

Production and characterisation of novel recombinant antibody fragments against sialic acid

A thesis submitted for the degree of Ph.D.

By

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This thesis is dedicated to my mother, Patricia Donohoe, my father Gerard Donohoe (R.I.P 2007) and my younger brother Tony Donohoe (R.I.P 2005)

"Our greatest glory is not in never failing, but in rising up every time we fail".

Ralph Waldo Emerson

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, that I have exercised reasonable care to ensure that the work is original, and does not to the best of my knowledge breach any law of copyright, and has not been taken from the work of others save and to the extent that such work has been cited and acknowledged within the text of my work.

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Abbreviations

ABEE	p-aminobenzoic acid ethyl ester
ACG	<i>Agrocybe cylindracea</i> galectin
AGP	α 1-acid glycoprotein
AMAC	2-aminoacridone
ANDSA	7-amino-naphthalene-1,3-disulphonic acid
APTS	8-aminopyrene-1,3,6-trisulphonate
B-cells	Bone marrow-derived cell
BMS	Bristol Myers Squibb
Bp	Base pair
BSA	Bovine serum albumin
BSM	Bovine submaxillary mucin
cDNA	Complementary DNA
CDR	Complementarity determining region
CE	Capillary electrophoresis
cfu	Colony forming units
CH	Constant domain of heavy chain
CHA-I	<i>Cepaea hortensis</i> agglutinin-I
CL	Constant domain of light chain
Clr	Calreticulin
Clx	Calnexin
CMAH	CMP-Neu5Ac-hydroxylase
CMP	Cytidine monophosphate
COM	Component Object Model
CP	Colony picker
Cys	Cysteine
CZE	Capillary zone electrophoresis
C γ	Constant gamma
C ν	Constant nu
dATP	Deoxyadenosine triphosphate
dCTP	Deoxycytidine triphosphate
dGTP	Deoxyguanosine triphosphate

DMB	1,2-diamino-4,5-methylenedioxybenzene
DNA	Deoxyribonucleic acid
DNaseI	Deoxyribonuclease I
dNTP	Deoxyribonucleotide triphosphate
DOGS	Degenerate oligonucleotide gene shuffling
dTTP	Deoxythymidine triphosphate
DWP	Deep-well plate
<i>E.coli</i>	<i>Escherichia coli</i>
EDC	<i>N</i> -ethyl- <i>N</i> -(dimethyl-aminopropyl) carbodiimide hydrochloride
ELISA	Enzyme-linked immunosorbent assay
EM	Electrophoretic mobility
EMS	Ethylenethanesulphonate
EOF	Electro-osmotic flow
ER	Endoplasmic reticulum
ERAD	ER-associated degradation pathway
ESI	Electrospray ionisation
Fab	Fragment antigen binding
Fc	Fragment crystallisable region
FCA	Freund's complete adjuvant
FC-L	<i>Fenneropenaeus chinensis</i> lectin
Ff	Filamentous
FPLC	Fast Performance Liquid Chromatography
GalNAc	<i>N</i> -acetylgalactosamine
GALT	Gut-associated lymphoid tissue
GC	Gas chromatography
GlcNAc	<i>N</i> -acetylglucosamine
GluNAc	<i>N</i> -acetylglutamic acid
GPI	Glycosylphosphatidylinositol
GU	Glucose units
HA	Haemagglutinin
HAMA	Human anti-mouse antibody

HAT	Hypoxanthine Aminopterin Thymidine
HBS	Hepes buffered saline
HD	Hanganutziu-Deicher
HEL	<i>Hericium erinaceum</i> lectin
HIV	Human Immunodeficiency Virus
HPAEC-PAD	High-performance anion-exchange chromatography with pulsed amperometric detection
HPLC	High-performance liquid chromatography
HRP	Horseradish peroxidase
HSA	Human serum albumin
IC50	Half maximal inhibitory concentration
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IgY	Immunoglobulin Y
IMAC	Immobilised metal affinity chromatography
IPTG	Isopropyl β -D-1-thiogalactopyranoside
J	Joining region
KDN	2-keto-3-deoxy-D-glycero-D-galacto-nononic acid
KDO	3-deoxy-octulosonic acid
KLH	Keyhole limpet haemocyanin
LC	Liquid chromatography
LFA	<i>Limax flavus</i> agglutinin
LIF	Laser-induced fluorescence
LiHa	Liquid handling arm
LPA	<i>Limulus polyphemus</i>
LPS	Lipopolysaccharide
mAb	Monoclonal antibody
MAH	<i>Maackia amurensis</i> hemagglutinin
MAL	<i>Maackia amurensis</i> leukoagglutinin
MALDI	Matrix-assisted laser desorption
Man	Mannose
ManNAc	<i>N</i> -acetyl-D-mannosamine
ManNGc	<i>N</i> -glycolyl-D-mannosamine

MgCl ₂	Magnesium chloride
MP	Microtitre plate
Mp3pos	Plate Holder
MPO	Myeloperoxidase
MS	Mass Spectrometry
MTT	3-methyl-1-p-tolyltriazene
MURA	Mutagenic and unidirectional reassembly
MWCO	Molecular weight cut-off
NADH	Reduced nicotinamide adenine dinucleotide
NANA	5- <i>N</i> -acetylneuraminic acid
NaOH	Sodium hydroxide
NCAM	Neural cell adhesion molecule
Neg	Negative
Neu	Neuraminic acid
Neu2,7an5Ac	5- <i>N</i> -acetyl-2-deoxy-2,7-anhydro-neuraminic acid
Neu4,5Ac ₂	5- <i>N</i> -acetyl-4- <i>O</i> -acetylneuraminic acid
Neu4,8an5Ac	4,8-anhydro- <i>N</i> -acetylneuraminic acid
Neu4Ac5Gc8Me	8- <i>O</i> -methyl-4- <i>O</i> -acetyl-5-glycolylneuraminic acid
Neu5,9Ac ₂	5- <i>N</i> -acetyl-9- <i>O</i> -acetylneuraminic acid
Neu5Ac	5- <i>N</i> -acetylneuraminic acid
Neu5Ac2en	5- <i>N</i> -acetyl-2-deoxy-2,3-didehydro-neuraminic acid
Neu5Ac8S	5- <i>N</i> -acetyl-8- <i>O</i> -sulpho-neuraminic acid
Neu5Ac9Lt	5- <i>N</i> -acetyl-9- <i>O</i> -lactyl-neuraminic acid
Neu5FPr	5- <i>N</i> -phenylacetylneuraminic acid
Neu5Gc	5- <i>N</i> -glycolyl-neuraminic acid
Neu5Gc8Me7,9Ac ₂	8- <i>O</i> -methyl-7,9-di- <i>O</i> -acetyl-5-glycolyl-neuraminic acid
Neu5Gc9Lt	5- <i>N</i> -glycolyl-9- <i>O</i> -lactyl-neuraminic acid
Neu5iBu	5- <i>N</i> - <i>iso</i> -butanoylneuraminic acid
Neu5PhAc	5- <i>N</i> -phenylacetylneuraminic acid
Neu5Pr	5- <i>N</i> -propionylneuraminic acid
Neu7Ac5Gc8Me	8- <i>O</i> -methyl-7- <i>O</i> -acetyl-5-glycolyl-neuraminic acid
Neuraminic acid	5-amino-3,5-dideoxy-D-glycero-D-galacto-2-nonulosonic acid
NHS	<i>N</i> -hydroxysuccinimide

NMR	Nuclear Magnetic Resonance
NPNA	<i>N</i> -propionylneuraminic acid
OD	Optical density
OPD	<i>O</i> -phenylenediamine
OPL	Ovine placenta lectin
OS	Operating system
OSM	Sheep submaxillary mucin
OVA	Ovalbumin
PAA	Polyacrylamide
pAb	Polyclonal antibody
PBS	Phosphate buffered-saline
PBST	Phosphate buffered-saline with 0.05% (v/v) Tween
PC	Personal Computer
PCL	<i>Phaseolus coccineus</i> lectin
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
Phe	Phenylalanine
pI	Isoelectric point
PMP	1-phenyl-3-methyl-5-pyrazolone
PNGase	Peptide N-glycanase
pNPP	p-nitrophenyl phosphate
RACHITT	Random Chimeragenesis on Transient Templates
RF	Rheumatoid factor
rhu-EPO	Recombinant human erythropoietin
Rmax	Theoretical maximal response
RNA	Ribonucleic acid
RoMa	Robotic arm
RoMa	Robotic manipulator
RPR	Random priming recombination
RT-PCR	Reverse transcription polymerase chain reaction
SCA	<i>Sambucus canadensis</i> agglutinin
scFv	Single-chain variable fragment
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis

SEC	Size exclusion chromatography
Ser	Serine
Sia	Sialic acid
SNA	<i>Sambucus nigra</i> agglutinin
SOC	Super optimal catabolite
SOE	Splice by overlap extension
SPR	Surface plasmon resonance
ssDNA	Single stranded DNA
StEP	Staggered extension process
SupE	Suppressor stain
T	Tween
TEMED	Tetramethylethylenediamine
TACAs	Tumour-associated carbohydrate antigens
TBA	2-thiobarbituric acid
T-cells	Thymus cells
TF	Thomsen-Friedenreich antigen (Gal β 1-3GalNAc α 1-O-Ser/Thr)
Thr	Threonine
TMB	Tetramethylbenzidine dihydrochloride
TML	<i>Trichomonas mobilensis</i> lectin
TOF	Time-of-flight
TMS	Trimethylsilyl
Trp	Tryptophan
TYE	Tryptone yeast extract medium
Tyr	Tyrosine
UGGT	Uridine 5'-diphosphate-glucose:glycoprotein glucosyltransferase
UV	Ultraviolet
V _H	Variable heavy chain
V _L	Variable light chain
WGA	Wheat germ agglutinin
TMS	Trimethylsilyl

Units

μg	Microgram
μL	Microlitre
μm	Micrometre
cm	Centimetre
Da	Daltons
g	Gram
k_a	Association rate constant ($\text{M}^{-1} \text{s}^{-1}$)
k_d	Dissociation rate constant (s^{-1})
K_D	Equilibrium binding constant (M)
kDa	Kilodaltons
kB	Kilobase
L	Litre
M	Molar
mg	Milligram
mL	Millilitre
min	Minute
mM	Millimolar
ng	Nanogram
$^{\circ}\text{C}$	Degrees Celsius
pfu	Plaque-forming unit
Rpm	Revolutions per minute
U	Unit
v/v	Volume per unit volume
w/v	Weight per unit volume
g	Centrifugal acceleration

Publications, Patent applications and Presentations

Publications

O'Kennedy, R Townsend, S; **Donohoe, G.G.**, Paul, L., Hearty, S., and Byrne, B. (2010). Speedy, Small, Sensitive, and Specific—Reality or Myth for Future Analytical Methods. *Anal.Lett*, **43**,1630-1648.

Byrne, B., **Donohoe, G.G.**, and O'Kennedy, R. (2007). Sialic acids: carbohydrate moieties that influence the biological and physical properties of biopharmaceutical proteins and living cells. *Drug Discov Today*, **12**,319-26.

Patent applications

Donohoe, G.G., Byrne, B., Hearty, S., and O'Kennedy, R. (2008). Anti-sialic acid antibody molecules. European Patent Application EP 2287202 (A1) and US Patent Application No: 20110034676

Manuscripts

Donohoe, G.G., Byrne, B., Hearty, S., and O'Kennedy, R. The generation of avian recombinant antibodies towards sialic acid. (Manuscript in preparation)

Byrne, B., Donohoe, G.G., Thompson, R., Gilmartin, N., Clarke, P., Hearty, S., and O'Kennedy, R. Monitoring of sialylation patterns on bovine fetuin by CAABLES (carbohydrate analysis by antibody/lectin sequencing)-based analysis.

Posters

Donohoe, G.G., Byrne, B., Leonard, P., Hearty, S., McDonnell, B., and O'Kennedy, R. (2009). High-throughput screening of scFv and Fab antibody fragments using Biacore A100. International Conference on Trends in Bioanalytical Sciences and Biosensors, Dublin, Ireland.

Invention disclosure

Byrne, B., **Donohoe, G.G.**, and O'Kennedy, R. (2008). Novel anti-Orencia antibody fragments. (submitted to Invent, DCU.)

Presentations

Byrne, B., **Donohoe, G.G.**, and O'Kennedy, R. (2006-2007-2008-2009), Centre for Bioanalytical Sciences, DCU and NUIG.

Abstract

Sialic acids are a family of acidic monosaccharides that typically reside as terminal moieties on *N*- and *O*-linked glycans. These sugars are actively involved in a plethora of biological phenomena, ranging from cell-cell adhesion and recognition, intracellular signalling events, pathogen attack, viral infection and inflammatory disease. In addition, many cancer-related antigens contain terminal sialic acids or altered sialylation patterns. The identification of sialic acid-specific antibodies is important in the fields of basic research and diagnostics. Therefore the primary objective of this thesis was the generation and characterisation of recombinant anti-sialic acid antibodies.

A single-chain antibody fragment (scFv) library was constructed from a chicken that was immunised with a novel synthetic sialic acid protein-conjugate (Neu5Gc-human serum albumin). The scFv library was biopanned using a second novel sialo-neoglycoconjugate (Neu5Gc-bovine serum albumin). Anti-sialic scFvs were isolated by phage-display and binding activity, multimeric status, and multivalent properties were assessed by ELISA, HPLC, FPLC, and SPR. One clone, AE8, displayed the strongest reactivity to a panel of different sialylated structures. In addition, SPR inhibition and kinetic analysis revealed that the AE8 scFv had nanomolar affinity for the Neu5Gc-BSA neoglycoconjugate.

The heavy chain gene of the AE8 scFv was assembled with a repertoire of error prone PCR-amplified light chain genes, that originated from the unpanned anti-sialic acid library. The mutant scFv library was biopanned using a depletion protocol and two ovalbumin (OVA) conjugates, Neu5Gc-**linker1**-OVA and **linker1**-OVA. ELISA analysis, SPR inhibition and 'off-rate' studies identified one mutant clone, E15, which displayed strong affinity for conjugates of sialic acid. A solution-phase SPR-based inhibition study demonstrated that the E15 scFv had an IC_{50} concentration of 1.6ng/mL for the Neu5Gc-BSA conjugate. When compared to the parental AE8 clone (IC_{50} of 5.7ng/mL) the mutant E15 scFv exhibited an improvement in sialic acid binding of approximately 3.5-fold. The generation of such highly specific anti-sialic acid antibodies in a recombinant format is exceedingly rare. Most anti-carbohydrate antibodies are not recombinant and display weak carbohydrate-protein interactions. This work shows for the first time, the generation and directed evolution of anti-carbohydrate scFvs that have nanomolar affinity for Neu5Gc-containing structures.

Chapter 1

Introduction

1.0 Introduction

1.1 Glycosylation

Carbohydrate side chains or glycans, (oligosaccharides and polysaccharides) are ubiquitous, diverse and structurally-complex molecules found on both the inside and surfaces of cells. These multiple branched structures, with their wide array of different linkages, can carry much more information per unit weight than either proteins or nucleic acids (Gabijs *et al.*, 2004) and are essential for cell viability and normal function. This is exemplified by the pivotal role they play in a wide range of biological processes: cell migration, adhesion, proliferation, transcriptional regulation, differentiation, oncogenic transformation, apoptosis and metastasis (Cobb *et al.*, 2005). Typically, they are the first cellular components encountered by approaching pathogens, cells, antibodies and other molecules.

Glycans exist as covalent linkages of saccharides attached to proteins (glycoproteins) and lipids (glycolipids) and form a dense sugar coat around the surfaces of cells known as the glycocalyx. It is estimated that 10% of the expressed genes in humans are proteins involved in the synthesis of glycans and more than half of all human proteins are glycosylated (Apweiler *et al.*, 1999). In addition the surfaces of many bacteria, viruses and lipids are also decorated with a dense array of complex glycans. Glycosylation is a ubiquitous co- and post-translational enzymatic modification that involves the attachment of glycans to the side chains of specific amino acids in the protein backbone. Typically, glycans are added in a sequential and competitive assembly line process, through the coordinated action of hundreds of different cellular enzymes. This process is neither template-driven nor under direct transcriptional control and is thus impossible to predict. In eukaryotes, glycosylation primarily occurs in the lumen of the endoplasmic reticulum (ER) and the Golgi apparatus (Ohtsubo and Marth, 2006). Within the ER membrane, bound chaperones calnexin (Clx) and calreticulin (Clr) direct nascent glycoproteins to a folding pathway. Clx and Clr retain unfolded glycoproteins within the ER until they are correctly folded and assembled, after which they are translocated to the Golgi apparatus where the glycans become increasingly oligomeric and branched.

Misfolded or unassembled glycoproteins are trafficked back via the ER-associated degradation (ERAD) pathway to the cytosol, where proteasomal degradation by peptide *N*-glycanase (PNGase) occurs.

The basic glycosylation machinery consists of glycosyltransferases that synthesise glycan chains through the transfer of nucleotide-activated sugar or lipid residues to the glycan and glycosidases that are involved in glycan trimming via the hydrolysis of glycosidic bonds. For example, uridine 5'-diphosphate-glucose:glycoprotein glucosyltransferase (UGGT) functions as a folding sensor recognising the innermost *N*-acetylglucosamine (GlcNAc) moiety on misfolded glycoproteins. The re-glucosylation of proteins by this enzyme results in unfolded proteins rebinding Clx and entering a cyclical pathway whereby the protein either folds correctly or is targeted for ERAD. The process is initiated by mannosidase I, a glycosidase that trims a mannose (Man) residue from an oligosaccharide precursor and generates a nascent glycoprotein with the glycan motif, GlcNAc₂Man₈. When GlcNAc₂Man₈ is re-glucosylated by UGGT to GlcNAc₂Man₈Glc₁, this glycan motif acts as a poor substrate for a second glycosidase, α -glucosidase II, which prefers the GlcNAc₂Man₈Glc₁ motif. Consequently, the unfolded glycoprotein readily rebinds to Clx and is eliminated as previously described. Each cell produces a distinct array of glycans (glycome) that depends upon the specific profile of the glycosyltransferases / glycosidases, their combinatorial expression level and their subcellular distribution within the Golgi apparatus. Glycosyltransferases account for approximately 1% to 2% of the gene products of an organism (Lairson *et al.*, 2008).

Glycosylation can substantially modify the structure, function, solubility and biological activity of proteins by steric influences involving intermolecular and intramolecular interactions. For example, large oligomeric glycan structures can cover much of the protein surface, thereby providing protection from cellular proteases (Rudd *et al.*, 2001). The predominant sugars found in glycoproteins are sialic acid, glucose, galactose, mannose, fucose, *N*-acetyl galactosamine (GalNAc) and GlcNAc. Glycoproteins on average carry >3 glycan sites, each site containing between 10 and 30 structures which are classified by their carbohydrate-peptide linkages (Alavi and Axford, 2008). Protein glycosylation includes *N*-glycans, *O*-glycans, *C*-glycans and glycosamino-glycans.

N-linked glycan chains account for up to 90% of the glycan structures seen in eukaryotic glycosylated proteins. They are attached to the protein core via the amide nitrogen of an Asparagine (Asn) residue that is part of the consensus sequence Asn-X-Serine (Ser) /-Threonine (Thr), where X can be any amino acid except proline.

For *O*-glycosylation, no consensus sites have been identified and glycans are attached via the hydroxyl groups of mainly Ser or Thr and, in some cases Tyrosine (Tyr) residues. Unlike *N*- and *O*-glycosyl linkages, *C*-glycans have a carbohydrate-protein linkage that does not involve an amino-acid functional group. This carbon-carbon bond involves the attachment of an α -mannosyl residue to the C-2 of tryptophan (Trp), as seen in the mammalian glycoproteins RNase2 and interleukin-12 (Spiro, 2002). The proteoglycans (examples include: heparan, chondroitin, dermatan and keratan) contain carbohydrate structures known as glycosaminoglycans; these long unbranched polymers consist of a repeating disaccharide unit. They are linked to serine and threonine, and are typically components of the extracellular matrix and cell surface. They are produced by a different biosynthetic pathway and are often highly sulphated.

Lipid glycosylation also occurs and can be seen in eukaryotic cell-surface glycolipids, which are anchored in the lipid bilayer by a glycosylphosphatidylinositol (GPI) membrane anchor. A heterogeneous glycan moiety is linked via mannose to the inositol headgroup of the phosphatidylinositol, which in turn is linked to the C-terminal carboxyl group of the protein. The glycolipids also include the subgroup glycosphingolipids of which the gangliosides (found in neuronal cell membranes) are an important class. Gangliosides contain the lipid component ceramide (composed of sphingosine and a fatty acid) linked by a glycosidic bond to an oligosaccharide chain containing hexose and *N*-acetylneuraminic acid (sialic acid) (Ohtsubo and Marth, 2006).

There has been a decrease in the use of mannose linkages and an increase in glycan complexity and the use of new saccharides like sialic acid with evolution. This is particularly evident in the *N*-glycan biosynthetic pathway. Yeast and fungi, for example, carry high-mannose *N*-glycans while invertebrates typically have hybrid *N*-glycans, whereas vertebrates carry complex *N*-glycans (Marth and Grewal, 2008).

1.2 Sialic acid structure and occurrence in nature

It was Gunnar Blix, who in 1936, isolated crystals from bovine submaxillary mucin (BSM) and later named this substance sialic acid. In 1940, Ernst Klenk crystallised a substance from a glycoprotein purified from the brain of patients with Tay-Sachs disease and named that substance neuraminic acid (5-amino-3,5-dideoxy-D-glycero-D-galacto-2-nonulosonic acid, Neu). Subsequently, Alfred Gottschalk proposed a coherent structure for sialic acid, and in 1957, Blix, Klenk and Gottschalk agreed the nomenclature of the substance. Sialic acid was designated as the family name for these substances and their core structure was called neuraminic acid. Thus, sialic acid (Sia) is the generic term given to a family of acetylated derivatives of neuraminic acid, a 9-carbon α -keto carboxylated acidic monosaccharide. Sia predominantly occupy the exposed terminal positions of the nonreducing end of the oligosaccharide chains of many glycoproteins and glycolipids. Thus, they are found in *N*-glycans, *O*-glycans and glycosphingolipids (Schauer, 2000).

The three major Sia types are 5-*N*-acetylneuraminic acid (Neu5Ac), 5-*N*-glycolyl-neuraminic acid (Neu5Gc) and 2-keto-3-deoxy-D-*glycero*-D-*galacto*-nononic acid (KDN). Neu5Ac is the most ubiquitous Sia, and the largest structural variations of the Sia are seen at carbon 5 (C-5). For example, Neu5Gc differs from Neu5Ac at C-5 by the addition of an oxygen atom to the *N*-acetyl group (Traving and Schauer, 1998). This change is catalysed by the enzyme cytidine monophosphate(CMP)-Neu5Ac hydroxylase (Tangvoranuntakul *et al.*, 2003). In contrast, KDN has a hydroxyl group at the C-5 position. In the last 50 years, 62 distinct sialic acid structures have been identified in nature, arising from acetylation, methylation, lactylation, sulphation and phosphorylation of the C-4, C-5, C-7, C-8, or C-9 hydroxyl groups (Traving and Schauer, 1998; Kamerling and Gewig, 2006; Murrey and Hsieh-Wilson, 2008). All of these types of substitutions are seen in the deuterostome lineage. The echinoderms (e.g. sea urchins and starfish) express these Sia in large amounts (Schauer, 2000). The most frequent vertebrate Sia modification is the esterification of the hydroxyl groups at C-7, C-8, C-9 and rarely at C-4 by acetyl groups (*O*-acetylation). Under physiological conditions, *O*-acetyl esters from C-7 and C-8 positions spontaneously migrate to the C-9 position, thereby making *O*-acetylation at the C-9 position the most common Sia biological modification (Schauer, 2004).

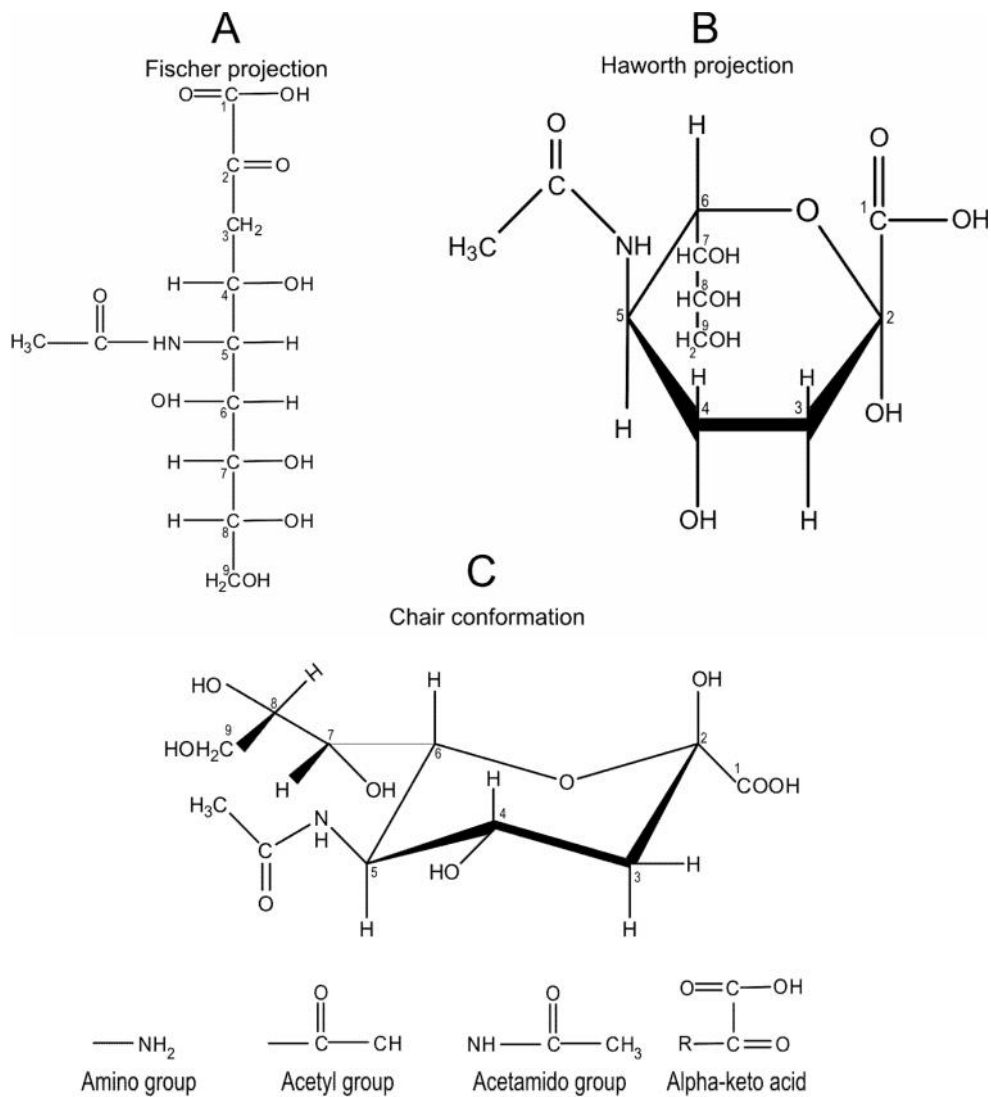


Figure 1.2.1 The chemical structure of sialic acid in different views. (A) open chain Fischer projection. (B) alpha-Neu5Ac (Haworth formula). (C) alpha-Neu5Ac (²C₅ chair conformation). Various groups which are components of the sugar are also listed.

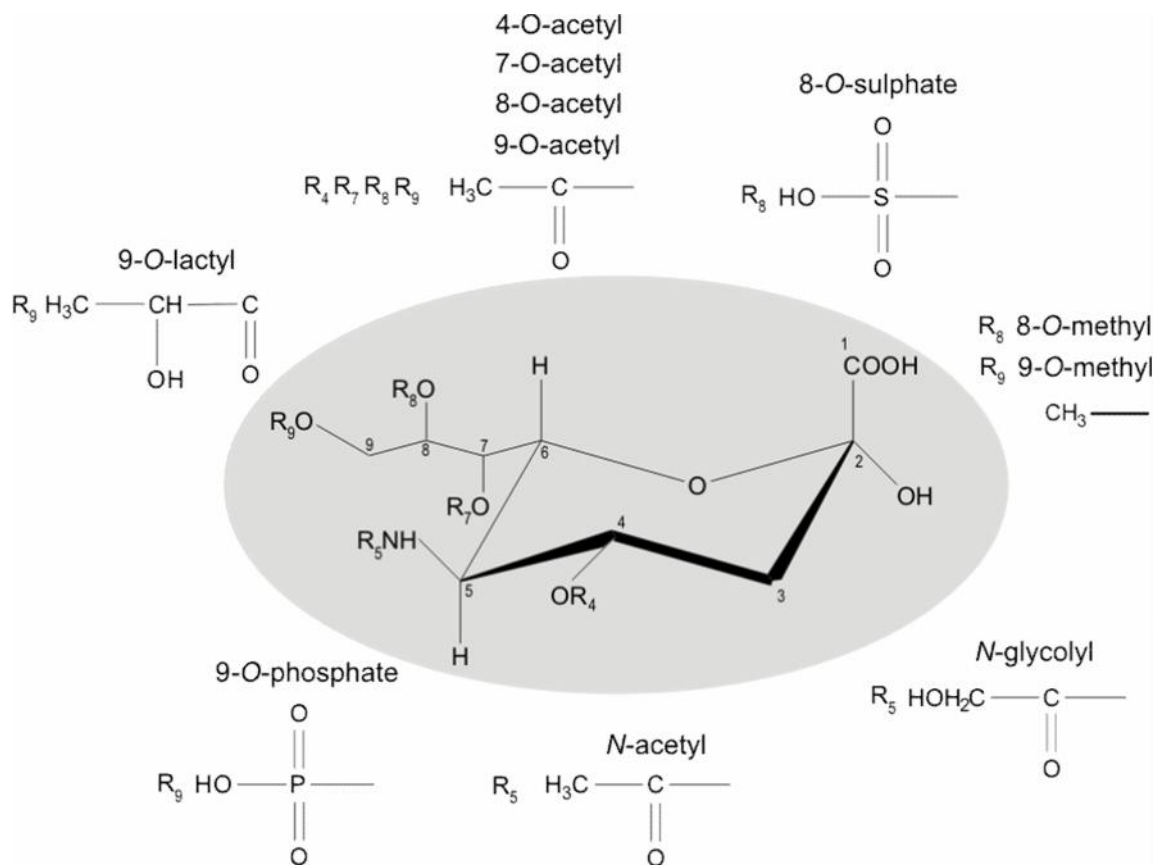


Figure 1.2.2 The different structures of the family of naturally occurring Sia. The common backbone structure of Sia is shown, and the carbon atoms are numbered. Various combinations of the R groups (1 to 9) yield molecular diversity of the sialic acids. 9-O-acetylation is the most frequent biological modification of Sia.

Sia can occur free in nature like the unusual Neu5Ac2en (5-*N*-acetyl-2-deoxy-2,3-didehydro-neuraminic acid) which has a double bond between C-2 and C-3 and has been found in animal fluids and human urine. Other unusual Sia include Neu2,7an5Ac (5-*N*-acetyl-2-deoxy-2,7-anhydro-neuraminic acid) which has an internal anhydro linkage (loss of water between two hydroxyl groups in the monosaccharide unit). This Sia has been identified in leeches where an intramolecular trans-sialidase cleaves the terminal Neu5Ac residue from a glycoprotein releasing Neu2,7an5Ac (Luo *et al.*, 1998).

In nature, Sia forms glycosidic bonds in the α -configuration. Typically the C-2 of the Sia is linked either to the 3- or 6-hydroxyl groups of galactose residues [$\alpha(2,3)$ / $\alpha(2,6)$] or the 6-hydroxyl groups of GalNAc residues. Other infrequent linkages can also occur: $\alpha(2,4)$ to Galactose (Gal), $\alpha(2,4)$ to GlcNAc, $\alpha(2,3)$ to GalNAc, $\alpha(2,6)$ to GlcNAc, and $\alpha(2,6)$ to Glucose (Glc). Rare Neu5Gc oligomers have been found in starfish. Unusually, these residues are linked via their hydroxyl glycolyl residue [-Neu5Gc(α 2-5O)Neu5Gc(α 2-)]. In addition, in some amphibians Sia are found as internal residues of the glycan chain (Fucose- $\alpha(2,6)$ -KDN- $\alpha(2,3)$ -Gal-) (Maes *et al.*, 1995). The sialyltransferases are localised throughout the Golgi and use a high energy donor form of Sia (CMP-Sia) as a substrate in glycoprotein and glycolipid biosynthesis (Roth *et al.*, 1986). Unusually, CMP-Sia differs from other Sia in that the anomeric carbon is in the β -configuration (Ambrose *et al.*, 1992). Sia can also form polymeric structures of linear oligo and polySia compounds comprising of up to 8 residues and more than 8 residues, respectively. These chains vary in the type of Sia monomers as well as in their type of linkage. So far $\alpha(2,8)$ -linkages, $\alpha(2,9)$ -linkages, alternating $\alpha(2,8)/\alpha(2,9)$ -linkages and α 2-5O-glycolyl linkages between Sia have been described (Yabe *et al.*, 2003).

Sia are thought to be evolutionarily ancient and may have emerged during the Cambrian expansion about 530 million years ago (Varki, 2007). The most complex forms and largest distribution of Sia occur in the deuterostome lineage (vertebrates, cephalochordates, ascidians, echinoderms). In addition, Sia has been demonstrated in some nondeuterostome eukaryotes (Protozoa, Protostomes and Fungi). The arthropods and molluscs express a similar class of glycoconjugates as those found in deuterostomes. However, whether or not they express Neu5Gc remains controversial (Angata and Varki, 2002).

In contrast, pathogenic fungi (*Candida albicans*, *Aspergillus fumigates*, *Cryptococcus neoformans* and *Sporothrix schenckii*) may express Neu5Ac, Neu5Gc and Neu5,9Ac₂ (5-*N*-acetyl-9-*O*-acetylneuraminic acid) (Alviano *et al.*, 1999). Gangliosides (Sia-containing glycolipids) are expressed in molluscs (octopuses and squid) and insects may express Neu5Ac in a stage-specific manner (Saito and Sugiyama, 2001; Viswanathan *et al.*, 2006).

The protozoan *Trypanosoma cruzi*, which causes Chagas disease, is known to be sialylated. Unusually, these organisms do not synthesise their own Sia but borrow it from the host's glycoproteins using the enzyme *trans*-sialidase (Angata *et al.*, 2002). Plants appear not to have the genes necessary for biosynthesis, activation or transfer of sialic acids. Although complex *N*-linked glycans are found in plants, these typically lack galactose and terminal sialic acid. However, there are a few controversial and sporadic reports in the literature that have claimed Sia exists in plants (Shah *et al.*, 2003; Seveno *et al.*, 2004). It may be that these reports are due to environmental contamination and/or incorrect identification of the chemically related sugar 3-deoxy-octulosonic acid (KDO) (Varki *et al.*, 2009).

In general, Sia is not widely expressed in bacteria. However, there are some species and strains that can synthesise and express Neu5Ac, *O*-acetylated Neu5Ac and KDN. Terminal sialic acid can be found in bacterial lipopolysaccharides (*O*-antigens) whereas the more abundant polysialic acid is found in several bacterial capsular polysaccharides (K-antigens). Bacterial expression of Neu5Gc has not been reported (Byres *et al.*, 2008; Varki, 2008). Interestingly, many Sia-expressing bacteria (*Escherichia coli* strain K1, *Neisseria meningitidis* group B/C and *Campylobacter jejuni*) can cause illness in humans and domestic animals (Angata and Varki, 2002).

1.3 Methodologies for the release and analysis of Sia

1.3.1 Enzymatic release of Sia

Most methods that identify Sia from biological material require that it is liberated and completely purified from the glycoconjugate, with its modifications intact before analysis. Typically, sialidases or mildly acidic conditions are employed for the non-destructive release of the Sia. Sialidases (also called 'neuraminidases') hydrolyse the glycosidic-linkage between the Sia molecules and the penultimate sugar of the carbohydrate chains of glycoconjugates. A wide variety of sialidases are available from different organisms (*Arthrobacter ureafaciens*, *Vibrio cholerae*, *Clostridium perfringens*) and their specificity depends on the type of glycosidic-linkage, the occurrence of *N*-glycolyl groups and whether the Sia is *O*-acetylated (Lamari *et al.*, 2002). For example, the sialidase from *A. ureafaciens* has broad glycosidic-linkage specificity [Neu5Aca(2,3)Gal β , Neu5Aca(2,6)Gal, Neu5Aca (2,6)GalNAc β , Neu5Aca(2,6)GlcNAc β and Neu5Aca(2,8)NeuAca]. This enzyme is widely used to non-selectively cleave Sia from a variety of *N*- and *O*- linked glycans (Iwamori *et al.*, 2005). The drawback of sialidase treatment is that the Sia is only released from the terminal ends of a glycoconjugate. In addition, Sia release is dependent upon its substitution pattern; for example; the addition of an *O*-acetyl group at C-4 renders the Sia resistant to cleavage by some sialidases and 9-*O*-acetylation can reduce sialidase cleavage rates by more than 50%. Furthermore, the sample matrix of biological material is quite complex and this can impede the action of sialidases (Schauer, 2000).

1.3.2 Hydrolytic release of Sia

Hydrolytic methods have incorporated an assortment of different acids and bases to release Sia from a variety of glycoconjugates. Typically, hydrolysis with organic acids in the range of 25-100mM will still leave the Sia's carboxyl and *N*-acyl groups intact. An in-depth study by Lamari and Karamanos, (2002) suggested that 25mM hydrochloric acid or trifluoroacetic acid at 80°C for two hours is optimal for the complete hydrolysis of the Sia linkages in a wide variety of glycoproteins. However, as with all hydrolytic procedures, a certain proportion of Sia is always lost; in the above method this is approximately 20% (Lamari and Karamanos, 2002).

Lagana *et al.* (1993) showed that a ten minute microwave (660W) with 2M acetic acid could be used to release Neu5Gc and Neu5Ac from rat serum and rat glands with only approximately 3% losses (Lagana *et al.*, 1993). A solution of 1% (v/v) acetic acid at 70°C for 60min has been used to release KDN from lipopolysaccharide (LPS) (Kiang *et al.*, 1997). Hara *et al.* (1987) have proposed the use of 25mM sulphuric acid but hydrolysates can be difficult to lyophilize (Hara *et al.*, 1987). In addition, the use of sulphuric acid and other strong acids (pH <3) and bases (pH >>7) can cause both the destruction of *O*-acetyl esters (de-*O*-acetylation) and the migration of *O*-acetyl groups to C-9. Using formic acid reduces the destruction of these groups, although hydrolysis is incomplete. Minimal destruction and complete release of 7-, 8- and 9-*O*-acetylated Sia can be achieved with 2M acetic or propionic acid at 80°C for two to three hours. When Sia is treated with mineral acids a fine balance needs to be struck between the competing factors of Sia destruction (caused by increasing acidity and temperature) and release. Sia liberated with volatile acids are usually dried in a vacuum concentrator (without heating) and reconstituted in de-ionised water. It is important that acid hydrolysis components do not interfere with the derivatisation reaction (Manzi, 2001).

1.3.3 Colorimetric methods

A number of colorimetric methods have been developed and have been employed for the detection and quantification of Sia (Pearce and Major, 1978). One of the most popular methods is the periodate-thiobarbituric acid assay. This method was independently developed by Warren in 1959 and Aminoff in 1961 to detect free Sia. In this procedure periodate oxidation of free Sia leads to the eventual formation of β -formyl pyruvic acid. This, in turn, reacts with 2-thiobarbituric acid (TBA) to yield a red chromophore with an absorption maximum at 549nm (Bhavanandan *et al.*, 1998). A severe disadvantage of this assay is that the presence of *O*-acetyl groups at the C-7, C-8 and C-9 carbons, prevents periodate oxidation of the Sia. Consequently, chromophore development is substantially reduced (Reuter and Schauer, 1994; Aminoff, 1961; Warren, 1959).

Other colorimetric assays include the resorcinol method which was first described by Svennerholm in 1957. In this assay the glycoconjugate is heated in the presence of a strong acid (HCl) which breaks the glycosidic bonds releasing free Sia which then reacts with resorcinol and copper (II) ions to form a chromogen (Crook, 1998; Svennerholm, 1957). However, these colorimetric methods are not highly specific as a range of different substances (2-deoxyribose, lactose, maltose, fructose and malondialdehyde) are known to interfere with the measurement (Jourdian *et al.*, 1971; Sobenin *et al.*, 1998). A variety of modifications that reduce interference and stabilise the colour reaction have also been described. For example, dimethyl sulphoxide has been used to stabilise the TBA chromophore (Skoza and Mohos, 1976). In addition, 2-deoxyribose interference can be reduced by measuring the TBA colour reaction at an additional wavelength of 532nm (Crook, 1998).

Jourdian *et al.* (1971) modified the Svennerholm method by introducing a periodate oxidation step prior to heating with resorcinol. This negated the interference from sugars, lipids and amino acids and allowed the determination of both free and bound Sia (Jourdian *et al.*, 1971). An ion-exchange column can be used to purify the Sia before colorimetric analysis, although this is a cumbersome step. Gerbaut *et al.* (1973) described a fully automated procedure based on the Warren / Aminoff technique that eliminated the extraction of the chromogen into an organic solvent (Gerbaut *et al.*, 1973). Other colorimetric methods include the acid ninhydrin procedure. This reagent interacts specifically with Sia to yield a stable chromophore that has an absorption maximum at 470nm. However, as ninhydrin also reacts with amino acids, they need to be excluded from the assay. The sensitivity of this method is comparable to that of the periodate-resorcinol method but it has the drawback that cysteine will non-specifically react with the acid ninhydrin reagent (Yao and Ubuka, 1987; Yao *et al.*, 1989).

1.3.4 Enzymatic assays

Enzymatic assays have also been used to determine Sia content and these offer the theoretical advantage of improved specificity. One such method is the acylneuraminate pyruvate lyase system. Here, Neu5Ac is converted to pyruvate and *N*-acetylmannosamine by the enzyme acylneuraminate-pyruvate-lyase. The liberated pyruvate is reacted with lactate dehydrogenase (LDH) and reduced nicotinamide adenine dinucleotide (NADH). The resulting decrease in NADH is measured spectrophotometrically at 340nm. Automated versions of this assay using the Cobas Mira™ auto analyser have been described for the detection of Sia in urine and plasma apolipoproteins (Crook *et al.*, 2002; Shishino *et al.*, 2005). A potential pitfall of this enzymatic method is that the presence of endogenous NADH oxidase will interfere with the assay.

A modified method is to couple the liberated pyruvate with pyruvate oxidase which generates hydrogen peroxide in the presence of the electron acceptor oxygen. Sia is subsequently quantified by the colour produced by the peroxidase-coupled reaction. Simpson *et al.* (1993) adapted this procedure for the determination of Sia in capillary blood samples using an ELISA plate reader to quantify the coloured end product (Simpson *et al.*, 1993). A drawback of these enzymatic systems is that the presence of endogenous pyruvate can result in an overestimation of the Sia content. Teshima *et al.* (1988) developed a different enzymatic method that avoids pyruvate interference. Here, *N*-acetylmannosamine generated from Neu5Ac is converted to *N*-acetyl-D-glucosamine by the enzyme acylglucosamine-2-epimerase. The latter is subsequently converted to *N*-acetylglucosaminic acid and hydrogen peroxide by the enzyme *N*-acetyl-hexosamine oxidase. The liberated hydrogen peroxide reacts with 4-aminoantipyrine and a dye in the presence of a peroxidase to produce a chromophore with a wavelength maximum at 590nm. However, this method suffers from ascorbic acid interference (Teshima *et al.*, 1988). A modification of this method converts *N*-acetylmannosamine to *N*-acyl-2-aminomannonic acid with the production of NADH by the action of purified *N*-acylmannosamine dehydrogenase. The appearance of NADH is measured spectrophotometrically at 340nm (Crook, 1998).

1.3.5 Gas Chromatography

Since the early 1960s, a number of gas-liquid chromatography (GC) methods have been developed for Sia detection. These procedures typically involve a variety of derivatisation schemes which convert the carboxyl groups into methyl esters and the free hydroxyl groups into methyl ethers, trimethylsilyl (TMS) ethers or acetyl esters (Reuter *et al.*, 1994). One such scheme involves the addition of *N*-trimethylsilylimidazole to a lyophilised sample of Sia which forms a volatile trimethylsilylated derivative (Elwood *et al.*, 1988). Other trimethylsilyl derivatives can be prepared with trimethylchlorosilane-hexamethyldisilazane-pyridine. The common per-*O*-trimethylsilyl ether methyl ester is generated by the addition of Sia to methanolic hydrogen chloride followed by TMS. Better GC separation of Sia was seen with the introduction of wall-coated open tubular fused silica capillary columns (Reuter and Schauer, 1994). GC in combination with Mass Spectrometry (MS; GC-MS) was originally used to characterise many of the *O*-substituted Sia (Casals-Stenzel *et al.*, 1975). The release of Sia from glycoconjugates can be carried out not only by mild acid hydrolysis but also by methanolysis. The latter procedure cleaves glycosidic bonds and causes less destruction of the Sia than the use of acid. It also allows for a single step GC analysis of monosaccharides as (methyl ester) methyl glycoside derivatives. However, methanolysis leads to the loss of the *N*- and *O*-acyl Sia substituents (deacylation). Although milder methanolysis conditions can be applied to minimize deacylation, this has the disadvantage of incomplete release of Sia, as a methyl ester methyl glycoside. Thus, a re-*N*-acetylation step is included in the derivatisation procedure (Kamerling and Vliegthart, 1982). However, this step can be time consuming and the reaction yield is difficult to control. Consequently, variations can occur in the estimation of the monosaccharide molar composition of the glycoconjugate. The need for re-*N*-acetylation can be eliminated by the use of a strong acylating agent (acid anhydride) to generate trifluoroacetate derivatives. Zanetta *et al.* (1999) applied this approach to the determination of Sia in glyco-proteins and glycolipids (Zanetta *et al.*, 1999). However, the use of small capillary columns is not feasible, as excess trifluoroacetic anhydride destroys the liquid phase. As a result, the method lacks sensitivity when small amounts of material are used (<100µg of glycoconjugate). This problem can be overcome by acylation with anhydrides of higher mass (pentafluoropropionic acid or heptafluorobutyric anhydrides) which produce less volatile compounds.

The heptafluorobutyrate approach allows the quantitative determination of Sia (e.g. KDN and Neu5Ac) from 1 µg of glycoconjugate to a sensitivity of 25 pmol. It also has the added advantage that samples can be analysed after derivatisation without additional purification steps (Zanetta *et al.*, 1999; Bohin *et al.*, 2005). An ultrasensitive method which couples GC and heptafluorobutyrate derivatives with MS has been used to identify new Sia structures (Zanetta *et al.*, 2001). Other chromatographic techniques that include high-performance liquid chromatography (HPLC), High-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD) and capillary electrophoresis (CE) are more widely used (Lamari and Karamanos, 2002).

1.3.6 High-performance anion-exchange chromatography with pulsed amperometric detection

High-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD) is a direct detection method that can separate glycans based on charge. The key steps of this technology are anion exchange chromatography, high pH and the application of a changing positive potential that is applied to an electrode (pulsed amperometry). The use of electrochemical-detection in glycan analysis is based on the generation of current by the oxidation of carbohydrates at the surface of a gold electrode and was first reported in 1983. Since then it has become routine and it is particularly useful for the separation of carbohydrates as no derivatisation step is required (Rohrer, 2000). A disadvantage of this system for the analysis of Sia relates to the highly alkaline buffer conditions used. Neu5Gc, Neu5Ac and KDN are stable in alkaline pH, but other Sia with *O*-acyl substituents are not (Lamari and Karamanos 2002).

In 1990, Manzi *et al.* reported the first HPAEC-PAD method for detection of *O*-acetylated Sia in the pmolar range using a CarboPac™ PA-1 column and acetic acid/sodium acetate as the eluent. The addition of sodium hydroxide to the column effluent prevented the loss of the Sia's *O*-acyl substituents. Most HPAEC-PAD Sia separations employ a CarboPac™ PA-1 column with an isocratic NaOH and sodium acetate solution, or a constant NaOH concentration and a gradient of sodium acetate (Manzi *et al.*, 1990).

When a sodium acetate gradient is employed, Neu5Gc is retained for a longer time on the column. This generates a smaller peak width for Neu5Gc which translates into higher sensitivity. Therefore, a sodium acetate gradient is recommended when Neu5Gc concentrations are low in comparison to other Sia in the sample. HPAEC-PAD has been used to determine Sia in a wide array of samples namely platelets, fibroblasts, gangliosides, thyroglobulin, human choriogonadotropin, α_1 -acid glycoprotein (AGP), Tamm-Horsfall protein and human serum. In addition, this method was used to characterise and determine the chain length of $\alpha(2,8)$ linked sialic acid homopolymers of Neu5Ac, Neu5Gc and KDN. This assay requires highly purified samples and many contaminants commonly found in biological samples such as lipid and protein can cause interference (Rohrer, 2000).

1.3.7 High performance liquid chromatography

The first High performance liquid chromatography (HPLC) separation of Neu5Ac and Neu5Gc was carried out by Roland Schauer's group in 1982 using an anion-exchange resin (Shukla *et al.*, 1982). Since then, HPLC has not only been used to quantify Sia, but also to reliably separate and identify a wide range of different Sia residues. A wide variety of column types, elution methods, derivatisation schemes and detection modes have been employed. A smaller number of HPLC methods which do not require Sia derivatisation have also been reported. For example, Ogawa and co-workers determined the Neu5Ac and Neu5Gc content of bovine vitronectin using *N*-acetyl glycine as an internal standard. HPLC was performed without pre-column or post-column labelling and with ultraviolet (UV) detection at 205nm (Ogawa *et al.*, 1993). Siskos and Spyridaki (1999) used a reversed-phase ion-pair HPLC method with a C18 column, tri-isopropanolamine ion-pair reagent and UV detection, for the separation of Neu5Ac and Neu5Ac2en in serum, urine and saliva (Siskos and Spyridaki, 1999).

Although derivatisation constitutes an additional step, it is far more widely employed, as it increases the specificity and intensity of the instrument signal, while reducing interferences. Hence, it provides far more accurate Sia quantitation. The group of Nikos K. Karamanos used *p*-toluene-sulphonyl-chloride to generate tosyl-derivatives and benzoic anhydride/pyridine to generate per-*O*-benzoylated derivatives that are highly UV-absorbent (Makatsori *et al.*, 1998). Typically, a sample clean-up step prior to derivatisation with ion-exchange chromatography is required to remove neutral monosaccharides and amino acids. In addition, after derivatisation a solid phase extraction with a Sep-Pack C₁₈ is used to remove excess reagents and non-tosylated or un-benzoylated derivatives. Both Neu5Ac and Neu5Gc Tosyl and per-*O*-benzoylated derivatives can be separated on a Supelcosil LC₁₈ column by isocratic elution using a mobile phase of water-acetonitrile and UV detection. The per-*O*-benzoylated derivative of Neu5Gc elutes later than that of Neu5Ac due to the extra benzoyl group on the *N*-glycolyl moiety. These HPLC methods have a limit of detection in the picomolar range and have been used to detect Sia in animal tissues, blood serum and human tissues (Lamari *et al.*, 2002).

Fu and O'Neill (1995) developed a method for the quantitative analysis of Sia using the derivatising agent 1-phenyl-3-methyl-5-pyrazolone (PMP). In this procedure, Sia is first released by neuraminidases or by mild acid treatment with TFA. Sia are then converted to their *N*-acyl mannosamine derivatives with neuraminic acid aldolase before being labelled with PMP after which the mannosamine derivatives are separated by reverse-phase HPLC and detected by UV absorption. Fu and O'Neill (1995) used this method to determine the Neu5Ac and Neu5Gc content of bovine fetuin, human serotransferrin and porcine thyroglobulin (Fu and O'Neill, 1995). Yasuno *et al.* (1999) also reported a similar assay, whereby Neu5Ac and Neu5Gc in bovine fetuin and 3'-sialyllactose were quantitatively converted to acid-stable mannosamine derivatives [*N*-acetyl-D-mannosamine (ManNAc) and *N*-glycolyl-D-mannosamine (ManNGc) respectively] by *N*-acetylneuraminic acid aldolase. In contrast to Fu *et al.* (1995), the fluorophore *p*-aminobenzoic acid ethyl ester (ABEE) was used to derivatise the Sia. The ABEE-converted Sia were analysed by reverse-phase HPLC on a C₁₈ honepak™ column with an isocratic running buffer (potassium borate and acetonitrile) and detected by fluorescence (Yasuno *et al.*, 1999).

One of the most commonly used fluorescent tags for the determination of Sia, is the compound 1,2-diamino-4,5-methylenedioxybenzene (DMB). Hara *et al.* (1986) reported the first reversed-phase HPLC fluorimetric separation of DMB-derivatised Neu5Ac and Neu5Gc isolated from human serum, urine and rat serum. This method had a 40 femtomole limit of detection for both Neu5Ac and Neu5Gc (Hara *et al.*, 1986). Since then Sia from many biological sources have been successfully identified and quantified using this method. In this derivatisation reaction using mild acidic conditions, the α -keto carboxylic acid group of the Sia reacts with DMB to form quinoxaline derivatives which are stabilised by reduction with a high concentration of sodium dithionite. It is only carbon C-1 and C-2 of the Sia that are involved in the DMB derivatisation reaction as the other Sia carbons remain intact (Lamari *et al.*, 2003). The addition of the DMB methylenedioxy group to the Sia results in an increase in mass and contributes to the fluorescence properties of the molecule (Anumula, 2006). The quinoxaline derivatives show strong fluorescence at 448nm (emission wavelength) on irradiation at 373nm (excitation wavelength). KDN elutes before Neu5Gc which in turn elutes before Neu5Ac when these DMB-derivatised samples are separated on a C₁₈ column using an isocratic methanol-acetonitrile-10mM acetate buffer (pH 5.0) (Ito *et al.*, 2002). Most known naturally occurring substituted Sia can be derivatised and separated by DMB-HPLC. The exceptions are 2,3-dehydro Sia and glycosides which do not have a free α -keto group available for reaction (Powell *et al.*, 2001).

A number of researchers have used an internal standard in DMB-HPLC to improve accuracy. Stanton *et al.* (1995) used Neu5Gc as an internal standard for the determination of Sia content in human pituitary gonado-tropins (Stanton *et al.*, 1995). To avoid potential interference from endogenous Sia, Hikita and co-workers (2000) generated an *N*-propionyl analogue of the ganglioside GM3, which was used as an internal standard for the quantification of Neu5Ac and Neu5Gc in the gangliosides isolated from the kidneys of bovine, dog and cat (Hikita *et al.*, 2000). Ito and co-workers used a similar approach; however, they chemically synthesised *N*-propionylneuraminic acid (NPNA) and used it as an internal standard for the quantification of Neu5Ac from human apolipoprotein E. They showed that the coefficient of variation values for the estimation of Neu5Ac with NPNA as an internal standard were significantly less than those calculated without NPNA (Ito *et al.*, 2002).

A major challenge in the quantitative analysis of *O*-acetylated Sia is their low abundance and instability. However, highly sensitive DMB-HPLC combined with mild acid conditions allows the complete cleavage of Sia, with no loss of the *O*-acetyl group (Butor *et al.*, 1993). DMB-HPLC has also been used to identify and to determine the degree of polymerization of polySia structures in bacteria, fish, mammalian brain and milk (Yabe *et al.*, 2003).

The fluorophore *o*-phenylenediamine (OPD) has also been used in Sia determination but is not as widely used as DMB. However, OPD has a number of advantages over DMB in Sia HPLC analysis. The OPD-HPLC method has a greater linear range (2-450 picomoles) in comparison to the DMB-HPLC method (3.5-28 picomoles). In addition, OPD is an inexpensive reagent and it does not require either 2-mercaptoethanol in the reaction mixture or an internal standard for quantitation. In addition, OPD in comparison to DMB retains its fluorescent stability for a significantly longer period of time (Anumula, 1995; Anumula, 2006).

1.3.8 Capillary Electrophoresis

Capillary electrophoresis (CE) is a high-resolution separation technique that requires only trace amounts of sample. It has been applied in a number of different modes to the separation and quantitation of Sia. The commonest and simplest form of CE is capillary zone electrophoresis (CZE). CZE separation in an uncoated fused-silica capillary is based on differences in solute size and charge (the charge-to-mass ratio) at a given pH. In CZE the electro-osmotic flow (EOF) of the buffer moves analytes towards the negative electrode (cathode). The movement of cations (positively charged species) depends on the sum of their EOF and electrophoretic mobility (EM). In contrast, the EM of anions (negatively charged species) attracts them to the positive electrode (anode) and as a result, their apparent migration depends on the net difference between the two driving forces of EOF and EM. It is possible to change the charge-to-mass ratio of many ions by adjusting the pH of the buffer medium to affect their ionization and hence, EM (Rassi, 1996). In the case of most carbohydrates they do not possess ionizable groups (phosphate, sulphate and carboxyl). However, their hydroxyl groups can be ionised but only in very alkaline conditions (pH>11) (Lamari and Karamanos, 2002).

Sia are unique in that they have a carboxyl group at the anomeric carbon, which is ionised at physiological pH and results in a negative charge above a pH of approximately 2.6 (De Bruyn *et al.*, 1978).

CZE has been widely employed in the analysis of many different biological materials for the detection of both underivatised and derivatised Sia. The majority of carbohydrate CE separations are accomplished primarily with borate complexation regardless of whether the carbohydrates are derivatised or underivatised. Borate complexation can magnify small structural differences among closely related carbohydrates and thus, allow a better resolution of multicomponent mixtures (Rassi, 1996). Kakehi *et al.* (1999a) analysed Neu5Ac monomer and oligomers without derivatisation by CZE with UV detection at 200nm. Dong *et al.* (2001) subsequently reported the separation of underivatised Neu5Ac and Neu5Gc employing a 50mM borate/50mM phosphate buffer (pH 8.95) at 10 kV with UV detection. The method was applied to the determination of Neu5Ac in blood sera and had a 39 fmol limit of detection (Dong *et al.*, 2001). Bao *et al.* (2007) separated and quantified 12 of the major sialyloligosaccharides from human milk by CE using a phosphate buffer (pH 7.05) that contained the ionic detergent sodium dodecyl sulphate (SDS) mixed with methanol, an organic modifier. Detection was by absorbance at 205nm and the applied voltage was 30kV with normal polarity (sample is loaded at the anode and detected at the cathode) (Bao *et al.*, 2007). Soga and Heiger (1998) developed an indirect UV detection CE method for the simultaneous separation of underivatised Neu5Gc, Neu5Ac and a panel of different monosaccharides. Their electrolyte solution (pH 12.1) contained 20mM of 2,6-pyridinedicarboxylic acid (UV marker ion) and cetyltrimethylammonium bromide which was used to reverse the direction of EOF (Soga and Heiger, 1998).

Since Sia lack chromophores or fluorophores, several CE methods using a variety of derivatisation schemes have been developed. Derivatisation cannot only increase detection sensitivity and separation efficiency. It also enhances specificity, as detection is performed at wavelengths where interferences from other sample components are avoided (Lamari *et al.*, 2003). The most widely used derivatisation method for Sia analysis is reductive amination. The advantage of this reaction is that it is efficient and may be used to attach a chromophore, fluorophore, or stable isotope tag to a single position of a glycan.

Under mild acid conditions, the carbonyl group of the reducing sugar reacts with the amino-group of the label and forms a Schiff base which is then reduced with sodium cyano-borohydride to a stable secondary amine (Stanton *et al.*, 1995). Reductive amination of Sia was reported by Kakehi *et al.* (1999b) who tested 9 different aminobenzene analogs. The 3-aminobenzoic acid (3-AA) and 3-aminobenzamide analogs were found to be the most suitable for the derivatisation of Sia-containing oligosaccharides. In this CE method, 3-AA derivatives and laser-induced fluorescence (LIF) detection was used to identify Sia released from the glycoproteins of fetuin, thyroglobulin and ribonuclease B. The use of mild derivatisation conditions ensured that labile Sia was not destroyed (Kakehi *et al.*, 1999b).

Guttman introduced the analysis of Sia derivatisation with 2-aminoacridone (AMAC) by CE with LIF detection (Guttman, 1997). Che and co-workers modified the technique for the simultaneous analysis of Neu5Ac and 11 other monosaccharides using borate buffer (pH 10.5) and UV detection at 260nm. In addition, the method was used to determine the Neu5Ac and Neu5Gc content of AGP, human urinary trypsin inhibitor and recombinant human erythropoietin (rhu-EPO) (Che *et al.*, 1999).

Other fluorophores used in CE-LIF Sia analysis have included 8-aminopyrene-1,3,6-trisulphonate (APTS). This reagent was used to label ManNAc by reductive amination. ManNAc was quantitatively generated from Neu5Ac by the action of *N*-acetylneuraminic acid aldolase. The assay had a detection limit of 100 picomole of Neu5Ac (Chen *et al.*, 1998). Mechref *et al.* (1995) used the UV absorbing and fluorescent tag 7-amino-naphthalene-1,3-disulphonic acid (ANDSA) to label sialo-oligosaccharides derived from gangliosides. This reaction involved the condensation of the carboxylic acid group of Neu5Ac with the amino group of ANDSA. This method used a noncomplexing electrolyte system where separation was controlled by altering the ionic strength of the running electrolyte while maintaining zero EOF through the use of polyvinyl alcohol-coated capillaries (Mechref *et al.*, 1995).

Honda *et al.* (2000) developed a novel method based on pre-capillary reductive *N*-methylation of a reducing carbohydrate followed by the introduction of a fluorescent tag. In this procedure, reducing sugars are converted to *N*-methylglycamines with methylamine and the reductant dimethylamine-borane complex.

The *N*-methylglycamines are subsequently labelled with 7-nitro-2,1,3-benzoxadiazole and analysed by CE-LIF. As the derivatisation conditions are mild, destruction or desialylation of the sialylated glycans is prevented (Honda *et al.*, 2000). Other chromophores (per-*O*-benzoylated derivatives) and fluorophores (PMP derivatives) as mentioned for HPLC have also been applied to CE-Sia analysis. Although the PMP reagent cannot be used to derivatise Sia directly due to the presence of an electron-withdrawing carboxyl group, other reducing sugars present within the glycan chains can undergo PMP derivatisation (Honda *et al.*, 2003). Suzuki *et al.* (2001) developed a CE method to detect UV-absorbing PMP derivatives of sialo-*N*-glycans, isolated from fetuin.

The attaching positions of Sia were determined using two neuraminidase preparations from *A. ureafaciens* and *Salmonella typhimurium* that have different linkage preferences (Suzuki *et al.*, 2001). Neu5Ac per-*O*-benzoylated derivatives were also applied to CZE-UV analysis with a phosphate buffer (pH 3.0) containing acetonitrile as an organic modifier. The method was used in the analysis of AGP and blood sera and was found to have a linear range from 5µg/ml to 5mg/ml with a detection limit of 2µM (Strousopoulou *et al.*, 2002).

1.3.9 Mass Spectrometry and Nuclear Magnetic Resonance

The structural organisation of glycoconjugates is extremely complex due to the presence of many saccharides with multiple and diverse substitutions, different glycosidic-linkages and multiple glycosylation sites. The elucidation of such complex structures poses a substantial analytical challenge (Anumula, 2006; Harvey *et al.*, 2006). Mass spectrometry (MS) is mainly used to determine the molecular weights and, hence, the composition of the glycans. In general it does not give details such as sequence or linkage information (Guerardel *et al.*, 2006). However, multistage tandem MS using collisional induced dissociation can provide limited structural information on glycans (Zaia, 2008). In contrast, Nuclear Magnetic Resonance (NMR) is a non-destructive spectroscopic technique that can provide information on the identity and anomeric configuration of glycan residues as well as their sequences and linkages. One dimensional NMR is useful for profiling small amounts of oligosaccharide samples, whereas the two-dimensional NMR techniques provides more detailed information, but requires larger sample amounts. A weakness of NMR is its inherent low sensitivity when compared to other methods as milligram amounts of material are generally required for analysis (Schweda *et al.*, 2008).

The development of ionization methods such as electrospray (ESI), matrix-assisted laser desorption (MALDI) and fast atom bombardment in conjunction with quadrupole, ion trap, time-of-flight (TOF) and tandem mass (MS/MS) spectrometry have greatly accelerated the use of MS-based technologies in sialobiological analysis. Moreover, these soft ionization methods combined with high-resolution separation techniques like CE, HPLC and GC can be applied to the analysis of limited amounts of oligosaccharides in biological materials (Miura *et al.*, 2007). Collectively a combination of these techniques (NMR, MALDI-TOF-MS, ESI-MS, fast atom bombardment-MS, GC-MS, HPLC-ESI-MS, CE-ESI-MS and liquid chromatography (LC)-ESI-MS/MS) have been used to identify the composition, sequence, branching pattern and linkage positions of sialoglycans.

In biological analysis the two major ionization methods used are MALDI and ESI. MALDI involves mixing the analyte molecules with an organic matrix such as 2,5-dihydroxybenzoic acid. A small volume is then dried on a metal target resulting in the formation of crystals after which the sample is placed in the vacuum source of a mass spectrometer. This ionization method has the advantage of simple preparation and a relatively high tolerance to salts and other contaminants. However, underivatized acidic (sialylated, sulphated or phosphorylated) carbohydrates can undergo fragmentation. ESI involves spraying a solution containing the analyte through a needle to which an electrical potential is applied. Based on charge, the very fine droplets produced by the needle are either attracted or repelled towards the mass spectrometer orifice while undergoing solvent evaporation. Fragmentation occurs less in ESI than in MALDI and better resolved peaks are seen due to the absence of matrix adducts. Huang and Riggin (2000) has shown that the analysis of underivatized sialylated *N*-glycans by LC-ESI-MS is superior to that of MALDI (Huang and Riggin, 2000).

For ESI, samples must be salt free and this is accomplished by LC either off- or on-line prior to ESI-MS analysis. Derivatization of carbohydrates is widely used in MS to increase their volatility, stability and to simplify mass spectra interpretation. For example, glycans are often reduced to alditols and permethylation of glycan alditols converts all hydrogen atoms bounded to oxygen and nitrogen to methyl groups which renders the glycans hydrophobic (Zaia, 2008). However, derivatization by permethylation has proved unsuitable for the analysis of Sia, as the harsh conditions can cause the destruction of *O*-acetyl groups. This makes it difficult to work out which positions were originally methylated (Toyoda *et al.*, 2008). Other problems associated with MS analysis of sialoglycans include the instability of the α -glycoside bond between the Sia and the adjacent sugar residue. When sialoglycans are analysed by MS (in either positive or negative ion modes) in-source and post-source processes may cause the release of Sia residues from the core oligosaccharide due to the presence of the free carboxyl group. In addition, the mass spectra observed for sialo-oligosaccharides often shows complex patterns (Kakehi *et al.*, 2002). Desialylation can be prevented by chemical modification of target Sia in glycoconjugates. One approach is to neutralise the negative charge of Sia carboxyl groups either by methyl esterification or by amidation.

Miura *et al.* (2007) developed a novel and quantitative method, for solid-phase methyl esterification of carboxy groups of sialylated oligosaccharides using 3-methyl-1-*p*-tolyltriazene (MTT). They applied MTT-based methyl esterification and MALDI-TOF-MS to the analysis of *N*-glycans isolated from porcine fibrinogen and showed no detectable loss of Sia residues (Miura *et al.*, 2007). Toyoda *et al.* (2008) found that methyl esterification or amidation may result in the incomplete derivatisation of $\alpha(2,3)$ -linked sialoglycans. They proposed a novel amidation method using acetohydrazide to overcome this problem. This procedure was applied to MALDI-TOF MS analysis of *N*-linked glycans released from bovine fetuin, which is rich in $\alpha(2,3)$ -linked sialoglycans. The resulting MS spectra unambiguously showed the complete derivatisation of the $\alpha(2,6)$ and $\alpha(2,3)$ -linked sialoglycans (Toyoda *et al.*, 2008). Galuska *et al.* (2007) applied MALDI-TOF-MS to the characterisation of oligo- and polySia species from colominic acid, neural cell adhesion molecule (NCAM) and bacterial capsular polysaccharides. This method was not only able to identify the type of Sia involved, but also differences in *O*-acetylation and the length of the oligo- and polySia chains. Moreover, $\alpha(2,8)$ -linked polySia chains could be easily discriminated from $\alpha(2,9)$ -linked species, due to differences in acid-catalysed lactone ring formation (Galuska *et al.*, 2007).

CE-ESI-MS was used to quantitatively determine the Neu5Ac and Neu5Gc content of human plasma, AGP, fetuin and transferrin (Ortner and Buchberger, 2008). Allevi and co-workers (2008) developed a new HPLC-ESI-MS/MS technique using isotopologues (molecular species that have identical elemental and chemical compositions but differ in isotopic content) of Neu5Ac and Neu5Gc as internal standards. In this method, underivatised Neu5Ac and Neu5Gc (derived from acid hydrolysis of bovine fetuin) were fully separated and quantified using a boronic-modified C₁₈ reverse phase column and 0.04% (v/v) aqueous formic acid as eluent (Allevi *et al.*, 2008). Hashii *et al.* (2007) developed an extremely sensitive nano flow liquid chromatography MS method to quantify DMB-labelled Sia. Using this method, femtomole amounts of Neu5Gc contamination were detected in a human cell line (HL-60RG) that was cultured with human serum (Hashii *et al.*, 2007). Royle *et al.* (2008) used a combination of techniques that included LC-ESI-MS/MS to compare the distribution of ocular mucins in humans, rabbits and dogs.

In this method, purified *O*-linked glycans were fluorescently labelled with 2-aminobenzamide (2-AB) and separated with normal phase HPLC. Elution positions were expressed as glucose units (GU) by comparison with the elution times of a dextran ladder. The GU value before and after glycosidase digestion was compared to a database of known *O*-glycan GU values. In addition, mass composition information and fragmentation spectra were obtained from LC-ESI-MS/MS analysis. This study showed that the most significant structural difference between human, rabbit and dog ocular glycans was the occurrence of large numbers of sialylated structures [Neu5Ac- α (2,3)-Gal] in humans, compared to the fucosylated structures found in rabbit and dog (Royle *et al.*, 2008). Using a similar set of techniques Peracaula *et al.* (2003) analysed the glycans of the glycoprotein, prostate-specific antigen. They showed that Sia [α (2,3) and α (2,6)-linked] was only expressed in normal PSA, whereas tumour PSA isolated from the prostate cancer cell line, LNCaP, was fucosylated and had no Sia in its glycan structures (Peracaula *et al.*, 2003).

Manzi *et al.* (1990) applied fast atom bombardment-MS to characterise Sia in both its native and derivatised states. Sia was derivatised by reduction and peracylation under mild acid conditions. Different positional isomers were identified using controlled degradation with periodate and tagging of fragments with ABEE. With this method, two new types of Sia were discovered. The 8-*O*-methyl-7,9-di-*O*-acetyl-5-glycolyl-neuraminic acid (Neu5Gc8Me7,9Ac₂) isolated from the starfish *Pisaster brevispinus* and the unusual 4,8-anhydro-*N*-acetylneuraminic acid (Neu4,8an5Ac) isolated from horse serum (Manzi *et al.*, 1990). Zanetta *et al.* (2001) used heptafluorobutyrate derivatives of methyl esters of Sia in GC-MS analysis and identified new 1,7-Sia lactones and *O*-acylated forms of Neu5Ac and KDN (Zanetta *et al.*, 2001).

Their analysis of a rare form of Sia, 8-*O*-methyl-5-glycolyl-neuraminic acid (Neu5Gc8Me) extracted from the gonads of the starfish, *Asterias rubens* revealed for the first time that this Sia was mono-*O*-acetylated at position 4 or 7 (8-*O*-methyl-4-*O*-acetyl-5-glycolyl-neuraminic acid, Neu4Ac5Gc8Me and 8-*O*-methyl-7-*O*-acetyl-5-glycolyl-neuraminic acid, Neu7Ac5Gc8Me) (Zanetta *et al.*, 2006). They also characterised

Sia found in human erythrocyte membranes and discovered new acetylated (5-*N*-acetyl-4-*O*-acetylneuraminic acid, Neu4,5Ac₂), lactylated (5-*N*-acetyl-9-*O*-lactyl-neuraminic acid, Neu5Ac9Lt) and sulphated (5-*N*-acetyl-8-*O*-sulpho-neuraminic acid, Neu5Ac8S) derivatives (Bulai *et al.*, 2003). Rinninger *et al.* (2006) used the same GC-MS technique to characterise the distribution of Sia in the brain, gut and liver of mice. They identified 13 different Sia which differed in their alkyl and acyl substituents. Among the Sia found were Neu5Gc and its lactylated derivative 5-*N*-glycolyl-9-*O*-lactyl-neuraminic acid (Neu5Gc9Lt) which were especially abundant (40-60% of total Sia) in the glycoproteins and glycolipids of mouse liver. In addition, six different mono-*O*-acylated forms were detected, and, for the first time, 5-*N*-acetyl-4-*O*-acetylneuraminic acid (Neu4,5Ac₂) was found in mouse brain (Rinninger *et al.*, 2006).

Haverkamp *et al.* (1982) applied proton NMR (¹H-NMR) spectroscopy to determine the number and position of *O*-acetyl substituents in Sia residues (Haverkamp *et al.*, 1982). A new monosialyl-tetrasaccharide [GalNAcβ(1,4)Neu5Acα(2,3)Galβ(1,4)Glc] isolated from the colostrum of a bottlenose dolphin was discovered by Uemura *et al.* (2005) using one-dimensional ¹H-NMR and MALDI-TOF (Uemura *et al.*, 2005). Guérardel and co-workers applied a combination of MALDI-TOF-MS, MS/MS and one-dimensional ¹H-NMR to show that the sequences of sialylated *O*-glycans isolated from zebrafish, contained an unusual α(2,3)-sialylation of an internal β-Gal- residue [Fuca(1,3)GalNAcβ(1,4)Neu5Gc / Neu5Acα(2,3)Galβ(1,3)GalNAc] (Guérardel *et al.*, 2006). Similarly, Viverge *et al.* (1997) used a combination of methods to characterise the sialyl oligosaccharides found in goat milk. Initially, ion-exchange chromatography was used to separate acidic and neutral oligosaccharides. The monosaccharide composition was determined by GC and the acidic oligosaccharides were converted to methyl glycosides derivatives for MS-GC analysis by methanolysis and trifluoroacetylation. Further elucidation of the structures by fast atom bombardment-MS and ¹H-NMR, identified two tetrasaccharide isomers containing Neu5Ac that was either α(2,3) or α(2,6) linked to galactose (3'- and 6'-sialylgalactosyl-lactose) (Viverge *et al.*, 1997).

1.4 Lectins

Lectins were originally defined as agglutinins which could discriminate between different types of red blood cells. These proteins or glycoproteins have three distinct characteristics: (a). The ability to bind carbohydrates; (b). they are of non-immune origin and (c). biochemical modifications of the carbohydrates to which they bind generally do not occur. Lectins were initially discovered in plants and in snake venom, but are now known to exist throughout nature and are found in both eukaryotic and prokaryotic organisms. They represent a highly diverse group of oligomeric proteins consisting of many unrelated protein families that have common biological properties. In addition, they vary widely in size, structure and molecular organization. Many lectins exhibit characteristic binding specificity to carbohydrate structures and have been utilised as biological research tools and diagnostic agents. For example, they have been used in affinity chromatography and electrophoresis, detection of blots of separated glycoproteins and to probe the glycan structures of proteins (Mandal and Mandal, 1990; Loris, 2002; Lehmann *et al.*, 2006; Wu *et al.*, 2008).

Several lectins isolated from various animals, plants, fungi and microorganisms have been reported to recognize Sia. Even though lectins have been detected in over a thousand species of plants, only a few plant lectins have the ability to bind Sia (Sharon and Lis, 2004). For many years wheat germ agglutinin (WGA) was the only plant lectin known to bind Sia. However, this lectin preferentially recognises GlcNAc and its $\beta(1,4)$ -linked oligomers. Sia binding occurs, because the configuration of the amino and hydroxyl groups at C-5 and C-4 of the pyranose ring of Sia is identical to the C-2 and C-3 groups of GlcNAc (Monsigny *et al.*, 1980; Shibuya *et al.*, 1987). Lectins isolated from the bark of the elderberry plant can also recognize Sia linked to oligosaccharides.

These lectins include: *Sambucus nigra* agglutinin (SNA, SNA1 and SNA1'), *Sambucus sieboldiana* agglutinin (SSA) and *Sambucus canadensis* agglutinin (SCA) (Shibuya *et al.*, 1989; Van Damme *et al.*, 1997). Although most plant tissues contain a single lectin, occasionally two or more lectins are found in the same tissue. For example, two Sia-specific isolectins were isolated from the seed of *Maackia amurensis*.

These lectins differ in their cell specificity and are designated as *Maackia amurensis* hemagglutinin (MAH) and *Maackia amurensis* leukoagglutinin (MAL) (Sharon and Lis, 2004). Chen and co-workers (2009) identified a new leguminous plant lectin (PCL) from the seeds of *Phaseolus coccineus* with specificity towards Sia.

Lectins may recognise a part of the sugar molecule, the whole monosaccharide, a glycosidic-linkage, or a sequence of sugars. However, most lectins classified as Sia-specific do not bind free Sia but only Sia that is linked to other monosaccharides (Sharon and Lis, 2004). For example, the SNA lectins show no inhibition with free Neu5Ac or Neu5Gc but preferentially bind *N*-glycans that contain $\alpha(2,6)$ -Sia-oligosaccharides (Neu5Ac/Gc $\alpha(2,6)$ Gal/GalNac) (Shibuya *et al.*, 1987). Similarly, the mushroom lectin PSL, can bind sialylated $\alpha(2,6)$ but not $\alpha(2,3)$ -linked *N*-glycans that contain the trisaccharide sequence Neu5Ac $\alpha(2,6)$ -Gal $\beta(1,4)$ -GlcNAc/Glc (Mo, 2000). In contrast, the MAL lectin has an $\alpha(2,3)$ Sia-linkage specificity and binds strongly to Neu5Ac $\alpha(2,3)$ -Gal $\beta(1,4)$ GlcNAc/Glc structures found on *N*-glycans. Whereas MAH has its highest affinity for disialylated tetrasaccharide (Neu5Ac $\alpha(2,3)$ Gal $\beta(1,3)$ -[Neu5Ac- $\alpha(2,6)$]GalNac) structures which are present on *O*-glycans (Imberty *et al.*, 2000). Interestingly, it has been proposed that MAL should be classified as a galactose-specific lectin as the presence of Sia only provides an acidic group that enhances the interaction. Experimental work has shown that MAL can tolerate substitution of *N*-acetyllactosamine with sialic acid at the 3 position of galactose. Furthermore, its affinity is enhanced by the removal of Sia and the addition of an acidic group (e.g. sulphate) at the 3 position of galactose. This has led researchers to propose that the interaction with Sia is purely coincidental and that Gal is the real binding partner (Wang and Cummings, 1988; Rudiger and Gabius, 2001).

Studies on the crystal structure of MAL have revealed that the Neu5Ac residue sits in a hydrophobic pocket created by the amino acids Phenylalanine (Phe), Tyrosine (Tyr), Alanine (Ala) and Lysine (Lys). In addition, the carboxyl group of Neu5Ac is required for binding, as this forms a salt bridge with the lysine side chain. In contrast, the C-8 and C-9 of the glycerol side chain ('glycerol tail') of Neu5Ac is not required for binding, while replacement of sialic acid's *N*-acetyl group with *N*-glycolyl does not abolish binding (Imberty *et al.*, 2000).

Practically all classes and subclasses of invertebrates examined have lectins, although their function within these organisms remains unclear (Sharon and Liz, 2004). It has been proposed that these lectins may participate in the innate immune response by inducing bacterial agglutination (Lehmann *et al.*, 2006). A number of Sia-specific lectins have been found in snails, worms, slugs, scorpions, spiders, beetles, shrimp and crabs (Angata and Varki, 2002; Sharon and Liz, 2004; Lehmann *et al.*, 2006). A plethora of Sia-specific lectins were isolated from the haemolymph of crabs. The best known example is limulin, a plasma lectin extracted from the serum of *Limulus polyphemus* (American horseshoe crab). Other examples include: *Scylla serrata* (mud crab), *Cancer antennarius* (California costal crab) and *Carcinoscopus rotunda* (Indian horseshoe crab) (Sharon and Liz, 2004). Mercy and Ravindranath (1993) showed that the preferred sugar of the *Scylla* lectin is Neu5Gc but not Neu5Ac (Mercy *et al.*, 1993). In contrast, limulin reacts with both free and protein bound Neu5Ac and Neu5Gc. The acetyl group at C-5, the hydroxyl group at C-4 and the C-1 equatorially oriented carboxyl group of Neu5Ac are all important for binding to limulin. Similar to MAL, the glycerol tail of Neu5Ac is not needed for binding. In addition, limulin can tolerate Sia substituents like the 9-*O*-acetyl group but has a preference for $\alpha(2,6)$ over $\alpha(2,3)$ -linked Sia. The activity of limulin requires the presence of Ca^{2+} in the medium. Limulin shows non-specific binding with *N*-acetylglucosamine and the Ca^{2+} -chelating sugar, glucuronic acid (Maget-Dana *et al.*, 1981; Muresan *et al.*, 1982; Armstrong *et al.*, 1996). In contrast, the crab lectin from *Cancer antennarius* has the ability to recognise *O*-acetylated Sia in sialoglycoproteins and gangliosides and is highly specific for 4-*O* and 9-*O*-acetylated Neu5Ac (Neu5,4Ac₂ and Neu5,9Ac₂) (Ravindranath *et al.*, 1985).

Both *Cepaea hortensis* agglutinin-I (CHA-I) which is found in the albumin gland of the garden snail and *Limax flavus* agglutinin (LFA) isolated from the slug *Limax flavus* are gastropod lectins (Sharon and Liz, 2004). The binding specificity of CHA-I was extensively tested using 32 different sialic acid analogues and 14 glycoproteins. Analysis showed a requirement for an intact *N*-acetyl group, trihydroxypropyl side chain and an axially-oriented carboxyl group. Unlike LPA and WGA the hydroxyl group at C-4 was not found to be important for binding. Inhibition studies demonstrated that CHA-I recognised Neu5Ac (0.6mM for 50% inhibition) and that the presence of the hydroxyl group in Neu5Gc decreased binding (12.5mM for 50% inhibition).

In contrast to the SNA and MAL lectins, CHA-I does not distinguish between sialic acid in $\alpha(2,3)$ or $\alpha(2,6)$ linkages. Histochemical investigations on formalin-fixed tissues confirmed that CHA-I has a high specificity for *O*-glycosidically linked sialic acids (Brossmer *et al.*, 1992; Fischer *et al.*, 2000; Gerlach *et al.*, 2002; Gerlach *et al.*, 2004).

Knibbs *et al.* tested 24 different Sia analogues for binding affinity towards LFA and showed that most of the functional groups positioned around the Sia ring are required for binding. Similar to CHA-I, modification or removal of the equatorial hydroxyl group at C-4 did not affect LFA binding. However, binding was substantially reduced (15-fold) when a C-4 epimer derivative was tested, thus suggesting that the axial hydroxyl group at C-4 sterically hinders the LFA binding interaction. Interestingly, the carboxyl group at C-1 only made a moderate contribution to binding as modifications decreased binding by only 5-fold. In common with CHA-I a change from 5-*N*-acetyl to 5-*N*-glycolyl reduced LFA binding. However, unlike MAL, the removal of Neu5Ac's glycerol tail eliminates binding to both LFA and CHA-I. The acetylation of Neu5Ac's 9-hydroxyl group results in a 20-fold decrease in LFA binding. The best inhibitor of LFA was CMP-Neu5Ac which contains the β -anomer of Neu5Ac. This is unusual, as the free form of Sia exists mostly in the β -anomeric ring structure (>93%) state. In contrast, glycoconjugate-bound Sia occurs specifically in the α -anomeric form. In common with limulin, LFA prefers $\alpha(2,6)$ over $\alpha(2,3)$ -linked Sia. In addition, both CHA-I and LFA but not limulin have affinity for colominic acid [$\alpha(2,8)$ -linked Neu5Ac] (Miller *et al.*, 1982; Knibbs *et al.*, 1993).

Other gastropod lectins include Achatinin-H, which was extracted from the hemolymph of the African giant land snail (*Achatina fulica*). Unlike the crab lectin from *Cancer antennarius*, Achatinin-H has a strong affinity for free β -Neu5,9Ac₂ and glycoconjugates containing terminal Neu5,9Ac₂- $\alpha(2,6)$ -linked to GalNac, but shows no inhibition with Neu5,4Ac₂. Thus, it is not the sugar's anomeric specificity but the *O*-acetyl group at C-9 that plays a major role in this lectin's-sugar interaction. Achatinin-H shows little reactivity towards the free monosaccharides Neu5Ac and Neu5Gc, but is inhibited by colominic acid. As with LFA and CHA-I the glycerol side chain of Neu5Ac is directly involved in binding.

Achatinin-H was used to identify an increase in 9-*O*-acetylated sialoglycoconjugates (9-*O*AcSA) on the erythrocytes of patients with visceral leishmaniasis (Sen and Mandal, 1995; Chava *et al.*, 2002; Pal *et al.*, 2004; Bandyopadhyay *et al.*, 2005).

Only a few Sia-binding lectins produced by different protozoa species have been reported. These include lectins from *Leishmania*, *Tritrichomonas*, *Babesia*, *Trypanosoma* and *Plasmodium* species. The lectin found in merozoites (daughter cell of the protozoan parasite) of *Plasmodium falciparum* binds to glycoporphins (A,B and C) on human erythrocytes and is specific for $\alpha(2,3)$ -linked Neu5Ac. This parasite is responsible for the most severe form of human malaria and is dependent upon the presence of Sia on host erythrocyte receptors for cell binding and invasion (Lehmann *et al.*, 2006). The *Tritrichomonas mobilensis* lectin (TML) unlike MAL-II and SNA-I does not discriminate between $\alpha(2,3)$ and $\alpha(2,6)$ -linked sialoglycoproteins, but has equal affinity for both types of linkages. In addition, it has an 8-fold higher affinity for free Neu5Ac compared to that of Neu5Gc. Unlike Achatinin-H, *O*-acetylation of Neu5Ac is not important for binding. TML's ability to bind Sia depends on a free carboxyl at C-1 and an unlinked hydroxyl group at C-8 (Babal *et al.*, 1994).

In mammalian cells, adhesion of the bacterium to the host tissue is mediated by hairlike, filamentous surface structures called fimbriae which carry bacterial adhesins. In several Gram-negative and Gram-positive bacteria, these lectin-like bacterial adhesins specifically mediate the interaction with membrane bound cell surface carbohydrates. Strains of *E. coli* may express many types of fimbriae (S-fimbriae and K99-fimbriae) that have different carbohydrate specificities (Sharon and Liz, 2004). For example, FanC is the major subunit of K99 (also known as type F5 fimbriae) and recognises Neu5Gc but not Neu5Ac-containing gangliosides [Neu5Gc $\alpha(2,3)$ -Gal $\beta(1,4)$ -GlcNAc] (Hanisch *et al.*, 1993; Garg *et al.*, 2007). Site directed *in vitro* mutagenesis studies have shown that positively charged amino acids Lys-132 and Arg-136 are essential for K99 binding to negatively charged Sia (Jacobs *et al.*, 1987). Unlike K99, it is the minor subunit of S-fimbriae, SfaS, which is the Sia-binding adhesin. SfaS binds glycoconjugates containing Neu5Gc $\alpha(2,3)$ -Gal $\beta(1,4)$ and Neu5Ac $\alpha(2,8)$ -Neu5Ac (Morschhauser *et al.*, 1990).

The latter glycan is a structural element found on the b-series gangliosides. S-fimbriae inhibition studies with Sia analogues have shown that binding is dependent on the carboxyl group at C-1 and the C-8 and C-9 hydroxyl groups (Hanisch *et al.*, 1993).

Kawagishi *et al.* (1994) reported the first Sia-binding lectin (*Hericium erinaceum* lectin, HEL) from fungi. This lectin is unusual in that it has strong affinity for free Neu5Gc but is weakly inhibited by sialoglycoconjugates (Kawagishi *et al.*, 1994). Other fungal Sia-binding lectins include, *Polyporus squamosus* agglutinin, which has a high specificity and affinity for the trisaccharide sequence Neu5Aca(2,6)-Gal β (1,4)GlcNAc/Glc of *N*-glycans (Mo, 2000). In contrast the galectin, *Agrocybe cylindracea* galectin (ACG) strongly interacts with glycoconjugates containing an α (2,3)-Sia linkage (Neu5Aca(2,3)-Gal β (1,3)/(1,4) (Ban *et al.*, 2005).

Sun *et al.* (2008) identified the *Fenneropenaeus chinensis* lectin (FC-L) from Chinese shrimp (Sun *et al.*, 2008). However, only the animal lectins LFA, LPA and a few plant lectins WGA SNA-I and MAL-II have been widely used in histochemical localization or lectin-affinity chromatography of sialylated glycoproteins (Varki *et al.*, 2009).

1.5 Antibodies

The traditional approach for the production of monoclonal antibodies (mAbs) has used the hybridoma fusion techniques which were first introduced in the 1970's by Kohler and Milstein (Kohler and Milstein, 1975). These techniques involve the injection of an immunogen (e.g., purified antigen or cells or cellular extracts carrying the antigen) into an animal (e.g., a mouse) so as to elicit a desired immune response (i.e., production of antibodies) in that host. When a sufficient serum antibody titre is reached, the primed animal is euthanised and the spleen which contains antibody-producing cells (immune B lymphoblasts) is removed. The spleen B-cell chromosomes encoding desired immunoglobulins are immortalised by fusing the spleen cells with myeloma cells (these cells grow indefinitely), generally in the presence of a fusing agent such as polyethylene glycol (PEG), which promotes cell adherence and exchange of nuclei.

The resulting cells, which include the desired hybridomas, are then grown in a selective medium, such as Hypoxanthine Aminopterin Thymidine (HAT) medium, in which unfused parental myeloma (don't contain the enzyme hypoxanthine guanine phosphoribosyl transferase) or lymphocyte cells (not immortal) eventually die. Thus, only the hybridoma cells (which are immortal) can grow in the HAT medium and survive. The supernatants of the hybridomas are screened for the presence of mAbs with the desired specificity. After testing, the positive cultures are cloned (usually by limiting dilution) to obtain a homogeneous cell clone secreting mAb (Little *et al.*, 2000).

In contrast, phage display and antibody gene libraries can be used to generate recombinant antibodies. This approach combines the generation of billions of phage particles (termed a 'library') that express at their surface an antibody fragment that has the potential to bind to the desired antigen. In addition, a fast screening procedure is used to identify those phage particles from the library that carry specific monoclonal antibody fragments towards the antigen of interest. In contrast to conventional tissue culture methods, the immunoglobulin genes, rather than the cells from which they were derived are immortalised (Siegel, 2002). Various recombinant antigen-binding fragments of immunoglobulins have been displayed on the surface of filamentous phage, these include Fv, scFv, Fab, and scAb. However, the two most commonly displayed antibody fragments are scFv and Fab (See Figure 1.2.3).

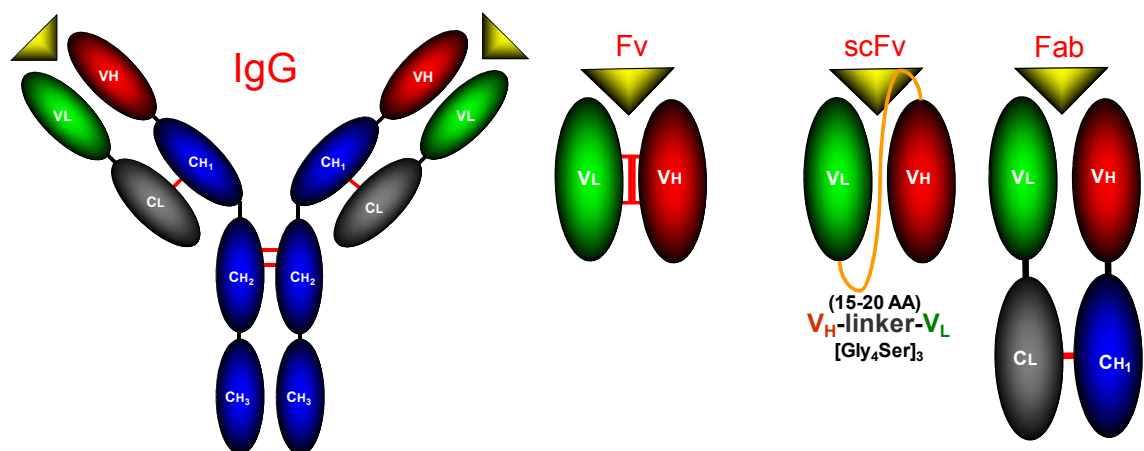


Figure 1.2.3. *The structure of IgG and various recombinant antigen binding fragments.*

The IgG antibody consists of two heavy and two light chains. The variable domains, situated at the end of the IgG molecule, are responsible for antigen binding. The Fab fragment consists of both variable domains and the constant regions of the complete light chain and the first domain of the heavy chain. The scFv fragment contains only the variable domains connected by a peptide linker.

