability and suitability of the immobilised surface for the development of a sensitive and reproducible assay.

2.7.5 *'Spiked' whole milk sample preparation*

A 5 mg portion of cephalexin hydrate was weighed out and diluted in 1 ml whole milk (well known brand locally purchased). The cephalexin milk solution was incubated at 37°C for 10 min and vortexed. Serial dilutions of stock cephalexin were prepared accordingly. Each of the samples to be assayed was placed in a water bath at 95°C for 3 min (Gustavsson, 2003) followed by centrifugation at 14,000 rpm at R.T. for 15 min. The aqueous phase was gently removed using a 200 µl pipette and diluted 1:2 with the appropriate antibody dilution.

2.7.6 *Inhibition assay for the detection of cephalexin using anti-cephalexin antibodies*

The cephalexin-thyroglobulin conjugate was immobilised on a CM5 sensor chip (TCC) surface as described in section 2.7.2 while cephalexin was directly immobilised to the CM5 sensor chip surface (DIC) as outlined in section 2.7.3. Each of the antibodies studied (i.e. polyclonal, monoclonal, and scFv) were diluted in HBS, PBS-T and prepared whole milk assay diluents. The antibody dilutions were optimised to yield a response ranging from 100-500 response units (RU). For each antibody both TCC and DIC chip surfaces were evaluated. Antibodies were passed across the TCC and DIC chip surfaces following pre-incubation with a range of cephalexin solution concentrations (i.e. from 100,000 ng/ml to 5 ng/ml) for 20 min at R.T. Each of the antibody-cephalexin standard samples were mixed thoroughly by inverting several times placed in the BIAcore 3000 auto-sampler and randomly passed over the appropriate sensor surface (in triplicate). The mean response (RU) was calculated and expressed as a ratio of RU/RU_0, where the 'spiked' sample response (RU) was divided by the response with no free cephalexin (RU_0).

Chapter 3

Production of polyclonal antibodies to cephalexin and the development of ELISA-based assay for the detection in milk

3.1 Polyclonal antibody production

The aim of the work described in this chapter was the production, purification and characterisation of polyclonal antibodies to cephalexin. The production of cephalexin-protein (i.e. thyroglobulin-, bovine serum albumin (BSA)-, ovalbumin-cephalexin) conjugates, and their subsequent use in the production of polyclonal antibodies to cephalexin is presented. Polyclonal antibodies produced were evaluated in the development of an enzyme–linked immunosorbent assay (ELISA) for the detection of cephalexin in PBS and whole milk sample matrices.

3.1.1 *Introduction to polyclonal antibody production*

The need for a rapid and reliable assay for the determination of cephalosporin antibiotics has already been described in chapter 1. European laws clearly set out Maximum Residue Limits (MRL's) for this class of compounds. The work presented in this thesis deals with cephalexin, a first generation semi-synthetic cephalosporin. Current analysis techniques are based on microbiological, receptor or enzyme-based screening methods that are inexpensive and broadly sensitive to a range of different antimicrobials but lack specificity. Confirmatory testing may also be carried out using chromatographic methods. High Performance Liquid Chromatography (HPLC) and Liquid chromatography (LC) are the most commonly used method for the specific determination of β-lactams in milk, as they are both sensitive and specific (Schenck *et al.*, 1998 and Samanidou *et al.*, 2003). However, LC/HPLC techniques involve extensive sample clean up when analysing complex biomatrices like milk (Fedeniuk and Shand, 1998). They also require trained operators, are expensive and require bulky equipment. Immunoassays may offer greater advantages in comparison to the microbial screening assays and HPLC/LC, as they are relatively inexpensive, sensitive, robust and highly specific. They also facilitate portability with assay transfer onto lateral flow devices or 'biochips' for field-testing or on farm testing.

Antibodies or Immunoglobulins (Igs) are produced by an organism's immune system as part of the humoral immune response. Upon initial exposure, mature B-lymphocytes present in the secondary lymphoid organs (e.g. spleen, lymph nodes) of the particular organism produce antibodies with multiple specificities (Chapter 1). Antibodies produced are specific to relatively small regions on the antigens surface, referred to as the epitopes or antigenic determinants. In the production of antibodies, antigens may be of various different chemical

classes ranging from biological to non-biological molecules. The relative molecular mass of the target antigen plays an important role in the subsequent immune response, as if it is relatively small molecular weight (<5,000 Da) for example, illicit drugs or some hormones, it may not be recognised by the organism's immune system. Such compounds are termed haptens. In antibody production this obstacle is overcome by coupling the hapten of interest to larger protein carrier. Antibodies will be produced that are specific to the desired hapten and also the carrier protein. Bovine serum albumin (BSA), ovalbumin (OVA), thyroglobulin (THY), and non-protein polymers e.g. dextran, are generally used for coupling to haptens to elicit an immune response. Antibodies are produced and circulated within the blood of the organism; such antibody populations are heterogeneous in nature, derived from multiple parental B-cells and are termed as polyclonal (i.e. mixture of antibodies reactive to many different epitopes).

Polyclonal antibodies can be raised quickly and at relatively low cost to the producer when a sufficient supply of target analyte is available. This is in contrast to the expense and expertise required for traditional hybridoma and recombinant antibody production techniques (Stapleton *et al.*, 2003). One of the main challenges when producing antibodies against haptens (e.g. cephalexin) is the need to conjugate such small molecules to larger ones (e.g. protein), so as to elicit an immune response and generate antibodies. Polyclonal antibody production techniques offer a relatively cheap and easy way to assess the potential immune response to a particular conjugated hapten. Once a serum titre is obtained against conjugated hapten, the antibody binding to free (i.e. un-conjugated) hapten may be evaluated in inhibition or competitive ELISA formats. There are potential drawbacks to using polyclonal antibody production techniques, depending on the desired application. These include, the facts that there is a finite supply of homogenous antibody populations, by their very nature they possess multiple epitope specificities and production varies from animal to animal, leading to problems with quality.

3.2 Results

3.2.1 *Characterisation of cephalexin-carrier protein conjugates*

Cephalexin-protein and cephalexin-dextran conjugates were synthesised using various carbodiimide-coupling strategies, as shown in section 2.2.1. Initially, this involved activating the carboxyl group on the cephalexin molecule, and subsequent reaction with amine groups on the relevant protein (designated cephalexin-activated ester coupling, figure 2.1 (A)). However, cephalexin conjugates produced by this method lacked stability. It was observed that cephalexin–protein conjugates produced by activating the carboxyl groups on the protein and subsequent reaction with the amine functional group on the cephalexin (designated protein-activated ester coupling, figure 2.1 (B)) showed increased stability.

Cephalexin (Ceph)-protein conjugates were characterised by indirect checkerboard ELISA (section 2.3.1.2) using a cephalexin-specific polyclonal antibody, previously described by Dillon *et al* (2003a), that was generously donated by Dr. Aart van Amerongan (designated AVAab). This was carried out to confirm the cephalexin had been covalently linked to the respective carrier protein. Figures 3.1 and 3.2 show typical results obtained for checkerboard ELISAs using Bovine Serum Albumen (BSA) and ovalbumin (Ova) - Ceph conjugates, with AVAab anti-cephalexin polyclonal antibody.

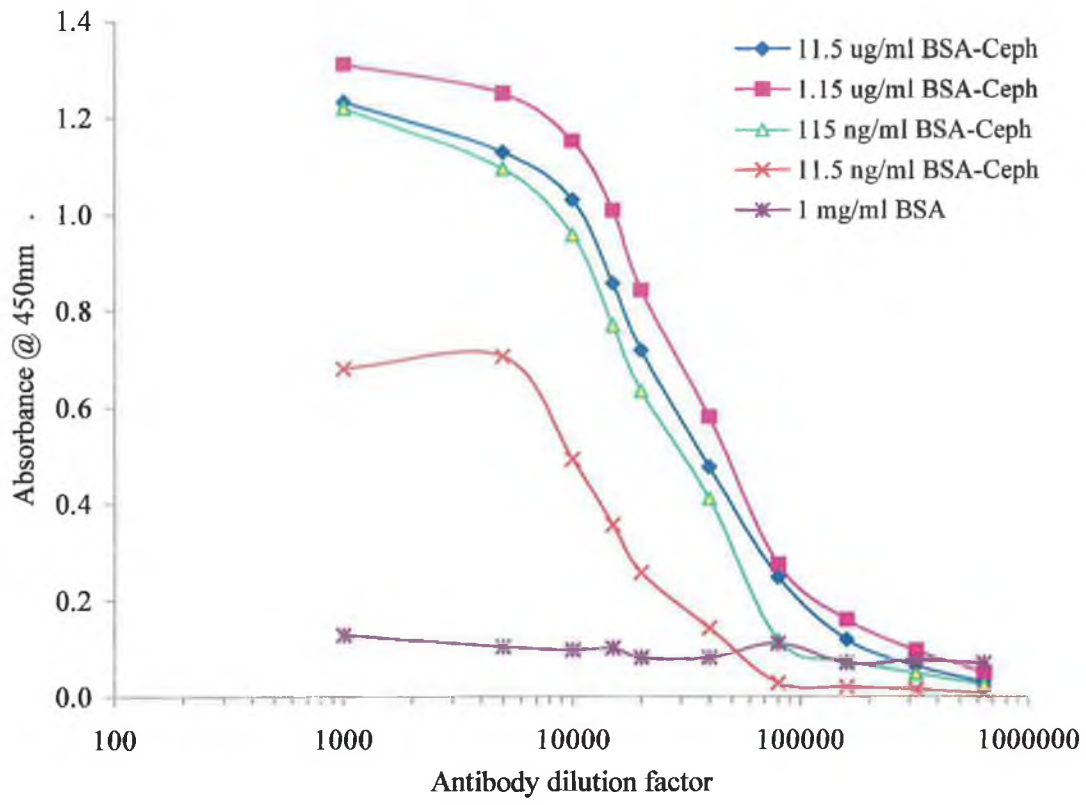


Figure 3.1. Results obtained from a checkerboard ELISA using various coating concentrations (11.5 ng/ml, 115 ng/ml, 1.15 µg/ml, and 11.5 µg/ml) of the BSA-cephalexin (BSA-Ceph) conjugate detected with varying dilutions of the AVAab polyclonal anti-cephalexin primary antibody. HRP-labelled goat anti-rabbit secondary antibody was used for the detection and the absorbance measured at 450 nm with OPD as substrate. Wells coated with 1 mg/ml BSA dissolved in PBS were used as negative controls. Each point represents the mean of two measurements.

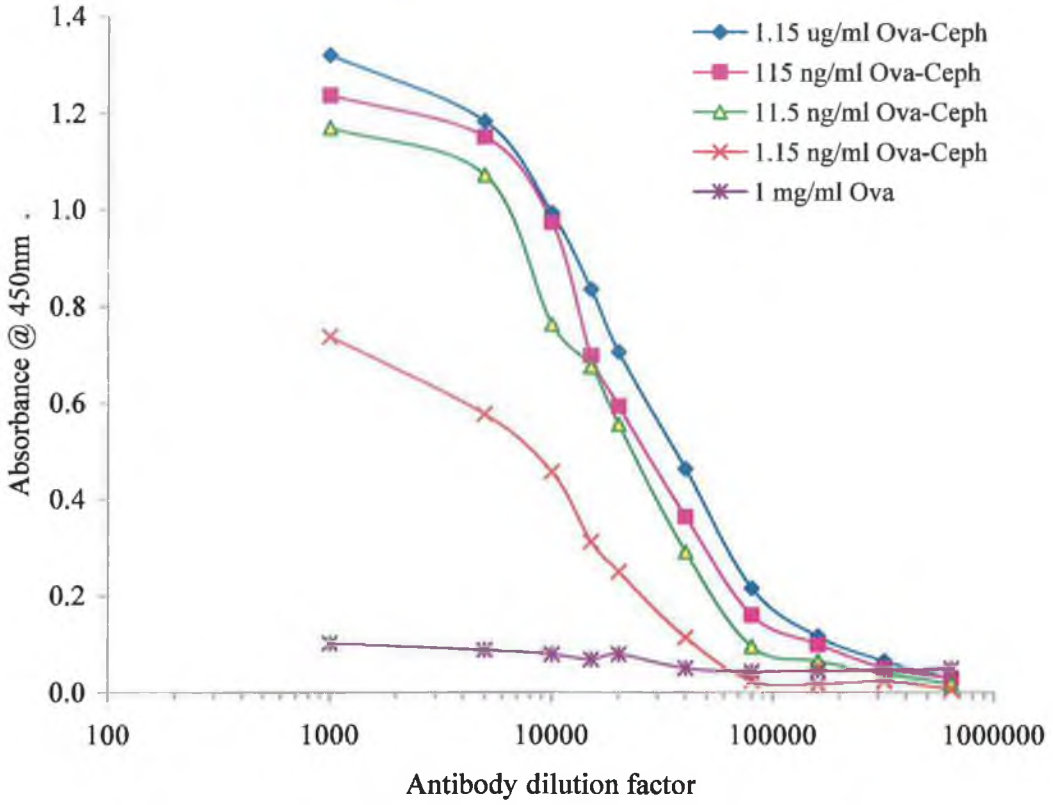


Figure 3.2. Results obtained from a checkerboard ELISA using various coating concentrations (11.5 ng/ml, 115 ng/ml, 1.15 μg/ml, and 11.5 μg/ml) of the Ovalbumin-cephalexin (Ova-Ceph) conjugate detected with the AVAab polyclonal anti-cephalexin primary antibody. HRP-labelled goat anti-rabbit secondary antibody was used for detection and the absorbance measured at 450 nm with OPD as substrate. Wells coated with 1 mg/ml ovalbumin dissolved in PBS were used as negative controls. Each point represents the mean of two measurements.

The protein concentrations of the cephalexin-conjugates were determined using a BCA (Bicinchonic acid) protein assay kit (Pierce) according to manufacturers' guidelines. BCA assays were carried out, as described in section 2.3.4. The protein concentration of each conjugate was estimated by constructing a calibration curve with relevant protein standards (i.e. BSA for BSA-Ceph conjugate). A typical result of a BCA assay carried out for thyroglobulin-cephalexin conjugate is shown in figure 3.3. UV photometric spectroscopy was used to give putative evidence that the cephalexin had covalently bound to protein, by comparison of the spectra for the individual components of the reaction (i.e. cephalexin and protein) and the relevant conjugates post dialysis (section 2.2.1.2). However, it was observed that the cephalexin molecule has a broad absorbance peak from 290nm to 200nm thus masking the protein absorbance maximum at 280nm.

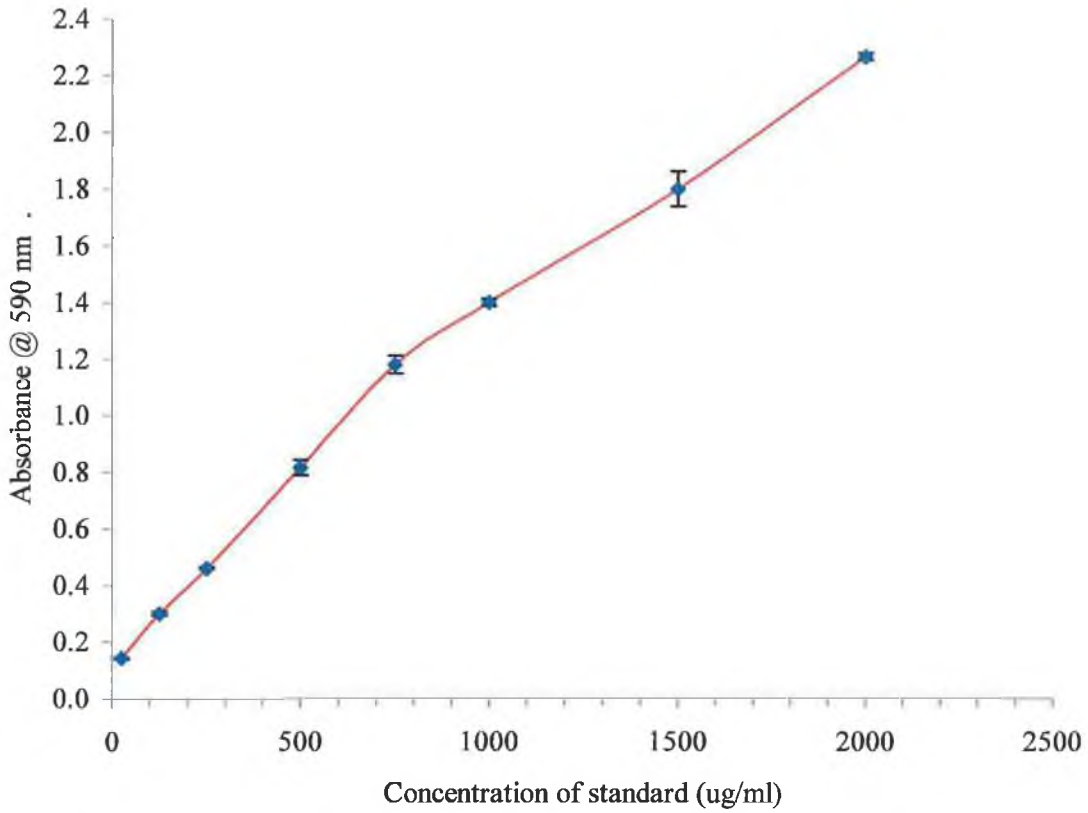


Figure 3.3. Plot of the results obtained from a typical BCA assay standard curve. The calibration curve was constructed to estimate the concentration of a thyroglobulin-cephalexin conjugate (Thyro-Ceph) and was prepared using thyroglobulin protein standards ranging from 0 µg/ml to 2,000 µg/ml. 25 µl of each standard was added to the wells of a 96-well plate and colour developed by the addition of 200 µl of working reagent. Following incubation at 37°C for 30 min the absorbancies were read at 590 nm. Each point represents the mean of two measurements.

3.2.2 *Production and purification of polyclonal antibody (designated anti-CephP1) to cephalexin.*

3.2.2.1 *Immunogen preparation and immunisation*

A 1 mg/ml solution of BSA-Ceph conjugate diluted in PBS was added to an equal volume of Freund's Complete adjuvant (FCA). The immunogen was prepared and immunisation carried out as described in section 2.2.2. Once a sufficient titre was obtained ($\geq$ 1/100,000) the animal was sacrificed and the serum removed.

3.2.2.2 *Serum titre of polyclonal anti-serum against thyroglobulin-cephalexin conjugate*

An indirect titre ELISA, as described in section 2.3.1.1, was used to determine IgG antibody titre. Serum was obtained from whole blood by centrifugation and serially diluted in PBS-T containing 2% (w/v) dried skimmed milk powder. 96 well plates were coated with a 13.2 μg/ml thyroglobulin-cephalexin conjugate and the serum dilutions were added in to the wells triplicate. The antibody titre for the purposes of this work was defined as the highest dilution that yielded a response distinguishable from the background plus three standard deviations. The polyclonal anti-serum demonstrated sufficiently high titre (> 1/100,000) as can be seen in figure 3.4 (A). A similar titre ELISA was carried out using cephalexin dextran (Ceph-Dex) conjugate and coating a 96-well plate with 1:1,000 and 1:10,000 dilutions of Ceph-Dex and carrying out the rest of the assay as above, with serial dilutions of serum ranging from 1:400 to 1:204,800. The rabbit was sacrificed and the terminal-bleed taken for subsequent antibody purification and characterisation.

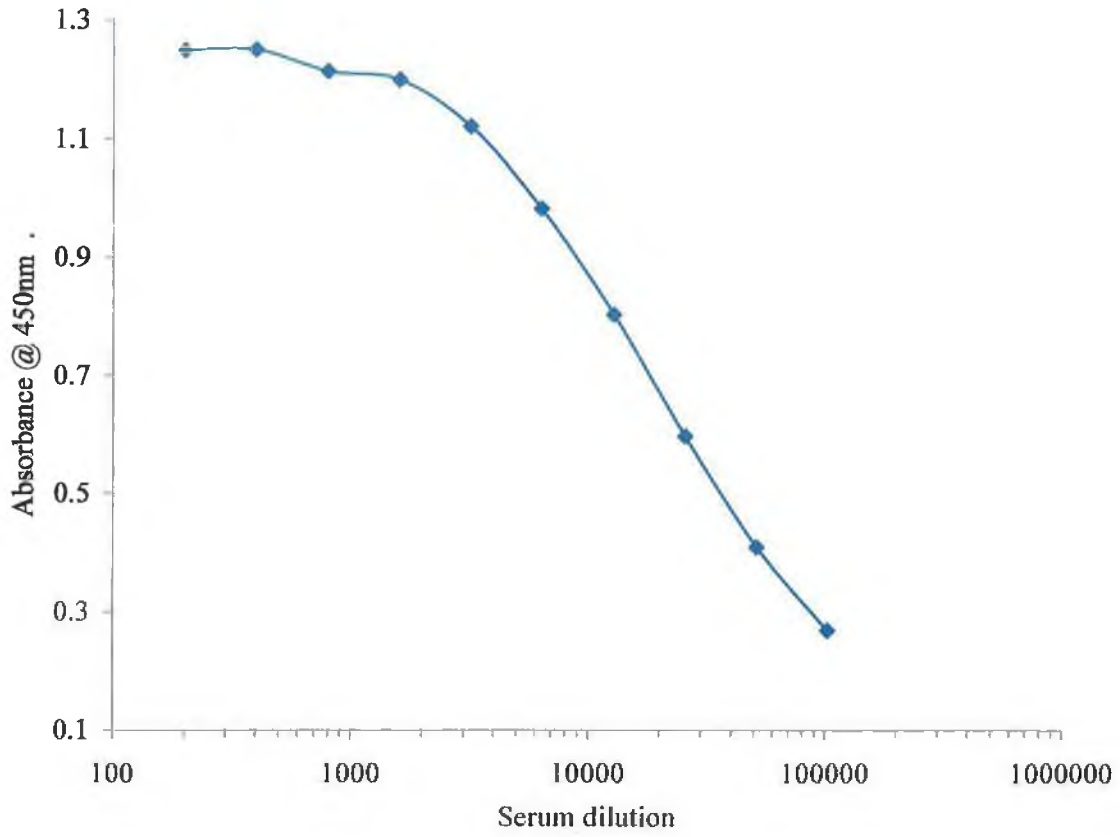


Figure 3.4. (A) Determination of antibody titre to cephalexin using an indirect ELISA (section 2.3.1.1). A 96-well Nunc immunoplate was coated with 13.2 µg/ml thyroglobulin-cephalexin (Thyro-Ceph) conjugate and serial dilutions of crude rabbit serum from 1/200 to 1/102,400, diluted in PBS-TM dilution buffer (section 2.1.6.3), were added. Three wells were coated with 10 µg/ml thyroglobulin and, following addition of 1/200 serum diluted as above, were used as a negative control. Each point represents the mean of three measurements.

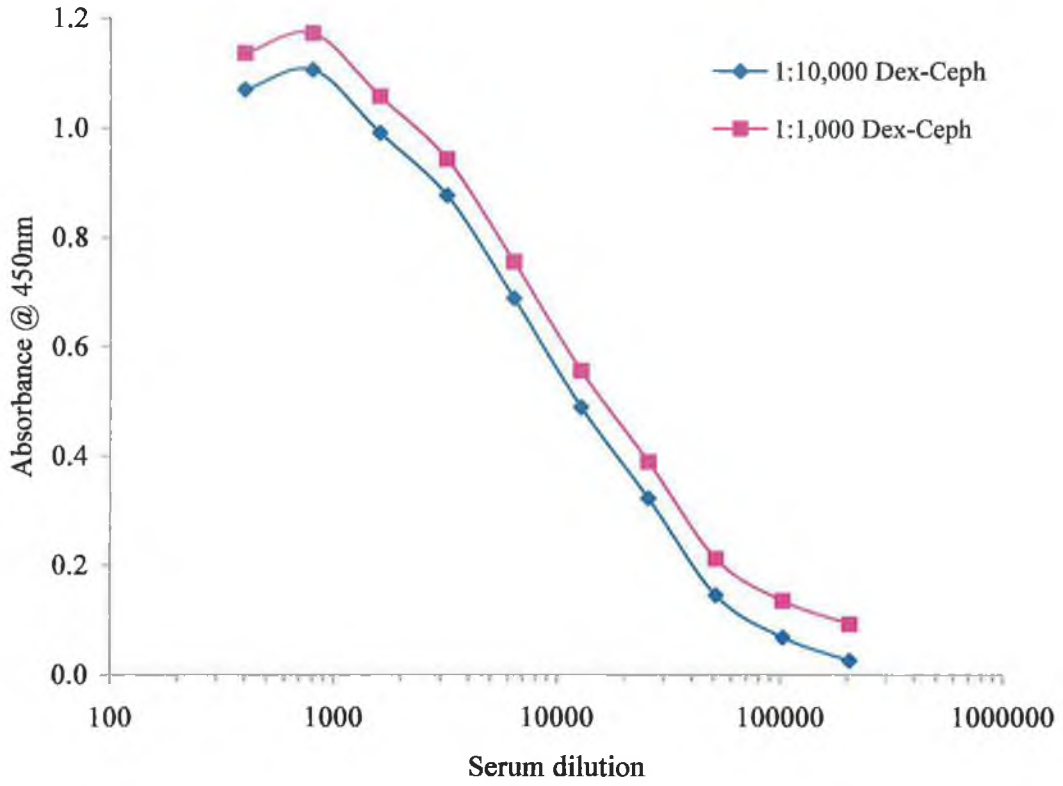


Figure 3.4. (B) Determination of antibody titre to cephalexin using an indirect ELISA (section 2.3.1.1). A 96-well Nunc immunoplate was coated with 1:1,000 and 1:10,000 dilutions of cephalexin dextran conjugate (Dex-Ceph) conjugate and serial dilutions of crude rabbit serum from 1/400 to 1/204,800, diluted in PBS-TM dilution buffer (section 2.1.6.3), were added. Three wells were coated with 10 µg/ml non-conjugated dextran and, following addition of 1/200 serum diluted as above, were used as a negative control. Each point represents the mean of three measurements.

3.2.2.3 *Purification of polyclonal anti-serum*

The crude rabbit serum was initially purified using saturated ammonium sulphate (SAS) precipitation, as described in section 2.2.3.1. Following dialysis against PBS the SAS-purified fraction was applied to a protein-G affinity chromatography column (section 2.2.3.2). The partially purified SAS precipitated antibody was passed through the column twice. The SAS portion was allowed to run through the column, by gravity, to maximise IgG binding to the protein-G immobilised phase via the Fc region. The column was then extensively washed so as to remove any non-specific impurities. A 0.1 M glycine solution, pH 2.2, was then used to elute the IgG fraction. Eluted fractions were subsequently neutralised with 2 M Tris base. A total of 16 fractions were taken and aliquots of each were transferred to a quartz cuvette and the absorbance at 280 nm was determined. Fractions with the highest absorbance values at 280 nm, as determined from figure 3.5, were pooled and dialysed overnight at 4°C against PBS.

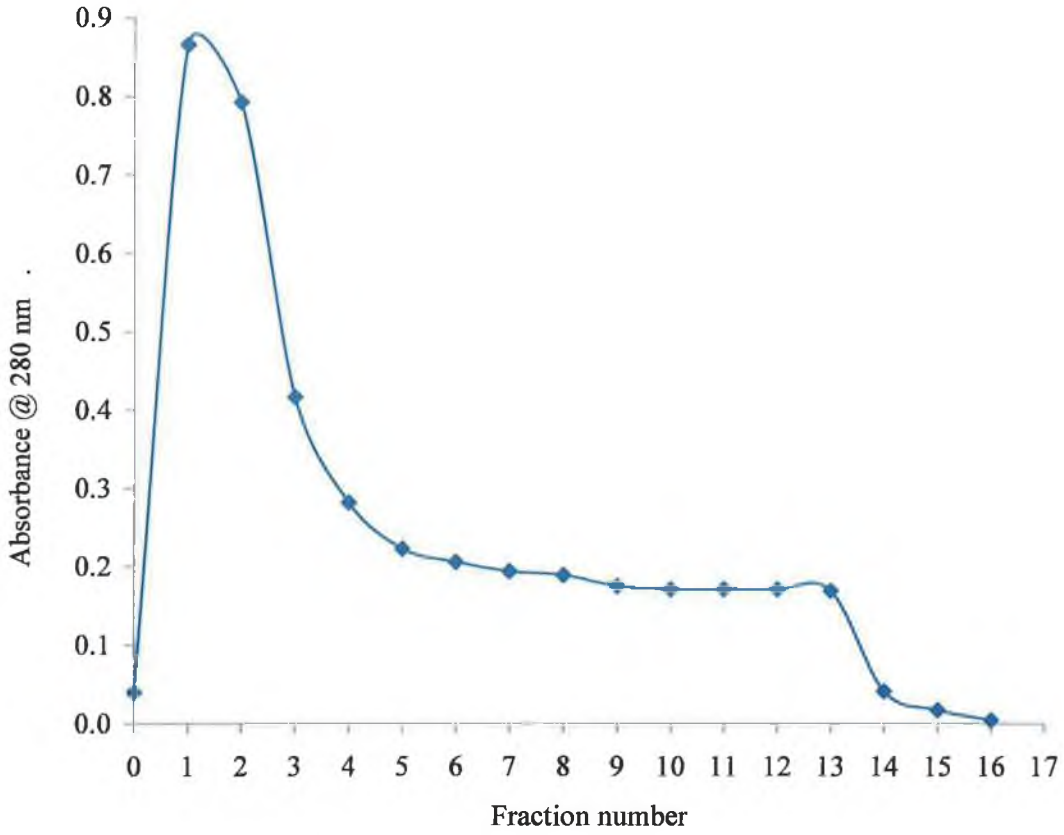


Figure 3.5. Elution profile of the protein-G column purified fractions for the SAS precipitated rabbit anti-*CephP1* polyclonal serum. Antibody was eluted from the column using a 0.1 M glycine solution, pH 2.2, and neutralised with a 2 M Tris base, pH 8. A total of 16x 1 ml (A small aliquot of each fraction was transferred to a quartz cuvette) fractions were collected. The total protein content of each fraction was determined by measuring the absorbance at 280 nm. Fractions 1-4 were pooled and dialysed overnight at 4°C against two 5L portions PBS to ensure complete buffer exchange.

3.2.2.4 *Characterisation of polyclonal antibody purification by SDS-PAGE, Western blotting and ELISA.*

The purity of the anti-*CephP1* antibody was analysed by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) under denaturing conditions and subsequent Western blotting analysis, probing with an anti-rabbit horseradish peroxidase (HRP)-labelled secondary antibody, as detailed in sections 2.3.2 and 2.3.3, respectively. The SDS-polyacrylamide gel picture (figure 3.6) shows the serum, SAS precipitate, and protein G-purified fractions. The affinity-purified antibody appears as two separate bands on the SDS-PAGE gel with approximate molecular weights of 50kDa and 25 kDa, representing the heavy and light chains, respectively. The heavy chain may be seen in the Western blot (figure 3.7). However; the light chain was not detected on the western blot as an HRP-conjugated goat anti-rabbit IgG (Fc) specific antibody was used. The activity of the purification fractions (i.e. serum, SAS precipitate and protein-G purified) was analysed using a screening ELISA (section 2.3.1.1) the result of which showed minimal loss of activity during purification (figure 3.8).

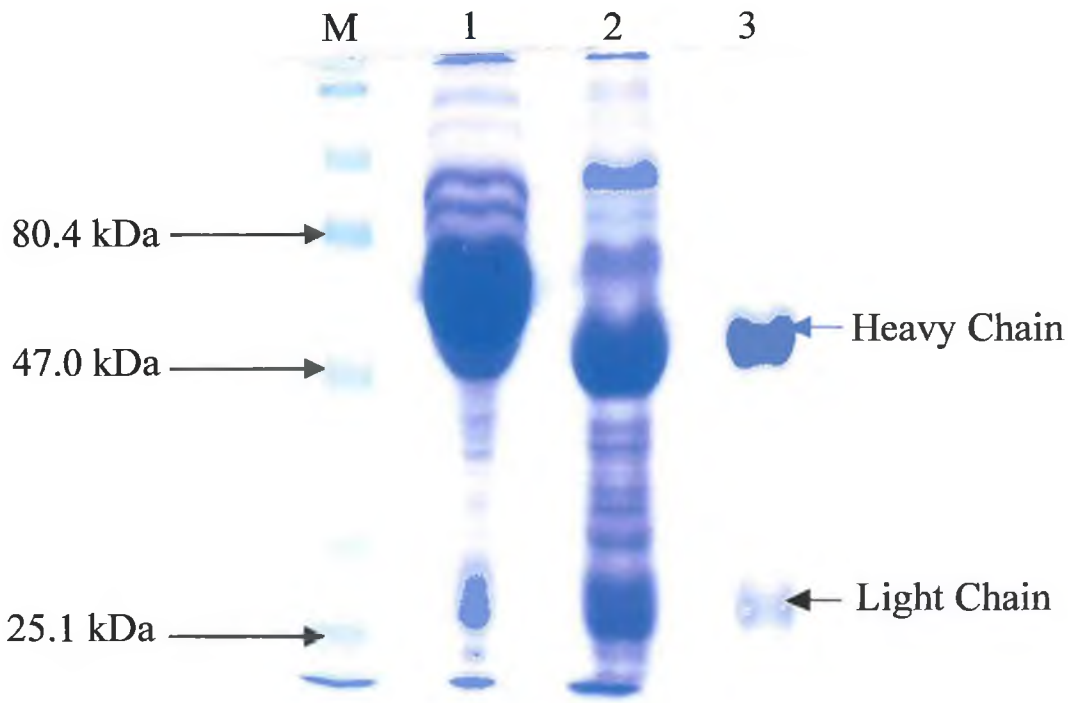


Figure 3.6. Denaturing SDS-PAGE gel of the polyclonal antibody following purification. The serum, SAS, and protein G-purified samples of the anti-cephalexin polyclonal antibody were loaded from left to right, respectively. Lane M, represents Blue Ranger pre-stained standard molecular weight marker, obtained from Pierce (Table 2.6); Lane 1 is the crude serum diluted 1:20 in PBS; Lane 2 is the SAS (saturated ammonium sulphate precipitate) diluted 1:2 in PBS; Lane 3 is neat protein-G affinity-purified pooled fractions. Heavy chain and light chain domains are highlighted at approximately 50 kDa and 25 kDa, respectively.

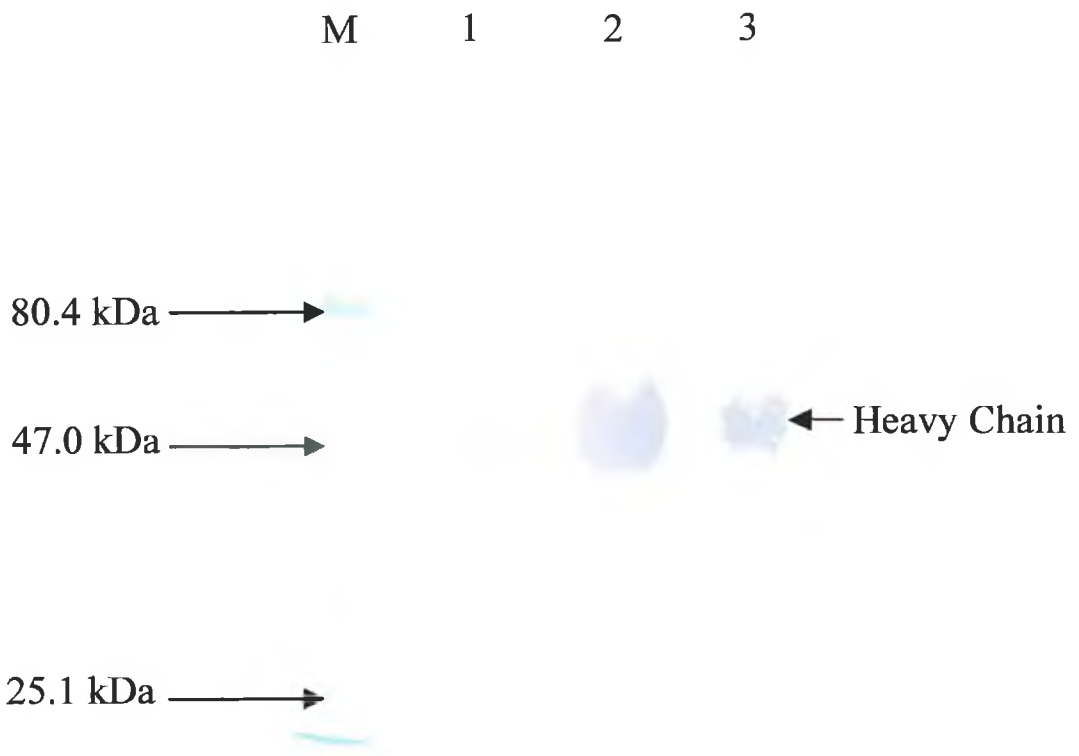


Figure 3.7. Western blot analysis of polyclonal antibody preparations following transfer from denaturing SDS-PAGE. The serum, SAS, and protein G-purified samples for anti-cephalexin polyclonal antibody were loaded, as in figure 3.6. Lane M, represents Blue Ranger pre-stained standard molecular weight marker, obtained from Pierce (Table 2.6); Lane 1 is the crude serum diluted 1:20 in PBS; Lane 2 is the SAS (saturated ammonium sulphate precipitate) diluted 1:2 in PBS; Lane 3 is neat protein-G affinity-purified pooled fractions. Heavy chain domain is highlighted at approximately 50 kDa. Following transfer to nitrocellulose membrane (section 2.3.3) heavy chain was detected, as highlighted above, using a HRP-labelled goat anti-rabbit IgG specific antibody, with subsequent addition of TMB substrate system. Light chain was not visualised as the HRP-labelled goat anti-rabbit IgG secondary antibody was Fc specific.

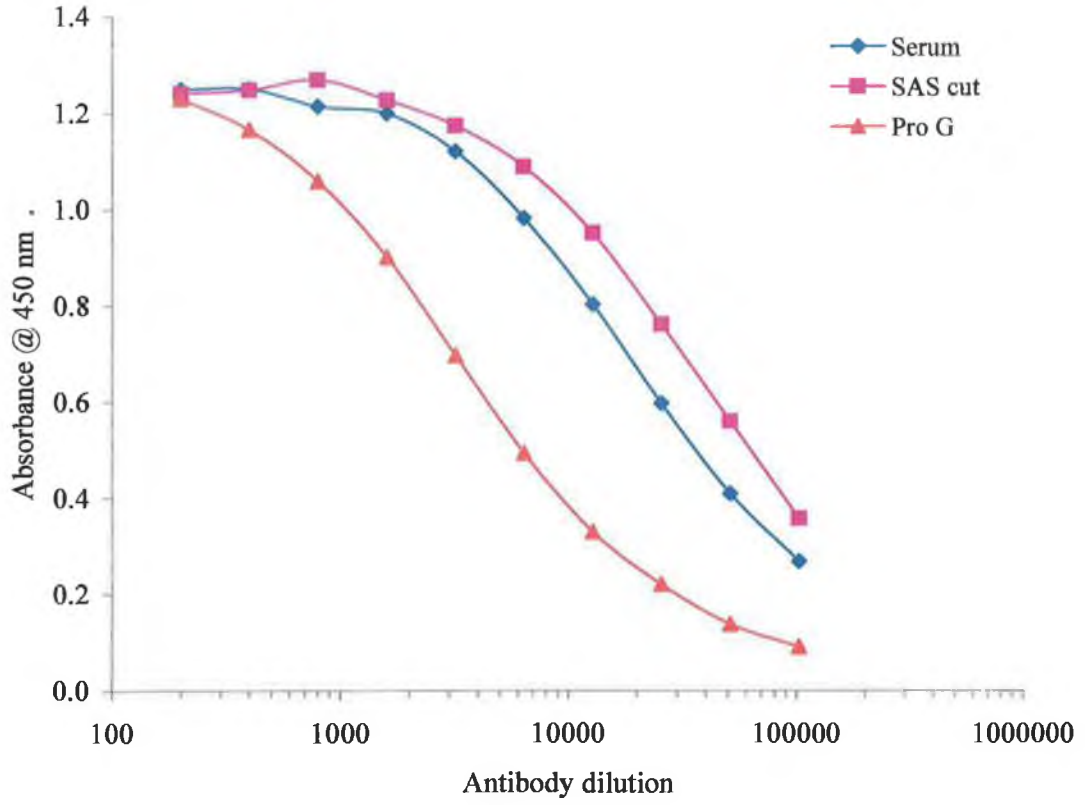


Figure 3.8. Antibody titres of serum, SAS and protein-G purified fractions using an indirect ELISA (section 2.3.1.1). A 96-well Nunc immunoplate was coated with 13.2 μg/ml thyroglobulin-cephalexin (Thyro-Ceph) conjugate and serial dilutions of crude rabbit serum, SAS precipitate and protein-G purified polyclonal antibody fractions. The dilutions ranged from 1/200 to 1/102,400, and were prepared in PBS-TM dilution buffer (section 2.1.6.3). Three wells were coated with 10 μg/ml thyroglobulin and following addition of 1/200 serum, diluted as above, were used as negative control. Each point represents the mean of three measurements.

3.2.3 *Development of ELISA for the detection of cephalexin using polyclonal antibody (anti-CephP1)*

3.2.3.1 *Checkerboard ELISA for determining the optimal conjugate coating concentration and antibody dilution.*

An indirect checkerboard ELISA was used to determine the optimal coating dilution of conjugate (described in section 2.3.1.2). Varying dilutions of a 1.32 mg/ml stock solution of thyroglobulin-cephalexin conjugate were prepared in PBS (pH 7.4) and coated on a 96 well microtitre plate. Dilutions of the protein G-purified anti-*CephP1* antibody were added to each of the conjugate dilutions. The results were plotted (Figure 3.9). The optimal coating concentration of thyroglobulin-cephalexin conjugate was found to be 1.32 µg/ml. The optimal polyclonal antibody (anti-*CephP1*) dilution, which gave the greatest change in absorbance per change in antibody dilution, was determined to be 1/1,500. These conditions were used in the development of an inhibition ELISA for the detection of free cephalexin.

3.2.3.2 *Development of an inhibition ELISA for the detection of cephalexin hydrate in spiked PBS and whole milk sample matrices.*

Inhibition assays for the detection of cephalexin hydrate were developed in spiked PBS and 'spiked' whole milk samples using the affinity-purified anti-*CephP1* polyclonal antibody. The optimal conjugate coating concentration and protein-G purified polyclonal antibody dilution (section 3.2.3.1) were used initially to develop an inhibition assay for the detection of cephalexin in 'spiked' PBS-T buffer containing 2% (w/v) skimmed milk powder. The Thyro-Ceph coating concentration used for subsequent inhibition assay development was 1.3 µg/ml and an antibody dilution of 1/1,500. Standard 'spiked' solutions of cephalexin hydrate were prepared by serial dilution of a 1 mg/ml stock solution (1:2) in the appropriate sample matrix (i.e. PBS-T 2% (w/v) skimmed milk powder (section 2.1.6.3) and whole milk) and incubating as described in section 2.3.1.3. The cephalexin standard and antibody mixtures were then added to the microtitre plate and incubated at room temperature with gentle mixing. Following incubation, the rabbit anti-*CephP1* polyclonal was detected with an HRP-conjugated goat anti-rabbit antibody (section 2.3.1.3). The inhibition assay carried out in PBS-T 2% (w/v) had a limit of detection for free cephalexin of approximately 30 ng/ml (taking the limit of detection to be the background (i.e. A_0) plus three standard deviations). The A_0 represents the relevant antibody dilution and no free cephalexin. The absorbance (A) for each 'spiked' sample was expressed as a ratio of the A_0 (i.e. antibody with no free

cephalexin). The assay was subsequently carried out using whole milk as diluent for the standard cephalexin concentrations. The limit of detection was found to be 20 ng/ml. Inter- and intra-day variability studies were carried out using the optimised assay conditions.

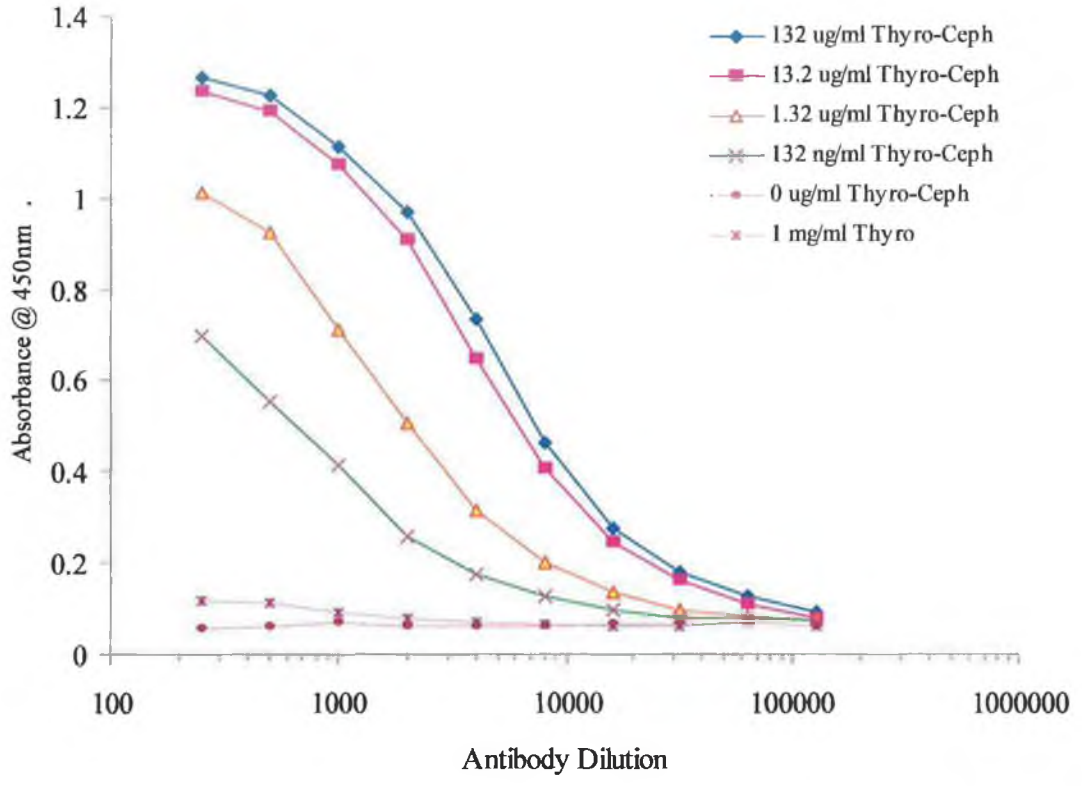


Figure 3.9. Results obtained from a checkerboard ELISA to determine the optimal conjugate coating concentration. A 96 well plate was coated using various concentrations of thyroglobulin-cephalexin (Thyro-Ceph) ranging from 0 µg/ml to 132 µg/ml. A 1 mg/ml solution of non-conjugated thyroglobulin (Thyro) was used as a negative control. The wells were subsequently blocked with PBS containing 5% (w/v) skimmed milk powder. Following this, serial dilutions of protein-G purified polyclonal antibody prepared in PBS-TM dilution buffer, were added. The anti-cephalexin polyclonal antibody was detected with HRP-labelled goat anti-rabbit polyclonal antibody (Sigma) and absorbance measured at 450 nm using OPD substrate.

3.2.3.3. *Intra-day assay variability studies of rabbit anti-cephalexin polyclonal (anti-CephP1) antibody in PBS.*

An inhibition ELISA format (section 2.3.1.3) was used in the development of an assay for the detection of free cephalexin hydrate in spiked phosphate buffered saline (PBS, 0.15 M NaCl, 2.5 mM potassium chloride, 10 mM disodium hydrogen phosphate and 18 mM sodium dihydrogen phosphate, pH 7.4). A 96 well microtitre plate was coated with a 1.3 μg/ml solution of thyroglobulin-cephalexin conjugate and blocked with a 5% (w/v) solution of milk marvel. Standard solutions of cephalexin hydrate were prepared in PBS and mixed with anti-*CephP1* polyclonal antibody. The linear range of detection was from 1.9 to 3906 ng/ml. The polyclonal anti-cephalexin antibody and standard cephalexin mixtures were incubated at 37°C for 30 min and then added to the wells of a microtitre plate. A HRP-conjugated goat anti-rabbit antibody was used to detect the presence of rabbit IgG after the plate was washed initially with PBST and then with PBS. To carry out intra-day variability studies, five sets (i.e. five separate plates) of cephalexin hydrate standards were prepared and incubated with antibody prior to being assayed on the same day. Each concentration point was prepared in triplicate on each plate. The intra-day assay results were plotted (figure 3.10) and a limit of detection for the assay was found to be 20 ng/ml. The coefficients of variation (CV's) were determined as a measure of the assay's precision, by expressing the standard deviation as a percentage function of the mean A/A_0 value. The A/A_0 was determined by dividing the absorbance mean for each concentration point by the absorbance mean of the A_0 (i.e. the absorbance obtained with antibody and no free antibiotic). CV's for the intra-day assay ranged from 2.85% to 9.80% (Table 3.1).

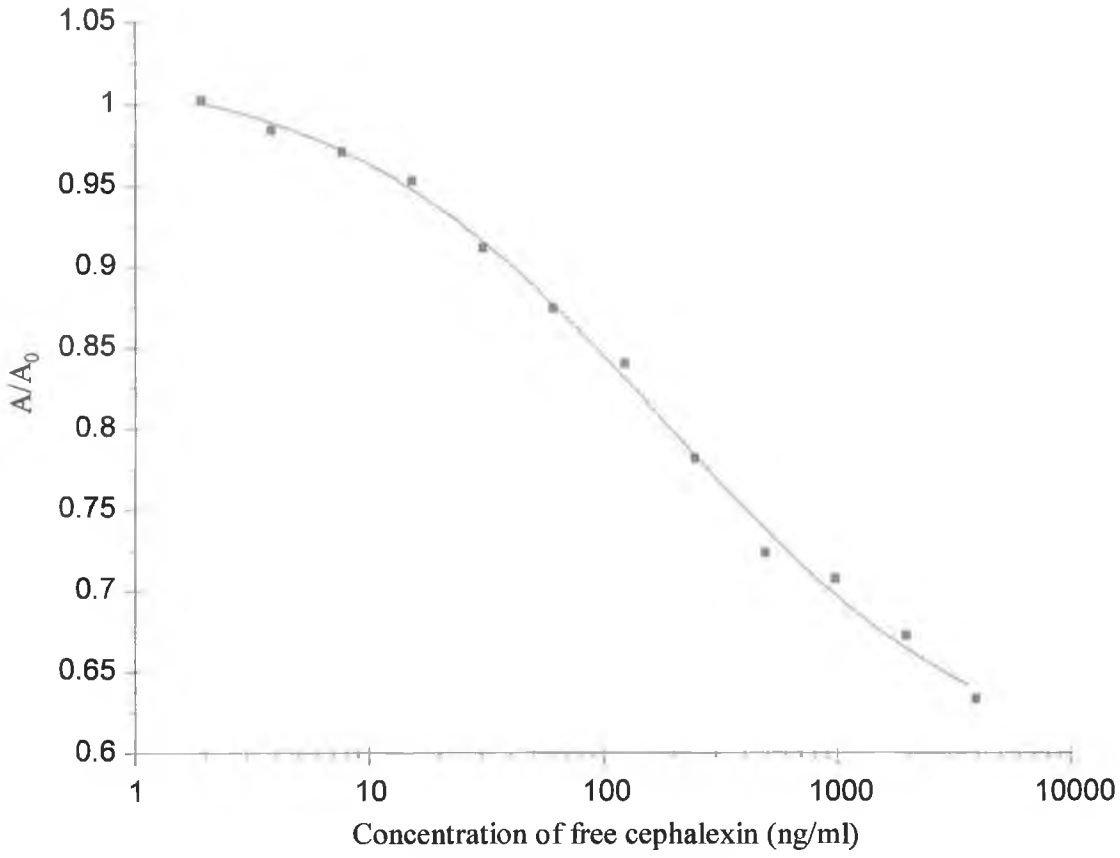


Figure 3.10. Inhibition ELISA assay calibration curve for rabbit anti-cephalexin polyclonal (anti-*CephP1*) antibody. A four-parameter equation was fitted to the intra-day data set using BIAevaluation 3.1 software. The mean A/A_0 represents the mean of five sets of data. The mean absorbance for each standard cephalexin concentration (A) is divided by the background, i.e. antibody with no free cephalexin (A_0). For each of the five replicates, each point was carried out in triplicate. The linear range of detection was found to be from 1.9 to 3906 ng/ml. The limit of detection (LOD) was estimated by taking three standard deviations away from the background (A_0). The LOD for the assay was 20 ng/ml.

Table 3.1. Calculated coefficients of variation for the rabbit anti-cephalexin polyclonal (anti-*CephP1*) antibody intra-day assay in PBS. The calculated mean A/A_0 values and standard deviation for each concentration point are shown.

Concentration of cephalexin (ng/ml)	Calculated mean $A/A_0 \pm$ S.D.	CV's %
3906.3	0.633 ± 0.053	8.4
1953.1	0.672 ± 0.030	4.5
976.6	0.707 ± 0.038	5.3
488.3	0.723 ± 0.071	9.8
244.1	0.781 ± 0.039	5.0
122.1	0.840 ± 0.066	7.9
61.0	0.874 ± 0.039	4.4
30.5	0.911 ± 0.062	6.8
15.3	0.952 ± 0.048	5.1
7.6	0.970 ± 0.037	3.9
3.8	0.984 ± 0.032	3.3
1.9	1.002 ± 0.029	2.9

3.2.3.4. *Inter-day assay variability studies of rabbit anti-cephalexin polyclonal (anti-CephP1) antibody in PBS.*

Inter-day assay variability studies were carried out for the anti-*CephP1* polyclonal antibody in 'spiked' PBS. Five sets of standards were set up across a concentration range of cephalexin as described for the intra-day assay. The variability study was carried out by repeating the polyclonal inhibition assay five times, over five consecutive days (Findlay *et al.*, 2000). The limit of detection for the assay was estimated by subtracting three standard deviations from the background (A_0). The inter-day assay for the detection of cephalexin hydrate was found to have a limit of detection of 20 ng/ml (figure 3.11), with CV's ranging from 4.9% to 13.7% (Table 3.2).

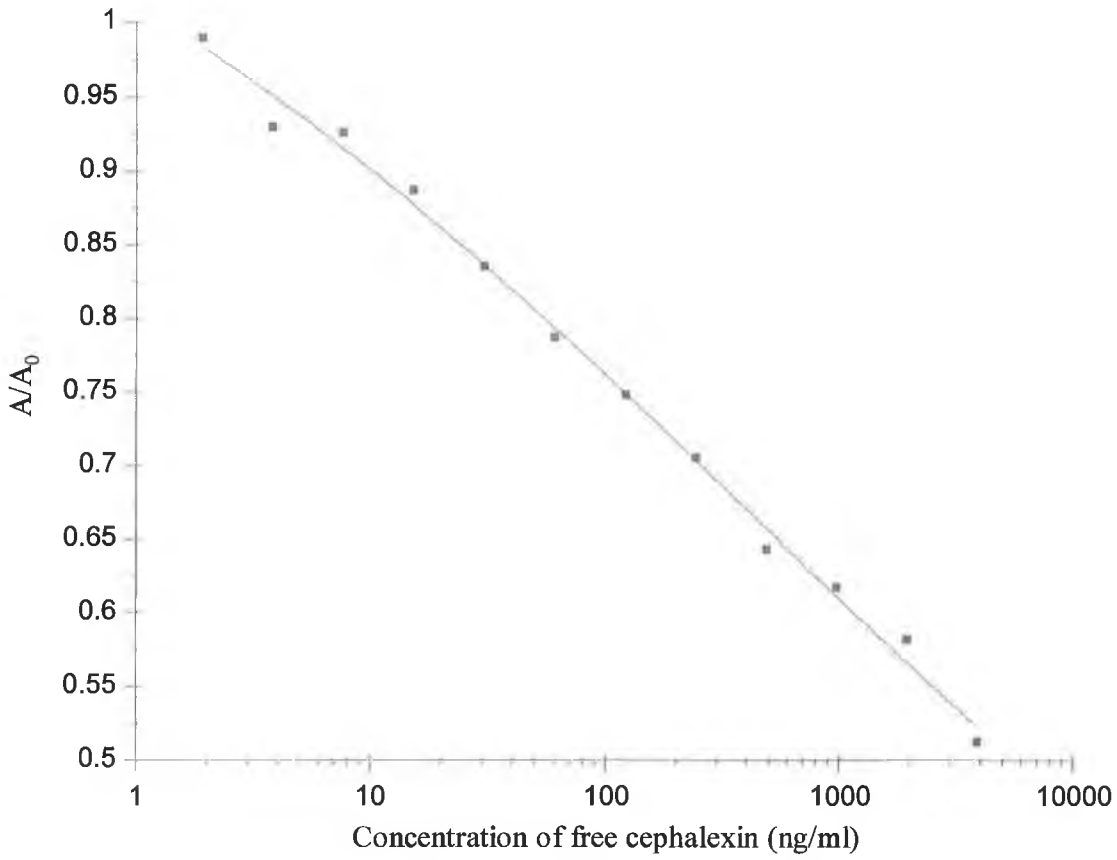


Figure 3.11. Inhibition ELISA assay calibration curve for rabbit anti-cephalexin polyclonal (anti-*CephP1*) antibody. A four-parameter equation was fitted to the inter-day data set using BIAevaluation 3.1 software. The mean A/A_0 represents the mean of five sets of data. The mean absorbance for each standard cephalexin concentration (A) is divided by the background, i.e. antibody with no free cephalexin (A_0). For each of the five replicates, each point was carried out in triplicate. The limit of detection (LOD) was estimated by taking three standard deviations away from the background (A_0). The LOD for the assay was 20 ng/ml.

Table 3.2. Calculated coefficients of variation, mean A/A_0 values and standard deviations for the rabbit anti-cephalexin polyclonal antibody inter-day assay, carried out in PBS.

Concentration of cephalexin (ng/ml)	Calculated mean $A/A_0 \pm$ S.D.	CV's %
3906.3	0.513 ± 0.025	5.0
1953.1	0.582 ± 0.048	8.3
976.6	0.617 ± 0.060	9.8
488.3	0.643 ± 0.088	13.7
244.1	0.705 ± 0.080	11.3
122.1	0.748 ± 0.074	9.9
61.0	0.787 ± 0.078	9.9
30.5	0.835 ± 0.073	8.7
15.3	0.887 ± 0.059	6.7
7.6	0.926 ± 0.065	7.0
3.8	0.930 ± 0.050	5.5
1.9	0.990 ± 0.048	4.9

3.2.3.5. *Inter-day assay variability studies of anti-cephalexin polyclonal antibody in whole milk.*

An inhibition ELISA (section 2.2.5), was developed to determine the free cephalexin levels in 'spiked' whole milk samples. A pint of a well-known brand of whole milk was purchased. This was stored at -20° C in 20.5 ml aliquots that were thawed and 'spiked' with cephalexin, as required. A 96 well microtitre plate was coated with a 1.3μg/ml solution of thyroglobulin-cephalexin conjugate and blocked with a 5% (w/v) solution of milk marvel in PBS (0.15 M NaCl, 2.5 mM potassium chloride, 10 mM disodium hydrogen phosphate and 18 mM sodium dihydrogen phosphate, pH 7.4). Five sets of standards, across a concentration range of cephalexin, were prepared in the whole milk and mixed with antibody diluted to 2 x the working dilution, also diluted in whole milk. The assay was carried out as described previously. The inter-day assay results were plotted (figure 3.12) and the limit of detection for the assay was found to be 20 ng/ml. The CV's for the intra-day assay ranged from 3.8% to 14.6% as shown in table 3.3.

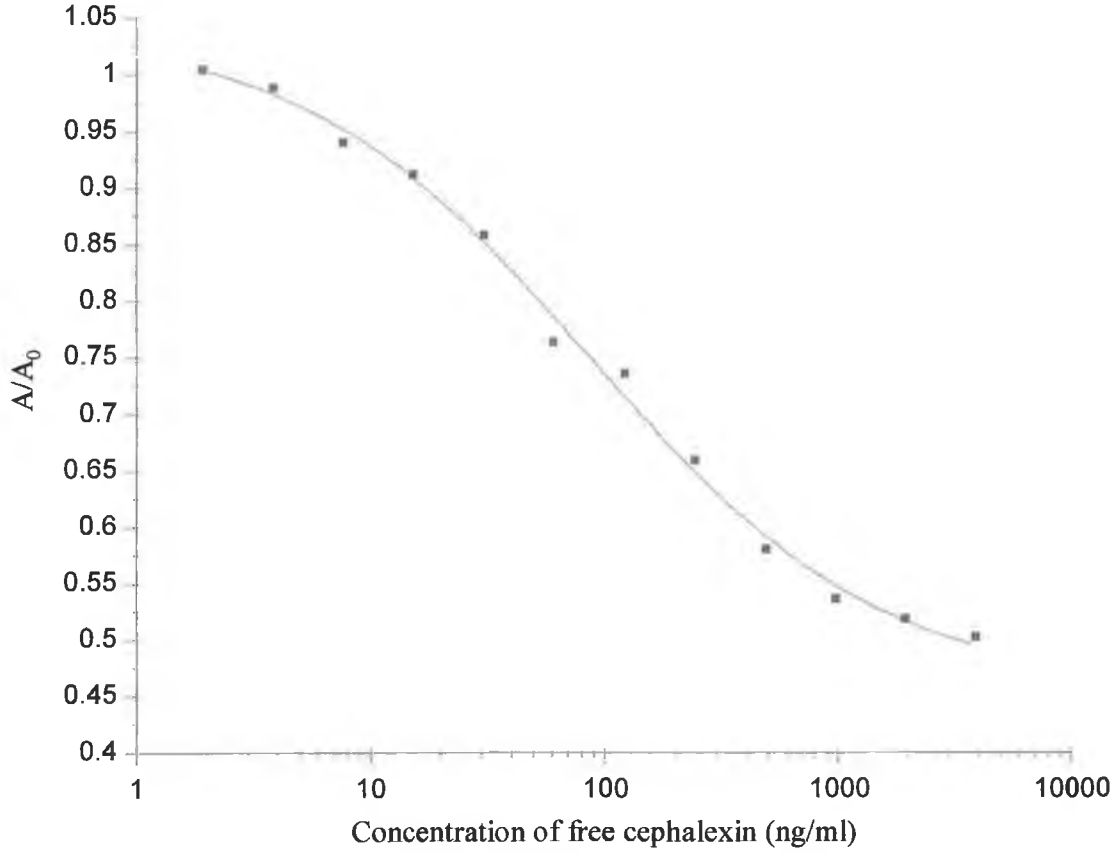


Figure 3.12. Inhibition ELISA assay calibration curve for rabbit anti-cephalexin polyclonal (anti-*CephP1*) antibody. A four-parameter equation was fitted to the inter-day data set using BIAevaluation 3.1 software. The assays were carried out in using cephalexin 'spiked' whole milk. The mean A/A_0 represents the mean of five sets of data. The mean absorbance for each standard cephalexin concentration (A) is divided by the background, i.e. antibody with no free cephalexin (A_0). For each of the five replicates, each point was carried out in triplicate. The limit of detection (LOD) was estimated by taking three standard deviations away from the background (A_0). The LOD for the assay was 20 ng/ml.

Table 3.3. Calculated coefficients of variation of inter-day assay study for the rabbit anti-cephalexin polyclonal antibody assay carried out in whole milk. The calculated mean A/A_0 values and standard deviation for each concentration point are shown.

Concentration of cephalexin (ng/ml)	Calculated mean $A/A_0 \pm$ S.D.	CV's %
3906.3	0.502 ± 0.063	12.6
1953.1	0.518 ± 0.076	14.6
976.6	0.536 ± 0.043	8.0
488.3	0.580 ± 0.063	10.8
244.1	0.658 ± 0.094	14.3
122.1	0.735 ± 0.094	12.7
61.0	0.763 ± 0.099	13.0
30.5	0.858 ± 0.074	8.6
15.3	0.911 ± 0.069	7.6
7.6	0.940 ± 0.068	7.2
3.8	0.988 ± 0.043	4.4
1.9	1.004 ± 0.038	3.8

3.2.4. *Cross-reactivity studies of the rabbit anti-cephalexin polyclonal (anti-CephP1) antibody.*

Cross reactivity studies were carried out with various related β-lactam compounds using an inhibition ELISA format. Since cephalexin is such a relatively small compound it is probable that the anti-cephalexin polyclonal antibody will cross-react with similar chemical structures. The extent of the cross reactivity and with which particular compound it occurs is critical to the performance of the immunoassay. Various related β-lactam antibiotic compounds including, cefazolin sodium salt, cephalosporin C zinc salt, cefuroxime sodium salt, oxacillin sodium salt, cefadroxil, amoxicillin tri-hydrate, cloxacillin sodium salt, 7-aminodesacetoxy cephalosporanic acid, cefoperazone sodium salt, (+)- 6 -aminopenicillanic acid, dicloxacillin sodium salt and cefotaxim sodium salt were chosen as potential cross reactants. The chemical structures of these compounds are outlined in table 3.4. The cross-reactivity characteristics of an immunoassay are invariably defined as the concentration of analyte that gives a 50% decrease (IC50) in the overall signal, from a concentration range of zero analyte (IC0) to the maximum concentration of analyte detected by the assay (IC100). The target analyte concentration at the IC50 is taken as a reference for calculating the percentage cross-reactivity (% CR) of a potential cross-reactant. For each potential cross-reactant an inhibition ELISA was carried out as described for the cephalexin immunoassay. The IC50 concentration of cephalexin was expressed as 100% of the cross-reactivity and each potential compounds IC50 value was expressed as a percentage of this value using the following formula:

$$\% \text{ cross-reactivity (CR)} = \frac{\text{Concentration of analyte at IC50}}{\text{Concentration of cross-reactant at IC50}} \times 100$$

The immunoassays were carried out in whole milk as a sample matrix, the resulting calculated cross-reactivity for each of the compounds, listed in table 3.4, are shown in table 3.5. An overlay plot of each inhibition assay is for cephalexin and potential cross-reactant is shown in figure 3.13.

