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**Generation of recombinant anti-*N*-glycolylneuraminic acid (Neu5Gc) scFv  
and its applications for detection and quantitation of Neu5Gc**

**A Thesis Submitted to the National University of Ireland for the  
Degree of Doctor of Philosophy**

**By**

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**January 2016**

**Glycoscience Group**

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## **Declaration**

I hereby declare that the work embodied in this thesis is presentation of my original research carried out at National University of Ireland for the fulfilment of the degree of PhD.

Rajesh Kumar Sharma

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The text and the scientific outcomes presented in this PhD thesis reflect only one aspect of the learning that I have gained during the course of the study. There is a whole lot of background that remains unnoticed. The major proportion of the PhD work has contributed to the development of intellectual capacity not limited to science but also to develop a strong character, which remains unaffected from the success and failure. Though the work within the document is represented by an individual, genuine effort and positive influence from various people have contributed towards the successful completion of this research work. All these people who I now wish to acknowledge have also helped in my overall intellectual development.

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besides me to provide continuous moral support. She has been a true driving force behind this accomplishment. I dedicate this work to my family.

## Abstract

Sialic acids are a family of 9 carbon  $\alpha$ -keto acid monosaccharides. Owing to their terminal position on glycans, sialic acids are involved in cell adhesion and recognition events, host pathogen interactions, immune regulation and in disease processes such as cancer progression. Sialic acids are highly diverse with more than 50 different forms reported to date. The two predominant forms of sialic acid expressed in the mammals are the *N*-acetylneuraminic acid (Neu5Ac) and *N*-glycolylneuraminic acid (Neu5Gc). Though humans can no longer synthesise Neu5Gc due to an evolutionary mutation in the enzyme responsible (CMAH), the presence of this non-human glycan has been frequently reported distributed on a number of human tissues, both healthy and malignant. Neu5Gc has also been reported as a contaminant in biopharmaceutical preparations, produced in mammalian cell systems, compromising their safety and efficacy. To date, no monoclonal antibody has been reported against Neu5Gc. Neu5Gc recognition molecules would have applications in detection and quantitation of Neu5Gc in clinical diagnosis (e.g. cancer screening) and in quality control for biopharmaceutical manufacturing. The research performed within this thesis was directed towards the construction and characterisation of anti-Neu5Gc antibody fragments for application in the detection and quantitation of Neu5Gc.

Derived from an immunised chicken, short and long-linker single chain fragment variable (scFv) phage display library were constructed and biopanned against the Neu5Gc. A panel of anti-Neu5Gc scFvs were identified and assessed for their binding specificity and affinity to Neu5Gc. Among the panel of anti-Neu5Gc scFvs, short-linker scFv-SL1A1, demonstrated the highest sensitivity for binding to Neu5Gc and was selected as lead candidate for further characterisation and assay development. By competitive ELISA, developed for detection and quantitation of Neu5Gc, scFv-SL1A1 detected Neu5Gc with an  $IC_{50}$  of 514 nM. The scFv-SL1A1 demonstrated high specificity for Neu5Gc in free as well as protein bound form. Further assessment of scFv on western blot and surface plasmon resonance supported the demonstration of Neu5Gc specificity. In western blotting platform, scFv-SL1A1 detected the presence of Neu5Gc on clinical samples and on

commercial biopharmaceutical preparations. Investigations to assess the suitability of scFv-SL1A1 for immunohistochemistry applications were performed. This thesis research reports the generation and characterisation of the first recombinant monoclonal anti-Neu5Gc scFv fragment.

## List of Abbreviations

AA	Amino Acid
BLAST	Basic Local Alignment Search Tool
BSA	Bovine Serum Albumin
CDR	Complementarity Determining Region
DNA	Deoxyribonucleic Acid
EDC	1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide
ELISA	Enzyme Linked Immunosorbent Assay
ELLA	Enzyme Linked Lectin Assay
ER	Endoplasmic Reticulum
FASTA	Fast Alignment
FR	Framework Region
Fuc	Fucose
Gal	Galactose
GalNAc	<i>N</i> -acetylgalactosamine
Glc	Glucose
GSP	Gene Specific Primer
GT	Glycosyltransferase
HPLC	High Performance Liquid Chromatography
HRP	Horseradish Peroxidase
K <sub>a</sub>	Association constant
K <sub>d</sub>	Dissociation constant
kDa	Kilo Dalton
Lac	Lactose
LB	Luria Bertani
LL	Long Linker
Man	Mannose
MES	2-( <i>N</i> -morpholino) ethanesulfonic acid
mL	Milli Liter
mM	Milli Molar
Neu5Ac	<i>N</i> -acetylneuraminic acid

Neu5Gc	<i>N</i> -glycolylneuraminic acid
NHS	N-Hydroxysuccinimide
nM	Nano Molar
PAA	Polyacrylic Acid
PBS	Phosphate Buffered Saline
pBSA	Periodated Bovine Serum Albumin
PCR	Polymerase Chain Reaction
PEG	Polyethylene Glycol
PTM	Post Translational Modification
RNA	Ribonucleic Acid
RT	Room Temperature
SB	Super Broth
scFv	Single Chain Fragment Variable
SD	Standard Deviation
SEM	Standard Error of Mean
SL	Short Linker
SOB	Super Optimal Broth
SPR	Surface Plasmon Resonance
TAE	Tris Acetate EDTA
TEMED	N,N,N',N'-Tetramethylethylenediamine
VH	Variable Heavy
VL	Variable Light
Xyl	Xylose
μL	Micro Liter
μM	Micro Molar

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# **Chapter 1**

## **Introduction**

## 1.1. General introduction

Glycans are ubiquitous in nature and have been found to coat cell surfaces of all the species studied to date (Varki, 2011a). Until recently, glycans were only considered as a source of energy. Among the four fundamental molecules of life, i.e. nucleic acids, proteins, lipids and carbohydrates, glycans remained largely under-appreciated (Moon, 2012). The central dogma of biology published by F. H. C. Crick in 1970 established that DNA, made up of nucleic acids, is the central storage of coded information, which is transcribed into an information transporter, mRNA, and finally translated into proteins (Crick, 1970). However, glycans are secondary gene products and are not encoded directly by the DNA. This also means that the structure and function of glycans cannot be predicted from the DNA sequence alone (Moon, 2012). In fact, the proteins that are encoded by DNA can be modified by glycans to increase the functional diversity of proteins. This was realised from the observation that the human genome with an estimated 20,000 to 25,000 genes gives rise to over 1 million different proteins that constitute the human proteome (Consortium, 2004, Jensen, 2004). This lack of correspondence in the size of genome and proteome clearly indicated that protein diversity is not the direct result of genome encoding. It was later realised that far greater complexity from genomic level to proteins is facilitated by post-translational modifications (PTM) of protein that it acquires during its translocation to various cellular destinations. PTMs are specific enzyme catalysed covalent processing events, at one or more specific amino acids on the protein chain, and provide organisms with adaptability to rapidly alter protein structure, function and activity in response to internal and external stimuli. It is estimated that there are 200 known PTMs of proteins catalysed by enzymes that comprise 5% of the total proteome (Walsh and Walsh, 2006). Among this huge variety of protein modifications, the most common types of PTMs are phosphorylation, acetylation, acylation, glycosylation, ubiquitination, sulfation, methylation, deamidation, and disulfide bond formation (Knorre et al., 2009, Mann and Jensen, 2003).

Glycosylation is one of the most prominent PTM of the eukaryotic proteins, and generates a diverse repertoire of glycoproteins (Eichler and Adams, 2005, Lis and Sharon, 1993). The glycans on individual glycoproteins and on cell surfaces are

involved in a myriad of processes that include cellular recognition, immuno modulation, host pathogen interaction, protein folding and cell adhesion and proliferation (Hart, 2013, Stanley et al., 2009, Lis and Sharon, 1993). Changes in glycan patterns and their aberrant expression has also been identified in many disease processes (Vasconcelos-dos-Santos et al., 2015, Dube and Bertozzi, 2005). Despite the involvement of glycans in various biological processes, the understanding of glycans is still not comparable to the developments made in protein and nucleic acid research. This is largely attributed to the structural complexity of glycans, and also to the relative lack of tools to probe and understand glycans (Moon, 2012).

Although some glycans were known to have immunogenic potential in humans, recent investigations have demonstrated their presence on drugs intended for human use (Ghaderi et al., 2010, Butler and Spearman, 2014). This has generated mounting safety concerns over the use of such drugs and have highlighted urgent need for the development of tools to detect glycans for analysing and monitoring the quality of biopharmaceuticals.

The initial part of this chapter will be an introduction to the glycans and their structural diversity and the role of glycans in biological processes. The later part details the research and development in glycan detection and the need to generate tools for glycan recognition.

## **1.2 Glycans and their structural diversity**

Glycans or carbohydrates are the most abundant biological molecules on the planet, and are one of the four fundamental molecules along with nucleic acids, proteins and lipids, that make up all living systems (Hart, 2013). Carbohydrates are hydrates of carbon and consists of carbon, hydrogen and oxygen in a stoichiometric proportion of  $C_n(H_2O)_n$ , where  $n \geq 3$  (Gabijs, 2009). Currently, polyhydroxyaldehydes or polyhydroxyketones or substances that yield such compounds on hydrolysis are termed as carbohydrates. Carbohydrates are also referred to as saccharides (derived from Greek word “*sakchar*” meaning sugar or sweetness) (Bertozzi and Rabuka, 2009).

Carbohydrates are divided into three types: monosaccharides, oligosaccharides and polysaccharides. Monosaccharides are the fundamental units of carbohydrates and they cannot be hydrolysed into simpler forms. Monosaccharides contain a carbonyl group that may be either an aldehyde or a ketone (Varki and Sharon, 2009). Monosaccharides that contain an aldehyde functional group are also termed as polyhydroxyaldehydes (aldose), while monosaccharides with a ketone functional group are called polyhydroxyketones (ketoses). The aldoses are reducing sugars, e.g. glucose (Glc), fucose (Fuc), galactose (Gal) and mannose (Man), while ketoses are non-reducing sugars, e.g. fructose (Fru). The monosaccharides can be joined together by a covalent bond, termed as glycosidic bond, to form complex carbohydrates (Varki and Sharon, 2009, Bertozzi and Rabuka, 2009).

The oligosaccharides are short-chain carbohydrates with two to twenty monosaccharide units, joined together by glycosidic bonds, e.g. sucrose is made up of Glc and Fuc. An oligosaccharide with two monosaccharide units is called a disaccharide while those with three monosaccharide units are called trisaccharides and so on. Polysaccharides, on the other hand, are made up of more than twenty monosaccharide units forming linear or branched sugar polymers, e.g. cellulose and glycogen (Boyle, 2005, Bertozzi and Rabuka, 2009), Figure 1.1. Carbohydrates containing a number of monosaccharide units linked by glycosidic linkages are also termed as glycans, a term used synonymous to polysaccharide (Gold et al., 1997). Glycans have high structural diversity, which arises from the structural complexity of constituent monosaccharides.

The structural diversity of monosaccharides arises from various isomeric forms. The asymmetric carbon (chiral) within the monosaccharides alone increases the number of stereoisomeric forms of a single monosaccharide. The number of asymmetric carbons in a monosaccharide can be calculated using the expression:  $n-2$  for aldoses and  $n-3$  for ketoses, where  $n$  is the total number of carbons (Bertozzi and Rabuka, 2009). In the case of Glc, which is an aldohexose with 6 carbon atoms, 4 asymmetric carbons can generate 16 ( $2^4$ ) stereoisomeric forms. Further, the orientation of OH group at the penultimate carbon (C-5) generates the D (OH group on right side) and L (OH group on left side) form of Glc, Figure 1.2. The D-glucose can also generate isomeric forms that differ in orientation of the OH group at a single asymmetric

carbon, such isomeric forms are termed as epimers. D-galactose is the C-4 and mannose is the C-2 epimer of D-glucose, Figure 1.1 (Gabijs, 2009, Bertozzi and Rabuka, 2009). Further, the monosaccharides with 5 or more carbons predominantly exist in cyclic structures, which are formed by the intramolecular reaction between the carbonyl and the hydroxyl group, giving rise to a hemiacetal derivative (Boyle, 2005, Bertozzi and Rabuka, 2009). The aldohexoses such as Glc can form a cyclic six membered ring, when C-1 reacts with the C-5 hydroxyl group, or a five membered ring by the reaction of C-1 with the C-4 hydroxyl group. The six membered ring is termed as furanose and five membered ring is called pyranose (Bertozzi and Rabuka, 2009). The C-1 carbon (as in glucose) forming hemiacetal is termed as the anomeric carbon and the presentation of an OH group at the anomeric carbon generates additional stereoisomeric forms. The orientation of the OH group, at the axial position forms the  $\alpha$ -D-glucose, and at the equatorial position forms the  $\beta$ -D-glucose (Gabijs, 2009, Bertozzi and Rabuka, 2009), Figure 1.3. The anomeric carbon in the  $\alpha$  or  $\beta$  configuration can link at any OH group of another monosaccharide to form a disaccharide. The structural characteristics, type of anomeric linkage, the position and presence or absence of branching in a sugar residue are responsible for the exponential increase in the complexity of glycans (Solís et al., 2001). To get an estimate of the coding capacity of glycans, it has been calculated that, with 20 monosaccharides  $1.44 \times 10^{15}$  unique hexasaccharides can be generated. While four nucleotide of DNA can generate  $4^6$  hexanucleotide and 20 amino acids can generate  $6.4 \times 10^7$  unique hexapeptides (Laine, 1997). Although not all the  $10^{15}$  combinations are synthesised in living systems, nevertheless the information storage and coding capacity of glycans is far greater than that of nucleic acids and amino acids.

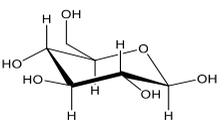
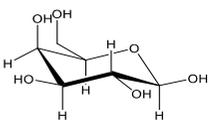
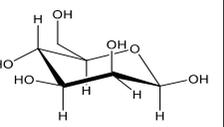
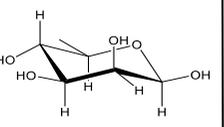
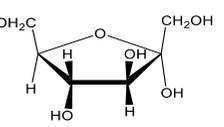
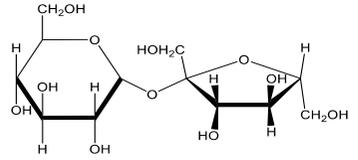
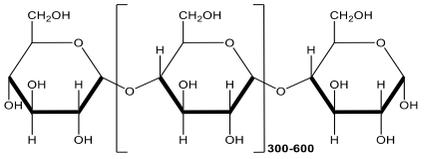
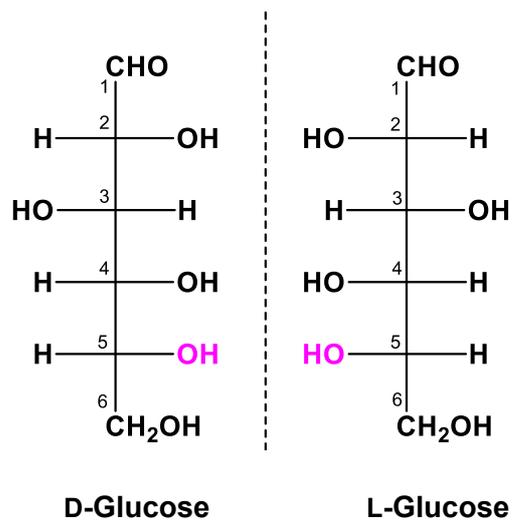
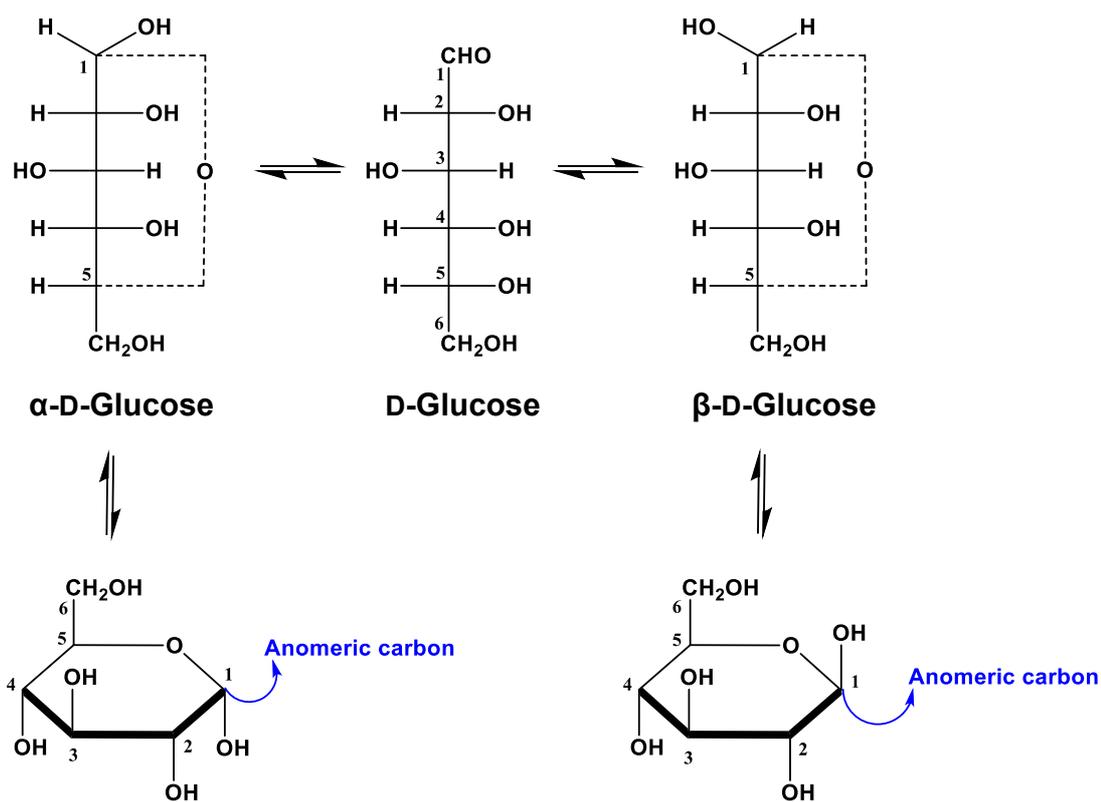
Monosaccharide structure	aldose				ketose
	Glucose	Galactose	Mannose	Fucose	Fructose
(Open-chain)	$  \begin{array}{c}  \text{CHO} \\    \\  \text{H} - \text{C} - \text{OH} \\    \\  \text{HO} - \text{C} - \text{H} \\    \\  \text{H} - \text{C} - \text{OH} \\    \\  \text{H} - \text{C} - \text{OH} \\    \\  \text{CH}_2\text{OH}  \end{array}  $	$  \begin{array}{c}  \text{CHO} \\    \\  \text{H} - \text{C} - \text{OH} \\    \\  \text{HO} - \text{C} - \text{H} \\    \\  \text{HO} - \text{C} - \text{H} \\    \\  \text{H} - \text{C} - \text{OH} \\    \\  \text{CH}_2\text{OH}  \end{array}  $	$  \begin{array}{c}  \text{CHO} \\    \\  \text{HO} - \text{C} - \text{H} \\    \\  \text{HO} - \text{C} - \text{H} \\    \\  \text{H} - \text{C} - \text{OH} \\    \\  \text{H} - \text{C} - \text{OH} \\    \\  \text{CH}_2\text{OH}  \end{array}  $	$  \begin{array}{c}  \text{CHO} \\    \\  \text{HO} - \text{C} - \text{H} \\    \\  \text{HO} - \text{C} - \text{H} \\    \\  \text{H} - \text{C} - \text{OH} \\    \\  \text{H} - \text{C} - \text{OH} \\    \\  \text{CH}_3  \end{array}  $	$  \begin{array}{c}  \text{CH}_2\text{OH} \\    \\  \text{C} = \text{O} \\    \\  \text{HO} - \text{C} - \text{H} \\    \\  \text{H} - \text{C} - \text{OH} \\    \\  \text{H} - \text{C} - \text{OH} \\    \\  \text{CH}_2\text{OH}  \end{array}  $
(Cyclic)					
Oligosaccharide structure	<p style="text-align: center;"><b>Sucrose</b></p> 				
Polysaccharide structure	<p style="text-align: center;"><b>Starch</b></p> 				

Figure 1.1 Representative glycan types depicted with their linear and cyclic structures.



**Figure 1.2** Glucose represented in its D and L isomeric form



**Figure 1.3** Pyranose form of glucose showing configuration of OH group on anomeric carbon.

### 1.3 Glycosylation

Glycosylation is the site-specific, enzyme-driven, covalent attachment of carbohydrates to proteins and lipids. Glycosylation is one of the major and most complex co- or post-translational modification of proteins (Spiro, 2002). In humans, more than 50 % of proteins are glycosylated (Apweiler et al., 1999). Glycans are complex carbohydrates that serve varied functions in many biological processes including cell-cell adhesion, receptor binding and its activation, protein maturation, targeting and turnover, immune regulation, host pathogen interaction and generating species specific determinants (Varki, 2006, Helenius and Aebi, 2004, Lowe and Marth, 2003, Varki, 2011a, Caramelo and Parodi, 2007). Glycosylation mainly occurs in the endoplasmic reticulum (ER) and Golgi complex, where the concerted effort of different enzymes attach and trim glycans on proteins (Lodish et al., 2000). Two major types of protein glycosylation occur in eukaryotes: *N*-linked glycosylation, where glycans are linked to asparagine on the polypeptide chain in the ER and *O*-linked glycosylation that occurs in the Golgi complex, where glycans are attached to the hydroxyl side chain of serine or threonine. However, other important but less common types of glycosylation such as  $\beta$ -GlcNAc,  $\alpha$ -Man and  $\alpha$ -Fuc also exist. The GlcNAc- $\beta$ -Ser/Thr type glycosylation is mainly found on nuclear and cytoplasmic proteins. This monosaccharide remains unextended unlike other forms of glycosylation (Stanley et al., 2009, Spiro, 2002). The Man- $\alpha$ -Ser/Thr modification is found on yeast cell wall proteins. Besides yeast, Man-Ser/Thr proteins modification in yet unknown anomeric configuration is also found on brain proteoglycans and  $\alpha$ -dystroglycans of peripheral nerves. The Fuc- $\alpha$ -Ser/Thr modification appear predominantly on proteins such as coagulation and fibrinolytic factors (Spiro, 2002).

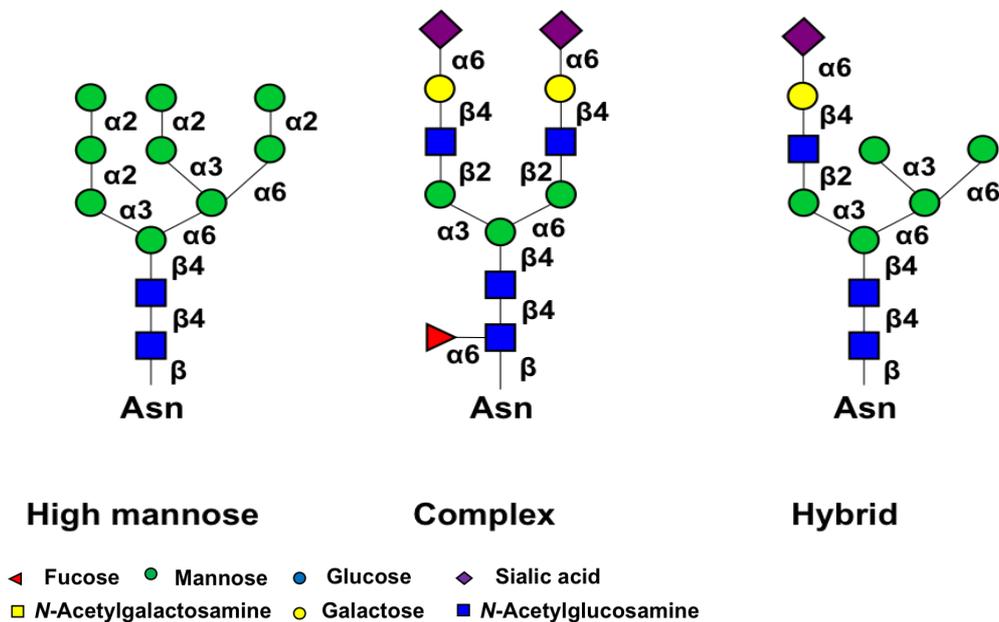
#### 1.3.1 *N*-linked glycosylation

*N*-linked glycosylation is the most common form of glycan modification of secretory proteins (Helenius and Aebi, 2004, Mechref and Novotny, 2002). The process of *N*-linked glycosylation starts in the ER and final processing events take place in the Golgi complex. The *N*-glycans have a common core consisting of Man<sub>3</sub>GlcNAc<sub>2</sub>-Asn and have three major types of structures:

A. Oligomannose glycan (High mannose), where mannose residues, attached to the *N*-acetylglucosamine (GlcNAc<sub>2</sub>) core, remain unsubstituted. These structures can contain 5 to 9 mannose residues.

B. Complex, where antennae formation is initiated by the addition of GlcNAc to mannose residues, which is catalysed by *N*-acetylglucosaminyltransferases.

C. Hybrid, these glycans have unsubstituted mannose- $\alpha$ -(1,6) arm which is substituted with more mannose, and a mannose- $\alpha$ -(1,3) arm with GlcNAc substitution to form antennae (Stanley et al., 2009), Figure 1.4.

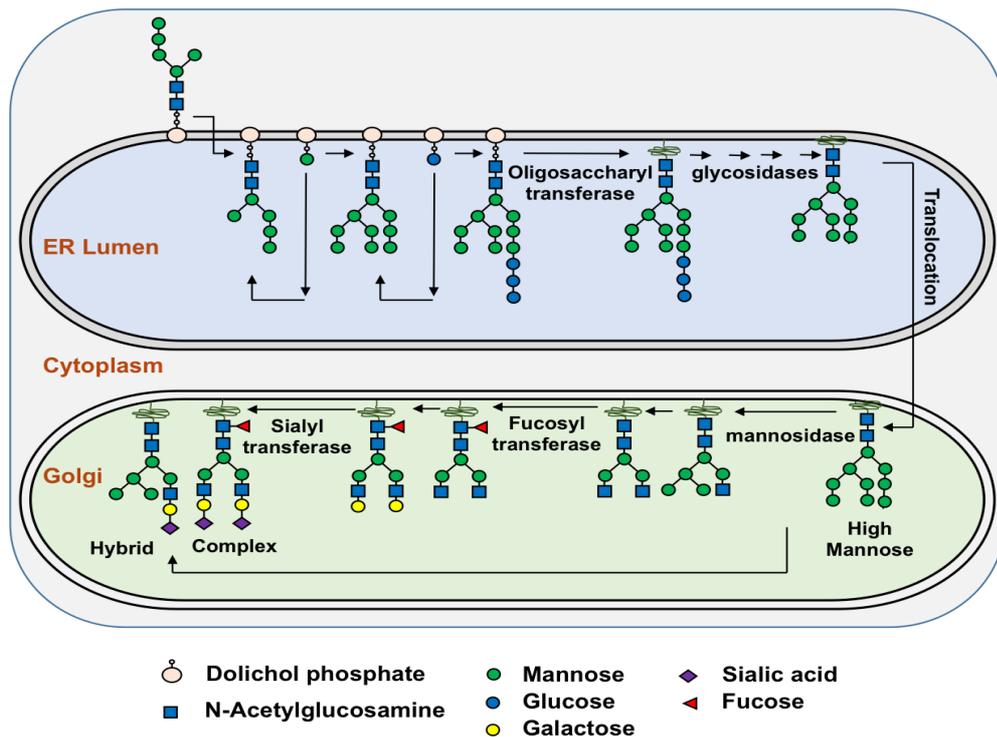


**Figure 1.4** Common *N*-linked glycan structures. All structures made using Consortium of Functional Glycomics ([www.functionalglycomics.org](http://www.functionalglycomics.org)) notations.

Glycosylation begins with the synthesis of mannose-rich glycans attached to the dolichol lipid on the cytosolic face of the ER and synthesis is completed once the glycans are flipped on the luminal side of ER. The mannose-rich glycan is then branched by addition of more mannose and glucose units to produce the Glc<sub>3</sub>Man<sub>9</sub>GlcNAc<sub>2</sub> structure. This precursor glycan is then transferred *en bloc* to asparagine residue on nascent proteins by the ER resident multi-subunit enzyme oligosaccharyltransferase (OGS). OGS transfers glycans from dolichol lipid donor to the nascent acceptor protein at the conserved Asn-X-(Ser/Thr) tripeptide sequence to form *N*-linked glycan precursors (Stanley et al., 2009, Ruddock and Molinari, 2006).

Further glycan processing events on the luminal side of the ER are catalysed by different enzymes that also help in protein folding. In the ER, during the process of folding, chaperones calreticulin and calnexin participate in the quality control of proteins. Calreticulin and calnexin are lectin chaperones that interact with and assist the folding of proteins carrying monoglucosylated glycans. In this process, glucosidase I and II removes the first and second glucose units respectively from  $\text{Glc}_3\text{Man}_9\text{GlcNAc}_2$  resulting in a  $\text{GlcMan}_9\text{GlcNAc}_2$  structure, which becomes an active ligand for calnexin and calreticulin. The removal of a third glucose by glucosidase II terminates the interaction with calnexin and calreticulin. After removal of the final glucose, the UDP glucose glycoprotein glucosyl transferase (UGGT) senses the protein folding status. Inadequately folded proteins are monoglucosylated by UGGT to again promote association with calnexin and calreticulin for correct refolding. However, if the proteins remains misfolded after repetitive refolding, the ER  $\alpha$  1,2-mannosidase II removes a mannose that leads to recognition and association with ER degradation-enhancing  $\alpha$  1,2-mannosidase-like protein (EDEP). This targets proteins for ER associated degradation (ERAD) (Hebert et al., 2005, Ellgaard and Helenius, 2003).

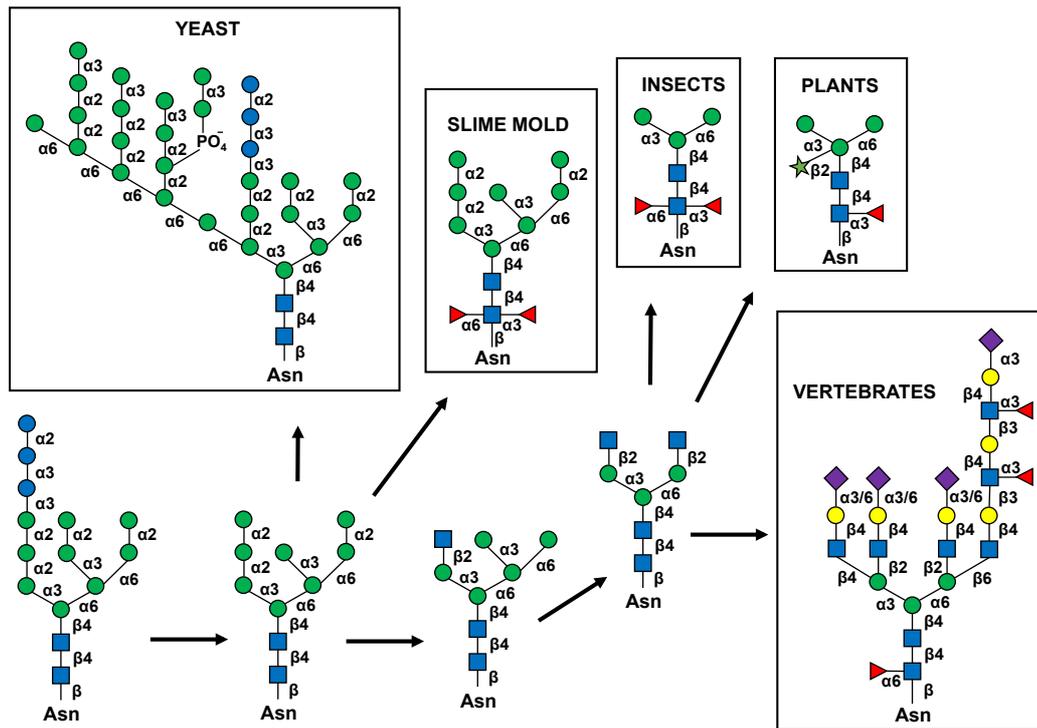
The correctly folded proteins with  $\text{Man}_9\text{GlcNAc}_2$ -linked structures are then translocated to the Golgi complex for final editing, where six mannose units are removed by the Golgi complex resident mannosidase enzymes to form the  $\text{Man}_3\text{GlcNAc}_2$  pentasaccharyl core, which can then be branched giving rise to complex and hybrid *N*-linked oligosaccharides (Moremen et al., 2012, Aebi, 2013), Figure 1.5.



**Figure 1.5** Mammalian *N*-linked glycosylation process. The *N*-linked glycosylation begins in ER by attachment of mannose-rich glycan to protein. The glucose attached to mannose are removed to signal proper folding of protein in the ER and the protein with attached glycan is translocated to Golgi complex. In Golgi complex the mannose residues are removed, followed by generation of hybrid and complex *N*-glycan structures by concerted efforts of various enzymes. All structures made using CFG notations.

Interestingly, in most of the eukaryotic species studied to date, the glycosylation pathway starts with a common  $\text{Glc}_3\text{Man}_9\text{GlcNAc}_2$  precursor. The Golgi complex then acts as an evolutionary engineer to generate species specific differences in glycosylation patterns. Yeast use this  $\text{Glc}_3\text{Man}_9\text{GlcNAc}_2$  precursor for further modification to generate the mannose-rich structures. While slime molds undergo mannose trimming to a certain extent to generate high mannose structures (Varki, 2011a). From insects onwards, mannose trimming is more prominent and the final  $\text{Man}_3\text{GlcNAc}_2$  core structure takes shape, the  $\text{GlcNAc}$  core in insects is found attached to  $\alpha$ -(1,3)-linked Fuc and  $\alpha$ -(1,6)-Fuc, however  $\text{Man}_3\text{GlcNAc}_2$  core remains largely unextended. In plants, the  $\text{Man}_3\text{GlcNAc}_2$  core is extended further to a limited extent. In addition to  $\beta$ -(1,2)-linked xylose residue on the  $\beta$ -linked mannose,  $\alpha$ -(1,3)-Fuc is attached to  $\text{GlcNAc}$  in plants. In vertebrates, the  $\text{Man}_3\text{GlcNAc}_2$  core structure becomes highly branched and extended giving rise to complex glycosylation patterns

often terminating with sialic acid (Varki, 2011a, Stanley et al., 2009), Figure 1.6. Besides generating glycan specific differences across different taxa, glycans also show intra-species differences in glycan patterns, typical example being blood group antigens and polymorphism that contribute towards differential susceptibility to various diseases within the same species (Marionneau et al., 2001, Bishop and Gagneux, 2007, Blackwell et al., 2009).



**Figure 1.6** Species specific glycosylation. The glycosylation in different species begin with a common mannose-rich oligosaccharide precursor. From yeast to invertebrates, the mannose content decreases however the branching and addition of glycan to the core increases. Thus beginning with a common precursor, the glycosylation machinery in different species generates a species specific glycan profile. Image adapted from (Varki, 2011a).

### 1.3.2 O-linked Glycosylation

*O*-linked glycosylation is the covalent attachment of glycans to the hydroxyl-group of serine/threonine by an *O*-glycosidic bond. *O*-linked glycosylation is exclusively a post-translational event that begins by attachment of a single monosaccharide unit followed by iterative elongation steps (Stanley et al., 2009). Proteins containing multiple clusters of *O*-linked glycans are termed as mucins and this type of

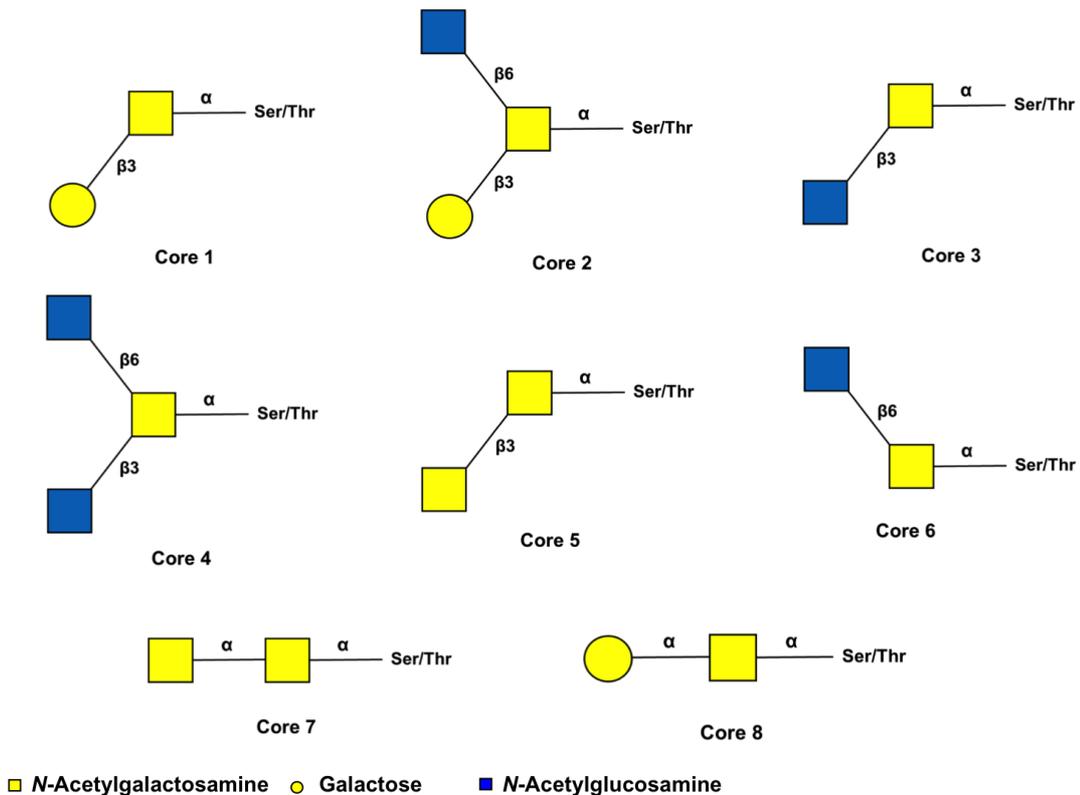
glycosylation is called mucin-type *O*-linked glycosylation. The term mucin was used for first time for the substances isolated from mucus, which were later recognised as mixture of proteins and carbohydrates (Hang and Bertozzi, 2005). Mucins are high molecular weight glycoproteins, where the carbohydrate component can comprise 80% of the total weight.

The biosynthesis of *O*-linked glycosylation is initiated in the Golgi complex by addition of GalNAc to the side chain of serine or threonine by a family of *N*-acetylgalactosaminyltransferases (Bennett et al., 2012). This structure, GalNAc- $\alpha$ -serine or threonine, is called the Tn-antigen and capping of this antigen by sialic acid ( $\alpha$ -(2,6)-linked sialic acid) by ST6GalNAc-I sialyltransferase enzyme generates sialyl-Tn-antigen (STn), which is commonly found in human cancers (Springer, 1984, Steen et al., 1998).

Further stepwise elongation of the GalNAc- $\alpha$ -serine or threonine is achieved by specific transferases to form 8 different mucin type core structures. The core 1 structure (Gal- $\beta$ -(1,3)-GalNAc) is generated by addition of Gal in  $\beta$ -(1,3) linkage to the Tn structure. This reaction is catalysed by core 1  $\beta$ 3-galactosyltransferase enzyme (C1  $\beta$ 3-GalT). The C1  $\beta$ 3-GalT is a unique enzyme as it requires expression of an ER resident molecular chaperone termed as Cosmc, a C1  $\beta$ 3-GalT specific molecular chaperone. The Cosmc specifically assists in C1  $\beta$ 3-GalT folding events. Studies have demonstrated that loss of Cosmc function results in expression cancer antigens (Tn and STn) (Aryal et al., 2010, Ju and Cummings, 2002, Ju et al., 2008, Wang et al., 2010).

The core 1 structure (Gal- $\beta$ -(1,3)-GalNAc) converted to core 2, by the addition of GlcNAc in  $\beta$ -(1,6) linkage to GalNAc, by core 2 GlcNAc transferase enzyme. The GlcNAc can be added to the Tn structure (GalNAc- $\alpha$ -serine or threonine) to generate core 3 structure (GlcNAc- $\beta$ -(1,3)-GalNAc) by the action of core 3 synthase enzyme. The core 3 structure can also be modified by addition of GlcNAc in  $\beta$ -(1,6) linkage to GalNAc to produce core 4 structure. Core 2 and core 4 GlcNAc containing structures can be extended further into type 1 *N*-acetylglucosamine (Gal- $\beta$ -(1,3)-GlcNAc) by  $\beta$ -(1,3)-GalT enzymes or to type 2 *N*-acetylglucosamine (Gal- $\beta$ -(1,4)-GlcNAc) by  $\beta$ -(1,4)-GalT enzymes.

The core 3 structure is extended by addition of galactose in  $\beta$ -(1,4) linkage by  $\beta$ -(1,4)-GalT enzyme. Finally the *O*-linked glycans can be terminated by the addition of sialic acid by ST3Gal and ST6Gal sialyltransferases to generate  $\alpha$ -(2,3) and  $\alpha$ -(2,6)-linkages or can be linked to fucose in  $\alpha$ -(1,2), by  $\alpha$ -(1,2)-fucosyl transferases enzyme. Core 5 to 8 have been identified on human tissues but the enzymatic machinery for their synthesis remains elusive (Steen et al., 1998, Stanley et al., 2009, Gill et al., 2011, Tarp and Clausen, 2008, Hanisch et al., 1993b). Figure 1.7 shows different core types identified to date.



**Figure 1.7** *O*-linked glycan core structures. All structures made using CFG notations.

#### 1.4 Decoding glycosylation *in vivo*

Glycans on cell surfaces potentially store a large amount of information (Rabinovich and Croci, 2012). This information is deciphered endogenously by a family of glycan binding proteins termed as lectins. The term lectin is derived from the Latin word *legere* meaning “to choose” or “select”, and has been used for proteins that bind to glycans without catalysing any glycan modification (Ghazarian et al., 2011, Stanley et al., 2009). Lectins bind to carbohydrates using well defined carbohydrate

recognition domains (CRDs) (Sharon and Lis, 2004). The CRD of lectins recognises glycans in multiple presentations, and can differentiate between different structural forms of glycans (Mody et al., 1995, Ghazarian et al., 2011). Lectins from animals have highly conserved CRD regions and are classified in structurally related families (Sharon and Lis, 2004). The major types of lectins are: L-type, P-type, C-type, Galactins or S-type, I-type lectins and R-type lectins (Stanley et al., 2009). The L-type lectins have a common jelly roll fold, these lectins were identified in leguminous plants e.g. concanavalin A from *Canavalia ensiformis* (ConA) and phytohaemagglutinin E from soyabean (PHA-E). The P-type lectins are common in that they recognise mannose-6-phosphate. The C-type lectins are a family of  $\text{Ca}^{2+}$  dependent glycan binding proteins and have primary and secondary structural homology in their CRDs e.g. asialo-glycoprotein receptor from liver (Van Vliet et al., 2008, Stanley et al., 2009). Galactins, formerly known as S-type lectins, are  $\beta$ -galactose containing glycoconjugate binding lectins and share primary structural homology in their CRD. The I-type lectins are immunoglobulin like lectins e.g. Siglecs, and are sialic acid binding lectins. The members of R-type lectins have the CRD region similar to the plant lectin ricin (Stanley et al., 2009).

Various lectins from bacteria have been identified, which help in establishing adhesion and interaction with host cell surfaces to facilitate pathogenesis. Since the primary function of microbial lectins is to adhere to host cell surfaces, these lectins are termed as adhesins. Viruses are also well known for their glycan binding proteins called haemagglutinins, that mediate binding of viruses to the host cells (Esko and Sharon, 2009).

## **1.5 Biological role of glycans**

Every cell that interacts with other cells or the microenvironment cannot do so without interacting with the outermost glycan layer, the glycocalyx (Varki, 2011a). Most of the glycosylated proteins, intracellular or secreted, perform their functions in context of their glycosylation. Further, glycosylation is central to protein quality control and recycling systems in the ER (Ferris et al., 2014). Besides covering proteins, glycans are also involved in cell-cell interaction, in the generation of unique species specific identification, host pathogen interactions and immunological functions (Moon, 2012, Stanley et al., 2009).

### 1.5.1 Glycans in host-pathogen interactions

Glycans often serve as the first contact point for pathogenic organisms to interact with host cells (Hooper and Gordon, 2001). Binding to cell surfaces is a prerequisite for microbial pathogenesis. For this purpose, microbes have evolved with various lectins that can specifically recognise and bind to cell surface glycans to initiate colonisation and infection. Bacteria adhere to host cell surfaces using bacterial adhesins (bacterial lectins), that bind to their complementary glycans expressed on host cells (Sharon and Lis, 1989). The bacterial cell surface lectins are expressed as elongated multi-subunit proteins termed as fimbriae or pilli (Sharon, 2006). The bacterial lectins can bind to terminal glycans or to internal glycan residues. The glycan-adhesin binding interactions are weak but the high surface density of adhesins and glycans increases the avidity of interaction. Besides this, the bacteria expresses multiple adhesins with a range of specificities against different glycan targets expressed on multiple tissue types (Esko and Sharon, 2009).

The binding of *Helicobacter pylori* to the gastric mucosa is well characterised for its glycan mediated binding. This Gram-negative bacterium establishes adhesion to host cells by directly interacting with gastric epithelial glycans terminating with Lewis<sup>b</sup> (Le<sup>b</sup>) (a human blood group antigen) using blood group antigen binding adhesin (BabA) (Moran et al., 2011, Boren et al., 1993). In the gastric mucosa *H. pylori* can also adhere to mucus by employing the sialic acid binding adhesin (SabA) (Hooper and Gordon, 2001, Aspholm et al., 2006). This glycan mediated colonisation of *H. pylori* results in chronic active gastritis, peptic ulcers and gastric adenocarcinoma. Similarly, another Gram-negative bacterium *C. jejuni* also seems to interact with Le<sup>b</sup> and intestinal H-2 antigen. However, the lectins responsible for binding to human blood group antigens have not been identified (Juge, 2012).

Similar to bacteria, viruses also bind to cells for gaining entry to establish infection. The hemagglutinin protein, a viral glycan binding protein is the most studied and characterised for its binding to sialic acid containing glycans. The specificity of hemagglutinin for sialylated glycans is linkage-specific and varies across the different subtypes of influenza virus. For example the influenza strain A and B, which infects humans, bind to  $\alpha$ -(2,6) linked sialic acid (Skehel and Wiley, 2000), while influenza viruses that infect chickens bind to  $\alpha$ -(2,3) linked sialic acid (Shelton

et al., 2011). Noroviruses and rotaviruses have been demonstrated to bind to histo-blood group antigens expressed on the cell surface of the small intestine (Le Pendu et al., 2014). Rotavirus has been well characterised for its binding to sialic acid on cellular glycans *via* its lectin-like protein, VP4 (outer capsid protein of the virion) (Dormitzer et al., 2002a, Dormitzer et al., 2002b). A representative list of microbial lectins with their glycan ligands are shown in Table 1.1.

Various parasites are known to use glycans for adhesion. The adhesins expressed by *Plasmodium falciparum* are also known to bind to sialylated glycoproteins to gain entry into red blood cells. The binding of the parasite to Neu5Ac- $\alpha$ -(2,3)-Gal- $\beta$  glycan is mediated by a 175 kDa erythrocyte binding protein (Orlandi et al., 1992).

**Table 1.1** Microbial lectins and their glycan binding partners

Microbes	Lectin	Carbohydrate ligand	References
<i>Actinomyces naeslundii</i>	fimbriae	Gal- $\beta$ -(1,3)-GalNAc- $\beta$ -	(Brennan et al., 1987)
<i>Bordetella pertussis</i>	filamentous hemag- glutinin (FHA)	sulfated glycolipids; heparin	(Brennan et al., 1991)
<i>Escherichia coli</i>	P fimbriae	Gal- $\alpha$ -(1,4)-Gal- $\beta$ -	(Godaly and Svanborg, 2007)
<i>Escherichia coli</i>	S fimbriae	gangliosides GM3, GM2	(Prasadarao et al., 1993)
<i>Escherichia coli</i>	Type-1 fimbriae	Man- $\alpha$ -(1,3)-(Man- $\alpha$ -(1,6)-Man	(Cohen, 2015)
<i>Pseudomonas aeruginosa</i>	type IV pili	Asialo GM1 and GM2	(Comolli et al., 1999)
<b>Viruses</b>			
Influenza C	hemagglutinin-esterase	9- <i>O</i> -acetyl-Neu5Ac $\alpha$ -	(Zimmer et al., 1992)
Herpes simplex	glycoproteins gB, gC, gD	3- <i>O</i> -sulfated heparan sulfate	(Ali et al., 2012)
HIV	gp120 V3 loop	heparan sulfate	(Connell and Lortat-Jacob, 2013)

### 1.5.2 Glycans in the immune system

The immune system is a defense mechanism that is highly adaptable and generates a pathogen specific immune response. The fundamental ability of the immune system is to discriminate self from non-self. The immune system specifically eliminates pathogens bearing non-self determinants. However, some pathogens escape immune recognition by either masking non-self antigens or by mimicking self antigens through molecular mimicry. Pathological conditions can also arise where tolerance to self-antigens can be violated by altered presentation of self-antigens. The immune system continuously scans the body for danger signals and this immune surveillance will engage with the glycocalyx of cells to access the cellular information related to its health (Maverakis et al., 2015).

The adaptive immune system can perform self distinction by employing various lectins for recognition of self glycan patterns. The lectins such as siglecs, are mainly involved in recognition of sialic acids and are associated with self-associated molecular pattern recognition (Paulson et al., 2012). Siglecs are mainly involved in self-recognition, as they recognise terminal sialic acid in specific linkages as self antigen. The most well-known example is siglec 2 or CD 22 which is present on B cells. The siglec 2 on B cells preferentially recognises sialic acid with an  $\alpha$ -(2,6)-Gal linkage on *N*-acetyllactosamine present on glycoproteins and CD 22 itself to induce tolerance against self antigens (Paulson et al., 2012, Marth and Grewal, 2008). The siglecs are also known to mediate cell-cell adhesion events, which are very well characterised in leucocyte binding to vascular endothelium for leucocyte trafficking (Ohtsubo and Marth, 2006).

Similarly, glycan binding proteins like galectins, expressed on all immune cells, recognise specific glycan epitopes to modulate adaptive and innate immune responses, ranging from self tolerance to mounting a wide spread immune response against pathogens (Rabinovich and Toscano, 2009). The galactins bind to glycans containing terminal  $\beta$ -linked Gal, and are known for their regulatory role in the immune system. Proteins from this family are known to maintain T cell homeostasis by fine tuning T cell signaling and activation (Boscher et al., 2011). This ability of galectins to control the T cells is exploited by tumours to promote an immunosuppressive microenvironment to escape attack by the immune system

(Rabinovich and Toscano, 2009). Galactins can also recognise surface glycosylation on parasites, viruses, bacteria and fungi to activate specific immune response (Rabinovich and Toscano, 2009).

From the previous discussion pertaining to role of glycan interactions in self recognition, and in regulation of immunological status by inducing tolerance against self antigens, it makes sense that any aberrant glycan synthesis and display can lead to evasion of regulatory mechanisms leading to autoimmune reaction. In autoimmune diseases like Tn syndrome, IgA neuropathy and Henoch–Schonlein purpura, altered self-antigens are generated due to improper folding of enzymes associated with glycan attachment (Ju and Cummings, 2005). Further, the exposure of otherwise shielded cryptic glycan epitopes can be recognised as non-self and results in induction of inflammatory processes, this is consistent with the observation that exposure of cryptic mannose in  $\alpha$ -mannosidase-II deficient mice induces an autoimmune syndrome similar to systemic lupus erythematosus (Green et al., 2007). In a similar instance, inflammation in rheumatoid arthritis is aggravated by activation of alternative complement pathway by mannose binding lectin (MBL), which binds to IgG0 (lacking terminal galactose) glycoform that aberrantly expose internal mannose residues in the absence of complete glycosylation (Malhotra et al., 1995).

### **1.5.3 Glycans in diseases**

#### **1.5.3.1 Congenital diseases of glycosylation**

The diseases related to multi-system, defective synthesis of glycoconjugates were first described under the umbrella of carbohydrate deficient glycoprotein syndromes and were later renamed as congenital disorders of glycosylation (CDG) (Jaeken and Carchon, 2000). Since the first clinical description of CDG in 1980, 100 disorders have been associated with defective glycan synthesis or glycosylation, with clinical scope ranging from a normal phenotype to multiple organ dysfunction leading to infantile lethality (Hennet and Cabalzar, 2015, Li et al., 2015). Within CDG, the genetic defects can severely affect the *N*-linked glycosylation of lysosomal proteins either due to failed assembly of lipid linked oligosaccharide in type 1 CDG or altered processing events in ER and Golgi complex in CDG type 2 (Freeze, 2001). Most CDGs lead to multisystem disorders that most frequently involve the central nervous

system (CNS) (Freeze et al., 2015).

### 1.5.3.2 Cancer

Most of the cell surface receptors responsible for growth and development, are glycosylated with glycans playing important roles in mediating key signalling events that regulate normal growth of cells (Vasconcelos-dos-Santos et al., 2015). The changes in normal glycosylation patterns have been associated with cancer, with some specific glycan phenotypes potentially found to promote migration and subsequent metastasis in advanced stages (Hakomori, 1985, Häuselmann and Borsig, 2014). In various cancers, the glycan markers and aberrant phenotypes have been identified (Table 1.2). Changes of glycosylation in the tumour microenvironment permit malignant cells to enhance mobility, cell adhesion and receptor activation, thereby contributing to an invasive phenotype (Kannagi, 1997, Fuster and Esko, 2005). Hakomori and Kannagi were the first to propose that incomplete synthesis or neo-synthetic process produce the aberrant glycan phenotypes associated with cancer (Hakomori and Kannagi, 1983). Synthesis of incomplete glycan structures like Thomsen-Friedenreich (Tf) and Tn structures are observed in various cancer types including breast cancer and colonic adenocarcinoma (Campbell et al., 1995, Kumar et al., 2005, Springer, 1984). Another common feature of malignant cells is the expression of highly branched *N*-glycans, which is termed as Warren-Glick phenomenon, that seems to be highly correlated to metastatic potential of tumour cells (Kobata, 1989). Oncogenesis seems to highly favour up-regulation of branched *N*-glycans and alterations of *N*-linked glycan structures have been identified in many human cancers including breast, hepatocellular carcinoma, pancreatic and prostate cancers (Vasconcelos-dos-Santos et al., 2015). The increased  $\beta$ -(1,6) branching of *N*-glycans in metastatic cancer is attributed to enhanced activity of  $\beta$ -(1,6)-*N*-acetylglucosaminyltransferase-5 (GnT5) (Dennis et al., 1987). The absence of activity of GnT5 impairs tumour growth and metastasis, as observed in mouse models incapable of producing GnT5 (Granovsky et al., 2000).

Increased  $\alpha$ -(2,6) sialylation due to overexpression of sialyltransferases, ST6Gal1 and ST6GalNac is also commonly observed in cancer (Marcos et al., 2011, Seales et al., 2005). The more advanced metastatic cancers also show *de novo* expression of sialyl Lewis a (Sle<sup>a</sup>) and sialyl Lewis x (Sle<sup>x</sup>), both these determinants are highly



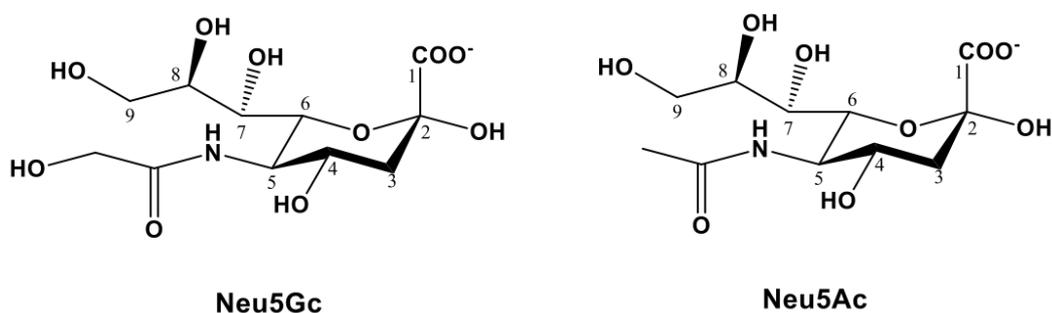
**Table 1.2** Glycans markers associated with various cancer.