Phage display libraries can be generated from immunised and non-immunised biological material or through synthetic means. Typically, cDNA is prepared from the mRNA (antigen-specific plasma cells are harvested from the bone marrow and/or spleen of the primed animal) of the rearranged immunoglobulin B-cell genes. The heavy and light chain gene segments are then amplified by the polymerase chain reaction (PCR). Subsequently, these antibody gene fragments are inserted into one of the genes encoding a phage (a virus that lives on *E. coli*) surface coat protein. Upon expression in *E. coli*, the coat protein fusions are incorporated into new phage particles that are assembled in the bacterium. Expression of the chimeric fusion product and its subsequent incorporation into the mature phage coat results in the display of the foreign proteins on the surface of filamentous bacteriophage (Pini and Bracci, 2000).

In a process known as biopanning, specific phage are selectively enriched by selection on an immobilised target, antibody fragments that have specific binding properties are identified from a vast pool of phage variants. Typically, affinity is enriched (matured) through multiple rounds of biopanning, a process that involves binding to reduced concentrations of the immobilised ligand. Phage that are weakly bound or have low affinity for the antigen are removed by stringent washing steps during biopanning. The high-affinity bound phage are removed from the surface of an immunotube/maxisorb plate by acid or trypsin elution, and re-amplified through infection of mid-logarithmic growth-phase *E. coli* cells. Typically, 4 to 6 rounds of biopanning and amplification are sufficient to select for phage displaying high-affinity antibody fragments. The hallmark of phage display is that it couples a polypeptide or peptide of interest (antibody's phenotype) to the DNA that encodes it (genotype), thus making it possible to select these two characteristics together (Hoogenboom, 2002).

It was George P. Smith who, in 1985, pioneered the concept of a library of molecular entities each with an artificially coupled phenotype-genotype and the concept of panning from such libraries. Smith cloned a fragment of the bacterial enzyme *Eco* RI into the gene encoding the pIII minor coat protein of the M13 bacteriophage virus. He showed that phages containing the *Eco* RI-pIII fusion gene could be enriched more than 1000 fold from a mixture containing wild type and non-binding phage using an immobilised endonuclease-specific polyclonal antibody. Moreover, these mutated phage were still infectious and capable of passing along their genetic information to their progeny (Smith, 1985). Soon thereafter, Smith and colleagues constructed the first library of phage in which pIII was fused to random peptide sequences. An antibody was again used as the interacting target protein, but this time it selected from 10^7 competing peptides, each of which could potentially form a specific complex with the antibody.

After successive rounds of enrichment, several peptides representing the antibody's interaction site (epitope) were recovered (Smothers *et al.*, 2002). Five years after the discovery of phage display, McCafferty *et al.* (1990) showed that an antibody gene (scFv fragment) could be directly inserted into the phage genome (Mc Cafferty *et al.*, 1990). Although phage display fusions have been made with the major coat protein, pVIII, the minor coat protein pIII is far more widely used. PIII has a number of distinct advantages over other phage coat proteins, these include: its ability to tolerate large insertions, its compatibility with monovalent display, and the wide availability of suitable vectors. A large number of moieties have been displayed on the surface of filamentous phage. These include peptides, antibody fragments, enzymes, protein scaffolds, cDNA libraries, protease inhibitors, transcription factors, cytokines, and extracellular domains of receptors (Hust and Dübel, 2005).

The most popular cloning vehicle used for antibody display is the filamentous bacteriophage. Filamentous phages constitute a large family of bacterial viruses that infect many Gram-negative bacteria. Typically, examples of filamentous (Ff) phage used for display are f1, fd and M13. The genomes of Ff phages are more than 98% identical and their gene products are interchangeable. These non-lytic phages infect strains of *E. coli* containing the F conjugative plasmid (F^+ *E. coli*).

The Ff phages are produced and secreted from infected bacteria without cell killing or cell lysis. The best characterised bacteriophage is M13. M13 is approximately 6.4nm in diameter and 930nm in length. The phage contains a circular single stranded DNA genome (6,400 nucleotides) that encodes 11 different genes (I-XI) which produce 11 different proteins (pI-pXI). Five of the proteins are structural components of the phage particle (pIII, pVI, pVII, pVIII, and pIX) three are involved with the assembly of the phage particle (pI, pIV and pXI), whereas the remaining three proteins (pII, pV and pX) are required to generate single stranded DNA (ssDNA). The most abundant phage proteins are pV and pVIII. Typically, an M13 phage has 27,000 copies of the pVIII protein which forms an envelope around the phage's ssDNA. The proximal tip or the pointed end of the phage particle is formed by the minor coat proteins pIII and pVI present in 3 or 5 copies. The same number of minor coat proteins pVII and pIX are found at the blunt or distal end. Phage assembly begins at the pVII-pIX end, and in the absence of either protein, no particle is formed. A packaging signal located at the pVII-pIX is necessary for the encapsulation of circular ssDNA into phage particles. This signal is positioned in the non-coding intragenic region of the phage genome (Clackson and Lowman, 2007).

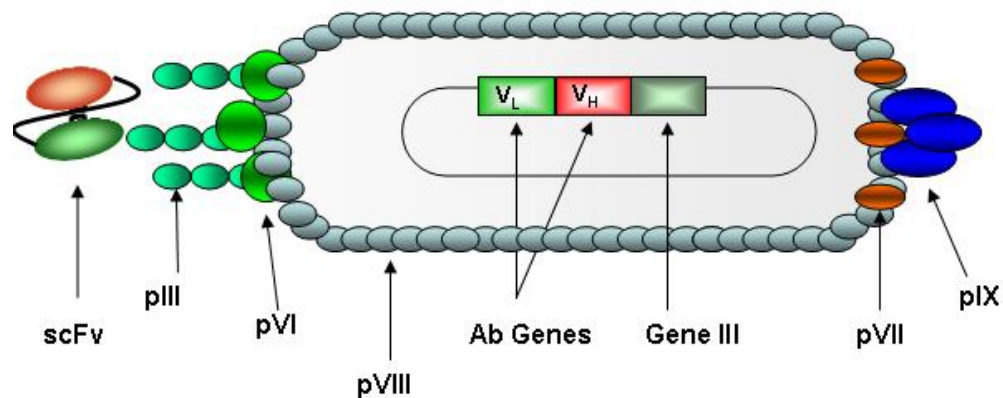


Figure 1.2.4. Diagrammatic representation of a filamentous phage expressing scFv on the gene III product. 2700 copies of the major coat protein (pVIII) encapsulate a single stranded DNA genome. The antibody genes are shown in-frame with gene III.

In the extracellular milieu, bacteriophage float freely until they make contact with a structure know as a sex pilus (F pilus) which resides on the surface of *E. coli*. The pilus is a protein tube assembled by the polymerisation of pilin subunits.

Its role is to connect two *E. coli* cells together during conjugation when DNA exchange occurs. The proteins required for the assembly and subsequent transfer of DNA during conjugation via the F pilus are encoded by a large plasmid, called the F conjugative (F') plasmid. The presence of F' plasmid in the host strain is absolutely essential for infection of *E. coli* by filamentous phage. F pili are low in abundance (only a few are found per cell) and, therefore, the rate of bacterial infection is improved by using large numbers of phage and a high cell density. However, if bacterial cells are grown past log phase, pilus expression is decreased and infectivity compromised. In addition, F pili do not assemble at low temperatures. Therefore, for efficient infection, Ff and *E. coli* cells are incubated at or above 34°C (Barbas, 2001).

The life cycle of M13 starts with the infection of *E. coli* cells by the attachment of the phage particles via their pIII capsid protein with the tip of the bacterium sex pilus. Functionally, pIII can be divided into three different domains (N1, N2 and CT) which are separated by two long flexible linkers that are rich in glycine amino acids. It is the N2 domain of the phage pIII protein that binds to the tip of the F pilus. Within the outer bacterial membrane, 3 Tol proteins (Q, R and A) are necessary for phage infection and for the translocation of phage DNA into the cytoplasm. Binding of N2 to the pilus releases N1 from N2, and allows N1 to interact with domain 3 of TolA. During infection, the phage particles which are attached to the pilus tip by the pIII protein are brought to the surface of the bacterium by pilus (retraction) depolymerisation (Clackson and Lowman, 2007). The major phage capsid protein (pVIII) integrates with the bacterial membrane and subsequently, the phage ssDNA is translocated into the bacterium cytoplasm. The viral phage ssDNA (the plus strand) is converted to a double-stranded, supercoiled molecule, known as the replicative form (RF), by the bacterial host enzymes. The RF serves as a template for phage gene expression, in particular pII, which is an enzyme needed for further viral replication. After formation of RF, the phage pII protein cleaves the positive strand within the intergenic region, which rolls off from the negative strand. Both strands are again replicated to produce double stranded RFs. This type of replication, known as the rolling circle method, continues until there are some 200 copies of the RF from inside the cell. During this time, pV, a single stranded binding protein, accumulates and sequesters the positive strand as it peels off the negative strand, thus preventing it from being converted to double-stranded DNA.

This ssDNA-pV complex is then assembled in the bacterial cytoplasmic membrane and nascent phage particles are subsequently secreted from the cell. The first progeny phage particles appear in the culture supernatant about 10min after infection (at 37°C). These numbers increase exponentially for about 40min, after which the rate becomes linear. About 1000 phage per cell are produced in the first hour. In subsequent generations bacteria produce about 100-200 phage particles. Under optimal conditions phage can be produced from bacterial cells that continue to grow and divide (Rohrbach and Dübel, 2003).

Two different systems, phage vectors and plasmid-based “phagemid” vectors have been developed for the expression of antibody/pIII fusion proteins. In phage vectors, the fusion gene sequence is inserted directly into the phage genome and lies between the secretion signal sequence and the coding sequence for wild-type pIII. Thus, every pIII fusion protein on the progeny *E. coli* phage has an antibody fragment (polyvalent display). This may offer an advantage in the first round of panning, where the desired binder is diluted by millions of phage with unwanted specificity. The avidity effect provided by the oligovalency of these phage improves the chances of a specific binder being enriched (Rohrbach and Dübel, 2003). In contrast, in the phagemid systems, both the replication and foreign fusion protein expression are independent of the phage genome. In this system, the displayed pIII fusion gene is cloned into a small plasmid under the control of a weak promoter. Phagemids make large amounts of recombinant chimeric pIII-foreign protein, but by themselves are unable to make phage. Helper phage (for example VCSM13 and M13K07) are required to provide all the proteins and enzymes required for phagemid replication, ssDNA production and packaging, and also the structural proteins forming the phage coat (Clackson and Lowman, 2007). Helper phage proteins act at the intergenic region (a phage derived origin of replication) of the phagemid DNA as if it was the RF form of a wild-type phage genome. When phagemid-carrying cells are super-infected with helper phage (process often called “phage rescue”), both the wild-type pIII from the helper phage genome, as well as the recombinant pIII from the phagemid, are expressed. In phage assembly wild type pIII is preferentially incorporated into the phage. Thus, the vast majority of phage (90%) do not express recombinant fragments. In contrast, only 10% or less of the phage particles display the pIII fusion protein. In theory, 3-5 pIII fusion proteins can be displayed by the phage.

However, only a very small percentage of phage will display two copies of the fusion protein, thus the display is classified as monovalent. This allows antibody fragments to be selected based on true binding affinities as avidity effects which would decrease the dissociation rate from the panning antigen are avoided. With the use of helper phage, some phage particles may display the desired pIII recombinant fragment and contain the helper phage genome. These phage are useless as they do not have the desired genetic information that encoded the pIII recombinant fragment. Consequently, these phage can not produce more recombinant pIII in subsequent amplification steps. The fraction of phage containing the helper phage genome can be reduced to approximately 1 in 1,000 by using a helper phage with a defective origin of replication or packaging signal. This ensures the preferential packaging of phagemid DNA over helper phage genome and thus the linkage between the displayed protein and its gene is preserved (Paschke, 2006).

A major advantage of phagemids is that their smaller size makes cloning easier. In contrast, phage vectors have a complex structure of overlapping genes, promoters and terminators which makes cloning more difficult. Moreover, the transformation efficiency of phagemids is two to three orders of magnitude better than phage vectors, thus facilitating the generation of large libraries in phagemids. Other important features of phagemid vectors include a plasmid origin of replication as well as phage-derived origin of replication. An antibiotic-resistance marker is also added to allow selection and propagation.

The pIII fusion is controlled by an upstream inducible promoter, typically *lacZ*, which is inhibited by glucose and induced by IPTG. The phagemid also contains an N-terminal secretion signal sequence (OmpA or PelB) which transports the recombinant protein into the bacterial periplasm. Histidine and or haemagglutinin tags are common features that facilitate protein purification and immunochemical detection (Pannekoek *et al.*, 1995).

Phagemids also allow for the soluble (undisplayed) production of the recombinant protein. This is achieved by placing an AMBER stop codon with the TAG sequence between the foreign protein gene and gIII and switching the bacterial strain. For example, when the phagemid is expressed in a nonsuppressor strain of *E. coli* (HB2151, TOP10F') the TAG codon is read as a stop signal.

This produces soluble recombinant protein in the bacterial periplasmic space that is not fused to pIII. In contrast, in suppressor *E. coli* strains (XL1-BLUE, TG1) the TAG codon is read as glutamic acid so that transcription is not stopped and recombinant pIII fusion protein is displayed on the phage (Pini and Bracci, 2000). Two different versions of pIII are found in phagemid constructs. Either, the full-size version (406 amino acids long with a molecular weight of 42kDa) or a truncated pIII form. The truncated version is made by deleting the pIII N-terminal domain. In the case of the pComb3X vectors the truncated pIII fragment (approx 20 kDa) is 208 amino acids long (198-406). As the N-terminal domain of pIII is required for infection, phage displaying only a truncated fused pIII are noninfectious. However, as the majority of pIII displayed is wild-type this allows for the propagation of phage that carry truncated pIII (Rohrbach and Dübel, 2003).

A number of mAbs that bind to Sia have been reported. Typically, it is the extended epitope formed by the underlying glycan chain which is necessary for mAb recognition. MAbs that recognise both Neu5Ac and Neu5Gc on the same underlying glycan are extremely rare (Padler-Karavani *et al.*, 2008). A significant number of mAbs have been generated that recognise sialic acid residues as components of gangliosides. Gangliosides are prominent glycosphingolipids of the plasma membrane that contain one or more sialic acid residues. They are involved in multiple cellular processes such as growth, differentiation and adhesion and as regulators of cell death signalling pathways (Roque-Navarro *et al.*, 2008). The GM₂ ganglioside is known to accumulate in Tay-Sachs disease and other types of lysosomal disorders (Kitamura *et al.*, 1995), whereas the ganglioside GM₃(Neu5Ac) [Neu5Ac α (2,3)-Gal β (1,4)-Glc β (1-1')Ceramide] has been shown to inhibit the proliferation and induce apoptosis in some cancer cells (Noll *et al.*, 2001). In contrast, the unusual GM₃(Neu5Gc) ganglioside [Neu5Gc α (2,3)-Gal β (1,4)-Glc β (1-1')Ceramide] which contains one terminal Neu5Gc residue is a tumour marker that is expressed in certain tumour cells, such as melanoma and breast tumours, but is otherwise absent from normal human tissues (Malykh *et al.*, 2001).

A large number of mAbs that are specific for sialic acid containing-gangliosides are of the IgM class (Carr *et al.*, 2000). These mAbs are produced from a primary immune response; thus, the B cells have not undergone class switching or affinity maturation and have not progressed to a memory phenotype.

Examples include: MK2-34, a murine mAb that recognises the GM₂ Neu5Gc-containing ganglioside [GM₂(Neu5Gc)] and the murine mAb MK1-16, which specifically detects GM₂(Neu5Ac) (Miyake *et al.*, 1988). The murine P3 immunoglobulin M (IgM) mAb was generated using a liposome that contained the ganglioside GM₃(Neu5Gc). Unlike MK2-34, P3 recognises terminal or internal Neu5Gc on a variety of different gangliosides (Malykh *et al.*, 2001). In addition, it also binds some sulphated glycolipids. Asaoka *et al.* (1992) generated two chicken mAbs HU/Ch6-1 and HU/Ch2-7 that detected GM₃(Neu5Gc) and the Hanganutziu-Deicher epitope HD5 [Neu5Ac α (2,3)-Gal β (1,4)-GluNac β (1-3)-Gal β (1,4)-Glc β (1-1')Ceramide]. These mAbs were generated by cell fusion of a thymidine kinase-deficient chicken B cell line with GM₃(Neu5Gc)-immunised chicken spleen cells (Asaoka *et al.*, 1992).

The first IgG anti-Neu5Gc-ganglioside mAb (14F7) was generated by Carr and co-workers in 2000. 14F7 is extremely specific for GM₃(Neu5Gc) and unlike P3, which is restricted to Neu5Gc, 14F7 recognises the disaccharide epitope Neu5Gc α (2,3)-Gal β (1,4). The 14F7 mAb shows no binding to GM₃(Neu5Ac), GM₂(Neu5Ac), GM_{1a}(Neu5Ac) and also GM₂(Neu5Gc). It belongs to the subclass IgG₁ and has binding affinity in the nanomolar range. In contrast, lectins have relatively low affinity for glycans with a K_d in the range 10^{-4} to 10^{-7} . The 14F7 mAb was produced by immunising mice with a vaccine formulation containing GM₃(Neu5Gc) hydrophobically conjugated to human very-low-density lipoproteins (Carr *et al.*, 2000). Crystal studies on the Fab fragment of 14F7 show that it contains a very long CDR H3 loop (16 amino acids) which divides the antigen binding site into two subsites. Typically, Fab-carbohydrate complexes have an antigen binding site that is located in a groove at the interface between the light and heavy chain variable domains. Thus, residues from both V_L and V_H contribute to ligand binding. In contrast, antigen contacts made in the Fab fragment of 14F7 are only with heavy chain amino acids. Its ability to discriminate GM₃(Neu5Gc) from GM₃(Neu5Ac) is due to location of the hydroxymethyl group (-CH₂-OH) which sits in a hydrophilic cavity, forming hydrogen bonds with the carboxyl group of Asp (Aspartate) H52, the indole NH of Trp H33 and the hydroxyl group of Tyr H50. The hydrophobic methyl group (-CH₃) of GM₃(Neu5Ac) cannot bind in such an environment. The positively charged Arg H98 residue forms a salt bridge with the carboxyl group of the Neu5Gc residue (Krengel *et al.*, 2004).

Interestingly, 14F7 may have value as a therapeutic agent as *in vitro* studies using murine myeloma cells have shown that it can induce a complement-independent cell death (Roque-Navarro *et al.*, 2008). In addition, experiments carried out on mice have shown it can inhibit the growth of solid tumours (Krengel *et al.*, 2004).

Chefalo *et al.* (2004) approach the problem of antibody generation towards Sia in a unique way. Their strategy is based on the need to overcome the immunotolerance problem of tumour-associated carbohydrate antigens (TACAs). In principle a specific immune response could occur, if a cancer patient is immunised with a synthetic vaccine that is composed of a TACA analog that contains artificial Sia residues. Treatment of the patient with the modified precursor sialic acid would then initiate the expression of the artificial TACA in place of the natural ones, on tumour cells. The immune response directed towards the artificial TACA should then eliminate the patient's tumours. This group studied the influence of various chemical modifications of Neu5Ac on its immunological properties. The following derivatives were tested: Neu5Ac, 5-*N*-propionylneuraminic acid (Neu5Pr), 5-*N*-iso-butanoylneuraminic acid (Neu5iBu), 5-*N*-phenylacetylneuraminic acid (Neu5FPr) and 5-*N*-phenylacetylneuraminic acid (Neu5PhAc). These *N*-acyl derivatives were coupled to the carrier protein KLH (~300 Sia residues were attached to each protein molecule) and HSA conjugates were used for ELISA analysis. The immunogenicity of the KLH-Sia-containing conjugates were assessed in C57BL/6 mice. Most of the unnatural Sia were much more immunogenic than Neu5Ac which showed little response. Neu5Pr showed the highest antibody titres followed by Neu5iBu and Neu5PhAc. Unusually, Neu5FPr which differs from Neu5Pr by a terminal fluorinated methyl group was weakly immunogenic as it produced only minimal IgM and no IgG titres. The other unnatural Sia produced IgM and different subclasses of IgG antibodies (IgG1, IgG2a and IgG3). In addition, repeated immunisations with Neu5PhAc and Neu5iBu caused a steady increase in the immune response. Chefalo and co-workers concluded that Neu5Ac is a poor murine immunogen. In contrast, certain artificial Sia are capable of inducing a strong immune IgG based response in mice (Chefalo *et al.*, 2004).

The group of Ajit Varki have generated a polyclonal monospecific anti-Neu5Gc antibody. This polyclonal antibody (pAb) was raised in chickens that were immunised with the GM₃ (Neu5Gc) ganglioside. This ganglioside was extracted from the lipid fraction of horse erythrocytes. The pAb was isolated from the total IgY fraction of the chicken egg yolks. Subsequently, a bovine asialofetuin affinity column was used to pre-clear the serum. Either, after which the flow-through fraction was passed over a column of immobilised bovine fetuin with a very low density of bound Neu5Gc (2.7% of total Sias). The pooled fractions were then applied to another fetuin column that was pretreated with mild periodate oxidation to truncate the side chains of Sias. The resulting IgY fraction was enriched for a subset of high-affinity antibodies against Neu5Ac. However, when this pAb was tested in immunohistochemical studies of embryos and tissues from knockout mice that were unable to produce Neu5Gc, (*Cmah-null*) a positive signal was obtained. This occurred even though MS studies had shown that no Neu5Gc was present in the embryos or tissues of the mice. It is thought that the pAb cross reacted with high densities of Neu5Ac contained in the mice tissues (Tangvoranuntakul *et al.*, 2003). In 2009, this group managed to overcome the non-specific binding of the pAb to Neu5Ac. In their new method, human type AB serum sialoglycoproteins were immobilised on a pre-clearing column. The affinity column was prepared by coupling chimpanzee serum. Crude immune IgY was applied to the pre-clearing column to remove non-specific or Neu5Ac-recognising IgY antibodies. The eluant was applied to the chimpanzee serum affinity column. Neu5Gc specific antibodies were eluted with free Neu5Gc. This pAb recognised Neu5Gc in $\alpha(2,3)$, $\alpha(2,6)$ and $\alpha(2,8)$ glycosidic-linkages. In addition, *O*-acetylation of Neu5Gc did not inhibit its binding. Moreover, there was little or no reactivity with the Neu5Ac-containing glycans. Flow cytometry studies using Chinese Hamster Ovary (CHO) cells which contain only 2-3% Neu5Gc, gave a clear positive signal. Moreover, no positive signal was seen with the previous problematic *Cmah-null* mice tissues which are Neu5Gc free (Diaz *et al.*, 2009).

1.6 The importance of sialic acid in disease and in the biopharmaceutical industry.

1.6.1 Sialic acid in disease

Pathogens have evolved complex sialylation patterns in order to masquerade as ‘self’ to avoid, evade or inhibit host innate immunity. For example sialylation (using a *trans*-sialidase) of the trypanosomal surface by Sia sequestered from the host glycoconjugates is thought to protect these parasites from the mammalian host’s or insect vector’s defence systems. Other pathogens, like *E. coli* K1 use *de novo* synthesis of their own bacterial Sia for cell surface sialylation. In contrast, *Neisseria gonorrhoeae* scavenges an extracellular sialyltransferase and CMP-Sia from human secretions for sialylation. Whereas, *Haemophilus influenzae* scavenges free Sia from its host to use for its own cell surface decoration (Vimr *et al.*, 2004).

It has been postulated that just as pathogens evolve their different Sia binding specificities for invasion, the host also counters with various Sia modifications to prevent this attack. The analogy often used is that of an arms race between host and pathogen. This may account for the large structural diversity seen in Sia. However, in the process of altering Sia to prevent one attacker it is possible that this modification may cause the host to be more susceptible to a different pathogen. Such an example is the 9-*O*-acetylation of Neu5Ac which prevents recognition by influenza A virus but this same structure is the preferred ligand for influenza C virus (Angata and Varki, 2002).

In 1924, the Romanian pathologist Hanganutziu noticed that serum taken from a patient who had received anti-tetanus horse serum strongly agglutinated sheep, horse, guinea-pig, rabbit, calf and pig red blood cells. Two years later Deicher replicated these results. These antibodies were referred to as the Hanganutziu-Deicher or HD antibodies. Subsequently these antibodies were detected in patients who had never received animal sera and in those with melanomas and various carcinomas, as well as under certain inflammatory or infectious conditions. Fifty years after the discovery of the HD antibodies, Higashi *et al.* (1977) and Merrick *et al.* (1978) demonstrated that the epitope recognised by these antibodies included the sialic acid residue in Neu5Gc-containing gangliosides (Higashi *et al.*, 1977; Merrick *et al.*, 1978).

The HD antigen is classified as a heterophile antigen (a substance that occurs in unrelated species of animals but has similar serologic properties) and chemically defined as a ganglioside and/or glycoprotein containing Neu5Gc. HD antigens have been identified in humans in association with a wide variety of malignancies, for example, gastric, breast, colorectal, nasopharyngeal and uterine cancer as well as in leukaemia, melanoma, retinoblastoma, hepatocellular carcinoma and malignant lymphoma. Typically anti-Neu5Gc antibodies contain three populations: the first recognises the saccharides of Neu5Gc-Gal, the second recognises the saccharides of Neu5Gc-Gal-GlcNac and the third recognises polypeptides in addition to Neu5Gc-Gal-GlcNac (Ezzelarab *et al.*, 2005).

Humans cannot synthesise Neu5Gc because of an irreversible mutation in the gene encoding CMP-Neu5Ac-hydroxylase (CMAH). CMAH is the gene responsible for the conversion of CMP-Neu5Ac to CMP-Neu5Gc. The human protein lacks the *N*-terminal 104 amino acids due to the deletion of 92bp in the human cDNA. However, monoclonal and polyclonal antibodies have shown the presence of HD antigens in humans. Furthermore, chemical analysis and MS studies have shown the accumulation of Neu5Gc in a variety of tumours (Malykh *et al.*, 2001). In addition, Neu5Gc is also found in small amounts in normal human tissue and high amounts of circulating antibodies towards Neu5Gc have been found in humans. This paradox is explained by the fact that humans absorb Neu5Gc through the diet. Mammalian foodstuffs like red meat and milk products contain Neu5Gc. This exogenous Neu5Gc glycoconjugate enters human cells via pinocytosis where it is released by lysosomal sialidase and subsequently transported by the lysosomal sialic acid transporter to the cytosol. Neu5Gc is then incorporated into human glycoconjugates and these sialoglycoconjugates are deposited in human cell types (in particular endothelium and epithelium). Therefore, within humans Neu5Gc is recognised as “non-self” by the immune system and is a ‘xeno-auto-antigen’ (i.e. an endogenous antigen that is found in more than one species and stimulates the production of auto-antibodies) but as ‘self’ by the cellular biochemical mechanism (Varki, 2007).

1.6.2 Sialic acid and the biopharmaceutical industry

Recombinant mAbs are emerging as key therapeutics in the biopharmaceutical industry. The carbohydrate chains and Sia content of these glycoproteins are extremely important as alterations may affect the mAb's conformation, leading to changes in intracellular transport and secretion, solubility and susceptibility to proteases. Hypersialylation has been applied to a number of mAbs (asparaginase, leptin, luteinizing hormone and cholinesterase) in order to increase their half-life by altering routes of clearance and receptor binding properties. Erythropoietin (EPO) is one of the best studied recombinant therapeutic proteins. EPO stimulates the proliferation and differentiation of erythroid precursor cells to produce mature erythrocytes, a process known as erythropoiesis. This naturally occurring glycoprotein is produced in the kidney and circulates in the blood to stimulate erythropoiesis in the bone marrow. Human-derived EPO is heavily glycosylated with one serine *O*-linked oligosaccharide which can accommodate up to two Sia residues, whereas the three asparagine *N*-linked oligosaccharides can accommodate up to four Sia residues each. This means EPO has the potential to carry 14 Sia residues. The removal of sialic acid from native EPO can increase its *in vitro* biologic activity, possibly by increasing its affinity to receptors on target cells. However, removal of sialic acid residues destroys the *in vivo* biologic activity of EPO which correlates with its rapid clearance from the circulation by interaction with hepatic galactosyl receptors (asialoglycoprotein receptor). The cDNA for EPO has been cloned and two additional consensus-attachment sites for glycans were added at asparagine residues 30 and 88. The resulting recombinant EPO (recHuEPO) has a total of five *N*-linked oligosaccharides. When this recombinant therapeutic (darbepoietin α or novel erythropoiesis stimulating protein) was administered to humans an increase in serum half-life was observed. The higher Sia content of darbepoietin α which can accommodate 22 Sia is a contributory factor for the increase in serum-life. RecHuEPO, which contains more extensively branched chains, exhibits greater *in vivo* biologic activity, suggesting a possible unique role for the complex *N*-linked oligosaccharides *in vivo*.

Other strategies for mAb hypersialylation have focused on the sialyltransferase enzyme which transfers Sia to nascent oligosaccharides. Researchers have overexpressed $\alpha(2,3)$ or $\alpha(2,6)$ -specific sialyltransferases derived from mouse or rat in engineered CHO cell lines. This process can increase the Sia content of the mAb by as much as 40%. Other methods to elevate Sia content have included feeding CHO cells with the Sia precursor ManNAc, but this approach has had limited success. Yet another method is to minimise the actions of cytosol-derived sialidases (cleave Sia) in the CHO-cell culture process. The addition of the transition state analogue, 2,3-dehydro-2-deoxy-*N*-acetylneuraminic acid, or small interfering RNA to the CHO culture supernatant, has been shown to suppress sialidase activity. It is also possible to introduce a thioglycoside linkage between the Neu5Ac and galactose residues. The replacement of an oxygen atom with a sulphur atom ensures that the Sia-Gal bond is resistant to sialidase cleavage (Byrne *et al.*, 2007).

A major concern in biopharmaceutical manufacturing is the presence of the aberrant Neu5Gc glycoepitopes in the glycoproteins of human biotherapeutics. Chinese hamster ovary cells (CHO)-derived products such as epoetin α , epoetin β and darbepoetin β contain approximately 1% to 1.4% of Neu5Gc residues relative to the total sialic acid content (Howard, 2008). It has been shown that Neu5Gc contamination can originate from one or all of the following: the mammalian expression system, animal-derived serum or the growth medium additives (Diaz *et al.*, 2009). As already mentioned Neu5Gc in humans is recognised as ‘non-self’ by the immune system and is classified as a ‘xeno-auto-antigen’. The immunological implications of this in humans are still not fully understood. Whether the Neu5Gc content of CHO-derived biotherapeutics may attract circulating Neu5Gc antibodies and thus reduce the biotherapeutics, pharmacokinetic, bioavailability or functionality in select patients, such as those with higher levels of circulating Neu5Gc antibodies, is currently unknown (Howard, 2008). However, it is still prudent that biomanufacturers strictly control the ratio of Neu5Ac to Neu5Gc, as an increase in Neu5Gc content may correlate with enhanced antigenicity (Fernandes, 2006).

1.7 Thesis Aims

The identification of sialic acid-specific antibodies is important in the fields of basic research and diagnostics. Moreover, understanding carbohydrate-protein interactions at the molecular level has considerable scientific value. Currently, only a limited set of monoclonal anti-sialic acid antibodies exist. The majority of these are not commercially available and were generated using hybridoma technology. Furthermore, they are typically of the immunoglobulin M (IgM) class and have low-affinity and are therefore unsuitable for immunoassays or in *vitro* diagnostics. Importantly, no recombinant avian anti-sialic acid antibody exists. Therefore, the primary objective of this thesis was the generation and characterisation of avian recombinant antibodies that recognised sialic acid. In addition, a secondary aim was the application of directed molecular evolution for the creation of improved anti-sialic acid antibody fragments. Finally, a third goal was the development of a high-throughput screening system for antibody discovery.

Chapter 2

Materials and Methods

2.1 Materials

2.1.1 Equipment

- Biometra T_{GRADIENT} PCR machine - LABREPCO,
101 Witmer Road, Suite 700,
Horsham, PA19044, USA.
- Nanodrop™ ND-1000 - NanoDrop Technologies, Inc.,
3411 Silverside Rd 100BC,
Wilmington, DE19810-4803, USA.
- Gene Pulser Xcell™
electroporation system - Bio-Rad Laboratories, Inc.,
2000 Alfred Nobel Drive,
Hercules, California 94547, USA.
- Roller mixer SRT1 - Sciencelab, Inc.,
14025 Smith Rd.
Houston, Texas 77396, USA.
- Safire 2 plate reader - Tecan Group Ltd.,
Seestrasse 103,
CH-8708 Männedorf, Switzerland.

- PX2 thermal cycler - Thermo Electron Corporation,
81 Wyman Street, Waltham,
Massachusetts (MA), 02454, USA.
- Biacore 3000 - GE Healthcare Bio-Sciences AB,
SE-751 84 Uppsala
Sweden.
- Vibra Cell™ sonicator - Sonics and Materials Inc.,
53 Church Hill Road,
Newtown, CT 06470-1614, USA.
- Shimadzu LC system - Shimadzu Europa GmbH.,
Albert-Hahn-Str. 6-10,
47269 Duisburg, Germany.
- ÄKTA™ Explorer 100 system - GE Healthcare UK Ltd.,
Amersham Place, Little Chalfont,
Buckinghamshire, England.

Robotic System Components

- | | | |
|---------------------------|---|---|
| Tecan freedom EVO 200 | - | Tecan Group Ltd.,
Seestrasse 103,
CH-8708 Männedorf, Switzerland. |
| Automated incubators | - | LiCONiC Instruments,
Trafford Park,
Manchester, M17 1LB UK. |
| Automated Centrifuge | - | Hettich AG,
Seestrasse 204a,
Bäch, Switzerland. |
| Colony Picker | - | KBiosciences,
Maple Park,
Essex road,
Hoddesdon,
Hertfordshire, EN11 0EX, UK. |
| Waste and reagent Bottles | - | VWR International,
Northwest Business Park,
Ballycoolin,
Dublin 15, Ireland. |

2.1.2 Reagents

All reagents were of analytical grade and purchased from Sigma-Aldrich Ireland Ltd (Dublin, Ireland), unless otherwise specified.

Table 2.1 Reagents used in the study along with their suppliers.

Reagent	Supplier
Bacteriological agar	Cruinn Diagnostics Ltd., Hume Centre, Parkwest Business Park, Nangor Road, Dublin 12, Ireland.
Yeast extract	
Tryptone	
DNA ligase	ISIS Ltd., Unit 1 & 2, Ballywaltrim Business Centre, Boghall Road, Bray, Co. Wicklow, Ireland.
Helper phage	
Restriction enzymes	
dNTPs	Medical Supply Company Ltd, Damastown, Mulhuddart, Dublin 15, Ireland.
GoTaq [®] DNA Polymerase	
RedTaq [®] DNA Polymerase	
FastDigest [®] Restriction Enzymes	Fermentas UK, Sheriff House, Sheriff Hutton Industrial Park, York YO60 6RZ, UK.
PageRuler [™] Plus Prestained Protein Ladder	AGB Scientific Limited - A VWR International Company, Orion Business Campus, Northwest Business Park, Ballycoolin, Dublin 15, Ireland.
PCR primers	Eurofins MWG Operon, 318 Worple Road, Raynes Park, London, SW20 8QU, UK.
Perfectprep Gel Cleanup Kit	Eppendorf AG, Barkhausenweg 1,

	Hamburg 22339, Germany.
Wizard Plus SV Miniprep™ kit	Promega, 2800 Woods Hollow Road, Madison, WI 53711, USA.
Platinum® Taq DNA Polymerase High Fidelity	Bio-sciences, Crofton Road, Dun Laoghaire, Dublin, Ireland.
Superscript III reverse transcriptase kit	
Trizol	
Neu5Gc/Ac-linker1-BSA, HSA and OVA conjugates	Carbohydrate Synthesis, Culham Science Centre, Oxford,UK.
Polyacrylamide (PAA) conjugates	Glycotech, 7860 Beechcraft Ave, Gaithersburg, Maryland 20879, USA.
Asialo bovine fetuin	GALAB Technologies GmbH, Max- Planck-Str. 1, D-21502 Geesthacht, Germany.
Bovine fetuin,	
Bovine alpha-1-acid glycoprotein,	
Porcine thyroglobulin	
Neu5Ac	Toronto Research Chemicals, Inc., 2 Brisbane Rd., North York, Ontario, Canada.
Neu5Gc	
Anti-M13 antibody (HRP-labelled)	GE Healthcare Bio-Sciences AB, SE- 751 84 Uppsala, Sweden.
Biacore CM5 research grade sensor chips	
Mouse anti-HA (HRP-labelled) mAb	Roche Diagnostics, Grenzacherstrasse 124, Basel 4070, Switzerland.
FPLC molecular protein standards	Phenomenex Ltd., Melville House, Cheshire SK10 2BN, UK.

2.1.3 Culture Compositions

All materials **unless otherwise stated** were purchased from Sigma and were of analytical grade.

2 x TYE is a culture medium formulation for *E. coli* cell expression.

2 x TYE Medium	Tryptone	16 g/l
	Yeast Extract	10 g/l
	NaCl	5 g/l

SOC is a culture medium formulation for the recovery of transformed *E. coli* cells following electroporation.

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

100 X 5O5 medium	Glycerol	50% (v/v)
	Glucose	5% (w/v)

2.1.4 Buffer Composition

Phosphate buffer saline (PBS) is a wash buffer high in salt content which removes non-specific ionic interactions in an ELISA.

PBS, 150mM, pH 7.4	0.15 M NaCl
	2.5 mM KCl
	10 mM Na ₂ HPO ₄
	18 mM KH ₂ PO ₄

The constituents were dissolved in 990 mL distilled and deionised water and adjusted to pH 7.4. The solution was then made up to a final volume of 1 Litre.

Phosphate buffered saline Tween (PBST)

PBST, 150 mM, pH 7.4	0.05 % (v/v) Tween 20 detergent
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Bovine serum albumin solutions (BSA)

Specified % (w/v) BSA dissolved in either PBS or PBST

Hepes buffered saline (HBS) is the running buffer and sample dilution buffer for Biacore analysis. This buffer is degassed and filtered through a 0.22 µm filter before use.

HBS	2.38 g HEPES
	8.77 g NaCl
	1.27 g EDTA
	0.5 mL Tween
	pH 7.4

The constituents were dissolved in 990 mL distilled and deionised water and adjusted to pH 7.4. The solution was then made up to a final volume of 1 Litre.

2.1.5 *E. coli* strains

All strains were purchased from Stratagene, La Jolla, California, USA.

E. coli TOP10 F strain: {*lacI*^q, *Tn10*(*Tet*^R)} *mcrA* Δ (*mrr-hsdRMS-mcrBC*) ϕ 80*lacZ* Δ M15 Δ *lacX74* *recA1* *araD139* Δ (*ara-leu*)7697 *galU* *galK* *rpsL* (*Str*^R) *endA1* *nupG*

E. coli XL1-Blue strain: *recA1* *endA1* *gyrA96* *thi-1* *hsdR17* *supE44* *relA1* *lac* [*F'* *proAB* *lacI*^q Δ M15 *Tn10* (*Tet*^R)].

2.1.6 Constituents of buffers for SDS-PAGE and Western blotting

Tris-HCl solution

1 M Tris-HCl	Trizma base	121.14 g/L
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Trizma base was dissolved in 800 mL of ultra-pure water. The pH was adjusted to pH 8.8 and 6.8 by adding 1M HCl. The solution was brought to 1 L volume by adding ultra-pure water. The solution was filtered through a 0.2 μ m filter and used for SDS-PAGE preparations.

Separation gel (12.5% (v/v))	1gel/6mL
1 M Tris-HCl, pH 8.8	1.5 mL
30% (w/v) acrylamide	2.5 mL
2% (w/v) methylamine bisacrylamide (Bis-Acrylagel)	1.0 mL
Ultra-pure water	934 μ L
10% (w/v) sodium dodecyl sulphate (SDS)	30 μ L
10% (w/v) ammonium persulphate (APS)	30 μ L
Tetramethylethylenediamine (TEMED)	6 μ L

12.5% (v/v) Stacking gel	1 gel/ 2.5 mL
1 M Tris-HCl, pH 6.8	300 μ L
30% (w/v) acrylamide	375 μ L
2% (w/v) Methylenebisacrylamide (Bis-Acrylagel)	150 μ L
Ultra-pure water	1.74 mL
10% (w/v) sodium dodecyl sulphate (SDS)	24 μ L
10% (w/v) ammonium persulphate (APS)	24 μ L
TEMED	2.5 μ L
Electrophoresis buffer	1 L
Trizma base	3.0 g
Glycine	14.4 g
SDS	1.0 g
Ultra-pure water	to 1 L

Sample treatment buffer (4X)	10 mL
0.5M Tris, pH 6.8	2.5 mL
Glycerol	2.0 mL
2-mercaptoethanol	0.5 mL
20% SDS (w/v)	2.5 mL
Bromophenol blue	20 ppm
Ultra-pure water	2.5 mL
0.5M Tris, pH 6.8	2.5 mL
Coomassie blue stain	1 L
Coomassie blue R-250	2.0 g
Methanol	450 mL
Acetic acid	100 mL
Ultra-pure water	450 mL
Coomassie destain solution	1 L
Acetic acid	100 mL
Methanol	250 mL
Ultra-pure water	650 mL

Transfer buffer	1 L
Trizma base	3.0 g
Glycine	14.4 g
Methanol	200 mL
Ultra-pure water	to 1 L

2.1.7 Constituents of buffers for Immobilised metal ion affinity chromatography (IMAC)

Sodium Hydroxide (NaOH) solution

100 mM NaOH	NaOH	4 g/L
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Appropriate amount of NaOH was dissolved in 10 mL of ultra-pure water and the solution was filtered through a 0.2 µm filter.

Sodium Acetate solution

100 mM Sodium acetate	CH ₃ COONa	8.2 g/L
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Appropriate amount of CH₃COONa was dissolved in 45 mL of ultra-pure water. The pH was adjusted to 4.4 using 1M HCl and the solution was filtered through a 0.2 µm filter.

Lysis Buffer	100 mL
150 mM PBS	100 mL
NaCl	2.92 g
Imidazole	0.136 g

Running Buffer	100 mL
150 mM PBS	100 mL
NaCl	2.92 g
Imidazole	0.136 g
Tween-20	1 mL
Neutralisation Buffer	1 mL
100mM NaOH	500 μ L
10X PBS, pH 7.4	500 μ L

2.2 Methods

2.2.1 Synthesis of the Neu5Gc-BSA and Neu5Gc-HSA conjugates.

The Neu5Gc-bovine serum albumin (BSA), Neu5Gc-human serum albumin (HSA), Neu5Gc-Ovalbumin (OVA) and Neu5Ac-OVA conjugates were custom synthesised by Carbohydrate Synthesis, U.K. An overview of the synthesis scheme is given below (Figure 2.2.1).

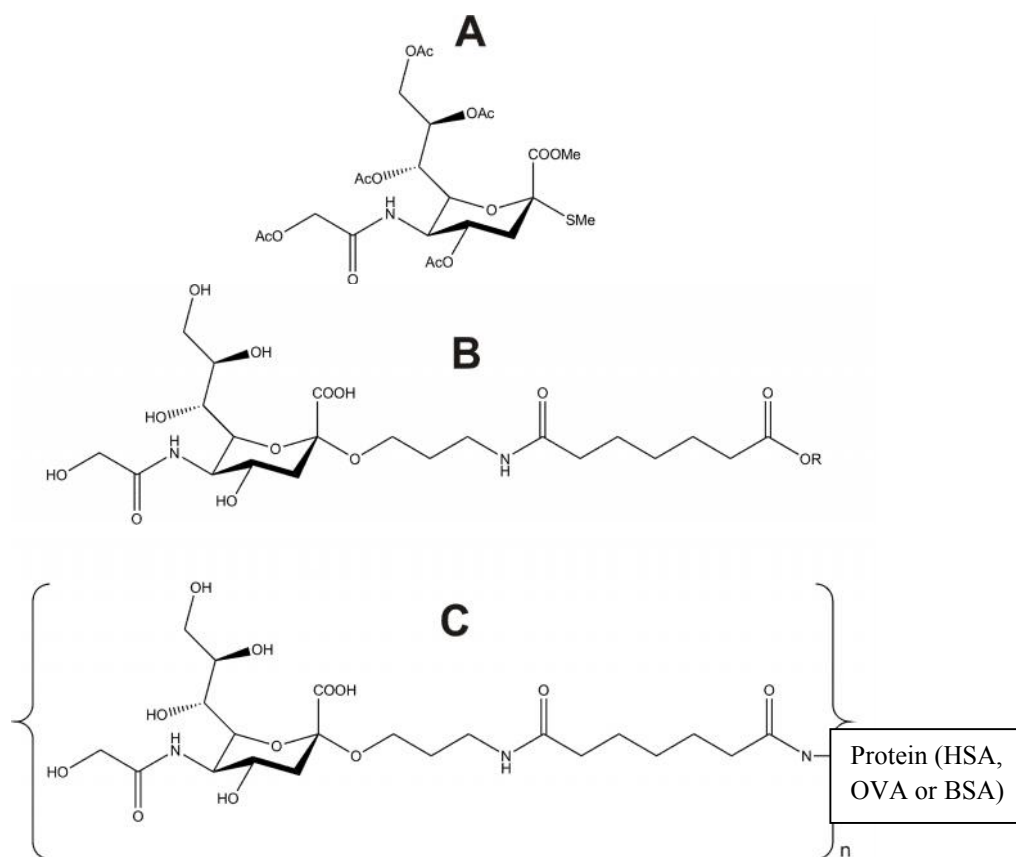


Figure 2.2.1 *The synthesis of Sia-containing conjugates.* A batch of a donor derivative (compound A) of Neu5Gc was synthesised from the free sugar. Compound A was further derivatized to form a new derivative with an activated ester at the end of a long chain (compound B). Compound B was reacted with the side chain amines of the protein's (HSA/BSA/OVA) lysine residues, forming amide bonds with the sugar derivative (compound C). The pre-activated form of compound B was checked by NMR to ensure structural correctness and by TLC to determine purity level. The final product, compound C, was checked by MALDI-MS to give an accurate figure for the degree of sugar substitution to the protein.

2.2.2 Immunisation of Leghorn chickens using the HSA-Neu5Gc conjugate.

All procedures involving the use of animals (*gallus domesticus*, breed Leghorn) were sanctioned by the local ethics committee at Dublin City University (Dublin, Ireland). In addition, these experiments were approved and licensed by the Irish Department of Health and Children (Dublin, Ireland) and were performed with the highest standards of care.

A single comb white male Leghorn chicken (*gallus domesticus*) (aged one month) was injected with 250µg/mL of the HSA-Neu5Gc conjugate (35 monosaccharide units of Neu5Gc per mole of HSA protein) and an equal volume of Freund's complete adjuvant (FCA). The chicken was injected subcutaneously (200µL) at four different sites. Following the first injection, the second, third and fourth boosts were given at two, three, and two weekly intervals, respectively. For boosting injections, an equal volume of Freund's incomplete adjuvant was used. A bleed was taken after the fourth boost and a serum-based polyclonal response was determined by ELISA. For comparative analysis a pre-bleed sample (taken from the same host pre-immunisation) was also selected.

2.2.3 Determination of the avian serum antibody titre using with the BSA-Neu5Gc conjugate.

Direct ELISA was used to determine the serum antibody titre from immunized chicken. A Maxisorp plate (Nunc A/S, Denmark) was coated overnight at 4°C with 5µg/mL of the BSA-Neu5Gc conjugate. The plate was blocked with 3% (w/v) BSA in PBS (pH 7.2) for 1 hour at 37°C. The plate was washed three times with PBSTween (PBST), (pH 7.2) followed by three times with 1x PBS, pH 7.2. A series of dilutions ranging from neat to 1 in 1,000,000 of the chicken serum, diluted in 1% (v/v) BSA 1x PBST (pH 7.2), were added to the ELISA plate in triplicate and incubated for 1 hour at 37°C. The plate was washed three times with 1x PBST (pH 7.2) followed by three times with 1x PBS (pH 7.2). 100µL of rabbit anti-chicken IgY, conjugated with horseradish peroxidase (HRP, Thermo Scientific) (1:2000 1% (v/v) BSA PBST) was added to the plate and then incubated for 1 hour at 37°C.

The plate was washed 3 times with 1x PBST (pH 7.2) and 1x PBS (pH 7.2) and 100 μ L of TMB substrate solution was added to each well. The plate was incubated at room temperature to allow chromophore development, after which the reaction was stopped by the addition of 100 μ L of 10% (v/v) HCl. The optical density (O.D.) was determined at 450nm with a Tecan Safire plate reader.

2.2.4 Determination of the avian serum response to the PAA-Neu5Gc conjugate.

To ensure that the avian polyclonal response was directed towards the Neu5Gc component of the conjugate and not the synthetic linker or protein element, the polyclonal serum was also tested against a synthetic carbohydrate that consisted of a multivalent biotinylated polyacrylamide (PAA) polymer that contained 0.2 moles of Neu5Gc per mole of PAA. The serum IgY response against the Neu5Gc antigen was measured by direct ELISA. A 96 well Maxisorp plate was coated overnight at 4°C with 5 μ g/mL of neutravidin prepared in coating buffer (1x PBS, pH 7.2). The plate was washed three times with 1x PBST (pH 7.2) and three times with 1x PBS (pH 7.2). 100 μ L of biotinylated-PAA-Neu5Gc (25 μ g/mL) was added to the plate and incubated for one hour at 37°C. The plate was then blocked with 3% (w/v) BSA solution in 1x PBS (pH 7.2) for 1 hour at 37°C. After washing three times with 1x PBST (pH 7.2) and three times with 1x PBS (pH 7.2), 100 μ L of serially-diluted serum (in 1% (v/v) BSA 1x PBST (pH 7.2) blocking buffer) was added to the relevant wells. After 1 hour at 37°C, the plates were washed as before and 100 μ L of rabbit anti-chicken IgY conjugated with HRP was added and the plate was then incubated for a further 1 hour. The plate was washed 3 times with 1x PBS (pH 7.2) and the washed 3 times with 1x PBST (pH 7.2), HRP product detection was conducted as previously described (Section 2.2.3).

2.2.5 Determination of the inhibition capacity of the PAA-Neu5Gc conjugate.

A Maxisorp Nunc plate was coated overnight at 4°C with 100µL of 5µg/mL of the BSA-Neu5Gc conjugate. The plate was blocked with 3% (w/v) BSA prepared in 1x PBS (pH 7.2) and incubated for 1 hour at 37°C. The plate was washed three times with 1x PBST (pH 7.2) and 1x PBS (pH 7.2). The Neu5Gc-BSA conjugate was added at varying concentrations to a 1:50,000 dilution of avian serum in 1% (v/v) BSA in 1x PBST (pH 7.2). Samples containing no conjugate (A_0) were diluted in 1x PBST (pH 7.2) to ensure the same serum concentration. Sample dilutions were incubated for 2 hours at 37°C and 100µL of sample was added to the relevant wells. After a 1 hour incubation at 37°C, the plates were washed three times with 1x PBST (pH 7.2) and three times with 1x PBS (pH 7.2) and 100µL of rabbit anti-chicken IgY conjugated with HRP was added and the plate was then incubated for 1 hour. The plate was washed three times with 1x PBST (pH 7.2) and three times with 1x PBS (pH 7.2) and HRP was detected as previously described (Section 2.2.3).

2.2.6 Isolation and quantification of total cellular RNA from the spleen and bone marrow of an immunised Leghorn chicken.

The immunised Leghorn chicken was sacrificed. Both the spleen and the femurs were immediately harvested and processed in a laminar flow hood (Gelaire BSB 4) that was thoroughly cleaned with 70% (v/v) industrial methylated spirits (IMS, Lennox) and RNaseZAP[®] (Invitrogen, USA). The bone marrow from the chicken femurs was washed out with 10mL of chilled TRIzol[®] reagent (Invitrogen, USA) using a 25 gauge needle and 5mL syringe. chilled TRIzol[®] reagent (10mL) was added to the avian spleen and all samples were fully homogenised using a sterile (autoclaved and baked overnight at 180°C) homogeniser (Ultra-Turrax model TP 18/10, IKA[®] Werke GmbH & Co. KG, Germany). The tubes were incubated at room temperature for 5 minutes and centrifuged (Eppendorf centrifuge 5810R) at 2232g for 10 minutes at 4°C. The supernatants were carefully removed and transferred to fresh 'RNase-free' 50mL Oakridge tubes (Thermo Fisher Scientific, USA). For each sample, 3mL of 'RNase-free' chloroform were added and tubes were shaken vigorously for 15 seconds, stored at room temperature for 15 minutes and subsequently centrifuged at 55,800 X g at 4°C for an additional 15 minutes.

Following centrifugation, the mixture separated into a lower phenol-chloroform phase, an interphase, and a colourless upper aqueous phase. The upper aqueous phase, containing the RNA, was carefully removed and transferred to a fresh 'RNase-free' 50mL Oakridge tube. For each sample, 15mL of propan-2-ol was added and tubes were shaken vigorously for 15 seconds, stored at room temperature for 10 minutes and centrifuged at 55,800 X g at 4°C for 30 minutes. RNA precipitated as a white gel-like pellet on the bottom and side of the tube. The supernatant was removed and the pellet was washed with 30mL of 75% (v/v) ethanol and centrifuged at 55,800 X g at 4°C for 10 minutes. This step was repeated and after removal of the supernatant, the RNA pellet was allowed to air dry for 5 minutes. The pellet was then resuspended in 250µL of 'RNase-free' water. The RNA concentrations were determined by spectrophotometric measurement at 260 nm with a NanoDrop™ spectrophotometer ND-1000 (Thermo Fisher Scientific, USA). The purity of the RNA preparation was assessed by measuring the ratio of absorbance at 260 nm and 280 nm. Furthermore, sample purity was assessed by analysis on a 1% (w/v) agarose gel. An aliquot of freshly isolated RNA was used for cDNA synthesis. The remaining RNA solution was precipitated at -20°C with 1/10 the volume of 'RNase-free' sodium acetate pH 5.2 (Sigma-Aldrich) and 2 times the total sample volume of 100% (v/v) ethanol. To enhance RNA precipitation, 'nuclease-free' Glycogen (Fermentas) was added at a final concentration of 1µg/µL.

2.2.7 Reverse transcription of total RNA to cDNA.

The SuperScript™ III First-Strand Synthesis System for RT-PCR, (Invitrogen, USA) was used to generate first strand-cDNA from 5 µg of total RNA using oligo dT₂₀ priming. All reactions were kept on ice at all times. Two 20X master mixes, hereafter referred to as 1 and 2, were made using the recipe below. Reaction mix 1, which contained 25µL was added to 8 tubes that were subsequently incubated at 65°C for 5 minutes (Biometra TGRADIENT PCR machine) and placed on ice for 1 minute. Reaction mix 2, which contained 25µL of reaction mix 2 was added to the same 8 tubes; the tubes were further incubated at 50°C for 50 minutes and the cDNA reaction was terminated by incubation at 85°C for 5 minutes. The samples were centrifuged briefly at 200 X g and 1 µL of RNase H was added to each tube. The tubes were then incubated at 37°C for 20 minutes, after which the cDNA was pooled, aliquoted (20µL) and stored at -20°C. To assess cDNA quality samples were run on a 1% (w/v) agarose gel.

<u>Mix 1 - Components</u>	<u>Stock Concentration</u>	<u>Volume per rxn</u>
RNA	X μ L (to give 5 μ g)	X μ L (to give 5 μ g)
Oligo-dT ₂₀	50 μ M	1 μ L
dNTPs	10mM	1 μ L
Sterile H ₂ O		Y μ L
Total Volume		10μL

<u>Mix 2 - Components</u>	<u>Stock Concentration</u>	<u>Volume per rxn</u>
RT Buffer	10x	2 μ L
MgCl ₂	25mM	4 μ L
Dithiothreitol (DTT)	10mM	2 μ L
RNase OUT	40U/ μ L	1 μ L
Superscript III RT	200U/ μ L	1 μ L
Total Volume		10μL

2.2.8 PCR primers used for the construction of the avian scFv.

The following sets of oligonucleotides were used to generate a chicken scFv library with a short linker from both the bone marrow and spleen. All primers were high purity, salt free and were purchased from Eurofins MWG Operon (Ebersberg, Germany).

Variable heavy chain (V_H) primers

CSCHo-F (sense), Short Linker

5' GGT CAG TCC TCT AGA TCT TCC GCC GTG AC GTT GGA CGA G 3'

CSCG-B (reverse)

5' CTG GCC GGC CTG GCC ACT AGT GGA GGA GAC GAT GAC TTC GGT CC
3'

Variable light chain (V_L) primers

CSCVK (sense)

5' GTG GCC CAG GCG GCC CTG ACT CAG CCG TCC TCG GTG TC 3'

CKJo-B (reverse)

5' GGA AGA TCT AGA GGA CTG ACC TAG GAC GGT CAG G 3'

Overlap extension primers

CSC-F (sense)

5' GAG GAG GAG GAG GAG GAG GTG GCC CAG GCG GCC CTG ACT CAG 3'

CSC-B (reverse)

5' GAG GAG GAG GAG GAG GAG GAG CTG GCC GGC CTG GCC ACT AGT
GGA GG 3'

2.2.9 Amplification of antibody variable domain genes using the pComb primers.

For V_H and V_L gene amplification, a 100µL PCR reaction contained the following: 1µL of cDNA, 60 pMole of CSCHo-F and CSCG-B, 5x PCR Buffer (Promega, USA), 1.5mM MgCl₂ (Promega, USA), 200µM dNTPs (Promega, USA), and 0.5µL GoTaq® DNA Polymerase (Promega, USA). For V_L gene amplification the PCR reaction components were the same except that 60 pmole of CSCVK and CKJo-B were used in place of the V_H primers. The Hybaid Thermal Cycler (Thermo Px2, Thermo Fisher Scientific, USA) was used for all PCR reactions. Touchdown PCR was performed with the following cycling conditions: 4 minutes at 94°C (initial denaturation), followed by 30 cycles of 15 sec at 94°C (denaturation), 30 sec at 60°C (annealing) – the annealing temperature of each cycle was decreased by 0.1°C, 45 sec at 72°C (extension) and the reaction was terminated after 5 minutes at 72°C (final extension).

2.2.9.1 Amplification of the variable heavy chain gene from the anti-sialic AE8 scFv.

For V_L gene amplification, a 100µL PCR reaction contained the following: 10ng of AE8 plasmid DNA, 60pmol of CSCHo-FL and CSCG-B, 5x PCR Buffer 1.5mM MgCl₂, 200µM dNTPs and 0.25µL of RedTaq® DNA Polymerase (Sigma, USA). The Hybaid Thermal Cycler (Thermo Px2, Thermo Fisher Scientific, USA) was used for all PCR reactions. Touchdown PCR was performed with the following cycling conditions: 4 minutes at 94°C (initial denaturation), followed by 30 cycles of 15 sec at 94°C (denaturation), 30 sec at 60°C (annealing) – the annealing temperature of each cycle was decreased by 0.1°C, 45 sec at 72°C (extension) and the reaction was terminated after 5 minutes at 72°C (final extension).

2.2.9.2 Amplification of the avian light chain library using error-prone PCR conditions.

For error-prone PCR V_L gene amplification, a 100µL PCR reaction contained the following: 5ng of the anti-sialic acid scFv library, 60pmol of CSCHo-FL and CSCG-B, 1x PCR buffer, 0.5mM MnCl₂, 7mM MgCl₂, 200µM dATP, 200µM dGTP, 1000µM dCTP, 1000µM dTTP and 0.5µL RedTaq® DNA polymerase. Standard PCR was performed with the following cycling conditions: 4 minutes at 94°C (initial denaturation), followed by 35 cycles of 15 sec at 94°C (denaturation), 30 sec at 60°C (annealing) – the annealing temperature of each cycle was decreased by 0.1°C, 45 sec at 72°C (extension) and the reaction was terminated after 5 minutes at 72°C (final extension).

2.2.10 Purification of V_H and V_L variable gene fragments using Promega® clean up Kit.

The amplified PCR products were analysed using a 1% (w/v) agarose gel and purified with the Wizard® SV Gel and PCR Clean-Up System (Promega, USA). The V_H and V_L bands were excised separately using sterile scalpels and transferred into separate clean 1.5mL micro-centrifuge tubes.

Subsequently, 3 volumes of binding buffer were added to every 1 volume of gel slice. The tubes were then incubated at 50°C for 10min or until the gel had completely dissolved. The binding buffer contains guanidine isothiocyanate which enhances DNA binding to the silica membrane columns. One volume of isopropanol equal to the original weight of the gel slice was then added and mixed to allow for precipitation of the DNA. The mixture was then transferred to a clean collection tube and centrifuged at 15,000 X g for 1 min to remove any residual buffer. To ensure the removal of salts and other buffer contaminants, the flow-through was discarded and the column was washed by centrifugation with 750µL wash buffer and again with 250µL wash buffer. The column was centrifuged at 15,000 X g for 2 min to ensure all residual ethanol was removed and the DNA was eluted from the column using 30µL molecular grade H₂O. The purified DNA was quantified using the Nanodrop™ ND-1000.

2.2.11 Splice by Overlap extension (SOE) PCR.

The V_H and V_L purified fragments were joined with a glycine-serine linker (Gly₄Ser)₃ using splice overlapping extension (SOE) PCR to produce an 750 bp amplicon. For SOE-PCR, a 100µL PCR reaction contained the following: 100ng of the V_L and V_H purified products, 60 pMolar of CSC-F and CSC-B, 10x PCR Buffer (Invitrogen, USA), 1.5mM MgSO₄ (Invitrogen, USA), 200µM dNTPs and 1µL Platinum® Taq DNA Polymerase (Invitrogen, USA). PCR was performed with the following cycling conditions: 5 minutes at 94°C (initial denaturation), followed by 30 cycles of 30 sec at 94°C (denaturation), 30 sec at 57°C (annealing), 1 minute at 72°C (extension) and the reaction was terminated after 10 minutes at 72°C (final extension). The resulting PCR products were run on a 1% (w/v) agarose gel and purified with the Wizard® SV Gel and PCR Clean-Up System as described previously (2.2.10).

2.2.12 SOE-PCR restriction digestion and ligation into pComb3XSS vector for phage display.

The scFv fragment and the cloning vector pComb3XSS were digested with the *Sfi* I restriction enzyme. Prior to digestion, the vector and scFv DNA concentrations were determined by UV absorbance at 260nm with the NanoDrop™ ND1000 spectrophotometer. For scFv digestion, a 100µL reaction contained the following: 12µg of gel-purified short linker scFv, 200 units of *Sfi* I, 10x NEBuffer 2 and 10x BSA. The pComb3XSS 100µL digestion reaction contained the following: 40µg of gel-purified vector, 240 units of *Sfi* I, 10x NEBuffer 2 and 10x BSA. The digestion of purified insert (scFv) and vector (pComb3XSS) was performed for 5 hours at 50°C. Following digestion, the cut pComb3XSS vector and the scFv fragment were purified from a 1% (w/v) agarose gel using the Wizard® SV Gel and PCR Clean-Up System and DNA quantification was determined at 260nm using the NanoDrop™ ND1000 spectrophotometer. The ligation of the scFv fragment with the pComb3XSS vector (ratio of vector to insert 2:1) was performed using T4 DNA ligase overnight at room temperature. The 200µL ligation mixture contained the following: 1.4µg of gel-purified and stuffer free pComb3XSS vector, 700ng of gel-purified scFv, 5x ligase Buffer, and 200 units of T4 DNA ligase. After ligation, the solution was precipitated at -20°C with 1/10 the volume of 'RNase-free' sodium acetate (pH 5.2), 2 times the volume of 100% (v/v) ethanol and 2µL of pellet paint® NF co-precipitant. After overnight precipitation, the sample was centrifuged (Hermle microcentrifuge 233-MK-2) at 19500 X g for 20 minutes at 4°C and the pellet was washed with 70% (v/v) ice-cold ethanol. The mixture was centrifuged at 19500 X g for 10 minutes at 4°C and the pellet was resuspended in 5µL of molecular grade water.

2.2.13 Electro-transformation of XL-1 blue *E. coli* cells with scFv-containing plasmid.

Commercially available electrocompetent XL-1 blue *E. coli* cells (Stratagene, USA) were transformed with the ligated scFv vector construct. This was achieved using a Gene Pulser Xcell electroporation system (Bio-Rad Laboratories, USA) with the controls set at 25 μ F, 1.25kV and the Pulse Controller at 200 Ω . The *E. coli* cells (50 μ L) were thawed on ice. The ligated product (2 μ L) was added to the cells, mixed, left to incubate for 30 seconds and immediately transferred to an ice-cold 0.2cm electroporation cuvette (Bio-Rad Laboratories, USA). The cuvette was tapped so that the suspension was at the base and was placed in the ShockPod and pulsed once. The cuvette was quickly removed from the chamber and 1mL of SOC medium was added immediately to the cuvette. The cells were quickly but gently resuspended with a sterile Pasteur pipette. The 1mL suspension was transferred to a 20mL sterile universal container containing 2mL of SOC medium. To facilitate recovery of the cells, the universal container was shaken for 1 hour at 37°C. The pComb3XSS transformants were plated on TYE plates, supplemented with 100 μ g/mL carbenicillin and 1 % (v/v) glucose. Untransformed XL-1 blue *E. coli* cells (negative control) were plated out in parallel on agar plates with 100 μ g/mL carbenicillin and 1 % (v/v) glucose. The plates were incubated overnight at 37°C. The pComb3XSS transformant colonies were scraped off the plates and used as library stocks. These cells were suspended in 20 % (v/v) glycerol, snap frozen in liquid nitrogen and stored at -80°C. Liquid nitrogen is hazardous, as little as 0.5g in a 1.5mL tube will generate a pressure of 4,053 pounds per square inch (psi) when it evaporates. If the cap fails the tube can turn into a projectile with an initial velocity of up to 296 miles per hour. Therefore, appropriate personal protective equipment (gloves, laboratory coat and protective face mask) should be used when handling this reagent.

2.2.14 Rescue of scFv-displaying phage.

The anti-sialic acid spleen and bone marrow libraries were propagated using 2 x 600 μ L inocula of cells (from the frozen glycerol stocks) into 2 x 600mL cultures of 2xTY (pH 7.2) containing 100 μ g/mL carbenicillin and 2% (w/v) glucose. These libraries were propagated at 37°C until mid-exponential phase of growth (O.D. \sim 0.600 at 600 nm). The cultures were spun down at 2057 X g at 4°C for 10 minutes. The pellets were resuspended in fresh 2xTY medium (600mL) containing 100 μ g/mL carbenicillin and 1×10^{11} plaque-forming units (pfu)/mL of M13KO7 helper phage. The cultures were incubated at 37°C for 30 minutes without agitation after which time they were propagated by shaking at and 37°C for 2 hours. Subsequently, carbenicillin (100 μ g/mL) and kanamycin (50 μ g/mL) were added and the cultures were grown overnight at 30°C. The cultures were centrifuged at 2057 X g for 15 minutes at 4°C and the supernatants transferred to clean sterile 250mL Sorvall centrifuge tubes (Thermo Fisher Scientific, USA). The phage particles were precipitated by the addition of polyethylene glycol 8000 (to 4% (w/v)) and NaCl (to 3% (w/v)). The PEG-NaCl solution was dissolved by shaking at 37°C for 10 minutes. The 250mL centrifuge tube was placed on ice for 1 hour at 4°C and centrifuged at 8228 X g for 25 minutes at 4°C. The phage/bacterial pellet was resuspended in 2mL Tris-EDTA buffer in 2 % (w/v) BSA solution. After the phage pellet was transferred to 1.5mL sterile centrifuge tubes, it was centrifuged at 17500 X g for 5 minutes at 4°C. The supernatant containing the phage scFv was placed on ice and stored at 4°C.

2.2.15 Enrichment of avian phage library via biopanning against immobilised Neu5Gc-BSA.

Maxisorp immuno-tubestm (Thermo Fisher Scientific, USA) were coated overnight at 4°C with 500µL of 100µg/mL Neu5Gc-BSA conjugate. The tubes were blocked with 4mL of 3 % (w/v) BSA in 1x PBS (pH 7.2) for 2 hours at room temperature. The blocking solution was removed and 500µL of rescued phage was added and incubated on a tube roller-mixer SRT1 for 2 hours at room temperature. The solution was removed and non-binding phage were discarded by washing three times with 1x PBST (pH 7.2) and 1x PBS (pH 7.2). Excess PBS was discarded and bound phage particles were eluted with 500µL of 10mg/mL type II porcine trypsin in 1x PBS (pH 7.2) solution. The immuno-tubestm were then incubated at 37°C for 30 minutes. Half the eluted phage particles (250µL) were stored at 4°C. The other 250µL of phage were infected into 2mL of mid-exponential phase XL-1 blue *E. coli* cells. After a static 30 minute incubation at 37°C, 20µL of culture was removed and serially diluted (10^{-1} - 10^{-12}) in 2xTY medium. Serial dilutions (10^{-8} - 10^{-4}) were spread on 2xTY agar plates containing 100µg/mL carbenicillin and incubated overnight at 37°C. The remaining culture was propagated at 37°C for 1 hour. Cells were harvested by centrifugation at 2057g for 10 minutes at 4°C. Library plates were prepared by resuspending the cell pellet in 600µL of fresh 2xTY medium and by spread-plating on TYE plates containing 1% (w/v) glucose and 100µg/mL carbenicillin. Plates were incubated overnight at 37 °C. Input titres were performed by infecting mid-exponential growth phase XL-1 blue *E. coli* cells (180µL) with 20µL of precipitated phage (stored at 4°C) for 15 minutes at 37°C and serial dilutions were performed (10^{-1} - 10^{-12}) and spread on TYE agar plates containing 100µg/mL carbenicillin and incubated overnight at 37°C. In the subsequent rounds (2, 3, 4, and 5) of bone marrow and spleen library biopanning, only 100mL of 2xTY medium was used for cell propagation. As anti-carbohydrate antibodies are known to have weak affinities two different phage elution strategies were followed namely (A) competitive elution and (B) standard trypsin elution. The competitive elution method was expected to generate clones which had low affinity for sialic acid. The standard trypsin elution method was performed as described above except that the Neu5Gc coating concentrations of the immuno-tubestm were reduced in rounds 2, 3, and 4 of biopanning to a concentration of 30µg/mL, 20µg/mL, and 10µg/mL, respectively.

In addition, to remove weakly bound phage, the washing of the immuno-tubestm was increased as follows: round two, 6 times 1x PBST (pH 7.2) and 1x PBS (pH 7.2), round three, 9 times 1x PBST (pH 7.2) and 1x PBS (pH 7.2) and round four, 12 times 1x PBST (pH 7.2) and 1x PBS (pH 7.2). For competitive elution the Neu5Gc-BSA and Neu5Gc-PAA conjugates were added to the immuno-tubestm and incubated overnight at 4°C. The next day the eluted phage were infected into 2mL of mid-exponential phase XL-1 blue *E. coli* cells and the biopanning protocol was followed as described above. For each successive round of biopanning using competitive elution, 500µL of the Neu5Gc-BSA and Neu5Gc-PAA conjugates in 1% (w/v) BSA 1x PBST (pH 7.2) were added to the immuno-tubestm at the following concentrations: round 2, 500µg/mL of Neu5Gc-BSA and 40µg/mL Neu5Gc-PAA, round 3, 300µg/mL of Neu5Gc-BSA and 20µg/mL Neu5Gc-PAA and round 4, 200µg/mL of Neu5Gc-BSA and 20µg/mL Neu5Gc-PAA. The immuno-tubestm coating concentration (50µg/mL) and washing (three times 1x PBST (pH 7.2) and 1x PBS (pH 7.2)) was kept the same for all rounds of competitive elution biopanning.

2.2.16 Enrichment of the mutagenised avian phage library via biopanning against immobilised Neu5Gc-OVA.

Biopanning was performed as described in section 2.2.1.5 with the following modifications. No competitive elution was performed. In addition, the non-depletion method was performed using the Neu5Gc-**Linker1**-OVA conjugate. The Neu5Gc-**Linker1**-OVA immuno-tubestm coating concentrations were 500µg/mL, 50µg/mL, 10µg/mL, 0.1µg/mL and 0.01µg/ml for biopanning rounds 1, 2, 3, 4 and 5, respectively. In the depletion method, phage were incubated for two hours at 37°C with the **Linker1**-OVA conjugate. Phage were transferred to a fresh immuno-tubetm that was previously coated with the Neu5Gc-OVA. **Linker1**-OVA depletion was performed in biopanning rounds 2, 4 and 5 only. The coating concentrations of Neu5Gc-**Linker1**-OVA were the same as previously described, whereas the **Linker1**-OVA conjugate was used at a concentration of 500µg/mL for each of the depletion biopanning round.

2.2.17 Polyclonal phage ELISA analysis.

For the determination of affinity maturation, a polyclonal phage ELISA was performed. A 96-well plate was coated overnight at 4°C with 100µL of 10µg/mL Neu5Gc-BSA. The plate was then blocked for 1 hour at 37°C with 3% (w/v) BSA in 1x PBS (pH 7.2). After blocking, the plate was washed three times with 1x PBST (pH 7.2) and 1x PBS (pH 7.2). Phage particles (100µL) from each round of biopanning (diluted 1:10 in 1% (w/v) BSA 1x PBST, pH 7.2) were assayed in triplicate. Plates were washed three times with 1x PBST (pH 7.2) and 1x PBS (pH 7.2) and 100µL of a 1:5000 dilution of HRP-conjugated mouse anti-M13 monoclonal antibody (GE Healthcare, U.K.) in 1% (w/v) BSA 1x PBST (pH 7.2) was added for 1 hour at room temperature. The plate was washed three times with 1x PBST (pH 7.2) and 1x PBS (pH 7.2) and HRP product detection was conducted as previously described (Section 2.2.3).

2.2.18 Production of soluble antibody fragments.

Antibody fragments without the pIII protein were produced by infecting phagemid DNA from rounds 3 and 4 of biopanning into *E. coli* TOP10F' cells (Stratagene, USA) at mid-logarithmic growth phase. After incubation for 30 minutes at 37°C, serial dilutions were prepared in 2xTY (10^{-2} to 10^{-10}), and plated on TYE plates containing 1% (w/v) glucose and 100µg/mL carbenicillin. Single colonies were inoculated into individual wells of a 96-well ELISA plate containing 200µL of 2xTY in the presence of carbenicillin (100µg/mL) and glucose (1.0% (w/v)). After an overnight incubation at 37°C, master plates of the original clones were prepared by adding glycerol (20% (w/v)) and storing at -80°C. These plates were used as a backup stock for each putative clone of interest. 20µL from the overnight subculture plates were inoculated into fresh 2xTY medium (180µL) containing 1 x 505 medium (0.5 % (v/v) glycerol, 0.05 % (v/v) glucose final concentration), 1mM MgSO₄ and 100µg/mL carbenicillin. The sterile 96 well plates were propagated at 37°C until an O.D₆₀₀ of approximately 0.6 was achieved. A final concentration of 1 mM Isopropyl-β-D-thiogalactoside (IPTG) was added to each individual well and the plates were induced overnight at 30°C. The overnight cultures were frozen at -80°C. The periplasmic scFv was extracted from the cells by three cycles of freeze-thaw.

Cell extracts were cleared by centrifugation (2057g, 10 minutes) and the lysates were diluted 1:5 in 1% (w/v) BSA 1x PBS (pH 7.2). ELISA-based analysis was performed as previously described (Section 2.2.3), with the exception that a 1:2000 dilution (1% (w/v)) BSA 1x PBST (pH 7.2) of rat anti-HA monoclonal antibody conjugated with peroxidase (Roche Diagnostics, USA) was used to detect scFvs that were specific for the sialic acid moiety of the Neu5Gc-BSA conjugate (10µg/mL).

2.2.19 Identification of soluble anti-sialic acid clones that recognise Neu5Gc in the context of a polyacrylamide backbone.

In order to identify scFvs that could not only bind Neu5Gc-BSA, but also recognise this monosaccharide in the context of an alternative backbone, positive clones identified from monoclonal scFv ELISA-analysis were tested against Neu5Gc-PAA. A 96 well Nunc maxisorb plate was coated overnight at 4°C with 5µg/mL of neutravidin in coating buffer 1x PBS (pH 7.2). The plate was washed three times with 1x PBS (pH 7.2) and 1x PBST (pH 7.2). Biotinylated-PAA-Neu5Gc (100µL of 25µg/mL solution) was added and the plate was incubated for one hour at 37°C. The plate was blocked with 3% (w/v) BSA solution in 1x PBS (pH 7.2) for 1 hour at 37°C. After washing three times with 1x PBS (pH 7.2) and 1x PBST (pH 7.2), 100µL of solubly-expressed scFv (diluted 1:5 in 1% (w/v) BSA 1x PBST, pH 7.2) were added to the plate. After a 1 hour incubation at 37°C, the plate was washed three times with 1x PBS (pH 7.2) and 1x PBST (pH 7.2) and 100µL of 1:2000 dilution (1% (w/v) BSA 1x PBST (pH 7.2)) of an anti-HA rat monoclonal antibody conjugated with peroxidase was added. The plate was subsequently incubated for 1 hour and washed 3 times with 1x PBS (pH 7.2) and 1x PBST (pH 7.2). HRP product formation was detected, as described previously (Section 2.2.3).

2.2.20 Cross reaction-analysis of the soluble anti-Neu5Gc clones with other mono and disaccharides.

The capacity of the anti-Neu5Gc clones to cross-react with other carbohydrate elements was assessed by analysis against the following structures: Neu5Ac-PAA, (Neu5Ac)₂-PAA, Neu5Gc-DOPE, glucose-PAA and galactose-PAA. The ELISA was performed as described in (Section 2.2.19) and the concentrations of all conjugates were 25µg/mL.

2.2.21 Sequence analysis of the anti-Neu5Gc and Neu5Ac binding clones.

To ensure the fidelity of the sequence data, three different samples (stab culture, plasmid prep and unpurified PCR products) of the same clone were sent for sequencing. Double stranded DNA sequencing of all clones was performed by Eurofins MWG Operon (Ebersberg, Germany). A panel of anti-sialic acid clones were grown in 1.5mL eppendorf stab cultures. In addition, purified plasmid was obtained from each clone using the Wizard® Plus SV Minipreps DNA Purification System. Furthermore, the scFv gene fragment was also amplified using colony pick PCR. For colony pick PCR, a 50µL PCR reaction contained the following: 2µL of an overnight culture, 60 pmole of CSC-F and CSC-B, 5x PCR Buffer, 1.5mM MgCl₂, 200µM dNTPs and 0.25µL GoTaq® DNA Polymerase. Touchdown PCR was performed with the following cycling conditions: 10 minutes at 94°C (initial denaturation), followed by 30 cycles of 30 sec at 94°C (denaturation), 30 sec at 56°C (annealing) – the annealing temperature of each cycle was decreased by 0.1°C, 1 minute at 72°C (extension) and the reaction was terminated after 10 minutes at 72°C (final extension).

2.2.22 Immobilised metal affinity chromatography (IMAC) purification.

Prior to further analysis by HPLC and surface plasmon resonance (SPR), the AE8 clone was purified by immobilised metal affinity chromatography (IMAC). A single colony of the AE8 clone was sub-cultured into 5mL of 2xTY containing 100µg/mL carbenicillin and 1% (w/v) glucose and grown overnight at 37°C. An overnight culture of 500µL was inoculated into 500mL of Terrific-broth, that contained 1x 505 medium, 50mL potassium phosphate solution, 1mM MgSO₄ and 100µg/mL carbenicillin. The culture was incubated at 37°C until an approximate OD₆₀₀ of 0.6 was reached.

The culture was then induced with 1mM IPTG and incubated at 30°C overnight. The following day, the culture was centrifuged at 2057g for 10 minutes at 4°C and the pellet was completely re-suspended in 30mL of ice-cold sonication buffer (1x PBS, 0.5M NaCl and 20mM imidazole) and then aliquoted (1mL) into 1.5mL Eppendorf tubes. Each individual sample was sonicated on ice for 45 seconds (130W with 40% amplitude) with 6 sec pulses for 3 minutes. The samples were then centrifuged at 17,500 X g for 10 minutes at 4°C. The lysates were pooled, filtered through a 0.2µM filter and stored at 4°C. All IMAC purifications were performed using PD-10 columns (GE Healthcare, U.K.). Two millilitres of nickel-nitrilotriacetic acid (Ni-NTA) resin (Qiagen, USA) were added to the column and allowed to form a packed bed. After equilibration with 30mL of running buffer (sonication buffer containing 1% (v/v) Tween), the pooled lysate was then added to the column and the flow-through was collected and stored at 4°C. The column was subsequently washed with 30mL of running buffer and the bound scFv was eluted by adding 20mL of 100mM sodium acetate (pH 4.4). Volumes of eluent (400µL) were each added to 50µL of 10X PBS (pH 7.2) and 50µL of 100mM NaOH before mixing. Individual fractions were tested for the presence of protein by O.D. measurement at 280nm. Those fractions that contained the eluted scFv were pooled and concentrated using a 5000Da molecular weight cut-off (MWCO) buffer exchange column (Sartorius, Germany). The scFv-containing sample was concentrated to a volume of 500µL by centrifugation (2057g) at 4°C. Five mL of 1x PBS were subsequently added to the column and, after an overnight incubation at 4°C, the sample was buffer exchanged and re-concentrated by centrifugation until the final volume was approximately 200µL. Protein concentration was determined as above.

2.2.23 Size exclusion chromatography-HPLC analysis of the anti-sialic acid clone.

HPLC size exclusion chromatography (SEC-HPLC) was used to determine the species composition, apparent molecular weight and to purify the monomeric fraction of the recombinant AE8 scFv. A Shimadzu LC system (Shimadzu Corporation, Japan), equipped with a Shimadzu CBM-20A controller, Shimadzu LC-20AB pumps, Shimadzu SPD-20A UV-Vis spectrophotometric detector, Shimadzu SIL-20A autosampler, Shimadzu FRC-10A fraction collector, Shimadzu CTO-20AC column oven and Shimadzu's LCsolution software for data handling. The experiments were carried out using the size exclusion Bio-Sep-SEC-S2000 column (Phenomenex; 300x7.8mm) protected with a guard column (Phenomenex; 35x7.8mm). The HPLC system was operated isocratically at room temperature using filtered and degassed 1x PBS (pH 7.2) as the mobile phase. Prior to sample analysis, the column was equilibrated for 45 minutes by gradually increasing the flow rate in increments of 0.1mL/min. All samples (20µL) were diluted in 1x PBS (pH 7.2) and assayed at a flow rate of 0.5mL/min with UV detection (280nm). The following protein standards (Agilent, USA) were used: bovine thyroglobulin (670kD), Human gamma globulin (IgG; 150kD), ovalbumin (44kD) and myoglobin (17kD). Samples were interspersed with water blanks to ensure that all residual protein was eluted. The monomeric AE8 scFv was isolated with the Bio-Sep-SEC-S2000 HPLC column by the collection of several fractions between 17.4 and 18.2minutes at a flow rate of 0.5mL/min.

2.2.24 Fast protein liquid chromatography analysis of the anti-sialic acid clone.

Fast protein liquid chromatography (FPLC) was used to estimate the molecular weight of the AE8 protein. The ÄKTA™ Explorer 100 system (GE Healthcare, USA) equipped with a UV-900 monitor, monitor pH/C-900, sample pump, fraction collector frac-950 and UNICORN™ software for data handling was used for protein analysis. The AE8 sample (100µL) was applied to a HiLoad™ 16/60 Superdex™ 200 Prep-grade FPLC column using filtered and degassed 1x PBS (pH 7.2) at a flow rate of 1mL/min. The following protein standards (Agilent, USA): bovine thyroglobulin (670kD), Human gamma globulin (IgG; 150kD), ovalbumin (44kD) and myoglobin (17kD) were used for the molecular weight estimation of the scFv.

2.2.25 Pre-concentration, immobilisation of neutravidin on a carboxy-methylated dextran chip and capture of biotinylated-Neu5Gc polyacrylamide (PAA).

For all BIAcore[®] 3000 (GE Healthcare, Sweden) experiments the running buffer used was filtered and degassed HEPES buffered saline (HBS) pH 7.4. Solutions of neutravidin (50µg/mL) (Thermo Fisher Scientific, USA) were prepared in 10mM sodium acetate (Sigma-Aldrich, USA) buffers that had been adjusted with 10% (v/v) acetic acid (Sigma-Aldrich, USA) to pH values 4.0, 4.2, 4.4, 4.6, 4.8, and 5.0. Protein (20µL) at each respective pH was sequentially passed over the underivatized carboxy-methylated dextran sensor chip surface (CM5, GE Healthcare, Sweden) at a flow-rate of 10µL/min. A pH of 4.6 was determined to be the optimal pH for neutravidin immobilisation as this yielded the largest change in response units (RU). Neutravidin was immobilised on the CM5 chip with the following protocol: 70µL of 400mM of 1-ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride (EDC) (GE Healthcare, Sweden) was mixed with 70µL of 100mM *N*-hydroxysuccinimide (NHS) (GE Healthcare, Sweden) and injected over the sensor chip surface for 10min at a flowrate of 10µL/min. A 50µg/mL solution of neutravidin was prepared in 10mM sodium acetate (Sigma-Aldrich, USA), pH 4.6 and injected over the activated chip surface for 24min at a flow-rate of 10µL/min. Unreacted NHS ester groups were capped and loose, non-covalently attached proteins were removed by injection of 1M ethanolamine hydrochloride (GE Healthcare, Sweden), pH 8.5, for 11min. Four 30 second sequential pulses of 5mM NaOH at a flow-rate of 10µL/min were used to remove any other loosely bound material. After neutravidin immobilisation, a 100µg/mL solution of biotinylated-Neu5Gc-PAA in HBS was passed over the chip surface at 10µL/min for 20min.

2.2.26 Surface plasmon resonance (SPR) analysis of the AE8 and E15 clones.

The sialic acid binding ability of the AE8 clone was assessed with the previously prepared Neu5Gc sensor chip (Section 2.2.25). A 1 in 100 dilution of the IMAC-purified AE8 clone in HBS was simultaneously passed over flow cells 1 and 2 of the sensor chip at a flow rate of 10 μ L/min for 7 minutes. Following on-line reference subtraction (2-1), the sensorgram indicated a response increase of 1,077.4 RU above baseline. Bound antibody was dissociated with a 30 second pulse of 10mM NaOH and the baseline was restored with the injection of HBS running buffer over the chip surface. The experiment was repeated five times and, on each occasion, the AE8 and E15 Neu5Gc binding responses were greater than 1000 RU. Figure 2.2.2 below illustrates the SPR assay setup.

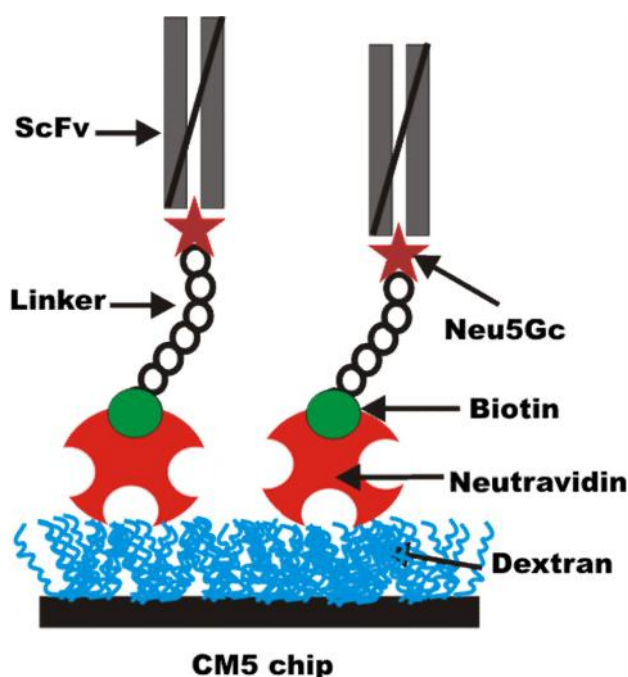


Figure 2.2.2 Assay format used for SPR studies on the AE8 clone.

2.2.27 Solution-phase Neu5Gc-binding assay.

To assess the ability of the anti-sialic acid scFv to bind the sialic acid conjugate in solution-phase, an inhibition binding assay was performed. The purified scFv was diluted 1 in 2000 in HBS buffer (pH 7.4). The Neu5Gc-BSA conjugate was also diluted in HBS buffer (pH 7.4) to the following concentrations: 2000ng/mL, 1000ng/mL, 500ng/mL, 250ng/mL, 125ng/mL and 62.5ng/mL. The AE8 sample (100 μ L) was mixed with 100 μ L of each of the Neu5Gc-BSA conjugate dilutions to yield the following free conjugate working concentrations: 1000ng/mL, 500ng/mL, 250ng/mL, 125ng/mL, 62.5ng/mL and 31.25ng/mL. The zero conjugate sample contained 100 μ L of 1 in 2000 dilution of the purified AE8 scFv in HBS buffer (pH 7.4) and 100 μ L of HBS buffer (pH 7.4). Samples were incubated for 1 hour at 37°C and then injected (40 μ L), in random order, over flow cells 1 and 2 of the Neu5Gc chip at a flow rate of 10 μ L/min for 4min and the change in response recorded. Bound antibody was removed by injection of 5 μ L of 5mM NaOH at a flow rate of 10 μ L/min for 30 seconds.

2.2.28 SPR kinetic studies.

SPR was used to determine the association and dissociation rate constants of the anti-sialic acid scFv. The rate constants were fitted with a pre-defined fitting algorithm using the Biaevaluation 4.1 software. To avoid mass-transfer limited binding, a smaller quantity (1 μ g/mL) of neutravidin (<10,000 RU) was immobilised (see section 2.2.25) on the sensor chip surface. Subsequently, for the capture step, a 40ng/mL solution of biotinylated-Neu5Gc-PAA in HBS buffer (pH 7.4) was passed over the chip surface at 10 μ L/min for 1 min. A final level of 28.6 RU of captured biotinylated-Neu5Gc-PAA was achieved. Furthermore, to rule out the contribution of avidity in the determination of the rate constants, only the monomeric HPLC-purified fraction of the anti-sialic scFv was used for Biacore kinetic analysis. The rate constants were calculated using different concentrations (6.67 μ g/mL, 4.44 μ g/mL, 2.96 μ g/mL, 1.98 μ g/mL, 1.32 μ g/mL, 0.88 μ g/mL, 0.59 μ g/mL and 0 μ g/mL) of monomeric scFv diluted in HBS buffer (pH 7.4). The kinject command was used to inject 90 μ L of each sample over flow cells 1 and 2 of the Neu5Gc sensor chip, at a flow rate of 30 μ L/min for 3min with a dissociation time of 12min.

The zero scFv sample was analysed twice and all samples were run in random order. To reflect the PBS composition of the HPLC eluted monomeric scFv, the zero scFv sample contained 1x PBS (pH 7.2) diluted 1 in 10 in HBS buffer (pH 7.4). Bound antibody was removed by injection of 5 μ L of 1.25mM NaOH at a flow rate of 30 μ L/minute for 10seconds. All sensorgrams were reference subtracted from flow cell 1 which contained a blank dextran surface. In addition, to remove systematic anomalies a blank run consisting of the zero scFv sample was subtracted from each of the sensorgrams.