Table 3.4. List of the potential cross-reactant (XR) structures tested in ELISA and the label assigned to each.

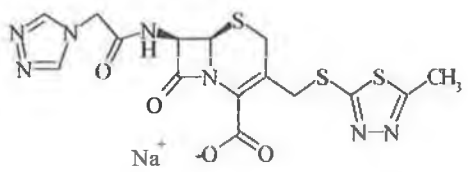
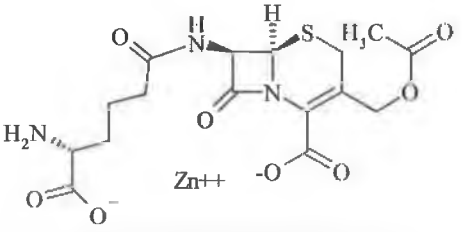
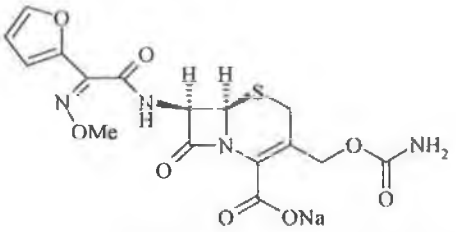
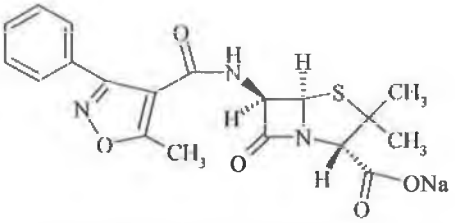
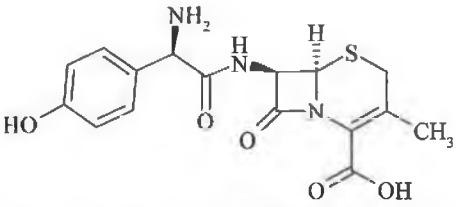
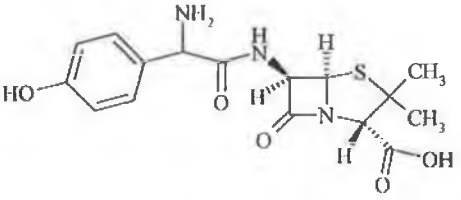
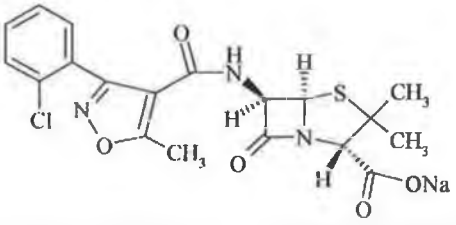
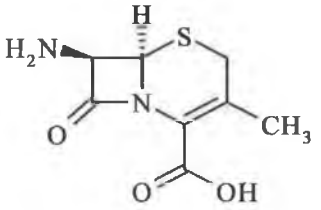
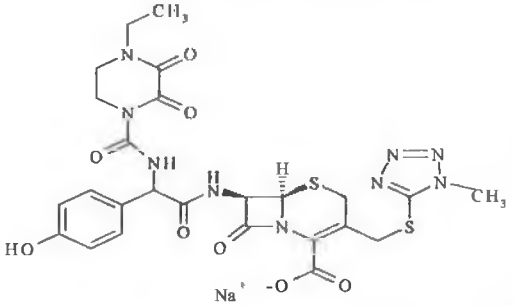
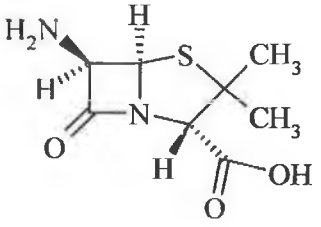
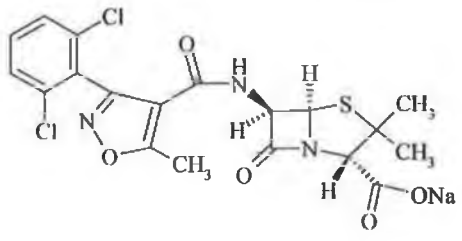
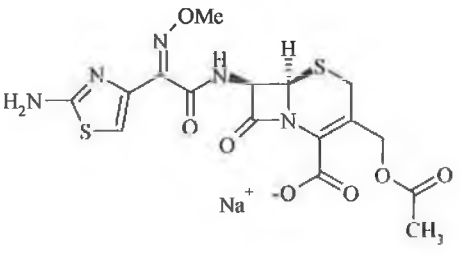
XR 1 – Cefazolin sodium salt 	XR 7 – Cephalosporin C zinc salt 
XR 2 – Cefuroxime sodium salt 	XR 8 – Oxacillin sodium salt 
XR 3 – Cefadroxil 	XR 9 –Amoxicillin tri-hydrate 
XR 4 – Cloxacillin sodium salt 	XR 10 -7-Aminodesacetoxy-Cephalosporanic Acid (ADAC) 
XR 5 – Cefoperazone sodium salt 	XR 11 –(+)-6-Aminopenicillanic Acid (APA) 
XR 6 – Dicloxacillin sodium salt 	XR 12 –Cefotaxim sodium salt 

Table 3.5. The calculated cross-reactivity (% CR) of selected β-lactam antibiotic compounds and the IC50 concentration of analyte (μg/ml) for the protein G-purified anti-*CephP1* antibody. The IC50 concentration of cephalexin was expressed as 100%, and the IC50 value for each potential cross-reactant was expressed as a percentage of this value. N/D means the concentration required to reach the IC50 value was too large (>1 mg/ml).

Compound	Polyclonal IC50 (μg/ml)	% CR (IC50)
Cephalexin	0.58	100.0
Cefadroxil	0.79	73.4
7-ADAC	6.18	9.4
Cefaperazone	87.90	0.7
Cephalosporin C	195.90	0.3
Cefotaxim	317.15	0.2
6-APA	401.60	0.1
Cefazolin	780.02	0.1
Oxacillin	N/D	---
Cloxacillin	N/D	---
Dicloxacillin	N/D	---
Cefuroxime	N/D	---
Amoxicillin	N/D	---

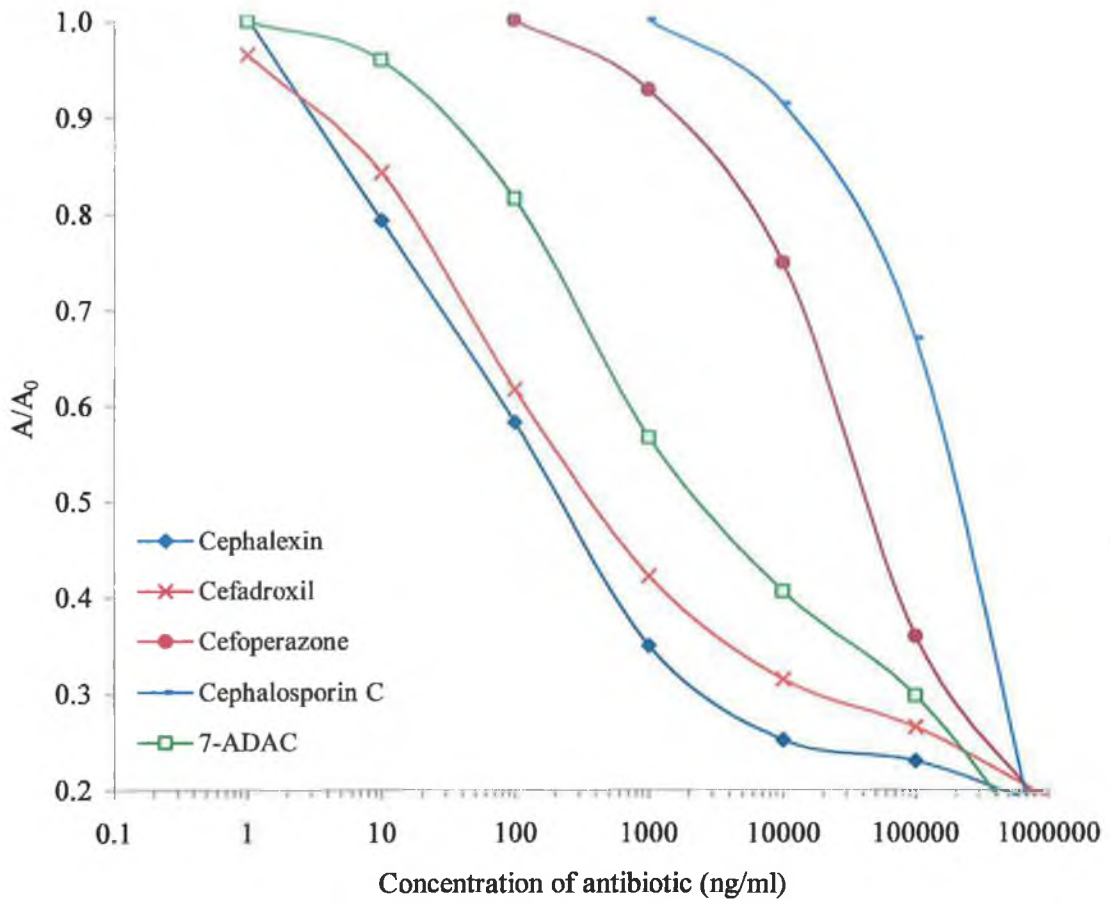


Figure 3.13. Overlay plot of selected polyclonal inhibition ELISA assay results using cephalexin, cefadroxil, cefoperazone, cephalosporin C and 7-ADAC. The assays were carried out using anti-*CephP1* polyclonal antibody (section 2.3.1.3). The A/A_0 represents the absorbance for each antibody and standard compound concentration (A) divided by the antibody alone, i.e. with no free compound (A_0). For each point above measurements were carried out in triplicate.

3.3. Discussion

This chapter describes the production and characterisation of polyclonal antibody to cephalexin. Cephalexin was conjugated to Bovine serum albumin (BSA-Ceph), ovalbumin (Ceph-Ova), thyroglobulin (Thyro-Ceph) and dextran (Dex-Ceph), as described in sections 2.2.1.2 and 2.2.1.3. Cephalexin-carrier conjugates were characterised by non-competitive ELISA (section 2.3.1.2) using a cephalexin-specific polyclonal antibody (designated AVAab) that was kindly donated by Dr. Aart van Amerongan. The non-competitive ELISA using AVAab was carried out to confirm the cephalexin had been covalently linked to the respective carrier protein and was still in a detectable configuration. Figures 3.1 and 3.2 show typical results obtained for checkerboard ELISAs using Bovine Serum Albumen (BSA) and ovalbumin (Ova) -Ceph conjugates, with AVAab anti-cephalexin polyclonal antibody. Ceph-BSA conjugate was used in the preparation of immunogen for the production of cephalexin-specific polyclonal antibody (Hermanson, 1996).

The polyclonal antibody was purified using saturated ammonium (SAS) precipitation, followed by protein-G affinity chromatography from rabbit serum. The protein concentration of protein-G affinity purified fractions was monitored by UV spectroscopy (figure 3.5). The purity of antibody preparation was assessed by SDS-PAGE (section 2.3.2) analysis and Western blotting (section 2.3.3). The specific activity of the polyclonal antibody against Thyro-Ceph conjugate was monitored during the purification process by ELISA, in order to show there was no adverse affects on antibody activity during the purification.

It was found that the purified anti-*CephP1* polyclonal antibody recognised all of the cephalexin conjugates in a non-competitive ELISA format. Polyclonal antibody was subsequently used in the development of inhibition ELISA assay for the detection of free cephalexin in solution (i.e. un-conjugated cephalexin). The anti-*CephP1* polyclonal antibody binding to cephalexin-conjugate (synthesised using protein-activated ester coupling as described in section 2.2.1.3) was inhibited in the presence of free cephalexin. Cephalexin conjugates synthesised using the cephalexin-activated ester coupling were unstable (i.e. formed precipitate during storage). These conjugates, when used as immobilised surfaces in inhibition ELISA with the polyclonal anti-cephalexin antibody, yielded assays with poor reproducibility. The protein-activated thyroglobulin-cephalexin conjugate was chosen to develop an inhibition ELISA for the detection of cephalexin.

In determining the optimum coating concentration of conjugated cephalexin and polyclonal antibody dilution, a checkerboard ELISA (section 2.3.1.2) was performed with affinity

purified polyclonal antibody and Thyro-Ceph conjugate. Initially an inhibition assay was developed, as described in section 2.3.1.3, using PBS to prepare cephalexin standards, following this cephalexin standards were prepared in whole milk (i.e. 'spiked' processed milk).

Cephalexin standards prepared in PBS and whole milk were used for the intra- and inter-day inhibition assay variability studies. The best comparative linear range of detection for the inhibition ELISA was found to be from 1.9 ng/ml to 3906 ng/ml. Intra- and inter-day assay variability studies in PBS had limits of detection (LOD) of be 20ng/ml. The LOD's were below the required European Union maximum residue limit (MRL), currently set at 100ng/ml for cephalexin in milk samples, as set out in EEC council regulation No 2377/90. Inter-day assay variability studies carried out in cephalexin 'spiked' whole milk (homogenised and pasteurised) showed the assay LOD was below the EU-MRL, at approximately 20 ng/ml for the inhibition assay. The limits of detection achieved for similar antibiotic compounds are comparable to those reported in this chapter using polyclonal antibodies.

Situ and Elliot (2005) reported a polyclonal immunoassay-based detection technique for the simultaneous detection of five banned antibiotic growth promoters. They developed the immunoassay for the detection of the antibiotics (i.e. bacitracin, spiramycin, tylosin, virginiamycin and olaquindox) in animal feedstuffs, thus a sample clean-up step was required. The minimal detectable concentrations (MDC) were 4 mg/Kg for olquindox, and 1 mg/Kg for bacitracin, spiramycin, tylosin and virginiamycin. These values correspond to 4,000 ng/ml and 1,000 ng/ml MDC in feedstuff extract, respectively. The polyclonal antibodies were generated in an analogous manner to that described in this chapter. Antibiotic-Human Serum Albumen (HSA) conjugates were produced and used as immunogen for each of the listed anti-microbial drugs. However, the ELISA assay format used was different to that reported in this chapter. Wells of 96-well plates were initially coated with antibiotic-specific antibody followed by the addition of HRP-labelled antibiotic standards, which were used to compare to unknown samples. A corresponding reduction in the response (i.e. absorbance) was attributed to the presence of antibiotic in the sample.

The cephalexin assays developed in this chapter also compare favourably with other distinct detection methods, in terms of sensitivity. In addition to assay sensitivity, each different β-lactam antibiotic has different maximum residue limits (MRL's) in milk, as set out by the EU, and therefore generic and broadly specific assay types are usually favoured. Current commercial antibiotic detection techniques vary in terms of their basic principles. Overall, they may be classified into four main types, microbial inhibition, receptor binding assays,

antibody-based assays (ELISA) and enzymatic assays. The more traditional microbial inhibition tests such as the *Bacillus stearothermophilus* disc assay are broadly specific for the β-lactam antibiotics and non- β-lactam antibiotics (Gilbertson *et al.*, 1995; Scannella *et al.*, 1997; Popelka *et al.*, 2003). Microbiological assays for the detection of antibiotic residues in milk utilise the genus Bacillus because of its high sensitivity to the majority of antibiotics. A host of commercial tests approved for screening utilise this principle, including the Delvotest SP (DSM Food Specialities, Delft, The Netherlands)(Althaus *et al.*, 2003). Such assays have good sensitivities to the β-lactam antibiotics on the whole, for example penicillin G, amoxicillin, ampicillin, cephapirin, and cetiofur can all be detected at or below their respective MRL's. However, they lack the ability to distinguish between the various antibiotic compounds within distinct groups. The Delvo® SP which is the standard rapid method of detection (Irish government, Department of Agriculture and Food, DAF) has a quoted sensitivity to cephalexin of 40-60 ng/ml. The anti-*CephP1* polyclonal antibody developed in this chapter is specific, as demonstrated by the minimal cross-reactivity with similar β-lactam compounds and it is capable of detection below that limit. However, PBS and milk assays performed poorly in terms of assay reproducibility, with relatively high standard deviation between assay replicates and individual measurements.

Some studies have found such rapid screening methods (e.g. microbial inhibitor, enzymatic assays and receptor binding assays) to be problematic also, in so far as they may lead to false positive results. One study carried out by Andrew (2000), suggests that results from Delvotest SP, Penzyme, Charm, and CITE Snap screening assays showed an increase in false positives between two distinct breeds of cattle. They also suggest that milk fat, protein content, and somatic cell counts were all associated with an increase in false positive results. This highlights a need for more robust and specific detection systems. Confirmatory testing is may be carried out using chromatographic methods (i.e. HPLC). These techniques can be costly and require trained operators. In addition, they involve extensive sample pre-treatment (Schenck and Callery, 1998). Zhi *et al.* (2001) developed an integrated flow-through immuno-analysis system for the detection of cephalexin in raw milk. This system was based on a polyclonal antibody specific for cephalexin and had a limit of detection (LOD) of 1 ng/ml. The system used a cephalexin-alkaline phosphatase conjugate in direct competition with analyte (i.e. cephalexin) in solution.

Chapter 4

Production of monoclonal antibodies to cephalexin and the development of an ELISA-based assay for detection in milk

4.1 Monoclonal antibody production

The aim of this chapter was the production, purification and characterisation of monoclonal antibodies against the β-lactam antibiotic cephalexin and their application in an ELISA-based assay for the detection of cephalexin in milk. Monoclonal antibodies were produced by murine somatic cell fusion procedures, subsequently purified and characterised by SDS-PAGE and Western blotting. Monoclonal antibodies with the desired characteristics (i.e. cephalexin-specific with a limit of detection (LOD) below the MRL) were identified and used in the development of an inhibition ELISA for the analysis of cephalexin in 'spiked' PBS and whole milk sample matrices. Cross-reactivity of this antibody with similar β-lactam antibiotic compounds was evaluated using solid-phase immunosorbent assay (ELISA).

4.1.1 *Production of monoclonal antibodies following in vivo immunisation*

BALB/c strain mice were initially immunised with cephalexin conjugates and boosted periodically over a period of several weeks as described in section 2.4.1. Specific serum antibody levels or titres were measured (Kemeny, 1992a) from tail bleed samples using an indirect screening enzyme-linked immunosorbent assay (ELISA). Upon obtaining a sufficient serum antibody titre (≥ 1/50,000) the animal was sacrificed and the lymphocytes extracted from the spleen and used for the cell fusion procedure. The spleen was used as it contains the highest percentage of B-lymphocytes of the major murine lymphoid organs (Table 4.1). The myeloma cells used as the fusion partner were originally derived from BALB/c lineage mice and selected on the basis of their hypoxanthine-guanine phosphoribosyltransferase (HGPRT) deficiency. Two of the most commonly used myeloma cell lines are Sp2/0-Ag14 (Shulman *et al.*, 1978) and X63/Ag8.653 (Brown and Ling, 1988), which were produced from a myeloma cell line following the injection of mineral oil into the peritoneal cavity of mice. Neither of these cell lines possesses the inherent genetic capacity to produce immunoglobulin. The technique employed the use of the HAT (Hypoxanthine, Aminopterin, Thymidine) selection system, described by Littlefield (1964). Myeloma cells that lack the enzyme hypoxanthine guanine phosphoribosyltransferase (HGPRT $^-$) are incapable of proliferating in the presence of HAT-supplemented medium. This is because aminopterin blocks the *de novo* synthesis of purines and pyrimidines required for DNA synthesis. Cells that are HGPRT $^-$ are incapable of using the salvage pathways for DNA synthesis as they do not possess the requisite enzyme systems (Figure 4.2).

Table 4.1. Sub-populations of B- and T- lymphocytes within the major murine lymphoid organs. (Adapted from Hudson and Hay, 1980).

Organ	% T-Cells	% B-Cells
Thymus	97	1
Lymph node	77	18
Spleen	35	38
Blood	70	24
Thoracic duct lymph	80	19

Spleenocytes are $HGPRT^+$ and therefore are capable of DNA synthesis using the salvage pathway. However, spleenocytes do not grow well in culture and naturally die off after a short period of time. Thus, in growth medium supplemented with HAT only hybridomas (i.e. fusion *hybrid* product of myel*oma* and lymphocyte cells) will proliferate. Cell fusion procedures in this work were carried out using polyethylene glycol (50% w/v) (Zola and Brooks, 1995). This facilitates membrane fusion between adjacent cells. Subsequent fused cell division leads to chromosome mixing. The cellular product of a fusion process consists of a heterogeneous population of un-fused myeloma cells and spleenocytes, fused myeloma/myeloma, lymphocyte / lymphocyte and the desired myeloma / B-lymphocyte hybrids. Early identification and isolation of specific monoclonal antibody-secreting myeloma / B-lymphocyte fusion products is essential. This may be achieved using appropriate screening techniques in conjunction with various cloning methods, as discussed in sections 4.1.2.1 and 4.1.2.2, respectively.

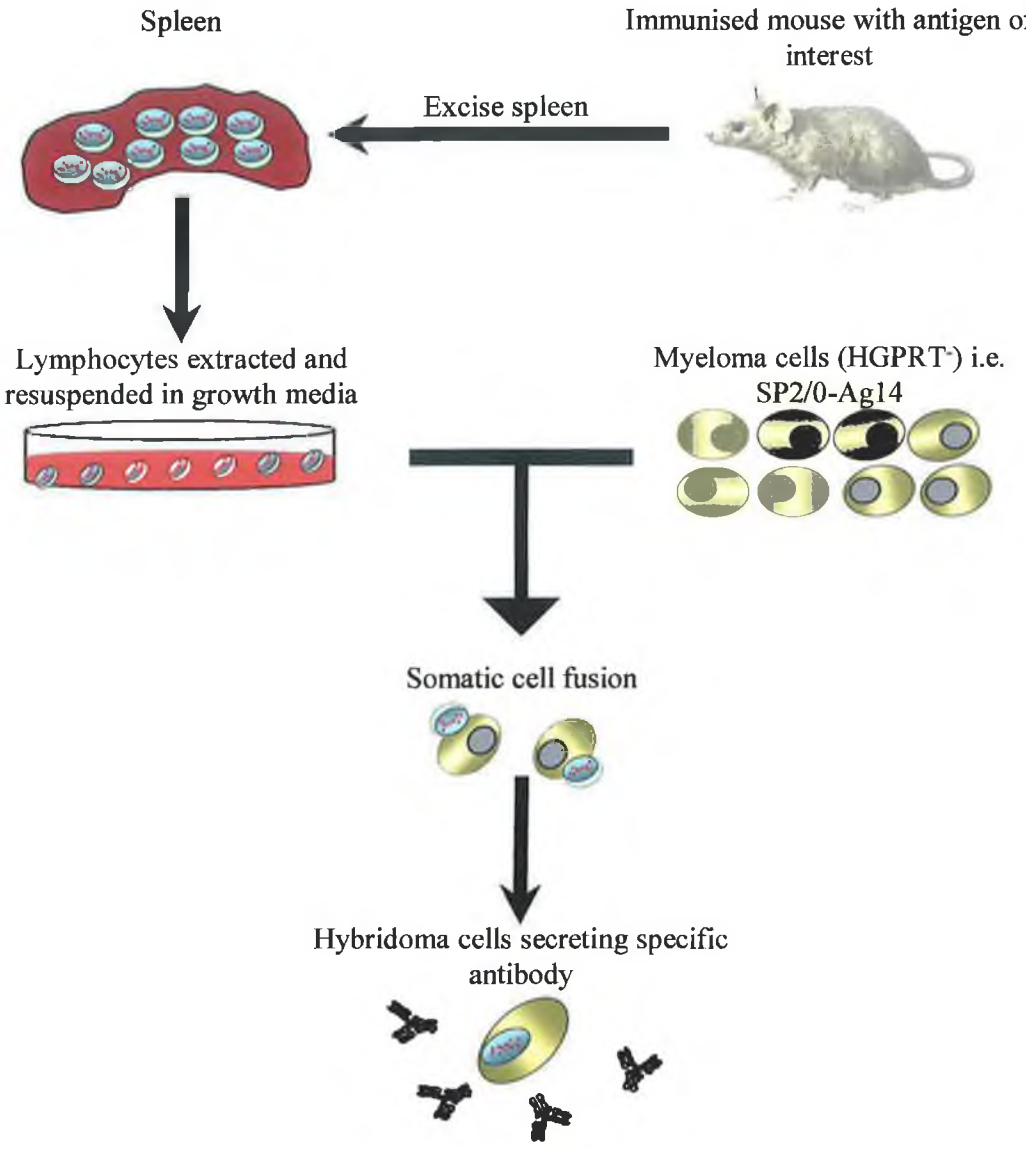


Figure 4.1. Overview of monoclonal antibody production by somatic cell fusion. B-lymphocytes are extracted from the spleen of a mouse immunised with the antigen of interest. The lymphocytes ($HGPRT^+$) are subsequently fused with a compatible murine myeloma cell line using appropriate fusogen (e.g. polyethylene glycol). Myeloma cells are $HGPRT^-$ and, thus, only proliferate *in vitro* if they have successfully fused with B-lymphocytes.

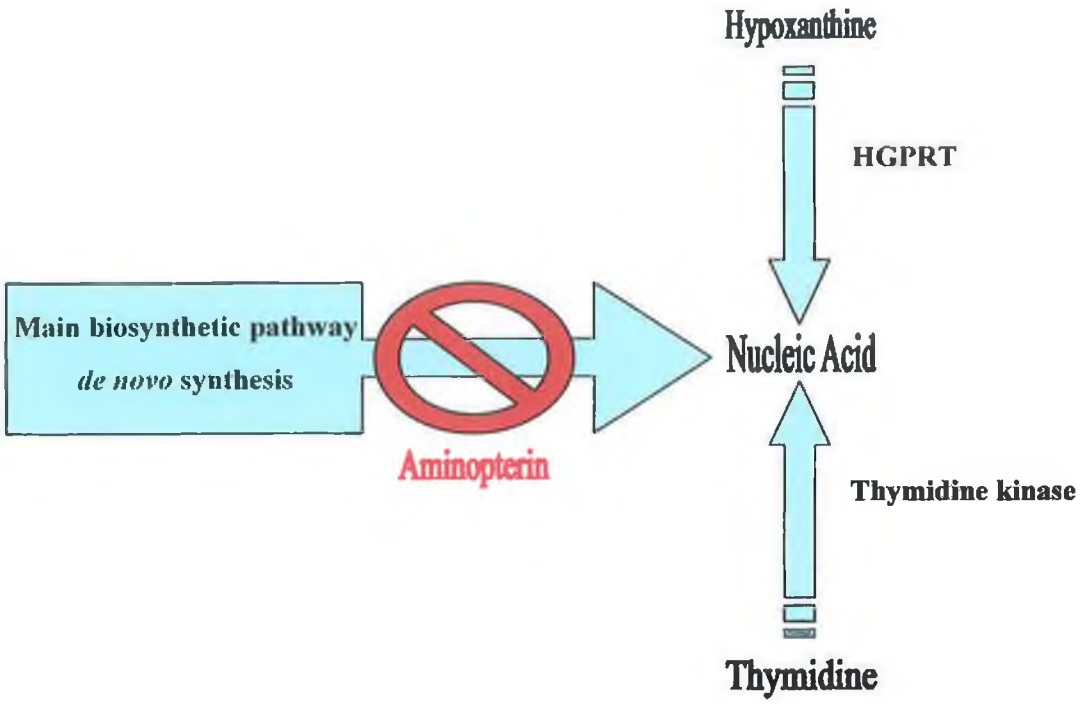


Figure 4.2. Principle of the HAT selection system. The main biosynthetic pathway for DNA synthesis is blocked by aminopterin (A). Cells possessing the requisite enzymes can synthesise nucleic acids using the salvage pathways, if provided with hypoxanthine (H) and thymidine (T). Cells lacking the enzymes hypoxanthine-guanine phosphoribosyltransferase (HGPRT) or thymidine kinase cannot synthesise nucleic acids via the salvage pathway and die off.

4.1.2 *Isolation of desired hybridoma clones*

4.1.2.1 *Screening*

The post-fusion cell mixture is typically cultured in 96-well culture vessels or master plates. The master plates contain a heterogeneous population of hybridomas i.e. secreting antibodies derived from different B-cells. Each of the hybridoma populations must be screened for specific antibody activity as early as possible using a suitable assay. A non-specific or non-secreting cell population can potentially outgrow hybridomas secreting the desired antibody, therefore, early identification and isolation is of paramount importance. Each hybridoma within a given well population can contribute anywhere between 0 & 100 % of the secreted antibody content. Enzyme-linked immunosorbent assay (ELISA) facilitates multiple screening of multiple wells and is the screening method of choice in many cases.

A direct ELISA format can be easily optimised and is suitable for initial screening of a very large number of wells. This was carried out by coating the antigen of interest onto an ELISA plate, adding test well supernatant (diluted in PBS-T to limit non-specific interactions) followed by an anti-species enzyme-labelled (e.g. HRP-labelled goat anti-mouse) secondary detection antibody. In the case of haptens (e.g. cephalexin), plates can be coated with conjugated hapten or non-conjugated hapten. Haptens may be directly linked to the surface of the ELISA plate via adaptor or linker molecules (Danilova, 1994). In this chapter, we used conjugated-cephalexin-coated plates for monoclonal antibody screening purposes. It is important to include adequate positive and negative controls to confidently identify hapten-specific antibodies, as opposed to carrier-specific antibodies within a given population. Ideally, a distinct hapten-conjugate from the one used for initial mouse immunisation should be used for screening of hybridomas, so as to limit the possibility of obtaining carrier specific antibodies. Initially supernatants were screened against cephalexin-activated Ova-Ceph conjugate followed by protein-activated thyroglobulin Thyro-Ceph conjugate, as outlined in Figure 4.3. The screening of hybridoma populations against Ova-Ceph conjugate was carried out as the Thyro-Ceph conjugate was used as immunogen. In addition, alternative functional groups on the cephalexin molecule were originally used for conjugate synthesis. The covalent attachment to the Thyro was via the primary amine and Ova via the carboxyl functional groups, on the cephalexin molecule. Hybridomas that were positive for both conjugates were selected for further analysis using inhibition displacement ELISA. The inhibition displacement ELISA format (section 2.3.1.3) is outlined in Figure 4.4. Such assay formats are favoured in the case of haptens because the monoclonal antibodies and antigen are allowed to interact in the solution phase.

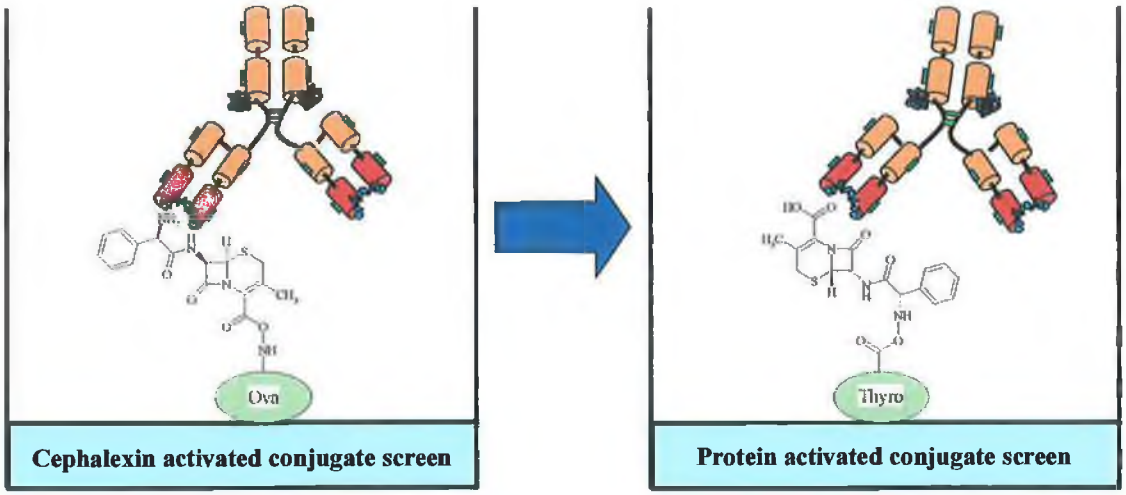


Figure 4.3. Schematic representation of the two stage initial hybridoma screening strategies employed for the identification of whole cephalexin molecule-specific antibodies. Firstly, hybridoma supernatant was screened against Ova-Ceph conjugate, prepared using the cephalexin-activated (above left) method i.e. where the covalent linkage to Ova was formed via the carboxylic acid functional group on the cephalexin molecule. Secondly supernatants were screened against protein-activated (above right) Thyro-Ceph conjugate, where the covalent bond between cephalexin and Thyro was formed using the primary amine functional group on the cephalexin molecule. This was carried out to maximise the potential of identifying antibodies specific to the whole cephalexin molecule by presenting alternative cephalexin epitopes.

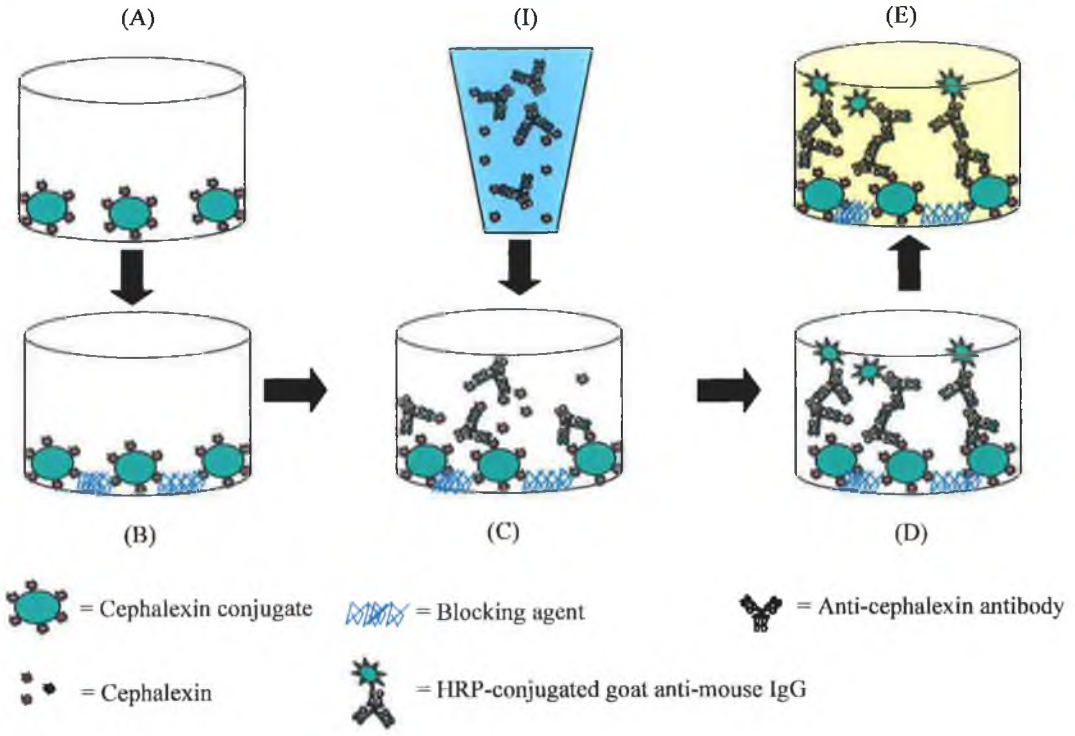


Figure 4.4. Schematic diagram of inhibition ELISA used to screen supernatants from hybridoma populations. (A) A 96 well plate was coated with cephalexin-protein conjugate (e.g. Thyro-Ceph); (B) The remaining adsorption sites are 'blocked' with a solution of 5% (w/v) milk marvel in PBS; (I) Supernatant containing monoclonal antibody was pre-incubated with free cephalexin standards for 30 minutes at 37°C in a 1.5 ml micro-centrifuge tube; (C) Pre-incubated samples are added to the 96 well plate; (D) The monoclonal antibody was detected with a horse radish peroxidase (HRP)-conjugated goat anti-mouse antibody; (E) HRP-substrate was added to 96 well plate and developed at R.T. and the absorbance measured at 450nm.

4.1.2.2 *Cloning of specific antibody-secreting hybridomas*

Following identification of the specific antibody-secreting hybridoma populations, the hybridomas must be isolated from the heterogeneous cell mixture to achieve monoclonality. There are many possible methods for doing this, but the simplest method is cloning by limiting dilution. Cloning by limiting dilution involves the repeated dilution and specific selection of positive antibody secreting hybrids (Harlow and Lane, 1988). This entails seeding the mixed population of hybridomas over a range of dilutions calculated to maximise the chances of only one cell being distributed to a particular well. A mathematical approach indicates that were less than 63 % of the wells seeded with concentrations of ≤ 1 cell/well, and Poisson distribution indicates (Lefkovitz, 1979; Eshhar, 1985) that each well will most

likely contain a single clone. The tendency of cells to clump, however, means that this mathematical explanation is not the most appropriate. In order to ensure 'monoclonality', it is best to repeat the cloning procedure again for several rounds, after which the hybridoma can be statistically considered monoclonal (Lietske and Unsicker, 1985).

One of the main problems with seeding the cells at such low concentrations is the need to add feeder layers to increase the seeding efficiency. The use of conditioned growth media such as Briclone™ can obviate the need for such layers. Cloning in semi-solid agar is the principle alternative to limiting dilution. The medium allows the cells to easily divide and grow to produce a viable, defined colony, without permitting cells to break away and cause disperse colony development (McCullough and Spier, 1990). Isolated colonies can then be picked off the semi-solid agar with a pipette tip and transferred to fresh liquid media. A more direct approach to cloning is one that involves the use of a Pelteir controlled pipette (Wewetzer and Seilheimer, 1995) to select a single colony and transfer it to a new well of a microtitre plate. This method increases confidence that only single hybridoma cells have been seeded and reduces the cloning period significantly in comparison to the traditional methods. Hybridomas secreting specific anti-cephalexin antibody were cloned out by limiting dilution, as outlined in figure 4.5.

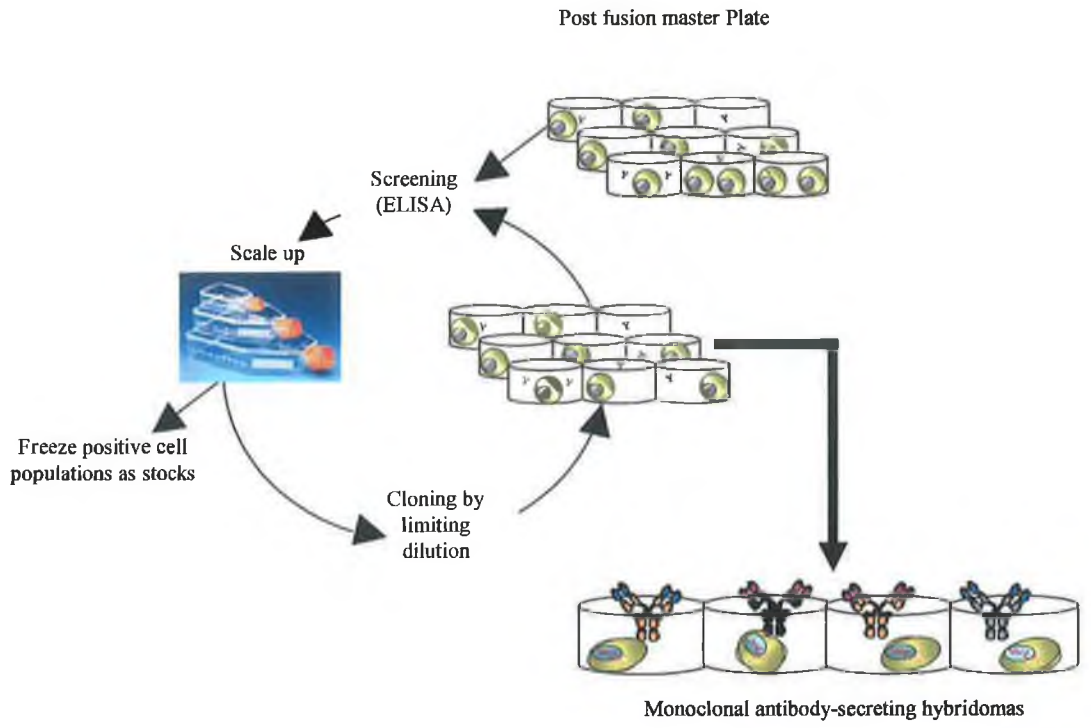


Figure 4.5. Overview of the post-fusion production of monoclonal antibodies. 'Spent' media is removed from the master plates and subsequently screened for specific anti-cephalexin antibody activity by ELISA. Positive populations are then scaled up. Frozen stocks of the positive hybridoma populations are made at this stage. The population is then cloned out by limiting dilution and seeded onto 96 well plates. Several cycles of the above process were carried out to achieve 'monoclonality'.

4.2 Results

4.2.1 *Monoclonal antibody-producing hybridoma production, isolation and screening*

A total of three mice were immunised with immunogens prepared using Keyhole Limpet Heamocyanin (KLH)-cephalexin (i.e. 1x mouse) and thyroglobulin-cephalexin (i.e. 2x mice) conjugates. Mice were immunised with respective immunogen, as outlined in section 2.4.1. The serum titre from each mouse was determined against an Ovalbumin-cephalexin (Ova-Ceph) conjugate using a non-competitive ELISA (section 2.3.1.1). The antibody titre has been classically described as the lowest dilution of sample that can be reliably detected at a predetermined cut-off (Ciclitira *et al.*, 1986). The titre for the purposes of this research was defined as the antibody dilution at 1.5x the background response (i.e. naïve serum, termed Mouse Neg.).

The initial serum screening was carried out using an Ova-cephalexin conjugate as opposed to either conjugate used as immunogen (i.e. Thyro-Ceph or KLH-Ceph) so as to rule out the possibility of selecting for a carrier protein-specific antibody. Serum samples from each mouse (i.e. Mouse 1 (KLH-cephalexin immunogen), Mouse 2 and 3 (Thyroglobulin-cephalexin immunogen) were serially diluted in PBS-T and added to the conjugate coated plate. The serum titre for mouse 1, 2 & 3 was approximately 1/50,000, in contrast to a negative control mouse serum (Mouse Neg.). The control serum was obtained from a mouse not previously immunised and showed negligible binding even at a 1:200 serum dilution (Figure 4.6).

On the day of the fusion (day 0), the spleens were removed and the lymphocytes harvested and pooled. After the somatic cell fusion (section 2.4.3.1) procedure, the cellular fusion product was seeded onto the wells of 96 well plates, at a density of 1.2 x 10^6 cells/ml and incubated for 8 days (termed master plates). The conditioned media (supernatant) from wells containing healthy colonies was removed for screening on day 8 post-fusion. Anti-cephalexin antibodies were screened for using an indirect non-competitive ELISA format. Post-fusion hybridoma populations are considered to be heterogeneous in nature (derived from different B-cells). Positive hybrid populations are propagated by incrementally seeding larger tissue culture vessels and subsequently isolated by cloning by limiting dilution. Cell dilutions were manipulated to achieve 1 or 0.5 cell/well seeding densities depending on the stability of the particular hybridoma cell line. A total of 11 positive wells were selected for scaling up to 24-well, 6-well and tissue culture flasks. An outline of the screening strategy employed is presented in figure 4.3.

The results of a typical initial master plate hybridoma supernatant screen are shown in figure 4.7. Hybridoma cell populations that yielded a high response, in terms of absolute absorbance, were selected from each master plate for further propagation, in order to obtain a functionally expressed and sufficiently high affinity antibody to cephalexin. Antibody binding was to the conjugated form of cephalexin at this stage. Ceph-conjugate, adsorbed onto the well surface, may present a dissimilar or mask the epitope to that of soluble antigen (i.e. cephalexin). Therefore, in order to characterise antibody binding to non-conjugated cephalexin, inhibition displacement ELISA (section 2.3.1.3.) were carried out.

The positive colonies detected in the initial screen were scaled up and subjected to four rounds of cloning by limiting dilution, as described in section 2.4.3.3. Positive cell populations were continuously screened by ELISA during the cloning process, to monitor cephalexin-specific antibody-secreting hybridomas. Following cloning out, hybridoma supernatant titres were ascertained, using an ELISA plate coated with thyroglobulin-cephalexin conjugate. In addition to this, the supernatants were screened against thyroglobulin (10 μg/ml in PBS, 0.15 M NaCl, 2.5 mM potassium chloride, 10 mM disodium hydrogen phosphate and 18 mM sodium dihydrogen phosphate, pH 7.4) and PBS supplemented with skimmed milk powder (section 2.1.6.4) as negative controls. This is shown in figure 4.8. No significant binding was observed with the negative controls. Crude supernatant dilutions for each clone were determined from this result and used to carry out preliminary inhibition assays using a series of free cephalexin concentrations, ranging from 1.9 to 500,000 ng/ml (Figure 4.9).

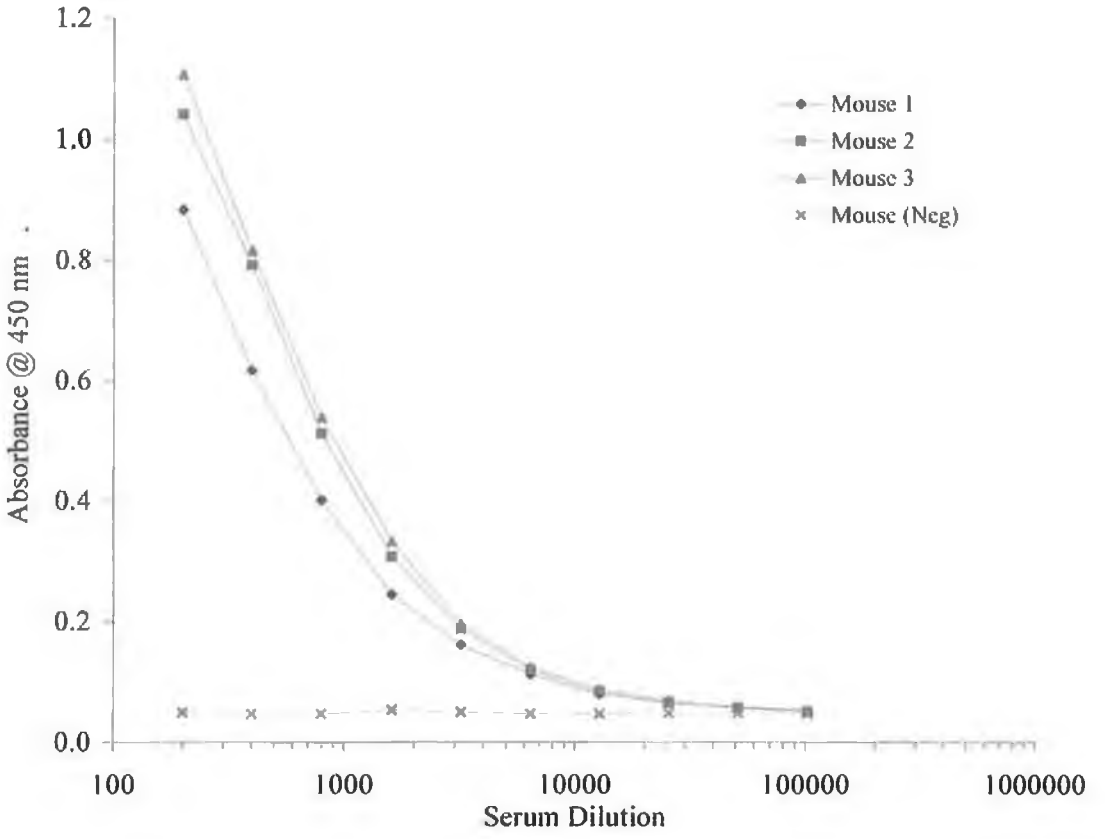


Figure 4.6. Plot of the results of a titre ELISA using mouse sera against a 10 μg/ml coating concentration of Ova-cephalexin conjugate. Mouse 1 was immunised with KLH-Cephalexin. Mouse 2 and mouse 3 were immunised with thyroglobulin-cephalexin. Mouse 3 was chosen for fusion after two boosts with thyroglobulin-cephalexin prior to removal of the spleen. Mouse (Neg) represents the response obtained from a non-immunised mouse serum.

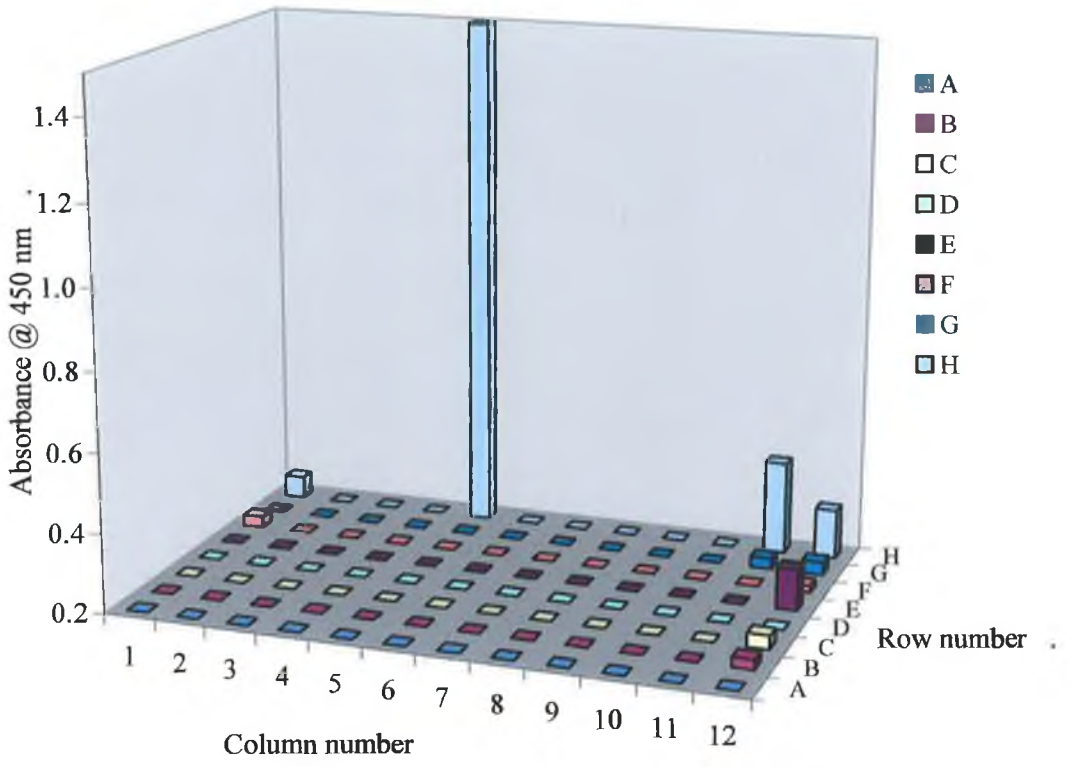


Figure 4.7. The results of a typical indirect screening ELISA using post-fusion master plate cell population supernatants. Supernatant was removed from the 96 well tissue culture plates containing post-fusion hybridoma cell populations and added to another 96 well plate coated with 10 μg/ml Ova-Ceph conjugate. Hybridomas in wells H5 and H11 showed the highest absorbance values (1.7 and 0.4 at 450 nm) for this particular master plate and were selected for scaling up and cloning by limiting dilution. The binding of murine IgG antibody to Ova-Ceph conjugate coated wells was detected using HRP-conjugated goat anti-mouse IgG secondary antibody. Wells A10 – A12 were used as negative control. Each well was coated using PBS and blocked with 5% (w/v) skimmed milk powder in PBS-T.

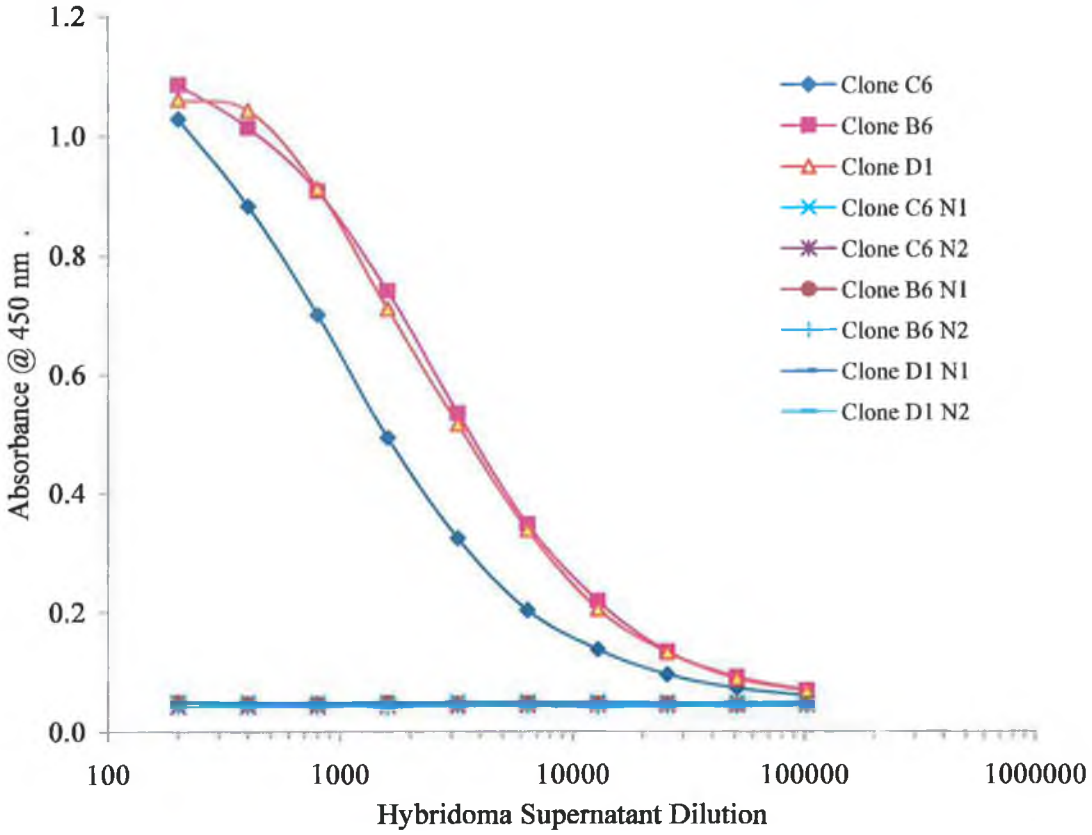


Figure 4.8. Results from an ELISA to determine the titre of monoclonal antibodies produced by positive clones C6, B6, and D1. After four rounds of cloning by limiting dilution, supernatant from culture vessels containing each clone was screened against a thyroglobulin-cephalexin conjugate. 96-well immunoplates were coated using Thyro-Ceph (1.3 μg/ml) and thyroglobulin (10 μg/ml). The thyroglobulin-coated wells comprised negative control (N1). In addition, the supernatant was also screened against a 5% (w/v) skimmed milk blocking solution (negative control (N2)).

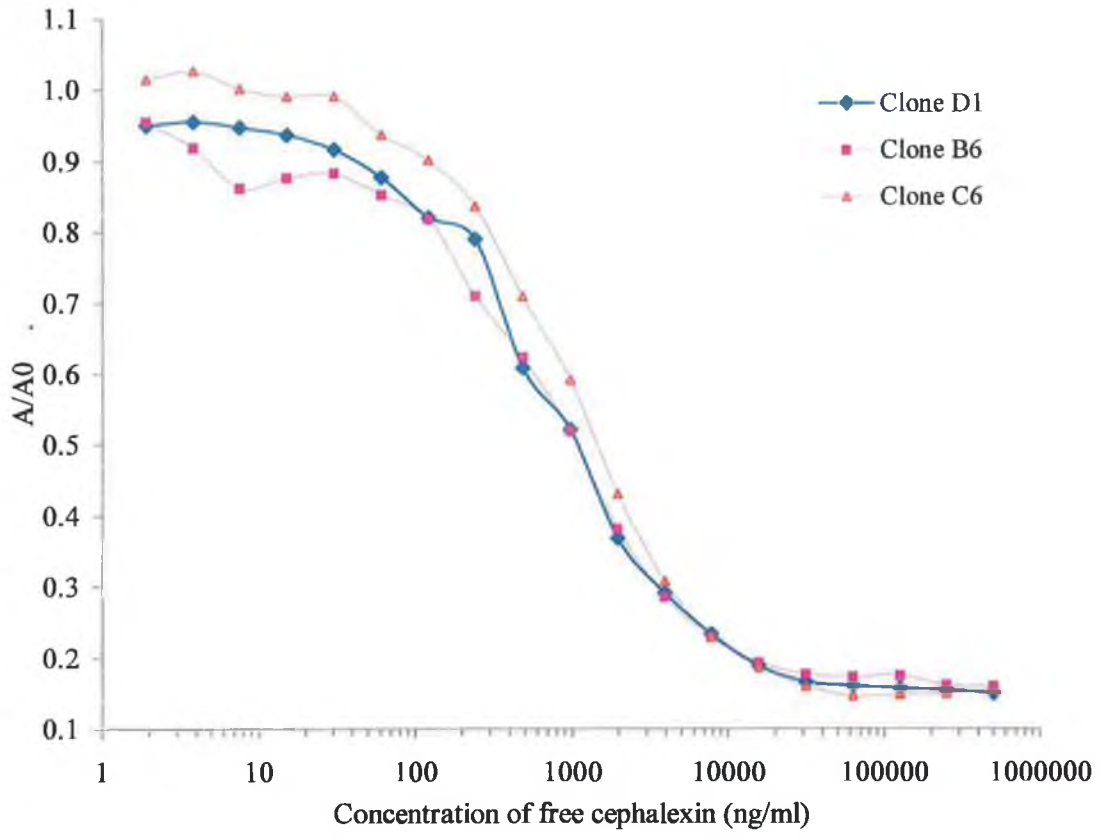


Figure 4.9. An overlay plot of the results from preliminary inhibition ELISAs using supernatant from positive cephalexin-conjugate-binding hybridoma clones D1, B6 and C6. Each assay was carried out using a thyroglobulin-cephalexin conjugate at a coating concentration of 1.3 μg/ml and the appropriate supernatant dilution (1/1,000 for C6, 1/1,500 for B6 and D1 clones) The optimum supernatant dilutions for each clone were obtained from the results of the titre experiment (figure 4.8) and were taken as the dilution at half the maximum absorbance.

4.2.2 *Antibody isotyping*

The most promising clones (C6 and B6) in terms of cell line stability and antibody characteristics were picked after being fully cloned out. Hybridoma supernatants (diluted 1:2 in PBST) were added to an immunoplate coated with a 1.3 µg/ml thyroglobulin-cephalexin. Following incubation and washing, diluted supernatant from the positive hybridoma populations was added and antibody subsequently detected using alkaline phosphatase (AP)-conjugated goat anti-mouse isotype-specific secondary antibodies (Immunokontact). Results show the positive clones (C6 and B6) to be type IgG2a containing a kappa (κ) light chain (Figure 4.10). Since both C6 and B6 hybrids were originally derived from the same master plate well, prior to cloning by limiting dilution and have similar inhibition assay characteristics it is highly probable that they are homologous. After determining the antibody isotype, C6 hybrid cell line was propagated and used to produce antibody for purification and assay development (from herein designated anti-*CephM1)*.

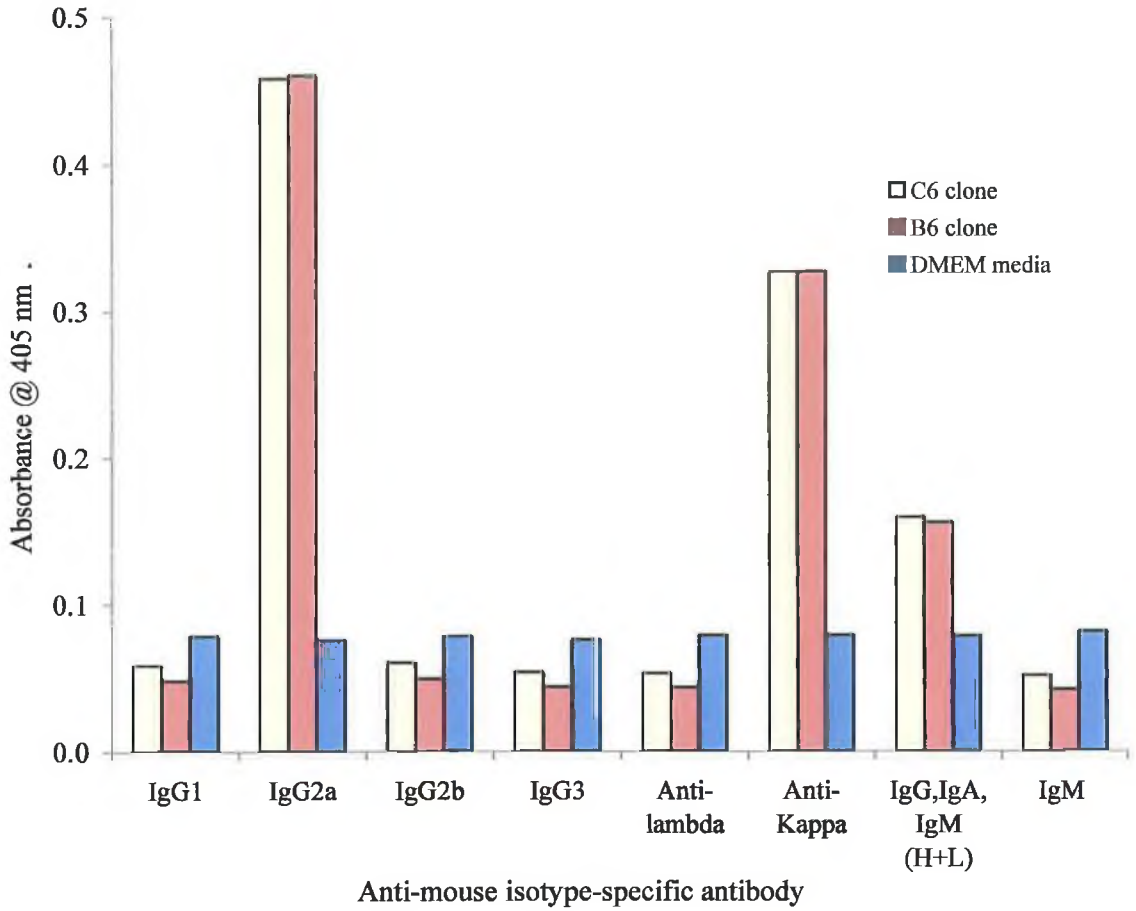


Figure 4.10. Isotype-specific ELISA for monoclonal antibody from positive cell populations C6 and B6. Hybridoma supernatant was screened using alkaline phosphatase (AP) conjugated goat anti-mouse isotype specific secondary antibodies and P-nitophenyl phosphate substrate system. The absorbance was recorded at 405 nm. Results indicate the isotype of the monoclonal anti-cephalexin antibody clones (anti-*CephM1*) to be IgG2a, containing a kappa light chain. The absorbance for DMEM growth media showed no significant response to any of the secondary antibodies used (DMEM media).

4.2.3 *Antibody purification*

Various techniques are available for the purification of monoclonal antibodies from tissue culture media. Invariably antibodies are purified using SAS precipitation (section 2.2.3.1) and affinity column chromatography. Protein A and G (section 2.2.3.2) covalently linked to sepharose are convenient affinity matrix for the affinity selection of various IgG isotypes. Recombinant forms of protein A & G are currently available which have reduced affinity to contaminating albumin proteins. Prior to affinity purification antibody containing supernatant may be concentrated by ultra-filtration to counteract relatively low expression into and concentrations in growth media. Low concentration yields of monoclonal antibody may be improved by using alternative *in vitro* production techniques. In general, *in vitro* cultured cells have two basic requirements, a constant supply of gaseous and water soluble nutrients and a means of removing toxic metabolites (e.g. ammonium ions and lactic acid) from surrounding culture medium. Various techniques address these needs in different ways. Overall, the choice of method will be dictated by the quantity and purity of the monoclonal required (Peterson, 1998). For the purposes of this research (i.e. small scale antibody) production, a static hybridoma suspension system was employed. In this technique, nutrients are provided by diffusion which requires continuous monitoring and exchange of growth media. Typically, depending on the particular growth characteristics of the cell line in question, yields of 5-50 μg/ml of antibody are achievable.

Supernatant from the anti-*CephM1* hybridoma cells was collected by continuously culturing cells in DMEM media and concentrated 10-fold using a stirred ultra-filtration cell. The concentrate was then applied to a protein-G sepharose affinity column, as described in section 2.2.3.2. The purification fractions were characterised by denaturing SDS-PAGE gel analysis and Western blotting, as described in sections 2.3.2 and 2.3.3, respectively. The results of the SDS-PAGE and Western blotting indicate that the antibody was successfully purified. The results are shown in figures 4.11 and 4.12, respectively. Heavy and light antibody polypeptide bands at approximately 50 kDa and 25 kDa can be clearly seen in both SDS-PAGE and Western blot. There were no detectable contaminants in the eluted antibody fraction.

