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**STUDIES ON
O-GLYCOSYLATION OF
MUCIN-TYPE PROTEINS
AND THEIR BINDING TO
ANTIBODIES, BACTERIAL
TOXINS AND VIRAL
RECEPTORS**

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An expert is a person who has found out by his own painful experience all the mistakes that one can make in a very narrow field.

Niels Bohr

ABSTRACT

Carbohydrates are ubiquitous on the surface of all cells in mammals where they are involved in interactions with the surroundings (extracellular matrix), other cells (including self and non-self) and microbes (bacteria and virus). Carbohydrate-protein interactions in nature are often mediated via multivalent binding where the combined strength of multiple receptor-ligand interactions results in a binding that is highly specific and strong.

In this thesis we have produced proteins that are densely decorated with carbohydrate determinants in order to study the glycosylation capacity of cell lines (paper I) and generate efficient binders of antibodies (paper II), bacterial toxins (paper III) and virus receptors such as the influenza hemagglutinin (paper IV).

P-selectin glycoprotein ligand-1 (PSGL-1) is a mucin-type protein that is heavily substituted with *O*-glycans. PSGL-1 genetically fused to mouse IgG_{2b} Fc forms a dimeric PSGL-1/mIgG_{2b} mucin-type fusion protein.

In paper I, PSGL-1/mIgG_{2b} was produced in Sf9 (*Spodoptera frugiperda*) and Hi-5 (*Trichoplusia ni*) cell lines. The mucin-type protein was used as a probe to analyze the *O*-glycosylation capacity of these cell lines, which today are used for the commercial production of recombinant proteins and vaccine components. Liquid chromatography-mass spectrometry (LC-MS) revealed that the *O*-glycosylation was more abundant and complex than previously reported which may limit their use for the production of therapeutic proteins.

The glycosylation of PSGL-1/mIgG_{2b} may be tailored by producing the protein in genetically engineered cell lines. Rational glycan design is achieved by transfecting cells with plasmids encoding PSGL-1/mIgG_{2b} together with specific glycosyltransferases that expand the glycosylation capacity of the cells.

In paper II, genetically engineered Chinese Hamster Ovary (CHO) cells were used to produce PSGL-1/mIgG_{2b} carrying blood group A and B determinants on type 1, 2 and 3 outer core saccharide chains. The multivalent mucins could adsorb chain type-specific anti-A antibodies, which indicate a prospective use of the mucins in immunoadsorption (IA) columns. IA columns are used to remove anti-A and anti-B reactive antibodies prior to organ transplantation across the blood group ABO barrier.

In paper III and IV, genetically engineered CHO cells were used to produce high affinity binders of Shiga toxin 1 and 2 (Stx1 and Stx2) and avian influenza hemagglutinin (H5). Biacore biosensor assays indicated that PSGL-1/mIgG_{2b} carrying the blood group P1 determinant in multiple copies bound with high affinity to Stx1 and Stx2, while PSGL-1/mIgG_{2b} presenting multiple copies of Sia α 2,3Gal on different *O*-linked cores bound with high affinity to avian influenza H5. It remains to be shown if PSGL-1/mIgG_{2b} can competitively inhibit and sterically block toxin and viral attachment to the cell surface.

In conclusion, PSGL-1/mIgG_{2b} carrying specific carbohydrates is a versatile tool that can be used in a range of applications where the multivalency confers a biologically relevant binding.

POPULÄRVETENSKAPLIG SAMMANFATTNING PÅ SVENSKA

Celler är rikligt dekorerade med socker på sin yta. Dessa socker är viktiga för cellens kommunikation med sin omgivning (extracellulär matrix), andra celler (kroppsegna och främmande) och mikroorganismer (bakterier och virus). Protein-socker bindningar i biologiska system förmedlas ofta via bindning där flera receptor-ligand par tillsammans bidrar till en stark och specifik bindning (multivalens). I denna avhandling har vi med hjälp av genmodifierade celler producerat proteiner som är rikligt dekorerade med sockerkedjor (glykoproteiner) i syfte att I) studera insektcellers förmåga att bilda olika sockerstrukturer på proteiner; II) skapa starkt bindande antigen till anti-socker antikroppar; III och IV) skapa starkt bindande sockerreceptorer till Shigaliknande toxiner och fågelinfluensa hemagglutinin. "P-selectin glycoprotein ligand-1" (PSGL-1) är ett kraftigt sockerbeklätt (*O*-glykosylerat) glykoprotein. Genetisk ligering av cDNA kodande för PSGL-1 och Fc delen av musens IgG_{2b} bildar proteinet PSGL-1/mIgG_{2b}. I delarbete I) undersöktes vilka *O*-glykaner som återfinns på PSGL-1/mIgG_{2b} producerat i insektcellerna Sf9 (*Spodoptera frugiperda*) och Hi-5 (*Trichoplusia ni*). Dessa cellinjer används idag i stor utsträckning för produktion av proteiner till forskning och vacciner. Analys av *O*-glykaner frisatta från PSGL-1/mIgG_{2b} visade att insektcellerna åstadkom en mer omfattande och komplex *O*-glykosyleringen än vad som tidigare rapporterats vilket kan begränsa deras användning för produktion av proteinläkemedel. Glykosyleringen av PSGL-1/mIgG_{2b} kan anpassas genom att producera proteinet i genetiskt modifierade cellinjer. Genom att sätta in gener som kodar för PSGL-1/mIgG_{2b} tillsammans med glykosyleringsenzymer, kan skräddarsydd glykosylering uppnås. I delarbete II) användes genetiskt modifierade Chinese Hamster Ovary (CHO) celler för att producera PSGL-1/mIgG_{2b} bärandes blodgrupp A och B på olika (typ 1, 2 och 3) sockerkedjor. De multivalenta glykoproteiner kunde binda upp kedjespecifika anti-A antikroppar. Ett möjligt framtida användningsområde för dessa glykoproteiner är immunoadsorptionskolonner. Sådana används idag för att ta bort anti-A och anti-B antikroppar i samband med organtransplantation över blodgruppsgränserna. I delarbete III) och IV) producerades PSGL-1/mIgG_{2b} i olika genmodifierade CHO cellinjer i syfte att skapa multivalenta sockerstrukturer som liknar de receptorer som Shiga toxin 1 och 2 (Stx1 och -2) och fågelinfluensavirusets hemagglutinin (H5) binder till på cellytan. Bindningsanalyser med s.k. "surface plasmon resonance" (Biacore) visade att PSGL-1/mIgG_{2b} som bar på sockerstrukturen P1 (Gal α 4Gal β 4GlcNAc) band starkt till Stx1 och Stx2 medan PSGL-1/mIgG_{2b} som bar på Sia α 2,3Gal på olika sockerkedjor band starkt till fågelinfluensahemagglutinin H5. Det återstår att se om dessa PSGL-1/mIgG_{2b} glykoproteiner kan hämma toxin- och virusbindning till cellytan. Sammanfattningsvis, multivalent presentation av specifika socker på PSGL-1/mIgG_{2b} gör att detta glykoprotein kan binda med biologisk relevant styrka till många bindningspartners. Detta gör PSGL-1/mIgG_{2b} till ett kraftfullt verktyg med många framtida potentiella terapeutiska och diagnostiska användningsområden.

LIST OF ORIGINAL PUBLICATIONS

- I. Mucin-type proteins produced in the *Trichoplusia ni* and *Spodoptera frugiperda* insect cell lines carry novel *O*-glycans with phosphocholine and sulfate substitutions
Stefan Gaunitz, Chunsheng Jin, Anki Nilsson, Jining Liu, Niclas G. Karlsson and Jan Holgersson
Glycobiology Advance Access published March 5, 2013
- II. Mucin-type fusion proteins with blood group A or B determinants on defined *O*-glycan core chains produced in glycoengineered CHO cells and their use as immunoaffinity matrices
Linda Lindberg, Jining Liu, **Stefan Gaunitz**, Anki Nilsson, Tomas Johansson, Niclas G. Karlsson, and Jan Holgersson
Glycobiology Advance Access published February 18, 2013
- III. Shiga-like toxin binds with high avidity to multivalent *O*-linked blood group P1 determinants on mucin type fusion proteins
Reeja Maria Cherian, **Stefan Gaunitz**, Anki Nilsson, Niclas G. Karlsson and Jan Holgersson
manuscript
- IV. Avian influenza H5 hemagglutinin binds with high avidity to sialic acid on different *O*-linked core structures on mucin-type fusion proteins
Stefan Gaunitz, Jining Liu, Anki Nilsson and Jan Holgersson
manuscript

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Abbreviations

Asn	Asparagine
BEVS	Baculovirus expression vector system
Blood group A	GalNAc α 1,3(Fuc α 1,2)Gal
Blood group B	Gal α 1,3(Fuc α 1,2)Gal
Blood group H	Fuc α 1,2Gal
CHO	Chinese Hamster Ovary cells
Core 3 enzyme, C3	β 1,3- <i>N</i> -acetylglucosaminyltransferase-6
dHex	Deoxyhexose
DMB	1,2-diamino-4,5-methylenedioxybenzene
ELISA	Enzyme-linked immuno sorbent assay
ER	Endoplasmic reticulum
ESI	Electrospray ionization
FAB	Fast atom bombardment
Fuc	Fucose
FUT1, H	α 1,2-fucosyltransferase-1
FUT2, Se	α 1,2-fucosyltransferase-2
GAG	Glycosaminoglycan
Gal	Galactose
GalA	Galacturonic acid
GalN	Galactosamine
GalNAc	<i>N</i> -acetylgalactosamine
GalNAcT	α 1,3- <i>N</i> -acetylgalactosyltransferase
GalT	α 1,3-galactosyltransferase
GalT5	β 1,3-galactosyltransferase-5
Gb3	Globotriaosylceramide
GBPs	Glycan-binding proteins
Glc	Glucose
GlcA	Glucuronic acid
GlcN	Glucosamine
GlcNAc	<i>N</i> -acetylglucosamine
HA	Hemagglutinin
HEK	Human embryonic kidney cells
Hex	Hexose
HexA	Hexuronic acid
HexNAc	<i>N</i> -acetylhexosamine

HPAEC-PAD	High-performance anion-exchange chromatography with pulsed amperometric detection
HPAIV	Highly pathogenic avian influenza viruses
HPLC	High-performance liquid chromatography
HUS	Hemolytic uremic syndrome
IA	Immunoabsorption
IdoA	Iduronic acid
LacdiNAc	<i>N, N'</i> -diacetyllactosamine
LacNAc	<i>N</i> -acetyllactoseamine
LC	Liquid chromatography
M1	Matrix protein 1
M2	Matrix protein 2
MALDI	Matrix-assisted laser-desorption ionization
Man	Mannose
MS	Mass spectrometry
NA	Neuraminidase
Neu5Ac	<i>N</i> -acetylneuraminic acid
Neu5Gc	<i>N</i> -glycolylneuraminic acid
NMR	Nuclear magnetic resonance
NP	Nucleoprotein
NS1	nonstructural protein 1
NS2	nonstructural protein 1
P1	Gal α 1,4Gal β 1,4GlcNAc
PAA	Poly(acrylic acid)amide
PB1	Polymerase basic 1
PB2	Polymerase basic 2
PC	Phosphocholine
P ^k	Gal α 1,4Gal β 1,4Glc
ppGalNAcT	UDP-GalNAc-polypeptide- <i>N</i> -acetylgalactosaminyltransferase
PSGL-1/mIgG _{2b}	P-selectin glycoprotein ligand-1/mouse IgG _{2b}
RP	Reverse-phase
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
Ser	Serine
Sia	Sialic acid
SPR	Surface plasmon resonance
STEC	Shiga toxin-producing <i>Escherichia coli</i>
Stx	Shiga toxin
Stx1	Shiga toxin 1
Stx2	Shiga toxin 2
Thr	Threonine

TOF	Time-of-flight
Type 1	Gal β 1,3GlcNAc
Type 2	Gal β 1,4GlcNAc
Type 3	Gal β 1,3GalNAc α
β 6GlcNAcT-I, C2	Core 2 β 1-6 <i>N</i> -acetylglucosaminyltransferase-1

1 General introduction to the field of glycobiology

Carbohydrates are one of the building blocks of life. Together with nucleic acids, proteins and lipids they are key components of all living organisms. Carbohydrates serve as building blocks, energy source and carrier of information. The field of glycobiology studies the structure, biosynthesis, biology, and evolution of oligosaccharides (glycans) in biological systems (Varki and Sharon, 2009).

Carbohydrates are the most common post-translational modification of proteins and contribute to expanding the complexity of the proteome beyond what is encoded in the genome. Carbohydrates have important roles in all stages of life including development, growth and maintaining a multicellular homeostasis. Carbohydrates are found on the cell surface and are involved in interaction with the surroundings (extracellular matrix), other cells (including self and non-self) and microbes (bacteria and virus). In addition, dynamic glycosylation of proteins are important for intracellular signaling. The following sections will give a brief introduction to those parts of glycobiology that are relevant for the presented papers in this thesis.

1.1 Carbohydrates as carriers of information

Carbohydrates have been called “the third alphabet of life” after nucleic acids and amino acids because they share several features with these molecules (Rüdiger and Gabius, 2009, Gabius, 2000). While the genetic code is composed of four-letter combinations, the sugar code is composed of carbohydrate monosaccharides that represent the “letters” of the alphabet and may be joined together in different oligosaccharides (glycans) or polysaccharides, “words” or “sentences”. The carbohydrate alphabet may actually carry more information than the nucleic acids and amino acids because the structure of carbohydrates is not only determined by the sequence of the monosaccharides but also dependent on the linkage between each monosaccharide. The linkage may vary in position and anomeric position. Further, the sugar ring size may also vary (five- or six-carbon ring). The carbohydrate sequence may be branched, something that is not observed in nucleic acids or proteins. Even though not all theoretical combinations of these structural differences are observed in nature (that would be an astronomical number), a combination of sequence, linkage position and branching are often needed for specific biological recognition by for example lectins. The glycan structures are not template derived; instead the structures are dynamic in space and time and dictated by the activity of enzymes in the ER and Golgi (see separate sections).

1.2 Protein glycosylation

Several forms of protein glycosylation are found in nature. Glycans are commonly attached to proteins via the amino acids asparagine and serine or threonine. Attachment to asparagine is mediated via the NH_2 group of the amino acid, and thus referred to as *N*-linked glycosylation. In an analogous manner *O*-linked glycosylation refers to glycan attachment to the OH group found in serine and threonine. The biosynthesis of *N*-linked and *O*-linked glycosylation is described in separate sections below. Both forms of glycosylation may consist of long and complex chains that may carry additional modifications such as sulfate or phosphocholine (paper I). The single *O*-GlcNAc glycosylation that competes with protein phosphorylation is involved in intracellular (cytosol and nucleus) signal transduction (Hart et al., 2011). *O*-Fuc and *O*-Glc glycosylation have been demonstrated to be important in development (Stanley and Okajima, 2010). The large carbohydrate polymers of proteoglycans are attached to the protein core with *O*-linked xylose (Couchman and Pataki, 2012) and *O*-linked mannose is quite prevalent in the brain (Stalnaker et al., 2011). *O*-linked mannose is also found on α -dystroglycan that is part of the dystrophin complex that connects the cytoskeleton and the extracellular matrix. The *O*-Man glycosylation is pivotal for the linkage of α -dystroglycan to laminin found in the extracellular matrix, the molecular basis for the contraction of muscles (Stalnaker et al., 2011). Mutations in these glycoproteins are therefore associated with muscular diseases (Stalnaker et al., 2011, Hewitt, 2009).

1.2.1 N-glycosylation

All *N*-glycans share a common core sequence, $\text{Man}\alpha 1-6(\text{Man}\alpha 1-3)\text{Man}\beta 1-4\text{GlcNAc}\beta 1-4\text{GlcNAc}\beta 1-\text{Asn-X-Ser/Thr}$ (X is any amino acid except proline) and are classified in three groups; high mannose (oligomannose), complex and hybrid (Figure 1B-D). High mannose has only mannose attached to the core, while complex type *N*-glycans have extensions (antennae) initiated with GlcNAc residues attached to the core. Hybrid structures have both mannose and antennae attached to the core (Figure 1). The addition of *N*-linked glycans to proteins is observed in all eukaryotic cells.

1.2.1.1 N-glycan synthesis

The *N*-glycan precursor is synthesized in the endoplasmic reticulum (ER) and consist of $\text{Glc}_3\text{Man}_9\text{GlcNAc}_2$ linked to the dolichol-P lipid (Dol-P) (Figure 1A). The precursor is conserved in all eukaryotic cells while the final trimming of the glycans differ considerably between plants, yeast, insects and mammals (Betenbaugh et al., 2004). The whole glycan precursor is transferred en bloc from the Dol-P lipid carrier to the polypeptide chain of the protein that is being synthesized and translocated through the ER membrane (Helenius and Aebi, 2004). The final structures found on mature

glycoproteins are then formed by the action of a large set of glycosyltransferases and glycosidases in the ER and Golgi.

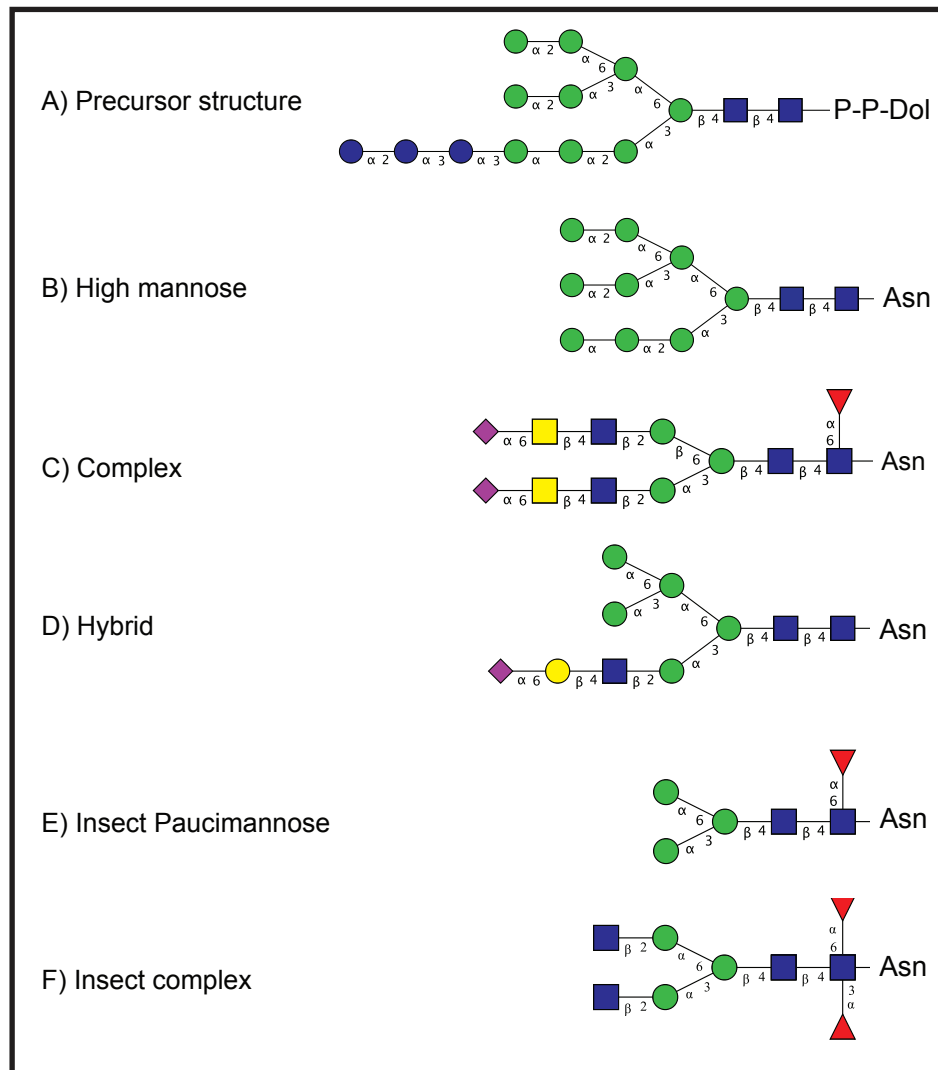


Figure 1 Examples of *N*-glycan types found in mammals (A-D) and insects (A and E-F).

1.2.1.2 Insect *N*-glycosylation

Insect and human *N*-glycosylation are different in several ways. *N*-glycans of insect cells are mostly of the high-mannose or paucimannose type with or without core α 1,3/6-fucosylation, although low amounts of hybrid- and complex types have been reported (Aoki et al., 2007, North et al., 2006, Ten Hagen et al., 2009, Shi and Jarvis, 2007, Ogonah et al., 1996, Hsu et al., 1997). The typical truncated paucimannosidic structure and a complex type *N*-glycan found in *Drosophila melanogaster* embryos are shown in Figure 1 E and F. Very low amounts of terminal sialic acid suggest a limited

and specialized expression pattern of this monosaccharide in *Drosophila* (Aoki et al., 2007). The *N*- and *O*-glycosylation of insect cells is described in more detail in the *Results and Discussion* section.

1.2.2 *O*-glycosylation

The most common form of *O*-linked glycosylation of proteins is initiated with the addition of GalNAc to serine or threonine in the ER/Golgi. This is also called mucin-type glycosylation due to the prevalence of *O*-GalNAc glycosylation in these proteins (*mucins*, see separate section). Different *O*-linked inner chain saccharide sequences, the “cores”, have been designated different numbers and are listed in Table 1. The cores may be further elongated with the terminal structures listed in Table 2.