<b>Cancer Type</b>	<b>Glycan biomarkers</b>	<b>References</b>
<b>Pancreatic cancer</b>	Sialyl-Le <sup>A</sup> , Rnase 1 with sialyl-Le <sup>x</sup> and sialyl-Le <sup>A</sup> forms fucosylated with complex bianttenary structures.	(Ballehaninna and Chamberlain, 2012, Peracaula et al., 2003a)
<b>Ovarian cancer</b>	Core 1 and core 2 type <i>O</i> -linked glycan, bi, tri and tetranntenary glycans with polylactosamine antennae endcapped with sialic acid. Sialyl-Le <sup>x</sup> epitope present on both <i>N</i> and <i>O</i> -linked glycans, mucin 1 with core 3.	(Wong et al., 2003, Wandall et al., 2010)
<b>Prostate cancer</b>	PSA being principal marker with loss of sialylation and contain fucosylated-GalNac, $\beta$ -(1,4)-GalNac-, MUC 1 with sialyl-Tn and core3.	(Peracaula et al., 2003b, Wandall et al., 2010)
<b>Liver cancer</b>	AFP with increased fucosylation and bi- and tri-anttenary structures, haptoglobin with increased fucosylation.	(Comunale et al., 2006, Kirwan et al., 2015)
<b>Breast cancer</b>	MUC1 with Tn, sialyl Tn, core 3 structures, <i>N</i> -linked glycans with trianttenary structures bearing increased sialyl- Le <sup>x</sup> expression.	(Kurian et al., 2008, Cazet et al., 2010, Zamagni et al., 1991)

## 1.6 Sialic acids

Sialic acids occupy the terminal positions on membrane bound and secreted glycoconjugates. Sialic acids are mediators of processes such as shielding pathogens from host recognition and self and non-self recognition (Varki and Gagneux, 2012, Jamieson et al., 1993, Varki, 2011b).

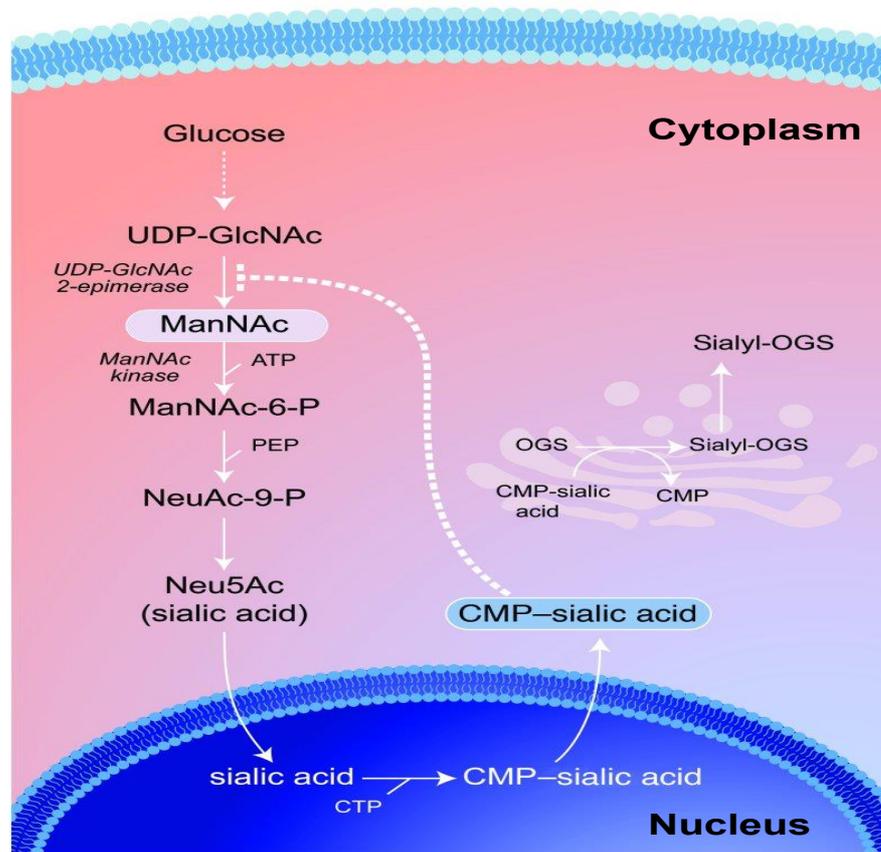
Sialic acids are  $\alpha$ -keto acid monosaccharides, made up of a 9-carbon backbone. Sialic acid was first isolated after cleavage of brain lipid (gangliosides) by Klenk in 1941 and then Blix isolated sialic acid from bovine submaxillary mucin (Blix et al., 1955, Klenk, 1941). The features of sialic acid are the presence of an amino group at C-5 and a carboxyl group at C-1 that presents a negative charge on the molecule at physiological conditions and makes it a strong organic acid with a pKa of 2.2 (Schauer and Kamerling, 1997). The unsubstituted form of sialic acid, neuraminic acid, is not found in nature and most commonly the amino group is acetylated to form *N*-acetylneuraminic acid (Neu5Ac), which is the most widespread form of sialic acid and almost exclusive form of sialic acid present in humans, Figure 1.9. The substitution of the hydroxyl group at the acetyl group on C-5, forms *N*-glycolylneuraminic acid (Neu5Gc), which is found in many animal species but not found in humans, except in cancer (Schauer et al., 1995).



**Figure 1.9** Common sialic acids Neu5Ac and Neu5Gc. The Neu5Gc differ from Neu5Ac in having a OH group at acetamido group at C-5.

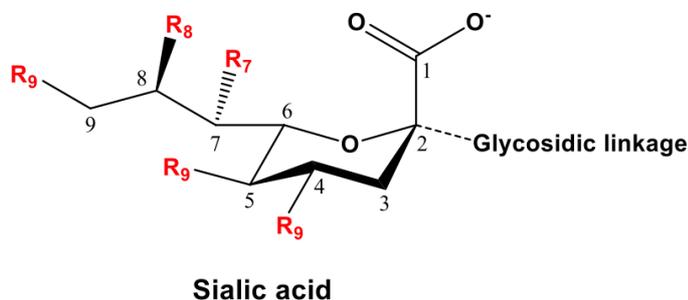
The biosynthesis of sialic acid spans across different cellular compartments within the cell. The synthesis starts in the cytosol by condensation of *N*-acetylmannosamine-6-phosphate (ManNAc-6-P) and phosphoenolpyruvate (PEP) to produce Neu5Ac-9-phosphate. The precursor of this reaction ManNAc-6-P is

produced from UDP-GlcNAc by a bifunctional enzyme UDP-*N*-acetylglucosamine-2-epimerase/*N*-acetylmannosamine kinase. The Neu5Ac-9-phosphate is dephosphorylated and transferred to the nucleus for activation. The activation takes place by transfer of cytidine monophosphate (CMP) from cytidine triphosphate (CTP) to form CMP-sialic acid (Figure. 1.10). In glycoconjugates sialic acid is always  $\alpha$ -linked but CMP-sialic acid is the only molecule known where the sialic acid linkage is  $\beta$ . The activated sialic acid is then transferred to the Golgi complex or the ER for attachment to oligosaccharides. Sialic acid can be modified after its attachment to oligosaccharides. In the cytosol, Neu5Ac linked to CMP can be hydroxylated to form Neu5Gc-CMP by the action of CMP-Neu5Ac hydroxylase enzyme, encoded by the *CMAH* gene (Schauer, 1991, Varki and Schauer, 2009).



**Figure 1.10** Sialic acid biosynthetic pathway. The synthesis starts with the conversion of glucose to UDP-GlcNAc. Enzyme UDP-GlcNAc 2-epimerase converts the UDP-GlcNAc to ManNAc, which then condenses with PEP to form NeuAc-9-P. The phosphate group is then removed and Neu5Ac is then translocated to nucleus for activation. The activated Neu5Ac is then translocated to the Golgi complex for attachment to oligosaccharides. Image reproduced with permission from American Society for Clinical Investigation (Galeano et al., 2007).

Sialic acids are amongst the most highly diverse sugars in nature, with more than 50 different forms. The most common form of structural variations in natural sialic acid occur at C-5, which can be substituted with an acetamido, hydroxyacetamido or hydroxyl group to form *N*-acetylneuraminic acid, *N*-glycolylneuraminic acid or deaminoneuraminic acid (KDN) respectively (Schauer and Kamerling, 1997). Mucins from human colon and bovine submandibular gland express di or tri-*O*-acetylated sialic acids. Certain sialic acid modifications have restricted expression, for example, 9-*O*-acetylation in gangliosides is restricted to the terminal  $\alpha$ -(2,8)-linked sialic acid (Varki, 1992). Further diversity arises by substitution of methyl, lactyl, phosphate and sulphate at the hydroxyl groups of Neu5Ac, Neu5Gc and KDN (Varki, 1992). Besides structural diversity, additional variety in sialic acids is generated by its attachment via a range of linkages to underlying sugars such as  $\alpha$ -(2,3) and  $\alpha$ -(2,6) to galactose,  $\alpha$ -(2,6)-linkage to GalNac,  $\alpha$ -(2,8) and  $\alpha$ -(2,9) to polysialic acid and on other hand modifications at the 4,5,7,8, and 9 positions within the backbone generates a variety of over 50 distinct sialic acids (Schauer, 2000, Angata and Varki, 2002), Figure 1.11.



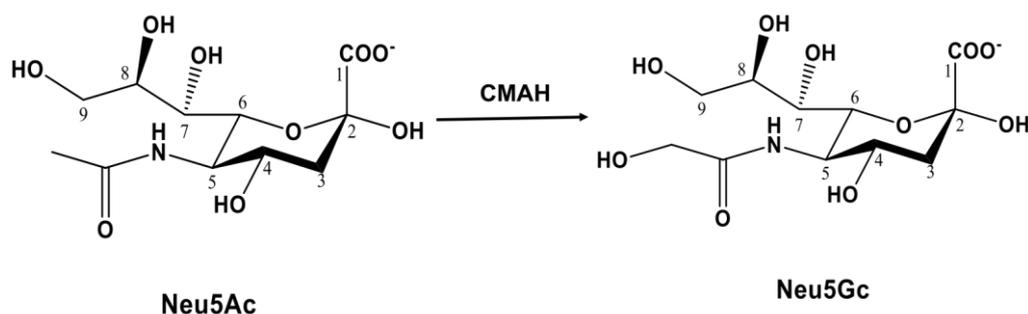
R	Substitutions
4	-OH, -OCOCH <sub>3</sub> ,
5	-OH, -NH <sub>2</sub> , -NHCOCH <sub>3</sub> , -NHCOCH <sub>2</sub> OH ,
7	-H, -OH, -OCOCH <sub>3</sub> ,
8	-H, -OH, -CH <sub>3</sub> , -OCOCH <sub>3</sub> , HSO <sub>4</sub> <sup>-2</sup> ,
9	-OH, -OCOCH <sub>3</sub> , HSO <sub>4</sub> <sup>-2</sup> , H <sub>2</sub> PO <sub>4</sub> <sup>-1</sup> , -OCOCH(OH)CH <sub>3</sub>

**Figure 1.11** Diversity of sialic acids. Substitutions at various sites generate different forms of sialic acids. The different forms of sialic acid are differentially expressed on various tissues.

### 1.6.1 Non-human sialic acid Neu5Gc

Neu5Ac and Neu5Gc are found most commonly in mammals. In most mammals except humans, Neu5Ac is converted to its hydroxylated version Neu5Gc by the enzyme cytidine monophosphate *N*-acetylneuraminic acid hydroxylase (CMAH), Figure 1.12. Humans lost the ability to synthesise Neu5Gc due to the inactivation of the gene for AMP-Neu5Ac hydroxylase (Varki, 2007, Varki, 2001). The human enzyme lacks the *N*-terminal 104 amino acids due to a deletion of a 92 base pair exon (Irie et al., 1998). In fact, humans have a population of circulating anti-Neu5Gc antibodies (Samraj et al., 2014). It was demonstrated that circulating anti-Neu5Gc antibodies in humans can activate complement associated cytotoxicity in cells expressing high levels of Neu5Gc (Padler-Karavani et al., 2008, Nguyen et al., 2005). However, studies have shown the presence of Neu5Gc in normal as well as diseased human tissues (Samraj et al., 2014).

Experimental observations in Neu5Gc knockout mice have suggested that Neu5Gc accumulated from a Neu5Gc-rich diet can be converted to CMP-Neu5Gc that in turn acts as substrate for sialyltransferases to allow incorporation of Neu5Gc into glycoproteins (Tangvoranuntakul et al., 2003, Hedlund et al., 2007). It has also been observed that human embryonic stem cells growing on mouse embryonic fibroblast feeder layers when exposed to human serum undergo complement fixation (Martin et al., 2005), indicating the incorporation of a non-human antigen accumulated from the components of growth media. At a mechanistic level, Neu5Gc incorporation into glycoproteins in the human colorectal adenocarcinoma cell (Caco-2) was shown to occur by uptake of Neu5Gc by micropinocytosis. This was followed by lysosomal uptake and delivery to the cytosol. Neu5Gc was then translocated to the nucleus for converting to CMP-Neu5Gc (Bardor et al., 2005). However, other mechanisms for the generation of Neu5Gc have been proposed by de-acylation and re-acylation of sialic acids, but a clear cut demonstration for Neu5Gc synthesis in humans is lacking (Varki, 2001).



**Figure 1.12** Synthesis of Neu5Gc from Neu5Ac. The Neu5Gc is synthesised from Neu5Ac by the action of enzyme cytidine monophosphate *N*-acetylneuraminic acid hydroxylase (CMAH). This CMAH enzyme is not functional in humans.

### 1.6.1.1 Neu5Gc in inflammation

First identified as Hanganutziu Deicher (HD) antigen in patients with serum sickness, Neu5Gc was eventually recognised as an immunogenic antigen conjugated to gangliosides in humans (Higashi et al., 1977, Merrick et al., 1978, Ezzelarab et al., 2005). Similarly, Paul-Bunnell antibodies in infectious mononucleosis were also reported to bind to Neu5Gc attached to glycoconjugates (Padler-Karavani et al., 2008). Further studies demonstrated that anti-Neu5Gc antibodies are generated in the first year of infants life in humans, by exposure to dietary acquired Neu5Gc and also by presentation of dietary Neu5Gc by commensal bacteria, non-typeable *Haemophilus influenzae* (Taylor et al., 2010). The metabolic incorporation of Neu5Gc can also serve as a ligand for pathogens which otherwise cannot infect humans. For example subtilase cytotoxin from Shiga toxic *E. coli* binds specifically to glycans terminating with Neu5Gc. This was demonstrated by mutation in the subtilase binding site that abrogated binding to Neu5Gc *in vitro* (Byres et al., 2008). Recent studies also suggest the possible involvement of Neu5Gc in autoimmune diseases like autoimmune hypothyroidism, Hashimoto's thyroiditis (Eleftheriou et al., 2014).

Neu5Gc being immunogenic in humans, can potentially lead to xenograft rejection. Neu5Gc mediated rejection has been demonstrated in CMAH knockout mouse, where the allotransplant of islets, showed transplant rejection that was mediated by anti-Neu5Gc antibodies (Padler- Karavani and Varki, 2011). However, the studies conducted in humans did not demonstrate rejection but showed a sustained anti-

Neu5Gc response. This was demonstrated in severely burnt human subjects, where the patients receiving dressings of live pig skin showed increased titres of anti-Neu5Gc antibodies in sera that was tested 10 years after the dressing, as compared to burnt patients who did not receive the dressing (Salama et al., 2015). This study though did not show evidence of rejection but clearly highlights the necessity of detailed investigation required for assessing the long term effect of sustained Neu5Gc exposure.

### **1.6.1.2 Neu5Gc in cancer**

Despite the fact that humans cannot synthesise Neu5Gc, various normal human tissues and cancers show the presence of this molecule (Malykh et al., 2001). The ganglioside, Neu5Gc-GM3, has been reported to be present in colon carcinoma, and is a known tumour associated antigen in breast cancer (Higashi et al., 1985, Marquina et al., 1996). The presence of Neu5Gc has been reported in various other types of cancers including breast cancer, lung cancer, melanomas and retinoblastoma (Malykh et al., 2001). Intriguingly, in a phase I trial on advanced breast cancer patients, it was demonstrated that vaccination of Neu5Gc-GM3 with the outer membrane protein complex of *Neisseria meningitidis* in proteoliposomes induced anti-Neu5Gc-GM3 antibody production. These antibodies demonstrated differential reactivity against mammary ductal carcinoma samples (Carr et al., 2003).

Although the role of Neu5Gc in cancer is not clear, it is hypothesised that display of Neu5Gc on cancer cell surfaces induces a weak immune response, which may not be sufficient to eliminate cancer cells, but may promote cancer by antibody mediated inflammation and angiogenesis, thus facilitating tumour invasion and metastasis (Padler-Karavani et al., 2011, Samraj et al., 2014). Recently, studies in Neu5Gc deficient (CMAH knockout) mice injected with anti-Neu5Gc antibodies demonstrated that anti-Neu5Gc antibodies interact with metabolically incorporated Neu5Gc derived from dietary intake to promote inflammation in a dose dependent manner. Further, this long term inflammation promoted carcinomas in mouse liver (Samraj et al., 2015). Previously, *in vitro* studies in mouse leukaemia cell line, L1210, showed that tumour development was impaired after shifting expression of sialic acid from Neu5Gc type to Neu5Ac (Casadesús et al., 2013). However, the study demonstrated the effect in cell lines, the effect in *in vivo* models is yet to be

determined. Moreover, extensive studies will be required to establish the role of Neu5Gc in cancer, specially in terms of establishing the cause and effect model.

### **1.6.1.3 Neu5Gc on therapeutic proteins**

The major biopharmaceutical drugs are recombinant glycoproteins. The glycan component of the therapeutic proteins, especially sialic acids, increases the circulating half-life and safety of drugs for human use (Byrne et al., 2007). The presence of sialic acid on the therapeutic proteins have outstanding impact on the stability and efficacy of the drug. The commercial production processes express high yields of therapeutic glycoproteins, which leads to production of therapeutic glycoproteins with inconsistent sialylation. Incomplete sialylation exposes the underlying glycans such as Gal, which are specifically recognised by the liver asialogal receptor and removed from the circulation (Morell et al., 1971). In addition to inadequate sialylation, the type of sialic acid also determines the quality and serum half-life of the drug. The majority of biopharmaceutical drugs are produced in non-human cell lines like CHO, NS0 and SP2/0 cells, which may lead to non-human glycosylation of therapeutic glycoproteins (Ghaderi et al., 2012). In early studies, the presence of Neu5Gc in erythropoietin (used for the treatment of anaemia) was reported, however it was shown that the presence of Neu5Gc had a weak immunogenic effect (Noguchi et al., 1995). But later it was suggested that, in humans with high levels of circulating anti-Neu5Gc antibodies, Neu5Gc can be neutralised and removed from circulation (Nguyen et al., 2005). This was demonstrated in recent studies in mouse administered with Cetuximab, where presence of Neu5Gc on the drug induced immune complexes *in vitro* that lead to reduced half-life of drug (Ghaderi et al., 2010). Although, the study is insufficient to explain the pharmacokinetics behaviour of Neu5Gc containing drugs in a human context, nevertheless, the study indicated towards the role of Neu5Gc in rapid drug clearance and paves the way for detailed future investigations. In accordance with regulatory guideline for the quality of biopharmaceutical drugs, ICH(Q)6B, determination of sialic acids (Neu5Ac and Neu5Gc) in biopharmaceutical preparation is required to demonstrate the quality attribute of drugs.

## 1.6.2 Detection of sialic acids

### 1.6.2.1 Sialic acid recognition using lectins

Various lectins from plants, mammals, bacteria, viruses and fungi are known to recognise sialic acids (Table 1.3). Some lectins from plant sources recognise sialic acids in various but specific linkages, for example *Sambucus nigra* agglutinin (SNA) recognises sialic acid in  $\alpha$ -(2,6) linkage to Gal and GalNAc (Shibuya et al., 1987). The lectins isolated from *Maackia amurensis* recognise sialic acid in  $\alpha$ -(2,3) linkage to Gal (Wang and Cummings, 1988). Another plant lectin, *Limax flavus* agglutinin, recognise multiple sialic acid forms (Knibbs et al., 1993).

Lectins from mammals, like selectins, bind to sialic acids with specific linkages to underlying sugars, yet another family of lectins; siglecs bind to different forms of sialic acids not only by virtue of their linkage to other sugars but also by side chains at carbon 7, 8 and 9 and an acetamido group at C-5 (Varki, 2007). Bacterial strains like *E. coli* which express S-fimbriae binds to Neu5Gc- $\alpha$ -(2,3)-Gal and to Neu5Ac- $\alpha$ -(2,8)-Neu5Ac structures, yet another bacteria *H. pylori* utilises lectin SabA to bind to  $\alpha$ -(2,3)-linked sialic acids irrespective of underlying glycan (Lehmann et al., 2006, Mahdavi et al., 2002). Sialic acid dependent interactions similar to those observed with lectins, have also been reported with fungal strains like *H. capsulatum* and *A. fumigatus*, but details regarding the structural specificity is not established (Lehmann et al., 2006). Various viruses like human influenza A virus, avian influenza virus, corona and reoviruses are now well known for their sialic acid specific binding for establishing infection and tissue tropism (Lehmann et al., 2006).

For assay development, lectins have been used for recognition of glycan features on tissues, organelles, gels, blots and in thin layer chromatography (Gemeiner et al., 2009, Sharon and Lis, 2003). Lectins have been used to develop binding assays in an ELLA (Enzyme Linked Lectin Assay) format in microtiter plates (Lambré et al., 1991). For high throughput analysis, the lectin microarray has emerged as a prominent technology platform for glycoprofiling (Gemeiner et al., 2009). The lectin microarray has been employed for elucidation of glycan features on bacteria, where clear differences in glycan patterns were observed in pathogenic and non-pathogenic bacteria (Hsu and Mahal, 2006). In lectin arrays, a panel of lectins immobilised onto

a chip are exposed to a pool of labelled glycoconjugates. The subsequent binding events are then detected by scanner (Pilobello and Mahal, 2007). In a reverse phase lectin array, in which sialylated glycans were immobilised onto chips and lectins from plants and viruses were then used to probe different sialylated glycans to reveal novel interactions with modified sialic acids (Song et al., 2011). However, lectin arrays also require labelling, which generates a high degree of variability in analysis (Gemeiner et al., 2009).

The lectins from different sources recognise sialic acids in various presentations, however their use for assay development *ex vivo* is limited due to poor stability, less sensitivity and most of them show cross-reactivity with different forms and presentations of sialic acids (Haab, 2012, Lehmann et al., 2006). All these shortcomings make lectins unsuitable for assay development for recognition of sialic acids.

**Table 1.3** A selection of sialic acid recognising lectins

Species/Source	Lectin	Specificity/ligand	References
<b>Plants</b>			
<i>Maackia amurensis</i>	MAL	Neu5Ac- $\alpha$ -(2,3)-Gal- $\beta$ -(1,4)-GlcNAc	(Knibbs et al., 1991)
<i>Maackia amurensis</i>	MAH	Neu5Ac- $\alpha$ -(2,3)-Gal- $\beta$ -(1,3)-Neu5Ac- $\alpha$ -(2,6)-GalNAc	(Kanami et al., 1994)
<i>Sambucus nigra</i>	SNA	Neu5Ac- $\alpha$ -(2,6)-Gal	(Shibuya et al., 1987)
<i>Sambucus canadensis</i>	SCA	Neu5Ac- $\alpha$ -(2,6)-Gal	(Shibuya et al., 1989)
<b>Gram-negative</b>			
<i>Escherichia coli</i>	SfaI, II, SFaS	Neu5Gc- $\alpha$ -(2,3)-Gal; Neu5Ac- $\alpha$ -(2,8)-Neu5Ac	(Hanisch et al., 1993a)
<i>Helicobacter pylori</i>	SabA	Sia- $\alpha$ -(2,3)	(Roche et al., 2004)
<i>Haemophilus influenzae</i>	HMW1	Sia- $\alpha$ -(2,3)	(St Geme, 1994)
<i>Neisseria subflava</i>	Sia-1	Neu5Ac- $\alpha$ -(2,3)-Gal- $\beta$ -(1,4)-Glc	(Nyberg et al., 1990)
<b>Mammals</b>			
bovine	Calcyclin	Neu5Gc	(Brinck et al., 1995)
ovine	Calreticulin	Neu5Gc, Neu5Ac	(Iglesias et al., 1996)
Macrophage	Siglec-1 (sialoadhesin)	Neu5Ac- $\alpha$ -(2,3)-Gal > Neu5Ac- $\alpha$ -(2,6)-Gal > Neu5Ac- $\alpha$ -(2,8)-Neu5Ac	(Varki and Angata, 2006, Crocker, 2005)
B-cell	Siglec-2 (CD22)	Sia- $\alpha$ -(2,6)-Gal	
Monocyte, Macrophage	Siglec-3 (CD33)	Sia- $\alpha$ -(2,6)-Gal > Sia- $\alpha$ -(2,3)-Gal	
<b>Fungi</b>			
<i>Hericium erinaceum</i>	HEL	Neu5Gc > Neu5Ac	(Kawagishi et al., 1994)
<i>Psathyrella vetutina</i>	PVL	Neu5Ac- $\alpha$ -(2,3)-Gal- $\beta$ -(1,4)-GlcNAc <sub>2</sub>	(Ueda et al., 2002)
<i>Aspergillus fumigatus</i>	HA-A	Neu5Ac- $\alpha$ -(2,6)-GalNAc? /laminin, fibronectin, fibrinogen, collagen	(Tronchin et al., 2002, Wasylnka and Moore, 2000)
<b>Viruses</b>			
Human influenza	HA	Neu5Ac- $\alpha$ -(2,6)-Gal	(Suzuki, 2005)
Avian influenza	HA	Neu5Ac- $\alpha$ -(2,3)-Gal	
Porcine rotavirus group A OSU	VP4	Neu5Gc-GM3 $\geq$ Neu5Ac-GM3	(Rolsma et al., 1998)
Rhesus rotavirus	VP4	Neu5Ac >> Neu5Gc	(Dormitzer et al., 2002a)

### 1.6.2.2 Chromatography and mass spectrometry

In chromatography-based methods, molecules or compounds are separated based on differential movement through the stationary phase. The differential movement is governed by the charge, molecular mass and polarity of the molecules. Various chromatography methods have been employed for the detection of sialic acids.

Sialic acids were first detected by employing thin layer chromatography followed by colourimetric analysis (Schauer and Corfield, 1982). Since then significant advancements have been made in detection of sialic acids by chromatography. A variety of detection approaches have now been developed for the detection of sialic acids by High Performance Liquid Chromatography (HPLC). In fluorimetric detection approach, sialic acid released by acid hydrolysis is labelled with an  $\alpha$ -keto acid specific fluorogenic reagent, 1,2-diamino-4,5-dimethoxybenzene (DDB), the labelled sialic acid is then separated using reverse phase-HPLC and detected by fluorescent detection (Hara et al., 1986). The sialic acid released from glycoconjugates can also be hydrolysed and per-*o*-benzoylated and separated on RP-HPLC and detected by UV absorbance (Karamanos et al., 1990). In an alternative method, a high throughput, high pH anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD) is used to detect unlabelled sialic acids, released by acid hydrolysis or enzymatic treatment (Hurum and Rohrer, 2011, Chemmalil et al., 2015).

Liquid chromatography coupled with mass spectrometry (LC-MS) is another methodology that has been employed for detection of sialic acids attached to gangliosides (Zarei et al., 2010). A label-free approach for sialic acid has been reported wherein non-derivatised sialic acid separated by hydrophilic interaction chromatography is detected by nano quantity analyte detector (NQAD) (Chemmalil et al., 2015). In MS-based methods, the sialylated glycans can be derivatised with *p*-toluidine and then detected by MADLI-TOF (Shah et al., 2013). Gas chromatography coupled with MS (GC-MS) has been employed to detect different structures of sialic acids from different tissues and fluids. For GC-MS analysis, sialic acid released post-acid treatment is esterified and converted to a volatile derivative using heptafluorobutyric anhydride and subsequently analysed by GC-MS (Zanetta et al., 2001).

Despite the sophistication of chromatography and MS methods, these techniques have their disadvantages. In LC-based methods, the release and derivatisation of sialic acids is variable and cannot differentiate sialic acid linkages to glycoconjugates (Chemmalil et al., 2015). MS-based methods also require derivatisation of sialic acids and the labile nature of sialic acid contributes to the loss of signal in sialylated glycans (Shah et al., 2013, Saba et al.).

### **1.6.2.3 Antibodies for Neu5Gc detection**

Recently, antibody based detection methods have been developed for Neu5Gc detection. Polyclonal anti-Neu5Gc chicken IgY preparations (Fujii et al., 1982), monoclonal anti-Neu5Gc chicken IgY (Asaoka et al., 1992), monoclonal anti-Neu5Gc developed in mouse (Sanai et al., 1988) and monoclonal anti-Neu5Gc human IgM and IgG (Nakamura et al., 1995) have been developed and some of them are commercialised for detection of Neu5Gc. However, all antibody based Neu5Gc detection methods derive anti-Neu5Gc antibodies from animal serum, which, from a production and quality point of view, are highly inconsistent in terms of lot-to-lot variation and there is no guarantee for generating same affinity antibody consistently. Other major drawbacks for serum derived anti-Neu5Gc antibody is that such antibodies recognise specific presentation of sialic acids in samples. In the same context a more recent monoclonal humanised anti-Neu5Gc-GM3 antibody, racotumomab (trade name Vaxira) is undergoing clinical trial for non-small cell lung cancer this antibody specifically recognises Neu5Gc-GM3 displaying cells and induces strong anti-metastatic effect (Vazquez et al., 2012). However, this antibody can recognise Neu5Gc in context of its attachment to gangliosides and sulfatides and therefore may not be useful for detection of other Neu5Gc presentations.

From the review of technologies and tools for detection of sialic acids, it is clearly evident that specific detection of Neu5Gc is beyond the scope of existing technologies. The HPLC based methods can specifically detect Neu5Gc, however extensive sample preparation, labelling steps and requirement of highly specialised instruments with expert operators for instrument handling and analysis makes this technique unsuitable for convenient analysis. A need therefore exists for development of antibody based recognition molecules that can specifically recognise Neu5Gc. These anti-Neu5Gc recognition molecules can be used to develop

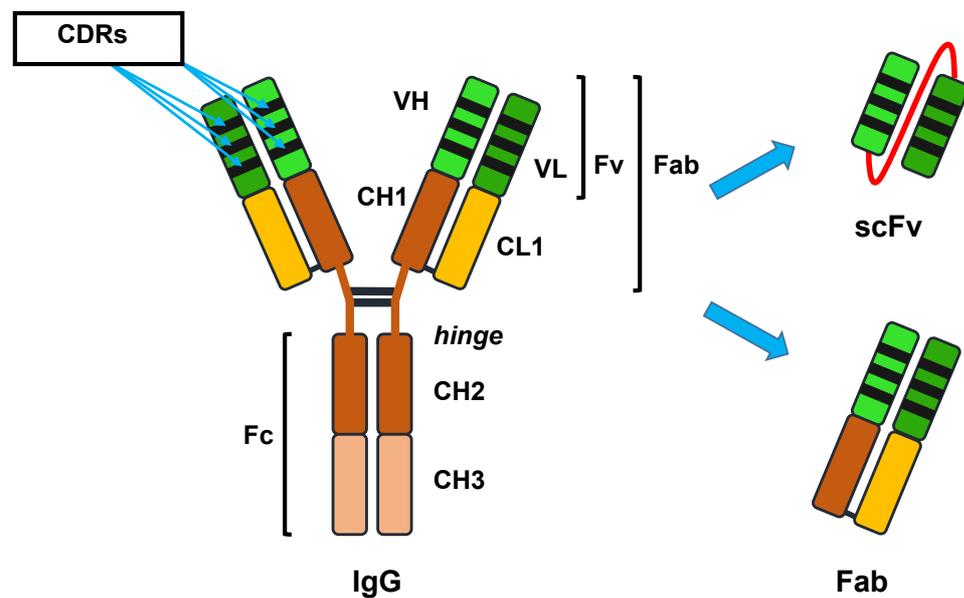
convenient ELISA based assays for detection and quantitation of Neu5Gc in various sample types. Further, these molecules can be used to develop applications for immunohistochemical analysis of tissues.

### **1.7 Antibody structure and antibody formats**

Antibodies are glycoproteins produced in response to foreign antigens by the host immune system. This property of the immune system is exploited to generate highly specific antibodies against an antigen of interest by immunising the host with the antigen. These antibodies can then be used to develop highly sensitive immunoassays for detection of antigen (Siddiqui, 2010). Antibodies produced by the immune system elicit two different responses upon encountering an antigen, first specific recognition and binding to antigen followed by stimulation of cytotoxic events to eliminate antigen displaying cells or organisms. Antibodies have two distinct regions, the highly variable amino-terminal region and a constant carboxy-terminal region (Janeway et al., 2001). The amino-terminal of the antibody facilitates recognition and binding events while the carboxy-terminal region mediates the effector function of antibody. The effector function includes complement dependent cytotoxicity and antibody dependent cellular cytotoxicity. The antibodies have a heterotetramer Y shaped structure consisting of two identical light chains and two identical heavy chains connected by disulfide bridges (Janeway et al., 2001). The light chain has a molecular weight of approximately 25 kDa and heavy chain approximately 50 kDa. The antigen binding variable region consists of both heavy and light chains. The light chain contains one variable and one constant (CL) region while heavy chains have three constant (CH1, CH2 and CH3) and one variable region (Janeway et al., 2001). The variable regions of both light and heavy chains are divided into hypervariable and framework regions. The variable region in heavy and light chain contains three hypervariable regions, which are flanked by framework regions. Together, the three hypervariable regions form the complementarity determining region (CDR) (Wu and Kabat, 1970, Griffiths et al., 1993). The CDR region is mainly responsible for antigen recognition and binding of antigen.

With the advancements in recombinant DNA technology, specially engineered antibodies fragments have been produced for immunoassay applications. Instead of full length antibodies, recombinant antibody fragment molecules have been widely

adopted for therapeutic and diagnostic applications due to their small size, better tissue penetration and cost effective production (Ahmad et al., 2012). Fragment antigen binding (Fab) and short chain fragment variable (scFv) are more commonly used and are precursors for generating more complex antibody fragments (Nelson, 2010). The Fab fragment consists of a variable and constant region of both heavy and light chain and has a molecular weight of approximately 50 kDa. The scFv is a recombinant antibody fragment and consists of variable heavy and light chains connected by a peptide linker (Ahmad et al., 2012), Figure 1.13.



**Figure 1.13** Antibody and antibody fragments. The typical Y shaped antibody is divided into fragment crystallisable (Fc) and Fab region. The variable light and heavy chain contain CDR regions for antigen binding. The Fab fragment consists of light and heavy chain variable region (VH and VL) and constant region (CH1 and CH2). The scFv fragment is only made up of variable light and heavy chain region connected by a peptide linker. Image adapted from (Nelson, 2010).

Houston and Bird first constructed scFvs independently in 1988 (Huston et al., 1988, Bird et al., 1988). The scFv was developed from a murine hybridoma cell line where it was demonstrated that the derived scFv retained the affinity of parent antibody (Bird et al., 1988). The linker region of scFv consists of usually 10 to 20 amino acids and is composed of hydrophilic amino acids to avoid intercalation of peptides during folding of scFv (Shen et al., 2008, Ahmad et al., 2012). The length of the linker has an effect on the folding of scFv. The scFvs with linker length of 12 or more amino

acids folds properly and forms a monomeric scFv (Todorovska et al., 2001). Reduction of linker length to less than 12 does not allow the scFv to fold, the unfolded scFv can associate to form multimeric scFv with two or more scFv associating together to form diabody with two scFv and tribodies with three scFv and tetrabodies with four scFvs (Todorovska et al., 2001). The scFvs are highly malleable and can be used to generate different scFv combinations. The bi-specific scFv for example is a combination of two scFvs, each having specificity for different antigens. Similarly, more scFvs with different specificities for multiple antigens can be joined to form multi-specific scFv (Weisser and Hall, 2009). Recently, bi-specific scFv with the Fc portion of antibody have been constructed, these scFv have demonstrated targeted reduction of tumour cell growth *in vivo* (Schneider et al., 2005, Weisser and Hall, 2009). The scFv has also been used for development of sensors for detecting contamination of uranium in ground water (Zhu et al., 2011). The scFv fragments, conjugated to various molecular probes, cytotoxic drugs, peptides radionuclides and liposomes have been developed for targeted drug delivery to applications in tumour cell imaging (Wu and Senter, 2005).

### **1.8 Phage display for construction and isolation of anti-glycan scFv**

The first demonstration of generation and selection of scFv using phage display technology was reported in 1990 (McCafferty et al., 1990). Since then, scFvs generated using phage display technology have also found applications as lectin mimetics in glycobiology. In 1994, the phage display technology was successfully employed to produce anti-carbohydrate scFv against bacterial cell wall (Dennissen et al., 2002). This was followed by generation of scFvs against heparin sulphate and chondroitin sulphate (van Kuppevelt et al., 1998, Smetsers et al., 2004). For smaller antigens like Tn antigen (GalNAc- $\alpha$ -1-Ser/Thr), which is highly expressed in carcinomas, it is difficult to generate anti-Tn antibodies using hybridoma technology. Using phage display, anti-Tn scFv was identified and further engineered to generate anti-Tn scFv-Fc (Kubota et al., 2010, Fukuda, 2012). Further, an scFv specific for chondroitin sulphate proteoglycan 4 (CSPG4) was identified from human derived scFv-phage display library and engineered with human IgG Fc region to generate anti-CSPG4 scFv-Fc. The CSPG4, which is highly expressed on malignant cells was specifically targeted by anti-CSPG4 scFv-Fc in both *in vitro* and *in vivo* experiments

and demonstrated inhibition of tumour growth (Wang et al., 2011). Besides applications as potential therapeutic agents, lectin mimic scFvs derived from an immunised chicken scFv-phage display library were also used to develop convenient ELISA based assays to detect non-human glycan, Gal- $\alpha$ -(1,3)-Gal (Cunningham et al., 2012).

### **1.8.1 Phage display**

Phage display is an *in vitro* selection process, wherein DNA encoding the protein of interest is fused with the coat proteins of bacteriophage, resulting in expression of fused protein on the surface of bacteriophage. George Smith reported this technology in 1985 by demonstrating the ability of bacteriophage to express and display foreign peptides on the surface of the phage particle (Smith, 1985). In phage display, each phage particle presents a single protein based on gene encoded within the phage and information within the gene is easily retrieved via direct linkage between genotype and associated phenotype. This provides an opportunity to select and amplify specific antibodies from a diverse collection of functional antibodies from a pool of phage, each displaying an unique antibody (Miersch and Sidhu, 2012). Thus, instead of engineering proteins sequentially and then expressing, purifying and analysing each engineered species separately, phage display library encompassing billions of variants can be constructed and analysed simultaneously. The phage display library can be produced either from antibody gene fragments isolated from naïve sources or derived from an immunised host of choice or by *in vitro* mutagenesis of complementarity determining regions (CDR) (Clackson et al., 1991, Griffiths et al., 1994, Hoogenboom and Winter, 1992). The scope of having a host of choice for immunisation, is one the major advantages of phage display over hybridoma technology which is restricted to use of mouse (Schmitz et al., 2000). For generation of a phage display library, there is greater freedom to select any host of choice provided that sufficient information about the immunoglobulin gene sequences are available.

#### **1.8.1.1 Selection of host for library generation**

Phage display libraries can be generated either from a naïve or an immune challenged host. Naïve libraries are constructed from an unimmunised host, where

variable genes from IgM mRNA are isolated from peripheral blood lymphocytes, bone marrow and spleen to generate a phage display library that presents binding sequences against self, non-immunogenic or toxic antigens. However, the affinity of antibodies isolated from naïve library is usually low and may require further affinity maturation *in vitro* (Hoogenboom, 1997). In contrast, libraries generated from immunised hosts are used to generate a phage display library specifically enriched in antibodies against the antigen of interest. Also, the antibodies isolated are already affinity matured in the host itself generating high affinity antibodies (Clackson et al., 1991). For generating phage libraries from immunised hosts, the selection of an appropriate host organism is of prime importance. Mouse, the most common host in hybridoma technology can also be used in phage display, but due to its phylogenetic closeness to humans, generation of antibodies against conserved mammalian epitopes is restricted by thymic tolerance (Smith, 1985). For generating antibody phage display libraries against mammalian epitopes, chickens can be used as an alternative host as they are evolutionarily distant to mammals. Especially, in context of phage display library generation, chickens are ideally suited as they generate their immunoglobulin repertoire from a single set of variable heavy and variable light germ line gene sequences (Tjoelker, 1993). The single immunoglobulin heavy and light chain loci of chickens have only one functional V and J fragment segment, which rearrange to produce repertoire of antibodies within the chicken (Davies et al., 1995, Tjoelker, 1993). This simplified germ line V gene system enables the generation of antibody fragments by using only four sets of PCR primers (Davies et al., 1995). In contrast, mouse with a complex germ line V gene diversification process requires a complex mix of PCR primers (Krebber et al., 1997). Further, like rabbits, the chickens can also be immunised with multiple sets of antigens in a single immunisation regime to generate distinct immune responses against each antigen without cross-reactivity (Finlay et al., 2005, Chiliza et al., 2008, Hof et al., 2008).

### **1.8.1.2 Biology of filamentous phage**

The main tool of phage display technology is filamentous bacteriophage. The filamentous bacteriophages are viruses that infect gram-negative bacteria using F conjugative pilus and do not kill the host cells during infection. The F<sub>f</sub> filamentous phage (f1, fd and M13) contain single stranded DNA (ssDNA) enclosed within a

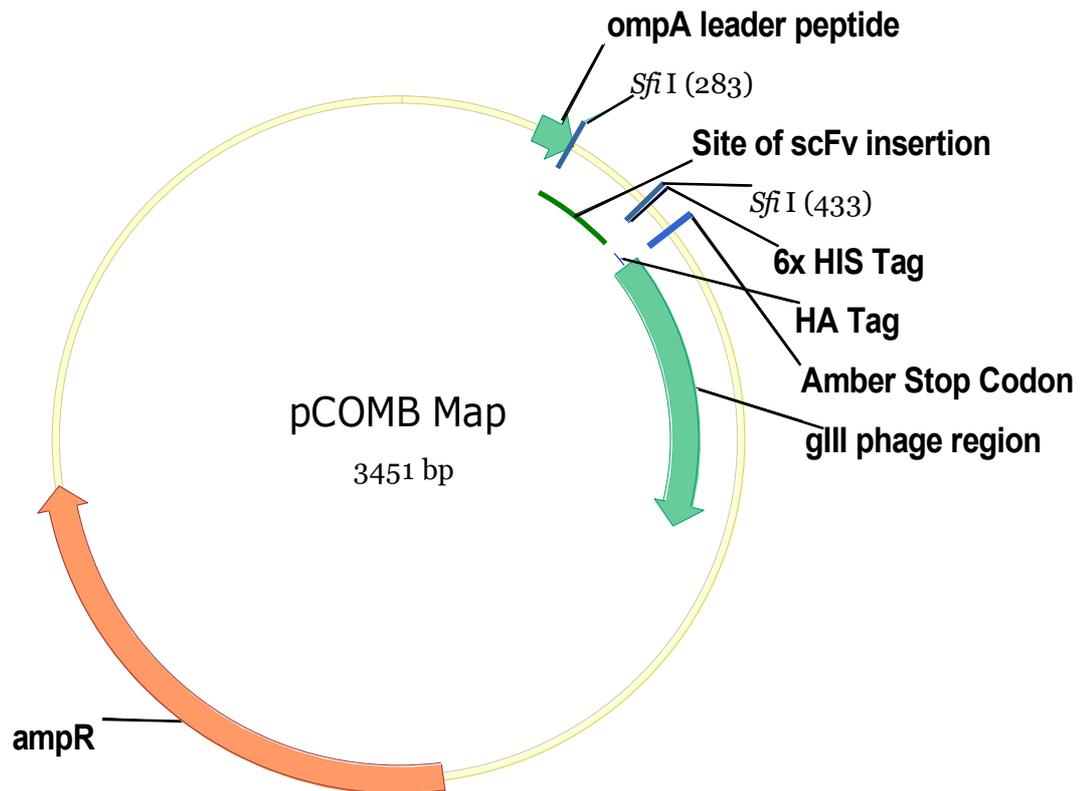
viral capsid. The phage genome contains 11 genes, coding for 11 different proteins (pI-pXI) present within the coat proteins (Marvin and Hohn, 1969). The phage produces around 2700 copies of the pVIII protein, the major coat protein of filamentous phage, and about 3 to 5 copies of pIII minor coat protein (Newman et al., 1977). Both these coat proteins can be used to display a protein or antibody of interest fused to the coat protein on the surface of M13 filamentous phage (Rakonjac, 2011). The major coat protein pVIII, is also used for assisting in detection by the anti-M13 antibody that binds specifically to pVIII (Dente et al., 1994). The filamentous phage initiates the infection by using the pIII protein to bind to the F pilus of bacteria. The pIII phage protein consists of 3 domains (N1, N2 and CT) separated by glycine-rich regions, which are essential for infection. Of the three, N2 domain binds to F pilus while N1 domain interacts with the bacterial membrane protein (TolA-D3), this helps the phage to establish proximity towards the bacterium surface where the major and minor phage capsid proteins are disassembled and the phage DNA is translocated into the cytoplasm. After gaining entry into bacteria the single stranded viral DNA is used by bacterial enzymes to synthesise the complementary DNA strand (Rakonjac, 2011). This forms the replicative form (RF) of viral DNA that can be used for replication and transcription. For making more copies of viral DNA, the pII protein cleaves the intergenic region of the RF that acts as a primer for new viral DNA synthesis, to generate 200 copies within a bacterium. The replication continues until pV protein reaches a critical level where it binds to newly synthesised viral ssDNA. The newly synthesised ssDNA-pV is assembled in the cytoplasm of the bacterium and the phage particle is subsequently secreted from the cell (Karlsson, 2004).

### **1.8.3 Phagemid vectors**

For display of proteins as fusion proteins to either pIII or pVIII, proper selection of phage vector is critical. The gene of interest can be directly inserted into the phage vector within the genome leading to a polyvalent display of proteins, which in turn results in increased avidity that may cause selection of low affinity binders. To avoid an avidity effect, a monovalent phage display process employing phagemid vector was developed, which is a hybrid of a phage and plasmid vector. Phagemid contains origin of replication of both M13 phage and *E. coli* in addition to pIII with multiple

cloning sites and selectable resistance (Mead and Kemper, 1987, Azzazy and Highsmith, 2002). This vector produces large quantities of pIII-fusion proteins however, it lacks other structural and non-structural gene components required for generating a complete phage particle (Azzazy and Highsmith, 2002). To assist in phage particle formation, helper phage (i.e. M13K07 and VCSM13) are introduced, that itself contains a defective origin of replication but supply all necessary components required for phage replication and assembly (Barbas et al., 2004). As mentioned above, the phagemid genome is packaged in the phage coat preferentially rather than in the helper phage because of a defective origin of replication site to ensure abundance of pIII fusion protein. Fusion protein with pIII generate phage particles with one or two copies of the displayed proteins, display of such few proteins per phage is helpful in generating high affinity binders (Schmitz et al., 2000). In contrast, proteins displayed as fusion partner with pVIII display multiple copies of pVIII fusion protein, which most likely leads to selection of low affinity but high avidity binders (Schmitz et al., 2000).

The modern phagemid vector, pComb3XSS, used for display also contains a *lac* promoter, which controls expression levels with *ompA* and *pelB* leader sequences directing the protein expression to the bacterial periplasm. The phagemid vector pComb3XSS is designed to produce phage particles in suppressor *E. coli* strains e.g. XL1-Blue, which allows for transcription of the pIII fusion protein during the process of selection, Figure 1.14. For soluble expression, the phagemid is transfected to a non-suppressor *E. coli* (TOP10F') where the amber stop codon (TAG) is read and the protein or antibody fragment is expressed in soluble form in the periplasmic space without the pIII gene product. The phagemid also introduces HA (hemagglutinin) and 6 X His (histidine) tags, that can be used for affinity purification of proteins.

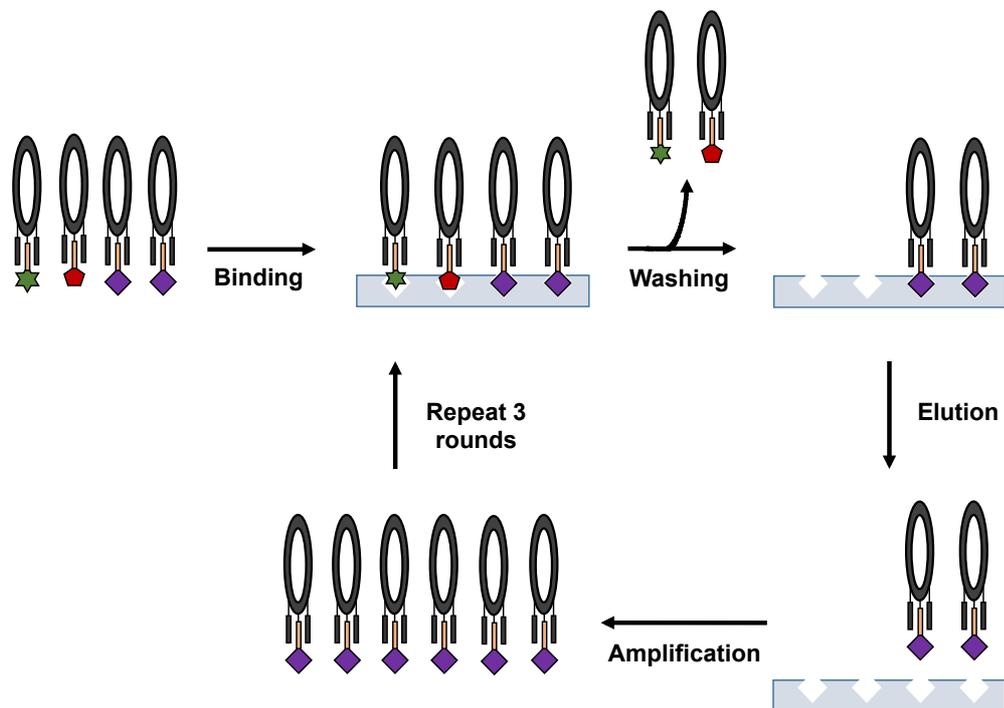


**Figure 1.14** Map of pComb3XSS phagemid vector. The vector has SfiI restriction site for introduction of scFv fragment. 6 X His and HA tags allow for purification and detection. The vector also contains an amber stop codon which is used to turn-off the expression of pIII fusion protein by switching to a non-suppressor strain of *E. coli* allowing production of soluble protein without subcloning.

#### 1.8.4 Phage display library generation and biopanning

For phage display library generation from immunised hosts, the RNA is isolated from the bone marrow and spleen, and cDNA is synthesised. From the cDNA pool, heavy and light chain genes are amplified by polymerase chain reaction (PCR) and the antibody fragment inserted into pIII encoding genes within the phagemid. The phagemid DNA is used to transform competent *E. coli* cells and after expression the coat protein fusions are integrated into new phage particles assembled in the bacterium. The particle assembly process is assisted by introducing helper phage that provide the necessary structural components for the initiation and packaging of phagemid DNA into phage particles. The phage particles are then harvested from the culture to isolate the phage library pool displaying antibody or antibody fragments. Using phage display more than  $10^{10}$  clones can be generated however it should be

noted that size of the library does not reflect the diversity within the library (Schmitz et al., 2000, Griffiths et al., 1994). For the selection of specific antibody fragment a process known as biopanning is employed (Clackson et al., 1991). During the biopanning process, the selection is achieved by immobilising the target antigen on a solid surface followed by incubation with phage library pool (McCafferty et al., 1990). The antibody phage selection can also be performed by exposing the phage library pool to cells expressing target marker or antigen and also by injecting phage pool into the animal and collecting tissues for analysis of bound phage (Pasqualini and Ruoslahti, 1996, Hawkins et al., 1992). The exposure to antigen results in capturing of antigen specific antibody phage. The non-specific phage are removed by washing and bound phage are eluted with a high salt or low pH solution or by enzymatic cleavage of a protease site in between antibody and pIII (Ward et al., 1996). The eluted phage are then allowed to re-infect bacteria for replication and amplification. Further rounds of panning with more stringent selection conditions; achieve the enrichment of antigen specific antibody phage population, Figure 1.15.



**Figure 1.15** Biopanning for selection and enrichment of scFv-phage. The antigen immobilised onto solid support is exposed to an scFv-phage display library pool. Specific phages bind to the presented antigen, and unbound and loosely bound phages are removed by washing. The bound phage are eluted and amplified in *E. coli*. The amplified phage pool is again subjected to biopanning for up to three rounds.

## **1.9 Scope and outline of thesis**

Glycans are proving to be one of the most information rich biomolecules and their potential information storage capability is far greater than that of nucleic acids and proteins. Understanding of their roles in various biophysical process and diseases is increasing. However, deciphering the information stored within glycans has lagged due to the lack of of suitable tools.

Chapter 2 deals with the generation of anti-Neu5Gc scFv using phage display technology. The detailed process for generation of scFv-phage display library from immunised chicken is described. A panel of short and long-linker scFv identified from the phage display library using biopanning approach are described. The expression and initial evaluation of identified scFv is also presented.

Chapter 3 describes the assay development and optimisation for Neu5Gc detection and quantitation using scFv. The scFv generated and identified in chapter 2 were evaluated for their binding to Neu5Gc. Among the identified scFv, short-linker scFv (SL1A1) was found to be better at binding Neu5Gc. Prior to assay development the specificity of the scFv was evaluated. Initial challenges with the assay development were addressed and optimised. The assay developed with scFv-SL1A1 was tested for its reproducibility and employed for the detection and quantitation of Neu5Gc in glycoproteins. The results were compared with the HPLC data.

Chapter 4 describes the explorative work done to assess the suitability of the anti-Neu5Gc scFv for developing applications in detection of Neu5Gc in western blot format. Detection of Neu5Gc in clinical and biopharmaceutical drugs was demonstrated in western blots. Initial kinetic evaluation of scFv on SPR was also carried out using Neu5Gc containing glycoproteins. The scFv was also evaluated for detection of Neu5Gc in tissues from healthy and cancer samples on a human tissue array. The research work presented in this chapter was to investigate the potential applications of anti-Neu5Gc scFv for Neu5Gc detection. The work is not conclusive but provides directions for future research work for developing applications using anti-Neu5Gc scFv in clinical setting.

Chapter 5 contains a discussion of the overall conclusion of the research work. Future research work with anti-Neu5Gc scFv is also proposed and discussed.

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## **Chapter 2**

### **ScFv-phage display library preparation and isolation of anti-Neu5Gc scFv**

## 2.1 Introduction

Phage display technology is one of the most powerful methodologies in the identification and development of antibody and antibody fragment molecules from immune and non-immune challenged libraries (Bazan et al., 2012). Phage display utilises molecular techniques, where the fusion of the desired gene library, be it antibody, peptide, or enzyme, with the pIII coat protein gene of M13 filamentous phage, results in the protein encoded by the gene of interest being expressed as a fusion protein on the surface of a phage particle (Barbas et al., 2004, Winter et al., 1994). This phenotypic association, established by the protein fragment displayed on the phage surface and corresponding gene encoding the displayed protein, allows for the generation, screening and enrichment of diverse libraries of proteins in a high throughput manner (Schmitz et al., 2000).

The phage display process begins with the generation of a naïve or immune challenged antibody or an antibody fragment library. Although naïve antibody and antibody fragment phage display libraries can be used to identify recognition molecules against a variety of target antigens, it has been reported that the affinities of the identified molecules remain usually low, with further rounds of affinity maturation required to increase affinity. In contrast, immune-challenged phage display libraries overcome this limitation by immunising the host with a target antigen to generate specifically enriched immunoglobulins and their further affinity maturation *in vivo*, thus increasing the chances of isolating a high affinity antibody or antibody fragments (Azzazy and Highsmith, 2002, Hoogenboom et al., 1998).