Chapter 3

Development of a high-throughput robotics system for automated antibody screening

3.1 Introduction

3.1.1 Background to robotic screening

There is an increasing demand for antibodies in various formats for target identification, validation programs and subsequent therapeutic applications. A major challenge for antibody production methods is how to identify more relevant targets at a faster rate and reduced cost. Recent technological advancements in automation and computing, combined with classical biological techniques, have led to the development of high-throughput screening systems for use in life sciences. These new automated technologies, which are based on linear robotic systems, have the potential to dramatically increase the volume, quality, and identification of antibody-based diagnostic and therapeutic reagents (O’Kennedy *et al.*, 2010).

Phage display is a powerful technique that links a protein displayed on the phage surface (phenotype) to its encoding DNA (genotype, which is integrated into the phage genome) and allows the analysis of large recombinant libraries for the presence of specific antibody fragments. The *in vitro* selection process, known as biopanning, involves the enrichment of the desired phage, through binding and elution from an immobilised target molecule. The phage particles are propagated in *E. coli* and typically, a number of cycles of biopanning and propagation are performed (Barbas, 2001). At the end of the process, single colonies are manually picked into microtitre plates to generate antibody-producing monoclonal phage cultures, and antibody specificity is tested by ELISA on solid-phase immobilised selection targets. A major drawback in this manual time-consuming and tedious screening procedure is that only a relatively small sample (~256 clones) of the enriched phage population are analysed. This leads to the possibility that high-affinity clones may be missed during the ELISA screening process. It is this part of the phage display process which is ideally suited for high-throughput work, using automated equipment (e.g. plate-washer, colony picker, incubator, spectrophotometer, centrifuge and a robotically-controlled work table). The key advantage of automated antibody-screening is that thousands of antibody-producing clones can be picked and screened for binding to their cognate antigen. Moreover, each clone can be electronically tracked and backed up during the process. Subsequent data analysis not only allows for rapid selection of multiple positive clones,

but also facilitates the facile identification and elimination of undesirable (non-specific, cross-reacting, low-affinity etc.) candidates. Thus, an automated high-throughput antibody screening system is ideally suited for the generation of superior antibody candidates.

3.1.2 Components of the high-throughput antibody robotic screening system

The high-throughput robotic antibody screening system was located at DCU (Figure 3.1.1). The major component of this system was the Tecan freedom EVO 200 liquid handling workstation (Tecan, UK). This workstation contained a work table, three robotic arms (eccentric gripper, centric gripper and a LiHa [liquid handling arm]), one shelf and a storage rack (hotel) for holding microtiter and deepwell plates, 2X Mp3pos (a plate holder on the worktable) and a number of reagent troughs (Tecan, UK). In addition, the external pieces of equipment are: (a) 2X StoreX (Incubator) (LiCONiC instruments, UK) (b) Centrifuge (Hettich AG, Switzerland) (c) Colony picker and pump (KBiosciences, UK) (d) A desktop PC, running Tecan freedom EVOware[®] software on a windows XP[®] operating system (Tecan, UK) and (e) Waste and reagent bottles (VWR, Ireland) (Figure 3.1.1).

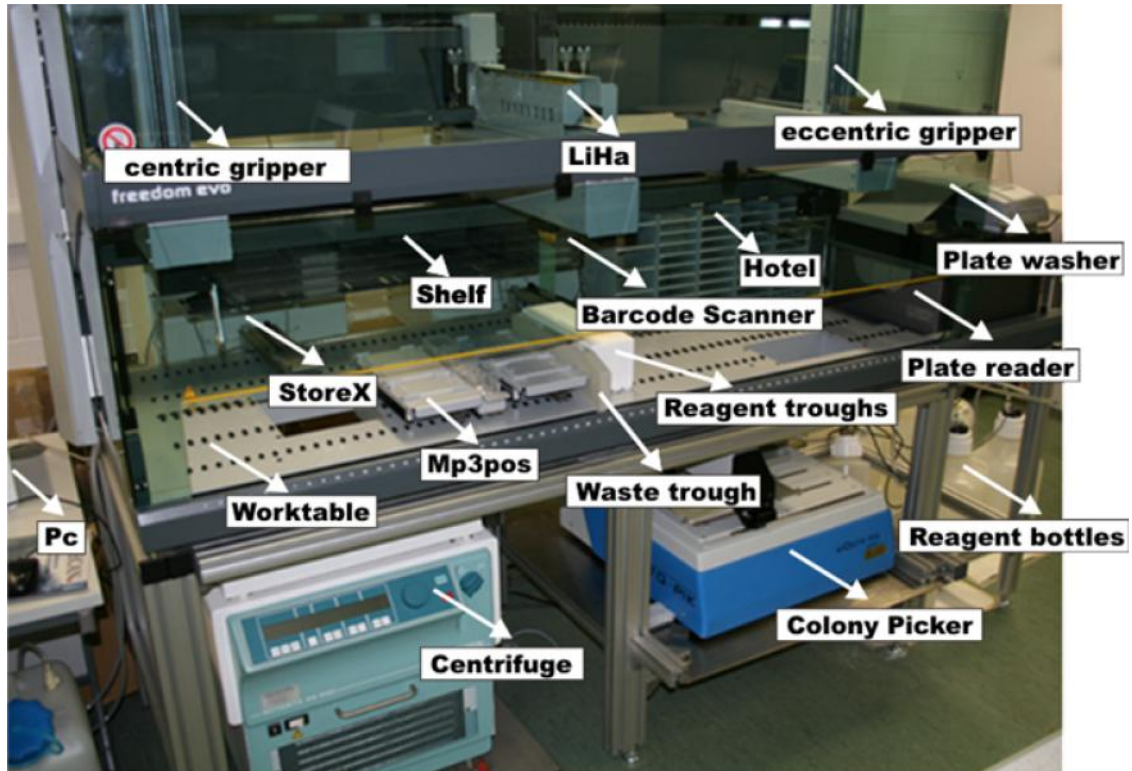


Figure 3.1.1 *Components of the Tecan robotics system.* The robotics system was custom installed ‘on site’ at DCU. The Tecan freedom EVO 200 liquid handling workstation constitutes the major component of the system. Other hardware components include the PC, colony picker, centrifuge, plate washer, plate reader and incubators (the incubators are located behind the instrument).

3.1.3 Flow diagrams of the processes used in the robotics system

An overall view of the different parts of the robotic antibody screening process is illustrated in Figure 3.1.2. This flow chart represents a modified form of the original plan. This plan was altered due to the inherent limitations of the robotic system. In summary, the screening procedure is made up of five major elements. Each element is termed a process. The initial process (process 1) transfers the 2mL deep-well plates (DWP) from the incubator to the Mp3pos and fills them with media. Subsequently, the plates are returned to the incubator. In process 2, the colony picker (cp) uses a camera to make an image of the bioassay dish (which contains the antibody-producing clones), in order to identify the location of each colony.

Process 3 transfers the DWPs from the incubator to the cp platform. The cp instrument uses pins to pick and inoculate a small amount of the colony into DWPs. Subsequently, the DWPs are returned to the incubator (Figure 3.1.2).

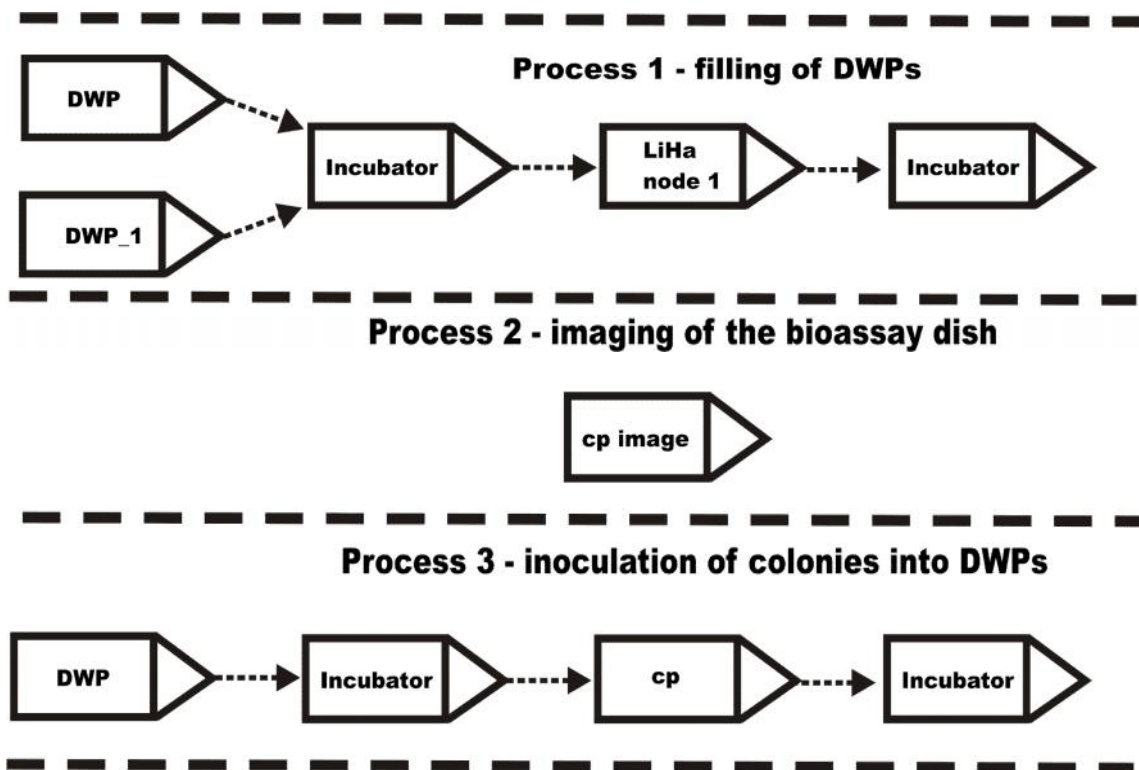


Figure 3.1.2 Flow diagram of the first 3 processes of the robotics system. Process 1 fills the DWPs and returns them to the incubator. Process 2 images the bioassay dish which contains individual *E. coli* colonies. Process 3 picks colonies from the bioassay dish and inoculates them into the DWPs.

Process 4 (Figure 3.1.3) performs the bulk of the screening and a large number of robotic movements are executed. Consequently, the script used are more complex than in processes 1, 2, 3 and 5. Overall, the functions of this process can be broken down into the following sub-components (a) Assign barcode id (b) Make backup of colonies (c) Induce clones (d) Add lysis solution (e) Perform ELISA. In the first step, a 384 microtiter plate (MP) is moved from the shelf position and the barcode label is scanned, after which the MP is moved to the Mp3pos. The lid from the MP is removed and placed in the hotel. The MP is subsequently filled with media and two DWPs are moved from the incubator, their barcode labels are recorded and the DWPs are placed on the second Mp3pos. Back-ups of the DWPs are inoculated into the 384 MP plates (LiHa node 1). The MP lid is removed from the hotel and placed on the plate. The DWPs are returned to the incubator and the 384 MP is returned to the shelf. The DWPs are moved back out of the incubator and placed on the Mp3pos. The induction solution is then added (LiHa node 2), after which the plates are returned to the incubator. After incubation, the plates are taken from the incubator and placed on the Mp3pos. The lysis solution is then added (LiHa node 3) and the DWPs are subsequently returned to the incubator. A 384 MP, which was previously coated with antigen and blocked (using the Biomeck, a separate liquid handling system), is taken from the shelf, the barcode is recorded and the plate is placed on the Mp3pos. Two DWPs are removed from the incubator and placed on the Mp3pos. Lysate from the DWPs is transferred to the MP (LiHa node 4). The DWPs are returned to the incubator and the MP is subsequently placed on the shelf. After a 1hour incubation step, the MP is placed on Mp3pos and subsequently transferred to the plate washer, where the lysate solution is removed and the plate washed (PBST and PBS). The plate is returned to the Mp3pos and the secondary solution is added (LiHa node 5). This process is repeated for both the substrate solution (LiHa node 6) and the stop solution (LiHa node 7). The plate is then transferred from the shelf to the Mp3pos, from where it is then transferred to the MP reader. The O.D. readings from the plate are stored in an excel output file, which also contains the MP's barcode. The plate is subsequently returned to the shelf.

In process 5 (Figure 3.1.4), the user sets a cut-off limit for the ELISA threshold and a visual basic programme then “cherry picks” those clones from the backup plates and inoculates them into new deep well plates.

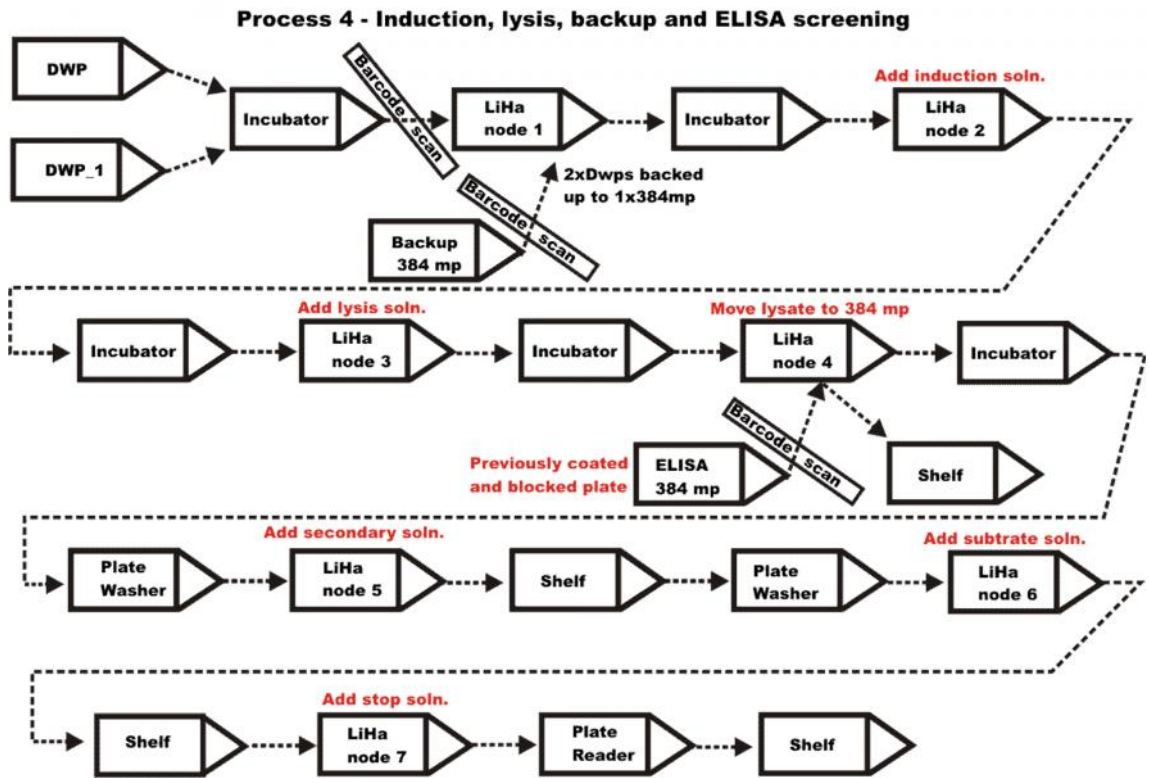


Figure 3.1.3 Flow diagram of the optimised process 4 of the robotics system. Process 4 performs the bulk of the work of the robotics system. Clones previously grown in DWPs are backed up to 384 MPs. After induction and lysis, the supernatant from individual clones is analysed by ELISA. The backup location and the ELISA results of each clone are electronically recorded during the process.

Process 5 - 'Cherry picking' of positive clones

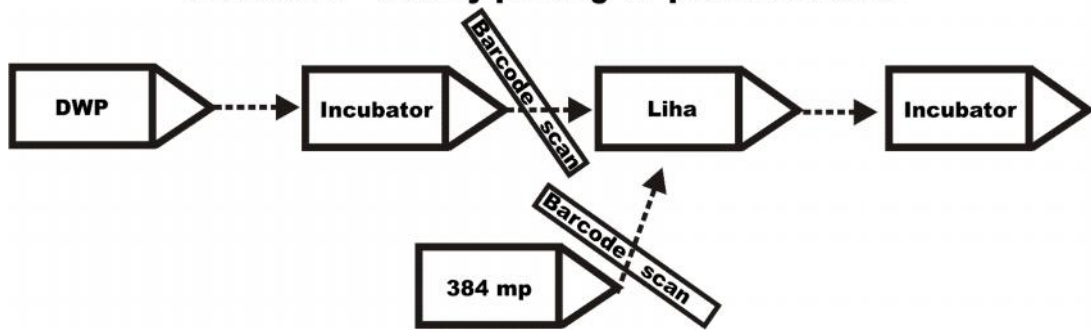


Figure 3.1.4 Flow diagram of process 5 of the robotics system. Process 5 'cherry picks' positive clones from stock plates and inoculates them into fresh DWPs for further analysis.

3.2 Results and Discussion

3.2.1 Robotic system hardware issues

The robotic system was assembled in DCU by a team of specialists from Tecan and Unitech in 2005. It took more than a year before the majority of the system was in place. However, since its installation, the robotics system has been plagued by a multitude of hardware problems and software failures.

3.2.1.1 The height of the centrifuge

The first problematic hardware issue to arise was the centrifuge system. After the system had been installed, the centric robotic arm was unable to reach far enough into the centrifuge to place a 384 MP into any of the bucket holders. To solve this problem a specially-designed steel plate was manufactured and placed underneath the centrifuge to reduce the distance between the centrifuge and the robotic arm (Figure 3.2.1).



Figure 3.2.1 *Centrifuge is raised with the addition of a steel plate.* The centrifuge was raised by placing a steel plate underneath the instrument. This enabled the centric robotic arm to place 384 MPs into the centrifuge.

3.2.1.2 The colony picker picking pins

A number of hardware issues occurred with the colony-picker (CP) instrument (Figure 3.2.2). The first problem encountered was that the tap supplying compressed air had insufficient pressure to operate the picking pins. To overcome this, an external compressor pump, which could deliver more than 5Bar, was purchased and installed. A second problem was identified during a picking test, related to the picking arm clearance of the drying-bath and brush bath-holders. After the CP had imaged the plate and when it started to pick colonies from the edges of the bioassay dish, the pin needles crashed into the brush holder and the drying bath. This was rectified by altering the travel height of the CP picking arm. A further issue arose when testing the 2mL DWPs, which had been filled with 500 μ L of medium. It was discovered that the CP needles were not of sufficient length to inoculate part of the bacterial colony into the DWPs. It was not an option to increase the medium level, as growing times would have been adversely affected. In addition, there was no capacity to increase the medium volume in the system as there was physically no more room available on the work table to add more media troughs. To overcome the problem, the manufacturers (KBiosciences) installed a new set of longer picking pins.

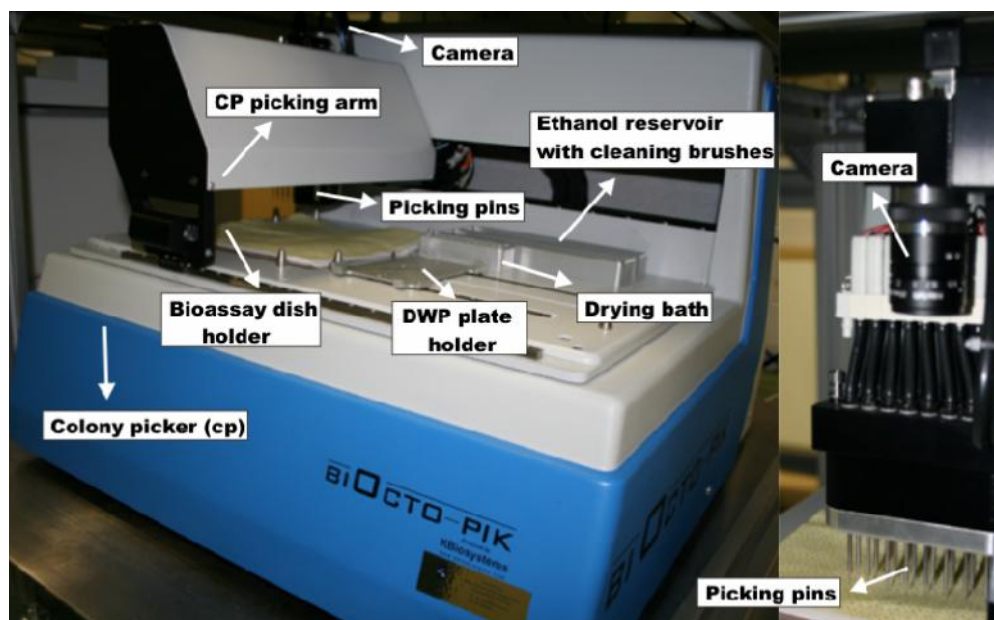


Figure 3.2.2 *Colony picker components.* The colony picker consists of a robotic picking arm, a set of 16 picking pins, a holder for the bioassay dish, a holder for a DWP, a camera, a drying bath and two brush holders.

3.2.1.3 The StoreX incubators, the deep well plates and lids

Two incubators (STX40) with a storage capacity of 20 DWPs each were supplied from LiCONiC instruments, UK (Figure 3.2.3). These third-party devices generated a large number of errors during the initial optimisation of the robotics system. After a substantial amount of troubleshooting, the specific problem was identified. In order to minimise contamination inside the incubator, DWPs were initially used with lids. The addition of the lid to the DWP results in a small increase in the height of the DWP. The DWPs are stored inside the incubator in cassettes (a steel holder divided into sections). When a DWP is taken from the incubator, using the lift assembly, the presence of the lid makes it impossible for the shovel to place the DWP on the transfer plate of the lift assembly. As a result the system crashes.

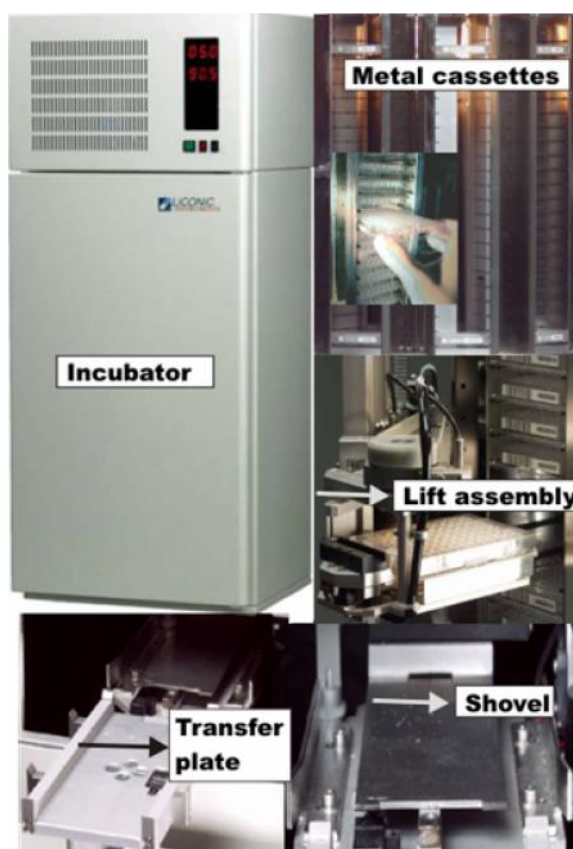


Figure 3.2.3 *The components of the StoreX incubators.* Two metal cassettes are used to hold the 20 DWPs and the lift assembly moves the plates to and from the cassettes. The lift assembly itself is composed of a transfer plate and a shovel.

3.2.1.4 Replacement of the liquid handling arm

The Tecan freedom EVO[®] automated workstation has a liquid-handling arm (LiHa) with eight variable-spacing pipetting tips. During a pipetting run with a 384 MP, it was discovered that one of the pipetting tips was out of alignment and this resulted in a system crash. The Tecan instrument contains calibration software, which allows the re-alignment of the pipette tips. However, application of the software did not rectify the pipette alignment issue. The LiHa arm operates with three different motors, which move the arm in the x,y or z axis. Investigation identified that one of the motors (y-motor), used to drive the LiHa arm, was faulty. The LiHa arm could not be repaired and was replaced (Figure 3.2.4).

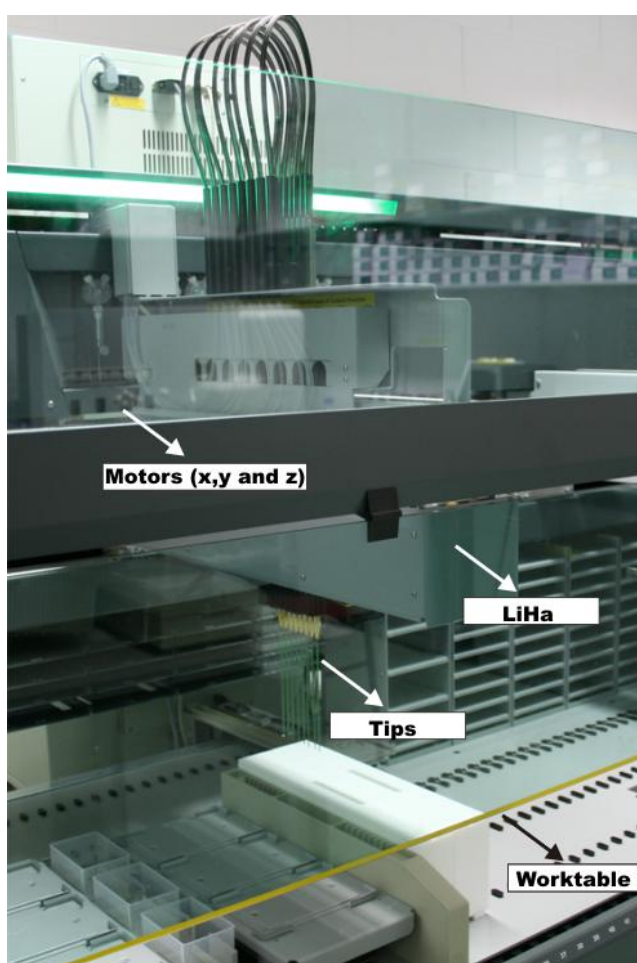


Figure 3.2.4 *The Tecan liquid robotic handling arm (LiHa).* The y-motor of the LiHa was discovered to be faulty and as the motor could not be repaired the robotic arm was replaced.

3.2.1.5 Other hardware issues

A number of other hardware issues were also discovered during testing. When testing the plate washer, a typical run generated more than 2L of waste. The capacity of the bottles with the original robotics system was only 5L. This meant that after every two iterations of the plate washer, the waste bottles would need to be emptied. This was rectified by purchasing larger bottles (25L) and reducing the number of prime steps in the plate washer programme (Figure 3.2.5A). Furthermore, during testing of the eccentric robotic arm, a number of collisions occurred. This resulted in the bending of the left finger gripper and as a result the arm was unable to place MPs into the hotel. The problem was rectified by replacing the left metal finger (Figure 3.2.5B).

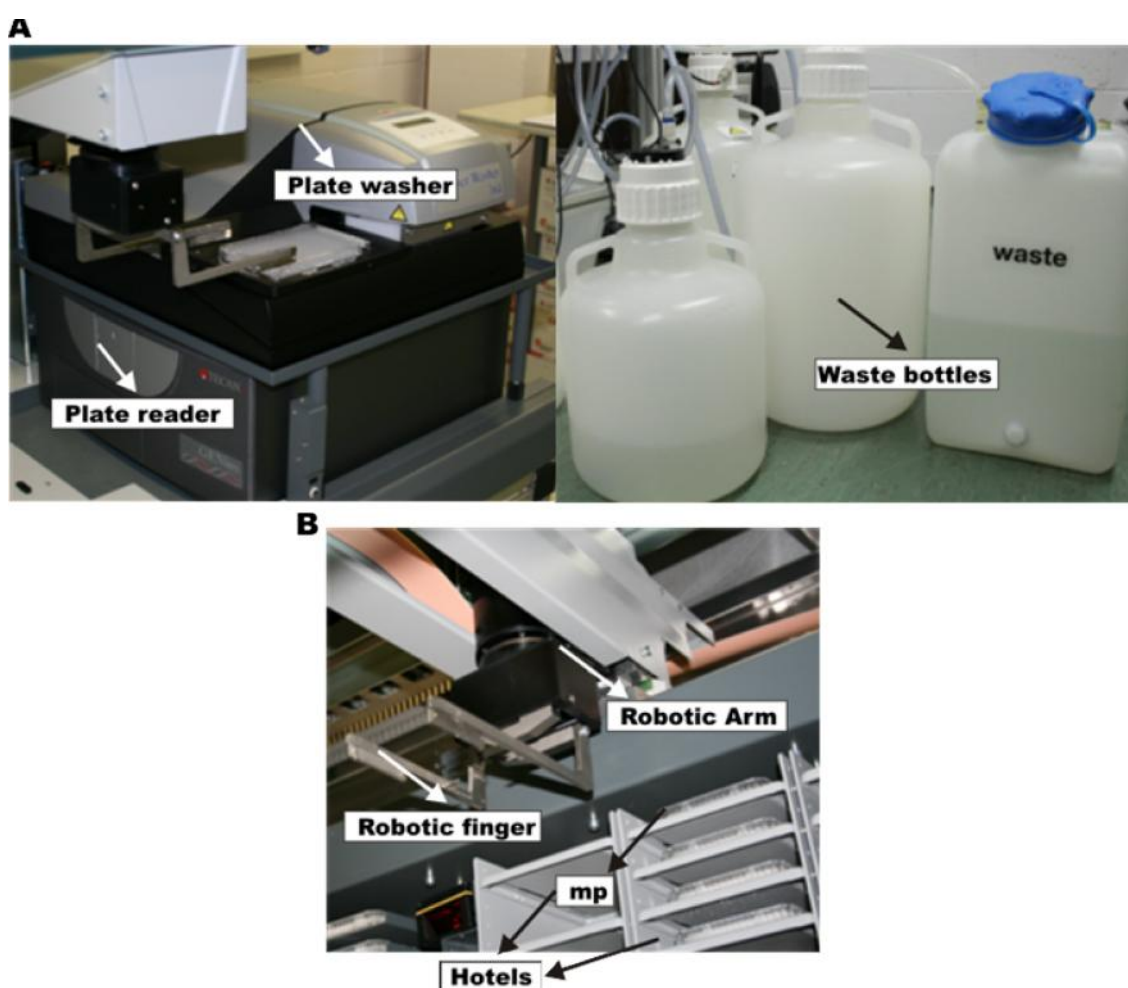


Figure 3.2.5 Plate reader, plate washer and waste bottles (A) and the eccentric robotic manipulator (RoMa) (B) of the Tecan system. Larger waste bottles were purchased for the system. The left robotic finger of the eccentric robotic arm was damaged and was subsequently replaced.

3.2.2 Robotic system software issues

Although the hardware issues of the robotics system caused a number of significant difficulties, once they were identified they were readily resolved. In contrast, software problems were far more numerous than hardware problems. Moreover, these problems were extremely difficult to troubleshoot and took considerable time and effort to resolve. Due to space constraints, only a selection of the major software issues will be discussed.

3.2.2.1 EVOware[®] software

The Freedom EVOware[®] software is available in two configurations: Freedom EVOware[®] Standard, the script-based option for simple control, and Freedom EVOware[®] Plus, which combines pipetting and advanced dynamic-scheduling in a single package. A data connection sends control signals from the software, via the PC, to the instrument and its peripherals. The plus package contains features which offer substantial improvements in throughput and operating speed, user-friendliness and data management. This software allows the user to develop programs and to quickly visualise how an experiment can be implemented. Within the software, the sequence of steps required to carry out a particular application is called a process. Each process consists of process steps, which are defined using the process editor. Process steps are created by dragging commands from the control bar onto the process editor. A process is then created by interlinking the process steps to create the workflow, which is required by the experiment. Each process step is associated with a single device, and devices can typically carry out several different functions. For example, a plate washer may have a washing function, a priming function and an initialisation function. This is handled by associating the process step with a command. Each command can be associated with pre-actions and/or post-actions, additional commands, which are carried out before and after the main command. Final position icons are used to specify the final position of the labware, after the process has finished. They are often associated with a storage device, such as a hotel. An example of an ‘on-the-fly’ device includes the barcode scanner, which is rigidly fixed to the instrument's deck and cannot move towards the labware to scan it. Instead, a plate robot (RoMa) is used to move the labware past the barcode scanner ‘on-the-fly’ while carrying out something else, for example, when moving the labware from one carrier (or process step) to another.

The barcode scanner is then instructed to perform a read operation when the plate robot presents the labware. The pipette command for the liquid handling arm has a large number of sub-commands, which are used for writing so-called scripts (a script is a sequence of pipetting commands).

In Freedom EVOware[®] Plus, when the pipette command is placed onto the process editor, this opens the scripting mode and the process editor is replaced by the script editor. The script editor is used to set up a pipetting (liquid handling) script (Figure 3.2.6/3.2.7).

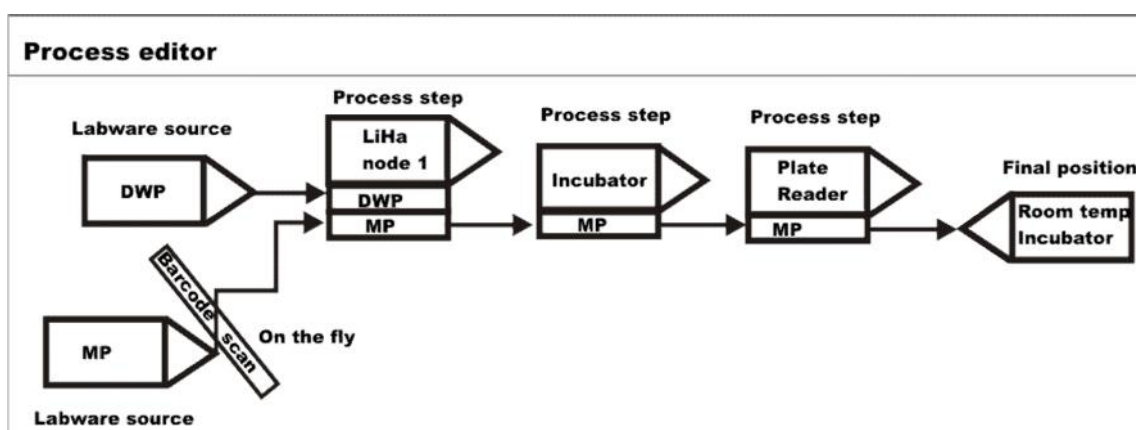


Figure 3.2.6 *Process editor showing an example process.* In this process, the MP is barcode scanned ‘on-the-fly’. After a LiHa pipetting step, involving both a DWP and MP, the latter is moved to the incubator and subsequently moved to the plate reader. After the plate has been read it is moved to its final position (hotel) for incubation at room temperature.

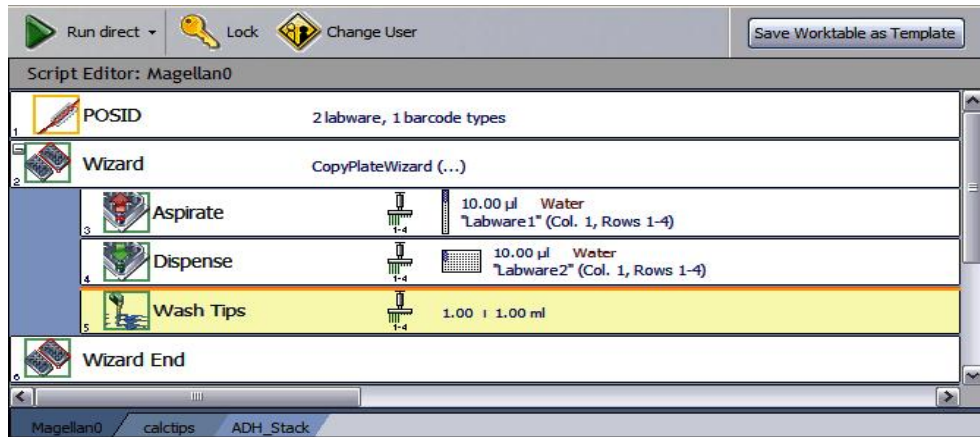


Figure 3.2.7 The *script editor with a simple pipetting script*. In this script, the copy plate wizard option is selected to aspirate 10µL of solution from labware 1 and dispense this volume into a second piece of labware (labware 2). At the end of the script the tips are cleaned.

3.2.2.2 Incubators (StoreX) driver issues

A software driver is a computer program specifically written for an operating system (OS), which allows hardware peripherals (e.g. incubator, centrifuge, plate washer, plate reader and colony picker) to communicate with a computer. When the hardware device sends data back to the driver, the driver may invoke routines in the original calling program. The driver for the StoreX was particularly problematic (Figure 3.2.8). Even after the DWPs and the lid issue had been resolved (See Section 3.2.1.3), a number of system crashes still occurred, due to the malfunctioning of the StoreX drivers (Version 3.6.3.0). At one stage, the system could not be started at all, due to the inability of the StoreX drivers to initialise. Another problem encountered was the inability of the StoreX driver to stop the StoreX from shaking, which resulted in a system crash and prevented the removal of the DWPs. StoreX problems were frequently observed when iterations of a process were executed. These problems were eventually overcome by obtaining and installing new executable files and a new build of the StoreX drivers (Version 3.8.6.0) from Tecan.

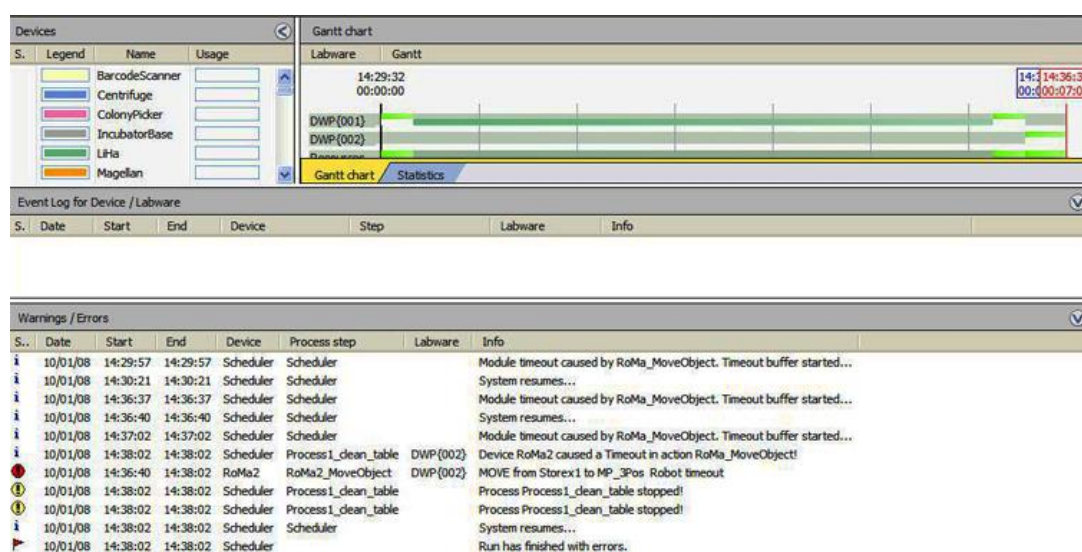


Figure 3.2.8 A typical StoreX error. The Gantt chart and error log at the end of a problematic run is illustrated. When this process was run, EVOware[®] issued a RoMa2 move object command, to move a DWP from Storex1 to the Mp3pos. This did not occur because of a communication problem with the StoreX driver (version 3.6.3) and the StoreX.

3.2.2.3 The transfer station and the centrifuge

When multiple iterations of process 4 were tested, a major problem with the transfer station occurred. The transfer station is an unused location on the worktable of the robotics instrument. This space is used to transfer labware from one RoMa arm to another, as not all locations on the instrument are accessible by both RoMas. This command is needed for mechanical reasons. In the process of transferring a DWP from the incubator to the centrifuge, the first step is to move DWP using RoMa2 to the Mp3pos. Subsequently the DWP is transferred from the Mp3Pos to the centrifuge using the RoMa arm. Thus, a transfer station is used when the labware has to be transferred to a location, which is only accessible by RoMa or RoMa2, but which cannot be accessed by both RoMa and RoMa2. On the worktable, the Mp3pos is the physical location of the transfer station. In a multiple iteration run of process 4, RoMa2 moved DWPs from the centrifuge and attempted to place them on the transfer station. However, as the Mp3Pos was already occupied with DWPs from a previous iteration, the system crashed (Figure 3.2.9).

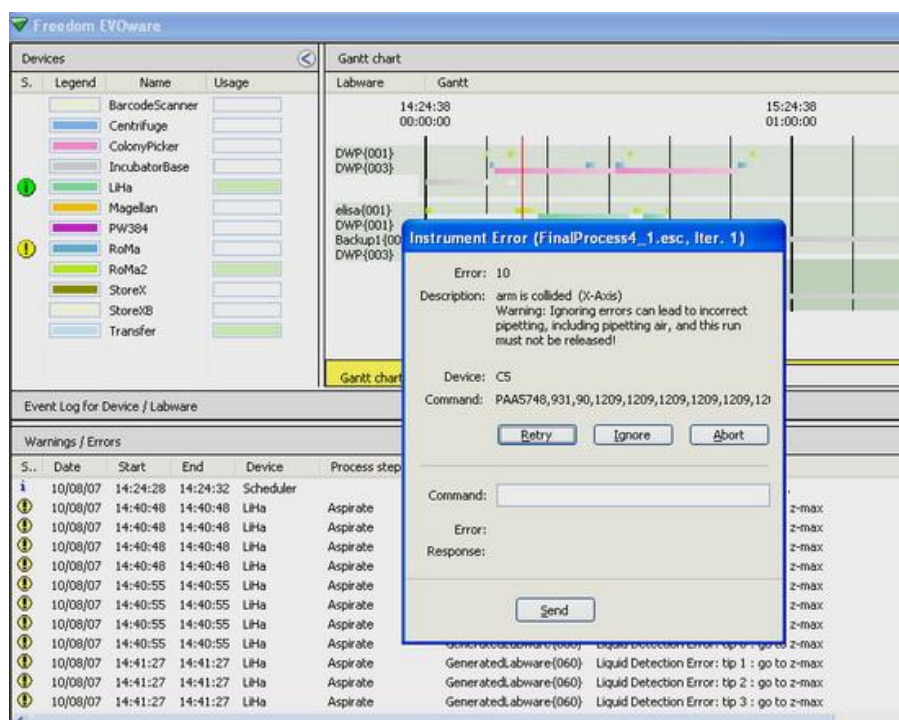


Figure 3.2.9 A system error occurred due to a transfer station problem. The RoMa arm caused a system crash when trying to place DWPs from the centrifuge onto the Mp3pos which was already occupied with DWPs from a different iteration of process 4.

In an attempt to overcome the transfer station issue and to speed up process 4, the transfer station was modified to allow four labware holding positions instead of two. To ensure that there was enough space to hold the DWPs and MPs during successive iterations, another Mp3pos carrier was added to the worktable. Process 4 was edited to allow the simultaneous processing of four DWPs instead of two. This modified process was tested and all four DWPs were transferred to the centrifuge successfully. However, after centrifugation and removal of some of the DWPs, RoMa attempted to place DWP_3 on top of DWP_2 which was already located on Mp3Pos1 (Figure 3.2.10).

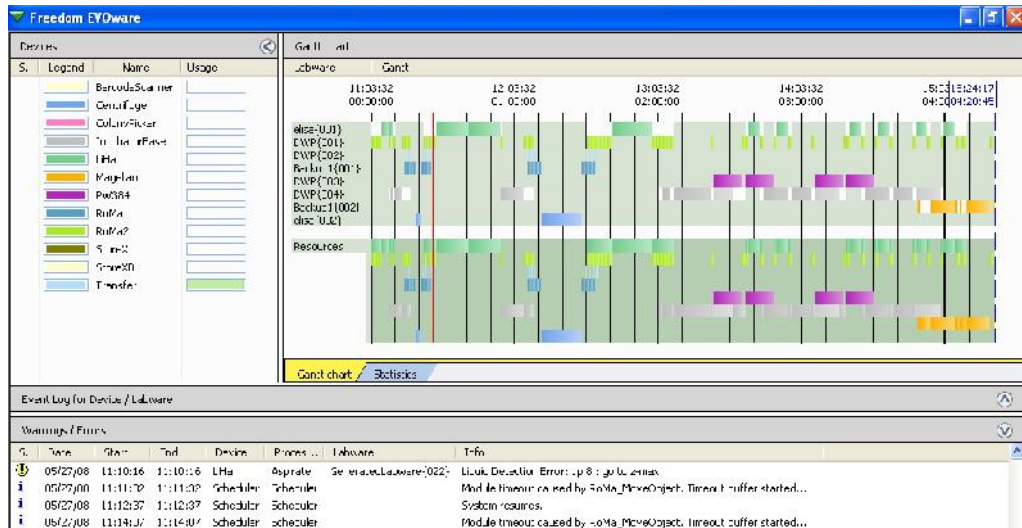
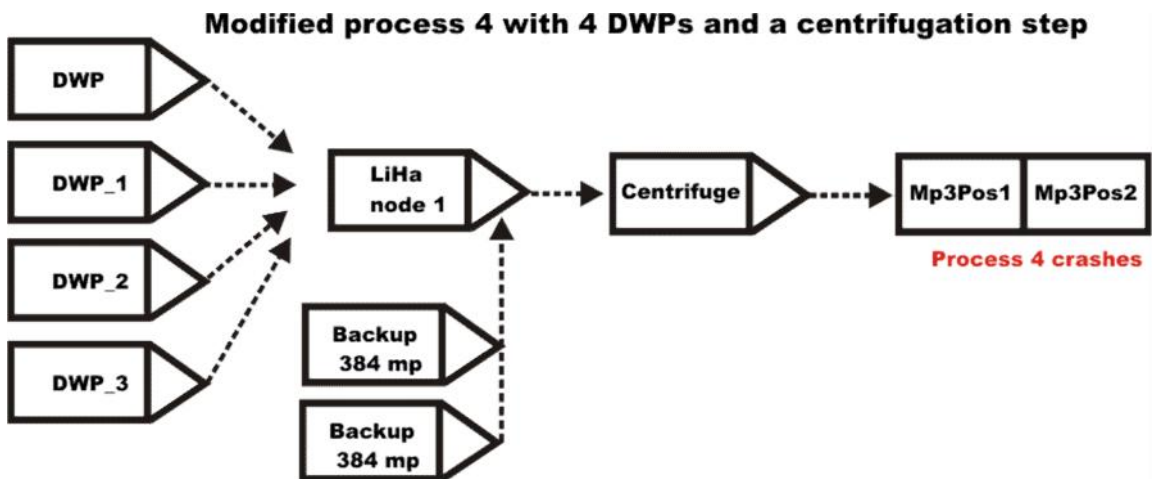


Figure 3.2.10 Modified process 4 and the system error event log. Process 4 was modified to use four DWPs instead of two. In addition, the transfer station was altered to hold four pieces of labware. When the process was run, it crashed after the centrifugation step, when RoMa tried to place a DWP on an already occupied Mp3Pos position.

It proved impossible to overcome this problem. An initial solution that was put forward was to move the StoreXs to the front of the instrument. This would allow RoMa to move the DWPs directly from the StoreXs into the centrifuge, thus avoiding the transfer station completely. However, this was not practical for a number of reasons; (1) there was limited space in front of the system, (2) there was the possibility of damage, especially to the incubator runners during lifting and (3) the lack of a table of an appropriate height to hold the StoreXs. The only solution left was to eliminate the centrifugation step from process 4. This was replaced by a LiHa step that adds a PopCulture reagent to the *E. coli* cultures. This reagent allows protein extraction and purification to be performed in the original DWP and thus eliminates the need for a centrifugation step (Figure 3.1.3).

3.2.2.4 The colony picker, its software driver and communication failure with EVOware[®]

During testing of the colony picker, a communication problem was discovered between EVOware[®], the colony picker and its software driver (Figure 3.2.11). This bug only occurred when the CP was run via EVOware[®] (remote mode) and did not occur when the CP was run as an independent device (i.e. in local mode). The software bug prevented the imaging of the bioassay dish and the picking of colonies into the DWPs. During the CP procedure, a bioassay dish is placed on a CP holder and subsequently imaged. Following this, a DWP is placed on the CP and colonies are picked from the bioassay dish into the DWP. During both the imaging and picking parts of the procedure, EVOware[®] constantly queries the status of the CP and the CP driver relays the CP status back to EVOware[®]. If the driver does not return the expected status, then the CP instrument will stop working and EVOware[®] will crash. Figure 3.2.11 outlines, in binary code, the communication process that occurs between EVOware[®], the CP and its software driver. When the CP is initially switched on, the CP driver responds to EVOware's[®] query with a binary code string of 0000001. This string indicates that the CP is ready and waiting to start. When the ST command is issued from EVOware[®], the CP responds with 0001001, meaning that it is now waiting for a source plate (i.e. the bioassay dish) before it can image. When EVOware[®] sends the S command (meaning the source plate is now on the CP), the CP images the bioassay dish.

Subsequently, the CP responds to the EVOware's[®] status query with the binary string 0000101, meaning that the system is busy (i.e. in this case, imaging the bioassay dish). When the binary string code changes to 0010001, this indicates that the CP is finished imaging and is now waiting on a destination plate (i.e. a DWP). When EVOware[®] issues the D command, the CP picks individual colonies from the bioassay dish into the DWP.

When EVOware[®] queries the system at this time, the CP responds with the binary string 0000101 (in this case meaning it is busy inoculating the DWP). This process is repeated for each new DWP. A software problem arose with the CP driver not returning the correct status and this resulted in an EVOware[®] crash. This software issue was eventually resolved when an update for the CP software was installed.

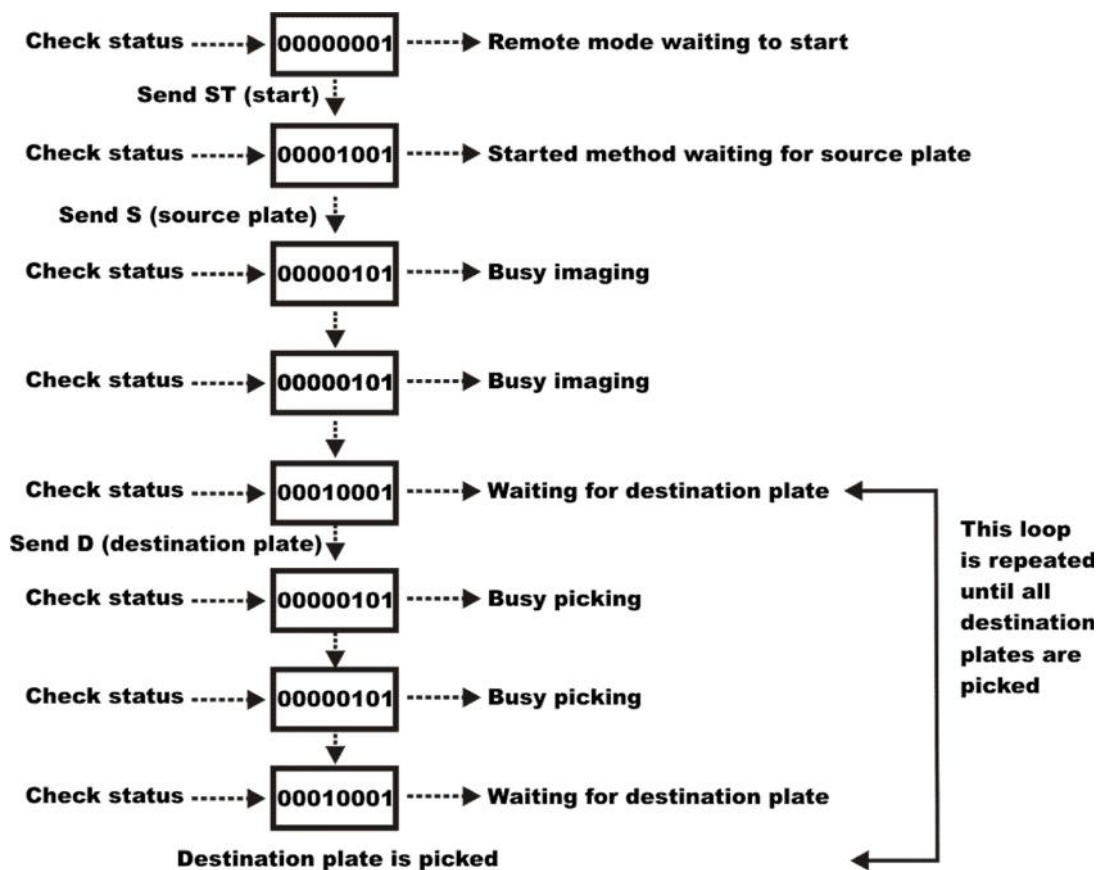


Figure 3.2.11 Flow diagram of the driver communication between the CP and EVOware[®]. A communication problem arose between the software of the CP and EVOware[®]. The CP software was found to be at fault, as it did not return the correct device status string during EVOware[®] queries. The installation of new CP software rectified this issue.

3.2.2.5 Process iterations and processes linking

The major advantage of a high-throughput system is the ability to run multiple samples. In EVOware[®] software, this is achieved by linking processes together and running iterations of those processes. However, a bug was discovered when iterations of process 1, 3 and 4 were tested. Figure 3.2.12 shows an error which occurred when using the runtime controller dialogue box to set the number of process iterations. Process 1 was set to 20 iterations, process2 = 1 iteration, process3 = 10 iterations and process4 = 10 iterations. An obscure error message was displayed “Error starting processes: A subscription cannot be stored unless its event class already exists”. Oddly this was overcome when process1 was set to 12 iterations, process2 = 1 iteration, process3 = 6 iterations and process4 = 6 iterations. The software engineers at Tecan could offer no explanation for this behaviour. A workaround for this bug was suggested, where DWP was to set to 001 and DWP_1 to 11 in the value (val) field box of the runtime controller, this allowed 10 iterations of both processes 3 and 4.

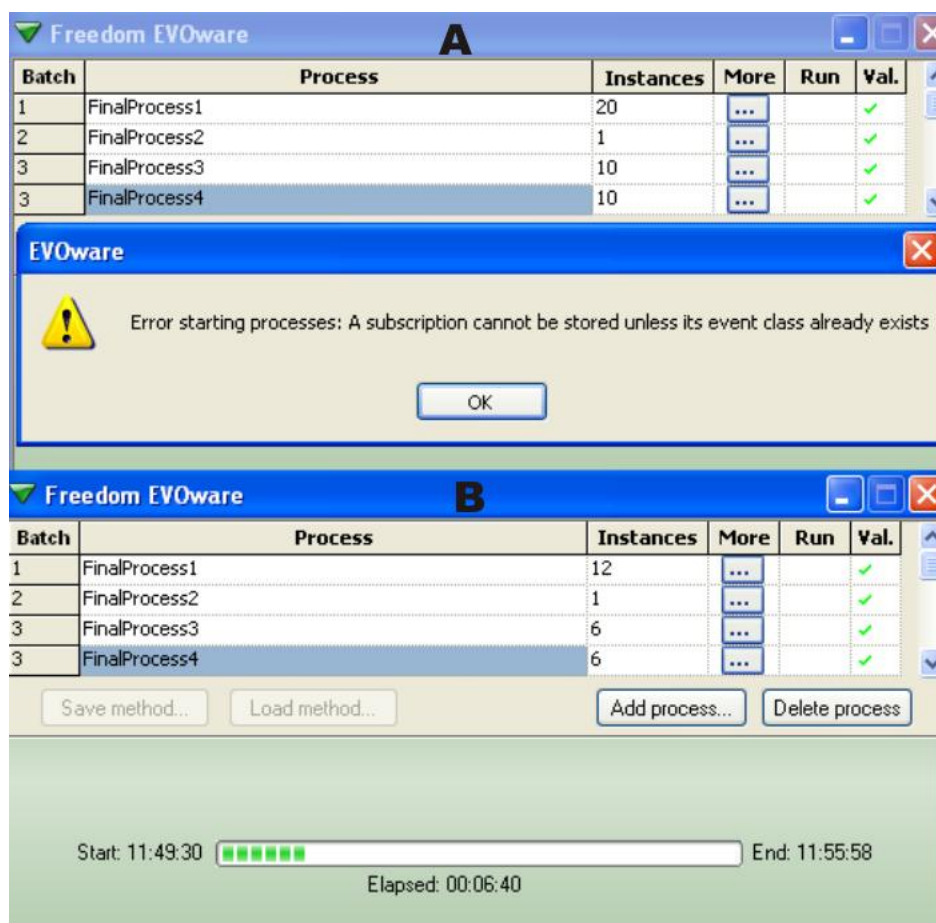


Figure 3.2.12 Runtime controller dialogue boxes and process iterations. Panel A shows the inability of EVOware[®] to run the four processes when process1 contained 20 iterations, process2: 1 iteration, process3: 10 iterations and process4: 10 iterations.

In contrast, panel B shows that EVOware[®] ran the four processes when process1 contained 12 iterations, process2: 1 iteration, process3: 6 iterations and process4: 6 iterations. EVOware[®] software specialists could offer no explanation for this behaviour. Another software bug related to the inability to link and run certain processes together using the runtime controller dialogue box. It proved impossible to identify any definitive reason for this behaviour. However, software engineers at TECAN suggested that a corruption in the database and/ or differences in worktable layout or labware could have prevented linking of the processes. To overcome this issue, all four processes were rebuilt using the exact same worktable and labware (Figure 3.2.13).

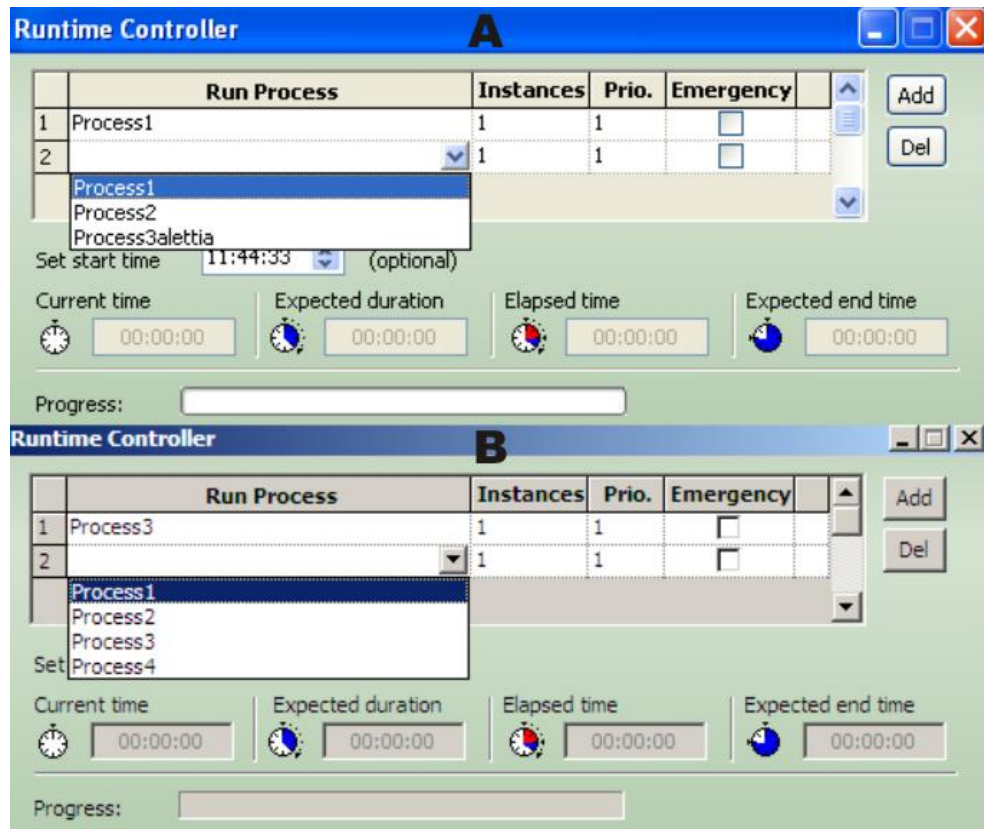


Figure 3.2.13 Runtime controller dialogue boxes and process linking. Panel A: Even though process 4 is in the EVOware[®] database, the runtime controller dialogue box will not show this process and therefore processes 1, 2 and 3 cannot be linked to process 4. Panel B: After rebuilding all four processes, using the same worktable and a new database, the runtime controller dialogue box allows all four processes to be selected and linked together.

3.2.2.6 Installation of a new operating system and reinstallation of EVOware® software

After an EVOware® software upgrade, it was discovered that the configure tool was no longer available. The configure tool is used to change fundamental instrument options. It controls the external devices, such as the CP, incubators, plate washer and centrifuge etc. The inability to use this tool meant that the settings on these devices could not be altered. In addition, access to user management functions was also unavailable. Removal of the software upgrade and its reinstallation did not rectify the situation. This procedure resulted in an even more serious issue, which was the inability to start any EVOware® processes. As no other alternative was available, a complete system reinstallation was undertaken on the advice of the TECAN software specialist. This procedure is not trivial and meant installing a new copy of the Microsoft® Windows® XP operating system (OS) on to the PC. In order to preserve the original data, the hard drive of the PC was divided, and a new copy of the OS was installed into a separate partition. In addition, to ensure no loss of data from the old system, a number of backup measures were undertaken. The first step was to export the database from the old system using the EVOware® export/import tool. This procedure was used to backup all processes and labware from the instrument. However, in order to backup specific devices and their drivers, a copy of the whole Tecan folder was saved to an external hard drive. Once the new OS was in place, the next step was to install the new version of EVOware® (2.1 SP3). It was at this stage that a number of problems arose. The first problem was the inability of the software to communicate with the instrument. This was eventually solved when Com Port 1 was chosen as the instrument's communication port (Figure 3.2.14). However, another problem arose when testing the devices which were attached to the instrument. Specifically, the centrifuge, the barcode scanner and the incubators could not communicate with the instrument (Figure 3.2.15). Each of the instrument peripherals was plugged into the edgeport device (which connects them to the PC) and after the installation of the edgeport driver the communication problem was partially fixed. The next step was to set proper com settings for each of the peripheral devices (Figure 3.2.16). Once this was completed, the peripheral devices communicated fully with the Tecan instrument. The last two software packages to be installed were the winwasher and the plate reader software. Although the old database was re-imported successfully, software problems still arose with some of the processes.

To overcome this, each process (Figures 3.1.1 and 3.1.2) was rebuilt from within the new version of EVOware[®]. When each process was tested, it ran without producing any errors. In addition, all processes could be linked together and run as a single unit.

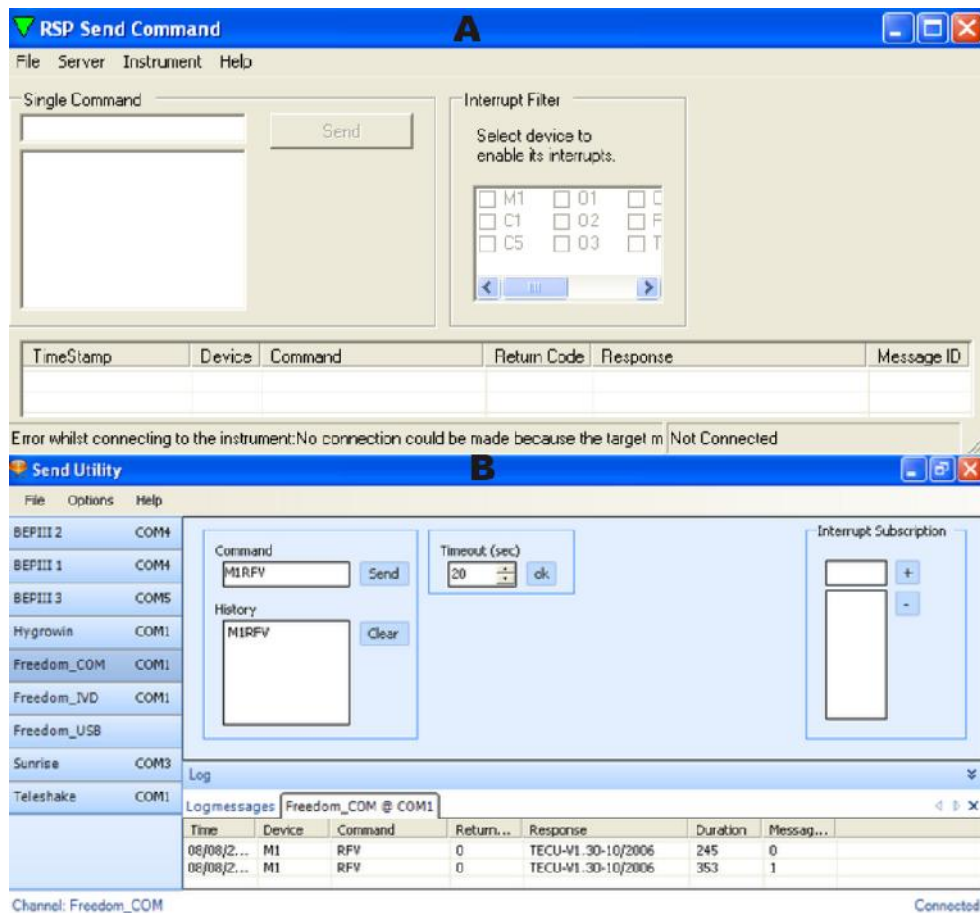


Figure 3.2.14 The EVOware[®] communication tool was used to check the status of the robotic instrument. Panel A: After installation of a new OS, the EVOware[®] software could not communicate with the instrument. Panel B: COM port 1 was identified as the proper instrument communication port. The command M1RFV was issued using the send utility and communication between EVOware[®] software and the instrument was observed.

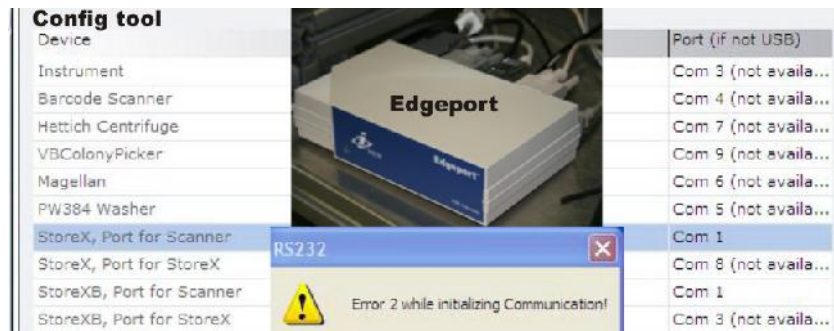


Figure 3.2.15 *There was no communication between the Tecan instrument and its peripheral devices. None of the peripheral devices (colony picker, centrifuge etc.) were detected by EVOware®. This problem was partially fixed when the driver for the Edgeport device was installed.*

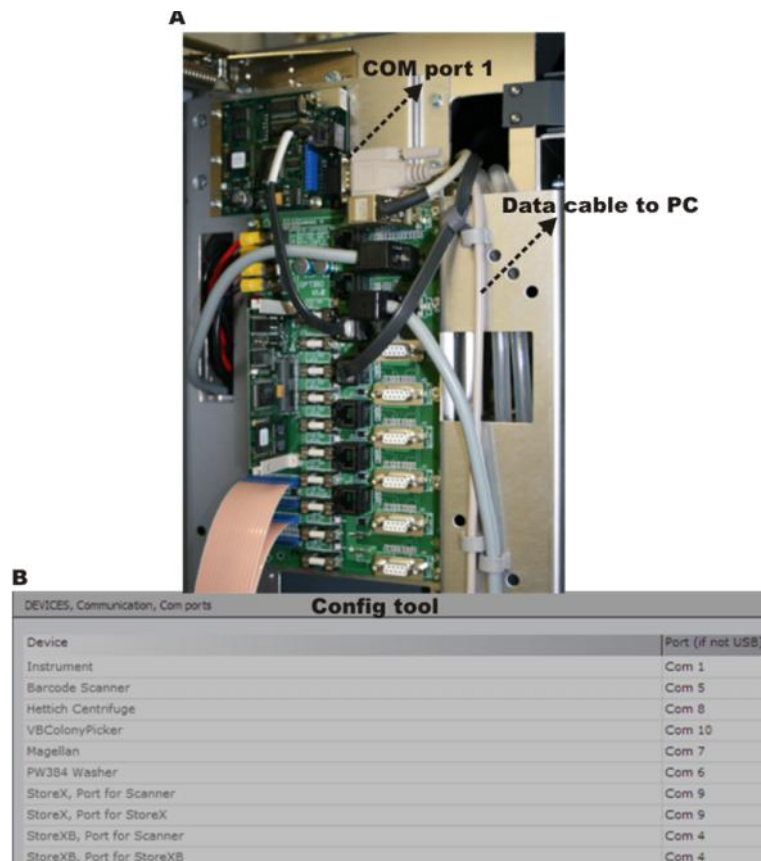


Figure 3.2.16 *Final Tecan COM port settings for the peripheral devices. Each peripheral device has its own com port. The robotic system instrument communicates to the PC via COM port 1 (panel A). The peripheral devices: barcode scanner, centrifuge, colony picker, plate reader, plate washer, incubator and incubatorB communicate on COM ports: 5,8,10,7,6,9 and 4, respectively (panel B).*

3.3 Conclusions

Recent advances in screening technology and automation have led to the development of high-throughput systems for use in life sciences. The development of a high-throughput system for the identification of novel affinity reagents from phage libraries has certain advantages. For example, such a system could enable the rapid selection of relevant antibody-producing clones. In addition, electronic-sample tracking and sample handling would allow for the easy identification and backup of superior antibody candidates. Moreover, extremely large numbers of antibody-producing cells could be analysed simultaneously. However high-throughput systems are often complex and, consequently, they are difficult to install, run and maintain. As exemplified in this chapter, installation and testing of a high-throughput antibody screening system was far from trivial.

After assembly and installation of the robotic antibody screening system at DCU, testing revealed a substantial number of hardware and software faults. Hardware problems included the insufficient length of the Tecan robotic arm and colony picker pins, the defective Y-axis motor of the robotic liquid handling arm and the misaligned robotic finger gripper. Although these, and other hardware issues, caused a number of significant difficulties, once they were identified they were readily resolved. In contrast, software problems were extremely difficult to troubleshoot and took far more time and effort to resolve. Major software problems occurred with the StoreX incubator drivers, Tecan EVOware[®] package and the colony picker drivers. In addition, corruption of the EVOware[®] database forced a complete PC reinstallation of the Windows[®] XP OS. Due to time constraints, no further work was performed on the robotic antibody screening system. However, all outstanding software and hardware issues were satisfactorily resolved.

Chapter 4

Generation of avian anti-sialic acid recombinant antibodies

4.1 Introduction

This chapter outlines the production and isolation of avian anti-sialic acid recombinant antibodies. It contains an in-depth description of a variety of different approaches that were used for anti-sialic acid recombinant antibody generation. The focus of this chapter is the use of phage display technology for the production of recombinant avian anti-sialic acid scFv fragments.

Phage display is a widely used method for the generation of recombinant antibodies. The display of protein libraries on filamentous phage, in combination with effective selection strategies, has proved to be very useful for the isolation of protein fragments with specific binding properties (Kramer *et al.*, 2003). In this technique, a target molecule is used to identify its cognate binding antigen from a library of billions of different binding partners. This powerful method can generate high-affinity recombinant antibody fragments with unique specificities for a wide variety of antigens (Barbas, 2001).

Phage display technology utilises bacterial viruses known as bacteriophage (or phage) that are modified to display specific binding fragments on their surface. Antibody gene fragments are typically cloned into a vector (phagemid) as a fusion to the gene encoding one of the phage surface coat proteins. These phage particles are recovered using solution or solid-phase selection with the cognate antigen. Those phage particles that bound to the antigen are isolated and re-infected into fresh male *E. coli* for further propagation and affinity maturation. The coding genetic material for the antibody fragment resides within the phage particle and, therefore, a direct link between the antibody's genotype and phenotype is maintained. This allows the subsequent isolation of the genes encoding the antibody fragment from the phage DNA (Barbas, 2001; O' Brien and Aitken, 2002).

The use of chickens, as opposed to mice and rabbits, has gained popularity as an alternative animal system for the generation of high-quality affinity reagents (Hoogenboom *et al.*, 1998; Finlay *et al.*, 2011). It was Leslie and Clem who in 1969 first described chicken IgY (Tizard, 2002). This immunoglobulin is often called chicken IgG as it is similar in structure and function to mammalian IgG (Bird and Thorpe, 2002).

However, despite these similarities there are some striking differences between these immunoglobulins. Chickens have three different immunoglobulin classes: IgA, IgY and IgM (Ratcliffe, 2006). In contrast, mammalian immunoglobulins are divided into five classes: IgA, IgD, IgE, IgG and IgM (Wang *et al.*, 1978). IgY constitutes approximately 75% of the total chicken immunoglobulin population. A typical immune response of a chicken begins with IgM production. This occurs 4-5 days following antigen exposure and disappears after 10-12 days. As the immune response progresses, the IgM-producing cells cease IgM production and start the production of IgY or IgA. This phenomenon is called “class switch”. Typically, IgY production reaches a peak 3 to 3 ½ weeks after immunisation and subsequently slowly decreases over time. IgA appears 5 days after antigen exposure and is predominantly found in the mucous secretions of the eyes, gut and respiratory tract (McCormack *et al.*, 1989; Masteller *et al.*, 1997). In the hen, IgY is transferred from the serum into the egg yolk to confer passive immunity to the embryo. This transmission of maternal immunity protects the chicks before their own immune capabilities are established (Schade *et al.*, 2005). A similar process occurs in mammals, where placental IgG transfer confers passive immunity to the foetus. IgY is predominantly present in the lipid-rich environment of the egg yolk, whereas IgM and IgA are mostly found in the egg white (Karlsson and Larsson, 2004). In older hens, the concentration of yolk IgY is significantly decreased (Barua *et al.*, 2001).

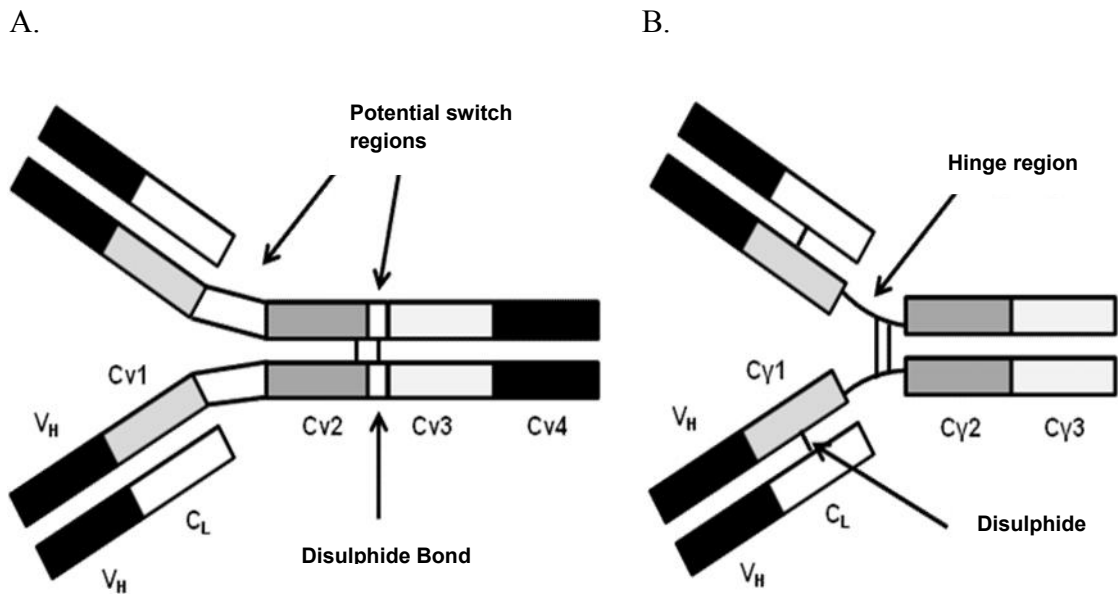


Figure 4.1.1 Structures of IgY and IgG A.) IgY: V_H, variable domain of heavy chain; C_L, constant domain of light chain; C_H1, C_H2, C_H3 and C_H4, constant domains of heavy chain B.) IgG: V_H, variable domain of heavy chain; V_L, variable domain of light chain; C_L, constant domain of light chain; C_H1, C_H2 and C_H3, constant domains of heavy chain. Disulphide bonds are indicated by lines connecting the two chains.

Similar to IgG, the Fc region of IgY mediates most biological effector functions, such as complement fixation and opsonisation (Tizard, 2002). However, unlike IgG, IgY can also mediate anaphylactic reactions, a function that is attributed to IgE in mammals (Karlsson and Larsson, 2004; Mine and Kovacs-Nolan, 2002; Mine and Yang, 2008). The DNA sequence of IgY shares a greater homology to that of human IgE (Tizard, 2002). Interestingly, the most hydrophobic part of IgY is the Fc fragment. As the Fc fragment is larger in IgY than in IgG, the IgY molecule is more hydrophobic than IgG (Schade *et al.*, 2005). In addition, the Fc region of IgY contains two carbohydrate chains as opposed to the one found in human IgG (Karlsson and Larsson, 2004; Jefferis, 2005). Human IgG contains only complex type oligosaccharides, whereas IgY contains three different types of *N*-linked oligosaccharides: oligomannose type (Man₅₋₉ GlcNAc₂), monoglucosylated oligomannose type (Glc₁Man₇₋₉ GlcNAc₂) and biantennary complex type (Ohta *et al.*, 1991; Jefferis, 2005). The greater carbohydrate content on IgY allows for the conjugation of larger amounts of enzyme or hapten.

For example, hydrazide carbohydrate chemistry can be used to conjugate 15-20 biotins per chicken IgY, in comparison to 7-10 biotins per rabbit IgG (Tizard, 2002; Narat, 2003). Similar to other full-length immunoglobulins with an antigen valency of 2, the IgY structural unit is a tetramer that is composed of two identical pairs of polypeptide chains (Figure 4.1.1). These are designated as "light" (L) and "heavy" (H), in reference to their molecular weight. The *N*-terminal portion of each chain defines a variable (V) region primarily responsible for antigen recognition. The C-terminal portion of each chain defines a constant (C) region (Tizard, 2002). Although IgY is similar in structure to that of IgG it is not identical. IgY contains two heavy chains with a molecular mass of 67-70kDa each, and two light chains with a molecular mass of 25kDa each. The IgY heavy chain is called epsilon (ν) and has one variable (V) region and four constant (C) regions (C ν 1-C ν 4). In contrast, the gamma (γ) IgG heavy chain has only three constant regions (C γ 1-C γ 3). Thus, IgY with the extra constant region has a greater molecular mass, 180kDa, compared to that of IgG (150kDa) (Narat, 2003). IgY is also less flexible than IgG due to the absence of the hinge region. In IgG, the hinge region is located in the heavy chains between the C_H1 and C_H2 domains and permits flexibility between the two Fab arms of the Y-shaped antibody molecule. The extra constant domain of IgY may be an evolutionary predecessor to the mammalian IgG hinge region. In addition, an IgY-like molecule is believed to be the evolutionary ancestor for both mammalian IgG and IgE (Tizard, 2002; Narat, 2003; Karlsson and Larsson, 2004; Mine and Yang, 2008). The pI range of IgY (5.7 - 7.6) is lower than that of IgG (6.1 - 8.5) (Schade *et al.*, 2005). IgY is more acidic than IgG and hence this may account for its greater stability in conditions of low pH (Hodek and Stiborova, 2003). IgY antibodies are resistant to high temperature and ionic strength and can be stored for over 10 years at 4°C without any significant loss in antibody activity. In addition, chicken antibodies still retain their activity after 6 months at room temperature, or 1 month at 37°C (Karlsson and Larsson, 2004; Hodek and Stiborova, 2003).

A major immunological difference between birds and mammals is the presence of the avian bursa of Fabricius (*Bursa cloacalis*), a specialised lymphoid organ involved in avian B-cell development. In chickens, the bursa is associated with the gut and facilitates exposure of developing B-cells to external antigens and bacterial flora (Masteller *et al.*, 1997).

In contrast, the foetal liver and bone marrow are the production sites for B-cells in humans and mice (Hill *et al.*, 1992; Morrow and Schlissel, 1992). In those mammals, the B-cell repertoire is constantly generated throughout life in the bone marrow (Kirman *et al.*, 1998). However, for chickens, the preimmune B-cell repertoire is mainly generated during development in gut-associated lymphoid tissue (GALT). Other important parts of the chicken immune system include the spleen, thymus, bone marrow, lymph nodes, circulating lymphocytes, lymphoid tissue in the alimentary tract and the Harderian gland (Carlander *et al.*, 1999; Casteleyn *et al.*, 2010). The Harderian gland is a lymphatic organ situated in the avian eye orbit. This is a dedicated plasma cell organ and functions as a site for terminal B-cell differentiation and as a site for immunoglobulin class switching (Mansikka *et al.*, 1989).

Chicken B-cell development can be divided into three stages: pre-bursal, bursal, and post-bursal (Masteller *et al.*, 1997). In the pre-bursal stage, the chicken simultaneously rearranges both its Ig heavy and light chain genes, in a non-sequential order, which initially takes place in the embryonic yolk sac (neonatal repertoire). Gene rearrangements continue over the course of approximately 10 days (Masteller *et al.*, 1997; Lanning *et al.*, 2003; Kohonen *et al.*, 2007). In contrast to mammalian development, there is no pre-B stage and no requirement for surrogate light chains (Kohonen *et al.*, 2007). After embryonic day ten, a single wave of preimmune committed B-cell progenitors, with their rearranged Ig genes, migrate into and colonise the bursa. The bursa provides the microenvironment necessary for the rapid growth of B-cells, as embedded within the bursa are about 10,000 lymphoid follicles. The B-cell precursors (stem cells) reside within these bursa follicles and undergo selective proliferation of in-frame Ig genes. This rapidly expands the receptor containing B-cell population (Masteller *et al.*, 1997; Kohonen *et al.*, 2007). The initial “pre-bursal” B-cell receptors have very limited diversity (Masteller *et al.*, 1997) and express on their surface undiversified IgM. Ig diversification occurs via somatic gene conversion and lasts 4-6 months after hatching. During hatching, B-cells are exposed to the contents of the digestive tract as the bursal lumen is connected to the gut via the bursal duct. Approximately 95% of the bursal B-cells undergo apoptosis, leaving only 5% that eventually migrate from the bursa. This high level of B-cell apoptosis may result from negative B-cell selection, as self-reactive B-cells are forced to undergo apoptosis.

In addition, immature bursal B-cells may have produced non-functional antigen receptors during somatic gene conversion and therefore these cells are also forced to undergo apoptosis (Bezzubova and Buerstedde, 1994; Masteller *et al.*, 1997; Tizard, 2002; Lanning *et al.*, 2003). Those B-cells that pass these development checkpoints proliferate rapidly within the bursal epithelial buds and begin to leave the bursa at embryonic day 18. It is these post-bursal B-cells which colonise the peripheral lymphoid tissues and serve as a source of antibodies against foreign antigens (Tizard, 2002; Davison *et al.*, 2008). The primary chicken antibody repertoire is generated during the late embryonic stage and for a short period after hatching. As the chick ages, its B-cells undergo further rounds of somatic gene conversion, which expands the antibody repertoire. Typically, after 5-7 weeks, both the bursa and antibody repertoire are fully matured (Davison *et al.*, 2008). When the chicken reaches sexual maturity (between 4 to 6 months after hatching) there is an involution of the bursa and the production of new B-cells ceases. The adult chicken is left with, and relies on, mainly quiescent IgM B-cells and a population of activated memory B-cells that reside within the peripheral lymphoid tissues (McCormack *et al.*, 1989; Tizard, 2002; Lanning *et al.*, 2003).

In the avian and mammalian immune systems, B-cells undergo different processes of regulated genetic alterations that diversify the coding potential of their Ig genes (Chua *et al.*, 2002; Davison *et al.*, 2008). These genes are not encoded in a functional form in the germline, but are assembled from gene segments by site-specific recombination (Scott *et al.*, 2010). Thus, each B-cell expresses one gene recombined from the germline gene segments. In mammals, this is accomplished by recombination and occurs early in the development of B-cells in the bone marrow. Recombination involves mixing and matching large clusters of pre-existing variable (V), diversity (D), and joining (J) gene segments. The random juxtaposition of different combinations (combinatorial diversity) and the imprecise joining of these segments (junctional diversity) leads to the expression of a functional B-cell-receptor on the cell surface that has a unique antigen-binding site (McCormack *et al.*, 1989; Masteller *et al.*, 1997; George, 2000). In humans, the antibody germline repertoire has been completely sequenced. Human germline antibody diversity is created from the combination of 51 V, 23 D and 6 J DNA segments that form functional variable heavy chain (V_H) genes.

In addition, 70 V (40 V κ and 30 V λ) and 5 J DNA segments form variable light chain (V_L) genes. Diversification also occurs by nucleotide deletions and additions in the splice junctions of the VDJ segments of V_H, the V_J segments of V_L and the CDR regions. In addition, the random association of different V_H and V_L domains creates further diversity. Interestingly, many human germline genes are never or only very rarely used during an immune response (Knappik *et al.*, 2000). Typically in mice and humans, after antigen-dependent activation of B-cells, somatic mutation and class switch recombination further diversifies the pool of B-cell-receptors (Han *et al.*, 2010). Somatic mutation, which is the dominant secondary genetic alteration mechanism, introduces mutations, small deletions and insertions at a high rate in the Ig variable region gene sequences. The combination of these genetic processes creates a highly-varied B-cell population (humans have $10^9 - 10^{10}$ unique B lymphocytes) capable of producing a wide range of antigen-specific antibodies (Honjo *et al.*, 2002; Silverman *et al.*, 2009).

Chickens have a very simple germline Ig locus, which is small in size, and is organised as a single functional V gene element that contains a single λ light chain isotype. In contrast, light chain diversity in mice and humans is generated from two independent genes (κ and λ), either of which can be recombined to form a functional Ig gene (Thompson and Neiman, 1987; Qin *et al.*, 2008). The chicken λ Ig light chain locus contains a V λ segment (V λ_1) and a single unique J λ segment (Parvari *et al.*, 1987; Thompson and Neiman, 1987; Qin *et al.*, 2008). In all chicken B-cells, the same V λ_1 segment is joined to the J λ segment, so while there is some junctional diversity at the V λ_1 J λ junction, the process of gene rearrangements generates only minimal light chain diversity. The chicken Ig heavy chain locus contains a single copy of the functional variable heavy gene (V_H) and a single J segment (J_H) gene. In addition, it contains 15 functional D_H elements. Many of the D_H elements are very similar to one another and there are several examples where these elements encode identical amino acid sequences. Thus, despite the use of alternative D_H elements and the presence of some junctional diversity, chicken VDJ_H rearrangements produce only a few different immunoglobulin configurations (Parvari *et al.*, 1987; Thompson *et al.*, 1987; Tizard, 2002; Ratcliffe, 2006; Qin *et al.*, 2008). Consequently, combinatorial joining of germline elements does not contribute significantly to antibody diversity in chickens.

In contrast, the primary antibody repertoire in rodents and primates is generated by both somatic rearrangement of multiple V(D)J gene segments and junctional diversity (Parng *et al.*, 1996; Ratcliffe, 2006).

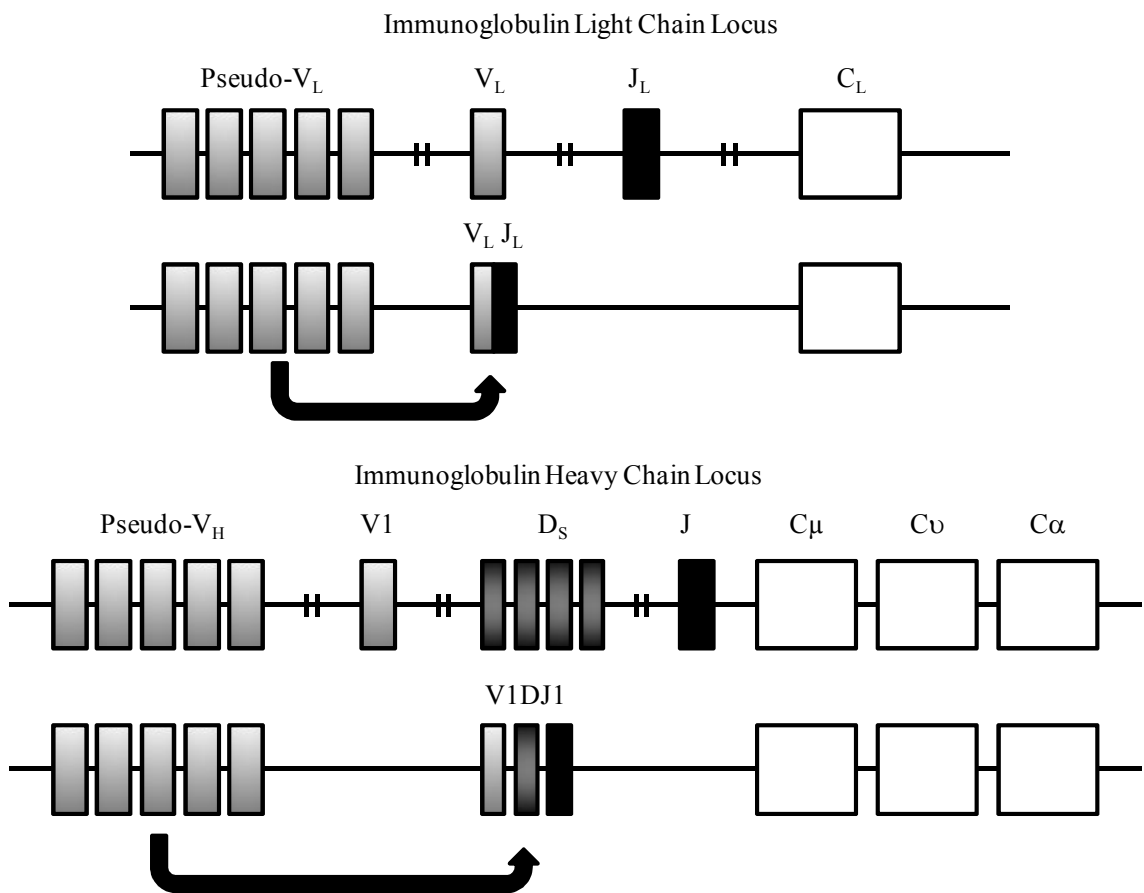


Figure 4.1.2 Development of avian B lymphocyte repertoire (Adapted from Fellah *et al.*, 2008).

Interestingly, chickens and other animals (horses, rabbits, cattle and swine) produce a diverse antibody repertoire by a different rearrangement mechanism, known as somatic gene conversion (Parvari *et al.*, 1987; Reynaud *et al.*, 1992; Parvari *et al.*, 1990; Arakawa *et al.*, 2004; Ratcliffe, 2006). In chickens, somatic gene conversion occurs when upstream groups of unexpressed pseudogenes are unidirectionally translocated into the heavy and light chain Ig variable regions (Thompson and Neiman, 1987). These transplanted pseudogene donors range in size from 8bp to 200bp and play a critical role in B-cell receptor diversification (Lanning *et al.*, 2003). Approximately 25 V_L pseudogenes are located upstream of the V_{λ1} segment, and up to 100 V_H pseudogenes are positioned upstream of the V_H segment (Sapats *et al.*, 2003).

Pseudogenes cannot behave as autonomous V genes as they lack transcriptional and promoter signals, leader exons and recombination signal sequences. In addition, they can also contain 5' or 3' truncations. However, they are still highly functional as they contain a sufficient amount of homology (in the framework regions) to promote gene exchange and a sufficient degree of divergence (in the hypervariable regions) to create a useful level of diversity (Reynaud *et al.*, 1983; Reynaud *et al.*, 1992; Ratcliffe, 2006).