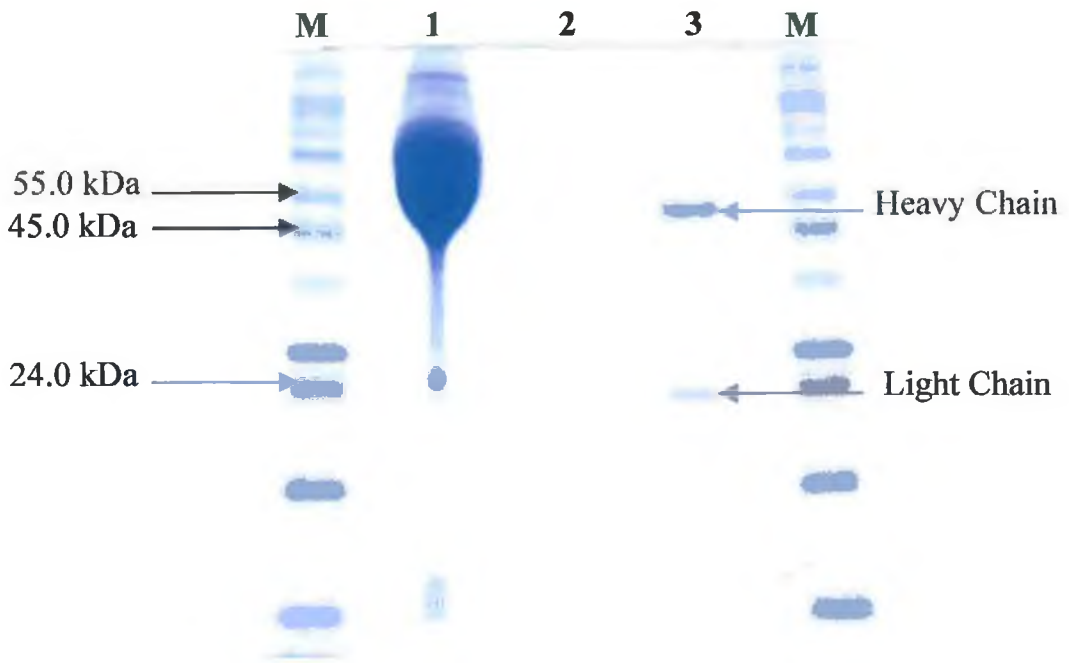


Figure 4.11. Results of a denaturing SDS-PAGE gel of the protein-G purified samples. The concentrated (10x) flow through, column wash, and protein G-purified samples for anti-*CephM1* monoclonal antibody were loaded from left to right respectively. Lane M, represents wide range standard molecular weight markers, obtained from Sigma (Table 2.6); Lane 1 is the concentrated flow through diluted 1:20 in PBS; Lane 2 is the column wash diluted 1:2 in PBS; Lane 3 is neat protein-G affinity-purified pooled fractions. Heavy chain and light chain domains are highlighted at approximately 50 kDa and 25 kDa respectively.

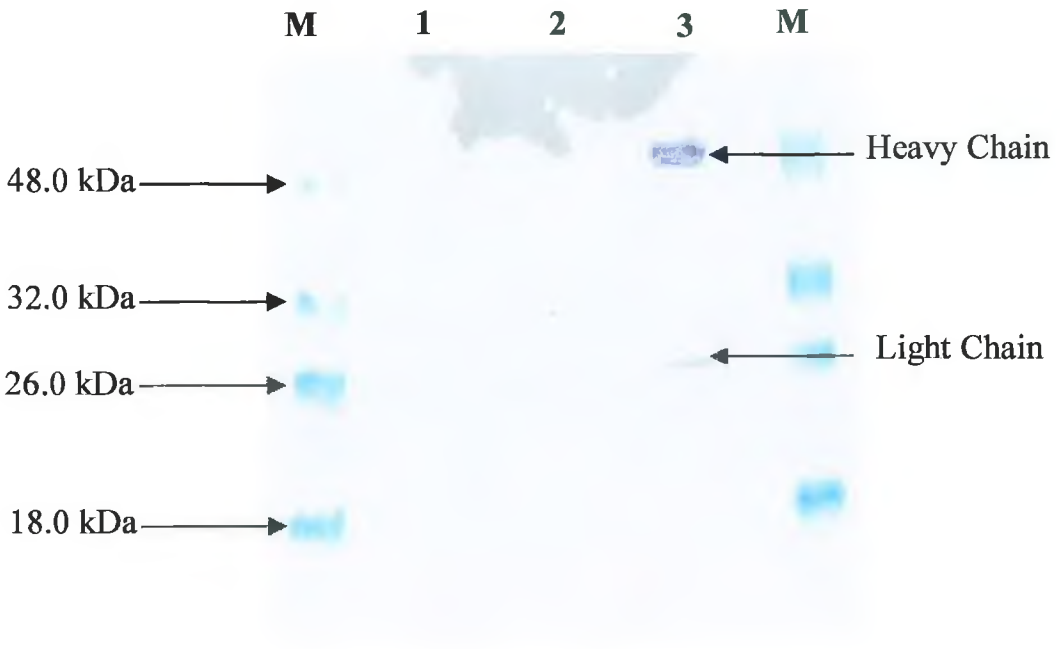


Figure 4.12. Results of Western blot analysis following transfer of the monoclonal purification samples from SDS-PAGE gel. The concentrated (10x) flow through, column wash, and protein G-purified samples for anti-*CephM1* antibody were loaded from left to right respectively. Pierce Blue ranger ™ pre-stained protein molecular weight standards were used. Lane M, represents Pierce Blue ranger standard molecular weight marker (Table 2.6); Lane 1 is the concentrated flow through diluted 1:20 in PBS; Lane 2 is the column wash diluted 1:2 in PBS; Lane 3 is the neat protein-G affinity-purified fraction. Heavy chain and light chain domains are highlighted at approximately 50 kDa and 25 kDa, respectively. Heavy and light antibody chains were detected, as highlighted above, using HRP-labelled goat anti-mouse IgG whole molecule specific antibody.

4.2.4 *Development of assay for the detection of cephalexin using monoclonal antibody*

Potential conformational changes arising from partial denaturation by passive adsorption onto hydrophobic microtitre wells was not envisaged to be problematic as cephalexin was covalently attached to protein surface and it is relatively small and inflexible,. The concentration of the adsorbed conjugate can play a vital role in the overall performance of the subsequent assay. Optimisation of the antigen coating and antibody concentration is of the utmost importance in the development of a solid-phase immunoassay. Potential problems may arise when the integrity of the adsorbed antigen is compromised (i.e. epitope masked) or the relative binding strength to the surface is not sufficient. The latter of the two generally arises from conditions where the concentration of protein (e.g. Thyro-Ceph) is higher than required, leading to saturation of the available surface spaces and weak inter-molecular (i.e. protein-protein) binding. Inter-molecular interactions are often weaker than those between treated surface and protein and results in the dissociation of 'supposedly' bound protein during the assay (Kemeny, 1992b).

Following protein G-purification of the anti-*CephM1* monoclonal antibody, it was then analysed using solid-phase immunoassay. A checkerboard ELISA, as described in section 2.2.4 was carried out to ensure that the purification process had no adverse effects on the ability of the antibody to recognise immobilised cephalexin conjugate and establish the optimal binding concentration of conjugate and purified monoclonal antibody for subsequent solution-phase binding studies i.e. inhibition displacement ELISA development.

A 96 well Nunc immunoplate was coated with varying concentrations of Thyro-Ceph in PBS. Following the blocking step, serial dilutions of protein G-purified anti-*CephM1* antibody prepared in PBS-TM were added to the appropriate wells. The resulting plot is shown in figure 4.13 and confirms that the purified antibody showed an increased titre, as expected. The monoclonal antibody retained binding capacity to adsorbed thyroglobulin-cephalexin conjugate.

Purified monoclonal anti-*CephM1* was evaluated for use in an assay for the detection of soluble cephalexin in whole milk. Cephalexin hydrate, the residue of cephalexin treatment was used to prepare standards in PBS-T supplemented with 2% (w/v) skimmed milk powder. This was carried out to confirm inhibition of antibody in the presence of soluble antigen and as a comparative study for the 'spiked' whole milk assay. The increased complexity of whole milk could have had potentially negative effects on assay performance, which needed to be fully elucidated. Inhibition displacement ELISA using 'spiked' PBS-T and whole milk

samples, as described in section 2.3.1.3. The antibody and conjugate concentrations used were determined from figure 4.13. Each assay was carried out using a 1/20,000 antibody dilution and 1.32 µg/ml solution of thyroglobulin-cephalexin coating concentration. Cephalexin standards ranging from 1.9 to 500,000 ng/ml were prepared in PBS-T and whole milk.

The resulting data was plotted using BIAevaluation software (version 3.1) and four-parameter equations fitted to each data set for analysis. The limit of detection for the assay was taken as the minimum inhibitory drug concentration which corresponds to the background (i.e. A_0) minus 3x standard deviations. The initial assays had limits of detection (LOD's) of 90 ng/ml and 60 ng/ml in PBST (Figure 4.14) and whole milk (Figure 4.15), respectively. The percentage accuracies were calculated using the following formula: (Back calculated value – Experimental value/ Back calculated value) x 100 + 100. The mean response (A/A_0), cephalexin concentration (ng/ml), back calculated values, percentage coefficient of variation (% CV) and percentage accuracy (% Accuracy) for the PBS-T and milk assays are presented in tables 4.2 and 4.3, respectively. An overlay plot of the PBST and milk assays using monoclonal antibody showed slight difference in the two sample matrices. The milk assay showed an apparent increased sensitivity at lower concentrations of free inhibitory cephalexin (Figure 4.15).

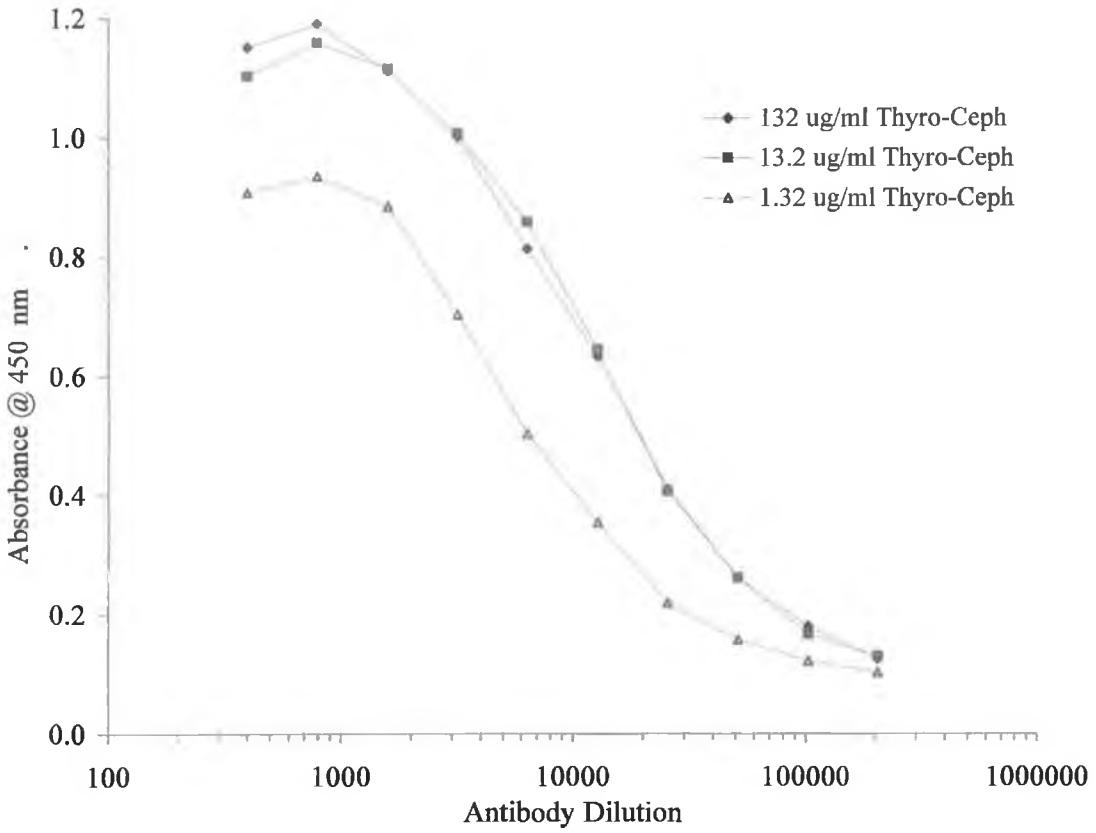


Figure 4.13. Results obtained from a checkerboard ELISA to determine optimal conjugate coating concentration. Wells were coated with concentrations of Thyro-Ceph conjugate (132 µg/ml, 13.2 µg/ml and 1.32 µg/ml). Wells were subsequently blocked with PBS containing 5% (w/v) skimmed milk powder and protein G-purified monoclonal anti-*CephM1* antibody serially diluted (ranging from 1/400 to 1/204,800) in PBST-M was added. Each measurement was carried out in duplicate. Wells coated with zero µg/ml conjugate showed no significant binding to monoclonal antibody and enzyme-labelled species-specific secondary antibody.

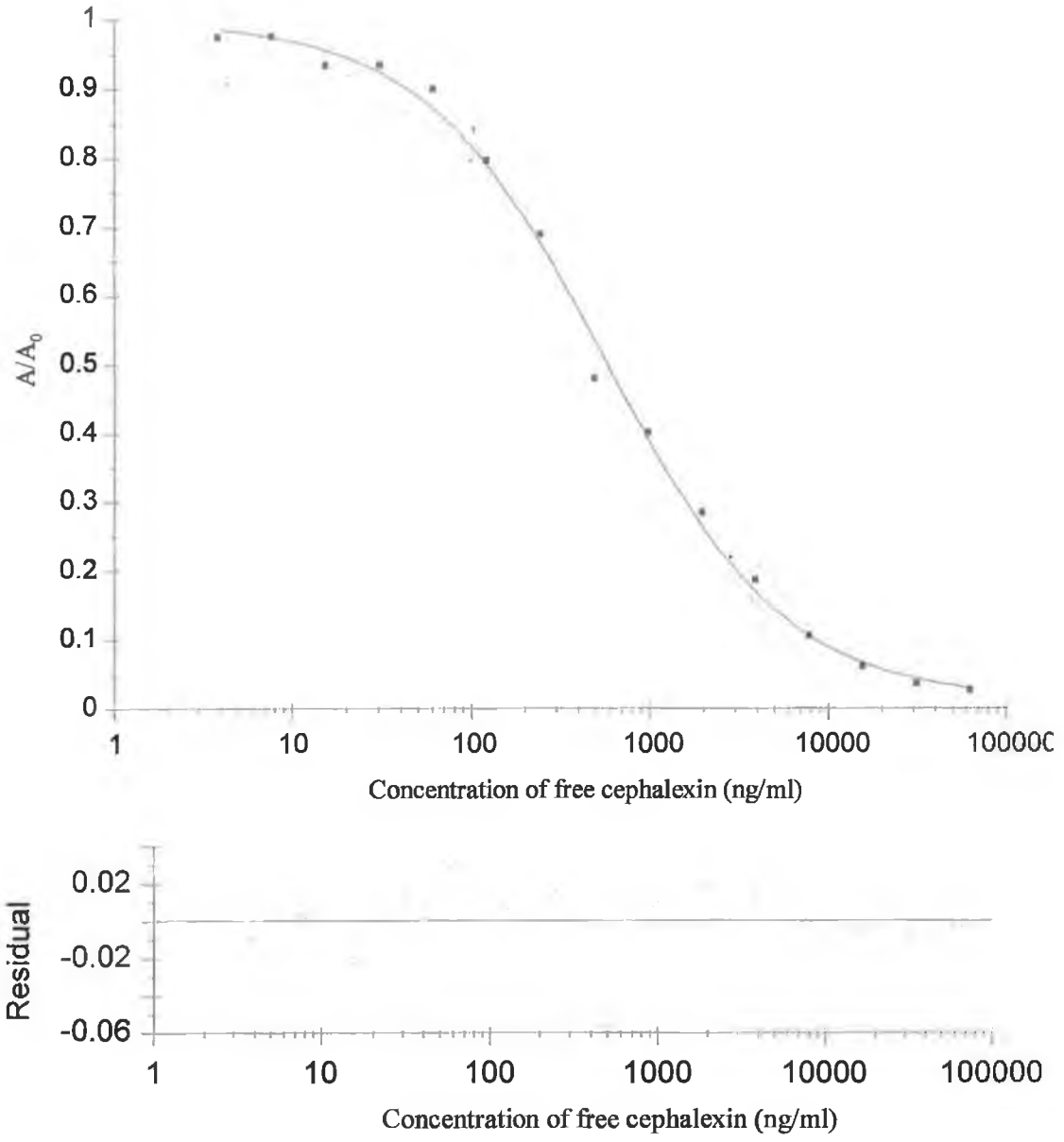


Figure 4.14. A typical inhibition ELISA in 'spiked' PBST using a 1/20,000 dilution of protein G-purified anti-*CephM1* (C6) and a 1.32 μg/ml solution of thyroglobulin-cephalexin coating concentration. Cephalexin standards were prepared in PBST by serial dilutions (1:2) ranging from 1.9 to 500,000 ng/ml. The linear range of detection was found to be from 61 to 7812 ng/ml. The limit of detection (LOD) was calculated by taking 3 times the standard deviation from the background (A_0), and was found to be 80 ng/ml.

Table 4.2. Tabulated values for mean response (A/A_0), cephalexin concentration (ng/ml) of 'spiked' PBST sample, back calculated values, percentage coefficient of variation (% CV) and percentage accuracy (% Accuracy) obtained for the monoclonal antibody inter-day assay in 'spiked' whole milk. Back calculated values were obtained by fitting a four-parameter equation to the data set using BIAevaluation software (version 3.1).

Calculated $A/A_0 \pm$ S.D.	Concentration of cephalexin (ng/ml)	Back Calculated value (ng/ml)	% CV's	% Accuracies
0.03 ± 0.003	62,500	77,743.9	3.45	119
0.04 ± 0.002	31,250	42,969.1	2.33	127
0.06 ± 0.002	15,625	17,896.5	1.65	112
0.11 ± 0.004	7,812.5	7,898.0	3.65	101
0.19 ± 0.001	3,906.3	3,401.1	0.46	85
0.28 ± 0.003	1,953.1	1,739.5	2.05	89
0.40 ± 0.004	976.6	934.4	2.37	95
0.48 ± 0.003	488.3	640.5	1.42	123
0.69 ± 0.008	244.1	227.2	3.32	92
0.80 ± 0.005	122.1	116.8	1.89	95
0.90 ± 0.004	61.0	43.4	1.32	59
0.93 ± 0.007	30.5	25.3	2.11	80
0.94 ± 0.006	15.3	25.0	1.76	138
0.98 ± 0.003	7.6	7.0	0.77	91
0.98 ± 0.003	3.8	7.5	0.99	149

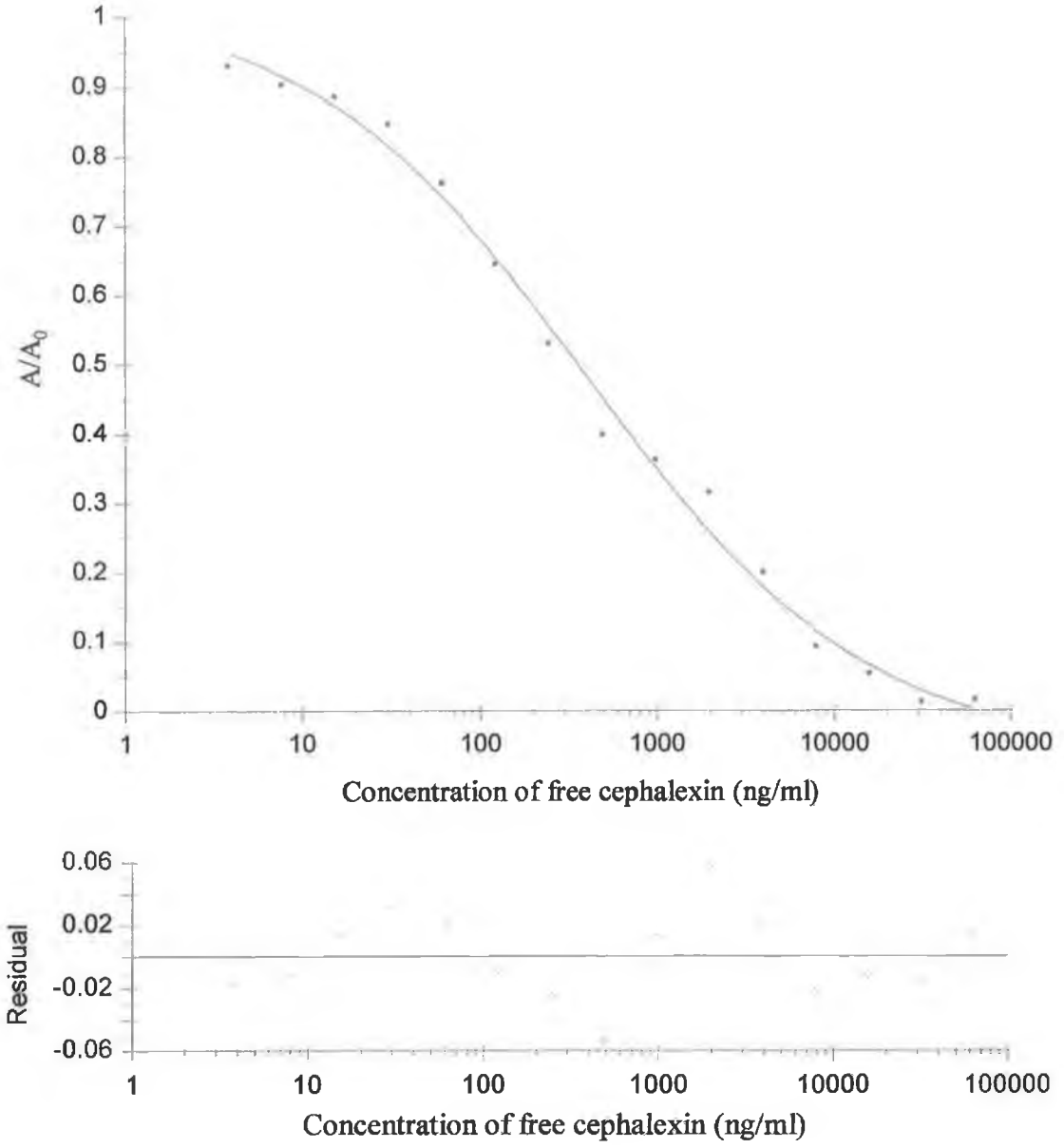


Figure 4.15. A typical inhibition ELISA in whole milk for 1/20,000 dilution of protein G-purified *anti-CephM1* and a 1.32 μg/ml solution of thyroglobulin-cephalexin coating concentration. Cephalexin standards were prepared in whole milk ranging from 1.9 to 500,000 ng/ml. The linear range of detection was found to be 30 to 15625 ng/ml. The limit of detection for the assay was found to be 60 ng/ml by taking 3 times the standard deviation from the background (A_0),

Table 4.3. Values for mean response (A/A_0), cephalexin concentration (ng/ml) of 'spiked' milk sample, back calculated values, percentage coefficient of variation (% CV) and percentage accuracy (% Accuracy) obtained for the inter-day assay in 'spiked' whole milk using anti-*CephM1* antibody. Back calculated values were obtained by fitting a four-parameter equation to the data set using BIAevaluation software (version 3.1).

Calculated $A/A_0 \pm$ S.D.	Concentration of cephalexin (ng/ml)	Back Calculated value (ng/ml)	% CV's	% Accuracies
0.02 ± 0.005	62,500	42,021.3	6.13	48
0.01 ± 0.003	31,250	45,572.7	4.34	131
0.05 ± 0.001	15,625	19,023.9	1.67	117
0.09 ± 0.002	7,812.5	10,558.9	2.09	126
0.20 ± 0.005	3,906.3	3,261.5	4.34	80
0.31 ± 0.004	1,953.1	1,282.2	2.47	47
0.36 ± 0.002	976.6	908.8	1.49	92
0.41 ± 0.001	488.3	674.7	9.36	127
0.53 ± 0.003	244.1	292.3	1.39	116
0.65 ± 0.009	122.1	131.1	4.26	106
0.76 ± 0.006	61.0	51.2	2.41	81
0.85 ± 0.005	30.5	21.2	2.06	56
0.89 ± 0.005	15.3	12.5	1.93	78
0.90 ± 0.011	7.6	9.4	4.38	119
0.93 ± 0.004	3.8	5.7	1.38	133

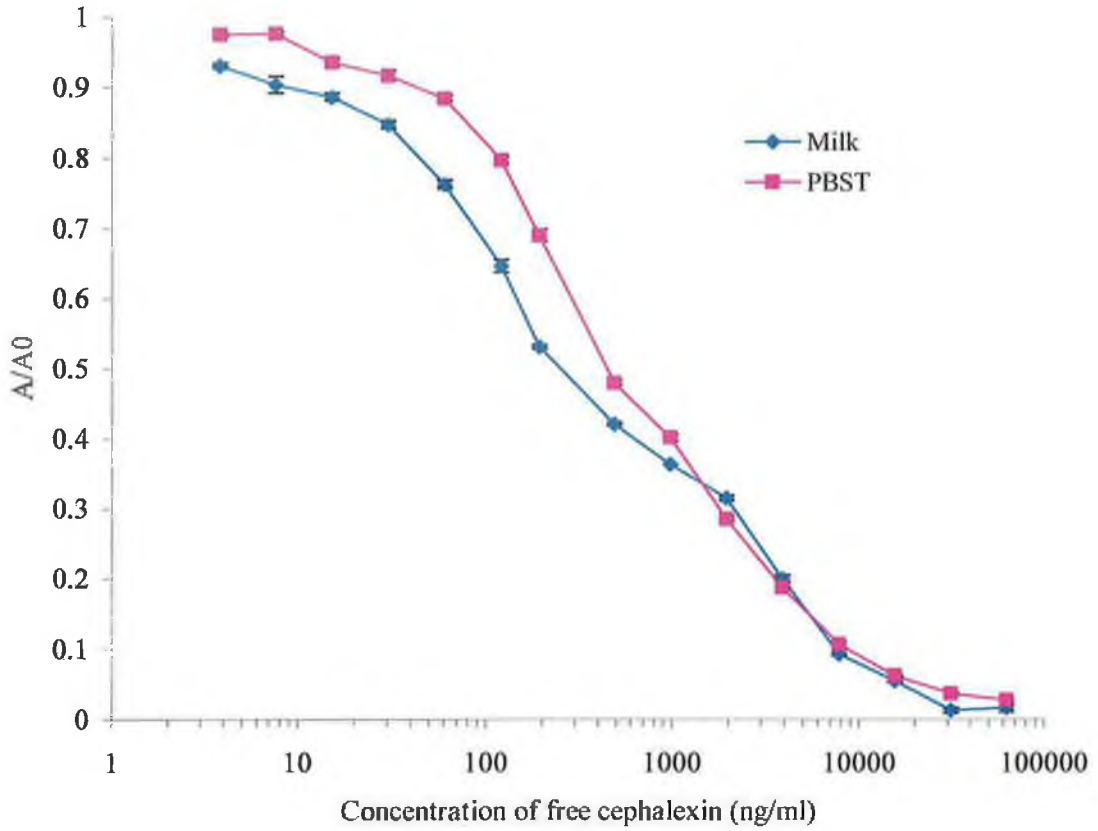


Figure 4.15. An overlay plot of inhibition ELISA using anti-*CephM1* and 'spiked' PBST and whole milk samples. The monoclonal assay was capable of detection of cephalexin in 'spiked' whole milk at the required maximum residue limit (MRL) of 100 ng/ml. The LOD for the inhibition assay was below this value and defined by taking 3 times the standard deviation from the background (A_0). The LOD was found to be 60 ng/ml (1.7×10^{-7} M), which corresponds to 6 ng/well.

4.2.5 *Cross-reactivity studies of the murine anti-cephalexin monoclonal (anti-CephM1) antibody*

As monoclonal antibody was raised using a different conjugate (i.e. Thyro-Ceph) and animal species to that used to produce polyclonal antibody (chapter 3), it was necessary to reassess the potential cross reaction with similar related compounds. The potential cross-reactivity of several related β-lactam antibiotics (table 3.4) to monoclonal antibody were evaluated using inhibition displacement ELISA format as previously described. Standards of each potential cross-reactant were prepared in whole milk and pre-incubated with monoclonal antibody prior to assay. The percentage cross-reactivity was determined as a ratio of the IC50 concentrations, as detailed in section 3.2.4. An overlay plot of the resulting assays is shown in figure 4.16. The IC50 values for each cross-reactant and the calculated percentage cross-reactivity (% CR) are listed in table 4.4.

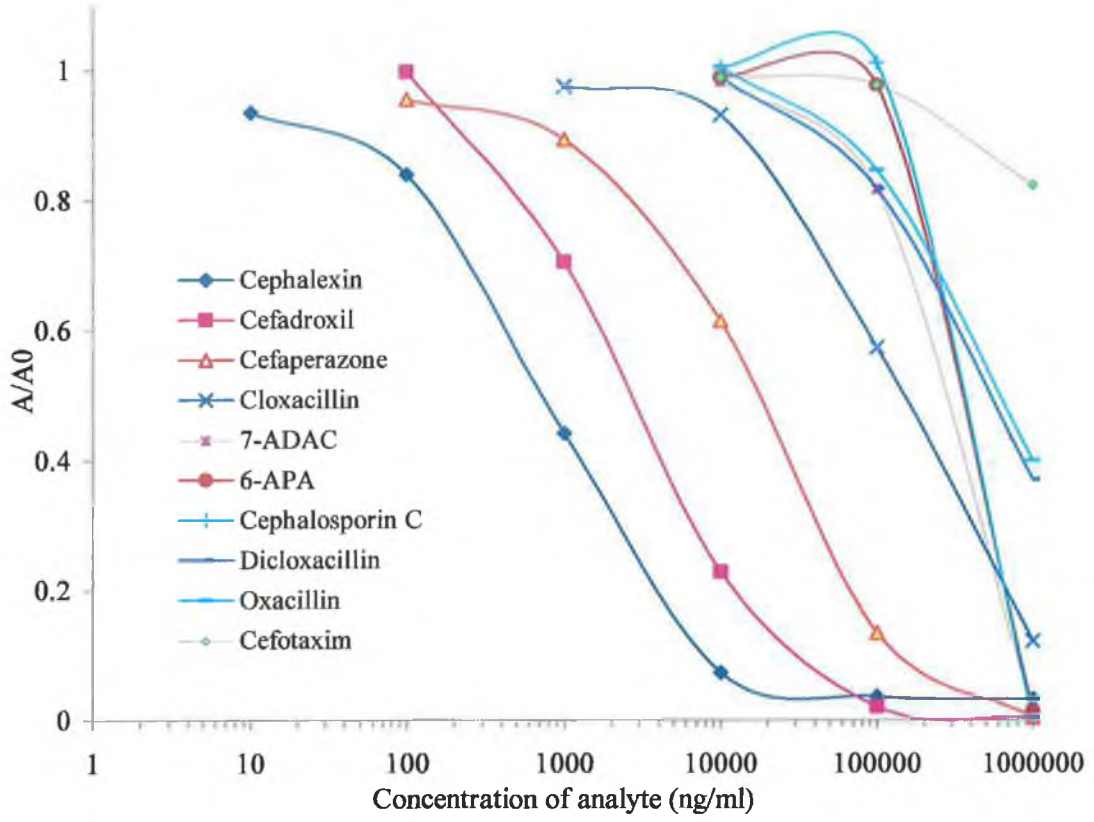


Figure 4.16. An overlay plot of inhibition ELISA in whole milk samples 'spiked' with the potential cross-reactant (as listed in above legend). The IC50 concentrations were calculated by fitting a four-parameter equation using BIAevaluation software. Each point represents the mean of 3 measurements.

Table 4.4. The tabulated results for the calculation for the percentage cross-reactivity (% CR) of monoclonal anti-cephalexin antibodies produced. The IC50 values were determined for each potential cross-reactant and expressed as a percentage of the IC50 concentration value obtained for the cephalexin assays.

Compound	IC50 (µg/ml)	%CR(IC50)
Cephalexin	0.74	100.00%
Cefadroxil	3.08	24.18%
Cefaperazone	13.23	5.62%
7-ADAC	245.58	0.30%
Cephalosporin C	320.38	0.23%
Cefotaxim	N/D	---
6-APA	311.39	0.24%
Cefazolin	N/D	---
Oxacillin	591.34	0.13%
Cloxacillin	125.98	0.59%
Dicloxacillin	506.66	0.15%
Cefuroxime	N/D	---
Amoxicillin	N/D	---

N/D = compounds showed no cross-reactivity with monoclonal antibody over the assay concentration range (max 1 mg/ml)

4.3 Discussion

In this chapter we report the production, purification and characterisation a monoclonal antibody against cephalexin. Thyro- and KLH-cephalexin conjugates were used to immunise balb/c mice. Mice were continuously boosted and serum antibody titres were determined using an enzyme-liked immunosorbent assay (ELISA). Typical antibody serum titres of 1/50,000 were obtained (Figure 4.6) during the immunisation protocol. There was very little difference between the serum titres of each of the thyroglobulin-cephalexin immunised mice (i.e. mouse 2 & 3), however; both had higher serum antibody titres in comparison with the KLH-Ceph conjugate immunised mouse. The spleens were removed and the lymphocytes extracted for *in vitro* somatic cell fusion procedures.

One week post-fusion, supernatant was removed from the hybridoma populations and used in a screening ELISA strategy that it was reasoned would isolate antibody secreting clones with greater affinity to soluble un-conjugated cephalexin. The stringent screening of hybrids did however, as an expected consequence; result in a decreased number of positive clones post fusion. Hybridoma cell populations with the highest absorbance values were selected (Figure 4.7) were scaled up and used in the development of an inhibition ELISA for the detection of cephalexin. After several rounds of cloning by limiting dilution, isolated monoclonal antibody secreting clones were characterised. An IgG2a-κ (anti-*CephM1*) was isolated and purified. The antibody specificity was determined by screening against various cephalexin conjugates with relevant controls, and confirmation of soluble cephalexin specificity was monitored by carrying out inhibition ELISA. It was discovered that binding of monoclonal antibody to immobilised conjugated cephalexin was inhibited over a range of soluble cephalexin concentrations. The antibody specificity was further analysed using a range of closely related β-lactam compounds (Figure 3.4), overall the antibody was found to be highly specific to cephalexin. However, the percentage cross-reactivity observed using cefadroxil and cefoperazone, in conjunction with results of the polyclonal antibody (Chapter 3) indicate that the probable antibody epitope is comprised mainly of the aromatic ring containing side chain. The lack of significant cross reaction with the core cephem structure of 7-amindesacetoxy-cephalosporanic acid would also corroborate this hypothesis.

The use of various coupling chemistries has been shown to have a determinant effect on the resulting epitope when generating antibodies toward such relatively small haptens. Other workers have reported difficulties associated with the selection of antibodies to small molecular weight haptens during screening procedures, resulting in the selection of antibodies sharing a combined hapten-protein epitope (Danilova, 1994; Keating, 1998; Killard, 1998). In

general the need to conjugate haptens for immunisation, selection and screening of antibodies and antibody fragments increases the likelihood of this problem (Moghadam *et al.*, 2001). The method employed to conjugate the hapten should mimic as closely as possible the structural and electronic properties of the non-conjugated hapten (Brun *et al.*, 2004).

Nagakura *et al.* (1990) suggested that monoclonal antibodies they produced toward cephalexin recognised at least three distinct epitopes. However, the cephalexin-protein conjugation production method they chose was also based on the alkaline hydrolysis, which opens the core beta lactam ring structure, hence, providing a molecular charge and structural conformation. The three different cephalexin-specific antibodies produced were IgM isotype. The antibodies were not evaluated in assay for the detection of cephalexin in milk. Nagakura *et al.* (1991) also produced monoclonal antibodies against ampicillin. One antibody (from clone Abp7) was found to be specific to ampicillin and cross-reacted to a significant degree with cephalexin. To the best of our knowledge this antibody was not used for detection of cephalexin.

Protein G-purified *anti-CephM1* showed inhibition in ELISA with standards of free cephalexin prepared in PBS-T and whole milk. The assays were linear over a range 61 ng/ml to 7812 ng/ml in PBS (figure 4.14) and 30 to 15625 ng/ml in whole milk (4.15). The limit of detection (LOD) for the inhibition assay was 60 ng/ml in whole milk prepared samples. The milk assay showed increased linearity which may be due to increased antibody solubility and stability in a more complex biological matrix. In comparison with available β-lactam testing kits available, in particular the Department of Agriculture and Food (DAF) approved standard, the Delvo® SP (Gistbrocades / DSM) microbial inhibition test, which has a stated sensitivity range of 40-60 ng/ml in milk, the sensitivity of the assay described in this chapter performs well. However, the anti-*CephM1* antibody specificity limits its application to the specific detection of cephalexin, as opposed to the broadly specific range of compounds detected by the Delvo® SP.

The lack of commercially available generic antibodies to antimicrobial compounds and the difficulty in generating such antibodies suggests that an assay incorporating several different antibodies would be the optimum approach for developing a multianalyte or broad specificity detection platform. Strasser *et al.* (2003) reported a semi-quantitative immunochemical rapid test for antimicrobials in milk using monoclonal antibodies. The method utilised several monoclonal antibodies raised against streptomycin, sulfadiazine, sulfamethazine and cloxacillin. The assay was capable of detecting the relevant antimicrobial in a multianalyte detection system in milk samples. They reported no observed matrix effects and very low

limits of detection i.e. 3.2 ng/ml for streptomycin, 9.0 ng/ml for sulfadiazine, 7.0 ng/ml for sulfamethazine and 0.8 ng/ml for cloxacillin. A similar approach to simultaneous detection of antibiotics in milk was reported by Knecht *et al.* (2004). Monoclonal antibodies against penicillin, cloxacillin, cephapirin, sulfadiazine, sulfamethazine, streptomycin, gentamycin, neomycin, erythromycin and tylosin were used for the simultaneous detection of respective analyte in a microarray-based system or parallel affinity sensor array (PASA). Protein conjugates of the haptens were immobilised as spots on disposable chips and integrated within a flow cell. An inhibition displacement assay format like the one in this chapter was used. The PASA system described was capable of detecting penicillin G at its MRL (4 ng/ml) and the remaining analytes could be detected well below their respective MRL values in milk.

In conclusion, the assay described in this chapter is capable of the specific detection of cephalexin in 'spiked' pasteurised whole milk. The assay performance also permits the detection of cephalexin at the current EUMRL, for cephalexin in milk (100 ng/ml). The assay is also comparable with similar work published in this field. Cliquet *et al.* (2001) raised broad specificity monoclonal antibodies against ampicillin-protein conjugates. One particular monoclonal antibody was capable of detecting a range of β-lactam antibiotics (ampicillin, penicillin G, oxacillin, dicloxacillin and carbenicillin) at 50 ng/ml concentrations. However, cephalexin was not assayed in this work and the competition ELISA formats were carried out using PBS as a sample matrix. Jin *et al.* (2005) described the production of monoclonal against gentamycin for the analysis of residues in serum and milk samples. The antibody was found to be highly specific with no observed cross-reactivity with other aminoglycosides. In addition, they reported a lower limit of detection for the milk assay (0.5 ng/ml) in comparison with PBS and serum 'spiked' samples, which were 0.9 and 1.0 ng/ml, respectively. The main benefit of the antibody presented in this chapter is its specific nature. As a working diagnostic reagent it would however need to be complemented with other antibodies for the broad detection of β-lactam antibiotics.

The *anti-CephM1* antibody, described in this chapter, was used in the development of a Biacore 3000™ SPR-based biosensor (Chapter 6) inhibition assay for the detection of cephalexin in milk. The hybridomas cell line that expresses anti-*CephM1* monoclonal antibody was also used in the production of a recombinant antibody fragment (scFv) libraries (Chapter 5). Future work arising from this research could encompass the evaluation of different assay formats and detection of cephalexin in different target samples, for example bovine kidney tissue extracts.

Chapter 5

Production and engineering of recombinant scFv antibodies to cephalexin

5.1 Introduction

5.1.1 *Recombinant antibody production*

The aim of this chapter was the production and isolation of recombinant scFv antibodies specific to cephalexin, and their subsequent use in the development of an immunoassay for the detection of cephalexin in milk. An existing naïve human combinatorial phage-display library (BMV library) was panned for cephalexin specific scFv-phage using cephalexin conjugates. Pre-immunised murine phage-display libraries were synthesised and anti-*CephM1* (chapter 4) antibody producing hybridoma cell line derived phage-displayed scFv were produced using the optimised phage-display system reported by Krebber *et al.* (1997). The isolated wild type (WT) phage-scFv derived from hybridoma cell line (anti-*CephM1*), scFv was expressed as single entity (i.e. not as phage-scFv fusion), purified and characterised. The WT-scFv was subsequently used as a template for the production of a mutant phage-display library, using random mutagenesis techniques. Mutant phage-displayed cephalexin-specific scFv were identified and expressed in soluble form, purified and compared to original WT-scFv in terms of their sequence information and assay performance. Two improved mutant scFv clones (R1*-DE-C5 and R2*-DE-H3) were identified, purified and characterised.

5.1.2 *The Krebber phage display system for production and isolation of specific single chain variable antibody fragments (scFv)*

Phage-display has been successfully utilised as a means of producing antibody fragments (e.g. scFv) and fragment libraries. Libraries may potentially contain vast quantities of heterogeneous Ab fragments (McCafferty *et al.*, 1990 and Winter *et al.*, 1994). In general, phage-display methodologies utilise non-lytic filamentous bacteriophage fd to express (display) specific scFv or Fab Ab fragments as phage protein coat fusions (Emanuel *et al.*, 2000). This technique combines the infectivity of the phage with the specificity of the antibodies. The fd phage infects strains of *E. coli* containing the F conjugative ('male') plasmid DNA (Azzazy and Highsmith, 2002). Therefore, antibody genes may be ligated into truncated-phage plasmids (i.e. phagemids), subsequent to transformation of *E. coli* and rescue of phage. Phage-displaying antibodies on their surface are then selected and enriched for analyte binding from the antibody population along with the genes encoding them.

Krebber *et al.* (1997) describes an optimised protocol for the generation of recombinant antibodies using a variety of genetic sources (e.g. hybridoma cell lines and lymphocytes). An overview of the major steps involved using the Krebber system is presented in figure 5.1. The

genetic source material may be immunised (immune) or a non-immunised (naïve libraries). The Krebber standardised method for the display of murine scFv antibody libraries was optimised for robustness, vector stability, tight control of scFv-gene III expression, and primer usage for amplification of heavy and light chain genes. The initial step in the process involves the cloning of antibody variable heavy and light chain antibody genes from a pool of genetic source material. A genetic source material rich in antibody genes (e.g. spleenocytes or hybridoma cell line), is used for RNA extraction, subsequent to cDNA synthesis using reverse transcription polymerase chain reaction (RT-PCR). The cDNA is then used as template pool for the amplification of antibody variable heavy (V_H) and light (V_L) chain genes. The Krebber system reported a more complete set of murine primers for the amplification of all potentially known V_H, V_κ, and V_λ sequences reported on the Kabat database (Kabat *et al.*, 1991) and extended primer sets, as described by Kettleborough *et al.* (1993); Ørum *et al.* (1993) and Zhou *et al.* (1994). In addition, V_L back primers have been engineered to encode a short FLAG peptide tag, which is located at the N-terminus of V_L chains.

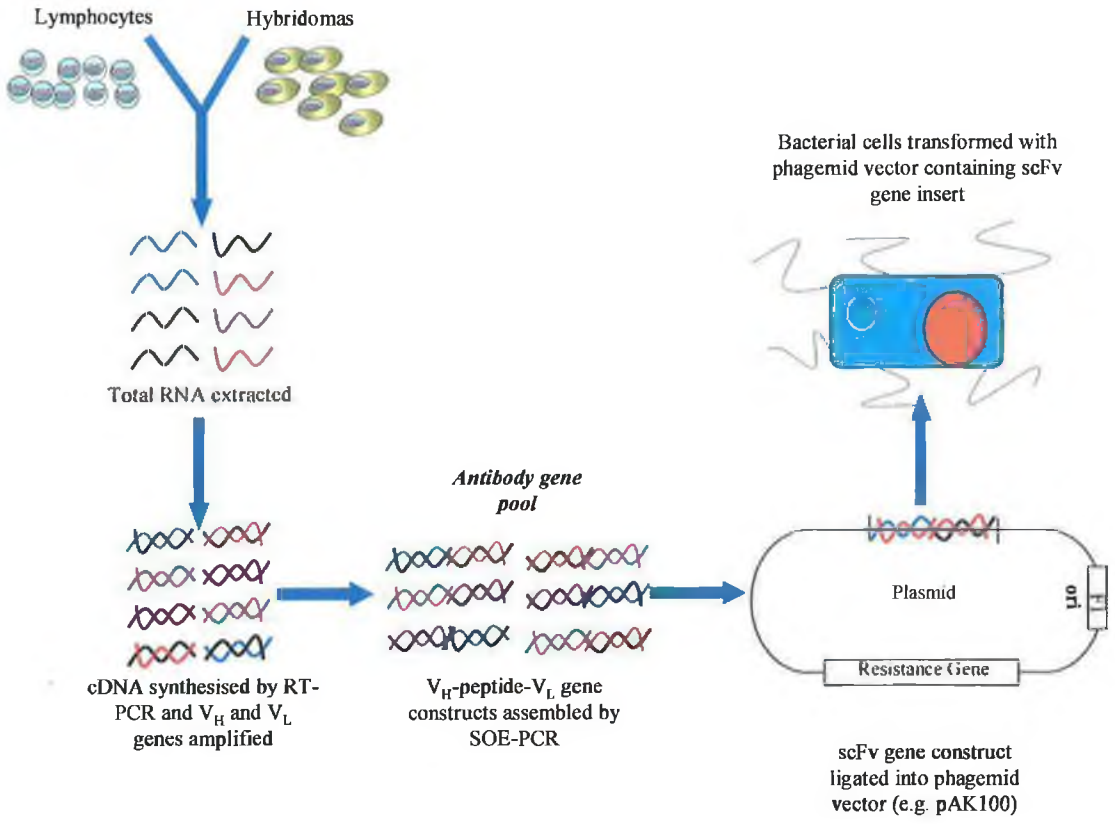


Figure 5.1. General schematic for production of phage display libraries, stepwise from initial extraction of mRNA, from either mixed populations of hybridomas or B-cells taken from the spleen of a pre-immunised animal. Both heavy and light chain cDNA are synthesised by Reverse Transcription PCR (RT-PCR) and annealed together using splice by overlap extension PCR. The combined H and L chain cDNA is then ligated into a phagemid vector and transfected into super-competent bacterial cells (e.g. XL1 blue). The scFv fragment is co-expressed with phage coat protein and preferentially packaged into phage particles using a helper phage (e.g. M13-K07).

The amplified V_H and V_L genes are assembled using a splice-by-overlap-extension polymerase chain reaction (SOE-PCR) as described by Horton *et al.* (1989). This system facilitates the directional cloning of scFv gene constructs by utilising a rare (i.e. in terms of restriction sites) restriction enzyme (*Sfi* 1) with an engineered series of compatible vectors (pAK100-600). The *Sfi* 1 restriction enzyme recognises eight bases in total, interrupted by a sequence of five unrecognised (N) nucleotides:

5′- GGCCN*NNNNGGCC-3′
3′- CCGGNNNN*NCCGG-5′

The benefit of such a rare restriction site is that its presence in potential antibody gene sequences is highly improbable. Restriction sites were engineered to have two distinct 'sticky' ends that facilitate cloning of scFv genes in a directional manner.

The vector series also contain tetracycline (*tet*A and *tet*R) resistance cassettes (approximately 2000 bp) in between two *Sfi 1* restriction sites (Figures 5.2 and 5.3) that allow the linear digested vector to be easily identified and purified. In general, truncated phage plasmid vectors (phagemids) consist of double stranded DNA, encoding a phage coat protein (e.g. pIII). They also contain an antibiotic resistance gene, an origin of replication and a promoter sequence (e.g. *lacZ*) (Garrard *et al.*, 1991 and Krebber *et al.*, 1997). The pAK100 phagemid vector which was optimised for phage-display contains a chloramphenicol (cam) resistance cassette, to aid in the selection of transformed *E. coli*, a phage origin of replication (F1 ori), a bacterial origin of replication (ColE1) as well as lac operon regulatory genes for the tight control of expression. The V_H and V_L chains for the scFv of interest may be integrated into pAK100 phagemid upstream of a myc affinity tag which is intersected by an amber stop codon (*), to facilitate the soluble expression of scFv in non-suppressor cells (e.g. *E. coli* JM83).

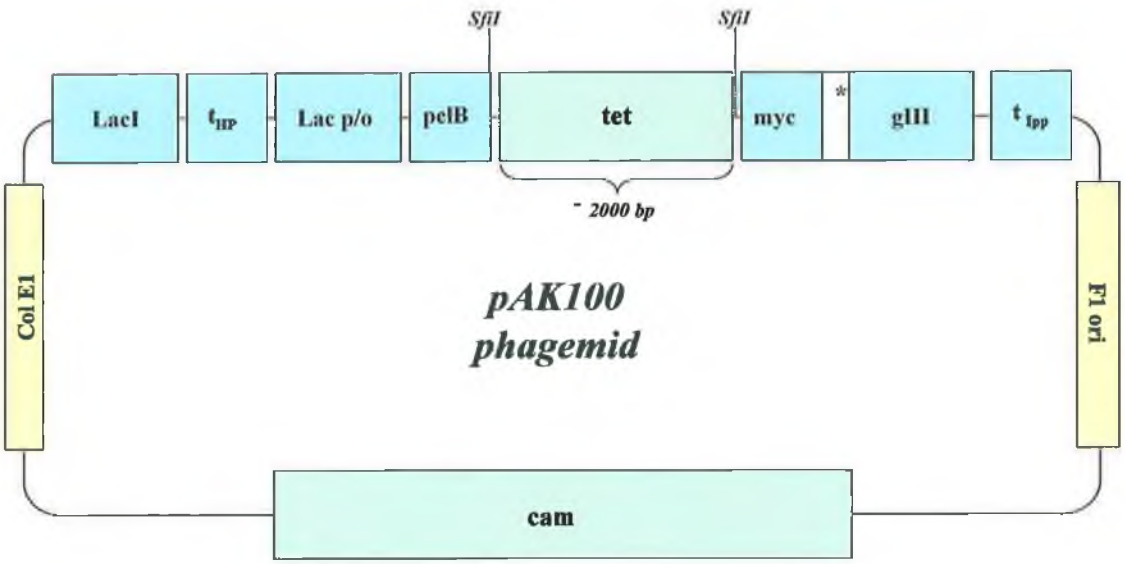


Figure 5.2. Diagram showing the pAK100 phagemid vector containing tetracycline (*tet*A and *tet*R) resistance cassette (approximately 2000 bp) in between two *Sfi 1* restriction sites. The scFv gene may be ligated in frame into the vector in place of the tetracycline resistance cassette upstream of the region encoding a myc detection tag, the phage coat protein gIII (250-406) followed by an amber codon (*). The pAK vectors also contains a pelB sequence, which adds an amino acid sequence to direct scFv-gIII fusion proteins to the periplasmic membrance in *E. coli*, subsequent to phage packaging. In addition pAK100 contains a chloramphenicol (cam) resistance cassette, to aid in the selection of transformed *E. coli*, a phage origin of replication (F1 ori), a bacterial origin of replication (ColE1) as well as lac operon regulatory genes for the tight control of expression.

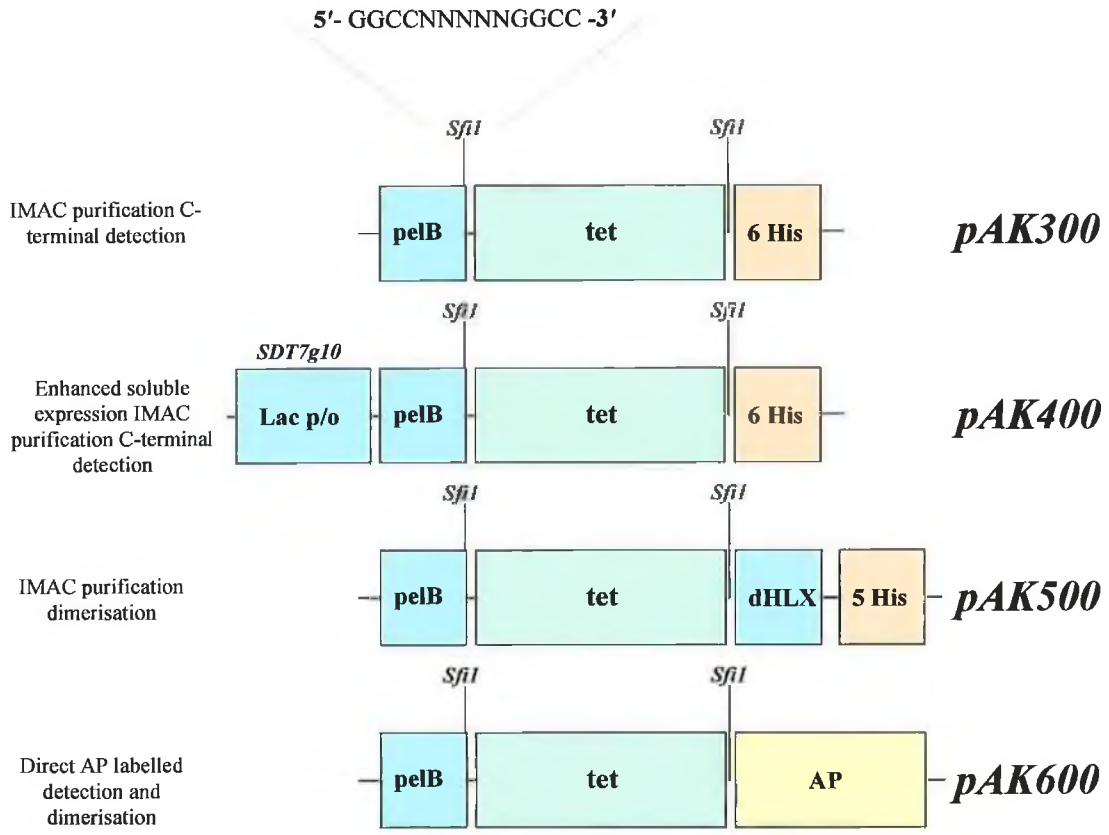


Figure 5.3. Diagram of the pAK100 related vectors that facilitate the introduction of modifications that allow for easier purification, enhanced soluble expression, dimerisation and enzyme-labelled dimerisation. All the pAK series of vectors contain tetracycline (*tet*A and *tet*R) resistance cassette (approximately 2000 bp) in between two *Sfi 1* restriction sites (restriction site sequence shown above). The scFv gene may be ligated in frame into the pAK300 and 400 vectors that facilitate soluble expression. The pAK400 contains a modified ribosomal binding sequence (i.e. Shine-Dalgarno, $SDT7_g10$) that results in enhanced protein expression. The pAK500 vector allows for dimerisation of antibody and pAK600 allows dimerisation and *in vivo* labelling with alkaline phosphatase (AP) for the direct detection of expressed scFv.

Transcribed antibody fragments may be fused at the N-terminus of the mature gene III protein (McCafferty *et al.*, 1990), as in the Krebber-based system (Figure 5.4), or at the N-terminus of a truncated pIII lacking the first two N-terminal domains (Barbas *et al.*, 1991 and Garrard *et al.*, 1991). The coat protein-antibody fusion protein is expressed and then incorporated into new phage particles within the *E. coli* by the superinfection with helper phage (e.g. M13KO7, VCS-M13, Hyperphage) (Rondot *et al.*, 2001) to package the DNA into phage particles. The pAK100 phagemid is designed for low level expression which is of paramount importance when dealing with phage-displayed libraries. Therefore, it is not a suitable vector for the large-scale production of soluble scFv. The Krebber system has the pAK400 engineered compatible vector to counter this. The pAK400 (Figure 5.3) is designed for the enhanced soluble expression and purification of antibody fragment by the incorporation of a Shine-Dalgarno ($SDT7_g10$) sequence. Other pAK series vectors facilitate purification and the introduction of modifications to scFvs. The scFv may be expressed as a dimer using the pAK500 and as a dimer with *in vivo* enzyme labelling (alkaline phosphatase) for direct detection using pAK600.

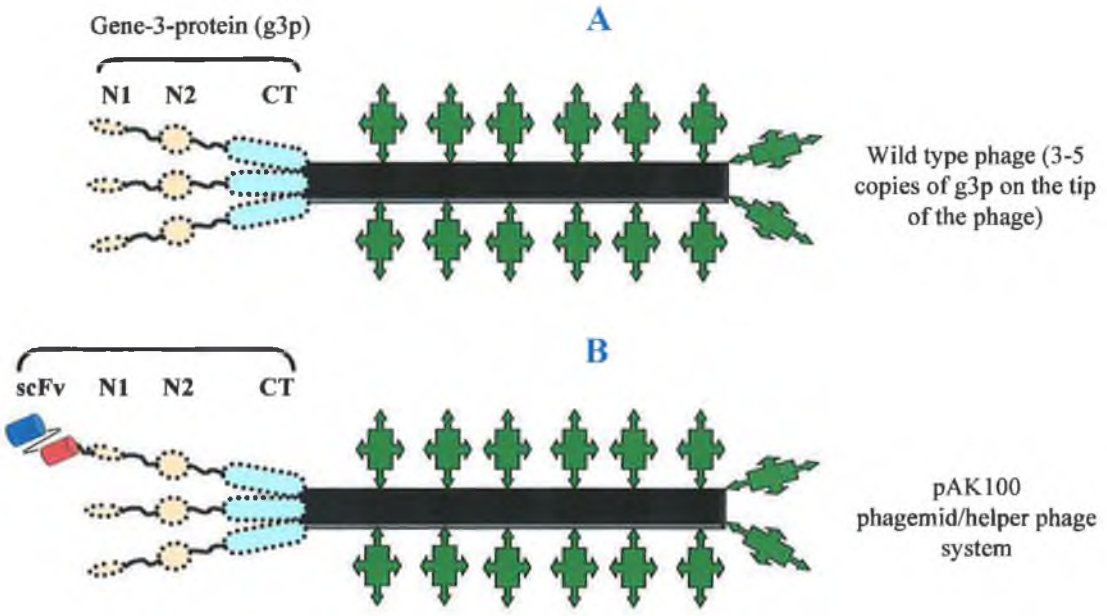


Figure 5.4. (A) The wild-type (WT) bacteriophage with mature gene-3-protein (gIIIp) protein, consisting of the C-terminal (CT), N2 and N1 terminal domains in contrast with gIIIp-scFv fusion containing phage produced using the Krebber protocol. (B) Phage-displaying scFv as a gIIIp protein fusion on its surface; the scFv antibody fragments are fused at the N1-terminus of the mature gene III protein.

5.2 Results

5.2.1 *Panning of a naïve human synthetic recombinant antibody library for anti-cephalexin-specific clones*

A naïve human recombinant antibody library (BMV library) obtained from Cambridge Antibody Technology (CAT) was panned using immunotubes coated with a cephalexin-BSA conjugate. A total of four rounds of panning were carried out, as described in section 2.5.5.4, using the BSA-cephalexin conjugate. No positive cephalexin-specific phages-scFv antibodies were obtained after screening of phage using ELISA (section 2.5.5.6). Some workers have described the isolation of hapten-specific scFv using human naïve phage-display libraries (Moghaddam *et al.*, 2001 and 2003) but also recognise that it is potentially problematic. Factors affecting the probability of isolating a hapten-specific scFv from human naïve libraries include the library source, panning strategy and hapten-conjugates. Another study (Van Wyngaardt *et al.*, 2004) using haptens showed that naïve semi-synthetic combinatorial phage-display libraries produced using chickens generated large antibody diversity with hapten binding capability.

5.2.2 *The production of scFv phage-display libraries against cephalexin using the Krebber system.*

The Krebber optimised phage-display library protocol was used to develop two libraries using mice immunised with cephalexin conjugates. Libraries developed from immunised hosts have a distinct advantage over naïve libraries, especially in the case of haptens, as the host immune system is primed towards specific antigen before library production proceeds.

5.2.2.1 *Phage-display library production*

Initially Balb/c mice were immunised and boosted with cephalexin conjugates. Mouse A and B were immunised using cephalexin activated BSA and protein-activated cephalexin-Ova conjugate (sections 2.2.1.2 and 2.2.1.3), respectively, in Freund's adjuvant. The serum titre from each mouse was analysed by ELISA (section 2.3.1.1) against thyroglobulin-cephalexin conjugate, at a concentration of 1 μg/ml. Serial dilutions of mouse serum prepared in PBS-T were added to Thyro-Ceph-coated immunoplates and detected using HRP-labelled rabbit anti-mouse secondary antibody. The antibody titre for the purposes of this work was defined as the highest dilution that yielded a response distinguishable from the background plus three standard deviations. Figure 5.5 shows that the serum antibody titres for two mice was

1/50,000 for BSA-cephalexin (mouse 1) conjugate and Ova-cephalexin (mouse 2). The mice were re-boosted one week prior to sacrifice and extraction of RNA from spleen (section 2.5.3.2).

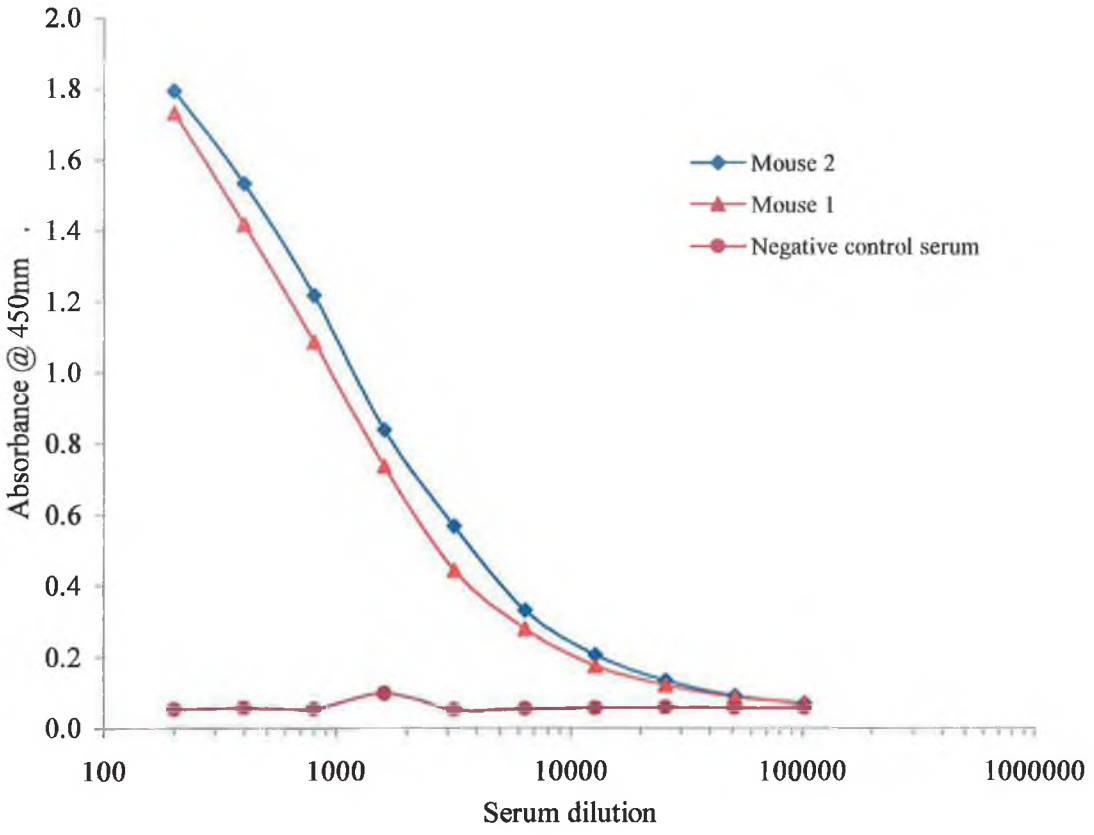


Figure 5.5. Plot of the results obtained from an ELISA to determine the anti-cephalexin antibody titre, using the serum obtained from the mouse 1 (immunised with BSA-cephalexin) and mouse 2 (immunised with OVA-cephalexin). Serum samples from both mice were screened against thyroglobulin-cephalexin conjugate-coated plates at a concentration of 1 μg/ml and negative control surface thyroglobulin at a coating concentration of 1 μg/ml. Negative control serum represents the serial dilutions of naïve mouse serum added to cephalexin-thyroglobulin-coated wells. No significant binding to thyroglobulin-coated wells was observed. The mice were boosted once more prior to sacrifice and spleen removal.

The spleens from mouse 1 and 2 were isolated under sterile conditions and the total RNA was extracted from the homogenized spleen tissue (section 2.5.3.2). The total RNA was used to synthesise cDNA with random hexamer, and oligo dT primers. Subsequently, using newly synthesised cDNA as template, heavy (V_H) and light (V_L) chain variable genes were amplified by PCR with the set of murine degenerate primers (section 2.5.4.1), as described by Krebber *et al.* (1997). Optimisation of the V_H and V_L chain amplification PCR was carried out using the Invitrogen PCR Optimizer™ kit. The kit covers a range of 300mM Tris-HCl and 75 mM ammonium sulphate buffers, differing in their respective $MgCl_2$ concentrations and pH. The optimum buffer for the amplification of the heavy chain PCR for mouse 1 had 2.0mM $MgCl_2$ and pH of 8.5 whereas, V_L amplification PCR product for mouse 1 was found to be unaffected by varying concentrations of $MgCl_2$ at pH 8.5. The PCR products of the V_H and V_L gene amplification were analysed by gel electrophoresis (1% (w/v) agarose) and the expected bands, at approximately 400 bp, were observed for V_L (375-402 bp) and V_H (386-440 bp) (Figures 5.6 and 5.7, respectively).