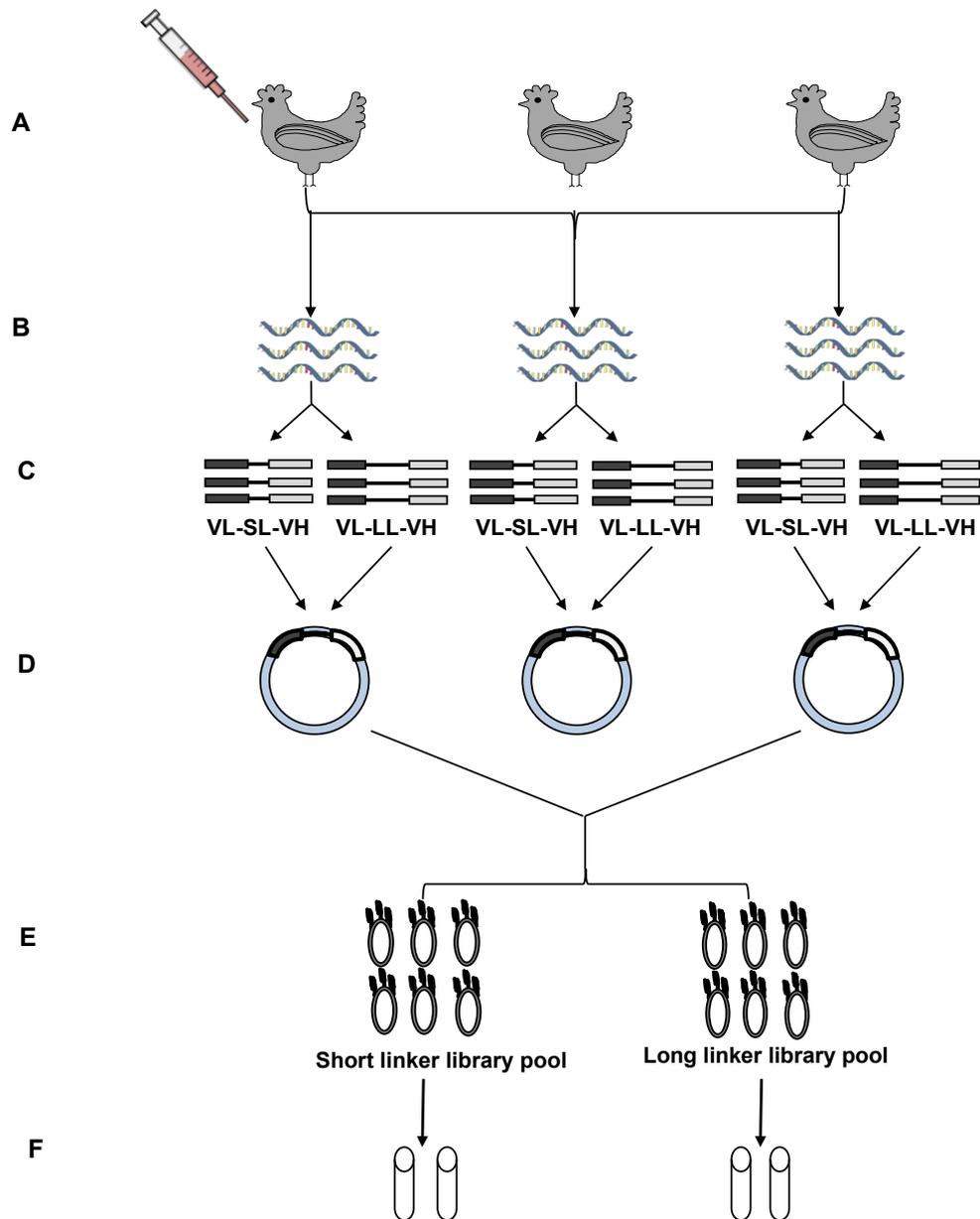
As a host, chickens are a preferable choice for the generation of immunised antibody fragment phage display libraries because of their amenability for selection of high affinity and specific antibodies against conserved mammalian proteins. Further, due to the relatively less diversity in germline genes of chickens compared to humans and mice, fewer primer sets are required for amplification of antibody genes (Leonard et al., 2007, Andris-Widhopf et al., 2000). The RNA isolated from spleen and bone marrow of an immunised host can be used to produce cDNA to generate antibody variable gene fragments, which can be inserted into phagemid vectors adjacent to the gene encoding coat protein of phage and finally expressed on the

surface of phage as a fusion protein after super infection by a helper phage (Oh et al., 2007).

For the generation of recognition molecules against Neu5Gc, white leghorn chickens were immunised with a mixture of glycoconjugates which contained equal concentrations of Neu5Gc-BSA, Neu5Gc-Biotin and Neu5Gc-GM3. During the immunisation regime, serum samples were collected at pre- and post- immunisation time points and evaluated by indirect ELISA to monitor the immune response against Neu5Gc conjugates. The chickens were killed and bone marrow and spleen harvested for RNA isolation. The RNA, converted to cDNA, was then used to produce light and heavy-chain gene fragments, which were fused to generate single chain fragment variable (scFv) antibody phage display library. The scFv comprises of a variable light (VL) and heavy chain (VH) region of antibody connected via a flexible linker.

Within this research, library generation was directed towards scFv generation for its ease of production and smaller insert size, which renders more stability to the scFv molecule compared to Fab fragments (fragment antigen binding) which are twice the size (50 kDa) and require extensive assembly and folding in a bacterial host (Willats, 2002, Skerra, 1993). Furthermore, scFv are less toxic to bacterial host resulting in better yields of scFv upon expression than Fab fragments (McCafferty et al., 1990). The scFv construction is initiated with PCR amplification of VL and VH chain fragments employing primers against the conserved framework sequences, that are present in the complementary determining region (CDR) of the antibody. The VL and VH chains were fused together by a short (GGSSRSS) or long (GGSSRSSSSGGGSGGGG) linker region (Andris-Widhopf et al., 2000). The length and composition of linker also determines the levels of aggregation and assembly of the VL and VH regions to form diabody with short-linker and monomeric scFv presentation with long-linker (Tang et al., 1996, Dall'Acqua and Carter, 1998). Fused VL and VH fragment were then cloned in the pComb3XSS phagemid vector for propagation in *E. coli* XL1-Blue. The bacterial culture was then superinfected with a helper phage to generate phage particles, which were isolated and stored as the scFv phage display library. The scFv-phage display library was subjected to rounds of biopanning and clones demonstrating a level of specificity and affinity to the target antigen were selected (Figure 2.1). In biopanning, the target

antigen can either be presented as immobilised on solid support (Barbas et al., 2004) or in solution phase on streptavidin-coated magnetic beads (Santala and Saviranta, 2004). In biopanning, the scFv-phage display library was exposed to target antigen (Neu5Gc) immobilised on a solid support, for specific scFv-phage to bind to immobilised antigen and permit the removal of non-specific scFv-phage by washing. The bound scFv-phage particles were then eluted and amplified in a bacterial host, prior to undergoing subsequent rounds of biopanning, selection and enrichment.



**Figure 2.1** Overview of strategy for immunised chicken phage display library generation. **(A)** White leghorn chickens immunised with a mixture of Neu5Gc-containing conjugates (Neu5Gc-BSA, Neu5Gc-Biotin, GM3-Neu5Gc). After immunisation, chickens were killed and bone marrow and spleen were removed and RNA was isolated from these organs. **(B)** The RNA was converted to cDNA. **(C)** The VL and VH chain with short (SL) or long-linker (LL) were PCR amplified. The VL and VH amplified fragments were fused together by overlap PCR to generate a fused short-linker (VL-SL-VH) and long-linker (VL-LL-VH) products. **(D)** The fused products were inserted into pComb3XSS phagemid vector and **(E)** transfected into *E. coli* XL1-BLUE for expression of long and short-linker scFv displaying phage particles. **(F)** Short-linker scFv-phage pool and long-linker scFv-phage pool were then subjected to biopanning in immunotubes.

## 2.2. Materials and Methods

### 2.2.1. Materials and reagents

SuperScript® III first strand synthesis kit for cDNA synthesis, SYBR® Safe DNA Gel Stain, TRIzol® and agarose for gel preparation were from Life Technologies (UK); QIAquick® gel extraction kit and RNeasy® Mini kit for total RNA extraction was purchased from QIAGEN (Manchester, UK). Plasmid purification kit was from Macherey & Nagel (GmbH. Co., Duren, Germany). GoTaq® Flexi DNA Polymerase and components for PCR amplification were from Promega (UK). Primers were sourced and custom synthesised from Eurofins (Ebersberg, Germany). ExoSAP-IT® was from Affymetrix (Santa Clara, CA, US). SfiI was sourced from Roche (Indianapolis, Indiana, US). BstOI and rabbit anti-chicken IgY-horseradish peroxidase (HRP) polyclonal antibody was purchased from Promega (UK). DNA Hyperladder™ 1Kb was from Bionline (Germany). Throughout this research, bacterial strains *E. coli* XL1-Blue (*RecA endA1 gyrA96 thi-1 hsdR17supE44 relA1 lac (F proAB lacIq Z M15 Tn10 (Tetr))*) competent cells were from Stratagene (Santa Clara, CA, US). *E. coli* TOP10 F' (*F[lacIq Tn10 (tetR)] mcrA Δ (mrr-hsdRMS-mcrBC) φ80lacZΔM15 ΔlacX74 deoR nupG recA1 araD139 Δ (ara-leu)7697 galU galK rpsL (StrR) endA1 λ-*) competent cells were from Life Technologies (UK). Components for media preparation, HRP-labeled monoclonal anti-HIS antibody, human serum albumin (HSA), bovine serum albumin (BSA), bovine fetuin, bovine asialofetuin and antibiotics were sourced from Sigma-Aldrich Co. (UK). Agar, Pierce™ Silver stain kit, Pierce™ Coomassie Plus™ protein assay and ELISA substrate 1-Step® Ultra TMB was sourced from Fisher Scientific, (UK). Syringe filters (0.22 μm and 0.45 μm) and 30 kDa centrifuge filters were purchased from Millipore (Bradford, US). Mouse anti-M13 monoclonal antibody-HRP was from GE Healthcare Life sciences (Buckinghamshire, UK). F8 Maxisorp loose Nunc™ immuno module were from Thermo Scientific (UK). Free sugars Neu5Gc, Neu5Ac and Gal-α-(1,3)-Gal were purchased from Dextra (UK). Glycoconjugates Neu5Gc-polyacrylamide (PAA), Neu5Ac-PAA and Neu5Gc-biotin were purchased from Dextra and Lectinity (Moscow, Russia). Glycine was purchased from Riedel-de Haën (UK). All other reagents were from Sigma-Aldrich Co. unless indicated otherwise and were of the highest grade available.

### **2.2.2. RNA and DNA integrity and concentration**

The concentration of RNA and DNA was estimated by absorbance at 260/280 nm on a NanoDrop 2000 (Thermo Scientific, UK). For concentration measurement 2  $\mu$ L of RNA/DNA sample was directly dispensed onto the pedestal and measurement was made at 260/280 nm. Prior to measurement of RNA/DNA, the NanoDrop 2000 was blanked with PCR mix without RNA/DNA. The sample holding pedestal of the instrument was cleaned with 4  $\mu$ L of nuclease free water before and after measurement. The integrity of RNA was assessed on a BioAnalyser 2100™ (Agilent Technologies, Palo Alto, CA, US).

### **2.2.3. Agarose gel electrophoresis for DNA visualisation**

During the process of library generation, DNA was separated on 0.8 to 3.0 % (w/v) agarose containing gels depending on the size of the DNA (Sambrook and Russell, 2001). Depending upon percentage of gel required agarose was weighed and added to TAE buffer (40 mM Tris base, 2 mM EDTA, 20 mM acetic acid, pH 8.5). The mixture was heated until the agarose had completely dissolved. DNA binding dye, SYBR® Safe was then added and mixed gently. SYBR® Safe is a variant of SYBR® Green I, both the dyes bind to DNA and are 25 fold more sensitive with low mutagenicity and toxicity than ethidium bromide (Jin et al., 1994, Holgado-Madruga et al., 1997). The gel solution was then poured into a gel casting mold with appropriate combs to form wells for sample loading. The DNA was separated at 80-120 volts and amplicon size estimated by direct comparison against the migration pattern of a DNA standard ladder (Hyper ladder™ 1 Kb).

### **2.2.4 DNA extraction and purification**

For extraction and purification of PCR amplified DNA, the DNA was separated using electrophoresis and a TAE agarose gel. The desired band of DNA was excised from the gel as and when indicated in library preparation Section 2.2.9 and purified using QIAquick® gel extraction kit according to manufacturer's protocol.

DNA extraction post-ligation reaction, was performed by ethanol precipitation by addition of 1/10 volume of 3 M sodium acetate, pH 5.2, to ligation reaction followed by addition of 2.5 volume of ice cold 100% ethanol. The precipitation reaction was

incubated overnight at -80 °C. The precipitated product was then centrifuged at 15,000 x g for 20 min and the pellet was washed twice with 70% ice cold ethanol. The recovered pellet was finally dried and resuspended in 20 µL of nuclease free water and quantified as per Section 2.2.2.

### **2.2.5 Isolation of plasmid/phagemid DNA**

Plasmid/phagemid DNA was propagated by overnight bacterial culture grown at 37 °C with shaking at 200 rpm under the selection of appropriate antibiotic. The culture was then centrifuged to harvest the bacteria (4000 x g, 4 °C, 30 min). Plasmid was then extracted from the bacterial pellet using plasmid DNA isolation kits from Macherey & Nagel according to the manufacturer protocols. Elution was performed with nuclease free water, and plasmid quantified as per Section 2.2.2.

### **2.2.6 Preparation of standard *E. coli* XL1-Blue stock**

For isolation of individual colonies, bacterial stocks were streaked onto Luria Bertani (10 g Tryptone, 5 g Yeast extract, 10 g NaCl) (LB) agar plates (20 g/L LB, 1.5% agar) containing tetracycline (10 µg/mL) and incubated overnight at 37 °C. Single colonies were picked and propagated with overnight shaking at 200 rpm at 37 °C in LB broth containing tetracycline (10 µg/mL). Overnight culture was then sub-cultured at 1:100 dilution and grown in LB media with selective antibiotic and shaking at 200 rpm at 37 °C until an optical density at 600 nm (OD<sub>600</sub>) reached approximately 0.6 to 0.8, The bacteria were then pelleted by centrifugation (4000 x g, 4 °C, 30 min). Bacterial pellet was then resuspended in 10 % glycerol and pelleted again by centrifugation as above. This step was performed 3 times, after which the pellet was resuspended in 10 % glycerol and aseptically transferred in 100 µL aliquots to 1.5 mL microcentrifuge tubes and stored at -80 °C until further use.

### **2.2.7 Preparation of helper phage (VCSM13) for phage display**

For helper phage VCSM13 preparation an overnight culture of *E. coli* XL1-Blue in super broth (SB) media (30 g/L Tryptone, 20 g/L, 10 g/L MOPS, pH 7.0) supplemented with 10 µg/mL of tetracycline was inoculated and cultured at 37 °C, with shaking at 200 rpm. The overnight culture was then 1:100 dilution and grown with shaking at 200 rpm at 37 °C until OD<sub>600</sub> of approximately 0.6 to 0.8 was achieved. The VCSM13 helper phage stock was diluted in phosphate buffered saline

(PBS) (137 mM NaCl, 2.7 mM KCl, 10 mM Na<sub>2</sub>HPO<sub>4</sub>, 1.8 mM KH<sub>2</sub>PO<sub>4</sub>, pH adjusted to 7.4 with phosphoric acid) at a range of dilutions and added 1 µL to 50 µL of log phase *E. coli* XL1-Blue culture. The mixture was incubated for 15 min at room temperature (20 °C to 25 °C). Post incubation, 3 mL of LB top agar was added and spread onto pre-warmed LB agar plate and incubated overnight at 37 °C. Single phage plaques were picked and inoculated into log phase *E. coli* XL1-Blue culture. The culture was incubated for 2 h at 37 °C, shaking at 200 rpm. Post-incubation, the culture was expanded by transferring into a 2 L culture flask containing 500 mL of pre-warmed SB media. Finally, tetracycline and kanamycin were added to achieve final concentration of 10 µg/mL and 50 µg/mL respectively, and infected culture grown overnight shaking at 200 rpm at 37 °C. The following day the culture was centrifuged (2500 x g, 4 °C, 30 min) to remove the bacterial pellet. The supernatant was harvested and helper phage were precipitated by adding 1/5 volume of 5 X PEG/NaCl (5 % w/v PEG-8000, polyethylene glycol, 2.56 M NaCl) to supernatant and incubating on ice for 1 h. The supernatant was then centrifuged (10,500 x g, 4 °C, 30 min) and the resulting pellet (helper phage) was resuspended in PBS. The resuspended phage solution was again centrifuged (11,500 x g, 4 °C, 5 min) to remove bacterial cell debris. Finally, the supernatant was filtered by 0.22 µm filter and stored at 4 °C until further use.

### **2.2.8 Chicken immunisation, serum titration and evaluation of polyclonal serum**

A pooled glycoconjugate preparation composed of Neu5Gc-BSA, Neu5Gc-biotin and GM3-ceramide, were combined at a ratio of 1:1:1. Three adult male leghorn chickens were immunised, at 14 day intervals, with 50 µg of the glycoconjugate preparation in a total volume of 400 µL. The immunogen was given at 4 separate sites, intramuscularly, into the breast muscle (100 µL per immunisation site). The first dose was prepared with complete Freund's adjuvant, and three subsequent doses were administered with incomplete Freund's adjuvant. Serum anti-Neu5Gc responses were evaluated, across all bleeds, by indirect ELISA.

#### **2.2.8.1 Titration of immunised chicken polyclonal serum against Neu5Gc**

Indirect ELISA was employed to confirm a polyclonal serum response against Neu5Gc. Titration of polyclonal chicken sera was performed to determine the

optimal dilution point of serum for subsequent ELISAs, which could be used throughout the characterisation studies to evaluate the specificity of polyclonal serum against Neu5Gc.

ELISA plate preparation: A 96 well immunoassay plate was coated overnight at 4 °C with 100 µL/well of Neu5Gc conjugated to polyacrylamide (Neu5Gc-PAA) at 1.5 µg/mL in 100 mM sodium bicarbonate buffer pH 9.6. The plate was washed twice with PBS, pH 7.4. Non-specific binding sites within the assay wells were then blocked with 0.5 % periodated BSA (pBSA) in PBS for 1 h at 37 °C. Post blocking, wells were washed 3 times with 250 µL/well of PBS containing 0.05 % (v/v) Tween 20 (PBS-T).

Serum samples were serially diluted from 10,000 to 320,000 fold in 0.1 % pBSA in PBS, and then plated out in 100 µL aliquots. Plates were then incubated at 37 °C for 1 h, and subsequently washed 3 times with 250 µL/well of PBS-T. Rabbit anti-chicken IgY-horseradish peroxidase (IgY-HRP), acting as a secondary antibody, was diluted 10,000-fold in PBS-T and added to each well (100 µL/well) and incubated for 1 h at 37 °C. Post incubation, the plate was washed 3 times with 250 µL/well of PBS-T, and tapped dried on paper towel. The reaction was visualised by the addition of 100 µL/well of the HRP substrate, tetramethylbenzidine (TMB), and incubated with shaking in the dark for 20 min at room temperature. The HRP-TMB reaction was stopped with the addition of 100 µL/well of 1 M H<sub>2</sub>SO<sub>4</sub>. Absorbance was then determined at 450 nm using a SpectraMax M5<sup>e</sup> plate reader. The polyclonal serum response was estimated based on absorbance reading changes with respect to suitable assay controls, which in this case was Neu5Ac-PAA

#### **2.2.8.2 Determination of specificity of polyclonal serum**

Competitive ELISA using free antigen was employed to determine the specificity of the polyclonal serum response. In competitive ELISA, Neu5Gc-PAA was immobilised as an antigen and diluted polyclonal serum was used to probe Neu5Gc in presence of free Neu5Gc sugar at a range of concentrations, thus creating competition for binding in between immobilised and free sugar. Reduction of binding signal was estimated as the percentage of binding against a control without competitor. A plot of percentage binding at specific sugar concentration generated an inhibition curve. Percentage binding was calculated by following expression 1.

### **Expression 1.** Calculation for percentage binding

$$\text{Percentage binding (\% B/Bo)} = \frac{\text{Absorbance with competitor (B)}}{\text{Absorbance without competitor (Bo)}} \times 100$$

Specificity was assessed based on comparison of inhibition curve generated using competing sugars other than Neu5Gc.

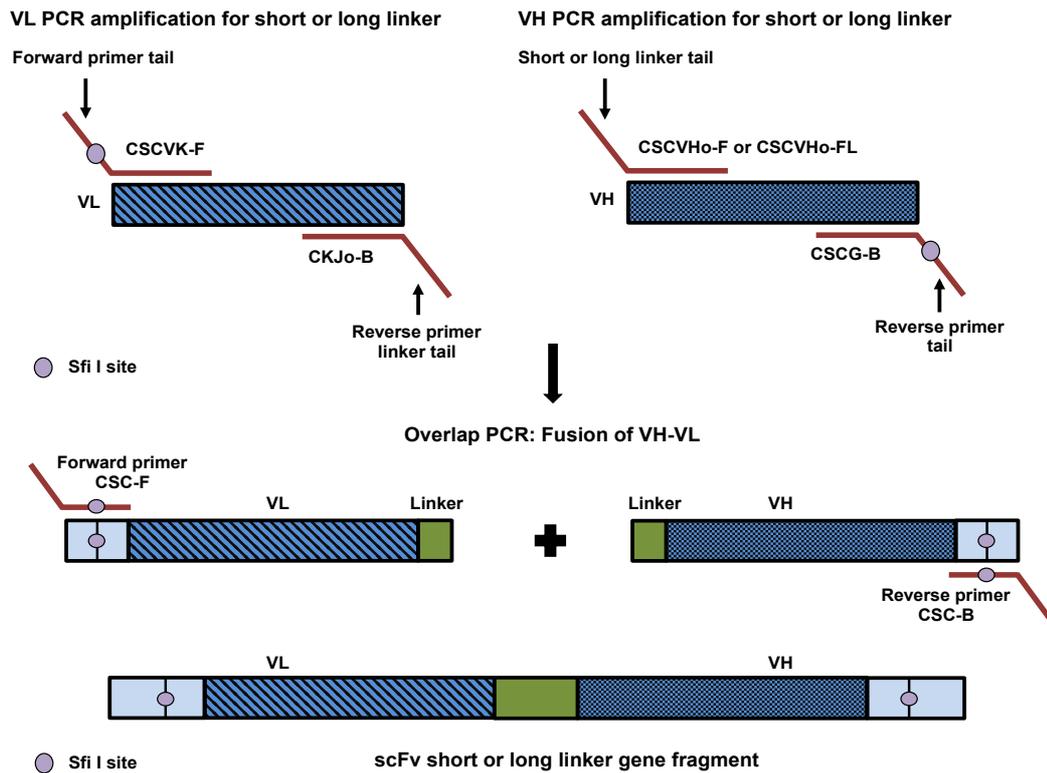
A 96 well immunoassay plate was prepared as detailed in Section 2.2.8.1, Neu5Gc free sugar was diluted from 10 mg/mL (30.7 mM) to 1 µg/mL (3.75 µM) in 0.1 % pBSA in PBS, pH 7.4. Similarly assay controls (free sugars: Neu5Ac, Gal-α-(1,3)-Gal, L-Fucose, L-Rhamnose; sialoglycoprotein: fetuin) were prepared in matched concentration ranges (1 µg/mL to 10 mg/ mL) in 0.1 % pBSA in PBS. To each well 50 µL of diluted free sugar was added, followed by addition of 50 µL of a 20,000 fold dilution of immunised chicken polyclonal serum.

After addition of samples and controls, the microplate was incubated at 37 °C for 1 h. Post incubation the microplate was washed 3 times with PBS-T (250 µL/well). 100 µL of rabbit anti-chicken IgY conjugated to HRP at 1:10,000 dilution in PBS-T was then added to each well and incubated for 1 h at 37 °C. After incubation the plate was washed 3 times with PBS-T as before, and tap dried on paper towel. Washing was followed by the addition of TMB solution (100 µL/well), with incubation in the dark for 20 min at room temperature. The reaction was stopped with 100 µL/well 1 M H<sub>2</sub>SO<sub>4</sub>. Absorbance at 450 nm was then measured.

### **2.2.9 Overview of antibody fragment generation and phage display**

To generate an scFv-phage display library, cDNA synthesised from RNA isolated from the spleen and bone marrow of immunised chickens was used. For scFv generation only VL and VH regions of the antibody were amplified. The VL and VH region of antibody consists of a constant framework region and hyper-variable regions that together form complementary determining regions (CDRs). PCR primers against framework regions were employed to amplify VL, VH and VHL fragments from the immune challenged cDNA template.

The PCR primers for VH and VHL (short-linker and long-linker respectively) can be used to introduce variable linker lengths between light and heavy chains. Here a short-linker (7 amino acids) and long-linker (18 amino acids) heavy chain fragments were generated. The VL, VH and VHL fragments were fused by an overlap PCR to generate a fused fragment of DNA containing variable light-heavy (VL-VH) and variable light-heavy long (VL-VHL) DNA fragments (Figure 2.2).



**Figure 2.2** Overview of scFv gene fragment generation. The VL and VH-chain fragments were amplified from immunised chicken cDNA. The primers used in this process incorporate a SfiI restriction site for cloning and a short or long-linker sequence containing overlap regions for subsequent overlap PCR. Amplified VL and VH (long or short-linker containing) fragments were then fused together by overlap PCR to generate full length short or long-linker scFv fragments. Image adapted from (Andris-Widhopf et al., 2000).

The fused fragment and phagemid vector pComb3XSS were then subjected to SfiI restriction digestion. The digested products were purified and ligated using T4-DNA ligase. Ligated products were used to transform *E. coli* XL1-Blue cells. Transformed cells pool were grown and expanded into fresh media wherein helper phage (VCSM13) was introduced to assist in phage particle formation. The cultures were expanded overnight and harvested the next day. The scFv-phage was isolated by

phage precipitation, as before using 5 X PEG/NaCl. Isolated phage were stored in PBS containing 0.1 % pBSA at 4 °C for use in biopanning.

### **2.2.9.1 Isolation of RNA and cDNA generation**

Post-immunisation (Section 2.2.8), bone marrow and spleen were removed from immunised white leghorn chickens and RNA was extracted and used in the construction of an antibody fragment phage display library. The spleen and bone marrow samples were weighed and 1 mL of TRIzol® reagent was added to 50 mg of bone marrow or spleen sample. Tissues were then homogenised with a sterile homogeniser (cleaned with RNase-free). Homogenised tissue was then incubated for 5 min allowing for disruption of nucleoprotein complexes. To 1 mL of homogenised tissue solution 200 µL of chloroform was added and shaken vigorously and incubated for 15 min at room temperature. Post incubation the mixture was centrifuged at 10,000 x g for 15 min at 4 °C. The solution separated into 3 phases with the lower mostly phenol/chloroform, a turbid protein interface and a clear aqueous RNA containing upper phase. The RNA containing phase was then removed and applied onto an RNeasy spin column. The column was then centrifuged at 8000 x g for 15 sec at 4 °C and the flow-through was discarded. To the column 350 µL of RW1 buffer was added followed by centrifugation at 8000 x g for 15 sec at 4 °C. On column DNase digestion was performed by following Qiagen's protocol, followed by elution in nuclease free water. The quantity of RNA was assessed on a NanoDrop 2000 and the quality of RNA was assessed on BioAnalyser 2100™ (Agilent Technologies, Palo Alto, CA, USA).

The cDNA synthesis was carried out using SuperScript® III first strand synthesis kit. This system employs an advanced version of M-MLV reverse transcriptase enzyme engineered to reduce RNaseH activity while remaining stable at a wider temperature range. The cDNA synthesis is a two-step process; in the first step RNA is primed with oligo dT<sub>20</sub> or gene specific primers (GSP) for annealing. In the second step cDNA is synthesised by addition of reverse transcriptase enzyme. In the current research work both oligo dT<sub>20</sub> and gene specific primers were used in parallel. Oligo dT<sub>20</sub> are short 20 mer nucleotides used to generate total cDNA from mRNA while GSP generate gene specific template of cDNA.

For cDNA preparation 850 ng of extracted RNA was added to a reaction mix comprising 1  $\mu$ L of primer (1  $\mu$ M Oligo dT<sub>20</sub> or GSP for light (CKJoB) or heavy chain (CSCGB) regions (Table 1), 1  $\mu$ L 10 mM dNTP and DEPC water to a final volume of 10  $\mu$ L). The reaction mixture was incubated at 65 °C for 5 min. After which, the reaction mixture was placed on ice for 1 min. To the reaction mixture 2  $\mu$ L of 10 X reaction buffer, 4  $\mu$ L 25 mM MgCl<sub>2</sub>, 2  $\mu$ L 100 mM DTT and 1  $\mu$ L 200 U/ $\mu$ L SuperScript® III RT) were then added, and incubated at 55 °C for 50 min. The reaction was then terminated by incubation at 85 °C for 10 min. The reaction product was then chilled on ice for 5 min and finally RNaseOUT was added (1  $\mu$ L/reaction) and incubated for 20 min at 37 °C, to eliminate any remaining RNA from the mixture. The cDNA was then quantified using NanoDrop 2000. Synthesised cDNA was stored at -80 °C until needed.

### 2.2.9.2 scFv antibody fragment library generation

For construction of a scFv library VL, VH and VHL fragments were amplified from the cDNA preparations i.e. oligodT<sub>20</sub> and GSP generated cDNA. The oligonucleotide primers used for the generation of scFv templates are shown in Table 2.1. The VL, VH and VHL amplification from both the cDNA preparations were compared and evaluated by visual inspection of PCR amplified product on agarose gel.

**Table 2.1** Oligonucleotide primers for scFv library construction

<b>Variable heavy (VH)</b>	<b>CSCVHoF sense</b>	5' GGT CAG TCC TCT AGA TCT TCC GCC GTG ACG TTG GAC GAG 3'
	<b>CSCGB antisense</b>	5' CTG GCC GGC CTG GCC ACT AGT GGA GGA GAC GAT GAC TTC GGT CC 3'
<b>Variable light (VL)</b>	<b>CSCVK sense</b>	5' GTG GCC CAG GCG GCC CTG ACT CAG CCG TCC TCG GTG TC 3'
	<b>CKJoB antisense</b>	5' GGA AGA TCT AGA GGA CTG ACC TAG GAC GGT CAG G 3'
<b>Variable heavy long (VHL)</b>	<b>CSCVHoF L sense</b>	5' GGT CAG TCC TCT AGA TCT TCC GGC GGT GGT GGC AGC TCC GGT GGT GGC GGT TCC GCC GTG ACG TTG GAC GAG 3'
	<b>CSCGB antisens</b>	5' CTG GCC GGC CTG GCC ACT AGT GGA GGA GAC GAT GAC TTC GGT CC 3'
<b>Overlap /fusion PCR</b>	<b>CSCF sense</b>	5' GAG GAG GAG GAG GAG GAG GTG GCC CAG GCG GCC CTG ACT CAG 3'
	<b>CSCB antisense</b>	5' GAG GAG GAG GAG GAG GAG GAG CTG GCC GGC CTG GCC ACT AGT GGA GG 3

For each VL gene 10 PCR amplification reactions were performed, 5 reactions were performed for each VH and VHL fragments. More reactions were performed for VL fragments to account for consumption in fusion PCR for generation of VL-VH and VL-VHL fragments. For the amplification of VL, VH and VHL the PCR reaction composition remained constant, with 2  $\mu$ L of cDNA as template, 5  $\mu$ L 5 X GoTaq green buffer 2  $\mu$ L of 25 mM MgCl<sub>2</sub>, 1  $\mu$ L of 10 mM dNTP, 1  $\mu$ L of 10  $\mu$ M each of sense and antisense primer, and 0.5  $\mu$ L of 5 U/ $\mu$ L GoTaq. The reaction volume was adjusted to 25  $\mu$ L with the addition of nuclease free water.

*PCR thermocycler conditions for VL amplification:*

Denaturation at 94 °C for 5 min then 30 cycles of denaturation at 94 °C for 15 sec, annealing at 56 °C for 15 sec and extension at 72 °C for 90 sec. After which, an extended extension at 72 °C was performed for 10 min, followed by cooling to 4 °C.

*PCR thermocycler conditions for VH/VHL amplification:*

Denaturation at 94 °C for 4 min then 30 cycles of denaturation at 94 °C for 15 sec, annealing at 58 °C for 20 sec and extension at 72 °C for 90 sec. After which, an extended extension at 72 °C was performed for 10 min, followed by cooling to 4 °C.

After amplification the amplified products for each VL, VH and VHL were pooled respectively, and concentrated using a 30 kDa centrifuge filter. Enzymatic clean-up using ExoSAP-IT was then performed (10  $\mu$ L for VL and 5  $\mu$ L for VH and VHL) for 45 min at 37 °C and an inactivation step at 85 °C for 15 min. ExoSAP-IT is a formulation of enzyme exonuclease I (digests small single stranded DNA) and shrimp alkaline phosphatase (SAP) (removes dNTPs) used for clean-up of PCR reaction by removing remaining primers and dNTPs (Olsen et al., 1991, Mu et al., 2002, Lehman and Nussbaum, 1964). After ExoSAP-IT treatment the amplified products were buffer exchanged against 400  $\mu$ L of nuclease free water 3 times in a 30 kDa centrifuge filter yielding a final template volume of 100  $\mu$ L for each of the amplified fragments and then used for overlap extension PCR.

### **2.2.9.3 Generation of fused short-linker (VL-VH) and long-linker (VL-VHL) scFv fragments by overlap extension PCR**

Amplified and buffer exchanged pools of VL, VH and VHL fragments were then fused together by overlap extension PCR, also known as fusion PCR. For each fusion short-linker (VL-VH) and long-linker (VL-VHL), 16 PCR reactions were performed. For each overlap extension PCR reaction 1  $\mu$ L of VL and 1  $\mu$ L of VH or VHL was added to 5  $\mu$ L of 5 X GoTaq green buffer, 2  $\mu$ L of 25 mM MgCl<sub>2</sub>, 1  $\mu$ L of 10 mM dNTP, 1  $\mu$ L of 10  $\mu$ M each of sense (CSCF) and antisense primer (CSCB), 0.5  $\mu$ L of 5 U/ $\mu$ L GoTaq and DEPC water to final volume of 25  $\mu$ L.

*PCR thermocycler conditions for overlap extension PCR:*

Denaturation at 94 °C for 5 min then 22 cycles of denaturation at 94 °C for 15 sec, annealing at 56 °C for 15 sec and extension at 72 °C for 120 sec. The reaction finally ended with 10 min extension at 72 °C followed by cooling to 4 °C for 20 min.

After overlap extension PCR, 16 PCR reaction products for short-linker (VL-VH) and long-linker (VL-VHL) were pooled and concentrated using a 30 kDa centrifuge filter up to a final volume of 100  $\mu$ L. The concentrated products were buffer exchanged against DEPC water twice using a 30 kDa centrifuge filter and finally concentrated to a volume of 50  $\mu$ L. Out of 50  $\mu$ L concentrated product, 30  $\mu$ L was loaded onto a 1.5 % (w/v) TAE agarose gel (Section 2.2.3) and separated by electrophoresis at 100 V. The scFv band at approximately 750 to 800 bp region was cut from the gel and gel extracted by QiaexII gel extraction (Section:2.2.4) kit.

### **2.2.9.4 SfiI digestion and ligation of fused fragments into pComb3XSS vector**

SfiI is a restriction endonuclease produced and isolated from *Streptomyces fimbriatus*. The enzyme recognises an octameric sequence having 5 non-recognised nucleotides 5'GGCCNNNN/NGGCC3' and generates 3' cohesive termini (Qiang and Schildkraut, 1984, Williams and Halford, 2001, Krebber et al., 1997). The recognition sequence is rare and not found in many organisms and specifically not in antibody sequences, thus eliminating chances of internal digestion within the antibody (Burmester and Plückthun, 2001, Li et al., 2011). SfiI digestion is used to incorporate the fused chains into the phagemid pComb3XSS for phage display.

For SfiI digestion, 10 µg of short-linker (VL-VH) and long-linker (VL-VHL) fused product and 10 µg of pComb3XSS vector was digested by addition of 360 units of SfiI (stock activity; 40 U/µL), 20 µL of 10 X Surecut buffer M, made up to a final reaction volume of 200 µL with DEPC water. The reaction was incubated for 3 h at 50 °C. SfiI digested products were pre-concentrated and buffer exchanged using 30 kDa centrifuge filters and separated on 1 % TAE agarose gel (Section 2.2.3) and extracted using QiaexII gel extraction kit (QIAGEN) (Section 2.2.4).

Post-gel extraction, SfiI digested fragments were ligated into SfiI digested pComb3XSS vector. The ligation mixture contained 1.4 µg of pComb3XSS, 700 ng of SfiI digested VL-VHL and VL-VH fragments, 40 µL of 5 X ligase buffer and 1 µL of T4 DNA ligase (stock activity; 5 U/µL). The reaction mixture was incubated for 2 h at room temperature. Each ligation was performed in triplicate. The ligated product was then precipitated as per Section 2.2.3. The three replicates of ligation reaction were finally pooled.

#### **2.2.9.5 Transformation of ligated product in *E. coli* XL1-Blue**

Competent *E. coli* XL1-Blue cells were used for transformation. Transformation of 20 µL of ligated product was performed by heat shock. For each transformation reaction, 300 µL competent cells were thawed on ice and 5.1 µL of β-mercaptoethanol was added and incubated for 10 min on ice with occasional gentle swirling. Ligated product was then added and mixed gently and incubated on ice for 30 min. The mixture was then heat pulsed at 42 °C in a water bath for 45 sec. The tubes were then immediately put on ice and for 2 min. 15 mL of super optimal catabolite (SOC) medium (30 g/L Tryptone, 20 g/L Yeast extract, 0.5 g/L NaCl, 2.5 mM KCl, 20 mM MgCl<sub>2</sub>, 20 mM Glucose) was added to transformed cells and incubated for 1 h at 37 °C with shaking at 200 rpm. After 1 h, 10 µL and 100 µL of culture was plated onto LB agar plate (20 g/L LB, 1.5 % Agar) containing 100 µg/mL ampicillin and 10 µg/mL of tetracycline. As a negative control, non-transformed *E. coli* XL1-Blue without insert was plated. The plates were incubated at 37 °C overnight.

### **2.2.9.6 Production of scFv displaying phage particles**

Following 1 h growth post-transformation in SOC media, 15 mL culture was transferred into 1 L culture flask containing 100 mL of SB media. The culture was supplemented with ampicillin and tetracycline to a final concentration of 100 µg/mL and 10 µg/mL respectively. The culture was then allowed to grow for 90 min shaking at 200 rpm at 37 °C. After 90 min, VCSM13 helper phage was added to a final concentration of 10<sup>12</sup> pfu/mL to each culture flask and the culture was incubated for 2 h with shaking at 200 rpm at 37 °C. Finally, kanamycin was added to each culture flask to a final concentration of 50 µg/mL and the culture was incubated overnight at 200 rpm at 37 °C.

Overnight culture was harvested by centrifugation at 3,300 x g for 20 min at 4 °C. To the culture supernatant, 1/5 volume of 5 X PEG/NaCl (5 % w/v PEG-8000, polyethylene glycol, 2.56 M NaCl) was added and the suspension was incubated for 1 h on ice to precipitate amplified phage. After centrifugation at 10,000 x g for 20 min at 4 °C, the supernatant was discarded and the scFv-phage pellet was resuspended in 0.5 % pBSA in TBS, pH 7.5. The resuspended phage pool was then sterile filtered (0.22 µm) and stored at 4 °C. This constituted the scFv-phage display library preparation.

### **2.2.9.7 BstOI restriction digestion for testing diversity in library**

BstOI restriction endonuclease recognises CC(A/T)GG sequences within the DNA (Roberts, 1990, Bloch and Grossmann, 1995). Differences in BstOI digestion pattern within the scFv-phage display library can be used to demonstrate and visualise sequence differences among the individual clones in a library. Therefore to test the scFv-phage display library for diversity BstOI restriction digestion was performed. Analysis initiated with colony PCR of single clones picked from plates used for transformants counting during library generation. For colony PCR, 25 colonies were picked up from each short and long-linker library and added to PCR tubes containing PCR reaction mixture. For each colony PCR the reaction mix comprised 5 µL of GoTaq green buffer, 2 µL of 25 mM MgCl<sub>2</sub>, 1 µL of 10 mM dNTP, 1 µL of 10 µM each of sense (ompseq) and antisense (gback) primer (Table 2.2), and 0.5 µL of 5 U/µL GoTaq. Final volume of 25 µL was achieved by addition of nuclease free water.

**Table 2.2** Oligonucleotide sequences for colony PCR.

<b>ompseq</b>	5' AAG ACA GCT ATC GCG ATT GCA G3'
<b>gback</b>	5' GCC CCC TTA TTA GCG TTT GCC ATC3'

*PCR thermocycler conditions for colony PCR:*

Denaturation at 94 °C for 5 min then 30 cycles of denaturation at 94 °C for 15 sec, annealing at 56 °C for 30 sec and extension at 72 °C for 2 min followed by cooling to 4 °C.

For restriction digestion 8 µL of PCR product was mixed with BstOI restriction enzyme mix: 2.4 µL of 10 X reaction buffer, 0.24 µL of 10 mg/mL BSA, 0.5 µL of BstOI (100 units/µL) and nuclease free water upto a final volume of 16 µL. Digestion reaction was carried out at 60 °C for 2 h. Finally reaction products were run on 3 % agarose gel for visualisation.

#### **2.2.10 Phage display and selection of scFv against Neu5Gc**

To select and enrich for specific scFv-phage particles against Neu5Gc biopanning was performed using both VL-VH and VL-VHL scFv-phage library preparations. Neu5Gc-PAA as antigen and pBSA for negative selection was coated onto immuno tubes at a concentration of 10 µg/mL in 100 mM sodium bicarbonate buffer, pH 9.6, overnight at 4 °C. The immuno tube was washed twice with 2 mL of TBS. After washing, the immunotube was blocked with 3 mL of 0.5 % pBSA in TBS for 1 h at 37 °C. Following blocking the tube was washed once with 2 mL of TBS-T and once with 2 mL of TBS. The scFv-phage library was then added (200 µL diluted to 2 mL in 0.1 % pBSA TBS), to pBSA coated immuno tube for negative selection and incubated with mixing at 37 °C for 1 h.

Post-negative selection the unbound phage pool was transferred to Neu5Gc-PAA coated immunotube for positive selection and incubated for 1 h at 37 °C. After incubation, the tube was washed 3 times with 2 mL of TBS-T and once with 2 mL of TBS. Bound scFv-phage were then eluted with 1 mL of 100 mM glycine-HCl, pH 2.2, for 10 min. The eluate was collected and neutralised in a 2 mL vial containing 1 mL of 1 M Tris, pH 8.0. Eluted phage pool (1.5 mL) was then added to 4 mL of log phase *E. coli* XL1-Blue culture and allowed to infect for 15 min without mixing at

room temperature. Phage infected bacteria was then made up to 10 mL with fresh SB media.

Phage titration was performed after each round of selection. 10  $\mu$ L of phage infection bacteria were diluted 100 X in SB media, 10  $\mu$ L and 100  $\mu$ L was spread plated onto LB agar plates containing ampicillin 100  $\mu$ g/mL and tetracycline 10  $\mu$ g/mL, this allowed for selection of only those *E. coli* XL1-Blue that were phage infected. Plates were incubated overnight at 37 °C. The number of colonies were counted the following day.

Tetracycline and ampicillin was added to the rest of the phage infection pool to a final concentration of 10  $\mu$ g/mL and 100  $\mu$ g/mL, respectively, and the culture was then grown at 37 °C for 1 h with shaking at 200 rpm. This culture was then transferred to 90 mL of fresh SB media containing 100  $\mu$ g/mL ampicillin and 10  $\mu$ g/mL tetracycline. The VCSM13 helper phage ( $10^{12}$  particles) were added to culture and incubated for a further 2 h at 37 °C at 200 rpm. Finally 50  $\mu$ g/mL kanamycin was added to the culture and grown overnight at 37 °C with shaking at 200 rpm. After overnight growth, the bacteria was harvested by centrifugation at 3,300 x g for 20 min at 4 °C, and the pellet was discarded. The supernatant containing the amplified phage was collected, and scFv-phage was precipitated by addition of 1/5 volume of 5 X PEG/NaCl followed by incubation on ice for 1 h. The precipitated phage was isolated by centrifugation at 15,000 x g for 20 min at 4 °C. The phage pellet was resuspended in 0.1 % of pBSA in TBS. Resuspended phage were centrifuged at 11,600 x g for 5 min at 4 °C to remove contaminating bacterial debris. After centrifugation the supernatant was stored as amplified polyclonal phage-scFv pool at 4 °C for further use. The amplified polyclonal scFv-phage pool was then used as the starting phage pool for the next round of selection. Selection was performed for up to three rounds, with increasing selection stringency mediated by a progressive increase in washing steps from 3 in pan 1, 6 in pan 2 and 9 in pan 3.

#### **2.2.11 Analysis of amplified polyclonal phage pool from three rounds of panning**

Indirect ELISA was employed to analyse amplified polyclonal phage pools from 3 rounds of panning. A 96 well immunoassay plate was coated overnight at 4 °C with 2.5  $\mu$ g/mL of Neu5Gc-PAA as antigen or Neu5Ac-PAA as assay control in 100 mM

sodium bicarbonate buffer, pH 9.6. The plate was washed once with 250  $\mu$ L/well PBS pH 7.4. Wells were blocked with 0.5 % HSA in PBS pH 7.4 for 1 h at 37 °C. The plate was washed 3 times with 250  $\mu$ L/well of PBS-T. Amplified polyclonal phage pool diluted 3 fold in 0.1 % HSA in PBS was then added (100  $\mu$ L/well). The plate was incubated for 1 h at 37 °C and then washed 3 times with PBS-T 250  $\mu$ L/well. Mouse anti-M13 monoclonal antibody conjugated to HRP was diluted 1:5000 in PBS-T and 100  $\mu$ L was added to each well and incubated for 1 h at 37 °C. After incubation the plate was washed 3 times with PBS-T. Washing was followed by addition of HRP substrate, TMB 100  $\mu$ L/well and incubated in the dark for 20 min at room temperature. The HRP-TMB reaction was stopped with the addition of 100  $\mu$ L/well 1 M H<sub>2</sub>SO<sub>4</sub>. Absorbance was measured at 450 nm and recorded on a SpectraMax M5<sup>e</sup> spectrophotometer (Molecular devices, Berkshire, UK).

### **2.2.12 Isolation and analysis of single scFv-phage clones**

From the third round of selection of both VL-VH and VL-VHL, individual clones (49 from VL-VH and 21 from VL-VHL) were picked, cultured and for binding to Neu5Gc in an indirect ELISA format.

In a 96 well culture block, 1 mL fresh SB media supplemented with 1 % (v/v) glucose, 100  $\mu$ g/mL of ampicillin and 10  $\mu$ g/mL tetracycline was inoculated with a single colony picked from plates from the third round of panning. The culture was grown overnight, shaking at 200 rpm at 37 °C. Following overnight growth, the culture was subcultured in 1 mL of fresh SB media supplemented with 1 % (v/v) glucose, 100  $\mu$ g/mL of ampicillin and 10  $\mu$ g/mL of tetracycline. The culture was grown up to log phase (OD<sub>600</sub> 0.6 to 0.8). VCSM13 helper phage (10<sup>12</sup> particles) was then added to the individual cultures to permit phage particle formation and the culture was grown for 2 h with shaking at 200 rpm at 37 °C. After incubation, kanamycin was added to achieve a final concentration of 50  $\mu$ g/mL and the culture was grown overnight with shaking at 200 rpm at 37 °C. The culture was centrifuged at 3,300 x g for 30 min at 4 °C. The pellet was stored for plasmid extraction and the supernatant was transferred to another sterile 96 well culture block. 1/5 volume of 5 X PEG/NaCl was added to the supernatant to induce phage precipitation. The cultures were incubated for 1 h on ice. After incubation the contents from each well were transferred into 1.5 mL centrifuge tubes. The tubes were centrifuged at 15,000

x g for 20 min at 4 °C. The supernatant was discarded and pellet was resuspended in 0.1 % pBSA in PBS pH 7.4. After resuspension, tubes were again centrifuged at 11,600 x g for 5 min at 4 °C. The supernatant was transferred to a sterile 1.5 mL centrifuge tubes and stored as amplified phage from single clones.

Isolated amplified single scFv-phage clones were analysed for binding to Neu5Gc on a 96 well microtiter plate with Neu5Ac as assay control. Briefly, 2.5 µg/mL of Neu5Gc-PAA as antigen or Neu5Ac-PAA as assay control, in 100 mM sodium bicarbonate buffer, pH 9.6, were coated overnight at 4 °C. The plate was washed once with PBS, 250 µL/well. Wells were blocked with 0.5 % HSA in PBS for 1 h at 37 °C. The plate was washed 3 times with 250 µL of PBS-T. Amplified phage pools were diluted 3 fold in 0.1 % HSA in PBS and then added at 100 µL/well. The plate was incubated for 1 h at 37 °C and post-incubation the plate was washed 3 times with PBS-T 250 µL/well. Mouse anti-M13 monoclonal antibody conjugated to HRP at a 1:5000 dilution in PBS-T was added at 100 µL/well and incubated for 1 h at 37 °C. After incubation the plate was washed 3 times with PBS-T 250 µL/well. Washing was followed by addition of HRP substrate, TMB at 100 µL/well and incubated in the dark for 20 min at room temperature. The HRP-TMB reaction was stopped with the addition of 100 µL/well of 1 M H<sub>2</sub>SO<sub>4</sub>. Absorbance measurement were then read at 450 nm and recorded on a SpectraMax M5<sup>o</sup> spectrophotometer.

### **2.2.13 Isolation and analysis of individual scFv clones for soluble expression**

After sequencing analysis, the unique sequences were identified and representative single clone for each unique sequence was picked for soluble scFv expression. The scFv on the phage surface is expressed as an scFv-pIII fusion protein. To allow for expression of scFv without the pIII protein, the pComb3XSS phagemid contains an amber stop codon (TAG) between the scFv and the viral pIII gene. The amber stop codon is suppressed in *E. coli* XL1-Blue and protein translation continues on to pIII, thus making it unsuitable for soluble scFv expression. For soluble scFv expression, pComb3XSS phagemid was transfected into a non-suppressor strain of *E. coli* i.e., TOP10F'. For phagemid extraction, sterile SB media with 100 µg/mL of ampicillin and 10 µg/mL of tetracycline was inoculated from the glycerol stock of short and long-linker clones. The culture was grown overnight at 37 °C, with shaking at 200 rpm. After overnight growth, the cells were pelleted by centrifugation (4000 x g at

4 °C for 20 min). Phagemid extraction was then performed on the bacterial pellet using a Nulceospin plasmid purification kit. Phagemid extraction was followed by a transformation reaction. Competent cells were thawed on ice and 100 µL of *E. coli* TOP10F' competent cells were aliquoted into 1.5 mL pre-chilled centrifuge tubes and finally 5 µL of phagemid preparation was added to respective tube. The tubes were then incubated on ice for 30 min. The mixture was then heat pulsed at 42 °C in a water bath for 45 sec. The tubes were then immediately put on ice and kept on ice for 2 min. For growth and revival of transformed bacteria, 1 mL of pre-warmed SOC media was added to the tubes and incubated for 1 h at 37 °C with shaking at 200 rpm. After 1 h of growth, 2 µL of culture was diluted to 200 µL with SOC media and 50 and 100 µL plated onto LB agar supplemented with 100 µg/mL of ampicillin and 10 µg/mL of tetracycline. Following growth, single colonies were taken and cultured overnight in 20 mL of SB media with antibiotics (100 µg/mL ampicillin and 10 µg/mL tetracycline), at 37 °C and 200 rpm shaking.

Overnight culture was then used as inoculum and diluted 50-fold in 200 mL of LB broth supplemented with 100 µg/mL ampicillin and 10 µg/mL tetracycline. The culture was grown with shaking at 200 rpm at 37 °C until log phase (approximately 0.4 to 0.8), at which point scFv expression was induced by the addition of 1 mM isopropyl-β-D-thiogalactoside (IPTG), post-induction the culture was grown with overnight shaking at 200 rpm at 37 °C. To isolate soluble scFv, bacterial cells were pelleted by centrifugation at 3,500 x g for 20 min at 4 °C and resuspended in equilibration buffer (50 mM Na<sub>2</sub>HPO<sub>4</sub>, 250 mM NaCl, 10 mM imidazole, pH adjusted to 7.4 with phosphoric acid). Periplasmic scFv was then released by sonication, cell debris was removed by centrifugation at 10,000 x g for 30 min at 4 °C, the pellet was discarded and the supernatant was filtered through a 0.45 µm syringe filter followed by another filter of 0.22 µm.

For soluble scFv purification, a 5 mL HisTrap (NiNTA) column and AKTA FPLC was used. Prior to purification the column was equilibrated with 3 column volumes (CV) buffer A (50 mM Na<sub>2</sub>HPO<sub>4</sub>, 250 mM NaCl, 10 mM imidazole, pH adjusted to 7.4 with phosphoric acid). After column equilibration, filtered supernatant was loaded onto the column at a flow rate of 1 mL/min and the flow through of the column was collected for analysis. Loading was followed by a 5 CV wash with

buffer A and 10% of buffer B (50 mM Na<sub>2</sub>HPO<sub>4</sub>, 250 mM NaCl, 250 mM imidazole, pH adjusted to 7.4 with phosphoric acid)., elution was performed in a 10 CV linear gradient from 0 to 100% of buffer B. Eluate was collected in multiple fractions and protein concentration was determined by Bradford assay (Bradford, 1976). Elution fractions showing highest purity on SDS-PAGE (Laemmli, 1970) were pooled and used for characterisation.

#### **2.2.14 Indirect ELISA of unique individual clones**

Indirect ELISA was employed to assess Neu5Gc binding of purified scFv. Neu5Gc-PAA and the assay control Neu5Ac-PAA at 2.5 µg/mL concentration in 100 mM sodium bicarbonate, pH 9.6 were coated (100 µL/well) overnight at 4 °C. The plate was washed once with PBS, 250 µL/well. Wells were blocked with 0.5 % HSA in PBS pH 7.4 for 1 h at 37 °C. The plate was washed 3 times with 250 µL/well of PBS-T. Clarified cell lysate was then applied to each well (100 µL/well). The plate was incubated for 1 h at 37 °C and then washed 3 times with PBS-T 250 µL/well. Anti-HIS antibody conjugated to HRP at 1:10000 dilution in PBS-T was added (100 µL/well) and incubated for 1 h at 37 °C. After incubation, the plate was washed 3 times with PBS-T 250 µL/well. Washing was followed by the addition of HRP substrate, TMB 100 µL/well and incubated in the dark for 20 min at room temperature. The HRP-TMB reaction was stopped with the addition of 100 µL/well of 1 M H<sub>2</sub>SO<sub>4</sub>. Absorbance measurements were then read at 450 nm on a SpectraMax M5<sup>e</sup> spectrophotometer.

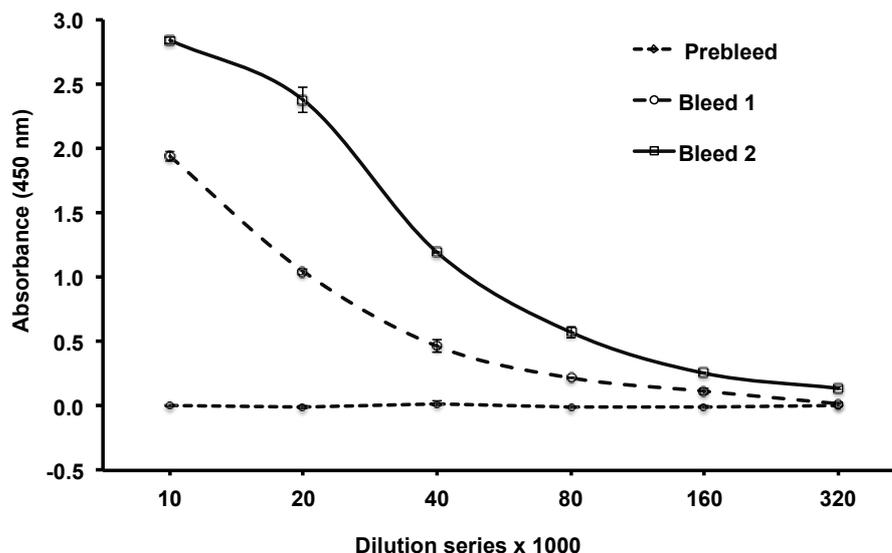
## **2.3. Results**

### **2.3.1 Immunised chicken polyclonal serum analysis**

The first consideration for selecting chickens as appropriate an host for anti-Neu5Gc scFv development was the fact that like in humans, non-human sialic acid Neu5Gc is also immunogenic in chickens. Besides this, chickens react strongly to mammalian epitopes which are otherwise self-tolerated by mammalian hosts such as mice. More importantly since the chicken immunoglobulin repertoire is generated by gene conversion of a single germline V region gene, from upstream V region pseudogenes, fewer primer sets were required for the generation of antibody fragments (Davies et al., 1995). The chickens were immunised with Neu5Gc-glycoconjugate mix and sacrificed at the end of the immunisation regime to permit harvesting of RNA from spleen and bone marrow.