Determinant	Structure
Tn antigen	GalNAc α Ser/Thr
Core 1 or T antigen	Gal β 1-3GalNAc α Ser/Thr
Sialyl-Tn antigen	Sia α 2-6GalNAc α Ser/Thr
Core 2	Gal β 1-3(GlcNAc β 1-6)GalNAc α Ser/Thr
Core 3	GlcNAc β 1-3GalNAc α Ser/Thr
Core 4	GlcNAc β 1-3(GlcNAc β 1-6)GalNAc α Ser/Thr
Core 5	GalNAc α 1-3GalNAc α Ser/Thr
Core 6	GlcNAc β 1-6GalNAc α Ser/Thr
Core 7	GalNAc α 1-6GalNAc α Ser/Thr
Core 8	Gal α 1-3GalNAc α Ser/Thr

Table 1 The *O*-glycan core structures found in mammals

1.2.2.1 *O*-glycan synthesis

In contrast to *N*-glycan synthesis, the *O*-glycosylation initiation is achieved by the addition of a nucleotide monosaccharide rather than the en bloc transfer of a large precursor carbohydrate (Hang and Bertozzi, 2005). The first transfer of GalNAc to the polypeptide chain is catalyzed by an UDP-GalNAc-polypeptide-*N*-acetylgalactosaminyltransferase (ppGalNAcTs) and takes place in the Golgi. A large family of ppGalNAcTs exists which may be explained by the fact that the ppGalNAcTs prefer different protein substrates and may be expressed differently in time and space (development and tissue tropism) (Hang and Bertozzi, 2005). No precise amino acid consensus sequence has been determined for the acceptor site of *O*-glycosylation, although proline amino acids near the Ser/Thr acceptor site are favorable. The synthesis of *O*-glycan core structures 1 to 4 is shown in Figure 2.

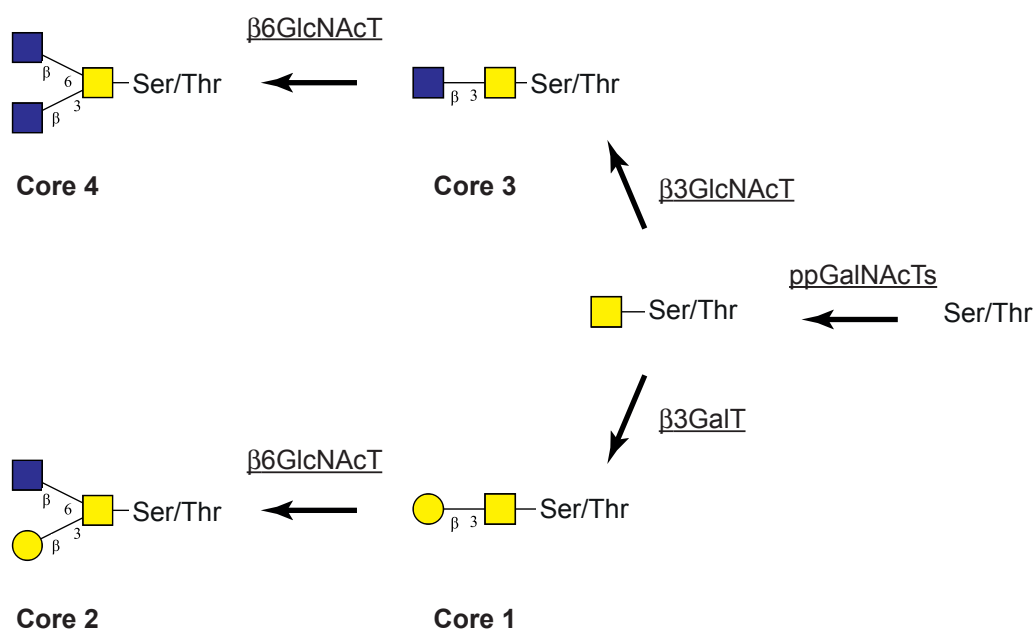


Figure 2 Synthesis of *O*-glycan core structures 1 to 4.

Determinant	Structure
Blood groups O, H	Fuc α 1-2Gal-
Blood group A	GalNAc α 1-3(Fuc α 1-2)Gal-
Blood group B	Gal α 1-3(Fuc α 1-2)Gal-
Linear B	Gal α 1-3Gal-
Blood group i	Gal β 1-4GlcNAc β 1-3Gal-
Blood group I	Gal β 1-4GlcNAc β 1-6(Gal β 1-4GlcNAc β 1-3)Gal-
Blood group Sd(a), Cad	GalNAc β 1-4(Sia α 2-3)Gal-
Blood group Lewis ^a	Gal β 1-3(Fuc α 1-4)GlcNAc-
Blood group Lewis ^x	Gal β 1-4(Fuc α 1-3)GlcNAc-
Blood group sialyl-Lewis ^x	Sia α 2-3Gal β 1-4(Fuc α 1-3)GlcNAc-
Blood group Lewis ^y	Fuc α 1-2Gal β 1-4(Fuc α 1-3)GlcNAc-
type 1 chain, neo- <i>N</i> -acetylglucosamine	Gal β 1,3GalNAc-
type 2 chain, <i>N</i> -acetylglucosamine (LacNAc)	Gal β 1,4GlcNAc-
type 3 chain	Gal β 1,3GalNAc α
type 4 chain	Gal β 1,3GalNAc β
type 6 chain	Gal β 4Glc
LacdiNAc	GalNAc β 1-4GlcNAc-
Chitobiose	GlcNAc β 1-4GlcNAc-

Table 2 Common terminal carbohydrate determinants that may be found on *N*-glycans, *O*-glycans or glycolipids

1.3 Function of *O*-GalNAc, mucin-type glycosylation

O-glycosylation serves many functions in biology and there is only room to mention a few areas of particular importance in this section. *O*-GalNAc glycosylation of proteins contributes to protein structure and stability and is involved in recognition (protein-carbohydrate binding). The extended structure of mucins and mucin-type proteins is highly dependent on dense glycosylation and is described in further detail in a separate section below. Highly *O*-GalNAc substituted proteins are also more resistant to proteolytic cleavage and are more heat-stable. Recognition associated with *O*-GalNAc includes cell growth and proliferation, glycoprotein clearance, glycoprotein trafficking, immunological recognition and signaling pathways (Tian and Ten Hagen, 2009, Patsos and Corfield, 2009b).

1.4 The mucosal barrier

Mucus secretions are part of the protective mucosa barrier of the auditory, gastrointestinal, respiratory, and the urogenital systems as well as the conjunctiva of the eyes (Perez-Vilar and Mabolo, 2007). The physical protection of the mucus membranes consists of the mucosal epithelium, its lamina propria and glycocalyx and secreted mucins (Patsos and Corfield, 2009a). The glycocalyx consist of membrane-associated glycoproteins, proteoglycans and glycolipids that are continuously renewed in order to maintain a defensive barrier (Moran et al., 2011). A highly hydrated mucus gel is covering the glycocalyx that consist mainly of large secreted glycoproteins, *mucins*. The mucins may be both membrane bound and secreted, where the latter form is disulfide-linked to form oligomers that may be as large as several million Dalton (Perez-Vilar and Mabolo, 2007). The name “mucin” was first used by Nicolas Theodore de Saussure 1835 when he described substances derived from mucus (Gottschalk, 1960). Eichwald and Hammarsten later showed that mucins consist of highly glycosylated proteins (Montreuil and Vliegenthart, 1995). The composition of the glycocalyx and mucus gel is dependent on the location and the requirements of the anatomical location (Ito, 1969, Perez-Vilar and Mabolo, 2007, Patsos and Corfield, 2009a). The mucus gel serves as a physical barrier that can trap particles but the highly glycosylated mucins may also, via their carbohydrate substitutions, specifically bind to bacteria and viruses (Patsos and Corfield, 2009a). Many bacteria and virus use carbohydrate receptors at the epithelial cell surface for attachment or uptake into the cells (Olofsson and Bergstrom, 2005, Moran et al., 2011). In addition, the mucus contains a range of anti-microbial proteins such as antibodies and host defense peptides including defensins, protegrins, collectins, cathlecidins, lysosymes and histatins (Kagnoff and Eckmann, 1997, Lu et al., 2002, Raj and Dentino, 2002).

1.5 Co-evolution of host and pathogens

It has been suggested that rapid evolution is shaping the glycosylation of the mucins in order to present carbohydrate decoy receptors that will trap microorganisms or mask microbial binding sites by capping with additional terminal carbohydrates. Likewise, strong selection pressure forces the microbes to evolve quickly in order to remain infectious. This has been proposed to be an example of an evolutionary arms race, where host and pathogen need to constantly evolve in order to avoid extinction (Varki et al., 2009c).

1.6 Mucins and mucin-type proteins

Mucins have the *O*-glycosylation sites (Ser/Thr) clustered together in mucin-domains that are found in variable number tandem repeats (VNTR) up to 10-100 times in the polypeptide chain (Hang and Bertozzi, 2005). This results in glycoproteins where the carbohydrates constitute more than half of the total weight. The tandem repeats are also rich in proline (Moniaux et al., 2001). Mucins have been proposed to have an extended structure as indicated by several studies (McMaster et al., 1999, Shogren et al., 1989, Gerken et al., 1989, Bansil et al., 1995). Many glycoprotein carry the tandem-repeats found in mucins and have thus been termed mucin-type or mucin-like proteins, although they may not be genetically related to proper mucins. Studies on the mucin-like protein CD43 (leukosialin, sialophorin) have shown that GalNAc α but not GalNAc β contribute to the elongated structure of this glycoprotein (Coltart et al., 2002, Live et al., 1999, Live et al., 1996). Another mucin-type protein is P-selectin glycoprotein ligand-1 (PSGL-1). In this thesis we have studied the PSGL-1 fused to the Fc part of mouse IgG_{2b} to form the PSGL-1/mIgG_{2b} fusion protein (Figure 10). The PSGL-1 /mIgG_{2b} is produced as a dimer that carry 32 tandem repeats and have 6 potential *N*- and 106 potential *O*-glycosylation sites (Cummings, 1999, Liu et al., 1997, Sako et al., 1993). The large size and the elongated shape of the protein make it suitable for interaction with other proteins. In this thesis PSGL-1/mIgG_{2b} was used as a tool to study *O*-glycosylation (paper I) and as a multivalent carrier of specific carbohydrate ligands in paper II-IV.

2 Protein-carbohydrate interactions

2.1 Lectins

Lectins are ubiquitous glycan-binding proteins (GBPs) found in all living organisms. Particularly plant lectins bind with high specificity to specific carbohydrate moieties. This feature has been extensively used for the analysis and purification of glycans. Since the lectins are so widespread the functions of lectins are also varied (Varki et al., 2009b). Microbial lectins include attachment factors and toxins, such as influenza hemagglutinin and Shiga toxins (Holgersson et al., 2009). These lectins are described in further detail in 6.4.1 and 7.3 and are the targets of novel carbohydrate-based inhibitors described in paper III and IV. The first mammalian lectin discovered was the asialoglycoprotein receptor, which is involved in the clearance of glycoproteins in the circulation. This is explained in further detail in section 5.4. Lectins are important in the innate immune system where C-type lectins and Siglecs are examples of pattern-recognition receptors (PRRs) that may have the capacity to regulate innate and adaptive immunity (Robinson et al., 2006).

2.2 The multivalency effect of protein carbohydrate interactions

Multivalency is a universal principle in nature to achieve strong but reversible binding and is important in recognition, adhesion and signaling processes (Fasting et al., 2012). Although an individual carbohydrate-protein interaction may be weak, multiplying the interaction in a multivalent or polyvalent fashion can radically strengthen the bond (Fasting et al., 2012). This type of interaction has been compared to the burr and Velcro binding that relies on the combined strength of multiple tiny hooks that entangle with loops to form a strong bond. The theoretical models of multivalency and its role in biological systems have been extensively reviewed (Mammen et al., 1998) (Fasting et al., 2012). A few examples of multivalent interactions mentioned by Mammen *et al* are viral (influenza) and bacterial (fimbriae of *E. coli*) adhesion to host cell surface, cell-cell adhesion (neutrophil binding to endothelial cells), binding of cells to polyvalent molecules (Fc receptor binding to multiple antibodies recognizing repetitive antigen on the surface of a bacterium) and interaction between polyvalent molecules (transcription factors binding to DNA sites) (Mammen et al., 1998). Additional abundant examples are given within the review (Mammen et al., 1998).

The strengthening of a multivalent bond (avidity) compared to a monovalent bond is mostly related to the decrease in the dissociation k_{off} value (k_d), instead of increase in association k_{on} value (k_a). This may be explained by the increase in re-binding. If one bond is dissociated the remaining bonds keep the unbound ligand and receptor in close proximity which facilitates re-binding. Multivalent interactions may or may not be

positively cooperative. An example of positively cooperative (synergistic) binding is the binding of O₂ molecules to hemoglobin where each additional binding becomes more favorable as the binding sites becomes occupied (Mammen et al., 1998). It should be noted that multivalency is not always associated with cooperative binding (Fasting et al., 2012). Even a non-cooperative (additive) or negatively cooperative (interfering) multivalent binding may confer stronger and more specific binding compared to monovalent binding (Fasting et al., 2012).

2.3 Chemical and biological approaches to achieve multivalency

Polyvalent presentation of ligands has been achieved in several ways, for example through the use of polyacrylamide polymers (PAA), dendrimers, neoglycoconjugates, nanoparticles, liposomes and glycoproteins (Mammen et al., 1998) (Chabre and Roy, 2009) (Fasting et al., 2012) (Gabius, 2000). Chemically synthesized multivalent molecules have several advantages in that they can be made more homogenous than glycoproteins and that the shape, spacing and presentation of the ligand may be modified in any desirable way. *In vivo* applications exclude some toxic scaffolds however and the synthesis of long carbohydrate chains is still complex (Oscarson, 2009). Another approach to produce multivalent molecules is to use genetic engineering of cell lines to produce multivalent glycoproteins. The production and practical application of multivalent glycoproteins with glycan tailored glycosylation has previously been a successful strategy in our laboratory. The heavily *O*-glycosylated P-selectin glycoprotein ligand-1 genetically fused to the Fc portion of mouse IgG_{2b} (PSGL-1/mIgG_{2b}) has been produced in cell lines that have been transfected with plasmids encoding glycosyltransferases. This allows the rational glycan design of PSGL-1/mIgG_{2b} presenting specific carbohydrates in a multivalent fashion. Glycoproteins inherently have larger variability of the carbohydrate epitopes compared to chemically synthesized molecules and care must be taken when choosing the expression system in order to avoid the production of proteins carrying carbohydrate determinants that are immunogenic, mediate binding to endogenous lectins or microbes that are not targeted.

2.4 Multivalent carbohydrate inhibitors

Carbohydrates are ubiquitous on all mammalian cell surfaces. Bacterial and viral tropism is therefore often conferred by attachment to species- or tissue-specific expression of glycans at the cell surface. In order to achieve strong and specific attachment to the cell surface the nature of the binding is often multivalent. It has therefore been suggested that potential carbohydrate based inhibitors of bacterial and viral attachment must be multivalent. An advantage of a large polyvalent inhibitor is the combined effect of competitive inhibition and steric hindrance (Mammen et al.,

1998). The high concentration of receptor analogues presented by the multivalent inhibitor competitively inhibits binding of a virus to the cell surface. In addition virus particles that have attached to a large polyvalent (flexible) inhibitor become shielded from the cell surface and may also become aggregated with other virus particles that have adhered to the polymer. The mechanism has been suggested to be similar to the inhibitory effect of natural mucins which bind specifically to certain viruses with the carbohydrate moieties and also provide a physical barrier (Mammen et al., 1998). Steric hindrance of viral or bacterial attachment to the cell surface does not have to be mediated via binding to attachment factors; rather, any factor on the viral or bacterial surface may serve as a target for steric inhibition such as influenza NA. In paper III and IV the binding of potential carbohydrate-based inhibitors of Shiga-like toxins and avian influenza hemagglutinin was investigated.

3 Tools to study glycosylation

3.1 Glycan structure analysis

Carbohydrates are dense in information and therefore several analytical methods are needed in order to elucidate all structural details of individual glycans and to determine the glycomic profile of glycan mixtures. This section will provide brief information of a few commonly used analytical methods used for structural elucidation. Specific information regarding some of the methods used in this thesis is found in section 9. *Methodological considerations.*

3.1.1 Western blot

Specific glycans on glycoproteins can be quickly detected with Western blot where separated and blotted glycoproteins are stained with monoclonal antibodies or lectins. A wide range of commercially available lectins and antibodies are available that can be used to determine the identity and linkage of (mostly terminal) glycans. Western blot is also used together with substrate-specific glycosidases. Enzymatic removal of carbohydrates is detected as a shift in migration in *e.g.* SDS-PAGE.

3.1.2 Monosaccharide analysis

The identification of individual monosaccharides in glycan or glycoprotein samples can be determined with several different techniques. The glycan is completely hydrolyzed into its monosaccharide constituents, which are identified by their retention times on a column compared to known standards. The monosaccharides can be separated by HPLC, but may require fluorescent labeling (see below) for detection, or by High Performance Anion Exchange Chromatography with Pulsed Amperometric Detection (HPAEC-PAD). HPAEC-PAD does not require any labeling of the monosaccharides for detection.

3.1.3 Mass spectrometry

Mass spectrometry (MS) is a sensitive analytical method to detect and identify molecules. In mass spectrometry the mass and charge ratio (m/z) of ionized molecules are accurately detected. All mass spectrometry instruments contain an ionizer, mass analyzer and detector. The ionization of the sample may be achieved in several ways. Common types of ionization techniques suitable for the analysis of biomolecules include fast atom bombardment (FAB), matrix-assisted laser desorption ionization (MALDI) and electrospray ionization (ESI). Different types of mass analyzers include sector-, time-of-flight- and quadrupole instruments. In this thesis a liquid chromatography column separated the glycans prior to electrospray ionization and mass

detection. Different derivatizations of glycans as a way to increase sensitivity and the information obtained are described in more detail elsewhere (Zaia, 2004). Mass spectrometry generates a wealth of structural information depending on the protocol. Glycoprotein analysis gives information on type of glycosylation and site of glycosylation, while glycan analysis reveals glycan branching, number and length of antennae, composition, substitutions and sequence of individual glycans (Mulloy et al., 2009). It should be noted that besides glycoproteins and released glycans, MS can be used to analyze the structure of glycolipids and GAG-derived glycans (Mulloy et al., 2009). Linkage position can be determined with partial methylation analysis where the fragmentation pattern is compared with known standards. However, this technique does not determine if the linkage is of α or β anomeric configuration.

3.1.4 High-performance liquid chromatography

N-glycan structural analysis can be performed with high-throughput analysis using high-performance liquid chromatography (HPLC). The *N*-glycans are released from the protein with *N*-glycosidases that cleaves the bond between Asn and the carbohydrate moiety. Derivatized glycans are separated with different columns using HPLC and the glycan structures are identified by comparing the elution position with known glycans. The structural determination can sometimes be automated by comparing the retention times with those of known structures.

3.1.5 Nuclear magnetic resonance

Nuclear magnetic resonance allows the full structural elucidation of unknown samples, including composition, linkage and anomericity (α/β). NMR requires a lot of source material for a full structural characterization due to the relatively low sensitivity. Important though is that the sample is not consumed in the analysis.

3.2 Glycan binding analysis

The interaction between glycan-binding proteins (GBPs) and glycans can be measured in several ways (Cummings and Esko, 2009). Binding constants (K_D) can be derived from near-equilibrium methods such as equilibrium dialysis, equilibrium gel-filtration and frontal affinity chromatography (Nakamura-Tsuruta et al., 2006, Bergstrom et al., 2012). Titration calorimetry can provide more complete thermodynamic information, but current instruments require ample material (Dam and Brewer, 2004). Surface plasmon resonance (SPR) measures the association and dissociation kinetics between an immobilized ligand and its receptor, but may also be used to measure equilibrium binding constants (affinity) (Olausson et al., 2011). This technique was used in paper III and IV and is explained in more detail in section 9.6.

ELISA has been extensively used to determine the K_D of the binding of GBPs to glycans. Usually this technique requires some sort of coupling of the glycan to biotin (for immobilization) or reporter group (for detection). In competition ELISA an inhibitory glycan is added to the well and the competition for GBP is measured as a function of concentration in order to obtain the IC_{50} value (and thus K_D). In glycan arrays the glycans are printed on a surface and GBPs is overlaid and allowed to bind. Bound GBP is detected with a fluorescent label that is conjugated either to the GBP directly or to an anti-GBP antibody. The binding is typically presented as a histogram where binding is measured in fluorescence units. Glycan arrays with hundreds of different glycans can give information of the specificity of the GBP but provide no binding constants (Paulson et al., 2006).

4 The ABO blood group system

4.1 Historical background

The ABO blood group antigen system was discovered by Karl Landsteiner already 1901 (Tagarelli et al., 2001). Landsteiner demonstrated that serum factors could agglutinate red blood cells from other humans and based on his observations divided humans into different blood groups. Landsteiner was awarded the Nobel prize in 1930 for his discovery (Tagarelli et al., 2001). Subsequent experiments have shown that the serum factors are antibodies and that the antigens on the red blood cells are glycan epitopes. In addition to the ABO system, several additional blood group systems have now been discovered (Storry and Olsson, 2009). The structural and genetic elucidation of the ABO antigens was largely revealed by Watkins (Watkins, 1966) and Kabat (Kabat, 1956) but the glycosyltransferase genes responsible for the biosynthesis of A and B were not cloned until 1990 by Yamamoto et al. (Yamamoto et al., 1990a, Yamamoto et al., 1990b).

4.2 Structures and distribution

The distribution of the blood group determinants is cell- and tissue-dependent. The expression of the ABO blood group determinants on different precursor type chains (type 1, 2, 3 and 4) has been extensively reviewed (Clausen and Hakomori, 1989) (Ravn and Dabelsteen, 2000, Holgersson et al., 1992). These studies have mainly been performed on extracted glycolipids or immunohistochemical staining of tissues with chain type-specific antibodies that do not show if the blood group determinants are carried by glycoproteins or glycolipids.