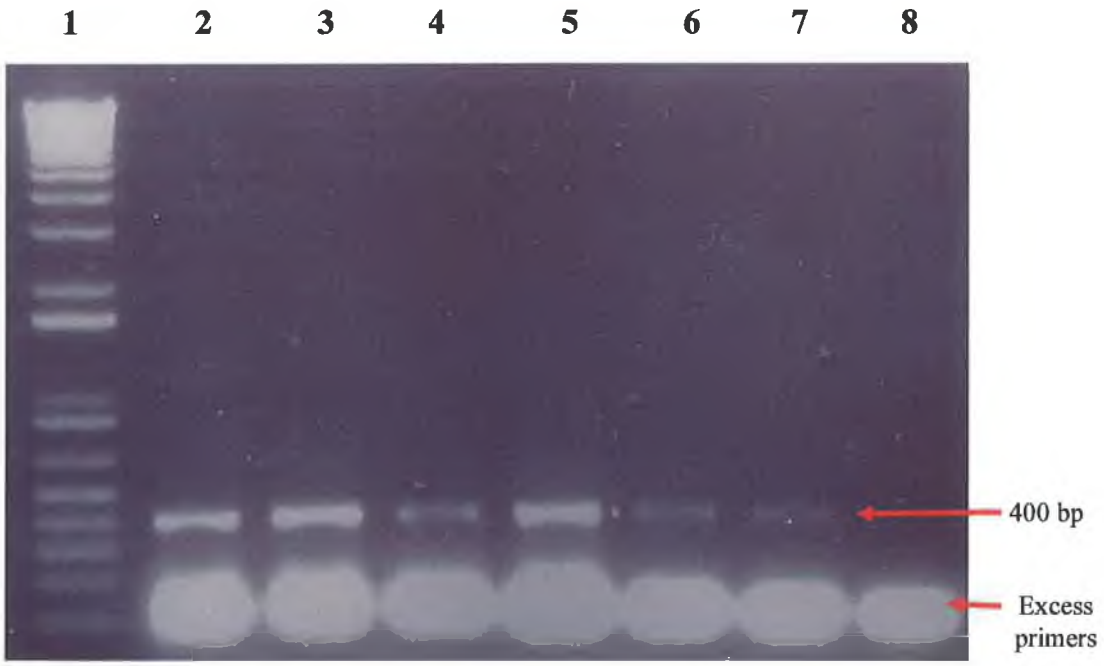


Figure 5.6. Picture of a typical 1% (w/v) agarose gel electrophoresis of buffer optimisation for mouse 1 heavy chain (V_H) amplification PCR. Lane 1, Gibco 1kb Plus DNA ladder; Lane 2, buffer A; Lane 3, buffer B; Lane 4, buffer C; Lane 5, buffer D; Lane 6, buffer F; Lane 7, buffer J; Lane 8, buffer N. From this result buffer B was chosen to carry out subsequent heavy chain PCR amplifications.

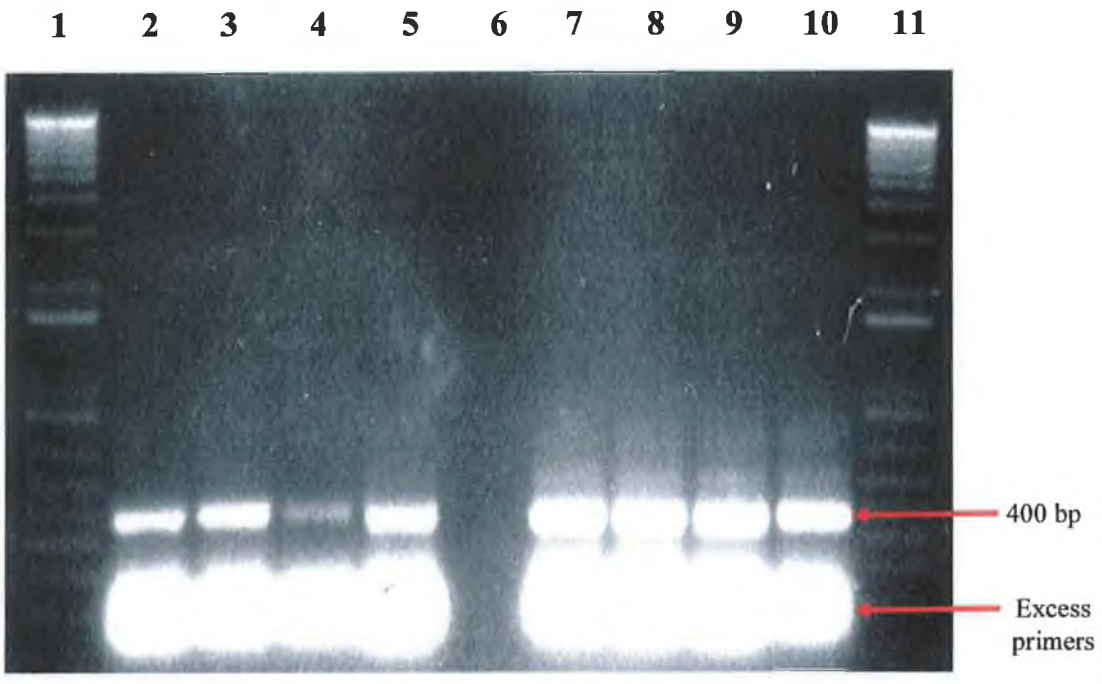


Figure 5.7. Picture of a typical 1% (w/v) agarose gel electrophoresis used during buffer optimisation for mouse 1, heavy (V_H) and light chain (V_L) amplifications. Lane 1, Gibco 1kb Plus DNA ladder; Lane 2, V_H buffer A; Lane 3, V_H buffer B; Lane 4, V_H buffer C; Lane 5, V_H buffer D; Lane 6, empty; Lane 7, V_L buffer A; Lane 8, V_L buffer B; Lane 9, V_L buffer C; Lane 10, V_L buffer D; Lane 11, Gibco 1 kb Plus DNA ladder. Buffer B was chosen to carry out subsequent V_H and V_L PCR amplifications.

The scFv gene constructs (V_L-$(G_4S)_4$-V_H) were synthesised using Splice-by-Overlap-Extension (SOE) PCR. The V_H and V_L chain PCR products were purified from a low melt 1% (w/v) agarose gel, as described in section 2.5.2.2. The amplified and purified V_H and V_L chain DNA for each mouse (1 & 2) were quantified with the 100 bp quantification marker (Promega) (Figure 5.8) and UV spectrophotometry.

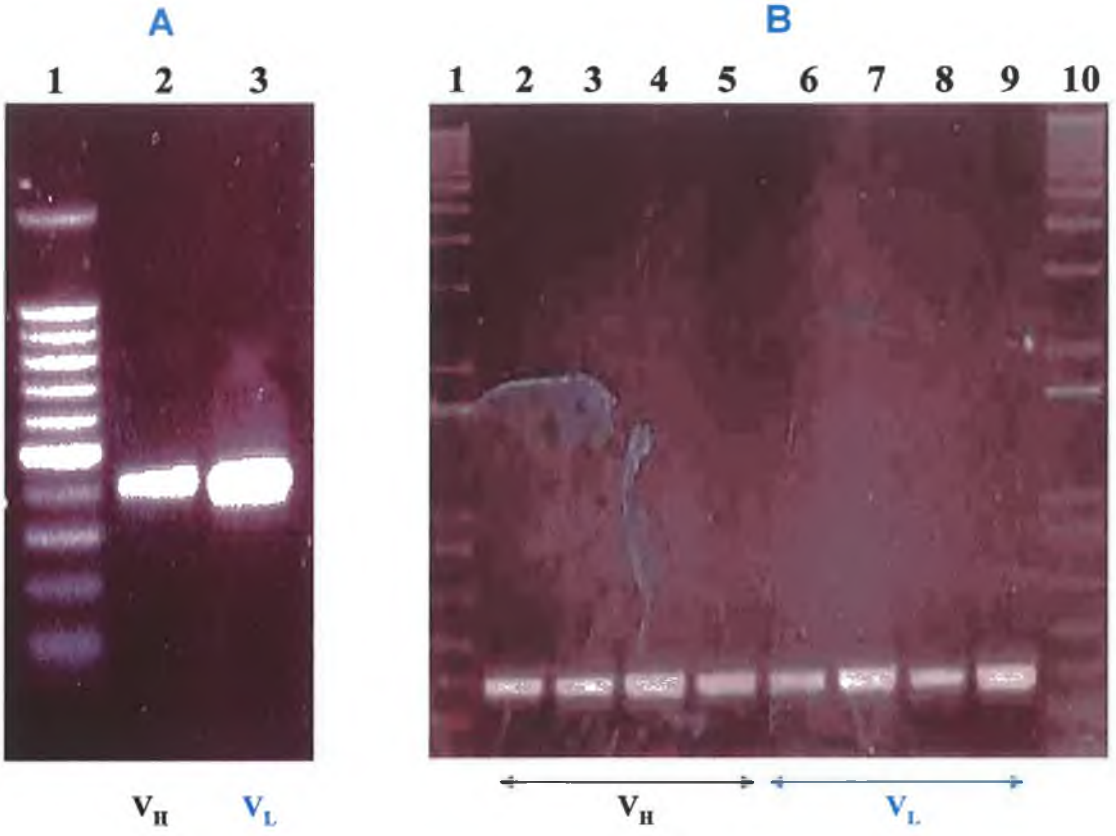


Figure 5.8. 1% (w/v) Agarose gel pictures; **A** shows pooled V_H and V_L chain PCR products purified from an agarose gel. The fragments were run on the gel with Promega 100bp marker (standard band concentrations, 500 bp = 150 ng/5µl, all other bands contain 50 ng/5µl). Lane 1, 100 bp Promega quantification ladder; Lane 2, heavy chain PCR product; Lane 3, light chain PCR product. The V_H and V_L chain stocks were prepared by pooling multiple PCR reactions following analysis on agarose gel (**B**). Lanes 1 & 10, Gibco 1 kb plus DNA ladder; Lanes 2-5, V_H reactions; Lanes 6-9, V_L reactions. The heavy and light chain pooled stocks were diluted to give a 10 ng/µl working stock for the subsequent SOE-PCR.

The V_H and V_L chain DNA stocks were both diluted to give a 10 ng/µl working stock concentration and joined by SOE-PCR (section 2.5.4.4). Splice by Overlap Extension (SOE) PCR was optimised using the Invitrogen PCR Optimizer™ kit buffer E (7.5 mM $MgCl_2$, pH 9.0) and sc_{for} and sc_{back} primers (Krebber *et al.*, 1997). This yielded predicted bands at approximately 800 bp, as shown in figure 5.9.

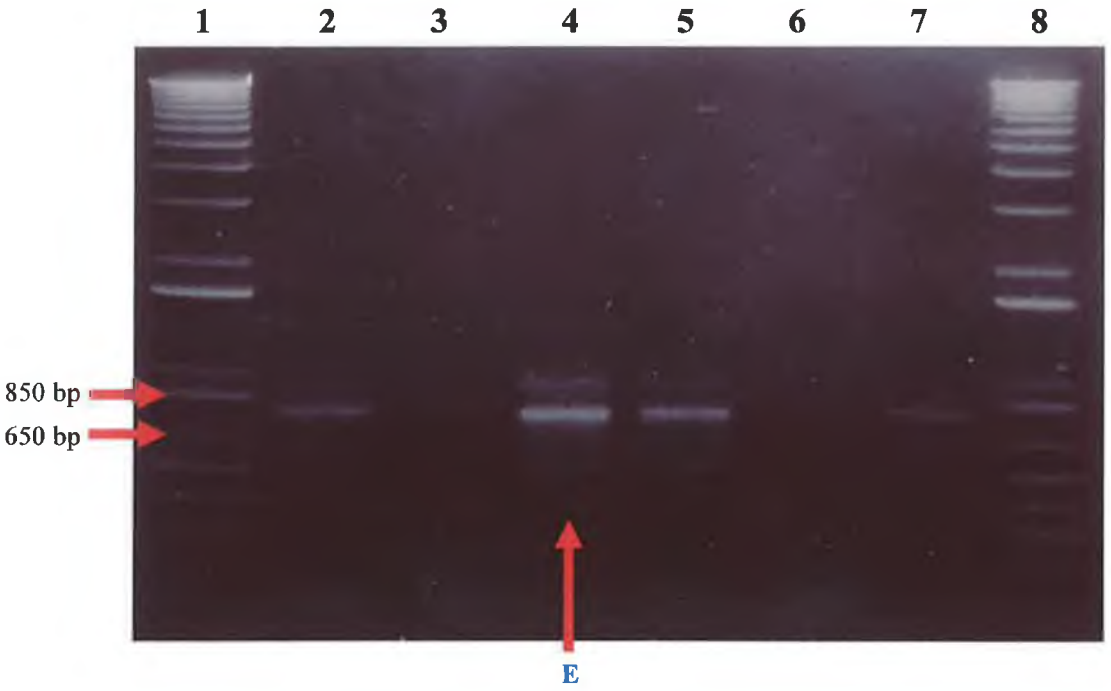


Figure 5.9. Picture of a 1% (w/v) agarose electrophoresis gel of SOE-PCR buffer optimisation reactions using mouse V_H and V_L chain genes. Lane 1, Gibco 1 kb Plus DNA ladder; Lane 2, SOE reaction in buffer A product; Lane 3, SOE reaction in buffer B; Lane 4, SOE reaction in buffer E; Lane 5, SOE reaction in buffer F; Lane 6, SOE in buffer F; Lane 7, SOE reaction in buffer I; Lane 8, buffer J; Lane 9, Gibco 1 kb Plus DNA ladder. Buffer E was chosen for further SOE-PCR reactions.

Bacterial stocks of XL1-blue (*E. coli*) containing pAK100 phagemid vector were grown as overnight cultures. The pAK100 was purified from cellular lysates as described in section 2.5.2.3. Purified vector and SOE-PCR product preparations were digested using *Sfi* 1 restriction enzyme (section 2.5.4.6) overnight at 50°C and the resulting products analysed using agarose gel electrophoresis. Cut pAK100 vector showed the expected bands at ~4000 bp and ~2000 bp, representing linear phagemid and tetracycline resistance cassette, respectively (Figure 5.10). The cut vector and SOE-PCR were purified from agarose gel and ligated overnight at 15°C using DNA ligase (section 2.5.4.7).

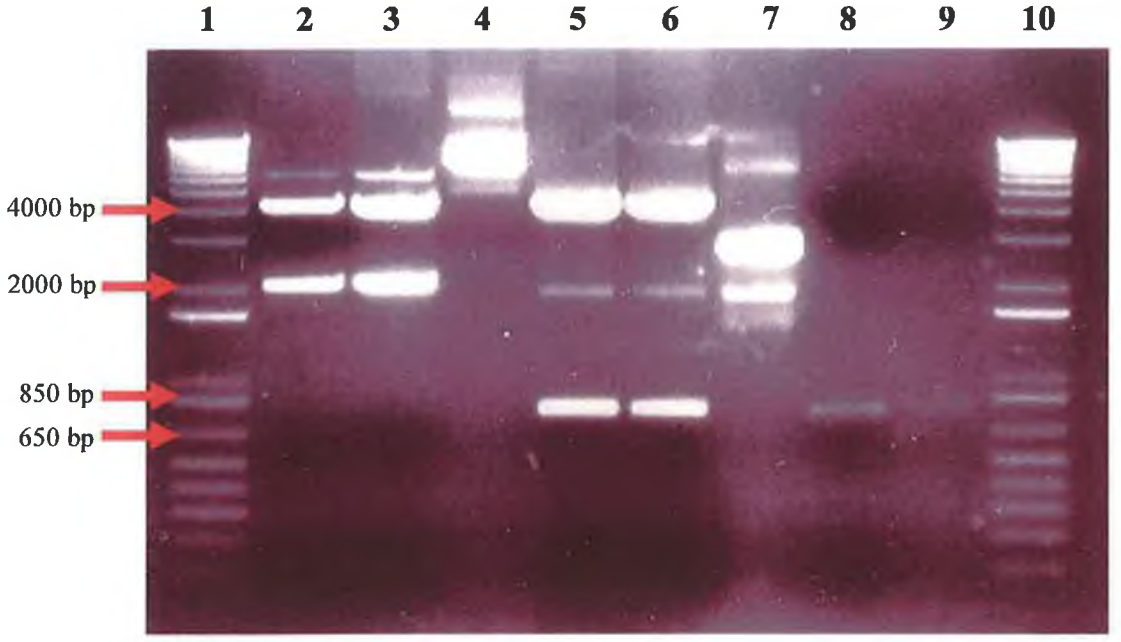


Figure 5.10. Picture of 1% (w/v) agarose electrophoresis gel of pAK100 vector and SOE-PCR *Sfi* 1 restriction digest products; Lane 1, Gibco 1 kb Plus DNA ladder; Lanes 2 & 3, identical pAK100 (containing Tet) digestions, o/n with *Sfi*1; Lane 4, undigested pAK100 (Tet) vector; Lanes 5 & 6, pAK100 (containing an scFv insert) digested o/n with *Sfi* 1; Lane 7, undigested pAK100 (SOE product); Lane 8, SOE-PCR product for mouse 1, digested o/n with *Sfi* 1; Lane 9, undigested SOE-PCR product; Lane 10, Gibco 1 kb Plus DNA ladder.

The ligation reaction products for mouse 1 and 2 derived genes were used in a subsequent transformation (2.5.4.9) of chemically competent XL1-blue (*E. coli*) cells prepared 'in house', as described in section 2.5.2.6. Selection of transformed cells containing the pAK100 and insert was achieved by plating on NE (Non-Expression) media containing chloramphenicol at a concentration of 25μg/ml. The chloramphenicol resistance gene is encoded for on the pAK100 vector. Therefore, any transformants that grow on the NE media plates supplemented with chloramphenicol were adjudged to contain the pAK100 vector. The numbers of transformants were found to be 4.6×10^3 and 3.2×10^3 cfu/ml, for mouse 1 and 2, respectively. Stocks of the transformed XL1-blue cells were produced and used for subsequent phage production.

5.2.2.2 *Panning of phage-display libraries*

Selection of specific scFv from phage-libraries involves the enrichment of specific binders by several rounds of panning with specific antigen. Alternative methods of panning and elution of specific phage-scFv were investigated. As the numbers of transformants for each library were relatively low, the libraries from mouse 1 and 2 were pooled. Phage particles were rescued and precipitated from bacterial stocks, as described in sections 2.5.5.1 and 2.5.5.2, respectively. The stock phages were panned using protein-activated thyroglobulin-cephalexin (Thyro-Ceph) immobilised conjugate for round one (section 2.5.5.4), followed by cephalexin-activated dextran-cephalexin conjugate for round two, and a third and final round of panning with Thyro-Ceph. Various phage elution strategies were used, including acidic phage elution (section 2.5.5.4), where bound phage were eluted from the coated immunotube by adding 800 μl 0.1 M glycine/HCl, pH 2.2, for 10 minutes. This was then neutralised using 48 μl of 2 M Tris/HCl, pH 8.5. XL1-blue *E. coli* in exponential growth phase were infected with eluted phages from each round of panning.

An alternative strategy using triethylamine and XL1-blue *E. coli* for eluting bound phage particles from the coated immunotubes was used (Wind *et al.*, 1997 and De Briun *et al.*, 1999). This method consisted of an initial elution of lower-affinity binding phage using XL1-blue cells in exponential growth phase. Following this remaining phages were eluted with triethylamine. The phages were then decanted into a solution of 1M Tris-HCl, pH of 7.5, for subsequent neutralisation. During each round of panning the phage particles are separated into two fractions, a low affinity phage fraction (i.e. XL1-blue elution) and a higher affinity phage fraction (i.e. 100 mM triethylamine). Each of the eluted phage stocks (i.e. low and high affinity) were screened and subjected to subsequent rounds of panning.

A sample was removed after each round of panning from both the bacterial and triethylamine elution's in order to carry out a titre of the phage. Following this, the eluted phage were either grown directly (i.e. in the case of the bacterial elution), or used to transfect exponentially growing XL1-blue *E. coli* (i.e. triethylamine elution), as outlined in section 2.5.5.5.

Following rounds of panning and elution prepared 'polyclonal' phages supernatants were screened for cephalexin specific phage-scFv by ELISA. In addition, a total of 183 random individual ('monoclonal') clones were picked and screened by phage ELISA (section 2.5.5.6). The phages were detected using a rabbit anti-fd bacteriophage antibody (Sigma), which was subsequently detected using an HRP-conjugated goat anti-rabbit secondary antibody. No cephalexin-specific phage-scFvs were obtained from immunised mice libraries. To ensure that the library clones contained the scFv gene constructs, random clones from round 3 panned stocks and untransformed (negative control) XL-1 blue *E. coli* were used in a PCR. The SOE sc_{for} and sc_{back} primers were used to amplify 800 bp scFv insert. The resulting products were run on a 1% (w/v) agarose gel and clearly showed 800 bp fragments were present in randomly picked clones (Figure 5.11).

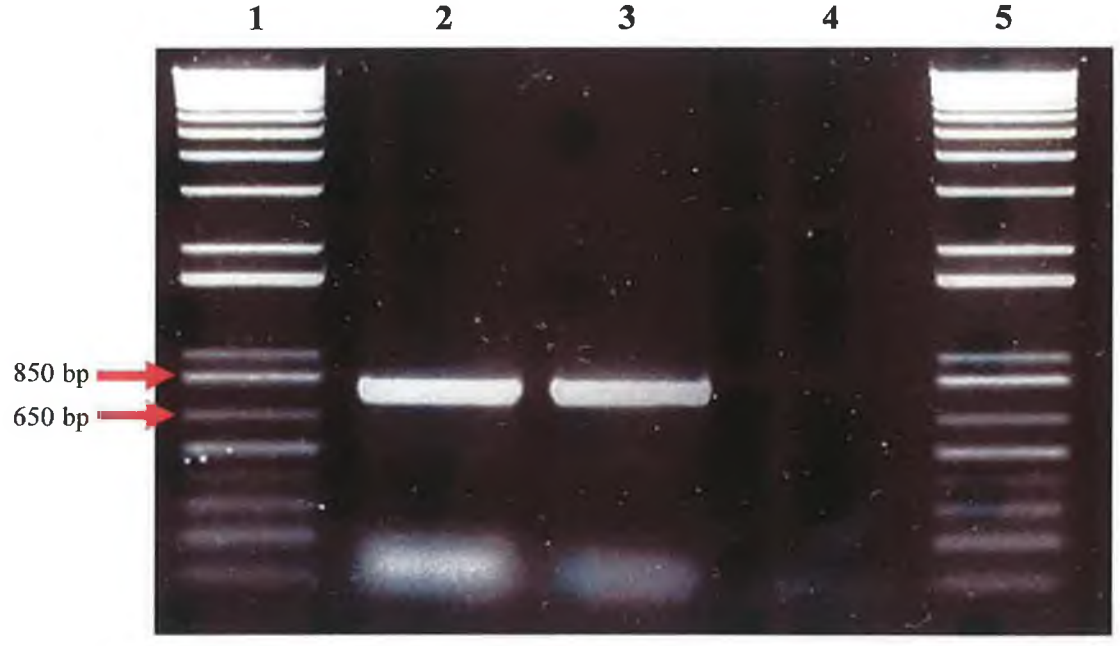


Figure 5.11. Picture of a 1% (w/v) agarose gel electrophoresis of the resulting SOE-PCR products of 2 random clones picked off a phage titre plate from the third round of panning. This experiment was to confirm the presence of vector (pAk100) containing the scFv gene insert (800 bp fragment). Lane 1 & 5, Gibco 1 kb plus DNA ladder; Lane 2, SOE-PCR product for clone 1; Lane 3, SOE-PCR product for clone 2; Lane 4, SOE-PCR product using untransformed X-L1 blue cells as negative control.

5.2.3 *The production of scFv against cephalexin from hybridoma cell line secreting monoclonal anti-CephM1 antibody using the Krebber system.*

The Krebber optimised phage-display library protocol was used to develop recombinant anti-cephalexin scFv, using the anti-*CephM1* monoclonal antibody (Chapter 4) secreting hybridoma cell line.

5.2.3.1 *The production of a phage-scFv antibody fragment from anti-cephalexin monoclonal antibody-secreting hybridoma cell line using the Krebber system.*

A phage-scFv specific to soluble cephalexin was obtained from the hybridoma cell line (Anti-*CephM1*). The protocol and vector series used were as previously described by Krebber *et al.* Initially, the hybridoma cell line developed in chapter 4 of this research was used as the source for total RNA extraction. Total RNA from the positive secreting cell line was used as a template for the synthesis of cDNA by RT-PCR, according to kit manufacturer's guidelines (Promega kit). V_H and V_L chain genes were amplified (Figure 5.12) using the listed set of degenerate primers, omitting λ light chain primers (anti-*CephM1* contains κ light chain), and purified prior to scFv gene construction by SOE-PCR (Figure 5.13).

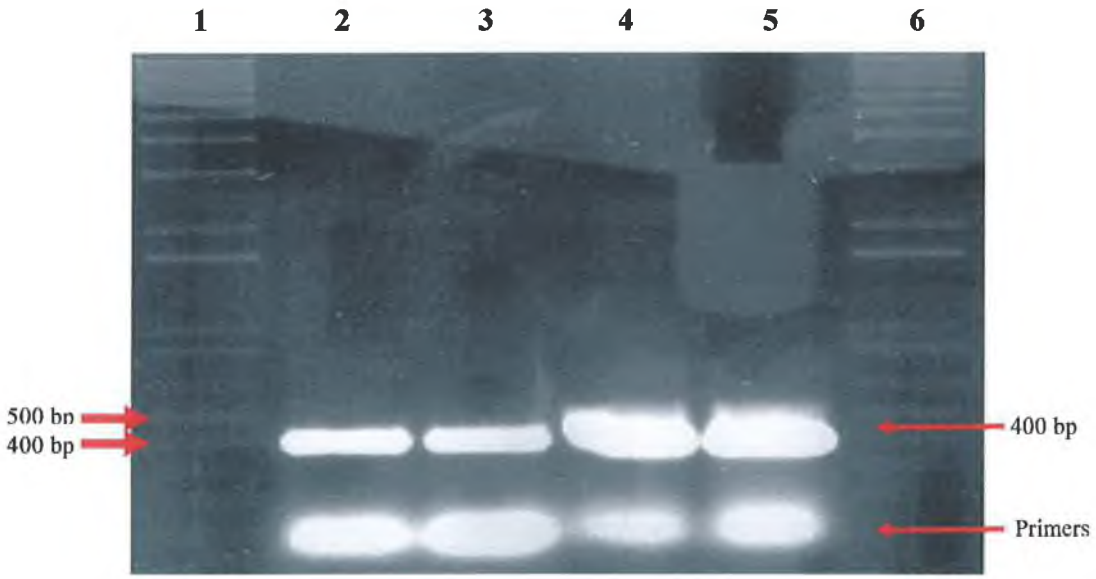


Figure 5.12. Picture showing 1% (w/v) agarose gel electrophoresis of V_H and V_L chain amplification PCRs. Lanes 1 & 6, Gibco 1kb Plus DNA ladder; Lanes 2 & 3, buffer A amplified hybridoma V_H chain; Lanes 4 & 5, buffer A amplified V_L. Both heavy and light chain amplification PCR reactions yielded discreet bands at approximately 400 base pairs (bp) as expected.

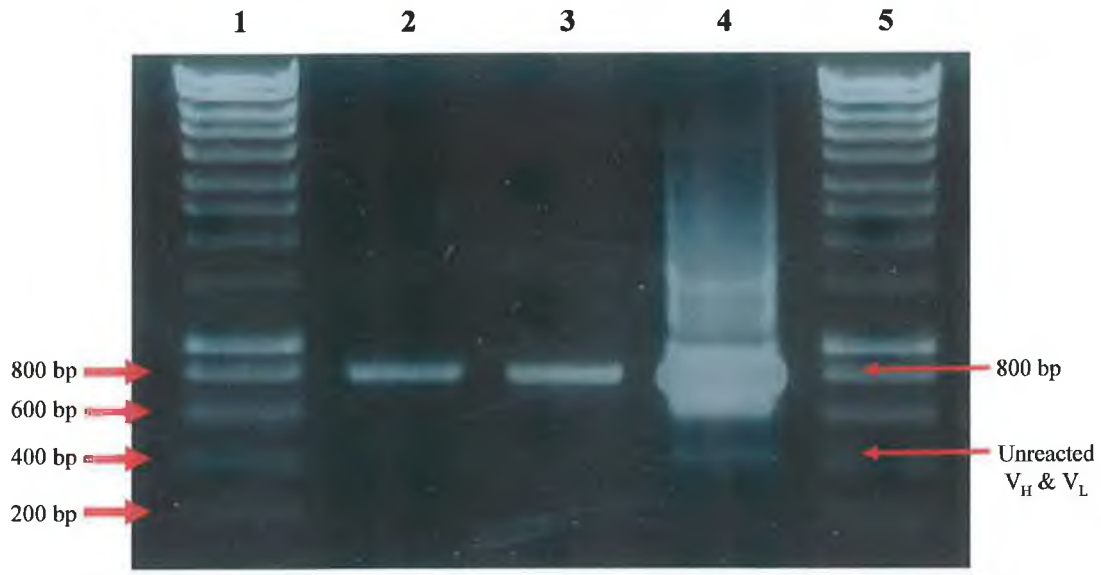


Figure 5.13. Typical 1% (w/v) Agarose gel of SOE-PCR product. Lanes 1 & 5, Hyperladder I DNA marker (Bioline); Lanes 2, 3 & 4 are SOE-PCR product using buffer A and varying concentration ratios of V_H and V_L chain template. The SOE-PCR reactions yielded discreet bands at approximately 800 base pairs (bp) as expected.

The resulting SOE-PCR gene constructs were digested with *Sfi* 1 restriction enzyme along with fresh pAK100 phagemid vector and subsequently ligated together. The *Sfi* 1 restriction digest products were gel purified (section 2.5.2.2) and analysed by agarose gel electrophoresis. The digested and undigested products were run *in situ*. Figure 5.14 shows the purified products of the *Sfi* 1 digested, pAK100 phagemid vector and SOE scFv gene constructs. Chemically competent *E.coli* (XL1 blue) cells were transformed with ligation reaction product (section 2.5.4.9) that was concentrated 10-fold (ethanol precipitation, section 2.5.2.4). Transformed cells were used for phage production and PEG concentration, as described in sections 2.5.5.1 and 2.5.5.2, respectively.

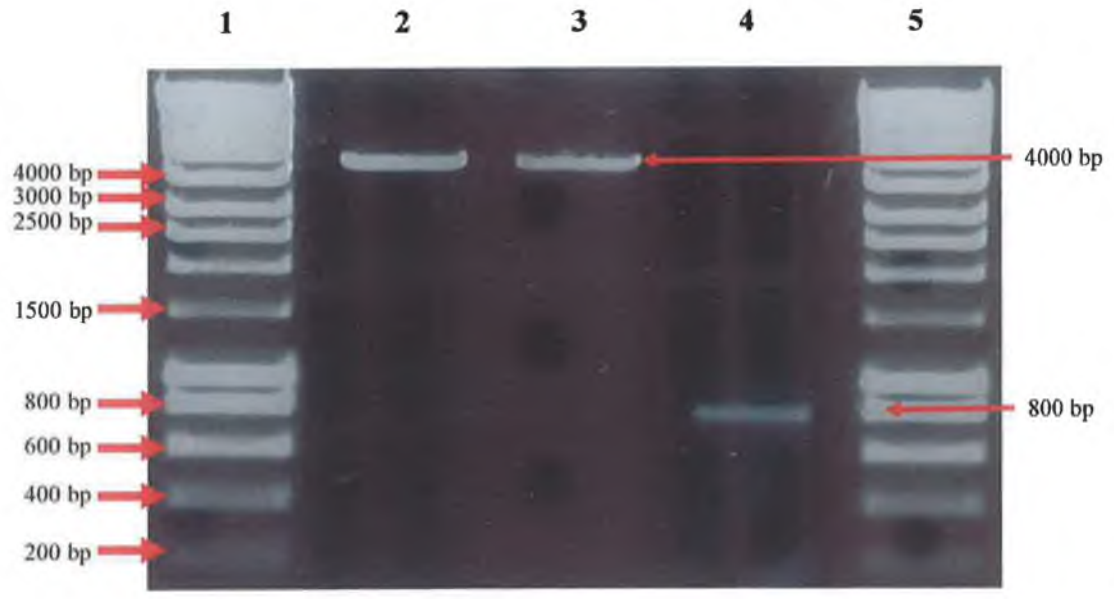
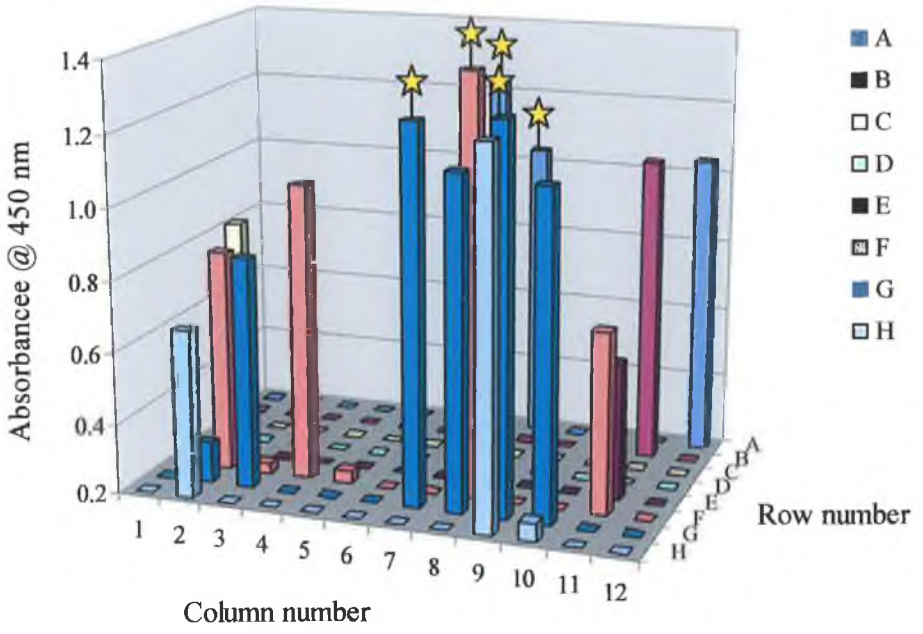


Figure 5.14. Picture of 1.5% (w/v) agarose gel electrophoresis of previously *Sfi* 1 digested and gel purified hybridoma SOE-PCR product and linear pAK100 phagemid vector. Lanes 1 & 5, Hyperladder I DNA marker (Bioline); Lanes 2 & 3, gel-purified linearised pAK100 vector. Lane 4 shows purified *Sfi* 1 digested SOE-PCR product.

The prepared phage-scFvs from hybridoma cell line were then subjected to two rounds of panning. The initial number of transformants was 6 x 10^4 cfu. The stock phages were panned (section 2.5.5.4) using three different conjugates of cephalexin (Thyroglobulin (T1), Bovine Serum Albumin (B1) and cephalexin-biotin-strepavidin-magnetic beads (MagCeph)). Panned and eluted phages were screened using ovalbumin-cephalexin conjugate by phage ELISA. This was to rule out potential selection of carrier protein (i.e. Thyro or BSA)-specific phage-scFv. Individual clones were randomly picked from spread plates and used to inoculate sterile 96-well plates containing media and screened individually (section 2.5.5.6). The transformed bacterial stocks from each round of panning were plated on 2xTY agar supplemented with chloramphenicol (resistance conferred by the presence of pAK100-scFv within the cell) and tetracycline (intrinsic resistance of the *E.coli* strain used) and used to determine the phage titre (section 2.5.5.3). Positive cephalexin binding phage-scFv clones were identified by ELISA, using ovalbumin-cephalexin and ovalbumin-coated plates. Results are shown in figures 5.15 and 5.16, for thyroglobulin-cephalexin panned and BSA-cephalexin panned phages, respectively.

(A)



(B)

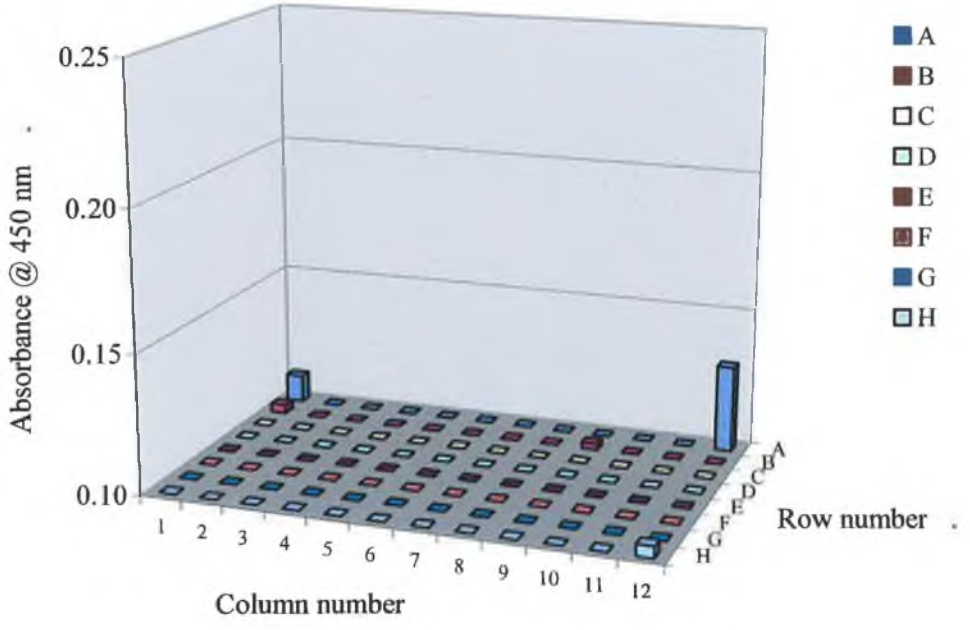


Figure 5.15. This figure represents a total 93 clones from round two panning. *(A)* The clones were panned using thyroglobulin-cephalexin and screened against ova-ceph (100µg/ml). *(B)* The same clones were also screened against unconjugated ovalbumin (100µg/ml). Clones picked for further characterisation are indicated with a star. Wells H10, H11 and H12 were negative control wells for A and B, in that they were not inoculated with phage-producing colonies but were subjected to the same addition of helper phage-containing media as other wells.

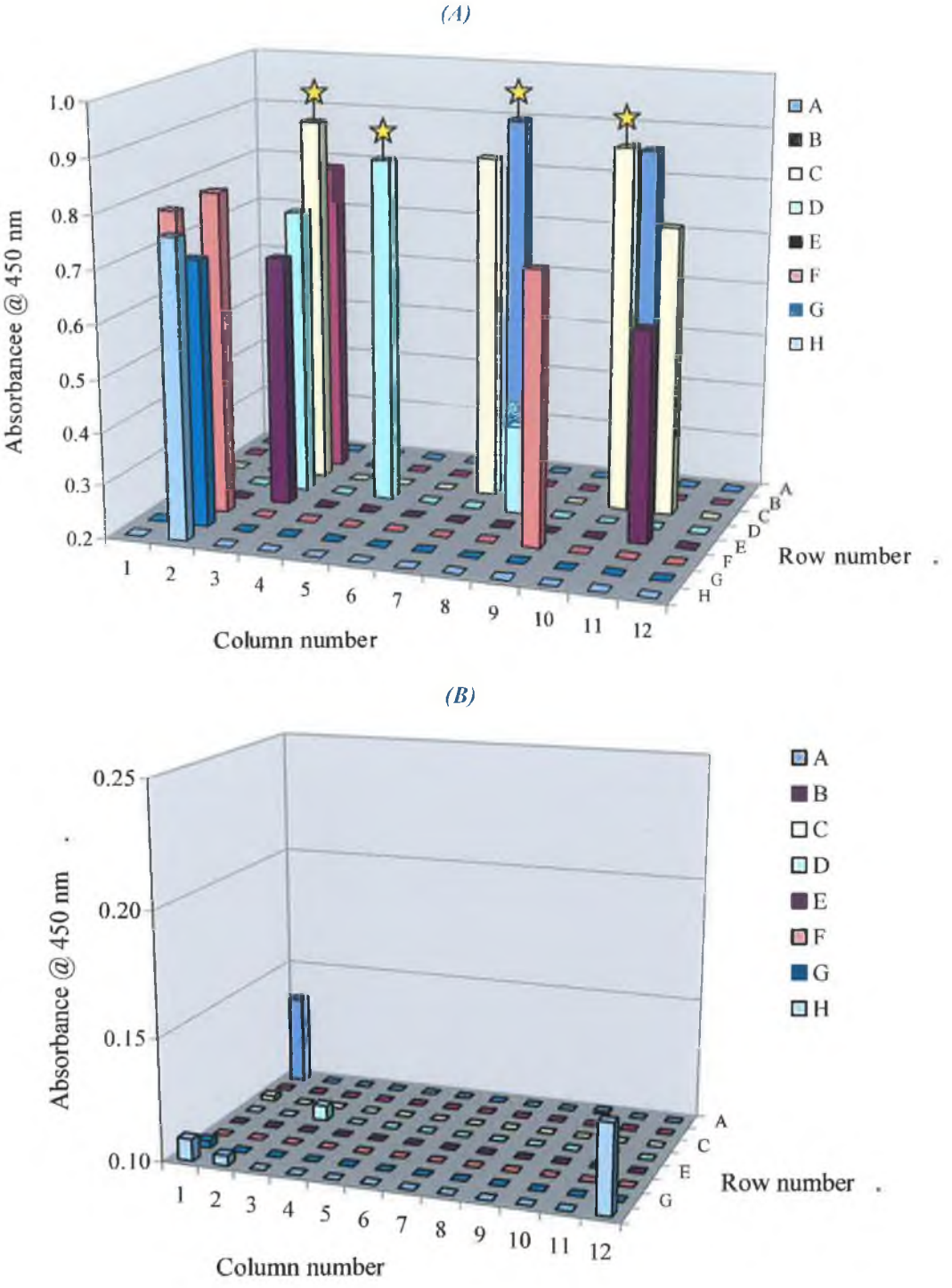


Figure 5.16. This figure represents a total 93 clones from round two panning. *(A)* The clones were panned using BSA-cephalexin and screened against ova-ceph (100µg/ml). *(B)* The same clones were also screened against unconjugated ovalbumin (100µg/ml). Clones picked for further characterisation are indicated with a star. Wells H10, H11 and H12 were negative control wells (A & B), in that they were not inoculated with phage-producing colonies but were subjected to the same addition of helper phage-containing media as other wells.

Four positive clones, picked from each conjugate (i.e. BSA- & Thyro-Ceph) round two pan stocks were further characterised. The clones were designated TP-A7, TP-F8, TP-G7, TP-G9, BP-A7, BP-C3, BP-C10, and BP-D5. Each clone was picked from master culture plates and used to inoculate 5 ml of growth media. Phage-scFvs were rescued as described in section 2.5.5.1. Prepared phage-scFvs were screened using phage ELISA (section 2.2.2.6) to determine the titre for each clone against ovalbumin-cephalexin-coated plates. The results of this experiment are shown in figures 5.17 and 5.18.

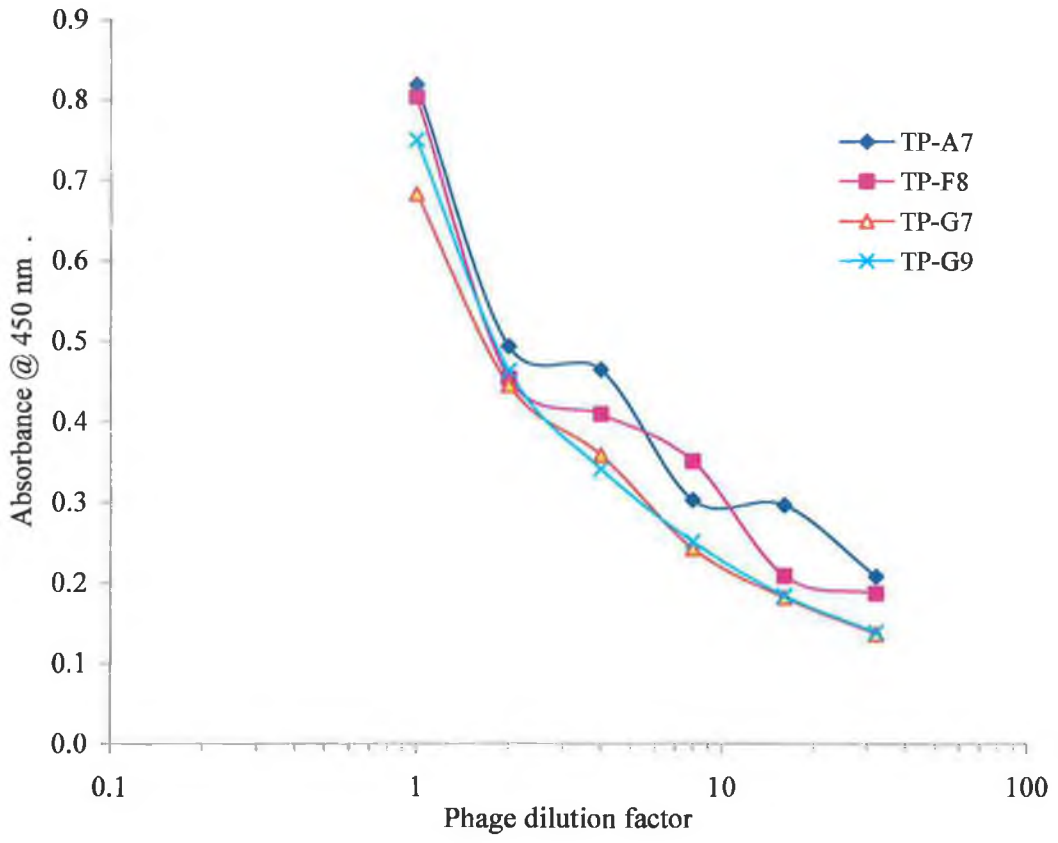


Figure 5.17. This figure represents a plot of the results of phage ELISA using round two thyroglobulin-cephalexin-panned individual clones (TP-A7, TP-F8, TP-G7 and TP-G9) and immunoplates coated with ovalbumin-cephalexin conjugate (100 µg/ml). Bound phage-scFv was detected using a rabbit anti-fd bacteriophage antibody followed by HRP-labelled goat anti-rabbit antibody. For each clone the supernatant was used following phage rescue.

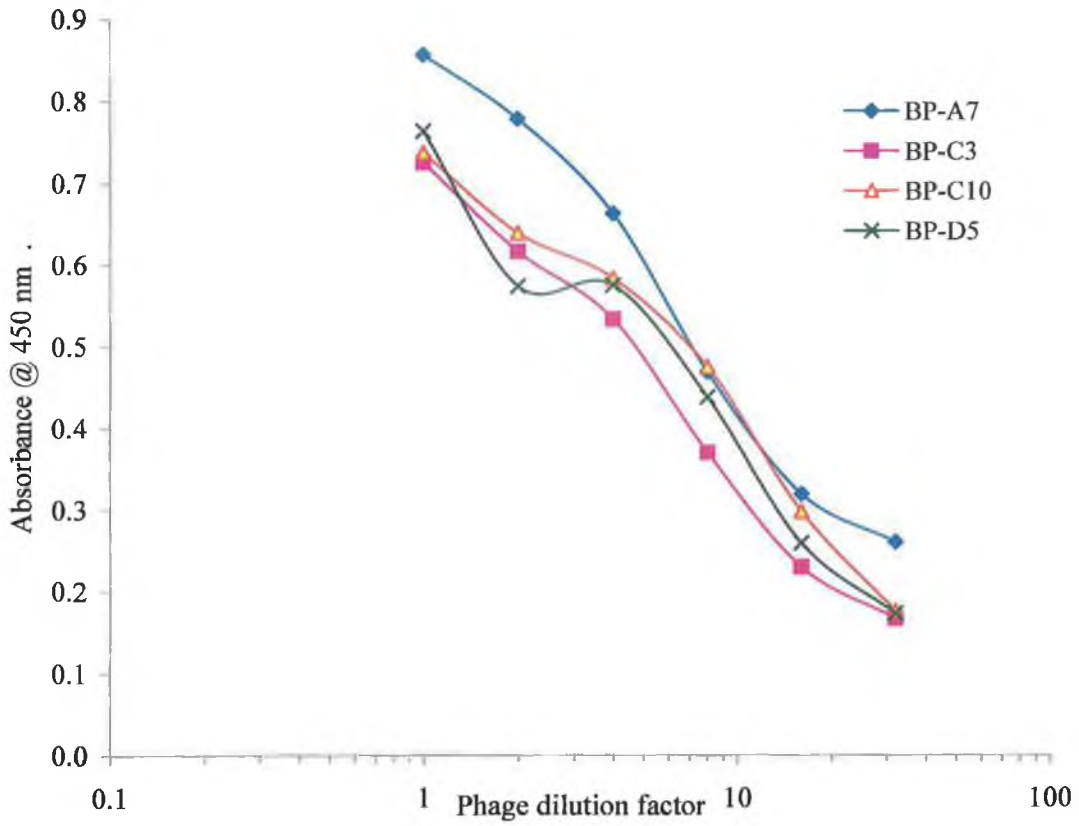


Figure 5.18. This figure represents a plot of the results of a phages ELISA using round two bovine serum albumin (BSA)-cephalexin-panned individual clones (BP-A7, BP-C3, BP-C10 and BP-D5) and immunoplates coated with ovalbumin-cephalexin conjugate (100 µg/ml). Bound phage-scFv was detected using a rabbit anti-fd bacteriophage antibody with subsequent addition of HRP-labelled goat anti-rabbit antibody. For each clone the supernatant was used following phage rescue.

In addition, prepared phage-scFvs from selected clones (BP-A7 & TP-G7) were used in inhibition ELISA with a set of cephalexin standards prepared in PBS-T. The assay was performed to confirm phage-scFv bound to free cephalexin (i.e. non-conjugated). The results of this are shown in figure 5.19.

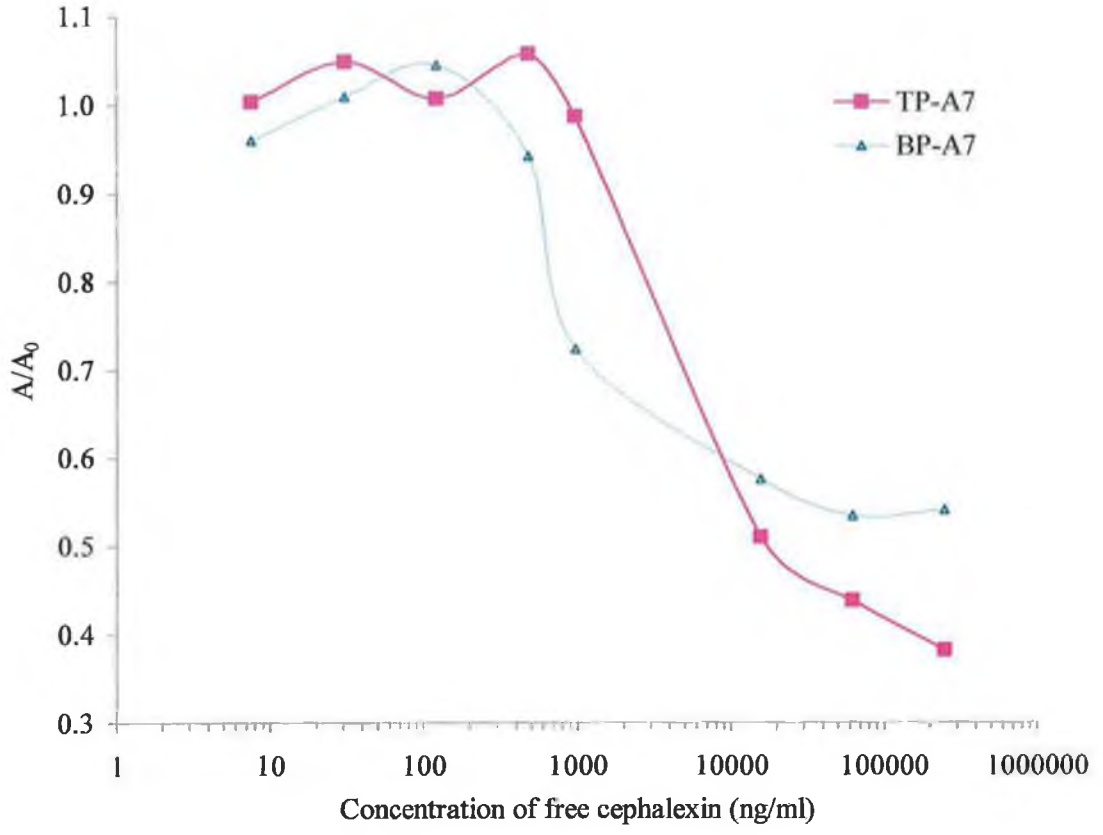


Figure 5.18. This figure represents a plot of the results of an inhibition ELISA using BP-A7 and TP-A7 phage-scFv clones. The ELISA was carried using Thyro-Ceph-coated 96-well plate (1μg/ml) and a 1:2 dilution of phage-scFvs pre-incubated with the appropriate free cephalexin standard. Bound phage-scFvs were detected with subsequent addition of rabbit anti-fd bacteriophage antibody followed by HRP-labelled goat anti-rabbit antibody. For each clone the supernatant was used following phage rescue.

5.2.3.2 *The production and purification of soluble scFv antibody fragment from anti-cephalexin monoclonal antibody-secreting hybridoma cell line using the Krebber system.*

The phage-scFv clones TP-A7 and BP-A7, described in the previous section (5.2.3.1), were chosen for the production of soluble scFv, by sub-cloning into the pAk400 vector for enhanced soluble expression. These clones showed the best inhibition ELISA results and were expressed as soluble scFv. The pAk400 containing *tet* resistance cassette and pAK100 vectors containing scFv gene constructs were purified from overnight cultures of the relevant clone (section 2.5.2.3) and digested using *Sfi* 1 restriction enzyme. The digested products were run on an agarose gel electrophoresis apparatus. The results of this are shown in figure 5.19. The linear pAK400 vector (4,000 bp) and scFv gene (800 bp) were purified from the gel (section 2.5.2.2) and ligated using T4 DNA ligase (section 2.5.4.7). Linearised pAK400 vector (as a control) was ligated without addition of the scFv gene and both experimental and control ligation mixes were precipitated using sodium acetate (3M) and 100% (v/v) ethanol (supplemented with pellet paint to aid in identification), as described in section 2.5.2.4. The ligation products for TP-A7 and BP-A7 clones and controls were used to transform chemically competent *E. coli* (JM83) cells, as described in section (2.5.4.9).

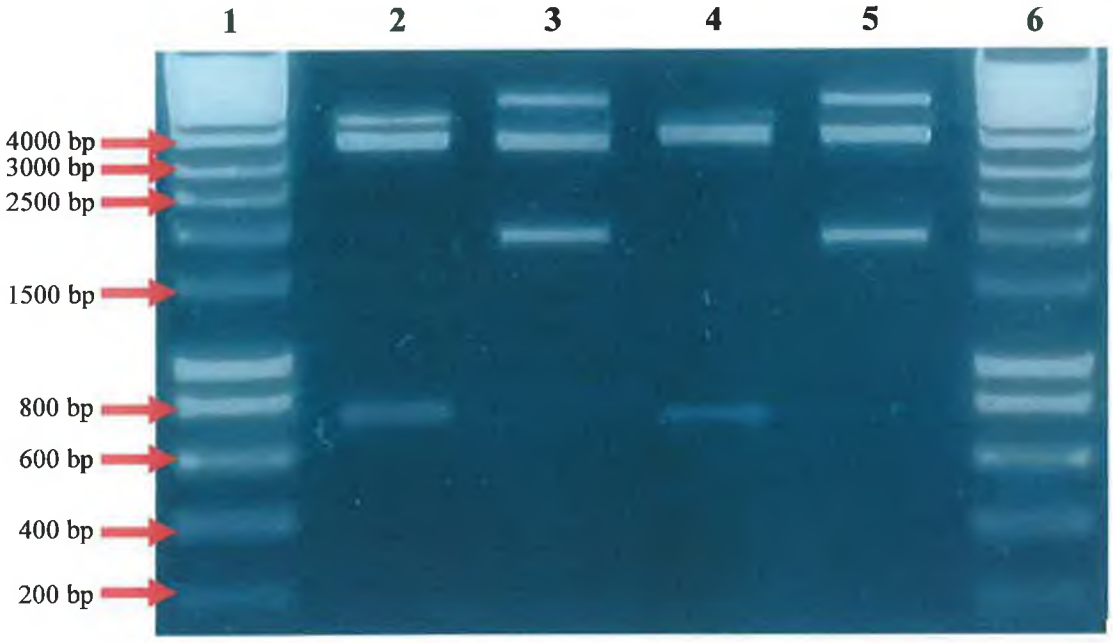


Figure 5.19. Picture of 1.5% (w/v) agarose gel electrophoresis using prepped pAK100 vector (TP-A7 and BP-A7 clones); and prepped pAk400 *Sfi* 1 digest products. Lanes 1 & 6, Hyperladder I DNA marker (Bioline); Lane 2, TP-A7 *Sfi* 1 digest product, showing scFv gene (800 bp) and linearised pAK100 (4,000 bp) bands; Lanes 3 & 5, show pAK400 *Sfi* 1 digest product, containing linear vector (4,000 bp) and *tet* resistance cassette (2,000 bp).

Individual colonies were picked from the pAK400 transformants for each original clone and used to inoculate cultures. Cultures were grown until the exponential phase was reached and subsequent soluble expression was induced using 1 mM IPTG (section 2.5.5.8). Cell lysates were prepared 4 hours and 20 hours post-IPTG induction and screened using an ELISA, as described in section 2.5.5.9. The results (Figure 5.20) of this experiment identified a positive scFv-expressing clone (designated wild type (CTh5)) from the original TP-A7 transformation and suggest longer post-IPTG induced expression incubation yields a larger amount of functional scFv antibody. The soluble scFv was detected using a combination of rabbit anti-poly histidine followed by HRP-labelled goat anti-rabbit antibody.

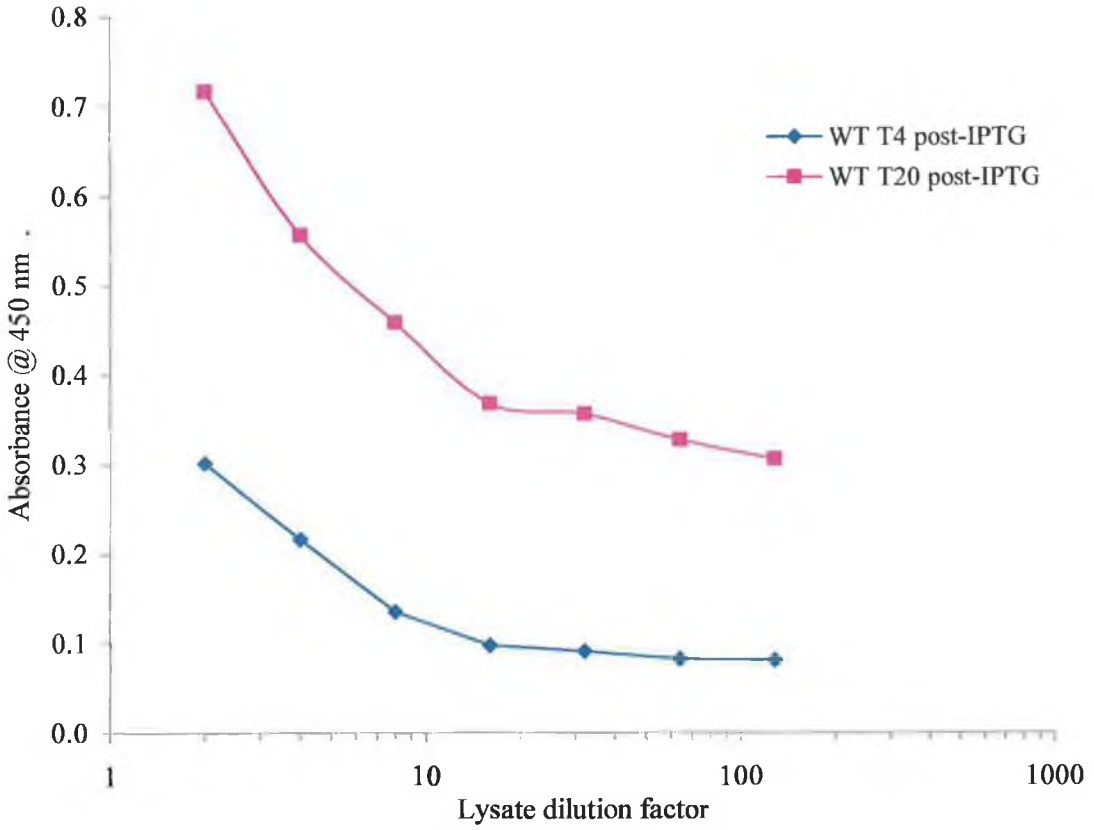


Figure 5.20. This figure represents a plot of the results of an ELISA using wild type clone cell lysates at 4 hours (WT T4 post-IPTG) and 20 hours (WT T20 post-IPTG) post-IPTG induction. Cell lysates were screened against thyroglobulin-cephalexin coated (1.3 µg/ml) wells, and soluble scFv was detected using rabbit anti-poly Histidine tag antibody followed by HRP-labelled goat anti-rabbit antibody.

Cell lysates from the WT-scFv clone (before soluble expression was induced using IPTG, 0 hours (T0), 4 hours (T4) post-IPTG and 20 hours (T20) post-IPTG induction) were analysed by denaturing SDS-PAGE analysis, as described in section 2.3.2. The results confirmed the appearance of an expected 30 KDa band after the cells had been exposed to IPTG (Figure 5.21). Further confirmation that the band at approximately 30 KDa was a scFv was obtained by carrying out Western blotting analysis, as outlined in section 2.3.3. Following transfer onto nitrocellulose membrane the cell lysates were probed with rabbit anti-FLAG (peptide tag) polyclonal antibody. This was subsequently detected with a HRP-labelled goat anti-rabbit antibody and a TMB substrate system. The results of this experiment showed a distinct band at approximately 30 KDa that corresponded to the expected scFv band (Figure 5.22).

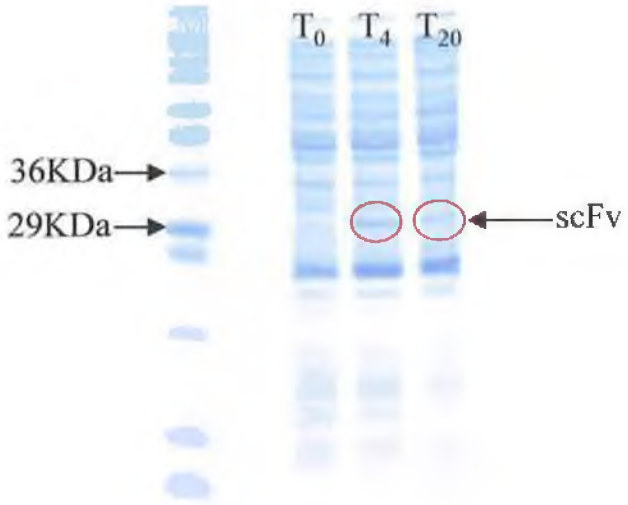


Figure 5.21. SDS-PAGE gel picture from a time-course expression experiment using the wild type pAK400 (WT CTh5) clone expression in Jm83 *E.coli*. The M lane contains Sigma Wide Range protein markers with the bands at 36 KDa and 29 KDa highlighted. Lane T_0 represents the WT cell lysate (1:5 dilution in PBS) prior to addition of 1mM IPTG and expression induction. T_4 lane is 4 hours post-induction with the addition of 1mM IPTG to culture media with the band representing the scFv encircled in red. The T_{20} lane represents the cell lysate 20 hours post-induction with the addition of 1mM IPTG, the bands representing the WT scFv are encircled in red.

