

‘GlycosaminoGlycome’ Microarrays: Applications in Studies of Recognition by Anti-Human Stem Cell Antibodies, Growth Factors and Morphogens

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by

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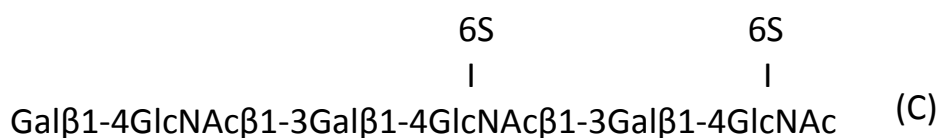
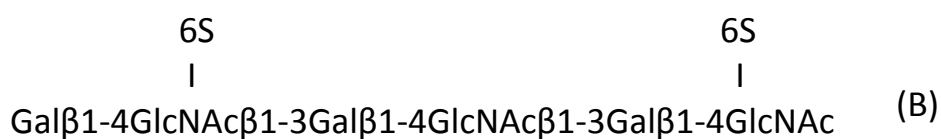
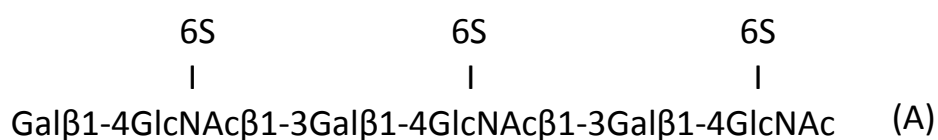
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Abstract

This thesis is focused on construction of microarrays of the GlycosaminoGlycome including glycosaminoglycan (GAG) polysaccharides [chondroitin sulphates, heparan sulphates, heparin, keratan sulphate (KS) and hyaluronan] and the derived oligosaccharides as neoglycolipids (NGLs), and their application to characterization of a stem cell antigen R-10G and investigation of GAG-binding preferences of growth factors and morphogens to which human stem cells respond.

R-10G antigen is of special interest as it is expressed on human embryonic stem (ES) and induced pluripotent stem (iPS) cells, but not on human embryonal carcinoma cells. Screening analysis using the GAG polysaccharide microarray showed strong binding of antibody R-10G specifically to bovine cornea KS, but little or no binding to other KS preparations or GAG polysaccharides. 'Designer' arrays were then constructed from KS oligosaccharides obtained by partial depolymerisation using keratanases and mild acid treatment. The oligosaccharides were fractionated by gel-filtration and HPLC, with monitoring by mass spectrometry (MS) before conversion to NGLs for antigenic analysis. KS oligosaccharide fractions terminating with Gal (keratanase II digestion products) rather than GlcNAc at the non-reducing end were strongly bound by R-10G, and the minimum chain length was hexasaccharide. Among oligosaccharides separated from the hexasaccharide fraction, Structure A (overleaf) assigned by NMR and Structure B assigned by MS/MS are antigen-positive, whereas Structure C is antigen-negative.



This suggests that the sulphate at the internal GlcNAc residue is not required and the sulphate at the non-reducing terminal GlcNAc residue is the key element.

A novel finding in screening of growth factors and morphogens using the GlycosaminoGlycome microarray is that KS is bound by fibroblast growth factor 2 and bone morphogenetic protein (BMP) 2, but not wingless-3a, sonic hedgehog and BMP-4. This opens the way into searching for a role for fully and undersulphated KS on stem cells.

Declaration of Originality

I, Nian Wu, hereby declare that the work presented in this thesis is my own work. Where I have used materials and results from other sources, this is clearly stated. This thesis has not been submitted for a degree to any other academic institution.

Part of the results described in Chapter 3 of this thesis has been published in one paper below.

Mulloy, B., Wu, N., Gyapon-Quast, F., Lin, L., Zhang, F., Pickering, M., Linhardt, R., Feizi, T., and Chai, W. (2016) Abnormally high content of free glucosamine residues identified in a preparation of commercially available porcine intestinal heparan sulfate. *Analytical chemistry*, 88(13), 6648-6652.