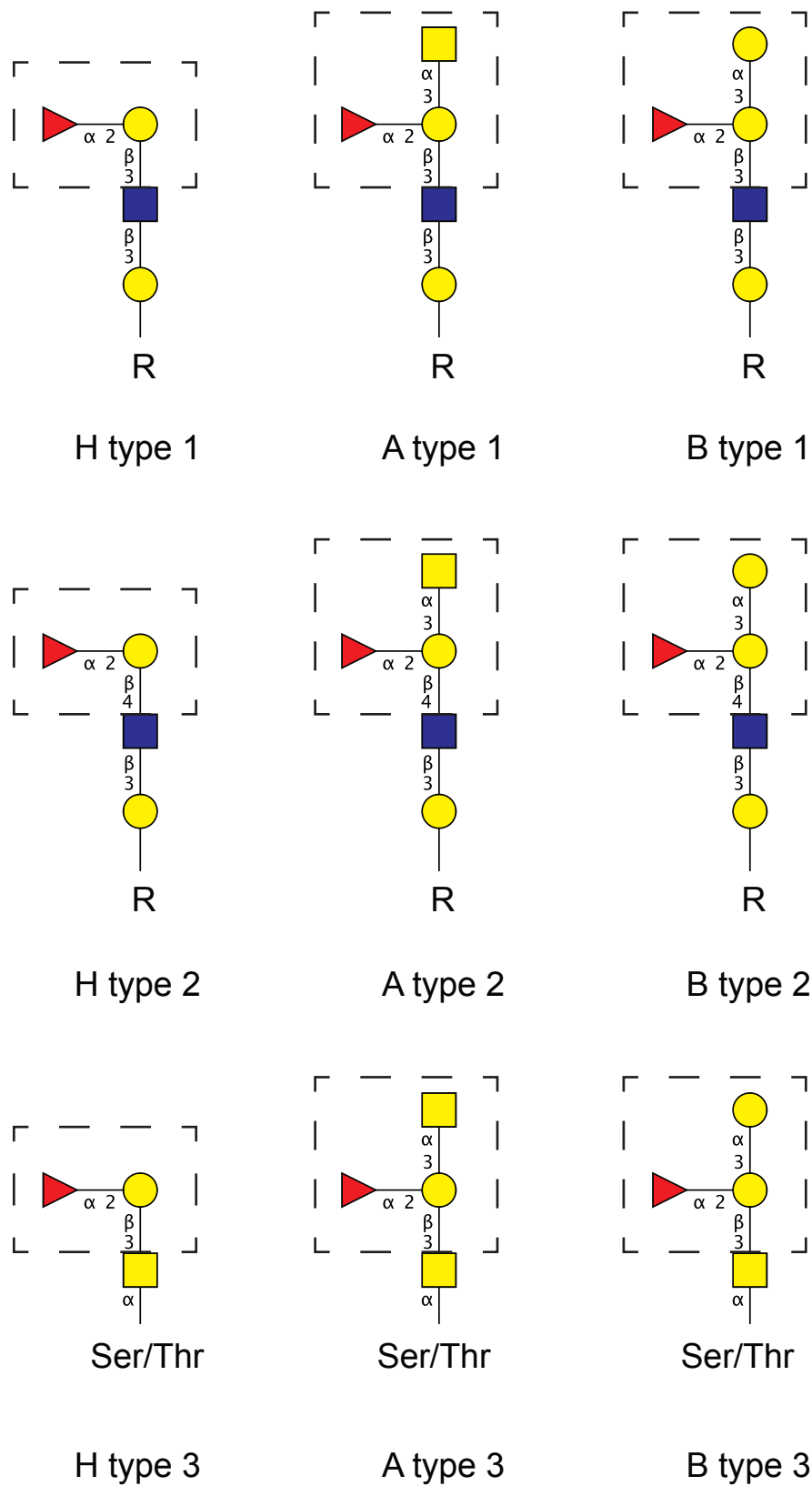


Figure 3 ABO (H) blood group determinants on different carbohydrate outer type chains

4.3 The medical importance of the ABO system

The ABO blood group antigen system has important medical implications. If blood is transfused between individuals of different blood groups, e.g. A to O, circulating IgM antibodies would immediately bind to donor red blood cells and trigger complement activation and lysis. This is a serious condition that can lead to hypotension, shock, renal failure and death (Storry and Olsson, 2009). Blood typing is thus important for blood- and serum transfusions.

4.4 Immunoabsorption

Prior to transplantation of kidney, heart and liver, blood group compatibility is ensured. However, due to the shortage of organs, kidney (and now also other organs) transplantation across the ABO barrier has recently been increasingly performed. The graft survival rates have been similar to those observed for ABO compatible kidneys (Genberg et al., 2008). ABO incompatible transplantation may be improved with immunoabsorption (IA) of ABO reactive antibodies using extracorporeal affinity columns carrying synthetic ABO determinants (Tyden et al., 2005). The A- and B-columns carry the trisaccharide determinants [A: GalNAc α 1-3(Fuc α 1-2)Gal and B: Gal α 1-3(Fuc α 1-2)Gal] covalently linked via a six-hydrocarbon spacer to Sepharose beads. Although, the columns were highly biocompatible and successfully reduced the anti-A/B titers (Rydberg et al., 2005), the trisaccharide blood group determinants may not remove all A/B reactive antibodies. Determining the fine antigen specificity of the anti-A/B antibodies before and after transplantation requires blood group determinants on several core chains (Lindberg et al., 2013). In paper II cell lines were engineered in order to produce glycoproteins carrying the blood group determinants on type 1, 2 and 3 outer core chains. Currently, the use of the glycoproteins carrying the blood group determinants in analytical (fine specificity of anti-A/B titers pre- and post-transplantations) and therapeutic use (immunoabsorption of anti-A/B antibodies) is being explored.

4.5 ABO synthesis

The blood group antigens may be found on several terminal chains, as demonstrated in Figure 3. The synthesis of the blood group determinants is described in detail in paper II.

5 Recombinant therapeutic proteins

5.1 *Increasing economical and medical importance*

Recombinant proteins such as hormones, growth factors, cytokines, enzymes, vaccine components and antibodies are examples of important licensed therapeutic proteins that account for an increasing share of the revenues in the pharmaceutical industry (Fernandez and Muyldermans, 2011). More than two hundred (of which ~100 are unmodified) therapeutic proteins have been approved for clinical use in the European Union and the USA. The total sales 2010 was 108 billion US\$, with monoclonal antibodies (mAbs) accounting for half of the sales (Dimitrov, 2012).

5.2 *Glycosylation affects protein function and stability*

Glycoproteins account for more than two thirds of the available therapeutic proteins on the market today (Li and d'Anjou, 2009). The biological function of glycoproteins is usually defined by the polypeptide chain, while the carbohydrate moiety influences stability, solubility, bioavailability, *in vivo* activity, pharmacokinetics, and immunogenicity (Li and d'Anjou, 2009). Mammalian cell lines (including human) are frequently used for the production of therapeutic proteins, even though non-human glycosylation may occur (Li and d'Anjou, 2009) (table 3). In contrast to most small molecule drugs, therapeutic proteins are heterogeneous. It has been realized that a vast number of variants may exist that differ with regard to a wide range of structural features including glycosylation, disulfide bond formation, modifications of amino acids, conformation and aggregation (Liu et al., 2008). The acceptable variability must be evaluated individually for each therapeutic protein with pre-clinical and clinical studies that demonstrates the importance of the glycosylation with regard to pharmacokinetics, bioavailability, clearance and potency (Li and d'Anjou, 2009). It should be noted that the glycosylation pattern of endogenous human proteins may be dynamic and that it is not always clear which “native” glycosylation that the recombinant therapeutic proteins should mimic (Hossler et al., 2009).

5.3 *Glycosylation dictates the effector function of IgG antibodies*

The carbohydrate moiety of therapeutic glycoproteins that are administrated in the circulation may bind to several receptors that influence their half-life and effector function. A prime example is antibodies, the effector function of which is highly dependent on the fine glycosylation of the Fc portion. Currently, all licensed antibodies are of the IgG class (Jefferis, 2009). While the Fab domains of antibodies mediate the antigen binding, the Fc portion interacts with receptors that activate the effector functions. The receptors include the Fc γ receptors (Fc γ RI, Fc γ RIIa, Fc γ RIIb, Fc γ RIIc,

Fc γ RIIIa, Fc γ RIIIb), the C1q component of the complement system and neonatal Fc receptor (FcRn) (Jefferis, 2009). Fc receptors are present on a wide range of leukocytes and the IgG binding can activate or inhibit inflammatory responses by the interaction of different IgG subclasses and glycosylation variants with specific Fc receptors (Nimmerjahn and Ravetch, 2005). The effector function may be fine-tuned with specific glycosylation of both IgG and the Fc receptor (Ferrara et al., 2006). Non-fucosylated Fc portion of IgG1 results in maximal Antibody-Dependent Cell-Mediated Cytotoxicity (ADCC) effector function, while non-glycosylated Fc of IgG2 have minimal effector function (Jefferis, 2007). Binding to C1q mediates activation of the complement cascade and neonatal Fc receptor controls the half-life of IgG in circulation and has a role in placental transport from mother to fetus (Basta, 2008, Jefferis, 2009).

5.4 Sialylation influences the half-life of glycoproteins in the circulation

The half-life in circulation of several therapeutic proteins is highly dependent on the sialylation. The asialoglycoprotein receptor (ASGP-R) is abundantly expressed on the cell surface of hepatocytes, and was initially identified and characterized by its ability to rapidly remove glycoproteins carrying terminal Gal or GalNAc from the circulation (Ashwell and Harford, 1982, Morell et al., 1971).

5.5 Specific glycosylation may improve future drugs

The half-life and effector function of therapeutic proteins is largely controlled by the specific glycosylation of the protein and the lectins to which they bind. Detailed studies of how antibodies exert their function have revealed a dynamic and complex system that regulates the effector function of antibodies. Almost thirty years ago it was noted that different glycoforms of antibodies were observed during different stages of rheumatoid arthritis (Parekh et al., 1985). We now know that the increased levels of G0 glycoforms (lacks terminal sialic acid and galactose) observed during disease are pro-inflammatory. The understanding of how glycosylation affects the function of therapeutic antibodies will hopefully in the near future lead to more efficient drugs with fewer side-effects.

5.6 Cell lines used for production of therapeutic proteins

Therapeutic proteins are currently produced in a range of cell lines, that each has their strength and weaknesses. Most of the current licensed therapeutic glycoproteins are produced in Chinese Hamster Ovary cells (CHO), mouse myeloma cells (NS0, SP/0) and hybridomas (Durocher and Butler, 2009) (table 3)

Expression system	Products	Positives	Negatives
Mammalian hamster: Chinese hamster ovary cells (CHO), BHK21 mouse myeloma cells: (NS0, SP2/0) human cells: HT1080m HEK-293 novel human cell lines: PER.C6, HKB11	<ul style="list-style-type: none"> antibodies biologically active large recombinant proteins with multiple PTMs, (Factor VIII) 	<ul style="list-style-type: none"> well characterized can produce large, complex proteins requiring post-translational modifications (PTMs) human-like glycosylation approval for human therapy from regulatory authorities mutant cell lines available 	<ul style="list-style-type: none"> safety (virus) costs non-human cell lines may produce Neu5Gc and Galα1,3Gal (hypersensitivity reactions) and may lack α2,6 sialyltransferase, α1-3/4 fucosyltransferase and N-acetylglucosamine transferase III.
Insect , baculo-virus expression system, <i>Spodoptera frugiperda</i> : Sf9, and Sf21. <i>Trichoplusia ni</i> (Hi-5), <i>Bombyx mori</i> , <i>Drosophila frugiperda</i> (S2)	Vaccine antigens (Cervarix, Ph.3 trials: Provenge, Flublok, Chimigen), virus-like particles	<ul style="list-style-type: none"> high expression levels safe production (no human pathogen risk) easy (no CO₂ needed) 	<ul style="list-style-type: none"> High-mannose and paucimannose, currently no therapeutic protein – risk of adverse reactions low sialylation immunogenic α1,3-fucose
Yeast : <i>Pichia pastoris</i> , <i>Sacharomyces cerevisiae</i> , <i>Hansenula polymorpha</i>	Vaccine antigens, non-glycosylated proteins: albumin, insulin, growth hormone	<ul style="list-style-type: none"> high yields easy to grow cheap mannose may bind to C-type lectins on immune cells 	<ul style="list-style-type: none"> Only approved for non-glycosylated proteins No Tyrosine O-sulfation N- and O-linked high-mannose is immunogenic in humans
Plants : <i>Lemna minor</i> , carrot cells	Edible oral vaccines, interferon alpha (Locteron)	<ul style="list-style-type: none"> Cheap safe ~ low yields 	<ul style="list-style-type: none"> proteolytic degradation gene silencing no sialylation immunogenic α1,3-fucose and β1,2-xylose
Transgenic animals: goat milk, chicken eggs,	Atryn, human anti-thrombin	<ul style="list-style-type: none"> high fidelity 	<ul style="list-style-type: none"> expensive non-human glycosylation

Table 3 Summary of the common expression systems and their suitability for the production of therapeutic proteins

5.6.1 Chinese Hamster Ovary cells (CHO)

CHO cells are the most widely used cells today and will continue to be so in the near future due to a history of regulatory approvals of licensed recombinant drugs and the continuous development of productivity of the cells. CHO cells have an active α 2,3 sialyltransferase and α 1,6 fucosyltransferase (N-linked core fucosylation). Genetic engineering is needed in order to produce glycoproteins with terminal α 2,6 sialylation or α 1,2/3/4 linked fucose (blood group determinants), respectively (Durocher and Butler, 2009, Xu et al., 2011). Low amounts of Neu5Gc and terminal Gal α 1,3Gal epitopes have been reported on proteins produced in CHO cells (Bosques et al., 2011). This is potentially problematic since these epitopes are not found in humans and are known to elicit immune responses (Chou et al., 1998, Ghaderi et al., 2010, Macher and Galili, 2008). However, the levels do seem to be low or non-detectable in comparison to murine cell lines (Xu et al., 2011, Bosques et al., 2011). The O-glycosylation capacity of CHO cells is limited unless genetically engineered. We have analyzed the heavily O-glycosylated mucin-like PSGL-1/mIgG_{2b} fusion protein produced in CHO cells in our laboratory and the main O-glycans are core 1 with- and without sialic acid (paper IV). However, in paper II we show that complex carbohydrates, such as blood group determinants may be produced if the CHO cells are transfected with cDNAs encoding the required glycosyltransferases.

5.6.2 Human cell lines

Production of recombinant therapeutic proteins in human cell lines generally results in glycoproteins with more human-like glycosylation that is devoid of immunogenic glycans commonly observed in other mammalian cell lines. Examples of human cell lines that produce licensed drugs include HEK-293, HT-1080, Namalwa, PerC6 and HKB11 (Durocher and Butler, 2009).

5.6.3 Insect cell lines

Recombinant protein production in insect cells is often performed with the baculovirus expression vector system (BEVS) that enables rapid production of large amounts of proteins (Roldao et al., 2011). Common cell lines include *Spodoptera frugiperda* derived Sf9/Sf21, *Trichoplusia ni* derived High-five cells, *Bombyx mori* (silkworm) and *Mamestra brassicae*. In paper I the *O*-glycosylation capacity of Sf9 and Hi-5 cells was investigated. Because the *N*-glycosylation (see *Results and Discussion*) and *O*-glycosylation (paper I) is significantly different compared to human glycosylation, no approved human drugs are currently produced in insect cells. However, vaccine components are currently being produced in insect cells and several novel insect cell-derived vaccines are in clinical trials (Cox, 2012).

5.6.4 Yeast

Several non-glycosylated approved therapeutic proteins such as insulin, growth hormone and albumin are produced in yeast cells such as *Pichia pastoris*, *Saccharomyces cerevisiae* and *Hansenula polymorpha* (Durocher and Butler, 2009). Glycoproteins produced in yeast are heavily mannosylated which targets them to dendritic cells via C-type mannose-specific lectin receptors which may or may not be desirable depending on intended use of the protein (Lam et al., 2007, Dasgupta et al., 2007, Gustafsson et al., 2011, Ahlen et al., 2012). Yeast cells have been extensively genetically engineered in order to produce proteins with human like *N*- and *O*-linked glycosylation (Hamilton and Gerngross, 2007). Yeast may thus be an important source of therapeutic proteins in the future due to the relative ease of large-scale cultures, high protein yields and more homogenous glycosylation (Hamilton and Gerngross, 2007).

5.6.5 Plants

Production of therapeutic proteins, “pharming”, is currently performed in several plants such as carrot cells and *Lemna minor*. Plants could be a suitable source of edible oral vaccines but so far the progress has been hampered by low yields (Davoodi-Semiromi et al., 2009). At present a few therapeutic proteins are approved for topical use but several novel therapeutic proteins are in clinical trials (Karg and Kallio, 2009).

5.6.6 Bacteria

A few licensed aglycosylated therapeutic proteins are produced in *E. coli* (Zhu, 2012). Although these cells are less commonly used than mammalian cells and are less optimized for the production of large proteins, aglycosylated antibodies may sometimes be advantageous (Simmons et al., 2002).

5.7 Several factors affect glycosylation of therapeutic proteins

The glycosylation of therapeutic glycoproteins does not only depend on the choice of cell line. Different culture conditions such as growth rate, temperature, pH, metabolic profile and dissolved oxygen, medium and nutrients, serum, glucose- and ammonium levels and choice of bioreactor all affect the glycosylation of therapeutic proteins (Zhu, 2012). Importantly, there is no expression system that is recommended in general for all glycoproteins. A systematic investigation of the vector system, cell line and product is needed in order to fully understand the impact of each factor for the final glycosylation (Hossler et al., 2009). The desired post-translational modifications dictate the choice of production system. The yields obtained of recombinant proteins are highly dependent on the optimization of vector design, transfection protocols and selection (gene amplification with selection drugs) and screening tools. Optimization steps of vectors include a strong promoter, the signal peptide, selected introns, codon optimization and chromatin opening elements (Zhu, 2012).

6 Influenza

6.1 *Classification and historical background*

In paper IV the *in vitro* binding of a potential inhibitor of avian influenza H5 hemagglutinin was investigated. The following section will give a background on influenza type A that has great impact on human health. Influenza virus belong to the family of *Orthomyxoviridae* and is divided into three types; A, B and C, (Baigent and McCauley, 2003, Cheung and Poon, 2007). Influenza A virus is enveloped and has a genome that consists of eight negative sense single stranded RNA segments that encode ten proteins. Influenza is classified by the two surface antigens it carries in the envelope, hemagglutinin (HA) and neuraminidase (NA). Seventeen HA subtypes (H1-H17) have been identified and ten NA subtypes (N1-N10) (Fouchier et al., 2005) (Sun et al., 2013). Although the main reservoir of influenza A is aquatic birds, the virus has a broad host tropism and also infects humans and other mammals. In contrast to the widespread infectivity in birds, only three influenza A strains have been fully adapted to humans the last century. Mankind largely lacked immunity against these novel virus variants that consequently caused worldwide pandemics; H1N1 (1918-1920, “Spanish flu”), H2N2 (1957), H3N2 (1968-present), H1N1 (1977-present) and H1N1 (2009) (Baigent and McCauley, 2003, Cheung and Poon, 2007). Although, the 1918, 1977 and 2009 pandemics were all caused by H1N1 strains, other virulence factors contributed to the severe outcome of the 1918 “Spanish flu” pandemic which caused more than 40 million deaths worldwide compared to the estimated ~285000 who died in the 2009 pandemic (Palese, 2004, Dawood et al., 2012). Occasionally other strains such as H5N1, H7N7, H9N2 and recently H7N9 have been isolated from human, although these strains display limited spread among humans (Cheung and Poon, 2007, Gao et al., 2013). Some of these strains are highly pathogenic, as described below.

6.2 *Disease*

Symptoms of influenza are characteristically high fever, chills, sore throat, headache, runny or stuffy nose, weakness, muscle pain and sometimes diarrhea (Gao et al., 2013). Although, more severe than a common cold, the disease is usually self-contained. Risk groups include children, elderly and individuals with pulmonary or cardiovascular complications and immune compromised individuals. Influenza infection is mainly spread via inhalation of aerosols but may also infect via the eyes. People above the age of 65 accounts for 90% of all deaths caused by seasonal influenza (Metersky et al., 2012).

6.2.1 Highly pathogenic avian influenza viruses and human adaption

Highly pathogenic avian influenza viruses (HPAIV), of which H5N1 is a prominent example, are of particular interest due to their sporadic outbreaks associated with high death rates. Since 2003 HPAIV have been confirmed in 570 individuals with a 60% fatal outcome (Kuiken et al., 2012). In comparison, the Spanish flu had maximum death rate of 7% (Morens et al., 2010). Fortunately, HPAIV does not yet spread readily between humans (Kuiken et al., 2012). The increased pathogenesis associated with HPAIV infection is not entirely understood. Both unique virulence factors of the virus and an inappropriate immune response contribute to a “cytokine storm” that induces a systemic inflammatory response syndrome that may lead to multi-organ failure and death (Tisoncik et al., 2012, Kuiken et al., 2012).

6.3 Life cycle of influenza

The infectious cycle of influenza is schematically depicted in Figure 5. In addition the figure shows how different anti-viral agents target different stages of influenza infection.

6.4 Structure of influenza virus

The influenza virus particle has an envelope that is derived from the host cell membrane and is acquired in the budding process (Nayak et al., 2004). Three proteins are found in the envelope; hemagglutinin (HA), neuraminidase (NA) and matrix protein 2 (M2). HA bind to the host cell surface receptor sialic acid and in addition mediate subsequent fusion of virus and host membranes (Skehel and Wiley, 2000). This protein is described in further detail below. NA is an enzyme that hydrolyzes sialic acid, the receptor of influenza. NA may have a role in both attachment and release from cells (Nayak et al., 2004). M2 is an ion channel protein (function described in Figure 5 legend). Additional components of influenza virus are shown in Figure 4.

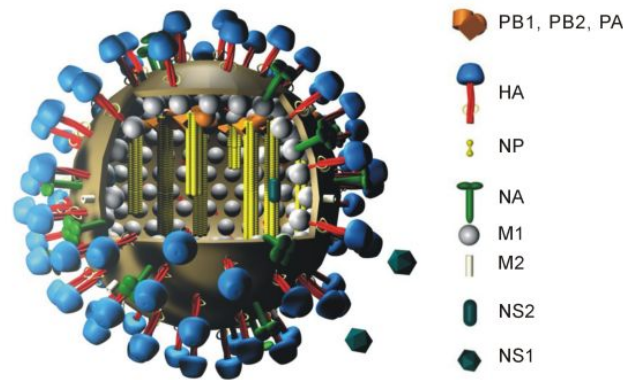


Figure 4 Schematic 3D model of an influenza virus particle. The proteins that build up the virus are indicated. Source: Wikipedia commons (public domain license).