In addition to gene conversion, chickens also diversify their B-cell receptors by somatic hypermutation (Arakawa *et al.*, 1998; Arakawa and Buerstedde, 2004). These repetitive processes occur in bursal follicles over several weeks and involve a large number of cell divisions. During bursal B-cell proliferation, some of the B-cells leave the organ and seed the periphery. Somatic gene conversion diversification of B-cells is not limited to the bursa, but also occurs in “post-bursal” B-cells which are undergoing an immune response in splenic germinal centres (Arakawa *et al.*, 1998; Arakawa and Buerstedde, 2004; Veistinen *et al.*, 2001). In the early antigen response phase, antigen-activated B-cells diversify their Ig genes by both gene conversion and somatic hypermutation (Arakawa and Buerstedde, 2004; Yang *et al.*, 2006). However, in the later stages when the immune response has waned (typically 14 days after immunisation) somatic gene conversion is down-regulated and most modifications are via somatic hypermutation. The mechanisms outline above take place over the lifetime of the B-cell, and this ensures that chickens can mount an antibody response against a diverse range of antigens (Arakawa and Buerstedde, 2004; Kurosawa and Ohta, 2011). The heavy and light chain variable regions of almost all diversified V genes in chickens mostly have identical sequences at their ends. For phage display, only a small set of oligonucleotide primers are required to recover the full chicken repertoire of V genes (Wyngaardt *et al.*, 2004). In addition, the loss of low-abundant transcripts is reduced when immune libraries are built from a very small set of oligonucleotide primers, as primer failure is less frequent. In contrast, mammalian immune libraries built from mice and humans require a large and complex set of different oligonucleotide primers. Hence, immune avian library construction tends to be simpler, faster and less expensive when compared to mammalian library construction (Andris-Widhopf *et al.*, 2000; Barbas, 2001; Wyngaardt *et al.*, 2004; Finlay *et al.*, 2005)

The use of avian hosts for antibody generation has several other significant advantages. For example, there are substantial phylogenetic differences between birds and mammals (Gasparyan, 2005). Birds are 300 - 350 million years removed from the human evolutionary line, whereas rabbits and mice are only 75 - 85 million years removed (Chowdhary and Raudsepp, 2000; Pace and Feschotte, 2007). This evolutionary distance is important. As the difference between the antigen and the immunised animal increases, the immune response generally increases. Therefore, the production of antibodies against conserved mammalian antigens is generally more successful in chickens, as opposed to mammals. IgY antibodies tend to recognise multiple antigenic epitopes in contrast to corresponding antibodies produced in mammals (Carlander and Larsson, 1999; Barbas, 2001; Carlander *et al.*, 2001; De Meulenaer and Huyghebaert, 2001; O' Brien and Aitken, 2002; Sapats *et al.*, 2003). Thus, a greater number of IgY antibodies can bind to the target antigen, and this generates an amplified signal in an immunoassay. Chicken antibodies can bind novel epitopes that are not recognised by mammalian antibodies. This gives access to a different antibody repertoire than that obtained from traditional mammalian antibodies. Furthermore, compared to mammals, far less antigen is needed to generate an efficient immune response in chickens, and sustained high-titres reduce the need for frequent injections. Moreover, a single chicken can be immunised with multiple antigens (Davies *et al.*, 1995; Andris-Widhopf *et al.*, 2000; Sapats *et al.*, 2003)

Antibodies generated from mammals can often cross-react with a number of different substances commonly found in patient serum or plasma samples. For example, the Fc portion of mammalian IgG can react with rheumatoid factor (RF), which is present in serum from patients with rheumatoid arthritis. This IgM antibody is also found in many other diseases as well as in some healthy individuals (Biro, 1968; Naot *et al.*, 1981). In a sandwich ELISA, where one antibody is used to capture the antigen and another labelled antibody is used to detect the bound antigen, a false-positive reaction can occur when the detection antibody binds to the capture antibody in the presence of RF but in the absence of the target antigen (Law, 1996; Miyashita *et al.*, 2010). Other interference factors include the human anti-mouse IgG antibody (HAMA). These antibodies are often produced when patients are treated with monoclonal mouse antibodies.

In a sandwich ELISA, HAMA can react with murine antibodies and IgG from other mammals causing erroneous results (Carlander *et al.*, 1999; Carlander and Larsson, 2001; Tate and Ward, 2004; Smith *et al.*, 2005).

Chicken antibodies do not react with either RF or HAMA and their use in immunoassays is a simple way to eliminate problems from those interference factors (Narat, 2003). The components of the complement system are also known to cause problems in immunoassays (Law, 1996). Mammalian capture antibodies that are bound to a solid surface are potent activators of the human complement system (Karlsson and Larsson, 2004). For example, human complement component C4 is known to strongly bind IgG (Campbell *et al.*, 1980). Thus, components of complement can potentially block the antigen-binding site and produce false-negative results (Tate *et al.*, 2004). Chicken antibodies do not activate human complement and hence these false negative results are avoided (Larsson *et al.*, 1992; Karlsson and Larsson, 2004).

Other problems associated with the use of mammalian antibodies include interactions with human and bacterial Fc receptors (Carlander and Larsson, 2001). Fc receptors play an important role in linking the effector mechanisms of innate immune cells with the adaptive immune system. Immune complex binding to Fc receptors initiates effector functions such as phagocytosis, antibody-dependent cellular cytotoxicity, degranulation of mast cells or neutrophils, and release of cytokines and cytotoxic mediators (Dunkelberger and Song, 2010). These Fc effector functions can be particularly problematic when using flow cell cytometry and living cells. Immune complex formations containing mammalian IgG can cause cell activation. Such immune complex activation may result in cytokine production and cause changes in the expression of cell surface proteins (Carlander and Larsson, 2001). These problems are avoided when chicken antibodies are used as they do not react with human Fc receptors. Mammalian IgG Fc interaction problems not only occur with human Fc receptors but can also occur with some bacterial proteins. Protein A from *Staphylococcus aureus* and protein G from *Streptococcus spp.* are capable of binding to the Fc portion of mammalian IgG. Therefore, mammalian antibody analysis of bacterial samples that contain these bacterial Fc-binding proteins may generate false-positive results.

In contrast, the use of chicken IgY antibodies does not generate erroneous results as they do not bind proteins A or G (Carlander *et al.*, 1999; Narat, 2003; Tate and Ward, 2004; Smith *et al.*, 2005). The production of pAbs from the egg yolk of immunised chickens has other advantages. For example, the collection of eggs from a laying hen is inexpensive, more convenient and safer than the traditional bleeding of animals. Furthermore, egg collection is much less invasive and is thus less stressful to the animal. Importantly, this animal-friendly approach offers greater compatibility with modern animal protection regulations (Lanning *et al.*, 2003; Kovacs-Nolan and Mine, 2004). In common with rabbits and mice, hens can easily be kept in conventional animal facilities. Animal care costs such as handling and feeding are considerably lower for a hen than for mammals such as rabbits.

A hen will start to lay eggs around 16-26 weeks of age (Mine and Kovacs-Nolan, 2002; Kovacs-Nolan and Mine, 2004). Typically, a laying hen produces approximately 5 eggs per week. The average volume of egg yolk is 15mL and contains 50-100mg of IgY, of which 2 to 10% are specific for antibodies (Rahimi *et al.*, 2007). Over the course of a year, a single hen can produce on average 250-280 eggs. Therefore, an immunised hen can provide over 30 grams of IgY per year (Kovacs-Nolan and Mine, 2004). Furthermore, in one week, a hen can supply as much antibody as that obtained from a rabbit in 3 months (Kim *et al.*, 2000). Thus, in comparison to rabbits, chicken pAbs are considerably less-expensive, while only large mammals such as cows or horses can produce more antibodies than a laying hen (Mine and Kovacs-Nolan, 2002; Leenaars and Hendriksen, 2005).

4.1.1 Chapter outline

This chapter describes in detail the multiple approaches taken to generate recombinant anti-sialic acid scFvs. In the initial approach, the semi-synthetic non-immune Tomlinson I and J phage display libraries were screened for sialic acid binders. In a second approach an “in-house” avian myeloperoxidase (MPO) library was also mined for anti-sialic scFvs. However, both these approaches were unsuccessful. Thus, the rest of this chapter describes the rational design and custom synthesis of new neoglycoconjugates (a protein to which defined carbohydrates are coupled), phage library construction, generation of recombinant antibody fragments and the isolation and characterisation of novel avian anti-sialic acid antibody fragments.

Although it was preferable to use laying hens for polyclonal antibody production, no hens were available at the start of this study. Therefore, a white male single comb Leghorn chicken (*Gallus domesticus*) was immunised with a sialic acid-containing neoglycoconjugate (Neu5Gc-HSA). After a 3 month immunisation course, the animal was sacrificed. Both the spleen and bone marrow were harvested and the ribonucleic acid (RNA) extracted using standard protocols. The amplified antibody variable genes were assembled by splice overlap extension polymerase chain reaction (SOE-PCR) and ligated into the Barbas pComb3XSS vector (Barbas, 2001). The recombinant phage display libraries were biopanned against a Neu5Gc-BSA neoglycoconjugate. A large proportion of the tested antibody fragments bound to sialic acid. The capacity of a subset of these scFvs to cross-react with other carbohydrate elements was investigated. Restriction digestion pattern analysis and nucleotide sequencing identified a number of unique scFv gene sequences. These scFv constructs were transformed and expressed in a non-suppressor strain of *E. coli*. Monomeric and dimeric scFv forms were identified by HPLC and FPLC analysis. Finally, the apparent dissociation constant of the monomeric scFv for sialic acid was determined by SPR.

4.2 Results

4.2.1 Screening of the Tomlinson I and J and MPO libraries

In the initial part of this project, the immunisation of an avian host was performed with a “homemade” conjugate. This conjugate consisted of neutravidin and a multivalent biotinylated PAA polymer that contained 0.2 moles of Neu5Gc per mole of PAA. The avian host was immunised over a period of three months. However, ELISA analysis showed no response to either PAA-Neu5Gc or a range of sialoglycoproteins (data not shown). This may be explained by the fact that PAA appears to be only weakly immunogenic and therefore, its immunostimulation and antigen presentation was not sufficient to generate a substantial immune response (Diano and Bivic, 2002; Enders *et al.*, 2007).

A second strategy used to identify anti-sialic acid antibodies was to screen the Tomlinson I and J libraries with a biotinylated PAA-Neu5Gc conjugate. These semi-synthetic non-immune libraries (Medical Research Council, Cambridge, England) contain a huge diverse repertoire of antibody specificities. These libraries were derived from a single framework scaffold that is comprised of IgV_H (V3-23/DP-47, JH4b) and IgV_L (O12/O2/DPK9, JK1) human germline gene segments (<http://lifesciences.sourcebioscience.com/clone-products/proteomic-resources/human-single-fold-scfv-libraries-i-plus-j.aspx>). The canonical (loop) structures encoded by these V_H and V_L segment pairings are found at a high frequency in the natural human antibody repertoire. Importantly, this human framework is known to express and fold efficiently in *E. coli* and bacteriophage (Leong *et al.*, 2007; An *et al.*, 2009). The third hypervariable regions of these heavy (CDRH3) and light chain (CDRL3) gene segments were synthetically randomised. These were designed to be as short as possible while still forming an antigen-binding surface. In addition, amino acid side chain diversity DVT (Aspartic acid, Valine and Threonine) and NNK (Asparagine and Lysine) is incorporated at 18 different positions in the CDR3 and CDR2 regions. The CDR1 region was kept constant. Antibody heavy and light chain gene coding segments are joined at random with a 15 amino acid serine/glycine linker (SGGGG)₃ (de Wildt *et al.*, 2000). The encoded scFv fragment is inserted into the gene III tail fibre of filamentous M13 bacteriophage (Barbas, 2001).

These phage particles are endowed with a phagemid genome (pIT2) which, in addition to the scFv segment, fused to the amino terminus of M13 pIII gene, contains both a phage and an *E. coli* origin of replication and a function for ampicillin resistance. In addition, the hexahistidine and *myc* C-terminal affinity tags located within the phagemid vector pIT2 allow for the detection of scFv fragments. Furthermore, purification of these scFv fragments is facilitated by the incorporation of human framework regions that bind to the generic capture ligands protein A (V3-23/DP-47, JH4b) or protein L (O12/O2/DPK9, JK1). These naïve semi-synthetic scFv libraries contain 1.47×10^8 (DVT encoded-library I) and 1.37×10^8 (NNK encoded-library J) phagemid clones in *E. coli* TG1 cells. The percentage of scFv inserts found within the I and J libraries are 88% and 96%, respectively (Kristensen and Winter, 1998; de Wildt *et al.*, 2000; Goletz *et al.*, 2002; Clackson and Lowman, 2007).

The Tomlinson I and J library stocks were amplified and phage particles rescued by superinfection with KM13 helper phage. After each round of biopanning, eluted phage were re-amplified by re-infection into exponentially growing cultures of *E. coli* TG1 cells. For each library, biopanning experiments were carried out against a biotinylated-Neu5Gc-PAA conjugate that was immobilised on neutravidin-coated immunotubes. ELISA analysis of precipitated phage populations from four separate rounds of biopanning was used to identify sialic acid-binding phage. No positive results were obtained by polyclonal phage ELISA, as phage optical density readings were the same or slightly lower than background (data not shown). To assess the binding of monoclonal phage scFvs, 192 different TG1 colonies were picked from the biopanned I and J libraries and grown in sterile culture plates. These clones were subsequently induced by IPTG and their supernatants containing the soluble scFvs were analysed by ELISA. However, none of the clones tested showed any positive sialic acid signal (data not shown). Consequently, these libraries did not appear to contain antibody fragments that could bind sialic acid in the context of the biotinylated-PAA-Neu5Gc conjugate.

A third approach used was to screen an “in-house” avian MPO immune library that was previously constructed for use in a different project. MPO is a haem-containing glycoprotein that is expressed at high levels in neutrophils, monocytes, and some populations of human macrophages. This enzyme plays a major role in the host defence function of these cells by generating strongly oxidant molecules.

Importantly, the surface of the MPO glycoprotein contains sialic acid linked $\alpha(2,3)$ to galactose (Yamada *et al.*, 1987; McMillen *et al.*, 2005; Hansson *et al.*, 2006). It was postulated that avian immunisation with MPO may have produced an immune response against sialic acid epitopes. Therefore, the MPO avian library was subjected to three rounds of biopanning using the biotinylated-PAA-Neu5Gc conjugate. However, ELISA screening of precipitated phage populations from each biopanning round did not identify any phage pools that were sialic acid-specific (data not shown). This library was analysed further by re-infecting output phage from each selection round into XL1-Blue, a bacterial strain of *E. coli*. In total, 192 randomly selected colonies were picked and cultured. ELISA analysis of supernatants containing phage displayed scFv did not identify any anti-sialic acid-binding phage clones (data not shown). These results indicated that the MPO scFv library and/or the biotinylated-Neu5Gc-PAA conjugate were not suitable for the isolation of anti-sialic acid antibody fragments.

In another approach, a novel sialo-neoglycoconjugate (Neu5Ac-HSA) was immunised into a hen. This multivalent sialic acid glycoconjugate was kindly donated by Dr. Arnberg of the Swedish University of Umeå. The HSA neoglycoconjugate contained 13 Neu5Ac residues per mole of HSA protein (Johansson *et al.*, 2007). However, after only two avian immunisations, a serum sample could not be taken as the hen did not survive. As none of the previous strategies for anti-sialic acid antibody generation were successful, a commercial company was employed to synthesis custom-made sialic acid-protein conjugates. Two different sialic acid conjugates, namely Neu5Gc-BSA and Neu5Gc-HSA, were initially custom synthesised by Carbohydrate Synthesis, U.K (see Section 2.2.1). The Neu5Gc-HSA conjugate contained 20 monosaccharide units of Neu5Gc per mole of HSA protein and was used for avian immunisations. In contrast, the Neu5Gc-BSA conjugate contained 35 monosaccharide units of Neu5Gc per mole of BSA protein and was used for ELISA, phage display and SPR-based analysis.

4.2.2 Avian anti-sialic acid recombinant antibodies.

4.2.2.1 Avian serum titre against Neu5Gc-BSA.

Prior to chicken sacrifice, a serum titre was performed to determine whether a sufficient response against the Neu5Gc-BSA antigen was achieved. A series of dilutions ranging from neat to 1 in 1,000,000 of the chicken serum, diluted in 1% (w/v) BSA, PBST (pH 7.2), were tested against Neu5Gc-BSA in a direct ELISA format. A titre in excess of 1/50,000 against Neu5Gc-BSA was observed from the ELISA, indicating a high level of specific mRNA for creation of a recombinant antibody library against the sialic acid antigen (Figure 4.2.2.1).

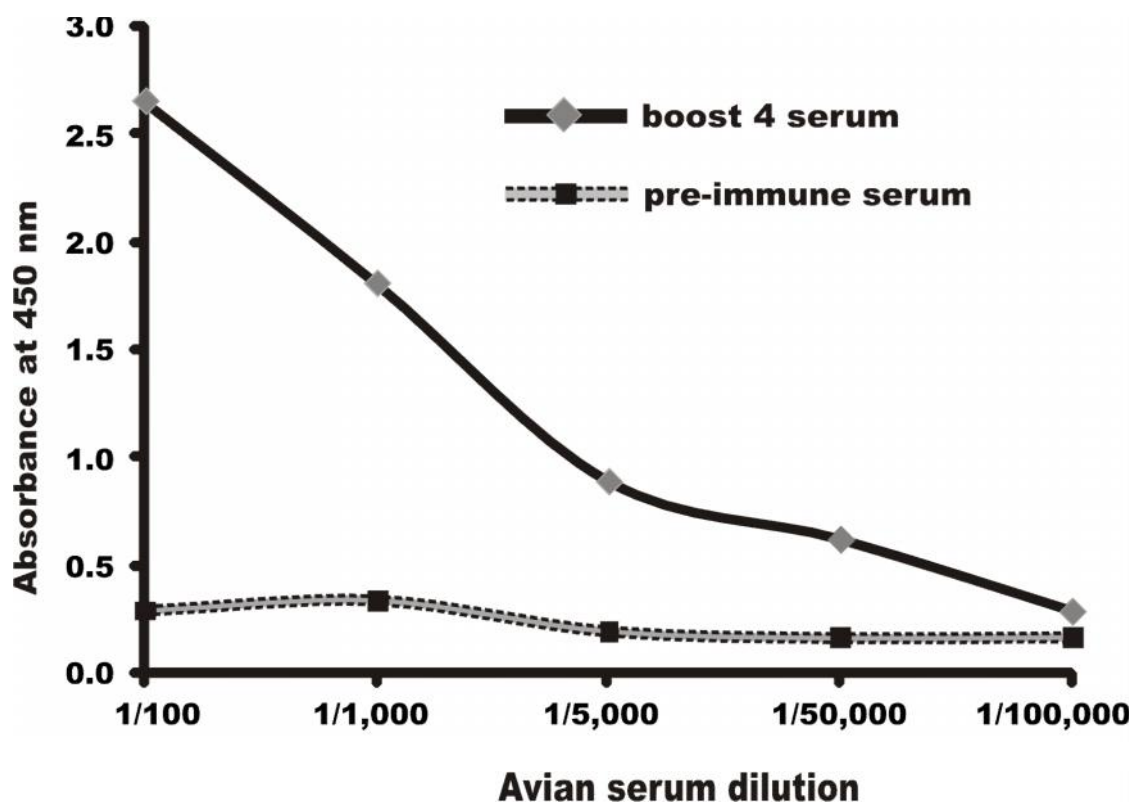


Figure 4.2.2.1 *Titre of an avian antiserum response to a Neu5Gc-BSA conjugate.* An ELISA plate was coated with 5 μ g/mL of the BSA-Neu5Gc conjugate. The plate was blocked with 3% (w/v) BSA in PBS (pH 7.2) and the diluted serum applied to wells in triplicate. A rabbit anti-chicken antibody, conjugated with horseradish-peroxidase (HRP) (1:2000) was used to detect any positively binding antibodies to sialic acid. The serum titre is greater than 1/50,000 but less than 1/100,000.

4.2.2.2 Avian serum titre against Neu5Gc-polyacrylamide (PAA).

To ensure that the avian polyclonal response was directed towards the Neu5Gc component of the conjugate and not the spacer arm or protein elements, the polyclonal serum was also tested against a synthetic carbohydrate that consisted of a multivalent biotinylated PAA polymer that contained a 20% molar ratio of Neu5Gc to PAA. This experiment demonstrated that avian pAbs could discriminate Neu5Gc in the context of two very different carriers with unrelated linkers, namely BSA-linker1-Neu5Gc and biotin-PAA-linker2-Neu5Gc (Figures 4.2.2.2/3).

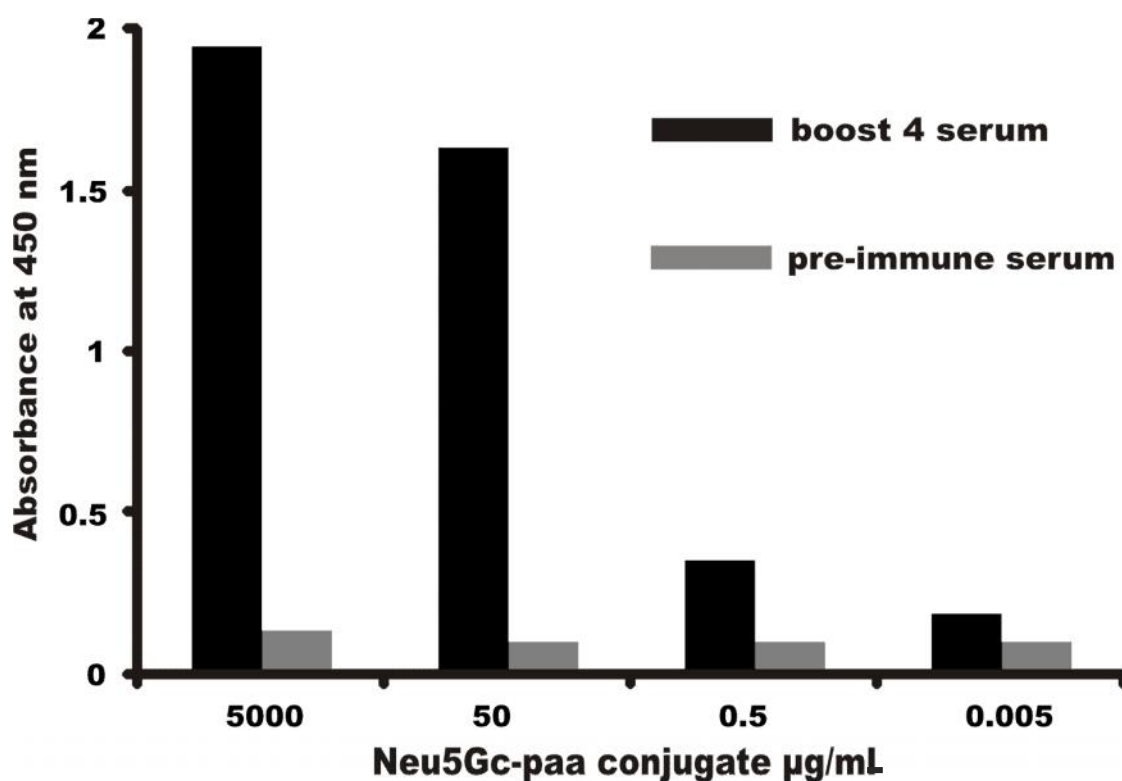


Figure 4.2.2.2 *Titre of an avian antiserum response to a Neu5Gc-PAA conjugate.* An ELISA plate was coated with $5\mu\text{g/mL}$ of neutravidin. After washing, $25\mu\text{g/mL}$ of the biotinylated-PAA-Neu5Gc conjugate was added to the plate. The plate was blocked with 3% (w/v) BSA in PBS (pH 7.2) and the diluted serum applied to wells in triplicate. A rabbit anti-chicken antibody, conjugated with HRP was used to detect any positively binding serum antibodies to the Neu5Gc-PAA conjugate.

4.2.2.3 Avian serum inhibition ELISA against Neu5Gc-BSA conjugate.

To verify that the avian polyclonal response for the Neu5Gc component was also competitive (e.g. able to differentiate between immobilised and solution-phase antigen), an inhibition ELISA with free Neu5Gc-BSA conjugate was performed using the polyclonal serum. ELISA analysis revealed that serum-based avian antibodies were able to compete between free and immobilised Neu5Gc-BSA (Figure 4.2.2.3).

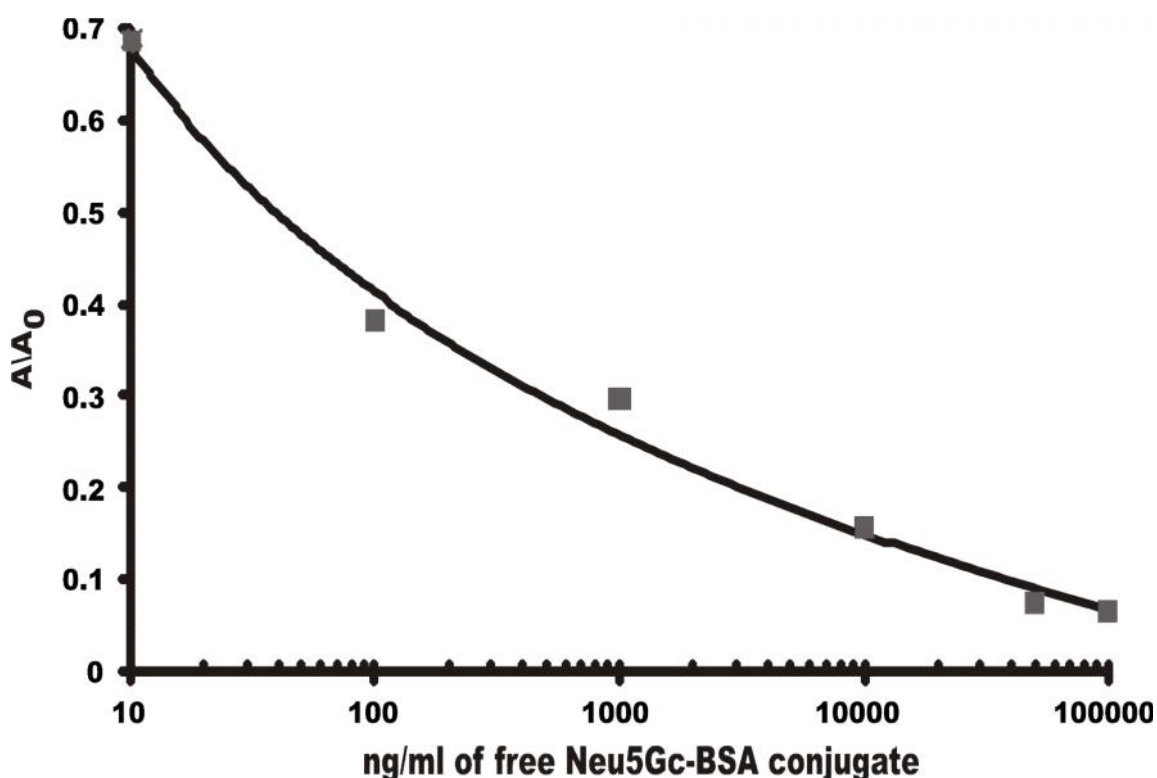


Figure 4.2.2.3 *Inhibition ELISA of the avian antiserum with free Neu5Gc-BSA conjugate.* An ELISA plate was coated with 5 μ g/mL of the BSA-Neu5Gc conjugate. The plate was blocked with 3% (w/v) BSA in PBS (pH 7.2). The Neu5Gc-BSA conjugate was added at varying concentrations to a 1:50,000 dilution of avian serum in 1% (v/v) BSA in PBST (pH 7.2). Samples containing no conjugate (A_0) were diluted in PBST (pH 7.2) to ensure equivalent serum concentration. The absorbance for each Neu5Gc-BSA conjugate concentration was plotted as a ratio of the absorbance without conjugate (A/A_0) versus the free Neu5Gc-BSA concentration (ng/mL).

4.2.2.4 Chicken variable heavy and light chain PCR amplification.

The preliminary testing of immune serum from the immunised chicken demonstrated a clear response to Neu5Gc, in the context of two very different carriers with unrelated linkers (Neu5Gc-BSA and Neu5Gc-PAA). It was also demonstrated that the serum antibodies had the capacity to recognise Neu5Gc-BSA free in solution, by inhibition ELISA. It was decided to isolate the antibody-encoding genes using a recombinant approach. In the first stage of recombinant antibody generation, the variable heavy (V_H) and variable light (V_L) genes were amplified from avian spleen and bone marrow cDNA. However, in addition to the target product (~400bp), a spurious non-specific PCR amplicon (>400bps) was also generated (Figure 4.2.2.4).

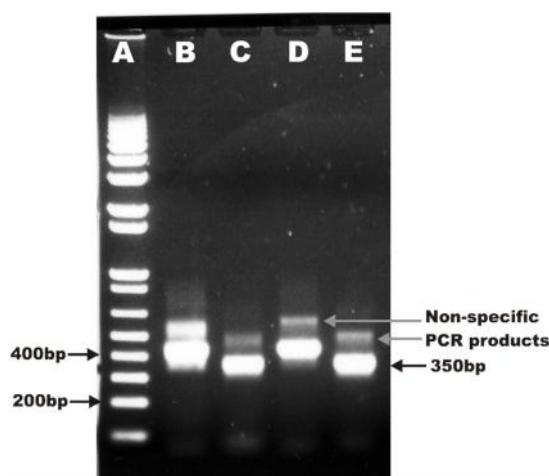


Figure 4.2.2.4 Initial amplification of the V_H (~400bp amplicon) and variable light V_L chain (~350bp amplicon) genes using the cDNA from a HSA-Neu5Gc-immunised chicken. The PCR reaction contained the following: 1 μ L of cDNA, 100 pmol of CSCHo-F and CSCG-B, 5x PCR buffer 1.5mM MgCl₂, 200 μ M dNTPs and 1 μ L GoTaq® DNA polymerase. For V_L gene amplification the PCR reaction components were the same except that 100 pmol of CSCVK and CKJo-B were used in place of the V_H primers. The PCR amplification profile was as follows: initial denaturation at 94°C for 5min, 35 cycles with denaturation at 94°C for 15s, annealing at 55°C for 30s, and extension at 72°C for 45s, followed by a final cycle of extension at 72°C for 5min. Lane A represents a 1 kb Plus DNA ladder (Invitrogen; 1 kb to 12 kb), lanes B and D represent V_H amplification and lanes C and E represent V_L amplification. Non-specific PCR products, that are larger than the desired amplicons can be seen for both V_H and V_L gene amplifications.

4.2.2.5 PCR optimisation of Chicken variable heavy and light chains.

Optimisation of PCR amplification of the V_H and V_L genes was achieved by increasing the annealing temperature of the PCR reaction (55°C to 59°C), reducing the quantity of GoTaq® DNA polymerase (1µL to 0.5µL per PCR reaction), decreasing the forward and reverse primer concentrations (100pmol to 60pmol per PCR reaction) and reducing the number of PCR cycles (35 to 30). In addition, a touchdown PCR thermocycling profile (see below) was used and the successful amplification of the avian variable domain genes in the absence of superfluous amplicons is shown in Figure 4.2.2.5

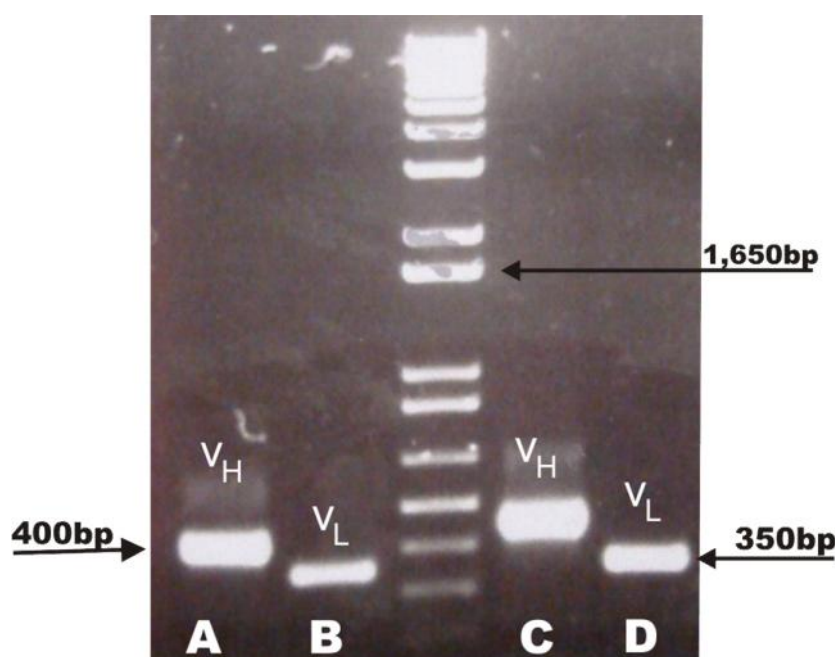


Figure 4.2.2.5 Optimised V_H (~400bp amplicon) and variable light V_L chain (~350bp amplicon) amplifications using the cDNA from a HSA-Neu5Gc-immunised chicken. The PCR reaction contained the following: 1µL of cDNA, 60pmol of CSCHO-F and CSCG-B, 5x PCR buffer 1.5mM MgCl₂, 200µM dNTPs and 0.5µL GoTaq® DNA polymerase. For V_L gene amplification the PCR reaction components were the same, except that 60pmol of CSCVK and CKJo-B were used in place of the V_H primers. The touchdown PCR amplification profile was as follows: initial denaturation at 94°C for 4min, 30 cycles with denaturation at 94°C for 15s, annealing at 59°C for 30s, and extension at 72°C for 45s. Subsequently the annealing temperature was decreased by 0.1°C per PCR cycle. This was followed by a final cycle of extension at 72°C for 5min. The middle lane represents a 1 kb Plus DNA ladder (Invitrogen; 1 kb to 12 kb), lane A and C represents V_H amplification and lanes B and D represents V_L amplification.

4.2.2.6 PCR Splice Overlap Extension (SOE) of variable avian heavy and light chain genes.

Following the successful amplification of the avian variable domain genes, single chain Fv fragments were assembled using a short 7-amino acid peptide glycine-serine linker in the orientation V_L -GQSSRSS- V_H by a two fragment SOE-PCR. However, in this second round of PCR not only was the target product (~750bp) amplified, but also an unwanted product of between 200-300bp (Figure 4.2.2.6).

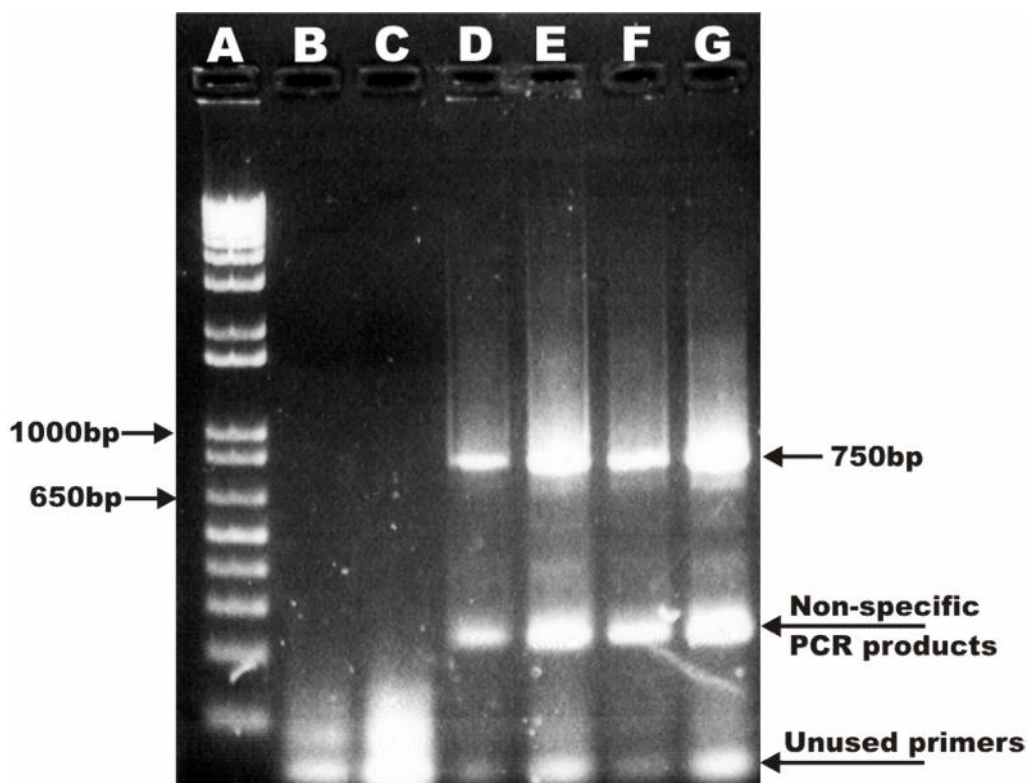


Figure 4.2.2.6 Initial optimisation of *SOE-PCR* of V_H and V_L fragments. For *SOE-PCR*, a 100 μ L PCR reaction contained the following: 100ng of the V_L and V_H purified products, 100 pmol of CSC-F and CSC-B, 10x PCR buffer, 1.5mM $MgSO_4$, 200 μ M dNTPs and 1 μ L Platinum® Taq DNA polymerase. The PCR was performed with the following cycling conditions: 5min at 94°C (initial denaturation), followed by 30 cycles of 30s at 94°C (denaturation), 30s at 55°C (annealing), 2min at 72°C (extension) and the reaction was terminated after 10min at 72°C (final extension). Lane A shows a 1 kb Plus DNA ladder (Invitrogen; 1 kb to 12 kb). Lanes B and C are no template controls. Lanes D/F and G/H show the amplification of V_H and V_L fragments to produce an *SOE* product of approximately 750bp. In addition, non-specific PCR products of between 200-300bp were also amplified.

4.2.2.7 Optimised avian SOE-PCR and digestion of the pComb3XSS vector

Optimisation of the SOE-PCR was achieved by increasing the annealing temperature of the PCR reaction (55°C to 57°C), reducing the PCR extension time (2 minutes to 1 minute) and decreasing the forward and reverse primer concentrations (100 pmol to 60 pmol per PCR reaction). *Sfi*I restriction digestion of the pComb3XSS vector yielded the expected stuffer fragment ~1600bp and the double-cut vector of ~3400bp (Figure 4.2.2.7). The pComb3XSS vector (Barbas, 2001) contains a double stuffer (SS) fragment between the two *Sfi*I cloning sites in the heavy and light chain regions for efficient cloning. This optimised PCR produced the desired 750 bp fusion product of the two chains alone, joined via a serine-glycine linker, to form the full length scFv gene fragment.

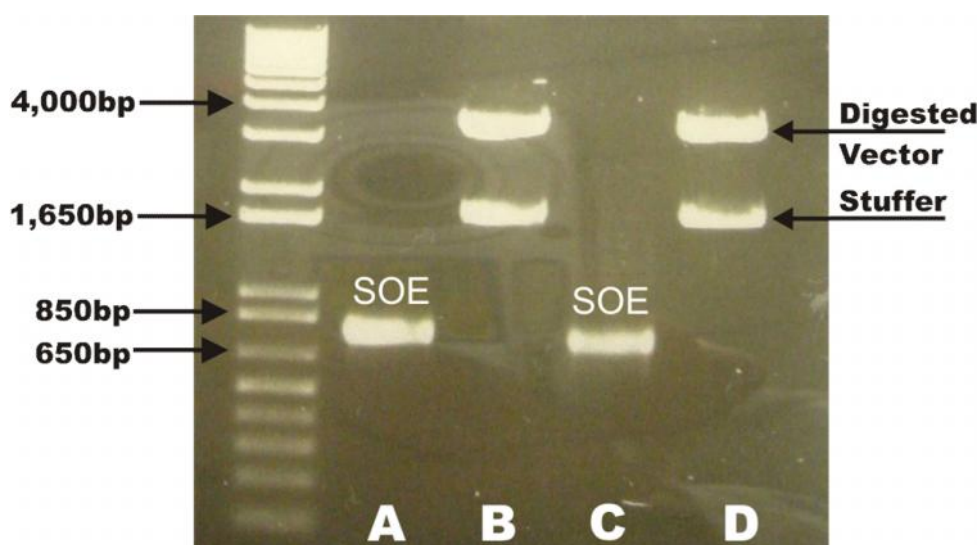


Figure 4.2.2.7 Optimised *SOE-PCR* of V_H and V_L fragments. A 1 kb DNA molecular weight marker was added to lane 1. For *SOE-PCR*, a 100 μ L PCR reaction contained the following: 100ng of the V_L and V_H purified products, 60 pmol of CSC-F and CSC-B, 10x PCR buffer, 1.5mM $MgSO_4$, 200 μ M dNTPs and 1 μ L Platinum® Taq DNA polymerase. PCR was performed with the following cycling conditions: 5 minutes at 94°C (initial denaturation), followed by 30 cycles of 30 sec at 94°C (denaturation), 30 sec at 57°C (annealing), 1 minutes at 72°C (extension) and the reaction was terminated after 10 minutes at 72°C (final extension). Lanes A and C show that the amplification of V_H and V_L fragments to produce an *SOE* product of approximately 750bp. Lanes B and D represent the digestion of the pComb3XSS vector to produce a stuffer fragment ~1600bp and the double-cut vector of ~3400bp.

4.2.2.8 Library construction and biopanning.

The SOE product was amplified in large-scale and subsequently concentrated using ethanol precipitation. The PCR product was quantified using the Nanodrop ND-1000™ and cloned into the pComb3XSS vector using the restriction enzyme *Sfi*I (See Section 2.2.12). The ligated product was transformed into electrocompetent XL1-Blue cells by electroporation using the protocol described in section 2.2.12. The transformed avian anti-sialic acid scFv library had a size of 1.2×10^7 cfu/mL. The library was subject to phage display biopanning against immobilised Neu5Gc-BSA antigen, as described in Section 2.2.15. Two different biopanning strategies (competitive and trypsin elution) were used for the isolation of putative anti-sialic phage binders. The goal was to isolate any sialic acid phage binders, even those with low-affinities for sialic acid. Tables 4.2.2.8a/b shows the phage input and output titres over the 4 rounds of biopanning of the avian anti-sialic scFv library.

Table 4.2.2.8a *The phage input and output titres over the 4 rounds of biopanning of the avian anti-sialic scFv library using the **competitive elution** strategy.*

Biopanning round	Colony forming units/mL
Input 1	1×10^{11}
Output 1	1×10^9
Input 2	1×10^{10}
Output 2	1×10^8
Input 3	1×10^9
Output 3	3×10^8
Input 4	1.0×10^7

Table 4.2.2.8b *The phage input and output titres over the 4 rounds of biopanning of the avian anti-sialic scFv library using the **trypsin elution** strategy.*

Biopanning round	Colony forming units/mL
Input 1	3×10^{12}
Output 1	2×10^{11}
Input 2	1×10^{11}
Output 2	6×10^9
Input 3	3×10^{11}
Output 3	1×10^7
Input 4	1×10^9

Phage output titres may give an indication of the success of the biopanning strategy, since increasing numbers of phage should remain bound to the antigen and this should result in higher output titres. A comparison of Tables 4.2.2.8a\b shows that for the trypsin method, phage numbers decrease after each round of biopanning, whereas the phage output titres for the competitive method remain relatively constant after each round of biopanning. Therefore, based only on phage output numbers, the competitive method appeared to be better. However, phage titres in general are not a reliable marker of biopanning success, as only a small fraction of the phage actually display correctly folded antibody fragments on their surface (Maynard and Georgiou, 2000; Barbas, 2001; O' Brien and Aitken, 2002; Clackson and Lowman, 2007). Therefore, it was not possible to say which elution strategy was more successful.

4.2.2.9 Avian polyclonal phage ELISA.

The precipitated input phage particles from the various rounds of biopanning were tested for sialic acid-binding using a polyclonal phage ELISA (section 2.2.17). BSA was also tested as a negative control to observe the degree of non-specific ‘background’ binding. The scFv-displaying phage particles were detected using a HRP-conjugated mouse anti-M13 antibody (GE Healthcare Life Sciences) and the absorbance was recorded at 450 nm, following a 10 minute incubation with TMB substrate. The sharp increase in absorbance from the first round onwards suggested the presence of sialic acid-specific scFv-harboring phage within the panned library. The largest enrichment of putative anti-sialic acid polyclonal phage pools was observed in the third round of selection. There was a slight decrease in anti-sialic acid antibody enrichment in round four. In contrast, round five showed a substantial decrease in anti-sialic acid reactivity. Additionally, negligible binding was observed against BSA. The spleen and bone marrow immune libraries were constructed and biopanned separately, thus the polyclonal phage ELISA illustrated the phage pools from both sources (Figure 4.2.2.9).

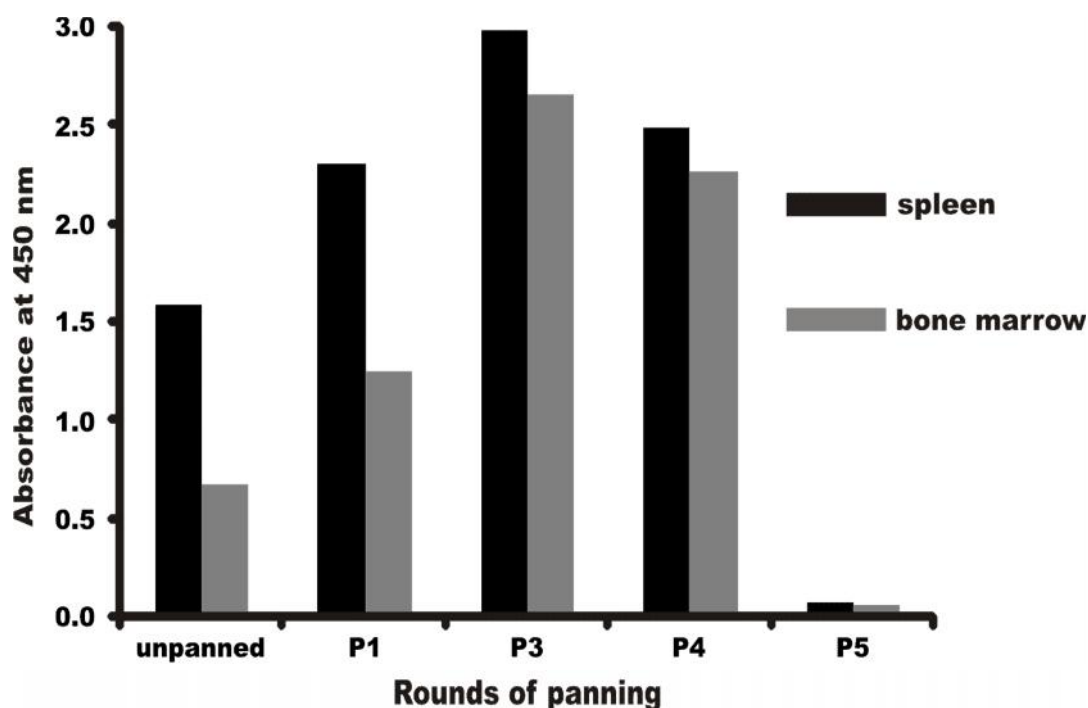


Figure 4.2.2.9 Polyclonal phage ELISA involving the phage pools of the unpanned library and the phage outputs from each of the successive rounds of biopanning. M13K07 helper phage was tested to identify any background non-specific binding. The same biopanning profile is seen for both the competitive and trypsin elution strategies.

4.2.2.10 Soluble expression and direct ELISA of avian anti-sialic acid scFv clones.

A soluble monoclonal ELISA was performed on clones that originated from the competitive or trypsin elution strategies (See Section 2.2.18.). Figures 4.2.2.10a\b show that greater than 90% of the clones analysed had ELISA signals which were more than three-fold higher than that of the negative control protein, BSA. Both methods produced equally large numbers of anti-sialic acid scFvs, so it was not possible to say if either method had any superior advantage.

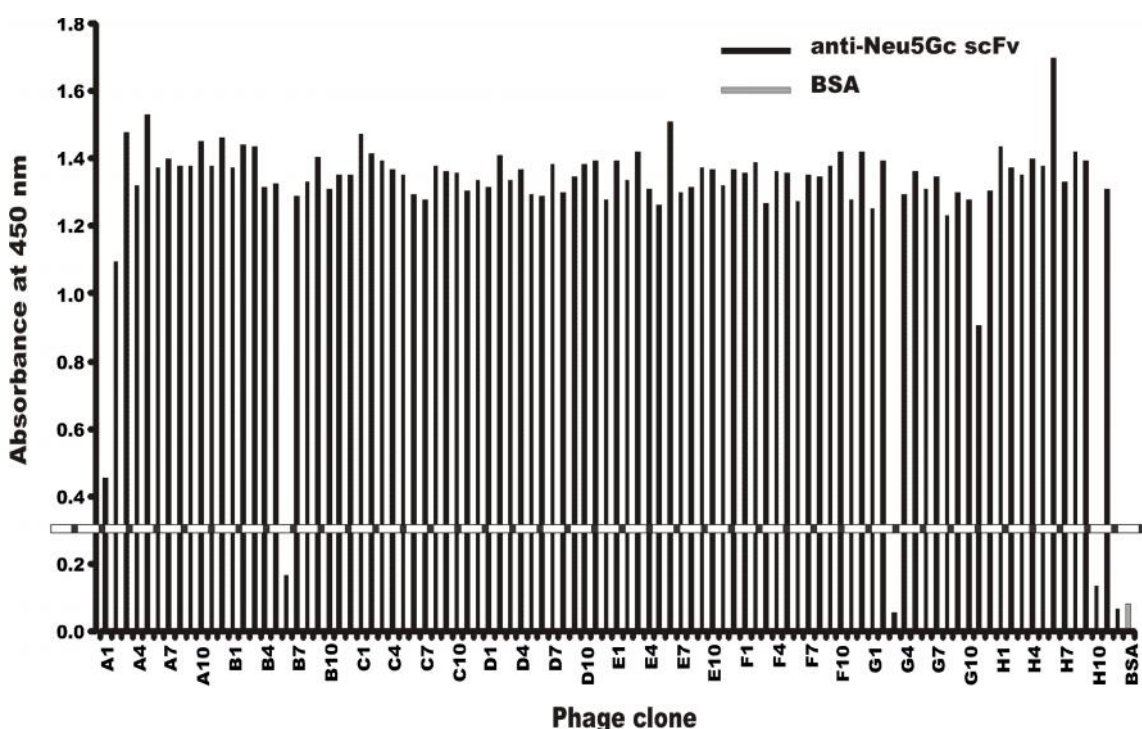


Figure 4.2.2.10a Soluble monoclonal ELISA of *competitively-eluted* phage clones from rounds three and four of biopanning. Each ELISA well was coated with 10 μ g/mL of Neu5Gc-BSA conjugate. Periplasmic fractions were diluted 1:5 in 1% (w/v) BSA, PBS (pH 7.2) and 100 μ L of each clone applied to the ELISA plate. A secondary rat anti-HA monoclonal antibody conjugated with peroxidase (1:2,000) was used to detect scFvs that were specific for the sialic acid moiety of the Neu5Gc-BSA conjugate. The ELISA threshold is marked with a horizontal line. The BSA carrier screening result represents the total average binding of all clones, when tested in parallel with BSA, as opposed to Neu5Gc-BSA.

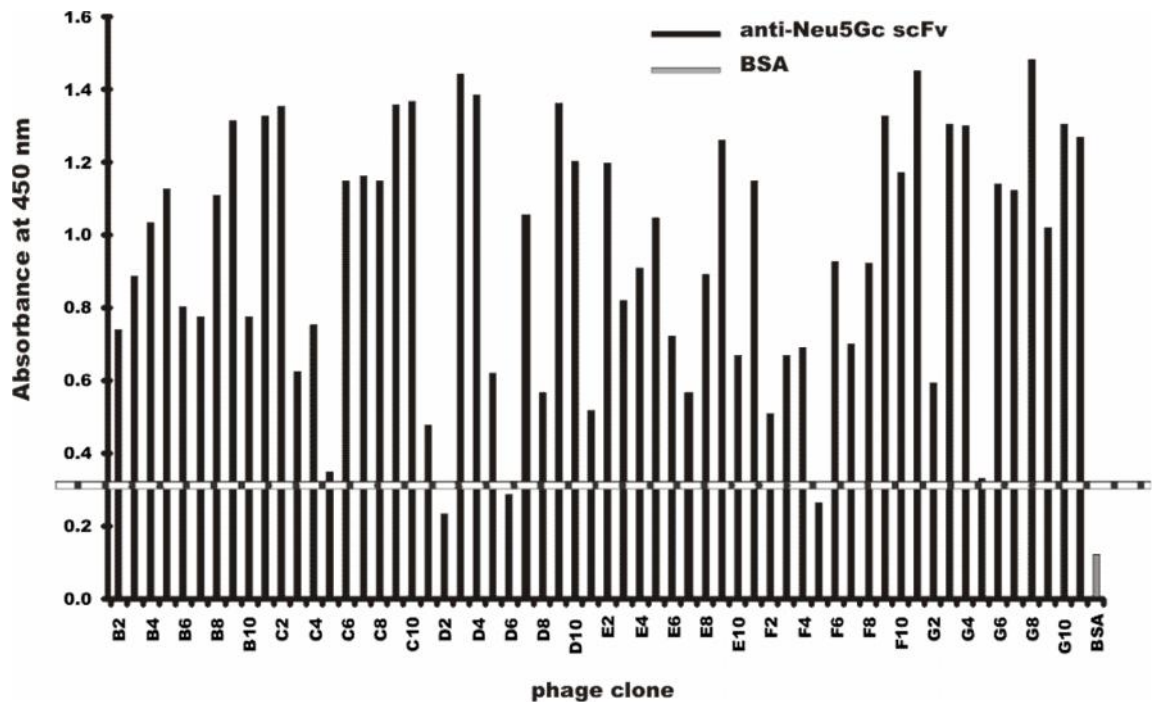


Figure 4.2.2.10b Soluble monoclonal ELISA of *trypsin-eluted* phage clones from rounds three and four of biopanning. Each ELISA well was coated with 10µg/mL of Neu5Gc-BSA conjugate. Periplasmic fractions were diluted 1:5 in 1% (w/v) BSA, PBS (pH 7.2) and 100µL of each clone applied to the ELISA plate. A secondary rat anti-HA monoclonal antibody conjugated with peroxidase (1:2,000) was used to detect scFvs that were specific for the sialic acid moiety of the Neu5Gc-BSA conjugate. The ELISA threshold is marked with a horizontal line. The BSA bar represents the total average binding of all clones to BSA.

4.2.2.11 Direct ELISA of avian anti-sialic acid scFv clones that recognise Neu5Gc in the form of both BSA-Neu5Gc and PAA-Neu5Gc.

ELISA-based analysis was used to assess the ability of soluble scFvs to recognise Neu5Gc in the context of Neu5Gc-linker1-BSA and a second conjugate, Neu5Gc-linker2-PAA. The PAA carbohydrate conjugates were pseudo-glycoproteins that were originally developed by Nicolai Bovin and have been widely used in carbohydrate-binding protein studies (Bovin, 1998; Ravn *et al.*, 2004; Huflejt, *et al.*, 2009). In this assay, neutravidin was used to capture the biotinylated-PAA-Neu5Gc conjugate.

From the original 90% of clones that bound to Neu5Gc-BSA, less than 10% of these clones recognised Neu5Gc in the context of the linker2-PAA-backbone. Thus, this scFv subset had the ability to recognise Neu5Gc without the requirement of a specific linker. Moreover, this experiment revealed that the scFvs were not simply binding to the linker region, but rather they had a real binding affinity for sialic acid (Figure 4.2.2.11).

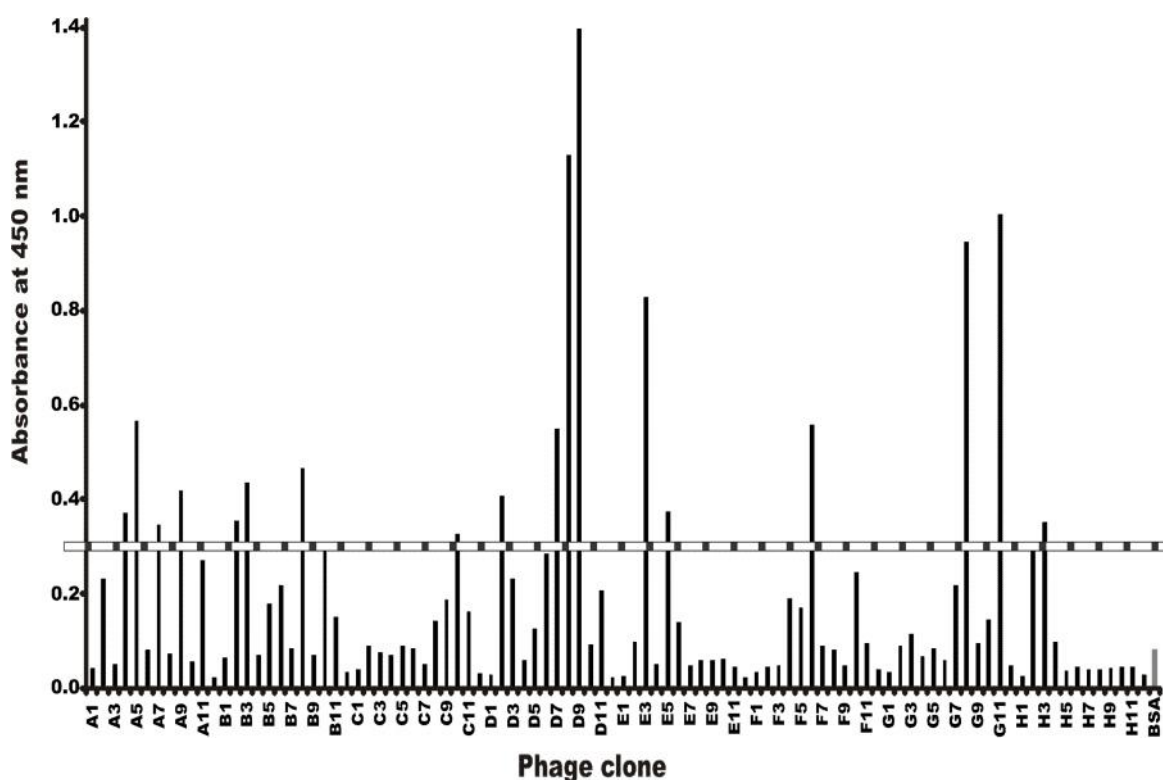


Figure 4.2.2.11 Soluble anti-sialic acid clones that recognise Neu5Gc in the form of both Neu5Gc-BSA and Neu5Gc-PAA. Each ELISA well was coated with 5µg/mL of neutravidin and subsequently, 100µL of biotinylated-PAA-Neu5Gc (25µg/mL) was added to the wells. Periplasmic fractions were diluted 1:5 in 1% (w/v) BSA, PBS (pH 7.2) and 100µL of each clone applied to the ELISA plate. A secondary rat anti-HA monoclonal antibody conjugated with peroxidase (1:2000) was used to detect scFvs that were specific for the sialic acid moiety of the Neu5Gc-BSA conjugate. The ELISA threshold is marked with a horizontal line. The BSA bar represents the total average binding of all clones to BSA. A selection of positive clones is shown.

4.2.2.12 Restriction digestion pattern analysis of 17 avian clones.

Restriction digestion mapping is a traditional method used for the evaluation of antibody sequence diversity. This procedure involves the digestion of DNA with restriction enzymes and comparison of the fragment patterns. A total of 17 scFv gene products, which had bound to BSA-Neu5Gc and PAA-Neu5Gc, were amplified by PCR and then digested using the high-frequency cutting enzymes *Bst*NI and *Alu*I. A number of clones had unique restriction patterns specific for their scFv gene sequence (Figure 4.2.2.12).

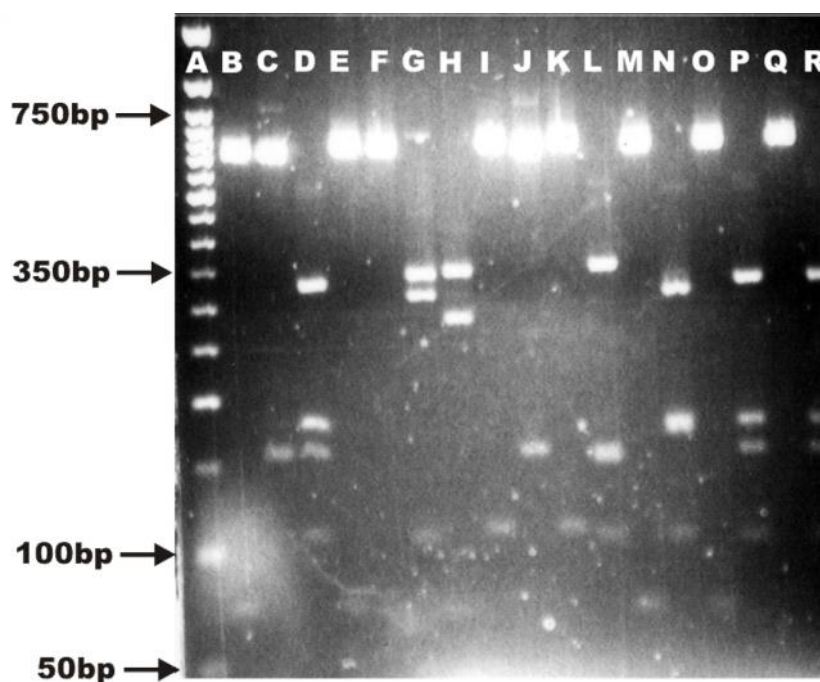


Figure 4.2.2.12 A restriction map profile of 17 different avian scFv inserts. Lane A shows a 50 base pair DNA molecular weight marker. Lanes B to R represent the avian scFv gene inserts digested after PCR amplification with a combination of the high-frequency cutting enzymes *Bst*NI and *Alu*I. Comparison of the restriction digestion fragment patterns indicates a high level of clonal diversity within the avian anti-sialic acid library.

4.2.2.13 Cross-reaction-analysis of the soluble anti-sialic clones with different mono and disaccharide structures.

A subset of the avian Neu5Gc-PAA/BSA binding clones were further analysed by cross-reactivity studies using different mono and disaccharide carbohydrate structures. The following structures were tested: Neu5Ac-PAA, (Neu5Ac)₂-PAA, Neu5Gc (Gc), glucose-PAA and galactose-PAA. An unrelated avian scFv served as a negative control in the immunoassay (Ayyar *et al.*, 2010). Each sugar conjugate had the same molecular weight (30kD) and contained the same 20:1 molar ratio of carbohydrate to PAA. In Figure 4.2.2.13, the binding response of 8 different scFvs to 5 different sugar-PAA conjugates and BSA is shown. As expected, the negative control, a protein-binding-avian scFv, did not react with any of the sugar-PAA conjugates. Overall, the AE8 scFv showed the strongest reactivity to all of the sialic acid structures.

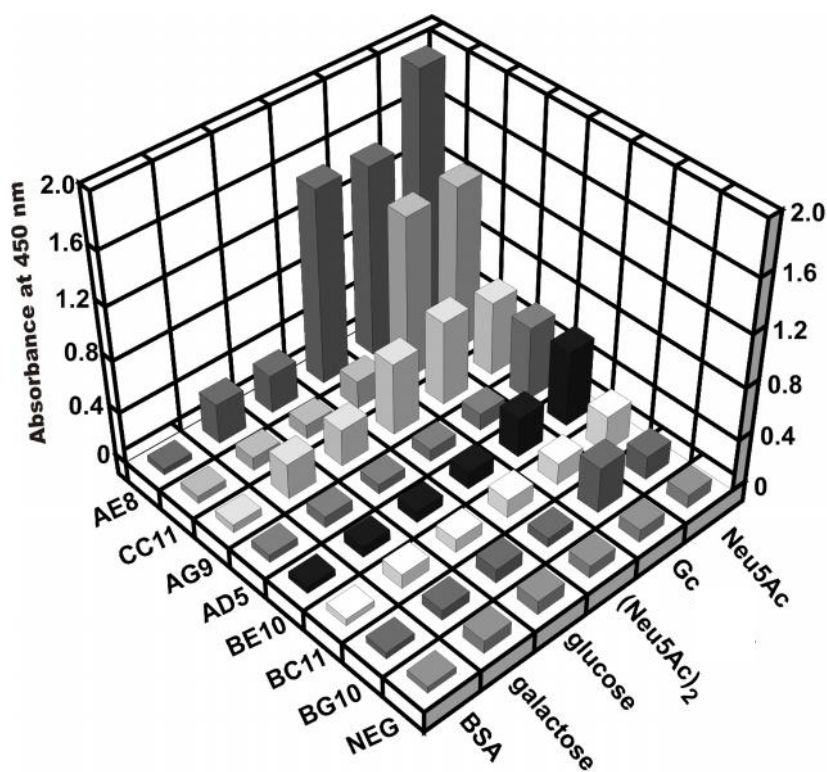


Figure 4.2.2.13 A cross-reaction study of soluble anti-sialic scFvs to different monosaccharides and sialic acids. The scFvs BG10 and BC11 bound relatively poorly to each of the sugar-PAA conjugates. In addition, AG9, AD5 and BE10 showed the same weak binding to Neu5Ac-PAA. In contrast, the AE8 and CC11 scFvs showed very strong binding to Neu5Ac-PAA and Neu5Gc-PAA. Moreover, these scFvs had no significant binding to galactose-PAA, glucose-PAA or BSA.

4.2.2.14 Deduced amino acid sequence alignment of four avian scFvs.

DNA sequence analysis was performed on four avian clones, AE8, CC11, AG9 and CD3. For comparison, clone CD3, which demonstrated no significant binding to sialic acid, was also included for control purposes. The deduced amino acid sequence alignment of the CDR V_H and V_L regions for each of the four different avian clones is shown in Figure 4.2.2.14. Each clone is genetically different and comparison of the CDRs show differences in both amino acid sequence and CDR length. Clone CD3, which had very weak affinity for sialic acid, shows the greatest differences in both the heavy and light chain CDR regions, whereas AE8 and AG9 show only subtle differences in CDRL3, CDRH1, CDRH2 and CDRH3 regions. Several amino acid differences between CC11 and AE8 occur in CDRL1 and CDRL3 and these may account for the inability of CC11 to recognise the di-sialic acid-PAA conjugate. Since the AE8 scFv had the strongest sialic acid reactivity, it was therefore chosen for further study.

CLONE	CDRL1	CDRL2	CDRL3
AE8	S G G G G S Y Y G	D N T N R P S	G S Y D R S A G Y V G I
AG9	S G G G G S Y Y G	D N T N R P S	G N F D T S A - - - - I
CD3	S G G S Y S - Y G	Q N T N R P S	G S F D S S V G M - - -
CC11	- - - - - K W Y G	D N T N R P S	G G Y D G S T D A - G I

	CDRH1	CDRH2	CDRH3
AE8	G F T F D S Y A M Y	I N R F G S S T G H G A A V	S V H G S C A S G T W C S P A S I D A
AG9	G F T F S S F N M I	I N R F G N S T G H G A A V	S V H G H C A S G Y W C S A A S I D A
CD3	G F S F S D R G M H	I Y D D G G T T Y Y G A A V	S A A G D A W A D D - - - - - I D A
CC11	G F D F S S Y Q M N	I N K F G T S T S R G A A V	S A Y G S C A S G S W C S A A S I D A

Figure 4.2.2.14 Comparative sequence analysis of avian scFvs. DNA sequencing of all clones was performed by Eurofins MWG Operon (Ebersberg, Germany) in triplicate. The complementary determining regions (CDRs) were determined according to Kabat and Wu (1991). The deduced amino acid sequences of the V_L and V_H CDRs are shown in single letter code. Amino acid residues that differ are highlighted in red. The dash symbol ('-') indicates no amino acid at that position.

4.2.2.15 HPLC-size exclusion chromatography.

The AE8 clone was purified by immobilised metal affinity chromatography (IMAC) (Section 2.2.22). Analysis of the IMAC-purified AE8 scFv by SDS-polyacrylamide gel electrophoresis revealed a number of contaminating protein bands (data not shown). Therefore, further purification of the scFv from IMAC-eluted material was performed using size exclusion chromatography (SEC). The initial IMAC cleanup step yielded a heterogeneous mixture of proteins. According to molecular weight estimates and SPR analysis (data not shown) of the HPLC fractions, the scFv dimer peak was observed at 16.6 minutes and a smaller monomeric peak eluted at 17.7 minutes (Figure 4.2.2.15).

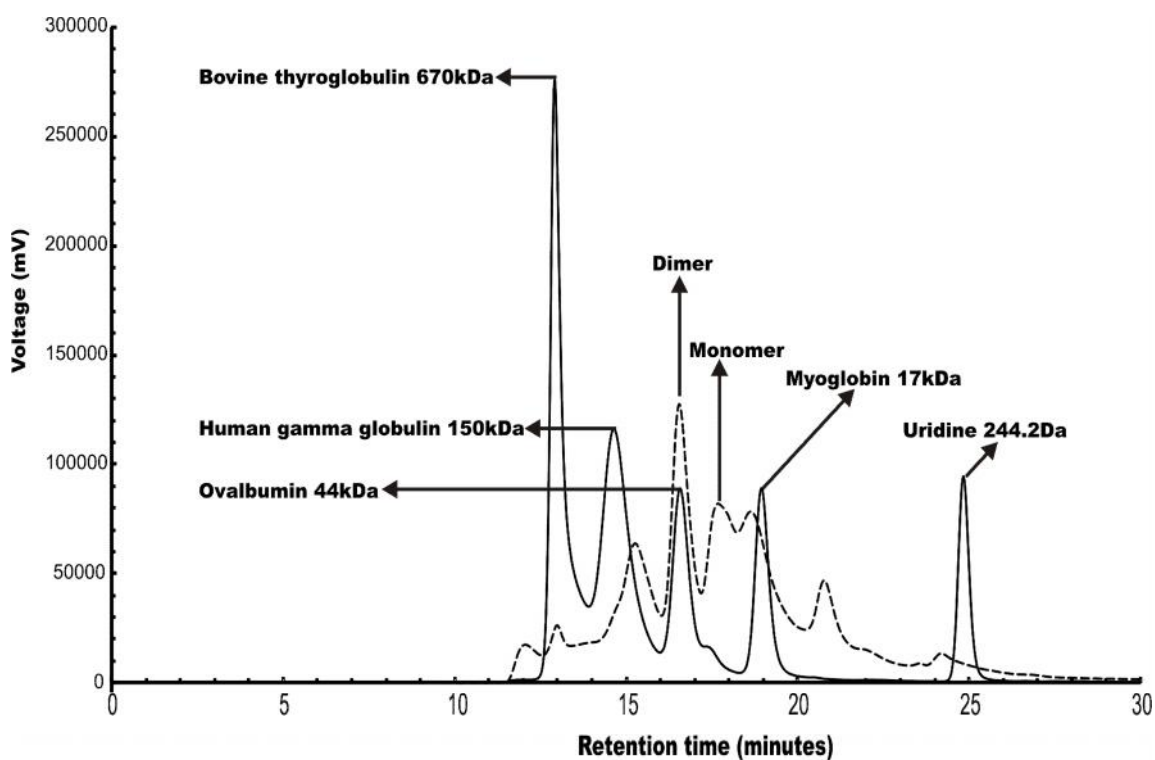


Figure 4.2.2.15 SEC-HPLC analysis of IMAC-purified AE8 scFv. The protein standards are represented by the black solid line. A neat sample of IMAC-purified AE8 (illustrated by the black dotted line) in PBS (pH 7.2) was selected for analysis. A Phenomenex 3000 SEC column was used with PBS (pH 7.2) mobile phase at a flow rate of 0.5mL/min and monitored by UV absorbance at 280nm.

4.2.2.16 Further HPLC analysis of the IMAC-HPLC-purified AE8 scFv fractions.

The composition of the previously purified HPLC scFv fractions was re-assessed by analysis with the same column and mobile phase (Figure 4.2.2.16). Fraction 4 contained a ‘clean’ dimer peak, whereas fraction 7 contained a small dimer but large monomeric peak. Subsequently, a neat sample of the AE8 scFv was purified by HPLC. In order to eliminate the scFv dimer and to capture only the monomeric species, fractions were collected starting from 17.3min up to 19.0min. As only a limited amount of protein was obtained, no further verification analysis by HPLC could be performed. The HPLC-purified monomeric AE8 sample was subsequently used for SPR kinetic analysis.

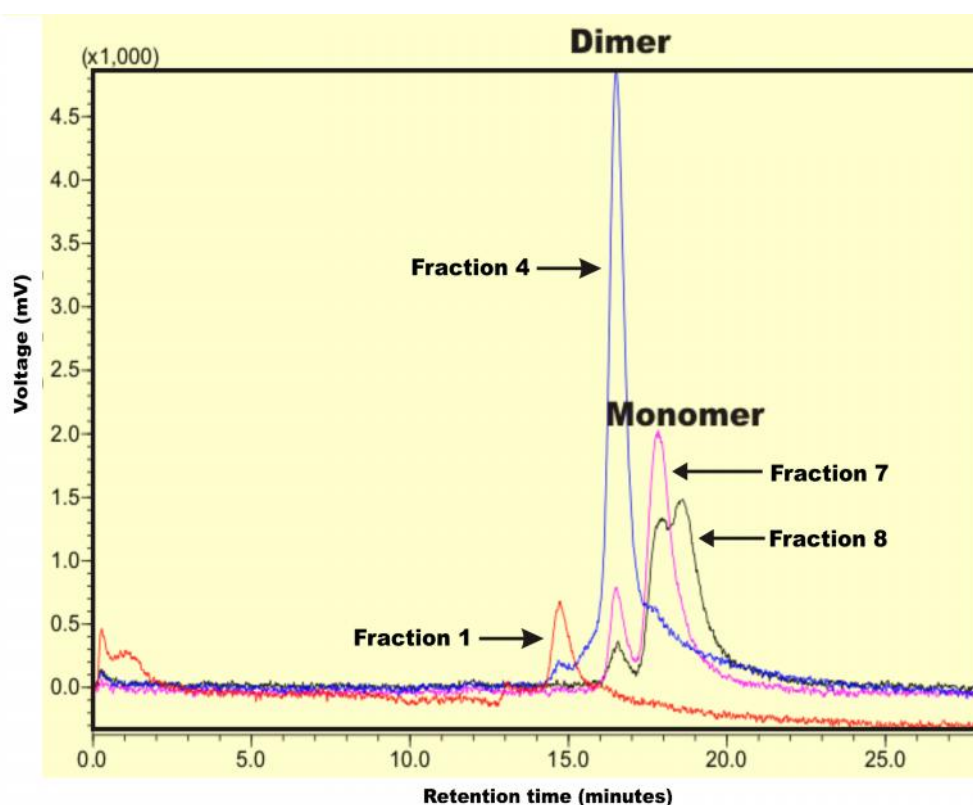


Figure 4.2.2.16 SEC-HPLC analysis of HPLC-purified AE8 fractions.

A total of 15 fractions were collected from the previous HPLC purification of the AE8 clone. Each fraction was analysed by HPLC using a Phenomenex 3000 SEC column and a mobile phase of PBS (pH 7.2) with a flow rate of 0.5mL/min. UV absorbance was monitored at 280nm. Fraction 4 contained a single symmetrical scFv dimer peak, whereas fraction 7 contained mostly monomeric scFv and only a small amount of dimeric scFv. In order to eliminate the scFv dimer and to only capture the monomeric species, fractions were collected starting from 17.3min up to 19.0min.

4.2.2.17 Fast protein liquid chromatography analysis of scFv clone AE8.

Fast protein liquid chromatography (FPLC) was used to estimate the molecular weight of a neat sample of IMAC purified AE8 protein. The ÄKTA™ Explorer 100 system (GE Healthcare, USA) was used for the analysis. Two major peaks were seen. The first peak (dimer) had an apparent molecular weight of approximately 60kDa and the second peak (monomer) had a molecular weight of 28kDa (Figure 4.2.2.17).

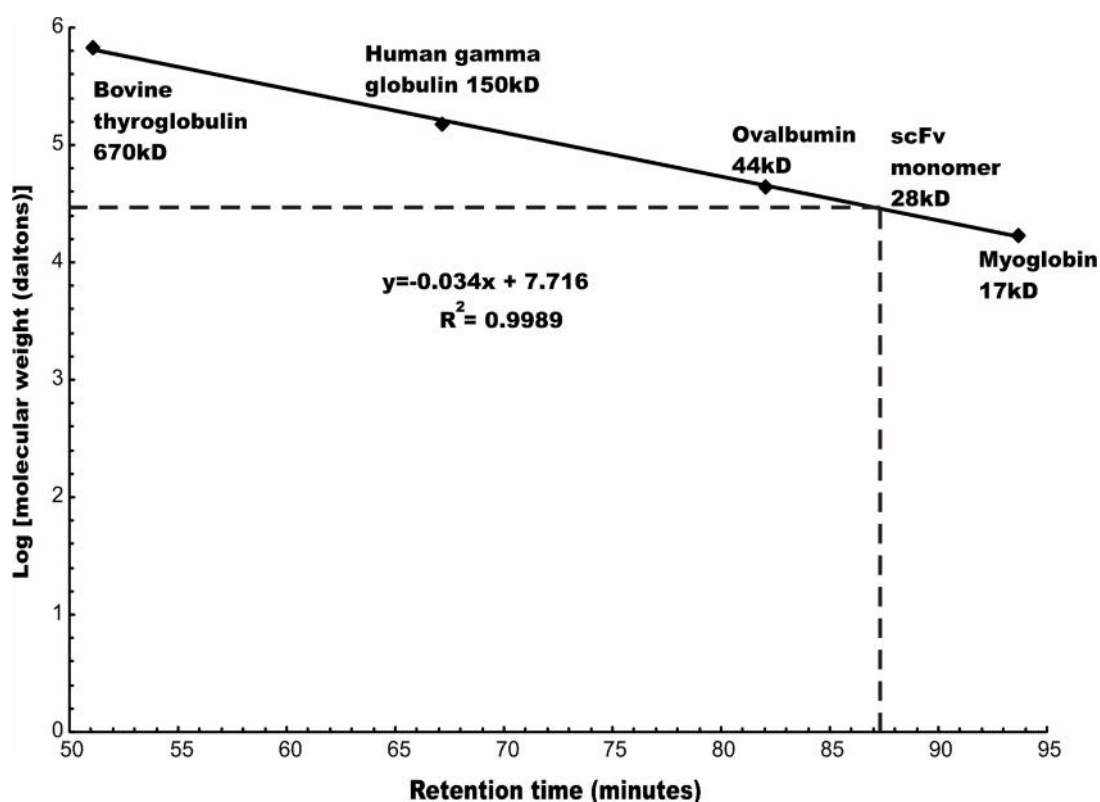


Figure 4.2.2.17 FPLC analysis of the IMAC-purified anti-sialic acid scFv (AE8). 100µL of the AE8 sample was applied to a HiLoad™ 16/60 Superdex™ 200 Prep-grade FPLC column using filtered and degassed PBS (pH 7.4) at a flow rate of 1mL/min. The retention time of the calibration standards indicated are bovine thyroglobulin, 670kDa, 51.09min; human gamma globulin, 150kDa, 67.15min; ovalbumin, 44kDa, 82.03min and myoglobin, 17kDa, 93.65min. The coefficient of determination for the standard curve ($y = -0.034x + 7.716$) was $R^2 = 0.9989$. The retention times of the monomeric and dimeric AE8 scFv fractions were 87.36 min and 78.51 min, respectively. The estimated molecular mass for the monomeric and dimeric scFv fractions were 28kDa and 60kDa, respectively.

4.2.2.18 Surface plasmon resonance analysis of the AE8 clone using the Biacore® 3000 biosensor.

Analysis of the binding and kinetic properties of the AE8 clone was performed using the Biacore® 3000 biosensor which monitors ‘label-free’ protein-antigen interactions in ‘real-time’ using the phenomenon of surface plasmon resonance (SPR). SPR is widely used for kinetic studies of biomolecular interactions, including carbohydrate–protein interactions. The basic assay format for AE8 SPR analysis was as follows: neutravidin was immobilised on the dextran surface of a Biacore® CM5 chip, biotinylated PAA-Neu5Gc conjugate was subsequently passed over and captured by neutravidin, after which the scFv was passed over the surface to check for binding to the sugar. As a negative control the scFv was also passed over a neutravidin surface which had no biotinylated sugar. Initially, a pre-concentration study was performed and a 10mM sodium acetate buffer at pH 4.6 was selected for immobilisation of neutravidin on the biosensor chip surface (Figure 4.2.2.18).

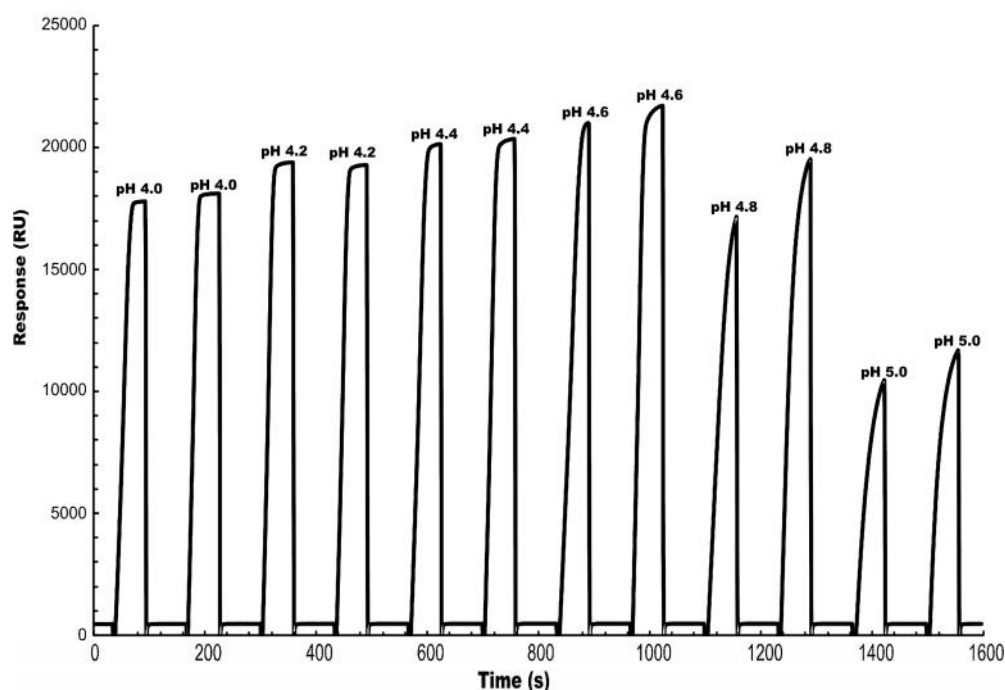


Figure 4.2.2.18 *Neutravidin pre-concentration analysis.* 50µg/mL solutions of neutravidin were prepared in 10mM sodium acetate buffers and adjusted with 10% (v/v) acetic acid to pH values 4.0, 4.2, 4.4, 4.6, 4.8, and 5.0. A 10mM sodium acetate buffer at pH 4.6 was chosen as the immobilisation buffer for neutravidin on the CM5 biosensor chip surface.

4.2.2.19 Neutravidin immobilisation on a CM5 dextran chip.

Although it was possible to use the ELISA format for sialic acid analysis, the SPR biosensor was chosen, since this allowed for the real-time detection and monitoring of binding between immobilised and free sialic acid conjugates and the soluble anti-sialic acid scFv. In addition, the biosensor only requires small amounts of sample and is far less time-consuming than ELISA (De Crescenzo *et al.*, 2008). Standard amine coupling chemistry was used to covalently attach the amine groups of neutravidin to the carboxyl groups of the dextran CM5 biosensor chip. Figure 4.2.2.19, shows the successful immobilisation of neutravidin on the surface of the biosensor chip.

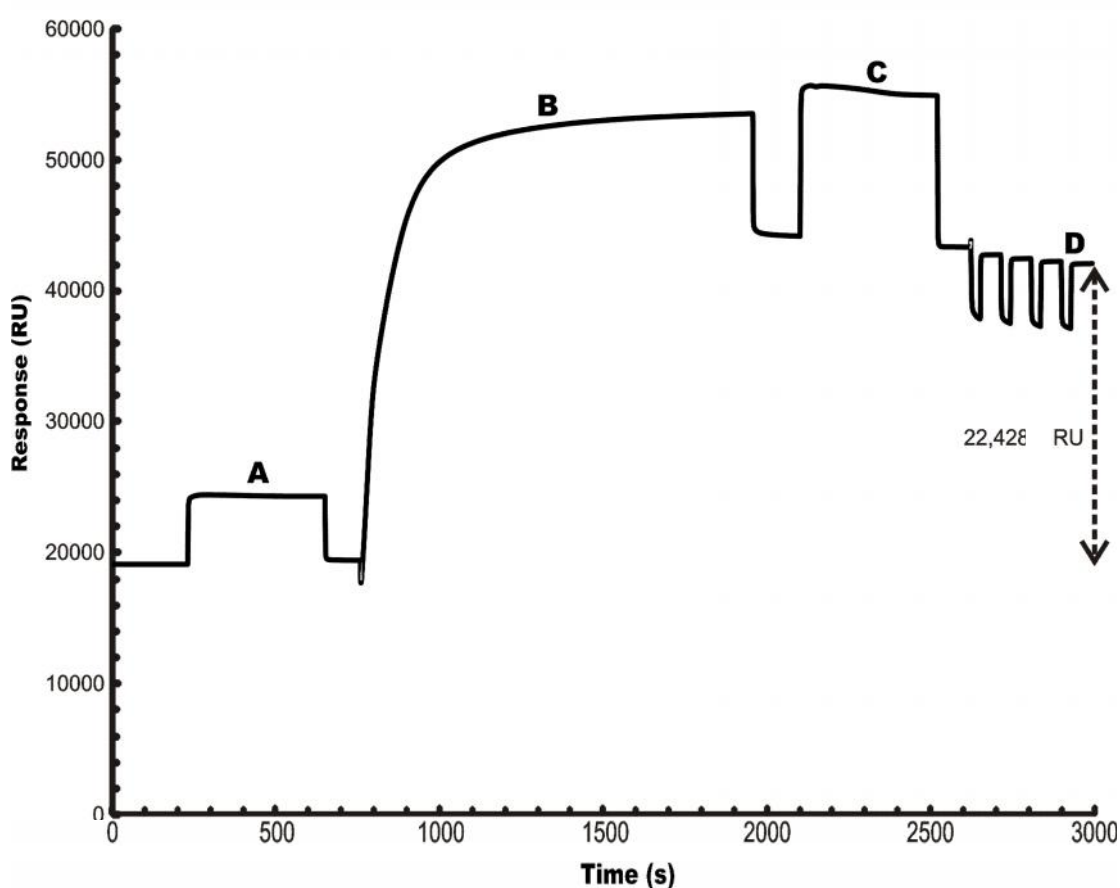


Figure 4.2.2.19 Immobilisation of 50 μ g/mL neutravidin onto the CM5 sensor chip surface. (A) EDC/NHS activation, (B) binding of neutravidin, (C) capping of unreacted groups and (D), regeneration pulses of 5mM NaOH. A final level of 22,428 RU of covalently attached neutravidin was achieved.

4.2.2.20 Capture of biotinylated-Neu5Gc-PAA on a neutravidin CM5 chip.

Neutravidin is a tetramer and forms a very stable complex with biotin, which was therefore used to capture the biotinylated-PAA-Neu5Gc conjugate. Due to the very low dissociation constant associated with the neutravidin/biotin interaction, the biotinylated conjugate is practically irreversibly bound to the sensor chip surface. This format had a number of distinct advantages. For example, all biotinylated molecules were in the same orientation and a high density of Neu5Gc was captured on the neutravidin surface. In addition, the biotinylated Neu5Gc sensor chip could better withstand harsh regeneration conditions. Furthermore, this chip could be stored for long periods of time with no significant loss in anti-sialic acid scFv binding capacity. The successful neutravidin capture of biotinylated-PAA-Neu5Gc is shown in Figure 4.2.2.20.

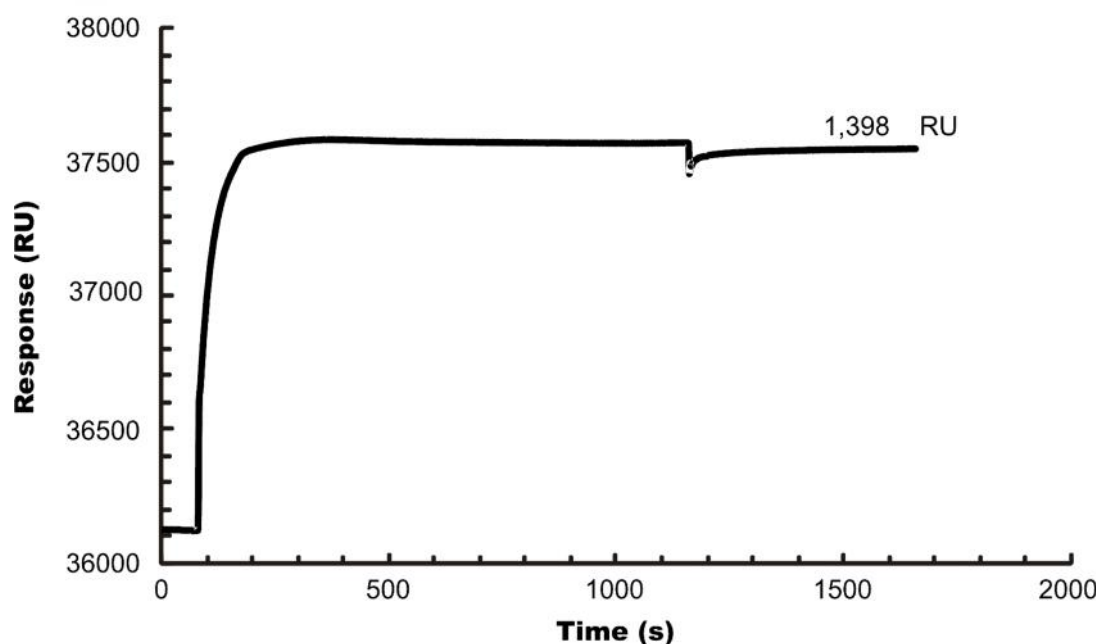


Figure 4.2.2.20 *The response profile of biotinylated-PAA-Neu5Gc binding to a CM5 chip with immobilised neutravidin. A 100 μ g/mL solution of biotinylated-Neu5Gc-PAA in HBS was passed over the chip surface at 10 μ L/min for 20 minutes. A final level of 1,398.8RU of captured biotinylated-PAA-Neu5Gc was achieved.*

4.2.2.21 Analysis of the AE8 clone with the Neu5Gc sensor chip.

After the capture of the biotinylated-PAA-Neu5Gc conjugate, a solution of the IMAC-purified AE8 scFv was passed over the Neu5Gc sensor chip. Figure 4.2.2.21 shows that the AE8 scFv recognised Neu5Gc in the context of the PAA backbone.

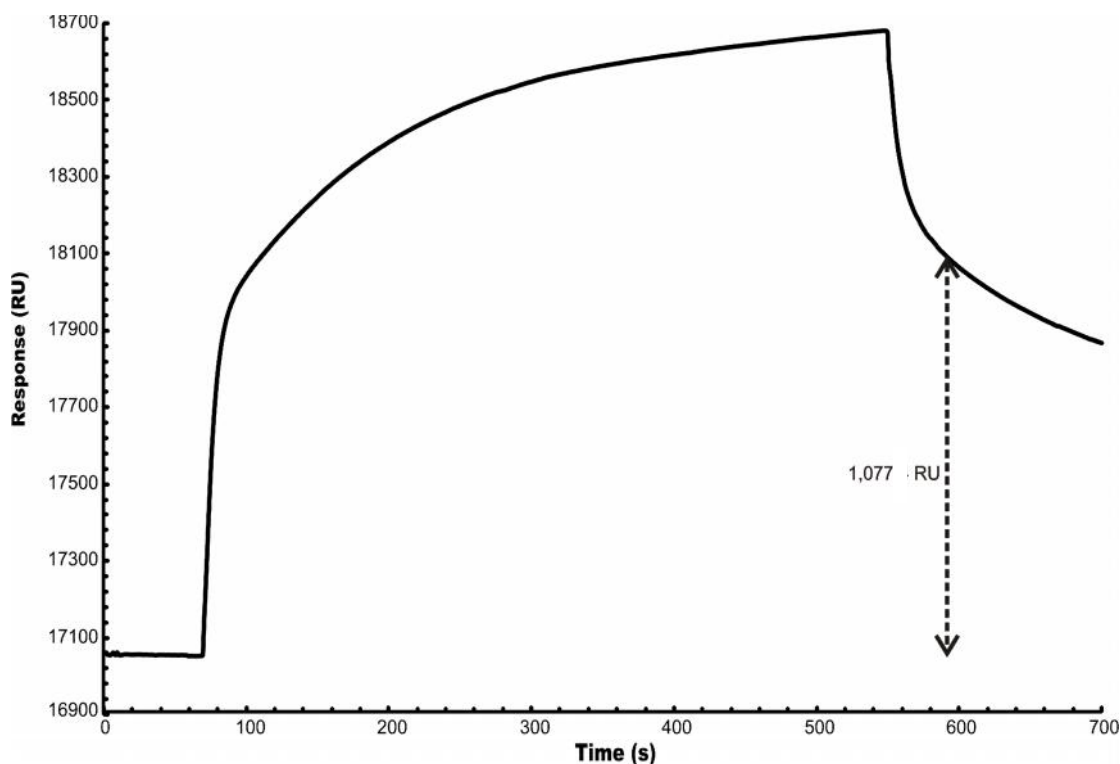


Figure 4.2.2.21 A reference-subtracted sensorgram depicting the binding of the AE8 recombinant anti-sialic scFv to Neu5Gc. The AE8 protein was purified by IMAC and a 1 in 100 dilution in HBS was passed over flow cells 1 and 2 of the Neu5Gc sensor chip at 10 μ L/min for 7 minutes. The net binding response achieved by AE8 was 1,077 RU. The reference subtracted control surface (flow cell 1) consisted of immobilised neutravidin with no biotinylated Neu5Gc-PAA.