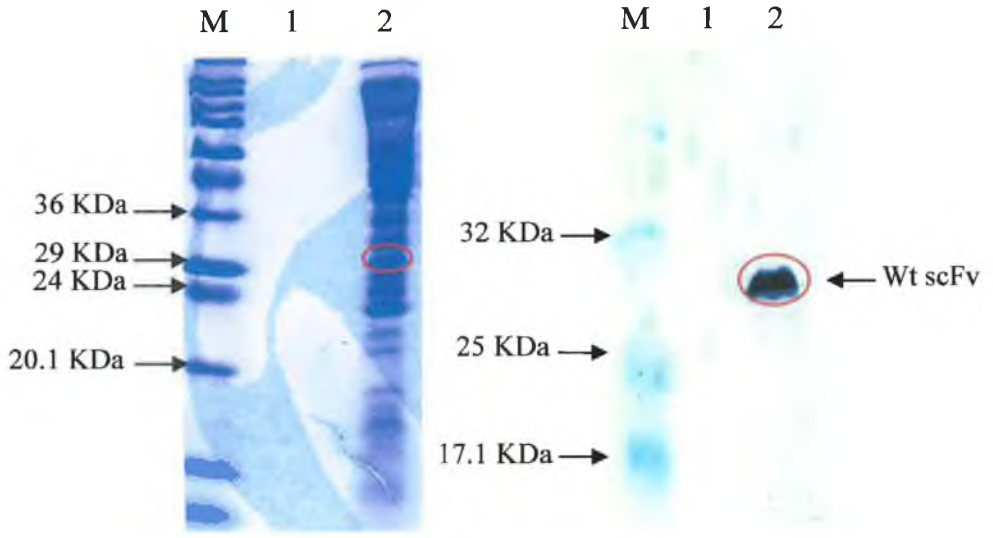


Figure 5.22. SDS-PAGE gel (above left) and Western blot (above right) analysis pictures of WT CTh5 scFv pAK 400 (JM83) cell expression (T20) lysates. The scFv bands were detected on the Western blot using a rabbit anti-FLAG polyclonal antibody. This was subsequently detected with a HRP-labelled goat anti-rabbit antibody and a TMB substrate system. The M lanes contain Sigma wide range protein markers for the SDS-PAGE gel and Pierce Blue-ranger pre-stained protein molecular weight markers for the Western blot. Lane 1 was empty and lane 2 contained WT clone cell lysates 20 hours post-induction with the addition of 1mM IPTG. The bands representing the WT scFv are encircled in red. The Western blotting analysis confirms that the picked clone was expressing soluble scFv.

Having identified and selected the WT CTh5 clone on the basis of its stable production of active scFv, it was necessary to optimise the purification from crude cell lysates and maximise the yield of pure and active scFv product. This would require a method that facilitated purification under native conditions to obtain optimum yield of correctly folded and active scFv. Immobilised metal affinity chromatography (IMAC) is a technique by which proteins or other molecules may be separated based on their ability to form coordination complexes with immobilised metal ions (Porath *et al.*, 1975; Hermanson *et al.*, 1992). Since the pAK400 expression vector incorporates a hexahistidine tag onto the C-terminal end of the scFv expression product, it provides a convenient method for detection and purification facilitated by complex formation between histidine residues and immobilised metal ion. In

general, histidine residues are infrequent, amounting to approximately only 2 % of the amino acid content of globular proteins. In addition, only half of these residues are normally exposed on the protein surface (Ueda *et al.*, 2003). Histidine residues are also advantageous in that they form relatively strong interactions with immobilised metal ions. The Ni-nitrilotriacetic acid matrix (NTA resin) currently supplied by Qiagen Ltd. (as Ni-NTA coupled to Sepharose®) is widely used for IMAC processes with hexahistidine tagged proteins (Figure 5.23). Overall the purification technique involves binding of his-tagged proteins to an immobilised Ni-NTA affinity matrix, washing to remove non-specifically bound material and subsequent elution of the histidine tagged protein. WT CTh5 soluble scFv was purified using IMAC, as described in section 2.5.6.8.

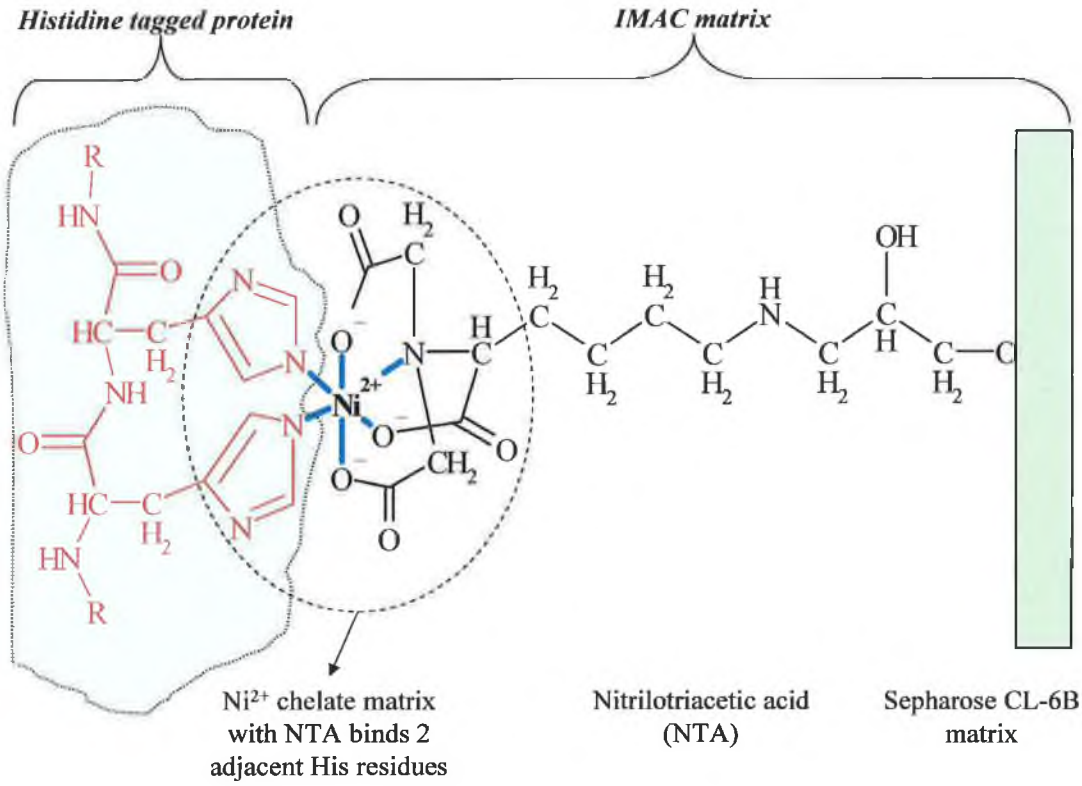


Figure 5.23. Outline of the principle interactions between histidine tag proteins (shown in red above) and nickel- nitrilotriacetic acid (Ni-NTA) immobilised matrices. The Ni^{2+} metal ion has four chelating sites with the NTA resin and two available chelating sites for two adjacent histidine residues from a 6x histidine tagged protein (chelating sites shown in blue). The Ni-NTA resin is coupled to sepharose CL-6B, thus comprising the Ni-NTA (IMAC) purification reagent as supplied by Qiagen Ltd., widely used for the purification of hexahistidine tagged proteins.

The WT CTh5 scFv was expressed, as outlined in section 2.5.5.8, and purified using IMAC with Ni-NTA (Qiagen Ltd.) reagent (described in section 2.5.6.8). SDS-PAGE and Western blot analysis were used to monitor the degree of purification and the functional scFv activity was assayed using ELISA. The SDS-PAGE analysis (Figure 5.24) showed that the IMAC purification yielded a clean and distinct band at 30 KDa as expected. Subsequent Western blotting analysis (Figure 5.25) and probing with anti-FLAG antibody showed that the expression product was successfully purified from cell lysate, with bands appearing in the original pre-IMAC lysate and the post-IMAC elution. No scFv was detected in the column flow through or wash fractions. The cephalexin activity of the WT CTh5 scFv purification fractions (i.e. flow-through, wash, and elution fractions) was analysed using a screening ELISA, the results of which are shown in figure 5.26.

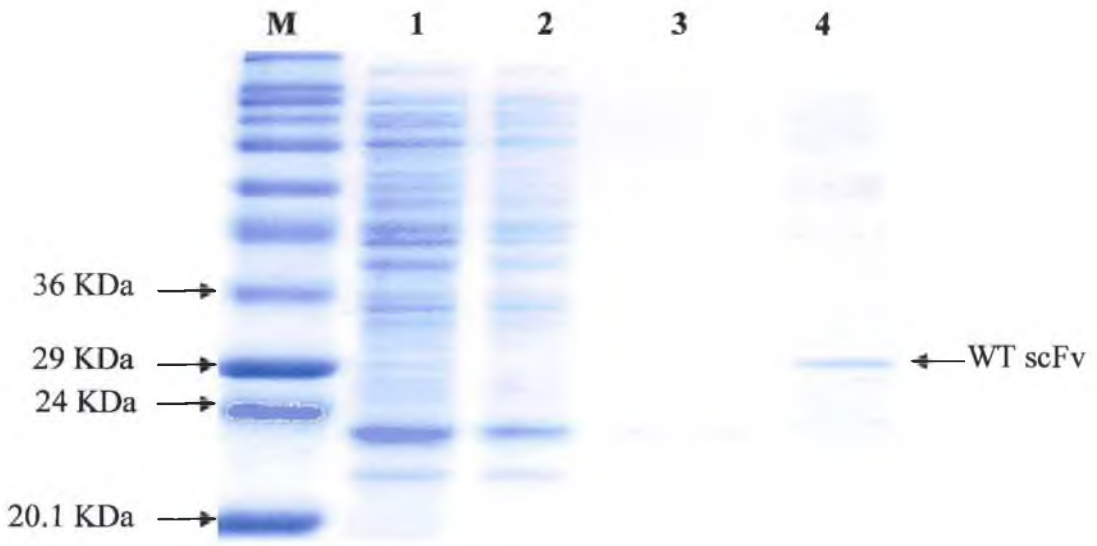


Figure 5.24. SDS-PAGE gel picture of IMAC Ni^{2+}-NTA purified soluble scFv from WT CTh5 clone expression lysates (*E. coli* JM83). The M lane contains Sigma Wide Range protein markers with the bands at 36 KDa and 29 KDa, 24 KDa and 20.1 KDa highlighted. Lane 1 represents the WT CTh5 cell lysate (1:5 dilution in PBS) prior to IMAC purification. Lane 2 represents the WT CTh5 cell lysate (1:5 dilution in PBS) after two passes through IMAC column (Flow through). Lane 3 represents the column wash run off (neat). Lane 4 represents WT CTh5 scFv eluate from IMAC column, showing the scFv band at approximately 30 KDa.

Figure 5.25. Picture of Western blot analysis of WT CTh5 clone expression and subsequent IMAC (Ni^{2+}-NTA) purification fractions. The M lane contains Pierce Blue ranger pre-stained protein markers with bands at 48 KDa, 32 KDa and 26 KDa, and 18 KDa highlighted. Lane 1 represents the WT CTh5 cell lysate (1:5 dilution in PBS) prior to IMAC purification. Lane 2 represents the WT CTh5 cell lysate (1:5 dilution in PBS) after two passes through IMAC column (Flow through). Lane 3 represents the column wash run off (neat). Lane 4 represents WT CTh5 scFv eluate from IMAC column, showing the distinct scFv band at approximately 30 KDa.

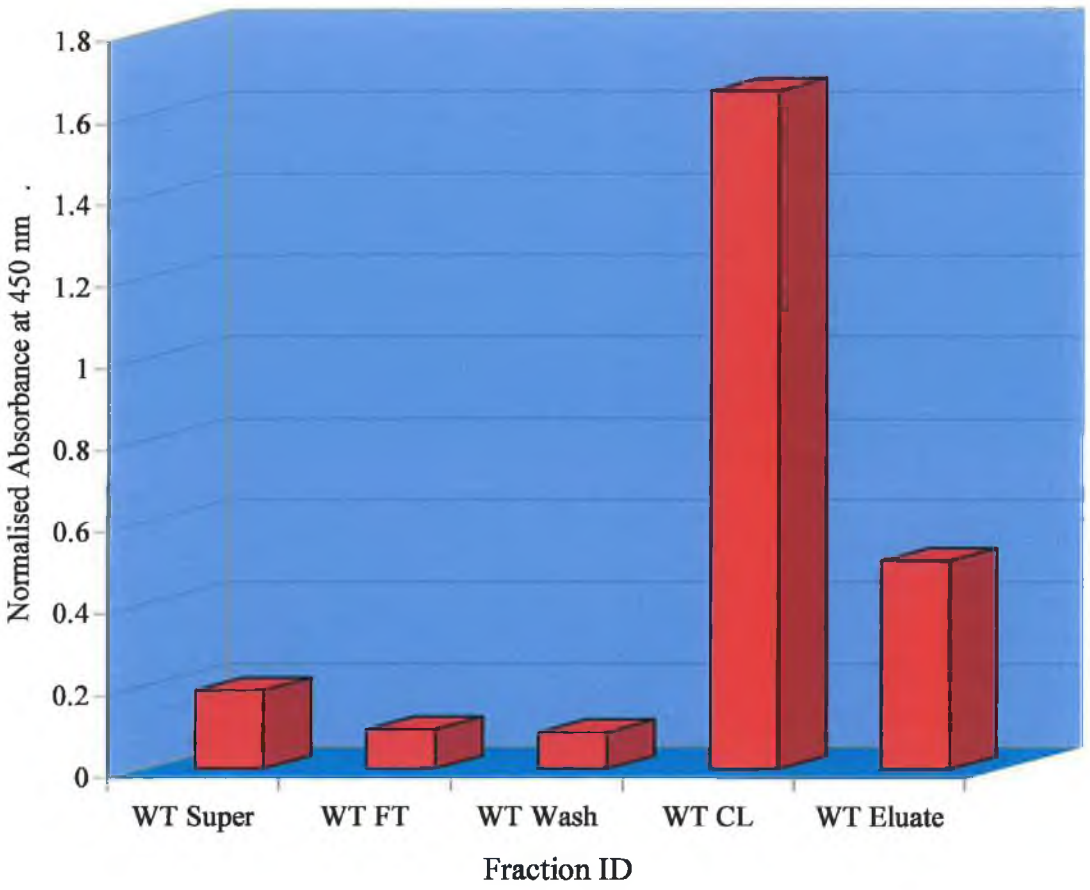


Figure 5.26. Typical result obtained for monitoring binding activity Ni^{2+}-NTA agarose resin immobilised metal affinity chromatography WT CTh5 scFv purification. A 96-well plate coated with 6µg/ml thyroglobulin-cephalexin conjugate was blocked with PBS containing 5% (w/v) milk marvel. 100µl aliquots of wild-type CTh5 supernatant (WT Super) (spent culture media) and each IMAC purification fraction, including column flow through (WT FT), wash (WT Wash), cleared cytoplasmic lysate (WT CL), and dialysed scFv IMAC eluate (WT Eluate) were added in duplicate to the 96-well plate and incubated. The scFv was subsequently detected with HRP-labelled monoclonal anti-poly histidine antibody, followed by addition of Sigma fast OPD substrate system. The normalised absorbance was calculated by taking the absorbance for each sample and subtracting background absorbance obtained from control surface (i.e. wells coated with thyroglobulin (6 µg/ml) protein).

The results of the SDS-PAGE, Western blotting and screening ELISA confirmed that the soluble scFv derived from WT CTh5 clone was successfully purified from crude cell lysates and that it retained binding activity to conjugated (Thyro-Ceph) cephalexin in ELISA. The purified scFv was used in the subsequent development of an ELISA for the detection of soluble cephalexin in 'spiked' whole milk samples.

5.2.3.3 *The development of an inhibition displacement ELISA using scFv antibody fragment from anti-cephalexin monoclonal antibody for the detection of cephalexin in whole milk samples.*

IMAC-purified WT CTh5 scFv antibody was used in the development of an ELISA for the detection of cephalexin in whole milk. Initially, the optimum secondary detection antibody and subsequent scFv dilution for use in the inhibition ELISA were determined. Plates were coated with a (protein-activated) thyroglobulin-cephalexin (1.3 μg/ml), as described in section 2.3.1.1. Serial dilutions of scFv were added and separately detected with both secondary antibodies i.e. anti-Histidine and anti-FLAG antibodies, used at 1/1,000 dilutions. This showed that the anti-histidine antibody gave a greater response in terms of corrected absorbance in comparison to the anti-FLAG antibody. Anti-histidine secondary antibody was used in subsequent ELISA experiments.

An inhibition ELISA was carried out using IMAC-purified WT CTh5 scFv and a range of free cephalexin concentrations. The scFv was diluted (1:100) in whole processed milk with the appropriate cephalexin standard. The linear range of detection for the assay was found to be from 100 to 100,000 ng/ml approximately (Figure 5.27). The limit of detection (LOD) was calculated by taking 3x standard deviations from the background (A_0), and was found to be 143.3 ng/ml. Results showed that the limit of detection of the assay, when carried out in 'spiked' processed milk, was not sufficiently low enough to detect free cephalexin hydrate at the required EUMRL value of 100 ng/ml.

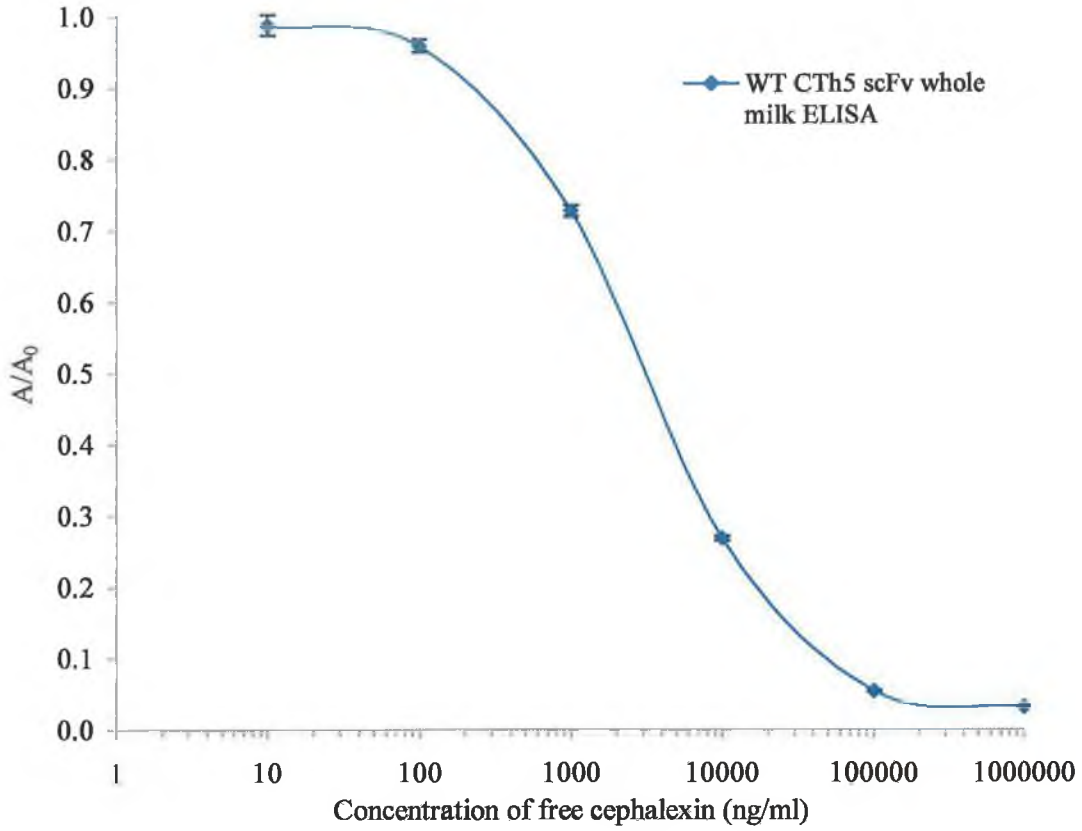


Figure 5.27. This figure represents a plot of the results of an inhibition ELISA in 'spiked' whole milk samples, using IMAC-purified WT CTh5 scFv and a range of free cephalexin concentrations from 1 ng/ml to 1,000,000 ng/ml. The scFv was diluted (1:100) in whole processed milk with the appropriate cephalexin standard. The linear range of detection for the assay was from 100 to 100,000 ng/ml. The limit of detection (LOD) was calculated by taking 3x standard deviations from the background (A_0), and was found to be 143.3 ng/ml.

5.2.3.4 *Bioinformatic analysis of wild type scFv antibody derived from anti-CephM1 antibody-secreting cell line.*

The wild type scFv gene construct was sequenced (Qiagen Genomic Services) using primers that were designed to anneal to sequence regions on the pAK400 vector (pAK_{for}: CGG CAG CCG CTG GAT TGT TAT TAC and pAK_{back}: CAT TTT TCA CTT CAC AGG TCA AGC). A 1.5 ml microcentrifuge tube containing wild type clone stab culture was prepared. The stab culture and primer sequence information were sent to Qiagen for primer synthesis and subsequent sequence analysis. The resulting nucleic acid sequence was analysed using the Chromaslite200 software package, and the Expasy nucleic acid translation tool (http://www.expasy.org/tools/dna.html). Once the amino acid sequence had been obtained, it was inputted into the IgBlast search engine at the National Institutes of Health, National centre for Biotechnology information website (http://www.ncbi.nlm.nih.gov/igblast/) that finds and aligns homologous antibody amino-acid sequences. Figure 5.28 shows the resulting wild type amino acid sequence, the variable light and heavy chain CDR regions, glycine / serine peptide linker peptide and the hexahistidine tag are all highlighted as shown. Using this site the variable heavy chain complemetarity determining regions (CDR's) were identified.

Wild type scFv

```
                *          20          *          40          *
                                                <----------VLCDR1-------->
GVGMADYKDIVLTQSPLSLPVSLGDQASISCRSSRNIVHSNGNTYLEWYLQKPGQSP

  60            *          80          *          100         *
        <VLCDR2>                                        <-VLCDR3->
KLLMYKVSNRLSGVPDRFSGSGSGTDFTLKISRVEAEDLGVYYCFQGSHVPWTFGGG

      120         *          140         *          160         *
                                                                <V
TKLEIKRGGGGSGGGGSGGGGSGGGGSEVMLVESGGGLVKPGGSLKLSCAASGFTFS

              180         *          200         *          220
HCDR1>                          <---------VHCDR2-------->
SYGMSWVRQTPEKTLEWVASISGGGNTYYPDSVKGRFTISRDNARNILYLQMSSLRS

  *             240         *          260         *
                <-----VHCDR3----->
EDTAMYYCARGLGYGKAFMDYWGQGTSVTVSSASGADHHHHHH-
```

Figure 5.28. Wild type amino acid sequence obtained using the Expasy DNA translation tool. The text highlighted in blue represents the particular number of the respective amino acid residue, where * represents ten residues. The complementarity determining regions (CDR) are highlighted in grey for both variable heavy and light chains. The 15 amino acid glycine serine repeat peptide linker and hexahistidine tag are highlighted in yellow. The variable heavy and light chain CDR regions were fully identified using the Kabat rules for identifying antibody CDR regions from linear amino acid sequences, which are summarised at the following website (http://www.rubic.rdg.ac.uk/abeng/cdrs.html).

The wild type scFv was also modelled in three dimensions using SWISS model and SWISS PDB viewer software (http://www.ca.expasy.org//SWISS-MODEL.html). Figures 5.29 shows a picture of the wild type scFv ribbon model with variable heavy and light chain domains coloured red and blue, respectively, for convenience.

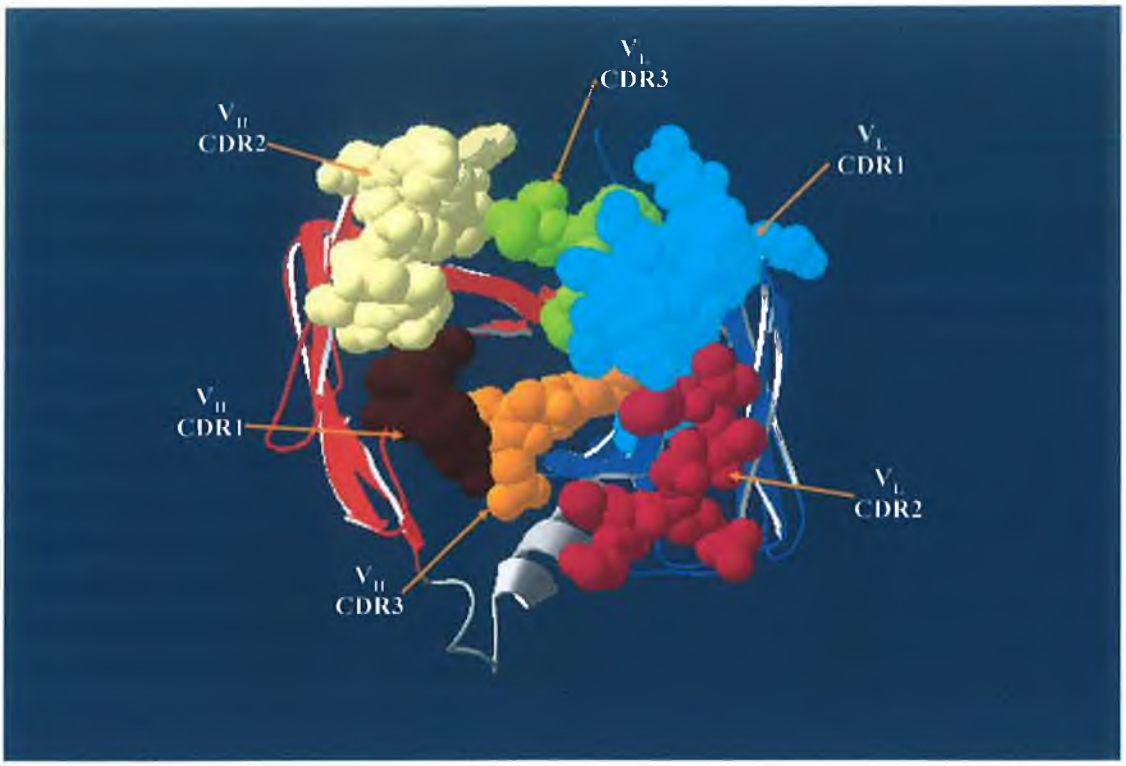


Figure 5.29. Picture of wild type scFv model (top view, with respect to antigen binding site) obtained using the SWISS-Model bioinformatics website and PDB viewer software. The heavy and light chain framework regions are shown as ribbon structures, and are highlighted in red and blue, respectively. The CDR regions are shown using a space fill model with the corresponding heavy and light chain CDRs highlighted in different colours for ease of distinction.

5.2.4 *The production of mutant scFv phage-display libraries using wild type scFv gene.*

The wild type scFv-expressing clone previously found to be cephalexin-specific and displaced by soluble cephalexin was used as a template in the production of mutant phage-display libraries. Mutant libraries were produced using random mutagenesis and error-prone PCR techniques as described in section 2.6. The wild type clone was grown overnight at 37°C, the cells were then pelleted by centrifugation and the vector DNA was purified (section 2.5.2.3) and used as template in multiple copy PCR reactions (section 2.5.4.4). The PCR products were run on a 1% (w/v) agarose electrophoresis gel (Figure 5.30).

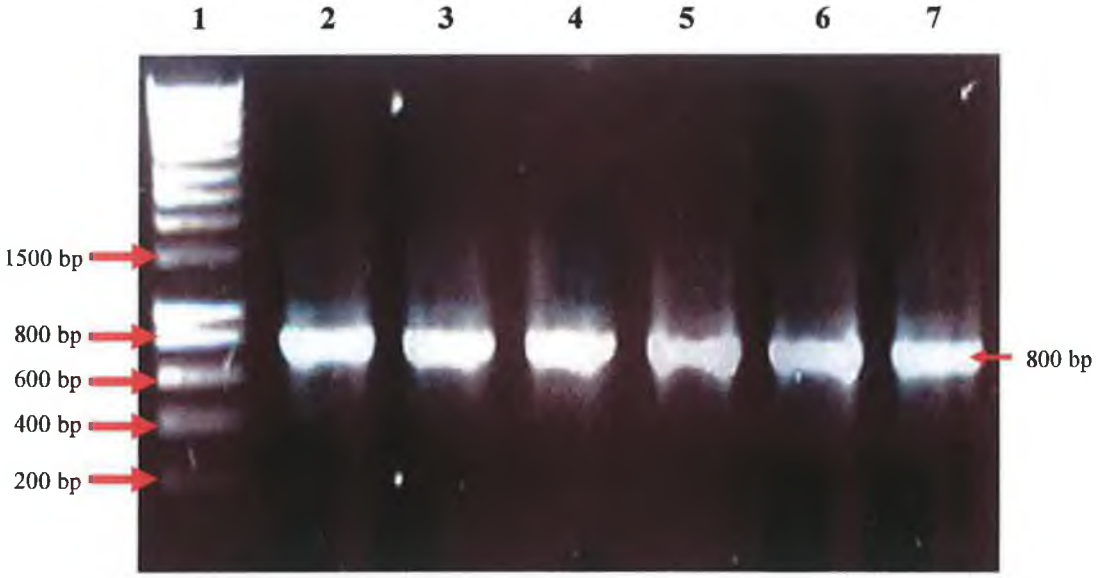


Figure 5.30. Picture of 1% (w/v) agarose gel loaded with multiple SOE PCR reaction products. The SOE-PCR reactions were carried out using mini-prepped pAK100 vector containing the wild type gene as template. Lane 1, Hyperladder I (Bioline) DNA marker; Lanes 2-7, multiple copies of the wild type PCR reaction.

The PCR products were pooled and concentrated using ethanol precipitation (section 2.5.2.4) and digested using DNase 1 endonuclease enzyme, as described in section 2.6.1. For the digest the optimised concentrations of the enzyme and PCR product were found to be 1 U/rxn and 4μg/rxn, respectively. The reaction time was also carried out over defined time intervals before addition of enzyme stopping buffer (EDTA). The optimised time was found to be 5 min. The products of the digest were analysed by agarose gel electrophoresis, as shown in figure 5.31.

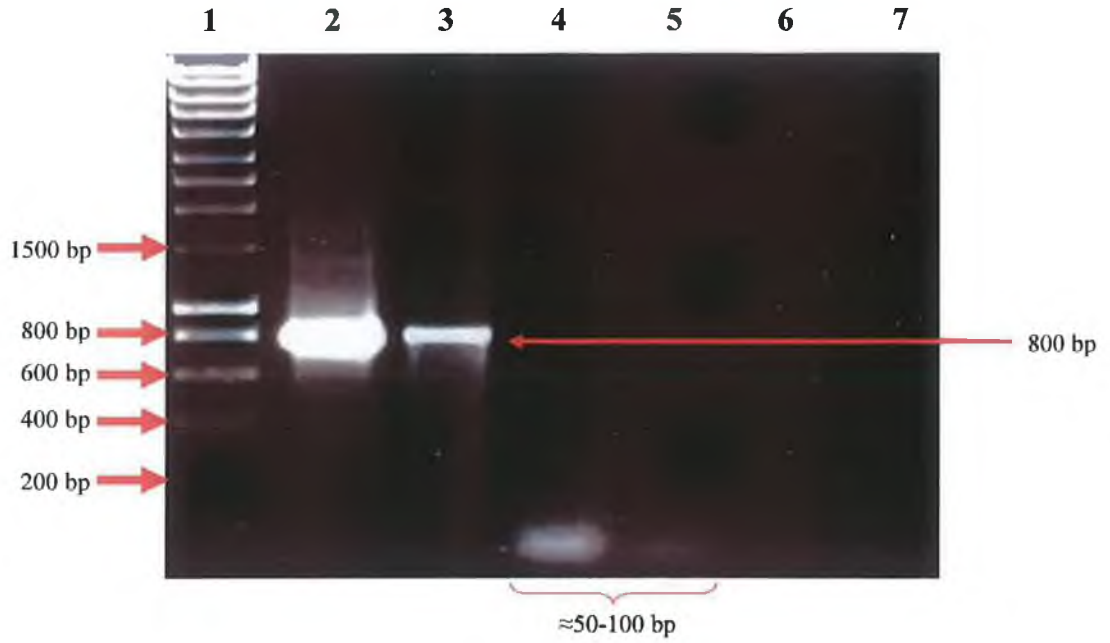


Figure 5.31. Picture of 1% (w/v) agarose gel loaded with undigested and DNase 1 digested wild type scFv gene (SOE PCR product). Samples were run on gel in order to assess the optimum digestion time. Lane 1, Hyperladder I (Bioline) DNA marker; Lane 2, undigested SOE PCR product stock; Lane 3, 0 min incubation at 37°C with DNase 1; Lane 4, Digest product after 5 min incubation; Lane 5, Digest product after 10 min incubation; Lane 6, Digest product after 15 min incubation with DNase 1.

All reactions were stopped after the allotted time using stop buffer as provided (section 2.6.1). Figure 5.31 demonstrates that the scFv gene (800 bp) was completely digested into smaller (50-100bp) fragments after 5 min incubation with the DNase 1 endonuclease enzyme. Following optimisation of the DNase 1 digest, multiple reactions were carried out and the reaction products pooled and concentrated using ethanol precipitation. The digested fragments were then used as primers for each other in an initial re-amplification (section 2.6.2). The product of this reaction varied in size from approximately 400-800 bp (Figure 5.32). The band at 800 bp was gel purified and used for error-prone PCR, as described in section 2.6.3.

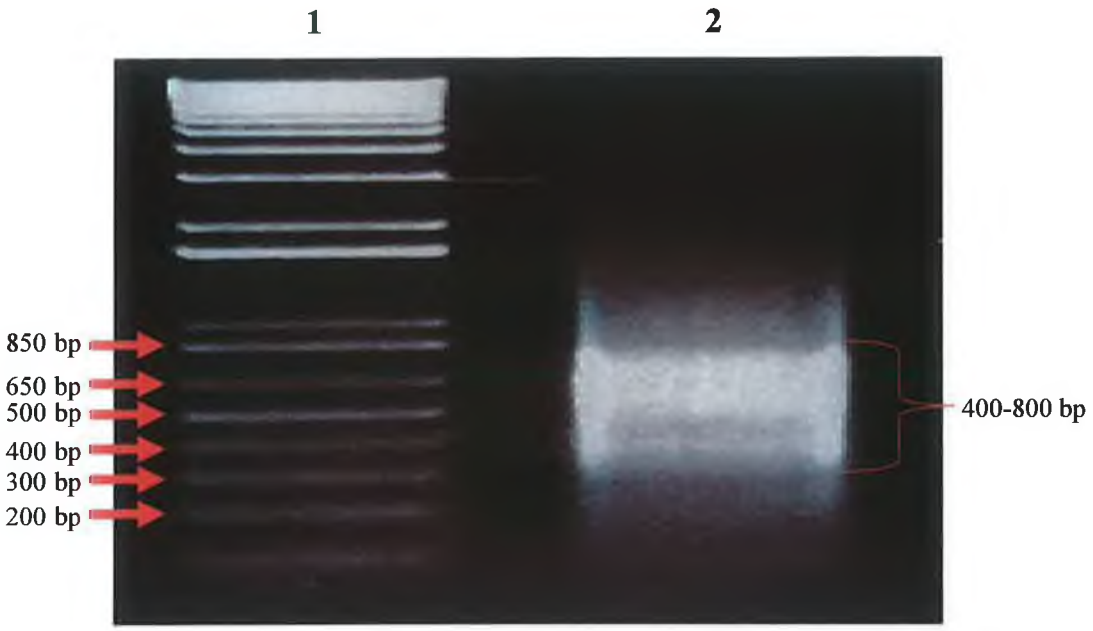


Figure 5.32. Picture of 1% (w/v) agarose gel loaded with re-amplified DNase 1 digestion product. The digested wild type scFv gene fragments were used as a mix of complementary primers and amplified, as described in section 2.6.2. Lane 1 was loaded with 1kb DNA ladder; Lane 2 contained re-amplification PCR product of the DNase 1 digested products.

The error-prone PCR was carried out using two similar PCR reactions. However, one was performed using $MgCl_2$ (i.e. R1*, designated shuffle library) and the other using $MnCl_2$ (i.e. R2*, designated error prone (EP) library), as described in section 2.6.3. To increase the potential introduction of mutations the PCR reactions were carried using 35 repeat cycles while using Red Taq (Sigma) which does not contain any proof reading capacity. Both PCR reactions yielded a discreet band at 800 bp, as shown in figure 5.33.

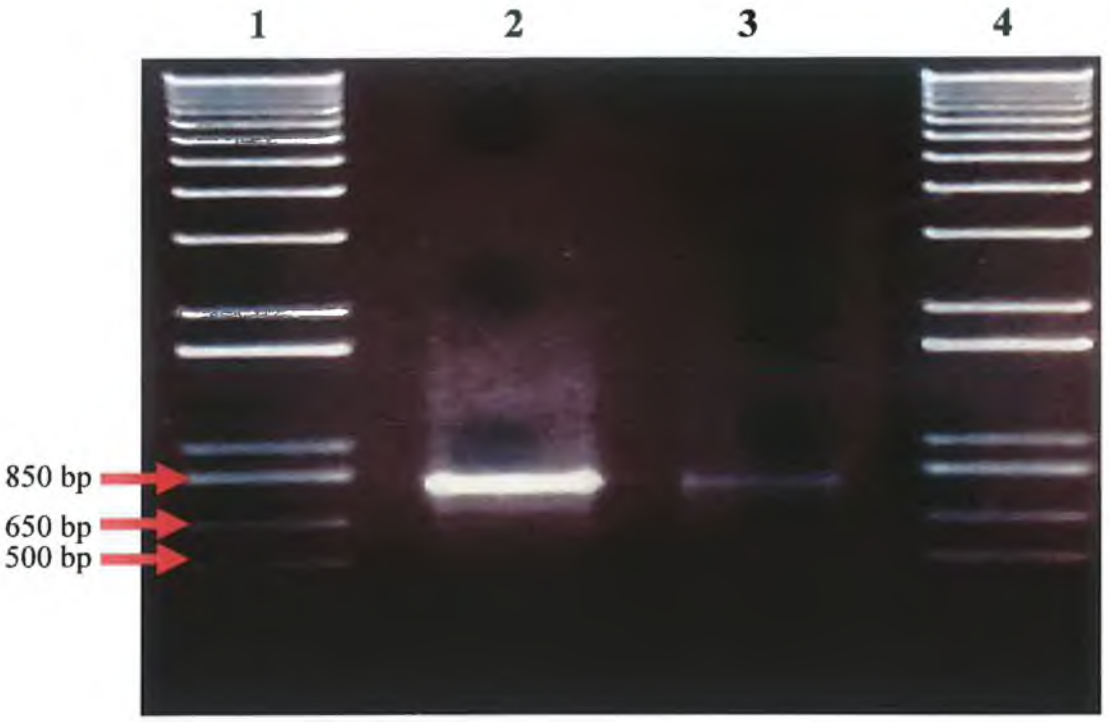


Figure 5.33. Picture of 1% (w/v) agarose gel loaded with shuffle and error-prone PCR products. The PCR reactions (R1* & R2*, section 2.6.3) were carried out using re-amplified wild type gene fragments as template. Lanes 1 & 4, Gibco 1kb plus DNA ladder; Lane 2 was loaded with shuffle PCR product; Lane 3 contains error-prone (EP)-PCR product.

The shuffle and EP-PCR products were purified by ethanol precipitation (section 2.5.2.4) and ligated into pAK100 vector after *Sfi* 1 restriction digest as described in section 2.6.4. The ligation products were used to transform electrocompetent *E. coli* (TOP10F′) as described in section 2.5.2.7. The electro-competent TOP10F′ *E.coli* cells were tested with commercial pUC18 plasmid and yielded a transformation efficiency of 10^9 transformants / μg pUC18 plasmid DNA. The number of transformants obtained for each library (i.e. shuffle (R1*) and error-prone (R2*)) was 1.4×10^7 and 2.3×10^6, respectively.

5.2.4.1 *Bio-panning Shuffle and Error-prone mutant scFv phage-display libraries.*

The R1* and R2* mutant phage-display libraries were panned for anti-cephalexin phage (section 2.6.5). A two phase elution strategy was employed. This entailed elution of conjugate bound phage-scFv using soluble cephalexin to elute binders with a higher affinity for the soluble drug. Secondly the remaining phages were eluted using acid shock, as described in section 2.5.5.4. Both libraries were subjected to three rounds of panning using BSA-cephalexin and thyroglobulin-cephalexin conjugates. The results of a polyclonal phage ELISA for each round eluate fractions is shown in figure 5.34.

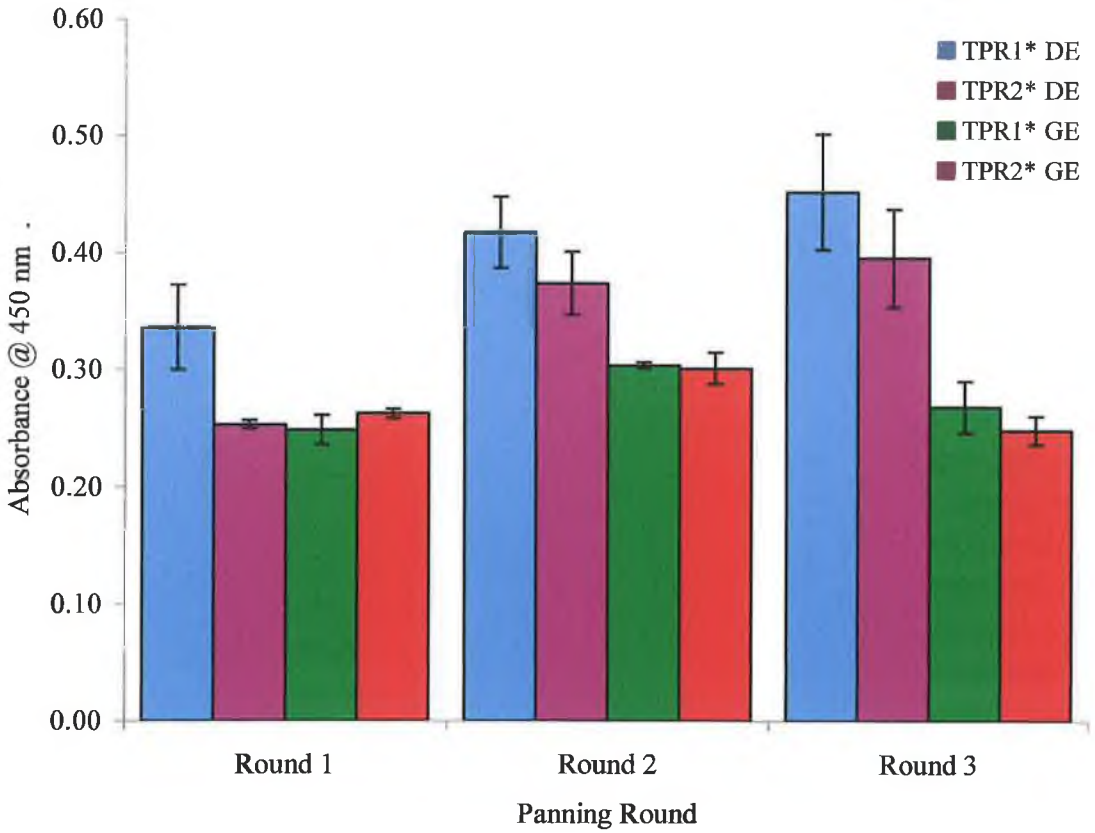


Figure 5.34. The plotted results of a polyclonal phage ELISA using eluted phages from the first, second and third rounds of panning with Thyro-Ceph (TP). The eluted phages from the two libraries (i.e. R1* and R2*) are shown as cephalexin eluate (DE) and glycine eluate (GE) fractions. The R1* library eluted phages for the Thyro-Ceph panned rounds are represented in blue and green above for the drug elution and glycine elution fractions, respectively. The R2* library phages eluate for the panned rounds are shown in purple and red for the drug eluate and glycine eluate fractions, respectively. No significant binding was observed to thyroglobulin and blocking solution control surfaces.

The cephalexin eluate (DE) and glycine eluate (GE) phages from the third round of panning for each library (i.e. R1* and R2*) were used to infect *E. coli* in exponential growth phase. Individual colonies were picked from agar plates containing transformed colonies from each library and DE or GE eluate phages. The colonies were randomly picked from streaked agar plates and used to inoculate sterile 96-well plates containing media and screened individually, as described in section 2.5.5.6. Positive cephalexin binding phage-scFv clones were identified by ELISA. Results of individual phage ELISA for randomly picked individual clones from the R1* library, drug (DE) and glycine (GE) eluted phages, are shown in figure 5.35. Phages were screened against thyroglobulin-cephalexin-coated (1.3 μg/ml) wells. Phage-scFv were detected using rabbit anti-fd-bacteriophage antibody, followed by HRP-labelled goat anti-rabbit antibodies. The corresponding R2* library results are shown in figure 5.36. In all ELISAs the original wild type phage-scFv were screened in well H12 as a positive control. The average absorbance for wild type phage-scFv on each plate ranged from 0.65 to 0.75, following colour development with Sigma fast OPD substrate system. The cut off point was set at an absorbance of 0.7 due to the large number of positive binders and the comparative value for the wild type phage. Thus, the wild type response is not visible on all of the resulting plots. The randomly picked free-drug eluted clones typically yielded a smaller percentage of positive clones when compared to the glycine-eluted clones for the shuffled mutant (R1*) library as clearly seen in figure 5.35.

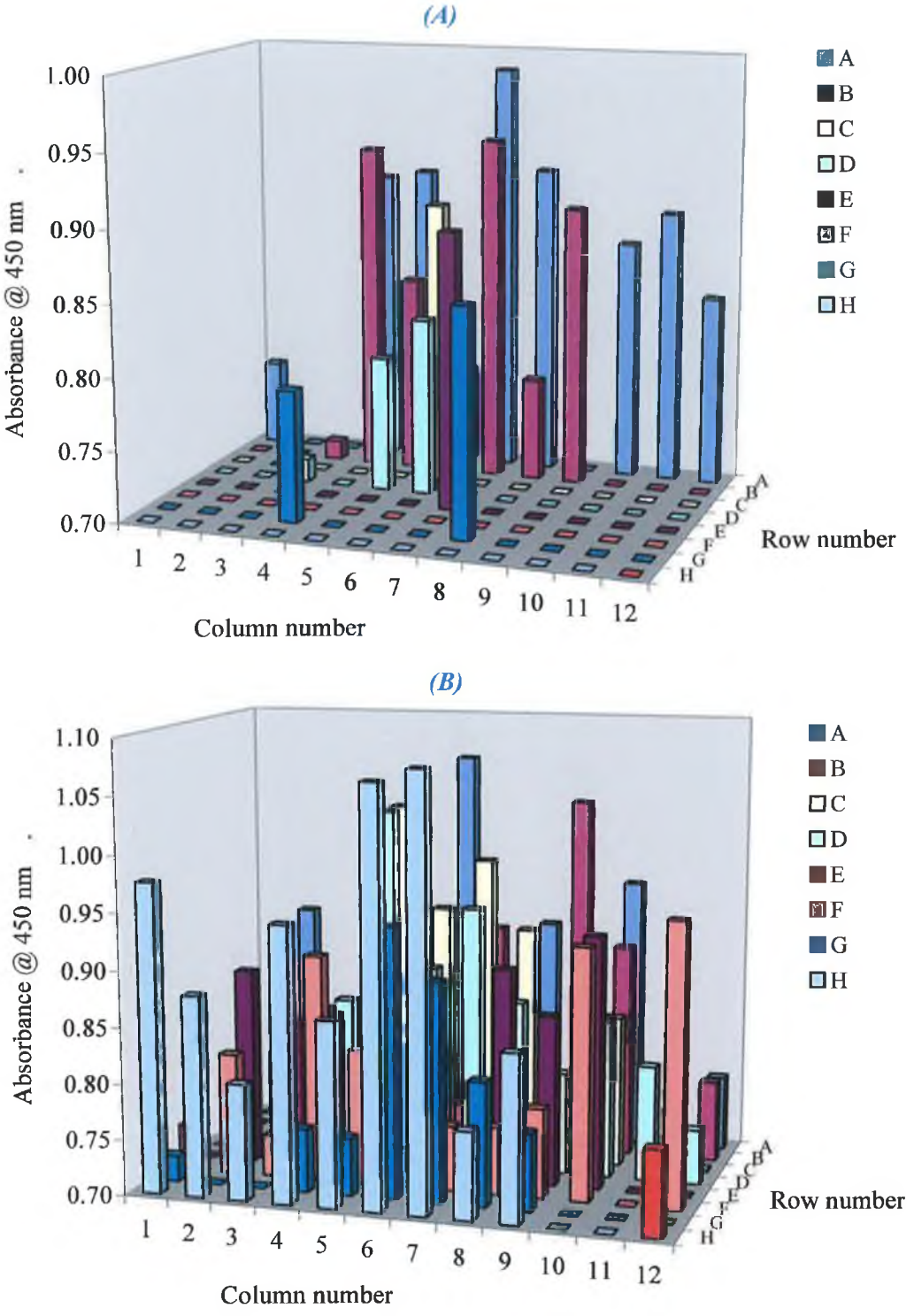


Figure 5.35. This figure represents a total 186 randomly picked clones from round three shuffle (R1*) mutant library, 93 clones were picked from each elution fraction (i.e. free-drug and glycine). *(A)* The 93 clones from the free drug eluted (DE) phage stocks; wells H10 & H11 were negative controls; well H12 (highlighted in red above) contained original wild type phage-scFv. *(B)* The 93 clones (with controls as for A) from the glycine eluted (GE) phage stocks. The cut off was set at an absorbance of 0.7 because of the large number of positive clones.

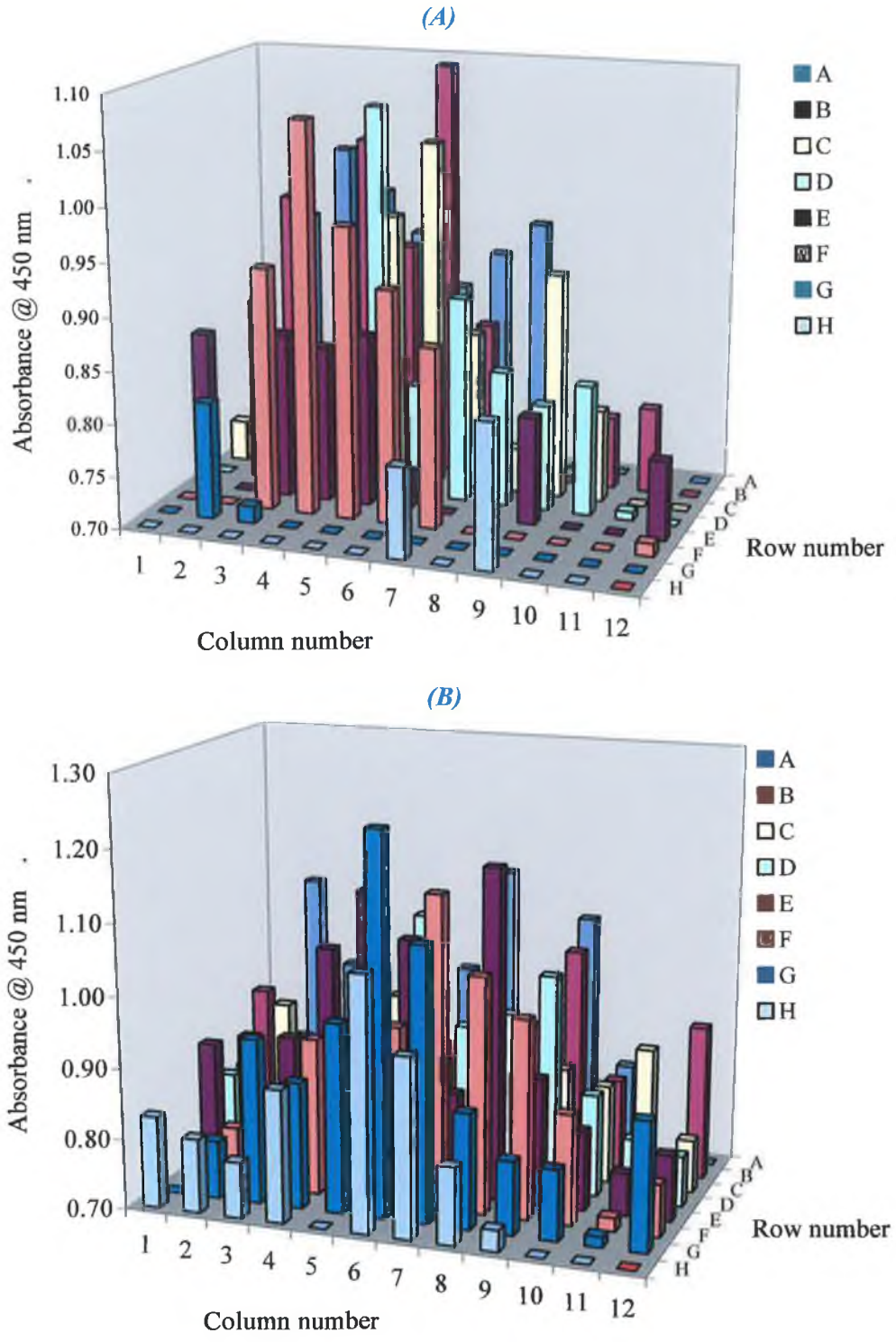


Figure 5.36. This figure represents a total 186 randomly picked clones from round three error-prone (R2*) mutant library, 93 clones were picked from each elution fraction (i.e. free-drug and glycine). *(A)* The 93 clones from the free drug eluted phage stocks; wells H10 & H11 were negative controls; well H12 (highlighted in red above) contained original wild type phage-scFv. *(B)* The 93 clones (with controls as for A) from the glycine eluted phage stocks. The cut off was set at an absorbance of 0.7 because of the large number of positive clones.

A total of 22 positive phage-scFv-producing clones were picked from the free-drug and glycine-eluted fractions of each mutant library (R1* and R2*). The clones were initially ranked on the basis of their respective absorbance in phage ELISA against cephalexin-conjugate-coated plates. Sample clones with low (≤0.5), medium (≤0.8) and high (≤1.0) absorbance were picked from the positive stock pool for further characterisation so as to limit the possibility of omitting a high affinity clone on the basis that it was relatively poorly expressed.

Preliminary inhibition displacement ELISAs with a range of free cephalexin concentrations were carried out using plates coated with Thyro-Ceph. This was done in order to ensure that the cephalexin conjugate positive mutants were binding to the soluble cephalexin prior to further selection, expression and purification. The assays were used to give an indication of the effects (if any) on assay performance using the selected clones. Figures 5.37 and 5.38 show the results of preliminary phage inhibition ELISA carried out for the clones picked from R1* mutant library. As observed from the results, some of the mutant clones picked had a dramatically reduced affinity for the soluble cephalexin, in particular, the glycine-eluted (GE) A1 and C1 clones from the R1* mutant library. The drug-eluted (DE) clones from the shuffle (R1*) library i.e. C5 and G2 (Figure 5.37), showed an increased sensitivity to cephalexin in comparison to the original wild type phage-scFv.

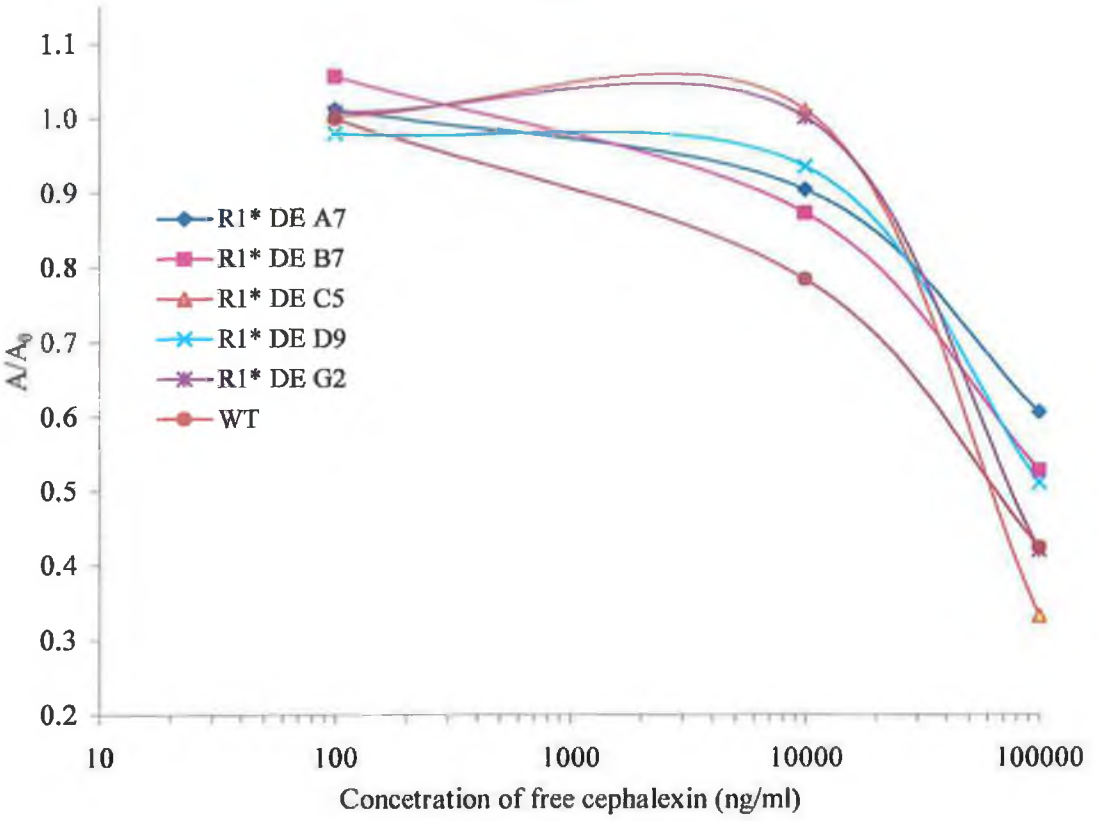


Figure 5.37. Plot of a preliminary inhibition displacement phage ELISA using selected clones from the cephalexin-eluted (DE) fraction of the shuffle (R1*) library. In addition, by way of comparison, the original wild type phage-scFv (WT) was screened. The assay was carried out using Thyro-Ceph-coated immunoplates and prepared phage from each clone (diluted 1:2 in PBS-T). Clones C5 and G2 showed noticeable increased slopes in comparison to the wild type phage-scFv plot.

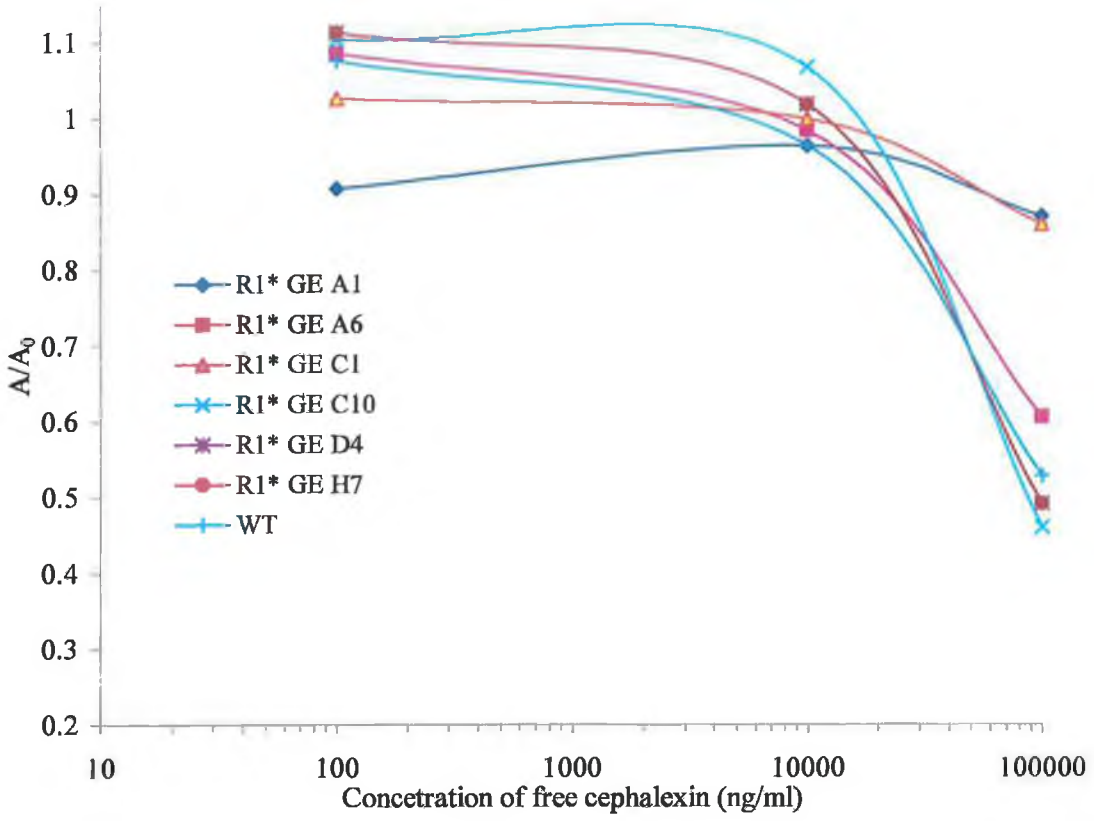


Figure 5.38. Plot of a preliminary inhibition displacement phage ELISA using picked clones from the glycine-eluted (GE) fraction shuffle (R1*) library. In addition, by way of comparison, the original wild type phage-scFv (WT) was screened. The assay was carried out using Thyro-Ceph coated immunoplates and prepared phage from each clone (diluted 1:2 in PBS-T). Clones C1 and A1 showed an apparent lowered affinity to cephalexin in comparison to the wild type phage-scFv.

5.2.4.2 *Soluble expression, purification and characterisation of recombinant mutant anti-cephalexin scFv antibody fragments.*

Six clones were picked for further characterisation on the basis of preliminary inhibition ELISA data. The six clones were picked from the positive drug-eluted stocks, three from each mutant library i.e. shuffle (R1*) and error-prone (R2*). The scFv gene-containing pAK100 vector for each clone was purified. The scFv gene constructs were excised using *Sfi* 1 restriction enzyme prior to sub-cloning into the pAK400 vector for enhanced soluble expression. Crude cell lysates were prepared from the pAK400 transformants for each of the picked clones and screened initially using ELISA. They were compared to wild type cell lysates in terms of the relative amount of expressed functional scFv. Two nascent clones (R1* DE C5 and R2* DE H3) were identified. They were selected for subsequent purification by immobilised metal affinity chromatography (IMAC), as described in section 2.5.6.8, prior to further characterisation and comparison with the original wild type.

The IMAC purification fractions were analysed using SDS-PAGE, Western blotting and ELISA. Figure 5.39 shows a picture obtained for an SDS-PAGE experiment. Crude cell lysates, Ni^{2+}-NTA column flow through, wash and eluted fractions were run for each clone. The gel picture shows strong and distinctive bands at approximately 30 KDa as expected for each mutant clone, with minimal impurities. A Western blotting experiment was carried out following SDS-PAGE analysis in order to confirm the bands were histidine tagged scFvs. After transferring the bands from the SDS gel (section 2.3.3) the nitrocellulose membrane was probed using mouse anti-polyhistidine antibody, followed by HRP-labelled goat anti-mouse antibody. The colour was developed using TMB liquid substrate. Figure 5.40 shows a picture obtained from the Western blot analysis. Bands were observed in the crude lysate and IMAC column eluate fractions for both mutant clones at approximately 30 KDa.

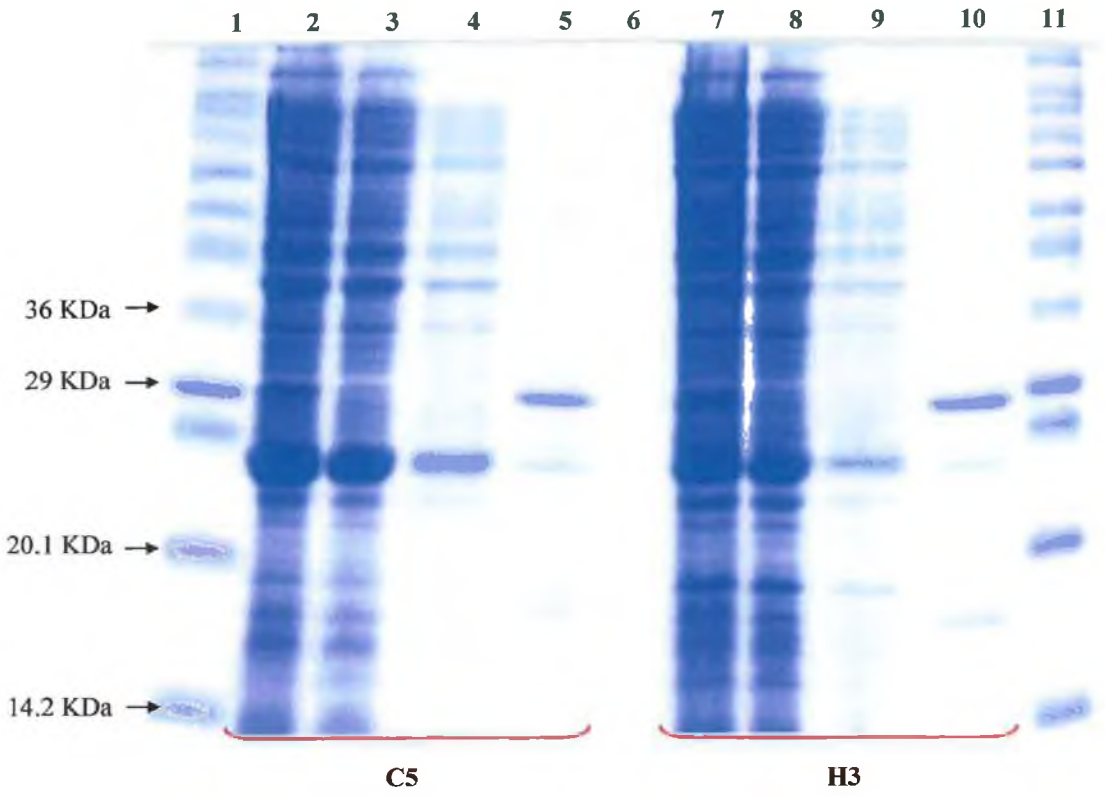


Figure 5.39. An SDS-PAGE gel image of Ni^{2+}-NTA immobilised metal affinity chromatography purification fractions from mutants R1*DE C5 and R2*DE H3 using PBS-T (0.5M NaCl) containing 10mM imidazole, running buffer. Lanes 1 and 11 are Sigma wide-range protein markers; Lane 6 is empty; Lane 2, C5 clone lysate (diluted 1:5); Lane 3, Column flow through (FT) for the C5 (diluted 1:5); Lane 4, column wash (neat); Lane 5, C5 IMAC column eluate (neat); Lane 7, H3 clone lysate (diluted 1:5); Lane 8, H3 column flow through (diluted 1:5); Lane 9, H3 column wash (neat); Lane 10, H3 eluate (neat). Strong distinct bands at approximately 30 kDa represent scFv protein, as confirmed by Western blotting analysis.