6.5 Hemagglutinin receptor specificity and biological role of HA

Hemagglutinin is abundant (~500) in the virus envelope of the influenza particle (Stegmann et al., 1987). The name hemagglutinin is derived from the early observation that HA can agglutinate red blood cells (Beaumont and Lorenzelli, 1972). HA is found as a homotrimer in the envelope of the virus particle where it may bind up to three sialic acid moieties. The receptor binding specificity that is conferred by HA has important biological consequences. Sia α 2,3Gal and Sia α 2,6Gal are terminal carbohydrate determinants that are ubiquitous on avian and mammalian cell surfaces. Different HA molecules have different specificities to Sia α 2,3Gal and Sia α 2,6Gal carbohydrates receptors, respectively. As little as a single amino acid substitution may change the specificity towards either of the carbohydrates (Rogers et al., 1983). Human adapted influenza binds to Sia α 2,6Gal, avian influenza binds to Sia α 2,3Gal and swine influenza may bind to both receptors (Suzuki et al., 2000, Matrosovich et al., 1997). The specificity of HA reflects the distribution of the different sialic acid linkages in the different hosts and also contributes to the observed tissue tropism (Suzuki et al., 2000). In humans, swine and horse influenza cause respiratory tract infection while in birds the infection is enteric. Interestingly, human mucus is rich in Sia α 2,3Gal and avian mucus has Sia α 2,6Gal (Olofsson et al., 2005). However, in the human eye the sialic acid linkage of the ocular surface (Sia α 2,3Gal) and the mucus (Sia α 2,6Gal) is similar to the distribution in the intestine of birds. This may explain the reports of human eye infections caused by avian strains of influenza (Fouchier et al., 2004). In conclusion, the distribution of Sia α 2,3Gal and Sia α 2,6Gal on different host cell surfaces and the mucus layer contributes to the observed host and tissue tropism of avian and human influenza. The effect that amino acid substitutions in the receptor binding site has on receptor specificity has been extensively studied and is review elsewhere (Skehel and Wiley, 2000).

The fine receptor specificity of human and avian influenza has been analyzed *in vitro*. In a comprehensive study, duck influenza preferentially bound to Sia α 2,3Gal β 1,3GlcNAc conjugates, while chicken and mammalian influenza bound with highest affinity to Sia α 2,3Gal β 1,4GlcNAc and Sia α 2,6Gal β 1,4GlcNAc core saccharide chains, respectively (Gambaryan et al., 2005). The technique that is used to measure the affinity and specificity of HA may influence the obtained results. Glycan array and ELISA assays have indicated that the optimal binding partner of highly pathogenic H5N1 hemagglutinin is Sia α 2,3Gal β 1,4(SO₃H-)GlcNAc β (Stevens et al., 2006)(Gambaryan et al., 2005). In a Biacore assay performed with the same recombinant H5 as the glycan array assay, there was no difference observed in affinity to Sia α 2,3Gal on different inner carbohydrate chains (Suenaga et al., 2012). Interestingly, a recent study that used totally different techniques to measure HA binding to the cell surface (optical tweezers, atomic force microscopy-based single-molecule force spectroscopy and molecular dynamics simulations) came to the conclusion that human influenza A X-31 bound equally well to cells expressing either terminal Sia α 2,3 alone or both Sia α 2,3 and Sia α 2,6 (Sieben et al., 2012). The affinity differences between different receptors may thus be lower than expected *in vivo* (Sieben et al., 2012).

6.6 High mutation rate of influenza

Small changes in the virus proteins, often affecting the glycosylation, “drift”, may allow the virus to escape from neutralizing antibodies and anti-viral drugs (Wolf et al., 2006). Strains with novel traits can easily be formed by genetic re-assortment of RNA segments in hosts co-infected with two different strains, referred to as genetic “shift” (Webster et al., 1992).

6.7 Anti-influenza treatments

6.7.1 Vaccines

A range of strategies to prevent or treat influenza has been attempted, with vaccination being the most successful so far. Due to the high mutation rate, “drift”, of human influenza the vaccine companies have to make new vaccine formulation each year to combat seasonal influenza (Kaiser, 2006). Efforts have been made to make broad-spectrum vaccines that would give long-lasting immunity (Kaiser, 2006, Gomez Lorenzo and Fenton, 2013). Other novel strategies involves DNA vaccination that is known to give a stronger cellular immune response and cell-based vaccine production systems that are cheaper and faster than egg production (Josefsberg and Buckland, 2012, Cox, 2012). The shortage of influenza antigen 2009 forced some vaccine companies to use higher doses of adjuvant that may have contributed to the rare unexpected side effect narcolepsy (Kothare and Wiznitzer, 2013). A major problem

with influenza vaccination is that elderly, one of the risk groups of influenza, have weaker immune responses to vaccinations (Weinberger and Grubeck-Loebenstein, 2012).

6.7.2 Antivirals

In the course of a pandemic, vaccine supplies may not be sufficient. In this situation there is a need for alternative anti-influenza treatments. Indeed, during the 2009 pandemic governments stock-piled the two most common anti-influenza drugs oseltamivir (Tamiflu; Roche/Genentech) and zanamivir (Relenza; GlaxoSmithKline) to a cost of US \$4 billion (Barik, 2012). A wide range of antiviral treatments of influenza has been attempted, targeting different steps of the infectious cycle, summarized in Figure 5. Current drugs include the M2 channel inhibitors (adamantanes) and neuraminidase inhibitors (Barik, 2012, Lagoja and De Clercq, 2008). Neuraminidase inhibitors are examples of drugs invented through rational drug design, and which resulted in a small molecule drug binding with high affinity to neuraminidase, preventing the hydrolysis of sialic acid receptors. Resistance to NA inhibitors have appeared (Moscona, 2005). A recent extensive Cochrane review of neuraminidase inhibitors reported a modest 21h reduction of symptoms and no reduction of hospitalizations (Jefferson et al., 2012). Several novel NA inhibitors are now in clinical trials (Barik, 2012). Other novel drugs in clinical trials include an RNA polymerase inhibitor (purine mimic), and hemagglutinin (inhibitory peptides) and neuraminidase (destroys host cell receptors) inhibitors (Barik, 2012, Lagoja and De Clercq, 2008). As noted above, the pathogenicity of influenza is often the result of the immune response. Anti-inflammatory treatments have therefore been attempted with a number of different drugs (Barik, 2012).

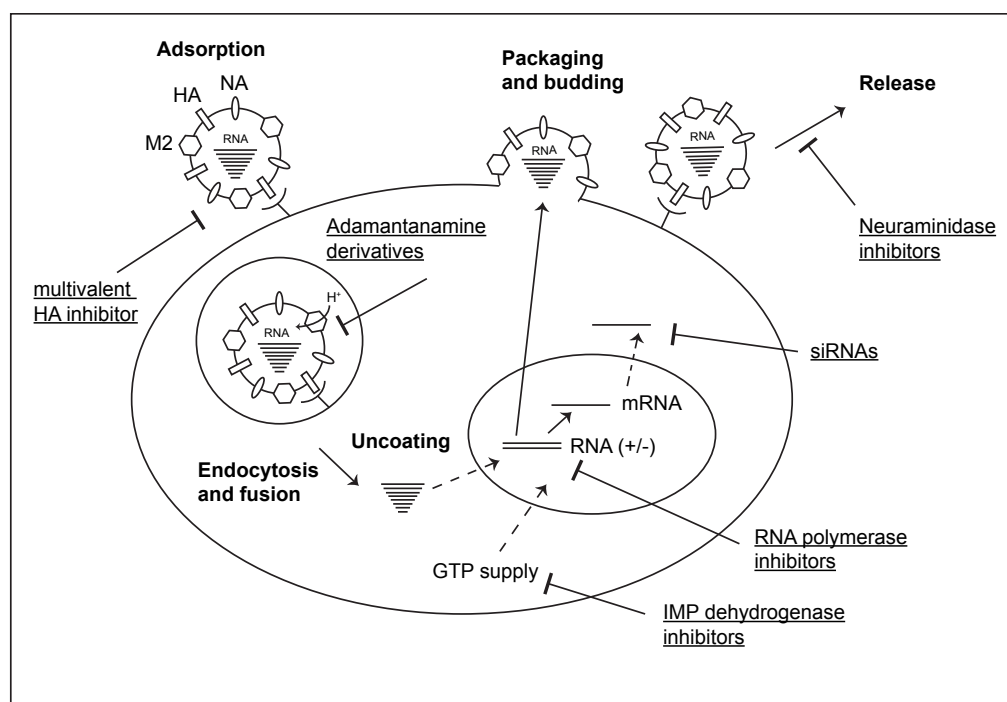


Figure 5 Illustration of influenza virus infectious cycle and the blocking of the different steps with anti-viral agents. Adsorption of virus to the cell surface is mediated by binding of multiple HA molecules to Sia α 2,3/6Gal- on the cells surface. An inhibitor of influenza attachment should be multivalent in order to be effective. In paper IV the binding of a novel multivalent avian HA inhibitor was investigated. The inhibitor is based on PSGL-1/mIgG_{2b} that present terminal Sia α 2,3Gal in multiple copies. After attachment, receptor mediated endocytosis leads to viral internalization. The low pH of the endosome induces a HA conformational change that fuses viral and endosome membranes. M2 allows proton transfer to the interior of the virus particle and release of RNA to the cytoplasm. Adamantan derivatives block this step (M2 inhibitors). RNA replication and transcription in the nucleus may be blocked by inosine 5'-monophosphate (IMP)- and RNA polymerase inhibitors. Viral mRNA may be targeted by small interfering RNAs (siRNAs). After packaging and budding at the cytoplasmic membrane the release may be blocked with neuraminidase inhibitors. Currently, only neuraminidase inhibitors and adamantane derivatives are approved for human therapy. Adapted from (De Clercq, 2006, Palese, 2004).

6.7.3 Novel multivalent carbohydrate inhibitors

It has been suggested that the influenza virus particle bind with high avidity to the cell surface due to the combined strength of many individual HA bonds to sialic acid receptors. A potential inhibitor must therefore be multivalent in order to be biologically relevant. This has also been shown in several studies (Bovin et al., 2004, Matrosovich and Klenk, 2003, Totani et al., 2003, Ogata et al., 2009). In paper IV we investigate the binding strength of a potential inhibitor of avian influenza H5N1 hemagglutinin in a Biacore assay.

7 Shiga toxin producing bacteria

7.1 Introduction

Shiga toxins are a family of toxins produced by *Shigella dysenteriae* (Shiga toxin) and some strains of *Escherichia coli* (Shiga-like toxins; Shiga toxin 1 and 2). Shiga toxins adhere to the cell surface of host cells and after cellular uptake inhibit protein synthesis by targeting the ribosomes leading to cell death. A common complication of Shiga toxin-producing *E. coli* (STEC) infections is hemolytic uremic syndrome (HUS). Limited treatment options are available for disease caused by STEC including the HUS. In paper III we analyze the binding between Shiga-like toxins and a recombinant mucin-like protein that may serve as a potential novel inhibitor of Shiga-like toxins. In this section, a brief background of Shiga toxins will be given although the detailed intracellular toxic mechanism of action and its use as a tool to study intracellular trafficking is beyond the scope of this thesis and can be found elsewhere ((Bergan et al., 2012) and references within).

7.2 Historical background

For historical reasons the members of the Shiga toxin family have been given several names that are sometimes used interchangeably. The Shiga toxin name is derived from the bacterium *Shigella dysenteriae*. The toxin was first described 1903 (Conradi, 1903, Neisser and Shiga, 1903) and was then termed “Neurotoxin” due to its ability to induce paralysis and death when injected into rabbits. Later it was discovered that some *E. coli* strains produce similar toxins termed “Verotoxins” and “Shiga-like toxins”. The toxin produced in *S. dysenteriae* is today referred to as Shiga toxin and the two *E. coli*-derived toxins are termed Shiga toxin 1 (Stx1) and Shiga toxin 2 (Stx2) or Shiga-like toxin 1 and 2 (SLT1/SLT2) (Bergan et al., 2012). Several *E. coli* strains produce variants of the Stx1 and Stx2 (Bergan et al., 2012).

7.3 Structure of Shiga toxins

The Shiga toxins belong to the bacterial AB₅ toxin family. The AB₅ toxin family includes additional members such as cholera toxin, heat-labile enterotoxins I and II, *Campylobacter jejuni* enterotoxin and pertussis toxin (Merritt and Hol, 1995). The toxins bind to different glycolipid receptors at the cell surface with the B pentamer while the A subunit has an enzymatic activity that confer their different toxic effector functions. Shiga toxin (Stx) and Shiga toxin 1 (Stx1) are almost identical while Shiga toxin 2 (Stx2) share less sequence homology (Bergan et al., 2012).

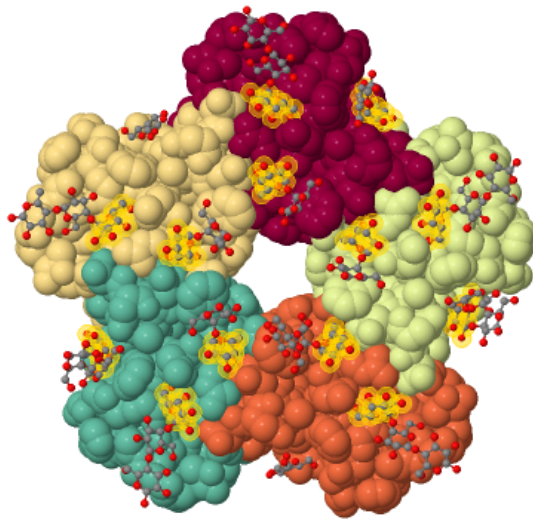


Figure 6 Space-fill 3D model of Stx1 B pentamer complexed with the Gb3 analogue 8-(methoxycarbonyl)-octyl trisaccharide (Pk-MCO), where the Pk terminus is the same as the carbohydrate portion of the glycolipid Gb3Cer. Each B subunit binds to three trisaccharides, fifteen in total. The terminal Gal α 1,4 moiety is marked in yellow. Source: Protein Data Bank (<http://www.rcsb.org/pdb/home/home.do>, last accessed April 24 2013), accession number 1BOS (Ling et al., 1998).

7.4 Binding

It is believed that the native receptor of Shiga toxins is the glycosphingolipid Gb3Cer (globotriaosylceramide). Each B subunit has three binding sites, thus theoretically resulting in a total of fifteen binding sites per pentamer. The carbohydrate moiety of Gb3Cer is Gal α 1,4Gal β 1,4Glc (also known as the P^k antigen) to which the B pentamer binds specifically. Removal of the Gb3Cer receptor protects mice challenged with Shiga toxin and cells that lack Gb3Cer are sensitized by incorporation of Gb3Cer in the cell membrane (Okuda et al., 2006, Waddell et al., 1990). Gb3Cer is expressed in humans on the surface of red blood cells, in the urinary tract, kidney epithelium and endothelium, but is also observed in a subset of cells in the intestine, platelets, and in a subset of B lymphocytes (Devenica et al., 2011, Engedal et al., 2011). In addition, Shiga toxins may bind to Gb3Cer on a subset of cells in the peripheral and central nervous system (Ren et al., 1999, Obata et al., 2008). It is not clear why Shiga toxin specifically targets the kidneys during HUS when the Gb3Cer receptor is found on a range of cells. Shiga toxins are not observed free in circulation, but appear to be bound to the surface of polymorphonuclear leukocytes (Brigotti et al., 2011). Therefore it has been proposed that Shiga toxins are delivered to the kidney by the leukocytes. The type

of presentation of Gb3Cer on the cell surface of kidney cells may also contribute to the binding specificity, see below. It is also possible that Shiga toxin can bind to other receptors on the cells in the kidney. It has been shown *in vitro* that Stx1 binds well to P1 (Gal α 1,4Gal β 1,4GlcNAc) ((Gallegos et al., 2012) and paper III) while Stx2 has been shown to bind well to NAc-Pk (Flagler et al., 2010). It is not known if these determinants are only carried by glycolipids (Yang et al., 1994, Gallegos et al., 2012). Both Stx1 and Stx2 appears to bind to Gb3Cer *in vivo* while *in vitro* binding of Stx2 to Gb3Cer or Pk (without ceramide) is weaker than that of Stx1 (Gallegos et al., 2012). It has been shown that Stx2 binding may depend on other parts of Gb3Cer than the carbohydrate moiety alone, such as other glycolipids, cholesterol and phosphatidylcholine (Gallegos et al., 2012). The difference in binding specificity may be important since Stx2 is more toxic *in vivo* and is more closely associated with development with HUS than Stx1. It has been noted that the clustering of Gb3Cer in lipid rafts is proportionally higher in kidney compared to other sites (Lingwood, 1994, Khan et al., 2009). One can thus speculate if the higher toxicity of the Stx2 toxin may be explained by the specific targeting of Stx2 to Gb3Cer in lipid rafts in the glomerular cells of the kidney. Once concentrated to the kidney, the lipid rafts may in addition contribute to a more efficient endocytosis of the toxin compared to no clustered endocytosis in other sites (Falguieres et al., 2001).

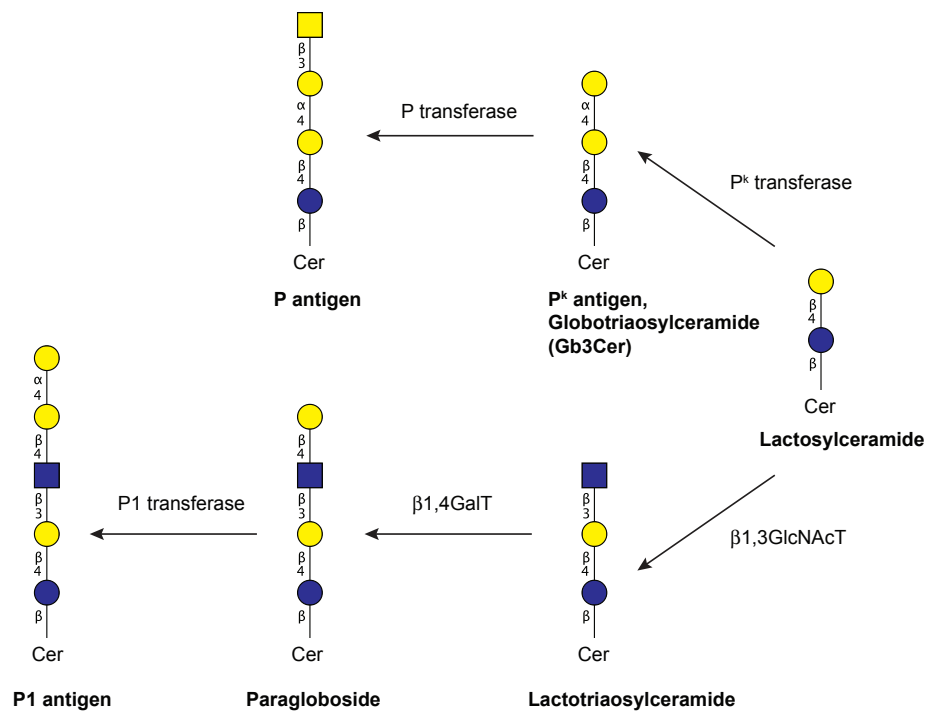


Figure 7 Synthesis of the human P blood group system. Gb3Cer (P^k antigen) is the native receptor of Shiga toxins. In paper III strong binding of Stx1 and Stx2 to PSGL-1/mIgG_{2b} carrying multiple copies of P1 determinant was shown in a Biacore assay.

7.5 Pathogenesis

The most common symptom of infections caused by STEC is hemorrhagic colitis. Infection occurs by ingestion of food or contact with infected humans or animals (Tarr et al., 2005). Risk groups include children and elderly that are more likely to develop complications, such as HUS. HUS is characterized by microvascular thrombosis with consequent thrombocytopenia, haemolytic anaemia and acute renal failure (Noris et al., 2012, Melton-Celsa et al., 2012). HUS is mostly associated with Shiga toxin-producing *E. coli* (STEC) that produces the Stx2 toxin. Occasionally outbreaks occur with STEC that cause considerably higher rates of HUS. This has been observed for O157:H7 and O104:H4 (Tarr et al., 2005, Frank et al., 2011), where the latter strain caused HUS in 22% of the patients (Frank et al., 2011). The mechanism of how STEC cause HUS is complex. In brief, during STEC infection, Shiga toxin triggers complement hyper activation, possibly via up regulation of P selectin on endothelial cells and direct involvement with complement regulatory molecules (Noris et al., 2012). Inflammation is induced and platelet activation leads to microvascular

thrombosis (Noris et al., 2012). Since STEC produce other virulence factors in addition to Shiga toxin, these may also contribute to disease.

7.5.1 Shiga toxin mechanism of action

The detailed description of the mechanism of action of Shiga toxins is beyond the scope of this thesis. Briefly, the A subunit inactivates the 60S ribosomal subunit which effectively inhibits protein synthesis in the target cell. This is achieved by the RNA *N*-glycosidase activity of the A subunit which removes an adenosine from the 3' region of the 28S rRNA. ((Mulvey et al., 2003) and references within).

7.6 Traditional treatment and novel inhibitors

Hospitalized STEC and HUS patients have traditionally been limited to supportive treatment, resorting to the notion that the best way to prevent HUS is to prevent STEC infection (Tarr et al., 2005). Recent studies have indicated that monoclonal anti-C5 antibodies that target the hyperactive complement system in HUS show promising results (Noris et al., 2012). Other means to treat STEC include different strategies to inhibit the Shiga toxin either by preventing attachment (receptor mimics (Kitov et al., 2008, Mulvey et al., 2003, Nishikawa et al., 2005), or monoclonal antibodies (Bitzan et al., 2009, Lopez et al., 2010)) or inhibiting the intracellular effector function of the toxin (inhibitory peptides and chloroquine (Nishikawa et al., 2006) (Dyve Lingelem et al., 2012)). The receptor mimics studies have indicated the need of a multivalent inhibitor in order to achieve inhibition *in vivo* since a clinical phase III trial with Synsorb-Pk showed no protection against HUS (Trachtman et al., 2003). No licensed drug against Shiga toxin is yet approved. In paper III we analyze the binding of Stx1 and Stx2 to a potential novel inhibitor that is based on a recombinant fusion protein that is presenting the P1 epitope in multiple copies.