4.2.2.22 Solution-phase Neu5Gc-binding assay.

To assess the ability of the anti-sialic acid AE8 scFv to bind the Neu5Gc conjugate in solution-phase, an inhibition binding assay was performed. In this assay, the AE8 scFv was prevented (inhibited) from binding to the immobilised PAA-Neu5Gc by the addition of variable amounts of solution-phase Neu5Gc-BSA conjugate. This experiment demonstrated that the AE8 scFv could bind to Neu5Gc independent of its carrier molecule, since it recognised Neu5Gc, in the context of two very different carriers, with structurally unrelated linkers, namely BSA-linker1-Neu5Gc and biotin-PAA-linker2-Neu5Gc. Furthermore, SPR analysis revealed that the AE8 recombinant scFv had an IC₅₀ concentration of 5.7ng/mL for the Neu5Gc-BSA conjugate. In addition, the AE8 scFv was shown to bind Neu5Gc conjugates when they were either captured on a solid phase or when they were free in solution (Figure 4.2.2.22).

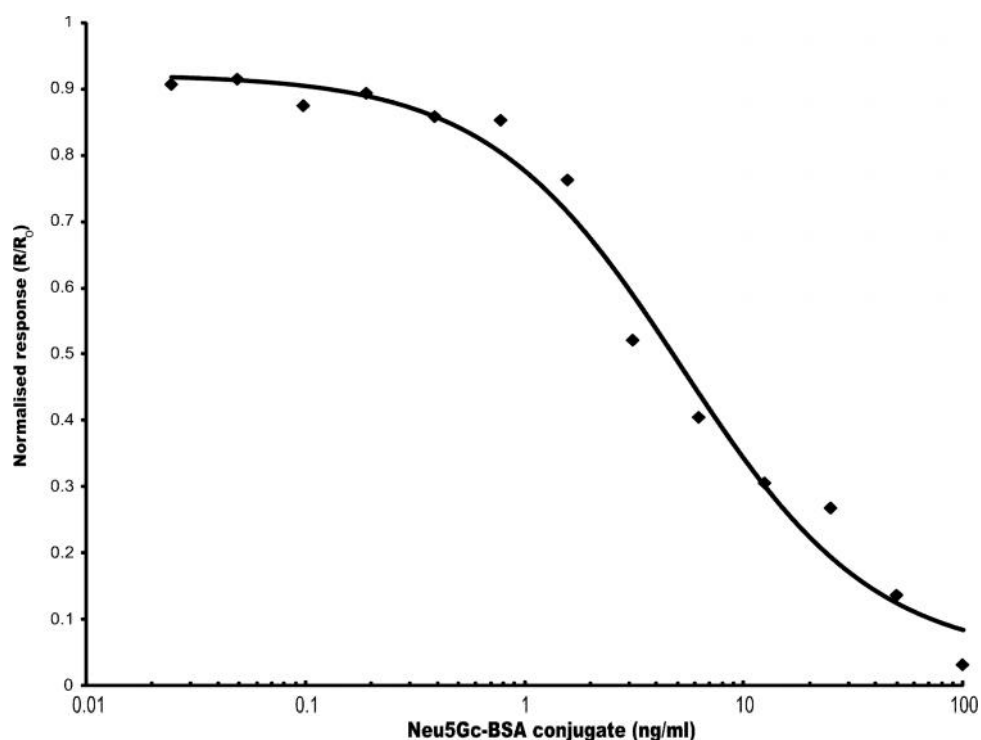


Figure 4.2.2.22 An SPR inhibition assay curve of the anti-sialic scFv (AE8), binding Neu5Gc-BSA in solution. The R/R_0 was calculated by dividing the RU response obtained at 13 different Neu5Gc-BSA conjugate concentrations (100ng/mL to 0.02ng/mL) by the RU response obtained from the AE8 sample with no Neu5Gc-BSA conjugate. A 4-parameter equation $[F(x) = ((A-D) / (1 + ((x/C)^B))) + D]$ was fitted

to the data set using BIAevaluation 4.1 software. Each point in the curve is the mean of three replicate measurements.

4.2.2.23 SPR kinetic studies on the AE8 clone.

SPR was used to determine the association (k_a) and dissociation (k_d) rate constants of the anti-sialic acid scFv. To rule out the contribution of avidity in the determination of the rate constants, only the monomeric HPLC-purified fraction of AE8 was used for Biacore[®] kinetic analysis. The k_a and k_d values were $1.19 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$ and $6.80 \times 10^{-3} \text{ s}^{-1}$ respectively. Thus, the calculated K_D value for the AE8 scFv/Neu5Gc interaction was 57nM (Figure 4.2.2.23). The AE8 scFv has a high-affinity for sialic acid. Moreover, the generation of such a highly specific anti-sialic acid antibody in a recombinant format is exceedingly rare (Kaltgrad *et al.*, 2007).

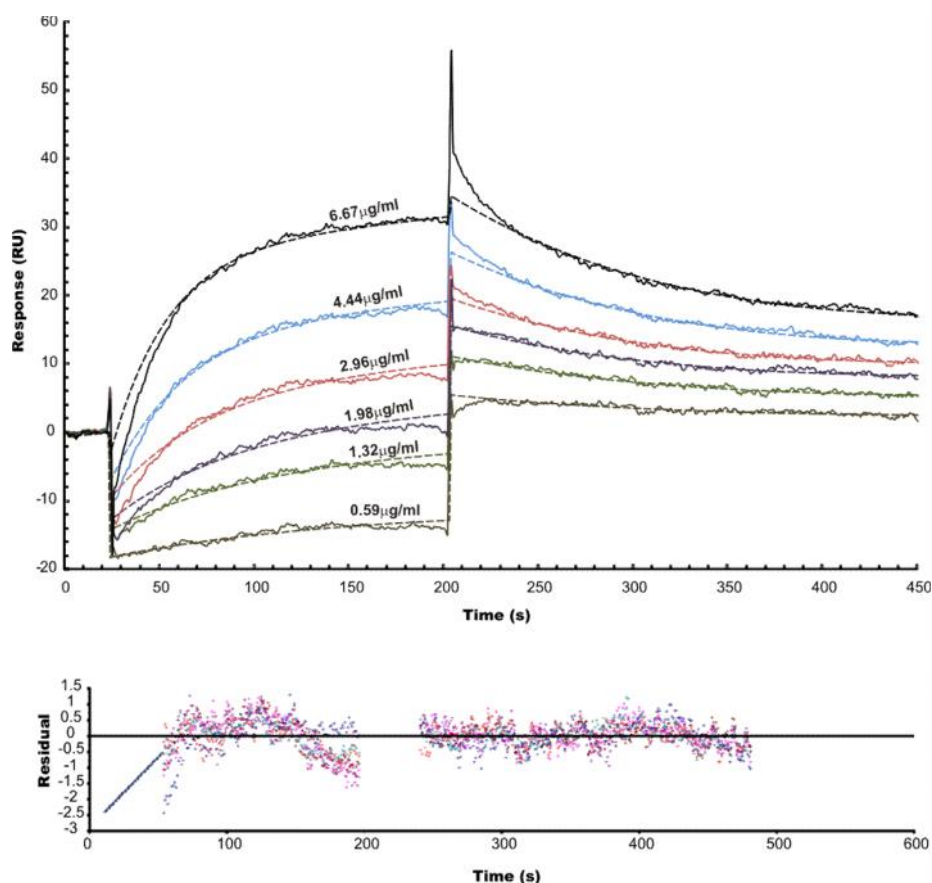


Figure 4.2.2.23 Curve fitting of monomeric AE8 scFv with BIAevaluation 4.1 using a 1:1 binding model. The fitted curves for each monomeric scFv concentration are represented by the dashed line, whereas the solid lines represent the actual RU change for each sample. The apparent K_D was estimated to be $57 \times 10^{-9} \text{ M}$. The residual plot is a measure of the ‘goodness of fit’.

4.3 Discussion

In this chapter, the rationale behind the design of the sialic acid immunisation and screening conjugates is discussed. In addition, the strategy employed for generation and characterisation of recombinant antibody fragment libraries, from a chicken, immunised with a novel neoglycoconjugate, Neu5Gc-HSA is also discussed. Furthermore, this chapter describes the selection of positively-binding sialic acid clones from the generated avian phage libraries. In addition, FPLC, HPLC, ELISA and SPR analysis of the AE8 anti-sialic acid recombinant antibody is described in detail.

4.3.1 Recombinant antibody generation

As mentioned in section 4.2.1 the preliminary work on the generation of anti-sialic acid antibody fragments utilised a number of different strategies. The first approach consisted of immunisation of an avian host with a mixture of neutravidin and a Neu5Gc multivalent biotinylated polyacrylamide polymer. Following the first immunisation, the chicken received a further 3 boosts with the neoglycoconjugate, HSA-Neu5Gc. ELISA analysis of the pre-immunisation and final boost chicken sera showed no significant increase in the polyclonal response (data not shown). Therefore, it was concluded that this particular cocktail of sialic acid could not generate anti-Sia antibodies. It has been reported in the literature that PAA is only weakly immunogenic (Vidal-Gomez and Gras 1978; Enders *et al.*, 2007; Diano *et al.*, 2002). Immune activation requires appropriate presentation and recognition of antigen (Schijns, 2002). Thus, it is possible that the PAA containing conjugate was incompletely processed by the chicken's circulating immune cells and therefore a significant anti-sialic acid immune response was not generated. However, the exact reasons for the failure of this 'home-made' conjugate to elicit a substantial immune response remain unclear.

A second approach focused on the use of synthetic libraries which are artificially built. These libraries (also termed single pot or universal libraries) were initially developed as an alternative to the use of animals for antibody production. Antibody diversity within synthetic libraries is achieved by the random *in vitro* assembly of V and D/J gene segments. Typically, PCR and randomised oligonucleotide primers are used to rearrange synthetic V_H and V_L gene segments (de Wildt *et al.*, 2000; Clackson and Lowman, 2007; An *et al.*, 2009). Most of the direct interaction with an antibody's

cognate antigen is with one or more of the six CDR loops. Therefore, synthetic libraries introduce diversity in the antibody-combining site by varying the length of any of these six CDR loops (Kumagai and Tsumoto, 2001). Unlike an immune B-cell derived library, synthetic libraries are not constrained by the forces of selection in the natural immune system and , therefore, may contain novel antibodies that recognise self antigens (e.g. carbohydrates) (Maynard and Georgiou, 2000). Thus, a key advantage of these libraries is the potential isolation of antibody fragments that bind to every possible antigen (Schirrmann *et al.*, 2011). However, in order to ensure a high probability that the required antibody fragments with the necessary affinity and specificity are isolated, these synthetic and semi-synthetic libraries need to be large in size (Barbas, 2001; Benatuil *et al.*, 2010). Typically, semi-synthetic libraries are built using un-rearranged V genes from pre-B-cells (germline cells) and one antibody framework region with genetically randomised CDR regions (Schirrmann *et al.*, 2011).

Therefore, a second strategy used to identify anti-sialic acid antibodies, was to screen the semi-synthetic Tomlinson I and J libraries. These combinatorial non-immune phage display libraries contain over 100 million different scFv fragments. They are artificially constructed by using PCR to randomly assemble the genes encoding the hypervariable regions from a naïve B-cell. By employing this process, a unique set of V_H and V_L genes are recombined, thereby increasing the likelihood of generating scFvs that can bind non-immunogenic or ‘self’ antigens like carbohydrates or haptens (Pansri, *et al.*, 2009; O’ Brien and Aitken, 2002).

However, even after four rounds of biopanning of the Tomlinson I and J libraries, no positive anti-sialic acid-binding was evident for either the polyclonal or monoclonal phage ELISA (data not shown). During successful biopanning experiments phage yields usually increase after each subsequent round of enrichment (O’ Brien and Aitken 2002; Barbas, 2001). However, no such enrichment of sialic acid-binding phage was observed. Therefore, these libraries do not appear suitable for the isolation of anti-sialic acid antibody fragments, when the screening conjugate is neutravidin-biotinylated-PAA-Neu5Gc. Theoretically, synthetic libraries should contain within them anti-carbohydrate antibodies of differing specificities and affinities.

Synthetic libraries are designed to encode structures with broad complementarity to many different antigens including those previously found to be relatively non-immunogenic (Schoonbroodt *et al.*, 2008; Deng *et al.*, 1995). However, any synthetic library will always have the disadvantage of containing a certain amount of non-functional clones. These clones arise from PCR errors, stop codons in the random sequence, or the inability to fold properly (Lee *et al.*, 2004; Sidhu, 2005). It is possible that non-functional mutant clones may have reduced the I and J library's diversity and thus impeded the isolation of phage harbouring anti-sialic acid scFvs. Moreover, affinity selection ultimately depends on the molecular interaction of the antibody-phage with the antigen within a vast background of interfering molecules. This interaction is critically dependent on the surface-coupled antigen and the biopanning conditions (pH, salt concentrations, competitors, antigen concentrations, phage inoculum, blocking reagents, washing steps, etc) (Sidhu, 2005; O' Brien and Aitken, 2002; Barbas, 2001). Any deleterious combination of these factors may have prevented the capture and subsequent enrichment of sialic acid-binding phage. Alternatively, these libraries may have had no pre-existing combination of V_L and V_H pairings that recognise Neu5Gc in the context of its polyacrylamide backbone.

Unlike protein targets, there are far fewer studies published that have demonstrated the successful isolation of anti-carbohydrate antibody fragments. Moreover, only a small subset of these studies has produced antibody fragments that bind sialylated antigens. Therefore, the generation and subsequent isolation of anti-sialic antibodies is a formidable technical challenge. Phage display methods developed to select antibodies specific for carbohydrates moieties have used a plethora of different biopanning protocols, screening antigens and library types. Specific examples include: Mao *et al.*, (1999) who constructed a naïve phage library from the peripheral blood leukocytes (PBL) of cancer patients and isolated scFvs that bound the sialyl Lewis^x {sLe^x, Neu5Aca(2,3)Galβ(1,4)[Fuca(1,3)]GlcNAcβ-R} tumour antigen. Rojas *et al.*, (2004) generated a light chain-shuffled scFv library from a murine monoclonal antibody (14F7) that had high specificity for GM3(Neu5Gc) ganglioside. The random combination of murine and human light chain variable regions with a single heavy chain variable region (14F7) produced functional scFv variants that had greater affinity for the Neu5Gc containing ganglioside.

Schoonbroodt *et al.*, (2008) constructed a semi-synthetic human antibody Fab phage library by varying the length and composition of anti-carbohydrate CDRH3 (≤ 6 -amino acids) binding consensus sequences. Antibody fragments were isolated against sulphated and non-sulphated sLe^x antigens. A common feature of these and other studies is the use of neoglycoproteins or naturally occurring glycoproteins that contain linear or branched chains of monosaccharide units. In contrast, the Tomlinson I and J libraries were screened with a conjugate that displayed single sialic acid moieties on the surface of an artificial polyacrylamide backbone. It was reported that positively charged residues within the antigen-binding site are crucial in stabilising the negative charge of the bound sugar molecule (Schoonbroodt *et al.*, 2008). Thus, it is possible that the presentation of single sialic acid moieties was not sufficient to allow adequate interaction with charged residues in the scFv binding pocket.

A third approach for the isolation of anti-sialic antibody fragments was to biopan an 'in-house' immune library. This avian library was originally constructed for the isolation of anti-MPO scFv fragments. MPO is a glycoprotein with a carbohydrate content of 3% - 5%. It is an enzyme that has antimicrobial functions and is synthesised by neutrophil and monocyte precursor cells. MPO contains 4 *N*-linked glycosylation sites, substituted with either high-mannose chains, or oligosaccharide chains that terminate with sialic acid $\alpha(2,3)$ linked to galactose (Hansson *et al.*, 2006; Yamada *et al.*, 1987). Thus, it was postulated that avian immunisation with the MPO sialoglycoprotein may have produced an immune response against sialic acid epitopes. Previous animal studies have shown that immunisations with glycoproteins can generate an immune response that results in the production of antibodies that target carbohydrate epitopes. For example, Noguchi *et al.*, (1995) reported the generation of avian polyclonal antisera against glycoproteins that contained sialic acid. These anti-carbohydrate pAbs were raised in chickens by immunisation with bovine fetuin and GM3(Neu5Gc) ganglioside, whereas Tangvoranuntakul *et al.*, (2003) also used GM3(Neu5Gc) ganglioside as an avian immunogen and isolated the first monospecific polyclonal anti-Neu5Gc chicken IgY antibody. Diaz *et al.*, (2009) improved the specificity of the anti-Neu5Gc pAb preparation by alteration of a two-step affinity purification process. Subsequent analysis of this pAb against a variety of different sialoglycoproteins revealed little or no cross-reactivity to Neu5Ac epitopes.

Dinh *et al.*, (1996) generated high affinity murine antibodies against Lewis antigens (Le^x and sLe^x) using the ganglioside immunogen (GM1) and biopanning with a Le^x-BSA neoglycoprotein. Ravn *et al.*, (2004) isolated recombinant antibodies to the Thomsen–Friedenreich tumour carbohydrate antigen (TF, Gal β 1-3GalNAc α 1-O-Ser/Thr) by immunisation of mice with TF-carrying asialoglycophorin. Biopanning was performed on immunotubes coated with asialoglycophorin and soluble TF α -PAA-biotin streptavidin-conjugated magnetic beads.

The MPO scFv library was subjected to four rounds of biopanning against a biotinylated-Neu5Gc-PAA conjugate that was immobilised on neutravidin-coated immunotubes. However, no positive signals were seen in either the polyclonal or monoclonal phage ELISA. It therefore appears that this library is not suitable for the isolation of anti-sialic acid antibody fragments when the screening conjugate is Neu5Gc-PAA. It is plausible that the low immunogenicity of carbohydrates, which are ‘self-antigens’, caused no immune response to be mounted against the sialic acid epitopes of MPO. Currently, several theories have been proposed to explain the mechanisms of immunological tolerance. These include: clonal anergy, clonal deletion, receptor editing, idiotype network or clonal ignorance (Iglesias, 2001; Parmentier *et al.*, 2004; Sioud, 2007). Any or all of these immune tolerance mechanisms may have blocked the action of carbohydrate reactive B-cell or T-cell clones. This would have led to the suppression of primary and secondary antibody responses to sialic acid and thus prevented the isolation of anti-sialic acid antibodies.

Another explanation may relate to library building. During this process, anti-sialic acid cDNA encoding heavy and light chain variable regions may not have been captured or amplified. There is a considerable amount of time and a multitude of steps required for recombinant antibody generation and errors at any stage of the process (animal immunisations, library construction and biopanning) may have negatively impacted on the isolation of anti-sialic acid clones. Furthermore, ensuring the quality and specificity of antibody-phage antigen interaction is key to successful biopanning. It is possible that during library screening, poor target-driven selection resulted in the successive amplification of background binding phage. This phenomenon arises when non-target binding phage have enhanced growth, infection and elution properties.

Consequently, clones with superior antigen-binding properties are lost and do not predominate in later rounds of selection (Menendez and Scott, 2005). In such a scenario, non-target, unreactive phage clones would have propagated and produced low polyclonal and monoclonal phage ELISA signals.

4.3.2 Conjugation and Immunisation

The quality of antigen used for immunisation is critical for successful antibody generation (Leenaars and Hendriksen, 2005). A major difficulty with carbohydrate immunogens is that they are not readily available and their isolation, purification and identification, is difficult, low yielding, tedious and mostly impractical (Seeberger and Werz, 2005). Furthermore, many carbohydrate antigens tend to be weakly immunogenic and typically invoke a T-cell-independent, short-lived humoral response, with little or no immunological memory. Consequently, even after repeated carbohydrate immunisations, only a few B-cell clones are activated. This produces a low titre and low affinity polyclonal IgM population. Furthermore, these heterogeneous IgM antibodies are characterised by poor antibody affinity maturation. Finally, the absence of T-cell stimulation of B-cells results in a failure to class switch to high affinity IgG antibodies (Ravindranath and Donald, 2001; Buskas *et al.*, 2004). Currently, a major challenge in carbohydrate vaccine development is the ability to tailor the selection and synthesis of carbohydrate immunogens, so that they direct the immune system to produce anti-carbohydrate antibodies of sufficient affinity and specificity. However, there are still many fundamental issues within this field that need to be resolved. For example, it is still not understood how carbohydrate antigens are processed and presented by the immune system. Consequently, designing carbohydrate immunogens with the proper epitopic binding sites for both B-cell and T-cell recognition is one of the most challenging research areas in immunology today (Rudd *et al.*, 2001; Roy, 2004; Buskas *et al.*, 2004; Ghazarian *et al.*, 2011)

Therefore, in this work a considerable amount of time was used to design and source sialic acid immunisation and screening conjugates. Importantly, the sialo-immunogen was systematically designed to elicit not only an IgM primary immune response, but also a T-cell-dependent response. It was reasoned that this would lead to the generation of anti-sialic acid antibodies with high affinity and specificity.

A commercial company that specialised in carbohydrate protein conjugation (Carbohydrate Synthesis, U.K.) chemically coupled Neu5Gc monosaccharides to BSA/HSA. For a variety of reasons, BSA was chosen as the screening neoglycoprotein. BSA is the most commonly used carrier protein and is widely available in a relatively pure form. Moreover, it is inexpensive, well characterised and has 59 lysine ϵ -amino groups of which 30–35 are typically available for derivatisation. Furthermore, it is not glycosylated and does not suffer from the solubility issues of keyhole limpet haemocyanin (KLH), which makes conjugate isolation and characterisation easier. BSA is also universally used in ELISA as a blocking buffer. (Law, 1996; Hermanson, 2008; Singh *et al.*, 2004). Thus, using a Neu5Gc-BSA conjugate for screening rather than for immunisations minimises non-specific BSA background binding.

Keyhole limpet haemocyanin (KLH), like BSA, is also widely used in the preparation of hapten-protein conjugates. However, for the reasons outlined below, KLH was not used as the Neu5Gc immunisation-protein carrier. KLH is a high mannose-type glycosylated protein with 4% carbohydrate content (containing: mannose, galactose, fucose, GlcNAc and GalNAc) (Harris and Markl, 2000; Geyer *et al.*, 2004; Ragupathi *et al.*, 2003). Therefore, it was possible that a sialic acid KLH conjugate could have generated unrelated endogenous carrier-specific anti-carbohydrate antibodies, and thus overly complicated cross-reactivity studies of putative anti-sialic acid clones. Other potentially problematic issues associated with KLH, include the inability to determine its exact molecular weight ($4.5 \times 10^5 - 1.3 \times 10^7$) (Lemus and Karol, 2008). This makes it difficult to calculate the molar ratio of hapten to carrier in KLH conjugates. Furthermore, even though animal immunisations with KLH can elicit strong B-cell responses, the resulting production of high titres of anti-KLH antibodies, may well suppress the formation of IgG antibodies against hapten epitopes. Herzenberg *et al.*, (1980) demonstrated such an effect: when immunising mice with KLH and KLH-dinitrophenyl, they observed KLH-induced suppression of the anti-dinitrophenyl response.

It was postulated that since HSA is a relatively weak antigen, immunisation with Neu5Gc-HSA rather than Neu5Gc-KLH, may generate a more targeted immune response to Neu5Gc, since less carrier-specific antibodies would be produced.

Indeed, the use of HSA as an immunogenic, carbohydrate-carrier protein has been described in several other studies. For example, the study of Pozsgay *et al.*, (1999) on *Shigella dysenteriae* type 1 showed that the immunogenicity of HSA neoglycoconjugates was dependent on both saccharide chain length and density. Conjugates that contained the longest carbohydrate chains with high carbohydrate loadings induced the highest IgG levels in mice. Alexander *et al.*, (2000) generated anti-fucopentose murine pAbs using fucopentose conjugated to HSA, whereas, Li *et al.*, (2010) investigated the effect of hapten density by immunising rabbits with low and high density HSA-Tn (GalNAc α 1-O-Ser/Thr) conjugates. They demonstrated that modulation of hapten density may be a simple way to control the selectivity and breadth of the induced antibody repertoire. HSA does not normally contain covalently attached sugars, although HSA glycation (non-enzymatic attachment of glucose) may occur (Wa *et al.*, 2007; Bunk, 1997). HSA was, therefore, chosen as the Neu5Gc immunisation protein carrier.

A further consideration in the rational design of the sialic acid immunisation and screening conjugates was the selection of an appropriate cross-linker bridge, to covalently attach the carbohydrate-hapten to the carrier protein. A number of different synthetic linkers (spacer 'arms') and conjugation chemistries are available. However, not all combinations will lead to the successful production of antibodies with the correct specificity. Interestingly, it has been suggested that the influence of the linker on immunogenicity may highly depend on the nature and intrinsic immunogenicity of the carbohydrate hapten. Moreover, the chemical makeup of the linker used to attach the saccharide to the carrier protein may have a deleterious effect on anti-carbohydrate antibody generation (Phalipon *et al.*, 2009). A number of studies have shown that certain synthetic linkers, when immunised into animals, generate substantial immune responses. This can be detrimental, as the linker response may suppress the carbohydrate hapten immune response. For example, research on the tumour-associated carbohydrate antigen, Lewis^y {Le^y, Fuca(1,2)Gal α (1,4)[Fuca(1,3)]GlcNAc β -R} has shown that the widely used cyclic maleimide linker is highly immunogenic and generates anti-linker antibodies that may well suppress formation of IgG antibodies against the Le^y epitope (Buskas *et al.*, 2004).

In addition, Ni *et al.*, (2006) investigated a novel carbohydrate vaccine for HIV-1 and discovered that the majority of IgG antibodies raised by oligomannose-KLH glycoconjugates targeted the maleimide-functionalised linker rather than the carbohydrate antigens. Furthermore, Boeckler *et al.*, (1996) examined linkers with different thiol-reactive moieties and showed that linkers containing maleimide moieties evoked a significant anti-linker immune response. In contrast, Phalipon *et al.*, (2008) showed that murine immunisation of a pentadecasaccharide, coupled to tetanus toxoid, using a linear maleimide linker, did not produce detectable anti-linker antibodies. In the current work, maleimide-functionalised linkers were not used to prepare the sialic acid immunisation and screening conjugates.

The length and placement of the linker may have a profound effect on the formation of specific and high-affinity antibodies and therefore, was also a key consideration in the design of the sialoconjugates. For example, a small molecule that is covalently tethered to a carrier protein may suffer a considerable amount of masking in an area of the hapten that is close to the site of linkage (Kim *et al.*, 2003b). Thus, it was important to ensure that the sialic acid moiety was at a distance from the carrier surface, to ensure access during the immune response. Typically, this is achieved by extending the hapten out in space via a linker/spacer arm. Linkers of many different lengths (short, medium and long) have been used during the last decades to produce high-affinity antibodies to a wide range of different targets. Some researchers have suggested that the optimal spacer arm is medium in length and contains 3 or 4–6 atoms (Kim *et al.*, 2003b; Sakensa *et al.*, 2006; Lei *et al.*, 2010). In contrast, a spacer arm that is too long may cause the hapten to fold back towards the protein surface. This could cause shielding of the hapten determinant groups and lead to a reduction in the exposure of the hapten to the immune system. However, solid experimental data supportive of these hypotheses are limited (Kim *et al.*, 2003b). Saksena *et al.* (2006) has reported that mice immunised with hexasaccharide neoglycoconjugates that contained only short (2 atoms) or medium (4 atoms) linkers, produced *Vibrio cholerae* specific, anti-lipopolysaccharide antibodies. In contrast, Lee *et al.* (2002) used long hydrophilic linkers (10-12 atoms) to attach synthetic GM3 trisaccharide haptens to the surface of BSA. These conjugates were used to biopan a scFv-phage library derived from the blood samples of human subjects diagnosed with a variety of cancers.

The authors isolated a fully human scFv that was specific for native GM3 on the surface of tumour cells. These and other studies show there are no set rules that define the optimal linker length for all types of conjugates.

In this work, sialic acid was attached to a protein carrier via a long 13 atom carbohydrate-hapten-spacer. It was postulated that the use of a long linker, may increase the relative visibility of the exposed Neu5Gc epitope to the host's immune system. This could potentially generate a more direct and specific immune response to the carbohydrate epitope. Furthermore, the use of a long flexible spacer may reduce steric hindrance and allow for greater interaction with the antibody-binding site. Such improved access to the sialic acid structure may also lead to the generation of antibodies with increased affinity. The location and type of sialic acid functional groups, for linker attachment, was also a key consideration in the design of the sialoglycoconjugates. During the conjugation process, it is vital that the structural and chemical integrity of the hapten is maintained. In addition, modification of hapten functional groups, which may have destabilising effects on the antigen-antibody immunocomplex, should be avoided (Hermanson *et al.*, 2008; Lemus and Karol, 2008). Although sialic acid is a small molecule it contains many functional groups that could serve as sites for covalent attachment of a spacer arm. However, the placement of a linker is limited, since the majority of these functional groups may be essential for Sia-protein binding. For example, the anionic carboxyl moiety located at the C1-position of the carbohydrate pyranose ring, is a key functional group that imparts a net negative charge and is also responsible for the acidic nature of sialic acid (Angata and Varki, 2002). Moreover, studies have shown that the C1-carboxyl group is an important structural element involved in Sia-protein binding. For example, lectin X-ray crystallographic studies of the MAL carbohydrate-binding cleft revealed that the carboxyl group of Neu5Ac forms an important salt bridge with a lysine side chain of the protein (Imberty *et al.*, 2000). Similarly, LFA, CHA-I and TML Sia-binding lectins, also require the presence of an intact axially-oriented C1-carboxyl group (Brossmer *et al.*, 1992; Mercy and Ravindranath 1993; Babál *et al.*, 1994).

Furthermore, analysis of viral Sia-binding proteins (influenza virus hemagglutinin, polyomavirus VP1 protein, rotavirus VP4 protein and adenovirus serotype 37 protein) have also demonstrated that the C1-carboxyl group of sialic acid is critical for protein recognition (Weis *et al.*, 1988; Frank and Von Der Lieth, 1997; Dormitzer *et al.*, 2002; Gee *et al.*, 2004). Therefore, the C1-carboxyl group was not selected as a site for linker attachment.

Other important functional groups on the sialic acid molecule include the *N*-acyl moiety located at the C5-position. The presence of an *N*-acetyl or *N*-glycolyl functional group at that position, determines the type of sialic acid molecule (Neu5Ac or Neu5Gc) (Angata and Varki, 2002). Examples of proteins that preferentially bind sialic acid when the C5-position contains the *N*-acetyl group include: the LFA lectin, sialoadhesin (Siglec-1) and myelin-associated glycoprotein (Siglec-4) (Knibbs *et al.*, 1991). In contrast, the Scylla lectin and the *E. coli* K99 S-adhesin protein, recognise Neu5Gc but not Neu5Ac (Hanisch *et al.*, 1993; Mercy and Ravindranath, 1993). Thus, placement of the linker at the C5-position could have prevented the isolation of Neu5Ac or Neu5Gc specific antibody fragments. Another part of the Sia molecule that contributes to the protein binding reaction is the glycerol tail of sialic acid [(CHOH)₂-CH₂OH]. Importantly, the interaction between sialic acid and certain proteins [TML, LFA, CHA-I, GM3(Neu5Gc) ganglioside and neuraminidase] is dependent upon the presence of this 3-carbon side chain (Brossmer *et al.*, 1992; ; Babál *et al.*, 1994; Gerlach *et al.*, 2002; Gerlach *et al.*, 2004; Krengel *et al.*, 2004; Das *et al.*, 2010). Therefore, the linker was not attached to any of the hydroxyl groups at the C7, C8 or C9-positions of the glycerol tail. Lectin studies involving, OPL I, LPA, WGA, limulin and *Cancer antennarius* have all shown that the C4-hydroxyl group is also important for binding (Ravindranath *et al.*, 1985; Knibbs *et al.*, 1991; Troncoso *et al.*, 2000). In contrast the C-6 region is important for recognition by *N*-acetylneuraminase lyase (*N*-acetylneuraminase pyruvate-lyase, EC 4.1.3.3), an enzyme involved in the metabolism of sialic acid (Zbiral *et al.*, 1992). In addition, analysis of the closely related sialic acid derivative, Neu5Ac2en, has shown that the C3 region is necessary for sialidase activity (Luo *et al.*, 1998). Thus, the C3, C4 and C6-positions of the sialic acid molecule, were also not selected as sites for the covalent attachment of the linker.

In the majority of glycolipids and glycoproteins, Sia is linked via its C2-carbon atom to other saccharides. For example, in humans the C2-position of Neu5Ac is α -linked to either the C3- or C6-position of galactose or to the C6-position of *N*-acetylgalactosamine and glucosamine. Moreover, terminal α 2,3 or α 2,6-linked sialyl galactose-capped oligosaccharides are found ubiquitously in nature (Angata and Varki, 2002; Varki, 2007; Cohen and Varki, 2010). Therefore, in order to mimic natural glycosides and to avoid those functional groups which may be important in Sia-protein binding, the C2-position of Neu5Gc was chosen as the point for the covalent attachment of a 13 atom spacer. Yet another important parameter to be considered in the design of the sialoconjugates was the number of saccharide units incorporated into the carrier protein after conjugation. Although there are many methods available for conjugation of carbohydrates to proteins, there is no universal protocol that can predict the saccharide content of the resulting carbohydrate-protein conjugate. Unlike protein and nucleic acid synthesis, there are still no robust methods of automated carbohydrate synthesis (Aich and Yarema, 2008). Furthermore, the conjugation of carbohydrates to proteins often requires laborious protecting and de-protecting group manipulations. Consequently, the preparation of neoglycoconjugates with narrow, well-defined carbohydrate-protein ratios is a time consuming and difficult process (Guo and Wang, 2009). In addition, subtle differences in the nature of the protein, carbohydrate, and linker parts, as well as their reactivity to the coupling chemistry, all affect the conjugation process, and therefore alter the carbohydrate-protein ratio. Typically, a trial-and-error approach is required to achieve the desired carbohydrate-carrier ratio, although, even with such an empirical approach, there is still no guarantee that the desired carbohydrate density will be obtained, since the reaction rate of the conjugation process can be variable (Glycotech, personal communication; Carbohydrate Synthesis, personal communication).

Furthermore, inconsistent carbohydrate-protein stoichiometries can lead to a wide variation in the immune response. In general, anti-carbohydrate antibodies have relatively low monovalent affinities for glycans, with dissociation constants typically in the micromolar to millimolar range (Astronomo and Burton, 2010). However, carbohydrate clustering can increase the number of exposed carbohydrate epitopes and thus greatly improve the immune response to the carbohydrate conjugate (Pozsgay *et al.*, 1999; Kircheis *et al.*, 2006; Li *et al.*, 2010).

This multivalent mechanism, coined as the “cluster effect,” describes the scenario where the overall affinity (avidity) for a multivalent sugar ligand is enhanced, compared with that of a simple monovalent monosaccharide (Davis, *et al.*, 2000). In general, the ability to present carbohydrate epitopes in a polyvalent context, with the correct sugar spatial arrangement, leads to an enhancement in the association constant that is typically greater than that expected on the basis of valency alone.

Although the benefits of multivalency are well established for both antibody and lectin-binding to carbohydrates, the molecular mechanisms underlying these phenomena are poorly understood (Davis, 2000; Arranz-Plaza *et al.*, 2002; Kiessling and Pohl, 1996). In order to emulate the multivalent carbohydrate surface and to overcome the inherent low affinity of sialic acid monosaccharides, conjugates were generated with a high density of sugar. It was anticipated that multivalent interactions would enhance the strength and specificity of the immune response. This reasoning is supported by reports in the literature, which show that the density of carbohydrate epitopes on the carrier is an essential parameter that affects the magnitude and type of anti-carbohydrate immune response. For example, Kircheis *et al.* (2006) investigated the effect of the SialylTn coupling density on the induced anti-carbohydrate immune response of rhesus monkeys. They showed that a low ratio of carbohydrate to protein (3:1) induced only a marginal anti-SialylTn response. In contrast, a ratio of approximately 16:1 resulted in significant anti-SialylTn IgG titres. As mentioned previously, the study of Pozsgay and colleagues, (1999) showed that HSA conjugates with high carbohydrate/protein ratios had enhanced immunogenicity. In addition, it has been reported that high antibody titres are usually obtained with hapten density of 15 to 30 molecules per carrier protein (Pozsgay *et al.*, 1999; Singh *et al.*, 2004; Lemus and Karol, 2008). Therefore, conjugates prepared for use in this study had relatively high loadings of carbohydrate. The Neu5Gc-HSA conjugate contained approximately 35 Neu5Gc monosaccharides per mole of HSA protein. In contrast, the Neu5Gc-BSA conjugate contained approximately 20 Neu5Gc monosaccharides per mole of BSA protein.

The selection of an appropriate animal host was also an important consideration for the successful generation of anti-sialic acid antibody fragments. To date, a significant number of anti-carbohydrate antibodies have been derived from murine hybridomas (Magnani, 1986; Miyake *et al.*, 1988; Vázquez *et al.*, 1998). However, as discussed previously in the introduction to this chapter, the avian host offered a number of distinct advantages for antibody generation. In addition, the immunogenicity of sialic acid was also a key factor in choosing the animal host. Unfortunately, gangliosides and glycoproteins that contain sialic acid are found in most animals (Kawai *et al.*, 1991; Schauer, 2000; Vallejo *et al.*, 2000). For example, glycoproteins and glycolipids containing Neu5Gc and Neu5Ac are particularly abundant in mouse liver (Hikita *et al.*, 2000). Furthermore, work by Chefalo *et al.*, (2004) has shown that Neu5Ac conjugates, injected into mice, elicit a poor immune response. In common with other carbohydrates, sialic acid is recognised as self by the immune system of most animals. Typically, when self-antigens are immunised into animal hosts they produce little or no antigenic response. The poor immunogenicity of carbohydrates presents a formidable challenge to the generation of anti-carbohydrate antibodies.

Moreover, even if an anti-carbohydrate response is elicited, the antibodies produced are typically low-affinity IgMs and these are generally not suitable for *in vitro* diagnostic applications. Fortunately, Neu5Gc appears to be absent from the normal tissues of chickens (Diaz *et al.*, 2009). Thus, the avian immune system offers a potential way to circumvent the problem of carbohydrate immunological tolerance. It was speculated that avian immunisation with Neu5Gc neoglycoconjugates could potentially provoke a strong T-cell-dependent IgG immune response. As previously mentioned, the work by Asaoka *et al.* (1992), Noguchi *et al.*, (1995) and Tangvoranuntakul *et al.*, (2003) had shown that chickens could be used as hosts for the successful generation of anti-Neu5Gc pAbs. It was thus reasonable to conclude that immunisation experiments should use an avian host with immunogenic Neu5Gc neoglycoconjugates. The success of this strategy is illustrated in Figure 4.2.2.1. A comparison of chicken pre-immune serum with immune serum revealed a significant immune response against the Neu5Gc-BSA neoglycoconjugate. The avian antiserum appeared to contain polyclonal anti-sialic acid antibodies. However, both BSA and HSA display approximately 76% sequence homology (Gelamo and Tabak, 2000).

Therefore, it is possible that pAb Neu5Gc binding may have required a common albumin amino acid sequence motif. Furthermore, the avian immunisation conjugate (Neu5Gc-HSA) and the antibody screening conjugate (Neu5Gc-BSA) both contained an identical 13 carbon atom spacer arm. Thus, to ensure that pAb binding was actually directed towards the Neu5Gc component of the conjugate and not the spacer arm or protein elements, the polyclonal serum was further tested against a synthetic polyacrylamide sialic acid antigen (Neu5Gc-PAA-biotin). Importantly, there are major differences between the albumin protein conjugates and the Neu5Gc-PAA-biotin conjugate. For example, the PAA sialic acid polymer is fully synthetic, smaller in size (30kDa) and contains Neu5Gc attached to the PAA backbone at a molar ratio of 20%. In addition, a shorter spacer arm (11 carbon atoms) is used to couple Neu5Gc to the PAA polymer. Importantly, this experiment (Figure 4.2.2.2) demonstrated that pAbs could discriminate Neu5Gc in the context of two very different carriers with structurally unrelated linkers, namely BSA-**linker1**-Neu5Gc and biotin-PAA-**linker2**-Neu5Gc. Consequently, these pAbs had the ability to bind Neu5Gc without the requirement of a specific linker. Therefore, these pAbs are not linker-specific but rather sialic acid-specific. Further analysis using a solid phase binding assay (Figure 4.2.2.3) showed that these serum-based antibodies were able to compete between free and immobilised Neu5Gc-BSA.

4.3.3 Immune library generation

One week after the chicken received its final immunisation, the animal was sacrificed and both the spleen and bone marrow were harvested. RNA was extracted from both sources and used as a template for cDNA synthesis. The amplification of full-length scFv gene fragments was carried out using consecutive PCR steps. In the primary PCR, variable domain genes were amplified from the cDNA template using the primers listed in Section 2.2.8. The PCR protocol as defined by Barbas (2001) was followed and Figure 4.2.2.4 shows the initial PCR amplification of avian V_H and V_L genes. In addition to the target product (~400bp), a spurious non-specific PCR amplicon (> 400bps) was also generated. The optimisation of a PCR reaction generally involves the sequential investigation of a number of different reaction variables. These variables typically include the PCR primers, annealing temperature, magnesium chloride concentration, buffer system, enzyme, template concentration and thermocycling conditions (Cobb and Clarkson, 1994; Rao *et al.*, 2008). The avian V_H and V_L PCRs were optimised by a reduction in the annealing temperature. In addition, the concentrations of DNA polymerase and oligonucleotide primers were also reduced. Furthermore, PCR cycling was reduced and a touchdown thermocycling profile was employed (Figure 4.2.2.5). The standard PCR touchdown strategy involves selecting an annealing temperature that is several degrees higher than the melting temperature of the primers. The annealing temperature is then gradually reduced in every PCR cycle until the target annealing temperature is reached. This method adjusts for any imbalance between the correct and incorrect annealing temperatures and thus enriches for the target amplicon, over any spurious products (Don *et al.*, 1991).

Following the successful amplification of the avian variable domain genes, single chain Fv fragments were assembled using a short 7-amino acid peptide linker in the orientation V_L - GGSSRSS- V_H by a two fragment Splice Overlap extension PCR. In this second round of PCR, both the target product (~750bp) and an unwanted product of between 200-300bp were amplified (Figure 4.2.2.6). This non-specific amplicon was eliminated by reducing the PCR reaction annealing temperature and extension time. In addition, the concentrations of both the forward and reverse SOE primers were also reduced.

However, unlike the V_H and V_L PCRs a touchdown thermocycling profile was not required. Purified pComb3XSS vector and SOE-PCR product were digested using the *SfiI* restriction enzyme. Barbas (2001) designed the pComb3 phagemid display system so that directional cloning of the scFv gene sequences could be accomplished with a single restriction endonuclease. *SfiI* recognises an 8-bp sequence (GGCCNNNN[^]NGGCC) and cuts (between 4th and 5th N) within the degenerate region of its interrupted palindromic recognition site, leaving an indeterminate “sticky” end. The freedom in the N nucleotide selection enables the design of different restriction sites that are recognised and cut by the same *SfiI* enzyme. Use of unique 5' (GGCCCAGG[^]CGGCC) and 3' (GGCCCAGGC[^]CGGCC) *SfiI* sites within the sense and reverse avian primers, allows scFv fragments to be ligated into the pComb3XSS cloning vector in the correct orientation (Gao *et al.*, 2002). The successful *SfiI* restriction digestion of the pComb3XSS vector is shown in Figure 4.2.2.7. Both the expected stuffer fragment (~1600bp) and the double-cut vector (~3400bp) are illustrated.

Ligated pComb3XSS-scFv gene sequences were transformed into electrocompetent *E.coli* XL-1 Blue cells. Dilutions of the electroporated bacteria were plated and the number of scFv-containing clones within the library was estimated. The complexity of an antibody library is defined as the number of clones bearing a suitable selectable marker (antibiotic resistance) and containing the full size antibody gene (Barbas, 2001). In contrast to naïve libraries, the use of hyper-immune animals as the source of immunoglobulin cDNA, reduces the need for very large (> billion recombinants) antibody libraries. Typically, an immune antibody library has already been enriched with antigen-specific antibodies. Furthermore, some of these antibodies may have undergone natural affinity maturation (Smith *et al.*, 2005). Even so, the overall immune library size is still an important determinant for successful selection against an antigen. In addition, when relatively large immune libraries are used, there is a higher probability that panels of antibodies that bind the same antigen are isolated. Typically, libraries derived from hyper-immune animals range in size from 10^7 to 10^8 transformants. However, smaller immune libraries are not recommended for biopanning, as diversity may be limited due to the absence of certain antibody sequences.

This in turn makes it more difficult to identify the rarest recombination species and also rapidly decreases the probability of isolating high affinity antibodies (Maynard and Georgiou, 2000; Barbas, 2001; O' Brien and Aitken, 2002; Clackson and Lowman, 2007). The size of the avian anti-sialic acid scFv library was 1.2×10^7 transformants and this was considered large enough for biopanning.

4.3.4 Biopanning

Several different biopanning strategies have been used to isolate phage displaying anti-carbohydrate-specific antibodies. In the conventional phage display method pure antigen is absorbed to a solid support (microtitre plates, immunotubes or magnetic beads). Following exposure of phage particles to the antigen, non-specific binders are removed in a washing step, and phage bound to the target are recovered by elution. The eluted phage are re-amplified in *E. coli* and used in subsequent rounds of biopanning. In this work, biopanning of the avian anti-sialic acid scFv library was performed in Maxisorp[®] immunotubes. These tubes have a surface area of 5cm^2 , in contrast, the ELISA plate has a much lower surface area (0.5cm^2). It has been reported that the effective antigen density in immunotubes is higher than that of ELISA plates (Lou and Marks, 2010; Katakura *et al.*, 2002). Anti-carbohydrate antibodies are widely known to have weak affinities. Therefore, the traditional immunotube was chosen for biopanning, as this allowed a higher density of neoglycoconjugate to be bound to the solid support. It was speculated that this would lead to avidity effects and therefore enable the isolation of sialic acid phage binders, even those with weak carbohydrate affinities. Other workers have reported the successful isolation of anti-carbohydrate antibody fragments by library selection on immunotubes. For example, Lee *et al.*, (2002) used a chemoenzymatically synthesised GM3-BSA neoglycoprotein biopanning reagent and successfully isolated scFvs that bound native GM3 on the surface of tumour cells. In contrast, Mao *et al.*, (1999) used immunotubes coated with the neoglycoconjugate sLe^x-BSA and isolated scFvs that bound the sLe^x epitope on pancreatic adenocarcinoma cells.

A second strategy employed to isolate anti-sialic phage binders was a modification of the phage elution method. Typically, antigen-bound phage are released by non-specific elution using a wide variety of methods. These include the use of extreme pH (0.2 M glycine-HCl, pH 2.2), high ionic strength solutions, reductants and specific proteases. Trypsinisation is a common method and this involves the proteolytic cleavage of the antibody fragment from the phage surface. It therefore allows the recovery of infective phage even under conditions where the phage has bound very strongly to its antigen. Alternatively, competitive elution may be a preferred option especially when the ligand of a particular target is available. In competitive elution, solution phase target molecules are used to compete bound phage away from the immobilised target (Lunder *et al.*, 2008; Vodnik *et al.*, 2011). It was speculated that competitive elution with a relatively large concentration of free sialic acid conjugate, in combination with a long immunotube incubation time, would enrich for anti-sialic acid phage antibodies. This reasoning was based on the premise that polyvalent binding reactions between the solution-phase multivalent conjugate and multimeric phage-displayed scFvs could occur. In biopanning, multiple antibody fragments may be present on single phage particles. For example, phage-displayed dimers are known to occur and result from the association of phage-displayed scFv with soluble scFv. This occurs when the displayed scFv is released from its pIII fusion partner by proteolysis in the periplasmic space (Barbas, 2001). Thus, these avidity effects may increase the overall binding of low-affinity anti-sialic scFv antibodies.

Tables 4.2.2.8a and 4.2.2.8b show the phage input and output titres over the 4 rounds of biopanning of the anti-sialic scFv library. The number of phage eluted following biopanning (output phage) can be used as an indication of biopanning success. In theory, if conventional biopanning is successful then increasing numbers of phage should remain bound to the antigen-coated immunotubes and this should result in higher output titres. The phage titre inputs for the competitive elution method are lower than that obtained with the trypsin method. In contrast, the phage titre outputs for the trypsin method decrease after each round of biopanning, whereas the phage output titres for the competitive method remain relatively constant after each round of biopanning. Typically inputs from a standard amplified library with a diversity of 10^8 range from $10^{12} - 10^{13}$ cfu/mL, whereas phage output titres can vary from $10^2 - 10^7$ cfu/mL.

It is not known why the input titres for the competitive elution drop below 10^{12} cfu/mL. The decreasing output titres from the competitive elution method may indicate that unlike the trypsin method, fewer anti-sialic acid binding-phage were captured. However, phage titres at each round of selection are not a reliable marker of biopanning success. Only a small fraction of the titred phage actually display correctly folded antibody fragments on their surface. In addition, phage that have a low propensity to interact with the target antigen but are able to replicate quickly will rapidly dominate the output titre. Furthermore, antibodies that bind to a contaminant with high affinity rather than the target antigen, can also dominate the selection process. Therefore, total phage titres do not reveal the amount of functional phage present (Maynard and Georgiou, 2000; Barbas, 2001; O' Brien and Aitken, 2002; Clackson and Lowman, 2007).

In the next stage of analysis, bone and spleen phage library preparations for each biopanning round were analysed by ELISA. As the viral input to the assay is mixed in composition, the assay is termed a polyclonal phage ELISA (O' Brien and Aitken, 2002). In this step, polyclonal mixtures of phage produced by re-propagation of the libraries after each round of selection were screened against the Neu5Gc-BSA neoglycoconjugate. An increase in ELISA signal as a function of biopanning round is indicative of enrichment for sialic acid binders (O' Brien and Aitken, 2002). Increases in specific ELISA signals over background were evident with phage pools obtained after just one round of selection (Figure 4.2.2.9). In addition, further enrichment of anti-sialic acid polyclonal phage pools were observed in the third round of selection. This round showed the strongest anti-sialic acid reactivity. There was a slight decrease in anti-sialic acid antibody enrichment in round four. In contrast, round five showed a substantial decrease in anti-sialic acid reactivity.

The competitively-eluted and trypsin-eluted bone and spleen polyclonal phage pools from biopanning rounds three and four were separately combined. These phage pools were subsequently infected into a non-suppressor (non-SupE) strain of *E. coli* (TOP10F'). The pComb3XSS phagemid vector contains an amber stop codon positioned between the scFv fragment and pIII genes. This allows for the expression of scFv fragments as either phage-bound pIII fusion protein (in SupE bacterial strains) or soluble recombinant protein (in non-SupE bacterial strains) that is not fused to pIII (Barbas, 2001; O' Brien and Aitken, 2002).

Therefore, binding to the target antigen may be evaluated by phage ELISA or soluble ELISA. It has been reported that soluble ELISA has less non-specific background problems and tends to be more accurate than phage ELISA. For example, in phage ELISA false positive signals occur when target antigen is only bound by the scFv-pIII fusion protein and not by the soluble scFv (Kirsch *et al.*, 2008). Thus, competitively-eluted or trypsin-eluted polyclonal phage preparations were infected into TOP10F' cells. Figures 4.2.2.10a/b shows a selection of the 392 random clones that were solubly expressed and screened by an indirect ELISA binding assay. Greater than 90% of the clones analysed, had ELISA signals which were more than three-fold higher, than that of the negative control protein, BSA. Therefore, based on the soluble monoclonal phage ELISA results, there appears to be no significant difference between the two elution strategies. Both methods produced equally large numbers of putative anti-sialic acid scFvs. Therefore, for anti-sialic acid scFv isolation, the traditional phage display method of trypsin elution can be used.

4.3.5 Clonal Selection and Screening

The anti-sialic acid scFv candidates identified by direct ELISA were further screened using an alternative synthetic Neu5Gc conjugate. The goal was to identify clones that recognised sialic acid in the context of different backbone structures. However, obtaining other polyvalent synthetic carbohydrate structures proved challenging, as there are only a handful of specialist companies that can supply these materials. One such company, Glycotech, provided a library of common and rare carbohydrate reagents. Their soluble polyacrylamide (PAA) carbohydrate conjugates were originally developed by Nicolai Bovin. These pseudo-glycoproteins have been widely used in carbohydrate-binding protein studies. For example, Kojima *et al.*, (2002) used Le^b-oligosaccharide conjugated with PAA to study the adhesion of *Helicobacter pylori* to cell surface receptors. In contrast, Rapoport *et al.*, (2006) used 22 different PAA-neoglycoconjugates to probe the carbohydrate-binding interaction of sialic acid-binding immunoglobulin-like lectins (Siglecs). Siglecs are a family of type I membrane proteins with variable numbers of immunoglobulin domains. These proteins have the ability to bind sialic acid-containing glycans (Kooyk and Rabinovich, 2008).

In a separate study, Padler-Karavani *et al.* (2008) used sialylated and non-sialylated PAA-glycopolymers to study the human immunological response to Neu5Gc. In common with proteins, these sugar-PAA conjugates can be directly coated onto the surfaces of polystyrene microtitre plates. However, it has been reported that only 2% of the conjugate is actually adsorbed on the polystyrene surface, whereas the rest (98%) is lost through ELISA washing steps (Galanina *et al.*, 2003). Therefore, in this study the avidin-biotin system was used to capture the PAA-sugar conjugates. The use of the avidin-biotin interaction within an immunoassay can substantially improve its performance. This is due to the exceptionally strong ($K_d = 1.3 \times 10^{-15}$ M at pH 5) bond that is formed between avidin and biotin (Haugland and You, 2002). However, avidin is a glycosylated protein and, therefore, its use in an immunoassay design to detect carbohydrate structures would have been challenging. Instead, neutravidin which is a chemically deglycosylated form of avidin was used to capture the biotin-PAA-sugar conjugates. The neutravidin-biotin approach allowed for a more uniform and dense sialic acid filling of the surface. In addition, the biotinylated-PAA-sugars were attached in a strict ligand orientation. This assay format was used to identify a number of soluble anti-sialic acid clones that recognised Neu5Gc when attached to either BSA or PAA. From the original 90% of clones that bound to Neu5Gc-BSA, less than 10% of these clones recognised Neu5Gc in the context of the PAA-backbone (Figure 4.2.2.11). This loss of recognition may have occurred due to alterations in the spatial arrangements of Neu5Gc when attached to PAA, as compared to BSA. A different type of linkage chemistry was employed in the attachment of Neu5Gc to PAA. In addition, Neu5Gc is attached to the PAA conjugate by a shorter linker arm. Furthermore, the Neu5Gc loading density on PAA differs from that of BSA. Importantly, these results revealed that these scFvs could bind Neu5Gc in the context of two very different carriers with structurally unrelated linkers, namely BSA-linker1-Neu5Gc and biotin-PAA-linker2-Neu5Gc. Therefore, these scFvs have the ability to recognise Neu5Gc without the requirement of a specific linker and consequently, these scFvs are not linker-only specific but rather sialic acid specific.

The diversity of clones within the avian library was assessed by restriction digestion pattern analysis. In this method, the gene encoding the scFv is amplified directly from bacterial colonies harbouring the phagemid using primers which flank the scFv gene.

The PCR product is then digested with restriction enzymes that cut frequently within V-genes. Agarose gel electrophoresis of the digested PCR products can then be used to visualise the pattern of bands that is unique to each clone. Analysis of these PCR “fingerprints” allows the identification of clones that are different, since those clones should carry a unique combination of V_H and V_L DNA sequences. In general, clones that are identical show the same banding pattern. The main strengths of this technique are its simplicity and low cost. However, it is limited by the resolving power of the agarose gel and in certain cases scFvs with the same fingerprint may actually represent different clones. This can occur when diversity in the library does not translate into a gain or loss of restriction sites (Barbas, 2001; O’ Brien and Aitken, 2002; Clackson and Lowman, 2007). A restriction map profile of 18 different anti-sialic acid avian scFv inserts is shown in Figure 4.2.2.12. Comparison of the restriction digestion fragment patterns revealed that the majority of clones appear to be different. This indicated that unique combinations of V_H and V_L DNA sequences occurred within a subset of the avian Neu5Gc-PAA/BSA binding clones.

The avian Neu5Gc-PAA/BSA binding clones were further analysed by cross-reactivity studies using different mono- and disaccharide carbohydrate structures. The goal was to further elucidate the carbohydrate-binding specificity of the soluble scFv fragments. To that end, a number of different sugar-PAA conjugates [Neu5Ac-PAA, (Neu5Ac)₂-PAA, Neu5Gc-PAA, glucose-PAA and galactose-PAA] were tested using an indirect ELISA. An unrelated avian scFv served as a negative control in the immunoassay (Ayyar *et al.*, 2010). Importantly, all the PAA conjugates were synthesised by the same supplier (Glycotect). In addition, each sugar conjugate had the same molecular weight (30kD) and contained the same 20:1 molar ratio of carbohydrate to PAA. In Figure 4.1.2.13, the binding response of 8 different scFvs to 5 different sugar-PAA conjugates and BSA is shown. As expected, the negative control, protein-binding-avian scFv, did not react with any of the sugar-PAA conjugates. The scFvs BG10 and BC11 bound relatively poorly to each of the sugar-PAA conjugates. In contrast, AG9, AD5 and BE10 showed the same weak binding to Neu5Ac-PAA. Unlike AD5, AG9 also showed weak binding to Neu5Gc-PAA and (Neu5Ac)₂-PAA. In contrast, the AE8 and CC11 scFvs showed very strong binding to Neu5Ac-PAA and Neu5Gc-PAA.

In addition, no significant binding to galactose-PAA or glucose-PAA was observed. Interestingly, AE8, unlike CC11, reacted strongly with (Neu5Ac)₂-PAA. Overall, the AE8 scFv showed the strongest reactivity to all of the sialic acid structures.

Although AE8 and CC11 recognised Neu5Gc in both the BSA and PAA forms, they also reacted strongly with Neu5Ac-PAA. This occurred even though the HSA neoglycoprotein used for immunisation was only chemically linked to Neu5Gc. It is possible that the HSA preparation was not 100% pure and was contaminated with serum sialoglycoproteins, like human IgG, which contains a significant proportion (>20%) of sialylated (Neu5Ac) glycan chains (Thobhani *et al.*, 2009). It is perhaps not surprising that AE8 and CC11 recognises both Neu5Gc and Neu5Ac. These two sialic acids are structurally exceptionally similar and differ from each other by an extremely subtle chemical modification. The difference between them is only the position of one oxygen atom. Neu5Ac contains an *N*-acetyl group (-CO-CH₃) whereas Neu5Gc has an *N*-glycoyl group (-CH₂-OH) (Tangvoranuntakul *et al.*, 2003). Thus, the combining sites of these scFvs appear to tolerate modifications of the *N*-acetyl group at C-5. Indeed, a number of lectins (limulin, CHA-I and LFA) also share this feature (Troncoso *et al.*, 2000). Moreover, it is widely known that many anti-carbohydrate antibodies can cross-react with other glycans. For example, BR55, a Le^Y binding anti-carbohydrate antibody, is known to cross-react with Le^X and DiLe^X (Manimala *et al.*, 2007). Therefore, it is unsurprising that the anti-sialic acid scFv clone, AE8 binds to both Neu5Gc and Neu5Ac structures (Figure 4.2.2.13).

DNA sequence analysis was performed on four avian clones, AE8, CC11, AG9 and CD3. For comparison, clone CD3, which had shown no significant binding to sialic acid was also included. The deduced amino acid sequence alignment of the CDR V_H and V_L regions for each of the four different avian clones is shown in Figure 4.2.2.14. Each clone is genetically different and comparisons of the CDRs, show differences in both amino acid sequence and CDR length. For example, all four clones showed large variations in the length and amino acid composition of CDRL3. AE8 has the longest CDRL3 followed by CC11, CD3 and AG9. In contrast, each of the sialic acid-binding clones (AE8, AG9 and CC11) showed little variation in CDRH3 length (19-amino acids). Furthermore, these anti-sialic acid clones contain only subtle amino acid differences in their CDRH3s.

This indicates that these CDRH3-conserved amino acids may be important in sialic acid binding. In contrast, the non-binding sialic acid clone, CD3, had a shorter CDRH3 (13-amino acids). In addition, the CDRH3 amino acid sequence of CD3 differed substantially from the sialic acid-binding clones. Each of the sialic acid-binding clones contained two cysteine (Cys) within the heavy chain CDR3. In contrast, CD3 contained no Cys residues within its CDRH3. Such Cys motifs are not commonly found in other chicken antibodies. Therefore, this indicates that these residues may be important for antigen recognition (Andris-Widhopf *et al.*, 2000). Moreover, there are reports that suggest the variable heavy chain plays an important role in carbohydrate-protein molecular interactions (MacKenzie and To, 1998; Rojas *et al.*, 2004; Pashov *et al.*, 2005). In addition, anti-carbohydrate antibodies that recognise sialic acid structures on gangliosides have been reported to have a relatively long CDR3 (Lopez-Requena *et al.*, 2007). It has been suggested that a longer CDR3 is a requirement to compensate for the smaller carbohydrate antigen-binding surface area (Schoonbroodt *et al.*, 2008; Gerstenbruch *et al.*, 2010). Therefore, it is possible, that differences in the CDR3 of CD3, may account for the inability of this scFv to recognise sialic acid.

Both, the AE8 and CC11 scFvs showed very strong binding to Neu5Ac-PAA and Neu5Gc-PAA. However, CC11 showed no significant binding to (Neu5Ac)₂-PAA. A number of amino acid differences between CC11 and AE8 occur in CDRL1 and CDRL3. In contrast, fewer differences are seen in CDRH1, CDRH2 and CDRH3. Overall, these changes probably account for the inability of CC11 to recognise the di-sialic acid-PAA conjugate. Many naturally occurring antibodies that bind carbohydrates have Arg and Lys residues in their hypervariable regions. The CDRL3 of AE8 has an Arg residue, whereas, CC11 contains a glycine residue at the same position. In addition, both AE8 and AG9 contain an Arg at the same location in CDRH2, whereas, CC11 contains a Lys at that position. In contrast, the non-binding sialic acid scFv, CD3, contains an Asp at that position. In addition, the AE8 scFv which showed the strongest reactivity to all of the sialic acid structures also contains the most number of Arg residues. Conversely, the non-binding sialic acid clone CD3, contained the least number of Arg residues.

It is possible that the locations and larger numbers of AE8 Arg residues contributed to its ability to strongly bind sialic acid. Arginine is a large and positively charged amino acid and acts as an important contact residue in the sialic acid-binding site of certain proteins and peptides (Lekakh *et al.*, 2001; Krenzel *et al.*, 2004; Müller *et al.*, 2006; Kiessling and Pohl, 1996; Matsubara *et al.*, 2008). For example, mutagenesis studies using a Neu5Gc-ganglioside binding protein (K99) have shown a binding pocket requirement for the charged amino acids Lys and Arg (Morschhäuser *et al.*, 1990). Other examples include neuraminidase (sialidase) from the influenza virus, which cleaves terminal sialic acid residues and allows the release of the virus from infected cells. Within the active site of this enzyme are three strategically arranged Arg residues (triarginyl cluster) that form strong charge-charge association with the negatively charged carboxylate group of Neu5Ac. In addition, a separate Arg residue forms a hydrogen bond with the carbonyl group of the *N*-acetyl group of Neu5Ac (Chan *et al.*, 1997; Haselhorst *et al.*, 2004). Matsubara *et al.*, (2008) showed that Arg residues in carbohydrate-binding peptides are important for GM1-ganglioside recognition. Whereas, Krenzel *et al.*, (2004) showed that carbohydrate-binding of an anti-GM3(Neu5Gc) Fab, may depend on three Arg residues located in CDRH3 loop of the Fab.

The AE8 scFv had the strongest sialic acid reactivity and was therefore chosen for further study. It has been reported that Top10F' *E. coli* cells produce scFv protein yields which are higher than those obtained with *E. coli* strains HB2151 and ER2537 (Lim *et al.*, 2004). Therefore, the AE8 scFv was produced and solubly expressed in *E. coli* TOP10F' cells. Incorporation of a carboxyl-terminal histidine affinity tag in the pComb3XSS phagemid vector enabled recombinant protein purification (Barbas, 2001). However, analysis of the IMAC-purified AE8 scFv, by SDS-polyacrylamide gel electrophoresis, revealed a number of contaminating protein bands (data not shown). It is possible that contaminating host cell *E. coli* proteins bound to the IMAC column and co-eluted with the AE8 scFv. Co-purification of *E. coli* proteins with the histidine-tagged recombinant protein is very common and occurs when *E. coli* histidine-rich proteins bind to the nickel-agarose column. For example, the *E. coli* host cell protein SlyD, contains a C-terminal histidine rich metal binding domain and is a common contaminant in nickel-IMAC preparations (Kipriyanov 2002; O'Brien and Aitken, 2002; Willems *et al.*, 2003; Moss *et al.*, 2003; Charlton 2004; Gräslund *et al.*, 2008).

Therefore, further purification of the scFv from IMAC-eluted material was performed using size exclusion chromatography (SEC). Analysis of the SEC-HPLC IMAC-purified AE8 scFv is shown in Figure 4.2.2.15. Since different protein peaks were observed in the chromatograph, multiple peak fractions were sampled and sialic acid-binding specificity determined by SPR. For SPR sialic acid-binding studies, neutravidin was immobilised on the dextran surface of a Biacore[®] CM5 chip, biotinylated-PAA-Neu5Gc conjugate was passed over the chip and subsequently captured by neutravidin. Each HPLC-purified protein fraction was checked for Neu5Gc binding; in addition, a neutravidin only reference surface was used to assess non-specific binding. SPR analysis demonstrated significant Neu5Gc binding for IMAC-eluted HPLC-purified protein fractions 4 and 7. Further analysis of these fractions by SEC-HPLC (Figure 4.2.2.16) showed that fraction 4 contained scFv dimers, whereas fraction 7 contained both scFv dimeric and monomeric species. It is unsurprising that scFv dimers were observed by SEC-HPLC analysis, since it is widely known that scFvs have a tendency to form higher order aggregates. This phenomenon primarily depends on the length of the scFv linker. For example, as the artificial linker is shortened, the V_H domain of the scFv cannot interact with its attached V_L domain in the natural Fv orientation. Instead, complementary V_H/V_L pairs from two separate scFv molecules combine to form a non covalent bivalent dimer, termed a diabody. If both scFv molecules are genetically identical a monospecific diabody is formed. The propensity of scFvs to form oligomeric structures is also influenced by expression conditions, pH, ionic strength and protein concentration (Ravn *et al.*, 2004; Johansson *et al.*, 2006; Johansson *et al.*, 2007).

Comparison of the dimeric and monomeric AE8 peak area fractions revealed that scFv diabodies predominate over monomeric scFv (Figure 4.2.2.16). Since this recombinant avian scFv library was built using a short 7-amino acid Gly-Ser-polypeptide linker it was not unexpected that a large percentage of the scFvs formed monospecific diabodies. Moreover, the avian scFv library was designed with the intention of scFv dimer formation, since it was speculated, that avidity effects may increase the overall binding of anti-sialic acid scFv antibodies. Indeed, it was observed that the monomeric fraction of AE8 had a lower SPR Neu5Gc-binding signal in comparison to its dimeric fraction. This gain in functional affinity (avidity) for the dimeric AE8 scFv is probably due to reduced off-rates which result from both multiple binding (scFv multivalency) and rebinding to the multivalent Neu5Gc conjugate.

FPLC analysis of the IMAC-purified anti-sialic acid AE8 scFv confirmed the presence of both monomeric and dimeric scFv forms. The estimated molecular size of the monomeric AE8 scFv was 28kDa, whereas the estimated molecular mass for the dimeric version was 60kDa (Figure 4.2.2.17). These results are consistent with the theoretical molecular mass of scFvs and diabodies. Diabodies are approximately twice the molecular mass of an scFv. These values are also similar to those reported in other scFv recombinant antibody studies (Brennan *et al.*, 2003).

SPR is widely used for kinetic studies of biomolecular interactions, including carbohydrate–protein interactions. Therefore, the AE8 scFv was further analysed using a solution-phase sialic acid SPR based inhibition study. Although it was possible to use the ELISA format, the SPR biosensor was chosen as it allowed for the real-time detection and monitoring of binding between immobilised and free sialic acid conjugates and the soluble anti-sialic acid scFv. In addition, the biosensor only requires small amounts of sample and is far less time-consuming than ELISA (De Crescenzo *et al.*, 2008). In the preliminary design of the SPR inhibition assay, the neoglycoconjugate, Neu5Gc-BSA was selected for immobilisation on the sensor chip surface. However, the carboxymethylated dextran surface of the CM5 sensor chip is highly negatively charged and this made it impossible to immobilise the strongly negatively charged Neu5Gc-BSA conjugate, due to charge-charge repulsion (data not shown) (O’Shannessy *et al.*, 1992). Therefore, neutravidin was selected as the immobilisation ligand and a pre-concentration study performed in order to select an efficient immobilization buffer (Figure 4.2.2.18). Neutravidin was successfully immobilised on the CM5 sensor chip surface (Figure 4.2.2.19) and the neutravidin/biotin interaction was used to capture the biotinylated-PAA-Neu5Gc conjugate (Figure 4.2.2.20). Due to the very low dissociation constant associated with the neutravidin/biotin interaction, the biotinylated conjugate is practically irreversibly bound to the sensor chip surface. This format had a number of distinct advantages. For example, all biotinylated molecules were in the same orientation and a high density of Neu5Gc was captured on the neutravidin surface. In addition, the biotinylated Neu5Gc sensor chip could better withstand harsh regeneration conditions. Furthermore, this chip could be stored for long periods of time with no significant loss in anti-sialic acid scFv binding capacity.

Importantly, SPR analysis also showed that the AE8 scFv bound Neu5Gc independent of its carrier molecule as it recognised Neu5Gc, in the context of two very different carriers, with structurally unrelated linkers, namely BSA-**linker1**-Neu5Gc and biotin-PAA-**linker2**-Neu5Gc. In addition, it also revealed that the AE8 recombinant scFv could bind the Neu5Gc conjugates when they were either captured on a solid phase or when they were free in solution (Figures 4.2.2.21\22). The SPR inhibition assay was used to determine the IC₅₀ concentration of the Neu5Gc-BSA conjugate. The IC₅₀ value was defined as the concentration of Neu5Gc-BSA conjugate that inhibited AE8 scFv binding to captured Neu5Gc-PAA by 50%. In the inhibition assay, the AE8 scFv is prevented from binding to captured Neu5Gc-PAA by the addition of variable amounts of solution-phase Neu5Gc-BSA conjugate. The lower limit of detection that can be achieved is directly influenced by the affinity of the AE8 scFv for the Neu5Gc conjugates. Hence, the greater the affinity of the scFv for the sialic acid conjugates the lower the IC₅₀ value that can be theoretically achieved. The SPR sensorgram in Figure 4.2.2.22 revealed a calculated AE8 IC₅₀ concentration of 5.7ng/mL for the Neu5Gc-BSA conjugate. This IC₅₀ value is extremely low for an anti-carbohydrate antibody. A large proportion of antibodies that bind carbohydrates are typically polyclonal IgMs with weak carbohydrate-binding affinities. In addition, lectins which are carbohydrate-binding proteins also have poor carbohydrate affinities with dissociation constants typically in the millimolar range (Ohlson *et al.*, 1997). In contrast, the AE8 scFv has an unusually high-affinity for sialic acid and this suggests that the parental B-cell clone(s) of this antibody was of the IgG class. Moreover, the generation of such a highly specific anti-sialic acid antibody in a recombinant format is exceedingly rare (Kaltgrad *et al.*, 2007).

A detailed SPR kinetic binding study of the interaction between the AE8 scFv and the Neu5Gc-BSA conjugate was performed (Figure 4.2.2.23). The goal was to accurately calculate the association and dissociation rate constants, as well as the equilibrium binding constant for the scFv/sialic acid interaction. For accurate and reproducible SPR kinetic data, a number of key experimental parameters required careful optimisation. One parameter was the amount of neutravidin to immobilise on the sensor surface, since this would ultimately affect the amount of captured biotinylated-PAA-Neu5Gc.

For accurate determination of the kinetic constants, a low ligand density is typically required, as this prevents the rebinding of the analyte during the dissociation phase. In addition, other factors like mass transport limitation (see below) and steric hindrance are also minimised (Hahnefeld *et al.*, 2004). A low density of neutravidin was immobilised on the sensor surface using short contact times and very low neutravidin concentrations. In addition, the biotinylated-PAA-Neu5Gc conjugate was significantly diluted and passed over the neutravidin immobilised sensor chip. A maximum response (R_{\max}) of 41.1 resonance units (RU) was achieved. The R_{\max} value describes the binding capacity of the sensor chip surface and depends on the ligand immobilisation levels. Typically, R_{\max} values of between 20-100RU are desirable for reliable determination of kinetic constants (Karlsson and Larsson, 2004).

A second factor considered in this experimental design was the effect of limited diffusion on the analyte/ligand interaction at the biosensor surface (often referred to as mass transport limitation). This can occur when the transport (diffusion) of an analyte to the vicinity of the sensor surface is slower than its binding to the ligand. Thus, the observed reaction rate reflects the transport rate on and off the sensor surface, rather than the intrinsic reaction rate. This effect can be minimised by using a low ligand density and increasing the flow rate at which the analyte injection is performed. A low ligand density reduces analyte consumption at the biosensor surface and therefore the supply of analyte to the sensor chip surface is not a limiting factor for the analyte ligand interaction. In addition, the use of fast flow rates ($\geq 30\mu\text{L}/\text{min}$) reduces the diffusion distance of the analyte from the bulk flow to the sensor chip surface. Importantly, mass transport limitations can result in calculated binding kinetic constants that are slower than the true rate constants (Karlsson and Larsson, 2004). Analysis of binding of the AE8 scFv with the immobilised PAA-Neu5Gc conjugate at different flow rates revealed no significant difference in the magnitude of the binding response (data not shown). Therefore, the kinetic assay did not appear to be mass transport limited.

A further parameter to be considered in the kinetic experiment design was avidity. In SPR, the interaction between the immobilised ligand and the injected analyte is presumed to follow a simple Langmuirian mode of binding that involves monovalent partners. In contrast avidity effects can cause deviations from a simple 1:1 binding model.

For example, a single analyte involved in multiple ligand binding interactions may cause the analyte/ligand complex to be artificially stabilised and therefore the observed apparent affinity is actually much larger than the true binding rate constant. Overall, avidity effects add a level of complexity to the biosensor surface-binding reaction that typically precludes accurate kinetic interpretation (Karlsson and Larsson, 2004; Katsamba *et al* 2006; Hahnefeld *et al.*, 2004; De Crescenzo *et al.*, 2008; Duverger *et al.*, 2010). Therefore, to exclude the effects of avidity, all scFv preparations were purified using a Phenomenex 3000 SEC column, equilibrated in PBS or HBS buffer. Analysis of the binding kinetics of the purified monomeric AE8 scFv fraction is shown in Figure 4.2.2.23. The quality of most of the sensor data is reasonable although a number of discrepancies can be observed. Visual inspection of the fitted overlay plots reveals differences in the observed and calculated data, particularly at the start of the association and dissociation phases. In general these differences are less pronounced for lower analyte concentrations. Analysis of the residual plot, which is a plot of the difference between the observed and calculated data, shows larger deviations in the association phase of the sensorgrams. The reason for this is unclear, however difficulties in obtaining extremely accurate concentration measurements of the purified AE8 scFv, may have negatively impacted on the determination of the association rate constants. The predicted R_{\max} value 39.7RU was slightly lower the actual R_{\max} value (41.1 RU). The χ^2 value which describes the goodness of fit should ideally be 1% or less of the theoretical R_{\max} , the calculated value was 1.5%. The relatively low χ^2 value obtained indicates that the binding model generated is accurate (Karlsson and Larsson, 2004). The k_a was $1.19 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$ and the k_d was $6.80 \times 10^{-3} \text{ s}^{-1}$ this gave a K_D of 57nM for the scFv/Neu5Gc interaction. The calculated kinetic values fall within the working range of the Biacore[®] 3000 biosensor ($k_a = 10^3 - 10^7 \text{ M}^{-1} \text{ s}^{-1}$, $k_d = 5 \times 10^{-6} - 10^{-1} \text{ s}^{-1}$ and $K_D = 10^{-4} - 2 \times 10^{-10} \text{ M}^{-1}$) (Jason-Moller *et al.*, 2006). Carbohydrate-protein interactions are typically characterised by intrinsically weak interactions (Arranz-Plaza *et al.*, 2002). Therefore, the observed nanomolar affinity of the AE8 scFv for the Neu5Gc neoglycoconjugate was unexpected. Moreover, only a handful of antibodies against Neu5Gc have been generated and most of those are not recombinant and recognise Neu5Gc in the context of gangliosides. In contrast, AE8 is a novel recombinant antibody fragment that has an unusual binding specificity for Neu5Gc neoglycocojugates that is in the nanomolar range.

In conclusion, this chapter describes in detail the multifaceted approaches used for the isolation of anti-sialic acid recombinant antibodies. In the initial approach, the non-immune Tomlinson I and J naïve libraries were screened against biotinylated-Neu5Gc-PAA. However, no anti-sialic clones were isolated from these libraries. In a second approach an “in-house” avian MPO phage display library was mined for anti-sialic acid scFvs using the same Neu5Gc conjugate. However, no specific sialic acid binders were isolated. In a third approach, novel sialic acid neoglycoconjugates were designed and commercially synthesised. These conjugates were used for avian immunisations (Neu5Gc-HSA) and phage display (Neu5Gc-BSA). This approach was successful and avian pAbs were identified that could competitively bind Neu5Gc in the context of neoglycoproteins and synthetic carriers. Subsequently, phage display libraries were constructed from the avian host. Biopanning with a novel Neu5Gc-BSA conjugate identified a large panel of putative anti-sialic acid scFvs. Further analysis revealed that only 10% of this panel recognised Neu5Gc in the context of both BSA and PAA. Furthermore, restriction digestion pattern analysis indicated that these novel anti-sialic scFvs had unique combinations of V_H and V_L DNA sequences. Cross-reactivity studies using different mono and disaccharide carbohydrate structures identified only two scFvs, AE8 and CC11 that had very strong binding to Neu5Ac-PAA and Neu5Gc-PAA. In addition, both these scFvs showed no significant binding to galactose-PAA, glucose-PAA or BSA. Therefore, these scFvs were sialic acid-specific. Unlike CC11, AE8 also reacted strongly with (Neu5Ac)₂-PAA. Moreover, the AE8 scFv had the strongest reactivity to all of the sialic acid structures.

Comparison of the deduced amino acid sequence alignment of AE8 and CC11, CDR V_H and V_L regions, showed that both clones were genetically different. AE8 had a longer CDRL3 and also contained more Arg residues in its CDRs. Both AE8 and CC11 had few amino acid differences in CDRH1, CDRH2 and CDRH3. This indicated that the conserved amino acids in the heavy chain CDRs may be important in sialic acid recognition. Analysis of the AE8 IMAC-purified protein preparation by SEC-HPLC, revealed the presence of scFv dimeric and monomeric species with estimated molecular sizes of 28kDa and 60kDa, respectively. These samples were analysed by SPR and the monomeric fraction was shown to have a lower SPR Neu5Gc-binding signal in comparison to its dimeric fraction.

A solution-phase sialic acid SPR based inhibition study revealed that the AE8 scFv had an IC_{50} of 5.7ng/mL for the Neu5Gc-BSA conjugate. In addition, an SPR kinetic study revealed that the monomeric AE8 scFv had a K_D of 57nM for the Neu5Gc-BSA. In contrast to the weak carbohydrate-protein interactions of other anti-carbohydrate antibodies, this is the first reported example of an anti-carbohydrate scFv that has nanomolar affinity for Neu5Gc-containing structures.

Chapter 5

Directed molecular evolution of the AE8 anti-sialic acid recombinant antibody

5.1 Introduction

This chapter outlines the application of directed molecular evolution for the creation of improved anti-sialic acid antibody fragments. It contains an in-depth description of the mutagenesis and functional selection protocols used to achieve antibody gene diversity. Furthermore, it describes in detail the isolation and characterisation of new mutant anti-sialic acid avian scFvs.

Directed molecular evolution is a powerful laboratory tool that is often used in biomolecular engineering. This biological optimisation process mimics Darwinian evolution and is used to both alter and optimise antibody function. The process involves two key steps (1) the generation of a large and genetically-diverse library of candidate mutants and (2) the identification of improved variants from within that library. In directed molecular evolution of proteins, iterative rounds of mutation and artificial screening are used to identify clones with high-fitness (e.g. superior antigen-binding characteristics) (Steipe, 1999).

Phage display is a rapid selection system that can be coupled to directed molecular evolution and is used to discriminate between different antibody variants. Importantly, this system has the advantage that at the molecular level, the nucleic acid of the antibody is directly linked to the encoded gene product. Therefore, antibody repertoires generated by DNA mutation and/or recombination methods can be displayed on the surface of filamentous phage and through biopanning (functional genetic selection), antibody variants that have improved properties may be isolated. Moreover, the identity of any selected protein variant can be directly determined by DNA sequencing (Barbas, 2001; O' Brien and Aitken, 2002).

Many different methodologies are available for the creation of random diversity (non-targeted mutagenesis) within protein libraries. These methods can be divided into two main groups: (a) diversity by recombination and (b) random diversity by disturbed DNA replication (Lai *et al.*, 2004; Stebel *et al.*, 2008). DNA recombination *in vitro*, as an evolutionary strategy, was first published by Stemmer in 1994. Stemmer named this method "DNA shuffling" (Stemmer, 1994). DNA shuffling, which is also known as "sexual PCR" or molecular breeding, is an *in vitro* DNA recombination method that is frequently used as a tool for protein evolution (Clackson and Lowman, 2007; Joern,

2003). In this method, an endonuclease, deoxyribonuclease I (DNase I), is used to cleave double-stranded target DNA into smaller-sized random fragments. After agarose gel purification, these DNA fragments are reassembled into a full-length sequence, using DNA polymerase and iterative cycles of denaturation, annealing and extension. In this PCR-like reaction, oligonucleotide primers are excluded. The purified digested DNA fragments prime replication of each other based on their homology (Figure 5.1.1). Library diversity is generated through recombination, where fragments from different parents anneal at a region of high sequence identity and are subsequently extended. Following the reassembly reaction, PCR amplification with primers is used to generate full-length chimeras suitable for cloning into an expression vector. In DNA shuffling, recombination occurs between substantially homologous but non-identical sequences (Kikuchi and Harayama, 2002).

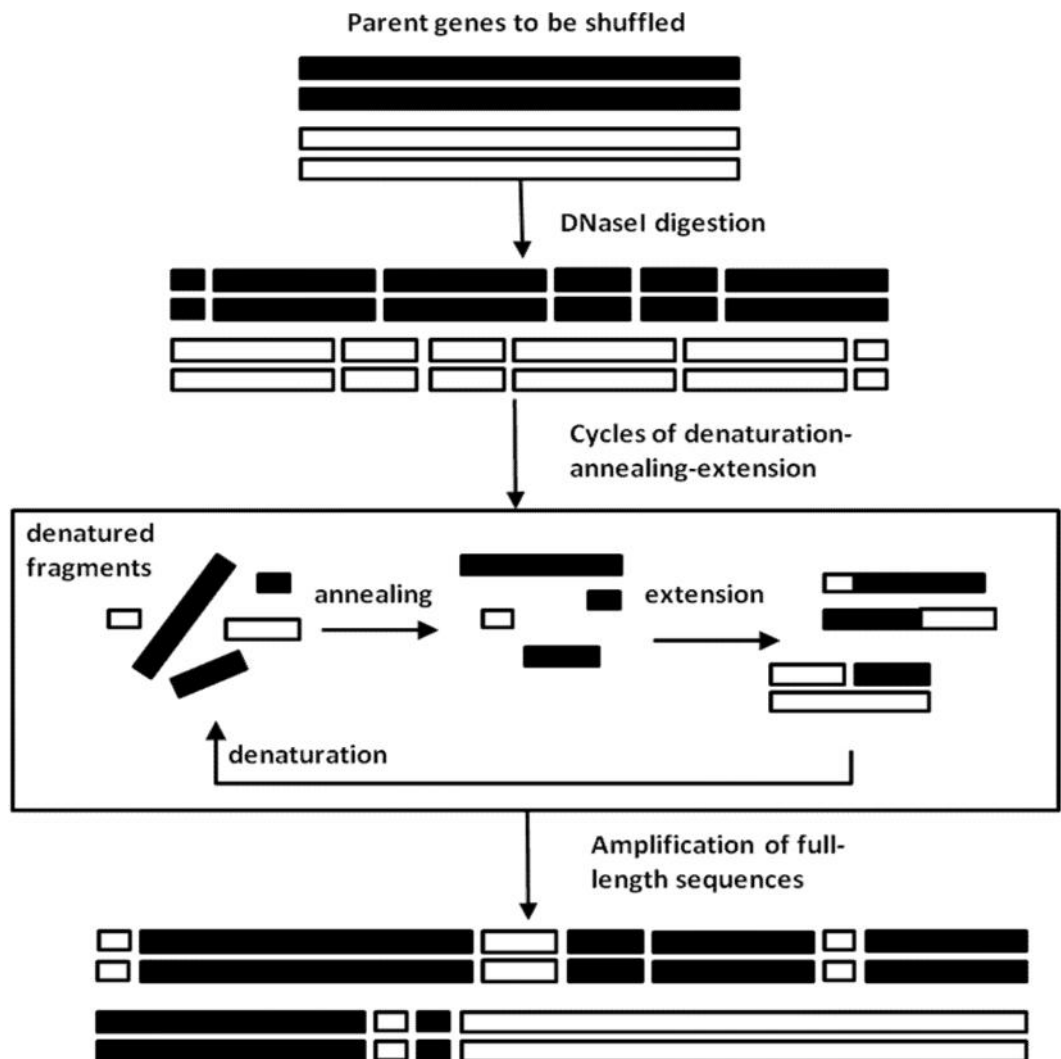


Figure 5.1.1 Schematic of DNA shuffling process.

Recombination methods take advantage of the fact that multiple “best” sequences can parent the next generation. Consequently, shuffling accelerates the isolation of multiple beneficial mutations that act synergistically. Moreover, deleterious mutations are eliminated (Vanhercke *et al.*, 2005). Antibody affinity may be significantly improved by applying a DNA shuffling approach. For example, Fermer and co-workers (2004) used DNA shuffling in combination with phage display to improve the affinity of an scFv clone (800 to 900-fold) that recognised a lung cancer marker (pro-gastrin-releasing-peptide). DNA shuffling can be applied to either the V_H genes or V_L genes or between V_H and V_L libraries (Fermer *et al.*, 2004).

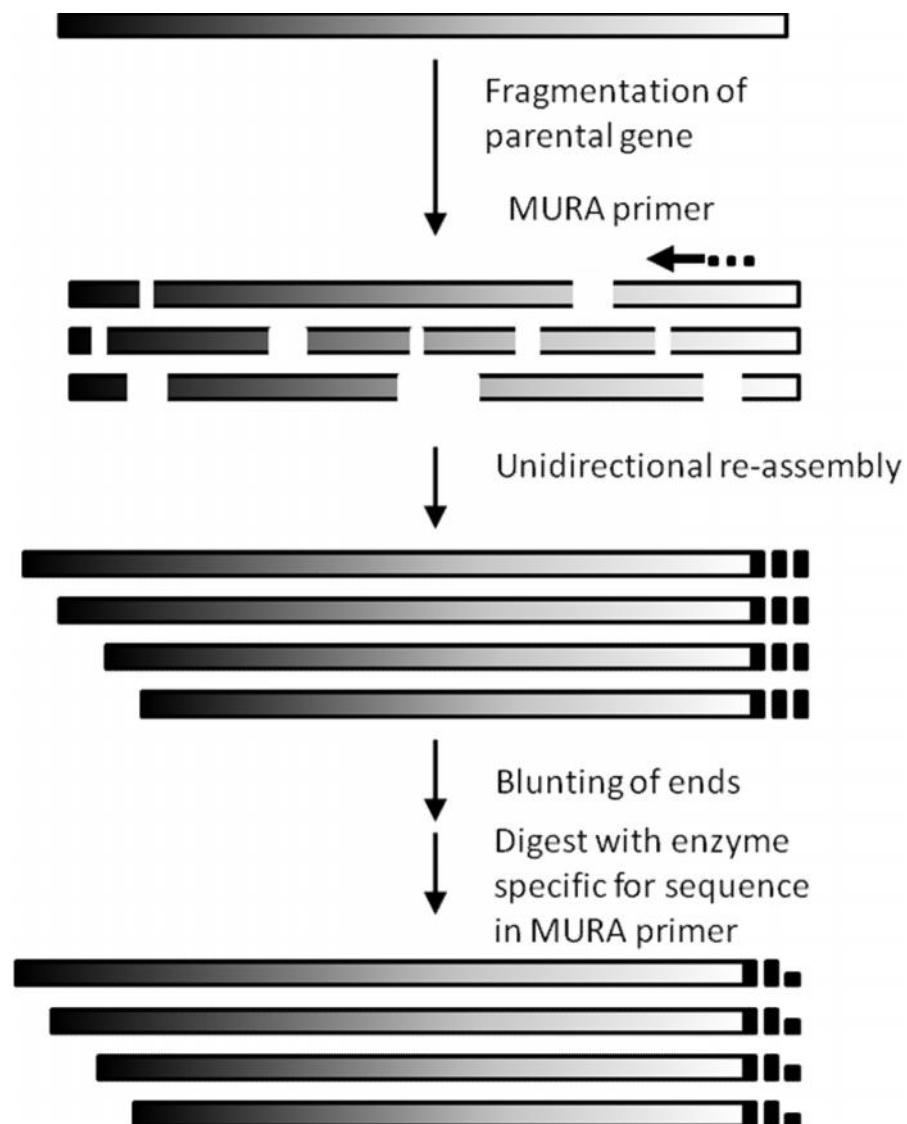


Figure 5.1.2 Schematic of Mutagenic and unidirectional re-assembly method (MURA).

A number of variations of the DNA shuffling technique have been developed. These methods have larger numbers of recombination events (crossover points) in regions of high sequence identity (Stebel *et al.*, 2008). Some examples include: mutagenic and unidirectional reassembly (MURA) and random chimeragenesis on transient templates (RACHITT) (Coco, 2003; Song *et al.*, 2002). The MURA protocol involves: (a) random fragmentation of the parental gene and fragment reassembly using a unidirectional primer, which contains an appropriate restriction site (b) separation of the fragments of interest by gel electrophoresis (c) formation of blunt and sticky termini and (d) ligation into an expression vector (Figure 5.1.2). Diversity is achieved by both DNA shuffling and incremental DNA truncation (Song *et al.*, 2002). In the RACHITT method (Figure 5.1.3), a parental top or bottom DNA strand containing dUTP is generated. This uracil DNA strand is used as a “transient template” to anneal target gene(s) fragments that were originally generated by *DNase I* digestion. A *Pfu* DNA polymerase is subsequently used to fill in and ligate gaps in the chimeric nucleotide sequence, and non-annealed 5' or 3' overhangs are removed. The uracil-containing strand is then eliminated by digestion with uracil-DNA glycosylase and the resulting chimeric strand is amplified by PCR (Coco, 2003). In all cases, these methods have the disadvantage that *DNase I* has to be removed from the reaction before reassembly.

Other homologous *in vitro* recombination techniques include: random priming recombination (RPR) and staggered extension process (StEP). In contrast to DNA shuffling, RPR and StEP do not utilise *DNase I* fragmentation. Rather, these methods recombine genes in a fragmentation-free PCR-based protocol (Chaparro-Riggers *et al.*, 2007). In RPR (Figure 5.1.4), random hexamer oligonucleotide primers are utilised to generate a large number of short DNA fragments complementary to different sections of the template sequence. The short DNA fragments prime one another based on homology, and are subsequently recombined and reassembled into full-length genes by repeated thermocycling in the presence of thermostable DNA polymerase (Shao *et al.*, 1998). The StEP method (Figure 5.1.5) is based on cross hybridisation of growing gene fragments during polymerase-catalysed primer extension. Unlike RPR, the StEP protocol uses very short annealing/extension steps. Following denaturation, primers anneal and extend in a step in which brief duration and suboptimal extension temperature limit primer extension.