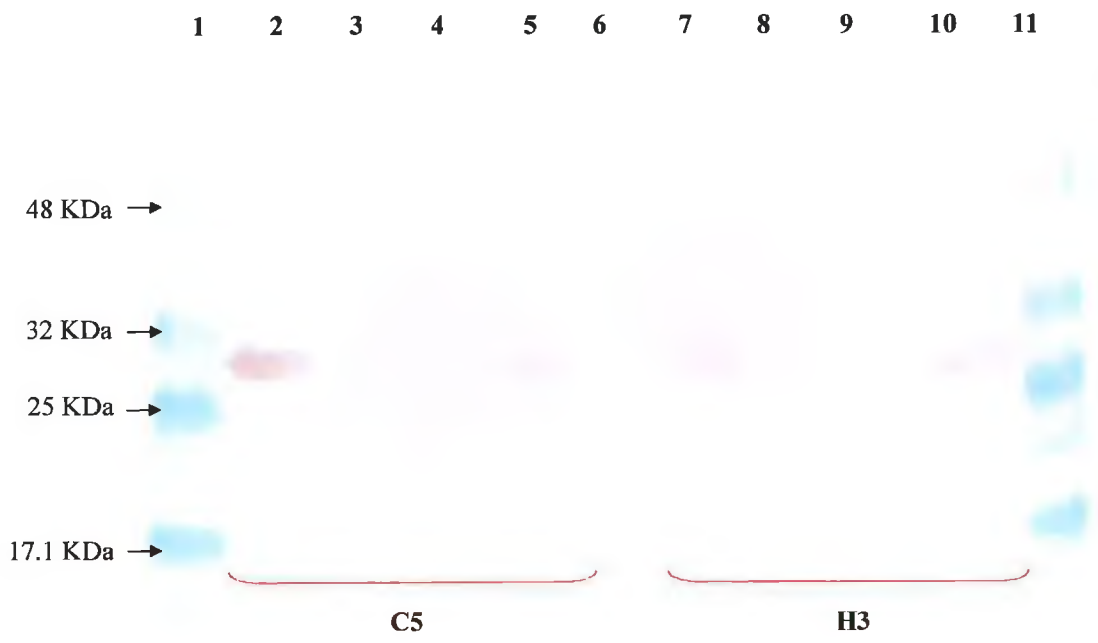


Figure 5.40. A Western blot for mutant clones C5 and H3 IMAC purification fractions. The IMAC purification was carried out, as described previously, where fractions were run on a 15% (w/v) SDS-PAGE gel and transferred to a nitrocellulose membrane using a semi-dry blotter. Lanes 1 and 11 represent Pierce Blue Ranger™ pre-stained protein markers; Lane 2, C5 lysate (diluted 1:5); Lane 3, column flow through (FT) for the C5 (diluted 1:5); Lane 4, column wash (neat); Lane 5, C5 IMAC column eluate using 100 mM sodium acetate, pH 4.5 (neat); Lane 6 is empty; Lane 7, H3 clone lysate (diluted 1:5); Lane 8 H3 column flow through (diluted 1:5); Lane 9, wash for the H3 column (neat); Lane 10, H3 eluate using 100mM sodium acetate, pH 4.5 (neat). The membrane was blocked using PBS-T supplemented with 5% (w/v) semi-skimmed milk powder. Subsequent scFv detection was carried out using 1:2,000 dilution of HRP-labelled monoclonal anti-poly histidine antibody. Colour development was achieved by the addition of TMB membrane substrate system. Both C5 and H3 scFv were detected (distinct bands at approximately 30 kDa) in the cleared lysates and IMAC eluate fractions.

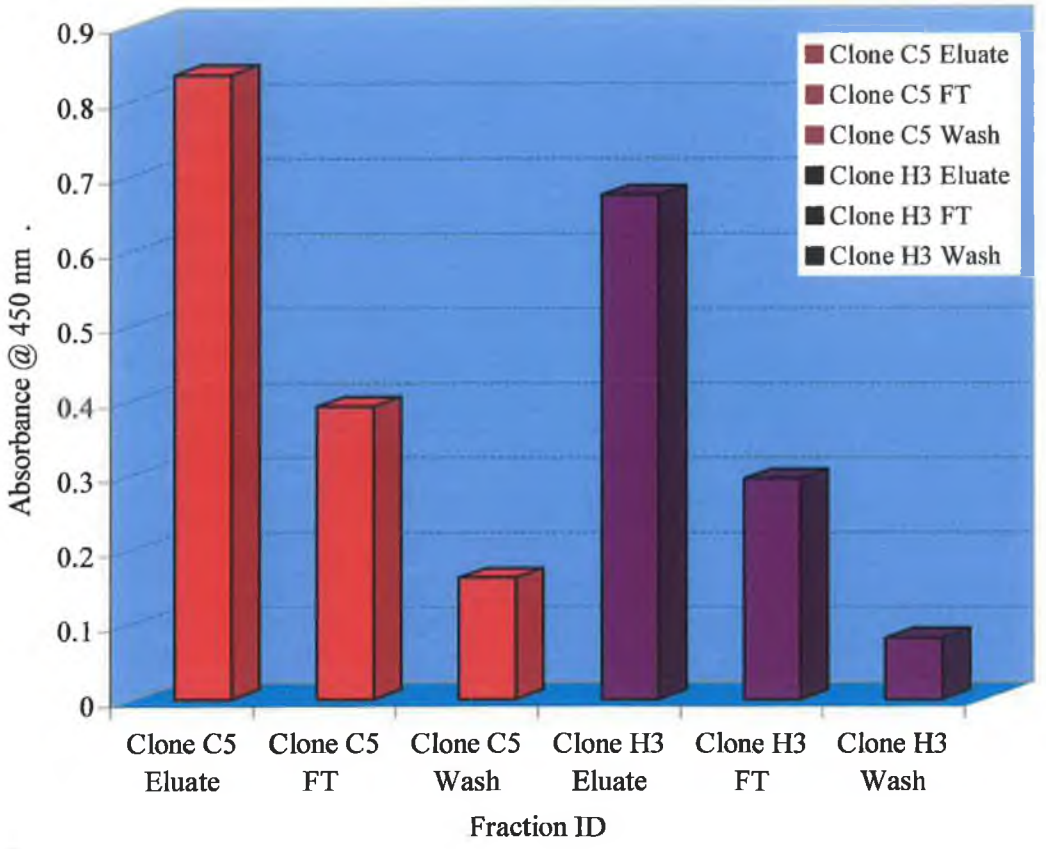


Figure 5.41. Result obtained for ELISA to monitor the binding activity of C5 and H3 scFvs to Thyro-Ceph-coated wells following Ni^{2+}-NTA agarose resin IMAC-purification. A 96-well plate coated with 6µg/ml thyroglobulin-cephalexin conjugate, blocked with PBS containing 5% (w/v) milk marvel. 100µl aliquots of each IMAC purification fraction, i.e. column flow through (FT) (diluted 1:2 with PBS-T), column washes (Wash) (neat), and dialysed scFv IMAC eluates (diluted 1:2 with PBS-T) were added in duplicate to the 96-well plate and incubated. The scFv was subsequently detected with HRP-labelled monoclonal anti-poly histidine antibody, followed by addition of Sigma fast OPD substrate system.

5.2.4.3 *Development of an inhibition ELISA for the detection of cephalexin using IMAC-purified scFv from mutant clones C5 and H3 and assay comparison with original wild type scFv.*

The mutant scFv produced by clones C5 and H3 (section 5.2.4.2) were used in the development of an inhibition ELISA for the detection on soluble cephalexin hydrate. Initially, the IMAC-purified scFv titres for original wild type, C5 (from shuffle mutant library R1*), and H3 (from error-prone mutant library R2*) clones were assessed (section 2.3.1.1) with immunoplates (Nunc) coated with thyroglobulin-cephalexin conjugate (Figure 5.42).

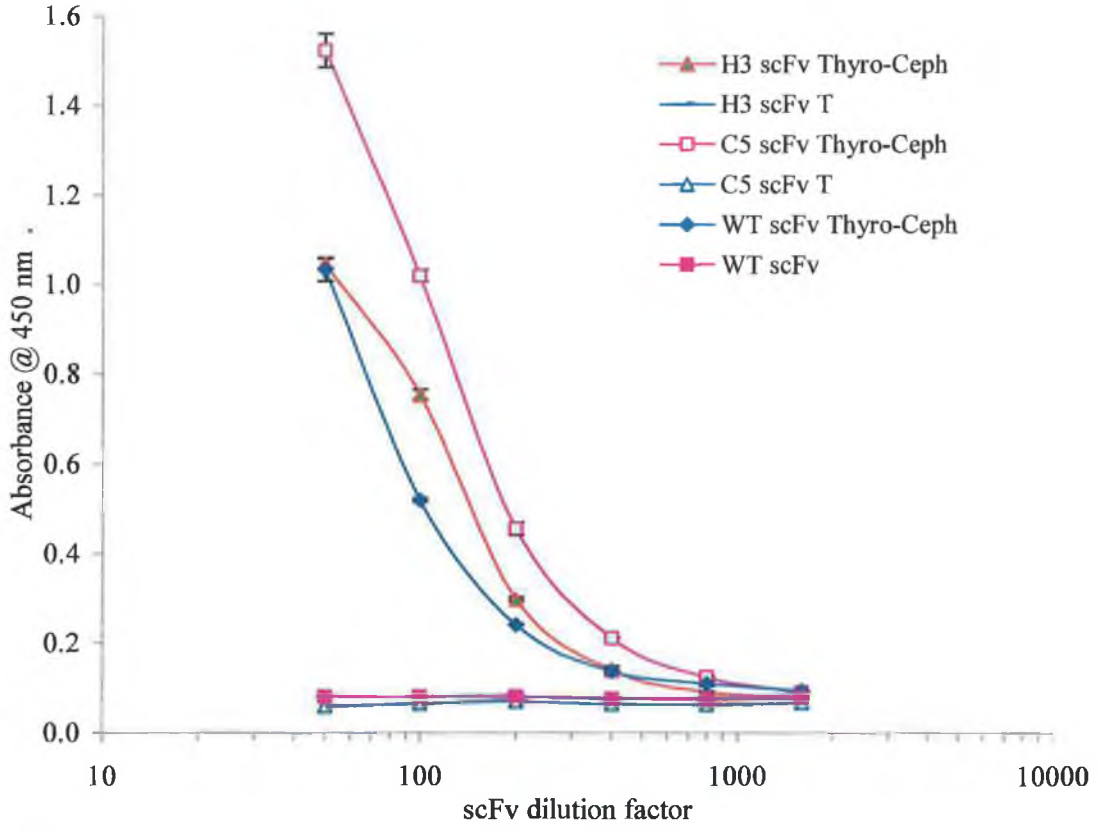


Figure 5.42. Results obtained for an ELISA to determine the scFv titre for wild-type (WT), C5 and H3 clone antibodies. The scFvs were screened against Thyro-Ceph (6 µg/ml) and thyroglobulin (T) - coated wells. From the plot the scFv dilution yielding an absorbance of 0.4 at 450 nm was chosen for each scFv for subsequent inhibition displacement ELISA development. The WT dilution was 1:150, C5 was 1:300 and H3 scFv dilution was 1:200. Overall the mutant clones expressed a larger amount of functional scFv in comparison to WT (observation). Each point represents the mean value of three measurements (n=3).

A control coating (thyroglobulin with no cephalexin attached) was used to rule out any non-specific binding interactions. The results of this experiment (Figure 5.42) showed the mutant C5 scFv displayed the highest titre of the three tested antibody preparations. None of the scFv displayed any significant binding to the control surface. Inhibition ELISAs were carried out, as described previously (section 5.2.3.3). The results of each assay are shown in figure 5.43.

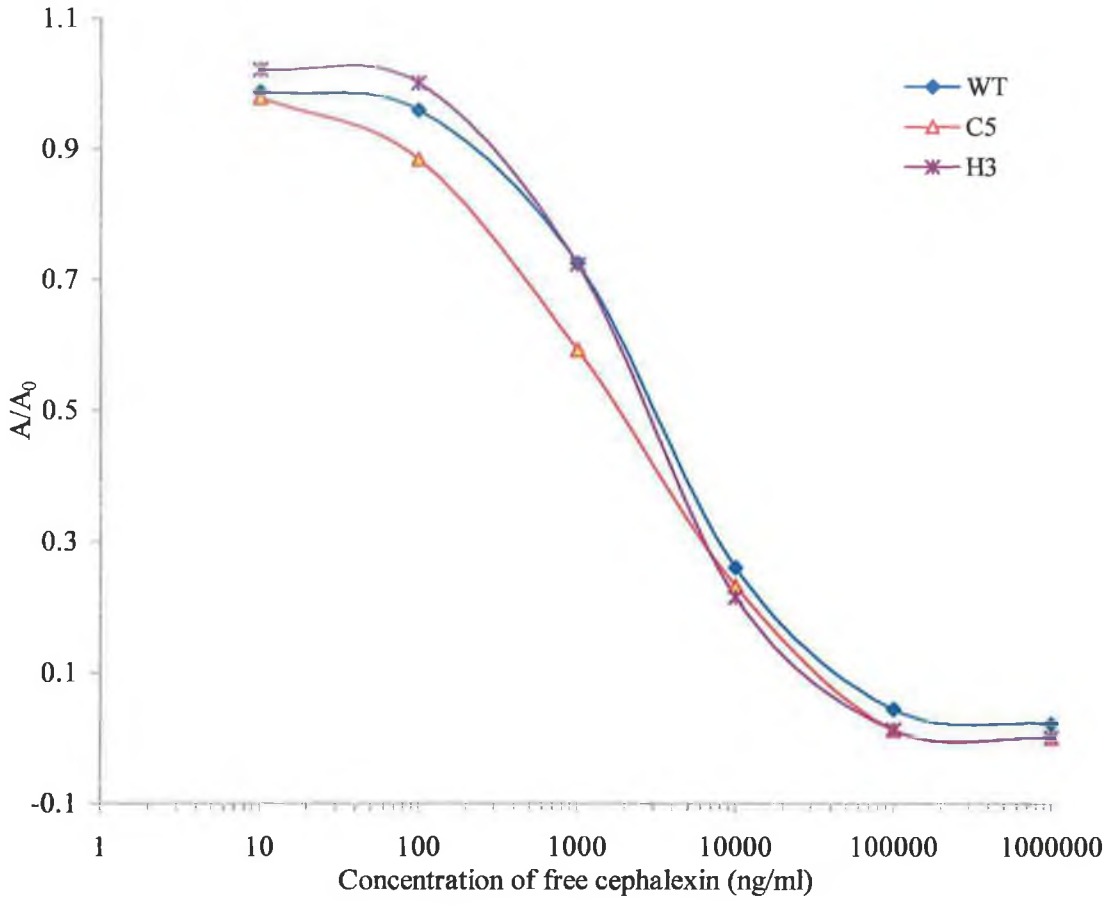


Figure 5.43. Overlay plot of the results obtained from displacement ELISA assays using wild-type (WT), H3 and C5 soluble scFv. The graph shows the improved assay sensitivity using C5 and H3 compared to WT. The resulting data was used in BIAevaluation and four-parameter equation fitted to each data set, and used to back calculate concentrations, and determine the IC50 values. This revealed a 2 fold improvement in sensitivity for clone C5 soluble scFv with respect to WT.

The limits of detection (LOD) for each of the assays were determined by subtracting 3 times the standard deviation obtained from the background (A0, no free drug). The LOD achieved with C5 scFv in comparison with wild type scFv showed a 1.8 fold improved sensitivity, from an absolute value of 143.3 ng/ml for the original wild type scFv to 77.9 ng/ml for the mutant C5 scFv. Although the H3 scFv displayed an increased sensitivity to free drug at concentrations of >1000 ng/ml, as observed by a 1.2 fold increase in the IC50 (in comparison with wild type scFv), at lower drug concentrations the comparative sensitivity was poor, with an assay LOD of 327 ng/ml. The standard deviation observed for assay point using the H3 scFv in ELISA was observably higher than for the original wild type and C5 scFv antibody fragments, which may suggest a lack of functional stability due to point mutations.

5.2.4.4 *Sequencing and bioinformatics analysis of mutant C5 and H3 scFv gene constructs.*

The mutant scFv clones C5 and H3 gene constructs were sequenced, as described in section 5.2.3.4. In addition, isolated mutant clone A1 (from R1* mutant library, glycine-eluted clones (GE)) was also sequenced. The A1 phage-scFv was previously found not to show competitive inhibition by free cephalexin (Figure 5.38), and was sub-cloned into pAK400 soluble expression vector prior to sequencing. The A1 clone was chosen for comparison, in order to assess potential mutations that may be detrimental to soluble cephalexin binding activity. All clones were sequenced from pAK400-containing *E. coli* (JM83) stab cultures with primers detailed in section 5.2.3.4.

The resulting nucleic acid sequences were translated into amino acid sequences using the Expasy translation tool (http://www.expasy.org/tools/dna.html). Subsequently the amino acid sequences were then co-aligned with the original wild type sequence (Figure 5.28) using the sequence alignment tool (http://www.prodes.toulouse.inra.fr/multalin). The resulting amino acid alignments for wild type (WT), C5 and H3 scFvs are shown in figures 5.44 (CDRs) and 5.45 (structural framework regions). Regions where there are mutations are highlighted in red (C5) and blue (H3). Amino acid deletions are represented by asterisks (*).

The C5 mutant clone was modelled using the SWISS-Model web site described in section 5.2.3.4. Using the SWISS-Model PDB viewer software, the CDR regions were highlighted in an analogous manner to the wild type model (Figure 5.29). The specific CDR regions where amino acid sequence mutations observed were highlighted, as shown in figure 5.46.

CDR $_{H1}$	WT-	S Y G M S
	C5-	[redacted] Y G M S
	H3-	S Y G M S
	A1-	S Y G M S
CDR $_{H2}$	WT-	S I S G G G N T Y Y P D S V K G
	C5-	S I S G G G N T Y Y P D S V K G
	H3-	S I S G G G N T Y Y P D S V K G
	A1-	S I S G G G N T Y Y P D S V K G
CDR $_{H3}$	WT-	G L G Y G K A F M D Y
	C5-	G [redacted] G Y G K A F M D Y
	H3-	G L G Y G K A F M D Y
	A1-	G L G Y G K A F M [redacted] Y
CDR $_{L1}$	WT-	R S S R N I V H S N G N T Y L E W
	C5-	R S S Q N I V H S N G N T Y L E W
	H3-	R S S Q N I V H S N G N T Y L E W
	A1-	R S S Q [redacted] I V H S N G N T Y L E W
CDR $_{L2}$	WT-	K V S N R L S
	C5-	K V S N R L S
	H3-	K V S N R L S
	A1-	K V S N R L S
CDR $_{L3}$	WT-	F Q G S H V P
	C5-	F Q G S H V P
	H3-	F Q G S H V P
	A1-	F Q G S H V P

Figure 5.44. This represents the scFv heavy and light chain complementarity determining region (CDR) amino acid sequence alignments for the wild-type (WT), C5 and H3 clones. The WT complete amino acid sequence is shown in figure 5.28. The point mutations for clone C5 and A1 are highlighted in red. Clone H3 has complete CDR amino acid sequence homology with the wild type. Mutations in the H3 clone are confined to scFv framework regions.

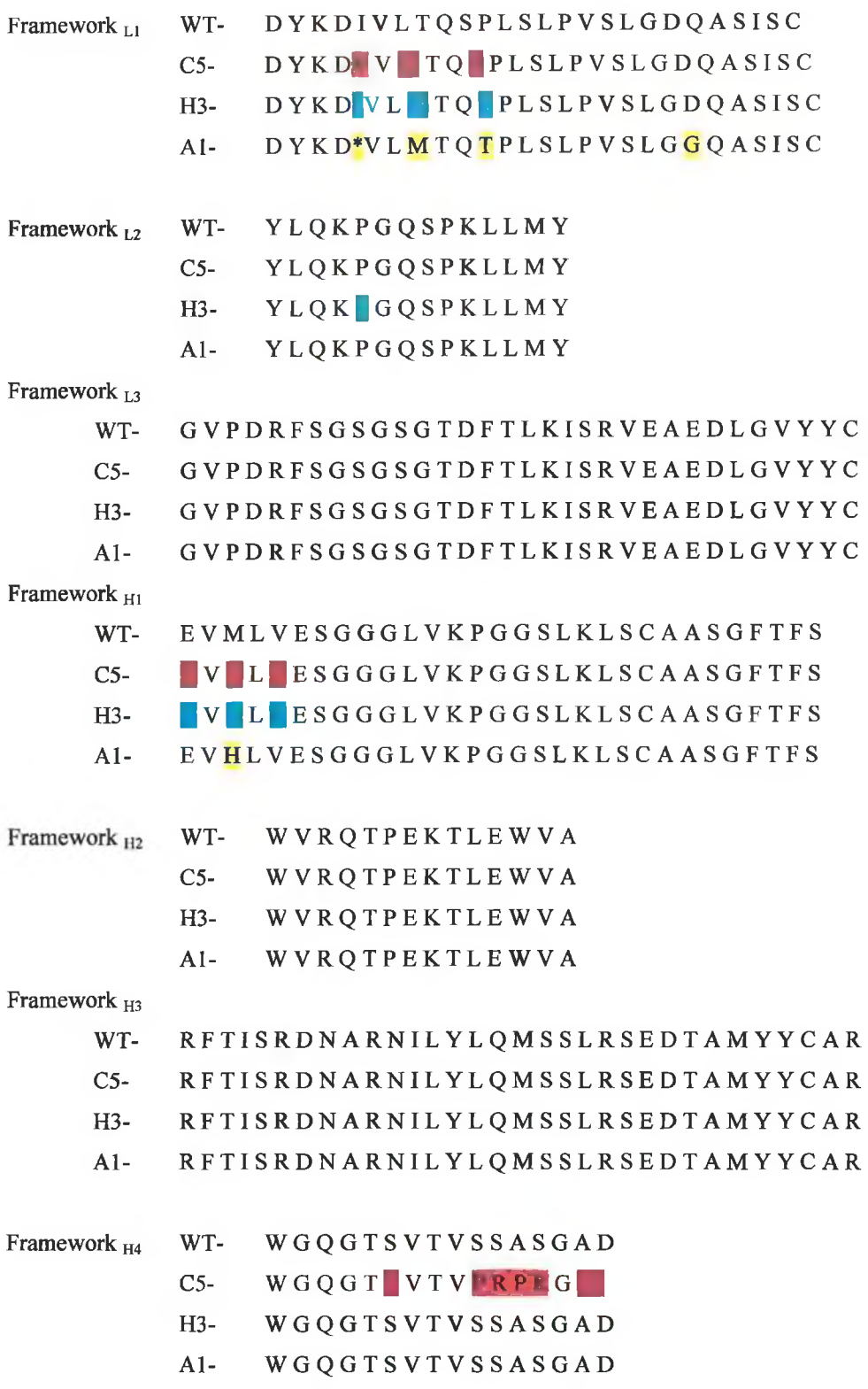


Figure 5.45. Representation of the scFv heavy and light chain framework region (FR) amino acid sequences. The sequences were aligned for the wild-type (WT), C5 and H3 clones. The point mutations for clone C5 are highlighted in red, for H3 clone in turquoise and for A1 in yellow, deletions are represented by an asterisk [*]. Heavy and light chain regions are designated H or L subscript with the corresponding number shown.

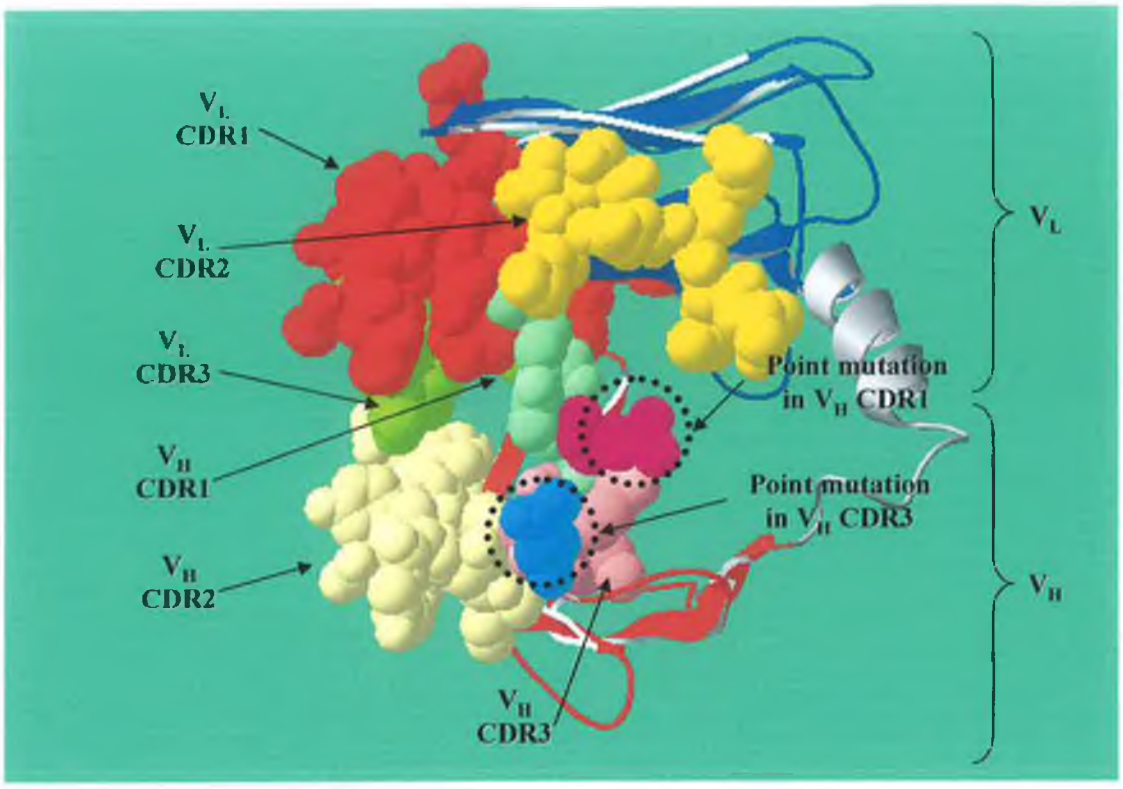


Figure 5.46. Picture of C5 scFv three dimensional model obtained using the SWISS-Model bioinformatics website and PDB viewer software. The heavy and light chain framework regions are shown as ribbon structures, and are highlighted in red and blue, respectively. The CDR regions are shown using a space fill model with the corresponding heavy and light chain CDRs highlighted in different colours for ease of distinction. The point mutations (highlighted by dashed black circle) in the heavy chain CDR1 (Purple) and CDR3 (blue) are shown.

5.3 Discussion

In summary, the BMV human naïve phage-scFv antibody library was panned with cephalexin conjugates. No soluble cephalexin-specific phages were isolated using this library. In the literature, there are reports of scFvs successfully isolated against hapten molecules using human naïve libraries. The likelihood of isolating a hapten (e.g. cephalexin)-specific phage, however, depends a great deal on the library size and the particular one used. In the pursuit of a scFv against cephalexin, two phage display libraries were produced using the method described by Krebber *et al.* (1997). The two libraries were produced from pre-immunised Balb/C mice.

Serum antibody titres for the immunised mice were monitored using ELISA and the animals were sacrificed, their spleens removed, and the RNA extracted for library construction. Total RNA from both mice (i.e. 1 & 2) was used to synthesise cDNA by reverse-transcription PCR (RT-PCR). Random hexamer primers were used for the RT-PCR initially. The resulting cDNA was used as a template for the heavy and light chain amplification PCR using the established set of degenerate primers (section 2.5.4.1). Variable heavy and light chain PCR products were gel-purified and genes were constructed by SOE-PCR. The gene constructs were ligated into the pAK100 phagemid vector and, subsequently, used to transform chemically competent *E. coli* (XL1 blue). The two phage display libraries produced contained 4.6×10^3 and 3.2×10^3 transformants each. In comparison with phage-display libraries reported in the literature, the number of transformants obtained for both libraries was low (usually of the order of 10^8 or 10^9 transformants). However, it was decided to pan the libraries since they originate from immunised mice and should have greater numbers of cepahexin-specific antibodies, as a result. Some authors have reported the isolation of specific phage scFv form such small libraries produced from pre-sensitised sources (Daly, 2001; Dillon *et al.*, 2003b; Leonard, 2003).

After several rounds of panning with various cephalexin conjugates, and investigating various bound phage elution strategies (Wind *et al.*, 1997 and De Briun *et al.*, 1999) no scFv were isolated that recognised the soluble (i.e. non-conjugated) β-lactam antibiotic residue. Two random clones were picked from library stock agar plates and used along with un-transformed cells to ensure that the cells did indeed contain pAK100 vector with scFv gene insert. The results of this experiment (Figure 5.11) showed that the library clones did contain the scFv gene construct. Although we can be sure the library clones did contain scFv gene constructs, the sequence diversity and hence the variety of binding function could not be fully enumerated without sequencing all the resulting transformants, which for the purposes of this

work was impracticable. The lack of potential cephalexin-specific scFvs within the two libraries is most probably due to the small library sizes obtained. The transformation of bacterial cells is well recognised as a critical limiting factor in terms of obtaining large libraries. It was decided in future library production work to apply an alternative electroporation-based (section 2.5.6.5) transformation protocol, used in the production of mutant phage display libraries (section 5.2.4)

At this point another approach was taken to obtain a cephalexin-specific scFv antibody fragment. The hybridoma cell line produced in chapter 4 of this work was used as a genetic source for scFv production (section 5.2.3). Positive phage-scFvs were produced and identified (Figures 5.15 and 5.16) and picked clones bound free-cephalexin which was demonstrated in inhibition ELISA (Figure 5.18). The scFv gene construct was purified from one of the picked clones and sub-cloned into the pAK400 vector for enhanced soluble expression. The scFv was successfully expressed as a single entity (i.e. no longer as a gIIIp fusion protein) in *E. coli* (JM83) cells. The expression was induced with IPTG and scFv production was monitored by SDS-PAGE and Western blotting (Figures 5.21 and 5.23). The cephalexin-specific scFv (termed wild type) was successfully purified by Ni^{2+}-NTA immobilised metal affinity chromatography (IMAC) and the purification process characterised using SDS-PAGE, Western blotting and ELISA.

Following isolation and purification of the wild type (WT) scFv by IMAC, an inhibition ELISA for the detection of cephalexin in 'spiked' processed milk samples was developed (section 5.2.3.3). The assay was not as sensitive as the parental monoclonal IgG antibody (Chapter 4) resulting in a limit of detection (LOD) of 143.3 ng/ml, compared to 60 ng/ml for the intact monoclonal IgG. It is probable that the reduced assay sensitivity may be due to avidity effects, going from a divalent intact IgG to a predominantly monovalent scFv. Kortt *et al.* (1994) suggest scFvs with 15 amino acid linker sequences are comprised mostly of the monomeric form. However, under certain concentration conditions (≈5 mg/ml) they found that dimeric and, in some cases, larger multimeric aggregates were formed. In addition, freezing was found to enhance aggregate formation.

The wild type scFv gene was sequenced (section 5.2.3.4) and the complementarity determining regions (CDR) were identified using the National (US) centre for Biotechnology information Ig Blast tool (http://www.ncbi.nlm.nih.gov/igblast/). The wild type scFv gene sequence was found to be 98.3% homologous to the murine sequence number AJ231205 from the cr1 germline gene sequence (Thiebe *et al.*, 1999), the heavy chain scored 95.8% homology with the Balb/c VH7183 gene family, as characterised by Williams *et al.* (2001). A

model of the WT scFv protein was produced using the SWISS-Model protein modelling website, the SWISS-Model system and PDB software (Pietsch, 1995; Guex and Pietsch 1997; Schwede *et al.*, 2003,).

The cephalexin-specific wild type (WT) scFv gene was isolated from the bacterial clone used to express the soluble scFv, and used as a template for random mutagenesis procedures (Coia *et al.*, 2001; Korpimäki *et al.*, 2002; Razai *et al.*, 2005). Intra-gene sequence shuffle (R1*) and error-prone PCR (R2*) phage-display libraries were constructed by ligation of mutated scFv gene constructs into the pAK100 phagemid vector. The ligation products were used to transform *E. coli* TOP10F′ electrocompetent cells and the number of transformants obtained was of the order of 1.4×10^7 for R1* and 2.3×10^6 for R2* libraries, respectively. The two libraries were panned using various cephalexin-conjugates. The conjugate bound phage-scFvs were initially competitively eluted by adding non-conjugated cephalexin and successively lowering the concentration followed by elution with glycine. The specific 'polyclonal' phage-scFv populations were more noticeably enriched over three rounds of panning using the free drug elution (DE) method (Figure 5.34). For example, in terms of absorbance, the response obtained for third round panned R2* drug eluted phages was 1.6-fold higher than the first round panned phages. The glycine-eluted phage for the same library (R2*) response for round one and three panned phage was the same.

Individual specific clones (i.e. 'monoclonal') were screened and positive phage-scFv identified by ELISA (Figures 5.35 and 5.36). In addition, binding of 22 selected clones to free cephalexin was observed in inhibition ELISA (Figures 5.37 and 5.38). Nascent phage-scFvs were singled out and sub-cloned into pAK400 vector for subsequent soluble expression. Two clones (C5 and H3) with the most promising improvements in terms of inhibition ELISA sensitivity were purified by IMAC chromatography. From the IMAC purification fractions were analysed by denaturing SDS-PAGE and Western blotting analysis (Figures 5.39 and 5.40). The IMAC-purified mutant scFv antibody fragments were used in an inhibition ELISA for the detection of cephalexin hydrate in 'spiked' processed milk samples (section 5.2.4.3), in parallel with the original wild type scFv. The mutant C5 clone scFv displayed two-fold increased assay sensitivity in comparison with wild type. The mutant H3 scFv was not as sensitive at lower cephalexin concentrations, but it did have a lower IC50 value. In comparison with the wild type the mutant H3 scFv assay IC50 value represented a 1.2 fold decrease. The H3 clone had point mutations in the framework regions which may contribute to a greater stability. However, there were no observed mutations in any CDR regions when compared to the WT clone.

Other workers have suggested that larger mutation rates are required to increase specificity (Saviranta *et al.*, 1998) by reducing cross reaction with similar target antigens. They described the production of a mutant anti-17β-estradiol Fab library using random mutagenesis techniques. They found that each mutant contained on average 24 amino acid changes. Drummond *et al.* (2005) suggest that a balance rate of mutation may be calculated to strike a balance between obtaining functional and unique clones.

The mutant clone C5 scFv ELISA limit of detection (LOD) was determined by subtracting 3 times the standard deviation obtained from the background and achieved a 1.8 fold improved sensitivity, from an absolute value of 143.3 ng/ml for the original wild type scFv, to 77.9 ng/ml for the mutant C5 scFv. The C5 scFv was the best scFv produced in terms of expression, functional stability and assay performance, capable of cephalexin detection below the 100 ng/ml EUMRL for milk. Harvey *et al.* (2006) reported the production and isolation of mutant scFvs with improved affinity and expression compared to original parental scFv. EP-PCR was used to incorporate mutations into the original gene sequence for a methamphetamine-specific scFv. Interestingly, they isolated scFv mutants with single amino acid point mutations in their V_HCDR1 and V_HCDR3 regions compared to parent scFv, with higher affinity to methamphetamine (up to 3-fold higher) and improved expression in *E. coli*.

The C5 scFv amino acid sequence with observed mutations in the variable heavy chain CDR1 and CDR3 regions (Figure 5.44) supports the established concept that the majority of antigen binding interactions are conferred by the variable heavy chain complementarity determining regions. In conjunction with this finding, the point mutations observed in the C5 and H3 protein framework regions may go some way to explaining the observed improvement in bacterial host expression in comparison with the wild type scFv clone. The H3 scFv did contain many similar framework mutations (Figure 5.45) to the C5 scFv. However, clone H3 scFv did not have analogous heavy chain CDR mutations and hence the assay improvement was not as considerable as for C5 scFv. The mutant clones had observably higher titres than the wild type. This may be attributed to increased expression of mutant clones or expression of more functional properly folded proteins.

Chapter 6

Biosensor-based detection of cephalexin

6.1 Biosensors an introduction

The IUPAC has previously introduced a more rigid definition of a biosensor (Thevenot *et al.*, 1999): i.e. "A biosensor is a self-contained integrated device which is capable of providing specific quantitative or semi-quantitative analytical information using a biological recognition element (i.e. antibody) which is in direct spatial contact with a transducer element. A biosensor should be clearly distinguished from a bioanalytical system, which requires additional processing steps, such as reagent addition. Furthermore, a biosensor should be distinguished from a bioprobe which is either disposable after one measurement, i.e. single use, or unable to continuously monitor the analyte concentration." This definition excludes many devices that have been successfully developed, for example optically-based immunosensors and disposable enzyme electrodes for the detection of blood glucose levels, as they do not fulfil the criterion of continuous monitoring or have additional preparation steps. However, for the sake of clarity this chapter will consider biosensors which fulfil the more traditional criterion, the direct spatial combination of biological recognition and transduction into a readable signal.

Human senses allow us to extract information from our environments e.g. from our sense of smell to our sight. The fundamental principles of these senses is the initial detection of an analyte (e.g. odorous molecules, light) via receptors (Figure 6.1), that then transduce this into an electrical impulse via neurotransmitters that can be processed in the brain. A biosensor combines a biological component (e.g. antibody, enzyme, whole cell, DNA etc.) integrated with a transducing element that in turn changes an event (e.g. antibody-antigen binding) into a signal (e.g. electric, photonic), which may be further processed, usually by a computer.

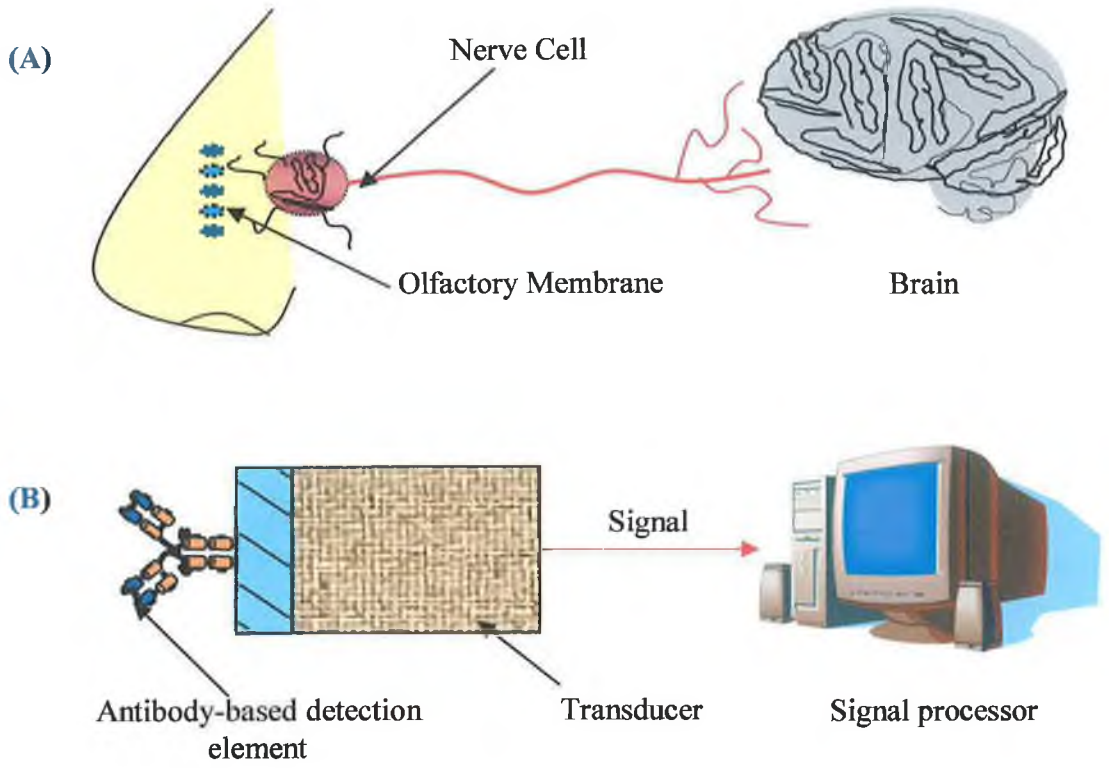


Figure 6.1. Human sense of smell signal path schematic (A) and the general biosensor schematic (B) with the corresponding elements labelled.

Since the first biosensor devices were reported in the 1960's, much interest was generated and details of many different types of biosensor have been published in the literature (Cornell *et al.*, 1997; Nice and Catimel, 1999; Preston and Mc Fadden, 2001; Sanders *et al.*, 2001; Vo-Dinh and Cullum, 2000a). There are a lot of potential variations in the choice of biocomponent and means of signal transduction available when developing a biosensor device. These are considerations that depend on the future application and performance criteria of the biosensor and its intended use in medical, industrial or environmental diagnostics. It may be possible to integrate any number of combinations of transducer and bio-component (Table 6.1), yielding a host of different possibilities.

Table 6.1. The biological recognition and transducer elements that may be combined in Biosensor design.

Biological Elements	Transducer Types
Whole Organisms	Potentiometric
Tissues	Amperometric
Cells	Conductometric
Organelles	Impedimetric
Membranes	Optical
Enzymes	Calorimetric
Enzyme Components	Acoustic
Receptors	Mechanical
Antibodies	
Nucleic Acids	

Modern antibody-based sensors have been developed utilising different optical phenomena as a means of detection and analysis for antibody-antigen interactions. There are many biosensors that rely on evanescent wave generation such as waveguide interferometers, resonant mirror (or frustrated total internal reflection (FTIR)), surface plasmon resonance (SPR), surface enhanced rayman scattering (SERS), and near field scanning optical microscopy (NSOM) (Attridge *et al.*, 1991; Liron *et al.*, 2002; Seydack, 2005).

In addition, many light-based biosensor technologies comprise of tapered optical fibres incorporating a bio-component. Most of the optical fibre devices reported utilise a fluorescence-based probe coupled to a biological recognition element. The technique was first developed for sub-micron fibre probes for chemical analysis (Tan *et al.*, 1992) and as a means of measuring refractive index changes using SPR (Ranot-Trioli *et al.*, 1996; Dorstalek *et al.*, 1997; Homola and Hongbo, 2001). One particular antibody-based study utilised an antibody for the detection of benzo [a] pyrene tetrol (BPT), a marker for exposure to a known carcinogen, benzo [a] pyrene (BaP) (Vo-Dinh *et al.*, 1987). It was fabricated using a micropipette puller to taper the optical fibres. The outside of the fibre was coated with a uniform layer of silver, 200nm thick, and the uncoated tip of the fibre was silanised and covalently bonded to the anti-BPT antibody. The bio-sensing device showed increased sensitivity and response time, attributed to the small tip area and it had an absolute limit of detection for BPT of approximately 300 zeptomoles (10^{-21}moles). Since the initial reports of this type of biosensor many researchers have applied the technology for the direct and indirect

analysis of a host of different compounds using a variety of bio-components (Barker *et al.*, 1998; Cullum *et al.*, 1999; Liu and Tan 1999; Vo-Dinh *et al.*, 2000b).

SPR-based sensors (Quinn and O'Kennedy, 1999; Homola, 2003) have become an integral part of biomolecular interaction analysis. In particular the Biacore ™ systems have been extensively reported in the literature, with approximately 90% of the 1998-1999 commercial biosensor publications citing the use of a Biacore instrument (Rich and Myszka, 2000), which seems to be a prevalent trend to date. Biacore sensors have been successfully applied in conjunction with antibodies to the detection of whole cells (Leonard *et al.*, 2003; Hearty, 2004), proteins and peptides (Yribarren *et al.*, 2003) and small molecules (i.e. haptens) (Brennan *et al.*, 2003; Tüdös *et al.*, 2003).

6.1.1 *Surface plasmon resonance (SPR)-based signal transduction*

SPR is a unique optical phenomena that has been successfully applied to optical-based sensing with numerous and varied applications. The initial historical applications of SPR were in the investigation of optical properties of thin metal films. Since then SPR has been incorporated into a variety of sensing devices, in particular biosensors, with great success. Many SPR-based sensors have been developed based on the 'Kretschmann configuration' (Kretschmann and Raether, 1968; Kretschmann, 1971), as shown in figure 6.2. SPR was used to probe changes in the refractive index (RI), within the evanescent field (EF) range. Thus, any physical event or change in mass that occurs on the surface of the sensor (e.g. antibody binding to antigen) changes the value for the refractive index and thus produce a measurable signal.

SPR may be achieved by varying the frequency of the incident light or by varying the angle of incidence of that light until there is a noticeable sharp drop in the overall intensity of the reflected light. The SPR condition is generated by evanescent field tunnelling of incident light energy into free electrons on the metal surface, thus creating plasmons. The incident p-polarised light is directed into the prism at an angle that is greater than the critical angle. Thus, incident light undergoes total internal reflection within the glass substrate (e.g. high refractive index media) when it is reflected at the boundary of the low refractive index material (i.e. sample medium). Hence, an evanescent field is generated normal to the sensor surface and decays exponentially into the sample medium. In effect the evanescent filed is sensitive to mass changes within a defined distance from the surface (200-300 nm), since refractive index changes are proportional to mass changes at the sensing surface. Using the apparatus set up in the Kretschmann geometry, for example, (Figure 6.2) transverse magnetic

polarised light passing through the prism with a particular angle above the critical angle reflects from the thin metal film. Resonance is observed by monitoring the position of the reflectance minimum.

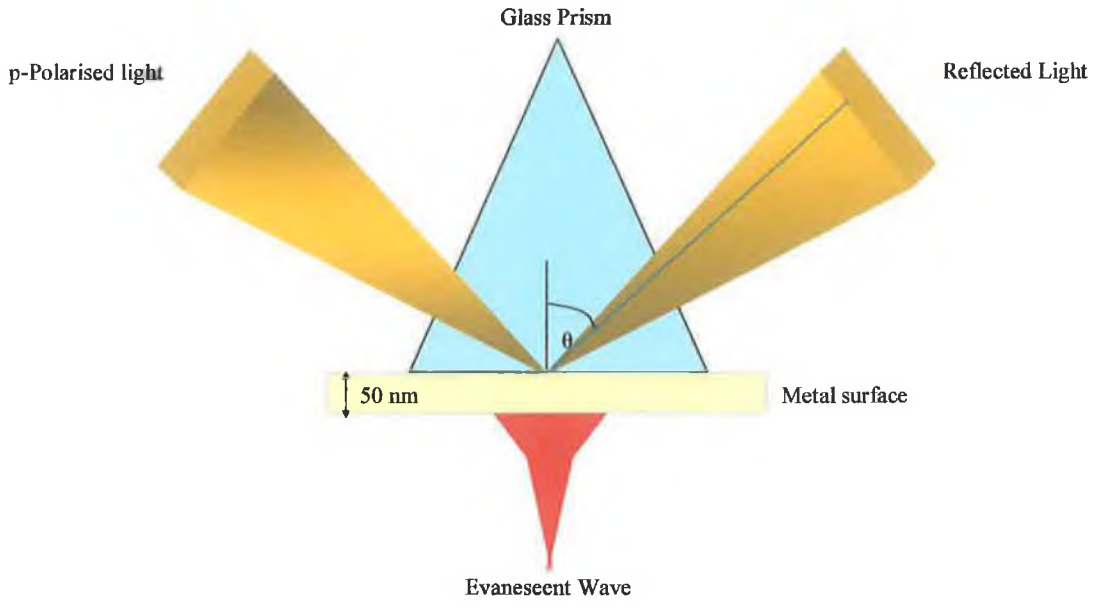


Figure 6.2. The basic 'Kretschmann configuration' apparatus setup for the monitoring of SPR optical phenomena. The SPR condition is generated by evanescent field tunnelling of incident light energy into free electrons on the inert thin metal surface (≈50 nm thickness). When the incident p-polarised light is directed into the prism at an angle (θ) that is greater than the critical angle it undergoes total internal reflection within the glass substrate (e.g. high refractive index media) when it is reflected at the boundary of the low refractive index material (i.e. sample medium). Thus an evanescent field is generated normal to the sensor surface and decays exponentially into the sample medium.

There are now a host of commercially available devices based on SPR signal transduction. Biacore™ AB (Uppsala, Sweden) has numerous automated instruments including, the Biacore™ 1000/2000/3000 series. The Biacore systems utilise SPR using the principles outlined in figure 6.3. Texas Instruments have also manufactured a waveguide-based SPR biosensor, the Spreeta™. They generally consist of several basic elements including, sensor chips, an optical detection unit, and integrated micro fluidics with appropriate control software. Other commercially available SPR-based biosensors include, the IAsys instrument (NeoSensors), the BIOS-1 system (Windsor Scientific Ltd), and the AnaLight Bio 200 (Farfield sensors Ltd.). SPR-based biosensor systems offer robust and reliable analysis techniques and have been extensively used for antibody characterisation and assay development. SPR biosensors comprise the majority of published material with regard to antibodies and their application in the detection of a variety of hapten compounds.

This chapter describes the use of the Biacore™ 3000 SPR-based real-time biosensor for the evaluation of anti-cephalexin polyclonal (Chapter 3), monoclonal (Chapter 4) and recombinant single chain Fv (scFv) antibodies (Chapter 5) in the development of a semi-quantitative inhibition assay for the detection of cephalexin hydrate, in milk. Conjugated and un-conjugated cephalexin was immobilised onto carboxymethylated dextran (CM5) Biacore chips, and evaluated in terms non-specific binding and assay performance with each anti-cephalexin antibody.

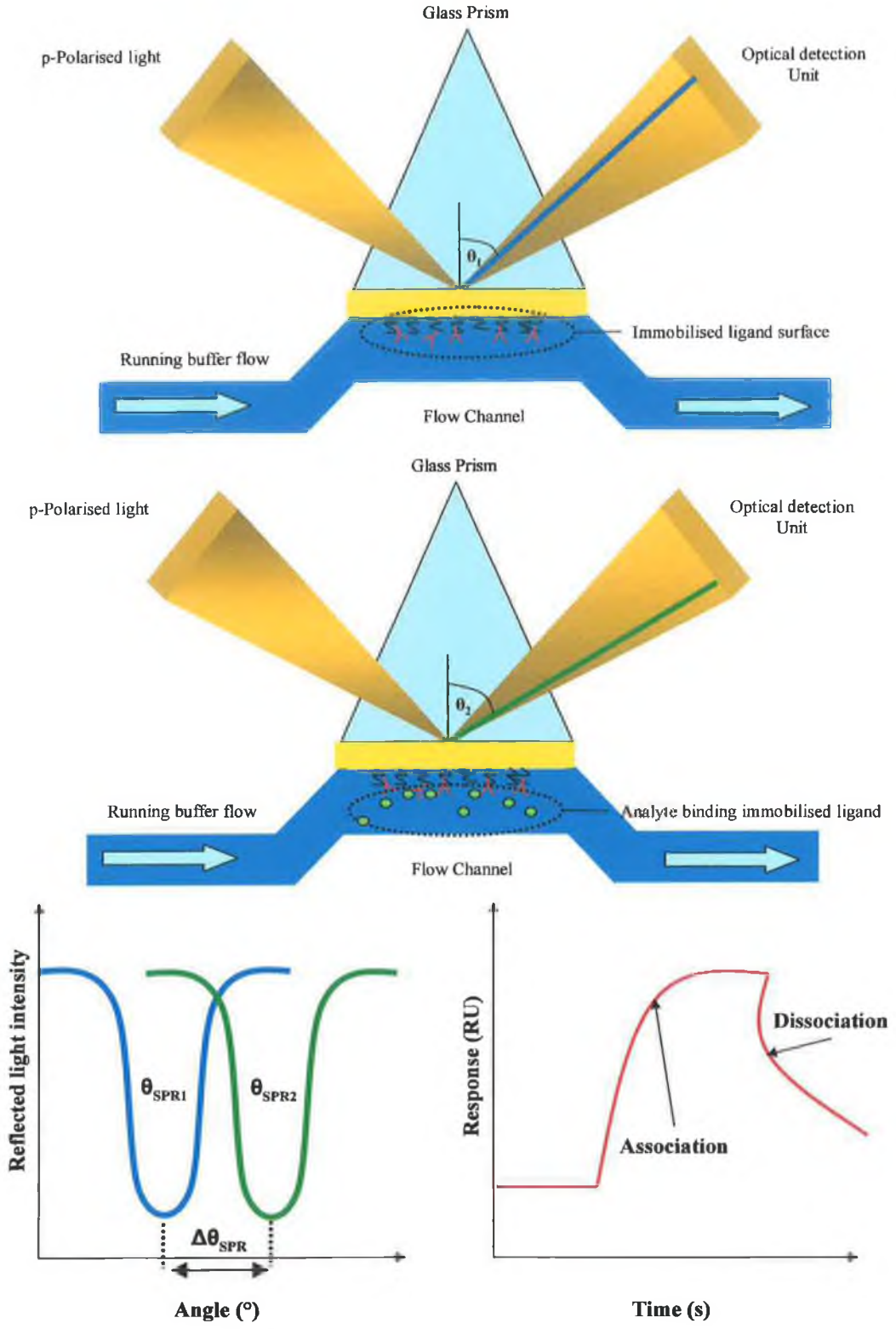


Figure 6.3. Diagrammatic representation of the Biacore SPR-based biosensor working principles. The SPR angle (— θ_{SPR1}) with immobilised ligand is altered by the binding of target analyte to give θ_{SPR2} (—). The 'real-time' difference between the SPR angles ($\Delta\theta_{SPR}$) is plotted as arbitrary response units (— RU) against time in seconds (s). The association and dissociation rates may be determined for the ligand and analyte binding.

6.2 Results

6.2.1 *The production of immobilised cephalexin-thyroglobulin conjugate sensor surface.*

The Biacore 3000 SPR-based biosensor was used in conjunction with carboxymethylated dextran (CM5) sensor chips, as described in section 2.7. Immobilisation of thyroglobulin-cephalexin conjugate was carried out by initial CM surface 'preconcentration' (section 2.7.1) followed by covalent attachment of cephalexin-thyroglobulin using NHS/EDC-mediated chemistry.

6.2.1.1 *Preconcentration studies using a carboxymethylated dextran (CM5) chip and thyroglobulin-cephalexin conjugate.*

The underivatised CM5 sensor chip surface has a net negative charge, therefore, in order to maximise the immobilisation of protein ligands, it is essential to maximise the electrostatic attraction between surface and ligand in solution. This is achieved by using low ionic strength buffers (e.g. 10 mM sodium acetate) that facilitates maintaining the protein ligand at a pH value below its isoelectric point (pI), thus giving the protein a net positive charge. Studies were carried out using 10 mM sodium acetate buffer at a range of low pH values so as to maximise or 'preconcentrate' the amount of conjugate (i.e. in terms of response units (RU)) electrostatically associated to the negatively charged CM5 chip surface. The optimum pH of 10 mM sodium acetate buffer was found to be 3.9, as shown in figure 6.4, which gave the maximum response of 10,000 RU.

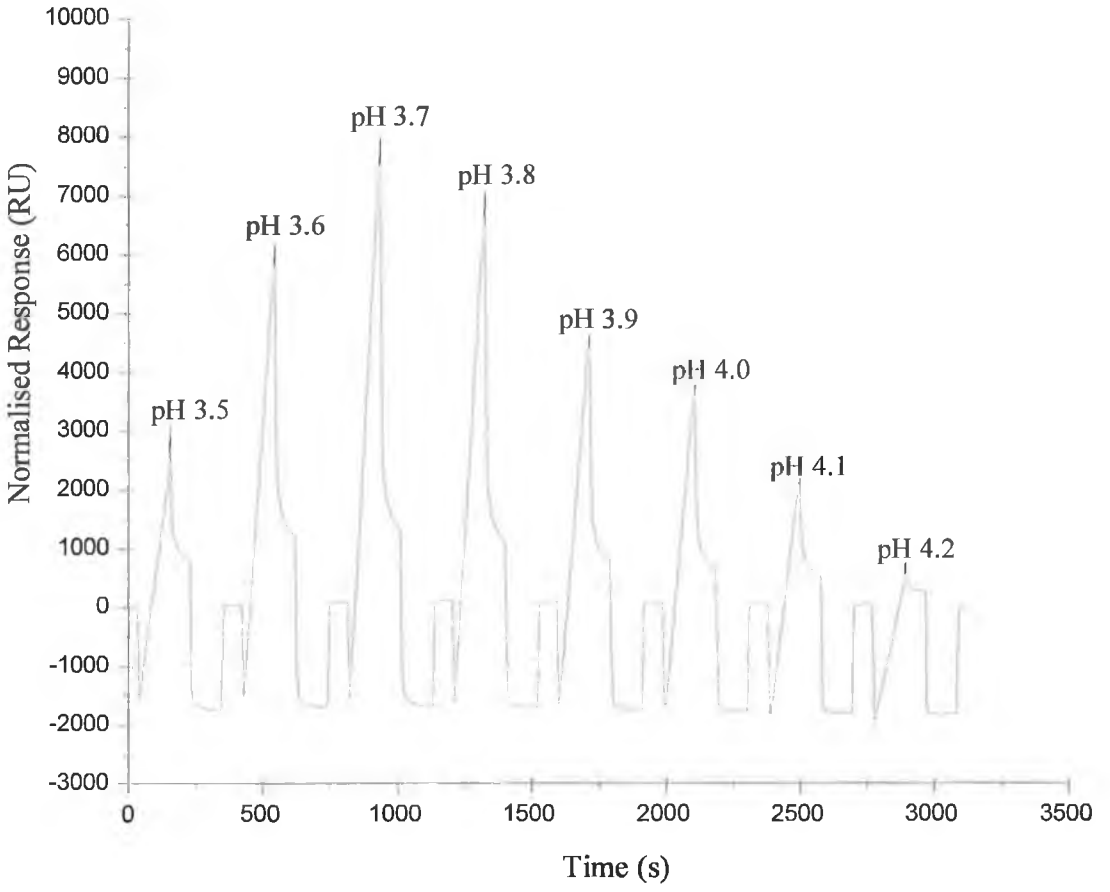


Figure 6.4. Cephalexin-thyroglobulin (20 µg/ml) was passed over a CM5 chip surface in 10mM sodium acetate buffer at a flow rate of 10 µl/min for 2 min, in order to facilitate electrostatic association between the negative charged dextran surface and the charged conjugate. Sodium acetate buffer (10 mM) with pH values ranging from 3.5 to 4.2 were used. After each injection of conjugate a regeneration step was required to remove residual conjugate. A regeneration solution of 5 mM NaOH was used to remove electrostatically associated conjugate. The sodium acetate buffer, with pH of 3.7, gave the largest response units (~10,000 RU). This progressively decreased as the pH increased.

6.2.1.2 *Immobilisation of Thyro-Ceph conjugate and thyroglobulin to the CM5 sensor chip flow cell surface and binding studies using polyclonal antibody.*

Following the determination of the optimum immobilisation buffer by 'preconcentration', the thyroglobulin-cephalexin conjugate and thyroglobulin itself (as a control surface) were immobilised onto two separate flow cells on the same sensor chip (section 2.7.2). Initially for the cephalexin conjugate, the CM5 chip surface was activated using 0.05 M NHS / 0.2 M EDC and followed by an injection of 50 µg/ml solution of the conjugate dissolved in the appropriate sodium acetate buffer (10 mM, pH 3.7). Any remaining un-reacted sites ('activated' carboxylic acid groups) were then 'capped' by passing a solution of 1M ethanolamine, pH 8.5 across the chip surface. Figure 6.5 shows a typical immobilisation sensorgram for thyroglobulin-cephalexin. The total amount of conjugate immobilised in terms of response units was approximately 14,000 (RU).

In order to assess the newly synthesised Thyro-Ceph (TCC) immobilised flow cell surface (section 2.7.2), cephalexin-specific polyclonal antibody (Chapter 3) diluted in PBS-T was injected over it and two control surfaces. Non-conjugated thyroglobulin protein was also immobilised, as described (section 2.7.2), yielding a final immobilised thyroglobulin response of 13,360 RU (Figure 6.6). This flow cell comprised control surface one. The polyclonal antibody was simultaneously injected over an unmodified carboxymethylated dextran flow cell surface, comprising control surface two. The sensorgrams obtained for the TCC, control one and two surfaces were plotted and are presented in figure 6.7. No significant binding response was observed (<5 RU) for the negative control surfaces, as expected compared to a binding response of 220 RU using polyclonal (anti-*CephP1*) antibody and the TCC flow cell surface.

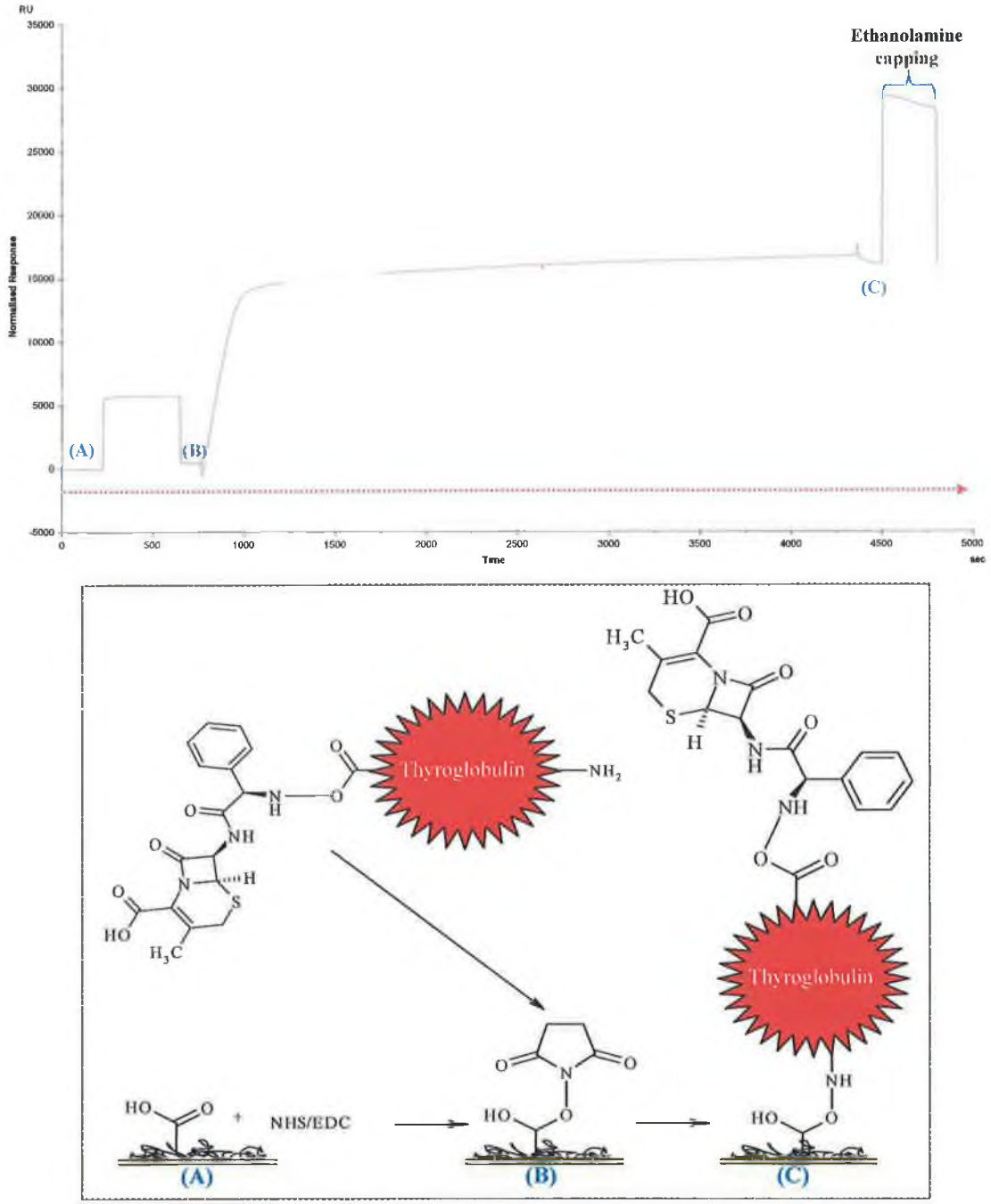


Figure 6.5. Sensorgram of thyroglobulin-cephalexin immobilisation (above top) and each of the corresponding main steps (A, B, C) are the schematically represented (above bottom). The carboxyl groups on the carboxymethylated dextran matrix surface (CM5) were activated using EDC and NHS (A), hence forming intermediate NHS ester groups (B). This was further reacted with thyroglobulin-cephalexin conjugate-containing amine groups (C). The surface was then treated with 1 M ethanolamine in order to remove electrostatically associated conjugate and block or 'cap' any unreacted carboxyl groups on the chip. A total of 14,000 RU of conjugate was covalently bound to the flow cell sensor surface.