8 Aims of the present study

The general aim of the thesis was to explore the *O*-glycosylation capacity of insect cells and to develop novel inhibitors of antibodies, bacterial toxins and viral receptors that bind carbohydrate determinants.

Specific aims:

- I. Determine the *O*-glycosylation capacity of *Spodoptera frugiperda* (Sf9) and *Trichoplusia ni* (High-five) cells by analyzing the *O*-glycosylation of the recombinant PSGL-1/mIgG_{2b} mucin-type fusion protein expressed in those cells.
- II. In glyco-engineered Chinese Hamster Ovary cells produce a novel multivalent immunoadsorber of chain type-specific anti-A and anti-B antibodies comprised of the PSGL-1/mIgG_{2b} mucin-type fusion protein with different blood group A and B determinants on different core saccharide chains.
- III. Produce a multivalent potential inhibitor of Shiga toxin by expressing the PSGL-1/mIgG_{2b} mucin-type fusion protein with blood group P1 determinants in glyco-engineered Chinese Hamster Ovary cells.
- IV. Produce a multivalent potential inhibitor of avian influenza hemagglutinin in glyco-engineered Chinese Hamster Ovary cells by expressing the PSGL-1/mIgG_{2b} mucin-type fusion protein substituted with terminal Sia α 2,3Gal.

9 Methodological considerations

9.1 Cell cultures

The insect cells Sf9 and Hi-5 were grown in serum-containing culture medium in T-flasks (small scale) and suspension cultures (large scale) in Erlenmeyer flasks. The cells were grown in a shaker-incubator at 27°C without CO₂ control. The excretion of PSGL-1/mIgG_{2b} to the culture medium was in the range of 1-2 mg/L. The insect cell culture conditions were not optimized and did not give yields that would be needed for large scale commercial production of recombinant proteins, such as with the baculovirus expression vector system (BEVS) (Drugmand et al., 2012). The mammalian cell cultures were grown under conditions that were monitored more closely. The blood group A producing cells (paper I) and 293-P, C-PSLex and C-P55 (paper IV) were grown in Wave bioreactors while blood group B producing cells (paper II) were grown in Erlenmeyer flasks. All mammalian cells were grown in serum-free culture medium. The Wave bioreactor is a single use bioreactor cell culture system that allows the control of temperature, CO₂, O₂ and the addition of culture medium. The glucose, glutamine and pH levels were monitored every day. The aeration and mixing of the cells is accomplished through a tilting table that creates waves inside the plastic culture bag. The production level of PSGL-1/mIgG_{2b} was more dependent on selecting high-producing clones rather than the culture technique (Wave vs. suspension cultures) and was between 5-40 mg/L for the different clones.

9.2 Constructs and transfections

In paper I the cDNA encoding the PSGL-1/mIgG_{2b} was cloned into the expression vector pIZ/V5-His (Invitrogen) as described previously (Gustafsson, 2005). The fusion protein expression is under the control of the opIE2 promoter, derived from the baculovirus *Orgyia pseudotsugata* multicapsid nuclear polyhedrosis virus (OpMNPV). Positive cells were batch-selected using zeocin selection agent. The vector has been demonstrated to allow the expression in a range of insect cells. However, we failed to establish a stable *Mamestra brassicae* cell line with this vector, even though a range of transfection techniques were tested (different liposome formulations, nucleoporation and magnetic nanoparticles). The vectors for the stable mammalian cell lines used in paper II are listed in the supplemental table S1 of the same paper. The backbone of the vectors was CDM8 with CMV or EF1 α promoters driving the expression of the desired gene (Liu et al., 2005). Since individual plasmids encoding PSGL-1/mIgG_{2b} and each of the glycosyltransferases were used, up to five selection drugs were used simultaneously (e.g. for selection and culture of the C-PB1 clone, paper II). The

transfections were carried out with Lipofectamine 2000 CP (Invitrogen) and single-cell clones were selected as described in the supplemental data of paper II.

9.3 Purification of PSGL-1/mIg_{G2b}

The purifications of the mucin-type fusion protein are described in detail in each paper. The relative ease of purification was dependent on the cellular origin and concentration of PSGL-1/mIgG_{2b}. The mucin-type fusion protein was purified with affinity- and gel filtration chromatography prior to dialysis against water and subsequent analysis. PSGL-1/mIgG_{2b} derived from insect cells was affinity purified on anti-IgG (whole molecule) agarose (Sigma-Aldrich) columns that capture the IgGFc-part of the fusion protein while PSGL-1/mIgG_{2b} derived from mammalian cell lines was purified on protein A columns (MabSelecSure, GE Healthcare). Protein A binds to the Fc-portion of antibodies. However, the Protein A columns proved to be less efficient for purification of the insect cell-derived fusion protein. It is possible that the glycosylation may affect the binding to the affinity columns. Other conceivable reasons for the difference in Protein A binding is the fact that cleared insect medium was loaded directly on the affinity columns and that the PSGL-1/mIgG_{2b} concentration in the medium was relatively low. The large-scale mammalian Wave cultures were grown in serum free culture medium. The cells were removed with microfiltration, concentrated (ultrafiltered) and buffer exchanged (diafiltered) to PBS before being loaded on the protein A column.

9.4 Specificity of antibodies

Monoclonal antibodies recognizing terminal carbohydrate determinants on the mucin-type fusion protein were used in paper I and II. In paper I, anti-Tn monoclonal antibodies used in Western blot indicated the presence of GalNAc α -Ser/Thr on PSGL-1/mIgG_{2b} produced in Hi-5 and CHO cells. In paper II, monoclonal antibodies were used to detect the blood group A determinants on the type 1, 2 and 3 saccharide chains and the blood group B determinant using Western blotting. The MS analysis provided the sequence of glycans carrying tentative blood group antigens, and the monoclonal anti-A antibodies could confirm the presence of the specific determinant (linkage information). Occasional discrepancies between Western blot and LC-MS are discussed in paper I and II.

9.5 Lectins

Lectins were used to detect specific terminal glycans on the mucin-type fusion protein. Lectin Western blot was used in paper I-II and IV, and in addition a lectin array chip was used in paper I. Lectins have preferred glycan binding partners but may also bind to secondary glycan targets. The risk of cross-reaction is increased if the conditions are

not optimal (some lectins requires metal ions such as Ca^{2+} , Zn^{2+} and Mn^{2+}) or if the ligands are supplied in very high concentration. We have noticed that the lectin binding may also depend on how the carbohydrate determinant is presented, that is, the inner carbohydrate chain and the protein that carries the carbohydrate (neoglycoconjugate vs. glycoprotein) may influence recognition. In the Western blot experiments positive and negative controls were therefore included. Since the lectins cannot give linkage information for individual glycans on the fusion protein, this strategy is more reliable if the glycosylation is less abundant.

9.6 Quantification of PSGL-1/mIgG_{2b}

The concentration of purified PSGL-1/mIgG_{2b} was determined with a sandwich enzyme-linked immuno sorbent assay (ELISA). ELISA plates were coated with a goat anti-mouse IgG₁ antibody upon which PSGL-1/mIgG_{2b} was added after blocking of non-specific binding sites. After washing, a goat anti-mouse horseradish peroxidase (HRP) conjugated antibody was used for detection. A dilution series of IgG_{2b} antibody with known concentration was used to determine the concentration of unknown PSGL-1/mIgG_{2b}. We have previously noticed that the assessed concentrations of PSGL-1/mIgG_{2b} and detections with Western blot have varied depending on which cell line PSGL-1/mIgG_{2b} was derived from. An example is the comparatively weak detection of PSGL-1/mIgG_{2b} with anti-PSGL-1 when produced in *Pichia pastoris* ((Gustafsson et al., 2011) and paper IV). The fusion protein produced in these cells is heavily glycosylated with mannose. We therefore suggest that the glycosylation of the fusion protein may partly mask the epitopes recognized by the antibodies. The concentration of PSGL-1/mIgG_{2b} determined with the ELISA method should therefore be interpreted with caution.

9.7 Surface Plasmon resonance

9.7.1 Biacore biosensor

Surface Plasmon Resonance (SPR) is a technique that measures the interaction of molecules in real-time in a label-free system. This technique was used to measure the interaction between the mucin-type fusion protein and recombinant avian influenza hemagglutinin (paper III) and Shiga like toxins (paper IV). In the Biacore instrument, one molecule is immobilized on the sensor chip surface (ligand) and the other molecule (analyte) is passed over the surface at different concentrations in a number of different injection cycles. Utilizing the SPR phenomenon, interaction of ligand and analyte is detected by the instrument via a change in the refractive index. The change in angle of reflected light is proportional to the mass of the analyte at the surface (Figure 8).

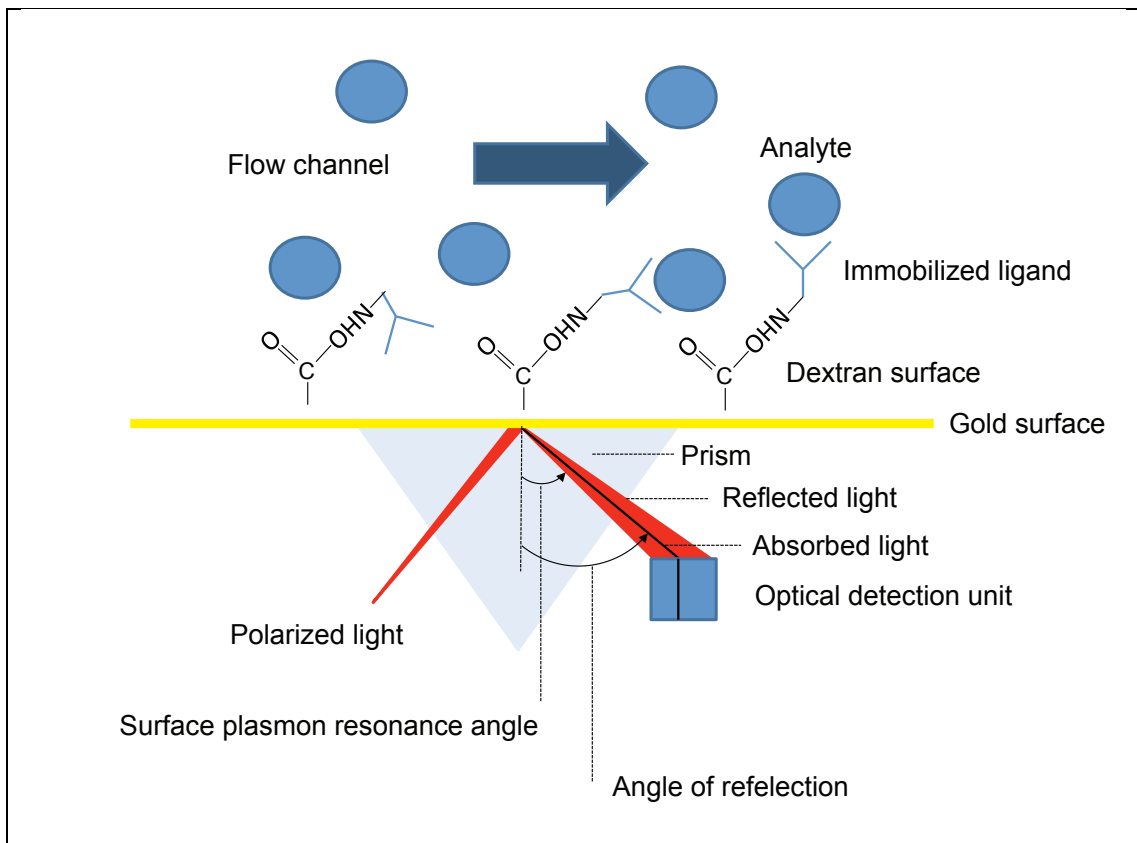


Figure 8 Illustration of how the SPR phenomenon is used in a SPR instrument (e.g. Biacore) in order to measure the interaction between an immobilized ligand and an analyte that is passed over the surface by a continuous flow of liquid. The interaction of the analyte in proximity of the surface causes a change in the refractive index. The resulting change in angle of reflected polarized light is detected and translated to a response signal that is plotted in a graph (sensorgram) on a computer screen in real-time.

The interaction of the ligand and injected analyte is plotted in a graph called a sensorgram with binding levels reported as response units (RU) (Figure 9).

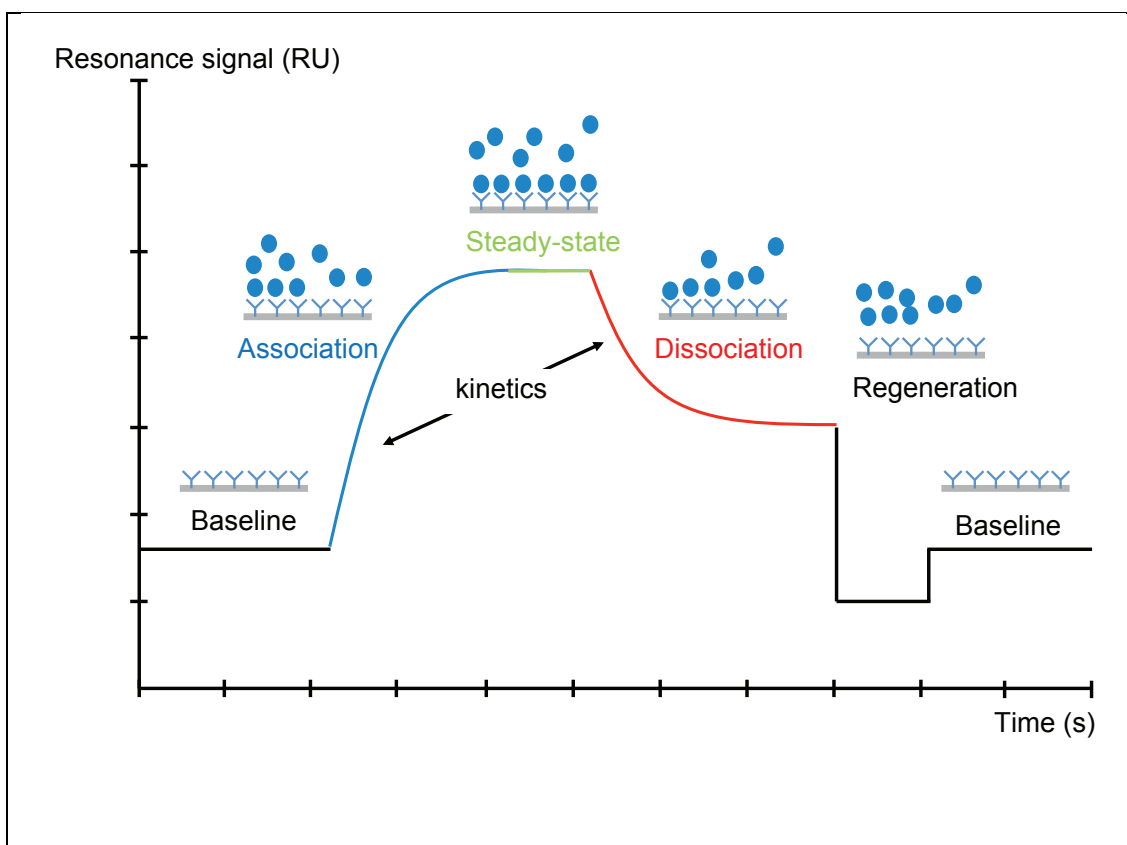


Figure 9 Schematic illustration of the different steps of an injection cycle in a sensorgram graph. Any change in molecule density at the chip surface is plotted as curve responses in the sensorgram. When buffer is flowing over the chip no response shift is recorded (flat line). This is referred to as the baseline. Injection of analyte results in build-up of analyte-ligand complexes that is plotted as a positive slope curve as more and more analyte becomes bound (association phase). As equilibrium is being reached the curve flats out and eventually reaches steady-state (flat curve). When analyte is no longer injected the analyte-ligand complexes starts to decay. The subsequent dissociation of analyte from the surface is recorded as a negative slope curve. In order to remove all bound analyte molecules, regeneration of the surface with regeneration buffer may be needed. When the baseline is restored to pre-injection levels a new injection cycle may be started.

Injection of analyte and subsequent running buffer, gives a binding curve with an association phase (analyte-ligand complex formation) and a dissociation phase (analyte-ligand complex dissolving) (Figure 9). In order to re-use the ligand surface for an additional injection cycle, all bound analyte must have been removed. If this is not achieved with buffer injection, the surface needs to be regenerated with more rigorous measures. The regeneration needs to be strong enough to remove all bound analyte but without removing or denaturing the immobilized ligand. From the binding curves, association- and dissociation kinetic constants, k_a and k_d , can be calculated by fitting the curves to theoretical binding models. The (affinity) equilibrium dissociation constant, K_D , can be derived from k_a and k_d or from curves that have reached equilibrium (steady-state). Affinity and kinetic constants in paper III and IV were determined with the

Scrubber 2 and BiaEvaluation softwares. The calculations are based on the equations listed below.

9.7.2 Determination of affinity and kinetic constants

Calculations of the affinity (how strong the complex is) and kinetics (how fast the interaction is) of the analyte-ligand interactions were calculated according to a 1:1 binding model in both paper III and IV. The mucins and the Shiga toxin / hemagglutinin have multiple binding sites, which are not accounted for in the simple 1:1 binding model. Even if a model adapted for multivalent interactions existed, it would not be feasible to use it because the exact valency of the mucins and the immobilized Shiga toxin and H5 is not known in this system. Calculations of K_D that are based on steady-state models should ideally only be performed when all curves have reached equilibrium. The concentration range of the injections should yield curves with curvature and reach saturation at high concentrations. If none of the curves are close to reaching saturation the calculated K_D value may be lower than the real K_D value. However, if the curves do not reach steady-state, the calculated K_D value will be higher than the real value.

Rate equations for 1:1 kinetics



$$\text{Association: } \frac{d[AB]}{dt} = k_a * [A] * [B] \quad (2)$$

$$\text{Dissociation: } \frac{-d[AB]}{dt} = k_d * [AB] \quad (3)$$

$$\text{Net rate equation: } \frac{d[AB]}{dt} = k_a * [A] * [B] - k_d * [AB] \quad (4)$$

$$\text{M/s} \quad \text{M}^{-1}\text{s}^{-1} \text{ M} \quad \text{M} \quad \text{s}^{-1} \quad \text{M}$$

Where

k_a = association rate constant [$\text{M}^{-1} \text{s}^{-1}$]

k_d = dissociation rate constant [s^{-1}]

Equilibrium constants

At equilibrium:

Association = dissociation

$$k_a * [A] * [B] = k_d * [AB] \quad (5)$$

$M^{-1}s^{-1} \quad M \quad M \quad s^{-1} \quad M$

The equilibrium constants:

$$K_A = \frac{k_a}{k_d} = \frac{[AB]}{[A] * [B]} \quad \text{the equilibrium association constant } [M^{-1}] \quad (6)$$

$$K_D = \frac{k_d}{k_a} = \frac{[A] * [B]}{[AB]} \quad \text{the equilibrium dissociation constant } [M] \quad (7)$$

9.7.3 Biacore nomenclature

A is the analyte in solution. The free concentration is constant by continuous flow

AB is the complex. Concentration of complex is measured as R in RU

B is the ligand on the surface. Total concentration can be expressed in RU as maximum binding capacity R_{max} . Free concentration is $R_{max} - R$



$$\frac{d[AB]}{dt} = k_a * [A] * [B] - k_d * [AB] \quad (8)$$

$$\frac{dR}{dt} = k_a * C * [R_{max} - R] - k_d * R \quad (9)$$

$RU/s \quad M^{-1}s^{-1} \quad M \quad RU \quad s^{-1} \quad RU$

A has one binding site and reacts with immobilized ligand.

B has n identical and independent binding sites.

9.7.4 Experimental design

Achieving high quality data from SPR experiments requires that many parameters have been considered. In addition to Biacore handbooks and courses, help can be found elsewhere. The www.sprpages.nl (last accessed March 24 2013) homepage written by Arnoud Marquart give ample information on how to avoid pitfalls and how to design experiments that yields reliable results. Rebecca L. Rich and David G. Myszka have

each year published a survey of the optical biosensor articles, showing both good and bad examples of experimental designs and results (Rich and Myszka, 2006). In paper III, the shortage of recombinant hemagglutinin (HA) in practice meant it had to be immobilized on the chip surface, rather than being flowed over the surface. The HA protein is meta-stable and the conformational change which is needed for membrane fusion during infection is triggered easily, especially by low pH (Skehel et al., 1982) (see separate section above about influenza hemagglutinin) therefore ionic strength regeneration was used. The instability of the chip surfaces precluded sequential runs of different analytes on the same surface while in paper III, the immobilized Stx1/2 was stable enough to allow direct comparison of successive analytes (PSGL-1/mIgG_{2b} and P^k-albumin).

9.8 Mass spectrometry

In paper I-IV we have used mass spectrometry to determine the composition, sequence and in some cases the linkage information of *O*-glycans released from PSGL-1/mIgG_{2b}. Successful sequencing of individual *O*-glycans in complex mixtures was demonstrated with both direct injection electrospray ionization mass spectrometry (ESI-MS) (paper I) and liquid chromatography mass spectrometry (LC-MS) (paper I-IV). In paper I ESI-MS was performed on a LCQ ion-trap instrument in positive mode on *O*-glycans released from PSGL-1/mIgG_{2b} that were derivatized by permethylation in order to increase ionization efficiency and facilitate the interpretation of the fragmentations (Kenny et al., 2013, North et al., 2009, Ashline et al., 2005). Since isomeric ions were not separated by a preceding column, the sequence of isomeric ions was deduced by successive MS-MS fragmentation that generated unambiguous fragments (paper I). Some disadvantages with the ESI-MSⁿ analysis included the relative insensitivity (0.5-1mg minimum glycoprotein starting material), the occasional problem of determining the sequence of isomeric glycans and the m/z $[M + 30]^+$ artifact that sometimes made the interpretation of the MS spectrum uncertain. LC-MS and LC-MS² analysis was used to characterize non-derivatized released *O*-glycans on a LTQ ion-trap mass spectrometer coupled to a graphitized column that often separated isomeric glycans (paper I-IV). The method proved to be robust, sensitive and versatile and gave as much structural information as ESI-MSⁿ and required much less starting material (as little as 10µg fusion protein). LC-MS allowed the detection of the substitutions sulfate and phosphocholine, which were not detected in the analysis of permethylated glycan derivatives (paper I). Carbohydrates carrying the charged substitutions were probably lost during the water: chloroform wash-up of the samples after the permethylation step. The charged residues may have migrated to the water phase, while the permethylated glycans stayed in the chloroform phase. By comparing the LC-MS² fragment spectra with known standards the linkage (paper I-IV), substitution- and monosaccharide identity of several *O*-glycans (paper I) were deduced.