In each cycle, partially extended primers randomly reanneal to different parent sequences, based on sequence complementarity. After multiple cycles, a full-length library of novel recombinant sequences is generated (Zhao *et al.*, 1998).

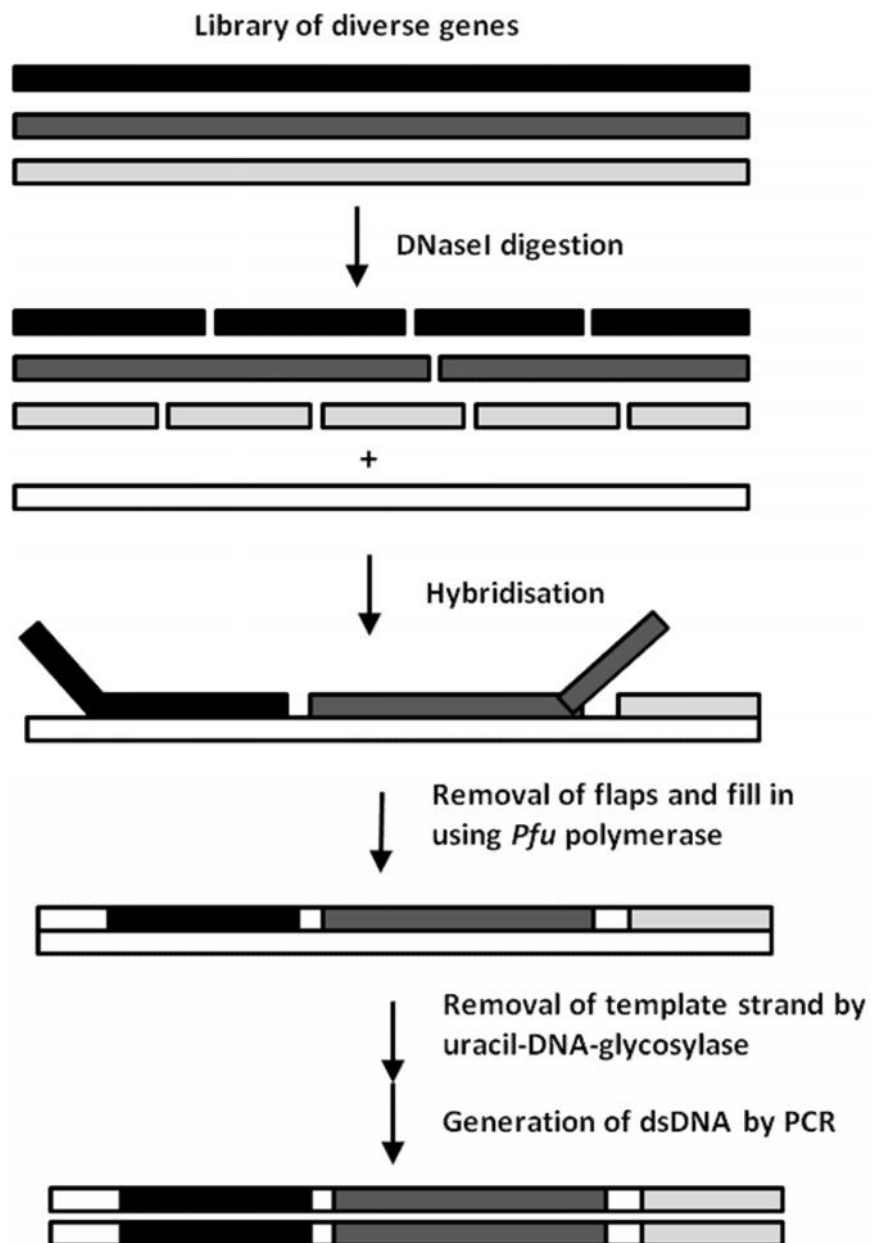


Figure 5.1.3 Schematic of the random chimeragenesis on transient templates (RACHITT) process.

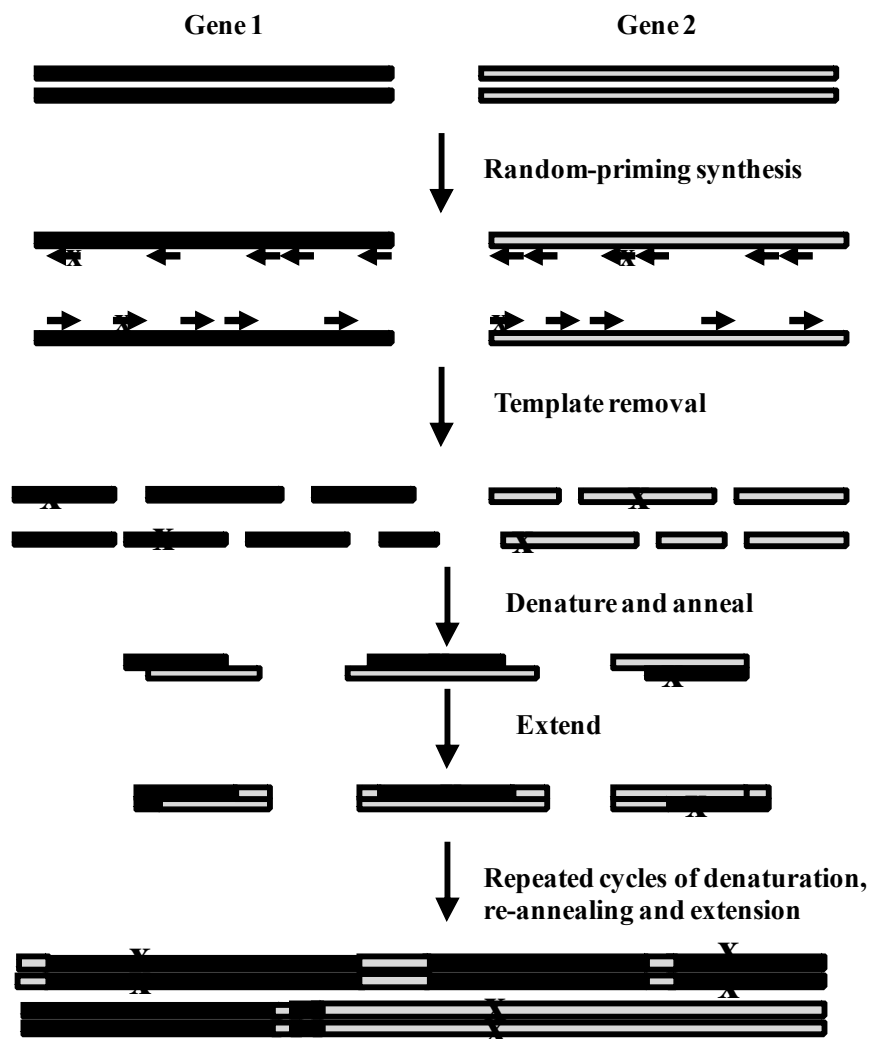


Figure 5.1.4 Schematic of random priming in vitro recombination (RPR).

Non-homologous *in vitro* recombination techniques make it possible to construct hybrid progeny even when the parental genes have little or no sequence homology. This process is useful as it allows the exploration of inaccessible yet functional relevant regions in the sequence space. In addition, it enables the generation of protein structural diversity which may not exist in nature (Tong *et al.*, 2010). For example, the method of degenerate oligonucleotide gene shuffling (DOGS) uses a group of carefully-designed complementary degenerate primers to individually amplify out conserved motifs found in the candidate genes (Figure 5.1.6). In the re-assembly procedure, chimeric fragments are recombined by using overlap-extended products. In addition, the library of fragments can be put together at different ratios, generating many biased libraries containing no parental genes (Gibbs *et al.*, 2001).

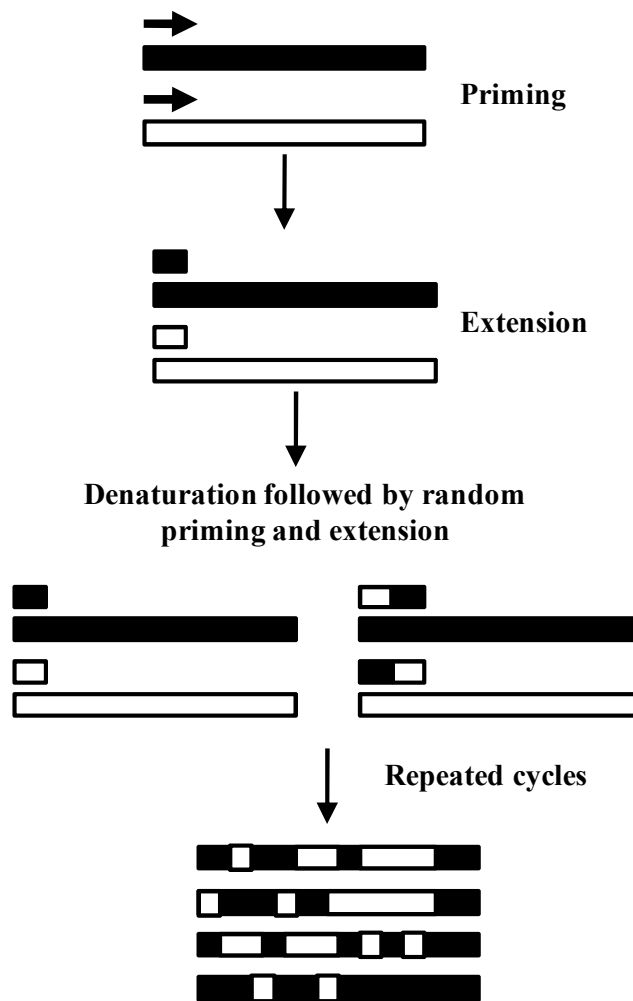


Figure 5.1.5 Illustration of StEP recombination for two gene templates.

Chain shuffling is an *in vitro* recombination-based method that exploits the natural architecture of the antibody. In this process of non-targeted diversification, a functional antibody chain is recombined (“shuffled”) with a repertoire of complementary chains. For example, a single variable heavy chain gene can be recombined with a variable domain library of light chain genes, or *vice versa*. The resulting combinatorial sub-library contains a vast number of antibodies with potentially different antigen-binding combinations (Ponsel *et al.*, 2011). Interestingly, during the process of natural antibody affinity maturation, only a restricted set of light and heavy chain pairings occur (Saini *et al.*, 2003).

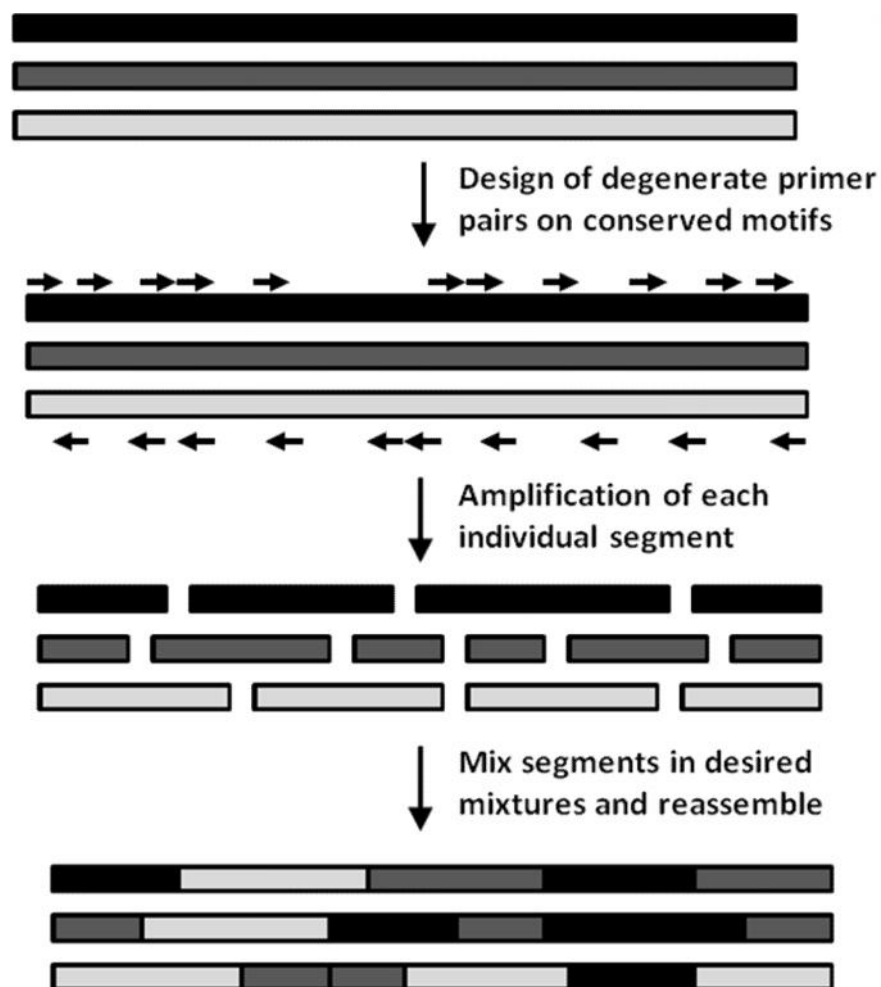


Figure 5.1.6 Schematic of Degenerate oligonucleotide gene shuffling (DOGS).

In contrast, *in vitro* chain-shuffling can generate unique and non-natural variable heavy- and light-chain combinations, which further add to the diversity of the shuffled antibody sub-library (Ponsel *et al.*, 2011). The generation of variable-chain sub-libraries requires the pre-isolation of an antigen-binding antibody. Therefore, prior to shuffling, a lead antibody candidate from a phage library screening campaign is isolated. Ultimately, the goal is to combine the best features of the parental antibody with other desirable traits from the shuffled library, such that all desirable characteristics are amalgamated into a single antibody. An optimised pairing of light and heavy chains can produce an improved antigen-binding site which does not match that of the original antibody. Thus, chain-shuffling can be used to optimise an antibody's affinity, as well as the elimination of unwanted cross-reactivity, whilst retaining the desired specificity. In addition, a novel antibody combining site may be produced that has a broader antigen recognition spectrum (Kang *et al.*, 1991; Ohlin *et al.*, 1996; Marks, 2004; Christensen *et al.*, 2009).

The second group of methods used to evolve protein function have in common some form of disturbed DNA replication. In these methods, sequence diversity is created in the form of point mutations, insertions or deletions. For example, DNA can be treated with physical and chemical mutagens such as UV irradiation or alkylating agents. The alkylation agent ethylenemethanesulphonate (EMS) can alkylate the guanidine residues of DNA and causes nucleotide transitions (e.g. GC to AT) in the DNA sequence (Stebel *et al.*, 2008). However, a disadvantage of the chemical mutagenesis method is that the mutagens used are often highly toxic and/or carcinogenic and they therefore, require extreme care during laboratory handling. Furthermore, they can produce extremely low frequencies of mutations and only certain types of mutations are generated (Steipe, 1999; Cirino *et al.*, 2003; Bradbury, 2010).

Other random mutagenesis strategies successfully used to randomise antibody genes include the propagation of the target gene in mutator strains. For example, the *E.coli* K12 mutator strains (XL1-red from Stratagene) are deficient in three DNA repair pathways, resulting in a 5,000-fold increase in mutation, compared to the wild-type strain. However, the bacterial mutator strain method can suffer from a low frequency of point mutations under standard conditions. In addition, a cultivation period longer than 24 hours is often required for introducing multiple mutations. Furthermore, mutator strains can mutate any genetic material, and this can include the cells' own genomic DNA, and/or the plasmid DNA. Mutation in regions other than the foreign gene of interest may cause unexpected deleterious effects to the host cell (Wang *et al.*, 2006; Agrawal and Wang, 2008; Lou and Marks, 2010).

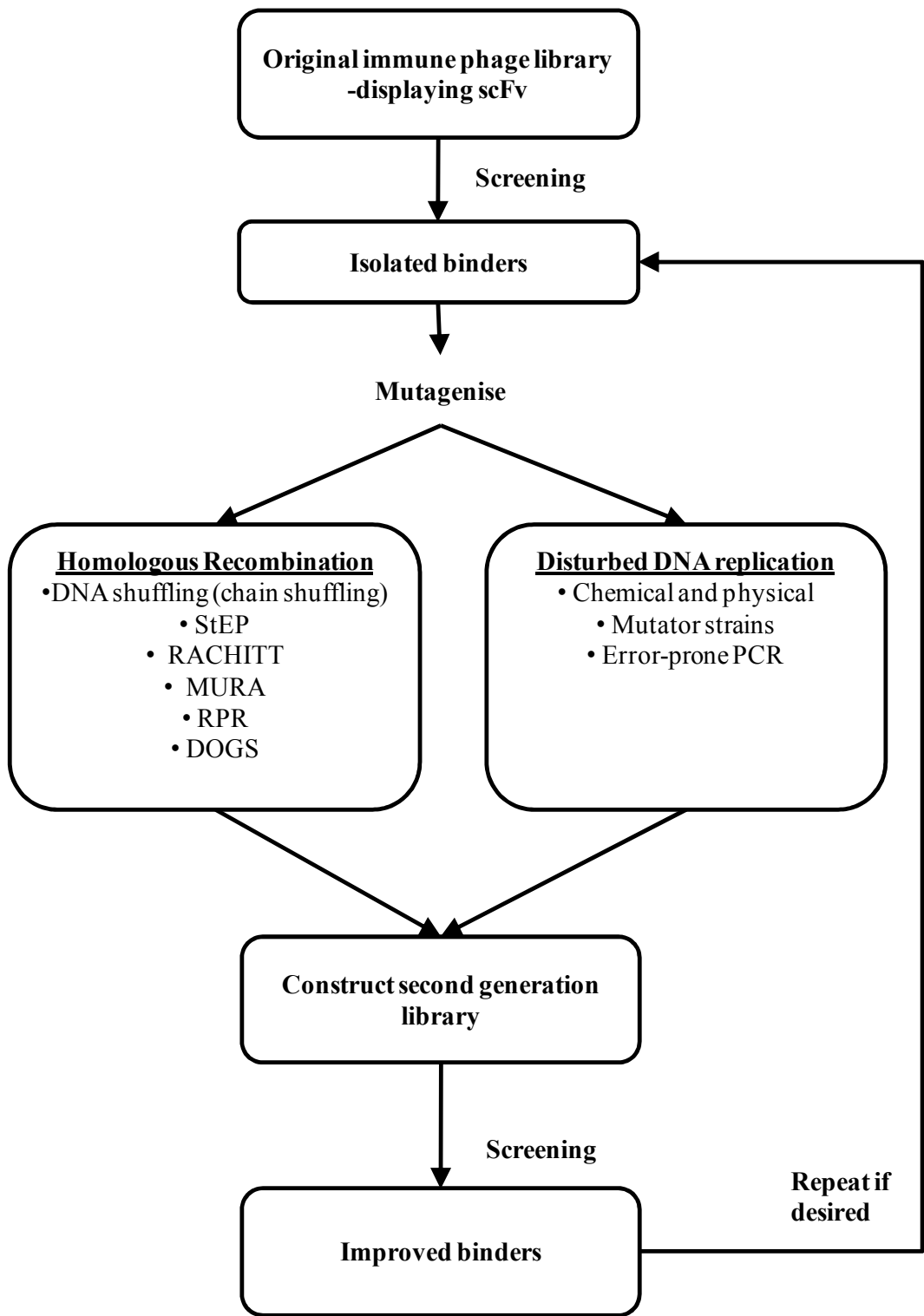


Figure 5.1.7 Overview of *in vitro* molecular evolution process for recombinant antibody manipulation.

Due to its simplicity and versatility, error-prone PCR has emerged as the most common technique used for random mutagenesis of antibody genes. Error-prone PCR is a DNA replication method that introduces random copying errors into the PCR process. This is achieved by using imperfect, and thus mutagenic or 'sloppy' reaction conditions. For example, if PCR is performed using a DNA polymerase that lacks proofreading activity, in combination with suboptimal PCR reaction conditions, then the random addition of incorrect nucleotides into newly synthesised daughter DNA strands can occur. Since the PCR reaction has an increased error rate randomly mutated PCR products are generated. This method has the following advantages: mutations can be precisely targeted to the amplified fragment, the error rate is easy to control, the technique is quick and easy to set up and the use of hazardous chemicals is avoided. Furthermore, the desired level of mutagenesis can be obtained in a relatively short period of time (Cadwell and Joyce, 1992; Martineau, 2002; Pritchard *et al.*, 2005; Vanhercke *et al.*, 2005; Stebel *et al.*, 2008; Ponsel *et al.*, 2011).

The selection of the appropriate reaction conditions is an important consideration during the setup of the error-prone PCR reactions. A key goal is to control the DNA mutation frequency. A mutation rate that is too high would most likely yield too many inactive clones. In contrast, if the mutation frequency was too low, then the amount of wild-type antibodies would be too high and the diversity of the sample too low (Melnikov and Youngman, 1999; Wang *et al.*, 2006; Martineau, 2002). The concentrations of both MgCl_2 and MnCl_2 are important variables in the error-prone PCR reaction. The addition of these divalent cations (Mn^{2+} and Mg^{2+}) at higher concentrations in comparison to standard PCR results in an increase in the nucleotide misincorporation rate of *Taq* DNA polymerase. For example, Mn^{2+} is often used at a final concentration of 0.5mM and this increases the error rate (approximately five-fold) of *Taq* DNA polymerase, by diminishing template specificity. Similarly, increasing the concentration of Mg^{2+} also generates a higher error rate (2-3-fold) by stabilising non-complementary base pairs. However, a manganese or magnesium concentration which is too high, often results in a substantial reduction in the efficiency of the PCR reaction. Another way to modify the PCR mutation rate is to alter the ratio of the four different dNTPs. These nucleotides may be present in the PCR reaction in either equimolar or unbalanced amounts.

A common method is to use dATP and dGTP at low concentrations and dTTP and dCTP at high concentrations. The unbalanced mixture of dNTPs promotes the incorporation of non-complementary nucleotides by *Taq* DNA polymerase into the growing DNA strands. Other factors that influence misincorporation events during PCR include; the initial DNA template copy number and the number of amplification cycles. The use of lower concentration of template in a PCR reaction necessitates a higher number of PCR cycles, which in turn results in more target duplications and consequently higher mutation frequencies in the amplified DNA. Finally, the type of DNA polymerase used in the PCR reaction will also affect the rate of mutagenesis (Cadwell and Joyce, 1992; Martineau, 2002; Fujii, 2004; Pritchard *et al.*, 2005; Vanhercke *et al.*, 2005; Stebel *et al.*, 2008; Rasila *et al.*, 2009; Tong *et al.*, 2010; Ponsel *et al.*, 2011).

The use of randomisation techniques such as chain-shuffling and error-prone PCR in combination, rather than in isolation, has the advantage of more closely mimicking the *in vivo* antibody maturation process. Moreover, a combination of these methods is often used in directed molecular evolution of antibody genes. Therefore, in this work, both error-prone PCR and chain shuffling were used to generate antibody variants with improved sialic acid-binding function (Tong *et al.*, 2010; Ponsel *et al.*, 2011).

5.1.1 Chapter outline

This chapter describes the application of directed molecular evolution for the generation of affinity-improved anti-sialic acid recombinant antibodies. The AE8 anti-sialic acid recombinant scFv was *in vitro* affinity matured, using directed molecular evolution and phage display. Two different techniques were selected to generate antibody variants with improved sialic acid binding function. They include light chain-shuffling and error-prone PCR.

The heavy chain gene from the AE8 scFv was randomly paired with a mutagenised anti-sialic acid cDNA library of light chain genes (light chain shuffled). The amplified antibody variable genes were assembled by SOE-PCR and ligated into the Barbas pComb3XSS vector (Barbas, 2001). The mutagenised and light chain shuffled phage library, was screened using two different biopanning strategies. In the standard method, library phage were biopanned against a single antigen (Neu5Gc-**linker1**-OVA). In contrast, the second method employed a negative (immunodepletion) biopanning strategy, in which the phage library was initially biopanned against **linker1**-OVA and subsequently biopanned against Neu5Gc-**linker1**-OVA. A large proportion of the tested antibody fragments bound to the sialoglycoconjugate. The capacity of a subset of these scFvs to cross-react with other sialic acid- containing structures was investigated.

SPR 'off-rate' ranking identified a reduced panel of novel mutant anti-sialic acid scFvs. One clone (E15 scFv) was selected for further study. Nucleotide and deduced amino sequences analysis of E15 and the parental scFv, AE8 revealed that these two scFvs contained amino acid differences in their CDRH-2, CDRL-3 and linker regions. Lastly, a solution-phase SPR inhibition assay showed that the mutant E15 scFv exhibited an improvement in sialic acid binding of approximately 3.5-fold compared to AE8.

5.2 Results

5.2.1 Amplification of the avian light chain library using error-prone PCR conditions

A directed molecular evolutionary strategy using random mutagenesis by error-prone PCR was selected for V_L gene diversification of the anti-sialic acid scFv library. Mutations were deliberately introduced into gene sequences using suboptimal PCR reaction conditions and a low-fidelity recombinant DNA polymerase. In addition, the PCR reaction contained Mn^{2+} , unbalanced dNTP concentrations and a relatively high concentration of $MgCl_2$. The ultimate goal was to mimic the process of somatic mutation and to increase the sequence diversity of the light chain library. The successful amplification of the avian V_L library under error-prone PCR conditions is shown in Figure 5.2.1.

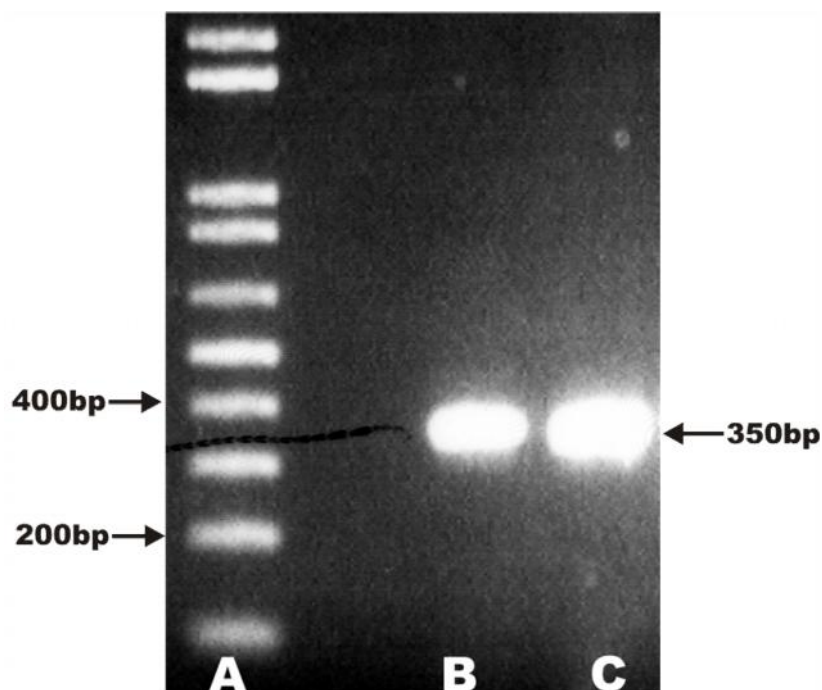


Figure 5.2.1 PCR amplification and mutagenesis of the light chain variable genes (~350bp amplicon) from the cDNA of a HSA-Neu5Gc-immunised chicken. For V_L amplification, the PCR reaction contained the following: 5ng of the anti-sialic acid scFv library, 60pmol of CSCHo-FL and CSCG-B, 1x PCR buffer, 0.5mM $MnCl_2$, 7mM $MgCl_2$, 200 μ M dATP, 200 μ M dGTP, 1000 μ M dCTP, 1000 μ M dTTP and 0.5 μ l RedTaq[®] DNA polymerase. Lane A shows a 1 kilobase DNA molecular weight marker and lanes B and C shows the amplification of the V_L genes in duplicate.

5.2.2 PCR amplification of the variable heavy chain gene from the anti-sialic AE8 scFv

In the next stage, the V_H gene of the anti-sialic acid AE8 clone was amplified using standard PCR conditions. Successful amplification of the avian, AE8 V_H gene is shown in Figure 5.2.2.

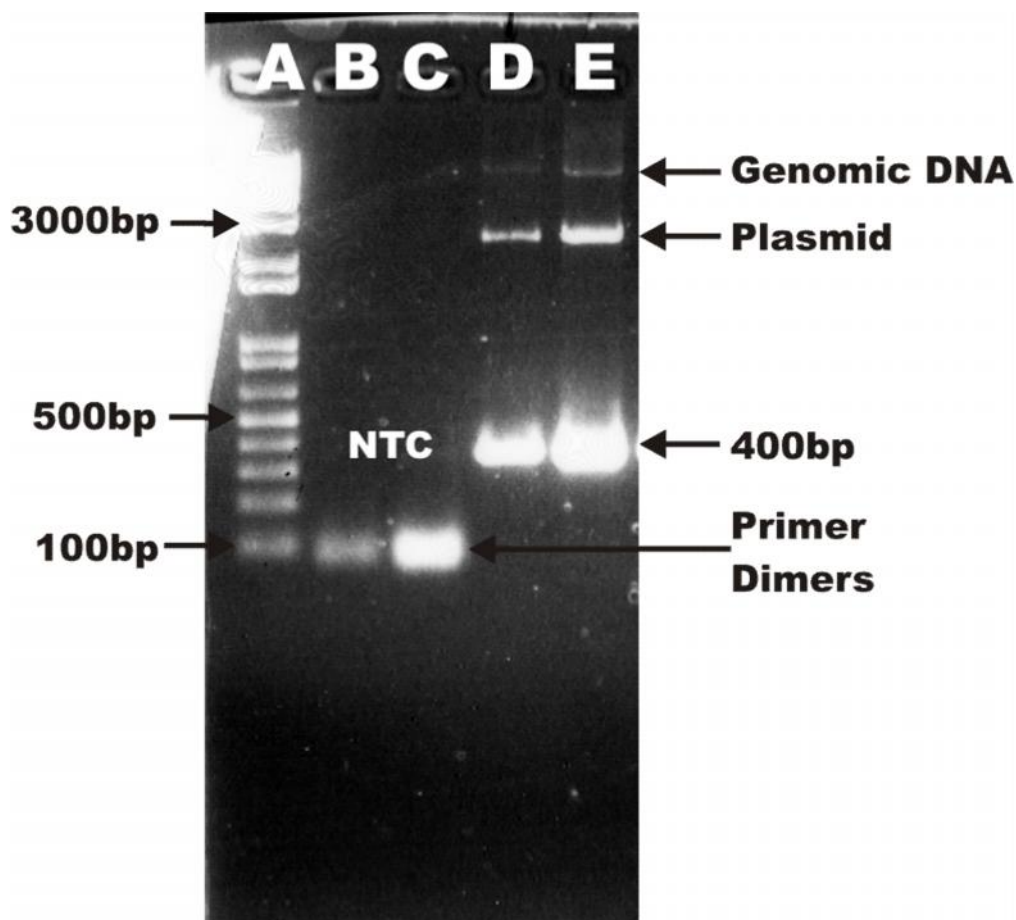


Figure 5.2.2 PCR amplification of the variable heavy chain gene from the plasmid DNA of clone AE8. The variable heavy V_H chain amplicon is approximately 400bp in length. The PCR reaction contained the following: 10ng of AE8 plasmid DNA, 60pmol of CSCHo-FL and CSCG-B, 5x PCR Buffer 1.5mM $MgCl_2$, 200 μ M dNTPs and 0.25 μ L Taq DNA polymerase. Lane A shows a 1 kilobase DNA molecular weight marker, lanes B and C are no template controls (NTC) and lanes D and E show the V_H gene amplification from the AE8 plasmid.

5.2.3 SOE of AE8 variable heavy and avian light chains.

Following the successful amplification of the avian variable domain genes, single chain Fv fragments were assembled using a long 18-amino acid peptide glycine-serine linker in the V_L -GQSSRSSSSGGGGSGGGG- V_H orientation by a two-fragment SOE-PCR. A long linker length was chosen in order to promote folding of the polypeptides into monomeric scFv. However, in this second round of PCR not only was the target product (~750bp) amplified, but two non-specific PCR products of between 500-650bp were also amplified (Figure 5.2.3).

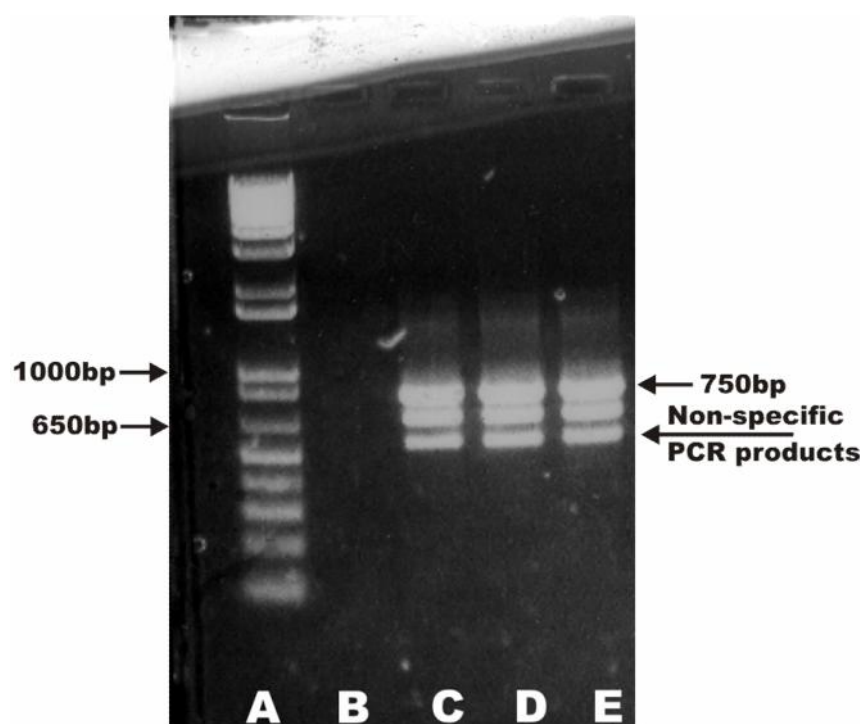


Figure 5.2.3 Initial SOE-PCR optimisation of the V_H and V_L fragments. For SOE-PCR, a 100 μ l PCR reaction contained the following: 100ng of the V_L and V_H purified products, 100 pmol of CSC-F and CSC-B, 10x PCR Buffer, 1.5mM $MgSO_4$, 200 μ M dNTPs and 1 μ L Platinum® Taq DNA Polymerase. The PCR was performed with the following cycling conditions: 5min at 94°C (initial denaturation), followed by 30 cycles of 30 sec at 94°C (denaturation), 30 sec at 59°C (annealing), 2min at 72°C (extension) and the reaction was terminated after 10min at 72°C (final extension). Lane A shows a 1 kilobase DNA molecular weight marker. Lanes C, D and E show the amplification of V_H and V_L fragments to produce an SOE product of approximately 750bp. In addition, two non-specific PCR products of between 500-650bp were also amplified

5.2.4 Chicken SOE of variable heavy and light chains.

Optimisation of the SOE-PCR was achieved by increasing the annealing temperature of the PCR reaction (57°C to 59°C) and reducing PCR extension time (2min to 1min).

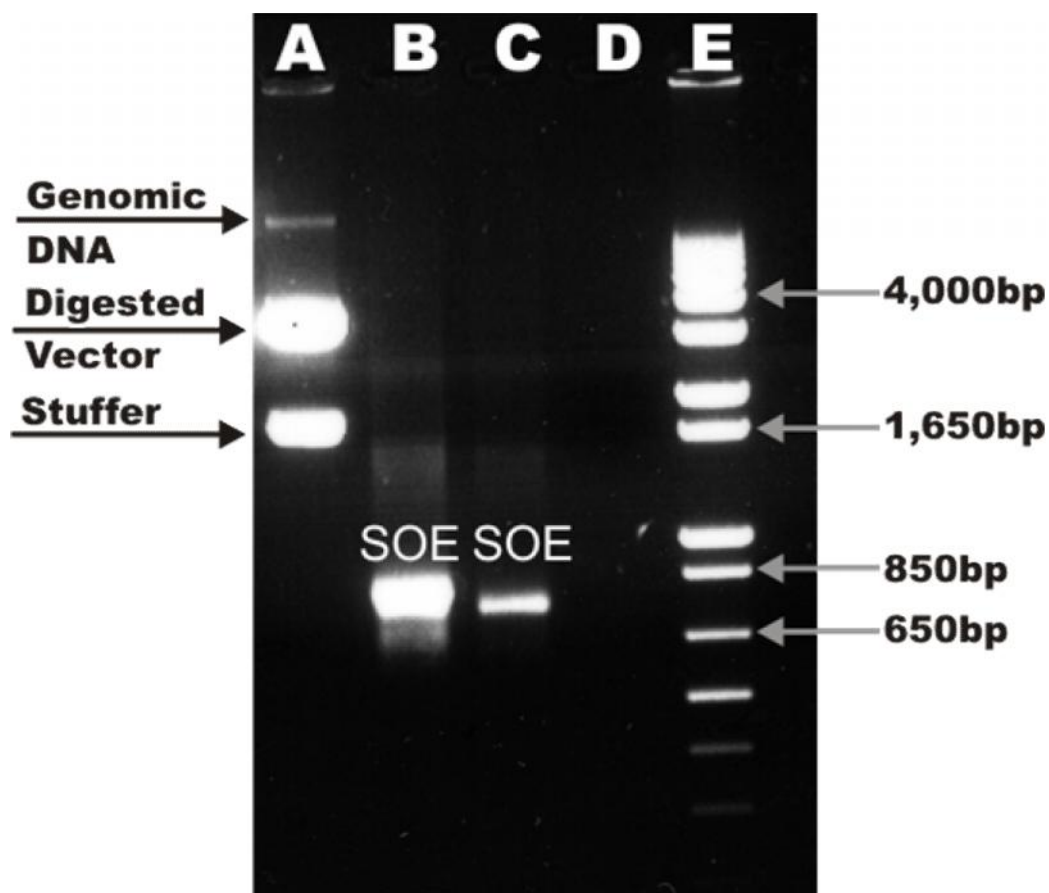


Figure 5.2.4 *SOE-PCR of V_H and V_L fragments.* For SOE-PCR, a 100 μ L PCR reaction contained the following: 100ng of the V_L and V_H purified products, 60pmol of CSC-F and CSC-B, 10x PCR Buffer, 1.5mM $MgSO_4$, 200 μ M dNTPs and 1 μ L Platinum® Taq DNA Polymerase. Lane A represents the digestion of the pComb3XSS vector to produce a stuffer fragment of ~1600bp and the double-cut vector of ~3400bp. Lanes B (high concentration) and C (low concentration) show the amplification of V_H and V_L fragments to produce an SOE product of approximately 750bp. Lane D is a blank lane. A 1 kb DNA molecular weight marker was added to Lane E.

5.2.5 Light chain-shuffled library construction and biopanning.

The SOE product was amplified on a large-scale and subsequently concentrated using ethanol precipitation. The PCR product was cloned into the pComb3XSS vector using the restriction enzyme *Sfi*I. The ligated product was transformed into electrocompetent XL1-Blue cells, by electroporation using the protocol described in section 2.2.13. The transformed, anti-sialic acid light-chain shuffled scFv library had a size of 5.2×10^7 cfu/mL. The AE8 light-chain shuffled library was biopanned using two new conjugates (Neu5Gc-**linker1**-Ovalbumin (OVA) and **linker1**-OVA)). In addition, a Neu5Ac-**linker1**-OVA conjugate was also synthesised. All multivalent conjugates were manufactured by Carbohydrate Synthesis (U.K.) using the same synthesis scheme as described previously (Section 2.2.1). Furthermore, each conjugate contained a 13 carbon atom spacer arm, which was identical in length and structure to the linker that was present in both the HSA and BSA neoglycoconjugates (Neu5Gc-**linker1**-HSA and Neu5Gc-**linker1**-BSA). The Neu5Gc-**linker1**-OVA conjugate contained 21 monosaccharide units of Neu5Gc per mole of OVA protein and was used for biopanning. The molar ratio of **linker1** on the **linker1**-OVA conjugate surface was 20 units/mole of OVA protein. This conjugate was used for subtractive selection during biopanning. In contrast, the Neu5Ac-**linker1**-OVA conjugate was used for ELISA and SPR-based analysis. This neoglycoconjugate contained 17 monosaccharide units of Neu5Ac per mole of OVA protein.

Two different methods were employed during the biopanning procedure. In the standard method, phage pools were biopanned against immobilised Neu5Gc-**linker1**-OVA antigen, as described in Section 2.2.16. In contrast, in the second method a subtractive selection step was used during biopanning. In this negative selection method, two different immunotubes were coated with different antigens, the first tube contained **linker1**-OVA and the second tube contained Neu5Gc-**linker1**-OVA. During biopanning, phage were initially incubated on the **linker1**-OVA tube and subsequently transferred to the Neu5Gc-**linker1**-OVA tube. This process was repeated during the different rounds of biopanning. The aim was to deplete (remove) those irrelevant phage that bound to the **linker1**-OVA components and to retain only the Neu5Gc binding phage. Ultimately, the goal was to isolate clones that only recognised the sialic acid component of the conjugate and not the spacer arm or protein elements.

Table 5.2.5., shows the phage input and output titres over the 5 rounds of biopanning of the light-chain shuffled, avian anti-sialic scFv library. Phage output titres may give an indication of the success of the biopanning strategy, since increasing numbers of phage should remain bound to the antigen and this should result in higher output titres. Table 5.2.5, shows that for the depletion and standard method, phage numbers are erratic. Therefore, it was not possible to say which biopanning strategy was more successful.

Table 5.2.5 The phage input and output titres over the 5 rounds of biopanning of the light-chain shuffled, avian anti-sialic scFv library. Phage output from the first round of biopanning (output 1) was split and panned either against Neu5Gc-OVA or (linker1-OVA / Neu5Gc-OVA). Linker1-OVA- depleted phage pools were initially added to an immunotube that contained an excess of the linker1-OVA conjugate. The phage were then added to a second immunotube that contained the Neu5Gc-OVA conjugate. In the second round of biopanning when the linker1-OVA depletion strategy was applied, phage output titres were extremely low (1×10^2 cfu/mL). Consequently, no linker1-OVA depletion was performed for round 3 of biopanning.

Biopanning round	Colony forming units/mL
Input 1	1×10^{14}
Output 1	1×10^9
Input 2	1×10^8
Output 2	1×10^5
Output 2 <u>linker1</u> -OVA depleted	1×10^2
Input 3	1×10^{10}
Output 3	1×10^5
Input 4	1×10^{11}
Input 4 <u>linker1</u> -OVA depleted	1×10^9
Output 4	1×10^5
Output 4 <u>linker1</u> -OVA depleted	1×10^4
Input 5	1×10^{11}
Input 5 <u>linker1</u> -OVA depleted	1×10^{10}
Output 5	1×10^5
Output 5 <u>linker1</u> -OVA depleted	1×10^4

5.2.6 Light chain-shuffled avian library polyclonal phage ELISA.

The precipitated input phage particles from the various rounds of biopanning were tested for sialic acid-binding using a polyclonal phage ELISA (Section 2.2.17). The scFv-displaying phage particles were detected using a HRP-conjugated mouse anti-M13 antibody (GE Healthcare Life Sciences). After the first round of Neu5Gc-OVA biopanning (P1), the phage output was split and only panned against Neu5Gc-OVA (no depletion) or **linker1**-OVA and Neu5Gc-OVA (**linker1**-OVA depleted). Non-depleted phage were added directly to an immunotube that contained Neu5Gc-OVA. In contrast, depleted phage were first added to an immunotube that contained an excess of **linker1**-OVA conjugate. The phage were then added to a second immunotube that contained the Neu5Gc-OVA conjugate. The largest enrichment of putative anti-sialic acid polyclonal phage pools was observed in the fourth and fifth rounds of selections (Figure 5.2.6).

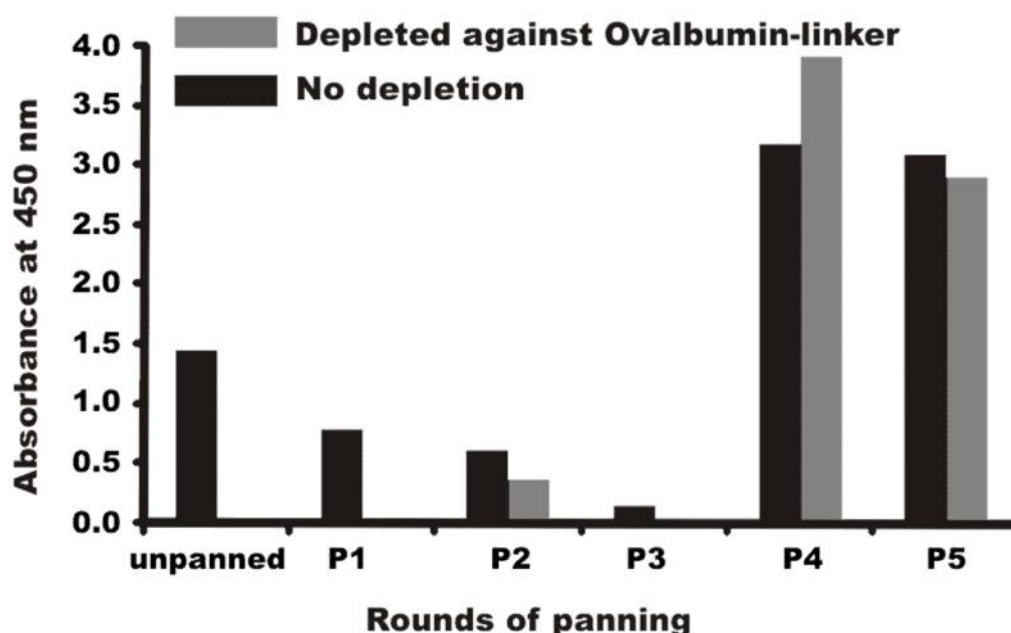


Figure 5.2.6 Polyclonal phage ELISA involving the phage pools of the unpanned library and the phage outputs from each of the successive rounds of biopanning. Bound phages were detected with an anti-M13-HRP-conjugated antibody. **Linker1**-OVA depletion was not performed in the third round of biopanning (P3) due to low phage output titres in the second round (P2) of biopanning. In rounds four and five of biopanning a sharp increase in the absorbance signal is seen and this suggests the presence of a considerable number of sialic acid-specific scFv-harboring phage.

5.2.7 Soluble expression and direct ELISA of light chain-shuffled anti-Neu5Gc scFv clones.

A soluble monoclonal ELISA was performed on clones that originated from the immunodepletion biopanning strategy (Section 2.2.18.). Figures 5.2.7 and 5.2.8 show that the majority of clones had ELISA signals which were more than three-fold higher than that of the negative control proteins, BSA or OVA. Thus, this panel of scFvs has the capacity to bind Neu5Ac or Neu5Gc-containing structures.

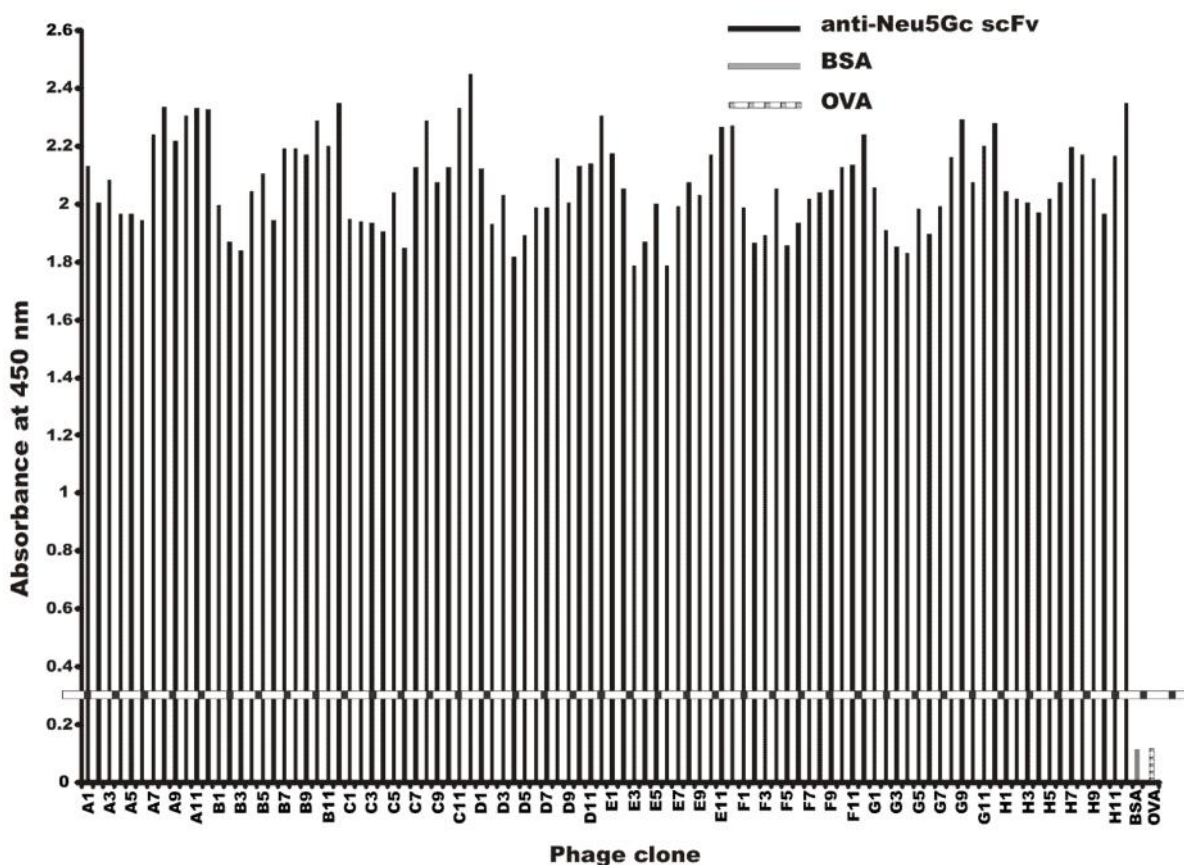


Figure 5.2.7 Soluble expression and direct ELISA of avian anti-Neu5Gc scFvs isolated from the light-chain shuffled library. The ELISA coating concentration of Neu5Gc-OVA was 10 μ g/mL and the blocking agent was 3% (w/v) BSA in PBS (pH 7.2). Ninety-six individual colonies were randomly selected and analysed by ELISA from the fourth and fifth rounds of biopanning. The ELISA threshold (three times the background) is marked with a horizontal line. The total average binding of all clones to BSA and OVA is also shown. All of the expressed clones showed significant binding to the Neu5Gc-OVA conjugate.

5.2.8 Soluble expression and direct ELISA of light chain-shuffled anti-Neu5Ac scFv clones.

The same ninety-six individual colonies that were previously analysed for Neu5Gc-OVA binding (Figure 5.2.7) were also assessed by direct ELISA for their capacity to bind the Neu5Ac-OVA conjugate. As demonstrated in Figure 5.2.8, all of the clones tested, had significant binding to the Neu5Ac-OVA conjugate. In addition, no significant binding to the negative control proteins (BSA and OVA) was observed. Therefore, these clones had the ability to recognise both forms of sialic acid in the context of the neoglycoconjugate.

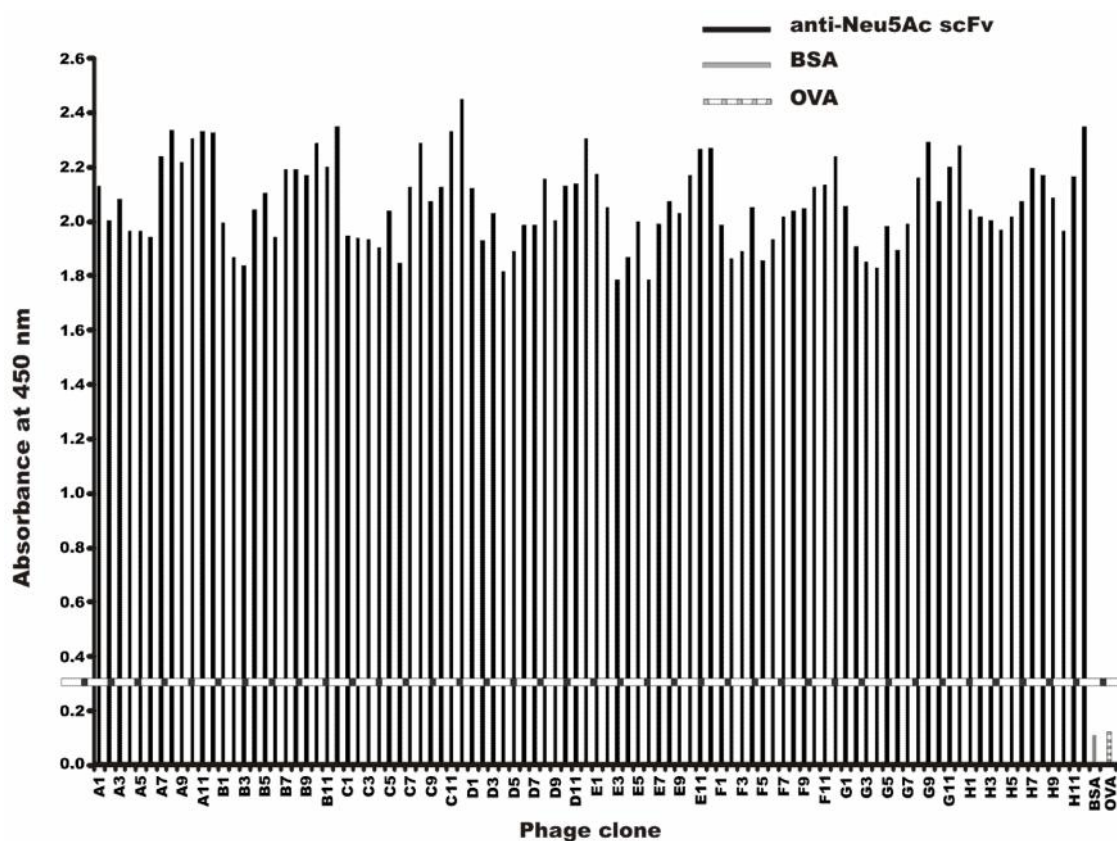


Figure 5.2.8 Soluble expression and direct ELISA of avian anti-Neu5Ac scFvs isolated from the AE8 light-chain shuffled library. The ELISA coating concentration of OVA-Neu5Ac was 10 μ g/mL and the blocking agent was 3% (w/v) BSA in PBS (pH 7.2). The ELISA threshold (three times the background) is marked with a horizontal line. The total average binding of all clones to BSA and OVA is also shown. All of the expressed clones showed significant binding to the Neu5Ac-OVA conjugate

5.2.9 Analysis of putative anti-sialic scFvs with the natural glycoproteins: fetuin, bovine submaxillary mucin and α_1 -acid glycoprotein.

In an attempt to isolate scFvs which recognised sialic acid when displayed on a protein in a natural context, four “naturally occurring” glycoproteins, namely bovine fetuin, bovine submaxillary mucin, α_1 -acid glycoprotein and porcine thyroglobulin, were selected for solid-phase ELISA analysis. These model-glycoproteins are sialylated and have been widely used to study carbohydrate-binding proteins (Aplin and Wriston 1981; Brossmer *et al.*, 1992; Matsuno and Suzuki, 2008; Thobhani *et al.*, 2009). In total, 384 different *E. coli* (TOP10F') colonies were selected from biopanning (non-depleted and **linker1**-OVA depleted) rounds 4 and 5. All clones were induced using IPTG and their supernatant-containing scFv was analysed by ELISA. However, none of the clones tested showed any significant binding to any of the four natural glycoproteins (data not shown).

5.2.10 Mining of the light chain-shuffled scFv library for Neu5Gc-specific binders

The same group of 384 scFv clones, which were screened against the natural glycoproteins, were also tested for binding reactivity towards the neoglycoconjugates, Neu5Gc-**linker1**-OVA and Neu5Ac-**linker1**-OVA. The aim was to isolate scFvs that only recognised Neu5Gc. However, no scFv clones were identified that were Neu5Gc positive and Neu5Ac negative. In addition, no scFv clones were found that were Neu5Ac positive and Neu5Gc negative. Rather, the majority of scFvs (>95%) bound to both Neu5Ac- and Neu5Gc-**linker1**-OVA neoglycoconjugates (data not shown).

5.2.11 Isolation of anti-sialic scFvs that do not bind the **linker1-OVA conjugate.**

A total of ninety-six clones that were previously shown to bind to Neu5Gc-OVA and Neu5Ac-OVA conjugates (Figures 5.2.7 and 5.2.8) were selected for **linker1**-OVA ELISA analysis. These scFv clones originated from biopanning rounds 4 and 5 of the **linker1**-OVA depletion strategy. The goal was to identify scFv clones that only recognised the Neu5Gc/Neu5Ac component of the conjugate and not the spacer arm or protein elements. Interestingly, when no **linker1**-OVA depletion steps were employed, all of the scFv clones analysed showed significant binding to the **linker1**-OVA conjugate (Figure 5.2.9).

In contrast, when the **linker1**-OVA immunodepletion steps were used, 10 of the 96 scFv clones tested did not show any significant binding reactivity to the **linker1**-OVA conjugate (Figure 5.2.10). Therefore, this anti-sialic acid scFv subgroup appeared to bind to Neu5Gc, independent of the linker used for sialic acid attachment.

5.2.12 Light chain-shuffled anti-sialic scFv clones biopanned with no immunodepletion step.

ELISA analysis was performed on clones from the AE8 light-chain shuffled library using the standard biopanning method with no **linker1**-OVA immunodepletion steps. All of the scFv clones had significant binding to the Neu5Gc-OVA and Neu5Ac-OVA neoglycoconjugates (data not shown). However, all ninety-six scFv clones tested had significant binding reactivity to the **linker1**-OVA conjugate (Figure 5.2.9).

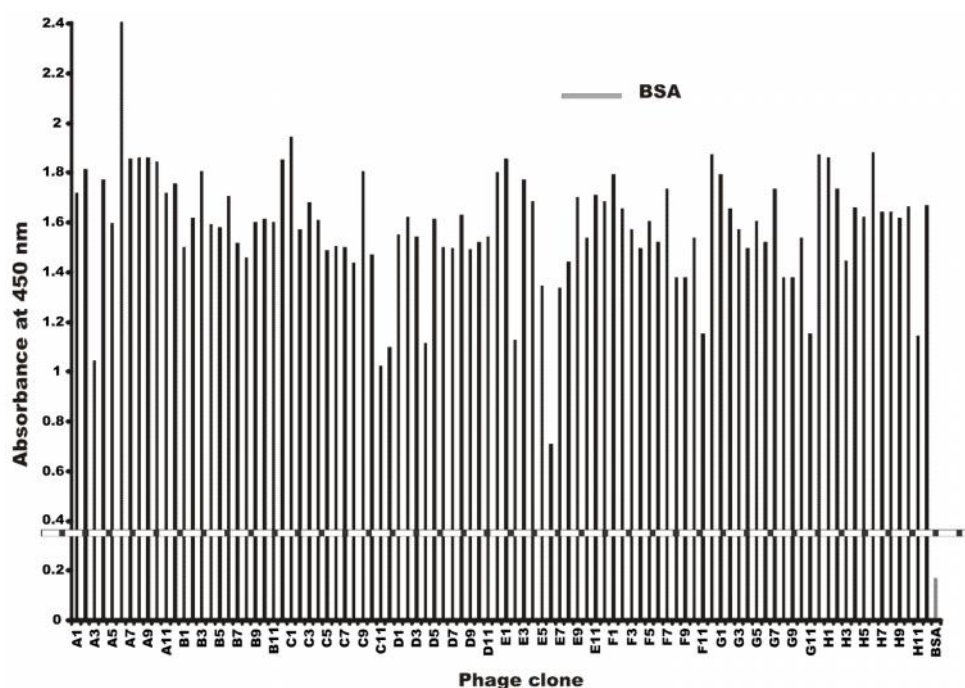


Figure 5.2.9 ELISA analysis of anti-sialic acid clones from rounds four and five of biopanning that were not OVA-linker depleted. The ELISA coating concentration of OVA-Linker was 25µg/mL and the blocking agent was 3% (w/v) BSA in PBS (pH 7.2). The linker-OVA binding threshold was arbitrarily set (3 times the BSA binding background) and is marked with a horizontal line. All of the ninety-six scFv clones appear to bind to the **linker1**-OVA. The total average binding of all scFv clones to BSA is also shown. All of the expressed scFv clones showed significant binding to the **linker1**-OVA conjugate.

5.2.13 Light chain-shuffled anti-sialic scFv clones from the linker1-OVA biopanning depletion strategy

In total, ninety-six individual clones were selected from the fourth and fifth rounds of biopanning using the linker1-OVA depletion strategy. These ninety-six clones were previously shown to bind both the Neu5Gc-OVA and Neu5Ac-OVA conjugates (Figures 5.2.7 and 5.2.8). Figure 5.2.10 demonstrates that of the 96 clones tested, only 10 clones did not show any significant binding reactivity to the linker1-OVA conjugate.

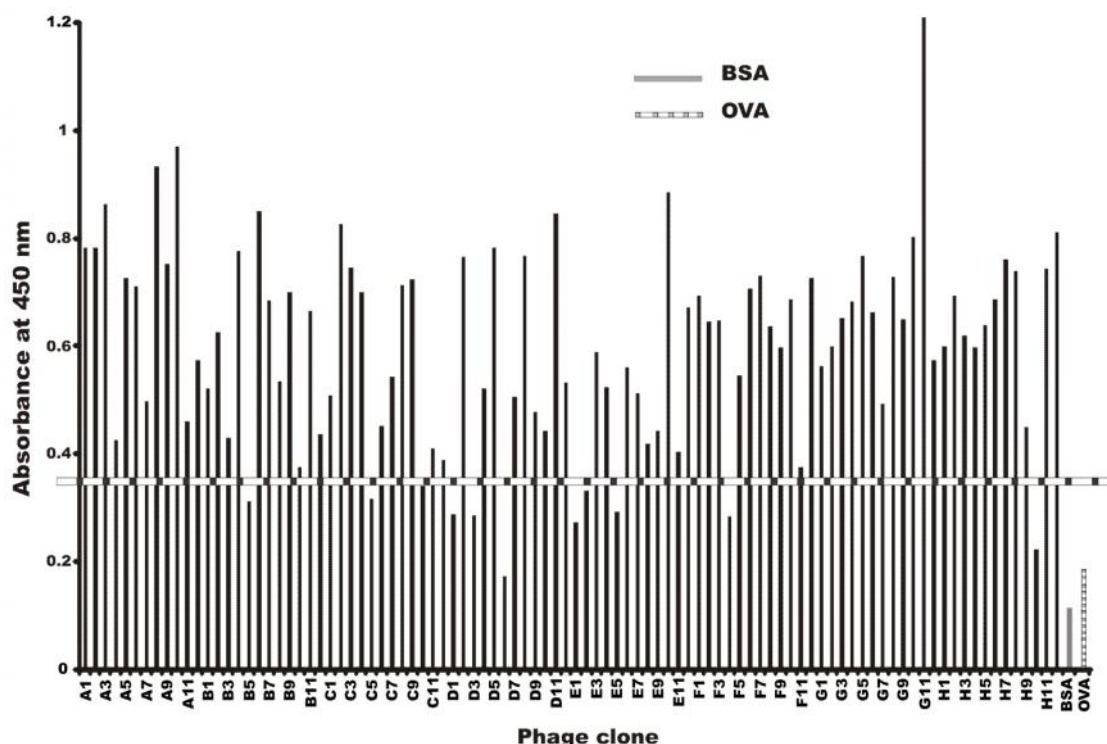


Figure 5.2.10 Identification of soluble anti-sialic acid clones from the AE8 light-chain library that bind the Neu5Gc/Ac-OVA conjugates but do not bind the linker1-OVA conjugate. The ELISA coating concentration of the linker1-OVA conjugate was 25 μ g/mL and the blocking agent was 3 % (w/v) BSA in PBS (pH 7.2). The ELISA threshold was set arbitrarily (3 times the BSA binding background) and is marked with a horizontal line. Clones above this threshold were defined as linker1-OVA binding positive, whereas clones below the threshold were defined as linker1-OVA binding negative. The total average binding of all clones to BSA and OVA is also shown. Only ten of the expressed clones did not bind significantly to the linker1-OVA conjugate.

5.2.14 Direct ELISA of light chain-shuffled anti-sialic scFv clones that recognise Neu5Gc in the context of two different sialoglycoconjugates: Neu5Gc-linker1-OVA and Neu5Gc-linker2-PAA.

ELISA-based analysis was used to assess the ability of soluble scFvs to recognise Neu5Gc in the context of a second conjugate, Neu5Gc-linker2-PAA. In this assay, neutravidin was used to capture the biotinylated-PAA-linker2-Neu5Gc conjugate. Analysis of the original ten clones, which were Neu5Gc/Ac-linker1-OVA positive and linker1-OVA negative, revealed that 4 clones (D14, D34, E15 and F46), recognised Neu5Gc in the context of the linker2-PAA-backbone (Figure 5.2.11). Thus, this scFv subset had the ability to recognise Neu5Gc in the context of a different linker. Therefore, these scFvs did not simply bind to the linker region, but rather, they had a real binding affinity for sialic acid.

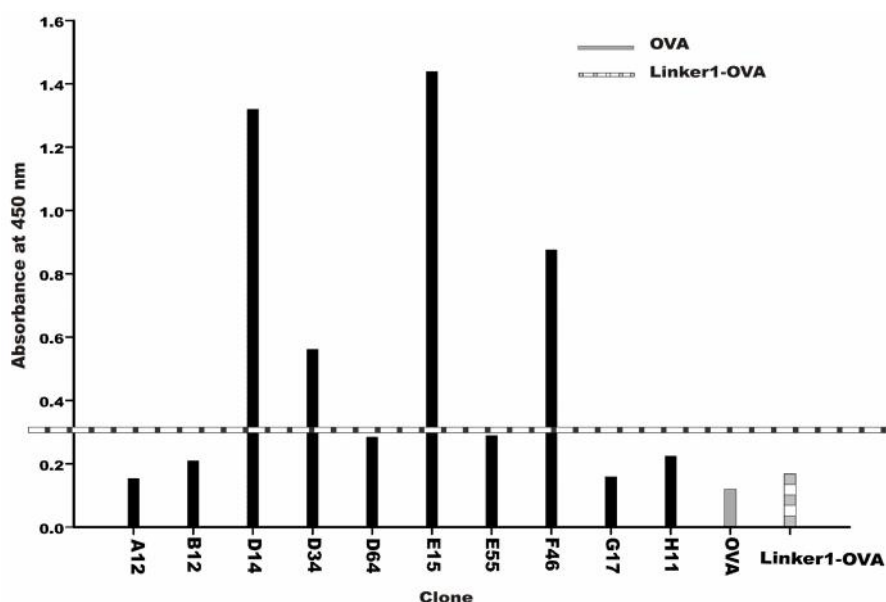


Figure 5.2.11 Soluble anti-sialic acid clones that recognise Neu5Gc in the form of Neu5Gc-linker2-PAA. Each ELISA well was coated with 5µg/mL of neutravidin and 100µL of biotinylated-PAA-Neu5Gc (25µg/mL). Periplasmic fractions were diluted 1:5 in 1% (w/v) BSA, PBS (pH 7.2) and 100µL of each clone applied to the ELISA plate. A secondary rat anti-HA monoclonal antibody conjugated with peroxidase (1:2000) was used to detect scFvs that were specific for the sialic acid moiety of the Neu5Gc-linker2-PAA conjugate. The ELISA threshold (3 times the OVA binding background) is marked with a horizontal line and the total average binding of all clones to OVA and linker1-OVA is also shown.

5.2.15 SPR ‘off-rate’ analysis of the light chain-shuffled anti-sialic scFv clones: D14, D34, E15 and F46.

Each of the avian anti-sialic clones; D14, D34, E15 and F46, were ranked on the basis of their respective ‘off-rate’ kinetics. ‘Off-rate’ selection is a tool that is widely employed to characterise the binding properties of different antibody fragments. In general, there is a correlation between the apparent ‘off-rate’ of an antibody and its affinity. Typically, antibodies that have similar association rates (k_a) but slower dissociation rates (k_d), have higher affinities since $K_D = k_d / k_a$. Therefore, antibodies which have improved affinities may be identified by measuring their ‘off-rates’ (k_d). Importantly, the association rate constant is concentration dependent, whereas the dissociation rate constant is concentration independent. Consequently, measurements of antibody ‘off-rates’ are not affected by differences in sample concentrations and therefore it is possible to measure ‘off-rates’ using an SPR instrument such as the Biacore[®] 3000, without purifying the antibody fragments.

For ‘off-rate’ SPR analysis, neutravidin was immobilised on the dextran surface of a Biacore[®] CM5 chip. A biotinylated PAA-linker2-Neu5Gc conjugate was passed over the chip surface and subsequently captured by neutravidin. Each scFv was passed over the chip surface to check for binding to the sugar. As a negative control, each scFv was also passed over a neutravidin-only surface that had no biotinylated sugar. Prior to ‘off-rate’ ranking analysis, a neutravidin pre-concentration buffer study identified a sodium acetate buffer of pH 4.6 and this was employed for immobilisation of neutravidin to the biosensor chip surface (data not shown). Standard amine coupling chemistry was used to covalently attach the amine groups of neutravidin to the carboxyl groups of the dextran CM5 biosensor chip. A level of approximately 21,600 RU of covalently attached neutravidin and ~1,200 RU of captured biotinylated-PAA-Neu5Gc was achieved (data not shown).

Preparations of crude antibody bacterial lysates from each of the avian anti-sialic clones: D14, D34, E15 and F46 were ranked on the basis of their respective ‘off-rate’ kinetics using the Biacore[®] 3000 instrument. Figure 5.2.12 shows the reference subtracted sensorgrams of each of the respective clones. For comparison purposes, bacterial lysate from the AE8 anti-sialic acid scFv was also tested in the same Biacore[®] 3000 run.

Each sensorgram was doubly reference subtracted in order to remove bulk shifts due to injection artifacts and system noise. The E15 and Ae8 scFvs had the slowest ‘off-rates’, whereas, F46, D14 and D34 antibodies had increased dissociation rates and consequently, these scFvs had faster ‘off-rates’. In comparison to the wild-type clone Ae8, the mutant clone, E15, appeared to have higher binding affinity for the Neu5Gc-**linker2**-PAA conjugate. Furthermore, this experiment confirmed that the anti-sialic acid scFvs were able to recognise Neu5Gc, independent of the linker used for sialic acid attachment. Importantly, this shows that these antibody fragments have a real binding affinity for sialic acid.

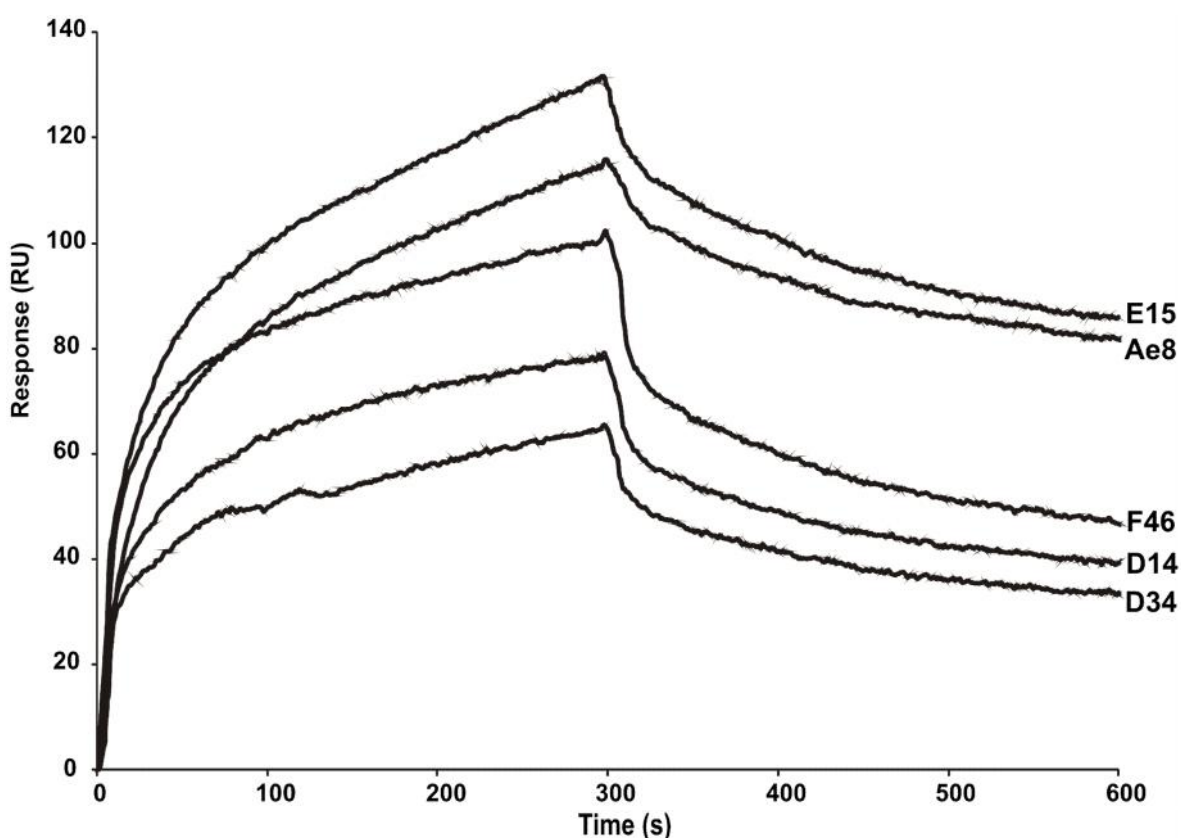


Figure 5.2.12 SPR ‘off-rate’ analysis of the interaction between recombinant anti-sialic scFvs and the Neu5Gc-**linker2**-PAA conjugate. Sensorgrams show the interactions between antibody material present in periplasmic extracts of a subgroup of the light chain-shuffled scFv library (E15, F46, D14 and D34).

This scFv subgroup was selected on the basis of their previous ELISA-scores (Figure 5.2.10). The Ae8 anti-sialic acid scFv was also included in this analysis for comparison purposes. Periplasmic fractions from each scFv clone were diluted 1:5 in HBS buffer (pH 7.4) that contained 12 mg/mL BSA and 12 mg/mL of CM-dextran.

Each scFv solution was passed over flow cells 1 and 2 of the Neu5Gc sensor chip at 10 μ L/min for 6 minutes. Flow cell 1 was used as a reference cell and contained a 'neutravidin-only' surface. In contrast, flow cell 2 contained biotinylated-PAA-Neu5Gc conjugate that was covalently attached to neutravidin. In order to remove systematic anomalies, a blank run consisting of a zero scFv sample was also subtracted from each of the sensorgrams.

5.2.16 Analysis of candidate avian anti-sialic clones with a solution phase SPR-based inhibition assay.

Each of the mutagenised recombinant avian anti-sialic scFvs was further characterised by a solution- phase SPR-based inhibition assay. The goal was to identify only those candidates which had the ability to bind the sialoglycoconjugate in solution. The inhibition assay is often used for hapten analysis. In this assay format, the measured response is inversely related to the concentration of analyte in the sample. The avian anti-sialic scFv clones were prevented (inhibited) from binding to immobilised Neu5Gc-PAA by the addition of a fixed amount of solution-phase Neu5Gc-OVA conjugate. A concentration of 10 μ g/mL of the sialoglycoconjugate was chosen for the SPR-based inhibition study. Previous Biacore[®] 3000 analysis had shown that, when the AE8 scFv (1:5 dilution in lysate) was incubated with 10 μ g/mL of Neu5Gc-OVA conjugate, complete inhibition of the SPR signal occurred.

Periplasmic fractions from each of the avian anti-sialic acid scFv clones were diluted 1:5 in HBS buffer (pH 7.4), containing 12mg/mL BSA and 12mg/mL of CM-dextran. Each scFv lysate sample was subsequently pre-incubated, with and without the Neu5Gc-OVA conjugate, at 37 $^{\circ}$ C for 30min. The scFv solutions were passed over flow cells 1 and 2 of the Neu5Gc sensor chip at 10 μ L/min for 6 minutes. Bound scFv was dissociated with a 30 second pulse of 10mM NaOH and the baseline restored with an injection of HBS running buffer over the surface. Figures 5.2.13a/b illustrates the reference-subtracted SPR sensorgrams of each of the respective scFvs. The E15 and AE8 scFvs showed complete inhibition at a concentration of 10 μ g/mL of the Neu5Gc-OVA conjugate (Figure 5.2.13a, Top). The D34 clone showed only slight inhibition with the same concentration of sialoglycoconjugate (Figure 5.2.13a, Bottom).

Furthermore, the D14 and F46 anti-sialic acid clones did not show full inhibition at the 10 μ g/mL concentration 9 (Figure 5.2.13b). Therefore, the E15 anti-sialic acid scFv was selected for further study.

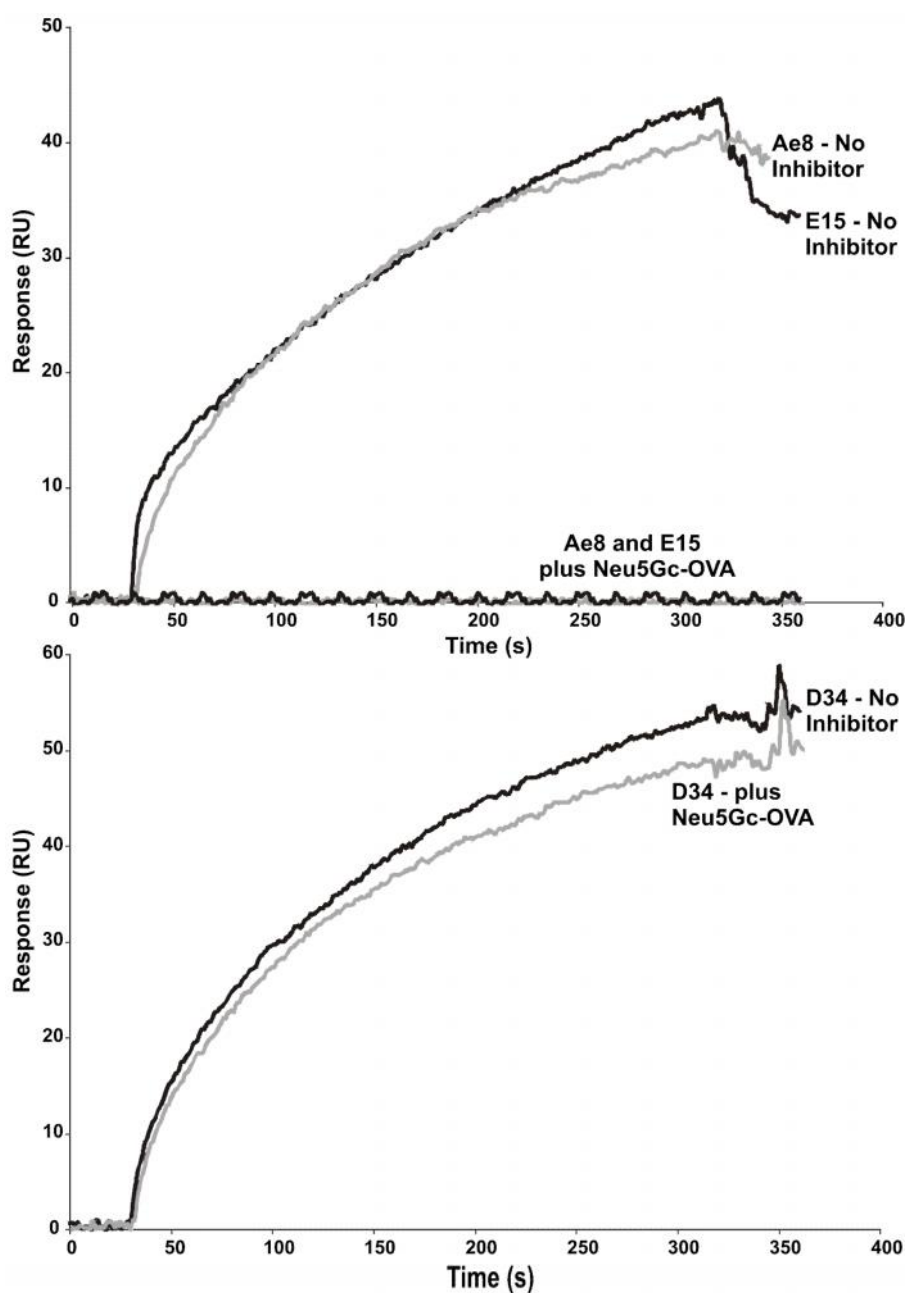


Figure 5.2.13a *Solution-phase SPR-based Neu5Gc-OVA inhibition of the AE8 and E15 anti-sialic acid scFvs.* Each scFv sample was incubated at 37°C for 30min either in the presence or absence of 10 μ g/ml Neu5Gc-OVA sialoglycoconjugate. The scFv solutions were passed over flow cells 1 and 2 of the Neu5Gc sensor chip at 10 μ L/min for 6 minutes. Flow cell 1 was used as a reference cell and contained a neutravidin-only surface.

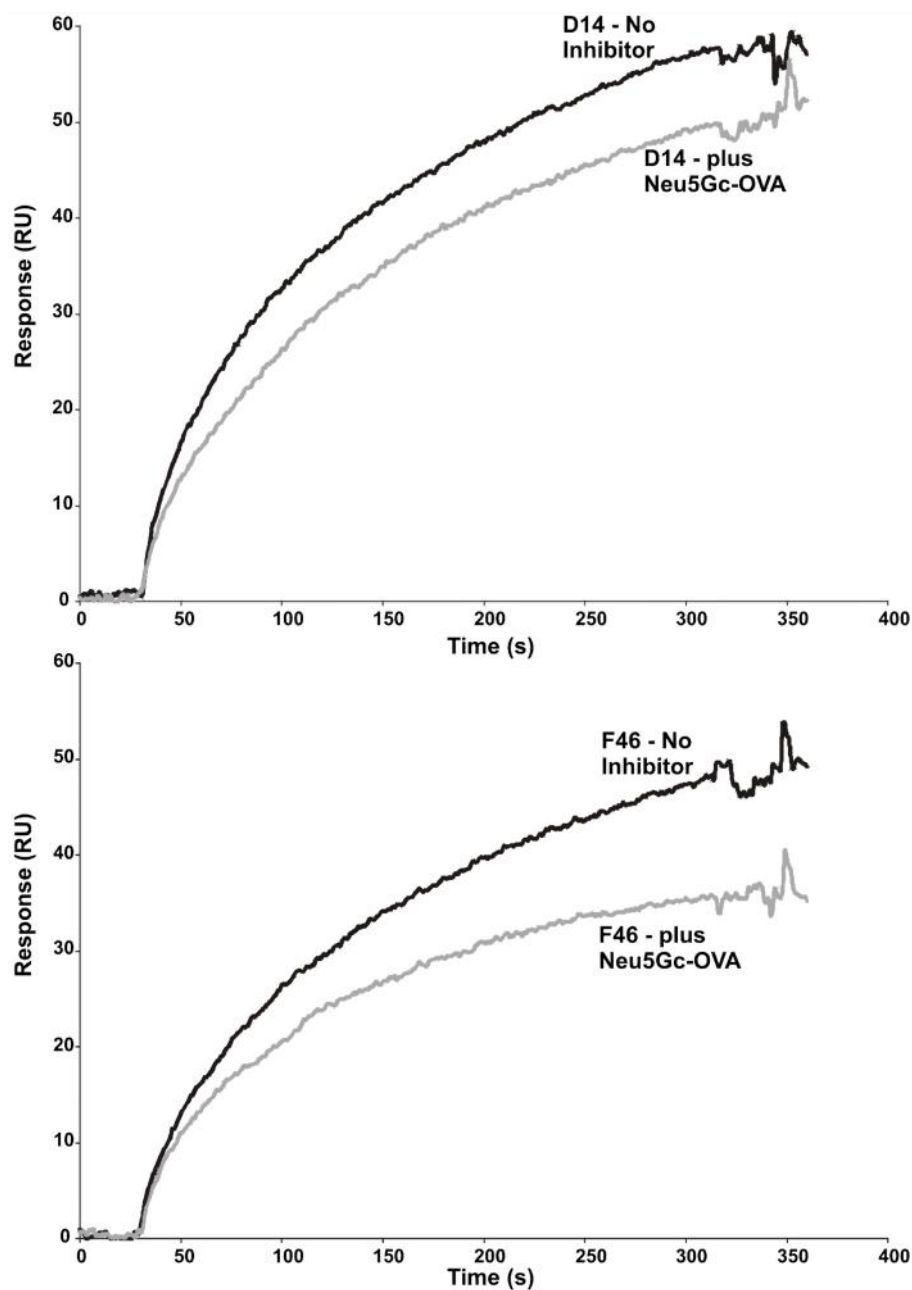


Figure 5.2.13b *Solution-phase SPR-based Neu5Gc-OVA inhibition of the D14 and F46 anti-sialic acid scFvs.* Each scFv sample was incubated at 37°C for 30min either in the presence or absence of 10µg/ml Neu5Gc-OVA sialoglycoconjugate. The scFv solutions were subsequently passed over flow cells 1 and 2 of the Neu5Gc sensor chip at 10µL/min for 6 minutes. Flow cell 1 was used as a reference cell and contained a neutravidin-only surface.

5.2.17 Deduced amino acid sequence alignment of the E15 anti-sialic acid avian scFv.

DNA sequence analysis was performed on the E15 anti-sialic acid scFv. Comparison of the deduced amino acid sequence alignment of the CDR V_H and V_L regions, for Ae8 and E15, is shown in Figure 5.2.14. The parental Ae8 scFv and the mutagenised-light chain shuffled E15 scFv are genetically different. Sequence comparison revealed differences in amino acid composition, in both the complementary determining regions and the linker regions. A single amino acid change, serine to glycine, occurred in the CDRL-3 region and a serine to asparagine, amino acid change, occurred in the CDRH-2 region of E15. Furthermore, a serine to proline change occurred in the linker region. A difference in amino acid sequence length was also seen within the linker regions. This was expected, since the recombinant E15 scFv was constructed with a long 18-amino acid peptide glycine-serine linker to prevent dimerisation, whereas the Ae8 scFv was constructed with a shorter 7-amino acid peptide linker. Both avian scFvs demonstrated strong binding affinity for conjugates of sialic acid.

	FW1	CDR-L1	FW2	CDR-L2	FW3
Ae8	GGTVKITC	SGGGGSYYG	WFQKSPGSAPVTVIY	DNTNRPS	NIPSRFSGSLSGSTNTLTITGVQAEDEAVYYC
E15	GGTVKITC	SGGGGSYYG	WFQKSPGSAPVTVIY	DNTNRPS	NIPSRFSGSLSGSTNTLTITGVQAEDEAVYYC
	CDRL-3	FW4	LINKER	FW1	
Ae8	GSYDRSAGYVGI	FGAGTTLTVL	GQSSRSS	AVTLDESGGGLQTPGGGLSLVCKASGFTFDSYAMY	
E15	GSYDRGAGYVGI	FGAGTTLTVL	GQSSRPSSSSGGGGSGGGG	AVTLDESGGGLQTPGGGLSLVCKASGFTFDSYAMY	
	CDRH-1	FW2	CDRH-2	FW3	
Ae8	GFTFDSYAMY	WVRQAPGKGLEWVAS	INRFGSSTGHGAAV	KGRATISRDNGQSTLRLQLNNLRAEDTGTYCAK	
E15	GFTFDSYAMY	WVRQAPGKGLEWVAS	INRFGNSTGHGAAV	KGRATISRDNGQSTLRLQLNNLRAEDTGTYCAK	
	CDRH-3	FW4	6XHIS	HA	
Ae8	SVHGSCASGTWCSPASIDA	WGHGTEVIVSSTSGQAGQ	HHHHHH	GAYPYDVPDYAS	
E15	SVHGSCASGTWCSPASIDA	WGHGTEVIVSSTSGQAGQ	HHHHHH	GAYPYDVPDYAS	

Figure 5.2.14 Comparative sequence analysis of the parental Ae8 and mutagenised E15, avian anti-sialic acid scFvs. DNA sequencing of both clones was performed by Eurofins MWG Operon (Ebersberg, Germany) in triplicate. The complementary determining regions (CDRs) and the framework regions (FW) were determined according to Kabat and Wu (1991). The deduced amino acid sequences of the V_L and V_H CDRs are shown in single letter code. Amino acid residues that differ are highlighted with a black box and white lettering. The location of two peptide affinity tags, hexahistidine (6XHIS) and haemagglutinin (HA) are also illustrated.

5.2.18 HPLC purification of the E15 scFv.

The E15 clone was purified by IMAC (Section 2.2.22). In common with AE8, analysis of the IMAC-purified E15 scFv by SDS-polyacrylamide gel electrophoresis revealed a number of contaminating protein bands (data not shown). Therefore, further purification of the scFv from IMAC-eluted material was performed using size exclusion chromatography (SEC). The initial IMAC cleanup step yielded a heterogeneous mixture of proteins. According to molecular weight estimates and SPR analysis (data not shown) of the HPLC fractions, the scFv dimer peak was observed at 16.3minutes and the monomeric peak eluted at 17.8minutes (Figure 5.2.15). In order to eliminate the scFv dimer and to capture only the monomeric species, fractions were collected starting from 17.5min up to 18.5min. The HPLC-purified monomeric E15 sample was subsequently used for SPR kinetic analysis.

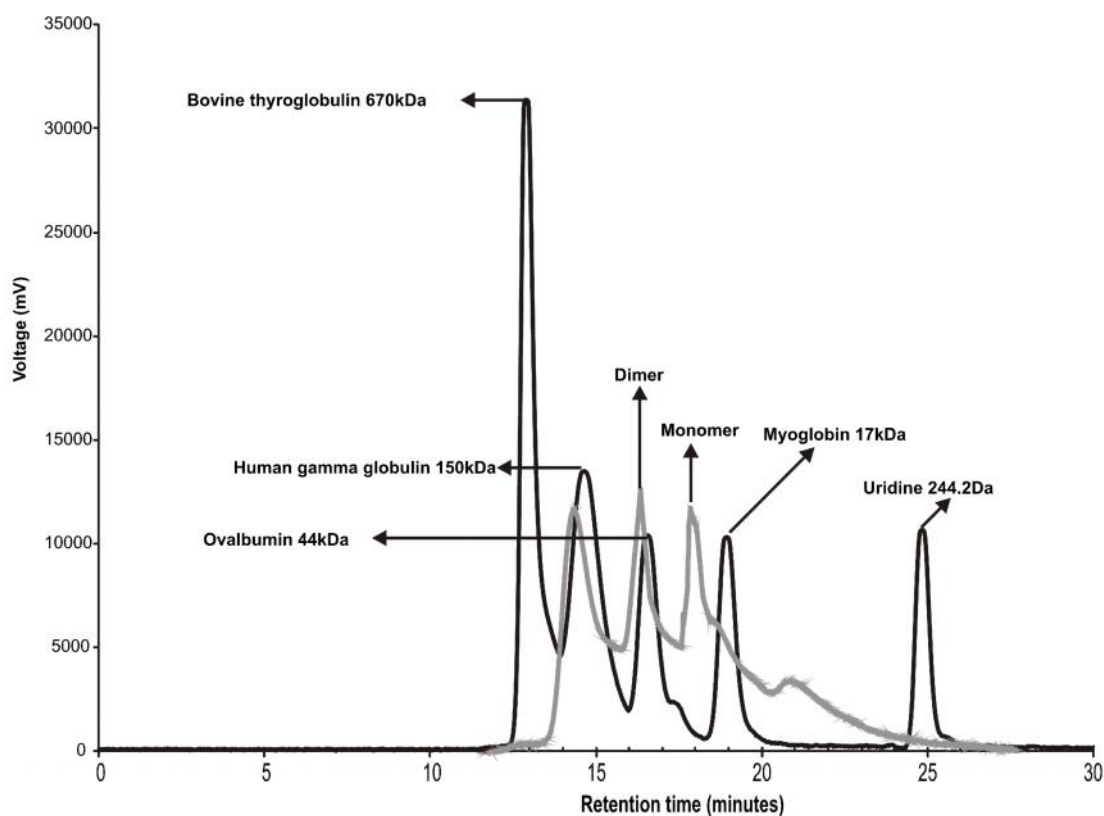


Figure 5.2.15 SEC-HPLC analysis of IMAC-purified E15 scFv. The protein standards are represented by the black solid line. A neat sample of IMAC-purified AE8 (illustrated by the gray solid line) in PBS (pH 7.2) was selected for analysis. A Phenomenex™ 3000 SEC column was used with PBS (pH 7.2) mobile phase at a flow rate of 0.5mL/min and monitored by UV absorbance at 280nm.