#### **2.3.1.1 Immunised chicken polyclonal serum analysis to assess immune response against Neu5Gc**

The serum collected from immunised chickens at defined time points and the polyclonal immune response against Neu5Gc was assessed and confirmed by indirect ELISA. In indirect ELISA, serum samples were tested across a dilution range to assess binding and recognition to the immobilised antigen (Neu5Gc). Specific immunogenic response from serum samples collected across the different time points during immunisation was observed and estimated based on absorbance value at 450 nm.



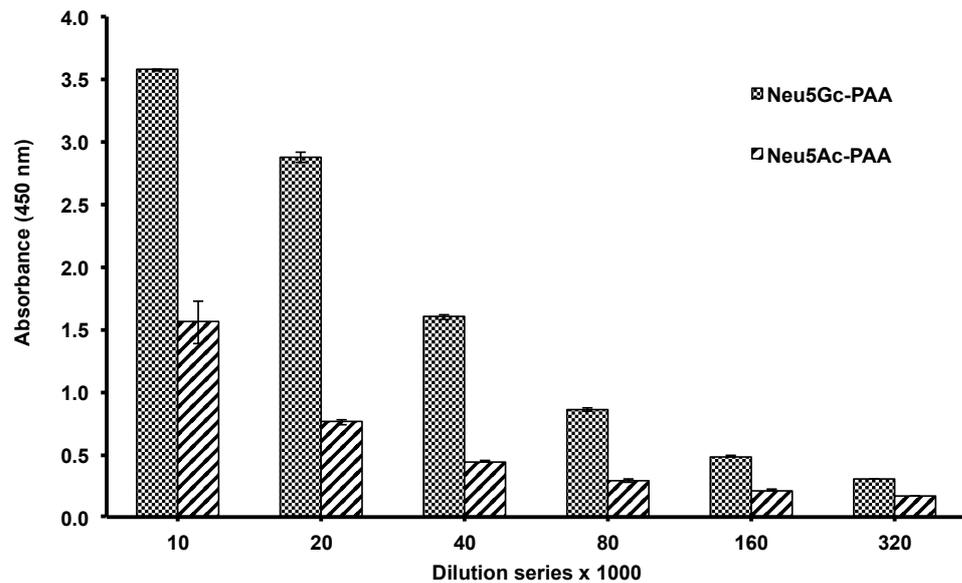
**Figure 2.3.** Chicken serum response to immunisation with Neu5Gc conjugates. Immune response across three bleeds collected at different time points determined by indirect ELISA from 10,000 to 320,000 fold dilution in 0.1 % pBSA in PBS. Serum samples collected across the collection time-points during the immunisation regime, demonstrated increased anti-Neu5Gc response from initial pre-bleed to final bleed 2 indicative of an immune response against the target antigen.

To monitor the immunisation process, serum samples were collected from chickens at defined time points. Serum samples collected after first immunisation were termed as pre-bleed and subsequent serum samples collected after two booster immunisations were called bleed 1 and bleed 2. These serum samples were tested for an immune response against Neu5Gc. Immunised chicken polyclonal serum analysis clearly showed an immune response against Neu5Gc increasing across three bleeds and throughout the dilution series in a concentration dependent manner. Bleed 2 sera showed the highest immune response against Neu5Gc and was selected for further titration and characterisations (Figure 2.3).

### 2.3.1.2 Titration of immunised chicken polyclonal serum

For serum antibody titrations, bleed 2 polyclonal chicken sera was used to detect Neu5Gc in an indirect ELISA assay. Serum samples were diluted at a range of serial dilution for estimation of polyclonal antibody titres. Serum antibody titer was reported as dilution factor of polyclonal chicken sera which showed an absorbance

value representing the steepest part of the dilution series (Figure 2.4). These dilutions were selected for further tests and characterisation activities.

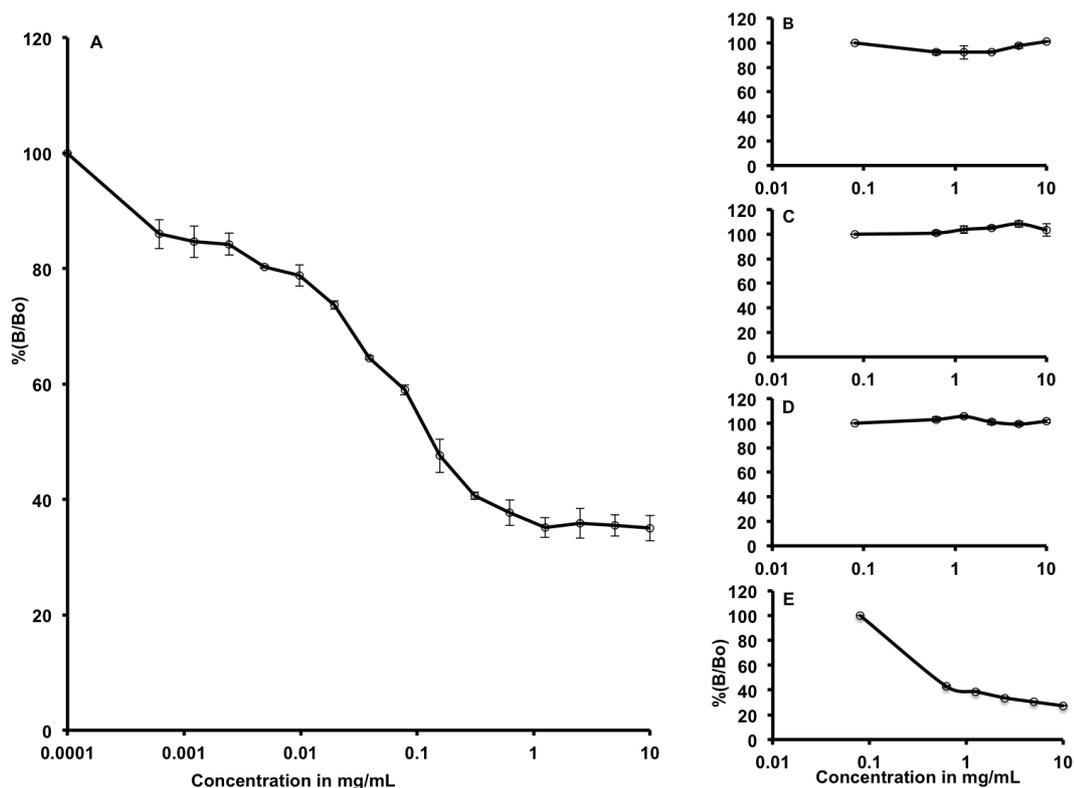


**Figure 2.4.** Serum antibody titration. Titration performed using bleed 2 diluted in 0.1 % pBSA in PBS in a dilution range of 10,000 to 320,000 fold revealed a concentration dependent signal for Neu5Gc-PAA with respect to assay control Neu5Ac-PAA.

Sera titration of bleed 2 indicated specific immunogenic response against Neu5Gc with respect to the Neu5Ac assay control. The signal against Neu5Gc was concentration dependant and at around 1/20,000 to 1/40,000 dilution was considered as steepest point or inflection point on the titration curve. Therefore 1/20,000 dilution was selected for further specificity analysis of the polyclonal chicken sera.

### 2.3.1.3 Specificity of immunised chicken polyclonal sera

Specificity of the polyclonal sera against Neu5Gc was determined in a competitive ELISA format. Neu5Gc-PAA was immobilised and diluted polyclonal sera was used to probe Neu5Gc in the presence of free Neu5Gc sugar at a range of concentrations (10 mg/mL to 1 µg/mL), creating a competition for binding between immobilised and free sugar. Depletion in binding signal was estimated as percentage of binding against a control without competitor. A plot of percentage binding at specific sugar concentration was used to determine specificity. Specificity was assessed based on comparison of inhibition, at a fixed concentration of competitor sugar and a plot generated (Figure 2.5).



**Figure 2.5** Specificity testing of immunised chicken polyclonal sera. (A) Free Neu5Gc showing concentration dependent inhibition of immunised chicken sera. (B) Free Gal- $\alpha$ -(1,3)-Gal, (C) L-Fucose and (D) L-Rhamnose failed to inhibit the sera binding to immobilised Neu5Gc. (E) bovine fetuin displaying Neu5Gc exhibited concentration dependent inhibition of sera binding. The inhibition study clearly shows the specificity of the immunised chicken polyclonal sera towards the free and protein bound Neu5Gc.

Competitive ELISA for polyclonal serum showed specific detection or binding to Neu5Gc as compared to assay controls Gal- $\alpha$ -(1,3)-Gal and monosaccharide sugars (L-fucose (Fuc) and L-rhamnose (Rha)). Bovine fetuin exhibited Neu5Gc specific inhibition of immunised chicken polyclonal sera indicating recognition of Neu5Gc in protein bound form as well.

### 2.3.2 Library generation and optimisation

Phage display library generation started with production of cDNA from RNA isolated from spleen and bone marrow of immunised chickens. The cDNA was further used to generate VL, VH and VHL chain fragments of DNA using primers specific to framework regions of antibody. The primers for VH and VHL (short-linker and long-linker respectively) also introduced a short-linker (7 amino acid) and

long-linker (18 amino acids) in heavy chain fragments. The VL, VH and VHL fragments were combined by an overlap PCR to generate a fused fragment of DNA containing VL-VH and VL-VHL DNA fragments. The fused fragment and phagemid vector (pComb3XSS) were then subjected to SfiI restriction digestion. The digested products were purified and ligated using T4-DNA ligase. Ligated products were transformed into *E. coli* XL1-Blue cells. The average library size for short and long-linker library was  $3.5 \times 10^6$  and  $3.3 \times 10^6$  respectively. Transformed cell pools were grown and expanded into fresh media wherein helper phage (VCSM13) was introduced to assist in phage particle formation. The culture was expanded overnight and harvested next day. The phage was isolated by phage precipitation. Isolated phage stored in pBSA in PBS pH 7.4 at 4 °C.

### **2.3.2.1 cDNA preparation**

cDNA preparations were performed using two approaches; (i) synthesis by OligodT<sub>20</sub> and (ii) synthesis by gene specific primer sets. Both these methods were employed to assess the suitability of one over the other for library generation.

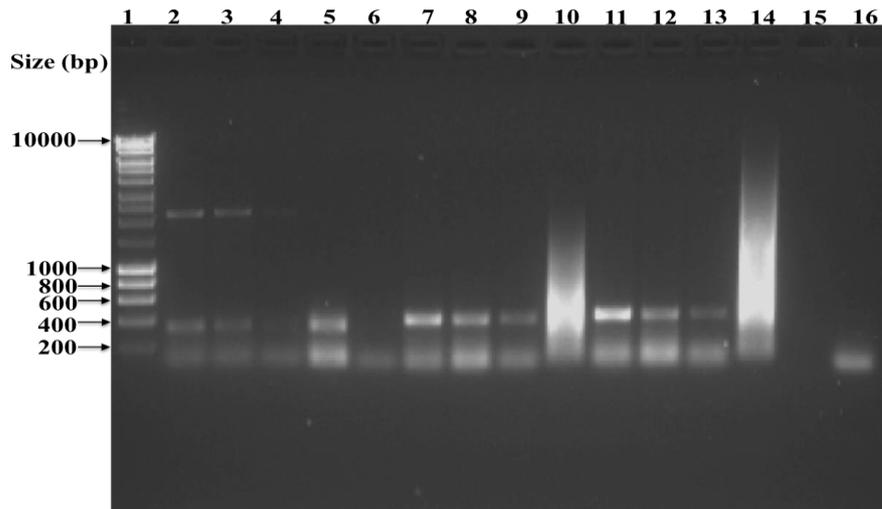
#### **(i) cDNA preparation using OligodT<sub>20</sub>.**

Oligo dT<sub>20</sub> are set of random primers that can bind to poly-A tail region of mRNA and thus can specifically convert mRNA to complementary cDNA. The cDNA preparation using OligodT<sub>20</sub> produces cDNA from all the available mRNA pool.

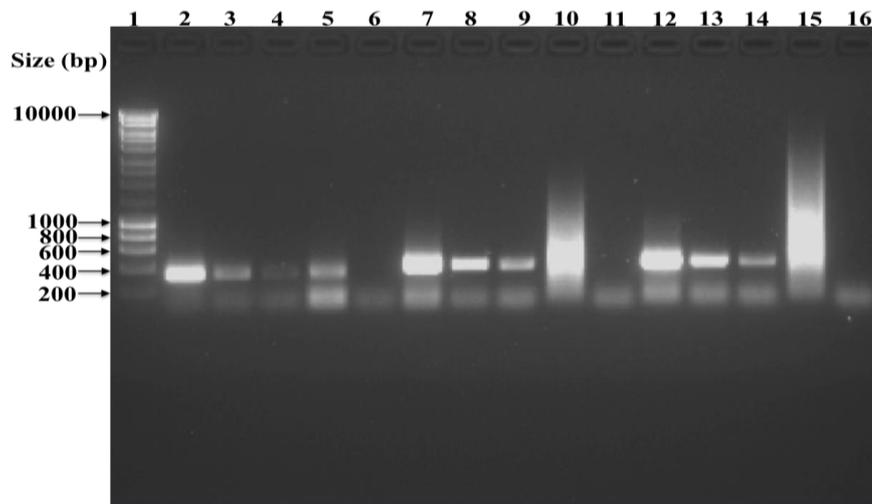
#### **(ii) cDNA preparation using specific primers.**

In this approach, primers specific to antibody framework regions were used for the production of cDNA. Only selected RNA that contain antibody framework regions convert to cDNA, rest of the RNA population degraded by RNases treatment.

The cDNA was produced using both the approaches was tested for amplification of VL, VH and VHL fragment of DNA.



**Figure 2.6** Amplification of VL, VH and VHL fragments from cDNA prepared using OligodT<sub>20</sub>. Lane 1: Hyper ladder™ 1 Kb, lane 2: VL neat template, lane 3: 2 fold diluted VL template, lane 4: 5 X diluted VL template, lane 5: positive control, lane 6: non template control, lane 7: VH neat template, lane 8: 2 fold diluted VH template, lane 9: 5 X diluted VH template, lane 10: positive control, lane 11: VHL neat template, lane 12: 2 fold diluted VHL template, lane 13: 5 X diluted VHL template, lane 14: positive control, lane 15: blank, lane 16: non-template control.

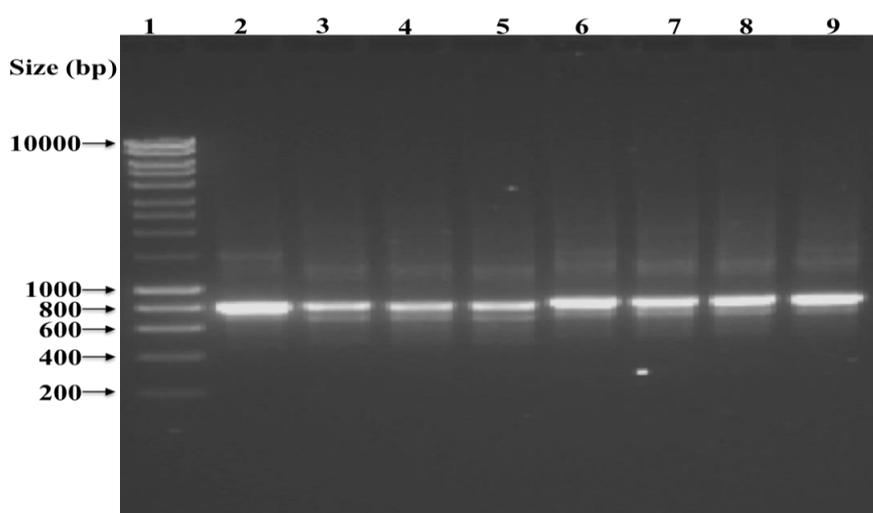


**Figure 2.7.** Amplification of VL, VH and VHL fragments from cDNA prepared using gene specific priming. Lane 1: Hyper ladder™ 1 Kb, lane 2: VL neat template, lane 3: 2 fold diluted VL template, lane 4: 5 X diluted VL template, lane 5: positive control, lane 6: non-template control, lane 7: VH neat template, lane 8: 2 fold diluted VH template, lane 9: 5 X diluted VH template, lane 10: positive control, lane 11: non-template control, lane 12: VHL neat template, lane 13: 2 fold diluted VHL template, lane 14: 5 X diluted VHL template, lane 15: positive control, lane 16: non-template control.

Visual inspection of the gel of amplified VL, VH and VHL fragments produced from both OligodT<sub>20</sub> primed (Figure 2.6) and gene specific priming approach (Figure 2.7) showed that cDNA template produced from gene specific primer approach performed better in PCR amplification of gene fragments (VL, VH and VHL) as compared to OligodT<sub>20</sub> generated cDNA template. Thus PCR product generated from cDNA synthesised by gene specific priming was used for further library generation.

### 2.3.2.2 Generation of fused short-linker (VL-VH) and long-linker (VL-VHL) scFv fragments by overlap PCR

Overlap PCR was employed for fusion of VL and VH for short-linker scFv generation, while VL and VHL fragments were randomly joined for long-linker scFv. Fused fragments were analysed on 1.5 % TAE agarose gel.

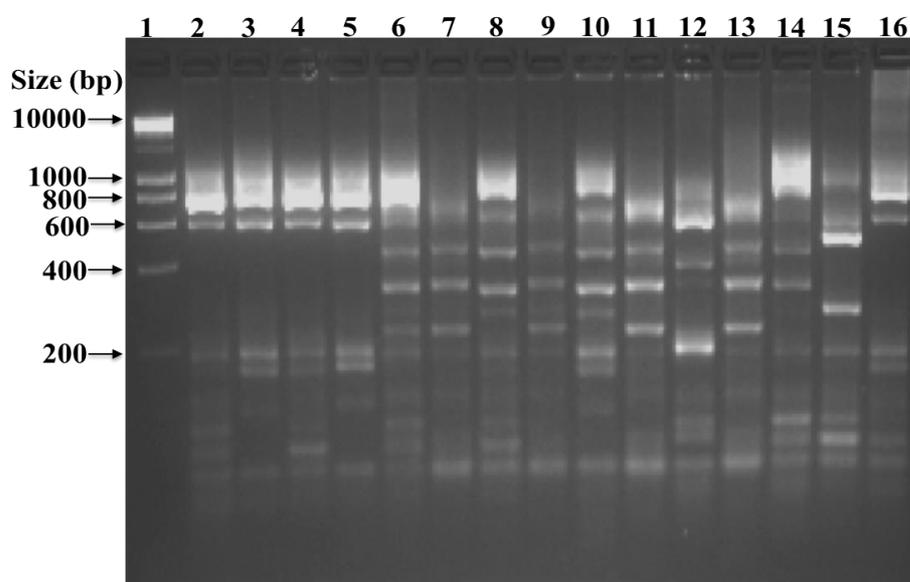


**Figure 2.8** Overlap PCR for generation of short VL-VH and long-linker VL-VHL fused product. Lane 1: Hyper ladder™ 1 Kb, lane 2-5: VL-VH and lane 6-9: VL-VHL fused product run on 1.5 % TAE agarose gel. Product at 750 to 800 bp indicative of successful fusion reaction

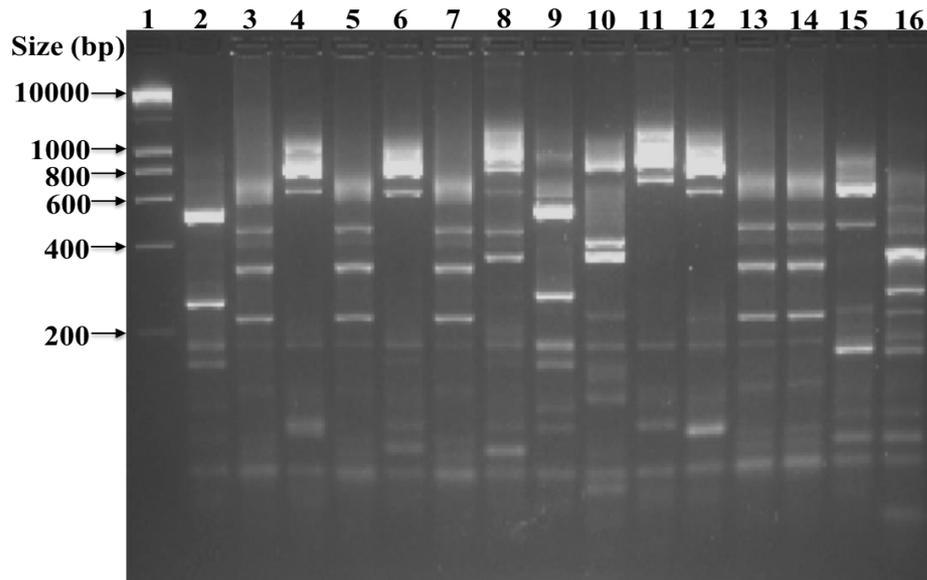
Overlap PCR generated short and long-linker scFv gene fragments. Figure. 2.8 shows fused product at 750 to 800 bp on 1.5 % TAE agarose gel. Analysis of overlap PCR product on agarose gel indicated a successful overlap reaction with good yield of fused product.

### 2.3.2.3 Testing library diversity by BstOI restriction digestion

Overlap PCR generated short, and long-linker full-length scFv fragments. The full-length scFv fragments were cloned into the pComb3XSS phagemid vector and an scFv phage display library was generated. For testing diversity of the scFv libraries single clones were picked and the scFv insert was amplified by colony PCR (Section 2.2.9.7). The amplified scFv insert was then digested with BstOI restriction enzyme. Differences in restriction digestion pattern were visualised after agarose gel electrophoresis to give an indication of the diversity of the library (Figure 2.9, 2.10).



**Figure 2.9** BstOI restriction digestion of representative short-linker clones to assess diversity across short-linker library. Restriction digested samples were loaded and run on a 3 % TAE agarose gel. Lane 1: Hyper ladder™ 1 Kb, lane 2-16: representative short-linker clones. Differences in banding pattern indicate diversity among individual clones across the library



**Figure 2.10** BstOI restriction digestion of representative long-linker clones to assess diversity across long-linker library. Restriction digested samples loaded and run on 3 % TAE agarose gel. Lane 1: Hyper ladder™ 1 Kb, lane 2-16: representative long-linker clones. Differences in banding pattern indicate diversity among individual clones across the library

Restriction digestion of representative short and long-linker clones by BstOI showed differences in banding pattern when separated on a 3 % TAE agarose gel. Differences indicated diversity of sequences among the clones within the library. There was some similarity in the restriction pattern for some clones, which probably indicate a potential *in vivo* selection of antibody fragments during the immunisation process. Other explanation could be a bias of PCR reaction towards certain sequences thus amplifying them preferentially over others.

#### **2.3.2.4 Panning and isolation of anti-Neu5Gc phage population**

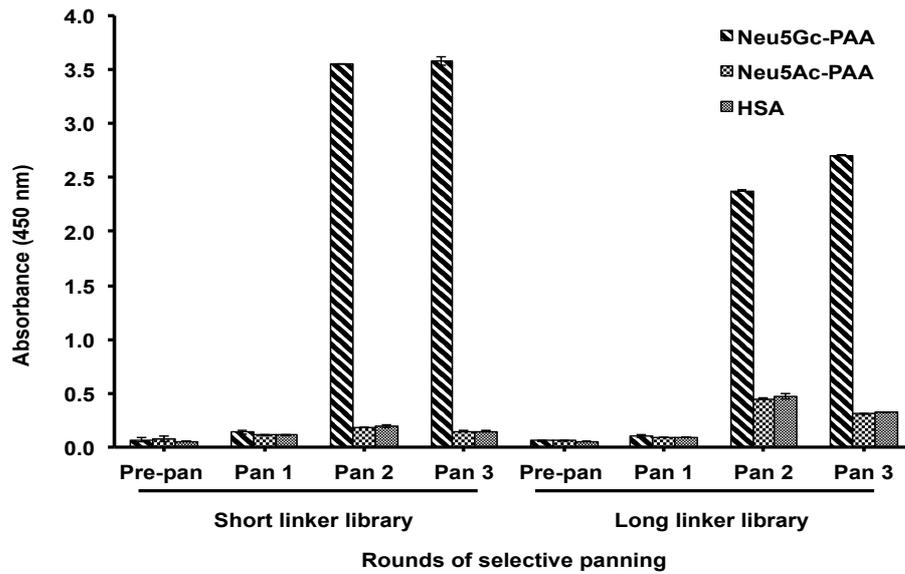
Three rounds of panning were performed for both short and long-linker library. Neu5Gc-PAA was used as antigen for selection and capture of specific scFv-phage. Washes with increasing stringency were performed with each successive round of panning to maximise the potential for selection of high affinity polyclonal scFv-phage population while removing non-specific, low affinity scFv-phage. After washing, bound scFv-phage were eluted and allowed to infect log phase *E. coli* XL1-Blue culture for amplification. Post-infection phage were titered by plating onto LB agar plates supplemented with tetracycline and ampicillin (Table 2.3). The plating of

cultures allows for monitoring the growth and screening of phage infected clones, as some clones may grow slower in culture than others. Therefore, growth of phage infected cells on solid growth media increases the chances of identifying high affinity clones, which may be represented in minority in liquid cultures (Oh et al., 2007).

**Table 2.3** Output titres from short and long-linker library.

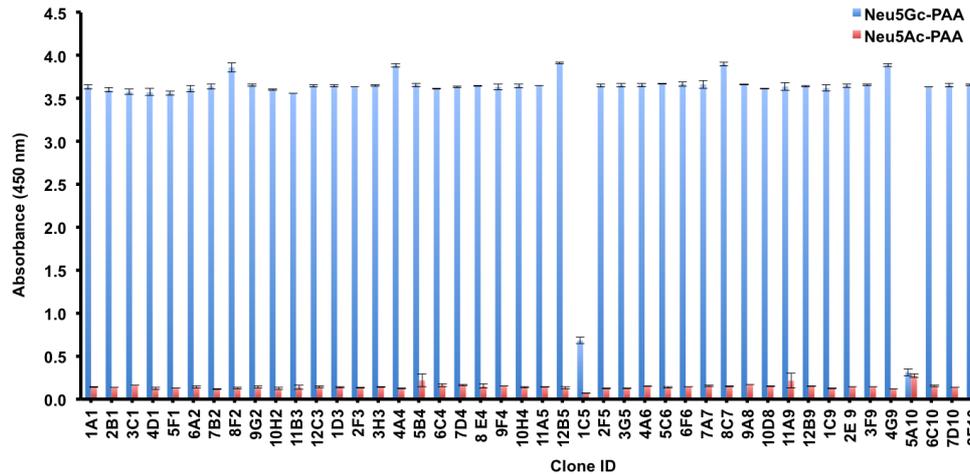
<b>Panning rounds</b>	<b>Titres</b>
Short-linker Pan 1	$2.1 \times 10^6$
Short-linker Pan 2	$2.8 \times 10^6$
Short-linker Pan 3	$1.0 \times 10^7$
Long-linker Pan 1	$3.0 \times 10^5$
Long-linker Pan 2	$6.0 \times 10^5$
Long-linker Pan 3	$9.9 \times 10^6$

Amplified phage pools obtained from three rounds of panning were analysed via indirect ELISA for assessing their binding to Neu5Gc (Figure 2.11). Polyclonal phage ELISA from the three rounds of panning assesses enrichment of antigen specific polyclonal phage population across rounds of panning. Ideally, higher signal for antigen of interest should be achieved at the end of multiple rounds of panning, indicative of selection and amplification of antigen specific phage population.



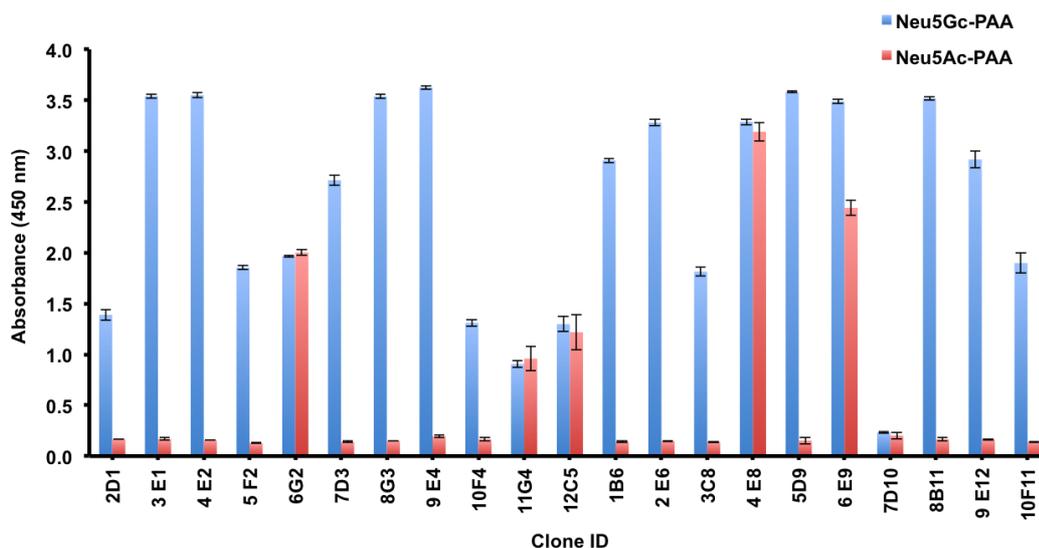
**Figure 2.11** Amplified phage pools from short and long-linker scFv-phage display library before (pre-pan) and after three rounds of selective panning. The panning products were analysed for enrichment of anti-Neu5Gc polyclonal scFv displaying phage pool on an antigen coated (Neu5Gc-PAA, 2.5  $\mu\text{g}/\text{mL}$ ) plate via indirect ELISA, detected with anti-M13-HRP secondary antibody. Signal obtained was compared against assay controls (Neu5Ac-PAA and HSA). Indirect ELISA revealed enrichment of anti-Neu5Gc scFv-phage population from round 2 onwards and stayed constant in third round of panning.

Three rounds of selective panning for both short and long-linker immunised chicken library showed specific enrichment of anti-Neu5Gc polyclonal scFv-phage population as compared to assay controls, Neu5Ac-PAA and HSA. The anti-Neu5Gc polyclonal scFv-phage population enriched during 3 rounds of panning will contain a diverse range of scFv constructs with different affinities and recognition capabilities towards Neu5Gc. Therefore, an assessment for binding to Neu5Gc on an individual clones basis was performed to identify clones that bind to Neu5Gc.



**Figure. 2.12** Analysis of 49 isolated scFv clones from pan 3 of the short-linker library. Each scFv-phage clone was applied to antigen coated (Neu5Gc-PAA, 2.5  $\mu\text{g}/\text{mL}$ ) 96 well microtiter plate blocked with 0.5 % HSA in PBS and incubated for 1 h at 37  $^{\circ}\text{C}$ . The plate was washed with PBS-T 3 times and bound scFv-phage detected with anti-M13-HRP conjugated antibody. The signal obtained was compared against assay control Neu5Ac-PAA. All the clones except one showed recognition of Neu5Gc compared to the assay control. All individual clones were sent for sequencing.

From pan 3 of the polyclonal phage pool of the short-linker library, 49 individual clones were picked and tested by indirect ELISA to assess for Neu5Gc recognition (Figure 2.12). Indirect ELISA showed that almost all clones recognised Neu5Gc specifically compared to the Neu5Ac control. Binding of all the clones also indicated that high level of selectivity and enrichment was achieved throughout the panning process. For further analysis all the clones were sent for sequencing.

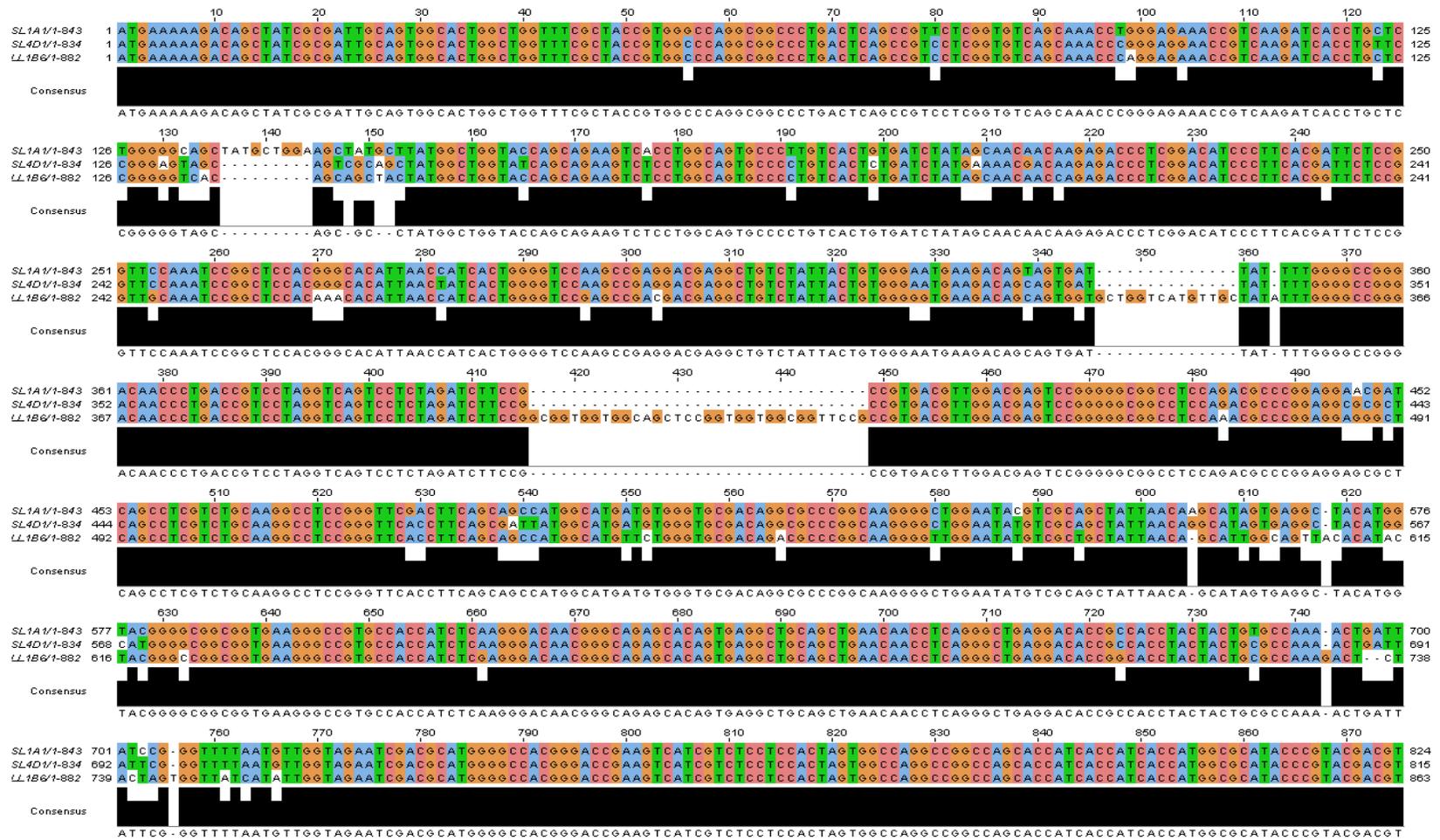


**Figure 2.13** Analysis of 21 isolated clones from pan 3 of long-linker library. Each scFv clone was applied to antigen coated (Neu5Gc-PAA, 2.5  $\mu\text{g}/\text{mL}$ ) 96 well microtiter plate blocked with 0.5 % HSA in PBS and incubated for 1 h at 37  $^{\circ}\text{C}$ . Plate was washed with PBS-T 3 times and bound phase detected with anti-M13-HRP conjugated antibody. The signal obtained was compared against assay control Neu5Ac-PAA. Most of the clones showed recognition of Neu5Gc against assay control. All individual clones were sent for sequencing.

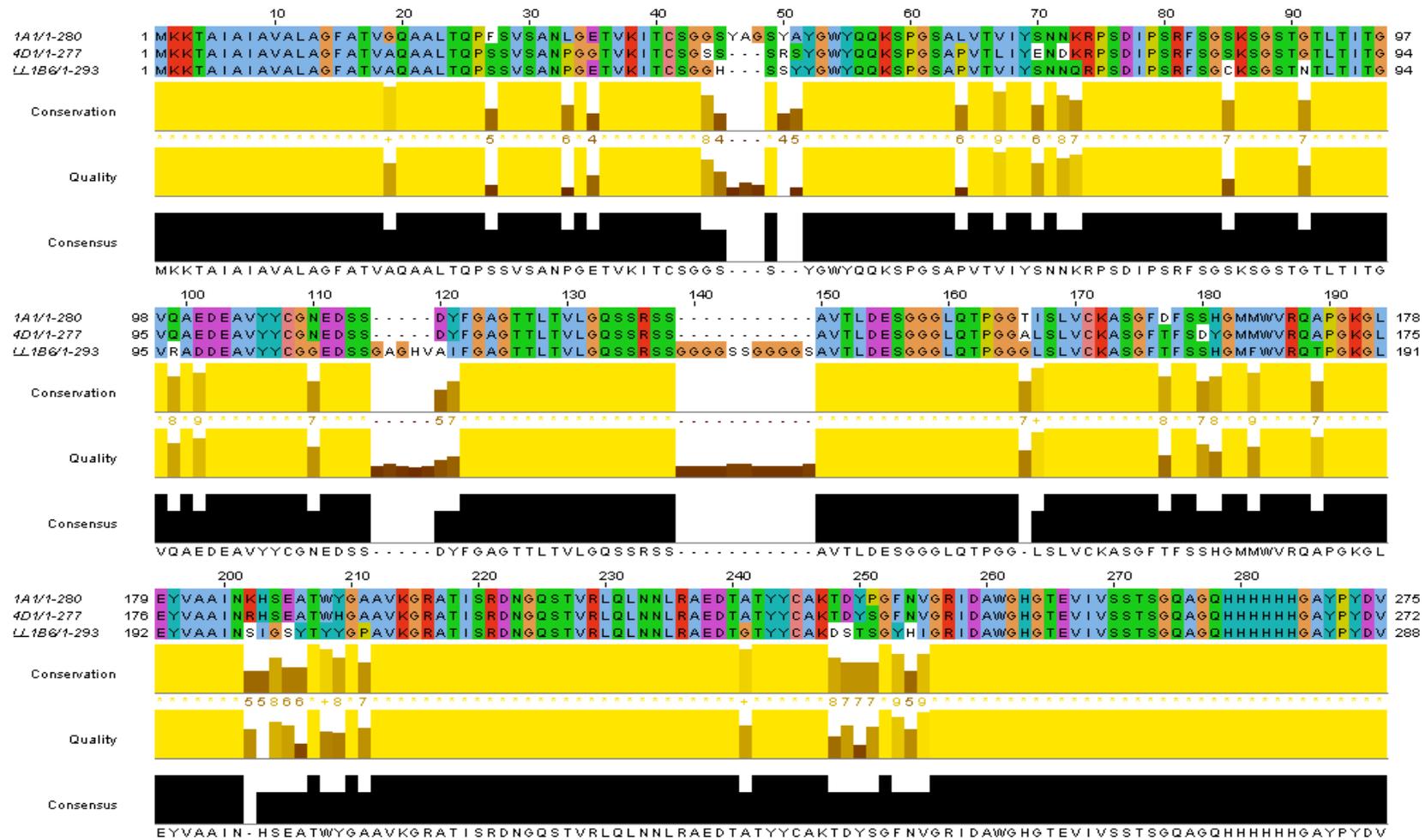
From pan 3 of the polyclonal phage from the long-linker library, 21 individual clones were picked and tested by indirect ELISA (Figure 2.13). Among 21 individual clones tested 16 clones showed specific recognition of Neu5Gc compared to the Neu5Ac control. All the clones from the long-linker library were sent for sequencing.

### 2.3.2.5 Sequence analysis of isolated clones

Individual clones selected from the third round of panning were sent for sequencing (Eurofins MWG operon). Multiple sequence alignment, translation, and recognition of complementarity-determining regions (CDRs) were performed using BLAST (<http://www.ncbi.nlm.nih.gov/BLAST>), ClustalW2 from EBI (<http://www.ebi.ac.uk/Tools/msa/>), and the CDR repository held at <http://www.bioinf.org.uk/abs> (Figure 2.14, 2.15 and 2.16 ).



**Figure 2.14** Alignment of short and long-linker scFv by CLUSTAL W2. The nucleotide alignment of short-linker scFv show similarity and differences among the sequences indicative of selection of unique sequences.