Some minor artifacts were noticed in the LC-MS spectra that were likely introduced during the release and wash up of the samples. They included a contaminating hexose ladder, de-*N*-acetylation m/z $[M - 42]^+$, *O*-acetylation m/z $[M - 42]^+$, peeling (sugars without GalNAc α 1) and loss of water m/z $[M - 18]^+$.

Mass spectrometry is not a quantitative method since the ionization efficiency of different glycans is not completely uniform. Occasionally, LC-MS failed to detect glycan structures the presences of which were proposed by Western blotting with monoclonal antibodies, for example the Tn antigen (GalNAc α 1Ser/Thr, paper I) and anti-A type 2 (paper II).

9.8.1 Interpretation of mass spectrometry data

The free software Glycoworkbench (free downloadable java tool, www.glycoworkbench.com) computer program was used to draw glycan cartoons and generate fragmentation *in silico* (Ceroni et al., 2007, Ceroni et al., 2008, Damerell et al., 2012). The annotated MS² spectra from paper I-IV have been submitted to the LC-MS² database (www.unicarb-db.com) for public access. The database contains the negative mode LC-MS² spectra of non-derivatized glycans presented in graphical format and as peak lists; in principle showing the “fingerprint” of each glycan.

9.9 DMB RP-HPLC

In paper I reverse-phase high performance liquid chromatography (RP-HPLC) on released DMB-labeled sialic acid was performed by the Glycotechnology Core Resource at the University of California, San Diego. Low amounts of Neu5Ac and Neu5Gc were detected in both Sf9 and Hi-5. This ultrasensitive technique has proved to be the source of some controversy. Zeleny *et al.* have reported that previous claims of sialic acid in a variety of sources (plants) may be due to inadvertent contamination during the sample work-up (Zeleny et al., 2006). In their study, they could detect sialic acid even in the blanks. The author’s conclusion was that the level of sialic acid in plants was in the same range as the background level of the method. Blanks (water) were treated in parallel to the insect derived samples and did not contain any detectable levels of sialic acid. The possible source of the sialic acid is further discussed in paper I.

10 Results and discussion

10.1 PSGL-1/mIgG_{2b} with tailored glycosylation produced in glyco-engineered cell lines

In this thesis all four papers involve the use of stable cell lines that produce a mucin-type fusion protein composed of the extracellular part of P-selectin glycoprotein ligand-1 fused to the Fc part of mIgG_{2b} to form the PSGL-1/mIgG_{2b} protein. The mucin-type protein was originally constructed in order to produce a multivalent scaffold that would allow the adsorption of specific anti-carbohydrate antibodies, in this case anti-Gal α 1,3Gal antibodies. The PSGL-1/mIgG_{2b} dimer has 6 potential *N*- and 106 potential *O*-glycosylation sites thus constituting a scaffold for a multivalent display of carbohydrates. The host cell determines the specific glycosylation of the mucin-type glycoprotein. The intended use of the glycoprotein, that is the desired glycosylation, dictates the choice of expression system. In addition, glyco-engineering of cell lines (CHO cells in particular) has proven to be a powerful technique to produce specific carbohydrate determinants. This is achieved by transfecting the cells with plasmids encoding specific glycosyltransferases that enables the cells to produce complex carbohydrates beyond their natural capacity (Figure 10). In paper I, PSGL-1/mIgG_{2b} was expressed in Sf9 and Hi-5 insect cell lines and was used as a probe to analyze the *O*-glycosylation capacity of these cells. In paper II, glyco-engineered CHO cell lines were used to produce the fusion protein carrying blood group determinants on different outer core saccharide chains. Paper III and IV also relied on the production of glyco-engineered CHO cells in order to produce the fusion protein substituted with the P1 carbohydrate determinant or terminal Sia α 2,3Gal determinants, which are binding partners of Shiga toxin and the avian influenza H5 hemagglutinin, respectively.

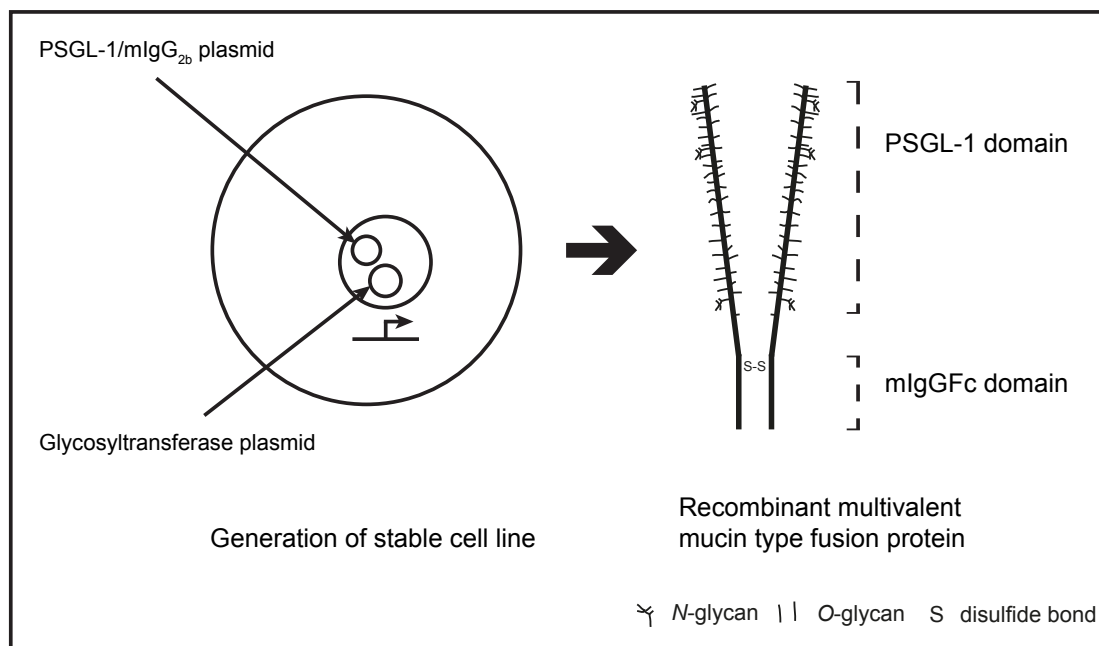


Figure 10 Overview of the establishment of cell lines expressing the mucin-type fusion protein PSGL-1/mIgG_{2b} with multiple copies of *O*-linked carbohydrates. The glycosylation of the mucin-type protein may be controlled either by producing the protein in cell lines with a desired glycosylation capacity or by modifying the glycosylation machinery of the cells by transfecting them with additional plasmids encoding specific glycosyltransferases.

10.2 The glycosylation in insect cells (paper I)

10.2.1 Aim and background

Sf9 (*Spodoptera frugiperda*) and Hi-5 (*Trichoplusia ni*) are two commercially important cell lines that are extensively used for the production of recombinant proteins for research use and increasingly as a source of human vaccine components (Roldao et al., 2011) (Cox, 2012). The aim of paper I was to investigate the *O*-glycosylation capacity of Sf9 and Hi-5 cells in order to explore their potential as expression systems of therapeutic proteins.

10.2.2 Production of PSGL-1/mIgG_{2b}

Hi-5 insect cells were engineered as described previously (Gustafsson, 2005), and Sf9 cells were generated in the same manner. The fusion protein was excreted to the cell medium and purified with affinity and gel filtration chromatography.

10.2.3 Structural characterization of carbohydrate epitopes on PSGL-1/mIgG_{2b}

O-glycans released from the heavily glycosylated PSGL-1/mIgG_{2b} protein produced in Sf9 and Hi-5 cells were characterized with ESI-MSⁿ and LC-MS². LC-MSⁿ analysis of

known standards together with monosaccharide analysis, lectin array chip and Western blot were used to give additional structural information.

10.2.4 Sf9 and Hi-5 O-glycosylation is complex and diverse

The structural analysis of *O*-glycans released from PSGL-1/mIgG_{2b} produced in Sf9 and Hi-5 cells revealed a large repertoire of diverse glycans, which is in contrast to previous findings of only smaller *O*-glycans in these cells such as the T- (Gal β 1,3GalNAc α Ser/Thr) and Tn antigen (GalNAc α Ser/Thr) (Lopez et al., 1999, Thomsen et al., 1990). We assigned 29 and 21 *O*-glycans in Hi-5 and Sf9, respectively. Some of the structures shared the sequence with previously reported *O*-glycans in *Drosophila melanogaster* embryos and salivary mucins from Wasps, but the majority had not been reported previously (Aoki et al., 2008, Aoki and Tiemeyer, 2010, Maes et al., 2005, Garenaux et al., 2011). Negative mode LC-MS of non-derivatized *O*-glycans proved to be a sensitive and specific method to assign the sequence of most glycans and for a few glycans also the linkage and identity of the terminal monosaccharides.

A Hi-5

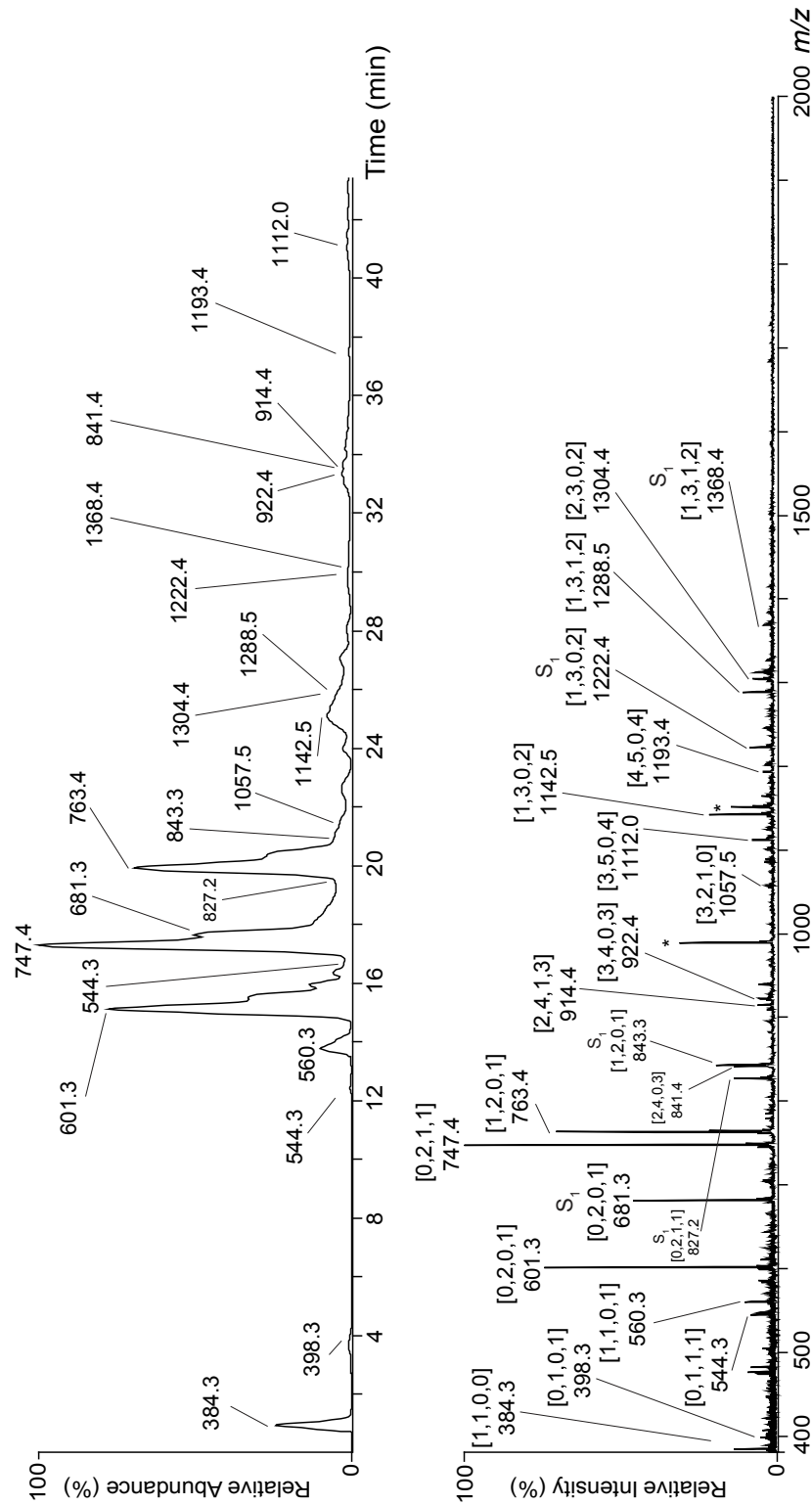
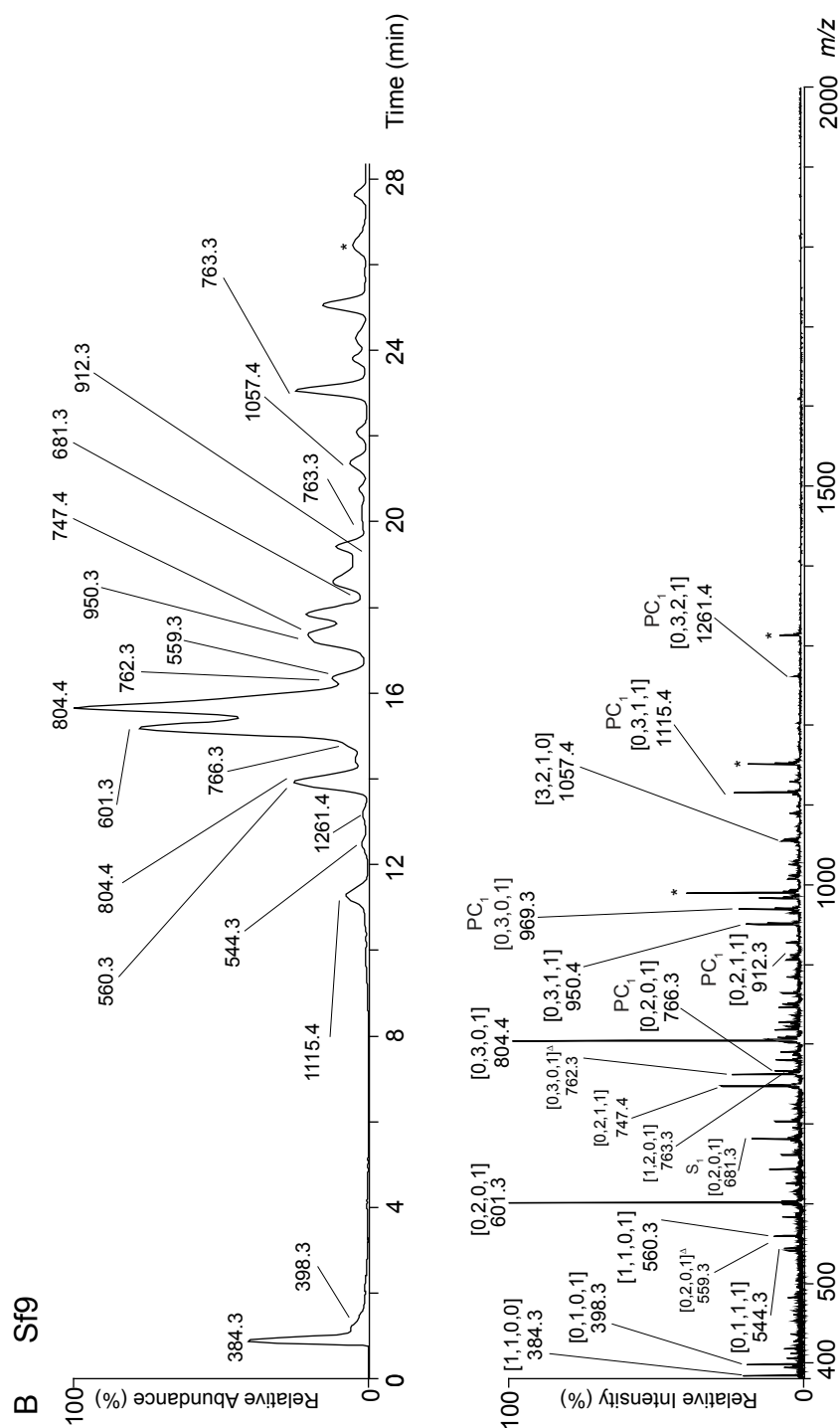


Figure 11A Negative mode LC-MS elution chromatograms and base-peak spectra of reduced non-derivatized O-glycans released from PSG-L-1/mIgG_{2b} produced in Hi-5 (A) and Sf9 (B). Carbohydrate compositions are assigned as [Hex, HexNAc, dHex, HexA] with reducing end GalNAc included as HexNAc. Substitutions are included with acronyms S (sulfate) and PC (phosphocholine). Symbol explanations: triangles, reducing end GalNAc; asterisks, contaminating hexose ladder. GalNAc may represent biological biosynthesis or be an artifact generated by the chemical work up of the sample prior to LC-MS.



10.2.5 A range of core structures

2 extended structures were detected (Figure 12). In comparison, the mammalian CHO cells produce *O*-glycans that are almost completely core 1 based, unless glyco-engineered (paper II-IV). The unusual addition of Fucose linked to the core GalNAc was noted and has previously been observed in Wasps and mutant nematodes (Garenaux et al., 2011, Palaima et al., 2010). The Tn antigen was detected in Hi-5 Western blot but was not observed in the mass spectrometric analysis of Sf9 and Hi-5 derived *O*-glycans.

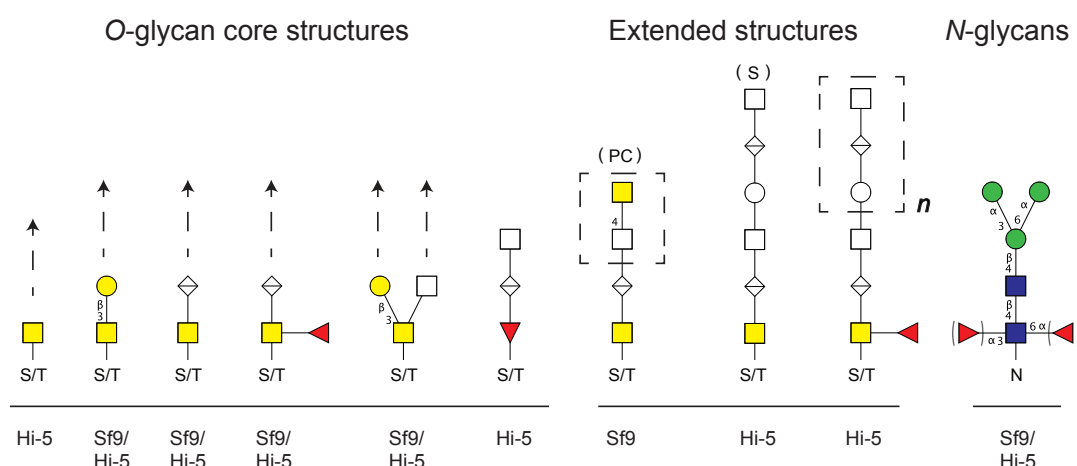


Figure 12 Examples of *O*-glycan cores and extended structures thereof derived from PSGL-1/mIgG_{2b} produced in Sf9 and Hi-5 cells. The occurrence of each structure in the cell lines is indicated. Dashed squares indicate the unusual GalNAc4HexNAc (Sf9) epitope and the HexNAc-HexA-Hex repeat (Hi-5).

10.2.6 Insect cell *O*-glycans are substituted with charged monosaccharides and functional groups

Glucuronic acid (GlcA) was prevalent (LC-MS and monosaccharide profile with HPAEC-PAD) in both Sf9 and Hi-5 derived *O*-glycans, while only trace amounts of sialic acid were detected (RP-HPLC of DMB derivatized sialic acid). Today there is a consensus that insect cells have very limited capacity to produce sialic acid unless genetically engineered (Voss et al., 1993, Marchal et al., 2001, Hillar and Jarvis, 2010). Previous studies of *O*-glycans in *Drosophila melanogaster* embryos and *Drosophila* Schneider-2 cells (S2) have also found glucuronic acid (Aoki et al., 2008, Breloy et al., 2008). One can speculate if these negatively charged hexuronic acid residues are a general theme of insects and that GlcA may have an analogous role to sialic acid in mammals. However, several observations indicate that this is an over simplification. GlcA was not detected in Wasp-derived *O*-glycans and GlcA does not seem to contribute to the negative surface charge of S2 cells (Breloy et al., 2008, Garenaux et al., 2011). Further, GlcA is detected in a range of positions in glycan chains, in contrast to the terminal position of sialic acid in mammals (paper I and (Aoki et al., 2008)). The capping of terminal residues with charged residues appears to be related to the specific

interaction with other molecules. The binding of a charged residue that fit into the hydrophobic pocket in the binding partner has been observed in several receptor-ligand pairs including carbohydrate-glycoprotein interactions (Yeh et al., 2001). The negatively charged sulfate substitution was detected on *O*-glycans derived from PSGL-1/mIgG_{2b} produced in both Sf9 and Hi-5 cells. Notably, the sulfate was only detected on terminal residues as indicated by LC-MS². The zwitterionic phosphocholine (PC) substitution was detected on *O*-glycans released from PSGL-1/mIgG_{2b} produced in Sf9 cells (LC-MS² in positive mode and anti-PC Western blot) but also in protein extracts of Sf9 cells that were not transfected with PSGL-1/mIgG_{2b} plasmids (anti-PC Western blot). To our knowledge, PC has not been reported previously on glycoproteins in insect cells. In Wasps a related phosphoethanolamine (2-aminoethyl phosphate group) substitution has been found on *O*-linked glycans (Maes et al., 2005). The role of PC in Sf9 cells is not known. However, PC is prevalent on nematode glycans and has been demonstrated to be a potent immune modulator (Lochnit et al., 2000, Harnett et al., 2008, Harnett and Harnett, 2008).