5.2.19 Sialic acid binding reactivity of the AE8 and E15 scFvs.

The E15 and AE8 avian anti-sialic acid scFvs were characterised by ELISA and SPR studies, using a diverse panel of synthetic and protein sialic acid-containing structures. This panel contained the following structures: neoglycoconjugates (BSA-Neu5Gc, OVA-Neu5Gc and OVA-Neu5Ac), sialo-PAA-conjugates (Neu5Ac-PAA, Neu5Ac α (2,8)-Neu5Ac-PAA and Neu5Gc-PAA), sialylated glycoproteins (fetuin, alpha-1-acid glycoprotein, bovine mucin and porcine thyroglobulin) and negative controls (BSA, OVA, OVA-linker1, PAA-linker2, glucose-PAA, galactose-PAA, dextran asialofetuin and ribonuclease B, a non-sialylated glycoprotein).

Each sugar PAA conjugate had the same molecular mass (30kDa) and contained the same 20:1 molar ratio of carbohydrate to PAA. Table 5.2.19 shows the binding reactivity of E15 and AE8 to the 23 different structures. Both scFvs bound to all Neu5Gc- and Neu5Ac-containing PAA conjugates and neoglycoconjugates. Importantly, neither scFv bound to any of the negative controls. However, no significant binding to any of the four naturally sialylated glycoproteins was observed. In addition, SPR analysis did not reveal any significant scFv inhibition with the unconjugated Neu5Ac and Neu5Gc monosaccharides. Overall, both E15 and AE8 showed the same binding pattern to all 22 structures.

Table 5.2.19 Analysis of the sialic acid binding reactivity of AE8 and E15.

Neoglycoconjugates, sialylated glycoproteins, control proteins and PAA-conjugates were analysed by direct ELISA (Sections 2.2.3 and 2.2.19) with antigen coating concentrations of 10 μ g/mL, 25-100 μ g/mL, 25-100 μ g/mL and 25 μ g/mL respectively. Solution phase inhibition SPR analysis was performed using the Neu5Gc sensor chip previously described in Section 5.2.15. For SPR inhibition analysis, each scFv sample was incubated at 37°C for 30min, either in the presence or absence of the target structure. The following sample groups were analysed: sialo-neoglycoconjugates (Neu5Gc-BSA, Neu5Gc-OVA and Neu5Ac-OVA), naturally sialylated glycoproteins (fetuin, alpha-1-acid glycoprotein, bovine mucin and porcine thyroglobulin), non-sialylated glycoproteins (OVA, OVA-linker1, BSA, ribonuclease B, asialofetuin) PAA conjugates (Neu5Ac-PAA, Neu5Ac α (2,8)Neu5Ac-PAA, Neu5Gc-PAA, PAA-linker2, glucose-PAA, galactose-PAA) and unconjugated sugars (Neu5Ac,

Neu5Ac α (2,8)Neu5Ac, Neu5Gc and dextran). Sample groups were tested at high (2mg/mL, 5mg/mL) and/or low (10 μ g/mL, 500 μ g/mL) concentrations.

Protein / Glycan / Conjugate /	AE8 scFv reactivity	Immunoassay format	E15 scFv reactivity	Immunoassay format
Neu5Gc- <u>linker1</u> -BSA	Positive	Direct ELISA Inhibition SPR	Positive	Direct ELISA Inhibition SPR
Neu5Gc- <u>linker1</u> -OVA	Positive	Inhibition SPR	Positive	Inhibition SPR
Neu5Ac- <u>linker1</u> -OVA	Positive	Inhibition SPR	Positive	Direct ELISA Inhibition SPR
OVA	Negative	Direct ELISA Inhibition SPR	Negative	Direct ELISA Inhibition SPR
BSA	Negative	Direct ELISA	Negative	Direct ELISA
OVA- <u>linker1</u>	Negative	Inhibition SPR	Negative	Inhibition SPR
Neu5Gc- <u>linker2</u> -PAA	Positive	Direct ELISA Direct/ Inhibition SPR	Positive	Direct/ Inhibition SPR
Neu5Gc- <u>linker3</u> -PAA	Positive	Inhibition SPR	Positive	Inhibition SPR
Neu5Ac- <u>linker2</u> -PAA	Positive	Direct ELISA Direct / Inhibition SPR	Positive	Direct / Inhibition SPR
Neu5Ac α (2,8)Neu5Ac- <u>linker2</u> -PAA	Positive	Inhibition SPR	Positive	Inhibition SPR
<u>linker2</u> -PAA	Negative	Direct / Inhibition SPR	Negative	Direct / Inhibition SPR
D-Glucose- <u>linker2</u> -PAA	Negative	Direct ELISA	Negative	Inhibition SPR
D-Galactose- <u>linker2</u> -PAA	Negative	Direct ELISA	Negative	Inhibition SPR
Dextran	Negative	Inhibition SPR	Negative	Inhibition SPR
Fetuin	Negative	Inhibition SPR	Negative	Inhibition SPR
Asialofetuin	Negative	Inhibition SPR	Negative	Inhibition SPR
Bovine Mucin	Negative	Inhibition SPR	Negative	Inhibition SPR
Alpha-1-acid glycoprotein	Negative	Inhibition SPR	Negative	Inhibition SPR
Porcine Thyroglobulin	Negative	Inhibition SPR	Negative	Inhibition SPR
Ribonuclease B	Negative	Inhibition SPR	Negative	Inhibition SPR
Neu5Ac	Negative	Inhibition SPR	Negative	Inhibition SPR
Neu5Gc	Negative	Inhibition SPR	Negative	Inhibition SPR

5.2.20 SPR kinetic studies on the E15 clone.

SPR was used to determine the association (k_a) and dissociation (k_d) rate constants of the E15 avian anti-sialic acid scFv. To rule out the contribution of avidity in the determination of the rate constants, only the monomeric HPLC-purified fraction of E15 was used for Biacore[®] kinetic analysis. However, the data obtained from multiple kinetic runs showed poor fits to the 1:1 (Langmuir) binding model ($A + B \leftrightarrow AB$). The apparent k_a and k_d values were: $4.67 \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$ and $1.33 \times 10^{-3} \text{ s}^{-1}$, respectively. Thus, the calculated K_D value for the E15 scFv/Neu5Gc interaction was 28nM (Figure 5.2.16).

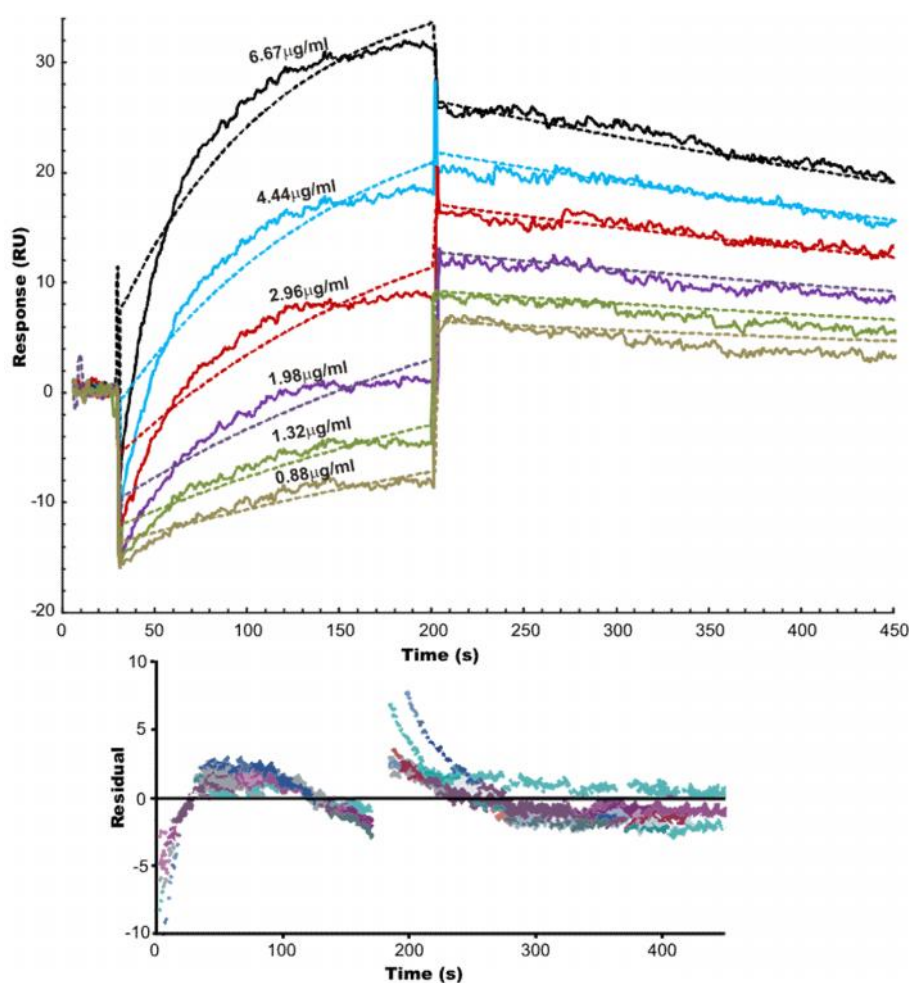


Figure 5.2.16 Curve fitting of monomeric E15 scFv with BIAevaluation 4.1 using a 1:1 binding model. The fitted curves for each monomeric scFv concentration are represented by the dashed line, whereas the solid lines represent the actual RU change for each sample. The apparent K_D was estimated to be $28 \times 10^{-9} \text{ M}$. The residual plot is a measure of the ‘goodness of fit’.

5.2.21 Sialic acid-binding affinity of the E15 recombinant scFv.

An SPR solution-phase Neu5Gc-binding assay was used to compare the relative sialic acid binding affinities of the E15 and AE8 scFvs. Analysis of their SPR inhibition binding curves showed that the two scFvs had different IC_{50} values and, therefore, different binding affinities for the Neu5Gc neoglycoconjugate. The parental AE8 scFv had an IC_{50} value of 5.7ng/mL, whereas the light chain shuffled E15 scFv had a lower IC_{50} value of just 1.6ng/ml (Figure 5.2.17). Overall, the E15 scFv exhibited an improvement in sialic acid binding of approximately 3.5-fold compared to the parental AE8 scFv. Furthermore, the E15 scFv had a narrower dynamic range.

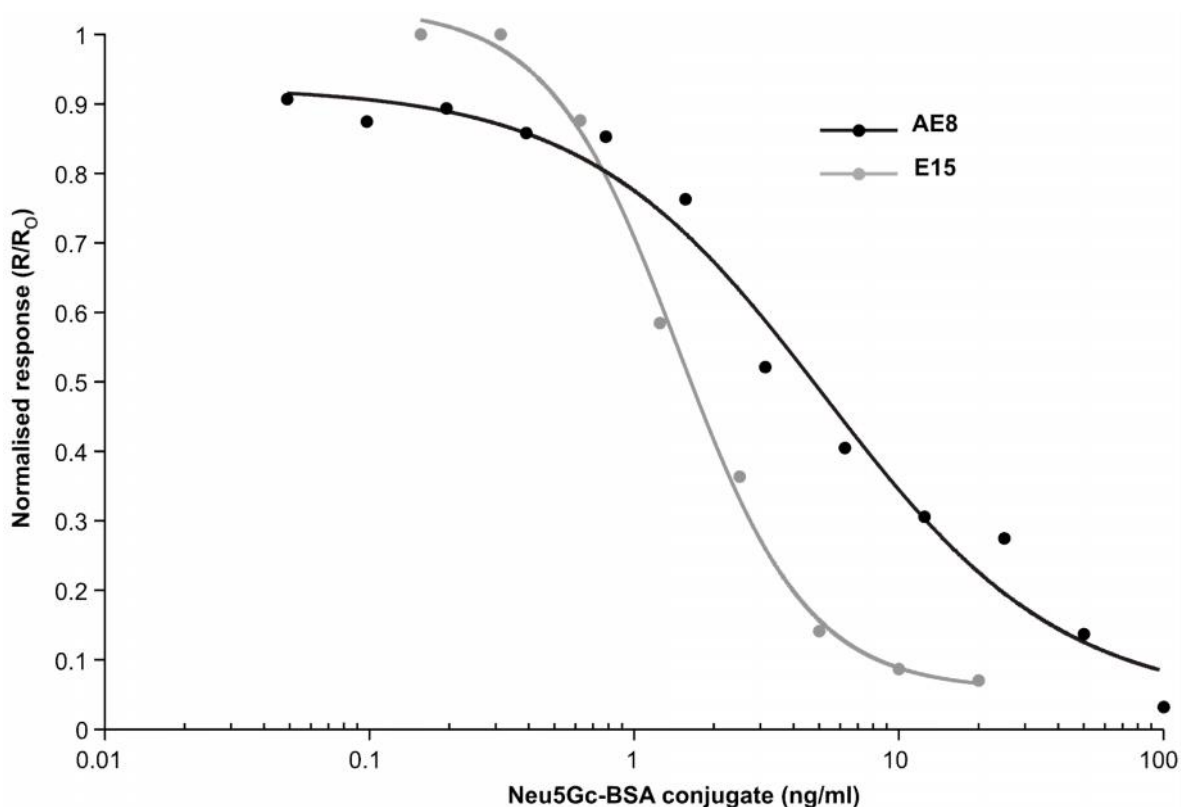


Figure 5.2.17 Comparison of the SPR inhibition assay curves, for the anti-sialic binding scFvs, E15 and AE8. The R/R_0 was calculated by dividing the RU response obtained at different Neu5Gc-BSA conjugate concentrations, by the RU response obtained from the scFv sample with no Neu5Gc-BSA conjugate. A 4-parameter equation was fitted to the data set using BIAevaluation 4.1 software. Each point in the curve is the mean of three replicate measurements.

5.3 Discussion

This chapter describes the rationale behind the application of directed molecular evolution for the creation of improved anti-sialic acid antibody fragments. In particular, the application of light chain shuffling and error-prone PCR techniques, to achieve antibody gene diversity is discussed. In addition, the biopanning strategies and the use of new sialo-neoglycoconjugates for the selection of positively-binding sialic acid clones, from the light chain and mutagenised avian phage library, is described. Furthermore, the specificity and affinity of the newly identified mutant E15 anti-sialic acid antibody is described in detail.

In this work an antibody fragment (AE8 scFv) that recognised sialic acid was *in vitro* affinity matured, using directed molecular evolution and phage display. However, before embarking on affinity maturation and *in vitro* screening, there were a number of key factors to be considered. One major factor, was where and how to place mutations into the variable genes of the parental AE8 anti-sialic acid scFv. In general, mutations can be introduced into an antibody gene sequence either randomly or at specific sites (site directed). The random mutagenesis approach is relatively simple and has the advantage of mimicking the *in vivo* process of somatic hypermutation (Lou and Marks, 2010).

A second approach is to use site-directed mutagenesis for antibody affinity maturation. In this method, mutations are “directed” or assigned to particular positions along the antibody gene sequence (Lou and Marks, 2010). An antibody’s affinity to its cognate antigen is mainly dependent on the conformation of the amino acids present in the CDRs. In addition, during *in vivo* somatic hypermutation, mutations tend to accumulate in CDRs, preferentially to framework residues (Chothia *et al.*, 1992; Betz *et al.*, 1993; Adams and Schier, 1999). Therefore, a common approach is to specifically target mutations in the CDRs of both the light and heavy chains of the antibody (Diaz *et al.*, 1999). However, rational site-directed mutagenesis of CDR residues for improved protein function is not trivial, and typically requires a detailed knowledge of protein structure (Steipe, 1999). A DNA sequence translates to a peptide sequence built of 20 different amino acids, which in turn is folded into a complex three dimensional structure. It is not obvious from the primary structure how individual amino acids are arranged in space.

Moreover, protein folding is a complex process that is still poorly understood (Madan *et al.*, 2008; Shental-Bechor and Levy, 2008). Consequently, predicting an amino acid sequence or even changes to an amino acid sequence, that would yield improvements in protein function, remains extremely challenging. Although analysis of the AE8 deduced amino acid sequence did point to some residues which may be important in sialic acid binding, this information was limited, and therefore not useful enough to devise a rational site-directed mutagenesis strategy. In contrast, the random mutagenesis technique does not require a structural model, or even an understanding of the molecular details of the antigen-antibody interaction (Forrer *et al.*, 1999; Fujii *et al.*, 2004). Therefore, the random mutagenesis approach (error prone PCR and light chain shuffling) was used to create a diverse library of anti-sialic acid scFv mutants.

In this work, a low-fidelity recombinant DNA polymerase (RedTaq[®]) was used for error-prone PCR. This thermostable enzyme lacks a 3' to 5' exonuclease activity and, therefore, has relatively low-fidelity in DNA replication. For instance, when *Taq* polymerase incorporates the wrong nucleotide into a DNA strand, it does not have the ability to remove and replace this nucleotide. For this reason, *Taq* polymerase is often used in mutagenic PCR experiments, since it has a naturally high intrinsic error rate of approximately 1×10^{-4} bps/kb under standard PCR conditions. Moreover, when this enzyme is used in error-prone PCR, an even higher error rate of between 1 to 20 nucleotides per 1000 nucleotides polymerised can be achieved (Cadwell and Joyce 1992; Cirino *et al.*, 2003; Chusacultanachai and Yuthavong, 2004; Vanhercke *et al.*, 2005). In this study error-prone PCR conditions were created by the addition of 0.5mM MnCl₂ to the reaction buffer. Testing of higher concentrations of MnCl₂ resulted in greatly reduced yields (data not shown). To increase the level of mutagenesis, the PCR reaction buffer also contained a relatively high concentration (7mM) of MgCl₂ (Cadwell and Joyce, 1992; Cirino *et al.*, 2003).

The goal of error-prone PCR is to produce a randomised DNA library, in which all potential mutations are equally represented. However, the inherent characteristics of DNA polymerases mean that certain types of nucleotide misincorporation errors are more common than others. For example, nucleotide substitutions during DNA replication occur more frequently between two purines (A,G) or two pyrimidines (C,T) (transition) than between a purine and pyrimidine (transversion).

Furthermore, in the case of *Taq* DNA polymerase, AT to GC transitions typically dominate the spectrum of possible nucleotide substitutions. This type of nucleotide transition is caused by the misincorporation of a G opposite a template T and results in an A to G base change. Subsequently, when this mutated coding DNA strand is duplicated, the misincorporated G generates a T to C base change. In *Taq*-based error-prone PCR methods, A and T residues are typically two- to fourfold more likely to be mutated compared to G and C residues. Such mutational bias can disproportionately alter the type of mutations observed in the randomised antibody library. Importantly, this form of transition bias can be reduced by lowering the initial PCR concentrations of dATP and dGTP (Cadwell and Joyce, 1992; Martineau, 2002; Cirino *et al.*, 2003; Chusacultanaichai and Yuthavong, 2004; Fujii, 2004; Pritchard *et al.*, 2005; Vanhercke *et al.*, 2005; Stebel *et al.*, 2008; Rasila *et al.*, 2009; Tong *et al.*, 2010; Ponsel *et al.*, 2011). Therefore, in this study an unbalance mixture of dNTPs with elevated concentrations of dTTP and dCTP (1mM) and lower concentrations of dGTP and dATP (0.2mM) were added to the PCR buffer mix. Furthermore, to ensure a sufficient level of mutation, a low concentration of the light chain anti-sialic acid library (5ng), was used as the input DNA template for error-prone PCR. In addition, a total 35 cycles of amplification was performed. Figure 5.2.1, shows the successful mutagenic amplification of the avian V_L genes from the original anti-sialic acid scFv library, using RedTaq[®] DNA polymerase and error-prone PCR conditions.

The introduction of mutations by shuffling the light chain variable region genes is a widely used method for obtaining high-affinity antibodies, particularly when the target antigens are proteins or small molecules (Ponsel *et al.*, 2011). In contrast, as only a relatively small number of anti-glycan antibodies have been generated from immunised animals, antibody redesign studies that have used chain shuffling to improve the affinity of anti-carbohydrate antibodies are rare. However, the utility of such an approach for the improvement of anti-carbohydrate antibodies was previously described by Deng *et al.* (1995). In that work, phage display was used in combination with chain shuffling and random mutagenesis to generate an scFv that had a 10-fold improved binding affinity for *Salmonella* serogroup B lipopolysaccharide. It is widely accepted that in many cases, the heavy chain of an antibody plays a major role in antigen recognition. In addition, the antibody heavy chain is usually more important than the light chain for determining antigen specificity.

Moreover, the CDR3 region of the variable heavy chain is generally considered to be the key determinant for antigen selectivity (de Wildt *et al.*, 1996; Xu *et al.*, 2000). Therefore, to prevent the complete abolition of sialic acid binding, a light chain shuffling strategy was undertaken in which a fixed anti-sialic acid heavy chain variable gene was shuffled with a library of light chain variable genes. The aim was to generate anti-sialic scFvs that had improved affinity and specificity. As previously described, the anti-sialic acid scFv library used in this study was prepared from the bone marrow and spleen tissue of a chicken that was immunised with a Neu5Gc-HSA neoglycoconjugate. The lead scFv clone (AE8) isolated from this library had shown very strong reactivity to a panel of different sialylated structures (neoglycoconjugates and sialylated PAA probes). Therefore, for light chain shuffling the heavy chain gene from the AE8 scFv was randomly paired with a mutagenised anti-sialic acid cDNA library of light chain genes. Figure 5.2.2 shows the successful amplification of the avian heavy chain variable gene from the plasmid DNA of the AE8 scFv.

In contrast to scFv generation in chapter 4, a long 18-amino acid peptide glycine-serine linker was chosen for the construction of the mutagenised-light chain shuffled scFv anti-sialic acid library. SPR analysis of the AE8 anti-sialic acid antibody fragment had previously shown that it was possible to generate an anti-carbohydrate scFv that had nanomolar affinity for Neu5Gc-containing structures in monomeric form. In the construction of the original anti-sialic acid library, a short chain 7 amino acid linker was chosen to increase the formation of scFv aggregates. The shortening of the linker peptide forces the scFv to cross-pair with other complementary fragments and this causes the formation of high order structures, such as dimers, trimers and tetramers (Kortt *et al.*, 2001; Sixholo *et al.*, 2011). Since these oligomers have increased valency, the resulting avidity effects, would aid the isolation of sialic acid phage binders, even those with weak carbohydrate affinities. However, as previously reported, the monovalent AE8 scFv had the ability to bind sialic acid with high affinity without the avidity gain conferred by scFv multivalency. Therefore, the generation of multivalent scFv structures for multivalent binding interactions through the use of a short chain amino acid linker did not appear necessary. Rather, the combined effects of mutagenesis, light chain shuffling and a longer linker length on sialic acid scFv binding affinity and specificity were investigated.

Following the successful amplification of the avian heavy and light chain variable regions, the V_L and V_H gene fragments were gel purified and spliced by overlap extension PCR. The assembled single chain Fv fragments were constructed with a V_L - V_H domain orientation. Figure 5.2.3 shows the initial SOE-PCR amplification of the V_L and V_H fragments. Although the target SOE product (~750bp) was amplified, two additional non-specific PCR products of between 500-650bp were also amplified. Optimisation of the SOE-PCR was achieved by increasing the annealing temperature of the PCR reaction (57°C to 59°C) and a reduction in the PCR extension time (2min to 1min) (Figure 5.2.4). In addition, the successful *Sfi*I restriction digestion of the pComb3XSS vector is also shown in Figure 5.2.4 and both the expected stuffer fragment ~1600bp and the double-cut vector of ~3400bp are illustrated. The gel-purified scFv DNA fragments were ligated into the *Sfi*I sites of the pComb3XSS phagemid vector. The ligated pComb3XSS-scFv gene sequences were subsequently transformed into electrocompetent *E.coli* XL-1 Blue cells. Dilutions of the electroporated bacteria were plated and the number of scFv-containing clones within the library was estimated. The transformed, anti-sialic acid mutagenised light-chain shuffled scFv library had a size of 5.2×10^7 cfu/mL. This library was considered to be of a sufficient size to proceed with biopanning.

Three new screening conjugates, which contained the carrier protein, ovalbumin (OVA), were used to mine the mutagenised-light chain shuffled scFv library. OVA is a 45kDa phosphoprotein that is most often used as a non-relevant carrier for immunoassay antibody screening, rather than as a carrier for immunisation. This egg albumin is the major protein in hen egg whites and comprises 75% of the total protein content. Importantly, OVA does not contain sialic acid and has 20 lysine ϵ -amino groups available for conjugation (Huntington and Stein 2001; Mao *et al.*, 2003, Hermanson, 2008). A number of anti-carbohydrate antibody studies have reported the successful use of OVA neoglycoproteins. For example, Williams *et al.* (1996) used an OVA carbohydrate screening conjugate to isolated recombinant Fab fragments to a plant cell wall polysaccharide, whereas Lo-Man *et al.* (1999) confirmed the specificity of a murine anti-Tn mAb with an OVA-Tn conjugate. In addition, Komagome *et al.* (2002) used sialic acid OVA conjugates to identify sialo-oligosaccharides required by the JC virus for host cell attachment.

Furthermore, OVA screening conjugates are especially useful for the detection of antibodies that are raised in chickens, as non-specific binding of chicken serum to OVA should not occur as OVA is seen as “self” by the chicken’s immune system and therefore chicken serum should not normally contain anti-OVA antibodies. Thus, OVA was chosen as a carrier protein for attachment of sialic acid. The Neu5Gc-**linker1**-OVA conjugate contained 20 monosaccharide units of Neu5Gc per mole of OVA protein and was used for biopanning. The second conjugate, **linker1**-OVA, contained 20 **linker1** units per mole of OVA protein and was used for negative selection during biopanning. A third conjugate, Neu5Ac-**linker1**-OVA was used to identify putative Neu5Ac-specific clones and contained 17 monosaccharide units of Neu5Ac per mole of OVA protein. All conjugates were synthesised by Carbohydrate Synthesis (U.K.) and each conjugate contained the same 13 carbon atom spacer arm. Furthermore, the linker used for the construction of the OVA conjugates was identical to that used in the synthesis of the HSA and BSA neoglycoconjugates.

In order to generate a panel of recombinant scFvs to sialic acid, the mutagenised and light chain shuffled phage library was screened using two different biopanning strategies. In the standard method, library phage pools were biopanned against a single antigen (Neu5Gc-**linker1**-OVA). In contrast, the second method employed a negative (immunodepletion) biopanning strategy, in which the phage library was initially biopanned against **linker1**-OVA and subsequently biopanned against Neu5Gc-**linker1**-OVA. The primary goal was to remove cross-reacting OVA and linker phage binders and to isolate scFv clones that only recognised the sialic acid component of the conjugate and not the spacer arm or protein elements. A secondary aim was to compare the effect of depletion and non-depletion biopanning on the ability of isolated scFv clones to bind to sialic acid in a natural glycoprotein context.

The phage input and output titres for each of the 5 rounds of biopanning, using two different phage display selection strategies, are shown in table 5.2.5. Typically phage input and output titres can be expected to vary between 10^{11} – 10^{13} cfu/mL and 10^2 – 10^7 cfu/mL respectively. In the initial round of biopanning (P1) the library was screened against a relatively large amount of neoglycoconjugate (Neu5Gc-**linker1**-OVA) and no depletion step was performed.

The goal was to avoid losing any specific sialic acid binders, as typically at the start of the biopanning process, a small heterogeneous subpopulation of specific phage binders are diluted into a very large population (e.g. billions) of non-specific phage. It is only after several rounds of biopanning that the specific phage binder subpopulation is substantially enriched such that phage which have high specificity and affinity for the target antigen predominate. The data present in table 5.2.5, show that the output titres were within the expected range. In contrast, the input titres were lower than expected. This may have resulted from the non-optimal growth of *E.coli*, or the sub-optimal infection of *E.coli* by bacteriophage. Overall, lower output titres were also seen when the depletion method was used. This was not unexpected, since, in this method, phage were sequentially incubated using two different immunotubes. It is probable that this method was more successful at eliminating non-specifically bound phage than the single incubation step protocol. Since phage output titres from the non-depleted or depleted biopanning methods were similar, it was therefore not known if either phage display selection strategy had any superior advantage. Moreover, as discussed previously, phage titres are typically not reliable indicators of biopanning success, as only a small proportion of phage may actually display correctly-folded antibody fragments on their surface (Barbas, 2001; O' Brien and Aitken, 2002; Kirsch *et al.*, 2005).

In the next stage of analysis, the depleted and non-depleted phage library preparations for each biopanning round were analysed by polyclonal phage ELISA. The Neu5Gc-**linker1**-OVA, neoglycoconjugate-screening antigen was used for analysis. Increases in specific ELISA signals over background (unpanned library) were evident with phage pools obtained from the depleted and non-depleted methods, for the fourth and fifth rounds of selection (Figure 5.2.6). In addition, for biopanning rounds four and five, the non-depleted method had a similar level of anti-sialic acid antibody enrichment. In contrast, the depleted method showed a moderate reduction in anti-sialic acid reactivity in biopanning round five. These initial results were encouraging as they indicated that the mutagenised and light chain -shuffled library contained putative anti-sialic acid binding clones.

Immunodepleted phage pools from the fourth and fifth rounds of biopanning were selected for monoclonal phage ELISA analysis. Phage pools were infected into a non-SupE strain of *E. coli* (TOP10F') and 96 clones were solubly expressed and screened by an indirect ELISA binding assay. The majority of randomly picked clones (>90%) demonstrated significant binding reactivity to both Neu5Gc-OVA and Neu5Ac-OVA neoglycoconjugates. In addition, no significant binding was observed against the negative control proteins, BSA and OVA (Figures 5.2.7/8). These data indicated that the scFv clones had significant, observable binding affinity for the sialic acid component of the OVA-neoglycoconjugate.

In an attempt to isolate scFvs that recognised sialic acid in the context of natural glycoproteins, a panel of 384 clones (picked from the non-depleted and **linker1**-OVA depleted biopanning rounds 4 and 5) were screened by ELISA for glycoprotein binding. A total of four different sialoglycoproteins (bovine fetuin, bovine submaxillary mucin, porcine thyroglobulin and bovine α_1 -acid glycoprotein) were chosen for ELISA analysis. These model sialoglycoproteins are commonly used in glycan studies and a wealth of literature relating to their carbohydrate structures is readily available. For example, bovine fetuin, a constituent of calf foetal serum and a highly glycosylated protein, has a complex carbohydrate structure that has been intensively studied since the early 1960s. This 48kDa glycoprotein has a carbohydrate content of approximately 30% and contains heteropolysaccharide units made up of sialic acid, galactose, *N*-acetylglucosamine, and mannose. Similar to other glycoproteins, bovine fetuin is not a single structural entity, but rather it exists as a mixture of different glycosylated and sialylated variants (glycoforms) (Green *et al.*, 1988; Nie, 1992; Hino *et al.*, 2003; Ding *et al.*, 2009). These glycoforms contain a heterogeneous population of glycans which are attached to multiple glycosylation sites within the protein backbone. The degree of glycan heterogeneity is not only dependent upon the location of the oligosaccharide chains (*N*- or *O*-linked), but is also a function of the number, linkage and composition of the carbohydrate units contained within those glycan chains. In comparison to amino acids, the combinatorial possibilities of glycans are truly impressive. For example, a 6 amino acid sequence using a 20 amino acid alphabet can theoretically generate 6.4×10^7 (20^6) distinct hexapeptides. In contrast, the total number of isomer permutations for a carbohydrate hexamer with an alphabet of 20 letters (monosaccharides) is a staggering 1.44×10^{15} linear or branched hexasaccharide structures (Weiss and Lyer, 2007).

Glycan heterogeneity within bovine fetuin is seen at many different levels. This single-chain polypeptide glycoprotein, contains 3 *N*-linked glycosylation sites (Asn81, Asn138 and Asn158), invariably occupied, and 4 partially occupied *O*-linked (Ser253, Thr262, Ser264 and Ser323) glycosylation sites. Approximately 80% of all bovine fetuin glycans are attached as *N*-linked complex glycans, and the remaining 20% are *O*-linked. The major terminal structures found on the *N*-linked and *O*-linked sialyloligosaccharides of this glycoprotein are Neu5Aca(2,3)Galβ(1,4)GlcNAc and Neu5Aca(2,3)Galβ(1,3)GalNAc, respectively. NMR studies have revealed that there are at least 23 distinct *N*-linked bovine fetuin glycoforms of which 3% are mono, 35% are di-, 54% are tri-, and 8% are tetrasialylated structures. Bovine fetuin also contains a mixture of α(2,3)- and α(2,6)-linked sialic acids. The major type of sialic acid found in bovine fetuin is Neu5Ac, whereas Neu5Gc is a minor component, and only accounts for 2-7% of total sialic acids. Furthermore, carbohydrate analysis has shown that this glycoprotein contains 17-18.8 moles of Neu5Ac and 0.49-0.63 moles of Neu5Gc per mole of glycoprotein (Berman, 1986; Green *et al.*, 1988; Nie, 1992; Hino *et al.*, 2003; Domann *et al.*, 2007; Ding *et al.*, 2009; Thaysen-Andersen *et al.*, 2009).

A second model protein chosen for scFv sialoglycoprotein binding was bovine submaxillary mucin (BSM). BSM belongs to a family of mucus proteins commonly known as mucins. These high molecular mass glycoproteins are often found within the saliva of mammals and are secreted from the submandibular (submaxillary) glands. BSM has a carbohydrate content of approximately 56% and unlike fetuin, contains mostly *O*-linked glycan chains. The most abundant carbohydrate component found on BSM is an *O*-linked disaccharide, Siaα(2,6)GalNAc, which is also known as Sialyl-Tn. A second *O*-linked sialylated structure, the trisaccharide, Siaα(2,6)GlcNAcβ(1,3)GalNAc, is also commonly found on this sialomucin. Due to the inability to precisely determine the molecular mass of certain glycoproteins, Sia content is often reported as nanomoles of sialic acid per mg of protein. BSM type I-S mucin contained 202nmol of Neu5Ac and 181nmol of Neu5Gc per mg of salivary glycoprotein. Thus, in common with fetuin, BSM is a sialic acid-rich glycoprotein, but unlike fetuin, it contains comparable amounts of Neu5Gc and Neu5Ac (Corfield *et al.*, 1991; Wu *et al.*, 1994; Stehling *et al.*, 1998; Robbe *et al.*, 2003).

Porcine thyroglobulin was also used to study the panel of putative anti-sialic acid scFvs. This sialic acid-rich glycoprotein contains between 15-20 *N*-linked glycosylation sites, which comprises 8-10% of its mass. The *N*-linked glycan chains are highly heterogeneous and contain a variety of complex biantennary and triantennary structures, some of which are partially capped with sialic acid. Two major groups of *N*-linked glycan chains can be distinguished: oligomannose type (unit A) and *N*-acetyllactosamine [Gal β (1,4)GlcNAc] type (unit B). Unit B chains are mostly mono- and disialylated α (1,6)fucosylated diantennary-structures, which terminate in α (2,6)-linked sialic acid (Neu5Ac or Neu5Gc) on the Man α (1,3) antennae. In contrast, the Man α (1,6) antennae is extremely heterogeneous. These antennae can terminate with Man, GlcNAc, or Gal residues. In addition, terminal Gal residues can be extended with either Gal α (1,3) or Neu5Ac/Gc α (2,6), or Neu5Ac α (2,3)-linked residues. The most abundant sialylated structure is Neu5Ac attached in an α (2,6) position [Neu5Ac α (2,6)Gal β (1,4)GlcNAc]. Only between 8 to 10% of the total Sia content of porcine thyroglobulin exists in the *N*-glycolyl form of sialic acid. Furthermore, carbohydrate analysis has shown that porcine thyroglobulin contains 25.1-25.2 moles of Neu5Ac and 2.0-2.4 moles of Neu5Gc per mole of glycoprotein (Yamamoto *et al.*, 1981; Tsuji *et al.*, 1981; Tsuji *et al.*, 1982; de Waard *et al.*, 1991; Rawitch *et al.*, 1993; Ikekita *et al.*, 1997; Charlwood *et al.*, 1999; Venkatesh *et al.*, 1999; Harvey, 2005; Zhang *et al.*, 2008; Wheeler *et al.*, 2009).

Bovine α_1 -acid glycoprotein (Orosomuroid, AGP) was also used to screen the mutagenised and light chain shuffled phage library, for scFv clones that could recognise naturally occurring sialylated glycan chains. Bovine AGP has a molecular mass of 33,800 Da and a carbohydrate content of 26.6%. In addition, it contains five *N*-glycosylation sites with di- and triantennary complex type glycans. The majority of the carbohydrate chains on bovine AGP are diantennary type. The diantennary structures contain not only different amounts of sialic acid (disialo, trisialo and tetrasialo) but also different forms of sialic acid. Similar to BSM, bovine AGP contains comparable amounts of Neu5Ac and Neu5Gc. Carbohydrate analysis by capillary electrophoresis has shown that bovine AGP contains 5.3-5.7 moles of Neu5Ac and 4.9–5.2 moles of Neu5Gc per mole of glycoprotein (Treuheit *et al.*, 1992; Hunter and Ganesm, 1995; Shiyan and Bovin, 1997; Che *et al.*, 1999; Nakano *et al.*, 2004; Balaguer and Neuss, 2006).

As discussed above, the four sialoglycoproteins, bovine fetuin, bovine submaxillary mucin, porcine thyroglobulin and bovine α_1 -acid glycoprotein, contain a diverse array of sialic acid structures. However, when a panel of 384 clones from the mutagenised and light chain shuffled phage library, were screened by ELISA for sialoglycoprotein reactivity, no significant binding was observed. These results indicate that the phage library may not contain anti-sialic acid scFvs that recognise sialic acid in its natural context (i.e. as part of a sialoglycoprotein). It is possible that the spatial conformation of the natural sialoglycan chains of the glycoproteins differed significantly from that of the artificial sialo-neoglycoconjugates, which were used for immunisations and biopanning. Such differences in sialic acid spatial conformation may result from the high flexibility and saccharide chain length of the natural sialoglycans. These differences could explain why the putative anti-sialic acid scFvs showed no specificity for the natural occurring sialylated glycoproteins.

The sialic acid-specificity of the same bank of 384 putative anti-sialic acid scFv clones was further investigated. Two different OVA-neoglycoconjugates (Neu5Gc-**linker1**-OVA and Neu5Ac-**linker1**-OVA) were used in an ELISA-based assay to identify scFv clones that were Neu5Gc-specific or Neu5Ac-specific. However, in common with the original anti-sialic acid phage library, only anti-Sia scFvs that bound to both Neu5Gc and Neu5Ac were recovered. Although the phage library was not exhaustively screened, these data suggested that a Neu5Gc-specific or a Neu5Ac-specific scFv combining site may not have resulted from the mutagenesis and light chain shuffling approaches. Although this result was not desirable it was not unexpected, as anti-Sia antibodies that bind only to the *N*-glycoyl form of sialic acid are exceedingly rare (Diaz *et al.* 2009)

‘Interface binders’ are often a problem during the isolation of anti-hapten specific antibodies. These types of antibodies do not bind to the target hapten molecules alone. Rather, they recognise the hapten antigen in the context of the conjugate and often bind in the vicinity of the point of conjugation. Such antibodies are referred to as ‘interface binders’ because they bind simultaneously to a portion of the hapten structure and a portion of the carrier protein (Charlton *et al.* 2001; Charlton and Porter, 2002; Kobayashi *et al.* 2008).

In this work, the sialo-neoglycoconjugates used for biopanning selections were constructed with an OVA protein and a synthetic linker (**linker1**-OVA). Therefore, in order to ascertain if the anti-Sia scFvs were interface binders, a panel of ninety-six Neu5Gc\Neu5Ac positive clones were selected for **linker1**-OVA ELISA analysis. The data presented in Figure 5.2.10, showed that approximately 10% of the clones had no significant binding reactivity to either the OVA protein or the **linker1**-OVA conjugate. Therefore, based on these results, it appeared that the combining sites of this scFv subgroup, specifically recognised the Sia component of the sialo-neoglycoconjugate. In contrast, when no **linker1**-OVA biopanning depletion steps were performed, all of the scFv clones analysed showed significant binding to the **linker1**-OVA conjugate (Figure 5.2.9). It is probable that these scFv clones were interface binders and bound to some or all constituent parts of the sialo-neoglycoconjugate (i.e. sialic acid, linker and OVA protein). These data reveal the importance of using depletion steps in a biopanning protocol. Depletion steps allow the removal of irrelevant binding motifs from the output of a screening campaign and, therefore, increase the chances of isolating antibodies that bind specifically to the target antigen.

The specificity of the ten panel, **linker1**-OVA negative, anti-sialic acid scFv subgroup, was further characterised by ELISA-based analysis, using a second Neu5Gc conjugate (Neu5Gc-**linker2**-PAA). The Neu5Gc-**linker2**-PAA conjugate differs from the Neu5Gc-**linker1**-OVA conjugate in a number of important aspects. Firstly, the linkage chemistry employed in the attachment of Neu5Gc to PAA differs from that of the OVA conjugate. Secondly, Neu5Gc is attached to the PAA conjugate by a shorter linker arm. Thirdly, the Neu5Gc loading density on PAA differs from that of OVA. Overall, it is reasonable to conclude, that the spatial presentation of Neu5Gc in the context of the PAA conjugate, is different from that of Neu5Gc in the OVA neoglycoconjugate. Even so, Figure 5.2.11 demonstrated that 4 clones (D14, D34, E15 and F46), recognised Neu5Gc in the context of the **linker2**-PAA-backbone. Importantly, these results revealed that these scFvs could bind Sia in the context of two very different carriers with structurally unrelated linkers, namely Neu5Gc-**linker1**-OVA and Neu5Gc- **linker2**-PAA. Therefore, these scFvs have the ability to recognise Neu5Gc without the requirement of a specific linker and consequently, these scFvs are not linker-only-specific but rather sialic acid-specific.

The binding affinity of the anti-sialic clones: D14, D34, E15 and F46 were further elucidated using a combination of ‘off-rate’ binding kinetics and solution phase SPR-based inhibition assays. The ‘off-rate’ binding screening strategy provided an effective means to directly identify mutants with apparently slower off rates, without the need for protein purification. As observed in Figure 5.2.12, all anti-sialic acid clones tested had different ‘off-rate’ profiles. The E15 mutant anti-sialic acid scFv and the parental AeE8 anti-sialic acid scFv displayed the slowest ‘off-rates’. Interestingly, the E15 mutant scFv had a slower ‘off-rate’ than that observed for the wild-type AE8 scFv. In contrast, the remaining mutant anti-sialic scFv clones: F46, D14 and D34 displayed faster ‘off-rates’. These initial data indicated that the mutant E15 scFv may have a higher binding affinity for sialic acid. Each of the scFv variants were further characterised using a solution phase SPR-based inhibition assay. In this assay, the scFv variants were prevented (inhibited) from binding to the immobilised PAA-Neu5Gc surface, by the addition of a fixed amount of Neu5Gc-**linker1**-OVA conjugate. The goal was to identify those scFv variants that had the best capacity to diminish or even abolish binding of the sialoglycoconjugate. SPR binding and inhibition sensorgrams for E15, AE8, F46, D14 and D34 are shown in Figures 5.2.13a/b. Both the parental AE8 scFv and the mutant E15 scFv displayed complete inhibition with the addition of 10µg/ml of the Neu5Gc- **linker1**-OVA sialoglycoconjugate (Figure 5.2.13a). In contrast, the mutant scFv clones D34, D14 and F46 either displayed minimal or no inhibition for the sialic acid glycoconjugate (Figures 5.2.13a/14b).

This suggests that these clones did not have the same propensity to bind the sialoglycoconjugate in solution as compared to AE8 and E15. It is not an uncommon occurrence during a phage library screening campaign, to identify clones that show high binding in a solid-phase ELISA, but have poor binding to the antigen in solution phase. The conformational integrity of the antigen is often partially disrupted (denatured) by coating the protein onto the polystyrene surface of the microtitre plate. In contrast, in a solution-phase binding assay, the native conformation of the antigen is often preserved (Vulliez-le Normand *et al.* 1997; Mutuberria *et al.* 2001; Vodnik *et al.* 2011). The goal of this screening process was to identify mutagenised light chain-shuffled scFvs, which had the ability to strongly bind sialoconjugates in both a solid-phase and a solution-phase context. Therefore, based on these criteria, the E15 anti-sialic acid mutant scFv was selected for further analysis.

A large and diverse panel of synthetic and sialic acid-containing structures were used to deduce the sialic acid binding specificity of both the mutant E15 and wildtype AE8 scFvs. The results are presented in table 5.2.19. In total, 22 different structures were tested using a combination of ELISA and SPR methods. Analysis was performed with five distinct sample groups: sialo-neoglycoconjugates, naturally sialylated glycoproteins, non-sialylated glycoproteins, PAA conjugates and unconjugated sugars. Table 5.2.19 shows that both the E15 and AE8 scFvs had strong binding reactivity to the sialo-neoglycoconjugates, Neu5Gc-**linker1**-BSA, Neu5Gc-**linker1**-OVA and Neu5Ac-**linker1**-OVA. In addition, both scFvs also bound strongly to the sialo-PAA conjugates, Neu5Gc-**linker2**-PAA and Neu5Ac-**linker2**-PAA. Furthermore, a third sialo-PAA conjugate Neu5Ac-**linker3**-PAA, which had a shorter linker length (9 atoms long), was also equally reactive. A disialy structure [Neu5Ac α (2,8)Neu5Ac-**linker2**-PAA] also showed strong reactivity when analysed by a solution-phase SPR-based inhibition assay. To ensure that scFv-binding was indeed specific to sialic acid, a series of negative control structures were tested using ELISA and/or SPR formats. Analysis showed that even at relatively high concentrations of BSA, OVA, OVA-**linker1** (2mg/ml) and **linker2**-PAA (500 μ g/ml) no significant binding or inhibition was observed. These results revealed that the protein or linker parts of the sialo-neoglycoconjugates, and the acrylamide and linker parts of the sialo-PAA conjugates, are most likely not part of the scFv-sialic acid binding interaction. Overall, these observations strongly support the assertion, that sialic acid is the epitope required for E15 and AE8 binding.

Two non-sialylated glycoproteins, ribonuclease B and asialofetuin were also tested for scFv reactivity. Ribonuclease B (molecular mass 15,500 Da) is a glycoprotein that contains a single *N*-glycosylation site at ASN-34. The carbohydrate chain is a mixture of high mannose type glycans. In contrast, desialylated fetuin contains the same oligosaccharide structures as fetuin, except that sialic acid is absent from the carbohydrate chains (An and Cipollo, 2011). Both E15 and AE8 showed no binding reactivity towards the non-sialylated glycoproteins even when relatively high concentrations (2mg/ml) were tested in the inhibition assay.

An inhibition solution-phase SPR test was used to analyse the binding of AE8 and E15 to the sialylated glycoproteins: fetuin, alpha-1-acid glycoprotein, bovine mucin and porcine thyroglobulin. However, SPR analysis of all four sialoglycoproteins revealed no detectable inhibition. These data appeared to suggest that the E15 and AE8 scFvs did not have the ability to recognise sialic acid in the context of a natural glycan chain. This lack of specificity in the natural context may relate to the difference in the conformation of sialic acid when attached to an overall carbohydrate structure. Presentation of sialic acid in the context of the artificial neoglyconjugate linker is most likely different, to that when Sia are presented as part of a natural oligosaccharide chain which is itself attached to the protein core of the glycoprotein. Indeed previous work by Berman (1984) has suggested, that the conformation of sialic acid is determined by its underlying sugar residues. Such context dependency binding effects are often observed with lectins, where changes in the glycan spatial conformation, alters the ability of the lectin to bind to the oligosaccharide chains of the glycoprotein. For example, the PSL, SNA lectins preferentially bind sialylated $\alpha(2,6)$ but not sialylated $\alpha(2,3)$ -linked *N*-glycans (Shibuya *et al.* 1987). Whereas the MAL lectin has an $\alpha(2,3)$ Sia-linkage specificity (Yamamoto *et al.* 2005). Berman (1984) has proposed that the difference in Sia-glycosidic linkage specificity, occurs because the sialylated $\alpha(2,6)$ isomers assume an extended conformation, with the sialic acid residues at the terminal of their oligosaccharide chain, whereas sialylated $\alpha(2,3)$ isomers appear to be folded backward towards the inner core. Similarly, it is likely that in this study, there is a different conformational arrangement of the sialic acid residue at the terminal ends of the sialoglycoproteins as compared to the neoglycoconjugates. This probably accounts for the inability of the AE8 and E15 scFvs to recognise the naturally occurring sialylated glycoprotein.

The ability of the AE8 and E15 scFvs to bind to unconjugated or “free” sialic acids (Neu5Ac or Neu5Gc) was also assessed by solution-phase SPR inhibition assays. However, SPR analysis with these free monosaccharides did not show any significant inhibition even at concentrations of up to 2mg/mL. Although this was not ideal, these data are consistent with other published reports on anti-carbohydrate binders. For example, it is known that some anti-carbohydrate antibodies do not recognise sugars or oligosaccharide units in their free form.

Rather these anti-carbohydrate antibodies recognise glycopeptidic epitopes that consist of specific oligosaccharide structures and adjacent amino acid residue(s) (Lisowska, 2002). Moreover, most lectins classified as Sia-specific do not bind free Sia but only Sia that is linked to other monosaccharides as part of *N*-linked or *O*-linked glycan chains. However, these lectins still bind to the sialoglycan chains of certain glycoproteins (Shibuya *et al.* 1987; Cummings *et al.* 2009). Based on the SPR inhibition data, it therefore appears that both AE8 and E15 also share this relatively common characteristic.

The discrepancy in binding of the free monosaccharides most likely relates to the nature of the scFv combining site. Indeed, the fact that both scFvs share practically the same pattern of sialic acid recognition (Table 5.2.19), coupled to the fact that they share >90% similarity in their amino acid sequences, (Figure 5.2.14) indicates that the design of their binding sites, in terms of specificity to sialic acid is probably very similar. Although the exact molecular features of their combining sites are not known, it is still possible to speculate on the sialic acid-scFv binding interaction. It is plausible that the shape of the scFv combining site cannot, to any significant degree, easily accommodate sialic acid in its free form. Perhaps the binding pocket is not shallow, or loose-fitting enough to easily allow passage of the free sialic acid molecule into the binding cavity. In contrast, when sialic acid is attached to a linker which is itself attached to a larger backbone structure, the conformation of the various sialic acid ring substituents (acetamido, carboxyl and hydroxyl groups as well as the glycerol side chain) around the pyranose ring are stabilised and fixed into a well-defined conformation. Moreover, as the sialic acid residue is anchored to a much larger structure, the scFv can more easily “grab hold” of the sugar molecule, which then pushes further into the binding pocket of the scFv. In addition, to the stabilising effect, the polyvalent structure of the neoglycoconjugate also provides numerous sialic acid contact points for scFv attachment. It is possible that the cluster arrangement of sialic acid residues on the glycoconjugate greatly enhanced the binding of the anti-sialic acid scFvs. Such multivalency or clustering of polyvalent sialic acid residues does not occur with the free monosaccharide and therefore, this may also account for the lack of scFv reactivity towards free Sia.

A detailed SPR kinetic binding study of the interaction between the mutant E15 scFv and the Neu5Gc-BSA conjugate was performed. The goal was to compare the binding kinetics of the mutant E15 scFv with the parental AE8 scFv. As outlined in chapter four, great care was taken to ensure the accuracy of the kinetic data. For example, to avoid mass transport effects, a low density of neutravidin and biotinylated-Neu5Gc-PAA was immobilised on the sensor surface. In addition, to exclude the effects of avidity, all scFv preparations were purified using a phenomenex™ 3000 SEC column equilibrated in PBS or HBS buffer. Analysis of the binding kinetics of the purified monomeric E15 scFv fraction is shown in Figure 5.2.16. The quality of the sensor data is poor. Visual inspection of the fitted overlay plots reveals large differences in the observed and calculated data, particularly in the association phases. Analysis of the residual plot, which is another means for assessing the quality of the fit, reveals large systematic (non-random) deviations in the association and dissociation phase of the sensorgrams. This indicated that the binding model did not capture the binding process observed in the experiment. Therefore, the calculated association and dissociation rate constants are not reliable. Although multiple SPR kinetic experiments were performed, each run also produced poor fits to the 1:1 (Langmuir) binding model. The exact reasons for this are unknown, however it has been reported that a wide range of variables (mass-transport limitations, drifting baseline, bulk refractive index artefacts, sample injection artefacts, protein rebinding artefacts, heterogenous immobilisation of antigen and inaccurate protein determination) can have a substantial negative impact on the kinetic experiment (Hahnefeld *et al.* 2004; Schuck and Zhao, 2010). It is possible that one or more of these variables were responsible for the poor SPR binding data.

Since a direct comparison of the affinity improvement of the E15 scFv relative to the AE8 scFv was not possible by SPR kinetics, a solution-phase SPR-based inhibition study was therefore employed to characterise the binding affinity of the mutant E15 scFv. In this assay, the E15 scFv was prevented from binding to captured Neu5Gc-PAA by the addition of variable amounts of solution-phase Neu5Gc-BSA conjugate. The IC_{50} value was defined as the concentration of Neu5Gc-BSA conjugate that inhibited E15 scFv binding to captured Neu5Gc-PAA by 50%. Thus, the greater the affinity of the E15 scFv for the sialic acid conjugates, the lower its Neu5Gc-BSA IC_{50} value.

The SPR sensorgram in Figure 5.2.17 revealed a calculated E15 IC₅₀ concentration of 1.6ng/mL for the Neu5Gc-BSA conjugate. In contrast, the parental AE8 scFv had an IC₅₀ value of 5.7ng/mL for the Neu5Gc-BSA conjugate. Therefore, the mutant E15 scFv exhibited an improvement in sialic acid binding of approximately 3.5-fold compared to the parental AE8 scFv. These data show that a directed molecular evolution approach can be successfully applied to the creation of affinity-improved anti-sialic acid antibody fragments.

The increased affinity of the E15 scFv most likely relates to its unique amino acid sequence. As shown in Figure 5.2.14 the E15 and AE8 scFvs are genetically different. However, a substantial part of the deduced amino acid sequence of AE8 and E15 is homologous. Thus, even though a combination of light chain-shuffling and PCR mutagenesis approaches were used, only a small number of DNA base changes occurred between the two sequences. It is probably that the mutant scFv library was highly diverse during the initial rounds of biopanning. However, with subsequent rounds of depletion and selection, the diversity of the scFv library was most likely reduced. It is of interest that the original AE8 heavy and light chains are mostly conserved within the E15 scFv. For example, no differences are seen between AE8 and E15 in their CDR-L1, CDR-L2, CDRH-1, CDRH-3 and FW regions. Rather, only single amino acid changes occur in the CDRH-2, CDRL-3 and linker regions. For example in the CDRH-2 region of E15 a serine to asparagine, amino acid change occurred. This conservative amino acid alteration originated from a single base substitution, AAC → AGC and gave rise to a missense mutation. In addition, a second conservative amino acid change, serine to glycine occurred in the CDRL-3 region of E15. Again this single base substitution, AGT → GGT gave rise to a missense mutation. Interestingly a third conservative mutation occurred in the linker region with a change in the amino sequence from serine to proline (TCC → CCC). Although, the dNTP ratio was altered to reduce the nucleotide transition bias, all nucleotide substitutions (A-G and T-C) in the E15 scFv, were transition type mutations. Without extensive sequencing of the original unpanned mutagenised and light chain shuffled scFv library, it is impossible to know if transition-type mutations predominate. This lack of sequence promiscuity indicates that the AE8 amino acid sequence was already thoroughly optimised for high affinity sialic acid recognition.

However, the E15 scFv sequence shows us, that affinity fine tuning is still possible, as just a few subtle amino acid changes, increased the nanomolar binding affinity of the AE8 anti-sialic scFv, by more than 3-fold. Whether or not, other amino acid changes could increase the sialic acid binding affinity even further, remains to be elucidated. The generation of the E15 scFv is quite significant, as this is the first report of an affinity-improved recombinant avian anti-sialic acid scFv.

In conclusion, this chapter described the successful application of a directed molecular evolutionary approach, for the generation of affinity-improved anti-sialic acid recombinant antibodies. A second anti-sialic acid library was constructed using a random mutagenesis approach. In order to prevent the complete abolition of sialic acid binding, a light chain shuffling strategy was undertaken, in which a fixed anti-sialic acid heavy chain (AE8) was shuffled with an error-prone PCR amplified library of light chains. Biopanning of this mutant scFv library identified a single scFv clone, E15, which had very strong binding reactivity to both sialo-neoglycoconjugates and the sialo-PAA conjugates. In addition, no significant binding or inhibition was observed when a range of control proteins (non-sialylated glycoproteins) and non-sialylated synthetic structures were tested. Importantly, these results demonstrated that the protein or linker parts of the sialo-neoglycoconjugates and the acrylamide and linker parts of the sialo-PAA conjugates, were most likely not part of the scFv-sialic acid binding interaction. Furthermore, a solution-phase SPR-based inhibition study revealed that the E15 scFv had an IC_{50} of 1.6ng/mL for the Neu5Gc-BSA conjugate. Compared to the AE8 clone (IC_{50} of 5.7ng/mL) the E15 scFv exhibited an improvement in sialic acid binding of approximately 3.5-fold. Analysis of the deduced amino acid sequence alignment of AE8 and E15, CDR V_H and V_L regions revealed that both clones were genetically different. Interestingly, three conservative amino acid changes (serine to asparagine, serine to glycine and serine to proline) were identified in the CDRH-2, CDRL-3 and linker regions. The generation of the E15 scFv is both significant and unique. Importantly, this is the first report of an affinity-improved recombinant avian anti-sialic acid scFv.

Chapter 6

Overall conclusions

6.1 Overall summary and conclusions

Sialic acids are a family of acidic monosaccharides that typically reside as terminal moieties on *N*- and *O*-linked glycans. In the last 50 years since their discovery, these biologically important nine carbon sugars have been extensively characterised. Sialic acids are actively involved in a plethora of biological phenomena, ranging from cell-cell adhesion and recognition, intracellular signalling events, pathogen attack, viral infection, inflammatory disease and cancer. Two important members of the sialic acid family are Neu5Ac and Neu5Gc. Neu5Ac is ubiquitous and is often found in eukaryotic cells either $\alpha(2,3)/\alpha(2,6)$ -linked to galactose, and/or $\alpha(2,6)$ -linked to *N*-acetyl-galactosamine (GalNAc). Many cancer-related antigens contain terminal sialic acids or altered sialylation patterns. In particular, human tumour tissues on average contain more Neu5Gc than is found in normal tissues. Therefore, the identification of sialic acid-specific antibodies is important in the fields of basic research and diagnostics. Moreover, understanding carbohydrate-protein interactions at the molecular level has considerable scientific value. Currently, no recombinant avian anti-sialic acid antibody exists. Therefore, the primary objective of this thesis was the generation and characterisation of recombinant antibodies that recognised sialic acid. In addition, a secondary aim was the application of directed molecular evolution for the creation of improved anti-sialic acid antibody fragments. Finally, a third goal was the development of a high-throughput screening system for antibody discovery.

The first part of this research project focused on the the development of a high-throughput screening system for antibody discovery. This system was highly complex and it took a team of engineers more than one year to install all of the components on site at DCU. Unfortunately, after its installation, the robotic system was plagued by a multitude of hardware problems and software failures. One such hardware problem was the inability of the robotic arm to place microtitre plates within the centrifuge. This was fixed by raising the centrifuge using a steel plate which was placed underneath the instrument.

A number of hardware issues also arose with the colony picker. An initial problem was the insufficient air pressure, which meant that the colony picking pins did not operate properly. This problem was rectified by adding an external compressor pump to the system.

A second issue related to the inability of the picking arm to clear the drying bath and the brush bath holders. This was fixed by altering the z-travel height of the picking arm. A third issue related to the colony picking pins. These were not of sufficient length to inoculate part of the bacterial colony into the deep well plates. This issue was resolved when a longer set of colony picking pins were installed.

A major hardware fault occurred with the robotic liquid handling arm. A motor that moved the arm in the Y-axis was found to be faulty. As this motor could not be fixed, the whole robotic arm was replaced. Although a number of other hardware issues also occurred, all of these were rectified. In contrast, software problems were extremely difficult to troubleshoot and took considerable time and effort to resolve.

The software issues included problems with incubator software drivers, colony picker software and Tecan EVOware[®] software. The software drivers for the incubators were particularly problematic as frequently they caused the whole Tecan instrument to crash. This problem was eventually solved when a new build of the StoreX drivers were released from Tecan. One reason for many of the software problems, related to the fact that the majority of peripheral devices were manufactured by companies not affiliated with Tecan. When the robotic system was set up at DCU, most of these peripheral devices had never been tested with Tecan EVOware[®] software. For example when the colony picker software was initially tested, a communication problem was discovered between EVOware[®], the colony picker and its software driver. This problem was only solved after the colony picker manufacturer (KBiosciences, UK) released a customised software driver update. Many bugs were also found with the Tecan EVOware[®] software. A severe problem occurred with the instrument after an EVOware[®] software upgrade was performed. This procedure caused EVOware[®] software to stop running. After a very lengthy procedure, the system was eventually fixed. Due to time constraints, no further work was performed on the robotic antibody screening system. However, all outstanding software and hardware issues were resolved.

The early work of chapter four, described the screening of two commercially available libraries, and the rational design and custom synthesis of sialic acid immunisation and screening neoglycoconjugates. The latter part of chapter four described phage library construction, generation of recombinant antibody fragments, and the isolation and characterisation of novel avian anti-sialic acid antibodies.

Initially the possibility of finding anti-sialic scFvs using two commonly available semi-synthetic libraries (Tomlinson I and J) was explored. However, none of the selected clones showed any significant sialic acid reactivity. Therefore, it appeared that these libraries were not suitable for the isolation of anti-sialic acid antibodies.

In the search for novel sialic acid binding proteins, animal immunisations were performed with a chemically-defined sialic acid-containing neoglycoconjugate. This approach was not without its risks, as many carbohydrate antigens are notoriously weakly immunogenic. Therefore, a considerable amount of time was invested in the design of the sialic acid immunisation and screening neoglycoconjugates. Neu5Gc monosaccharides were attached to HSA and BSA protein carriers using a 13 atom linker. In addition, the C2-position of Neu5Gc was covalently attached to a spacer arm. The HSA and BSA neoglycoconjugates contained 35 and 20 moles of Neu5Gc per mole of protein, respectively.

As previously described (Chapter 4.1) chickens offer many advantages for recombinant antibody library generation. An important advantage in the context of this project was the absence of Neu5Gc from the normal tissues of chickens (Diaz *et al.*, 2009). Thus, the avian immune system offered a potential way to circumvent the problem of carbohydrate immunological tolerance. Furthermore, the work by Asaoka *et al.*, (1992), Noguchi *et al.*, (1995) and Tangvoranuntakul *et al.*, (2003) had previously demonstrated that chickens could be used as hosts for the successful generation of anti-Neu5Gc pAbs. Therefore, a chicken was immunised with a novel, bespoke Neu5Gc-HSA neoglycoconjugate. This strategy was successful, as analysis of pre- and post-immunised chicken serum revealed a significant immune response against the Neu5Gc-BSA neoglycoconjugate. Furthermore, analysis with a sialylated PAA polymer, demonstrated that the avian pAbs could discriminate Neu5Gc in the context of two very different carriers with unrelated linkers, namely BSA-**linker1**-Neu5Gc and PAA-**linker2**-Neu5Gc. These data confirmed that chickens are advantageous hosts for the generation of anti-sialic acid antibodies.

These results also confirmed that the antigenicity of Neu5Gc was not compromised, even after covalent attachment of its C2-carbon to a 13 atom linker, which was itself attached to the protein carrier. Furthermore, this work revealed that a sialic acid density of 35 moles per mole of carrier protein was sufficient for the production of high anti-sialic acid avian antibody titres. These results are consistent with previous reports, which have suggested that high antibody titres are usually obtained with hapten densities of between 15 to 30 moles per mole of carrier protein (Pozsgay *et al.*, 1999; Singh *et al.*, 2004; Lemus and Karol, 2008).

The anti-sialic acid scFv library was constructed from the antibody variable-region genes, of B-cells derived from the spleen and bone marrow tissues, of a chicken that was immunised with a Neu5Gc-HSA neoglycoconjugate. The scFv library was screened using a novel Neu5Gc-BSA conjugate. Two different biopanning elution techniques were assessed: (a) competitive elution and (b) standard trypsin elution. Analysis of the monoclonal phage ELISA results revealed that both methods produced equally large numbers of anti-sialic acid scFvs. Therefore, these findings indicated that either the trypsin or competitive elution approaches could be used to retrieve anti-sialic acid phage binders from an avian immune library. Interestingly, of the original 90% of clones that bound to Neu5Gc-BSA, less than 10% of these clones recognised Neu5Gc in the context of the PAA-backbone. This loss of recognition probably occurred due to alterations in the spatial arrangements of Neu5Gc when attached to PAA, as compared to BSA.

Cross-reactivity studies using different mono and disaccharide carbohydrate structures identified two scFvs, AE8 and CC11 that had very strong binding to Neu5Ac-PAA and Neu5Gc-PAA conjugates. Comparison of the deduced amino acid sequence alignment of both clones, showed that they were genetically different. In addition, these clones had large differences in their amino acid sequence composition in both CDRL1 and CDRL3. In contrast, fewer differences were seen in CDRH1, CDRH2 and CDRH3. Both clones contained relatively long CDR3 regions. Interestingly, it has been reported that a number of anti-carbohydrate antibodies, which recognised sialic acid structures on gangliosides also contain a relatively long CDR3 (Lopez-Requena *et al.*, 2007).

Furthermore, it has been suggested that a longer CDR3 compensates for the smaller carbohydrate antigen-binding surface area (Gerstenbruch *et al.*, 2010). It is therefore possible that the CDR3 length of AE8 and CC11 played an important role in sialic acid binding.

Overall the AE8 scFv had the strongest sialic acid reactivity. In addition, this clone contained the largest number of arginine (Arg) residues. Arg is a large and positively charged amino acid, and acts as an important contact residue in the sialic acid binding site of certain proteins. Examples include, K99, a Neu5Gc-ganglioside binding protein, the sialidase protein of influenza, and the *N*-glycolyl GM3 ganglioside binding Fab. It is possible that both the locations and larger numbers of AE8 Arg residues may have contributed to its ability to strongly bind sialic acid.

SEC-HPLC and FPLC were used to determine the species composition and apparent molecular mass of the recombinant AE8 scFv. Analysis showed that the AE8 IMAC-purified protein contained both dimeric (60kDa) and monomeric (28kDa) scFv species. This was not unexpected as the avian scFv library was built using a short 7-amino acid Gly-Ser-polypeptide linker. The original intention was to promote scFv dimer formation, since it was speculated, that avidity effects may increase the overall binding of anti-sialic acid scFv antibodies. Such an effect was observed in an SPR binding study, which showed that the dimeric scFv fraction had a higher SPR Neu5Gc-binding signal than the monomeric fraction. This gain in functional affinity for the dimeric AE8 scFv was probably due to reduced 'off-rates' which resulted from both multiple binding (scFv multivalency) and rebinding to the multivalent Neu5Gc conjugate. The presence of scFv dimers was consistent with other literature reports, which have described the formation of scFv oligomers, when either short or long linkers are used for library construction (MacKenzie and To, 1998; Johansson *et al.*, 2006; Johansson *et al.*, 2007).

An inhibition assay demonstrated that the AE8 scFv had an IC₅₀ concentration of 5.7ng/mL for the Neu5Gc-BSA conjugate. Furthermore, an SPR kinetic study revealed that the AE8 scFv had a K_D of 57nM for the Neu5Gc-BSA conjugate. The generation of such a highly specific anti-sialic acid antibody in a recombinant format is exceedingly rare (Kaltgrad *et al.*, 2007). In contrast, most anti-carbohydrate antibodies are not recombinant and display weak carbohydrate-protein interactions.

This work is significant, as it showed for the first time, the generation of an anti-carbohydrate scFv with nanomolar affinity for Neu5Gc-containing structures.

The worked described in chapter five, focused on a mutagenesis approach for the generation of improved anti-sialic acid antibody fragments. In the latter half of the chapter, the use of new sialo-neoglycoconjugates for the isolation of positively-binding sialic acid clones was described. In addition, a detailed assessment of the specificity and affinity of these mutant clones was also presented.

The AE8 scFv had previously shown the strongest sialic acid reactivity and was therefore selected as a template for mutagenesis experiments. No structural sialic acid binding information was available for this clone. Therefore, a random mutagenesis approach was used for antibody gene diversification. In order to prevent the complete abolition of sialic acid binding, a light chain shuffling strategy was undertaken, in which a fixed anti-sialic acid heavy chain (AE8) was shuffled with a library of light chains. Mutagenesis was performed using both error-prone PCR and light chain shuffling techniques.

The mutant scFv library was biopanned using a depletion protocol and more than 90% of the clones tested, showed significant binding reactivity to Neu5Gc-**linker1**-OVA and Neu5Ac-**linker1**-OVA. However, only ten of these clones had no significant binding reactivity, to either the OVA protein or the **linker1**-OVA conjugate. These data showed the importance of using depletion steps in a biopanning protocol. Interestingly, only four of these ten clones recognised Neu5Gc in the context of a PAA-backbone. These data revealed that these 4 clones had the ability to recognise Sia without the requirement of a specific linker and consequently, these scFvs were not linker-only-specific but rather sialic acid-specific. SPR inhibition and ‘off-rate’ studies identified one mutant clone, E15, which in comparison to AE8 appeared to have greater affinity for sialic acid.

A large panel of synthetic and sialic acid-containing structures were used to investigate the sialic acid binding specificity of both the parental AE8 and the mutant E15 scFv clones. The E15 and AE8 scFvs showed strong binding reactivity to a range of sialo-neoglycoconjugates and sialo-PAA conjugates.

Importantly, no significant binding or inhibition was observed when a group of control proteins (non-sialylated glycoproteins) and non-sialylated synthetic structures were tested. Overall, these results indicated that the protein, acrylamide and linker parts of the conjugates were most likely not involved in the scFv-sialic acid binding interaction.

The ability of the AE8 and E15 scFvs to bind either natural sialoglycoproteins and/or free sialic acid monosaccharides, was assessed by solution-phase SPR inhibition assays. However, no significant inhibition was observed even at relatively high antigen concentrations. Sialic acid conformational differences may have accounted for the inability of the scFvs to recognise the naturally-occurring sialylated glycoproteins. In addition, the discrepancy in binding of the free monosaccharides most likely related to the nature of the scFv-combining site. It was possible that the shape of the scFv-combining site could not to any significant degree accommodate sialic acid in its free form. This trait also occurs in some anti-carbohydrate antibodies and lectins, which do not recognise sugars or oligosaccharide units in their free form. Rather, they only recognise glycopeptidic epitopes that consist of specific oligosaccharide structures and adjacent amino acid residue(s) (Shibuya *et al.*, 1987; Lisowska, 2002; Cummings and Etzler, 2009).

A solution-phase SPR-based inhibition study demonstrated that the E15 scFv had an IC_{50} concentration of 1.6ng/mL for the Neu5Gc-BSA conjugate. Compared to the AE8 clone (IC_{50} of 5.7ng/mL) the E15 scFv exhibited an improvement in sialic acid binding of approximately 3.5-fold. These data demonstrated that an error-prone PCR and light chain-shuffling approach could be used to improve the affinity of an anti-sialic acid scFv.

Importantly, comparison of the deduced amino acid sequence alignment of AE8 and E15 revealed that both clones were genetically different. Although the majority of their sequences were similar, three amino acid changes were observed in CDRH-2, CDRL-3 and the linker region. The lack of sequence difference between AE8 and E15, coupled with the modest improvement in sialic acid binding, indicated that the original AE8 scFv structure was already substantially optimised for high affinity sialic acid binding.

However, analysis of the E15 sequence also demonstrated that a few subtle amino acid changes increased the nanomolar binding affinity of AE8 by more than 3-fold. The generation of the E15 clone is unique, in that it represents the first report of an affinity-improved recombinant avian anti-sialic acid scFv.

This thesis work has identified many promising areas for further research in the field of recombinant anti-sialic acid antibody production. For example, one such avenue for future research, would be to identify those amino acids that are responsible for the improvement in sialic acid binding. It is possible that molecular modelling, site directed mutagenesis and X-ray crystallography studies could be used to identify those scFv residues, which play critical and non-critical roles in the binding of sialic acid. In addition, a second potential area of research relates to the anti-sialic acid phage libraries. It is possible that re-screening of the phage libraries using appropriate sialylated glycoprotein(s) would enable the isolation of new scFv clones, which would have the ability to recognise sialic acid in the context of a natural glycoprotein. Ultimately, it is possible that such newly identified recombinant antibody fragments could be incorporated into a rapid test for the estimation of a glycoprotein's sialic acid content.

In summary, the work presented in this thesis has demonstrated that a rational approach could be applied to the design of sialic acid immunogens. Importantly, this research showed that an anti-sialic acid immune response could be elicited, when appropriate choices were made for immunological variables such as: type of carrier protein, site of conjugation of the sugar moiety, the density of carbohydrate hapten, the linker length and the selection of the correct animal model. In addition, this work emphasised the utility of recombinant antibody technology for the production of anti-sialic acid scFvs. Finally, this research demonstrated that a directed evolution approach, which employed recombination and mutagenesis, could be used to affinity-improve a sialic acid binding protein fragment.

Chapter 7
Bibliography

- Adams, G. P. and Schier, R. (1999) Generating improved single-chain fv molecules for tumor targeting. *J. Immunol. Methods* **231**, 249-260
- Agrawal, A. F. and Wang, A. D. (2008) Increased transmission of mutations by low-condition females: Evidence for condition-dependent DNA repair. *PLoS Biol.* **6**, 30
- Aich, U. and Yarema, K. J. (2008) Glycobiology and immunology, 1-53. In: Guo, Z., and Boons, G.J. Editors, Carbohydrate-Based Vaccines and Immunotherapies, John Wiley & Sons, USA
- Alavi, A. and Axford, J. S. (2008) Sweet and sour: The impact of sugars on disease. *Rheumatology* **47**, 760-770
- Alexander, J., del Guercio, M. F., Maewal, A., Qiao, L., Fikes, J., Chesnut, R. W., Paulson, J., Bundle, D. R., DeFrees, S., and Sette, A. (2000) Linear PADRE T helper epitope and carbohydrate B cell epitope conjugates induce specific high titer IgG antibody responses. *J. Immunol.* **164**, 1625-1633
- Allevi, P., Femia, E. A., Costa, M. L., Cazzola, R., and Anastasia, M. (2008) Quantification of *N*-acetyl- and *N*-glycolylneuraminic acids by a stable isotope dilution assay using high-performance liquid chromatography-tandem mass spectrometry. *J. Chromatogr. A* **1212**, 98-105
- Alviano, C. S., Travassos, L. R., and Schauer, R. (1999) Sialic acids in fungi: A minireview. *Glycoconj. J.* **16**, 545-554
- Ambrose, M. G., Freese, S. J., Reinhold, M. S., Warner, T. G., and Vann, W. F. (1992) ¹³C NMR investigation of the anomeric specificity of CMP-*N*-acetylneuraminic acid synthetase from *Escherichia coli*. *Biochemistry* **31**, 775-780
- Aminoff, D. (1961) Methods for the quantitative estimation of *N*-acetylneuraminic acid and their application to hydrolysates of sialomucoids. *Biochem. J.* **81**, 384-392
- An, Z., Forrest, G., Moore, R., Cukan, M., Haytko, P., Huang, L., Vitelli, S., Zhao, J. Z., Lu, P., Hua, J., Gibson, C. R., Harvey, B. R., Montgomery, D., Zaller, D., Wang,

- F., and Strohl, W. (2009) IgG2m4, an engineered antibody isotype with reduced fc function. *MAbs* **1**, 572-579
- An, Y. and Cipollo, J. F. (2011) An unbiased approach for analysis of protein glycosylation and application to influenza vaccine hemagglutinin. *Anal. Biochem.* **415**, 67-80
- Andris-Widhopf, J., Rader, C., Steinberger, P., Fuller, R., and Barbas, C. F.,3rd. (2000) Methods for the generation of chicken monoclonal antibody fragments by phage display. *J. Immunol. Methods* **242**, 159-181
- Angata, T. and Varki, A. (2002) Chemical diversity in the sialic acids and related alpha-keto acids: An evolutionary perspective. *Chem. Rev.* **102**, 439-469
- Anumula, K. R. (1995) Rapid quantitative determination of sialic acids in glycoproteins by high-performance liquid chromatography with a sensitive fluorescence detection. *Anal. Biochem.* **230**, 24-30
- Anumula, K. R. (2006) Advances in fluorescence derivatization methods for high-performance liquid chromatographic analysis of glycoprotein carbohydrates. *Anal. Biochem.* **350**, 1-23
- Aplin, J. D. and Wriston, J. C.,Jr. (1981) Preparation, properties, and applications of carbohydrate conjugates of proteins and lipids. *CRC Crit. Rev. Biochem.* **10**, 259-306
- Apweiler, R., Hermjakob, H., and Sharon, N. (1999) On the frequency of protein glycosylation, as deduced from analysis of the SWISS-PROT database. *Biochem. Biophys. Acta* **1473**, 4-8
- Arakawa, H., Kuma, K., Yasuda, M., Furusawa, S., Ekino, S., and Yamagishi, H. (1998) Oligoclonal development of B cells bearing discrete Ig chains in chicken single germinal centers. *J. Immunol.* **160**, 4232-4241
- Arakawa, H. and Buerstedde, J. M. (2004) Immunoglobulin gene conversion: Insights from bursal B cells and the DT40 cell line. *Dev. Dyn.* **229**, 458-464

- Armstrong, P. B., Swarnakar, S., Srimal, S., Misquith, S., Hahn, E. A., Aimes, R. T., and Quigley, J. P. (1996) A cytolytic function for a sialic acid-binding lectin that is a member of the pentraxin family of proteins. *J. Biol. Chem.* **271**, 14717-14721
- Arranz-Plaza, E., Tracy, A. S., Siriwardena, A., Pierce, J. M., and Boons, G. J. (2002) High-avidity, low-affinity multivalent interactions and the block to polyspermy in *Xenopus laevis*. *J. Am. Chem. Soc.* **124**, 13035-13046
- Asaoka, H., Nishinaka, S., Wakamiya, N., Matsuda, H., and Murata, M. (1992) Two chicken monoclonal antibodies specific for heterophil hanganutzium-deicher antigens. *Immunol. Lett.* **32**, 91-96
- Astronomo, R. D. and Burton, D. R. (2010) Carbohydrate vaccines: Developing sweet solutions to sticky situations? *Nat. Rev. Drug Discov.* **9**, 308-324
- Ayyar, B. V., Hearty, S., and O'Kennedy, R. (2010) Highly sensitive recombinant antibodies capable of reliably differentiating heart-type fatty acid binding protein from noncardiac isoforms. *Anal. Biochem.* **407**, 165-171
- Babál, P., Pindak, F. F., Wells, D. J., and Gardner, W. A., Jr. (1994) Purification and characterization of a sialic acid-specific lectin from *Tritrichomonas mobilensis*. *Biochem. J.* **299**, 341-346
- Balaguer, E. and Neususs, C. (2006) Glycoprotein characterization combining intact protein and glycan analysis by capillary electrophoresis-electrospray ionization-mass spectrometry. *Anal. Chem.* **78**, 5384-5393
- Ban, M., Yoon, H. J., Demirkan, E., Utsumi, S., Mikami, B., and Yagi, F. (2005) Structural basis of a fungal galectin from *Agrocybe cylindracea* for recognizing sialoconjugate. *J. Mol. Biol.* **351**, 695-706
- Bandyopadhyay, S., Mukherjee, K., Chatterjee, M., Bhattacharya, D. K., and Mandal, C. (2005) Detection of immune-complexed 9-O-acetylated sialoglycoconjugates in the sera of patients with pediatric acute lymphoblastic leukemia. *J. Immunol. Methods* **297**, 13-26

- Bao, Y., Zhu, L., and Newburg, D. S. (2007) Simultaneous quantification of sialyloligosaccharides from human milk by capillary electrophoresis. *Anal. Biochem.* **370**, 206-214
- Barbas, C. F. I. (2001) Phage Display: A Laboratory Manual, 1st Edition, 736 pp, Cold Spring Harbor Laboratory Press
- Barua, A., Furusawa, S., Yoshimura, Y., and Okamoto, T. (2001) Effects of forced molting on the IgY concentration in egg yolk of chickens. *Poult. Sci.* **38**, 169-174
- Benatuil, L., Perez, J. M., Belk, J., and Hsieh, C. M. (2010) An improved yeast transformation method for the generation of very large human antibody libraries. *Protein Eng. Des. Sel.* **23**, 155-159
- Berman, E. (1984) Structural and conformational analysis of sialyloligosaccharides using carbon-13 nuclear magnetic resonance spectroscopy. *Biochemistry* **23**, 3754-3759
- Berman, E. (1986) Reinvestigation of the carbohydrate chains of calf fetuin using ¹³C-n.m.r. spectroscopy. *Carbohydr. Res.* **152**, 33-46
- Betz, A. G., Rada, C., Pannell, R., Milstein, C., and Neuberger, M. S. (1993) Passenger transgenes reveal intrinsic specificity of the antibody hypermutation mechanism: Clustering, polarity, and specific hot spots. *Proc. Natl. Acad. Sci. U. S. A.* **90**, 2385-2388
- Bezzubova, O. Y. and Buerstedde, J. M. (1994) Gene conversion in the chicken immunoglobulin locus: A paradigm of homologous recombination in higher eukaryotes. *Experientia* **50**, 270-276
- Bhavanandan, V. P., Ringler, N. J., and Gowda, D. C. (1998) Identification of the glycosidically bound sialic acid in mucin glycoproteins that reacts as "free sialic acid" in the warren assay. *Glycobiology* **8**, 1077-1086

- Bird, C. R. and Thorpe, R. (2002) Purification of immunoglobulin Y (IgY) from chicken eggs, 1009-1011, In: Walker, J. M., Bird, C. R., Thorpe, R., Editors. The Protein Protocols Handbook, 2nd Edition, Humana Press
- Biro, C. E. (1968) A method for the production of rheumatoid factor in rabbits. *Immunology* **15**, 455-458
- Boeckler, C., Frisch, B., Muller, S., and Schuber, F. (1996) Immunogenicity of new heterobifunctional cross-linking reagents used in the conjugation of synthetic peptides to liposomes. *J. Immunol. Methods* **191**, 1-10
- Bohin, A., Bouchart, F., Richet, C., Kol, O., Leroy, Y., Timmerman, P., Huet, G., Bohin, J. P., and Zanetta, J. P. (2005) GC/MS identification and quantification of constituents of bacterial lipids and glycoconjugates obtained after methanolysis as heptafluorobutyrate derivatives. *Anal. Biochem.* **340**, 231-244
- Bovin, N. V. (1998) Polyacrylamide-based glycoconjugates as tools in glycobiology. *Glycoconj. J.* **15**, 431-446
- Bradbury, A. R. M. (2010) The use of phage display in neurobiology. *Curr Protoc Neurosci.* **5**, Unt 5.12
- Brennan, J., Dillon, P., and O'Kennedy, R. (2003) Production, purification and characterisation of genetically derived scFv and bifunctional antibody fragments capable of detecting illicit drug residues. *J. Chromatogr. B. Analyt Technol. Biomed. Life. Sci.* **786**, 327-342
- Brossmer, R., Wagner, M., and Fischer, E. (1992) Specificity of the sialic acid-binding lectin from the snail *Cepaea hortensis*. *J. Biol. Chem.* **267**, 8752-8756
- Bulai, T., Bratosin, D., Pons, A., Montreuil, J., and Zanetta, J. P. (2003) Diversity of the human erythrocyte membrane sialic acids in relation with blood groups. *FEBS Lett.* **534**, 185-189
- Bunk, D. M. (1997) Characterization of the glycation of albumin in freeze-dried and frozen human serum. *Anal. Chem.* **69**, 2457-2463

- Buskas, T., Li, Y., and Boons, G. J. (2004) The immunogenicity of the tumor-associated antigen Lewis (Y) may be suppressed by a bifunctional cross-linker required for coupling to a carrier protein. *Chemistry* **10**, 3517-3524
- Butor, C., Diaz, S., and Varki, A. (1993) High level *O*-acetylation of sialic acids on *N*-linked oligosaccharides of rat liver membranes. differential subcellular distribution of 7- and 9-*O*-acetyl groups and of enzymes involved in their regulation. *J. Biol. Chem.* **268**, 10197-10206
- Byres, E., Paton, A. W., Paton, J. C., Lofling, J. C., Smith, D. F., Wilce, M. C., Talbot, U. M., Chong, D. C., Yu, H., Huang, S., Chen, X., Varki, N. M., Varki, A., Rossjohn, J., and Beddoe, T. (2008) Incorporation of a non-human glycan mediates human susceptibility to a bacterial toxin. *Nature* **456**, 648-652
- Cadwell, R. C. and Joyce, G. F. (1992) Randomization of genes by PCR mutagenesis. *PCR Methods Appl.* **2**, 28-33
- Campbell, R. D., Dodds, A. W., and Porter, R. R. (1980) The binding of human complement component C4 to antibody-antigen aggregates. *Biochem. J.* **189**, 67-80
- Carlander, D., Stalberg, J., and Larsson, A. (1999) Chicken antibodies: A clinical chemistry perspective. *Ups. J. Med. Sci.* **104**, 179-189
- Carlander, D. and Larsson, A. (2001) Avian antibodies can eliminate interference due to complement activation in ELISA. *Ups. J. Med. Sci.* **106**, 189-195
- Carr, A., Mullet, A., Mazorra, Z., Vazquez, A. M., Alfonso, M., Mesa, C., Rengifo, E., Perez, R., and Fernandez, L. E. (2000) A mouse IgG1 monoclonal antibody specific for *N*-glycolyl GM3 ganglioside recognized breast and melanoma tumors. *Hybridoma* **19**, 241-247
- Casals-Stenzel, J., Buscher, H. P., and Schauer, R. (1975) Gas-liquid chromatography of *N*- and *O*-acylated neuramic acids. *Anal. Biochem.* **65**, 507-524

- Casteleyn, C., Doom, M., Lambrechts, E., Van den Broeck, W., Simoens, P., and Cornillie, P. (2010) Locations of gut-associated lymphoid tissue in the 3-month-old chicken: A review. *Avian Pathol.* **39**, 143-150
- Chan, T., Xin, Y., and von Itzstein, M. (1997) Synthesis of phosphonic acid analogues of sialic acids (Neu5Ac and KDN) as potential sialidase inhibitors. *J. Org. Chem.* **62**, 3500-3504
- Chaparro-Riggers, J. F., Loo, B. L., Polizzi, K. M., Gibbs, P. R., Tang, X. S., Nelson, M. J., and Bommarius, A. S. (2007) Revealing biases inherent in recombination protocols. *BMC Biotechnol.* **7**, 77
- Charlton, K., Harris, W. J., and Porter, A. J. (2001) The isolation of super-sensitive anti-hapten antibodies from combinatorial antibody libraries derived from sheep. *Biosens. Bioelectron.* **16**, 639-646
- Charlton, K. A. and Porter, A. J. (2002) Isolation of anti-hapten specific antibody fragments from combinatorial libraries. *Methods Mol. Biol.* **178**, 159-171
- Charlton, K. A. (2004) Expression and isolation of recombinant antibody fragments in *E. coli*. *Methods Mol. Biol.* **248**, 245-254
- Charlwood, J., Birrell, H., Organ, A., and Camilleri, P. (1999) A chromatographic and mass spectrometric strategy for the analysis of oligosaccharides: Determination of the glycan structures in porcine thyroglobulin. *Rapid Commun. Mass Spectrom.* **13**, 716-723
- Chava, A. K., Chatterjee, M., Sundar, S., and Mandal, C. (2002) Development of an assay for quantification of linkage-specific *O*-acetylated sialoglycans on erythrocytes; its application in indian visceral leishmaniasis. *J. Immunol. Methods* **270**, 1-10
- Che, F. Y., Shao, X. X., Wang, K. Y., and Xia, Q. C. (1999) Characterization of derivatization of sialic acid with 2-aminoacridone and determination of sialic acid content in glycoproteins by capillary electrophoresis and high performance liquid chromatography--ion trap mass spectrometry. *Electrophoresis* **20**, 2930-2937

- Chefalo, P., Pan, Y., Nagy, N., Harding, C., and Guo, Z. (2004) Preparation and immunological studies of protein conjugates of *N*-acylneuraminic acids. *Glycoconj. J.* **20**, 407-414
- Chen, F. T., Dobashi, T. S., and Evangelista, R. A. (1998) Quantitative analysis of sugar constituents of glycoproteins by capillary electrophoresis. *Glycobiology* **8**, 1045-1052
- Chen, J., Liu, B., Ji, N., Zhou, J., Bian, H. J., Li, C. Y., Chen, F., and Bao, J. K. (2009) A novel sialic acid-specific lectin from *Phaseolus coccineus* seeds with potent antineoplastic and antifungal activities. *Phytomedicine* **16**, 352-360
- Chothia, C., Lesk, A. M., Gherardi, E., Tomlinson, I. M., Walter, G., Marks, J. D., Llewelyn, M. B., and Winter, G. (1992) Structural repertoire of the human VH segments. *J. Mol. Biol.* **227**, 799-817
- Chowdhary, B. P. and Raudsepp, T. (2000) HSA4 and GGA4: Remarkable conservation despite 300-myrr divergence. *Genomics* **64**, 102-105
- Christensen, P. A., Danielczyk, A., Ravn, P., Larsen, M., Stahn, R., Karsten, U., and Goletz, S. (2009) Modifying antibody specificity by chain shuffling of V / V between antibodies with related specificities. *Scand. J Immunol.* **69**, 1-10
- Chua, K. F., Alt, F. W., and Manis, J. P. (2002) The function of AID in somatic mutation and class switch recombination: Upstream or downstream of DNA breaks. *J. Exp. Med.* **195**, F37-41
- Chusacultachai, S. and Yuthavong, Y. (2004) Random mutagenesis strategies for construction of large and diverse clone libraries of mutated DNA fragments. *Methods Mol. Biol.* **270**, 319-334
- Cirino, P. C., Mayer, K. M., and Umeno, D. (2003) Generating mutant libraries using error-prone PCR. *Methods Mol. Biol.* **231**, 3-9
- Clackson, T. and Lowman, H. B. (2007) Phage Display: A Practical Approach, pp. 332, Oxford University Press, Oxford

- Cobb, B. D. and Clarkson, J. M. (1994) A simple procedure for optimising the polymerase chain reaction (PCR) using modified taguchi methods. *Nucleic Acids Res.* **22**, 3801-3805
- Cobb, B. A. and Kasper, D. L. (2005) Coming of age: Carbohydrates and immunity. *Eur. J. Immunol.* **35**, 352-356
- Coco, W. M. (2003) RACHITT: Gene family shuffling by random chimeragenesis on transient templates. *Methods Mol. Biol.* **231**, 111-127
- Cohen, M. and Varki, A. (2010) The sialome--far more than the sum of its parts. *OMICS* **14**, 455-464
- Corfield, A. P., Corfield, C. D., Veh, R. W., Wagner, S. A., Clamp, J. R., and Schauer, R. (1991) Characterization of the major and minor mucus glycoproteins from bovine submandibular gland. *Glycoconj. J.* **8**, 330-339
- Crook, A. M. (1998) Serum sialic acid. should it be measured clinically and if so how? *Clin. Lab.* **44**, 979-987
- Crook, M. A., Kargbo, S., and Lumb, P. (2002) Measurement of urine total sialic acid: Comparison of an automated ultraviolet enzymatic method with a colorimetric assay. *Br. J. Biomed. Sci.* **59**, 20-23
- Cummings, R. D. and Etzler, M. E. (2009) R-type lectins. In: Varki, A., Cummings, R.D., Esko, J.D., Freeze, H.H., Stanley, P., Bertozzi, C.R., Hart, G.W., Etzler, M.E., Editors. *Essentials of Glycobiology*. 2nd Edition. Cold Spring Harbor Laboratory Press
- Das, K., Aramini, J. M., Ma, L. C., Krug, R. M., and Arnold, E. (2010) Structures of influenza A proteins and insights into antiviral drug targets. *Nat. Struct. Mol. Biol.* **17**, 530-538
- Davies, E. L., Smith, J. S., Birkett, C. R., Manser, J. M., Anderson-Dear, D. V., and Young, J. R. (1995) Selection of specific phage-display antibodies using libraries derived from chicken immunoglobulin genes. *J. Immunol. Methods* **186**, 125-135