**Figure 2.15** Alignment of amino acid sequence translated from nucleotide sequence of short and long-linker scFv by CLUSTAL W2. Amino acid alignment of three scFv showed presence of unique amino acid sequences among three scFv.

```

                                CDR-L1
1A1      MKKTAIAIAVALAGFATVGQAALTQPFSVSANLGETVKITCSGGSYAGSYAYGWYQQKSP 60
4D1      MKKTAIAIAVALAGFATVAQAALTQPSSVSANPGGTVKITCSGSS---SRSYGWYQQKSP 57
LL1B6    MKKTAIAIAVALAGFATVAQAALTQPSSVSANPGETVKITCSGGH---SSYYGWYQQKSP 57
*****
                                CDR-L2                                CDR-L3
1A1      GSALVTVIYSNNKRPSDIPSRFSGSKSGSTGTLTITGVQAEDEAVYYCGNEDSS-----D 115
4D1      GSAPVTLIYENDKRPSDIPSRFSGSKSGSTGTLTITGVQAEDEAVYYCGNEDSS-----D 112
LL1B6    GSAPVTVIYSNNQRPSDIPSRFSGCKSGSTNTLTITGVRADDEAVYYCGGEDSSGAGHVA 117
*** **:*:*:*:*:*****.*****.*****:*:*****.****

                                Flexible Linker                                CDR-H1
1A1      YFGAGTTLTVLGQSSRSS-----AVTLDESGGGLQTPGGTISLVCKASGFDFFSS 164
4D1      YFGAGTTLTVLGQSSRSS-----AVTLDESGGGLQTPGGALS LVCKASGFTFSD 161
LL1B6    IFGAGTTLTVLGQSSRSSGGGGSSGGGSAVTLDESGGGLQTPGGGLSLVCKASGFTFSS 177
*****
                                CDR-H2
1A1      HGMMWVRQAPGKLEYVAAINKHSEATWYGA AVKGRATISRDNQSTVRLQLNNLRAEDT 224
4D1      YGMMWVRQAPGKLEYVAAINRHSEATWHGA AVKGRATISRDNQSTVRLQLNNLRAEDT 221
LL1B6    HGMFWVRQTPGKLEYVAAINSIGSYTYYGPAVKGRATISRDNQSTVRLQLNNLRAEDT 237
:*:*:*:*:*:***** ..*:*:*****

                                CDR-H3
1A1      ATYYCAKTDYYPGFNVGRIDAWGHGTEVIVSSTSGOAGQH HHHHHGAYPYDVPDYAS 280
4D1      ATYYCAKTDYSGFNVGRIDAWGHGTEVIVSSTSGOAGQH HHHHHGAYPYDVPDYAS 277
LL1B6    GTYYCAKDSTSGYHIGRIDAWGHGTEVIVSSTSGOAGQH HHHHHGAYPYDVPDYAS 293
***** .*:*:*****

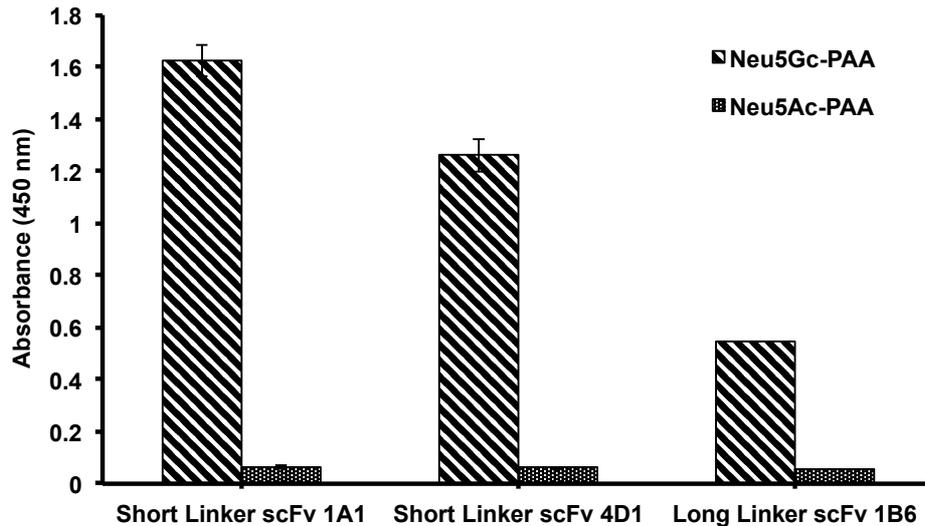
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**Figure 2.16.** Alignment of amino acid sequence of short and long-linker scFv with identified CDR regions. The identified CDRs and framework regions showing differences in amino acid sequences which indicates sequence uniqueness.

After sequencing of short and long-linker clones, based on similarity and differences, 2 unique clones (SL1A1 and SL4D1) were identified from the short-linker and 3 (LL1B6, LLX and LLY) were identified from long-linker library. However, the LLX and LLY clones failed to grow in liquid cultures and were therefore excluded from the sequence analysis. Alignment of amino acid sequences of the short (SL1A1 and SL4D1) and long-linker (LL1B6) clones showed differences across the framework and CDR regions (Figure 2.16). The CDRs are mainly responsible for recognition and binding to antigen. Differences in CDR sequences determine the unique antigen binding characteristics of the scFv molecules. In the VL chain, CDR L1 and CDR L2 differed across short and long-linker scFv. The CDR L3 was similar in the Short-linker scFv while the long-linker scFv differed by one amino acid substitution (isoleucine in place of tyrosine). The CDR H2, H1 and H3 were different across the short and long-linker scFv (Figure 2.16).

#### **2.3.2.6 Analysis of soluble scFv by indirect ELISA**

Phagemid from the selected short and long-linker clones were extracted and transfected into *E. coli* TOP10 F' for soluble expression of scFv. The clones were cultured and induced for scFv expression and the scFv was isolated by affinity purification. The soluble scFv were expressed in TOP10F' by induction with 1 mM IPTG in an overnight culture. The cells were harvested and lysed to extract scFv. The lysate was then clarified by centrifugation and tested by indirect ELISA to show the binding capabilities of the individual short and long-linker scFv (Figure 2.17).



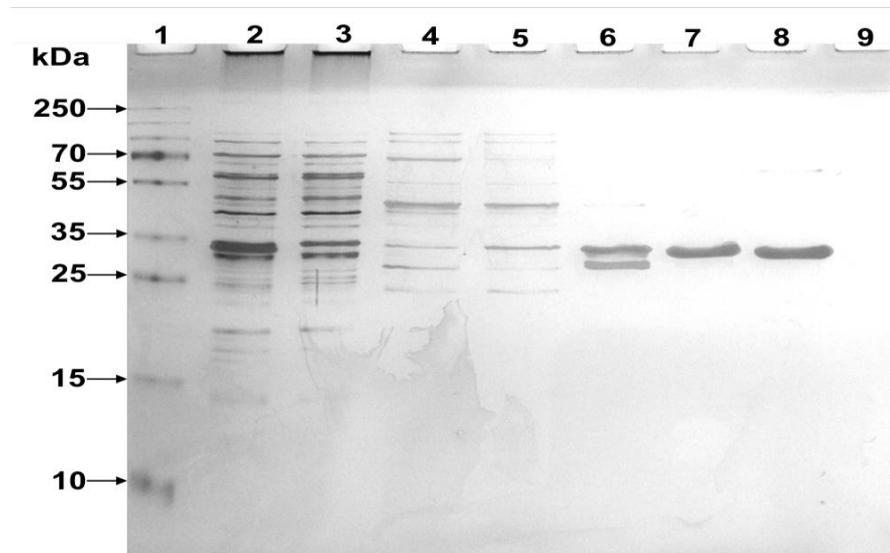
**Figure 2.17** Short and long-linker soluble scFv analysed by indirect ELISA for anti-Neu5Gc binding. The clarified lysate obtained from short and long-linker scFv production cultures were applied onto antigen coated (Neu5Gc-PAA, 2.5 µg/mL) 96 well microtiter plate blocked with 0.5 % HSA in PBS and incubated for 1 h at 37 °C. The plate was washed with PBS-T 3 times and bound scFv was detected with anti-His-HRP conjugated antibody. The signal obtained from binding to Neu5Ac-PAA was compared against the assay negative control, Neu5Ac-PAA.

Analysis of short and long-linker scFv showed successful production of an anti-Neu5Gc scFv, as indicated by specific recognition of Neu5Gc in indirect ELISA. The variability in level of recognition of Neu5Gc was attributed to differential expression or concentration differences of scFv. Since the aim of this experiment was to confirm production of scFv, crude sample as opposed to purified sample was used to perform a rapid assessment of scFv.

### 2.3.2.7 Expression and purification of soluble scFv

Clones identified from the short-linker (SL1A1 and SL4D1) and long-linker (LL1B6, LLX and LLY) libraries were transformed into TOP10F' cells for soluble scFv production. Clones SL1A1, SL4D1 and LL1B6 expressed successfully, however, LLX and LLY failed to express functional or stable scFv. Therefore, the short-linker (SL1A1 and SL4D1) and long-linker (LL1B6) clones showing successful expression of scFv were carried forward for testing and characterisation. After soluble expression, scFv was purified via nitrilotriacetic acid (Ni-NTA) affinity

chromatography and the purification of the protein was confirmed by electrophoresis on a 12 % SDS-PAGE using reducing conditions (Figure 2.18).



**Figure 2.18** Reducing 12 % SDS-PAGE with silver staining showing increasing purity of scFv SL1A1 during purification process. Lane 1: Molecular mass marker, lane 2: load, lane 3: flow-through, lane 4: Buffer A wash, lane 5: 10 % B wash, lane 6: Elution fraction 1, lane 7: Elution fraction 2, lane 8: Elution fraction 3.

## 2.4. Discussion

This chapter details the process of generation and identification of anti-Neu5Gc scFv antibody fragments from spleen and bone marrow isolated from chickens immunised with Neu5Gc. The first step towards the development of antibody-based recognition molecules against epitopes on mammalian proteins requires the immunisation of a suitable host organism with a target protein bearing epitope of interest. Within this current research project, chickens were selected as a suitable host as chickens lack Neu5Gc and have the ability to generate a strong immune response against conserved mammalian antigens which is attributed to vast phylogenetic difference between avian and mammalian species (Schauer et al., 2009, Davies et al., 1995, Carlander et al., 1999). Further, antibodies from mammalian sources show cross-reactivity with other secondary mammalian antibodies used in the assay and therefore increase the background binding in assays. In contrast, the chicken derived antibodies are advantageous in assay development, for chicken antibodies do not show cross-reactivity with other mammalian antibodies (Carlander et al., 1999). Furthermore, the mechanism of diversification of chicken antibodies *in vivo* is relatively less complex compared to mammalian species as both light and heavy chains immune diversification are derived from single conserved germline sequences (Tjoelker, 1993). This germline sequence is altered by a series of gene conversion events, where gene fragments from upstream pseudo genes are swapped into the functional V region gene. This feature of chicken's immune system, with just a single functional V region gene, simplifies the process of amplification of VH and VL as fewer primer sets are required compared to mammals (Davies et al., 1995, Yamanaka et al., 1996).

For library generation the chickens were immunised with Neu5Gc (Neu5Gc glycoconjugate mix containing Neu5Gc-BSA, Neu5Gc-biotin and Neu5Gc-GM3) and the serum samples were collected at specified time points. Prior to library generation, the polyclonal serum derived from immunised chicken was tested for its anti-Neu5Gc immunogenic response in an indirect ELISA (Figure 2.3). The indirect ELISA demonstrated a strong immune response against Neu5Gc as compared to Neu5Ac (negative control). The immune response for Neu5Gc increased from first immunisation (pre-bleed) to second booster immunisation dose (bleed 2). The

immune response against Neu5Gc is consistent with the fact that chickens cannot synthesise Neu5Gc and they generate an immune response against this glycan epitope (Samraj et al., 2014, Noguchi et al., 1995). The polyclonal serum was further characterised to assess the specificity of immune responses against Neu5Gc. Before specificity assessment, the polyclonal serum antibody titers in bleed 2 against Neu5Gc were determined over a range of concentrations from 10,000-fold to 320,000-fold by indirect ELISA. The polyclonal serum antibody titration demonstrated that bleed 2 showed a concentration dependent signal for Neu5Gc as opposed to Neu5Ac negative control. The absorbance for dilution factor of 20,000-fold showed was the steepest point across the titration series (Figure 2.4). The dilution factor that shows the steepest point on the titration curve is selected for competitive ELISA based specificity testing to generate a sensitive inhibition curve. After serum antibody titration, the specificity of polyclonal serum was tested in a competitive ELISA using free and protein (bovine fetuin) bound Neu5Gc. The disaccharide Gal- $\alpha$ -(1,3)-Gal and monosaccharide sugars (L-fucose (Fuc) and L-rhamnose (Rha)) were used as negative control. The competitive ELISA demonstrated inhibition of binding signal to Neu5Gc in the presence of free and protein bound Neu5Gc, while no inhibition was observed in presence of other control sugars (Figure 2.5). This clearly demonstrated the specificity of the polyclonal serum against Neu5Gc.

The bone marrow and spleen are rich sources of lymphocytes and antibody producing memory B-cells. These cells were harvested and total RNA was isolated (Andris-Widhopf et al., 2000, Finlay et al., 2006, Choi et al., 2012, Carlander et al., 1999). The RNA isolated from immunised chickens was then used to generate scFv-phage display libraries. To generate and screen scFv against Neu5Gc, scFv was constructed and displayed on filamentous bacteriophage by phage display technology (Andris-Widhopf et al., 2000, McCafferty et al., 1990). Phage display technology employs a filamentous bacteriophage to display the proteins or antibody fragments as fusion protein attached to pIII coat protein of phage (Barbas et al., 2004). The cDNA generated from RNA of immunised chickens was used to generate VL and VH gene fragments with long and short-linkers. Initial amplification of VL, VH and VHL fragments for library generation is dependent on quality of cDNA. Therefore, in library generation process, cDNA was synthesised by two approaches;

by OligodT<sub>20</sub> priming and by gene specific primers. The cDNA synthesis with OligodT<sub>20</sub> results in synthesis of total cDNA, while with gene specific priming approach only primer specific cDNA pool is synthesised. The cDNA synthesised from both OligodT<sub>20</sub> and gene specific priming was evaluated for amplification of VL, VH and VHL fragments by PCR followed by agarose gel electrophoresis. The cDNA synthesised by gene specific primers showed better amplification of VL, VH and VHL fragments as compared to cDNA synthesised by OligodT<sub>20</sub> (Figure 2.6, 2.7). Since the aim of the library generation was to specifically capture immunoglobulin gene fragments, targeted cDNA synthesis by gene-specific primer approach proved successful. Gene specific synthesis of cDNA has been reported to be successful for capturing rare transcripts as compared to other methods (Frohman et al., 1988). Although genes from immunoglobulins cannot be considered rare, gene specific priming can be used to preferentially produce cDNA of immunoglobulin genes for targeted library generation. In contrast, OligodT<sub>20</sub> assisted synthesis of cDNA is prone to internal poly A priming thus generating truncated cDNA populations (Nam et al., 2002), which can explain the low amplification of VL, VH and VHL gene fragments. Gene fragments amplified (VL, VH and VHL) post cDNA synthesis were joined by a linker region by employing overlap PCR reaction to generate variable light and heavy chain fused product termed as full length scFv fragment. The generation and amplification of VL-VH and VL-VHL fused product was confirmed by agarose gel electrophoresis (Figure 2.8).

The scFv fragments were cloned into the pComb3XSS phagemid vector and transformed into *E.coli* XL1-Blue to allow for display of the scFv fragment on the surface of phage. The library size post-transfection was  $3.5 \times 10^6$  for short-linker and 3.3 fold  $10^6$  for long-linker scFv clones. The library sizes were close to the reported library sizes of  $10^6$  to  $10^7$  for successful phage display library generation (Andris-Widhopf et al., 2000). The vector pComb3XSS used here in library generation is designed to express proteins and antibodies in phage bound form (recombinant protein displayed as a fusion partner with phage coat protein) or in soluble form depending on the use of suppressor or non-suppressor strains of *E. coli* and their effect on the inserted amber stop codon (Barbas et al., 1991). Additionally, the 6 X histidine and hemagglutinin (HA) tags were also introduced for ease of purification and detection of recombinant protein upon soluble expression.

The short and long-linker scFv-phage display library contained scFvs that bind to Neu5Gc with different affinities and specificities. To assess the diversity within the scFv-phage display library, full length scFv insert was restriction digested with BstOI restriction enzyme. Visual differences in fragmentation pattern were indicative of diversity captured within the library (Figure 2.9, 2.10).

As both short and long-linker library represent a diverse pool of scFv, biopanning was performed against Neu5Gc. The selection of antigen and its presentation is critical for successful identification of a recognition molecule (scFv). Neu5Gc was presented as Neu5Gc-BSA, Neu5Gc-biotin and Neu5Gc-GM3 during the immunisation of chickens and thus employing same antigens in the biopanning process could potentially lead to selection of non-specific scFv against the conjugated carrier, thus Neu5Gc was presented on a PAA backbone. In biopanning, the iterative process of selection and amplification selects for and enriches specific phage populations that bind to Neu5Gc. The biopanning was performed for 3 rounds and the amplified phage pool obtained after each round of panning was analysed for Neu5Gc binding in an indirect ELISA (Figure 2.11). The amplified phage pools clearly showed the enrichment of Neu5Gc binding scFv-phages across the 3 rounds of panning for both short and long-linker library. In contrast, no binding was observed for Neu5Ac-PAA that was used as negative control. The short and long-linker amplified polyclonal scFv-phage pool from pan 3 was analysed for Neu5Gc binding by indirect ELISA (Figure 2.12, 2.13). Of the 70 single clones tested, 48 clones out of 49 with a short-linker and 16 clones out of 21 with a long-linker showed binding to Neu5Gc as compared to Neu5Ac. All the single clones tested were also analysed by direct nucleotide sequencing. In combination, both single clone ELISA and nucleotide sequencing are used to reduce the number of single scFv clones to be examined and further tested (Andris-Widhopf et al., 2000). The nucleotide sequences of short and long-linker clones were aligned and to assess the similarity and differences among the Neu5Gc binding clones. From sequence analysis of all the short and long-linker clones, 2 unique sequences emerged from short-linker library (SL1A1 and SL4D1) and 3 unique sequences were identified from long-linker library (LL1B6, LLX and LLY). Since the long-linker clones LLX and LLY failed to express in soluble scFv form, both the clones were excluded from further sequence analysis.

The CDR region for SL1A1, SL4D1 and LL1B6 were identified and compared for uniqueness at the individual amino acid level (Figure 2.15, 2.16). The light chain CDR L1 and L2 were different for both the short and long-linker clones. Both the short-linker clones had similar CDR L3 region. The heavy chain CDR H1, H2 and H3 were different among the short and long-linker clones. The unique short and long-linker clones were then expressed in soluble form without its fusion partner pIII phage protein. Soluble expression of scFv leads to accumulation of scFv in the periplasmic space within the host *E. coli* permitting cells to be lysed and scFv extracted and purified by metal affinity chromatography. Both short and long-linker scFv were tested for their ability to recognise Neu5Gc by indirect ELISA (Figure 2.17). The analysis of short and long-linker scFv demonstrated binding to Neu5Gc as opposed to Neu5Ac. Overall an immunised chicken derived phage display library was successfully generated and was used to isolate two short and one long-linker anti-Neu5Gc scFv. The scFv identified against Neu5Gc are to be carried forward in the development of an ELISA-based assay for the detection of Neu5Gc in free and bound form (Chapter 3).

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## **Chapter 3**

### **ELISA-based assay development & characterisation of scFv**

### 3.1 Introduction

This chapter focuses on development and optimisation of a competitive ELISA assay for the detection and quantitation of the non-human sialic acid, Neu5Gc. Various assays are available to detect and quantify sialic acids including colorimetric and fluorimetric assays, which can measure the free and bound form of sialic acid (Warren, 1959, Skoza and Mohos, 1976, Shukla and Schauer, 1982, Jourdian et al., 1971). However, these assays cannot distinguish between Neu5Gc and Neu5Ac. This shortcoming of colorimetric and fluorimetric assays is overcome by sensitive and specific detection of Neu5Gc by HPLC and MS based methods. In HPLC based methods, either Neu5Gc is released, hydrolysed, benzoylated and detected by ultraviolet detection (reverse-phase HPLC) or the released Neu5Gc is labeled with a fluorophore and separated on HPLC with fluorescent detection (Hara et al., 1987, Karamanos et al., 1990, Hurum and Rohrer, 2011, Kawabata et al., 2000). MS is another method, which in itself or in combination with HPLC (LC/MS) is also used for the specific detection of Neu5Gc (Zarei et al., 2010, Wang et al., 2014). However, both the HPLC and MS based methods are not cost effective, require complex sample preparation and need a highly skilled operator for operational running and data analysis (Lee et al., 1990, Ruhaak et al., 2010).

Lectins have been utilised in the development of an enzyme linked lectin assay (ELLA) for the detection of sialylation on transferrin (Gornik and Lauc, 2007). Various plant and animal derived lectins can detect sialic acids in different presentations for example SNA binds to  $\alpha$ -(2,6) and MAA binds to  $\alpha$ -(2,3)-linked Neu5Gc or Neu5Ac (Song et al., 2011). However, poor specificity and cross-reactivity of lectins to glycan epitopes renders them unsuitable for routine assay applications (Lehmann et al., 2006, Haab, 2012).

Recently, an ELISA based assay using affinity purified polyclonal anti-Neu5Gc antibodies isolated from chickens immunised with Neu5Gc has been developed and applied for detection of Neu5Gc. It is able to detect Neu5Gc in healthy human tissues like prostate, spleen and brain; in cancer tissues from melanoma, neuroblastoma and ovarian carcinoma, and on cetuximab, an FDA approved biopharmaceutical drug (Tangvoranuntakul et al., 2003, Diaz et al., 2009, Ghaderi et al., 2010). However, polyclonal anti-Neu5Gc antibodies cannot be manufactured

with defined affinity, consistently and reliably at production scale, making them inappropriate for routine assay development and standardisation. To overcome this limitation, three scFv molecules were generated and identified by phage display technology, demonstrating specificity to Neu5Gc (Chapter 2) and used to develop a sensitive assay for detection and quantitation of Neu5Gc.

In the initial phase of assay development, immunised chicken polyclonal serum was used to develop and optimise the assay parameters for Neu5Gc detection (Section 3.2.1). The knowledge gained from sera-based assay optimisation was employed in scFv-based assay development. The three scFv identified were first tested for their suitability by assessing their binding to Neu5Gc in a competitive ELISA. In competitive ELISA, free antigen (Neu5Gc) is used to inhibit the binding of antibody (scFv) to immobilised antigen. A concentration dependent decrease in signal generates an inhibition curve. The absorbance signal is normalised by dividing the binding response for each concentration of free antigen by the maximum binding response (sample without free antigen). The inhibition curve can then be used to calculate the concentration of antigen in the test samples. The free antigen, Neu5Gc, used for generating the inhibition curve is a highly acidic sugar and can vary the pH of buffers, resulting in pH dependent rather than concentration dependent inhibition response. Therefore, studies were carried out to evaluate the effect of Neu5Gc concentration on buffer pH and also to identify suitable buffering capacity to maintain pH specified for the assay. Further, the scFv binding to Neu5Gc was also evaluated at a wider pH range and at different buffering capacity. To prevent the pH effects due to free Neu5Gc in assay operation, the glycoprotein bovine transferrin which carries Neu5Gc, was evaluated for use in inhibition and generation of an inhibition curve. The concentration of Neu5Gc in bovine transferrin as determined by HPLC analysis can then be utilised, permitting the determination of Neu5Gc in an unknown test sample. A competitive ELISA based assay using bovine transferrin as a standard for inhibition is presented and validated.

## **3.2 Material & Methods**

Rabbit anti-chicken IgY-horse radish peroxidase (HRP) polyclonal antibody was purchased from Promega (UK). Monoclonal anti-HIS HRP-labeled antibody (antibody against 6 X histidine tag), human serum albumin (HSA), bovine serum albumin (BSA), bovine fetuin, bovine asialofetuin, bovine transferrin and human transferrin were sourced from Sigma-Aldrich Co., (Dublin, Ireland). ELISA substrate 1-Step® Ultra TMB (tetramethylbenzidine) was sourced from Fisher Scientific, (UK). Syringe filters 0.22 and 0.45 µm, F8 Maxisorp immuno module were from Thermo Scientific, (Dublin, Ireland). Free sugars Neu5Gc, Neu5Ac and Gal- $\alpha$ -(1,3)-Gal and glycoconjugate Neu5Gc-polyacrylamide (PAA) were purchased from Dextra, (Reading, UK) and Lectinity Holdings, Inc (Russia) respectively. All other reagents were from Sigma-Aldrich Co. unless indicated otherwise and were of the highest grade available.

### **3.2.1 Polyclonal assay development and optimisation with immunised sera**

Chicken sera obtained after immunisation with Neu5Gc-glycoconjugate mix was tested for its immunogenic response against Neu5Gc (Section 2.2.8). The sera was then examined for its suitability for the development of an assay for the detection of Neu5Gc. The assay was designed as a competitive ELISA, wherein the immunised chicken polyclonal sera was allowed to bind to the immobilised antigen, Neu5Gc-PAA, in the presence of known concentrations of free antigen (Neu5Gc). The free antigen acts by competing for the binding of anti-Neu5Gc polyclonal antibodies to the immobilised antigen in a concentration dependent manner (increasing concentration of free Neu5Gc decreases endpoint binding signal for immobilised Neu5Gc). The binding signal is determined by the measurement of the signal generated by anti-chicken IgY-HRP secondary antibody on reaction with tetramethylbenzidine (TMB) substrate. During assay development the parameters of antigen coating concentration (Section 3.2.1.1), blocking agent (Section 3.2.1.2) and Tween 20 concentration (Section 3.2.1.3) were optimised.

#### **3.2.1.1 Optimisation of antigen coating concentration**

To determine appropriate antigen coating concentration for ELISA assay, indirect ELISA was performed at three antigen coating concentrations. A 96 well

immunoassay plate was coated overnight at 4 °C with 100 µL/well of Neu5Gc-PAA at 1.0, 2.5 and 5.0 µg/mL in 100 mM sodium bicarbonate buffer, pH 9.6. The plate was then washed twice with PBS, pH 7.4. The wells were then blocked with 0.5 % w/v pBSA (periodated BSA) in PBS, pH 7.4, for 1 h at 37 °C and then washed 3 times with 250 µL/well of PBS-T.

The immunised chicken serum sample was serially diluted from 10,000 to 320,000 fold in 0.1 % pBSA in PBS pH 7.4, and then aliquoted in 100 µL aliquots. Plates were then incubated at 37 °C for 1 h, and washed 3 times with 250 µL/well of PBS-T. Rabbit anti-chicken IgY-HRP was diluted 1,000-fold in PBS-T and added to each well (100 µL/well) and incubated for 1 h at 37 °C. The plate was then washed 3 times with 250 µL/well of PBS-T, and tapped dried on paper towel. Then, 100 µL of the HRP substrate, TMB, was added to each well and incubated with shaking in the dark for 20 min at room temperature. The HRP-TMB reaction was stopped with the addition of 100 µL/well of 1 M H<sub>2</sub>SO<sub>4</sub>. Absorbance was then read at 450 nm on a SpectraMax M5e plate reader.

### **3.2.1.2 Optimisation and evaluation of blocker and standard diluent**

For assessment of blocking agents, BSA and pBSA were selected for testing. A 96 well immunoassay plate was coated overnight at 4 °C with 100 µL/well of Neu5Gc-PAA at 2.5 µg/mL in 100 mM sodium bicarbonate buffer, pH 9.6. Coating with Neu5Ac-PAA at the same concentration was used as an assay control. The plate was then washed twice with PBS. Wells were blocked with 0.5 % w/v of either BSA or pBSA in PBS for 1 h at 37 °C and then washed 3 times with 250 µL/well of PBS-T.

As the diluent used for sera dilution also contained blocking agents, consistency was maintained by diluting immunised chicken serum sample serially to 40,000 and 160,000 fold in pBSA (0.1 % and 0.5 % w/v) and BSA (0.1 % and 0.5 % w/v) in PBS. The diluted samples were then aliquoted 100 µL/well, incubated at 37 °C for 1 h, and washed 3 times with 250 µL/well of PBS-T. Rabbit anti-chicken IgY-HRP, acting as a secondary antibody, was diluted 1,000-fold in PBS-T and added to each well (100 µL/well) and incubated for 1 h at 37 °C. The plate was then washed and tapped dried on paper towel. Then, 100 µL of the HRP substrate, TMB, was added to each well and incubated with shaking in the dark for 20 min at room temperature.

The HRP-TMB reaction was stopped with the addition of 100  $\mu\text{L}$ /well of 1 M  $\text{H}_2\text{SO}_4$ . Absorbance was then read at 450 nm on a SpectraMax M5e plate reader.

### **3.2.1.3 Determination of Tween 20 concentration in wash buffer**

To determine optimal detergent concentration in wash buffer indirect ELISA was employed. The 96 well plate was coated with Neu5Gc-PAA at 2.5  $\mu\text{g}/\text{mL}$  in 100 mM bicarbonate buffer pH 9.6. Assay controls Neu5Gc-PAA and pBSA were also coated in matched concentrations. The immunised chicken polyclonal sera, diluted serially upto 40,000 fold in 0.1 % pBSA, was used to probe surface immobilised antigen. All other steps of the ELISA procedure remained same as Section 3.2.1.1, with the exception of testing wash buffers with different concentrations of Tween 20 (0.05 % to 1 %) in PBS, pH 7.4, for washing the plate after each incubation. To maintain consistency of Tween 20 concentration, rabbit anti-chicken IgY-HRP secondary antibody was diluted 1,000-fold in corresponding Tween 20 (0.05 % to 1 %) containing PBS and added 100  $\mu\text{L}$ /well.

### **3.2.2 Monoclonal, scFv ELISA for the detection and quantification of Neu5Gc**

As described in Chapter 2, sequence analysis of scFv antibody fragments (monoclonal) highlighted two Short-linker (SL1A1 and SL4D1) and one long-linker (LL1B6) scFv antibody fragments which were carried through for assay development (Section 2.3.2.5). The analysis were aimed to assess the binding scFv to Neu5Gc. The scFv showing better binding was selected for further characterisation and assay development.

#### **3.2.2.1 Titration of scFv Short-linker and long-linker antibody fragments**

Short (SL1A1 and SL4D1) and long-linker (LL1B6) scFv were titrated to determine optimal protein concentration required to measure anti-Neu5Gc binding by indirect ELISA. For indirect ELISA, a 96 well immunoassay plate was coated overnight at 4  $^{\circ}\text{C}$  with 100  $\mu\text{L}$ /well of Neu5Gc conjugated to polyacrylamide (Neu5Gc-PAA) at 2.5  $\mu\text{g}/\text{mL}$  in 100 mM sodium bicarbonate buffer pH 9.6. Neu5Ac-PAA at the same coating concentration was used as assay control. After coating overnight, the plate was washed twice with PBS pH 7.4. Wells were then blocked with 0.5 % human serum albumin (HSA) in PBS pH 7.4 for 1 h at 37  $^{\circ}\text{C}$ . Post blocking, wells were washed 3 times with 250  $\mu\text{L}$ /well of PBS-T. The optimal scFv concentrations were

determined by serially diluting the affinity purified scFv stock solution in 0.1 % HSA in PBS at a range of dilutions (10,000 fold to 320,000 fold). The diluted scFv preparations, acting as the monoclonal primary antibody molecule, were applied (100  $\mu$ L/well) to the immunoassay plate and incubated for 1 h at 37 °C. The immunoassay plate was then washed 3 times with 250  $\mu$ L/well PBS-T. Anti-HIS-HRP, as secondary antibody, was diluted 10,000-fold in PBS-T and added to each well (100  $\mu$ L/well) and incubated for 1 h at 37 °C. Post incubation, the plate was washed 3 times with 250  $\mu$ L/well of PBS-T, and tapped dried on paper towel. Washing was followed by addition of 100  $\mu$ L/well of the HRP substrate TMB, and incubated with shaking in the dark for 20 min at room temperature. The HRP-TMB reaction was stopped with the addition of 100  $\mu$ L/well of 1 M H<sub>2</sub>SO<sub>4</sub>. Absorbance was then read at 450 nm and recorded on a SpectraMax M5e plate reader.

### **3.2.2.2 Specificity and generation of inhibition curve against Neu5Gc**

In competitive ELISA the binding of anti-Neu5Gc short and long-linker scFv to Neu5Gc was inhibited using free Neu5Gc. Reduction in binding signal was estimated as percentage of binding against a control without competitor. A plot of percentage binding over the free antigen concentration range permitted the generation of an inhibition curve. Specificity was assessed based on comparison of inhibition curves generated using competitors other than Neu5Gc. This comparative study was conducted to identify the best scFv candidate amongst short and long-linker scFv for further characterisation and assay development.

Briefly, a 96 well immunoassay plate was prepared as detailed in Section 3.2.2.1, ELISA plate preparation. Neu5Gc free sugar was diluted from 10 mg/mL (30.7 mM) to 1  $\mu$ g/mL (3.75  $\mu$ M) in 0.1 % HSA in PBS pH 7.4. Similarly assay controls (free sugars: Neu5Ac, Gal- $\alpha$ -(1,3)-Gal; sialoglycoproteins: fetuin and asialofetuin) were prepared in matched concentration ranges (1  $\mu$ g/mL to 10 mg/ mL) in 0.1 % HSA in PBS pH 7.4. To each well 50  $\mu$ L of diluted free sugar was added, followed by the addition of 50  $\mu$ L of diluted short and long-linker scFv.

After addition of the samples and controls, the microtiter plate was incubated at 37 °C for 1 h. Post incubation the microtiter plate was washed 3 times with PBS-T (250  $\mu$ L/well). Then 100  $\mu$ L of anti-HIS-HRP at 1:10,000 dilution in PBS-T was added to each well and incubated for 1 h at 37 °C. After incubation the plate was

washed 3 times with PBS-T, and tapped dried inverted on paper towel. Washing was followed by the addition of TMB solution (100  $\mu$ L/well), with incubation in the dark for 20 min at room temperature. The reaction was stopped with 100  $\mu$ L/well of 1 M H<sub>2</sub>SO<sub>4</sub>. Absorbance measurement was then recorded at 450 nm.

### **3.2.3 Selection of scFv-SL1A1 for assay development**

From specificity testing and inhibition curve comparison of short (SL1A1 and SL4D1) and long-linker (LL1B6) scFv, SL1A1 Short-linker scFv was selected as the optimal molecule for the detection of Neu5Gc and carried forward for further characterisation and assay development. The assay development regime was designed to optimise parameters including, blocking reagents, pH and buffers that could influence ELISA based assay development.

#### **3.2.3.1 Evaluation of blockers for use in Neu5Gc detection by scFv-SL1A1**

The study for blocker assessment was conducted in two phases. In phase I of this study various blockers were used in place of antigen for coating plates to assess their cross-reactivity with scFv in an indirect ELISA format. A 96 well immunoassay plate was coated with 100  $\mu$ L/well of 2.5  $\mu$ g/mL pHSA, pBSA, BSA and HSA in 100 mM sodium bicarbonate buffer pH 9.6 and incubated overnight at 4 °C. The plate was washed twice with 250  $\mu$ L/well of PBS pH 7.4. The plate was then blocked with corresponding blocking buffer (0.5 % pBSA, pHSA, BSA and HSA) in PBS for 1 h at 37 °C. Blocking was followed by 3 washes with 250  $\mu$ L/well of PBS-T. After washing, 100  $\mu$ L of scFv-SL1A1 at a concentrations range (2.5 to 400 ng/mL in PBS pH 7.4) were added for each blocking condition across the plate. The plate was incubated for 1 h at 37 °C and post incubation, the plate was washed 3 times with 250  $\mu$ L/well of PBS-T. Anti-HIS-HRP at 1:10,000 dilution in PBS-T was added (100  $\mu$ L/well) and incubated for 1 h at 37 °C. After incubation the plate was washed 3 times with PBS-T 250  $\mu$ L/well. Washing was followed by addition of HRP substrate, TMB, 100  $\mu$ L/well and incubated in the dark for 20 min at room temperature. The reaction was stopped with 100  $\mu$ L/well of 1 M H<sub>2</sub>SO<sub>4</sub>. The absorbance was recorded at 450 nm on SpectraMax M5e (Molecular devices).

In phase II of the study, the blockers selected from the first phase were compared in an indirect ELISA format. The 96 well immunoassay plate was prepared as detailed

in Section 3.2.2.1 except that post coating two different blockers selected from the first phase of testing (0.5 % pBSA and HSA) were used for blocking. The scFv-SL1A1 diluted across a concentration range (20 ng/mL to 0.3 ng/mL) in both 0.1 % pBSA and HSA in PBS pH 7.4, were applied onto each well (100  $\mu$ L/well). The washing of plate post incubations and detection with secondary antibody was the same as detailed in Section 3.2.2.1.

### **3.2.3.2 Evaluation of pH effects on binding of scFv-SL1A1**

As the Neu5Gc is an acidic sugar, it can change the pH of standard assay buffers used in ELISA. To study the pH effect of Neu5Gc, the study was conducted in two phases. In Phase I, the studies were conducted to assess the impact of free Neu5Gc on pH of the standard PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na<sub>2</sub>HPO<sub>4</sub>, 1.8 mM KH<sub>2</sub>PO<sub>4</sub>, pH adjusted to 7.4 with phosphoric acid) used in the assay. For this study, 50 mg of free Neu5Gc was dissolved in 1 mL of deionised water and then diluted to a concentration of 2.5 mg/mL and 0.25 mg/mL in PBS with increasing buffering capacity (10 mM to 200 mM phosphate buffer pH 7.5). The diluted Neu5Gc was dispensed (100  $\mu$ L/well) in a 96 well microtiter plate. As a control, matched concentrations of PBS (10 mM to 200 mM phosphate buffer pH 7.5) without Neu5Gc was dispensed in 100  $\mu$ L aliquots for reference. The plate was incubated at 37 °C for 1 h. Post incubation the pH in each well was measured with micro pH meter.

In phase II, the binding characteristic of scFv-SL1A1 was studied across a wider pH range (pH 2.5 to pH 7.5). To study the binding of scFv-SL1A1 in different pH conditions, the scFv was diluted to 10 ng/mL in 0.1 % pBSA in PBS with pH ranging from pH 2.5 to pH 7.5, in steps of 0.5 pH units. These preparations were then tested by ELISA against Neu5Gc, immobilised at 2.5  $\mu$ g/mL as well as against a control (Neu5Ac-PAA at 2.5  $\mu$ g/mL).

For indirect ELISA, a 96 well immunoassay plate was coated overnight at 4 °C with 100  $\mu$ L/well of 2.5  $\mu$ g/mL of Neu5Gc-PAA or 2.5  $\mu$ g/mL of Neu5Ac-PAA (as control) in 100 mM sodium bicarbonate buffer pH 9.6. The plate was washed twice with 250  $\mu$ L/well of deionised water. Wells were blocked with 0.5 % pBSA in PBS pH 7.4 for 1 h at 37°C. After incubation, the plate was washed 3 times with 250  $\mu$ L/well of PBS-T and then washed with 250  $\mu$ L/well of deionised water to ensure

removal of traces of buffer. The scFv-SL1A1 at 10 ng/mL concentration in 0.1 % pBSA in PBS with pH range of 2.5 to 7.5 was applied (100  $\mu$ L/well) to each well. The plate was incubated for 1 h at 37 °C and post incubation the plate was washed 3 times with 250  $\mu$ L/well of PBS-T and twice with deionised water to remove traces of PBS-T that may alter the pH during the assay. Anti-HIS-HRP at 1:10,000 dilution in PBS-T was added (100  $\mu$ L/well) and incubated for 1 h at 37 °C. After incubation the plate was washed 3 times with PBS-T 250  $\mu$ L/well. Washing was followed by addition of 100  $\mu$ L/well of the HRP substrate, TMB, and incubated with shaking in the dark for 20 min at room temperature. The HRP-TMB reaction was stopped with the addition of 100  $\mu$ L/well of 1 M H<sub>2</sub>SO<sub>4</sub>. Absorbance was then read at 450 nm and recorded on a SpectraMax M5e plate reader. During the ELISA the pH of each control well was measured by micro pH electrode to ensure proper control of pH during the experiment.

#### **3.2.4 scFv-SL1A1 inhibition curve generation using free Neu5Gc**

Inhibition studies employ competitive ELISA for specificity testing and for detection and quantitation of target analyte in various matrices. The parameters optimised in indirect ELISA experiments were used throughout. As standard, a 96 well immunoassay plate, was coated with Neu5Gc-PAA (2.5  $\mu$ g/mL) overnight, followed by washing with 250  $\mu$ L/well of PBS pH 7.4 and then blocked with 250  $\mu$ L/well of 0.5 % pBSA in PBS pH 7.4 for 1 h at 37 °C. Post blocking, the plate was washed 3 times with 250  $\mu$ L/well of PBS-T.

As an inhibitor, free Neu5Gc sugar was diluted over a range of concentration (30.7 mM to 3.75  $\mu$ M) in 0.1 % pBSA, pH 7.4, and 50  $\mu$ L added per well, with each well in triplicate for each concentration, followed by addition of 50  $\mu$ L scFv-SL1A1 at 10 ng/mL. The plate was then incubated for 1 h at 37 °C, followed by 3 washes with 250  $\mu$ L/well of PBS-T. Finally, anti-HIS-HRP antibody at 1:10,000 dilution in PBS-T was added at 100  $\mu$ L/well and incubated for 1 h at 37 °C. After which the plate was washed 3 times with PBS-T, followed by addition of HRP substrate, TMB (100  $\mu$ L/well) and incubated in the dark for 20 min at room temperature. The reaction was stopped with 100  $\mu$ L/well of 1 M H<sub>2</sub>SO<sub>4</sub>. The signal intensity was read at absorbance of 450 nm on SpectraMax M5e (Molecular devices). A plot of percentage binding at

specific sugar concentration generated an inhibition curve. Percentage binding was calculated by the following expression.

**Expression 2.** Calculation for percentage binding

$$\text{Percentage binding (\% B/Bo)} = \frac{\text{Absorbance with competitor (B)}}{\text{Absorbance without competitor (Bo)}} \times 100$$

### 3.2.4.1 scFv-SL1A1 inhibition curve generation using bovine transferrin

Bovine transferrin was evaluated as an alternative standard to free Neu5Gc for generating the inhibition curve, as it displays non-human sialic acid Neu5Gc on the terminal positions of its glycan chains and should not present pH issues observed with free Neu5Gc. As a consideration for commercial assay development, bovine transferrin would provide a more cost effective option than free Neu5Gc and permitting Neu5Gc to be presented in its natural conformation on a complex protein.

First, a dilution range of bovine transferrin was optimised for use in competitive assay. Bovine transferrin was diluted serially in 2 fold (12.4 mg/mL to 756 ng/mL) and 3 fold (12.4 mg/mL to 2.6 ng/mL) step dilutions for upto 15 dilutions. Since 1 mole of bovine transferrin contain 2.31 moles of Neu5Gc (as determined by in house HPLC) the following expression was used to calculate the concentration of Neu5Gc to be presented in the assay.

**Expression 3.** Calculation for Neu5Gc on protein

$$\text{Molarity of protein (M}_p\text{)} = \frac{\text{Amount of protein (mg)}}{\text{Molecular weight of protein (g/mol)}} \times 1000$$

$$\text{Molar concentration of Neu5Gc} = 2.31 \times M_p$$

To evaluate bovine transferrin as a standard inhibitor, competitive ELISA was employed. A 96 well microtiter plate was coated with Neu5Gc-PAA. After coating, the plate was washed and blocked with pBSA (detailed in Section 3.2.3.2). Blocking was followed with washing 3 times with 250  $\mu$ L/well of PBS-T. As an inhibitor, bovine transferrin was diluted over a range of concentrations in 2 fold (12.4 mg/mL to 756 ng/mL, Neu5Gc dilution range:  $36 \times 10^4$  nM to 22.1 nM) and 3 fold dilution

series (12.4 mg/mL to 2.6 ng/mL, Neu5Gc dilution range:  $36 \times 10^4$  nM to 0.08 nM) in 0.1 % pBSA pH 7.4. Fifty microliter of diluted bovine transferrin was added into each well followed by addition of 50  $\mu$ L of 10 ng/mL of scFv-SL1A1. The plate was washed and bound scFv was detected with anti-HIS-HRP and signal recorded as described in Section 3.2.3.2.

#### **3.2.4.2 Evaluation of the effect of different buffers**

To this point assays were performed using a modified PBS (137 mM NaCl, 2.7 mM KCl, 150 mM Na<sub>2</sub>HPO<sub>4</sub>, 27 mM KH<sub>2</sub>PO<sub>4</sub>) to replace the buffer to normal PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na<sub>2</sub>HPO<sub>4</sub>, 1.8 mM KH<sub>2</sub>PO<sub>4</sub>), competitive ELISA was performed using both the buffers to assess any impact on assay.