10.2.7 Insect glycosylation compared to other organisms

Paper I extends the list of known *O*-glycans in Sf9 and Hi-5 cells and insects in general. The majority of the detected structures will be recognized as foreign if introduced in humans. In particular, the PC modification found on some Sf9 derived *O*-glycans might cause adverse reactions. This emphasizes that the glycosylation of unmodified insect cells is unsuitable for the production of therapeutic glycoproteins. Human *O*-glycosylation is very complex, based on many core structures (table 1), and the cores are usually extended with additional determinants not detected in insect cells such as a range of blood group determinants, poly-*N*-acetylactosamine structures and terminal sialic acid. Actually, insect cells have more in common with nematodes and plants with regard to glycosylation. These organisms largely lack sialic acid synthesis and display mainly less complex *N*-glycosylation (truncated, paucimannose type) compared to humans, and terminally linked PC is a prevalent feature of nematodes (Harnett et al., 2010, Poltl et al., 2007). The *O*-glycans of nematodes also share some structural features with insects because several GlcA containing structures are the same or very similar to those detected in paper I.

While human *N*-glycosylation is often complex with extensive branching and capping with terminal *N*-acetylactosamine and sialic acid, insects mainly produce non-sialylated truncated *N*-glycans with fucose α 1,3 or α 1,6-linked to the chitobiose core. Fucose α 1,3-linked to core GlcNAc is non-human and potentially immunogenic (Altmann, 2007). However, detailed analysis on *Drosophila* embryos has revealed the presence of hybrid-, complex biantennary, and triantennary *N*-glycans (Aoki et al., 2007). Very low levels of terminal sialic acid suggest a limited and specialized expression pattern in *Drosophila* (Aoki et al., 2007). Studies on *S. frugiperda* cells

suggest the presence of hybrid *N*-glycans (Ogonah et al., 1996), while the analysis of a recombinant murine antibody in *T.ni* cells indicated the presence of complex type (terminal Gal and GlcNAc), hybrid, and paucimannosidic type *N*-glycans (Hsu et al., 1997). The wide range of *O*-glycan structures detected in both Sf9 and Hi-5 lead us to suggest that the efforts to “humanize” these cell lines with regard to their *N*-glycosylation (Harrison and Jarvis, 2006), (Palmberger et al., 2012, Aumiller et al., 2012, Aumiller et al., 2003, Jarvis et al., 1998) should also be expanded to include their *O*-glycosylation. This may involve both the addition of human glycosyltransferases but possibly, mainly the removal of unwanted glycosyltransferases. This type of work would benefit greatly by the availability of the genome sequences of these cells. The *Bombyx mori* genome and transcriptome is now available but corresponding efforts have not reached as far with the Sf9 and Hi-5 cells (Li et al., 2012, Xia et al., 2009, Xiang et al., 2010).

Since we use the recombinant PSGL-1/mIgG_{2b} protein as a probe to investigate the *O*-glycosylation capacity of Sf9 and Hi-5 cells, one may ask if the detected glycans are representative of the endogenous glycosylation. The answer depends on what kind of endogenous protein the comparison would be made with because the glycosylation of different proteins vary greatly. The cell lines are germ line derived and thus potentially less differentiated compared to cells derived from tissues. In the artificial cell culture system the glycosylation does not have biological constraints as cells would in living organisms. It is possible that the lack of inhibitory or stimulatory signals from the surrounding environment can lead to increased or decreased glycosylation.

In conclusion, Paper I has revealed that the *O*-glycosylation of Sf9 and Hi-5 insect cells is more complex than previously realized. This further underscores that unmodified Sf9 and Hi-5 cells may not be suitable for the production of therapeutic glycoproteins.

10.3 Mucin-type proteins as adsorbers of anti-A and anti-B antibodies (paper II)

10.3.1 Aim and background

The overall aim of paper II was to produce blood group A and B determinants on different outer core saccharide chains presented in multiple copies on the PSGL-1/mIgG_{2b} mucin-type protein. We suggest that the multivalent presentation of the carbohydrate epitopes increase their avidity to chain type-specific anti-A and anti-B antibodies compared to A and B determinants of low valency. The mucins may be used

as efficient adsorbers of chain-specific anti-A and anti-B antibodies and as substrates for the quantification and specificity-determination of ABO antibodies.

10.3.2 Establishment of glycan-tailored CHO cells expressing the A or B blood group determinants on different outer core saccharide chains

The cells were transfected with plasmids encoding PSGL-1/mIgG_{2b}, core 2 (β 1,6GlcNAc-T1) or core 3 (β 1,3GlcNAc-T6 and β 1,3Gal-T5) enzymes together with α 1,2Fuc-T1 or α 1,2Fuc-T2 and the A or B gene-encoded α 1,3GalNAcT or α 1,3Gal-T, respectively. The complete transfection schedule is shown in Figure 13. The fusion proteins were excreted to the cell medium and purified with affinity and gel filtration chromatography.

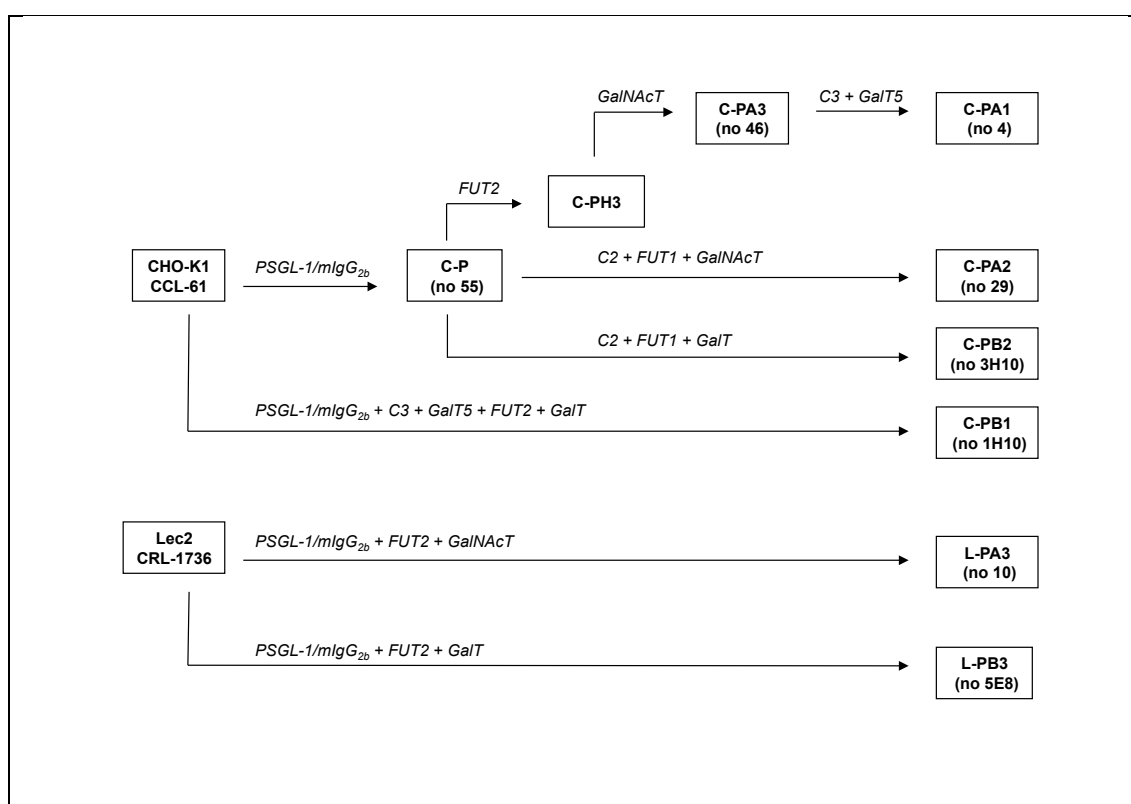


Figure 13 Transfection table to generate stable transfectants of CHO-K1 and Lec2 cells. Transfected cDNAs are indicated above the arrows, and cell lines selected for large-scale cultivation and further analysis are given in squares (clone name in parenthesis). Abbreviations as follows: FUT1, α 1,2-fucosyltransferase-1; FUT2, α 1,2-fucosyltransferase-2; C2, β 1,6-*N*-acetylglucosaminyltransferase-1; C3, β 1,3-*N*-acetylglucosaminyltransferase-6; GalT5, β 1,3-galactosyltransferase-5; GalNAcT, α 1,3-*N*-acetylgalactosyltransferase; GalT, α 1,3-galactosyltransferase.

10.3.3 Structural characterization of carbohydrate epitopes on PSGL-1/mIgG_{2b}

The glycosylation of purified mucin-type proteins was characterized with anti-A/B and lectin Western blot and LC-MS² on released *O*-glycans. The annotation of the blood group A determinants was largely facilitated by the availability of chain-specific anti-A

antibodies (anti-A type 1, 2 and 3, respectively) and knowledge of the available glycosyltransferases in the well-studied CHO cells. The blood group B determinants were determined with anti-B Western blot (unspecified chain) and LC-MS². The combined results indicated that A and B type determinants were produced on type 1 (Gal β 1,3GlcNAc-), type 2 (Gal β 1,4GlcNAc-) and type 3 (Gal β 1,3GalNAc α) chains. The obtained tentative blood group determinants detected in each clone are shown in Figure 14. The production of blood group determinants based on core 3 (conversion from core 1 to core 3 by transfection with β 1,3GlcNAc-T6) was efficient while blood group determinants based on core 2 (conversion from core 1 to core 2 by transfection with β 1,6GlcNAc-T1) was less prevalent. Fucosylation of the core 1 chain was also inefficient. We suggest that competition with endogenous sialyltransferases is accountable for the decreased conversion rates.

10.3.4 Glyco-engineering of cell lines

The use of glyco-engineered cell lines for the production of PSGL-1/mIgG_{2b} with tailored glycosylation has been successful in our laboratory (Liu et al., 1997), (Liu et al., 2005), (Lofling et al., 2002, Lofling et al., 2008, Gustafsson et al., 2011, Holgersson et al., 2005, Holgersson and Lofling, 2006, Lofling and Holgersson, 2009), and paper II-IV in this thesis). Paper II represents our most ambitious effort so far and demonstrates that “rational design” of blood group determinants on different outer core chains by transfection of cell lines with plasmids encoding specific glycosyltransferases is feasible. Since glycans are secondary gene products, the observed *O*-glycans on the PSGL-1/mIgG_{2b} are the result of competitive actions of both recombinant- and endogenous glycosyltransferases. Competition between different glycosyltransferases may be overcome by knocking-out the corresponding genes or by using mutant cell lines (the Lec2 cell line used in paper II has impaired sialic acid glycosylation). Heterogeneity of glycosylation of glycoproteins is commonly observed in recombinant therapeutic glycoproteins and on human endogenous glycoproteins (Liu et al., 2008, Hossler et al., 2009). This was also seen in paper II where each cell line produced a range of *O*-glycans, of which a major or minor subset carried blood group determinants.

Cell clone	Tentative structures carrying blood group determinants			
C-PA1-4				
C-PA2-29				
C-PA3-46				
C-PB1-1H10				
C-PB2-3H10				
L-PB3-5EH				

Figure 14 Tentative *O*-linked blood group determinants released from PSGL-1/mIgG_{2b} produced in glyco-engineered CHO-K1 and Lec2 cells. Cartoons are drawn according to nomenclature suggested by Varki et al. (Varki et al., 2009a)

10.3.5 Mucins carrying blood group determinants on different outer core saccharide chains can adsorb chain type-specific anti-A and -B antibodies

In paper II, we show that a Sepharose column with the fusion protein carrying A type 1 determinants adsorb anti-A type 1 and 2 chain-specific antibodies from pooled blood group O serum while the absorption of anti-A type 3 and 4 antibodies was less efficient. Sepharose columns with A type 1 or type 2 synthetic tetrasaccharides showed similar amounts of adsorption. The most efficient adsorption of chain type-specific antibodies was seen with a Sepharose column with conjugates of A type 1, 2, 3 and 4 tetrasaccharides. These results are in agreement with previous studies with tri- and tetrasaccharide based Sepharose columns and support the notion that chain-specific anti-A or -B antibodies exist in the blood of humans and that their removal from the blood is improved by the use of the A and B tetrasaccharide based on type 1-4 (Lindberg et al., 2012).

10.3.6 Future applications

The glycan-tailored mucins have already been used as substrates in ELISA assays to detect chain type-specific anti-A or -B antibodies (Lindberg et al., 2012). This work is ongoing together with optimization work on the immunoadsorption columns and other future applications. The successful transfer of these applications to a clinical setting relies on several factors including the feasible production of large quantities of correctly glycosylated mucins and the optimization of the suggested protocols.

10.4 Shiga-like toxin binds with high avidity to multivalent O-linked blood group P1 determinants on mucin type fusion proteins (paper III)

10.4.1 Aim and background

Shiga-like toxin (Stx1/2) producing *Escherichia coli* (STEC) usually cause diarrhea and hemorrhagic colitis but are also associated with the hemolytic uremic syndrome (HUS). Cellular attachment and uptake is mediated with the B pentamer of the toxin that binds with multiple binding sites to the glycolipid Gb3Cer (blood group P^k antigen, Gal α 1,4Gal β 1,4Glc β -Cer) on the cell surface (Ling et al., 1998, Mulvey et al., 2003). Our hypothesis was that Shiga toxins might bind to the P1 determinant (Gal α 1,4Gal β 1,4GlcNAc β), in addition to the P^k determinant since the structures are similar. The aim of paper III was to produce a high affinity inhibitor of Shiga toxins carrying multiple copies of P1 determinants on the PSGL-1/mIgG_{2b} mucin-type protein.

10.4.2 Production of PSGL-1/mIgG_{2b} expressing Shiga toxin receptor mimics

The synthesis pathway of the P1 determinant in glyco-engineered CHO cells (C-PP1) is outlined in Figure 15. The α 4GalT glycosyltransferase cDNA was cloned from pigeon while the other plasmids were available in the laboratory. The fusion protein was excreted to the cell medium and purified with affinity and gel filtration chromatography.

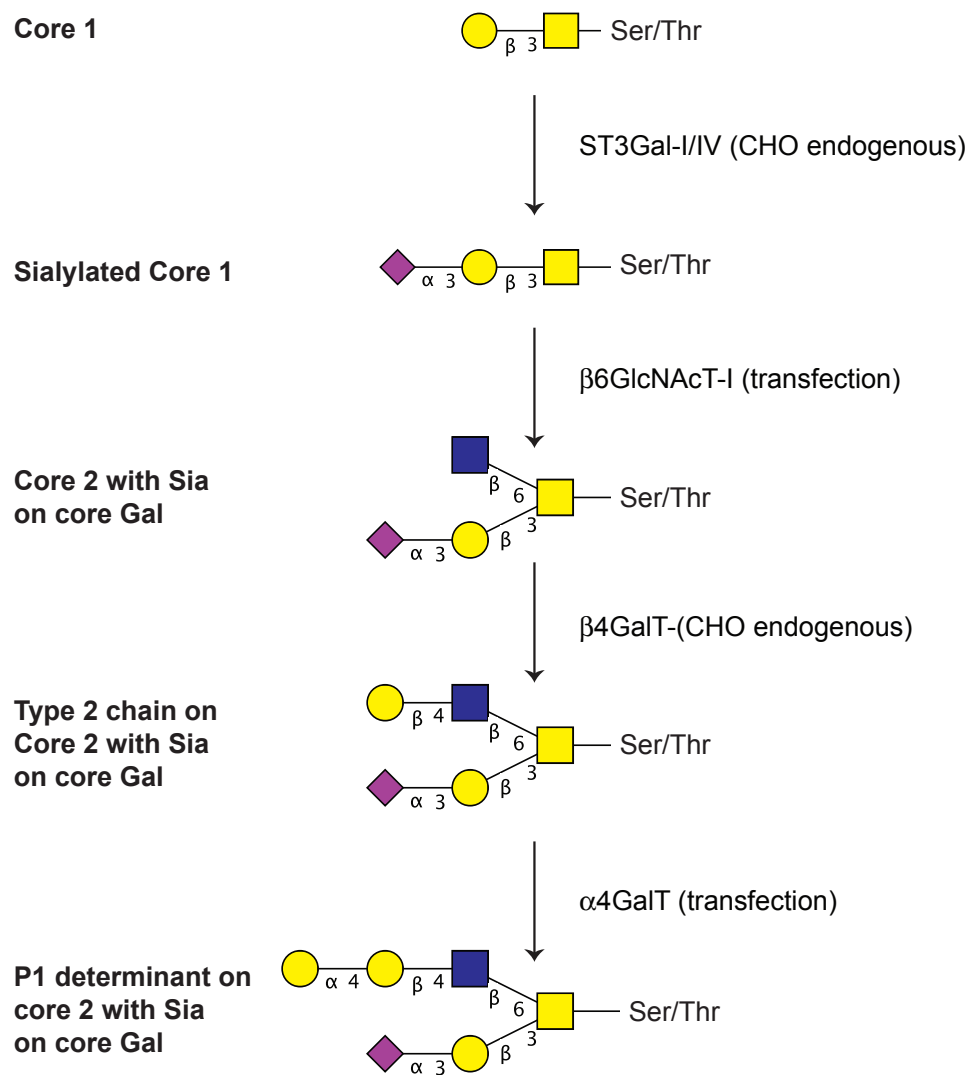


Figure 15 Overview of the synthesis of the terminal P1 blood group determinant on O-glycans carried by PSGL-1/mIgG_{2b} produced in glyco-engineered CHO cells (C-PP1) (paper III).

10.4.3 PSGL-1/mIgG_{2b} carry the P1 determinant on a core 2 saccharide chain

PSGL-1/mIgG_{2b} produced in C-PP1 was shown to carry the P1 blood group determinant with anti-P1 Western blot and LC-MS². A significant ion in the LC-MS spectrum of *O*-glycans released from PSGL-1/mIgG_{2b} had a composition corresponding to a tentative core 2 structure elongated with two additional hexoses and one sialic acid residue. Based on the MS² fragments and knowledge of the available glycosyltransferases in the C-PP1 cell line we suggest that this ion represents the Gal α 1,4Gal β 1,4GlcNAc β 1,6(Sia α 2,3Gal)GalNAc₆ol.

10.4.4 Stx1 and Stx2 bind with high affinity to PSGL-1/mIgG_{2b} carrying P1 in multiple copies

The binding of Shiga-like toxins (Stx1/2) to P1 PSGL-1/mIgG_{2b} (C-PP1) in Western blotting was compared to the binding to P^k- and P1 neoglycoconjugates. Stx1 bound strongly to P^k-albumin and C-PP1, while only weak binding was observed to P1-albumin. Stx2 did not bind any of the glycoconjugates in Western blotting. The stronger binding of C-PP1 than P1-albumin may be due to its higher ligand epitope density. To further investigate the binding of the Shiga-like toxins a Biacore assay was developed where Stx1 or Stx2 was immobilized on a CM5 dextran chip and P^k-albumin or C-PP1 were flowed over the surface and binding was measured in real time on a Biacore 2000 instrument and plotted in a graph (Figure 16). The SPR technique is explained in the *Methodological considerations* section. The K_D value (equilibrium dissociation constant) was determined with steady-state calculations on the curves with a 1:1 binding model (550-600 s). P^k-albumin and C-PP1 K_D values to Stx1 were in the low nanomolar range (39 nM and 16 nM, respectively). The shape of the binding- and dissociation curves and the low K_D values lead us to suggest that the binding of P^k-albumin and C-PP1 to Stx1 is multivalent. The binding to Stx2 was markedly reduced in comparison. The binding curves had another shape and did not reach equilibrium. The K_D values of the Stx2 binding were determined from simulated association and dissociation curves with a 1:1 binding model (plotted in orange color, C-D). The K_D of C-PP1 binding to Stx2 was 25 times weaker (379 nM) compared to Stx1 while the K_D value of P^k-albumin binding to Stx2 was 3 times weaker (101 nM) than for Stx1. Previous studies on monovalent Gb3Cer have reported K_D levels in the millimolar range while the most efficient polyvalent (polymers) inhibitors *in vivo* have shown K_D levels *in vitro* to Stx1 and Stx2 in the low nanomolar range (Gallegos et al., 2012, Watanabe et al., 2004). It should be noted that different K_D values have been reported of Stx1 and Stx2 depending on the analysis method used (Mulvey et al., 2003), (Gallegos et al., 2012), (Kitova et al., 2007, Flagler et al., 2010).

To summarize, C-PP1 is a good binding partner of Stx1 but bind with lower affinity to Stx2. Our hypothesis that Shiga toxins might bind to P1, in addition to the P^k

determinant was thus confirmed. The potential use of C-PP1 as an inhibitor of Shiga toxins is discussed in the separate section, *Multivalent PSGL-1/mIgG_{2b} as an inhibitor of Shiga toxins and influenza hemagglutinin*.