- Davis, B. G. (2000) Recent developments in glycoconjugates. *J. Chem. Soc. Perkin Trans.* **22**, 3215-3237
- Davison, F., Kaspers, B., and Schat, K.A. (2008) *Avian Immunology*, 1st Edition, pp.496, Elsevier
- De Bruyn, P. P., Michelson, S., and Becker, R. P. (1978) Nonrandom distribution of sialic acid over the cell surface of bristle-coated endocytic vesicles of the sinusoidal endothelium cells. *J. Cell Biol.* **78**, 379-389
- De Crescenzo, G., Boucher, C., Durocher, Y., and Jolicoeur, M. (2008) Kinetic characterization by surface plasmon resonance-based biosensors: Principle and emerging trends. *Cell. Mol. Bioeng.* **1**, 204-215
- De Meulenaer, B. and Huyghebaert, A. (2001) Isolation and purification of chicken egg yolk immunoglobulins: A review. *Food Agric. Immunol.* **13**, 275-288
- de Waard, P., Koorevaar, A., Kamerling, J. P., and Vliegthart, J. F. (1991) Structure determination by ¹H NMR spectroscopy of (sulfated) sialylated *N*-linked carbohydrate chains released from porcine thyroglobulin by peptide-N4-(*N*-acetyl-beta-glucosaminyl) asparagine amidase-F. *J. Biol. Chem.* **266**, 4237-4243
- de Wildt, R. M., Finnern, R., Ouwehand, W. H., Griffiths, A. D., van Venrooij, W. J., and Hoet, R. M. (1996) Characterization of human variable domain antibody fragments against the U1 RNA-associated A protein, selected from a synthetic and patient-derived combinatorial V gene library. *Eur. J. Immunol.* **26**, 629-639
- de Wildt, R. M., Mundy, C. R., Gorick, B. D., and Tomlinson, I. M. (2000) Antibody arrays for high-throughput screening of antibody-antigen interactions. *Nat. Biotechnol.* **18**, 989-994
- Deng, S. J., MacKenzie, C. R., HIRAMA, T., Brousseau, R., Lowary, T. L., Young, N. M., Bundle, D. R., and Narang, S. A. (1995) Basis for selection of improved carbohydrate-binding single-chain antibodies from synthetic gene libraries. *Proc. Natl. Acad. Sci. U. S. A.* **92**, 4992-4996

- Diano, M. and Bivic, A. L. (2002) Raising highly specific polyclonal antibodies using biocompatible support-bound antigens. In: Walker, J. M., Bird, C. R., Thorpe, R., Editors. *The Protein Protocols Handbook*, 2nd Edition, Humana Press, 945-952
- Diaz, M., Velez, J., Singh, M., Cerny, J., and Flajnik, M. F. (1999) Mutational pattern of the nurse shark antigen receptor gene (NAR) is similar to that of mammalian Ig genes and to spontaneous mutations in evolution: The translesion synthesis model of somatic hypermutation. *Int. Immunol.* **11**, 825-833
- Diaz, S. L., Padler-Karavani, V., Ghaderi, D., Hurtado-Ziola, N., Yu, H., Chen, X., Brinkman-Van der Linden, E. C., Varki, A., and Varki, N. M. (2009) Sensitive and specific detection of the non-human sialic acid *N*-glycolylneuraminic acid in human tissues and biotherapeutic products. *PLoS ONE* **4**, 4241
- Ding, W., Nothhaft, H., Szymanski, C. M., and Kelly, J. (2009) Identification and quantification of glycoproteins using ion-pairing normal-phase liquid chromatography and mass spectrometry. *Mol. Cell. Proteomics* **8**, 2170-2185
- Dinh, Q., Weng, N. P., Kiso, M., Ishida, H., Hasegawa, A., and Marcus, D. M. (1996) High affinity antibodies against Lex and sialyl Lex from a phage display library. *J. Immunol.* **157**, 732-738
- Domann, P. J., Pardos-Pardos, A. C., Fernandes, D. L., Spencer, D. I., Radcliffe, C. M., Royle, L., Dwek, R. A., and Rudd, P. M. (2007) Separation-based glycoprofiling approaches using fluorescent labels. *Proteomics* **7**, 70-76
- Don, R. H., Cox, P. T., Wainwright, B. J., Baker, K., and Mattick, J. S. (1991) 'Touchdown' PCR to circumvent spurious priming during gene amplification. *Nucleic Acids Res.* **19**, 4008
- Dormitzer, P. R., Sun, Z. Y., Wagner, G., and Harrison, S. C. (2002) The rhesus rotavirus VP4 sialic acid binding domain has a galectin fold with a novel carbohydrate binding site. *EMBO J.* **21**, 885-897

- Dong, X., Xu, X., Han, F., Ping, X., Yuang, X., and Lin, B. (2001) Determination of sialic acids in the serum of cancer patients by capillary electrophoresis. *Electrophoresis* **22**, 2231-2235
- Dunkelberger, J. R. and Song, W. C. (2010) Complement and its role in innate and adaptive immune responses. *Cell Res.* **20**, 34-50
- Duverger, E., Lamerant-Fayel, N., Frison, N., and Monsigny, M. (2010) Carbohydrate-lectin interactions assayed by SPR. *Methods Mol. Biol.* **627**, 157-178
- Elwood, P. C., Reid, W. K., Marcell, P. D., Allen, R. H., and Kolhouse, J. F. (1988) Determination of the carbohydrate composition of mammalian glycoproteins by capillary gas chromatography/mass spectrometry. *Anal. Biochem.* **175**, 202-211
- Enders, S., Bernhard, G., Zakrzewicz, A., and Tauber, R. (2007) Inhibition of L-selectin binding by polyacrylamide-based conjugates under defined flow conditions. *Biochem. Biophys. Acta* **1770**, 1441-1449
- Ezzelarab, M., Ayares, D., and Cooper, D. K. (2005) Carbohydrates in xenotransplantation. *Immunol. Cell Biol.* **83**, 396-404
- Fellah, J. S., Jaffredo, T., and Dunon, D. (2008) Development of the avian immune system. 51-66. In: Davison, F., Kaspers, B., Schat, K.A. Editors, Avian Immunology, 1st Edition, Academic Press
- Fermer, C., Andersson, I., Nilsson, K., and Nilsson, O. (2004) Specificity rescue and affinity maturation of a low-affinity IgM antibody against pro-gastrin-releasing peptide using phage display and DNA shuffling. *Tumour Biol.* **25**, 7-13
- Fernandes, D. L. (2006) Biopharmaceutical sialylation. *EBR*, 100-104
- Finlay, W. J., deVore, N. C., Dobrovolskaia, E. N., Gam, A., Goodyear, C. S., and Slater, J. E. (2005) Exploiting the avian immunoglobulin system to simplify the generation of recombinant antibodies to allergenic proteins. *Clin. Exp. Allergy* **35**, 1040-1048

- Finlay, W. J., Bloom, L., and Cunningham, O. (2011) Phage display: A powerful technology for the generation of high specificity affinity reagents from alternative immune sources. *Methods Mol. Biol.* **681**, 87-101
- Fischer, E., Wagner, M., and Bertsch, T. (2000) *Cepaea hortensis* agglutinin-I, specific for *O*-glycosidically linked sialic acids, selectively labels endothelial cells of distinct vascular beds. *Histochem. J.* **32**, 105-109
- Forrer, P., Jung, S., and Pluckthun, A. (1999) Beyond binding: Using phage display to select for structure, folding and enzymatic activity in proteins. *Curr. Opin. Struct. Biol.* **9**, 514-520
- Frank, M. and von der Lieth, C. (1997) Comparison of the conformational behavior of sialyllactose complexed with the two viral attachment proteins influenza A hemagglutinin and the murine polyomavirus. *Journal of Molecular Modeling* **3**, 408-414
- Fu, D. and O'Neill, R. A. (1995) Monosaccharide composition analysis of oligosaccharides and glycoproteins by high-performance liquid chromatography. *Anal. Biochem.* **227**, 377-384
- Fujii, R., Kitaoka, M., and Hayashi, K. (2004) One-step random mutagenesis by error-prone rolling circle amplification. *Nucleic Acids Res.* **32**, 145
- Gabius, H. J., Siebert, H. C., Andre, S., Jimenez-Barbero, J., and Rudiger, H. (2004) Chemical biology of the sugar code. *ChemBiochem* **5**, 740-764
- Galanina, O. E., Mecklenburg, M., Nifantiev, N. E., Pazynina, G. V., and Bovin, N. V. (2003) GlycoChip: Multiarray for the study of carbohydrate-binding proteins. *Lab. Chip* **3**, 260-265
- Galuska, S. P., Geyer, R., Muhlenhoff, M., and Geyer, H. (2007) Characterization of oligo- and polysialic acids by MALDI-TOF-MS. *Anal. Chem.* **79**, 7161-7169

- Gao, C., Mao, S., Kaufmann, G., Wirsching, P., Lerner, R. A., and Janda, K. D. (2002) A method for the generation of combinatorial antibody libraries using pIX phage display. *Proc. Natl. Acad. Sci. U. S. A.* **99**, 12612-12616
- Garg, R., Tolbert, M., Oakes, J. L., Clemente, T. E., Bost, K. L., and Piller, K. J. (2007) Chloroplast targeting of FanC, the major antigenic subunit of *Escherichia coli* K99 fimbriae, in transgenic soybean. *Plant Cell Rep.* **26**, 1011-1023
- Gasparyan, V. K. (2005) Hen egg immunoglobulin Y in colloidal gold agglutination assay: Comparison with rabbit immunoglobulin G. *J. Clin. Lab. Anal.* **19**, 124-127
- Gee, G. V., Tsomaia, N., Mierke, D. F., and Atwood, W. J. (2004) Modeling a sialic acid binding pocket in the external loops of JC virus VP1. *J. Biol. Chem.* **279**, 49172-49176
- Gelamo, E. L. and Tabak, M. (2000) Spectroscopic studies on the interaction of bovine (BSA) and human (HSA) serum albumins with ionic surfactants. *Spectrochim. Acta A Mol. Biomol. Spectrosc.* **56**, 2255-2271
- George, A. J. (2000) Use of biosensors to measure the kinetics of antibody-antigen interactions. *Methods Mol. Med.* **40**, 363-372
- Gerbaut, L., Rey, E., and Lombart, C. (1973) Improved automated determination of bound *N*-acetylneuraminic acid in serum. *Clin. Chem.* **19**, 1285-1287
- Gerlach, D., Wagner, M., Schlott, B., Zahringer, U., and Schmidt, K. H. (2002) Chemical and physicochemical characterization of the sialic acid-specific lectin from *Cepaea hortensis*. *FEMS Microbiol. Lett.* **214**, 61-68
- Gerlach, D., Schlott, B., and Schmidt, K. H. (2004) Cloning and expression of a sialic acid-binding lectin from the snail *Cepaea hortensis*. *FEMS Immunol. Med. Microbiol.* **40**, 215-221
- Gerstenbruch, S., Brooks, C. L., Kosma, P., Brade, L., Mackenzie, C. R., Evans, S. V., Brade, H., and Muller-Loennies, S. (2010) Analysis of cross-reactive and specific

- anti-carbohydrate antibodies against lipopolysaccharide from *Chlamydomonas reinhardtii*. *Glycobiology* **20**, 461-472
- Geyer, H., Wuhrer, M., Kurokawa, T., and Geyer, R. (2004) Characterization of keyhole limpet hemocyanin (KLH) glycans sharing a carbohydrate epitope with *Schistosoma mansoni* glycoconjugates. *Micron* **35**, 105-106
- Ghazarian, H., Itoni, B., and Oppenheimer, S. B. (2011) A glycobiology review: Carbohydrates, lectins and implications in cancer therapeutics. *Acta Histochem.* **113**, 236-247
- Gibbs, M. D., Nevalainen, K. M., and Bergquist, P. L. (2001) Degenerate oligonucleotide gene shuffling (DOGS): A method for enhancing the frequency of recombination with family shuffling. *Gene* **271**, 13-20
- Goletz, S., Christensen, P. A., Kristensen, P., Blohm, D., Tomlinson, I., Winter, G., and Karsten, U. (2002) Selection of large diversities of antiidiotypic antibody fragments by phage display. *J. Mol. Biol.* **315**, 1087-1097
- Green, E. D., Adelt, G., Baenziger, J. U., Wilson, S., and Van Halbeek, H. (1988) The asparagine-linked oligosaccharides on bovine fetuin. structural analysis of *N*-glycanase-released oligosaccharides by 500-megahertz ¹H NMR spectroscopy. *J. Biol. Chem.* **263**, 18253-18268
- Guérardel, Y., Chang, L. Y., Maes, E., Huang, C. J., and Khoo, K. H. (2006) Glycomic survey mapping of zebrafish identifies unique sialylation pattern. *Glycobiology* **16**, 244-257
- Guo, Z. and Wang, Q. (2009) Recent development in carbohydrate-based cancer vaccines. *Curr. Opin. Chem. Biol.* **13**, 608-617
- Guttman, A. (1997) Analysis of monosaccharide composition by capillary electrophoresis. *J. Chromatogr. A* **763**, 271-277
- Hahnefeld, C., Drewianka, S., and Herberg, F. W. (2004) Determination of kinetic data using surface plasmon resonance biosensors. *Methods Mol. Med.* **94**, 299-320

- Han, L., Masani, S., and Yu, K. (2010) Cutting edge: CTNNB1 is dispensable for Ig class switch recombination. *J. Immunol.* **185**, 1379-1381
- Hanisch, F. G., Hacker, J., and Schrotten, H. (1993) Specificity of *S fimbriae* on recombinant *Escherichia coli*: Preferential binding to gangliosides expressing NeuGc alpha (2-3)gal and NeuAc alpha (2-8)NeuAc. *Infect. Immun.* **61**, 2108-2115
- Hansson, M., Olsson, I., and Nauseef, W. M. (2006) Biosynthesis, processing, and sorting of human myeloperoxidase. *Arch. Biochem. Biophys.* **445**, 214-224
- Hara, S., Yamaguchi, M., Takemori, Y., Nakamura, M., and Ohkura, Y. (1986) Highly sensitive determination of *N*-acetyl- and *N*-glycolylneuraminic acids in human serum and urine and rat serum by reversed-phase liquid chromatography with fluorescence detection. *J. Chromatogr.* **377**, 111-119
- Hara, S., Takemori, Y., Yamaguchi, M., Nakamura, M., and Ohkura, Y. (1987) Fluorometric high-performance liquid chromatography of *N*-acetyl- and *N*-glycolylneuraminic acids and its application to their microdetermination in human and animal sera, glycoproteins, and glycolipids. *Anal. Biochem.* **164**, 138-145
- Harris, J. R. and Markl, J. (2000) Keyhole limpet hemocyanin: Molecular structure of a potent marine immunoactivator. A review. *Eur. Urol.* **37**, 24-33
- Harvey, D. J. (2005) Ionization and fragmentation of *N*-linked glycans as silver adducts by electrospray mass spectrometry. *Rapid Commun. Mass Spectrom.* **19**, 484-492
- Harvey, D. J., Dwek, R. A., and Rudd, P. M. (2006) Determining the structure of glycan moieties by mass spectrometry. *Curr. Protoc. Protein Sci.* **12**, Unit 12.7
- Haselhorst, T., Wilson, J. C., Thomson, R. J., McAtamney, S., Menting, J. G., Coppel, R. L., and von Itzstein, M. (2004) Saturation transfer difference (STD) ¹H-NMR experiments and in silico docking experiments to probe the binding of *N*-acetylneuraminic acid and derivatives to *Vibrio cholerae* sialidase. *Proteins* **56**, 346-353

- Hashii, N., Kawasaki, N., Nakajima, Y., Toyoda, M., Katagiri, Y., Itoh, S., Harazono, A., Umezawa, A., and Yamaguchi, T. (2007) Study on the quality control of cell therapy products. determination of *N*-glycolylneuraminic acid incorporated into human cells by nano-flow liquid chromatography/Fourier transformation ion cyclotron mass spectrometry. *J. Chromatogr. A* **1160**, 263-269
- Haugland, R. P. and You, W. W. (2002) Coupling of antibodies with biotin. 355-363. In: Walker, J. M., Bird, C. R., Thorpe, R., Editors. *The Protein Protocols Handbook*, 2nd Edition, Humana Press
- Haverkamp, J., van Halbeek, H., Dorland, L., Vliegthart, J. F., Pfeil, R., and Schauer, R. (1982) High-resolution ¹H-NMR spectroscopy of free and glycosidically linked *O*-acetylated sialic acids. *Eur. J. Biochem.* **122**, 305-311
- Hegde, V. L. and Venkatesh, Y. P. (2007) Generation of antibodies specific to D-mannitol, a unique haptenic allergen, using reductively aminated D-mannose-bovine serum albumin conjugate as the immunogen. *Immunobiology* **212**, 119-128
- Hermanson, G. T. (2008) *Bioconjugate Techniques*, 2nd Edition, pp. 1323, Academic Press
- Herzenberg, L. A., Tokuhisa, T., and Herzenberg, L. A. (1980) Carrier-priming leads to hapten-specific suppression. *Nature* **285**, 664-667
- Higashi, H., Naiki, M., Matuo, S., and Okouchi, K. (1977) Antigen of "serum sickness" type of heterophile antibodies in human sera: Identification as gangliosides with *N*-glycolylneuraminic acid. *Biochem. Biophys. Res. Commun.* **79**, 388-395

- Hikita, T., Tadano-Aritomi, K., Iida-Tanaka, N., Toyoda, H., Suzuki, A., Toida, T., Imanari, T., Abe, T., Yanagawa, Y., and Ishizuka, I. (2000) Determination of *N*-acetyl- and *N*-glycolylneuraminic acids in gangliosides by combination of neuraminidase hydrolysis and fluorometric high-performance liquid chromatography using a GM3 derivative as an internal standard. *Anal. Biochem.* **281**, 193-201
- Hill, L. M., Guzick, D., Hixson, J., Peterson, C. S., and Rivello, D. M. (1992) Composite assessment of gestational age: A comparison of institutionally derived and published regression equations. *Am. J. Obstet. Gynecol.* **166**, 551-555
- Hino, M., Kijima-Suda, I., Nagai, Y., and Hosoya, H. (2003) Glycosylation of the alpha and beta tubulin by sialyloligosaccharides. *Zoolog Sci.* **20**, 709-715
- Hodek, P. and Stiborova, M. (2003) Chicken antibodies – superior alternative for conventional immunoglobulins. *Proc. Indian Natn Sci Acad. B* **69**, 461-468
- Honda, S., Okeda, J., Iwanaga, H., Kawakami, S., Taga, A., Suzuki, S., and Imai, K. (2000) Ultramicroanalysis of reducing carbohydrates by capillary electrophoresis with laser-induced fluorescence detection as 7-nitro-2,1,3-benzoxadiazole-tagged *N*-methylglycamine derivatives. *Anal. Biochem.* **286**, 99-111
- Honda, S., Suzuki, S., and Taga, A. (2003) Analysis of carbohydrates as 1-phenyl-3-methyl-5-pyrazolone derivatives by capillary/microchip electrophoresis and capillary electrochromatography. *J. Pharm. Biomed. Anal.* **30**, 1689-1714
- Honjo, T., Kinoshita, K., and Muramatsu, M. (2002) Molecular mechanism of class switch recombination: Linkage with somatic hypermutation. *Annu. Rev. Immunol.* **20**, 165-196
- Hoogenboom, H. R., de Bruine, A. P., Hufton, S. E., Hoet, R. M., Arends, J. W., and Roovers, R. C. (1998) Antibody phage display technology and its applications. *Immunotechnology* **4**, 1-20
- Hoogenboom, H. R. (2002) Overview of antibody phage-display technology and its applications. *Methods Mol. Biol.* **178**, 1-37

- Howard, M. A. (2008) DYNEPO[®]: An erythropoiesis-stimulating agent with a difference? *Nephrol Hypert* **22**, 1-2 3 4 5
- Huang, L. and Riggan, R. M. (2000) Analysis of nonderivatized neutral and sialylated oligosaccharides by electrospray mass spectrometry. *Anal. Chem.* **72**, 3539-3546
- Huflejt, M. E., Vuskovic, M., Vasiliu, D., Xu, H., Obukhova, P., Shilova, N., Tuzikov, A., Galanina, O., Arun, B., Lu, K., and Bovin, N. (2009) Anti-carbohydrate antibodies of normal sera: Findings, surprises and challenges. *Mol. Immunol.* **46**, 3037-3049
- Hunter, A. P. and Games, D. E. (1995) Evaluation of glycosylation site heterogeneity and selective identification of glycopeptides in proteolytic digests of bovine alpha 1-acid glycoprotein by mass spectrometry. *Rapid Commun. Mass Spectrom.* **9**, 42-56
- Huntington, J. A. and Stein, P. E. (2001) Structure and properties of ovalbumin. *J. Chromat. B.* **756**, 189-198
- Hust, M. and Dübel, S. (2005) Phage display vectors for the *in vitro* generation of human antibody fragments. *Methods Mol. Biol.* **295**, 71-96
- Iglesias, A. (2001) Maintenance and loss of self-tolerance in B cells. *Springer Semin. Immunopathol.* **23**, 351-366
- Ikekita, M., Yajima, H., Murata, T., Takatera, K., Watanabe, T., Sonoyama, M., Tezuka, Y., Ishii, T., and Tarutani, O. (1997) Carbohydrate analysis of porcine thyroglobulin isoforms with different iodine contents. *Biochim. Biophys. Acta* **1336**, 557-565
- Imberty, A., Gautier, C., Lescar, J., Perez, S., Wyns, L., and Loris, R. (2000) An unusual carbohydrate binding site revealed by the structures of two *Maackia amurensis* lectins complexed with sialic acid-containing oligosaccharides. *J. Biol. Chem.* **275**, 17541-17548

- Ito, M., Ikeda, K., Suzuki, Y., Tanaka, K., and Saito, M. (2002) An improved fluorometric high-performance liquid chromatography method for sialic acid determination: An internal standard method and its application to sialic acid analysis of human apolipoprotein E. *Anal. Biochem.* **300**, 260-266
- Iwamori, M., Kaido, T., Iwamori, Y., Ohta, Y., Tsukamoto, K., and Kozaki, S. (2005) Involvement of the C-terminal tail of *Arthrobacter ureafaciens* sialidase isoenzyme M in cleavage of the internal sialic acid of ganglioside GM1. *J. Biochem.* **138**, 327-334
- Jacobs, A. A., Simons, B. H., and de Graaf, F. K. (1987) The role of lysine-132 and arginine-136 in the receptor-binding domain of the K99 fibrillar subunit. *EMBO J.* **6**, 1805-1808
- Jason-Moller, L., Murphy, M., and Bruno, J. (2006) Overview of biacore systems and their applications. *Curr. Protoc. Protein Sci.* **19**, 19.13
- Jefferis, R. (2005) Glycosylation of recombinant antibody therapeutics. *Biotechnol. Prog.* **21**, 11-16
- Joern, J. M. (2003) DNA shuffling. *Methods Mol. Biol.* **231**, 85-89
- Johansson, R., Ohlin, M., Jansson, B., and Ohlson, S. (2006) Transiently binding antibody fragments against Lewis X and Sialyl-Lewis X. *J. Immunol. Methods* **312**, 20-26
- Johansson, S. M., Nilsson, E. C., Elofsson, M., Ahlskog, N., Kihlberg, J., and Arnberg, N. (2007) Multivalent sialic acid conjugates inhibit adenovirus type 37 from binding to and infecting human corneal epithelial cells. *Antiviral Res.* **73**, 92-100
- Jourdain, G. W., Dean, L., and Roseman, S. (1971) The sialic acids. XI. A periodate-resorcinol method for the quantitative estimation of free sialic acids and their glycosides. *J. Biol. Chem.* **246**, 430-435
- Kabat, E. A. and Wu, T. T. (1991) Identical V region amino acid sequences and segments of sequences in antibodies of different specificities. relative contributions

- of VH and VL genes, minigenes, and complementarity-determining regions to binding of antibody-combining sites. *J. Immunol.* **147**, 1709-1719
- Takehi, K., Kinoshita, M., Hayase, S., and Oda, Y. (1999a) Capillary electrophoresis of *N*-acetylneuraminic acid polymers and hyaluronic acid: Correlation between migration order reversal and biological functions. *Anal. Chem.* **71**, 1592-1596
- Takehi, K., Funakubo, T., Suzuki, S., Oda, Y., and Kitada, Y. (1999b) 3-aminobenzamide and 3-aminobenzoic acid, tags for capillary electrophoresis of complex carbohydrates with laser-induced fluorescent detection. *J. Chromatogr. A* **863**, 205-218
- Takehi, K., Kinoshita, M., and Nakano, M. (2002) Analysis of glycoproteins and the oligosaccharides thereof by high-performance capillary electrophoresis-significance in regulatory studies on biopharmaceutical products. *Biomed. Chromatogr.* **16**, 103-115
- Kaltgrad, E., Sen Gupta, S., Punna, S., Huang, C. Y., Chang, A., Wong, C. H., Finn, M. G., and Blixt, O. (2007) Anti-carbohydrate antibodies elicited by polyvalent display on a viral scaffold. *Chembiochem* **8**, 1455-1462
- Kamerling, J. P. and Vliegthart, J. F. G. (1982) Gas-liquid chromatography and mass spectrometry of sialic acids. **10**, 95-125. In Schauer, R. ed., *Sialic acids. Chemistry, metabolism and function*, Springer
- Kamerling, J. P. and Gerwig, G. J. (2006) Structural analysis of naturally occurring sialic acids. *Methods Mol. Biol.* **347**, 69-91
- Kang, a. S., Jones, T. M., and Burton, D. R. (1991) Antibody redesign by chain shuffling from random combinatorial immunoglobulin libraries. *Proc. Natl. Acad. Sci. U. S. A.* **88**, 11120-11123
- Karlsson, R. and Larsson, A. (2004) Affinity measurement using surface plasmon resonance. *Methods Mol. Biol.* **248**, 389-415

- Katakura, Y., Lim, E. T., Tsujii, S., Omasa, T., and Suga, K. I. (1998) The importance of ionic strength as a parameter in screening peptide ligands from a phage display library. *J. Ferment. Bioeng.* **85**, 447-450
- Katsamba, P. S., Navratilova, I., Calderon-Cacia, M., Fan, L., Thornton, K., Zhu, M., Bos, T. V., Forte, C., Friend, D., Laird-Offringa, I., Tavares, G., Whatley, J., Shi, E., Widom, A., Lindquist, K. C., Klakamp, S., Drake, A., Bohmann, D., Roell, M., Rose, L., Dorocke, J., Roth, B., Luginbuhl, B., and Myszka, D. G. (2006) Kinetic analysis of a high-affinity antibody/antigen interaction performed by multiple biacore users. *Anal. Biochem.* **352**, 208-221
- Kawagishi, H., Mori, H., Uno, A., Kimura, A., and Chiba, S. (1994) A sialic acid-binding lectin from the mushroom *Hericium erinaceum*. *FEBS Lett.* **340**, 56-58
- Kawai, T., Kato, A., Higashi, H., Kato, S., and Naiki, M. (1991) Quantitative determination of *N*-glycolylneuraminic acid expression in human cancerous tissues and avian lymphoma cell lines as a tumor-associated sialic acid by gas chromatography-mass spectrometry. *Cancer Res.* **51**, 1242-1246
- Kiang, J., Szu, S. C., Wang, L. X., Tang, M., and Lee, Y. C. (1997) Determination of 2-keto-3-deoxyoctulosonic acid (KDO) with high-performance anion-exchange chromatography (HPAEC): Survey of stability of KDO and optimal hydrolytic conditions. *Anal. Biochem.* **245**, 97-101
- Kiessling, L. L. and Pohl, N. L. (1996) Strength in numbers: Non-natural polyvalent carbohydrate derivatives. *Chem. Biol.* **3**, 71-77
- Kikuchi, M. and Harayama, S. (2002) DNA shuffling and family shuffling for *in vitro* gene evolution. *Methods Mol. Biol.* **182**, 243-257
- Kim, M., Higashiguchi, S., Iwamoto, Y., Yang, H., Cho, H., and Hatta, H. (2000) Egg yolk antibody and its application. *Biotechnol. Bioprocess Eng.* **5**, 79-83

- Kim, Y. J., Cho, Y. A., Lee, H., Lee, Y. T., Gee, S. J., and Hammock, B. D. (2003a) Synthesis of haptens for immunoassay of organophosphorus pesticides and effect of heterology in hapten spacer arm length on immunoassay sensitivity. *Anal. Chim. Acta* **475**, 85-96
- Kim, K. K., Escobedo, J. O., St Luce, N. N., Rusin, O., Wong, D., and Strongin, R. M. (2003b) Postcolumn HPLC detection of mono- and oligosaccharides with a chemosensor. *Org. Lett.* **5**, 5007-5010
- Kipriyanov, S. M. (2002) Generation of antibody molecules through antibody engineering. *Methods Mol Biol.* **207**, 3-25
- Kircheis, R., Vondru, P., Zinocker, I., Haring, D., Nechansky, A., Loibner, H., and Mudde, G. C. (2006) Immunization of rhesus monkeys with the conjugate vaccine IGN402 induces an IgG immune response against carbohydrate and protein antigens, and cancer cells. *Vaccine* **24**, 2349-2357
- Kirman, I., Zhao, K., Wang, Y., Szabo, P., Telford, W., and Weksler, M. E. (1998) Increased apoptosis of bone marrow pre-B cells in old mice associated with their low number. *Int. Immunol.* **10**, 1385-1392
- Kirsch, M., Zaman, M., Meier, D., Dubel, S., and Hust, M. (2005) Parameters affecting the display of antibodies on phage. *J. Immunol. Methods.* **301**, 173-185
- Kirsch, M. I., Hulseweh, B., Nacke, C., Rulker, T., Schirrmann, T., Marschall, H. J., Hust, M., and Dübel, S. (2008) Development of human antibody fragments using antibody phage display for the detection and diagnosis of venezuelan equine encephalitis virus (VEEV). *BMC Biotechnol.* **8**, 66
- Kitamura, K., Livingston, P. O., Fortunato, S. R., Stockert, E., Helling, F., Ritter, G., Oettgen, H. F., and Old, L. J. (1995) Serological response patterns of melanoma patients immunized with a GM2 ganglioside conjugate vaccine. *Proc. Natl. Acad. Sci.* **92**, 2805-2809

- Knappik, A., Ge, L., Honegger, A., Pack, P., Fischer, M., Wellnhofer, G., Hoess, A., Wolle, J., Pluckthun, A., and Virnekas, B. (2000) Fully synthetic human combinatorial antibody libraries (HuCAL) based on modular consensus frameworks and CDRs randomized with trinucleotides. *J. Mol. Biol.* **296**, 57-86
- Knibbs, R. N., Goldstein, I. J., Ratcliffe, R. M., and Shibuya, N. (1991) Characterization of the carbohydrate-binding specificity of the leukoagglutinating lectin from *Maackia amurensis*. comparison with other sialic acid-specific lectins. *J. Biol. Chem.* **266**, 83-88
- Knibbs, R. N., Osborne, S. E., Glick, G. D., and Goldstein, I. J. (1993) Binding determinants of the sialic acid-specific lectin from the slug *Limax flavus*. *J. Biol. Chem.* **268**, 18524-18531
- Kobayashi, N., Kato, Y., Oyama, H., Taga, S., Niwa, T., Sun, P., Ohtoyo, M., and Goto, J. (2008) Anti-estradiol-17beta single-chain fv fragments: Generation, characterization, gene randomization, and optimized phage display. *Steroids* **73**, 1485-1499
- Kohler, G. and Milstein, C. (1975) Continuous cultures of fused cells secreting antibody of predefined specificity. *Nature* **256**, 495-497
- Kohonen, P., Nera, K. P., and Lassila, O. (2007) Avian model for B-cell immunology--new genomes and phylotranscriptomics. *Scand. J. Immunol.* **66**, 113-121
- Kojima, N., Seino, K., Sato, Y., and Mizuochi, T. (2002) Carbohydrate carriers affect adhesion of *H. pylori* to immobilized leb-oligosaccharide. *FEBS Lett.* **517**, 32-36
- Komagome, R., Sawa, H., Suzuki, T., Suzuki, Y., Tanaka, S., Atwood, W. J., and Nagashima, K. (2002) Oligosaccharides as receptors for JC virus. *J. Virol.* **76**, 12992-13000
- Kortt, A. A., Dolezal, O., Power, B. E., and Hudson, P. J. (2001) Dimeric and trimeric antibodies: High avidity scFvs for cancer targeting. *Biomol. Eng.* **18**, 95-108

- Kovacs-Nolan, J. and Mine, Y. (2004) Avian egg antibodies: Basic and potential applications. *Avian Poult. Biol. Rev.* **15**, 25-46
- Kramer, R. A., Cox, F., van der Horst, M., van der Oudenrijn, S., Res, P. C., Bia, J., Logtenberg, T., and de Kruif, J. (2003) A novel helper phage that improves phage display selection efficiency by preventing the amplification of phages without recombinant protein. *Nucleic Acids Res.* **31**, 59
- Krengel, U., Olsson, L. L., Martinez, C., Talavera, A., Rojas, G., Mier, E., Angstrom, J., and Moreno, E. (2004) Structure and molecular interactions of a unique antitumor antibody specific for *N*-glycolyl GM3. *J. Biol. Chem.* **279**, 5597-5603
- Kristensen, P. and Winter, G. (1998) Proteolytic selection for protein folding using filamentous bacteriophages. *Fold. Des.* **3**, 321-328
- Kumagai, I. and Tsumoto, K. (2001) Antigen Antibody binding. In: *eLS*. John Wiley & Sons Ltd.
- Kurosawa, K. and Ohta, K. (2011) Genetic diversification by somatic gene conversion. *Genes* **2**, 48-58
- Lagana, A., Marino, A., Fago, G., and Martinez, B. P. (1993) A hydrolysis method using microwaves: Determination of *N*-acetyl- and *N*-glycolylneuraminic acids in biological systems by fluorometric high-performance liquid chromatography. *Anal. Biochem.* **215**, 266-272
- Lai, Y. P., Huang, J., Wang, L. F., Li, J., and Wu, Z. R. (2004) A new approach to random mutagenesis *in vitro*. *Biotechnol. Bioeng.* **86**, 622-627
- Lairson, L. L., Henrissat, B., Davies, G. J., and Withers, S. G. (2008) Glycosyltransferases: Structures, functions, and mechanisms. *Annu. Rev. Biochem.* **77**, 521-555
- Lamari, F. N. and Karamanos, N. K. (2002) Separation methods for sialic acids and critical evaluation of their biologic relevance. *J. Chromatogr. B. Analyt Technol. Biomed. Life. Sci.* **781**, 3-19

- Lamari, F. N., Kuhn, R., and Karamanos, N. K. (2003) Derivatization of carbohydrates for chromatographic, electrophoretic and mass spectrometric structure analysis. *J. Chromatogr. B. Analyt Technol. Biomed. Life. Sci.* **793**, 15-36
- Lanning, D., Osborne, B. A., and Knight, K. L. (2003) Immunoglobulin genes and generation of antibody repertoires in higher vertebrates: A key role for GALT. 433-448. In: Honjo, T., Alt, F.W., Neuberger, M.S. *Molecular Biology of B Cells*, Academic Press
- Larsson, A., Wejaker, P. E., Forsberg, P. O., and Lindahl, T. (1992) Chicken antibodies: A tool to avoid interference by complement activation in ELISA. *J. Immunol. Methods* **156**, 79-83
- Law, B. (1996) *Immunoassay: A Practical Guide*, pp. 226, CRC Press
- Lee, K. J., Mao, S., Sun, C., Gao, C., Blixt, O., Arrues, S., Hom, L. G., Kaufmann, G. F., Hoffman, T. Z., Coyle, A. R., Paulson, J., Felding-Habermann, B., and Janda, K. D. (2002) Phage-display selection of a human single-chain Fv antibody highly specific for melanoma and breast cancer cells using a chemoenzymatically synthesized G(M3)-carbohydrate antigen. *J. Am. Chem. Soc.* **124**, 12439-12446
- Lee, C. V., Liang, W. C., Dennis, M. S., Eigenbrot, C., Sidhu, S. S., and Fuh, G. (2004) High-affinity human antibodies from phage-displayed synthetic fab libraries with a single framework scaffold. *J. Mol. Biol.* **340**, 1073-1093
- Leenaars, M. and Hendriksen, C. F. (2005) Critical steps in the production of polyclonal and monoclonal antibodies: Evaluation and recommendations. *ILAR J.* **46**, 269-279
- Lehmann, F., Tiralongo, E., and Tiralongo, J. (2006) Sialic acid-specific lectins: Occurrence, specificity and function. *Cell Mol. Life Sci.* **63**, 1331-1354
- Lei, H., Shen, Y., Song, L., Yang, J., Chevallier, O. P., Haughey, S. A., Wang, H., Sun, Y., and Elliott, C. T. (2010) Hapten synthesis and antibody production for the development of a melamine immunoassay. *Anal. Chim. Acta* **665**, 84-90

- Lekakh, I. V., Bovin, N. V., Bezyaeva, G. P., and Poverenny, A. M. (2001) Natural hidden autoantibodies react with negatively charged carbohydrates and xenoantigen bdi. *Biochemistry* **66**, 163-167
- Lemus, R. and Karol, M. H. (2008) Conjugation of haptens. *Methods Mol. Med.* **138**, 167-182
- Leong, M. K., Chen, C., Shar, K. C., and Shiuan, D. (2007) Selection and characterization of lipase abzyme from phage displayed antibody libraries. *Biochem. Biophys. Res. Commun.* **361**, 567-573
- Li, Q., Rodriguez, L. G., Farnsworth, D. F., and Gildersleeve, J. C. (2010) Effects of hapten density on the induced antibody repertoire. *ChemBiochem* **11**, 1686-1691
- Lim, K. P., Li, H., and Nathan, S. (2004) Expression and purification of a recombinant scFv towards the exotoxin of the pathogen, *Burkholderia pseudomallei*. *J. Microbiol.* **42**, 126-132
- Lisowska, E. (2002) The role of glycosylation in protein antigenic properties. *Cell Mol. Life Sci.* **59**, 445-455
- Little, M., Kipriyanov, S. M., Le Gall, F., and Moldenhauer, G. (2000) Of mice and men: Hybridoma and recombinant antibodies. *Immunol. Today* **21**, 364-370
- Lo-Man, R., Bay, S., Vichier-Guerre, S., Deriaud, E., Cantacuzene, D., and Leclerc, C. (1999) A fully synthetic immunogen carrying a carcinoma-associated carbohydrate for active specific immunotherapy. *Cancer Res.* **59**, 1520-1524
- Lopez-Requena, A., Rodriguez, M., de Acosta, C. M., Moreno, E., Puchades, Y., Gonzalez, M., Talavera, A., Valle, A., Hernandez, T., Vazquez, A. M., and Perez, R. (2007) Gangliosides, Ab1 and Ab2 antibodies II. light versus heavy chain: An idiotype-anti-idiotype case study. *Mol. Immunol.* **44**, 1015-1028
- Loris, R. (2002) Principles of structures of animal and plant lectins. *Biochim. Biophys. Acta.* **1572**, 198-208

- Lou, J. and Marks, J. D. (2010) Affinity maturation by chain shuffling and site directed mutagenesis. In: Kontermann, R., Dübel, S. *Antibody Engineering*, 2nd Edition, Springer, 377-396
- Lunder, M., Bratkovic, T., Urleb, U., Kreft, S., and Strukelj, B. (2008) Ultrasound in phage display: A new approach to nonspecific elution. *BioTechniques* **44**, 893-900
- Luo, Y., Li, S. C., Chou, M. Y., Li, Y. T., and Luo, M. (1998) The crystal structure of an intramolecular trans-sialidase with a NeuAc alpha2-3Gal specificity. *Structure* **6**, 521-530
- MacKenzie, R. and To, R. (1998) The role of valency in the selection of anti-carbohydrate single-chain Fvs from phage display libraries. *J. Immunol. Methods* **220**, 39-49
- Madan, D., Lin, Z., and Rye, H. S. (2008) Triggering protein folding within the GroEL-GroES complex. *J. Biol. Chem.* **283**, 32003-32013
- Maes, E., Wieruszkeski, J. M., Plancke, Y., and Strecker, G. (1995) Structure of three kdn-containing oligosaccharide-alditols released from oviducal secretions of *Ambystoma tigrinum*: Characterization of the carbohydrate sequence fuc (alpha 1-5) [fuc (alpha 1-4)] kdn (alpha 2-3/6). *FEBS Lett.* **358**, 205-210
- Maget-Dana, R., Veh, R. W., Sander, M., Roche, A. C., Schauer, R., and Monsigny, M. (1981) Specificities of limulin and wheat-germ agglutinin towards some derivatives of GM3 gangliosides. *Eur. J. Biochem.* **114**, 11-16
- Magnani, J. L. (1986) Carbohydrate sequences detected by murine monoclonal antibodies. *Chem. Phys. Lipids* **42**, 65
- Makatsori, E., Karamanos, K.,N., Anastassiou, E.,D., Hjerpe, A., and Tsegenidis, T. (1998) A method to quantitate total sialic acid, glucosamine, and galactosamine in blood serum and glycoconjugates by HPLC. *J. Liq. Chrom. & Rel. Technol.* **21**, 3031-3045

- Malykh, Y. N., Schauer, R., and Shaw, L. (2001) *N*-glycolylneuraminic acid in human tumours. *Biochimie* **83**, 623-634
- Mandal, C. and Mandal, C. (1990) Sialic acid binding lectins. *Experientia* **46**, 433-441
- Manimala, J. C., Roach, T. A., Li, Z., and Gildersleeve, J. C. (2007) High-throughput carbohydrate microarray profiling of 27 antibodies demonstrates widespread specificity problems. *Glycobiology* **17**, 17C-23C
- Mansikka, A., Sandberg, M., Veromaa, T., Vainio, O., Granfors, K., and Toivanen, P. (1989) B cell maturation in the chicken harderian gland. *J. Immunol.* **142**, 1826-1833
- Manzi, A. E., Diaz, S., and Varki, A. (1990) High-pressure liquid chromatography of sialic acids on a pellicular resin anion-exchange column with pulsed amperometric detection: A comparison with six other systems. *Anal. Biochem.* **188**, 20-32
- Manzi, A. (2001) Acid hydrolysis for release of monosaccharides. *Curr. Protoc. Mol. Biol.* **17**, 17.16
- Mao, S., Gao, C., Lo, C. H., Wirsching, P., Wong, C. H., and Janda, K. D. (1999) Phage-display library selection of high-affinity human single-chain antibodies to tumor-associated carbohydrate antigens Sialyl Lewis X and Lewis X. *Proc. Natl. Acad. Sci. U. S. A.* **96**, 6953-6958
- Mao, X., Wang, K., Du, Y., and Lin, B. (2003) Analysis of chicken and turkey ovalbumins by microchip electrophoresis combined with exoglycosidase digestion. *Electrophoresis* **24**, 3273-3278
- Marks, J. D. (2004) Antibody affinity maturation by chain shuffling. *Methods Mol. Biol.* **248**, 327-343
- Marth, J. D. and Grewal, P. K. (2008) Mammalian glycosylation in immunity. *Nat. Rev. Immunol.* **8**, 874-887
- Martineau, P. (2002) Error-prone polymerase chain reaction for modification of scFvs. *Methods Mol. Biol.* **178**, 287-294

- Masteller, E. L., Pharr, G. T., Funk, P. E., and Thompson, C. B. (1997) Avian B cell development. *Int. Rev. Immunol.* **15**, 185-206
- Matsubara, T., Iida, M., Tsumuraya, T., Fujii, I., and Sato, T. (2008) Selection of a carbohydrate-binding domain with a helix-loop-helix structure. *Biochemistry* **47**, 6745-6751
- Matsuno, K. and Suzuki, S. (2008) Simple fluorimetric method for quantification of sialic acids in glycoproteins. *Anal. Biochem.* **375**, 53-59
- Maynard, J. and Georgiou, G. (2000) Antibody engineering. *Annu. Rev. Biomed. Eng.* **2**, 339-376
- McCafferty, J., Griffiths, A. D., Winter, G., and Chiswell, D. J. (1990) Phage antibodies: Filamentous phage displaying antibody variable domains. *Nature* **348**, 552-554
- McCormack, W. T., Tjoelker, L. W., Barth, C. F., Carlson, L. M., Petryniak, B., Humphries, E. H., and Thompson, C. B. (1989) Selection for B cells with productive IgL gene rearrangements occurs in the *Bursa of fabricius* during chicken embryonic development. *Genes Dev.* **3**, 838-847
- McMillen, T. S., Heinecke, J. W., and LeBoeuf, R. C. (2005) Expression of human myeloperoxidase by macrophages promotes atherosclerosis in mice. *Circulation* **111**, 2798-2804
- Mechref, Y., Ostrander, G. K., and el Rassi, Z. (1995) Capillary electrophoresis of carboxylated carbohydrates. I. selective precolumn derivatization of gangliosides with UV absorbing and fluorescent tags. *J. Chromatogr. A* **695**, 83-95
- Melnikov, A. and Youngman, P. J. (1999) Random mutagenesis by recombinational capture of PCR products in *bacillus subtilis* and *acinetobacter calcoaceticus*. *Nucleic Acids Res.* **27**, 1056-1062

- Menendez, A. and Scott, J. K. (2005) The nature of target-unrelated peptides recovered in the screening of phage-displayed random peptide libraries with antibodies. *Anal. Biochem.* **336**, 145-157
- Mercy, P. D. and Ravindranath, M. H. (1993) Purification and characterization of *N*-glycolyneuraminic-acid-specific lectin from *Scylla serrata*. *Eur. J. Biochem.* **215**, 697-704
- Merrick, J. M., Zadarlik, K., and Milgrom, F. (1978) Characterization of the Hanganutziu-Deicher (serum-sickness) antigen as gangliosides containing *N*-glycolyneuraminic acid. *Int. Arch. Allergy Appl. Immunol.* **57**, 477-480
- Miller, R. L., Collawn, J. F., Jr, and Fish, W. W. (1982) Purification and macromolecular properties of a sialic acid-specific lectin from the slug *Limax flavus*. *J. Biol. Chem.* **257**, 7574-7580
- Mine, Y. and Kovacs-Nolan, J. (2002) Chicken egg yolk antibodies as therapeutics in enteric infectious disease: A review. *J. Med. Food* **5**, 159-169
- Mine, Y. and Yang, M. (2008) Recent advances in the understanding of egg allergens: Basic, industrial, and clinical perspectives. *J. Agric. Food Chem.* **56**, 4874-4900
- Miura, Y., Shinohara, Y., Furukawa, J., Nagahori, N., and Nishimura, S. (2007) Rapid and simple solid-phase esterification of sialic acid residues for quantitative glycomics by mass spectrometry. *Chemistry* **13**, 4797-4804
- Miyake, M., Ito, M., Hitomi, S., Ikeda, S., Taki, T., Kurata, M., Hino, A., Miyake, N., and Kannagi, R. (1988) Generation of two murine monoclonal antibodies that can discriminate *N*-glycolyl and *N*-acetyl neuraminic acid residues of GM2 gangliosides. *Cancer Res.* **48**, 6154-6160
- Miyashita, N., Kawai, Y., Yamaguchi, T., Ouchi, K., Kobashi, Y., and Oka, M. (2010) Evaluation of false-positive reaction with ELISA for the detection of *Chlamydophila pneumoniae*-specific IgM antibody in adults. *Jpn. J. Infect. Dis.* **63**, 150-151

- Mo, H., Winter, H. C., and Goldstein, I. J. (2000) Purification and characterization of a Neu5Acalpha2-6Galbeta1-4Glc/GlcNAc-specific lectin from the fruiting body of the polypore mushroom *Polyporus squamosus*. *J. Biol. Chem.* **275**, 10623-10629
- Monsigny, M., Roche, A. C., Sene, C., Maget-Dana, R., and Delmotte, F. (1980) Sugar-lectin interactions: How does wheat-germ agglutinin bind sialoglycoconjugates? *Eur. J. Biochem.* **104**, 147-153
- Morrow, T. and Schlissel, M. (1992) The purification of B-cell precursors from mouse fetal liver. *Curr. Top. Microbiol. Immunol.* **182**, 55-64
- Morschhauser, J., Hoschutzky, H., Jann, K., and Hacker, J. (1990) Functional analysis of the sialic acid-binding adhesin SfaS of pathogenic *Escherichia coli* by site-specific mutagenesis. *Infect. Immun.* **58**, 2133-2138
- Moss, J. A., Coyle, A. R., Ahn, J. M., Meijler, M. M., Offer, J., and Janda, K. D. (2003) Tandem IMAC-HPLC purification of a cocaine-binding scFv antibody. *J. Immunol. Methods* **281**, 143-148
- Müller, A., Severi, E., Mulligan, C., Watts, A. G., Kelly, D. J., Wilson, K. S., Wilkinson, A. J., and Thomas, G. H. (2006) Conservation of structure and mechanism in primary and secondary transporters exemplified by SiaP, a sialic acid binding virulence factor from *Haemophilus influenzae*. *J. Biol. Chem.* **281**, 22212-22222
- Muresan, V., Iwanij, V., Smith, Z. D., and Jamieson, J. D. (1982) Purification and use of limulin: A sialic acid-specific lectin. *J. Histochem. Cytochem.* **30**, 938-946
- Murrey, H. E. and Hsieh-Wilson, L. C. (2008) The chemical neurobiology of carbohydrates. *Chem. Rev.* **108**, 1708-1731
- Mutuberria, R., Arends, J., Griffioen, A. W., and Hoogenboom, H. R. (2001) Phage display technology for target discovery in drug delivery research, 255-273. In: Molema, G., Meijer, D. K. F. Editors, Drug Targeting: Organ-Specific Strategies, Wiley-VCH Verlag GmbH, Weinheim

- Nakano, M., Kakehi, K., Tsai, M. H., and Lee, Y. C. (2004) Detailed structural features of glycan chains derived from alpha1-acid glycoproteins of several different animals: The presence of hypersialylated, *O*-acetylated sialic acids but not disialyl residues. *Glycobiology* **14**, 431-441
- Naot, Y., Barnett, E. V., and Remington, J. S. (1981) Method for avoiding false-positive results occurring in immunoglobulin M enzyme-linked immunosorbent assays due to presence of both rheumatoid factor and antinuclear antibodies. *J. Clin. Microbiol.* **14**, 73-78
- Narat, M. (2003) Production of antibodies in chickens. *Food Tech. Biotech* **41**, 259-267
- Ni, J., Song, H., Wang, Y., Stamatou, N. M., and Wang, L. X. (2006) Toward a carbohydrate-based HIV-1 vaccine: Synthesis and immunological studies of oligomannose-containing glycoconjugates. *Bioconjug. Chem.* **17**, 493-500
- Nie, Z. (1992) Fetuin: Its enigmatic property of growth promotion. *Am. J. Physiol.* **263**, C551-62
- Noguchi, A., Mukuria, C. J., Suzuki, E., and Naiki, M. (1995) Immunogenicity of *N*-glycolylneuraminic acid-containing carbohydrate chains of recombinant human erythropoietin expressed in chinese hamster ovary cells. *J. Biochem.* **117**, 59-62
- Noll, E. N., Lin, J., Nakatsuji, Y., Miller, R. H., and Black, P. M. (2001) GM3 as a novel growth regulator for human gliomas. *Exp. Neurol.* **168**, 300-309
- O'Brien, P. M. and Aitken, R. (2002) Antibody Phage Display: Methods and Protocols, Methods in Molecular Medicine. **178** pp. 86, Humana Press
- Ogawa, H. K., Takeuchi, Y., Uchibori, H., Matsumoto, I., and Seno, N. (1993) Determination of *N*-acetylneuraminic acid and *N*-glycolylneuraminic acid in glycoproteins by high-performance liquid chromatography without derivatization. *J. Chromatogr.* **612**, 145-149

- Ohlin, M., Owman, H., Mach, M., and Borrebaeck, C. A. (1996) Light chain shuffling of a high affinity antibody results in a drift in epitope recognition. *Mol. Immunol.* **33**, 47-56
- Ohlson, S., Strandh, M., and Nilshans, H. (1997) Detection and characterization of weak affinity antibody antigen recognition with biomolecular interaction analysis. *J. Mol. Recognit.* **10**, 135-138
- Ohta, M., Hamako, J., Yamamoto, S., Hatta, H., Kim, M., Yamamoto, T., Oka, S., Mizuochi, T., and Matsuura, F. (1991) Structures of asparagine-linked oligosaccharides from hen egg-yolk antibody (IgY). occurrence of unusual glucosylated oligo-mannose type oligosaccharides in a mature glycoprotein. *Glycoconj. J.* **8**, 400-413
- Ohtsubo, K. and Marth, J. D. (2006) Glycosylation in cellular mechanisms of health and disease. *Cell* **126**, 855-867
- O'Kennedy, R., Townsend, S., Donohoe, G. G., Leonard, P., Hearty, S., and Byrne, B. (2010) Speedy, small, sensitive, and specific—Reality or myth for future analytical methods. *Anal. Lett.* **43**, 1630-1648
- Ortner, K. and Buchberger, W. (2008) Determination of sialic acids released from glycoproteins using capillary zone electrophoresis/electrospray ionization mass spectrometry. *Electrophoresis* **29**, 2233-2237
- O'Shannessy, D. J., Brigham-Burke, M., and Peck, K. (1992) Immobilization chemistries suitable for use in the BIAcore surface plasmon resonance detector. *Anal. Biochem.* **205**, 132-136
- Pace, J. K., and Feschotte, C. (2007) The evolutionary history of human DNA transposons: Evidence for intense activity in the primate lineage. *Genome Res.* **17**, 422-432

- Padler-Karavani, V., Yu, H., Cao, H., Chokhawala, H., Karp, F., Varki, N., Chen, X., and Varki, A. (2008) Diversity in specificity, abundance, and composition of anti-Neu5Gc antibodies in normal humans: Potential implications for disease. *Glycobiology* **18**, 818-830
- Pal, S., Ghosh, S., Mandal, C., Kohla, G., Brossmer, R., Isecke, R., Merling, A., Schauer, R., Schwartz-Albiez, R., Bhattacharya, D. K., and Mandal, C. (2004) Purification and characterization of 9-O-acetylated sialoglycoproteins from leukemic cells and their potential as immunological tool for monitoring childhood acute lymphoblastic leukemia. *Glycobiology* **14**, 859-870
- Pannekoek, H., Meijer, M., Gaardsvoll, H., and Anton, Z. (1995) Functional display of proteins, mutant proteins, fragments of proteins and peptides on the surface of filamentous (bacterio) phages: A review. *Cytotechnology* **18**, 107-112
- Pansri, P., Jaruseranee, N., Rangnoi, K., Kristensen, P., and Yamabhai, M. (2009) A compact phage display human scFv library for selection of antibodies to a wide variety of antigens. *BMC Biotechnol.* **9**, 6
- Parmentier, H. K., Lammers, A., Hoekman, J. J., De Vries Reilingh, G., Zaanen, I. T., and Savelkoul, H. F. (2004) Different levels of natural antibodies in chickens divergently selected for specific antibody responses. *Dev. Comp. Immunol.* **28**, 39-49
- Parng, C. L., Hansal, S., Goldsby, R. A., and Osborne, B. A. (1996) Gene conversion contributes to Ig light chain diversity in cattle. *J. Immunol.* **157**, 5478-5486
- Parvari, R., Ziv, E., Lentner, F., Tel-Or, S., Burstein, Y., and Schechter, I. (1987) Analyses of chicken immunoglobulin light chain cDNA clones indicate a few germline V lambda genes and allotypes of the C lambda locus. *EMBO J.* **6**, 97-102
- Parvari, R., Ziv, E., Lantner, F., Heller, D., and Schechter, I. (1990) Somatic diversification of chicken immunoglobulin light chains by point mutations. *Proc. Natl. Acad. Sci. U. S. A.* **87**, 3072-3076

- Paschke, M. (2006) Phage display systems and their applications. *Appl. Microbiol. Biotechnol.* **70**, 2-11
- Pashov, A., Perry, M., Dyar, M., Chow, M., and Kieber-Emmons, T. (2005) Carbohydrate mimotopes in the rational design of cancer vaccines. *Curr. Top. Med. Chem.* **5**, 1171-1185
- Pearce, E. I. and Major, G. N. (1978) The colorimetric analysis of sialic acid in human saliva and bovine salivary mucin. *J. Dent. Res.* **57**, 995-1002
- Peracaula, R., Tabares, G., Royle, L., Harvey, D. J., Dwek, R. A., Rudd, P. M., and de Llorens, R. (2003) Altered glycosylation pattern allows the distinction between prostate-specific antigen (PSA) from normal and tumor origins. *Glycobiology* **13**, 457-470
- Phalipon, A., Mulard, L. A., and Sansonetti, P. J. (2008) Vaccination against shigellosis: Is it the path that is difficult or is it the difficult that is the path? *Microb. Infect.* **10**, 1057-1062
- Phalipon, A., Tanguy, M., Grandjean, C., Guerreiro, C., Belot, F., Cohen, D., Sansonetti, P. J., and Mulard, L. A. (2009) A synthetic carbohydrate-protein conjugate vaccine candidate against *Shigella flexneri* 2a infection. *J. Immunol.* **182**, 2241-2247
- Pini, A. and Bracci, L. (2000) Phage display of antibody fragments. *Curr. Protein Pept. Sci.* **1**, 155-169
- Ponsel, D., Neugebauer, J., Ladetzki-Baehs, K., and Tissot, K. (2011) High affinity, developability and functional size: The holy grail of combinatorial antibody library generation. *Molecules* **16**, 3675-3700
- Powell, L. D., Varki, A. P., and Freeze, H. H. (2001) Release of saccharides from glycoconjugates. *Curr. Protoc. Immunol.* **8**, Unit 8.15

- Pozsgay, V., Chu, C., Pannell, L., Wolfe, J., Robbins, J. B., and Schneerson, R. (1999) Protein conjugates of synthetic saccharides elicit higher levels of serum IgG lipopolysaccharide antibodies in mice than do those of the O-specific polysaccharide from *Shigella dysenteriae* type 1. *Proc. Natl. Acad. Sci. U. S. A.* **96**, 5194-5197
- Pritchard, L., Corne, D., Kell, D., Rowland, J., and Winson, M. (2005) A general model of error-prone PCR. *J. Theor. Biol.* **234**, 497-509
- Qin, T., Ren, L., Hu, X., Guo, Y., Fei, J., Zhu, Q., Butler, J. E., Wu, C., Li, N., Hammarstrom, L., and Zhao, Y. (2008) Genomic organization of the immunoglobulin light chain gene loci in *Xenopus tropicalis*: Evolutionary implications. *Dev. Comp. Immunol.* **32**, 156-165
- Ragupathi, G., Koide, F., Sathyan, N., Kagan, E., Spassova, M., Bornmann, W., Gregor, P., Reis, C. A., Clausen, H., Danishefsky, S. J., and Livingston, P. O. (2003) A preclinical study comparing approaches for augmenting the immunogenicity of a heptavalent KLH-conjugate vaccine against epithelial cancers. *Cancer Immunol. Immunother.* **52**, 608-616
- Rahimi, S., Shiraz, Z. M., Salehi, T. Z., Karimi Torshizi, M. A., and Grimes, J. L. (2007) Prevention of salmonella infection in poultry by specific egg-derived antibody. *Int. J. Poult. Sci.* **6**, 230-235
- Rao, R. S., Kumar, C. G., Prakasham, R. S., and Hobbs, P. J. (2008) The taguchi methodology as a statistical tool for biotechnological applications: A critical appraisal. *Biotechnol. J.* **3**, 510-523
- Rapoport, E. M., Pazynina, G. V., Sablina, M. A., Crocker, P. R., and Bovin, N. V. (2006) Probing sialic acid binding Ig-like lectins (siglecs) with sulfated oligosaccharides. *Biochemistry* **71**, 496-504
- Rasila, T. S., Pajunen, M. I., and Savilahti, H. (2009) Critical evaluation of random mutagenesis by error-prone polymerase chain reaction protocols, *Escherichia coli* mutator strain, and hydroxylamine treatment. *Anal. Biochem.* **388**, 71-80

- Rassi, E. Z. (1996) High-Performance Capillary Electrophoresis of Carbohydrates, Beckman Instruments, Fullerton, CA
- Ratcliffe, M. J. (2006) Antibodies, immunoglobulin genes and the bursa of fabricius in chicken B cell development. *Dev. Comp. Immunol.* **30**, 101-118
- Ravindranath, M. H. and Donald, M. L. (2001) Antigens: Carbohydrates. *In: eLS*. John Wiley & Sons Ltd
- Ravindranath, M. H., Higa, H. H., Cooper, E. L., and Paulson, J. C. (1985) Purification and characterization of an *O*-acetylsialic acid-specific lectin from a marine crab *Cancer antennarius*. *J. Biol. Chem.* **260**, 8850-8856
- Ravn, P., Danielczyk, A., Jensen, K. B., Kristensen, P., Christensen, P. A., Larsen, M., Karsten, U., and Goletz, S. (2004) Multivalent scFv display of phagemid repertoires for the selection of carbohydrate-specific antibodies and its application to the thomsen-friedenreich antigen. *J. Mol. Biol.* **343**, 985-996
- Rawitch, A. B., Pollock, H. G., and Yang, S. X. (1993) Thyroglobulin glycosylation: Location and nature of the *N*-linked oligosaccharide units in bovine thyroglobulin. *Arch. Biochem. Biophys.* **300**, 271-279
- Reuter, G. and Schauer, R. (1994) Determination of sialic acids. *Methods Enzymol.* **230**, 168-199
- Reynaud, C. A., Dahan, A., and Weill, J. C. (1983) Complete sequence of a chicken lambda light chain immunoglobulin derived from the nucleotide sequence of its mRNA. *Proc. Natl. Acad. Sci. U. S. A.* **80**, 4099-4103
- Reynaud, C. A., Imhof, B. A., Anquez, V., and Weill, J. C. (1992) Emergence of committed B lymphoid progenitors in the developing chicken embryo. *EMBO J.* **11**, 4349-4358

- Rinninger, A., Richet, C., Pons, A., Kohla, G., Schauer, R., Bauer, H. C., Zanetta, J. P., and Vlasak, R. (2006) Localisation and distribution of *O*-acetylated *N*-acetylneuraminic acids, the endogenous substrates of the hemagglutinin-esterases of murine coronaviruses, in mouse tissue. *Glycoconj. J.* **23**, 73-84
- Robbe, C., Capon, C., Flahaut, C., and Michalski, J. C. (2003) Microscale analysis of mucin-type *O*-glycans by a coordinated fluorophore-assisted carbohydrate electrophoresis and mass spectrometry approach. *Electrophoresis* **24**, 611-621
- Rohrbach, P. and Dübel, S. (2003) Antibody phage display libraries for use in therapeutic gene targeting. 581-596. In: Curiel, D.T. and Douglas, J. T., Editors, *Vector Targeting for Therapeutic Gene Delivery*, John Wiley & Sons, USA
- Rohrer, J. S. (2000) Analyzing sialic acids using high-performance anion-exchange chromatography with pulsed amperometric detection. *Anal. Biochem.* **283**, 3-9
- Rojas, G., Talavera, A., Munoz, Y., Rengifo, E., Kregel, U., Angstrom, J., Gavilondo, J., and Moreno, E. (2004) Light-chain shuffling results in successful phage display selection of functional prokaryotic-expressed antibody fragments to *N*-glycolyl GM3 ganglioside. *J. Immunol. Methods* **293**, 71-83
- Roque-Navarro, L., Chakrabandhu, K., de Leon, J., Rodriguez, S., Toledo, C., Carr, A., de Acosta, C. M., Hueber, A. O., and Perez, R. (2008) Anti-ganglioside antibody-induced tumor cell death by loss of membrane integrity. *Mol. Cancer. Ther.* **7**, 2033-2041
- Roth, J., Taatjes, D. J., Weinstein, J., Paulson, J. C., Greenwell, P., and Watkins, W. M. (1986) Differential subcompartmentation of terminal glycosylation in the golgi apparatus of intestinal absorptive and goblet cells. *J. Biol. Chem.* **261**, 14307-14312
- Roy, R. (2004) New trends in carbohydrate-based vaccines. *Drug Discovery Today: Technologies* **1**, 327-336
- Royle, L., Matthews, E., Corfield, A., Berry, M., Rudd, P. M., Dwek, R. A., and Carrington, S. D. (2008) Glycan structures of ocular surface mucins in man, rabbit and dog display species differences. *Glycoconj. J.* **25**, 763-773

- Rudd, P. M., Elliott, T., Cresswell, P., Wilson, I. A., and Dwek, R. A. (2001) Glycosylation and the immune system. *Science* **291**, 2370-2376
- Rudiger, H. and Gabius, H. J. (2001) Plant lectins: Occurrence, biochemistry, functions and applications. *Glycoconj. J.* **18**, 589-613
- Saini, S. S., Farrugia, W., Ramsland, P. A., and Kaushik, A. K. (2003) Bovine IgM antibodies with exceptionally long complementarity-determining region 3 of the heavy chain share unique structural properties conferring restricted VH + vlamba pairings. *Int. Immunol.* **15**, 845-853
- Saito, M. and Sugiyama, K. (2001) Major and c-series gangliosides in lenticular tissues: Mammals to molluscs. *Comp. Biochem. Physiol. B. Biochem. Mol. Biol.* **130**, 313-321
- Saksena, R., Ma, X., Wade, T. K., Kovac, P., and Wade, W. F. (2006) Length of the linker and the interval between immunizations influences the efficacy of *Vibrio cholerae* O1, ogawa hexasaccharide neoglycoconjugates. *FEMS Immunol. Med. Microbiol.* **47**, 116-128
- Sapats, S. I., Heine, H. G., Trinidad, L., Gould, G. J., Foord, A. J., Doolan, S. G., Prowse, S., and Ignjatovic, J. (2003) Generation of chicken single chain antibody variable fragments (scFv) that differentiate and neutralize infectious bursal disease virus (IBDV). *Arch. Virol.* **148**, 497-515
- Schade, R., Calzado, E. G., Sarmiento, R., Chacana, P. A., Porankiewicz-Asplund, J., and Terzolo, H. R. (2005) Chicken egg yolk antibodies (IgY-technology): A review of progress in production and use in research and human and veterinary medicine. *Altern. Lab. Anim.* **33**, 129-154
- Schauer, R. (2000) Achievements and challenges of sialic acid research. *Glycoconj. J.* **17**, 485-499
- Schauer, R. (2004) Sialic acids: Fascinating sugars in higher animals and man. *Zoology* **107**, 49-64

- Schijns, V. E. J. C. (2002) Antigen delivery systems and immunostimulation. *Vet. Immunol. Immunopathol.* **87**, 195-198
- Schirrmann, T., Meyer, T., Schutte, M., Frenzel, A., and Hust, M. (2011) Phage display for the generation of antibodies for proteome research, diagnostics and therapy. *Molecules* **16**, 412-426
- Schoonbroodt, S., Steukers, M., Viswanathan, M., Frans, N., Timmermans, M., Wehnert, A., Nguyen, M., Ladner, R. C., and Hoet, R. M. (2008) Engineering antibody heavy chain CDR3 to create a phage display fab library rich in antibodies that bind charged carbohydrates. *J. Immunol.* **181**, 6213-6221
- Schuck, P. and Zhao, H. (2010) The role of mass transport limitation and surface heterogeneity in the biophysical characterization of macromolecular binding processes by SPR biosensing. *Methods Mol. Biol.* **627**, 15-54
- Schweda, E. K., Twelkmeyer, B., and Li, J. (2008) Profiling structural elements of short-chain lipopolysaccharide of non-typeable *Haemophilus influenzae*. *Innate Immun.* **14**, 199-211
- Scott, G. B., de Wynter, E. A., and Cook, G. P. (2010) Detecting variable (V), diversity (D) and joining (J) gene segment recombination using a two-colour fluorescence system. *Mob DNA* **1**, 9
- Seeberger, P. H. and Werz, D. B. (2005) Automated synthesis of oligosaccharides as a basis for drug discovery. *Nat. Rev. Drug Discov.* **4**, 751-763
- Sen, G. and Mandal, C. (1995) The specificity of the binding site of AchatininH, a sialic acid-binding lectin from *Achatina fulica*. *Carbohydr. Res.* **268**, 115-125
- Svennerholm, L. (1957) Protein-bound Sialic Acids in Human, Hog, and Horse Kidneys. *Biophys. Acta*, **24**, 604
- Seveno, M., Bardor, M., Paccalet, T., Gomord, V., Lerouge, P., and Faye, L. (2004) Glycoprotein sialylation in plants? *Nat. Biotechnol.* **22**, 1351-2

- Shah, M. M., Fujiyama, K., Flynn, C. R., and Joshi, L. (2003) Sialylated endogenous glycoconjugates in plant cells. *Nat. Biotechnol.* **21**, 1470-1471
- Shao, Z., Zhao, H., Giver, L., and Arnold, F. H. (1998) Random-priming *in vitro* recombination: An effective tool for directed evolution. *Nucleic Acids Res.* **26**, 681-683
- Sharon, N. and Lis, N. (2004) Lectins, 2nd Edition., pp. 784, Springer, Netherlands
- Shental-Bechor, D. and Levy, Y. (2008) Effect of glycosylation on protein folding: A close look at thermodynamic stabilization. *Proc. Natl. Acad. Sci. U. S. A.* **105**, 8256-8261
- Shibuya, N., Goldstein, I. J., Broekaert, W. F., Nsimba-Lubaki, M., Peeters, B., and Peumans, W. J. (1987) The elderberry (*Sambucus nigra* L.) bark lectin recognizes the Neu5Ac(alpha 2-6)Gal/GalNAc sequence. *J. Biol. Chem.* **262**, 1596-1601
- Shibuya, N., Tazaki, K., Song, Z. W., Tarr, G. E., Goldstein, I. J., and Peumans, W. J. (1989) A comparative study of bark lectins from three elderberry (*Sambucus*) species. *J. Biochem.* **106**, 1098-1103
- Shishino, K., Yoshioka, Y., Hashimoto, K., Ochi, M., Aono, K., Murase, M., Osawa, H., Makino, H., and Saheki, S. (2005) A simple precipitation method for measuring sialic acid in apolipoprotein B-containing lipoproteins in plasma. *Ann. Clin. Biochem.* **42**, 298-300
- Shiyan, S. D. and Bovin, N. V. (1997) Carbohydrate composition and immunomodulatory activity of different glycoforms of alpha1-acid glycoprotein. *Glycoconj. J.* **14**, 631-638
- Shukla, A. K., Scholz, N., Reimerdes, E. H., and Schauer, R. (1982) High-performance liquid chromatography of *N,O*-acylated sialic acids. *Anal. Biochem.* **123**, 78-82
- Sidhu, S.S. (2005) Phage Display in Biotechnology and Drug Discovery, pp. 768, CRC Press

- Siegel, D. L. (2002) Recombinant monoclonal antibody technology. *Transfus. Clin. Biol.* **9**, 15-22
- Silverman, G. J., Gronwall, C., Vas, J., and Chen, Y. (2009) Natural autoantibodies to apoptotic cell membranes regulate fundamental innate immune functions and suppress inflammation. *Discov. Med.* **8**, 151-156
- Simpson, H., Chusney, G. D., Crook, M. A., and Pickup, J. C. (1993) Serum sialic acid enzymatic assay based on microtitre plates: Application for measuring capillary serum sialic acid concentrations. *Br. J. Biomed. Sci.* **50**, 164-167
- Singh, K. V., Kaur, J., Varshney, G. C., Raje, M., and Suri, C. R. (2004) Synthesis and characterization of hapten-protein conjugates for antibody production against small molecules. *Bioconjug. Chem.* **15**, 168-173
- Sioud, M. (2007) An overview of the immune system and technical advances in tumor antigen discovery and validation. *Methods Mol. Biol.* **360**, 277-318
- Siskos, P. A. and Spyridaki, M. H. (1999) Determination of sialic acids in biological fluids using reversed-phase ion-pair high-performance liquid chromatography. *J. Chromatogr. B Biomed. Sci. Appl.* **724**, 205-212
- Sixholo, J., Van Wyngaardt, W., Mashau, C., Frischmuth, J., Du Plessis, D. H., and Fehrson, J. (2011) Improving the characteristics of a mycobacterial 16 kDa-specific chicken scFv. *Biologicals* **39**, 110-116
- Skoza, L. and Mohos, S. (1976) Stable thiobarbituric acid chromophore with dimethyl sulphoxide. application to sialic acid assay in analytical de-O-acetylation. *Biochem. J.* **159**, 457-462
- Smith, G. P. (1985) Filamentous fusion phage: Novel expression vectors that display cloned antigens on the virion surface. *Science* **228**, 1315-1317
- Smith, J., Kontermann, R. E., Embleton, J., and Kumar, S. (2005) Antibody phage display technologies with special reference to angiogenesis. *FASEB J.* **19**, 331-341

- Smothers, J. F., Henikoff, S., and Carter, P. (2002) Phage display. affinity selection from biological libraries. *Science* **298**, 621-622
- Sobenin, I. A., Tertov, V. V., and Orekhov, A. N. (1998) Optimization of the assay for sialic acid determination in low density lipoprotein. *J. Lipid Res.* **39**, 2293-2299
- Soga, T. and Heiger, D. N. (1998) Simultaneous determination of monosaccharides in glycoproteins by capillary electrophoresis. *Anal. Biochem.* **261**, 73-78
- Song, J. K., Chung, B., Oh, Y. H., and Rhee, J. S. (2002) Construction of DNA-shuffled and incrementally truncated libraries by a mutagenic and unidirectional reassembly method: Changing from a substrate specificity of phospholipase to that of lipase. *Appl. Environ. Microbiol.* **68**, 6146-6151
- Spiro, R. G. (2002) Protein glycosylation: Nature, distribution, enzymatic formation, and disease implications of glycopeptide bonds. *Glycobiology* **12**, 43R-56R
- Stanton, P. G., Shen, Z., Kecorius, E. A., Burgon, P. G., Robertson, D. M., and Hearn, M. T. (1995) Application of a sensitive HPLC-based fluorometric assay to determine the sialic acid content of human gonadotropin isoforms. *J. Biochem. Biophys. Methods* **30**, 37-48
- Stebel, S. C., Gaida, A., Arndt, K. M., and Müller, K. M. (2008) Directed protein evolution. 631-656. In: Walker, J. M., Rapley, R., Stebel, Sabine C., Gaida, A., Arndt, K. M., Müller, K. M. Editors, *Molecular Biomethods Handbook*, 2nd Edition, Humana Press
- Stehling, P., Gohlke, M., Fitzner, R., and Reutter, W. (1998) Rapid analysis of *O*-acetylated neuraminic acids by matrix assisted laser desorption/ionization time-of-flight mass spectrometry. *Glycoconj. J.* **15**, 339-344
- Steipe, B. (1999) Evolutionary approaches to protein engineering. *Curr. Top. Microbiol. Immunol.* **243**, 55-86

- Stemmer, W. P. (1994) DNA shuffling by random fragmentation and reassembly: *In vitro* recombination for molecular evolution. *Proc. Natl. Acad. Sci. U. S. A.* **91**, 10747-10751
- Strousopoulou, K., Militsopoulou, M., Stagiannis, K., Lamari, F. N., and Karamanos, N. K. (2002) A capillary zone electrophoresis method for determining *N*-acetylneuraminic acid in glycoproteins and blood sera. *Biomed. Chromatogr.* **16**, 146-150
- Gräslund, S., Nordlund, P., Weigelt, J., Hallberg, B. M., Bray, J., Gileadi, O., Knapp, S., Oppermann, U., Arrowsmith, C., Hui, R., Ming, J., dhe-Paganon, S., Park, H. W., Savchenko, A., Yee, A., Edwards, A., Vincentelli, R., Cambillau, C., Kim, R., Kim, S. H., Rao, Z., Shi, Y., Terwilliger, T. C., Kim, C. Y., Hung, L. W., Waldo, G. S., Peleg, Y., Albeck, S., Unger, T., Dym, O., Prilusky, J., Sussman, J. L., Stevens, R. C., Lesley, S. A., Wilson, I. A., Joachimiak, A., Collart, F., Dementieva, I., Donnelly, M. I., Eschenfeldt, W. H., Kim, Y., Stols, L., Wu, R., Zhou, M., Burley, S. K., Emtage, J. S., Sauder, J. M., Thompson, D., Bain, K., Luz, J., Gheyi, T., Zhang, F., Atwell, S., Almo, S. C., Bonanno, J. B., Fiser, A., Swaminathan, S., Studier, F. W., Chance, M. R., Sali, A., Acton, T. B., Xiao, R., Zhao, L., Ma, L. C., Hunt, J. F., Tong, L., Cunningham, K., Inouye, M., Anderson, S., Janjua, H., Shastry, R., Ho, C. K., Wang, D., Wang, H., Jiang, M., Montelione, G. T., Stuart, D. I., Owens, R. J., Daenke, S., Schutz, A., Heinemann, U., Yokoyama, S., Bussow, K., and Gunsalus, K. C. (2008) Protein production and purification. *Nat. Methods* **5**, 135-146
- Sun, J., Wang, L., Wang, B., Guo, Z., Liu, M., Jiang, K., Tao, R., and Zhang, G. (2008) Purification and characterization of a natural lectin from the plasma of the shrimp *Fenneropenaeus chinensis*. *Fish Shellfish Immunol.* **25**, 290-297
- Suzuki, S., Tanaka, R., Takada, K., Inoue, N., Yashima, Y., Honda, A., and Honda, S. (2001) Analysis of sialo-*N*-glycans in glycoproteins as 1-phenyl-3-methyl-5-pyrazolone derivatives by capillary electrophoresis. *J. Chromatogr. A* **910**, 319-329

- Tangvoranuntakul, P., Gagneux, P., Diaz, S., Bardor, M., Varki, N., Varki, A., and Muchmore, E. (2003) Human uptake and incorporation of an immunogenic nonhuman dietary sialic acid. *Proc. Natl. Acad. Sci. U. S. A.* **100**, 12045-12050
- Tate, J. and Ward, G. (2004) Interferences in immunoassay. *Clin. Biochem. Rev.* **25**, 105-120
- Teshima, S., Tamai, K., Hayashi, Y., and Emi, S. (1988) New enzymatic determination of sialic acid in serum. *Clin. Chem.* **34**, 2291-2294
- Thaysen-Andersen, M., Mysling, S., and Hojrup, P. (2009) Site-specific glycoprofiling of *N*-linked glycopeptides using MALDI-TOF MS: Strong correlation between signal strength and glycoform quantities. *Anal. Chem.* **81**, 3933-3943
- Thobhani, S., Yuen, C. T., Bailey, M. J., and Jones, C. (2009) Identification and quantification of *N*-linked oligosaccharides released from glycoproteins: An inter-laboratory study. *Glycobiology* **19**, 201-211
- Thompson, C. B. and Neiman, P. E. (1987) Somatic diversification of the chicken immunoglobulin light chain gene is limited to the rearranged variable gene segment. *Cell* **48**, 369-378
- Tizard, I. (2002) The avian antibody response. *Semin. Avian Exot. Pet* **11**, 2-14
- Tong, Z., Zhenfang, W., Hui, C., Qi, W., Zizhong, T., Junbo, G., Lihua, W., Chunmei, W., and Chunmei, L. (2010) Progress in strategies for sequence diversity library creation for directed evolution. *J. Biotechnol.* **9**, 9277-9285
- Toyoda, M., Ito, H., Matsuno, Y. K., Narimatsu, H., and Kameyama, A. (2008) Quantitative derivatization of sialic acids for the detection of sialoglycans by MALDI MS. *Anal. Chem.* **80**, 5211-5218
- Traving, C. and Schauer, R. (1998) Structure, function and metabolism of sialic acids. *Cell Mol. Life Sci.* **54**, 1330-1349

- Treuheit, M. J., Costello, C. E., and Halsall, H. B. (1992) Analysis of the five glycosylation sites of human alpha 1-acid glycoprotein. *Biochem. J.* **283** (Pt 1), 105-112
- Troncoso, M. F., Iglesias, M. M., Isecke, R., Todel, C. W., and Brossmer, R. (2000) Specificity of the binding site of the sialic acid-binding lectin from ovine placenta, deduced from interactions with synthetic analogues. *Glycoconj. J.* **17**, 705-711
- Tsuji, T., Yamamoto, K., Irimura, T., and Osawa, T. (1981) Structure of carbohydrate unit A of porcine thyroglobulin. *Biochem. J.* **195**, 691-699
- Tsuji, T., Yamamoto, K., Konami, Y., Irimura, T., and Osawa, T. (1982) Separation of acidic oligosaccharides by liquid chromatography: Application to analysis of sugar chains of glycoproteins. *Carbohydr. Res.* **109**, 259-269
- Uemura, Y., Asakuma, S., Nakamura, T., Arai, I., Taki, M., and Urashima, T. (2005) Occurrence of a unique sialyl tetrasaccharide in colostrum of a bottlenose dolphin (*Tursiops truncatus*). *Biochim. Biophys. Acta* **1725**, 290-297
- Vallejo, V., Reyes-Leyva, J., Hernandez, J., Ramirez, H., Delannoy, P., and Zenteno, E. (2000) Differential expression of sialic acid on porcine organs during the maturation process. *Comp. Biochem. Physiol. B. Biochem. Mol. Biol.* **126**, 415-424
- Van Damme, E. J., Roy, S., Barre, A., Rouge, P., Van Leuven, F., and Peumans, W. J. (1997) The major elderberry (*Sambucus nigra*) fruit protein is a lectin derived from a truncated type 2 ribosome-inactivating protein. *Plant J.* **12**, 1251-1260
- van Kooyk, Y. and Rabinovich, G. A. (2008) Protein-glycan interactions in the control of innate and adaptive immune responses. *Nat. Immunol.* **9**, 593-601
- van Wyngaardt, W., Malatji, T., Mashau, C., Fehrsen, J., Jordaan, F., Miltiadou, D., and du Plessis, D. H. (2004) A large semi-synthetic single-chain fv phage display library based on chicken immunoglobulin genes. *BMC Biotechnol.* **4**, 6

- van Wyngaardt, W., Malatji, T., Mashau, C., Fehrsen, J., Jordaan, F., Miltiadou, D., and du Plessis, D. H. (2004) A large semi-synthetic single-chain fv phage display library based on chicken immunoglobulin genes. *BMC Biotechnol.* **4**, 6
- Vanhercke, T., Ampe, C., Tirry, L., and Denolf, P. (2005) Reducing mutational bias in random protein libraries. *Anal. Biochem.* **339**, 9-14
- Varki, A. (2007) Glycan-based interactions involving vertebrate sialic-acid-recognizing proteins. *Nature* **446**, 1023-1029
- Varki, A. (2008) Multiple changes in sialic acid biology during human evolution. *Glycoconj. J.* **26**, 231-245
- Varki, A., Cummings, R. D., Esko, J. D., Freeze, H. H., Stanley, P., Bertozzi, C. R., Hart, G. W. and Etzler, M. E. (2009) Editors. Essentials of Glycobiology. 2nd Edition. Cold Spring Harbor Laboratory Press
- Vazquez, A. M., Perez, A., Hernandez, A. M., Macias, A., Alfonso, M., Bombino, G., and Perez, R. (1998) Syngeneic anti-idiotypic monoclonal antibodies to an anti-NeuGc-containing ganglioside monoclonal antibody. *Hybridoma* **17**, 527-534
- Veistinen, E. and Lassila, O. (2001) Bursa of fabricius. In: *eLS*, Wiley
- Venkatesh, S. G. and Deshpande, V. (1999) A comparative review of the structure and biosynthesis of thyroglobulin. *Comp. Biochem. Physiol. C. Pharmacol. Toxicol. Endocrinol.* **122**, 13-20
- Vidal-Gomez, J. and Gras, J. (1978) The immunogenicity of dinitrophenylated polyacrylamide and the adjuvant effect of polyacrylamide. *Allergol. Immunopathol.* **6**, 311-320
- Vimr, E. R., Kalivoda, K. A., Deszo, E. L., and Steenbergen, S. M. (2004) Diversity of microbial sialic acid metabolism. *Microbiol. Mol. Biol. Rev.* **68**, 132-153
- Viswanathan, K., Tomiya, N., Park, J., Singh, S., Lee, Y. C., Palter, K., and Betenbaugh, M. J. (2006) Expression of a functional *Drosophila melanogaster*

- CMP-sialic acid synthetase. differential localization of the *Drosophila* and human enzymes. *J. Biol. Chem.* **281**, 15929-15940
- Viverge, D., Grimmonprez, L., and Solere, M. (1997) Chemical characterization of sialyl oligosaccharides isolated from goat (*Capra hircus*) milk. *Biochim. Biophys. Acta* **1336**, 157-164
- Vodnik, M., Zager, U., Strukelj, B., and Lunder, M. (2011) Phage display: Selecting straws instead of a needle from a haystack. *Molecules* **16**, 790-817
- Vulliez-le Normand, B., Saul, F. A., Martineau, P., Lema, F., Hofnung, M., and Bentley, G. A. (1997) Maltodextrin-binding protein hybrids carrying epitopes from the preS2 region of hepatitis B virus: Expression, antibody-binding and preliminary crystallographic studies. *Protein Eng.* **10**, 175-180
- Wa, C., Cerny, R. L., Clarke, W. A., and Hage, D. S. (2007) Characterization of glycation adducts on human serum albumin by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. *Clin. Chim. Acta* **385**, 48-60
- Warren, L. (1959) The thiobarbituric acid assay of sialic acids. *J. Biochem* **234**, 1971-5
- Wang, A. C., Tung, E., Fudenberg, H. H., and Hadji-Azimi, I. (1978) Immunoglobulin evolution: Chemical study of clawed toad (*Xenopus laevis*) heavy and light chains. *J. Immunogenet.* **5**, 355-364
- Wang, W. C. and Cummings, R. D. (1988) The immobilized leucoagglutinin from the seeds of *Maackia amurensis* binds with high affinity to complex-type asn-linked oligosaccharides containing terminal sialic acid-linked alpha-2,3 to penultimate galactose residues. *J. Biol. Chem.* **263**, 4576-4585
- Wang, T. W., Zhu, H., Ma, X. Y., Zhang, T., Ma, Y. S., and Wei, D. Z. (2006) Mutant library construction in directed molecular evolution: Casting a wider net. *Mol. Biotechnol.* **34**, 55-68

- Weis, W., Brown, J. H., Cusack, S., Paulson, J. C., Skehel, J. J., and Wiley, D. C. (1988) Structure of the influenza virus haemagglutinin complexed with its receptor, sialic acid. *Nature* **333**, 426-431
- Weiss, A. A. and Lyer, S. S. (2007) Glycomics aims to interpret the third molecular language of cells. *microbe* 2007;2:489–97. *Microbe*, 489-497
- Wheeler, S. F., Domann, P., and Harvey, D. J. (2009) Derivatization of sialic acids for stabilization in matrix-assisted laser desorption/ionization mass spectrometry and concomitant differentiation of alpha(2 --> 3) and alpha(2 --> 6)-isomers. *Rapid Commun. Mass Spectrom.* **23**, 303-312
- Willems, A., Leoen, J., Schoonoghe, S., Grooten, J., and Mertens, N. (2003) Optimizing expression and purification from cell culture medium of trispecific recombinant antibody derivatives. *J. Chromatogr. B. Analyt Technol. Biomed. Life. Sci.* **786**, 161-176
- Williams, M. N., Freshour, G., Darvill, A. G., Albersheim, P., and Hahn, M. G. (1996) An antibody fab selected from a recombinant phage display library detects deesterified pectic polysaccharide rhamnogalacturonan II in plant cells. *Plant Cell* **8**, 673-685
- Wu, A. M., Csako, G., and Herp, A. (1994) Structure, biosynthesis, and function of salivary mucins. *Mol. Cell. Biochem.* **137**, 39-55
- Wu, A. M., Lisowska, E., Duk, M., and Yang, Z. (2008) Lectins as tools in glycoconjugate research. *Glycoconj. J.* **26**, 899-913
- Xu, J. L. and Davis, M. M. (2000) Diversity in the CDR3 region of V(H) is sufficient for most antibody specificities. *Immunity* **13**, 37-45
- Yabe, U., Sato, C., Matsuda, T., and Kitajima, K. (2003) Polysialic acid in human milk. CD36 is a new member of mammalian polysialic acid-containing glycoprotein. *J. Biol. Chem.* **278**, 13875-13880

- Yamada, M., Hur, S., Hashinaka, K., Tsuneoka, K., Saeki, T., Nishio, C., Sakiyama, F., and Tsunasawa, S. (1987) Isolation and characterization of a cDNA coding for human myeloperoxidase. *Arch. Biochem. Biophys.* **255**, 147-155
- Yamamoto, K., Tsuji, T., Irimura, T., and Osawa, T. (1981) The structure of carbohydrate unit B of porcine thyroglobulin. *Biochem. J.* **195**, 701-713
- Yamamoto, K., Ito, S., Yasukawa, F., Konami, Y., and Matsumoto, N. (2005) Measurement of the carbohydrate-binding specificity of lectins by a multiplexed bead-based flow cytometric assay. *Anal. Biochem.* **336**, 28-38
- Yang, S. Y., Fugmann, S. D., and Schatz, D. G. (2006) Control of gene conversion and somatic hypermutation by immunoglobulin promoter and enhancer sequences. *J. Exp. Med.* **203**, 2919-2928
- Yao, K. and Ubuka, T. (1987) Determination of sialic acids by acidic ninhydrin reaction. *Acta Med. Okayama* **41**, 237-241
- Yao, K., Ubuka, T., Masuoka, N., Kinuta, M., and Ikeda, T. (1989) Direct determination of bound sialic acids in sialoglycoproteins by acidic ninhydrin reaction. *Anal. Biochem.* **179**, 332-335
- Yasuno, S., Kokubo, K., and Kamei, M. (1999) New method for determining the sugar composition of glycoproteins, glycolipids, and oligosaccharides by high-performance liquid chromatography. *Biosci. Biotechnol. Biochem.* **63**, 1353-1359
- Yoshio, K., Guoqiang, Z., Satoshi, U., Tetsuo, F., Takeshi, O., Michimasa, K., and Ken-ichi, S. (2002) Analysis and optimization of biopanning process of phage display libraries; biological systems engineering. , 271-284. In: Marten, M.R., Park, T.H., and Nagamune, T. Editors, Biological Systems Engineering, 830, American Chemical Society
- Zaia, J. (2008) Mass spectrometry and the emerging field of glycomics. *Chem. Biol.* **15**, 881-892

- Zanetta, J. P., Timmerman, P., and Leroy, Y. (1999) Gas-liquid chromatography of the heptafluorobutyrate derivatives of the *O*-methyl-glycosides on capillary columns: A method for the quantitative determination of the monosaccharide composition of glycoproteins and glycolipids. *Glycobiology* **9**, 255-266
- Zanetta, J. P., Pons, A., Iwersen, M., Mariller, C., Leroy, Y., Timmerman, P., and Schauer, R. (2001) Diversity of sialic acids revealed using gas chromatography/mass spectrometry of heptafluorobutyrate derivatives. *Glycobiology* **11**, 663-676
- Zanetta, J. P., Srinivasan, V., and Schauer, R. (2006) Analysis of monosaccharides, fatty constituents and rare *O*-acetylated sialic acids from gonads of the starfish *Asterias rubens*. *Biochimie* **88**, 171-178
- Zbiral, E., Kleineidam, R. G., Schreiner, E., Hartmann, M., Christian, R., and Schauer, R. (1992) Elucidation of the topological parameters of *N*-acetylneuraminic acid and some analogues involved in their interaction with the *N*-acetylneuraminate lyase from *Clostridium perfringens*. *Biochem. J.* **282**, 511-516
- Zhang, Y., Go, E. P., and Desaire, H. (2008) Maximizing coverage of glycosylation heterogeneity in MALDI-MS analysis of glycoproteins with up to 27 glycosylation sites. *Anal. Chem.* **80**, 3144-3158
- Zhao, H., Giver, L., Shao, Z., Affholter, J. A., and Arnold, F. H. (1998) Molecular evolution by staggered extension process (StEP) *in vitro* recombination. *Nat. Biotechnol.* **16**, 258-261