This study was performed in a 96 well immunoassay plate coated with Neu5Gc-PAA. After coating the plate was washed and blocked with pBSA (Section 3.2.3.2). The bovine transferrin as a standard was diluted (12.4 mg/mL to 7.8 ng/mL, Neu5Gc dilution range:  $36 \times 10^4$  nM to 0.24 nM) in 0.1 % pBSA in modified and normal PBS. All the reagents used in the assay were prepared in modified and normal PBS for maintaining consistency throughout the assay. Bovine transferrin dilution series was added, followed by addition of scFv-SL1A1 and incubated for 1 h at 37 °C (Section 3.2.4.1). The plate was washed and bound scFv was detected with anti-HIS-HRP and signal recorded as described in Section 3.2.4.1.

#### **3.2.4.3 Assessing specificity of anti-Neu5Gc scFv-SL1A1 on glycoproteins**

For assessment of specificity of scFv-SL1A1 to Neu5Gc, an inhibition assay was performed with bovine transferrin and human transferrin. Bovine and human transferrin have over 70 % similarity in protein sequence and an almost similar glycosylation profile except that glycans on bovine transferrin terminate with Neu5Gc while glycans on human transferrin terminate with Neu5Ac (Baldwin, 1993, Lambert et al., 2005, Retzer et al., 1996). Therefore, human transferrin was selected as a control for it presents Neu5Ac in its natural conformation. Besides bovine transferrin, bovine fetuin was also tested in parallel as a competitive inhibitor. Bovine fetuin also displays Neu5Gc on the terminal ends of its glycan chains and can also be used to present Neu5Gc to scFv-SL1A1.

For specificity testing, a 96 well microtiter plate was coated with Neu5Gc-PAA. After coating, the plate was washed and blocked with pBSA (Section 3.2.4.1). Blocking was followed with washing 3 times with 250  $\mu$ L/well of PBS-T. As an inhibitor or competitor, bovine transferrin and human transferrin were diluted over a range of concentration in 3 fold dilution series (12.4 mg/mL to 7.8 ng/mL, Neu5Gc dilution range:  $36 \times 10^4$  nM to 0.24 nM) in 0.1% pBSA pH 7.4. Bovine fetuin was also diluted in a similar range (10 mg/mL to 6.2 ng/mL, Neu5Gc dilution range:  $9.5 \times 10^4$  nM to 0.05 nM) in terms of protein concentration but has less of Neu5Gc (0.46 mole of Neu5Gc per mole of bovine fetuin). 50  $\mu$ L of diluted bovine and human transferrin and bovine fetuin were added into each well followed by addition of 50  $\mu$ L of 10 ng/mL of anti-Neu5Gc scFv-SL1A1. The plate was then incubated for 1 h at 37  $^{\circ}$ C. The plate was washed and bound scFv was detected with anti-HIS-HRP and signal recorded as described in Section 3.2.4.1.

#### **3.2.4.4 Assay composite curve for quantification**

The assay developed using bovine transferrin as standard was evaluated for its reproducibility in 10 independent assay runs to generate a composite curve. The assay performance was analysed over a period of days to permit examination of variability arising due to buffer preparation, day-to-day environmental changes, operator handling, standard and scFv dilution.

For composite curve generation, the following procedure was performed in 10 independent runs. In a 96 well microtiter plate, 100  $\mu$ L of Neu5Gc-PAA (2.5  $\mu$ g/mL) was coated overnight in bicarbonate buffer pH 9.6 at 4  $^{\circ}$ C. The plate was then washed twice with 250  $\mu$ L/well of PBS pH 7.4. The plate was then blocked with 250  $\mu$ L/well of 0.5 % pBSA in PBS pH 7.4 and incubated for 1 h at 37  $^{\circ}$ C. Incubation was followed with washing 3 times with PBS-T. As a Neu5Gc competitor, bovine transferrin was diluted in 3 fold dilution series (12.4 mg/mL to 7.8 ng/mL, Neu5Gc dilution range:  $36 \times 10^4$  nM to 0.24 nM) in 0.1 % pBSA in PBS pH 7.4. 50  $\mu$ L of diluted bovine transferrin was added in wells in triplicate, followed by addition of 50  $\mu$ L anti-Neu5Gc scFv at 10 ng/mL concentration in 0.1 % pBSA in PBS pH 7.4. The plate was then incubated for 1 h at 37  $^{\circ}$ C. The plate was then washed 3 times with 250  $\mu$ L/well of PBS-T. Finally, anti-HIS-HRP antibody at 1:10,000 dilution in PBS-T was added at 100  $\mu$ L/well and incubated for 1 h at 37  $^{\circ}$ C. After incubation the plate

was washed 3 times with 250  $\mu\text{L}$ /well of PBS-T. Washing was followed by addition of HRP substrate, TMB (100  $\mu\text{L}$ /well) and incubated in the dark for 20 min at room temperature. The reaction was stopped with 100  $\mu\text{L}$ /well of 1 M  $\text{H}_2\text{SO}_4$ . The plate was washed, and bound scFv was detected with anti-HIS-HRP and signal recorded as described in Section 3.2.4.1.

For composite curve generation, inhibition data from 10 independent assays was fitted to 4 parameter logistic curve using GraphPad Prism software version 6.0c. The composite curve was then used to predict the concentration of Neu5Gc used in the 10 inhibition assays. The predicted concentration of Neu5Gc was then plotted against the actual concentration of Neu5Gc used in the assay. The data was fitted with linear regression model using GraphPad Prism software version 6.0c.

#### **3.2.4.5 Quantitation of Neu5Gc from bovine transferrin standard curve and compare with HPLC data**

Competitive ELISA was employed to estimate Neu5Gc substitution ratio on bovine transferrin, Section 3.2.4.4. To determine Neu5Gc substitution ratio, bovine transferrin was diluted from 671 nM to 25 nM in 0.1 % pBSA in PBS (137 mM NaCl, 2.7 mM KCl, 10 mM  $\text{Na}_2\text{HPO}_4$ , 1.8 mM  $\text{KH}_2\text{PO}_4$ , pH 7.4). 50  $\mu\text{L}$  of bovine transferrin was added in wells in triplicate, followed by addition of 50  $\mu\text{L}$  anti-Neu5Gc scFv at 10 ng/mL concentration in 0.1 % pBSA in PBS. Rest of the procedure was same as in Section 3.2.4.4. The signal obtained for test concentration of bovine transferrin was interpolated from the standard curve.

To determine the Neu5Gc substitution ratio calculated from ELISA, Neu5Gc content of bovine transferrin was assessed by DMB-HPLC (4,5-Methylenedioxy-1,2-phenylenediamine dihydrochloride). For HPLC analysis, 50  $\mu\text{g}$  of bovine transferrin and Neu5Gc standard diluted from 5 nmol to 0.05 nmol was acid hydrolysed with 2N HCl for 3 hr at 80  $^\circ\text{C}$ . Following acid hydrolysis, the sample and standard was spun at 14,000 g for 20 min in a 10 K spin filter and washed with 200  $\mu\text{L}$  of distilled water. The sample and standard were then placed in a speed vac for drying. In the meantime DMB labeling solution (7 mM DMB, 1.4 M  $\text{CH}_3\text{COOH}$ , 0.75 mM  $\beta$ -mercaptoethanol, 18 mM  $\text{Na}_2\text{S}_2\text{O}_4$  in distilled water) was prepared. The DMB labeling solution was added to the vacuum dried sample and standard, and incubated for 2.5 h at 50  $^\circ\text{C}$  in the dark for derivatisation. The sample and standard were then

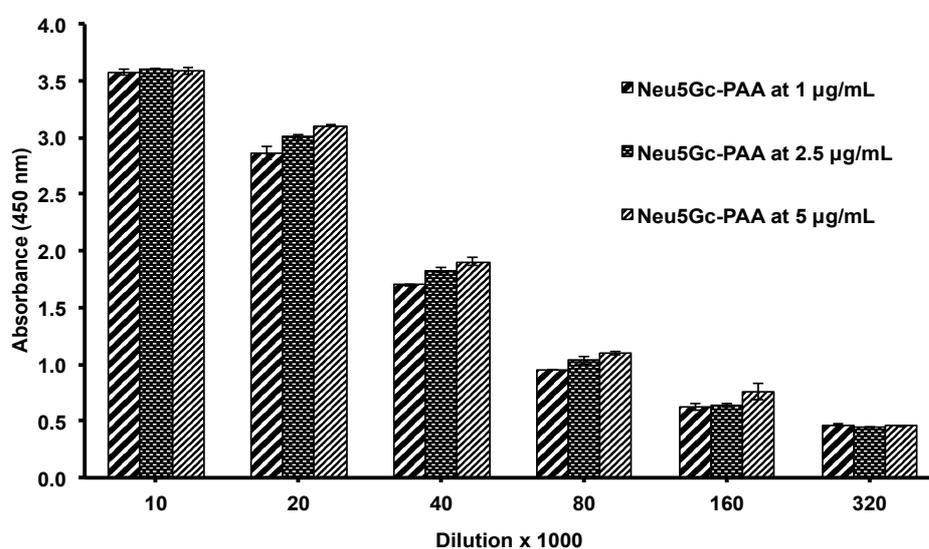
cooled and spun at 14,000 *g* for 1 min. Post-derivatisation, 10  $\mu\text{L}$  of Neu5Gc standard at a range of concentrations were injected into a GlycoSep R HPLC column pre-equilibrated with mobile phase (acetonitrile:methanol:water; 9:7:84), at a flow rate of 0.5 mL/min with separation time of 45 min. This was followed by injection of 10  $\mu\text{L}$  of derivatised sample. The area under the peak was calculated and plotted against Neu5Gc concentration to generate a standard curve. The Neu5Gc substitution for bovine transferrin was interpolated from the Neu5Gc standard curve.

The bovine transferrin inhibition curve generated was also used to determine the Neu5Gc substitution ratio on bovine fetuin. The bovine transferrin was diluted from 31.5 nM to 3.5 nM in 0.1 % pBSA in PBS (137 mM NaCl, 2.7 mM KCl, 10 mM  $\text{Na}_2\text{HPO}_4$ , 1.8 mM  $\text{KH}_2\text{PO}_4$ , pH 7.4). 50  $\mu\text{L}$  of bovine transferrin was added in wells in triplicate, followed by addition of 50  $\mu\text{L}$  anti-Neu5Gc scFv at 10 ng/mL concentration in 0.1 % pBSA in PBS. Rest of the procedure was the same as in Section 3.2.4.4. The signal obtained for the test concentration of bovine transferrin was interpolated from the standard curve.

### 3.3 Results

#### 3.3.1 Antigen coating optimisation

To minimise the use of antigen required for anti-Neu5Gc immunoassay using immunised chicken polyclonal serum, assays were performed to identify the minimal concentration of coating antigen. Immunoassay plates coated with antigen concentrations ranging 1  $\mu\text{g}/\text{mL}$  to 5  $\mu\text{g}/\text{mL}$  were used in the assay and the immunised chicken polyclonal sera diluted from 10,000 to 320,000-fold. The bound serum antibodies were detected using an anti-chicken IgY-HRP, outcome shown in Figure 3.1.

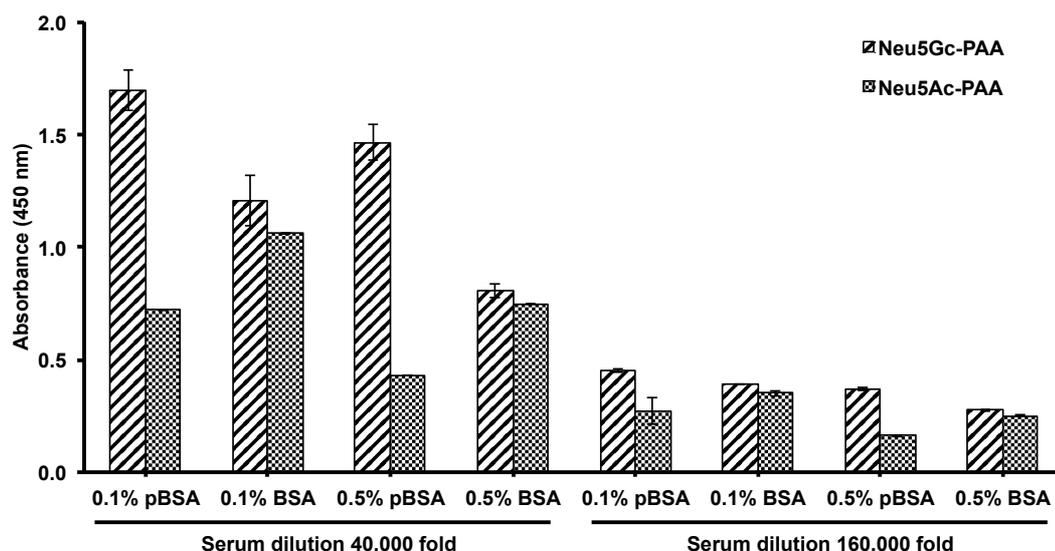


**Figure 3.1** Optimisation of antigen coating concentration. Immunoassay plate was coated with 1  $\mu\text{g}/\text{mL}$ , 2.5  $\mu\text{g}/\text{mL}$  and 5  $\mu\text{g}/\text{mL}$  of antigen. Immunised chicken polyclonal serum diluted serially from 10,000 fold to 320,000 fold was applied and detected with rabbit anti-chicken-IgY-HRP (Section 3.2.1.1). Bar chart represents signal obtained across the three antigen coating concentration. Detection of Neu5Gc was consistent across the dilution series. Error bars indicate  $\pm$  SD.

Data from antigen optimisation showed that Neu5Gc can be detected with reasonable signal at low antigen coating concentrations. Signal intensity for Neu5Gc detection at 1.0  $\mu\text{g}/\text{mL}$ , 2.5  $\mu\text{g}/\text{mL}$  antigen concentration was comparable to that achieved with 5  $\mu\text{g}/\text{mL}$  coating. For further assay development 2.5  $\mu\text{g}/\text{mL}$  was selected as the optimal antigen concentration for coating.

### 3.3.2 Blocker and standard diluent optimisation/evaluation

Both pBSA and BSA as blocking agents were tested in an indirect ELISA format wherein post antigen coated immunoassay plates were blocked. Polyclonal serum was diluted serially to 40,000 fold and 160,000 fold in PBS containing 0.1 % and 0.5 % of either pBSA or BSA. Figure 3.2 shows the comparison of assay signal versus background signal.



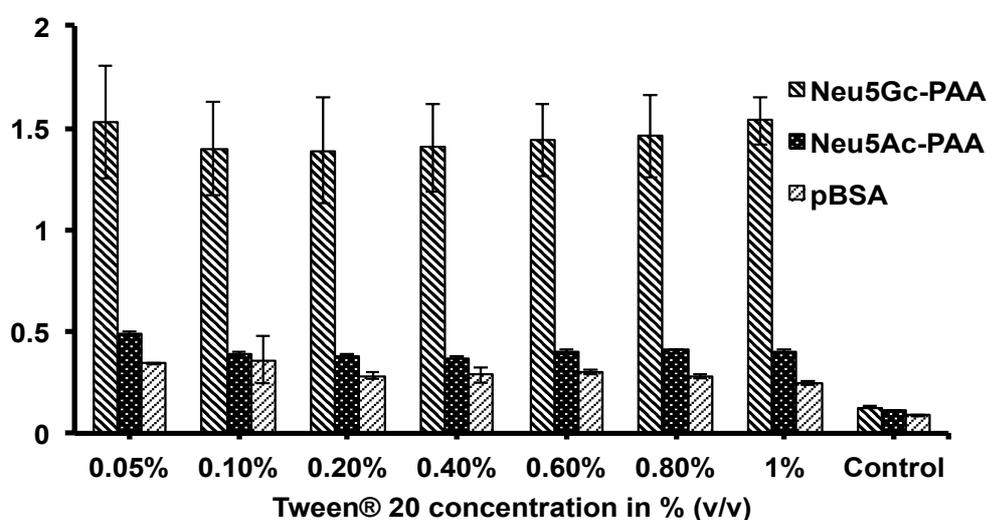
**Figure 3.2** Assessment of blocker for Neu5Gc assay with immunised chicken polyclonal serum. Blockers and dilution matrix evaluated by indirect ELISA (Section 3.2.1.2). Bar chart represents signal and background obtained with different blockers. With pBSA as blocker and as component in the dilution buffer the background signal was less as compared to BSA as blocker and component in dilution reagent. Error bars indicate  $\pm$  SD

Blocker testing clearly demonstrated that 0.5 % pBSA was better as compared to 0.5 % BSA in reducing background in anti-Neu5Gc immunoassay using immunised chicken polyclonal serum at both 40,000 fold and 160,000 fold dilution. Moreover, the presence of pBSA in the dilution buffer at the tested concentration showed a decrease in the background signal as compared to BSA which was not able to decrease the background signal. This was expected as Neu5Gc-BSA as a neoglycoconjugate was also used for immunisation of chicken and therefore antibodies against BSA backbone can also contribute to the background signal. It was also observed that background with 0.5 % pBSA containing dilution buffer showed less background than buffer containing 0.1 % pBSA, but signal above

background for Neu5Gc was comparable at both the concentrations of pBSA in the dilution buffer. Hence for further assays, 0.1 % pBSA was used for dilution of polyclonal serum.

### 3.3.3 Tween 20 optimisation

The wash buffer in immunoassays employs non-ionic detergents like Tween 20 to reduce background signal (Esser, 1991). To assess the impact of Tween 20 on background or non-specific signal, wash buffer with varying concentrations of Tween 20 was evaluated in an indirect ELISA using immunised chicken polyclonal serum to detect Neu5Gc immobilised on an immunoassay plate. The antigen Neu5Gc-PAA and controls Neu5Ac-PAA and pBSA were immobilised onto immunoassay plate. Immunised chicken polyclonal serum diluted to 40,000 fold was used as a primary antibody to probe immobilised Neu5Gc. The bound chicken polyclonal antibody was detected with anti-IgY-HRP. During the assay, wells in immunoassay plate were washed with wash buffers (PBS-T) containing increasing concentration (0.05 % to 1 %) of Tween 20.

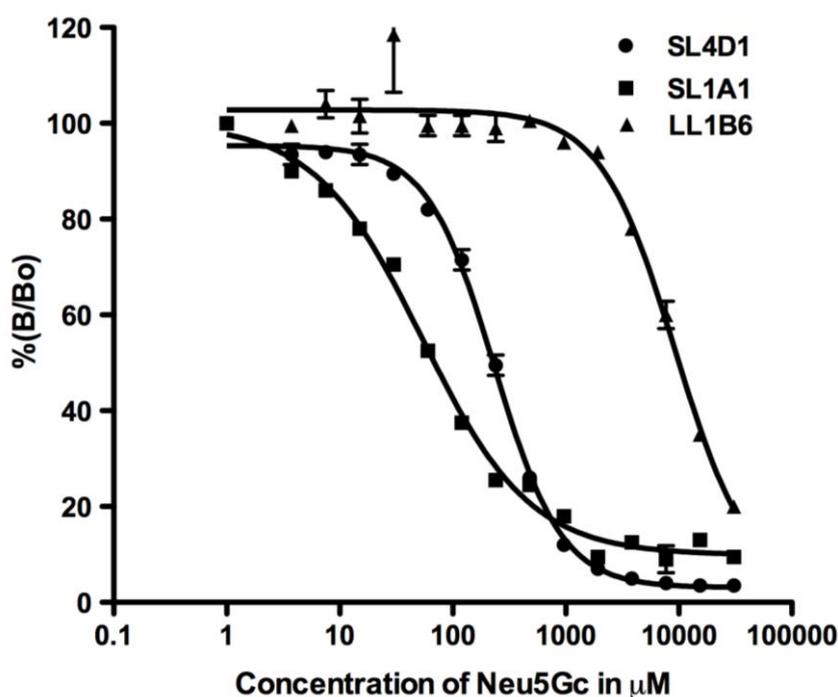


**Figure 3.3** Optimisation of Tween 20 concentration in wash buffer. To assess the impact of Tween 20 concentration on assay background, an indirect ELISA using 40,000 fold immunised chicken polyclonal serum for detecting Neu5Gc was performed and washing steps throughout the assay were done with different concentration of Tween 20 in washing buffer (Section 3.2.1.3). Bar chart represents signal obtained with use of PBS-T with different concentration of Tween 20 as wash buffer. Signals for Neu5Gc were consistent across the different concentrations of Tween 20 indicating no impact on assay background. Error bars indicate  $\pm$  SD.

Data from Tween 20 optimisation, Figure 3.3, showed that the effect of Tween 20 on background signal was minimal across the concentrations tested. There was slight improvement at increased Tween 20 concentration but overall the background reduction was insignificant with higher Tween 20 concentrations. At 1 % Tween 20 concentration, the background was slightly less with low inter-replicate variation but use of high concentration of detergents is not recommended for routine assay development. Therefore, 0.05 % Tween 20 was deemed suitable for use throughout assay development.

### 3.3.4 Competitive ELISA and specificity testing of scFv

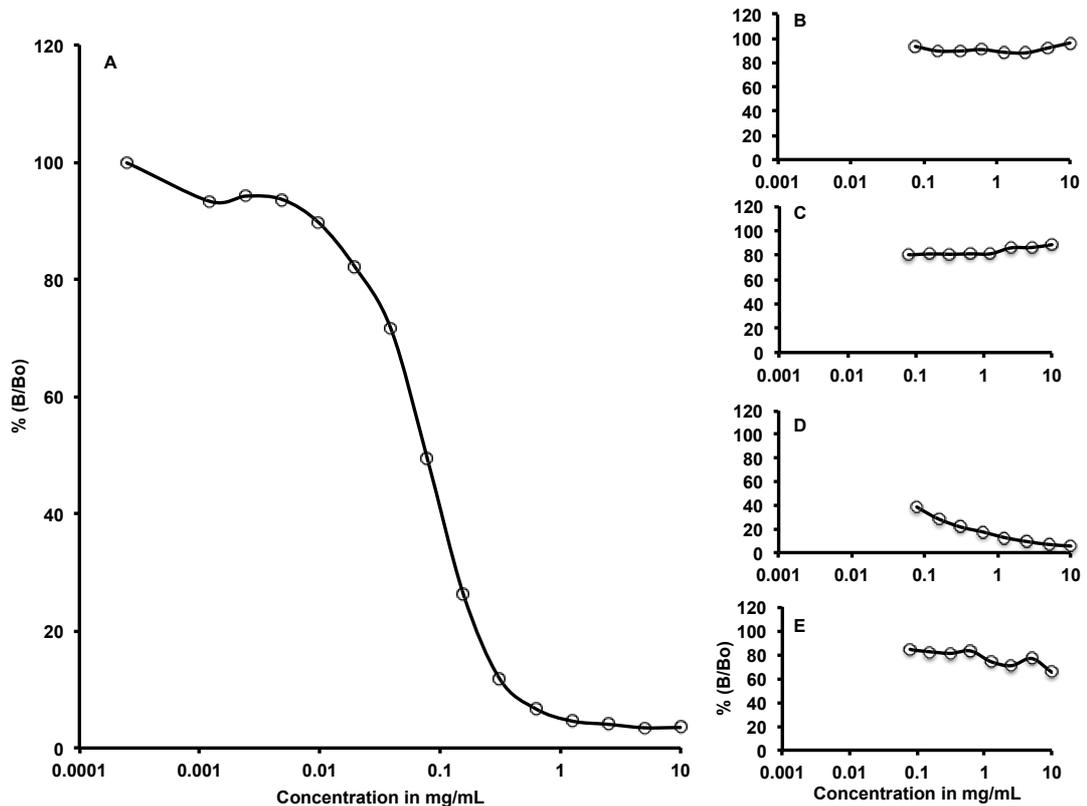
Of the identified short-linker and long-linker scFvs from Chapter 2, SL1A1 scFv demonstrated higher sensitivity for Neu5Gc free sugar as compared to SL4D1 and LL1B6, which showed less sensitive standard curves, Figure 3.4.



**Figure 3.4** Comparison of inhibition curves for short (SL1A1 and SL4D1) and long (LL1B6) linker scFv performed by ELISA as per Section 3.2.2.2. Comparison of inhibition curve for short and long-linker scFv demonstrated that scFv-SL1A1 was more sensitive for detection of Neu5Gc than SL4D1 and LL1B6. Error bars indicate  $\pm$  SD.

The competitive ELISA assay demonstrated that scFv-SL1A1 was more sensitive than SL4D1 and LL1B6 for the detection of Neu5Gc. The LL1B6 scFv was least sensitive for Neu5Gc detection. The scFv-SL1A1 was further evaluated for

specificity of Neu5Gc detection by competitive ELISA. Specificity testing included glycoconjugates and glycoproteins both with and without Neu5Gc. Specificity testing indicated detection of Neu5Gc over glycoprotein fetuin as opposed to asialofetuin control. Similarly, no response for Neu5Ac and Gal- $\alpha$ -(1,3)-Gal was observed, Figure 3.5.



**Figure 3.5** Demonstration of specificity of scFv-SL1A1 to Neu5Gc. (A) Free Neu5Gc showing concentration dependent inhibition of scFv. (B) Free Neu5Ac and (C) Gal- $\alpha$ -(1,3)-Gal failed to inhibit scFv binding to immobilised Neu5Gc. (D) Neu5Gc displayed on bovine fetuin showing concentration dependent inhibition of scFv. (E) Weak inhibition response observed with asialofetuin. The scFv was inhibited by free Neu5Gc and glycoprotein bound Neu5Gc (bovine fetuin) while no inhibition was observed with rest of the negative controls indicative of Neu5Gc specific binding of scFv.

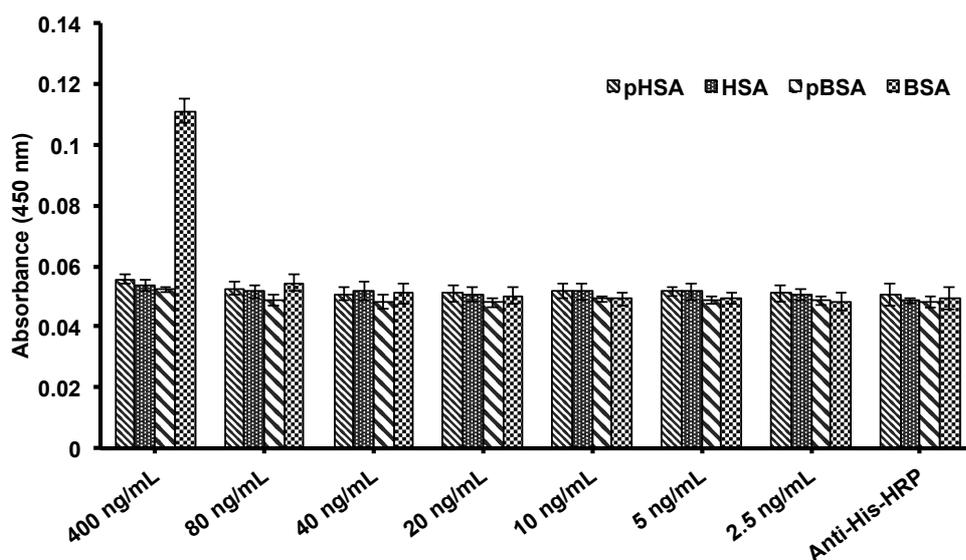
The scFv-SL1A1 recognised Neu5Gc in free as well as protein bound form presented on bovine fetuin, demonstrating that scFv can detect Neu5Gc in its natural presentation. There was weak response for asialofetuin, which can be attributed to the presence of traces of free Neu5Gc in commercial preparations. For free Neu5Ac

and Gal- $\alpha$ -(1,3)-Gal no response was observed indicative of specificity of scFv-SL1A1 and that inhibition of scFv was Neu5Gc mediated.

### **3.3.5 Optimisation of blocking buffer for use in assay development employing scFv-SL1A1**

An effective blocker should prevent non-specific binding, reduce background, block non-specific binding to adsorbed antigen and should not cross react with antibodies used in the immunoassay (Gibbs and Kennebunk, 2001). Most frequently BSA is used to block unoccupied adsorption active surfaces in immunoassay plates. However, for assays related to detection of carbohydrate antigens, impurity of glycoproteins in commercial BSA preparations can cross-react with anti-carbohydrate antibodies to generate a non-specific signal. Furthermore, the BSA is a protein isolated from a bovine source which are known to express Neu5Gc and can contribute to the signal for the detection of Neu5Gc using anti-Neu5Gc antibodies. To overcome this, commercial high grade BSA was periodate-treated to permit its use in assay development.

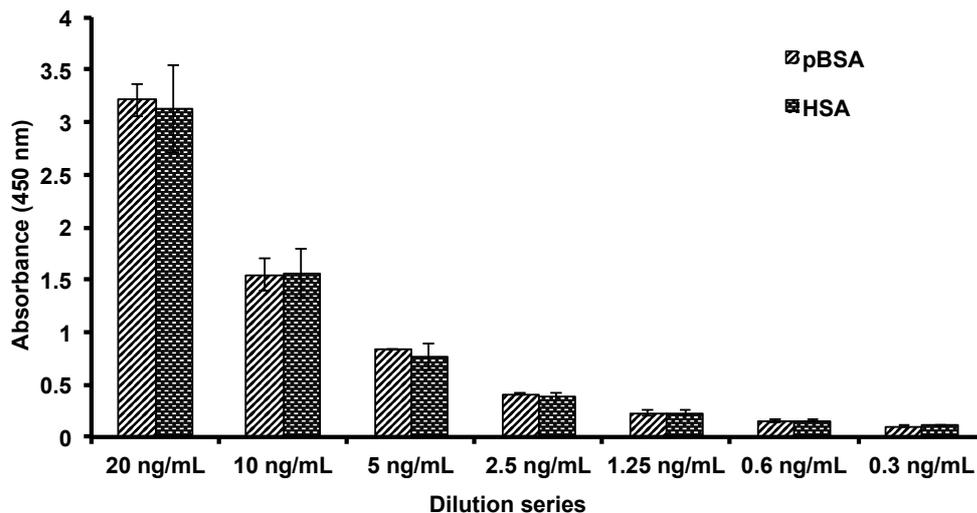
In the current assay development exercise for Neu5Gc detection, various blockers were assessed for their impact on background signal. In phase I of the study the different blocking solutions were tested to assess for any non-specific binding with scFv-SL1A1 in an indirect ELISA. To test for the cross-reactivity of antibodies used in Neu5Gc detection assay against blockers, immunoassay plates were coated with blockers (pBSA, pHSA, BSA, HSA) and a mock anti-Neu5Gc ELISA procedure was performed (Section 3.2.3.1).



**Figure 3.6** Testing different blockers for cross-reactivity. For testing different blockers for their cross-reactivity with scFv-SL1A1, blockers (pBSA, pHSA, BSA and HSA) were used to coat immunoassay plates. The scFv-SL1A1 was then applied and anti-HIS-HRP was used to detect bound scFv (Section 3.2.3.1). Bar chart represents background signal/non-specific binding to scFv obtained in presence of different blockers. The scFv-SL1A1 showed binding to BSA blocked wells while rest of the blockers did not show non-specific binding. Error bars indicate  $\pm$  SD

Data from blocker assessment, Figure 3.6, clearly suggests that scFv-SL1A1 did not show binding to pHSA, pBSA and HSA. A small signal close to background was observed for BSA at 400 ng/mL of scFv-SL1A1, this could be attributed to presence of small amounts of Neu5Gc containing immunoglobulin as an impurity in commercial BSA preparations. The pBSA is generated by mild periodate treatment to oxidise glycans, thereby avoiding cross-reactivity against anti-carbohydrate antibodies and hence being suited as a blocker in carbohydrate immunoassays.

Since, both pBSA and HSA showed no cross-reactivity in blocker assessment, pBSA and HSA were compared by indirect ELISA (Section 3.2.3.1), Figure 3.7.



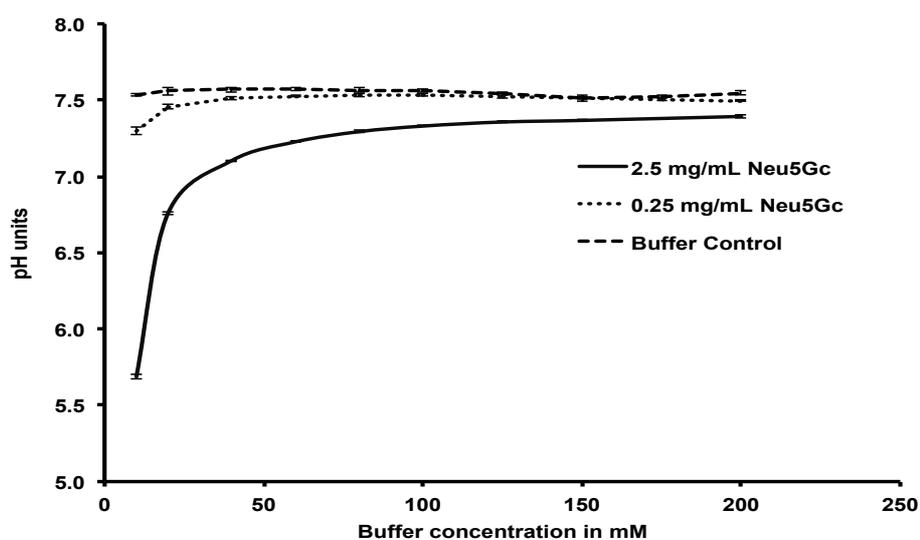
**Figure 3.7** Testing blockers in an indirect ELISA. Blockers pBSA and HSA were compared in an indirect ELISA for assessing impact on Neu5Gc detection (Section 3.2.3.1). Bar chart represents signal obtained for Neu5Gc in presence of blockers pBSA and HSA. In the presence of both the blockers pBSA and HSA signal obtained for Neu5Gc was almost comparable. Error bars indicate  $\pm$  SD

Data for pBSA and HSA evaluation showed that both the protein solutions are suitable for blocking and sample dilution. Assay performance in terms of signal intensity was similar for both the proteins. Overall pBSA can be used for blocking as an alternative to more expensive HSA. Further assay development was performed using pBSA for blocking and sample dilution.

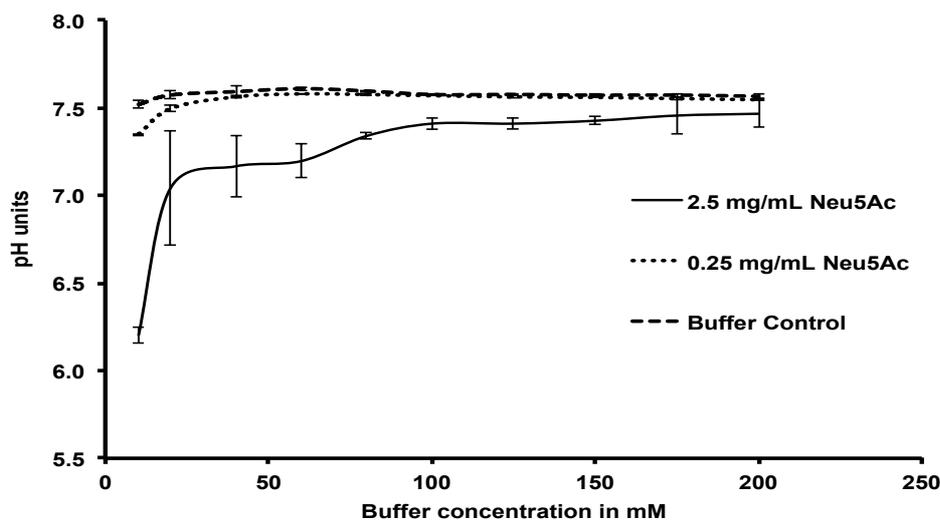
### 3.3.6 Evaluation of pH effects and testing of influence of pH on scFv-SL1A1 binding

In a competitive ELISA, free antigen in solution is used to create competition between immobilised and free antigen for binding to a specific binding partner. In the current assay, free Neu5Gc was used as free antigen to compete with immobilised Neu5Gc-PAA for binding to scFv-SL1A1. Since Neu5Gc attains negative charge by donating its hydrogen, this release of hydrogen makes the solution acidic, thus altering the binding properties of the scFv probably by inducing changes in ionisation states of the amino acid present in the binding pockets (Strauch et al., 2014).

Therefore, to develop Neu5Gc detection assays based on anti-Neu5Gc recognition molecules, a pH study was performed to assess the impact of pH change on the Neu5Gc detection assay. The pH optimisation study was conducted in two phases. In phase I the ability of free Neu5Gc to change the pH of PBS was estimated at a range of PBS buffering capacity. Typical PBS contains 10 mM phosphate buffer for maintaining pH. In this study PBS with buffering strengths ranging from 10 mM to 200 mM phosphate were used to determine suitable buffering strengths for assay development. The pH effect was also studied with free Neu5Ac in the same buffering strengths range. For pH study, free Neu5Gc and Neu5Ac solutions were prepared at 2.5 and 0.25 mg/mL in PBS with buffering strength ranging from 10 mM to 200 mM (Section 3.2.3.2). The pH of solutions containing Neu5Gc and Neu5Ac was recorded by a pH probe against a buffer control without Neu5Gc and Neu5Ac, Figure 3.8 and 3.9. Concentration of phosphate buffer at which pH was close to 7.4 was used for carrying out further assays.



**Figure 3.8** Effect of free Neu5Gc on assay buffer pH. Free Neu5Gc sugar was diluted serially from 50 mg/mL stock to assay concentration of 2.5 mg/mL and 0.25 mg/mL in PBS, pH 7.5, with range of buffer capacities (10 mM to 200 mM) and aliquoted out (100  $\mu$ L/well) on microtiter plate. PBS with different buffering capacity without Neu5Gc served as assay control. The plate was incubated at 37  $^{\circ}$ C for 1 h. Post incubation the pH in each well was measured with micro pH meter. Data from pH study revealed lowering of pH by free Neu5Gc at typical assay concentrations in buffers with low buffering capacity. Data points not marked to show error bars indicating  $\pm$ SD.

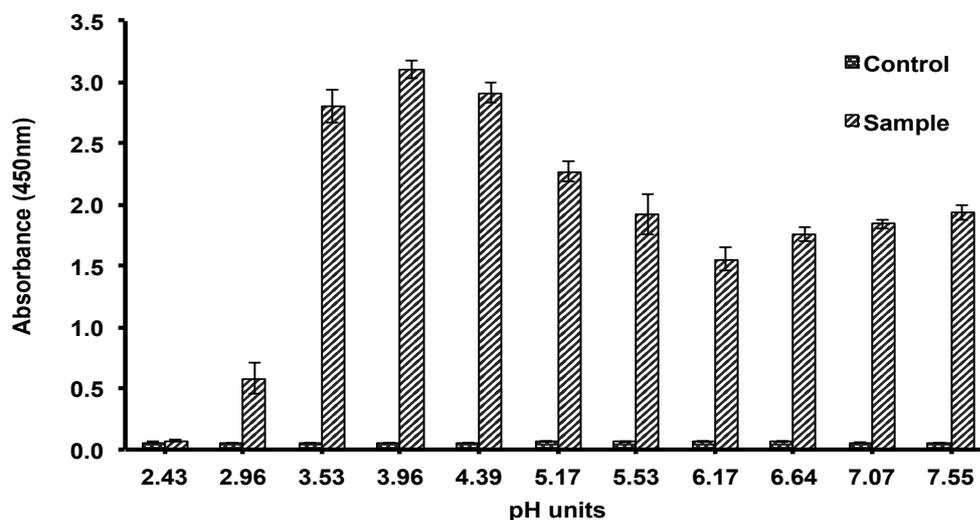


**Figure 3.9** Effect of free Neu5Ac on assay buffer pH. Free Neu5Ac sugar was diluted serially from 50 mg/mL stock to assay concentration of 2.5 mg/mL and 0.25 mg/mL in PBS, pH 7.5, with range of buffer capacities (10 mM to 200 mM) and plated out (100  $\mu$ L/well) on microtiter plate. PBS with different buffering capacity without Neu5Ac served as assay control. The plate was incubated at 37  $^{\circ}$ C for 1 h. Post incubation the pH in each well was measured with a micro pH meter. Data from the pH study revealed lowering of pH by free Neu5Ac in buffers with low buffering capacity. Data points not marked to show error bars indicating  $\pm$ SD.

The pH study for two different concentrations of Neu5Gc in varying PBS buffering capacity clearly indicated a change in pH of buffer to a significant extent, Figure 3.8. A similar effect on pH was also observed with free Neu5Ac, Figure 3.9, but was less as compared to Neu5Gc. This pH change can alter the behavior of scFv in presence of high concentrations of free Neu5Gc in a Neu5Gc detection assays. PBS at 150 mM concentration was able to maintain its buffering capacity, therefore 150 mM PBS (137 mM NaCl, 2.7 mM KCl, 150 mM Na<sub>2</sub>HPO<sub>4</sub>, 27 mM KH<sub>2</sub>PO<sub>4</sub>) was used in all following ELISA based assays.

In Phase II, the effect of pH on the binding characteristic of anti-Neu5Gc scFv was evaluated at a wider pH range (2.5 to 7.5) directly by ELISA. An immunoassay plate coated with antigen (Neu5Gc-PAA) and negative control (Neu5Ac-PAA) was incubated with the scFv-SL1A1 preparation diluted serially to 10 ng/mL in PBS (137 mM NaCl, 2.7 mM KCl, 150 mM Na<sub>2</sub>HPO<sub>4</sub>, 27 mM KH<sub>2</sub>PO<sub>4</sub>) across the pH range, with increments of 0.5 pH (Section 3.2.3.2). The bound scFv was detected with anti-

HIS-HRP secondary antibody. The impact of pH on anti-Neu5Gc scFv binding was variable across the pH range, Figure 3.10.

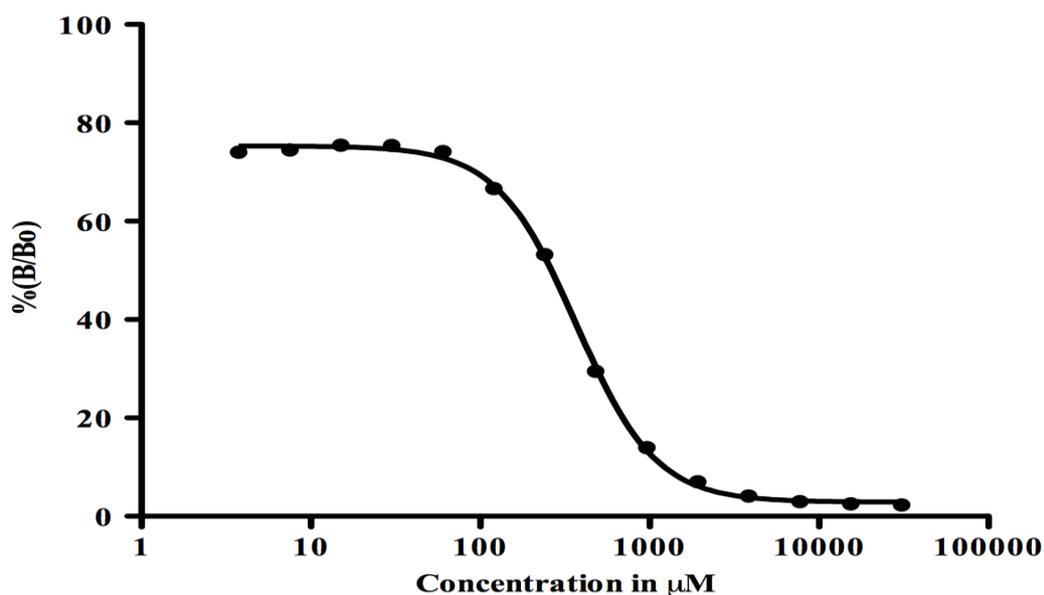


**Figure 3.10** Binding characteristic of scFv-SL1A1 at different pH. scFv diluted in PBS (137 mM NaCl, 2.7 mM KCl, 150 mM Na<sub>2</sub>HPO<sub>4</sub>, 27 mM KH<sub>2</sub>PO<sub>4</sub>) buffer with pH ranging from 2.5 to 7.5 and binding to Neu5Gc assessed by indirect ELISA (Section 3.2.3.2) Bar chart represents signal obtained for Neu5Gc at different pH. SL1A1 scFv can clearly detect Neu5Gc at broader range of pH (3.5 to 7.5) but not below 3.5. Error bars indicate  $\pm$  SD

Binding signal increased with increase in pH from 2.5 pH units to 4.0 pH units. From pH 4.0 to 6.0 binding signal decreased and then was stable above pH 6.5. It was demonstrated that the scFv-SL1A1 can bind to Neu5Gc at a broad pH range of 3.5 to 7.5 but not below 3.5 pH units. This also indicated that scFv-SL1A1 is robust and can sustain the changes in pH of buffer used in the assay, this characteristic can be advantageous in terms of tolerating buffering changes which may be inadvertently introduced in day to day buffer preparations.

### 3.3.7 Inhibition curve using free Neu5Gc, Competitive ELISA

The aim was to generate a standard curve, using known concentrations of free Neu5Gc to permit the quantification of Neu5Gc in an unknown sample. Free Neu5Gc, over a range of concentrations is used as an inhibitor in a competitive ELISA, wherein immunoassay plates coated with a fixed concentration of immobilised Neu5Gc was measured with scFv-SL1A1 in the presence of free Neu5Gc. The depletion in binding of scFv, caused by increasing concentrations of free Neu5Gc, with immobilised antigen permits the generation of an inhibition assay curve, Figure 3.11. The inhibition data was fitted with 4 parameter logistic curve using GraphPad Prism software version 6.0c.

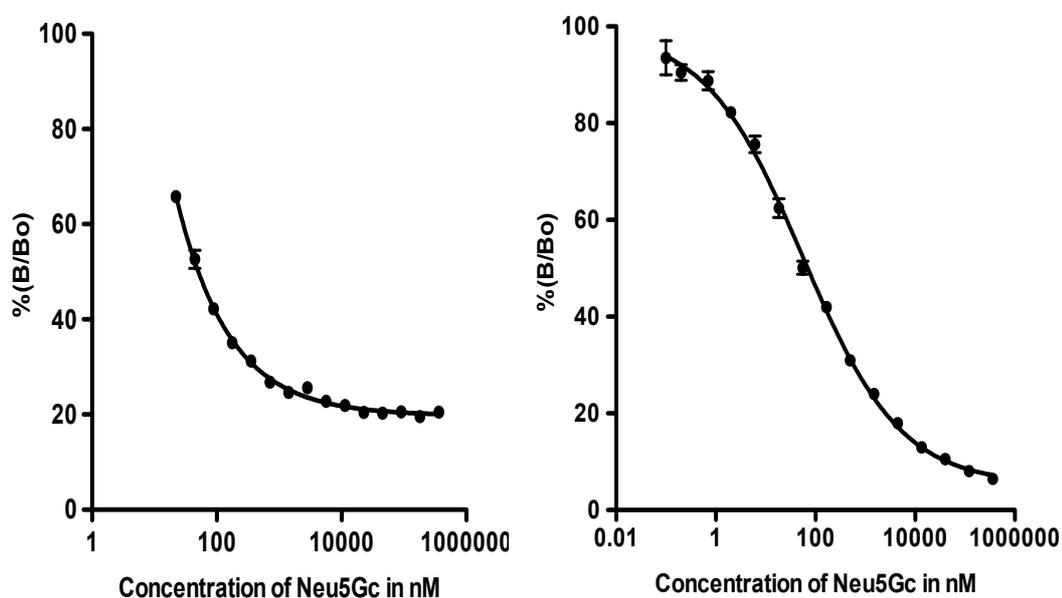


**Figure 3.11** Inhibition with free Neu5Gc. Performed by competitive ELISA using free Neu5Gc at a concentration range to inhibit scFv-SL1A1 (Section 3.2.4). Free Neu5Gc showing dose dependent inhibition of scFv-SL1A1 with an IC<sub>50</sub> of 221 μM. Error bars indicate ± SD.

Inhibition with free Neu5Gc generated a typical dose response curve with concentration dependent decrease in scFv binding to immobilised antigen. The IC<sub>50</sub>, which is the concentration of test compound or inhibitor that shows half maximal response was calculated to be 221 μM.

### 3.3.8 Assessment of bovine transferrin as standard in ELISA

Neu5Gc being an acidic sugar, can lead to pH changes of the assay conditions at high concentrations, which can result in the altered binding of scFv-SL1A1. To overcome pH effect attributed to the use of free Neu5Gc, bovine transferrin was evaluated as an alternative for the generation of the ELISA standard curve. The bovine transferrin was serially diluted in steps of 2 fold or 3 fold in a series upto 15 dilutions. Both dilution series were examined for the generation of a standard curve by competitive ELISA, data shown in Figure 3.12. The criteria for evaluation was based on generation of a concentration dependent sigmoidal dose response relationship between competitor (Neu5Gc on bovine transferrin) and scFv-SL1A1.

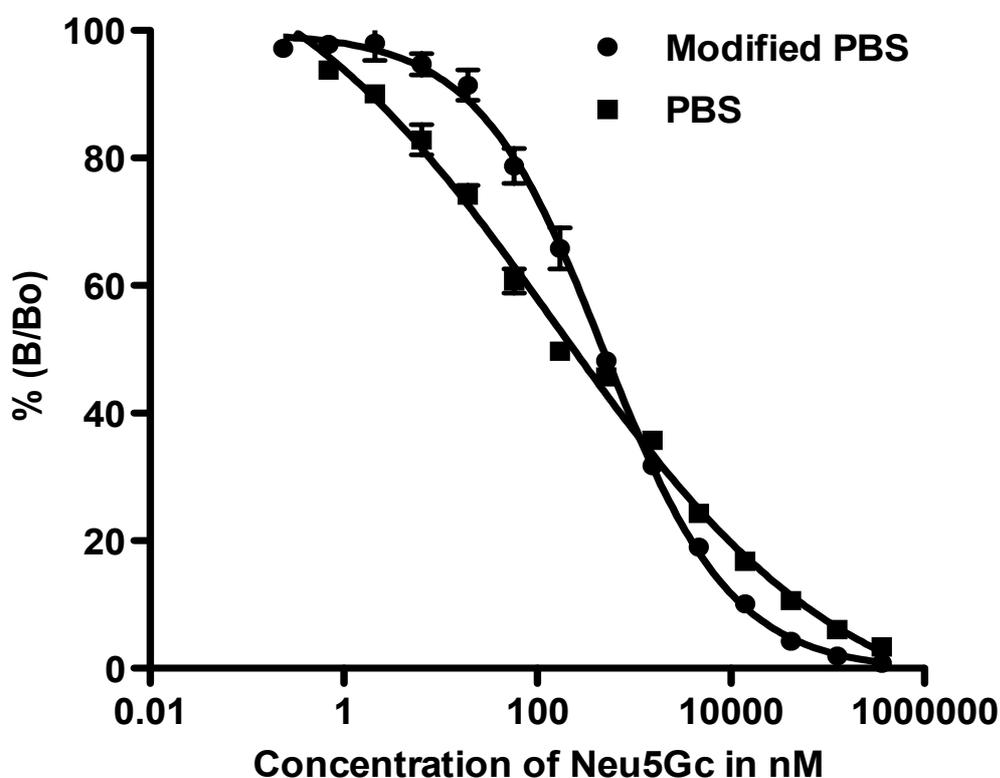


**Figure 3.12** Identification of appropriate dilution series for inhibition curve generation using bovine transferrin. Bovine transferrin was serially diluted in 2 fold (12.4 mg/mL to 756 ng/mL, Neu5Gc dilution range:  $36 \times 10^4$  nM to 22.1 nM ) and 3 fold (12.4 mg/mL to 2.6 ng/mL, Neu5Gc dilution range:  $36 \times 10^4$  nM to 0.08 nM) dilution series was used to inhibit scFv-SL1A1 in competitive ELISA (Section 3.2.4.1). Data showed a smooth and clear dose response/inhibition curve with 3 fold dilution series of bovine transferrin as compared to 2 fold dilution series. Error bars indicate  $\pm$  SD

Data from 2 fold serial dilution series of bovine transferrin (competitor) shows a steep decline in signal intensity over the dilution range, the bottom plateau of competitor excess was met but top plateau for antibody excess was not achieved.

Thus 2 fold dilution series could not represent desired dose response relationship within 15 serial dilution steps. In contrast, 3 fold dilution series showed a clear sigmoidal dose response relationship within 15 serial dilution steps. Therefore, 3 fold dilution series was considered appropriate for generation of a standard curve for Neu5Gc quantitation.

Further, to test whether modified PBS buffer and normal PBS buffer show any influence on assay using bovine transferrin, competitive ELISA was performed with both modified and normal PBS. The inhibition curve generated with use of the both the buffers is shown in Figure 3.13.



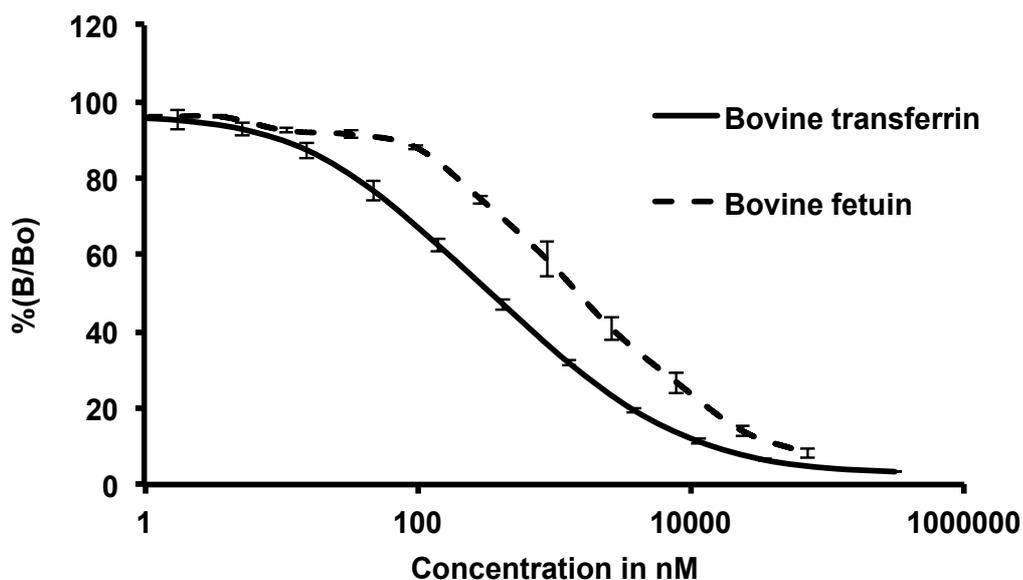
**Figure 3.13** Competitive ELISA performed with modified and normal PBS to assess the impact of buffering strength on competitive ELISA (Section 3.2.4.2). Inhibition curve generated with both modified and normal PBS showed dose dependent inhibition with minimal differences. Error bars indicate  $\pm$  SD.

The inhibition assay data showed minimal differences in the assay in the presence of modified and normal PBS indicating that the assay tolerated changes in buffer composition. Besides this, it also showed that normal PBS can be used with bovine

transferrin as an inhibitor. Further, the data also suggested that the Neu5Gc detection assay with anti-Neu5Gc scFv-SL1A1 is robust and can tolerate day to day variability in buffer preparations in terms of pH and ionic strength of buffer. Since, there were no pH effects associated with bovine transferrin as inhibitor, the normal PBS was used for inhibition assays using bovine transferrin

### **3.3.9 Test specificity of scFv on glycoproteins**

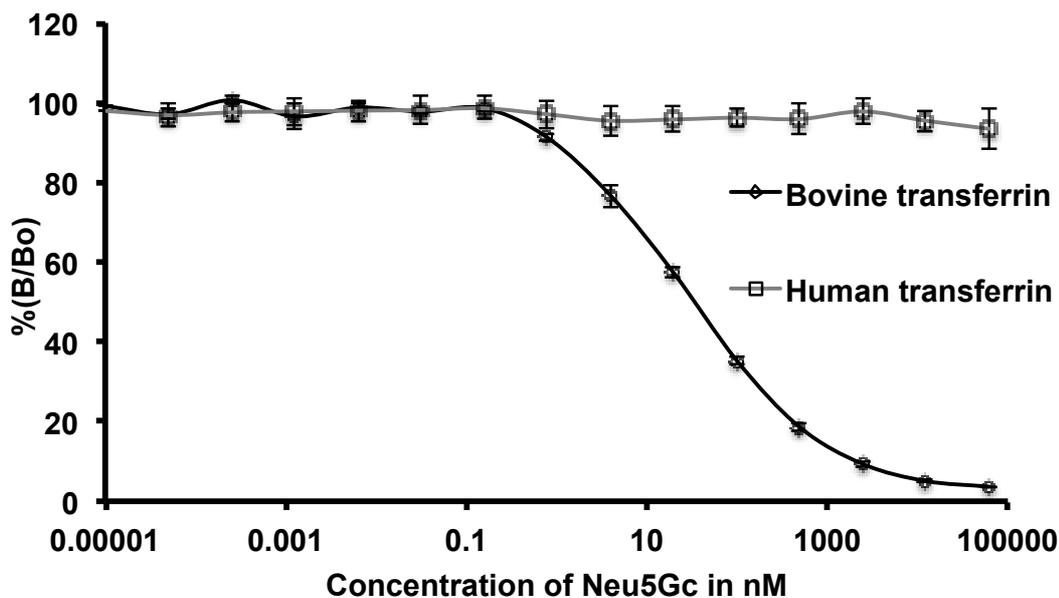
Anti-Neu5Gc scFv can detect Neu5Gc in its natural presentation on glycoproteins as evident from the bovine transferrin inhibition assay. Further, to assess the Neu5Gc specificity of scFv, bovine fetuin was tested in a parallel inhibition assay with bovine transferrin. Like bovine transferrin, bovine fetuin also displays Neu5Gc on the terminal positions of its glycans and can be used to further confirm the Neu5Gc specific inhibition of scFv-SL1A1. Ideally if the scFv-SL1A1 recognises Neu5Gc on both bovine transferrin and bovine fetuin then inhibition curve should be parallel. To assess this, a competitive ELISA was performed with bovine transferrin and bovine fetuin diluted in almost the same concentration ranges in a 14 step 3 fold serial dilution series. The diluted proteins were used to competitively inhibit scFv-SL1A1 from binding to immobilised Neu5Gc Figure 3.14.



**Figure 3.14** Competitive inhibition of scFv-SL1A1 by bovine transferrin and bovine fetuin. Bovine fetuin and bovine transferrin used at a concentration range to inhibit scFv in a concentration dependent manner to demonstrate recognition of the same antigen on glycoproteins (Section 3.2.4.3). Both bovine transferrin and bovine fetuin showed dose dependent and parallel inhibition curve indicating detection of Neu5Gc in the glycoprotein bound form. Data points not marked to show error bars indicating  $\pm$ SD.

Data from inhibition of bovine transferrin and bovine fetuin showed a parallel concentration dependent inhibition of scFv-SL1A1 indicating that scFv recognised the same epitope over both the glycoproteins. Although bovine fetuin, like transferrin inhibited scFv-SL1A1 in a dose dependent manner it showed less sensitive inhibition than transferrin, this was consistent with the reports that bovine fetuin contains less Neu5Gc than bovine transferrin. The data also indicated that scFv-SL1A1 can detect Neu5Gc on different glycoproteins.

The human transferrin and bovine transferrin have conserved sequences that show over 70 % homology (Retzer et al., 1996, Baldwin, 1993). Both the proteins have complex *N*-glycan structures, except that the glycans in bovine transferrin terminate with both Neu5Gc and Neu5Ac, while human transferrin glycans terminate with Neu5Ac exclusively. Thus human transferrin acted as a suitable negative control assessing specificity of scFv-SL1A1 against Neu5Gc. Figure 3.15 shows inhibition data for bovine and human transferrin.

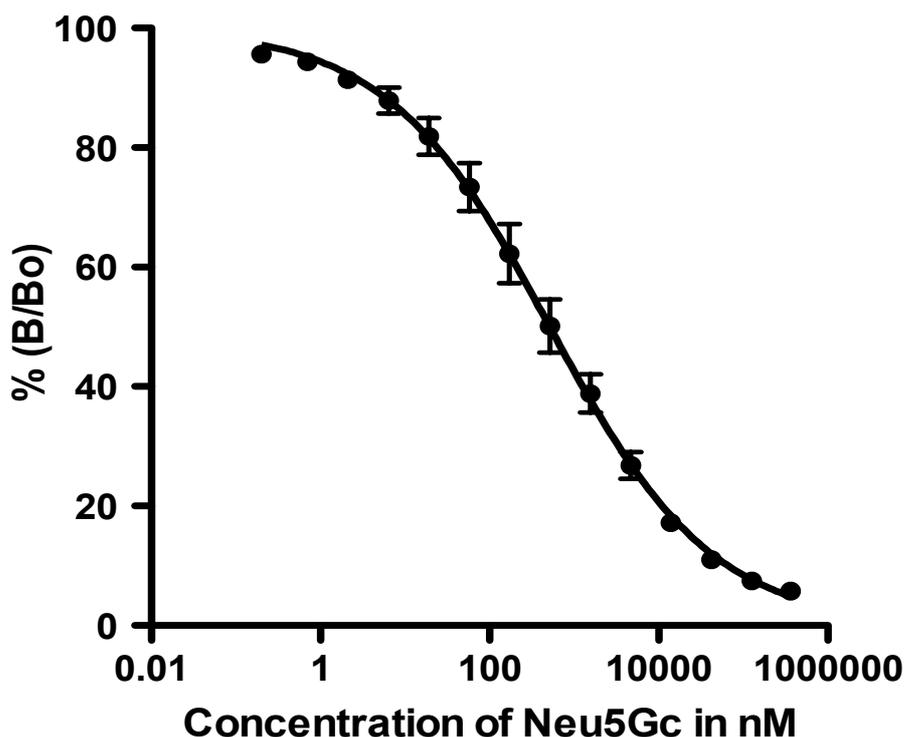


**Figure 3.15** Specificity testing of scFv-SL1A1. Bovine and human transferrin were used as competitors at a concentration range in a competitive ELISA to test the specificity of scFv-SL1A1 (Section 3.2.4.3). The data showed that human transferrin was not able to inhibit scFv-SL1A1 as compared to bovine transferrin demonstrating Neu5Gc specificity of SL1A1 scFv. Data points not marked to show error bars indicating  $\pm$ SD.

The inhibition data demonstrates that human transferrin failed to inhibit scFv-SL1A1 binding to Neu5Gc as opposed to bovine transferrin which clearly inhibited SL1A1 binding to Neu5Gc. The specificity data established that scFv-SL1A1 specifically binds to Neu5Gc without showing any cross-reactivity against Neu5Ac or underlying glycans.

### 3.3.9 Reproducibility of competitive ELISA

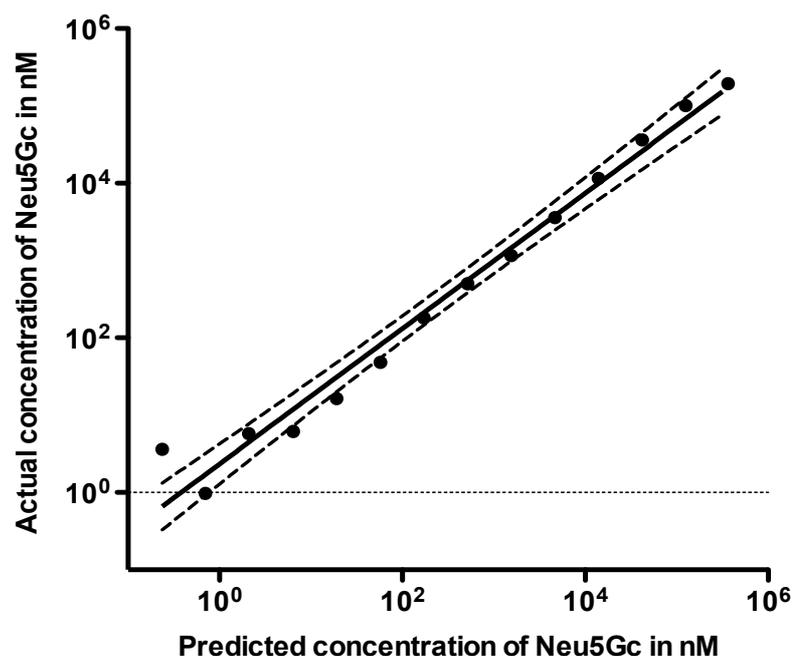
To demonstrate the reproducibility of inhibition assay using bovine transferrin as Neu5Gc standard, inhibition assay was performed 10 times over a period of 10 days. The assay, run over a number of different days takes into account the variability and fluctuations encountered due to handling, buffer preparation, sample preparation and stability of the scFv itself. Throughout the duration of the assay, buffers and samples were prepared on the day of assay. The inhibition curve was generated in triplicate each day for 10 days, the composite curve was generated by compiling the data collected over 10 days, Figure 3.16.



**Figure 3.16** Composite curve for anti-Neu5Gc scFv-SL1A1. Competitive ELISA performed in 10 independent runs over a period of 10 days (Section 3.2.4.4). Composite curve from 10 independent assays demonstrated reproducibility of inhibition assay. Error bars indicate  $\pm$  SEM.

The inhibition data was fitted to log inhibitor versus normalised response with a variable slope model using GraphPad Prism software version 6.0c. The composite inhibition curve from 10 independent inhibition assays demonstrated minimal variability of the assay. The scFv-SL1A1 showed an IC<sub>50</sub> of 514 nM. Data clearly suggested that scFv-SL1A1 can be used to reliably detect and quantify Neu5Gc.

For testing the accuracy of the inhibition curve in predicting the Neu5Gc concentration, data from 10 independent inhibition curves was compiled and Neu5Gc concentration was predicted. The average concentration predicted from 10 independent inhibition curves was then fitted against actual concentration of Neu5Gc used in the inhibition assay, by linear regression, Figure 3.17.



**Figure 3.17** Analysis of accuracy of Neu5Gc detection. Neu5Gc concentration predicted from 10 independent experiments and plotted against actual Neu5Gc concentration shows high correlation. This is indicative of assay reproducibility in terms of prediction of Neu5Gc in 10 independent experiments. Error bars indicate  $\pm 95\%$  confidence interval.

The predicted concentration data fitted against actual concentration showed a positive correlation of concentration values ranging from 12.4 mg/mL to 7.8 ng/mL (Neu5Gc dilution range:  $36 \times 10^4$  nM to 0.24 nM). This clearly indicated that competitive ELISA based assay using scFv-SL1A1 detected Neu5Gc reliably and accurately.

### **3.3.10 Quantitation of Neu5Gc from bovine transferrin standard curve and compare with HPLC data**

Competitive ELISA was performed to generate a Neu5Gc standard curve using bovine transferrin fitted using GraphPad Prism software version 6.0c. The standard curve was then used to estimate the Neu5Gc substitution ratio in test samples of bovine transferrin and bovine fetuin. To confirm the results from competitive ELISA, a HPLC method was employed to determine Neu5Gc substitution ratio for bovine transferrin. Table 3.1 shows comparative data obtained from literature, competitive ELISA and in house HPLC.

**Table 3.1** Comparison of estimated substitution ratio of Neu5Gc on glycoproteins by HPLC and competitive ELISA.

<b>Sample</b>	<b>HPAE-PAD</b>	<b>DMB-HPLC</b>	<b>ELISA</b>
Bovine transferrin	1.9	2.31	1.7
Bovine fetuin	0.54	NA	0.25

The substitution is expressed as moles of Neu5Gc per mole of protein.

Data from this comparative study shows that Neu5Gc substitution ratio estimated using scFv-SL1A1 in a competitive ELISA format using bovine transferrin were close to substitution values generated by DMB-HPLC. However, using the bovine transferrin standard curve, Neu5Gc substitution values estimates for bovine fetuin were half of the reported values.

### 3.4 Discussion

Glycans are one of most abundant molecules on cell surfaces, by virtue of their position, they mediate cell-cell interaction, host pathogen interaction, immune regulation and are involved in pathogenesis of various diseases (Varki and Lowe, 2009, Ohtsubo and Marth, 2006, Maverakis et al., 2015). Despite this, glycans have remained under appreciated, largely due to the fact that the study of glycans and their functional correlation is limited by lack of highly sensitive and specific tools to identify and analyse glycans (Walt et al., 2012). *N*-glycolylneuraminic acid, Neu5Gc, is a non-human glycan that is frequently found on various cancers in humans (Samraj et al., 2014). Further, presence of this glycan epitope on biopharmaceuticals leads to immunogenic reactions and reduced drug half-life (Ghaderi et al., 2010). Thus, detection and quantitation of Neu5Gc is not only important in cancer but also as a quality control tool for manufacturing of safe and efficacious drugs. The current research was aimed to develop a convenient and sensitive assay using a chicken derived monoclonal anti-Neu5Gc scFv-SL1A1.

Since the first report of ELISA technique in 1971 (Engvall and Perlmann, 1971), binding assays based on ELISA are still one of the most convenient, robust and cost effective assay. Over the years, ELISA has evolved into various assay formats adapted for research specific applications (Aydin, 2015). Recently, the assay has also been modified for detection of glycans using glycan binding proteins such as lectins (Gornik and Lauc, 2007). The current assay development activity was directed towards development of a competitive ELISA based assay for specific detection and quantitation of Neu5Gc. Given the small size of Neu5Gc, it is less likely to have more than one potential epitope for antigen binding. Hence, excluding sandwich type ELISAs formats for its detection in which the antigen is sandwiched in between a capture antibody and a detection antibody (Kanefusa et al., 1977). In contrast, the competitive ELISA method is advantageous for smaller antigens with one epitope for antigen recognition, wherein an antibody (scFv) competes for immobilised versus free antigen (Yorde et al., 1976). Further, competitive ELISA is highly sensitive for detection of antigens in a complex mixture of antigens with compositional differences (Gan and Patel, 2013). This feature of the competitive ELISA is relevant for most of the glycan epitopes including Neu5Gc.

In the initial phase of assay development, Neu5Gc was detected by the immunised chicken polyclonal serum, however this detection strategy is not novel and has been reported previously (Diaz et al., 2009). The aim of assay development using serum was to first to characterise the immunised chicken polyclonal sera for confirming its immunogenic response against Neu5Gc for library generation (Chapter 2) and second to optimise various assay related parameters that can be directly incorporated into assay development using anti-Neu5Gc scFv. Initial optimisation from immunised chicken polyclonal sera was directed to identify reasonable antigen coating concentration to reduce use of expensive sugar conjugate (Neu5Gc-PAA). The Neu5Gc-PAA was tested at 3 coating concentrations (5, 2.5 and 1  $\mu\text{g}/\text{mL}$ ) and 2.5  $\mu\text{g}/\text{mL}$  was found to be suitable for assay development (Figure 3.1). The assay was also optimised in terms of concentration of Tween 20 for use in wash buffers. Wash buffers with varying concentrations (0.05 to 1 %) of Tween 20 were employed and background signal was compared to identify the optimal concentration of Tween 20. Increasing the concentration of Tween 20 showed a slight reduction in background in a serum based assay, so typical concentration of 0.05 % was considered optimum (Figure 3.3). Further, from a scFv based assay point of view, it is likely that background would be less than serum based assay and 0.05 % Tween 20 would be sufficient for reducing background.

For immunoassays related to glycan detection, BSA is not considered a viable blocker as most of the commercial BSA preparations contain glycans that can cross-react with anti-glycan antibodies (Aspholm et al., 2006). This observation is of relevance in the current assay development exercise as BSA is a protein from bovine source and may contain traces of Neu5Gc that could possibly react with anti-Neu5Gc polyclonal antibodies and anti-Neu5Gc scFv. Consequently, in immunoassay development for the detection of glycan epitopes, BSA is replaced by pBSA, in which glycans are oxidised by sodium meta periodate treatment. Both the blockers (BSA and pBSA) were assessed at different concentrations for blocking. The BSA and pBSA were also included in the dilution reagents and tested at different concentrations in the Neu5Gc immunoassay. From the study it was concluded that a combination of 0.5 % pBSA as blocker and 0.1 % pBSA in sample dilution buffer showed reduced background as compared to similar combination of BSA (Figure

3.2). These conclusion were consistent to reported observation (Aspholm et al., 2006).

The parameters optimised with immunised chicken polyclonal sera were employed for development of anti-Neu5Gc scFv based assay. At the end of library generation (Chapter 2) 2 Short-linker (SL1A1 and SL4D1) and 1 long-linker (LL1B6) scFv were identified that bound to Neu5Gc specifically. For focused assay development for Neu5Gc, the 3 scFv were first compared for their sensitivity for Neu5Gc detection in a competitive ELSIA. Out of 3 scFv tested, scFv-SL1A1 showed higher sensitivity for detection of Neu5Gc as compared to SL4D1 and LL1B6. The long-linker scFv LL1B6 showed significantly less sensitivity for Neu5Gc than 2 Short-linker scFvs (Figure 3.4). This can be attributed to formation of monospecific diabody by short-linker scFvs therefore enhancing binding and detection of Neu5Gc. Similar effects have been reported for short-linker scFv by various research groups, wherein linker lengths of less than 12 amino acids promote dimer formation (Holliger et al., 1993, Kortt et al., 1997).