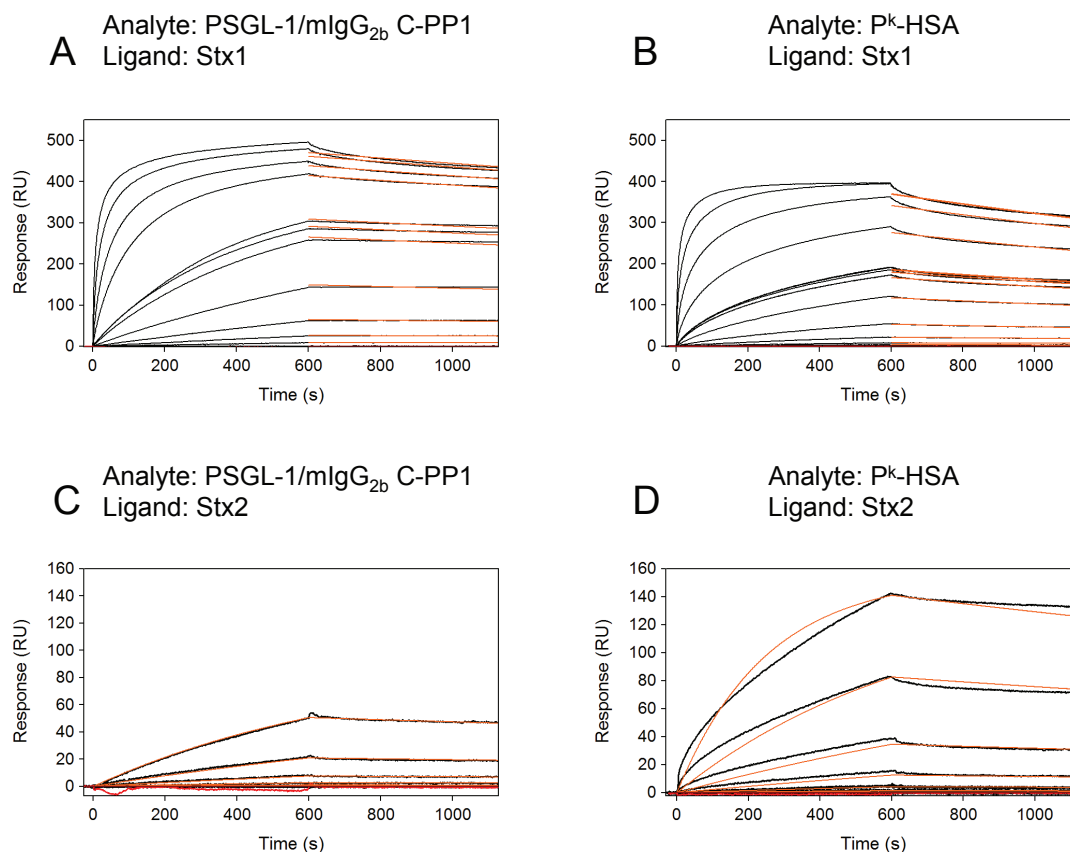


Figure 16 Biacore sensorgrams demonstrating C-PP1 PSGL-1/mIgG_{2b} and Pk-albumin binding to immobilized Stx1 and Stx2 on a CM5 chip. Simulated binding plots in orange color based on a 1:1 binding model are overlaid the binding curves in A-B (dissociation curves) and C-D (association – and dissociation curves). Negative controls with irrelevant glycosylation are shown in red (2700 nM C-P55 in A and C, 2700 nM Sia-LacNAc in B and D). Concentrations of injected analytes: 0.14, 0.4, 1.2, 3.7, 11, 33, 100, 300, 900 and 2700 nM. Stx1 and Stx2 immobilization levels were approximately 2800 and 2000 RU, respectively. Reference channel- (activated/deactivated) and blank (buffer) subtraction was used to reduce buffer bulk effects.

10.5 Avian influenza H5 hemagglutinin binds with high avidity to sialic acid on different O-linked core structures on mucin-type fusion proteins (paper IV)

10.5.1 Aim and background

In paper IV we wanted to investigate if PSGL-1/mIgG_{2b} presenting terminal Sia α 2,3Gal in multiple copies on different saccharide cores may be a good binding

partner for avian hemagglutinin with the ultimate goal of producing a novel inhibitor of highly pathogenic avian influenza such as H5N1.

10.5.2 Production of PSGL-1/mIgG_{2b} in different cell lines expressing avian influenza receptor mimics

In paper IV stable cell lines previously engineered were used. 293-P and C-P55 cell lines were established by transfection with a plasmid encoding PSGL-1/mIgG_{2b} ((Liu et al., 2005) and paper II). C-PSLex was established by transfecting CHO-K1 cells with plasmids encoding PSGL-1/mIgG_{2b}, β 1,6GlcNAc-T1 and FUT-VII transferases as described previously (Gustafsson and Holgersson, 2006). The fusion protein was excreted to the cell medium and purified with affinity and gel filtration chromatography.

10.5.3 PSGL-1/mIgG_{2b} carry avian influenza receptor mimics on different carbohydrate chains

LC-MS and LC-MS² analysis on released *O*-glycans indicated that C-P55 and 293-P produce the fusion protein with mostly sialylated core 1 structures (Sia α 2,3Gal β 1,3GalNAcol and minor amounts of Gal β 1,3(Sia α 2,6)GalNAcol). In addition 293-P cells produced a significant ion representing tentative lactosamine on core 2 with one or both chains terminated with sialic acid (Sia α 2,3Gal β 1,4GlcNAc β 1,6(Gal β 1,3)GalNAcol or Gal β 1,4GlcNAc β 1,6(Sia α 2,3Gal β 1,3)GalNAcol). The latter ion is dominant in the LC-MS spectrum of *O*-glycans released from PSGL-1/mIgG_{2b} produced in in C-PSLex. Western blot with *Maackia amurensis* lectins (MAL-I and MAL-II) and *Sambucus nigra* agglutinin (SNA) indicated presence of Sia α 2,3Gal (MAL-I/II) on the mucins derived from all cell lines while no Sia α 2,6Gal (SNA) was indicated.

10.5.4 Avian influenza H5 hemagglutinin binds with high avidity to PSGL-1/mIgG_{2b} carrying terminal Sia α 2,3 on different *O*-linked core structures

The binding of PSGL-1/mIgG_{2b} to recombinant H5 (A/H5N1/Vietnam/1203/2004) was measured in a Biacore biosensor assay in a similar manner as in paper III. H5 was immobilized to the dextran surface on a CM5 chip and the interaction to PSGL-1/mIgG_{2b} that was flowed over the surface was recorded and plotted in real-time in a sensorgram graph with a Biacore 2000 instrument. The binding curves were surprisingly similar between the mucins produced in the different cell lines (Figure 17, A-C). Binding of positive control Sia α 2,3LAcNAc-albumin was also observed (Figure

17, D), while PSGL-1/mIgG_{2b} negative controls with irrelevant glycosylation did not bind in a concentration dependent manner (paper IV). The calculated K_D (equilibrium dissociation constant) values of the mucin-H5 interactions calculated with a 1:1 steady-state binding model was in the low nanomolar range for all mucins (C-P55 0.8 nM; 293-P 0.7 nM and C-PSLex 7 nM). The increased slope at higher concentrations of Sia α 2,3LAcNAc-albumin (Figure 17, D) indicates heterogeneity in the system. This makes K_D calculation more uncertain. Excluding the two top concentrations yields a K_D value that is slightly higher than the values obtained for PSGL-1/mIgG_{2b} but still in the low nanomolar range (29 nM). The calculated K_D values for the fusion proteins and Sia α 2,3LAcNAc-albumin may be lower than the “real” K_D values because the surfaces did not appear to reach saturation even at the highest concentration of injected ligands. Taken together the Biacore binding analyses indicate that the mucin-type fusion proteins produced in C-P55, 293-P and C-PSLex are good binding partners of recombinant avian HA. The low nanomolar affinity is considerably stronger than the millimolar range K_D values observed between HA and the monovalent binding partners (Sauter et al., 1992, Sauter et al., 1989, Toogood et al., 1991). The binding results give an indication that recombinant H5 may bind to Sia α 2,3Gal on different carbohydrate chains, although we could not show this directly due the presence of several different sialylated *O*-glycans on PSGL-1/mIgG_{2b} produced in each cell line (paper IV). In support of this hypothesis a recent study using the same H5 strain as in paper IV showed that poly(acrylic acid)amide (PAA) conjugates carrying Sia α 2,3 on core 1 and type 2 chain (Gal β 1,GlcNAc β) bound with similar affinity in a Biacore single cycle kinetics assay (2.9 nM and 1.6 nM respectively) while no binding to Sia α 2,6 on type 2 chain was observed (Suenaga et al., 2012). Further, in a glycan array experiment H5 (A/H5N1/Vietnam/1203/2004) bound well to the heavily glycosylated α 1-acid glycoproteins (orosomucoid, carries both Sia α 2,3 and Sia α 2,6) and several Sia α 2,3Gal terminated glycans carrying sulfate substitutions, while no Sia α 2,6 binding was observed. Taken together, our and other studies indicate that H5 bind to several receptors carrying terminal Sia α 2,3, while Sia α 2,6 binding is absent, and that multivalent presentation of the receptor yields a strong binding.

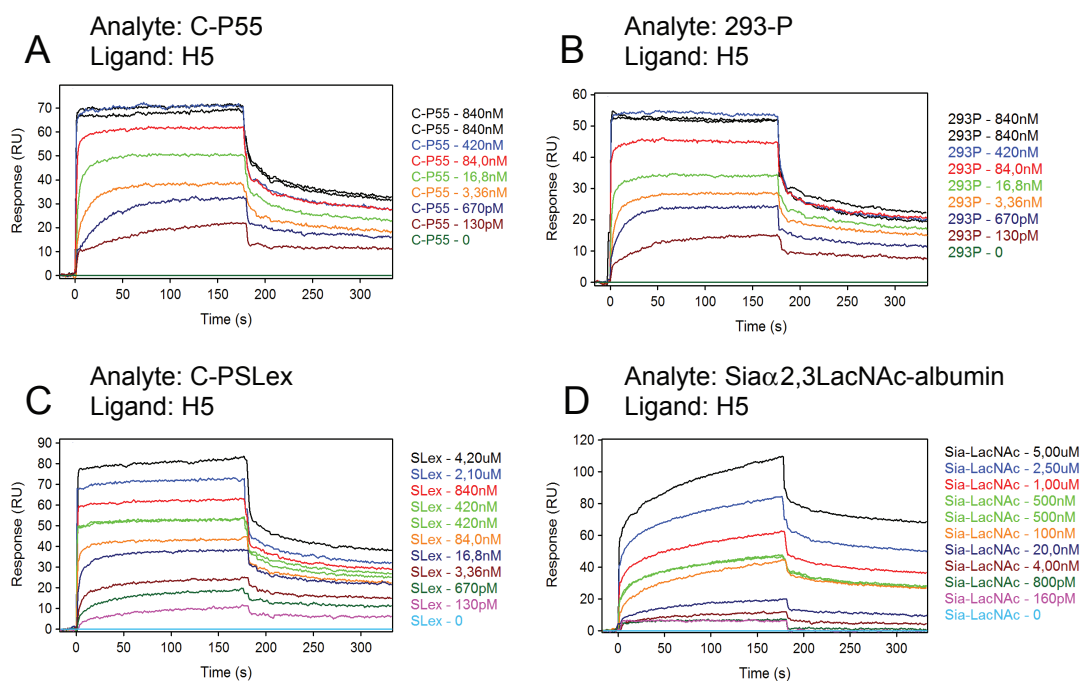


Figure 17 Biacore sensorgrams demonstrating PSGL-1/mIgG_{2b} (produced in C-P55, 293-P and C-PSLex) and Sia α 2,3-LacNAc-albumin binding to immobilized recombinant H5 (A/H5N1/Vietnam/1203/2004 on a CM5 chip). Reference channel- (activated/deactivated) and blank (buffer) subtraction was used to reduce buffer bulk effects.

10.6 PSGL-1/mIgG_{2b} as a multivalent inhibitor of Shiga-like toxins and avian influenza

10.6.1 Mechanism of action

The concept of multivalent carbohydrate inhibitors of Shiga-like toxins and influenza has been demonstrated in several studies but has not resulted in any approved drugs yet (Watanabe et al., 2004, Bovin et al., 2004, Matrosovich and Klenk, 2003, Ogata et al., 2009, Totani et al., 2003, Skehel and Wiley, 2000). The ideal proposed mechanism of action is similar for both inhibitors, where the combined effect of competitive inhibition and steric hindrance may block the attachment to the cell surface and possibly cause aggregation. It remains to be shown if PSGL-1/mIgG_{2b} may have this effect on Shiga-like toxins and influenza virus *in vitro* and *in vivo*.

10.6.2 Positive effect of inhibitor

A Shiga toxin inhibitor may not cure the infection or the HUS complication but buy time that allows anti-inflammatory treatments to reduce the cytokine storm that is taking place during HUS (Karmali, 2004, Noris et al., 2012). It would be preferable to reduce the load of toxin in an early stage of disease, before the toxin may enter the blood stream and cause systemic effects. Oral prophylactic use of Shiga toxin inhibitors have therefore been proposed (Karmali, 2004). Because an inhibitor of influenza HA

only blocks the initial phase of the infectious cycle treatment must be prophylactic or in early phase of disease. However, granted the inhibitor is highly efficient, the complete blocking of infection may protect both individuals and limit human-to-human spread. In contrast NA inhibitors that mainly inhibit the release of influenza virus during budding alleviate disease symptoms but does not limit human to human spread.

10.6.3 Achieving high concentration of inhibitor in a large surface area is a challenge

The surface area of the intestine is very large which may limit the inhibition of the Shiga-like toxin in the gut. By using a multivalent inhibitor, the local concentration can increase dramatically compared to a monovalent inhibitor. In animal models of STEC infections oral treatment with linear acrylamide polymers with highly clustered Gb3 trisaccharides bound to both Stx1 and Stx2 and decreased the disease symptoms (Watanabe et al., 2004). The monovalent inhibitor of Shiga toxin, Synsorb-Pk, notably failed in clinical trials (Trachtman et al., 2003). In a similar manner it is conceivably difficult to prevent even a single virus from attaching to cells in the respiratory tract. In contrast, the eye surface area is only $\sim 2 \text{ cm}^2$ (compare with $\sim 50 \text{ cm}^2$ of the proximal airways and $\sim 2 \text{ m}^2$ of the distal airways) and might thus be a more manageable area to protect if topical treatment with anti-influenza drugs is envisioned (Tsubota and Nakamori, 1995). Since the eyes may be an entrance port of highly pathogenic avian influenza there is indeed an interest in protecting the eyes (Belser et al., 2012) (Fouchier et al., 2004). Inhibition of oculotropic adenoviruses with multivalent constructs carrying sialic acid has been successfully shown *in vitro* (Aplander et al., 2011, Spjut et al., 2011, Johansson et al., 2007).

10.6.4 *In vivo* considerations

It has been observed that the Shiga toxin is not free in the circulation but is mainly bound to polymorphonuclear leukocytes (Brigotti et al., 2011). It remains to be seen if this limits the use of Shiga toxin inhibitors *in vivo*. By inhibiting influenza HA with an identical receptor analogue the risk of HA escape mutations is decreased (Bovin et al., 2004), but at the same time the inhibitor may be destroyed by NA hydrolysis. Modification of sialic acid on multivalent inhibitors, such as acetylation may prevent hydrolysis short term for some strains, but will likely generate escape mutants (Pritchett and Paulson, 1989). The concentration of inhibitor must therefore likely be high in order to overwhelm the binding of HA and hydrolysis activity of NA. This may be accomplished by increasing the concentration or the size and substitution grade of the inhibitor. It should be realized that the influenza virus binding (HA) and release (NA) molecules act in concert on the virus surface. Studies of NA inhibitor resistant influenza have indicated a weaker binding of HA to sialic acid receptors and

consequently a reduced role of NA during release of novel virus particles (Pritchett and Paulson, 1989). The combined use of several antiviral drugs such as HA and NA inhibitors may thus be needed in the future in order to reduce the rate of escape mutations (Barik, 2012). The effect of a multivalent inhibitor *in vivo* may not be easy to predict by *in vitro* experiments. Mammen et al previously showed that a polyvalent inhibitor bound poorly to HA-trimers but was an efficient inhibitor of influenza virus hemagglutination (Mammen et al., 1998). Binding of a single inhibitory molecule may be enough to cover the viral surface with the polymer that sterically prevents the virus from cross-linking erythrocytes (hemagglutination). Thus steric inhibition may be more important than competitive inhibition. In order to avoid unwanted side effects, non-human carbohydrates should not be present on inhibitors that are exposed to the immune system. Comprehensive structural characterization of the glycosylation is therefore needed on all licensed therapeutic glycoproteins. The Fc-portion of PSGL-1/mIgG_{2b} contributes to a long half-life in circulation but care must be taken to avoid unwanted effector functions.

10.6.5 Other application of multivalent carbohydrate inhibitors

In addition to synthetic Gb3 mimics, other suggested inhibitors of Shiga toxins are probiotic bacteria expressing Gb3 on the surface and monoclonal antibodies (James, 1992, MacConnachie and Todd, 2004). A dialyzer with immobilized glycoconjugate polymers for removal of Shiga toxin has been developed (Miyagawa et al., 2006). The concept has only been tested *in vitro* so far, though. Other possible applications of PSGL-1/mIgG_{2b} expressing the P1 epitope include the inhibition of P-fimbriated uropathogenic *E coli*, *Pseudomonas aeruginosa* (PA-I lectin), and *Staphylococcus aureus* (enterotoxin B) since the P1 determinant is the receptor of these microbes and toxins (Bock et al., 1985, Gilboa-Garber et al., 1994), (Tikkanen et al., 1995, Tikkanen et al., 1996, Chatterjee et al., 1995). PSGL-1/mIgG_{2b} that carries multivalent sialic acid may also be a suitable inhibitor of oculotropic adenoviruses (Aplander et al., 2011, Spjut et al., 2011, Johansson et al., 2007).

10.6.6 PSGL-1/mIgG_{2b} compared to chemical and naturally occurring inhibitors

Multivalency may be achieved by both organic chemistry and molecular biology. Chemists may be able to effectively synthesize homogenous polymers with high substitution grades with tailored scaffolds; however the synthesis of complex carbohydrates remains challenging. Glyco-engineering of CHO cells offers the advantage of producing multivalent glycoproteins with longer carbohydrate chains while chemical synthesis is often limited to tri- or tetrasaccharides. Longer carbohydrate chains may confer more specific binding compared to trisaccharides.

Indeed, it was shown that blood group A/B on type 1-4 tetrasaccharides were more efficient adsorbers of anti-A/B chain type-specific antibodies than A and B trisaccharide determinants (Lindberg et al., 2012). Paper II-IV represents examples of rational glycan design of PSGL-1/mIgG_{2b} with specific glycosylation for specific purposes. Another possible strategy may be to make use of naturally occurring glycoproteins, such as those found in milk. The glycoproteins may be suitable for several applications, including inhibition of toxins or microbial attachment (Rasooly et al., 2012, Gustafsson et al., 2006). Challenges for recombinant glycoprotein inhibitors include the high costs and the comprehensive structural characterization that is needed to ensure a homogenous reproducible product.

11 Conclusions

- Analysis of the recombinant PSGL-1/mIgG_{2b} mucin-type fusion protein in *Spodoptera frugiperda* (Sf9) and *Trichoplusia ni* (Hi-5) cells revealed that the *O*-glycosylation of these cells is more abundant and complex than previously reported (paper I).
 - The abundance of non-human *N*- and *O*-linked glycans produced by Hi-5 and Sf9 insect cells may limit their use as an expression system of therapeutic proteins
- CHO cells were successfully glyco-engineered in order to produce PSGL-1/mIgG_{2b} that carried *O*-linked blood group A- and B determinants on type 1, type 2 and type 3 outer core saccharide chains (paper II).
 - We showed that the mucins could adsorb chain type-specific antibodies from blood.
 - In the future the mucins may therefore be used in immunoadsorption columns to remove chain-specific anti-A and anti-B antibodies prior to organ transplantation and as substrates for the quantification and specificity-determination of ABO antibodies.
- PSGL-1/mIgG_{2b} carrying multivalent *O*-linked carbohydrate receptors of Shiga-like toxin and avian influenza H5 was successfully produced (paper III and IV).
 - PSGL-1/mIgG_{2b} carrying the P1 determinant bound with low nanomolar affinity to Stx1 in a Biacore assay, while Stx2 bound with lower affinity.
 - PSGL-1/mIgG_{2b} carrying Sia α 2,3Gal on mainly core 1 or lactosamine bound with similar low nanomolar affinities to avian H5 hemagglutinin.

12 Future projects

12.1 Paper I

The complete *O*-glycome and *N*-glycome determined from total cell extracts of Sf9 and Hi-5 cells would be of general interest but was beyond the scope of paper I. A deeper understanding- and manipulation of the glycosylation machinery of these cell lines would also benefit from the complete genome sequence as demonstrated previously for silk worm (Xiang et al., 2010, Li et al., 2012, Xia et al., 2009). As of today it is not clear which enzymes that synthesize most of the detected structures and the substitutions detected in paper I. Recent progress in transient and stable “knock-down” and “knock-out” technologies of specific genes may prove to be powerful techniques to study the action of specific glycosyltransferases (Kim et al., 2012, Malphettes et al., 2010). In paper I a general purpose culture medium was used (with serum), it may therefore be interesting to compare the glycosylation pattern observed with other defined cell media (such as serum-free medium), although the cell culture medium effect on glycosylation have been explored previously (Ikonomou et al., 2003, Drugmand et al., 2012)

12.2 Paper II

Different applications of the mucin-type fusion proteins carrying blood group antigens are being explored such as immunoadsorbents and ELISA substrates.

Future work in this field of glyco-engineering will benefit greatly from the recent initiative that provides expression constructs for more than 80% of all mammalian glycosylation enzymes for free to the research community (Repository of Glyco-Enzyme Expression <http://glycoenzymes.ccruc.uga.edu/>, last accessed April 17 2013).

12.3 Paper III and IV

Additional Biacore experiments with different set-ups (that is immobilize PSGL-1/mIgG_{2b} and flow Shiga toxin or hemagglutinin over the surface) and ELISA assays may be used to validate the binding data from paper III and IV. Immobilizing PSGL-1/mIgG_{2b} would also facilitate the evaluation of PSGL-1/mIgG_{2b} as an inhibitor, since a pre-mixing step before injection may then be performed with different amounts of monovalent binding partners or PSGL-1/mIgG_{2b}. The ability of PSGL-1/mIgG_{2b} to inhibit erythrocyte hemagglutination caused by influenza virus may also be used as method to evaluate the inhibitory potential. The protective effect of PSGL-1/mIgG_{2b} on Shiga toxin sensitive Vero cells or influenza virus sensitive MDCK cells may also be necessary future *in vitro* assays.

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