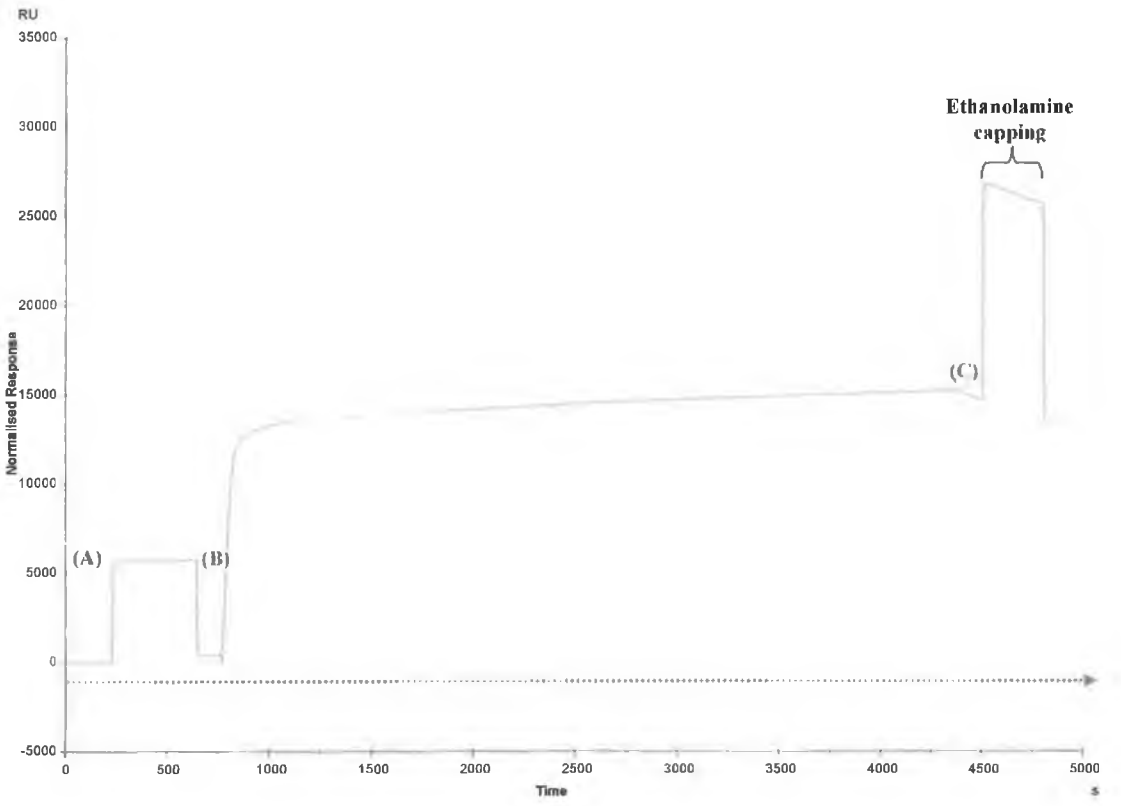


Figure 6.6. Sensorgram of unconjugated thyroglobulin immobilisation highlighting each of the main steps on the sensorgram (A, B, C) The carboxyl groups on the carboxymethylated dextran matrix surface (CM5) were activated using EDC and NHS (A), hence forming intermediate NHS ester groups (B). This was further reacted with thyroglobulin protein solution (50 µg/ml), thus forming covalent bonds with primary amine groups present on protein and EDC-activated sensor surface (C). The surface was then treated with 1M ethanolamine in order to remove electrostatically associated protein, and block or 'cap' any un-reacted carboxyl groups on the chip. A total of 13,360 RU of protein was covalently bound to the flow cell sensor surface.

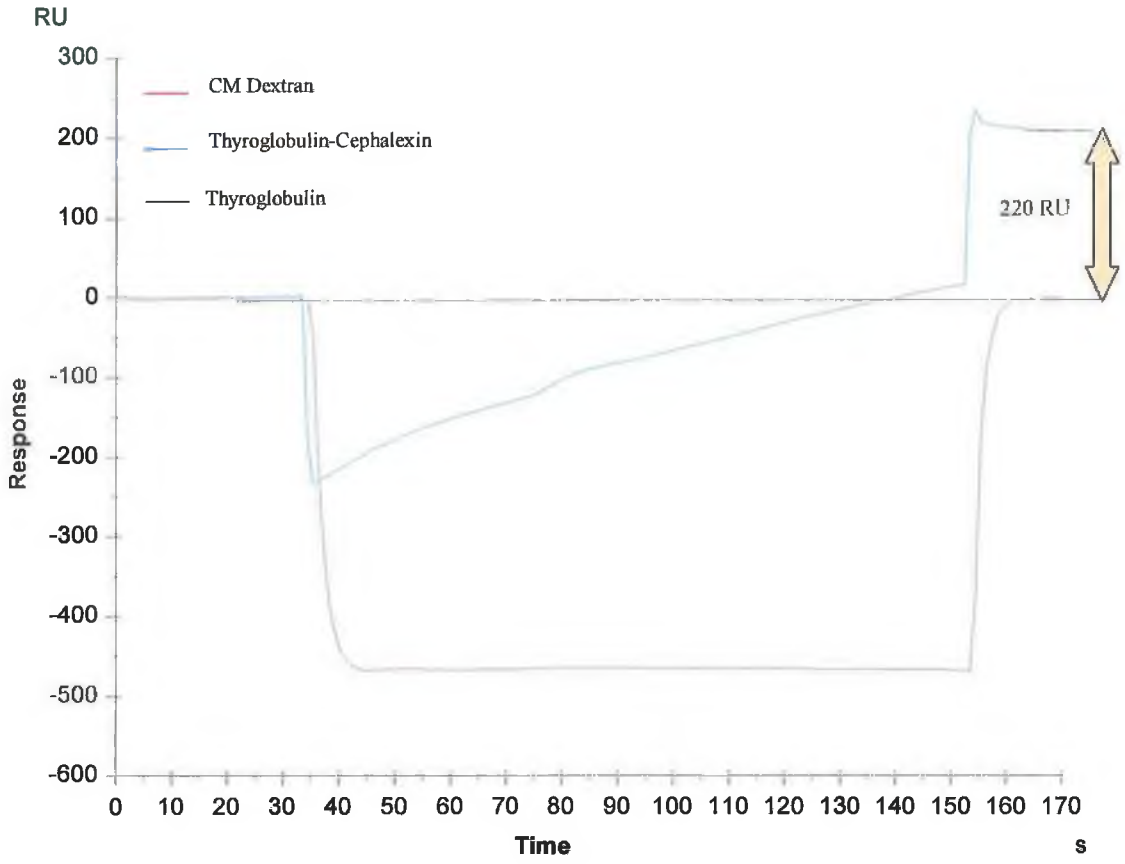


Figure 6.7. Overlay sensorgram plots of the response obtained using a CM dextran (–), immobilised thyroglobulin (–), and immobilised thyroglobulin-cephalexin surfaces (–). The dotted line represents the experimental baseline. A 1:100 dilution of anti-cephalexin polyclonal antibody diluted in PBS-T was injected over each surface at a flow rate of 5 µl/min for duration of 2 minutes. The response units (RU) for each sensorgrams have been normalised to zero. The immobilised thyroglobulin-cephalexin conjugate gave the greatest response (220 RU) as expected. Responses of <5 RU were obtained for each negative control surface.

6.2.1.3 *Evaluation of immobilised thyroglobulin-cephalexin conjugate surface stability by successive binding and regeneration using anti-cephalexin antibodies.*

In order to assess the immobilised cephalexin-conjugate surface stability, polyclonal antibody (Chapter 3, anti-*CephP1*) was repeatedly bound and surface regenerated. Initially, the regeneration solution was optimised from a range of sodium hydroxide and hydrochloric acid solutions (30 mM → 1 mM), as described in section 2.7.4. The 5 mM sodium hydroxide was found to be optimum for the regeneration of the immobilised conjugate surface following binding of anti-*CephP1* polyclonal antibody. A total of 50 antibody binding and surface regeneration cycles were carried out using 5 mM NaOH to remove bound polyclonal antibody. There was very little (4.5%) loss of surface activity over the course of the regenerations as can be seen in figure 6.8. After >200 regeneration cycles (observation, data not shown) the immobilised thyroglobulin-cephalexin surface activity was still below the acceptable loss of activity limit, of 20% (Wong *et al.*, 1997). This confirms its stability and applicability.

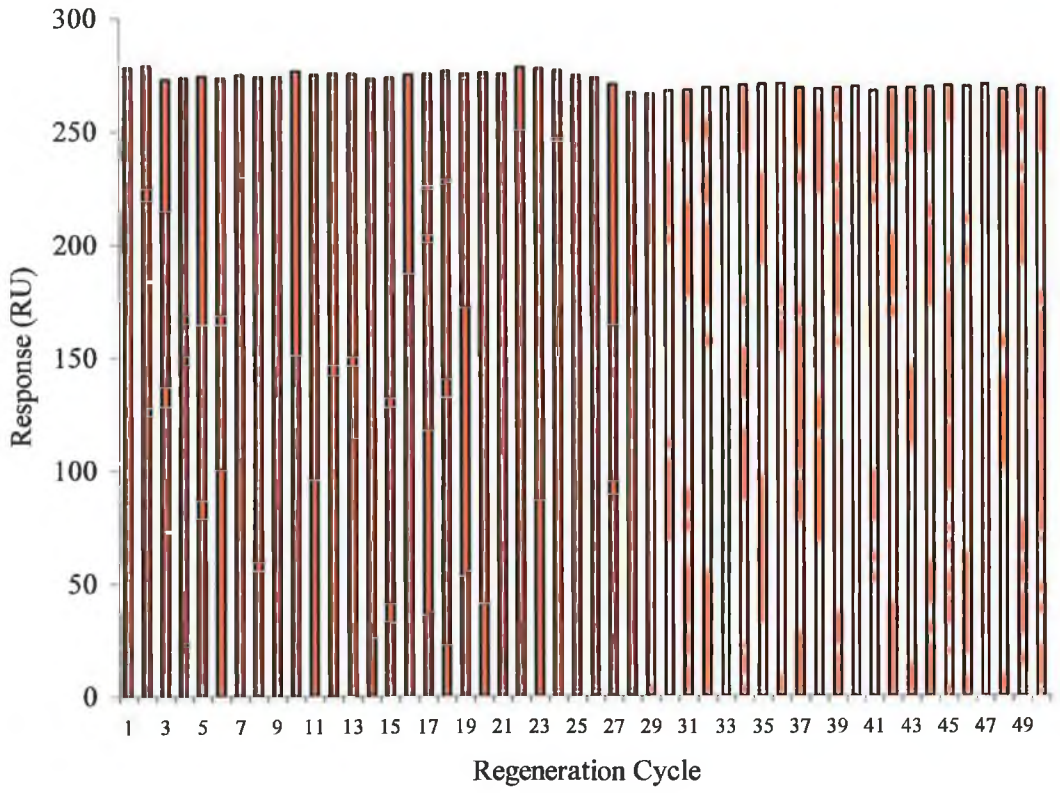


Figure 6.8. Regeneration studies of immobilised thyroglobulin-cephalexin surface polyclonal anti-cephalexin antibody binding. There was a 4.5% overall loss of surface binding activity over 50 regenerations from initial binding response of 278.8 RU to final polyclonal binding response of 265.9 RU. The surface showed minimal surface activity loss.

6.2.2 *The production of a directly immobilised cephalexin-CM5 sensor chip surface.*

In addition, to the immobilised conjugate (thyroglobulin-cephalexin) surface (TCC), cephalexin was also directly immobilised to a CM5 sensor chip surface. The directly immobilised cephalexin (DIC) flow cell was prepared as described in section 2.7.3. Initially, preconcentration studies were carried out using sodium acetate buffer across a range of acidic pH values (pH 3→ 6.6) in order to obtain sufficient attraction between the negatively charged CM dextran surface and the cephalexin in solution. The isoelectric point (pI) of cephalexin ranges from 4.5 – 5 approximately in water (www.rxlist.com). At prolonged exposure to alkaline pH (>7) the β-lactam ring will readily hydrolyse to form the biologically inactive forms. Therefore, only acidic pH buffers were used in pre-concentration studies. No observable 'pre-concentration' was observed (data not shown) across the above pH range. There was an apparent repulsion between soluble cephalexin and the un-modified CM5 chip surface. In order to circumvent this it was decided to alter the CM5 chip surface chemistry in order to maximise the electrostatic attraction to soluble cephalexin and hence the immobilisation efficiency. The CM5 chip surface was chemically altered in terms of net charge and binding functionality (i.e. –COOH → $-NH_2$) by reacting with ethylene diamine (1M, pH 8.5), as described in section 2.7.3, and cephalexin was covalently attached through its carboxylic acid functionality (Figure 1.4) by subsequent NHS/EDC-mediated chemistry. The resulting immobilisation sensorgram and an outline of the chemistries involved are shown in figure 6.9.

A similar reference flow cell was prepared as described for the DIC cephalexin flow cell. However, the cephalexin was omitted. This flow cell surface was prepared for use as a negative control and for 'real-time' reference subtraction which is facilitated using the Biacore 3000 control software. In order to obtain an accurate estimate of the amount of surface activity (Figure 6.9) the response obtained for the control or reference subtraction flow cell, as shown in figure 6.10, (447.6 RU) was subtracted from the 982.8 RU value observed for the DIC cephalexin flow cell. This yielded a response value of 535.2 RU. This value was much lower in comparison to cephalexin-protein immobilisation. It was expected, however, due to the relative molecular mass difference between the conjugated and free cephalexin.

The DIC test and control surfaces were used in binding studies using anti-cephalexin antibodies at various concentrations in order to confirm antibody binding and identify any potential non-specific interactions.

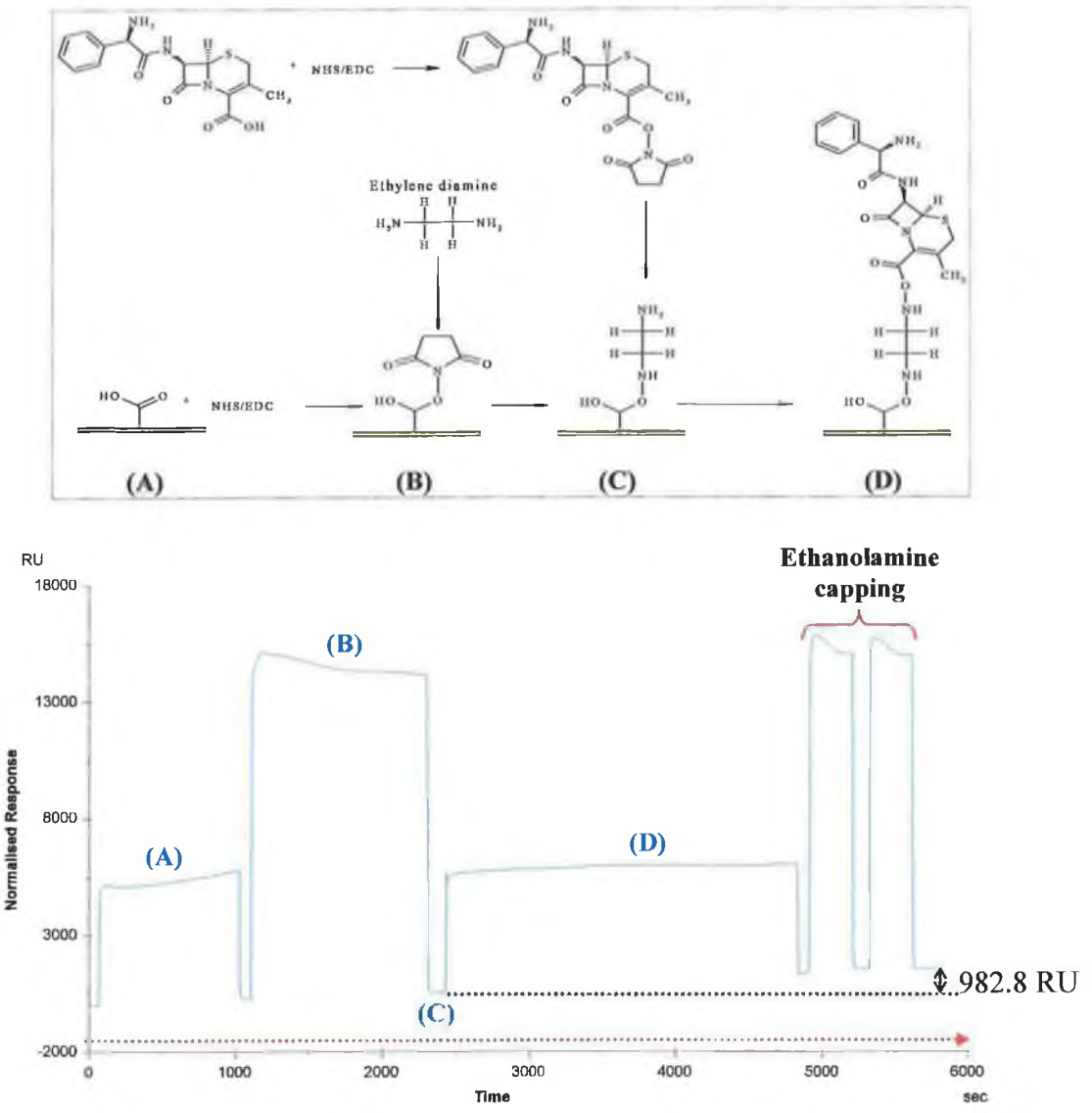


Figure 6.9. Chemistry schematic (top) and sensorgram (bottom) for the direct immobilisation of cephalexin on a CM5 Biacore sensor chip (DIC). (A) shows the initial activation of the CM5 chip surface using 100 mM NHS / 400 mM EDC for 16 min at a flow rate of 5 µl/min. Following activation, the surface was subjected to a 20 min pulse with a 1 M pH 8.5 ethylene diamine solution at 5 µl/min (B), so as to convert the surface to amine functionality (C). Cephalexin hydrate (200 µg/ml), dissolved in 100 mM NHS / 400 mM EDC, was then injected over the surface for 40 min at 5 µl/min (D). The surface was then 'double-capped' using two 5 min injections of 1 M ethanolamine hydrochloride, pH 8.5, at a flow rate of 10 µl/min, resulting in a final response of 982.8 RU.

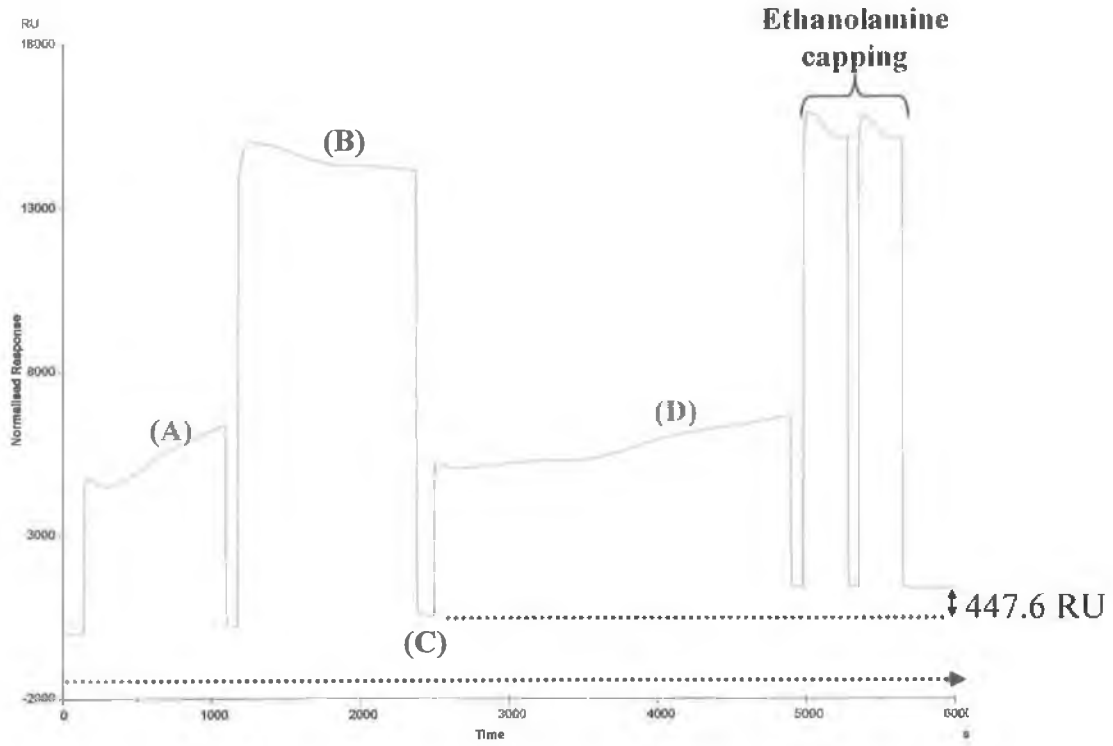


Figure 6.10. Sensorgram for the direct immobilisation control flow cell surface CM5 Biacore sensor chip (DIC). (A) Shows the initial activation of the CM5 chip surface using 100 mM NHS / 400 mM EDC for 16 min at a flow rate of 5 µl/min. Following activation, the surface was subjected to a 20 min pulse with a 1 M ethylene diamine, pH 8.5, solution at 5 µl/min (B), so as to convert the surface to amine functionality (C). A 100 mM NHS / 400 mM EDC solution was then injected over the surface for 40 min at 5 µl/min (D). The surface was then 'double-capped' using two 5 min injections of 1 M ethanolamine hydrochloride, pH 8.5, at a flow rate of 10 µl/min, resulting in a final response of 447.6 RU.

6.2.2.1 *Binding studies using monoclonal, polyclonal and recombinant single chain Fv (scFv) anti-cephalexin antibodies on a directly immobilised cephalexin (DIC) sensor chip.*

The directly immobilised cephalexin (DIC) flow cell surface, prepared as described in section 6.2.1.3, was evaluated for cephalexin-specific antibody binding. Protein G-purified monoclonal and polyclonal anti-cephalexin antibody dilutions were prepared in sterile-filtered HBS and injected over DIC and reference control flow cells for 1 min at a flow rate of 10 µl/min. A flow rate of 10 µl/min was used for throughout unless otherwise stated. The surface was regenerated using a 5-20 mM sodium hydroxide solution, depending on the antibody used and sample buffer (i.e. HBS, milk). The results showed that monoclonal (Figure 6.11) and wild type recombinant scFv antibodies (Figure 6.12) bound to the DIC with very little binding to the reference flow cell surface. Recombinant antibody was in the form of unpurified supernatant from wild type scFv-expression culture media. Hence, these results confirm that the directly immobilised cephalexin was still recognised by each of the antibodies.

In contrast the protein G-purified polyclonal antibody preparation (Figure 6.13) showed large non-specific binding to the control flow cell surface, comprising, on average, 70% of the overall response. This was an unexpected result, as it was reasoned that the DIC chip surface would reduce all non-specific binding with all antibody preparations in HBS and milk running buffers in comparison with the conjugate immobilised chip surface. There was minimal non-specific interaction between polyclonal anti-cephalexin antibody and the un-modified CM5 dextran and immobilised thyroglobulin surfaces (Figure 6.7). The reduction in non-specific interactions was observed, however, using monoclonal and recombinant anti-cephalexin antibodies.

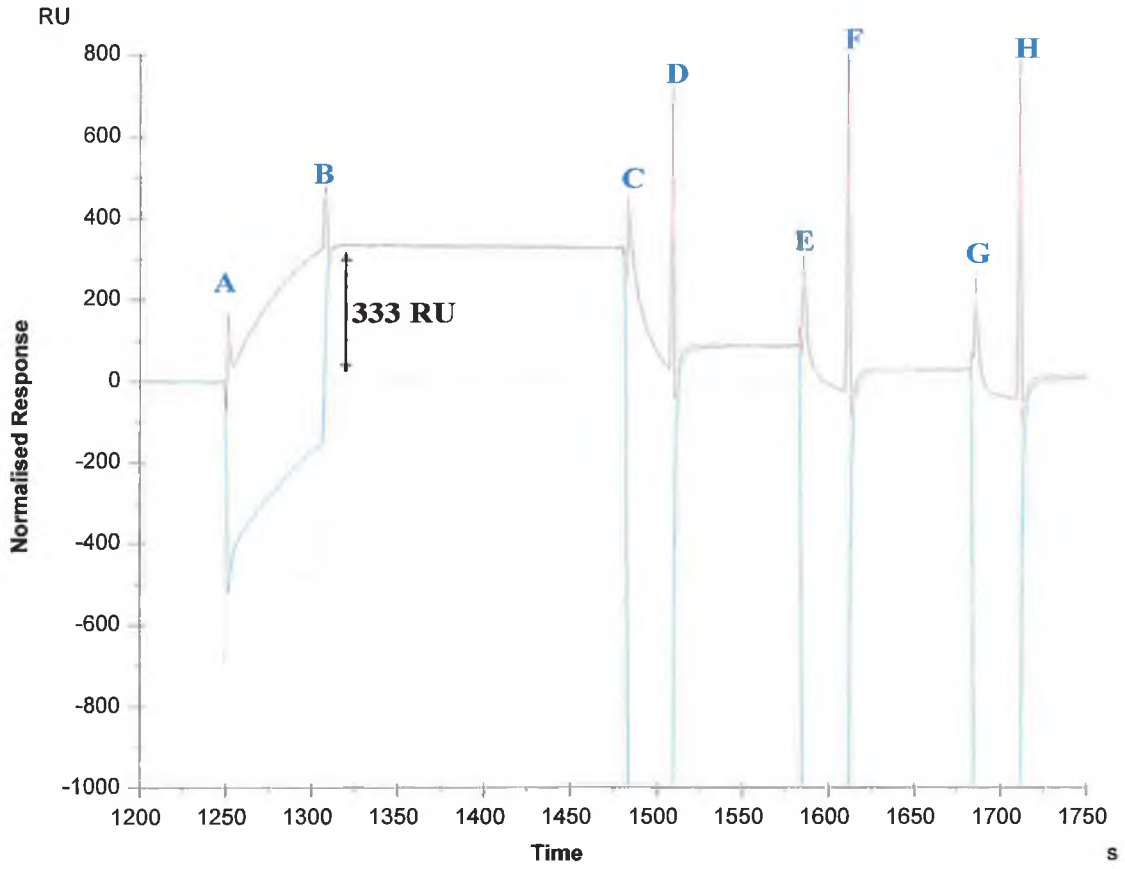


Figure 6.11. Overlay plot of protein-G purified monoclonal anti-cephalexin antibody binding sensorgrams for directly immobilised cephalexin flow cell (blue), control flow cell (grey) and the 'real-time' reference subtraction (red). Antibody was injected over the surface in order to ensure it bound to the directly immobilised cephalexin. The protein-G purified monoclonal antibody was diluted 1:20 in HBS and injected (A = injection start) over each surface for a 1 min pulse. (B = injection finish). Immediately following antibody association a standard report point response difference of 333 RU was measured. The surface was regenerated using three 30 s injections (starting at points C, E, & G) of 20 mM NaOH. The points D, F & H represent the end of each 30 s regeneration pulse.

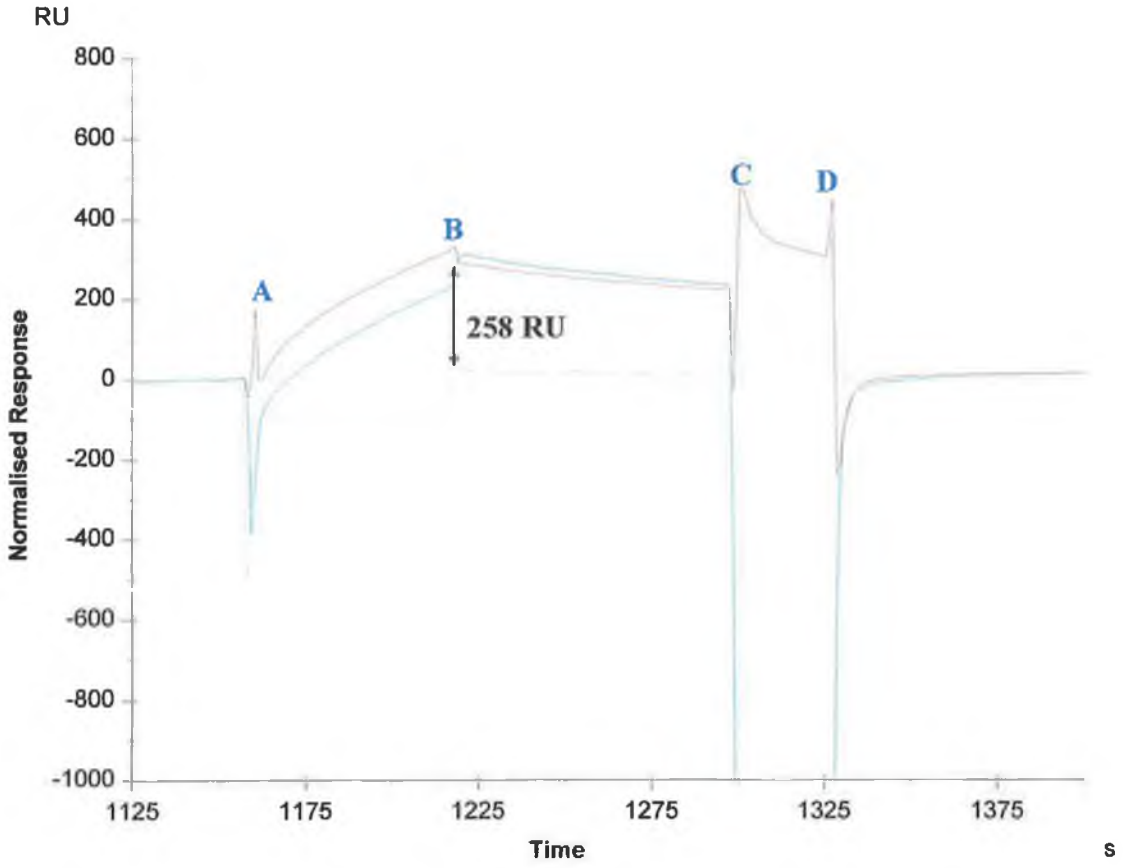


Figure 6.12. Overlay plot of wild type scFv anti-cephalexin antibody expression culture media (supernatant) binding sensorgrams for directly immobilised cephalexin flow cell (blue), control flow cell (grey) and the 'real-time' reference subtraction (red). Antibody was injected over the surface in order to ensure it bound to the directly immobilised cephalexin. The wild type supernatant was diluted 1:5 in HBS and injected (A = injection start) over each surface for a 1 min pulse. (B = injection finish). Immediately following antibody association a standard report point response difference of 258 RU was measured. The surface was regenerated using a 30 s injection (starting at point C) of 40 mM NaOH. D represents the end of the 30 s regeneration pulse.

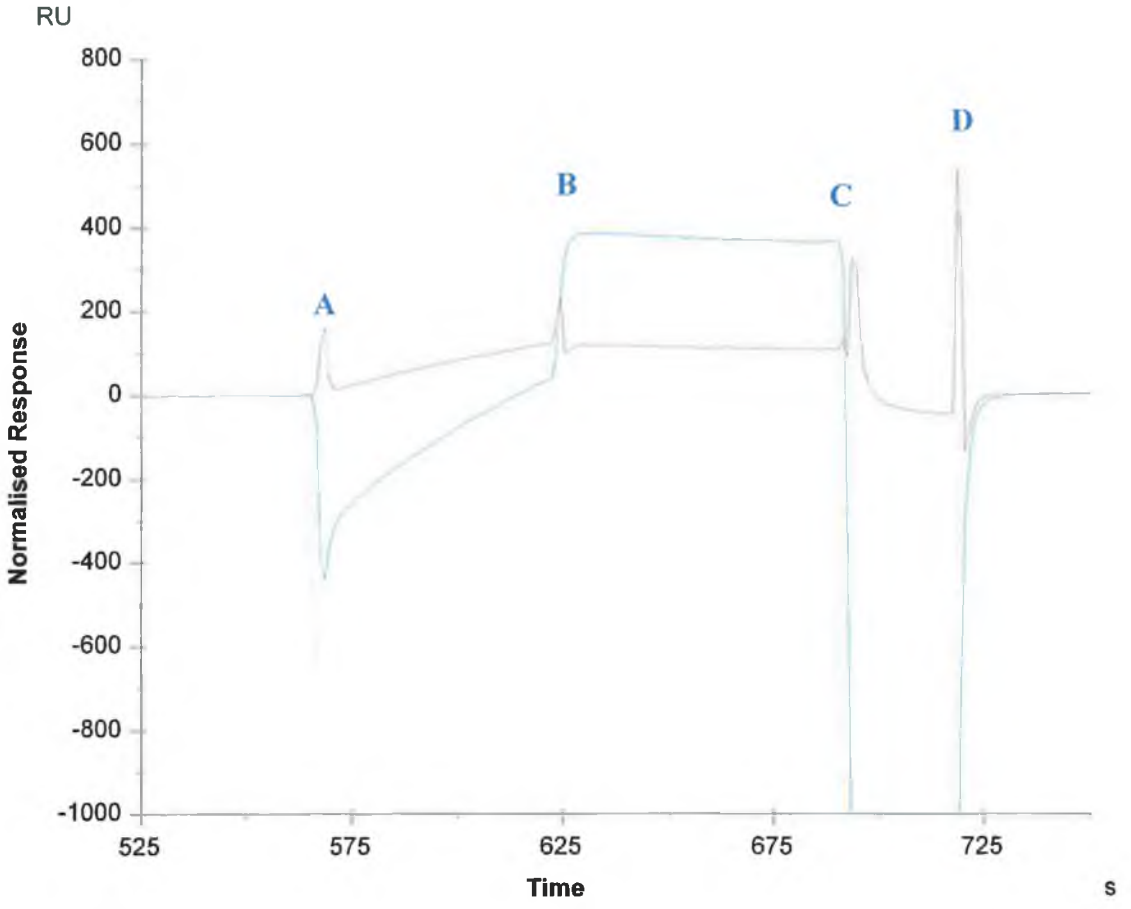


Figure 6.13. Overlay plot of protein-G purified polyclonal anti-cephalexin antibody binding sensorgrams for directly immobilised cephalexin flow cell (purple), control flow cell (grey) and the 'real-time' reference subtraction (red). Antibody was injected over the surface in order to ensure it bound to the directly immobilised cephalexin. The protein-G purified polyclonal antibody was diluted 1:100 in HBS and injected (A = injection start) over each surface for a 1 min pulse. (B = injection finish). Immediately following antibody association a standard reference-subtract response of 117 RU was measured. The DIC flow cell signal was 381 RU (purple line). However, the response observed with the reference flow cell was 263 RU (grey line). This comprised approximately 70% of the overall signal. The surface was regenerated using a 30 s injection (starting at point C) of 10 mM NaOH. D represents the end of the 30 s regeneration pulse.

6.2.2.2 *Binding studies using prepared milk samples with cephalexin conjugate (TCC) and directly immobilised cephalexin (DIC) sensor chip surfaces.*

Following demonstration of binding of each antibody preparation (i.e. polyclonal, monoclonal and recombinant), diluted in PBS-T, to DIC and TCC chip surfaces, an evaluation of the interactions between prepared milk samples and immobilised sensor surfaces (i.e. TCC and DIC) was carried out. Mammalian milk is highly complex and generally composed of water, protein, carbohydrate, fat and a variety of vitamins and minerals. Therefore, the potential for non-specific interactions with a mass-sensitive biosensor device (e.g. Biacore 3000) must be critically evaluated in order to develop a viable assay. The milk used in this work was a well known brand of full-fat processed (i.e. pasteurised and homogenised) milk purchased from the DCU campus shop. In order to assess the potential non-specific interactions with immobilised control and cephalexin CM5 flow cells (i.e. TCC and DIC chips), various milk sample preparation steps were carried out. Blank milk samples (i.e. no cephalexin added) and 'spiked' milk samples were prepared, as described in section 2.7.5, prior to injection over each sensor chip surface.

One set of the milk samples was prepared by removing the fat content (termed de-fatted). A second set of milk samples was placed in a water bath at 95° C for 3 min (Gustavsson, 2003) followed by centrifugation at 14,000 rpm at R.T. for 15 min to remove fat and whey protein (termed de-fatted and de-wheyed). Milk binding studies were carried out using prepared processed milk samples (i.e. de-fatted milk and de-wheyed / de-fatted milk) by injecting each sample set over the relevant sensor chip surface (i.e. DIC / TCC and relevant control reference flow cells) in parallel for one min and the response recorded (Figure 6.14). Any residual bound material was easily removed with a 30 s injection of 20 mM NaOH. Four replicate measurements were carried out and an average response was plotted for each sample preparation method, as shown for the DIC chip in figure 6.15. The non-specific binding observed for milk sample preparations with both flow cell surfaces (DIC and reference flow cells) were minimal and are better illustrated in figure 6.15. The maximum non-specific binding response was obtained for the de-fatted milk samples (40-45 RU). There was little difference in binding between DIC and reference flow cell surfaces for the de-fatted de-wheyed sample. Diluting the de-fatted de-wheyed sample had minimal effect on the response obtained. Undiluted (neat) and diluted 1:2 (prepared samples with PBS-T) average responses for de-fatted de-wheyed sample ranged from 5→10 RU (Figure 6.15).

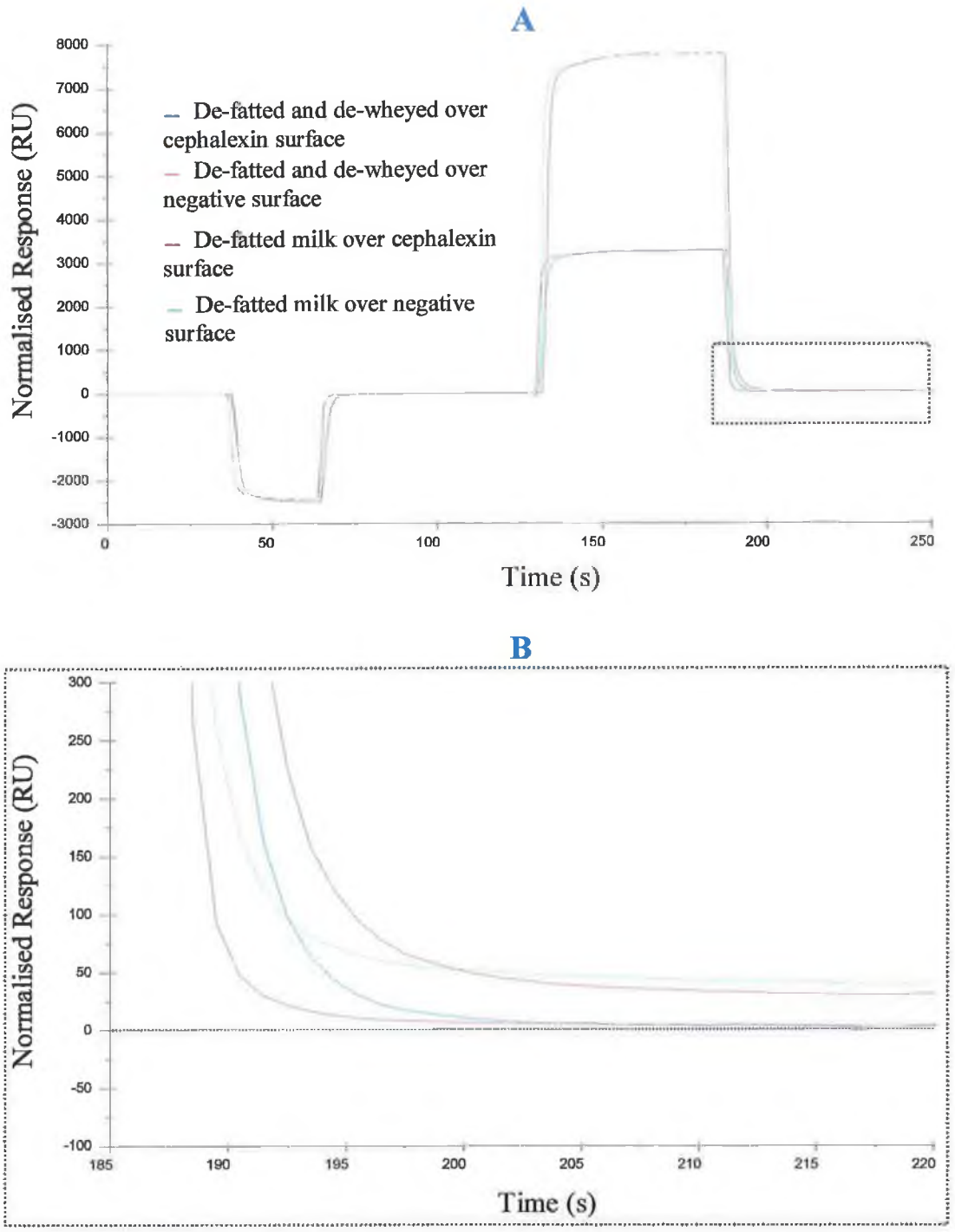


Figure 6.14. **(A)** Overlay plot of sensorgrams obtained for each of the prepared processed milk samples containing no antibody (de-fatted milk, de-wheyed and de-fatted milk) injected over directly immobilised cephalexin and negative control (i.e. reference) flow cell surfaces. Initially the surface was pulsed with the 5 mM NaOH regeneration solution. The net refractive index change (i.e. response) using the de-fatted and de-wheyed milk is significantly lower than the de-fatted milk samples as expected. **(B)** Above highlights the non-specific interactions with both surfaces (DIC and reference flow cells) were minimal.

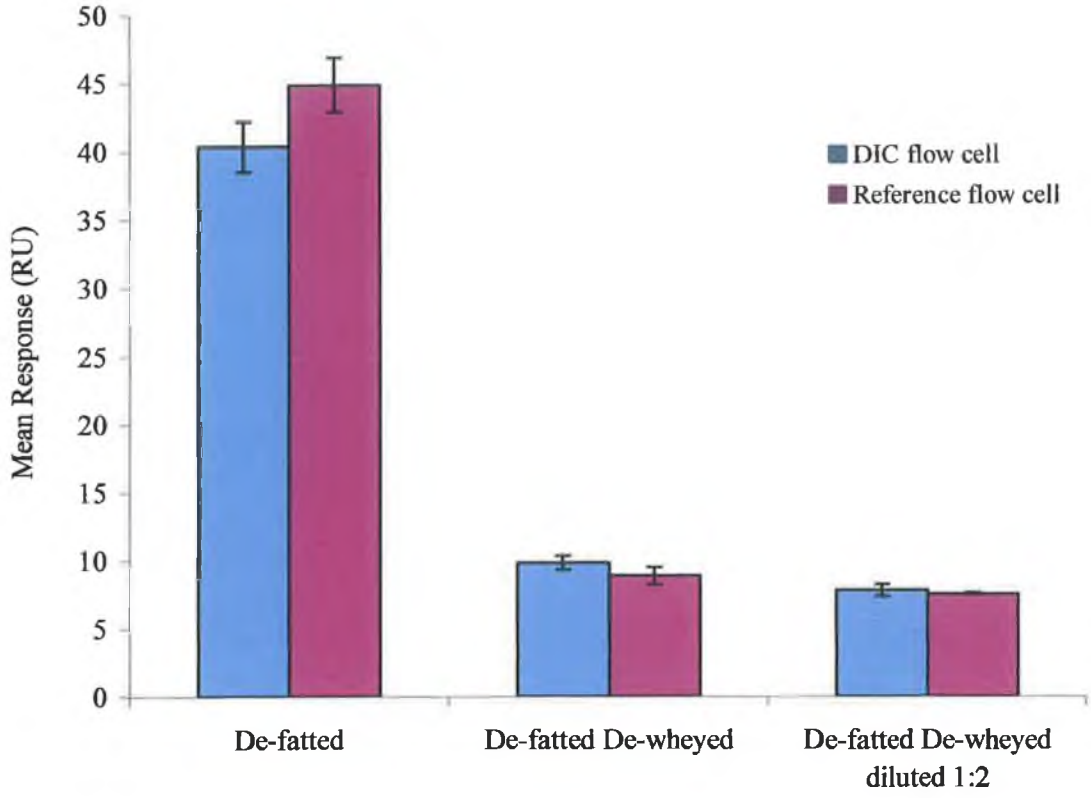


Figure 6.15. Plot of the mean (n = 4) responses obtained for each of the prepared processed milk samples containing no antibody (de-fatted milk; de-fatted milk and de-wheyed; de-fatted and de-wheyed diluted 1:2 with PBS-T) injected over directly immobilised cephalexin and negative control (i.e. reference) flow cell surfaces. Each surface was pulsed with 20 mM NaOH to remove any residual material. The highest non-specific binding response was observed for the de-fatted milk samples (40-45 RU). There was little difference in binding between both flow cell surfaces, and the de-fatted de-wheyed neat and diluted 1:2 prepared samples with PBS-T (ranged from 5→10 RU).

In comparison with the immobilised thyroglobulin-cephalexin conjugate surface prepared in section 6.2.1.2, the directly immobilised cephalexin flow cell surface showed a significant decrease, (~ 90% and 95% signal reduction) for defatted and de-fatted / de-wheyed milk samples, respectively. The DIC flow cell surface had approximately 11-fold less non-specific binding than the conjugate (TCC) flow cell (i.e. 40 RU obtained for DIC and 440 RU obtained for TCC using de-fatted milk) (Figure 6.16).

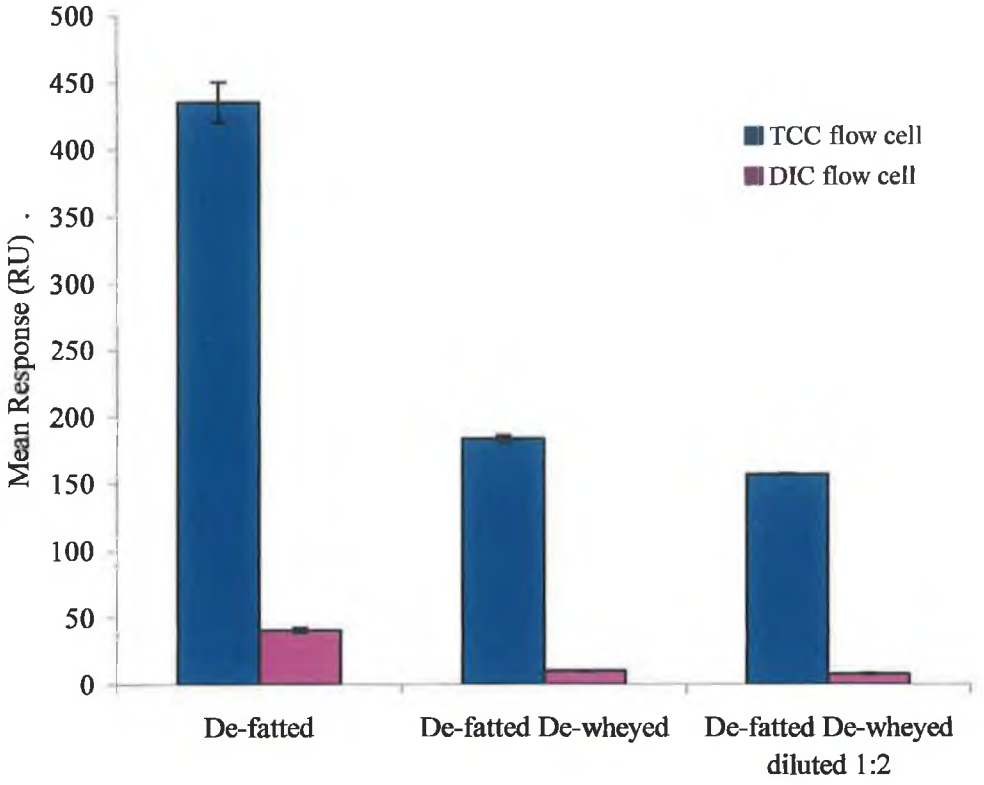


Figure 6.16. Plot of the mean (n=4) responses obtained for each of the prepared processed milk samples containing no antibody (de-fatted milk; de-wheyed and de-fatted milk; de-fatted and de-wheyed diluted 1:2 with PBS-T) injected over the immobilised thyroglobulin-cephalexin conjugate (TCC) directly immobilised cephalexin (DIC) flow cell surfaces. Each surface was pulsed with 20 mM NaOH to remove any residual material. The figure shows the decreased non-specific binding of milk components to the DIC and reference flow cells in comparison with the immobilised cephalexin conjugate surface. The Y-error bars shown indicate the standard deviation for each point.

6.2.3 *The development and validation of a Biacore 3000-based inhibition assay for the detection of Cephalexin hydrate-'spiked' PBS and processed milk.*

The Biacore biosensor 3000 has a number of advantages over conventional detection technologies, in that all analyses are carried out in 'real time' with no need for labelling, the system is automated, relatively rapid and facilitates multiple analyses using a single chip. SPR-based sensors in general essentially detect mass changes. Therefore, the specificity of the antibody used must be determined and all potential sources of non-specific interactions must be ruled out. Biacore-based assays for the detection of small molecules or haptens are invariably developed using an inhibition assay format. An inhibition assay format, as outlined in figure 6.17, was employed for each antibody and immobilised chip surface (i.e. thyroglobulin-cephalexin and directly immobilised cephalexin CM5 chips) described in this chapter. A range of cephalexin standards were prepared and injected over the relevant sensor surface. The response obtained for each measured sample was plotted as a ratio of the standard cephalexin concentration (RU) and the zero cephalexin (RU_0) sample.

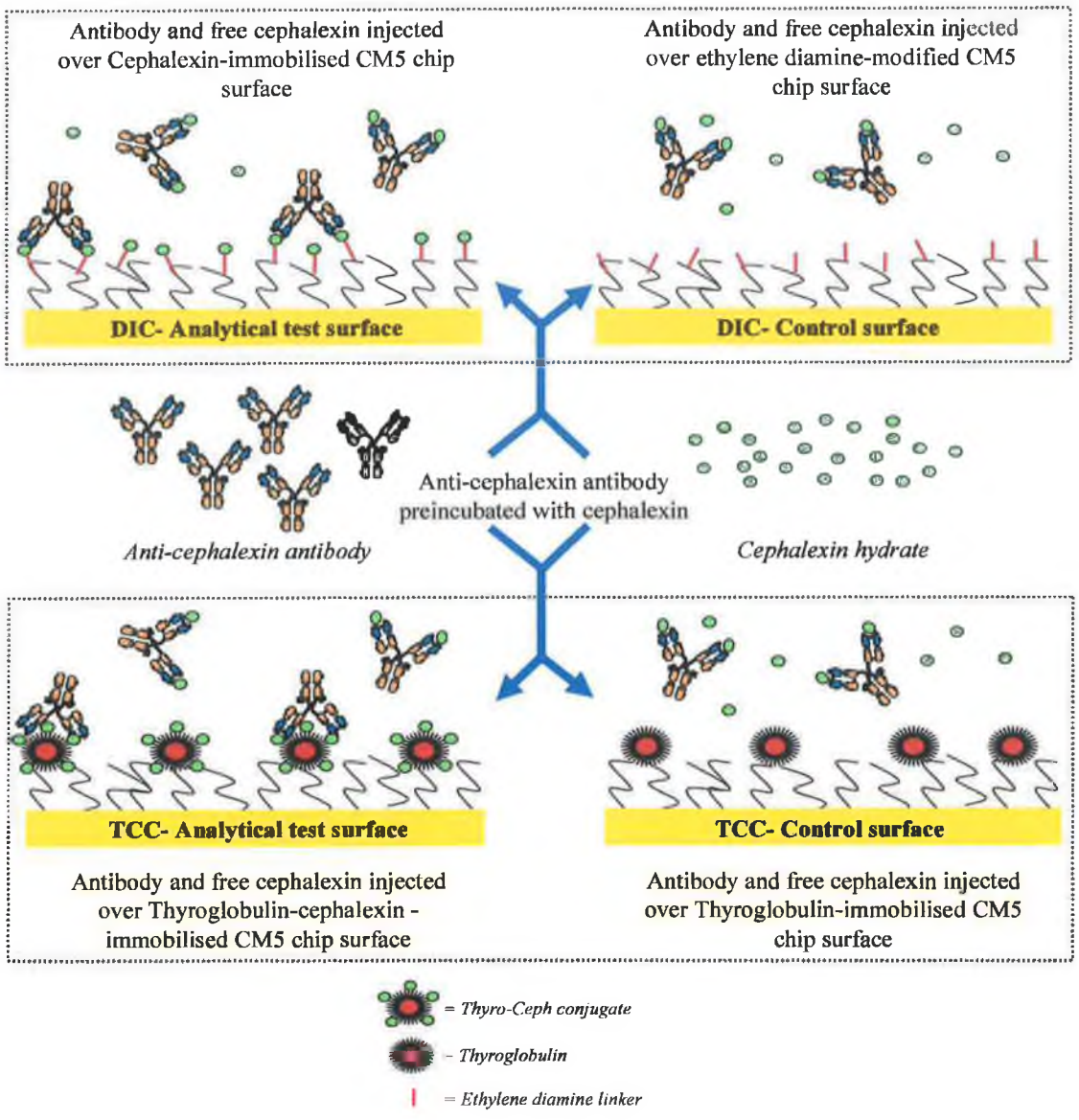


Figure 6.17. Schematic outline of the inhibition format used in the development of a Biacore 3000 SPR-based biosensor assays for the detection of cephalexin hydrate 'spiked' samples. Anti-cephalexin antibodies were pre-incubated with a range of free cephalexin standards, 0.1 mg → 0 mg (RU_0), and injected over DIC (above top) and TCC (above bottom) analytical and control surfaces and the response recorded.

As outlined in figure 6.17, anti-cephalexin antibodies were pre-incubated with a range of free cephalexin concentrations (section 2.7.6) and injected across the DIC or TCC flow cells and corresponding control surfaces. This assay format was employed with immobilised conjugate (TCC) and directly immobilised (DIC) cephalexin CM5 chip surfaces, as discussed previously (section 6.2.2.1). The degree of inhibition was inversely proportional to the observed response and each particular known free cephalexin concentration point was plotted as a ratio of its response (RU) divided by the response with no or 0 mg/ml free cephalexin, (denoted as RU_0).

The Biacore inhibition assay was developed initially using 'spiked' PBS-T as a sample matrix prior to carrying out the analysis for cephalexin in processed whole milk. The antibody dilutions were optimised to give the largest possible signal (RU), yet still at a sufficiently low concentration to be the limiting factor of the assay (i.e. yielding a typical response between 200-300 RU). The anti-*CephP1* polyclonal antibody (1:100 dilution) was pre-incubated with a range of free cephalexin standards for a 20 min period. The antibody and free cephalexin mixtures were then injected across the immobilised thyroglobulin-cephalexin surface in triplicate and the response recorded. Figure 6.18 shows a typical overlay plot taken from a set of replicate (triplicate) measurements using the TCC chip experimental and reference flow cell surfaces and polyclonal (anti-*CephP1*) antibody reported in chapter 3.

Similar studies were carried out using the DIC chip experimental and reference flow cells. Monoclonal antibody (anti-*CephM1*) was pre-incubated with a range of cephalexin standards prepared in PBS-T and were then injected over both flow cells. The results of this are shown in figure 6.19. On-line reference subtraction, where reference (i.e. treated with same chemistry as DIC surface with no cephalexin added) flow cell 3 was subtracted from DIC surface on flow cell 4. This was applied using the Biacore control software. Each surface was subjected to a 30 s injection of 20 mM NaOH after each measurement in order to regenerate the surface. The overlaid sensorgrams (Figures 6.18 & 6.19) were differentially coloured with the corresponding cephalexin standard concentration shown. There was a proportional decrease in response for each consecutive increase in concentration of soluble cephalexin.

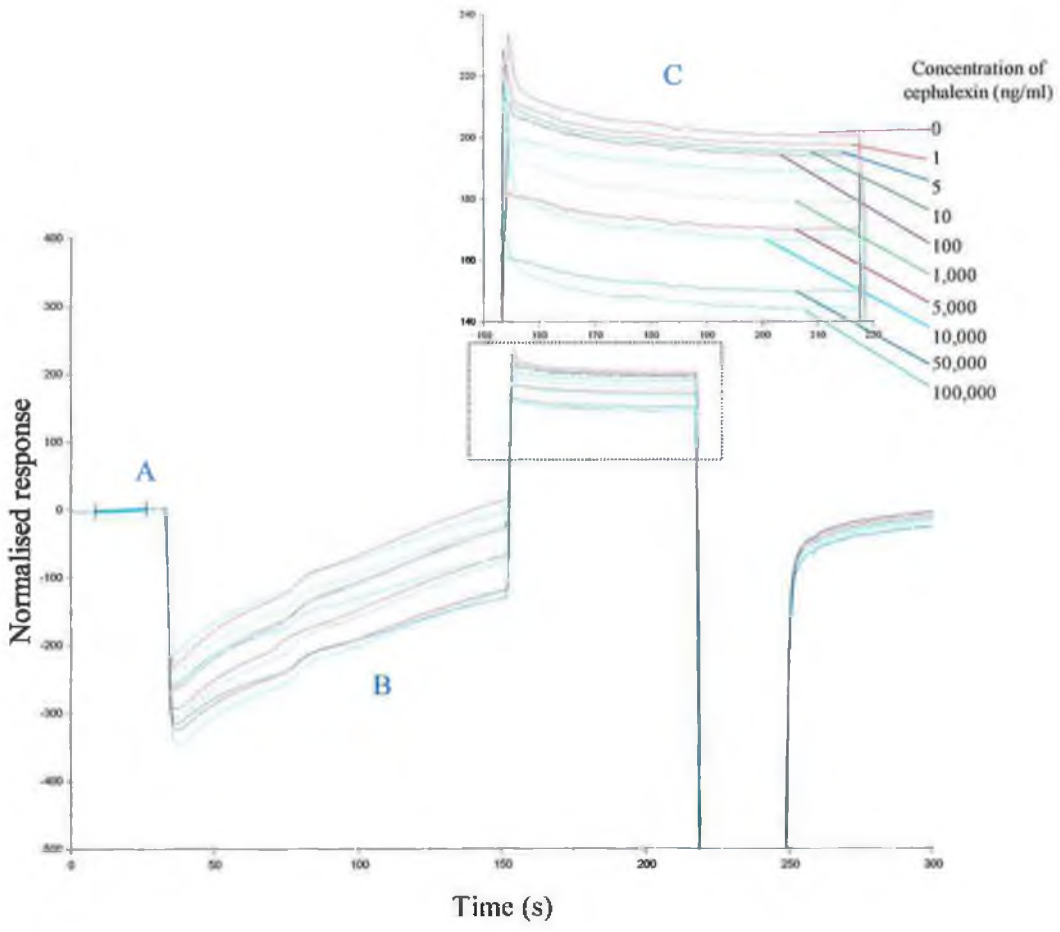


Figure 6.18. Overlay plot showing the decreasing response units (RU) per increase in the concentration of free cephalexin using the polyclonal antibody (anti-*CephP1*) on the immobilised thyroglobulin-cephalexin (TCC) flow cell surface. The baseline for each point was normalised to 0 RU. (A) Shows an initial drop, due to a refractive index change. (B) The subsequent increase due to the anti-*CephP1* antibody binding to the immobilised thyroglobulin-cephalexin surface. The plateau (C) shows the decreasing amount of antibody bound to the chip surface (RU) with increasing concentrations of cephalexin.

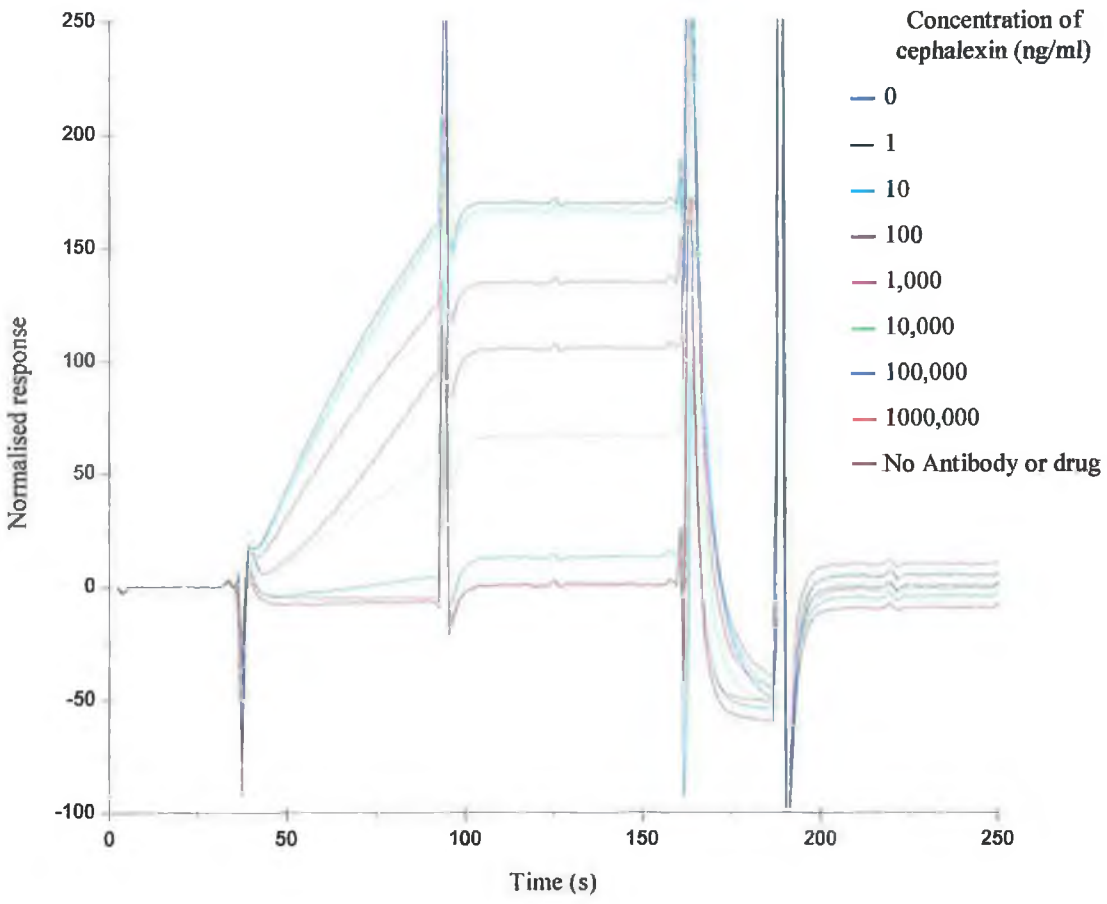


Figure 6.19. Overlay plot showing the decreasing response units (RU) per increase in the concentration of free cephalexin using the monoclonal antibody on the directly immobilised cephalexin (DIC) flow cell surface. The baseline for each point was normalised to 0 RU. Cephalexin standards were pre-mixed with anti-*CephM1* monoclonal antibody subsequent to injection over the DIC and reference surface. The sensorgrams represent the response difference between the DIC and reference flow cells. Following each injection of antibody and free drug the surfaces were regenerated using 20 mM NaOH.

6.2.3.1 *Comparative study of monoclonal antibody-based inhibition assay using thyroglobulin-cephalexin (TCC) and directly immobilised cephalexin (DIC) sensor chip surfaces.*

The TCC and DIC prepared sensor chip surfaces were evaluated in terms of inhibition assay performance. This was carried out using monoclonal anti-*CephM1* antibody (Chapter 4), and a range of cephalexin standards prepared in PBS-T. A range (i.e. 1:10 serial dilutions) of standards was prepared in order to cover a broad concentration range for each of the cephalexin-specific antibodies. Assays were carried out as described in section 2.7.6, using pre-treated 'spiked' milk samples (section 2.7.5). Results from milk control experiments using the TCC and DIC surfaces (Figure 6.16) had already indicated that there was a significant reduction in the background response using the DIC as opposed to the TCC chip. In order to further evaluate both DIC and TCC chips it was necessary to assess the inhibition assay performance using the two immobilised sensor chip surfaces. Inhibition assays carried out using the anti-*CephP1* on the TCC chip (Figure 6.18) and anti-*CephM1* on the DIC chip (Figure 6.19) had indicated that both surfaces facilitated the development of inhibition assays. As observed, there was a proportional drop in response for a range of free cephalexin concentrations using each chip surface. However, inhibition assays carried out using monoclonal anti-*CephM1* antibody showed increased assay sensitivity at the lower concentrations of free cephalexin (Figure 6.20) using the DIC, as opposed to the TCC sensor chip.

Assays developed in processed milk samples with the DIC chip (data not shown) showed significantly reduced standard deviation values for each replicate measurement in contrast to the TCC chip. The larger standard deviations obtained using the TCC may be as result of the fact that a larger fraction (i.e. at worst 50%) of the response obtained was due to non-specific binding with the reference flow cell on the TCC sensor chip. Therefore it had an intrinsically lower signal to noise ratio. It was reasoned that the DIC chip would be the more reliable surface to develop a reproducible Biacore biosensor-based assay for the detection of cephalexin in milk.