While scFv-SL1A1 showed sensitive detection of Neu5Gc, its specificity towards free and protein (bovine fetuin) bound Neu5Gc was tested in a competitive ELISA (Figure 3.5). The assay also included asialofetuin and other sugars (Neu5Ac and Gal- $\alpha$ -(1,3)-Gal) as controls. The scFv showed a clear dose dependent decrease in binding to immobilised Neu5Gc in presence of free Neu5Gc and bovine fetuin indicating Neu5Gc specific response. While there was no response for Neu5Ac and Gal- $\alpha$ -(1,3)-Gal, weak response was observed for asialofetuin. The response for asialofetuin can be explained from the fact that commercial preparations of asialofetuin do contain traces of sialic acid. For improved assay a dialysed preparation of asialofetuin should be used in Neu5Gc detection assays. From the above observations it was clear that scFv-SL1A1 was sensitive and specific for detection of Neu5Gc. Further assay development was carried out using scFv-SL1A1.

In assay development using scFv-SL1A1, various blockers (BSA, pBSA, HSA and pHSA) were compared initially for cross-reactivity to scFv at a range of scFv concentrations. The study showed that BSA cross-reacted with higher concentration of scFv as compared to the rest of the blockers (Figure 3.6). This is consistent with the observation of Aspholm et. al, where they observed that binding of *H. pylori* to

sialic acid was reduced in the presence of BSA while pBSA showed no interference to sialic acid binding (Aspholm et al., 2006). Following cross-reactivity assessment pBSA and HSA were used as blockers for comparison in an indirect ELISA. The results demonstrated that both pBSA and HSA were suitable for blocking and signal intensity for Neu5Gc detection was also similar (Figure 3.7). However, the pBSA being less expensive than HSA and pHSA was selected as a blocker for Neu5Gc detection assay.

In competitive ELISA based assays for Neu5Gc, free Neu5Gc competes with immobilised Neu5Gc for binding to anti-Neu5Gc scFv to generate a dose dependent inhibition curve. The Neu5Gc is an acidic sugar and can change the pH of buffers significantly in its free form. This change in pH of immunoassay buffers can alter the performance of assay significantly. To circumvent this problem studies were conducted to assess the impact of free Neu5Gc on buffer pH and to identify suitable buffering strength for the inhibition assays using free Neu5Gc (Figure 3.8). Further, scFv-SL1A1 was also tested for its binding characteristic at a range of pH units (Figure 3.10) in an indirect ELISA assay. The effect of Neu5Ac on buffer pH was also evaluated (Figure 3.9). The pH studies demonstrated that at 0.25 mg/mL and 2.5 mg/mL concentration both Neu5Gc and Neu5Ac decreased the pH of PBS, with the decrease in pH more drastic at 2.5 mg/mL concentration. The pH decreasing effect of Neu5Gc was slightly more than Neu5Ac. From this study, it was concluded that routine PBS which has a buffering strength of 10 mM phosphate was not suitable for use in the competitive ELISA. Instead PBS with buffering strengths from 60 mM to 150 mM phosphate buffer can be used for competitive ELISA. To make sure that the pH remains stable and above 7.0, PBS with a buffering strength of 150 mM was used for competitive ELISA using free Neu5Gc. From the binding characteristic study of anti-Neu5Gc scFv it was clear that scFv can remain stable during the assay and can detect Neu5Gc at a wider pH range (3.5 to 7.55). At a pH below 3.5 scFv binding was diminished. This is first study that reports the pH effect of Neu5Gc in assay conditions.

After gaining knowledge from pH studies the buffering strength of PBS was increased to 150 mM in the competitive ELISA using free Neu5Gc for generation of a Neu5Gc standard curve using 10 ng/mL of scFv-SL1A1. The scFv showed a dose dependent inhibition response in presence of free Neu5Gc at range of concentrations

resulting in an IC<sub>50</sub> of 221  $\mu$ M (Figure 3.11). Free Neu5Gc is an expensive sugar and also requires modified buffers to maintain pH during the assay. This not only increases the cost of the assay but also exposes other proteins to higher ionic strength buffers in cases where the Neu5Gc concentration have to be determined for unknown protein samples. To address this issue bovine transferrin was evaluated as an alternative to free Neu5Gc to generate a standard curve. The Neu5Gc substitution ratio for bovine transferrin is known and can also be estimated by in house HPLC methods, therefore it made sense to use bovine transferrin for standard curve generation. Initially, bovine transferrin was diluted in 2 fold and 3 fold step dilution series from starting concentration of 12.4 mg/mL using upto 15 dilutions. Both the dilution series showed inhibition but inhibition with 2 fold step dilution was more dramatic and did not follow a sigmoidal dose response relationship in contrast to 3 fold step dilution where a dose response relationship was achieved (Figure 3.12). For further assay development bovine transferrin was used in 3 fold dilution series.

To test the impact of modified PBS (150 mM buffering strength) on competitive ELISA using bovine transferrin, ELISA was performed in both modified and normal PBS. The testing showed that there was no impact of buffering strength on inhibition assay and also demonstrated the robustness of scFv-SL1A1 in terms of tolerating day to day variations that may arise in buffer preparation (Figure 3.13). Besides bovine transferrin, bovine fetuin was also used in an inhibition assay and showed a dose dependent inhibition profile parallel to bovine transferrin inhibition (Figure 3.14). This data confirmed that scFv-SL1A1 can detect Neu5Gc on glycoproteins in a concentration dependent manner. Further, parallel inhibition profiles observed for both bovine fetuin and bovine transferrin also indicated that the same antigen (Neu5Gc) is being detected on both the glycoproteins irrespective of underlying glycans.

Successful inhibition testing with bovine transferrin showed that scFv-SL1A1 can recognise Neu5Gc in its natural form, to further confirm that scFv is indeed binding to Neu5Gc, human transferrin was used as competitor in a competitive ELISA. The human transferrin shows 70 % sequence similarity with that of bovine transferrin and also have almost similar complex glycans except that bovine transferrin contains both Neu5Gc and Neu5Ac on its glycan terminus, in contrast human transferrin exclusively contain Neu5Ac (Baldwin, 1993, Lambert et al., 2005). Thus using

human transferrin will allow for a natural presentation of Neu5Ac and underlying glycans to scFv-SL1A1. The assay using both bovine transferrin and human transferrin demonstrated inhibition of scFv-SL1A1 with bovine transferrin while no inhibition response was observed for the human transferrin. This data strongly implied that the scFv-SL1A1 is specific to Neu5Gc, and underlying glycans or Neu5Ac do not interact with scFv (Figure 3.15). This is an advantage over existing lectins like SNA and MAA, which detect both Neu5Ac and Neu5Gc only in context to their linkage to underlying glycans (Rogerieux et al., 1993).

For the development of a competitive ELISA based assay using scFv it is imperative to assess the reproducibility of the assay over a number of different days to account for variability that may be introduced inadvertently from variations arising from buffer preparation, temperature, antibody stability etc. To demonstrate reproducibility, the competitive ELISA was performed 10 times with fresh buffers prepared each day either from buffer stock solutions or from mixing individual ingredients, scFv dilution were prepared each day from stock solution and competitor bovine transferrin was diluted from stock solution for each assay. Inhibition data over 10 assay runs was used to construct a composite curve (Figure 16). The scFv-SL1A1 showed an IC<sub>50</sub> of 514 nM. Further the standard curve prepared from 10 assay runs were used to back calculate the concentration of Neu5Gc to get the predicted concentration of Neu5Gc. The predicted or calculated concentration of Neu5Gc was then plotted against the actual concentration of Neu5Gc used in the assay. The predicted and actual concentrations showed high positive correlation ( $R^2$  0.94) demonstrating that inhibition curve predicted the Neu5Gc concentration with high accuracy (Figure 3.17).

The competitive ELISA was finally applied to estimate the Neu5Gc substitution ratio for bovine transferrin and bovine fetuin. For bovine transferrin the Neu5Gc substitution ratio were also assessed by DMB-HPLC. The ELISA assay predicted 1.5 moles of Neu5Gc per mole of bovine transferrin which was close to the DMB-HPLC measurements (2.31 moles of Neu5Gc per mole of bovine transferrin) (Table 3.1).

### 3.5 References

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## **Chapter 4**

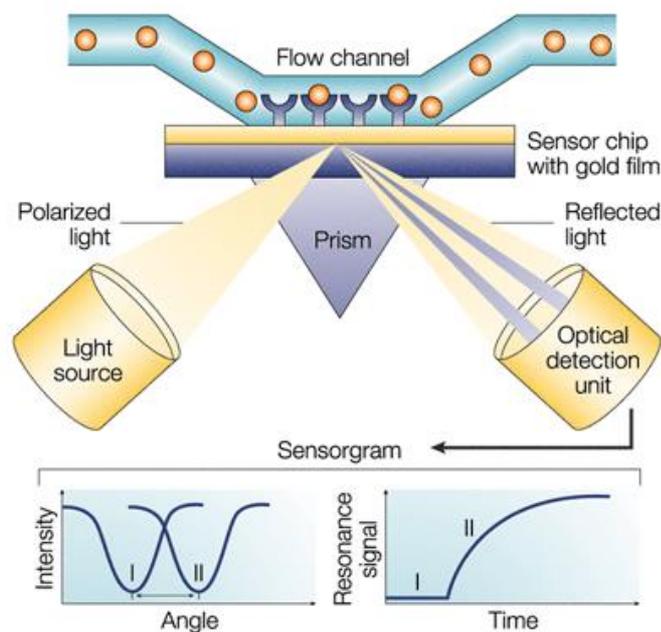
**Potential applications of scFv for detection of Neu5Gc using  
western blot, SPR and immunohistochemistry**

## 4.1 Introduction

This chapter details the application of scFv-SL1A1 for the detection of Neu5Gc across a number of assay formats. ScFv-SL1A1 has been demonstrated to bind to Neu5Gc (Chapter 2) and to be suited for application in a direct assay for the detection and quantification of Neu5Gc (Chapter 3).

The suitability and application of scFv-SL1A1 for detection of Neu5Gc on western blotted glycoproteins, therapeutic proteins and multiple myeloma serum samples was evaluated. Western blot (immunoblot) is a routinely used technique for the immunospecific detection of an antigen, first introduced in 1979 by Towbin *et al* for separation and identification of proteins (Towbin et al., 1979). The technique was later termed as ‘western blotting’ by W. Neal Burnette (Burnette, 1981). In western blotting, a mixture or complexed pool of protein is subjected to gel electrophoresis, resulting in protein separation due to molecular weight, isoelectric point, electric charge or a combination of these factors. The protein from the gel is then transferred to a membrane, in a process termed as blotting, which is then incubated with labelled antibodies specific to the protein of interest (Jensen, 2012). After removal by washing of the unbound antibodies, the membrane is ‘developed’ to detect the bound primary detection molecule, be they antibody, lectin, peptide or nucleic acid.

To further refine and study the kinetic interaction of scFv-SL1A1, Surface Plasmon Resonance (SPR) analysis was carried out. In principle, SPR is a physical phenomenon that occurs when plane polarised light above a critical angle is incident on the thin conducting film placed at the interface between the media of different refractive indices (Tang et al., 2010, Guiducci, 2011). The typical SPR instrument comprises of a near infrared light source, a prism, a detector and a sensor chip that is usually made up of a carboxymethyl dextran matrix linked to a thin layer (~ 50 nm) of gold substrate on the sensor surface (Daghestani and Day, 2010), Figure 4.1.



**Figure 4.1** Illustration of SPR functioning. SPR measures the changes in refractive index near the gold surface. The binding events occurring with the surface immobilised ligand changes refractive index near the gold surface. The change in refractive index shifts the SPR angle from the I to the II position, which is reflected as change in resonance signal per unit time on a sensorgram. Figure reused from (Cooper, 2002), with permission from Nature publishing group.

During operation, plane polarised light from a light source is focussed on the back of a gold layer, through a prism. At a certain critical angle of incident light, total internal reflection (TIR) occurs and the reflected light passes through a prism and is detected by a light detector. During TIR not all the light is reflected, some part of the light is absorbed which is observed as a sharp decrease in intensity of reflected light due to absorption of light energy by the surface electrons (surface plasmons) of the gold layer (Tudos and Schasfoort, 2008, Guiducci, 2011). The decrease in intensity of the reflected light is maximum at an angle above the critical angle, and is called the SPR angle. The surface plasmon phenomenon is affected by the change in refractive index close to the gold layer. Therefore, a change in mass occurring near the gold sensor surface changes the refractive index near the surface, which in turn shifts the angle of incident light at which SPR occurs. The change in SPR angle is translated into resonance units and is reflected as a sensorgram (Guiducci, 2011, Van Der Merwe, 2001). Typically, the gold sensor chip is coated with a hydrophilic layer of dextran hydrogel that allows covalent attachment of a ligand by amine, thiol,

aldehyde or maleimide chemistry. The binding partner of the surface attached ligand is then passed over the sensor surface, the dynamic binding and dissociation events occurring near the sensor surface are recorded in real time. The SPR is therefore used for label-free detection of analyte and aids in rapid, real-time monitoring of biomolecular reactions using very small quantities of label-free sample (Lausted et al., 2011). In current SPR analysis of scFv-SL1A1, bovine fetuin and bovine transferrin were used. Both these proteins carry Neu5Gc and therefore selected as reference proteins and were passed over a fixed concentration of immobilised scFv-SL1A1, presented on an SPR sensor chip to determine kinetic interactions.

ScFv-SL1A1 was also evaluated for its suitability in immunohistochemistry (IHC) analysis. Neu5Gc occurs on normal and cancer tissues. Its incorporation on human tissues has been attributed to dietary intake of food rich in Neu5Gc. Initial IHC protocol optimisation was performed on rat spinal cord tissue. The rat spinal cord tissue was stained with scFv-SL1A and secondary antibodies anti-HA-FITC and anti-HIS FITC. The IHC protocol was then optimised on a human tissue array containing 5  $\mu\text{m}$  sections of matched normal and malignant tissues of lung, breast, prostate, colon and liver. The study was performed to explore the potential application of scFv-SL1A1 for detection of Neu5Gc in clinical histochemistry.

## **4.2 Material**

HRP-labelled monoclonal anti-HIS antibody, human serum albumin (HSA), bovine serum albumin (BSA), ovalbumin, bovine fetuin, bovine asialofetuin, apo-transferrin bovine, human transferrin, bromophenolblue, DTT, glycerol and 0.2  $\mu\text{m}$  Immobilon™-P PVDF (Polyvinylidene fluoride) membrane for western blotting were from Sigma-Aldrich Co., (Dublin, Ireland). Syringe filters 0.22, 0.45  $\mu\text{m}$  were purchased from Millipore, (Dublin, Ireland). Carboxymethyl dextran slide was purchased from Reichert Technologies Life Sciences (New York, USA). NuPAGE® denaturing gel and ProLong® Diamond Antifade mountant medium was from Life Technologies (UK). EDC, NHS and PageRuler™ Plus prestained protein ladder were from Thermo Fisher Scientific, (UK). Multi-normal and tumor tissue array, 12 cases, 24 samples (1.5 mm) slide (ab178229) was purchased from Abcam®. Glass tubes for SPR were from Apex (North Carolina, USA).

### **4.3 Application of scFv-SL1A1 for detection of Neu5Gc on western blotted glycoproteins**

#### **4.3.1 Methods**

##### **4.3.1.1 Detection of Neu5Gc by scFv-SL1A1 in western blot platform**

For western blotting, the glycoproteins were first resolved by SDS-PAGE and then transferred onto PVDF membrane. The glycoproteins were resolved either on a 4-12 % gradient precast NuPAGE® denaturing gel or on 12 % denaturing gel prepared in-house. For both precast (NuPAGE®) and in-house gel runs, the protein samples were mixed with 6 X reducing buffer (350 mM tris, 10 % w/v SDS, 50 mM bromophenol blue, 18 mM DTT, 9 % v/v glycerol) and heated in a heat block for 10 min at 95 °C. The protein samples were then allowed to cool down and centrifuged 5000 x g for 30 sec. The molecular weight marker and protein samples were then loaded into the wells. The proteins were then resolved for 60 min at constant voltage of 150 V.

After resolving proteins on SDS-PAGE, the proteins were transferred onto PVDF membrane by western blotting. Prior to transfer, the PVDF membrane was soaked in methanol for 5 min. The PVDF membrane and blotting paper were incubated in transfer buffer (25 mM tris base, 200 mM glycine, 20 % methanol) for 30 min. The blotting paper was placed on the anode plate, the PVDF membrane was kept over the blotting paper and then gel was placed on PVDF membrane. The blotting paper was placed on the gel and the sandwich assembly was then covered by the cathode plate. The transfer was performed at 15 V for 20 min. After transfer, the PVDF membrane was blocked with the 2 % pBSA in PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na<sub>2</sub>HPO<sub>4</sub>, 1.8 mM KH<sub>2</sub>PO<sub>4</sub>, pH adjusted to 7.4 with phosphoric acid) for 90 min. The membrane was then washed 3 times with PBS-T (PBS containing 0.05 % Tween 20) and incubated with 400 ng/mL of scFv-SL1A1 in PBS-T for 1 h at RT. The membrane was then washed 3 times with PBS-T and incubated with 1:5000 of anti-HIS-HRP in PBS-T for 1 h at RT. The membrane was washed 3 times with PBS-T and 2 times with PBS. The membrane was then developed with addition of 15 mL of SIGMA FAST DAB Peroxidase Substrate (one 3, 3'-Diaminobenzidine and one urea hydrogen peroxide tablet in 15 mL of deionized water).

#### **4.3.1.2 Demonstration of specificity scFv-SL1A1 on western blotted glycoproteins**

From a 25 mg/mL stock, 100  $\mu$ L bovine transferrin and bovine fetuin were taken and mixed with 100  $\mu$ L of 2 M HCl to get a final protein concentration of 12.5 mg/mL in 1 M HCl. The protein, diluted in HCl was heated for 2 h at 80 °C to release sialic acids. The acid treated samples were then neutralised by addition of 200  $\mu$ L of 1 M Tris-HCl, pH 7.5. For blotting, 1  $\mu$ g each, of non-acid treated bovine transferrin and bovine fetuin, and 1, 2 and 4  $\mu$ g of acid treated bovine transferrin (~ 76-81 kDa), and bovine fetuin (~ 48.4 kDa) were loaded onto 12 % reducing SDS-PAGE gel and then transferred to a PVDF membrane. The blot was developed as in Section 4.3.1.1. A separate 12 % reducing SDS-PAGE gel was loaded with 0.8, 2.0 and 4.0  $\mu$ g of acid-treated and 0.8  $\mu$ g of non-treated proteins (acting as controls) for visualisation of proteins post-acid treatment.

#### **4.3.1.3 Estimation of loading quantity of glycoprotein for Neu5Gc detection**

A preliminary study with western blot was performed to estimate the appropriate loading quantity of bovine transferrin and bovine fetuin, for detection of Neu5Gc using scFv-SL1A1. For estimation, the bovine transferrin and bovine fetuin were blotted onto PVDF membrane at a protein loading range of 3  $\mu$ g to 0.05  $\mu$ g per well. The substitution ratio of Neu5Gc per mole of bovine transferrin is, 2.31 mole of Neu5Gc per mole of protein (as determined by DMB-HPLC in-house; Chapter 3). In bovine fetuin, per mole of bovine fetuin contains 0.54 moles of Neu5Gc (Dionex technical note 41). The substitution values for Neu5Gc on both the glycoproteins were used to calculate the quantity of Neu5Gc loaded per well.

Taking the substitution value of Neu5Gc into account, 3  $\mu$ g to 0.05  $\mu$ g per well of bovine transferrin translated into a Neu5Gc loading of 87.7 nmole to 1.5 nmole per well. For bovine fetuin 3  $\mu$ g to 0.05  $\mu$ g per well of protein load represented a Neu5Gc load of 36.5 nmole to 0.6 nmole. For western blotting, both the proteins were resolved on 12 % SDS-PAGE gel and blotted onto PVDF membrane. The blot was developed as in Section 4.3.1.1.

#### **4.3.1.4 Application of scFv-SL1A1 for determination of Neu5Gc in clinical and biopharmaceutical samples**

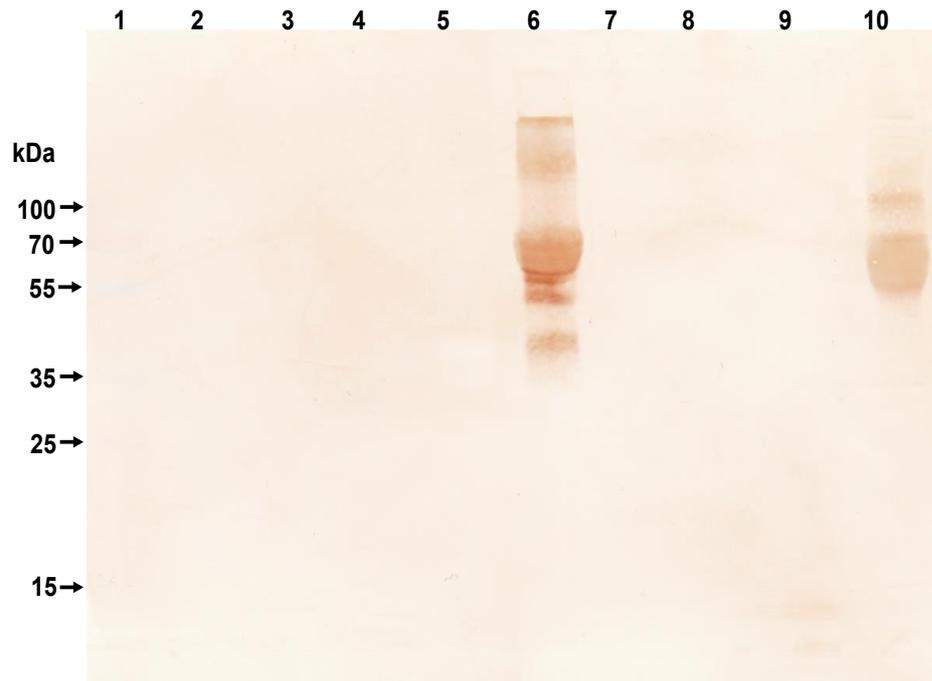
Serum samples obtained from multiple myeloma patients and healthy controls were tested for the presence of Neu5Gc using scFv-SL1A1. Prior to western blotting, the serum samples were delipidised, albumin depleted and enriched for the immunoglobulin fraction. For Neu5Gc detection, 10 µg of immunoglobulin enriched serum from multiple myeloma patients and healthy controls was loaded and ran on a reducing 4-12 % SDS-PAGE NuPAGE® gel. As a Neu5Gc positive control, 1 µg of bovine transferrin and as negative control, 1 µg of human transferrin was loaded on the gel. The resolved proteins were then transferred onto PVDF membrane and probed for the presence of Neu5Gc using scFv-SL1A1 as the primary antibody. The PVDF membrane was then developed as detailed in Section 4.3.1.1.

ScFv-SL1A1 was also used to detect the presence of Neu5Gc on FDA approved biopharmaceutical preparations, Mabthera (~144.5 kDa), Cetuximab (~152 kDa) and Avastin (~149 kDa). As a Neu5Gc positive control, bovine transferrin was used, with pBSA (~ 66.5 kDa) and HSA(~ 66.5 kDa) serving as negative controls. For analysis, 5 µg of each protein was loaded and resolved on a 12 % reducing SDS-PAGE and then blotted on PVDF membrane. The scFv-SL1A1 as primary antibody was then used to detect the Neu5Gc. The PVDF membrane was then developed as detailed in Section 4.3.1.1.

### **4.3.2 Results**

#### **4.3.2.1 Analysis of scFv-SL1A1 for detection of Neu5Gc on glycoproteins**

Bovine transferrin and bovine fetuin were used as Neu5Gc positive control while BSA, pBSA, HSA, ovalbumin,  $\alpha$ 1-AGP and asialofetuin were used as negative controls. 10 µg of each protein was resolved on reducing SDS-PAGE and then transferred on PVDF membrane. The Neu5Gc was then probed with scFv-SL1A1 and bound scFv detected by anti-HIS-HRP antibody, Figure 4.2.



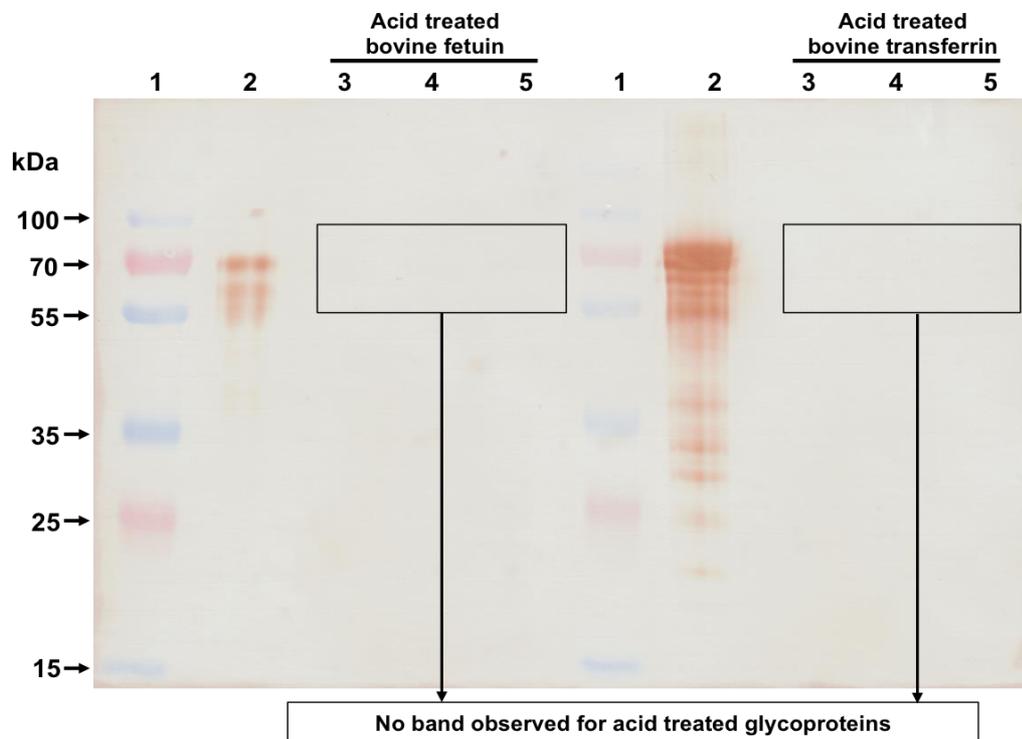
**Figure 4.2** Western blot PVDF membrane transfer of proteins resolved on reducing 12 % SDS-PAGE gel. The scFv-SL1A1 used to detect Neu5Gc on blotted proteins. The bound scFv was detected with anti-HIS-HRP antibody. Lane 1: Molecular mass marker, lane 2: HSA (~ 66.5 kDa), lane 3: pBSA, lane 4: BSA (~ 66.5 kDa), lane 5: Ovalbumin (~ 45 kDa), lane 6: Bovine transferrin (~ 76-81 kDa), lane 7: Human transferrin, lane 8:  $\alpha$ 1-AGP, lane 9: Asialofetuin and lane 10: Fetuin (~ 48.4 kDa). Each well loaded with 10  $\mu$ g of protein. In lane 6 and lane 10, signal was observed for Neu5Gc on bovine transferrin and bovine fetuin respectively.

The western blotting demonstrated that scFv-SL1A1 detected the presence of Neu5Gc on bovine transferrin and bovine fetuin, while there was no signal for human transferrin and asialofetuin (bovine fetuin without sialic acids). Similarly, no signal was obtained for BSA, pBSA, HSA,  $\alpha$ 1-AGP and ovalbumin. As discussed previously, both human and bovine transferrin have over 70 % of sequence similarity and contain complex *N*-glycans structures that terminate with sialic acids (Baldwin, 1993). While *N*-glycans in bovine transferrin contain both Neu5Gc and Neu5Ac, *N*-glycans in human transferrin exclusively contain Neu5Ac, thus detection of Neu5Gc on bovine transferrin, but not on human transferrin, demonstrated the specificity of scFv-SL1A1 in western blot format. Additionally, the Neu5Gc was also detected on bovine fetuin but not on asialofetuin in which sialic acids were removed. This data

showed that scFv-SL1A1 can also be applied to detect Neu5Gc on western blotted glycoproteins.

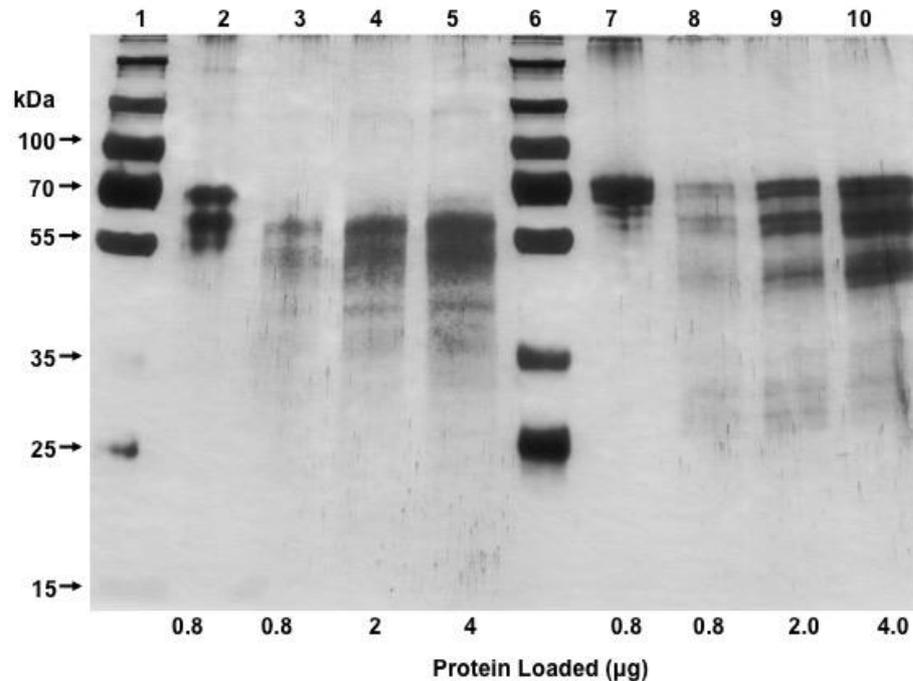
#### 4.3.2.2 Specificity testing of scFv-SL1A1 on western blotted glycoproteins

To further assess the specificity of detection of Neu5Gc by scFv-SL1A1, Neu5Gc containing glycoproteins, bovine transferrin and bovine fetuin, were subjected to mild acid treatment for release of Neu5Gc by acid hydrolysis. The acid treated glycoproteins were then neutralised, resolved on reducing SDS-PAGE and then blotted onto PVDF membrane, Figure 4.3. Non-acid treated glycoproteins were used as Neu5Gc positive controls.



**Figure 4.3** Western blot PVDF membrane of 12 % SDS-PAGE protein gel. The scFv-SL1A1 was used to detect Neu5Gc on bovine transferrin and bovine fetuin treated with acid to remove sialic acids. Lane 1 and 6: Molecular mass marker, lane 2: 1 µg of non-acid treated bovine fetuin, lane 3-5: 1, 2, 4 µg of acid treated bovine fetuin, lane 7: 1 µg of non-acid treated bovine transferrin, lane 8-10: 1, 2, 4 µg of acid treated bovine transferrin. Neu5Gc detected on non-acid treated glycoproteins, no signal obtained for Neu5Gc after removal of sialic acid by acid treatment.

To assess whether acid treated bovine fetuin and bovine transferrin were intact post-acid treatment, the proteins with their corresponding non-acid treated controls were analysed by silver staining of a 12 % reducing SDS-PAGE gel, Figure 4.4.

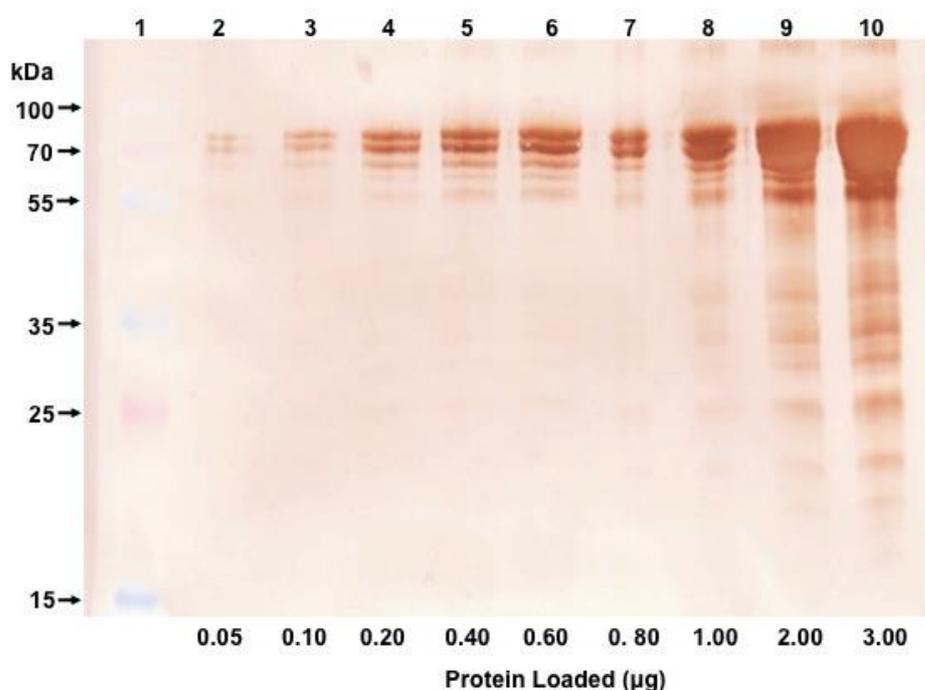


**Figure 4.4** Reducing 12 % SDS-PAGE with silver staining showing non-acid treated and acid treated bovine fetuin and bovine transferrin. Lane 1 and 6: Molecular mass marker, lane 2: 0.8 µg of non-acid treated bovine fetuin, lane 3-5: 0.8, 2 and 4 µg of acid treated bovine fetuin, lane 7: 0.8 µg of non-acid treated bovine transferrin, lane 8-10: 0.8, 2 and 4 µg of acid treated bovine transferrin.

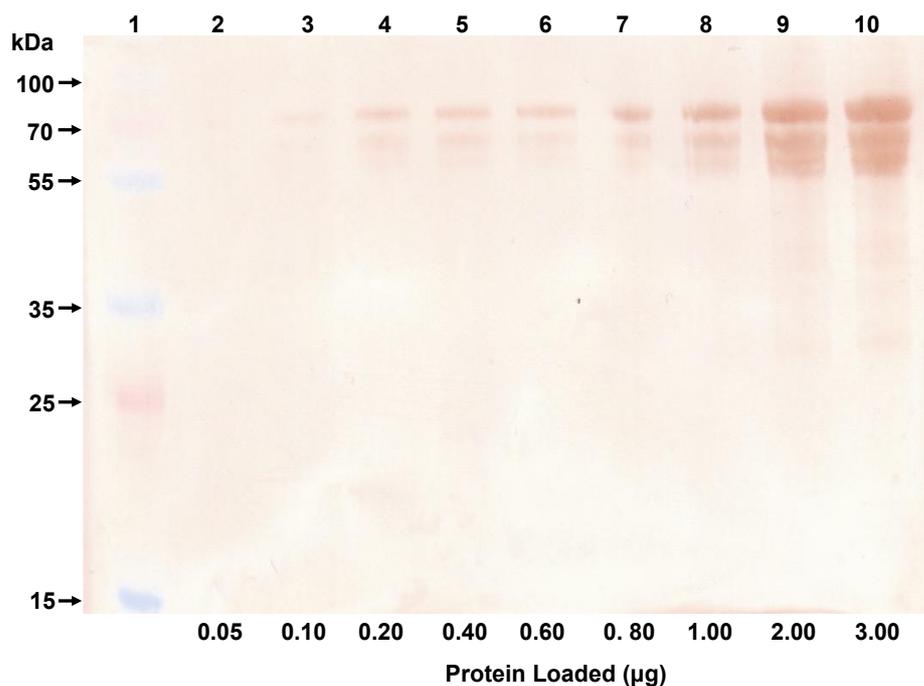
Western blotting analysis of acid treated and non-treated bovine fetuin and bovine transferrin indicated loss of Neu5Gc specific signal for acid treated glycoproteins against non-treated controls. This suggested that the signal was specific to Neu5Gc as protein backbone alone without sialic acid had no response on western blot. Further, the silver stained 12 % reducing SDS-PAGE gel showed that both the proteins were intact after acid treatment. Though a small change in molecular weight was observed for acid treated glycoproteins as compared to non-acid treated control, which can be attributed to removal of charged sialic acids.

### 4.3.2.3 Estimating loading quantity for Neu5Gc detection

Preliminary studies were conducted to test scFv-SL1A1 for detection of Neu5Gc over a range of protein loading quantity for bovine transferrin and bovine fetuin. Both the glycoproteins were blotted onto PVDF membrane at a range of 3  $\mu\text{g}$  to 0.05  $\mu\text{g}$  per well. The approximate amount of Neu5Gc loaded into each well of the gel was calculated from Expression 1. The calculated Neu5Gc loading range for bovine fetuin translated into 36.5 nmole to 0.6 nmole and for bovine transferrin 87.7 nmole to 1.5 nmole. Both glycoproteins were resolved on 12 % reducing SDS-PAGE gel followed by blotting on PVDF membrane. The Neu5Gc on blotted proteins was detected by scFv-SL1A1, Figure 4.5 and 4.6.



**Figure 4.5** Western blot PVDF membrane of reducing 12 % SDS-PAGE of bovine transferrin. The scFv-SL1A1 was used to detect Neu5Gc, at range of loading quantities. Lane 1: Molecular mass marker, lane 2-10: increasing concentration of Neu5Gc in nmole (1.5, 2.9, 5.8, 11.7, 17.5, 23.4, 29.2, 58.5 and 87.7). The quantity of Neu5Gc was calculated from quantity of protein loaded per well using Expression 1.



**Figure 4.6** Western blot PVDF membrane of reducing 12 % SDS-PAGE of bovine fetuin. The scFv-SL1A1 was used to detect Neu5Gc, at range of loading quantities. Lane 1: Molecular mass marker, lane 2-10: increasing concentration of Neu5Gc in nmole (0.6, 1.2, 2.4, 4.9, 7.3, 9.8, 12.2, 24.4 and 36.5 nmoles). The quantity of Neu5Gc was calculated from quantity of protein loaded per well using Expression 1.

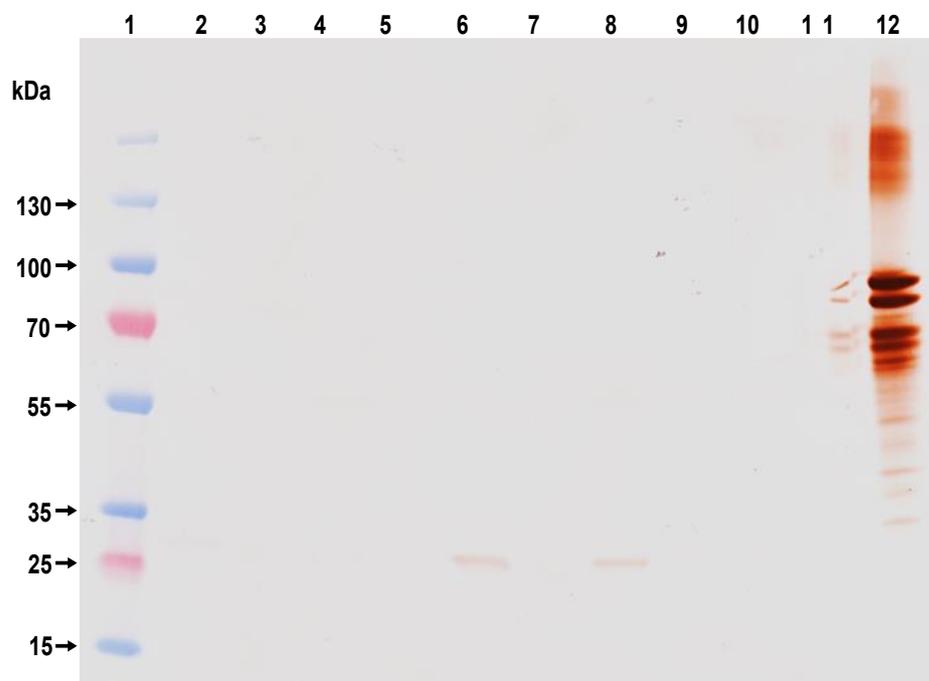
The data showed that scFv-SL1A1 could distinctly detect low nano moles of Neu5Gc on western blotted glycoproteins. For Neu5Gc presented on bovine transferrin, the scFv-SL1A1 showed detection to the lowest quantity loaded (1.5 nmole). For bovine fetuin, the Neu5Gc was detected to the minimum loading of 1.2 nmole.

However, it should be noted that, the exact estimate of the Neu5Gc detection and loading quantity cannot be made from the experiment, as rigorous validation for 100 % transfer of the protein on PVDF membrane will be required.

#### **4.3.2.4 Potential identification of Neu5Gc in clinical samples**

In a first trial for the application of anti-Neu5Gc scFv-SL1A1 for the detection of Neu5Gc in clinical samples, sera obtained from multiple myeloma patients and healthy controls were tested for presence of Neu5Gc using scFv-SL1A1. Serum samples of healthy and multiple myeloma patients were pre-processed (delipidisation,

albumin depletion and immunoglobulin enrichment) and resolved on a 4-12 % reducing SDS-PAGE gel and then transferred onto PVDF membrane. ScFv-SL1A1 was then used to detect Neu5Gc on blotted proteins, Figure 4.7.

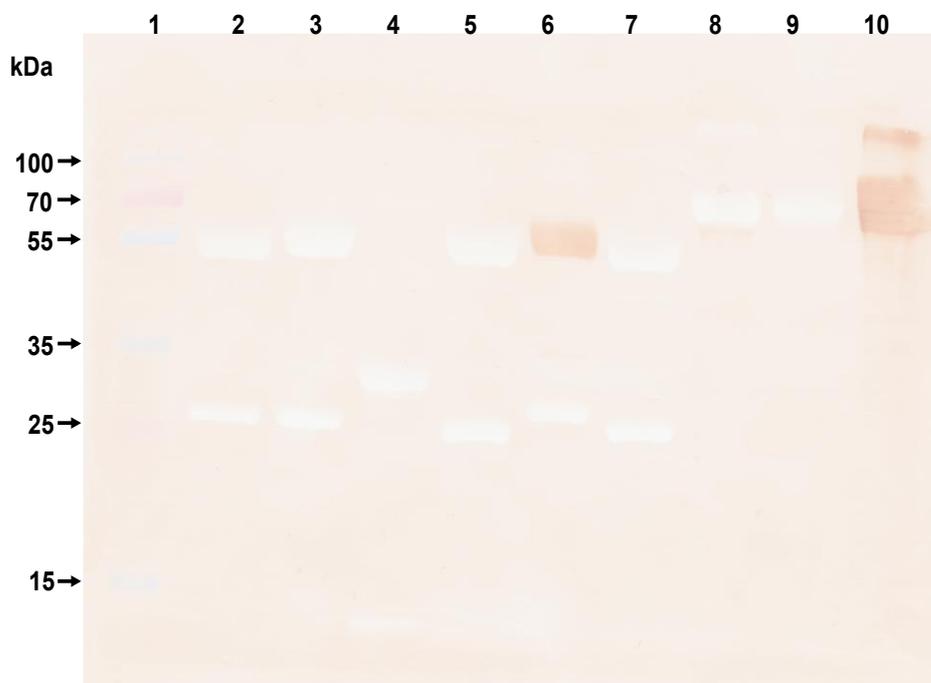


**Figure 4.7** Serum samples from healthy and multiple myeloma patients resolved on reducing 4-12 % SDS-PAGE gel and blotted on PVDF membrane. Lane 2-4, 6-8: Patient serum, lane 5, 9 and 10: Healthy control serum, lane 11: Human transferrin and lane 12: Bovine transferrin. ScFv-SL1A1 bound to the light chain of antibody present in multiple myeloma serum samples (lane 6 and 8).

The scFv-SL1A1 showed binding in 2 serum samples of multiple myeloma as compared to normal serum samples, where no binding was observed. The binding of scFv-SL1A1 to light chain of antibody isolated from multiple myeloma serum samples indicated the presence of aberrant glycans potentially containing Neu5Gc, which is consistent with the reported observation of aberrant glycosylation of antibodies in multiple myeloma. However, presence or expression of Neu5Gc specifically on antibody in multiple myeloma has not been reported (Narimatsu et al., 2014, Toda et al., 2009).

Further, scFv-SL1A1 was also applied to screen Neu5Gc on FDA approved biopharmaceutical preparations. The biopharmaceutical preparations were resolved on a 12 % reducing SDS-PAGE and then blotted onto PVDF membrane. ScFv-

SL1A1 was then used to detect the presence of Neu5Gc, Figure 4.8. Bovine transferrin was used as Neu5Gc positive control and BSA and HSA were used as negative controls.



**Figure 4.8** Biopharmaceutical drugs resolved on 12 % reducing SDS-PAGE and blotted on PVDF membrane probed with scFv-SL1A1 for the presence of Neu5Gc. Lane 1: Molecular mass marker, lane 2-4: mAbs, lane 5: Mabthera, lane 6: Cetuximab, lane 7: Avastin, lane 8: pBSA, lane 9: HSA and lane 10: bovine transferrin. The scFv showing the presence of Neu5Gc on Cetuximab (lane 6).

ScFv-SL1A1 showed binding to the heavy chain of Cetuximab, while no binding was observed on the rest of the mAb drugs included in the study. The binding indicated towards the presence of Neu5Gc on Cetuximab, which is consistent with similar observations from other research groups (Ghaderi et al., 2010). The white bands observed on the blot were attributed to high amount of protein loaded on the gel (Mahmood and Yang, 2012).

## **4.4 SPR analysis of scFv-SL1A1**

### **4.4.1 Methods**

#### **4.4.1.1 Conditioning of Reichert SPR instrument**

Reichert SPR instrument was set up prior to use by performing wash and preconditioning steps to clean and prime the instrument for use. The flow channels, sampling ports and syringe were washed once with 0.5 % SDS, 2 washes with deionised water, 2 washes with 50 mM glycine HCL pH 9.5 and at least 5 washes with deionised water. The system was then conditioned with running buffer PBS-T (137 mM NaCl, 2.7 mM KCl, 10 mM Na<sub>2</sub>HPO<sub>4</sub>, 1.8 mM KH<sub>2</sub>PO<sub>4</sub>, 0.05 % Tween® 20 pH adjusted to 7.4 with phosphoric acid). All the buffers were filtered (0.22 µm) and degassed before use.

#### **4.4.1.2 pH scouting for scFv-SL1A1 immobilisation**

pH scouting study was performed to determine the optimum pH for maximum immobilisation of scFv-SL1A1 on the surface of carboxymethyl dextran sensor chip. In pH scouting studies, 10 mM sodium acetate (CH<sub>3</sub>COONa) buffer at a range of pH (pH 4.0, 4.5, 5.0 and 5.5) was used to dilute scFv-SL1A1 to a final concentration of 20 µg/mL. The diluted scFv-SL1A1 was aliquotted (500 µL) into sampling glass vials and injected at a flow rate of 20 µL/min with a binding time of 1 min and dissociation time of 10 sec. After each injection of scFv-SL1A1, running buffer was injected to condition the sensor surface and to remove any traces of previous injection. The immobilisation at specific pH was assessed by comparing the binding response on the sensor surface.

#### **4.4.1.3 Activation of sensor chip by EDC/NHS coupling of scFv-SL1A1 to sensor**

For immobilisation of scFv on the carboxymethyl dextran sensor chip, amine-coupling chemistry using EDC (1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide) /NHS (N-Hydroxysuccinimide) was employed.

EDC in the presence of NHS activates the carboxyl group on the sensor surface to form esters. The NHS esters then react with the primary amines, which are present on the N-terminus of the protein and on the side chain of lysine (Murphy et al., 2006). 1 M ethanolamine, pH 8.0, is then used to block the remaining unbound NHS

esters (Fischer, 2010).

For covalent attachment of scFv-SL1A1, the carboxymethyl sensor chip was docked and conditioned with running buffer at 25 °C. The detector scan was done to estimate proper docking. For activation of the carboxymethyl dextran sensor chip, a 1:1 ratio of 0.4 M EDC and 0.1 M NHS were injected through both channels at a flow rate of 25 µL/min for 7 min. Activation was followed by injection of 10 µg/mL of scFv-SL1A1 in 10 mM sodium acetate buffer, pH 4.0, through the left channel only on the sensor surface at a flow rate of 25 µL/min for 10 min. For blocking excess activated groups on the sensor surface, 1 M ethanolamine HCl, pH 8.5, was injected through both the channels for 10 min at a flow rate of 25 µL/min. The sensor surface was then washed with running buffer.

#### **4.4.1.4 Kinetic assessment and detection of Neu5Gc using of scFv-SL1A1**

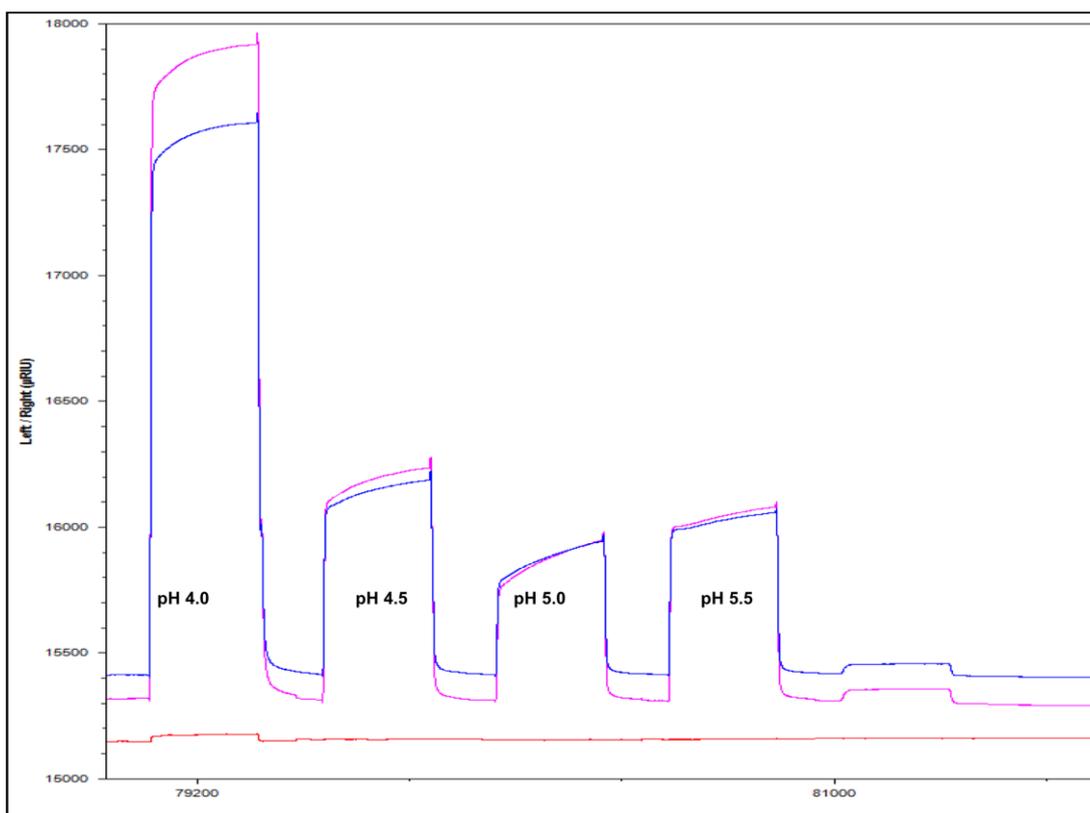
Bovine transferrin and bovine fetuin were used for the kinetic assessment of scFv-SL1A1. Bovine transferrin was serially diluted in a 3 fold step dilution series at a concentration range of 500 µg/mL to 228 ng/mL in SPR running buffer and injected sequentially on the sensor surface at flow rate of 25 µL/min and allowed to associate and dissociate for 6 min each. As a negative control, 62.5 µg/mL of human transferrin was injected at flow rate of 25 µL/min and allowed to associate and dissociate for 6 min each.

Kinetic analysis was also performed using bovine fetuin. To a preconditioned sensor chip with immobilised scFv-SL1A1, bovine fetuin diluted serially in a concentration range of 1.5 mg/mL to 244 ng/mL was injected sequentially on the sensor chip. The parameters of flow rate, association and dissociation were the same as for bovine transferrin. As a negative control, asialofetuin and  $\alpha$ 1-AGP were injected at a concentration of 125 µg/mL and 62.5 µg/mL. Prior to use, the asialofetuin stock was dialysed against deionised water to remove traces of sialic acids that may remain in commercial asialofetuin preparations. Dialysed asialofetuin was then diluted to assay concentration for use in SPR. The analysis of data for determination of association and dissociation constant were performed with the Scrubber 2 data analysis package (BioLogic Software, Australia).

## 4.4.2 Results

### 4.4.2.1 pH scouting

The pH scouting data demonstrated that scFv-SL1A1 at pH 4.0 showed higher binding or interaction with the carboxymethyl sensor surface, Figure 4.9. Thus pH 4.0 was used for immobilisation of scFv-SL1A1 to the sensor surface.

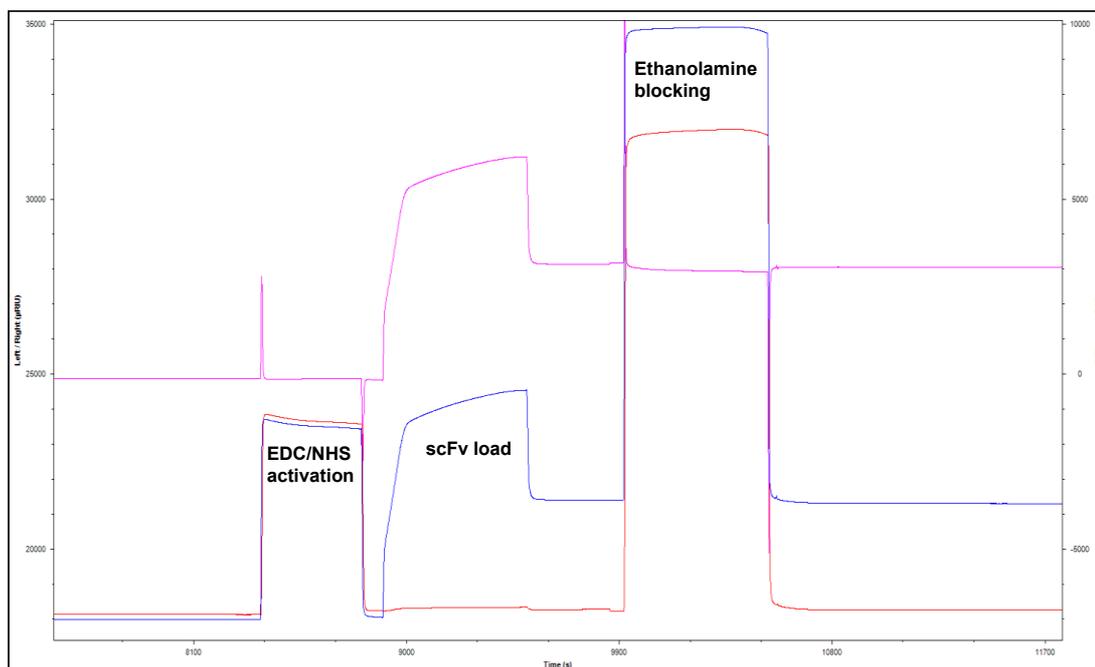


**Figure 4.9** pH scouting of scFv SL1A1. The pH scouting demonstrated higher binding of scFv-SL1A1 to sensor chip at pH 4.0 as compared to rest of the pH values. Red: Signal of reference/control channel, Blue: Signal sample/Test channel and Pink: Blue – Red (response of analyte)

### 4.4.2.2 EDC/NHS activation

The carboxymethyl sensor chip was first activated by injection of 1:1 solution of EDC/NHS followed by injection of 10  $\mu\text{g}/\text{mL}$  scFv-SL1A1 in 10 mM sodium acetate, pH 4.0, and blocked by 1 M ethanolamine, pH 8.5, Figure 4.10. The SPR data showed that scFv immobilised on the surface (pink line), compared to the reference channel (red line). The resultant signal (pink line), which is the difference in between the reference channel (red line) and test channel (blue line), was stable

after immobilisation and ethanolamine blocking indicating stable immobilisation without leaching or shedding of scFv-SL1A1.



**Figure 4.10** EDC/NHS activation of carboxymethyl sensor chip and immobilisation of scFv-SL1A1. The scFv immobilised on the sensor surface as indicated by difference of signal in between reference and test channel. Red: Signal of reference/control channel, Blue: Signal sample/Test channel and Pink: Blue – Red (response of analyte)

#### 4.4.2.3 Kinetic assessment of scFv-SL1A1

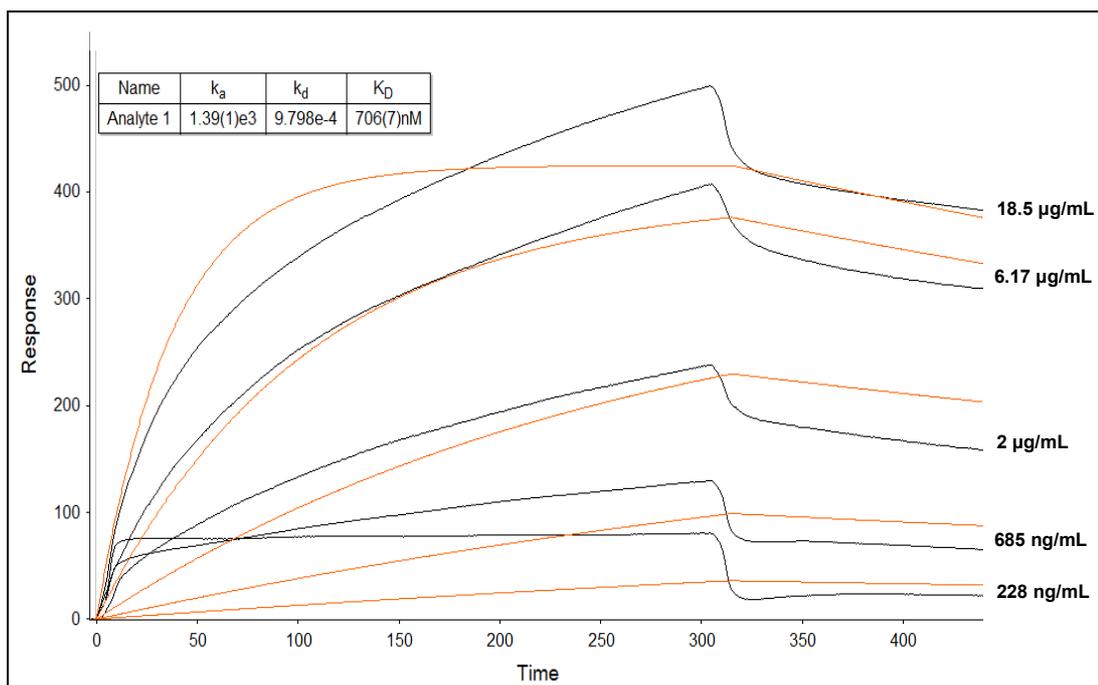
Free Neu5Gc is an acidic sugar and alters the pH of the assay buffers and was shown to alter the binding characteristics of the scFv (Section 3.3.6). To overcome this, bovine transferrin and bovine fetuin were used to present Neu5Gc to scFv-SL1A1 for kinetic studies.

Both these proteins contain Neu5Gc and demonstrate Neu5Gc dependent binding to scFv-SL1A1, as confirmed by inhibition assay previously (Section 3.3.9). For analysis, the scFv-SL1A1 was covalently attached to the carboxymethyl sensor chip using EDC/NHS coupling chemistry. Bovine transferrin and bovine fetuin diluted at a series of concentrations were injected through the sensor chip. Human transferrin, human  $\alpha$ 1-AGP and asialofetuin were used as negative controls. For kinetic analysis, the SPR post process data was analysed onto Scrubber 2 for estimating association, dissociation rate constants and affinity of the scFv-SL1A1.

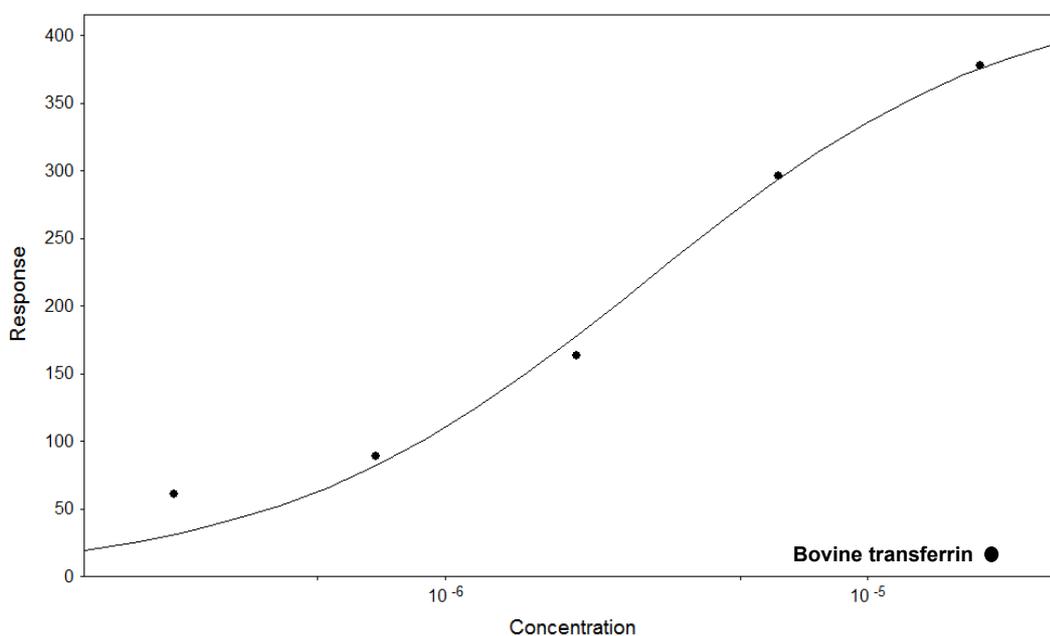
The kinetic titration of bovine transferrin was performed at 228 ng/mL to 500 µg/mL. As a negative control, 62 µg/mL of human transferrin was injected on the sensor chip. In kinetic titration, the increasing concentration of analyte is injected without regeneration of the sensor chip in between subsequent injections (Karlsson et al., 2006). The kinetic titration demonstrated a concentration dependent increase in association of bovine transferrin to scFv-SL1A1. As expected, human transferrin failed to show any interaction with scFv-SL1A1.

The kinetic titration data with calculated association and dissociation rate constants is shown in Figure 4.11 and association data in Figure 4.12. The calculated binding affinity ( $K_D$ ) of scFv-SL1A1 with bovine transferrin was  $706 \pm 250$  nM. The kinetic titration data was fitted using the global fitting function in Scrubber 2. Concentrations above 18 µg/mL did not show concentration dependent binding, this was attributed to accumulation of protein on the surface, which was evident from slow dissociation of the protein from sensor chip. Also, at high concentrations of protein, bulk shift was observed, resulting in distortion of the kinetic analysis.

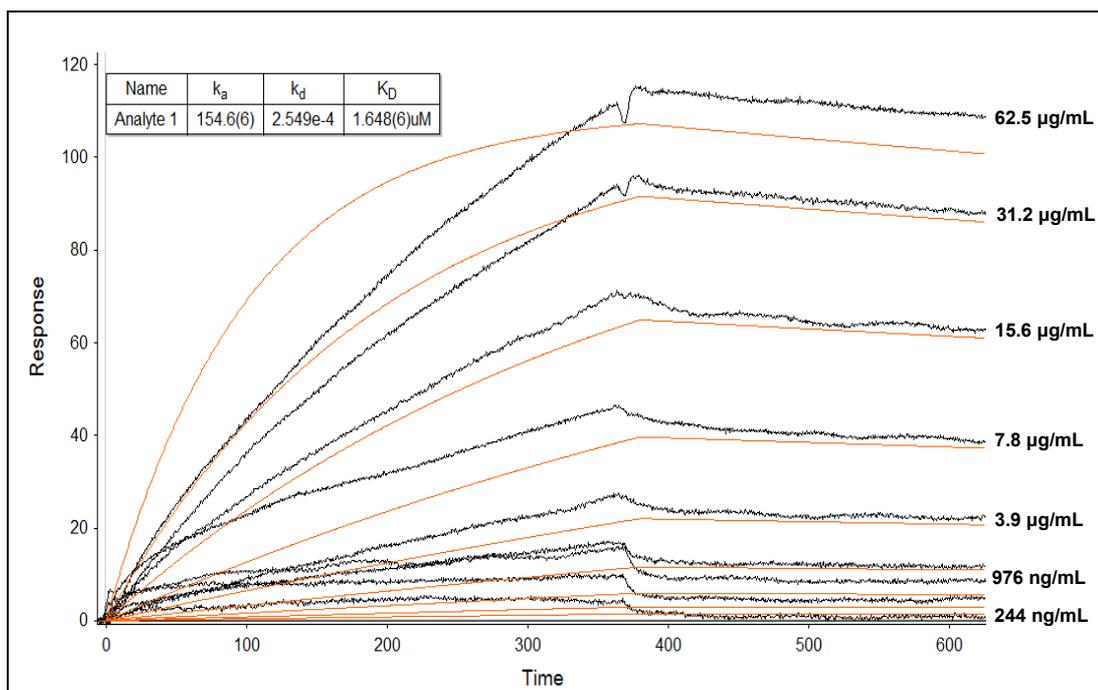
The kinetic titration of bovine fetuin was performed from 244 ng/mL to 500 µg/mL. As a negative control, asialofetuin and human  $\alpha$ 1-AGP were injected at 62 and 125 µg/mL concentration. Fetuin showed concentration dependent increase in association, while no response was observed with negative controls. The kinetic titration data for bovine fetuin is shown in Figure 4.13 and association data in Figure 4.14. For bovine fetuin, the calculated binding affinity was  $1.6 \pm 0.8$  µM. Similar to bovine transferrin, fetuin also showed major bulk shifts in kinetic analysis and therefore concentration above 62 µg/mL were excluded from analysis.



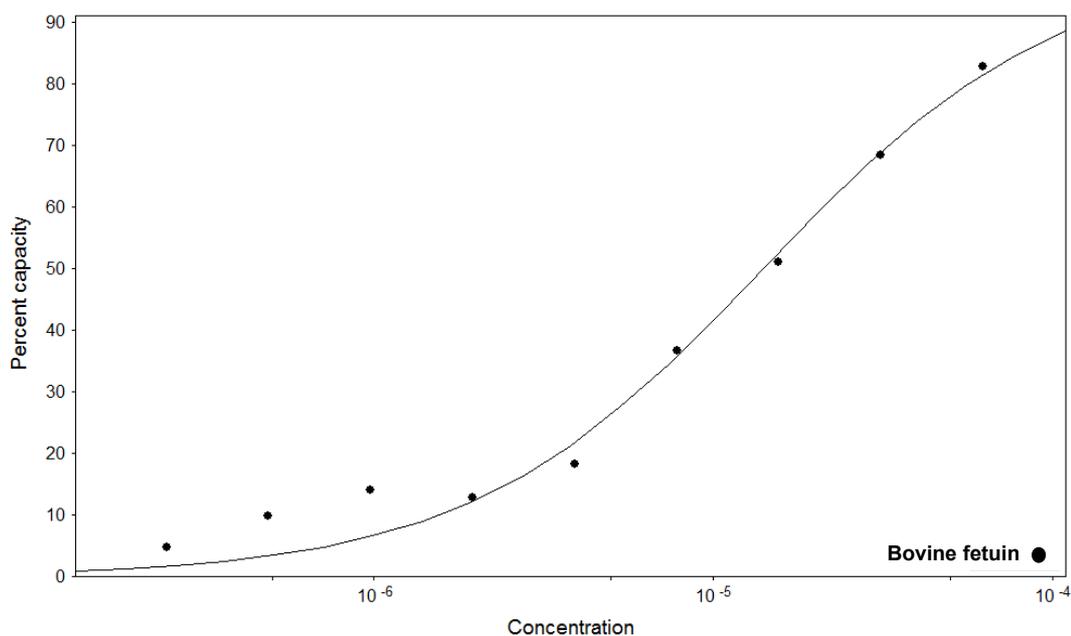
**Figure 4.11** Kinetic titration data using bovine transferrin. Incremental injection series of bovine transferrin (228 ng/mL to 18 µg/mL) on the sensor chip with immobilised scFv-SL1A1. The sensogram showing actual and simulated data.



**Figure 4.12** Kinetic titration data fitted to show association.



**Figure 4.13** Kinetic titration data using bovine fetuin. Incremental injection series of bovine fetuin (244 ng/mL to 62  $\mu$ g/mL) on the sensor chip with immobilised scFv. The sensogram showing actual and simulated data.



**Figure 4.14** Kinetic titration data fitted to association of bovine fetuin.

## **4.5 Application of scFv-SL1A1 for IHC analysis**

### **4.5.1 Methods**

#### **4.5.1.1 Assessment of IHC approach for detection of Neu5Gc on tissue**

For the assessment and potential determination of Neu5Gc within tissue samples utilising scFv-SL1A1, frozen sections of rat spinal cord tissues were used. For tissue staining, the pre-prepared rat spinal cord tissue slides were warmed on a slide warmer for 5 min. The slides were then washed 3 times, for 3 min each, in PBS-T (137 mM NaCl, 2.7 mM KCl, 10 mM Na<sub>2</sub>HPO<sub>4</sub>, 1.8 mM KH<sub>2</sub>PO<sub>4</sub>, 0.05 % Tween® 20 pH adjusted to 7.4 with phosphoric acid) on a shaker at RT. The slides were then blocked with 1 mL of 2 % pBSA in PBS for 1 h on a shaker at RT, followed by 3 washes, of 3 min each, with PBS. The slides were then incubated with 1 mL of 50 µg/mL scFv-SL1A1 diluted in 0.1 % pBSA in PBS, pH 7.4, for 1 h shaking at RT, followed by three washes, of 3 min each, with PBS.

Two secondary antibodies (anti-HIS-FITC and anti-HA-FITC), required for detection, were assessed and titrated for signal development, recognising the terminal tags contained within scFv-SL1A1. Assessment also permitted the observation and steps required to reduce background signal and non-specific binding on the tissue sample. Secondary antibodies, anti-HIS-FITC (diluted 100 and 500-fold) and anti-HA-FITC (diluted 200 and 500-fold) in PBS-T were assessed for all tissue types. The diluted secondary antibodies were then added to each slide (1 mL per slide) and incubated in the dark for 1 h on a shaker at RT. The slides were then washed twice with PBS-T, and twice with PBS. The slides were then mounted with ProLong® Diamond Antifade mountant medium overnight in the dark at RT. As a control for background signal, rat spinal cord tissue slides incubated only with secondary antibody were used. The slides were then imaged with an epifluorescence microscope (BX51, Olympus) and data was acquired by Cell B software (Olympus Italia, Segrate, Italy).

#### **4.5.1.2 Examination of IHC for detection of Neu5Gc on human tissue**

The optimisation of the IHC protocol for detection of Neu5Gc using scFv-SL1A1 was performed on human tissue array slides (according to manufacturer, the tissues were fixed in 10 % formalin for 24 h). For optimisation on human tissues, anti-HIS-

FITC and anti-HA-FITC antibodies were compared for their background signal at 500-fold dilution.

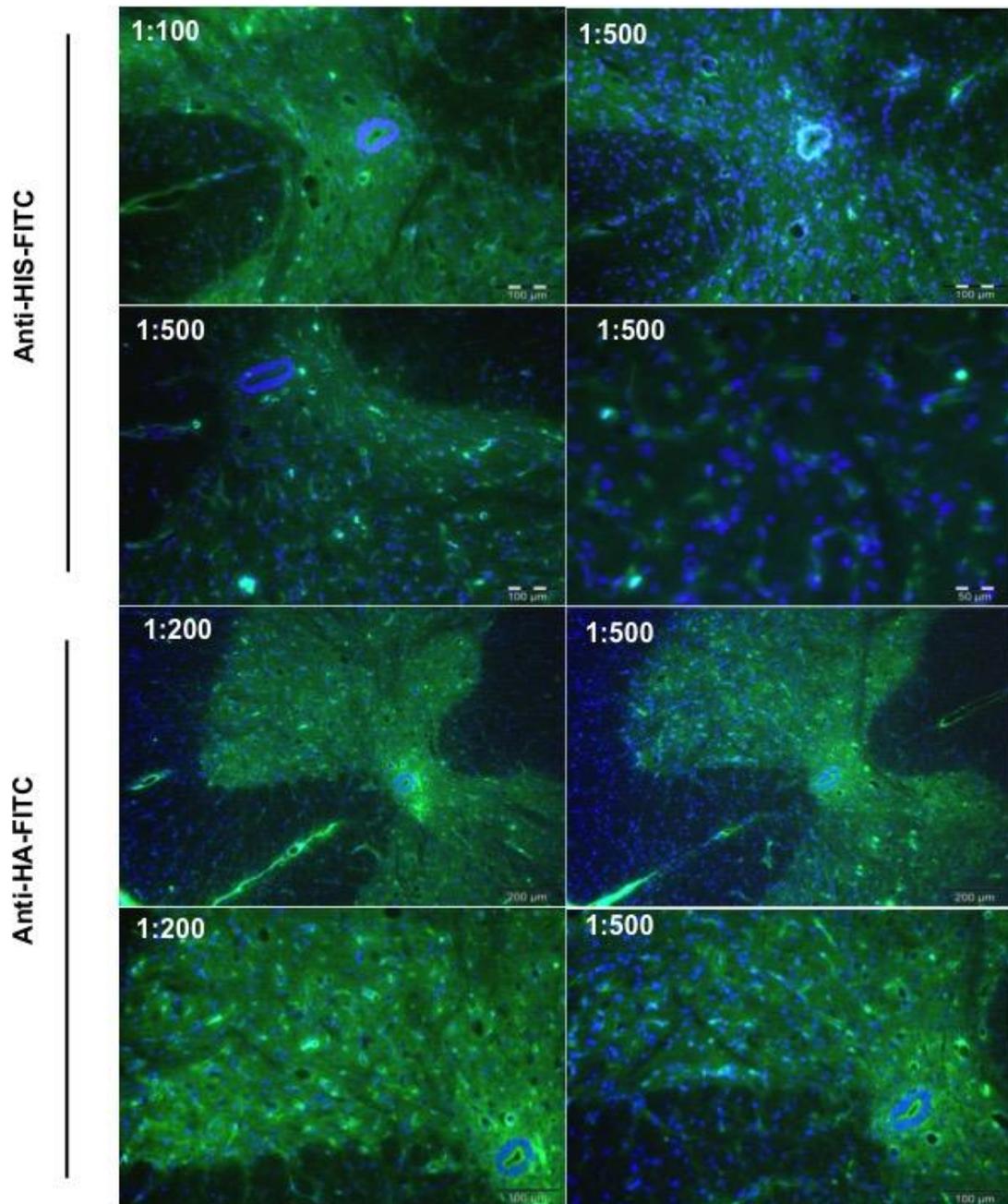
For staining, the tissue array slide was kept at RT for 5 min and then heated at 65 °C for 30 min. The slides were then dewaxed with 3 washes of xylene for 5 min each. The slides were then allowed to air dry to remove remaining xylene and then rehydrated by washing twice with decreasing concentrations of ethanol (100 %, 95%, 70%, 50% and 30 %) for 2 min each. The slides were then washed 3 times with PBS-T for 3 min each, followed by blocking with 1mL of 2 % pBSA for 1 h on a shaker at RT. The slides were then washed 3 times with PBS for 3 min each on a shaker at RT, followed by addition of 1 mL of 50 µg/mL scFv-SL1A1 in 0.1 % pBSA in PBS, pH 7.4, and incubated for 1 h with shaking at RT. The slides were then washed 3 times with PBS. Secondary antibodies, anti-HIS-FITC and anti-HA-FITC were diluted 500-fold in PBS-T and added to each slide (1 mL per slide) and incubated in the dark for 1 h on a shaker at RT. After incubation, the slides were washed twice with PBS-T, and twice with PBS. The slides were then mounted with ProLong® Diamond Antifade mountant medium overnight in the dark at RT. As a control for background signal, human tissue array slides incubated only with secondary antibody were used. The slides were imaged with epifluorescence microscope (BX51, Olympus) and data was acquired by Cell B software (Olympus Italia, Segrate, Italy).

## **4.5.2 Results**

### **4.5.2.1 Method optimisation with rat spinal cord tissue**

For initial assessment of the secondary antibodies, anti-HIS-FITC and anti-HA-FITC, were titrated on rat spinal cord tissue. The rat tissue has been reported to contain Neu5Gc (Muchmore, 1992). Rat tissue can therefore be used as a positive control for detection of Neu5Gc using scFv-SL1A1. For titration, the rat spinal cord tissue was incubated with scFv-SL1A1, the bound scFv was detected with either anti-HIS-FITC or anti-HA-FITC, Figure 4.15. The anti-HIS-FITC detected the bound scFv but also demonstrated binding to tissue as compared to anti-HA-FITC, which bound more specifically to the scFv only. The scFv-SL1A1 showed a strong signal in blood capillaries, which were clearly visible with both anti-HIS and anti-HA-FITC. This initial IHC with rat spinal cord tissue demonstrated that scFv-SL1A1

interacted and bound, in what appeared to be a specific manner, on rat spinal tissue samples. Both the secondary antibodies at higher concentration (1 in 100 of anti-HIS-FITC and 1 in 200 of anti-HA-FITC) showed high background. At 1 in 500 dilution of both the secondary antibodies the background signal was less and structures like blood capillaries were prominently evident. However, anti-HIS-FITC at 1 in 500 dilution showed non-specific binding to tissue at some places as compared to anti-HA-FITC at same concentration. Therefore, the anti-HA-FITC at 1 in 500 concentration was found optimum for signal development.

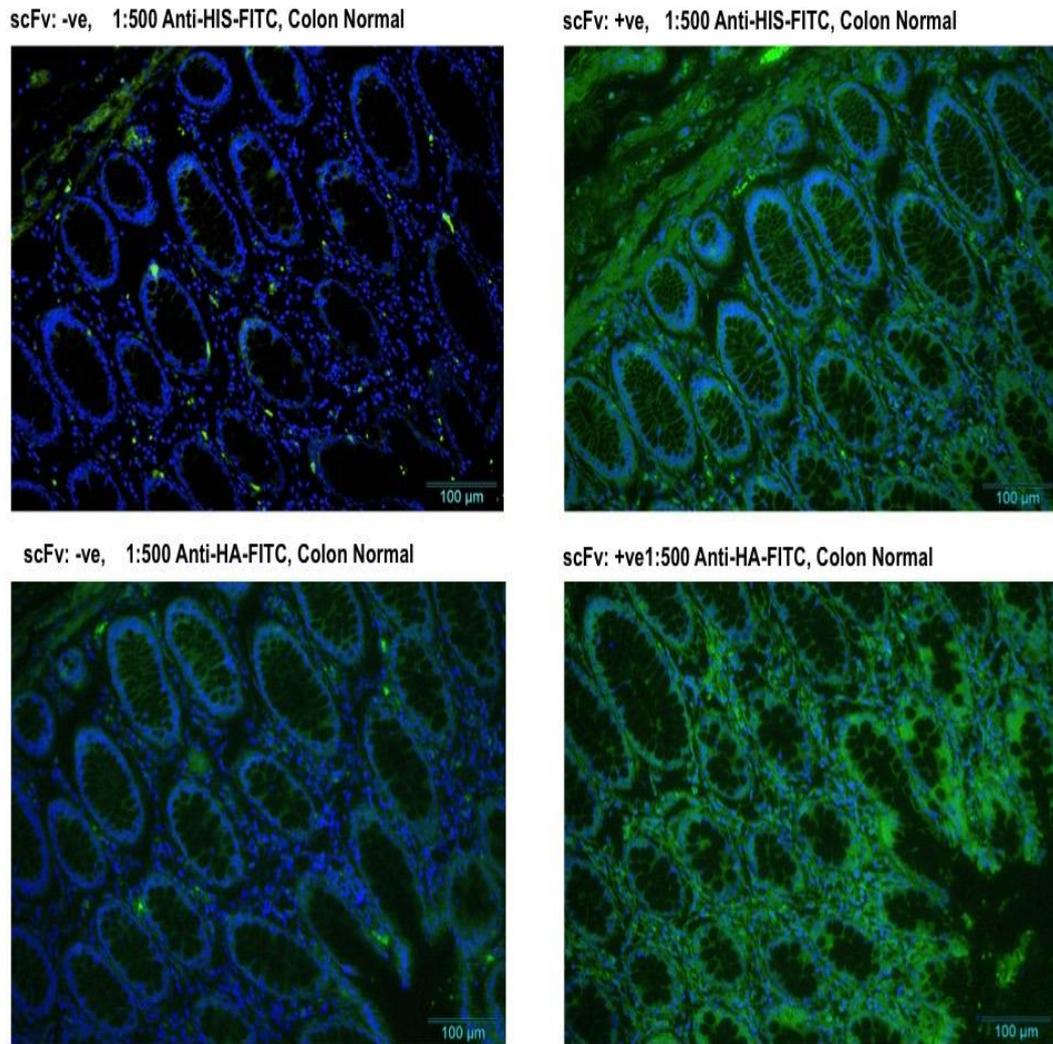


**Figure 4.15** Titration of secondary antibody and detection of scFv-SL1A1 on rat spinal cord tissue. Bound scFv-SL1A1 scFv was detected by different concentrations of anti-HIS and anti-HA-FITC. Staining demonstrates that 500-fold dilution of secondary antibody is sufficient for obtaining signal.

#### 4.5.2.2 Optimisation of IHC method on human tissue

Once the titration and suitability of secondary antibody concentration and conditions of primary incubation were identified, scFv-SL1A1 was then used to assess the suitability of approach using a human tissue array. The secondary antibody

concentration optimised on rat spinal cord tissue was directly employed on human tissue array. The background generated by both the secondary antibodies was compared for control slides where tissues were only incubated with secondary antibody but not with scFv-SL1A1, Figure 4.16.



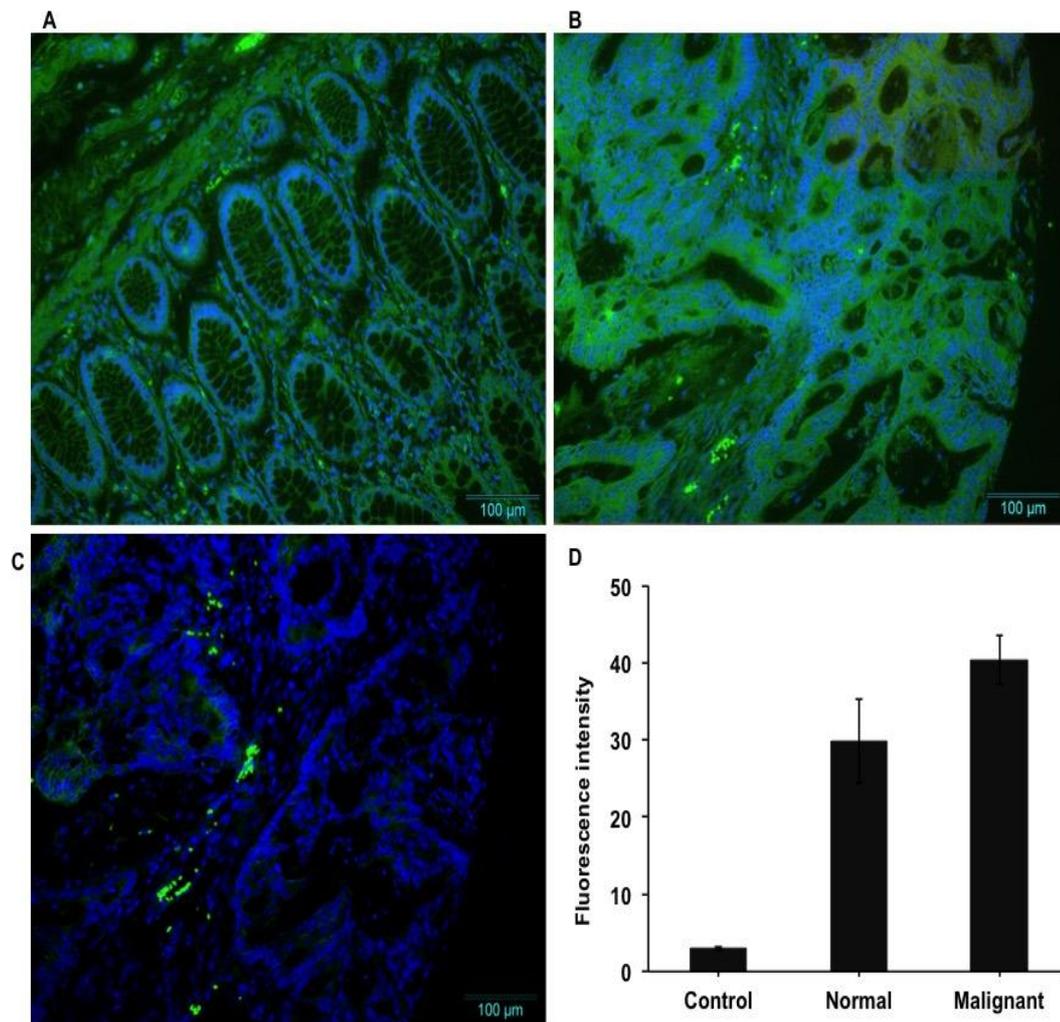
**Figure 4.16** Optimisation of secondary antibody for use in IHC of human tissues. The normal human intestinal colon tissue was probed with scFv-SL1A1. The bound scFv was detected with 500-fold diluted anti-HIS-FITC and anti-HA-FITC. The background signal was compared against secondary antibody control slide (scFv –ve and 1:500 of secondary antibody). The staining showed less background with anti-HIS-FITC as compared to anti-HA-FITC.

Tissue staining demonstrated that, anti-HIS-FITC secondary antibody produced less background staining as compared to anti-HA-FITC. The anti-HA-FITC interacted

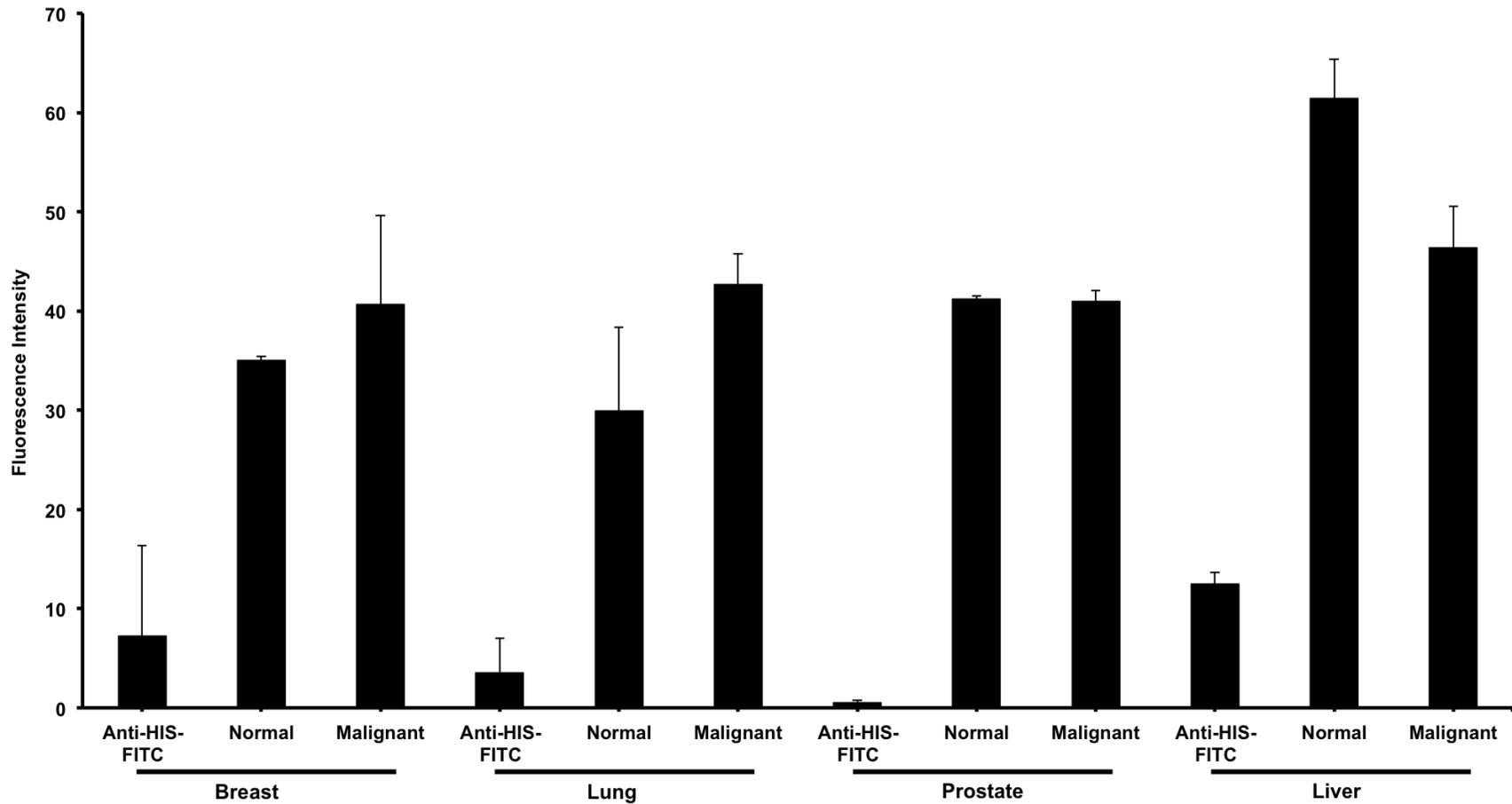
with tissue non-specificity, hence it was deemed unsuitable as a secondary antibody for human tissues.

#### **4.5.2.3 scFv-SL1A1 for IHC comparing normal and metastatic tissue**

Once secondary reagent optimisation was performed, scFv-SL1A1 was used to profile normal and malignant human tissue samples using a tissue array, consisting of matched healthy and malignant tissues from lung, prostate, intestinal colon, breast and liver. The aim of this IHC study was to identify potential differences in normal and malignant tissues, which may be attributed to Neu5Gc, on the basis of the specificity demonstrated previously in ELISA assays (Chapters 2 and 3). For staining, the tissues were heated for 30 min at 65 °C and dewaxed in xylene and then rehydrated in decreasing concentration of ethanol and immunostained with 50 µg/mL of scFv-SL1A1 and detected using 1:500 of anti-HIS-FITC (Section 4.5.1.2). The bound scFv was then detected by anti-HIS-FITC. The tissues were then imaged under epifluorescence microscope and fluorescence intensity measurement for control, normal and malignant tissues were performed using Image J 1.48 software package, Figure 4.17 and 4.18 (Schneider et al., 2012).



**Figure 4.17** Sections of normal and malignant human colon tissue stained with scFv-SL1A1. The blue colour indicates nucleus stained with DAPI and green colour shows staining by scFv-SL1A1 (primary), which was detected by anti-HIS-FITC (secondary). A. Normal, B. Malignant colon tissue, C. Secondary antibody control without scFv, D. Fluorescence intensity measurement data suggesting higher amount of Neu5Gc in malignant tissue as compared to normal tissue. Error bars indicate  $\pm$  SD for duplicate measurements.



**Figure 4.18** Fluorescence intensity measurement data of normal and malignant human tissues stained with scFv-SL1A1 (primary), anti-HIS-FITC (secondary).

Both the normal and malignant tissues stained using scFv-SL1A1 (primary) and anti-HIS-FITC (secondary) indicated towards the presence of Neu5Gc, though further imaging and suitable controls will be required for validation. Despite the fact that humans do not express Neu5Gc, the presence of this glycan has been reported on normal and cancerous tissues, suggested to be salvaged from dietary sources (Samraj et al., 2014). In a variety of normal and malignant tissues represented on the human tissue array, the scFv bound to all with varying intensities which implied the presence of Neu5Gc. This result is consistent with several observations where Neu5Gc expression has been reported on normal human tissues (Amon et al., 2014). However, the binding intensities between matched normal and malignant tissues stained with scFv-SL1A1 was variable on the human tissue array. In lung and colon tissues, fluorescence intensity measurement data indicated higher scFv binding in malignant tissue as compared to normal tissue. On human breast tissue, differences in scFv binding to the normal and malignant tissues were less evident. In prostate tissue, the fluorescence intensity measurement showed no differences in Neu5Gc expression on normal and malignant tissue. On liver tissue, staining for Neu5Gc was higher for normal tissue as compared to malignant tissue. Though differences in Neu5Gc staining of normal and malignant tissues were observed in some tissue types, a conclusive evidence for relative over or under expression of Neu5Gc on malignant and normal tissues was not evident. However, in the light of current research, the evidence for over expression of Neu5Gc on malignant cancer tissues as compared to normal is lacking, as IHC data from other research groups also do not show quantitative differences between healthy and malignant tissues (Amon et al., 2014, Samraj et al., 2014, Diaz et al., 2009). This shows that detection of Neu5Gc by monoclonal scFv-SL1A1 on both normal and malignant human tissues is consistent with current literature. Moreover, the tissue sections on human tissue array were paraffin embedded, which are not suitable for Neu5Gc detection as gangliosides are lost in the paraffin-embedding process (Samraj et al., 2014). Nevertheless, the staining of Neu5Gc by scFv-SL1A1 demonstrated the potential application of scFv-SL1A1 for use in IHC analysis, subject to increased sample testing and validation. The use of monoclonal scFv-SL1A1 is advantageous over current detection methods employing antibodies isolated from chickens or humans, these antibodies are polyclonal and lack absolute specificity for Neu5Gc.

## 4.6 Discussion

Neu5Gc is a non-human sialic acid, which is immunogenic in humans and is frequently expressed in human cancers (Malykh et al., 2001, Amon et al., 2014). The current detection and imaging methods for the non-human sialic acid Neu5Gc, employ immunised chicken derived polyclonal antibodies (Diaz et al., 2009). However, polyclonal antibodies when compared against monoclonal antibodies do not have defined specificities (Frank, 2002). Moreover, producing polyclonal antibodies by immunising the host repeatedly does not permit consistent quality of antibody for assay design or diagnostic application.

In the current research work, a monoclonal chicken derived anti-Neu5Gc scFv-SL1A1 was produced (Chapter 2), characterised and used to develop an ELISA based assay for the detection and quantification of Neu5Gc (Chapter 3). The aim of this chapter was to assess the suitability of scFv-SL1A1 across a number of applications where Neu5Gc detection and monitoring would improve beyond current approaches, both as potential research and diagnostic tool.

Initially, scFv-SL1A1 was assessed, utilising glycoproteins with or without Neu5Gc, by western blot. Glycoproteins, bovine transferrin and bovine fetuin containing Neu5Gc were used as positive controls. The human transferrin and human  $\alpha$ 1-AGP are glycoproteins with complex *N*-glycans which terminate with Neu5Ac as opposed to Neu5Gc, making them suitable as Neu5Gc negative controls (del Castillo Busto et al., 2005, Imre et al., 2008). Asialofetuin, which is bovine fetuin treated specifically to remove sialic acids leaving the rest of the underlying glycans intact was also used as a control. Albumins, (BSA, pBSA, HSA and ovalbumin) were also tested as Neu5Gc negative controls. Although the BSA itself do not contain Neu5Gc but commercial BSA preparations contain impurities including immunoglobulins, which can contain Neu5Gc and may bind to scFv-SL1A1. Therefore, pBSA in which glycans are oxidised by periodate treatment, was also used as additional negative controls. Among the proteins blotted, scFv-SL1A1 detected the presence of Neu5Gc on bovine transferrin and bovine fetuin, while Neu5Gc was not detected on the rest of the proteins (Figure 4.2). This demonstrated the suitability of scFv-SL1A1 to detect Neu5Gc on western blotted proteins. To further support Neu5Gc specific detection using scFv-SL1A1, bovine fetuin and bovine transferrin were acid treated

to remove sialic acids, and then transferred by western blot. The transfer was then probed for the presence of Neu5Gc by scFv-SL1A1, which resulted in a positive signal for untreated protein while no signal was observed for the acid treated protein preparations, Figure 4.3. This result confirmed the Neu5Gc specificity of scFv-SL1A1.

Preliminary studies were conducted to assess if it would be possible to utilise western blot as a semi-quantitative approach for the presence of Neu5Gc. For this study, 3 µg to 0.05 µg of bovine transferrin and bovine fetuin were subjected to western blotting, followed by detection with scFv-SL1A1, Figure 4.5, 4.6. As the Neu5Gc substitution of both the glycoproteins is known, the protein concentration can be translated to Neu5Gc quantity loaded per well using expression 1. The study demonstrated the detection of Neu5Gc for all the Neu5Gc loading quantities for bovine transferrin. For bovine fetuin, minimum detection for Neu5Gc was upto 1.2 nmoles. However, extensive validation for exact determination for the lowest limit of detection of scFv-SL1A1 on western blot is required. Further the limit of detection also depends on the signal development substrate used in the assay. Using chemiluminiscent substrates like west pico can push detection limits to low pico gram levels. Thus studies using different substrates are also required for assessing limits of detection.

ScFv-SL1A1 was then further examined, testing it as a potential QC tool against therapeutic proteins approved by FDA of which one, Cetuximab, had previously been reported as carrying Neu5Gc. The presence of Neu5Gc on therapeutic glycoproteins has been reported in the past, however the Neu5Gc mediated immune effects by Neu5Gc containing biopharmaceuticals was recently demonstrated in mouse models (Noguchi et al., 1996, Hokke et al., 1990, Ghaderi et al., 2010). In the case of Cetuximab, upon administration of Neu5Gc in a deficient mouse model, Neu5Gc mediated immune complex formation and rapid clearance of the drug from circulation was observed (Ghaderi et al., 2010). These studies with Cetuximab, employed polyclonal chicken derived IgY for the detection of Neu5Gc. As discussed previously, the polyclonal antibodies have variable specificities and cannot be produced with consistent quality, which may compromise the reliability of the Neu5Gc detection. This clearly highlights the need for monoclonal antibody or antibody fragment molecules for specific detection of Neu5Gc on therapeutic

products intended for human use. In keeping with the above necessity, scFv-SL1A1 was tested to detect Neu5Gc on three FDA approved therapeutics; Mabthera, Avastin and Cetuximab. Preparations of these therapeutics were western blotted and probed with scFv-SL1A1, using bovine transferrin as a positive control. The scFv-SL1A1 bound to the heavy chain of Cetuximab implying towards presence of Neu5Gc (Figure 4.8). This is consistent with the reports for presence of Neu5Gc on this therapeutic drug (Ghaderi et al., 2010). Binding to the other mAbs was not observed, as these drugs were produced in CHO cell lines that show relatively less expression of Neu5Gc. Cetuximab on the other hand is produced in mouse myeloma cells (SP2/0), in which Neu5Gc expression is relatively higher than CHO cell lines (Ghaderi et al., 2010). Although the detection of Neu5Gc on Cetuximab by scFv-SL1A1 was in congruence with reported findings, confirmation of presence of Neu5Gc on Cetuximab will be required by orthogonal analytical techniques like HPLC.

The use and application of scFv-SL1A1 as a potential tool for clinical diagnostics was then assessed with the examination of immunoglobulins derived from multiple myeloma patients. A number of reports have highlighted the presence of aberrant glycan structures in multiple myeloma (Aurer et al., 2007, Narimatsu et al., 2014). However, there is no specific report on the presence of Neu5Gc in multiple myeloma. To test for the presence of Neu5Gc, the immunoglobulin fraction from healthy and multiple myeloma patients were separated by SDS-PAGE and then transferred onto PDVF and probed with scFv-SL1A1. Within this study, scFv-SL1A1 binding was observed at ~25 kDa, corresponding to the light chain of immunoglobulins of multiple myeloma patients, in 2 out of 6 samples (Figure 4.7). The binding thus suggested towards presence of Neu5Gc. This result indicated that occurrence of Neu5Gc may not be a common feature of multiple myeloma. With a lot of literature suggesting a possible role of a Neu5Gc rich diet in the uptake and incorporation of Neu5Gc in human glycoproteins, a knowledge of dietary habits of patients may provide an explanation to the occurrence of Neu5Gc in some multiple myeloma patients. However, additional analysis with HPLC and analysis of Neu5Gc positive samples, with sialidase pretreatment, on western blot will be required to develop confidence in the findings. Nevertheless, potential detection of Neu5Gc in multiple myeloma patients using scFv-SL1A1, demonstrated the suitability of scFv

for development of clinical applications to detect Neu5Gc. Overall the evaluation of scFv-SL1A1 in western blot format clearly demonstrated that this monoclonal anti-Neu5Gc scFv can be used to develop applications for detection of Neu5Gc in clinical and non-clinical samples. However, extensive validation studies for western blot and confirmation by HPLC will be required to develop potential quality control and clinical applications using scFv-SL1A1 for detection and quantitation of Neu5Gc by western blot.

To further understand and profile the interaction of scFv-SL1A1, evaluation of interaction kinetics was performed on SPR. Bovine transferrin and bovine fetuin, spanning a range of concentrations (244 ng/mL to 500 µg/mL) were sequentially injected across immobilised scFv-SL1A1. As negative controls, human transferrin, asialofetuin and human  $\alpha$ 1-AGP were also assessed. The kinetic evaluation was performed by kinetic titration method, in which regeneration of the sensor chip is not performed between subsequent sample injections (Karlsson et al., 2006). This method is used to avoid denaturation or inactivation of the coated molecule, scFv-SL1A1, which may occur after repeated regeneration cycles. The kinetic titration with both bovine transferrin and fetuin showed a concentration dependent increase in interaction with scFv-SL1A1 (Figure 4.12, 4.14), while no interaction signal was observed for the control glycoproteins

However, with increases in concentrations above 18 µg/mL for bovine transferrin and 62.5 µg/mL for bovine fetuin, the increase in signal was no longer proportional to protein concentration. Factors leading to this may be slow dissociation or accumulation of protein on the sensor chip surface. These bulk shift observations resulted in distorted kinetic analysis of scFv-SL1A1, and were therefore removed from further analysis. The calculated binding affinity ( $K_D$ ) for bovine transferrin determined to be  $706 \pm 250$  nM and  $1.6 \pm 0.8$  µM for bovine fetuin (Figure 4.11, 4.13). However, further experiments with Neu5Gc disaccharide and trisaccharide conjugates will be required to determine exact kinetic assessment on scFv Neu5Gc interaction. Nevertheless, the data generated from SPR experimentation demonstrated that scFv-SL1A1 is interacting with Neu5Gc in a specific manner, though the interaction on complex proteins was not enough to generate conclusive kinetic data. This demands further detailed studies to evaluate the kinetics of the scFv-SL1A1.

ScFv-SL1A1 was also evaluated for IHC analysis of human tissues. Reported within a number of studies, it is now accepted that Neu5Gc is expressed on normal and cancerous human tissues, albeit at difference densities during and throughout disease state conditions (Tangvoranuntakul et al., 2003, Samraj et al., 2015). Previously, these studies have used polyclonal anti-Neu5Gc for immunohistochemistry, which may not be favourable for Neu5Gc detection. The polyclonal antibodies against Neu5Gc detect Neu5Gc only in a specific attachment and sometimes in context of underlying glycans (Diaz et al., 2009). To explore the potential application of scFv-SL1A1 as a primary detection antibody to permit the staining of tissues for detection and distribution of Neu5Gc, scFv-SL1A1 was assessed using both rat spinal cord and human tissue samples. For optimum detection, staining with secondary antibodies anti-HIS-FITC and anti-HA-FITC were titrated to permit the determination of optimal concentrations for minimal background signal. For titration, the rat spinal cord tissue was incubated with scFv-SL1A1 and bound scFv-SL1A1 detected by anti-HIS-FITC and anti-HA-FITC at 1:100 and 1:500 dilution (Figure 4.15). As a secondary antibody control, representative tissue slides were incubated with secondary antibody alone with the absence of the primary, scFv-SL1A1. The staining results demonstrated that anti-HIS-FITC and anti-HA-FITC at 1:500 dilution were sufficient to acquire images, producing acceptable signal with low background. However, the anti-HIS-FITC antibody showed non-specific staining of rat tissue as when compared to anti-HA-FITC secondary antibody.

On rat spinal cord tissue, the staining was noted to identify and label the blood capillaries, consistent with fact that rat express Neu5Gc on tissues. After demonstrating staining using scFv-SL1A1 and the secondary antibodies on rat spinal tissue, this combination was then progressed onto examining a human tissue array consisting of matched normal and malignant tissue.

The human tissue array contained each sample tissue in duplicate. For initial analysis, secondary antibodies anti-HIS-FITC and anti-HA-FITC were used at 1:500 dilution, and were compared for their background signal (Figure 4.16). The tissue array was incubated with scFv-SL1A1 and bound scFv detected with anti-HIS-FITC and anti-HA-FITC. Tissue incubated with secondary antibody only was used as a secondary control throughout. The study demonstrated that anti-HIS-FITC resulted

in less background staining than anti-HA-FITC. Thus anti-HIS-FITC at 1:500 dilution was used for further IHC with the human tissue array.

The human tissue array consisted of healthy and malignant lung, colon, breast, prostate and liver tissue. ScFv-SL1A1 was employed to label Neu5Gc on tissues, detected by tagged secondary antibody, and relative signal measured to estimate differences in Neu5Gc expression between matched normal and malignant tissues. It is believed that the scFv-SL1A1:secondary combination indicated the presence of Neu5Gc within both healthy and malignant tissues. For lung, colon and breast tissues the relative staining with scFv-SL1A1 was higher in malignant tissues, however the differences were not significant (Figure 4.17 and 4.18). In contrast, liver tissue showed higher staining with scFv-SL1A1 in normal tissue than matched malignant tissue (Figure 4.18). For prostate, no differences were observed in between normal and malignant tissues (Figure 4.18). The results from liver and prostate tissue will require detailed investigations as Neu5Gc expression may not be a common feature even within the same cancer type. Also, the fluorescence intensity measurements were made using random points on stained tissues, however the cancer cells may be concentrated at specific regions, this may explain unusual staining results from liver and prostate tissues. An expert guidance by a pathologist will be required to interpret and express staining data. In case of colon and prostate tissue, the observation with scFv-SL1A1 staining were similar to previous reports, where presence of Neu5Gc has been shown in both normal and cancerous tissue (Samraj et al., 2014). Overall, the staining data did not show major differences in Neu5Gc expression level in normal and malignant tissues. This observation is not unusual, as Neu5Gc expression is preferentially accumulated at the interface of the cancer but may not be highly expressed overall (Diaz et al., 2009). Neu5Gc has been demonstrated in normal and cancer tissue with no quantitative study to suggest the over expression in cancer tissues (Diaz et al., 2009, Tangvoranuntakul et al., 2003). These initial findings also suggest the potential presence of Neu5Gc more prominently in colon and prostate, this is consistent with similar findings from other research groups (Samraj et al., 2015). However, to draw solid conclusions that staining on the tissue by scFv-SL1A1 is indeed Neu5Gc mediated, additional inhibition to show specificity will be required. Preliminary IHC analysis using scFv-SL1A1, indicated that scFv-SL1A1 appears to bind to Neu5Gc on the tissues and can be used as a potential candidate for

development of IHC assay for clinical applications. Further, being smaller than a full length antibody and therefore offering better penetration of tissues, the scFv-SL1A1 could in theory be a better antibody for IHC method development. However, to develop any immunohistochemical application using scFv-SL1A1, validation of the staining should be performed on a larger group of randomised samples and comparison with current validated techniques will be required (Fitzgibbons et al., 2014).

## 4.7 References

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## **Chapter 5**

### **Conclusions and future perspectives**

## 5.1 Overall conclusions

Glycans are structurally complex and diverse. In various capacities, the glycans modulate a myriad of biological processes, from mediating cellular cross-talk and regulation of the immune system to progression of diseases such as cancer (Lis and Sharon, 1993). Various technological advancements have paved the way for study of glycans at structural and functional levels. However, the pace of research in glycobiology as compared to proteomics and genomics is still far behind partly due to lack of specific anti-glycan recognition molecules (Moon, 2012).

The non-human sialic acid Neu5Gc is immunogenic in humans and has been reported to be present on normal secretory epithelia, endothelia and in diseased states like cancer (Samraj et al., 2014). The Neu5Gc can also be incorporated into biopharmaceutical drugs during production process, leading to Neu5Gc mediated immune complex formation and reduced circulating half-life of drugs, this has been demonstrated in CMAH knockout mouse models (Ghaderi et al., 2010). Also, the biological roles of Neu5Gc in cancer and immune response is not known and this knowledge gap can be filled by utilising anti-Neu5Gc recognition molecules. Currently, there is a lack of sustainably produced, robust, specific and reproducible recognition molecule against Neu5Gc. The research in this thesis was focussed on generation of recombinant anti-Neu5Gc scFvs for the development of assays for specific detection and quantitation of Neu5Gc.

To generate and identify anti-Neu5Gc scFv, chicken derived short and long-linker phage display libraries were generated by phage display technology (Chapter 2). A population of 49 short-linker and 21 long-linker scFv-phage clones were isolated and screened for Neu5Gc binding, which was followed by nucleotide sequencing and alignment analysis. This resulted in identification of two unique short-linker scFvs (SL1A1 and SL4D1) and three unique long-linker scFvs (LL1B6, LLY and LLX). The clone LLY and LLX failed to grow in liquid cultures and were excluded from further analysis. The rest of the short (SL1A1 and SL4D1) and long-linker (LL1B6) scFvs were analysed for binding to Neu5Gc. Both the short and long-linker scFvs showed binding to Neu5Gc. The amino acid sequences of SL1A1, SL4D1 and LL1B6 were compared and CDRs were predicted. The CDR regions showed clear differences amongst the selected scFvs. Overall, a short and long-linker phage

display library was established from an immunised chicken and panned successfully to identify three scFv clones that showed binding to Neu5Gc.

Among the three scFv clones identified SL1A1, SL4D1 and LL1B6, scFv-SL1A1 showed better binding to Neu5Gc and was therefore used for ELISA based assay development (Chapter 3). Prior to assay development, first the scFv-SL1A1 was analysed for its specificity for Neu5Gc and then various assay variables such as antigen coating, blockers, buffer conditions and pH of the assay were optimised. ScFv-SL1A1 showed specific recognition of Neu5Gc in free as well as protein bound form. Further, among different blockers evaluated for the assay development, pBSA was selected as ideal blocker as it showed less background in assays.

The free Neu5Gc decreases the pH of standard assay buffers due to its acidic nature and may therefore alter the binding of scFv to Neu5Gc. Studies were carried out, to optimise the buffering strength of assay buffers, and to investigate the binding characteristic of scFv to Neu5Gc at a range of pH. The decrease in pH during the assay can adversely affect the binding of scFv to Neu5Gc resulting in false inhibition response. Studies with PBS buffer of different buffering capacity showed that PBS with 150 mM buffering capacity controlled the pH and was suitable for inhibition assay using free Neu5Gc. Further, the evaluation of scFv binding at different pH showed that scFv was able to bind to Neu5Gc at pH range of 3.5 to 7.5. However, at lower pH scFv binding to Neu5Gc was increased, which may also contribute to false positive signals in assays.

To eliminate the concern of altered pH due to free Neu5Gc, bovine transferrin was evaluated for generation of Neu5Gc standard inhibition curve. As the acidic effect of sialic acid in protein bound form is counteracted by the overall charge of amino acids within the protein, bovine transferrin was considered as suitable choice for presentation of Neu5Gc to scFv. The substitution ratio of Neu5Gc on bovine transferrin was determined using DMB labelling of released sialic acid followed by HPLC analysis. The substitution ratio was used to calculate the molar concentration of Neu5Gc used in the inhibition assay. The data from inhibition assay using bovine transferrin demonstrated a dose dependent inhibition of scFv-SL1A1. The inhibition assay was also performed with human transferrin, which exclusively has Neu5Ac and no Neu5Gc. No inhibition was observed in the assay, which further

demonstrated the specificity of scFv-SL1A1 to Neu5Gc. Inhibition assays with bovine fetuin also showed concentration dependent inhibition of scFv-SL1A1 with a parallel inhibition profile to bovine transferrin.

For assessing the reproducibility of the Neu5Gc detection and quantitation by scFv-SL1A1, the inhibition assay using bovine transferrin was performed 10 times in independent experiments to generate a composite standard curve. The standard curve showed high reproducibility across 10 assay runs demonstrating that the assay can tolerate the variations that can arise in routine buffer preparations and handling of the assay. Further, the Neu5Gc substitution ratio for bovine transferrin calculated by inhibition assay using scFv-SL1A1 was compared with HPLC quantitation data. The Neu5Gc substitution ratio determined by inhibition assay and DMB-HPLC were comparable.

This is the first report of a recombinant scFv that can be used to detect and quantitate Neu5Gc. This anti-Neu5Gc scFv can be used to develop applications for rapid detection and quantitation of Neu5Gc in various sample types and across various assay formats.

Chapter 4 of this research work presented the initial investigation into the applications of anti-Neu5Gc scFv-SL1A1 in various clinical and non-clinical samples including serum samples and tissues biopsies. The applications of scFv-SL1A1 in western blot format, tissue arrays with matched healthy and cancer samples and preliminary kinetic evaluation by SPR were described.

ScFv-SL1A1 was used to evaluate its suitability for detection of Neu5Gc on western blotted proteins with (bovine fetuin and bovine transferrin) or without Neu5Gc (BSA, pBSA HSA, ovalbumin, human transferrin, bovine asialofetuin and human  $\alpha$ 1-AGP). The scFv showed binding to Neu5Gc containing proteins, bovine fetuin and bovine transferrin, while no signal was obtained for rest of the proteins implying the presence of Neu5Gc. The removal of sialic acid by acid hydrolysis from bovine fetuin and bovine transferrin abolished binding of scFv-SL1A1.

Further, in the western blot format the scFv-SL1A1 was used to probe Neu5Gc in clinical samples such as multiple myeloma and on FDA approved biopharmaceutical drugs. The scFv showed prominent binding to the light chain of antibody in two

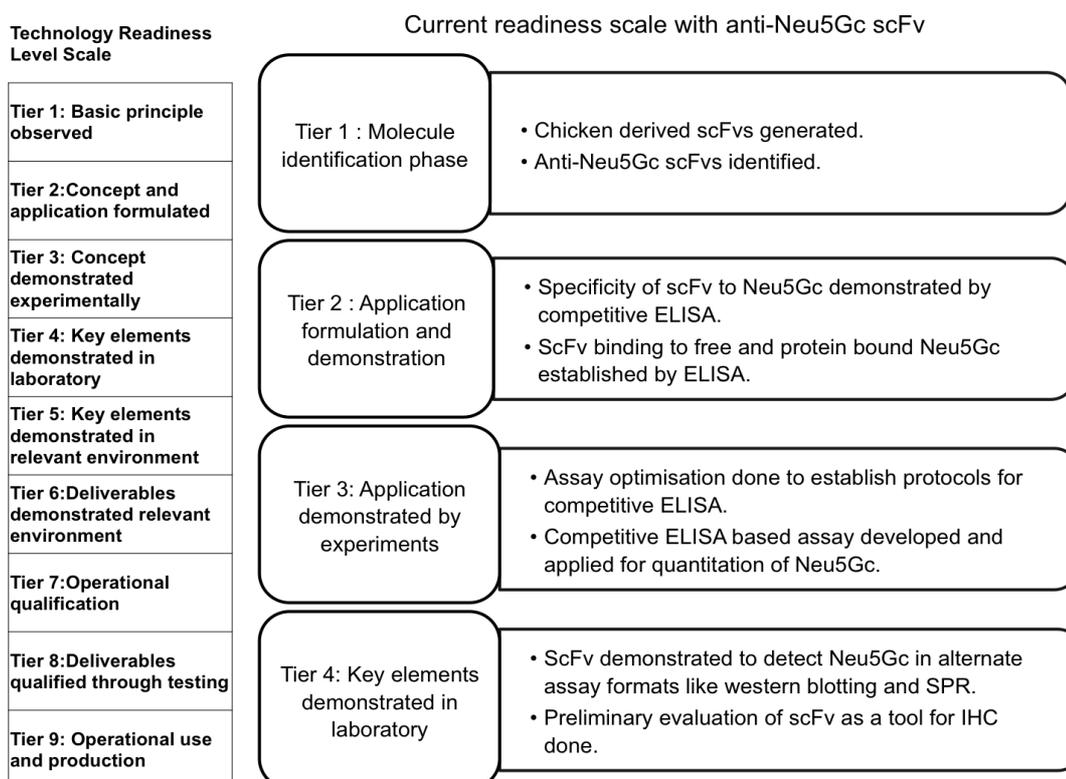
multiple myeloma patient samples out of six patient samples and no binding to healthy controls. This is the first report suggesting the presence Neu5Gc in antibodies isolated from multiple myeloma patients. However, to support these observations, validation using HPLC analysis of the sialic acid content will be required. The scFv also showed binding to Cetuximab, an FDA approved biopharmaceutical drug used for the treatment of metastatic colorectal cancer. The binding of scFv suggested the presence of Neu5Gc on this biopharmaceutical. These experiments show the potential of scFv-SL1A1 for quality control procedures for production of safe biopharmaceuticals.

The preliminary kinetic titration studies of scFv binding to Neu5Gc were conducted using bovine transferrin and bovine fetuin on SPR. The scFv showed concentration dependent increase in association kinetics. However, at high concentrations the dose dependent increase in association kinetics was not observed. This was attributed to the progressive accumulation of protein on the surface of sensor chip due to slow dissociation kinetics and bulk transfer effects.

The evaluation of scFv on human tissue array with matched healthy and cancer samples suggested that scFv can be potentially used to develop applications in IHC. The comparative staining of cancer and healthy tissues with scFv-SL1A1 showed differences in scFv binding within matched healthy and cancer tissues. However, this data is preliminary and further inhibition studies will be required for evaluating the suitability and specificity of scFv-SL1A1 for IHC use.

## 5.2 Future perspectives

One of the potential research area that has been highlighted in a recent review of the current state of research in glycoscience is the need for specific anti-glycan probes for research (Moon, 2012). Such probes can also find applications in diagnostics, analytics and therapeutics. The scFv developed in this work is the first recombinant antibody fragment that can specifically detect Neu5Gc in free as well as bound form. The readiness level of scFv as a tool for specific detection and quantitation of Neu5Gc is illustrated in Figure 5.1. The scFv has been applied for probing Neu5Gc on biopharmaceutical drugs by western blotting and has shown potential in initial investigations for further assay development. This scFv can be applied for detection of Neu5Gc in various assay formats. However, extensive research work needs to be done to qualify scFv for commercial assay development. The following sections give a brief overview of the future research that could be done to develop further applications using anti-Neu5Gc scFv.



**Figure 5.1.** Readiness level of anti-Neu5Gc scFv for incorporation in to technologies for Neu5Gc detection and quantitation.

### 5.2.1 Assay development and characterisation

In chapter 3, a convenient assay based on competitive ELISA was developed for the detection and quantitation of Neu5Gc. The Neu5Gc attached to glycoproteins and glycolipids occur in different forms such as *O*-acetylated Neu5Gc and in different presentations like Neu5Gc attached to underlying glycans with  $\alpha$ -(2,3)-,  $\alpha$ -(2,6)- and  $\alpha$ -(2,8)-linkages (Klein and Roussel, 1998, Inoue et al., 2001). This requires additional specificity studies of scFv-SL1A1 using different forms and presentations of Neu5Gc. The studies can be extended to other short and long-linker scFvs that were identified from chicken scFv-phage display library.

Further, to enhance the sensitivity and dynamic range of the assay, fluorescent and chemiluminescent probes can be evaluated for signal development (Gibbs and Kennebunk, 2001). Extensive reagent suitability and matrix compatibility studies will generate knowledge about the appropriate reagent and matrix pairs for Neu5Gc detection assay. As recommended with ligand binding assay method development and validation, selectivity and cross-reactivity, inter and intra lab accuracy, precision and recovery studies will be required in order to apply the ELISA based Neu5Gc detection for diagnostic analysis (Food and Administration, 2007, Sittampalam et al., 2004). Further, testing of the validated assay onto a large set of clinical samples and biopharmaceuticals drugs with known amounts of Neu5Gc, as estimated by validated analytical techniques like HPLC, would pave the way for integration of assay for routine determination of Neu5Gc.

#### 5.2.1.1 Characterisation of scFv using SPR

The kinetics of scFv-SL1A1 binding to Neu5Gc were evaluated by presenting Neu5Gc onto glycoproteins such as bovine transferrin and fetuin, however, a single glycoprotein can have different presentations of Neu5Gc and therefore kinetic experiment with glycoproteins are not definitive reflection of Neu5Gc and scFv interaction in isolation. Further, the slow dissociation observed during the kinetic titration studies with the scFv can also be attributed to the multivalent presentation of Neu5Gc on the protein surface, which may cause the build up of protein on the sensor chip surface causing distortion of kinetic data. To overcome multivalent interactions, the kinetics of scFv should be evaluated by presenting Neu5Gc in the form disaccharide, trisaccharides and complex glycan structures. Moreover, Neu5Gc

with different underlying linkage could be used for gaining insights into the potential linkage specificity or bias of scFv. The kinetic studies using SPR can also be extended to analysis of other short and long-linker scFvs.

### **5.2.2 Applications in quality control in biopharmaceutical industry**

In chapter 4, the scFv-SL1A1 showed binding to Cetuximab suggesting the presence of Neu5Gc. The presence of Neu5Gc causes rapid clearance of drugs in mouse models (Ghaderi et al., 2010) raising concern that Neu5Gc can potentially compromise the safety and efficacy of drugs. The need to have tools for rapid and convenient detection of Neu5Gc is urgent and highlights the potential application of scFv for quality control of biopharmaceutical drugs. Future studies with scFv-SL1A1 can be directed to use validated inhibition assay to detect and measure Neu5Gc in biopharmaceutical drugs. The validated method for Neu5Gc detection with scFv-SL1A1 can then be integrated into quality control procedures and process monitoring for consistent and safe biopharmaceutical manufacturing.

### **5.2.3 Developing IHC applications using scFv-SL1A1**

The application of scFv in chapter 4 for detection of Neu5Gc on tissue arrays in preliminary experiments showed differential binding of scFv on various healthy and cancer tissues. The studies can be further extended to include detailed specificity studies using inhibition experiments with glycoproteins and Neu5Gc-containing neoglycoconjugates. The tissues could also be treated with sialidases to remove sialic acid from the tissue to assess scFv specificity. However, this should be noted that sialidases remove all types of sialic acids and may also have issues with tissue penetration that may prevent removal of total sialic acids.

Once the specificity of the scFv on tissues is demonstrated, the scFv can be tested on tissues that are already tested and validated for the presence of Neu5Gc in other laboratories using IHC methods. A rigorous IHC method development and validation approach accompanied with larger set of clinical tissues samples would be required for the qualification of scFv for IHC use (Fitzgibbons et al., 2014).

Once the binding characteristics of the scFv-SL1A1 are firmly established on tissues and cells, the application of the scFv can be further extended to study the role of Neu5Gc in cancer using cancerous cell lines that expresses Neu5Gc. The mouse

lymphocytic leukaemia cells (L1210) express high amount of Neu5Gc-GM3 ganglioside and it has been demonstrated that knockout of Neu5Gc in cells impair tumour development *in vitro* (Casadesús et al., 2013). The anti-Neu5Gc scFv can be used for blocking the display of Neu5Gc on L1210 cells to further elucidate the role of Neu5Gc in cancer biology. The same methodology can be applied to other invasive cancer types displaying Neu5Gc.

#### **5.2.4 scFv engineering**

In chapter 2, the CDR regions of short and long-linker scFvs were identified. *In silico* molecular modelling studies and data from NMR interaction experiments can be used to identify the specific amino acids that mediate the recognition and binding events with Neu5Gc. The current panel of scFvs can also be engineered by mutagenesis to generate libraries of scFv variants that could potentially bind to other variants or presentations of sialic acids (Thakkar et al., 2013, Ahmad et al., 2012). After thorough characterisation of scFvs in terms of linkage specificity and binding to specific sialic acid types, the scFvs can be integrated into microarray format for high throughput profiling of Neu5Gc containing glycans.

ScFv fused with fluorescent proteins have been successfully produced and applied in direct immunodetection assays (Griep et al., 1999). From a diagnostic application point of view anti-Neu5Gc scFvs can be fused with fluorescent proteins for development of direct and live immunodetection technologies. Such direct detection technologies eliminate the need of secondary reagents and allow single step detection of target epitopes.

### 5.3 References

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