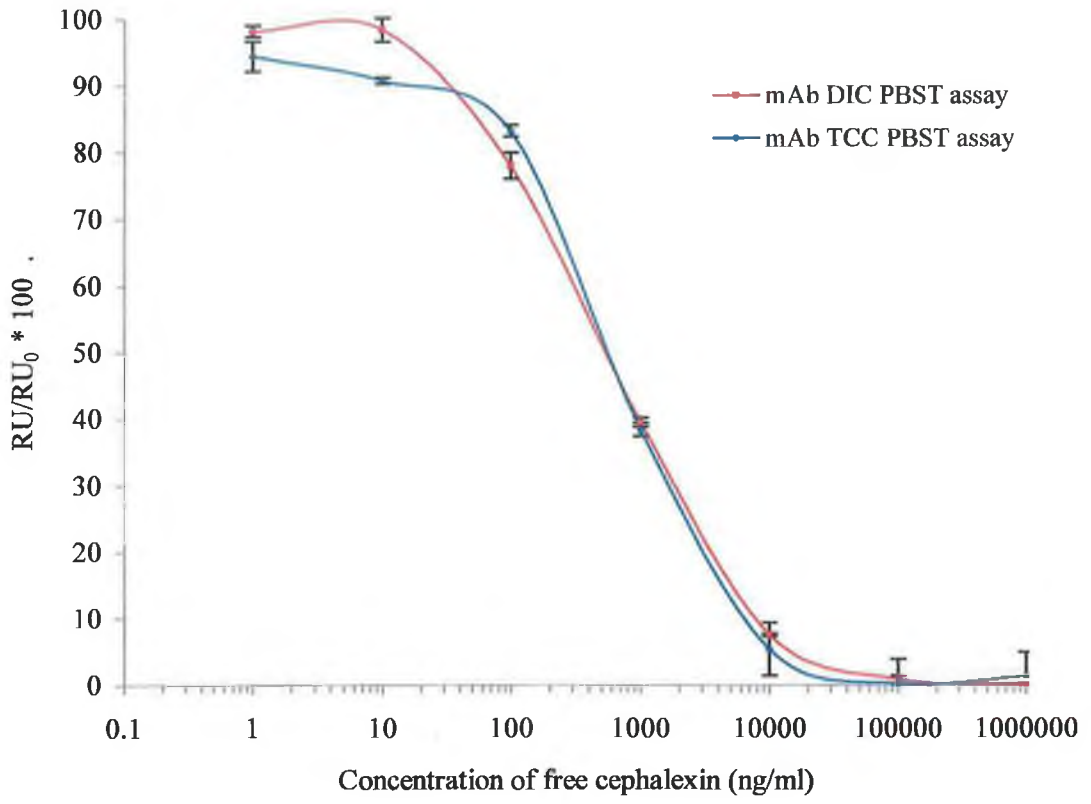


Figure 6.20. Overlay plot of Biacore inhibition assays using monoclonal anti-*CephM1* antibody. All cephalexin standards were prepared in PBS-T buffer. The standards were pre-incubated with antibody at R.T. for 15 min and injected across the surface (i.e. thyroglobulin-cephalexin (TCC) immobilised and directly immobilised cephalexin (DIC) flow cells). Each point represents the mean of three measurements, where the background response was subtracted from the experimental response. The TCC sensor surface (blue) begins to level off at 50 ng/ml, in contrast, the DIC sensor surface (red) does not begin to plateau until 10 ng/ml. The standard deviation (SD) for each point (n = 3) is highlighted.

6.2.3.2 *The development of Biacore-based inhibition assay for the detection of cephalexin in milk using polyclonal monoclonal and recombinant antibodies and a directly immobilised cephalexin (DIC) sensor chip surface.*

Inter-day assay reproducibility studies were carried out by performing each assay over a 3-day period (n = 3 for each individual point per assay) according to Wong *et al.* (1997). Four-parameter equations were fitted to each data set in order to construct calibration curves and obtain assay recoveries (i.e. back calculated values) using Biaevaluation software. Assay limits of detection were taken to be the background (i.e. RU_0, antibody with no soluble cephalexin) minus three standard deviations. The coefficient of variation and percentage accuracies were calculated using equation 6.1 and equation 6.2, respectively.

Equation 6.1.

$$\text{Accuracy (\%)} = \frac{\text{Experimental concentration of Cephalexin} - \text{Back calculated value}}{\text{Back calculated value} * 100 + 100.}$$

Equation 6.2.

$$\text{Coefficient of Variation (\%)} = \frac{\text{Standard deviation}}{\text{Mean } (RU/RU_0)}$$

Initially, polyclonal (anti-*CephP1*) antibody was evaluated in the development of Biacore-based assay for the detection of cephalexin in milk. Protein G-purified polyclonal anti-cephalexin antibody (Chapter 3) diluted in HBS buffer was found to bind non-specifically to the directly immobilised (DIC) reference flow cell (section 6.2.2.1, figure 6.13). The inhibition assay results (Figure 6.21) showed that a proportion of the protein G-purified polyclonal antibody was inhibited from binding to the immobilised cephalexin over a defined range of soluble cephalexin concentrations. However, a significant proportion of the response was due to non-specific binding (Figure 6.22) of the polyclonal and the milk sample matrix. Thus high individual standard deviation values were obtained, and hence, the assay exhibited intrinsically poor reproducibility and sensitivity. The limit of detection for the assay (i.e. based on three times the standard deviation subtracted from the mean RU_0) was 156 ng/ml. The standard deviation value obtained for the RU_0 was ± 8.3 RU.

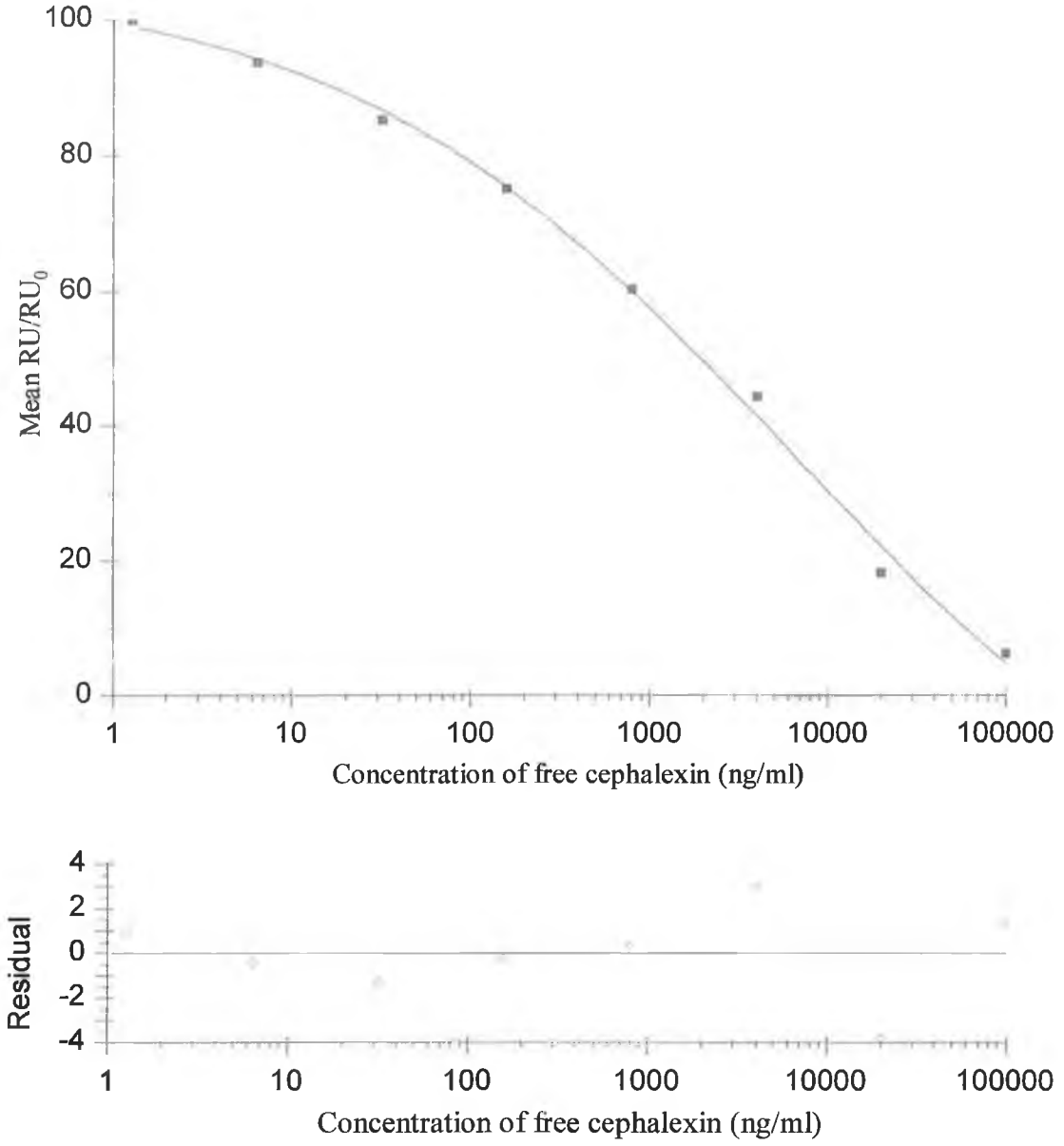


Figure 6.21. Graph representing the Biacore 'spiked' milk inhibition assay using polyclonal (anti-*CephP1*) antibody. De-fatted and de-wheyed 'spiked' milk was pre-incubated with antibody (1:100 working dilution) at R.T. and subsequently injected over the directly immobilised sensor chip (i.e. reference and cephalexin flow cells) utilising the online reference subtraction function to obtain data points. Each point represents the mean (n = 4) response (RU) divided by the response with no free cephalexin present (RU_0). Measurements were carried out in triplicate. The standard deviation values obtained for each replicate data point were unacceptably large. The residual values shown above represent the 'closeness of fit' of the data set to the four-parameter calibration curve. The limit of detection for the assay was calculated by taking 3 times the standard deviation from the mean RU_0 and was found to be 156 ng/ml.

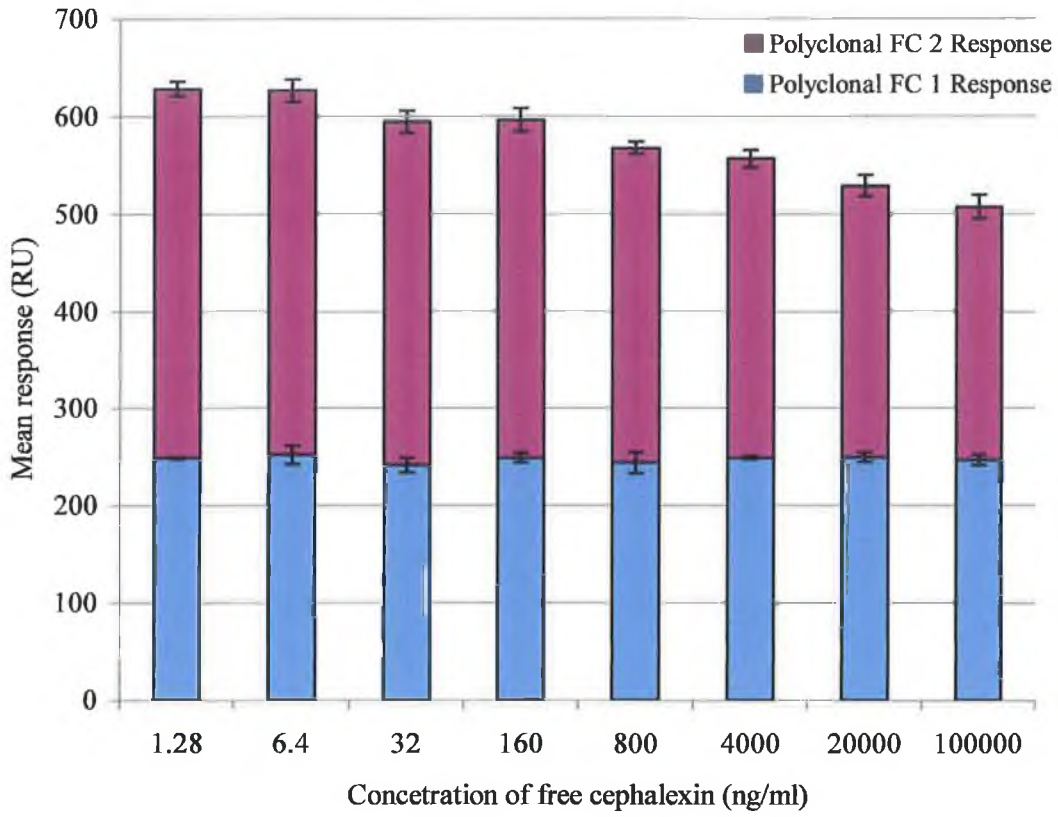


Figure 6.22. Bar plot of the mean response values obtained for the Biacore 'spiked' milk inhibition assay using polyclonal (anti-*CephP1*) antibody. The mean response for each flow cell is illustrated above. The directly immobilised cephalexin flow cell (red) shows the proportional decrease as the concentration of cephalexin increases. The mean response to the reference flow cell (blue) demonstrates little fluctuation across the range of cephalexin concentrations tested. The results illustrated the large degree of non-specific interactions observed using the anti-cephalexin polyclonal antibody. This contributes to the large standard deviations observed for each individual measurement in milk.

Similar inhibition assay development studies were carried out using the monoclonal (Chapter 4, anti-*CephM1*), wild type (WT) scFv, mutant C5 and H3 scFv antibodies described in chapter 5 of this thesis. Inter-day assay reproducibility studies were performed on the optimised inhibition assays. Assays were carried out on three consecutive days (Wong *et al.*, 1997). Protein G-purified monoclonal anti-cephalexin antibody was diluted in de-fatted and de-wheyed milk as described previously. The milk was 'spiked' with a range of cephalexin

concentrations from 0→ 100,000 ng/ml. Each sample preparation was assayed in triplicate on each separate day and the mean RU/RU_0 for each day determined. For the monoclonal antibody, a four-parameter equation was fitted to the data set, as shown in figure 6.23. The limit of detection for the assay was 72 ng/ml. The mean response (RU/RU_0), back calculated values, percentage coefficients of variation (CV) and accuracies are shown in table 6.2.

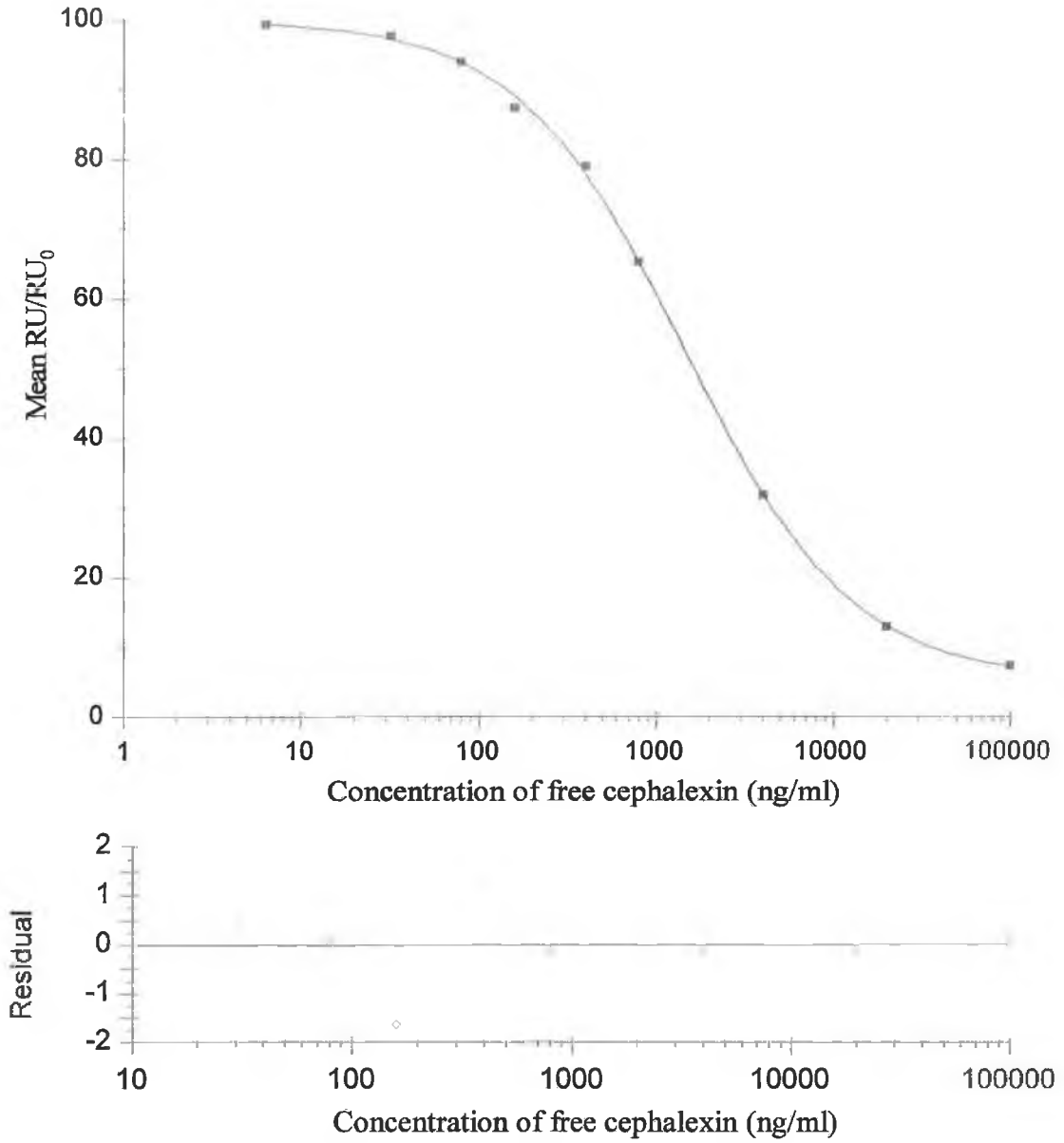


Figure 6.23. Biacore inhibition inter-day assay studies using monoclonal anti-*CephM1* antibody. The limit of detection (LOD) taken as the background minus 3 times standard deviations and was 72 ng/ml. Thus the monoclonal antibody-based inhibition assay provides a suitable analytical method for the detection of cephalexin below its respective MRL (100 ng/ml) in milk samples.

Table 6.2. Values obtained for the Inter-day Biacore inhibition assay in 'spiked' milk samples with monoclonal (anti-*CephM1*) antibody. Back calculated values were obtained by fitting a four-parameter equation to the data set using Biaevaluation software. The percentage accuracy and CV's were calculated using equations 3.1 and 3.2.

Response $RU/RU_0*100 \pm$ S.D.	Concentration of Cephalexin (ng/ml)	Back Calculated value (ng/ml)	% CV's	% Accuracies
7.37 ± 0.48	100,000	91,996.3	6.55	91
12.93 ± 0.49	20,000	20,319	3.80	101
31.74 ± 1.62	4,000	4,035.4	5.11	101
65.05 ± 1.46	800	804.8	2.24	101
78.91 ± 0.28	400	370.4	0.36	92
87.25 ± 1.80	160	189.9	2.06	115
93.80 ± 1.30	80	78.8	1.38	97
97.63 ± 1.49	32	25.2	1.52	72
99.28 ± 1.29	6.4	5.4	1.30	82

For each scFv antibody (e.g. WT, C5 & H3), the assays were carried out in an analogous manner. However, intra-day studies were used. Assays were carried out in triplicate within a single day and four-parameter equations fitted to the data sets. The results for the WT assay are shown in figure 6.24. The limit of detection for the assay was 111 ng/ml. The mean response (RU/RU_0), back calculated values, percentage coefficients of variation (CV's) and accuracies are shown in table 6.3. The mutant C5 and H3 scFv antibody-based assays results are plotted in figures 6.25 and 6.26, respectively. The back calculated values, percentage CV's and accuracies are shown in tables 6.4 (C5 scFv) and 6.5 (H3 scFv). The C5 clone scFv gave the best assay performance in terms of detection limit in milk samples, with a LOD of 84 ng/ml, and therefore, is a suitable assay for detection of cephalexin in milk below the defined MRL.

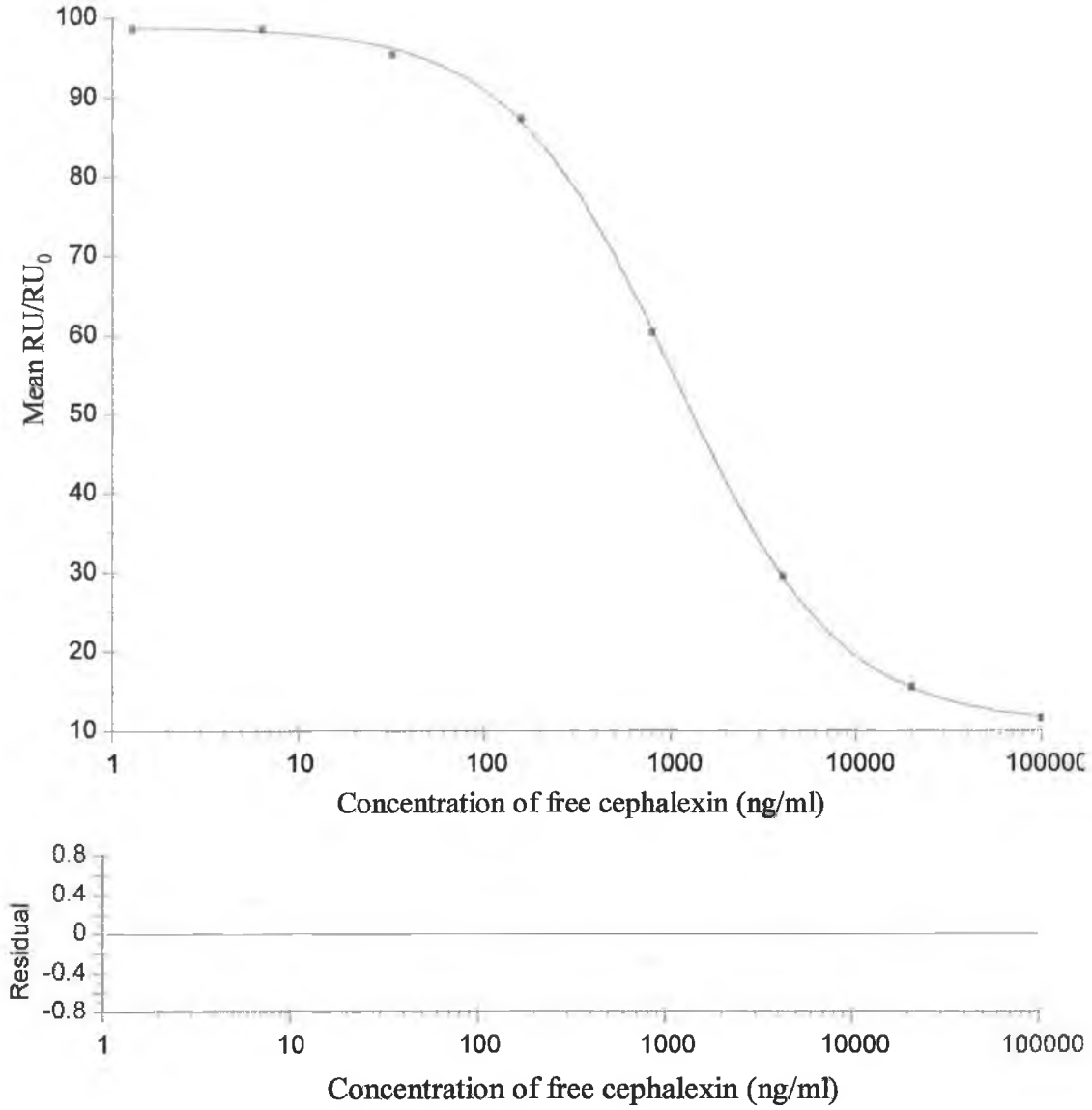


Figure 6.24. Biacore 3000 inter-day assay studies plot using the WT scFv antibody. The assay was carried out in whole milk on a directly immobilised chip. The calculated mean RU/RU_0*100 values are plotted against each of the cephalexin concentration points. Each point is a mean representation of 9 measurements. The assay limit of detection (i.e. background minus 3 times the standard deviation) was 111 ng/ml.

Table 6.3. WT scFv Biacore 3000 inhibition assay carried out in whole milk on a directly immobilised chip. The calculated mean RU/RU_0 values, standard deviations and coefficients of variation for each concentration point are shown.

Response $RU/RU_0*100 \pm$ S.D.	Concentration of Cephalexin (ng/ml)	Back Calculated value (ng/ml)	% CV's	% Accuracies
11.63 ± 0.82	100,000	122,895.2	7.06	81
15.59 ± 0.33	20,000	19,198.6	2.14	104
29.48 ± 0.42	4,000	3,975.2	1.44	101
60.28 ± 0.07	800	809.9	0.12	98
87.29 ± 0.03	160	151.9	0.04	105
95.35 ± 0.27	32	40.2	0.29	80
98.56 ± 1.40	6.4	2.6	1.42	146
98.67 ± 3.45	1.3	1.4	3.46	92

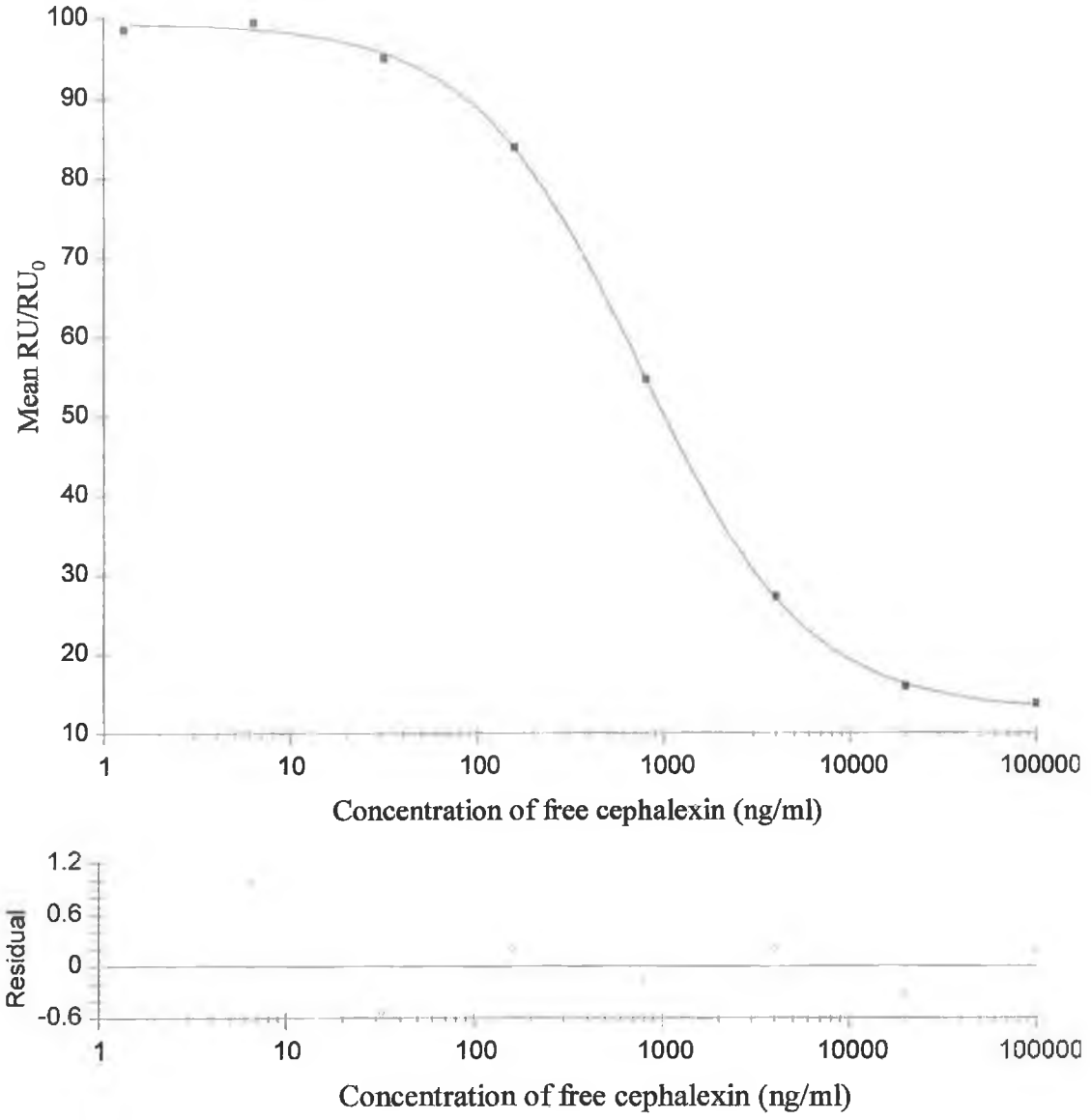


Figure 6.25. Results of a Biacore 3000 inhibition displacement assay carried out in 'spiked' whole milk samples. The assay was carried out using the C5 mutant scFv antibody. The assay was carried out in whole milk on a directly immobilised cephalexin chip. The calculated mean RU/RU_0*100 values are plotted against each of the cephalexin concentration points. Each point is a mean representation of 9 measurements with the respective residuals shown in the lower portion of the figure. The assay limit of detection (i.e. background minus 3 times the standard deviation) was 84 ng/ml.

Table 6.4. C5 mutant scFv Biacore 3000 inhibition assay carried out in whole milk on a directly immobilised chip. The calculated mean RU/RU_0 values, standard deviations and coefficients of variation for each concentration point are shown. Some values could not be calculated, due to poor fit to the calibration curve (---).

Response $RU/RU_0*100 \pm$ S.D.	Concentration of Cephalexin (ng/ml)	Back Calculated value (ng/ml)	% CV's	% Accuracies
13.68 ± 0.45	100,000	76,214.2	3.28	131
15.86 ± 0.94	20,000	22,256.1	5.94	90
27.14 ± 0.67	4,000	3,956.4	2.47	101
54.47 ± 0.05	800	801.5	0.10	100
83.69 ± 1.85	160	159.2	2.21	101
95.89 ± 3.21	32	33.4	3.35	96
99.43 ± 0.81	6.4	---	0.81	---
98.67 ± 3.45	1.3	5.9	2.03	22

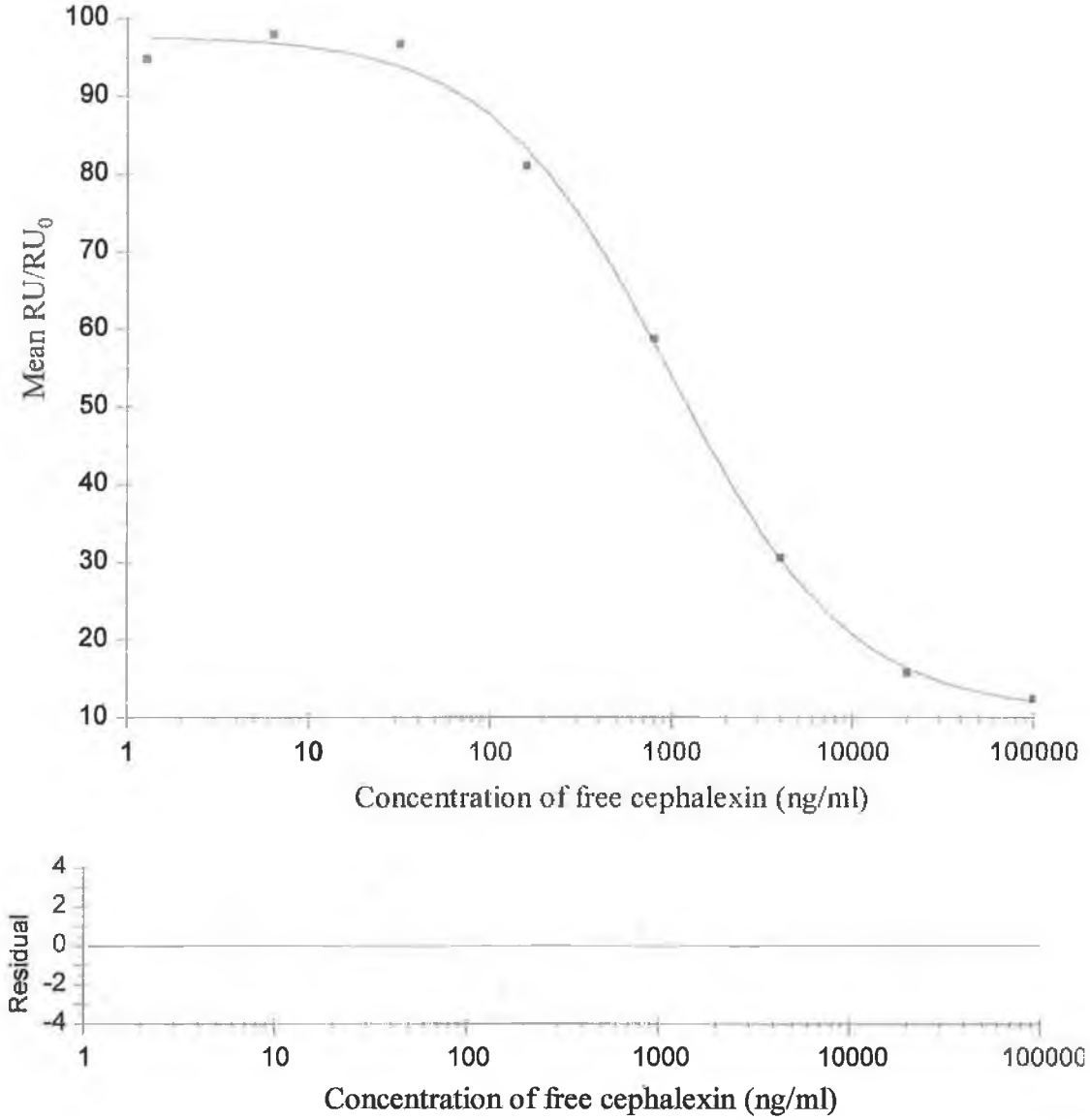


Figure 6.26. Biacore 3000 displacement inhibition assay plot of the H3 scFv antibody assays. The assay was carried out in whole milk on a directly immobilised chip. The calculated mean RU/RU_0*100 values are plotted against each of the cephalexin concentration points. Each point is a mean representation of 9 measurements. The assay limit of detection (i.e. background minus 3 times the standard deviation) was 146 ng/ml.

Table 6.5. H3 scFv Biacore 3000 inhibition assay carried out in whole milk on directly immobilised chip. The calculated mean RU/RU_0 values, standard deviations and coefficients of variation for each concentration point are shown. 'Low' represents a point at which the experimental RU/RU_0 value does not intersect the four-parameter fit and thus no back calculated value could be obtained. Some values could not be calculated, due to poor fit to the calibration curve (---).

Response $RU/RU_0*100 \pm$ S.D.	Concentration of Cephalexin (ng/ml)	Back Calculated value (ng/ml)	% CV's	% Accuracies
12.42 ± 1.30	100,000	79,956.2	10.48	125
15.82 ± 2.77	20,000	22,744.3	17.54	88
30.54 ± 2.86	4,000	3,934.9	9.36	102
58.79 ± 2.94	800	775.1	5.00	103
80.97 ± 1.79	160	193.1	2.22	83
96.65 ± 3.50	32	6.6	3.62	484
97.86 ± 2.81	6.4	---	2.87	---
94.82 ± 9.26	1.3	21.3	9.77	6.1

An overlay plot (Figure 6.27) of the three resulting calibration curves obtained for the WT, C5 and H3 mutant scFvs was constructed to highlight the improved linear range of the C5 mutant in comparison to the WT and H3 mutant scFvs. The C5 mutant proved to be the most sensitive scFv antibody to cephalexin spiked samples, having a limit of detection of 84 ng/ml. The mutant H3 clone assay suffered from poor reproducibility, with relatively high standard deviation values recorded for each point. In addition to intra-day assay studies, inter-day assay studies were also carried out using C5 mutant scFv, extra assay points were also added to the range of cephalexin 'spiked' standards. The resulting calibration plot of this experiment is shown in figure 6.28 and calculated values in table 6.6.

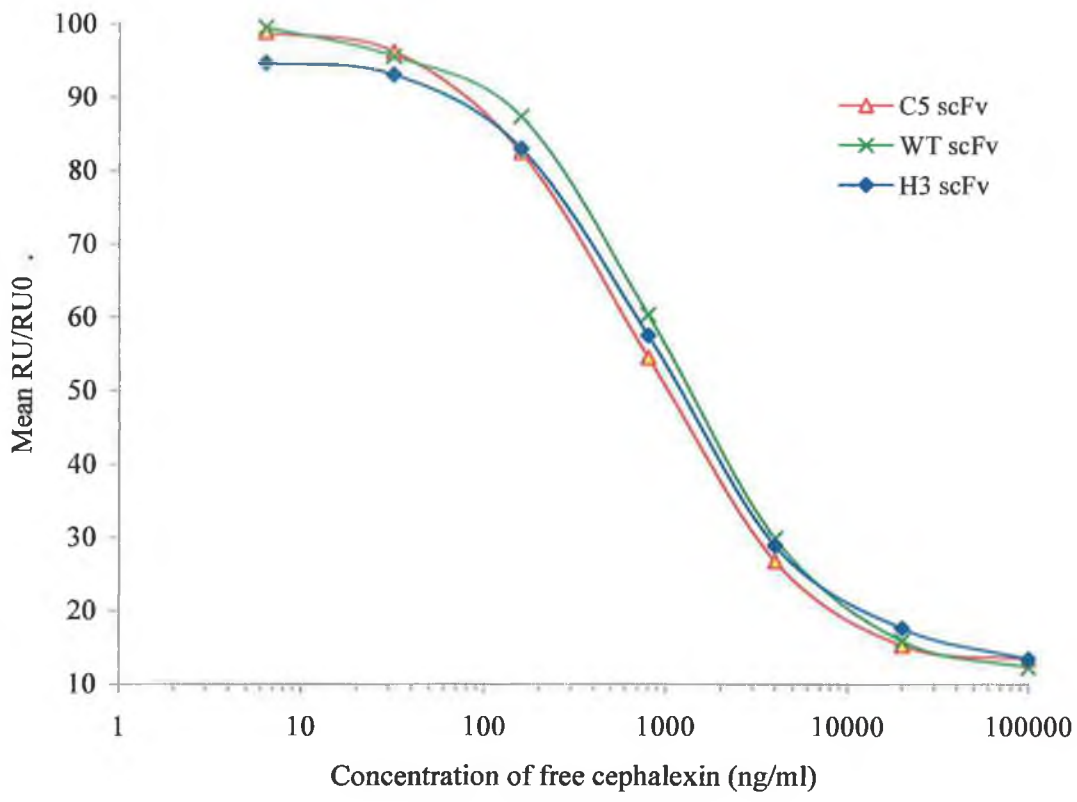


Figure 6.27. Overlay plot of the Biacore 3000 displacement inhibition assays for the IMAC-purified WT, C5 and H3 clone scFvs. The assays were carried out using 'spiked' whole milk samples on a directly immobilised cephalexin chip. The calculated mean RU/RU_0*100 values were plotted against each of the cephalexin concentration points.

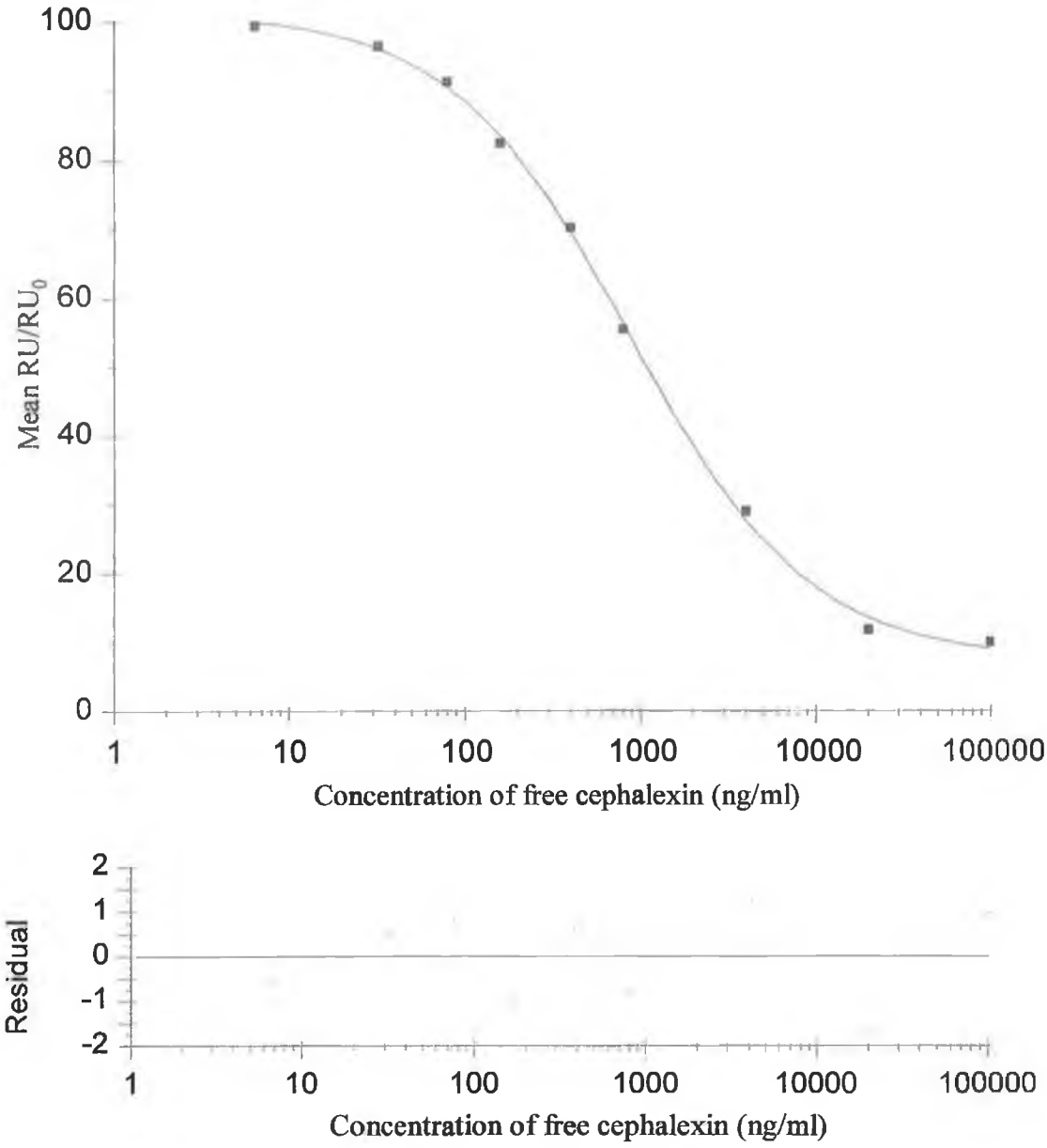


Figure 6.28. Biacore inhibition inter-day assay studies using the IMAC-purified C5 clone scFv anti-cephalexin antibody. The assay was carried out, as described previously, but including additional measurement points. The limit of detection (LOD) taken as the background minus 3 times the standard deviation and was 84 ng/ml. Thus, the C5 mutant clone scFv assay is suitable for the detection of cephalexin below its respective MRL (100 ng/ml) in milk.

Table 6.6. Values obtained for the Inter-day Biacore inhibition assay in 'spiked' milk samples with C5 scFv antibody. Back calculated values were obtained by fitting a four-parameter equation to the data set using Biaevaluation software. The percentage accuracy and CV's were calculated using equations 6.1 and 6.2.

Response $RU/RU_0*100 \pm$ S.D.	Concentration of Cephalexin (ng/ml)	Back Calculated value (ng/ml)	% CV's	% Accuracies
10.00 ± 0.92	100,000	58,577.1	10.01	170
11.83 ± 1.01	20,000	30,309.9	7.82	66
28.92 ± 2.25	4,000	3,650.4	8.59	110
55.33 ± 3.01	800	833.5	5.30	96
70.04 ± 2.69	400	386.2	3.70	104
82.33 ± 1.22	160	173.7	1.42	92
91.22 ± 0.69	80	72.2	0.77	111
96.43 ± 1.66	32	28.4	1.73	113
99.34 ± 1.71	6.4	9.2	1.74	70

6.3. Discussion

This chapter describes the development of specific assays for the detection of cephalexin in milk using a Biacore 3000™ system. One of the first demonstrations of the use of Biacore systems to detect foreign substances in food (Steresjö *et al.*, 1995) utilised polyclonal antibodies raised against sulfamethazine. The assay was successfully applied to the detection of sulfamethazine in HBS, raw and skimmed milk spiked samples. In this research, CM5 sensor chips were used in the production of immobilised cephalexin-thyroglobulin (TCC) and directly immobilised cephalexin (DIC) surfaces. Thyroglobulin-cephalexin was covalently immobilised to the carboxymethylated-dextran surface (CM 5) Biacore chip flow cell surface at a level of 14,000 RU. The reference flow cell for the TCC sensor chip consisted of immobilised thyroglobulin (13,360 RU). The stability of the immobilised TCC surface was evaluated by carrying our successive antibody binding and regeneration cycles. The DIC sensor chip surface yielded 983 RU during the immobilisation of cephalexin. The reference flow cell for the DIC chip consisted of a flow cell exposed to the exact same chemistries with the exception of cephalexin. The DIC reference flow cell yielded a response of 448 RU, therefore, the net directly immobilised cephalexin was taken as equivalent to 535 RU. The TCC, DIC and relevant control surfaces were evaluated in terms of antibody-binding and non-specific interactions with milk as a sample matrix prior to assay development.

Milk sample pre-treatment was also investigated as a means of reducing the background response observed when using a complex sample matrix. The milk was fractionated into lipid and aqueous phases by initial centrifugation at 13,000 rpm and the aqueous phase decanted and used for analysis (termed 'de-fatted'). This pre-treatment step resulted in a slightly lower non-specific binding response against both TCC and DIC flow cells (Figure 6.16). An additional sample pre-treatment step involving heating samples in a water bath at 95°C for 3 min and then centrifugation at 13,000 rpm for 15 min (termed 'de-fatted' and 'de-wheyed'). This sample treatment further reduced the non-specific binding with TCC and DIC flow cell surfaces. The milk sample pre-treatment method employed was a modified version of that reported by Gaudin *et al.* (2001) and Gustavsson (2003). The DIC sensor surface and milk sample pre-treatment resulted in an average 95% decrease in non-specific response in comparison with the TCC surface. The directly immobilised cephalexin flow cell surface was found to have the lowest background response to milk samples in comparison with the thyroglobulin-cephalexin immobilised (TCC) flow cell surface.

The polyclonal anti-*CephP1* (Chapter 3), monoclonal anti-*CephM1* (Chapter 4) and recombinant scFv antibodies (WT, C5 & H3, Chapter 5) were used to develop an inhibition assay for the detection of cephalexin. The inhibition assay format (Figure 6.17) was chosen due to the relative small molecular weight of the target analyte in this case (i.e. cephalexin). Inhibition assays are generally reported in the literature when using SPR-based biosensor devices for the detection of low molecular weight analytes. Initial polyclonal and monoclonal antibody assays showed, as expected, proportional decreases in response to TCC and DIC flow cells with subsequent increases in soluble (or free) cephalexin (Figures 6.18 & 6.19). A comparative study using both DIC and TCC sensor chip surfaces and monoclonal antibody was carried out in order to assess any potential difference in inhibition assay performance between the two surfaces. The assays were carried out in an identical manner for both surfaces using monoclonal antibody and cephalexin-'spiked' PBS-T. The results of this further suggested, in conjunction with non-specific interaction analysis, that the DIC sensor chip was the optimal format for further assay development. The inhibition assay using polyclonal anti-*CephP1* antibody was unexpectedly found to have intrinsically high variation with a large degree of binding to the reference flow cell. Online reference subtraction was employed in assay development and highlighted the fact that a large percentage ($\leq$50% at worst) of polyclonal response was non-specific in nature (Figure 6.22). This high non-specific response was only observed using protein G-purified polyclonal antibody. The apparent 'sticky' nature of the purified polyclonal antibody contributed to the high relative standard deviation and in turn the higher estimation of the limit of detection in comparison with its performance in the ELISA-based assay (20 ng/ml).

Monoclonal anti-*CephM1* antibody described in chapter 4 of this thesis was also used to develop a Biacore inhibition assay for the detection of cephalexin in milk. Inter-day assay variability studies were carried out using protein G-purified monoclonal and 'spiked' milk that had been pre-treated by 'de-fatting' and 'de-wheying'. In contrast to the polyclonal antibody-based assay, minimal non-specific interaction (<2%) was observed with reference flow cell surface. The limit of detection was below the defined MRL value at 72 ng/ml (Figure 6.23). The monoclonal assay was reproducible and had low coefficients of variation (<10%), in addition the calculated percentage accuracies (i.e. sample recovery) were acceptable over the concentration range of standards (Table 6.2).

Other beta-lactam antibiotic Biacore-based detection techniques have been reported in the literature with varying degrees of success in terms of sensitivities. Gaudin *et al.* (2001) reported a polyclonal antibody-based assay for the detection of penicillin residues in milk using the Biacore X system. The assay was not capable of detecting cephalosporins among

other beta-lactams, due to the antibodies used. The authors do, however, suggest that the particular antibody is the limiting factor in applying such biosensor systems to the analysis beta-lactam antibiotics in biological matrices. The antibody (polyclonal anti-ampicillin) binding response to open and closed ring ampicillin was different due the unstable nature of some beta lactam antibiotic ring structures (may be readily opened by hydrolysis, enzymatic or chemical means). This is particularly true of ampicillin, which they used to directly immobilise onto Biacore CM5 chip surface. The assay sensitivity was dependant on the particular antibody binding response to open and closed forms of the antibiotic.

A generic beta-lactam Biacore-based detection approach was taken by Cacciatore *et al.* (2004). They report the use of immobilised digoxigenin antibody, a penicillin binding protein (PBP2x*) to form complexes with a range penicillin and cephalosporins and a digoigenin-labelled (DIG-Amp) ampicillin conjugate. An inhibition assay format was used where sample was pre-incubated with PBP2x*, followed by incubation with DIG-Amp. In the presence of free beta-lactam there would be a decrease in the binding of PBP-DIG-Amp complex to the immobilised digoxigenin antibody. This method was successful for detecting a wide range beta-lactam antibiotics at quoted MRL values in milk samples. These included benzylpenicillin, ampicillin, amoxicillin, cloxacillin, cephalexin, and cefoperazone.

IMAC-purified recombinant antibodies (WT, C5, H3) described in chapter 5 were also used in the development of a Biacore inhibition assay for the detection of cephalexin. The optimum scFv in terms of assay performance was found to be the one expressed by mutant C5. This corroborates the findings for comparative ELISA-based assays, as described in chapter 5, section 5.2.4.3. The WT and H3 scFvs were capable of detecting cephalexin over a range of concentrations, yet had limits of detection (111 & 146 ng/ml, respectively) above the defined MRL of 100 ng/ml for cephalexin in milk. This was best illustrated in the overlay plot of the three different assays, as shown in figure 6.27.

The monoclonal and mutant C5 scFv (Figure 6.28) antibody-based inhibition assays presented in this chapter offer a reliable analytical method for the specific detection of cephalexin hydrate in 'spiked' processed milk. The limits of detection (LOD) of the monoclonal and C5 scFv antibody-based assays were found to be 72 ng/ml (Figure 6.23) and 84 ng/ml respectively. The detection limits for the monoclonal and C5 scFv antibodies are approximately equivalent to those described for the ELISA assays (60 ng/ml and 78 ng/ml respectively). The detection limits for each assay are below the required sensitivity as set out by the European Union maximum residue limit (MRL) of 100 ng/ml for cephalexin in milk (EEC council regulation No 2377/90).

Chapter 7

Overall conclusions

7.1 Overall conclusions

The purpose of this work was to develop an assay for the detection of the β-lactam antibiotic residue, cephalexin, in milk. Initial studies focused on the production and characterisation of polyclonal antibody to cephalexin, as described in chapter 1. Cephalexin-based protein conjugates were produced and characterised by non-competitive ELISA using a cephalexin-specific polyclonal antibody (designated AVAab) that was kindly donated by Dr. Aart van Amerongan. The non-competitive ELISA using AVAab was carried out to confirm the cephalexin had been covalently linked to the respective carrier protein and was still in a detectable configuration. A polyclonal antibody was produced that bound free and conjugated cephalexin. This confirmed that the cephalexin conjugates produced were satisfactory immunogens for the production of cephalexin-binding antibodies.

The polyclonal antibody was purified using saturated ammonium (SAS) precipitation, followed by protein-G affinity chromatography from rabbit serum. The purity of antibody preparation was assessed by SDS-PAGE analysis and Western blotting. It was found that the purified anti-*CephP1* polyclonal antibody recognised all of the cephalexin conjugates in a non-competitive ELISA format. Polyclonal antibody was subsequently used in the development of an inhibition ELISA assay for the detection of free cephalexin in solution (i.e. un-conjugated cephalexin). The anti-*CephP1* polyclonal antibody binding to cephalexin-conjugate was inhibited in the presence of free cephalexin. Initially an inhibition assay was developed using PBS to prepare cephalexin standards, following this cephalexin standards were prepared in whole milk (i.e. 'spiked' processed milk).

The best comparative linear range of detection for the inhibition ELISA was found to be from 1.9 ng/ml to 3906 ng/ml. Intra- and inter-day assay variability studies in PBS had limits of detection (LOD) of be 20ng/ml. The LOD's were below the required European Union maximum residue limit (MRL), currently set at 100ng/ml for cephalexin in milk samples, as set out in EEC council regulation No 2377/90. Inter-day assay variability studies carried out in cephalexin 'spiked' whole milk showed the assay LOD was below the EU-MRL, at approximately 20 ng/ml for the inhibition assay. The limits of detection achieved were comparable to other reported methods for antibiotic residue detection.

The Delvo® SP which is the standard rapid method of detection (Irish government, Department of Agriculture and Food, DAF) has a quoted sensitivity to cephalexin of 40-60 ng/ml. The anti-*CephP1* polyclonal antibody described in this chapter is specific, as demonstrated by the minimal cross-reactivity with similar β-lactam compounds and it is

capable of detection below that limit. However, PBS and milk assays performed poorly in terms of assay reproducibility, with relatively high standard deviation between assay replicates and individual measurements. In addition, there was a finite supply of homogenous antibody population which would limit its application in such assays.

Chapter 4 described the production, purification and characterisation a monoclonal antibody against cephalexin. The spleens of previously immunised mice were removed and the lymphocytes extracted for *in vitro* somatic cell fusion procedures. Hybridoma cell populations secreting antibody with the highest absorbance values were selected, scaled up and used in the development of an inhibition ELISA for the detection of cephalexin. An IgG2a-κ antibody subtype (anti-*CephM1*) was isolated and purified. It was discovered that binding of monoclonal antibody to immobilised conjugated cephalexin was inhibited over a range of soluble cephalexin concentrations. The antibody specificity was further analysed using a range of closely related β-lactam compounds and was found to be highly specific to cephalexin. However, the percentage cross-reactivity observed using cefadroxil and cefoperazone, in conjunction with results of the polyclonal antibody (Chapter 3) indicate that the probable epitope consists mainly of the three double bonded oxygens and the aromatic ring-containing side chain. The lack of significant cross reaction of both anti-*CephP1* and anti-*CephM1* antibodies with the core cephem structure of 7-amindesacetoxy-cephalosporanic acid indicated this to be the case.

Protein G-purified *anti-CephM1* showed inhibition in ELISA with standards of free cephalexin prepared in PBS-T and whole milk. The assays were linear over a range 61 ng/ml to 7812 ng/ml in PBS and 30 ng/ml to 15625 ng/ml in whole milk. The limit of detection (LOD) for the inhibition assay was 60 ng/ml in whole milk prepared samples. The milk assay showed increased linearity which may be due to increased antibody solubility and stability in a more complex biological matrix. The anti-*CephM1* antibody specificity limits its application to the specific detection of cephalexin, as opposed to the broadly specific range of compounds detected by the Delvo® SP. The lack of commercially available generic antibodies to antimicrobial compounds and the difficulty in generating such antibodies suggests that an assay incorporating several different antibodies is the optimum approach for developing a multianalyte detection technique. In conclusion, the assay is capable of the specific detection of cephalexin in 'spiked' pasteurised whole milk. The assay performance also permits the detection of cephalexin at the current EUMRL, for cephalexin in milk (100 ng/ml). The main benefit of the monoclonal antibody (anti-*CephM1*) is its specific nature. If used as a working diagnostic reagent it would however need to be complemented with other antibodies for the detection of a broad range of β-lactam antibiotics.

Recombinant scFv antibody production and engineering techniques based on phage-display methodologies were used, as described in chapter 5. In summary, the BMV human naïve phage-scFv antibody library was panned with cephalexin conjugates and two immunised phage display libraries were produced (Krebber *et al.*, 1997). No scFv were isolated that recognised the soluble (i.e. un-conjugated) β-lactam antibiotic residue. Results indicated showed that the immune library clones did contain scFv gene construct. However, the sequence diversity and hence the variety of binding function was most probably too small. This could not be fully enumerated without sequencing all the resulting transformants, which for the purposes of this work was impracticable. The lack of potential cephalexin-specific scFvs within the two libraries may be due to the small library sizes obtained.

The hybridoma cell line produced in chapter 4 of this work was used as a genetic source for scFv production. Positive phage-scFvs were produced, identified and bound free-cephalexin, as demonstrated in inhibition ELISA. The scFv gene construct was purified from one of the picked clones and sub-cloned into the pAK400 vector for enhanced soluble expression. The expression was induced with IPTG and scFv production was monitored by a SDS-PAGE and Western blotting. The cephalexin-specific scFv (termed wild type) was successfully purified by Ni^{2+}-NTA immobilised metal affinity chromatography (IMAC).

Following isolation and purification of the wild type (WT) scFv by IMAC, an inhibition ELISA for the detection of cephalexin in 'spiked' processed milk samples was developed. The assay was not as sensitive as the parental monoclonal IgG antibody (Chapter 4) resulting in a limit of detection (LOD) of 143.3 ng/ml, compared to 60 ng/ml for the intact monoclonal IgG. It is probable that the reduced assay sensitivity may be due to avidity effects, going from a divalent intact IgG to a predominantly monovalent scFv.

The wild type scFv gene was sequenced and the complementarity determining regions (CDR) were identified using the National (US) centre for Biotechnology information Ig Blast tool (http://www.ncbi.nlm.nih.gov/igblast/). The wild type scFv gene sequence was found to be 98.3% homologous to the murine sequence number AJ231205 from the cr1 germline gene sequence (Thiebe *et al.*, 1999), the heavy chain scored 95.8% homology with the Balb/c VH7183 gene family, as characterised by Williams *et al.* (2001). A model of the WT scFv protein was produced using the SWISS-Model protein modelling website, the SWISS-Model system and PDB software (Pietsch, 1995; Guex and Pietsch 1997; Schwede *et al.*, 2003).

The cephalexin-specific wild type (WT) scFv gene was used as a template for random mutagenesis procedures (Coia *et al.*, 2001 and Korpimäki *et al.*, 2002; Razai *et al.*, 2005).

Intra-gene sequence shuffle (R1*) and error-prone PCR (R2*) phage-display libraries were constructed by ligation of mutated scFv gene constructs into the pAK100 phagemid vector. The ligation products were used to transform *E. coli* electrocompetent cells. The number of transformants obtained was of the order of 1.4×10^7 for R1* and 2.3×10^6 for R2* libraries, respectively. The two libraries were panned and conjugate bound phage-scFvs were initially competitively eluted by adding un-conjugated cephalexin followed by elution with glycine.

Nascent phage-scFvs were singled out and sub-cloned into pAK400 vector for subsequent soluble expression. Two clones (C5 and H3) with the most promising improvements in terms of inhibition ELISA sensitivity were purified by IMAC chromatography. IMAC-purified mutant scFv antibody fragments were used in an inhibition ELISA for the detection of cephalexin hydrate in 'spiked' processed milk samples (section 5.2.4.3). The mutant C5 clone scFv displayed two-fold increased assay sensitivity in comparison with wild type. The mutant H3 scFv was not as sensitive at lower cephalexin concentrations, but it had a higher titre and a lower IC50 value compared to original wild type (1.2 fold lower).

The comparative (wild type and C5 mutant) amino-acid sequence point mutation (section 5.2.4.4) for the CDR_{H1} consisted of the replacement of a serine (S) with an asparagine (N) residue. The two residues are polar and hydrophilic. However, for the CDR_{H3} point mutation a wild type leucine (L) was replaced by a glutamine (Q) residue. This represents a change from a non-polar hydrophobic (L) to a polar hydrophilic (Q) based residue. From sequencing results it was found that the H3 clone had point mutations in the framework regions. However, there were no observed mutations in any CDR regions when compared to the WT clone. The C5 scFv amino acid sequence had point mutations in the variable CDR_{H1} and CDR_{H3} regions. This supports the established concept that the majority antigen binding interactions are conferred by the variable heavy chain complementarity determining regions. The mutant clone C5 scFv ELISA limit of detection was found to be 1.8 fold lower from a value of 143.3 ng/ml for the original wild type scFv, to 77.9 ng/ml for the mutant C5 scFv. The C5 scFv was the best scFv produced in terms of expression, functional stability and assay performance, capable of cephalexin detection below the 100 ng/ml EUMRL for milk.

The polyclonal (Chapter 3), monoclonal (Chapter 4) and recombinant scFv (Chapter 5) antibodies produced in this work were used to develop assays for the detection of cephalexin in milk using a BIAcore 3000™ system. In this research, CM5 sensor chips were used in the production of immobilised cephalexin-thyroglobulin and directly immobilised cephalexin surfaces. The TCC, DIC and relevant control surfaces were evaluated in terms of antibody binding and non-specific interactions with milk as a sample matrix prior to assay

development. Milk sample pre-treatment was also investigated as a means of reducing the background response observed when using a complex sample matrix. Sample treatment further reduced the non-specific binding with TCC and DIC flow cell surfaces. The milk sample pre-treatment method employed was a modified version of that reported by Gustavsson (2003). The DIC sensor surface and milk sample pre-treatment resulted in an average 95% decrease in non-specific response in comparison with the TCC surface. The directly immobilised cephalexin flow cell surface was found have the lowest background response to milk samples in comparison with the thyroglobulin-cephalexin immobilised (TCC) flow cell surface.

The inhibition assay format (Figure 6.17) chosen was generally reported in the literature when using SPR-based biosensor devices for the detection of low molecular weight analytes. A comparative study using both DIC and TCC sensor chip surfaces and monoclonal antibody inhibition assays performance between the two surfaces suggested, in conjunction with non-specific interaction analysis, that the DIC sensor chip was optimum for further assay development. The inhibition assay using polyclonal anti-*CephP1* antibody was found to have intrinsically high variation with a large degree of binding to the reference flow cell. Online reference subtraction was employed in assay development and highlighted the fact that a large percentage (≈50% at worst) of polyclonal response was non-specific in nature (Figure 6.22). This high non-specific response was only observed using protein-G purified polyclonal antibody. The apparent 'sticky' nature of the purified polyclonal antibody contributed to the high relative standard deviation and in turn the higher estimation of the limit of detection in comparison with its performance in the ELISA-based assay. The polyclonal antibody was not applicable to developing a reproducible assay using this system.

Inter-day assay variability studies using anti-*CephM1* monoclonal antibody, in contrast to the polyclonal antibody-based assay, showed minimal non-specific interaction (<2%) was observed with DIC reference flow cell surface. The limit of detection was below the required MRL value at 72 ng/ml. The monoclonal assay was reproducible and had low coefficients of variation (<10%), in addition the calculated percentage accuracies (i.e. sample recovery) were acceptable (i.e. within 20% of actual value) over the concentration range of standards.

IMAC-purified recombinant antibodies (WT, C5, H3) described in chapter 5 were also used in the development of a BIAcore inhibition assay for the detection of cephalexin. The optimum scFv in terms of assay performance was found to be the one expressed by mutant C5. This corroborates the findings for comparative ELISA-based assays, as described in section 5.2.4.3. The WT and H3 scFvs were capable of detecting cephalexin over a range of

concentrations, yet had limits of detection (111 & 146 ng/ml, respectively) above the MRL for cephalexin in milk of 100 ng/ml. The monoclonal and mutant C5 scFv antibody-based inhibition assays developed in this work offer a reliable analytical method for the specific detection of cephalexin hydrate in 'spiked' processed milk. In addition, the mutant libraries produced may offer a potential source of specific scFv antibodies to different β-lactams.

Future work arising from this research, and currently underway, is the incorporation of the antibodies described into novel assay formats including the development of fluorescent-based arrays. The application of such arrays in a 'user-friendly' portable sensing device for 'on-site' measurement of antibiotic levels in individual cows is currently being investigated. This work involves a multidisciplinary collaboration between biologists, physical chemists, physicists and engineers. It highlights the need for teams of experts for the generation of 'state-of-the-art' nanotechnology-based devices that can have significant impacts on animal and human health. It is envisaged that such a device will facilitate more stringent control on the use antibiotics hence should limit their potential to enter the food chain via bovine milk and associated products.

Chapter 8

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Appendix 1

Council Regulation (ECC) No 2377/90
Of the 26th June 1990
laying down a community procedure for the establishment of maximum residue limits of veterinary medicinal products in foodstuffs of animal origin
(OJ L 224, 18.8.1990, p14)

***adapted from* ANNEX 1**
List of pharmacologically active substances for which maximum residue limits have been fixed

	Pharmacologically active substance(s)	Marker residue	Animal species	MRLs	Target tissues	Other provisions
▼M91	Cefacetrile	Cefacetrile	Bovine	125 µg/kg	Milk	For intra-mammary use only
▼M71	Cephalexin	Cephalexin	Bovine	200 µg/kg 200 µg/kg 200 µg/kg 1000 µg/kg 100 µg/kg	Muscle Fat Liver Kidney Milk	
▼M100	Cefalonium	Cefalonium	Bovine	20 µg/kg	Milk	
▼M87	Cefapirin	Sum of cephapirin and Desacetylcephapirin	Bovine	50 µg/kg 50 µg/kg 100 µg/kg	Muscle Fat Kidney	