Most of the results described in Chapter 4 have been presented at Gordon Research Seminar on Glycobiology and Gordon Research Conference on Glycobiology (March 2017) and the abstract has been published as below.

Wu, N., Silva, L., Liu, Y., Zhang, Y., Fu, L., He, W., Jiang, P., Zhang, F., Linhardt, R., Kawasaki, T., Mulloy, B., Feizi, T., and Chai, W. (2017) Characterization of a keratan sulphate antigen expressed on human-induced pluripotent stem and embryonic stem cells by glycosaminoglycan designer microarrays.

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Abbreviations

Abbreviations for carbohydrates and related

CS	chondroitin sulphate
Fuc	fucose
GAG	glycosaminoglycan
Gal	galactose
GalNAc	N-acetylgalactosamine
Glc	glucose
GlcA	glucuronic acid
GlcNAc	N-acetylglucosamine
GPI	glycosylphosphatidylinositol
HA	hyaluronan
HEP	heparin
Hex	hexose
HexNAc	N-acetylhexosamine
HS	heparan sulphate
KS	keratan sulphate
LacNAc	N-acetylactosamine
NA	N-acetylated
Neu5Ac	N-acetylneuraminic acid
NeuGc	N-glycolylneuraminic acid
NS	N-sulphated
S	sulphate
SA	sialic acid
Xyl	xylose

Abbreviations for amino acids

Asn	asparagine
Ser	serine
Thr	threonine

Abbreviations for stem cells

EC	embryonal carcinoma
ES	embryonic stem
HSC	haematopoietic stem cells
iPS	Induced pluripotent stem

Abbreviations for antibodies, antigens and the related

Ig	immunoglobulin
mAb	monoclonal antibody
TRA	tumour-rejection antigen
SSEA	stage specific embryonic antigen

Abbreviations for growth factors, morphogens and related

Arr	Arrow
BMP	bone morphogenetic protein
Ci	Cubitus interruptus
CW	Cardin-Weintraub
Dhh	Desert hedgehog
Dsh	Dishevelled
FGF	fibroblast growth factor
FGFR	fibroblast growth factor receptor
Fz	Frizzled
Hh	Hedgehog
Ihh	Indian hedgehog
Ptc	Patched
Shh	Sonic hedgehog
Smo	Smoothed
TGF β	transforming growth factor β
TK	tyrosine kinase
Tkv	Thick veins
TwHh	Tiggy-winkle hedgehog
Wnt	int/Wingless family

Other abbreviations

ADHP	<i>N</i> -aminoacetyl- <i>N</i> -(9-anthracenylmethyl)-1,2-dihexadecyl- <i>sn</i> -glycero-3-phosphoethanolamine
AOPE	aminoxy-functionalized DHPE
BSA	bovine serum albumin
CID	collision-induced dissociation
DHPE	1,2-dihexadecyl- <i>sn</i> -glycero-3-phosphoethanolamine
ES-MS	electrospray mass spectrometry
HBS	Hepes buffered saline
HPLC	high performance liquid chromatography
HPTLC	high performance thin layer chromatography
MPLC	medium pressure liquid chromatography
MS	mass spectrometry
MALDI	matrix-assisted laser desorption/ionization
NGL	neoglycolipid
NHS	N-hydroxysuccinimide
NMR	nuclear magnetic resonance
PBS	phosphate buffered saline
PGC	porous graphitized carbon
TLC	thin-layer chromatography

Chapter 1

Introduction

1.1 General Introduction to Glycans

Every cell within multicellular organisms is coated with a dense and complex layer of glycans. Most secreted molecules and extracellular matrices of multicellular organisms are also glycosylated. The cell surfaces and matrices secreted by unicellular organisms such as bacterial biofilms are also rich in glycans.

1.1.1 Glycans and glycoconjugates

Glycans are commonly attached to proteins or lipids as glycoconjugates existing in or on eukaryotic cells. The common classes of mammalian glycoconjugates include glycoproteins, proteoglycans, glycosphingolipid (GPI)-anchored glycoproteins, and glycosphingolipid (Figure 1.1).

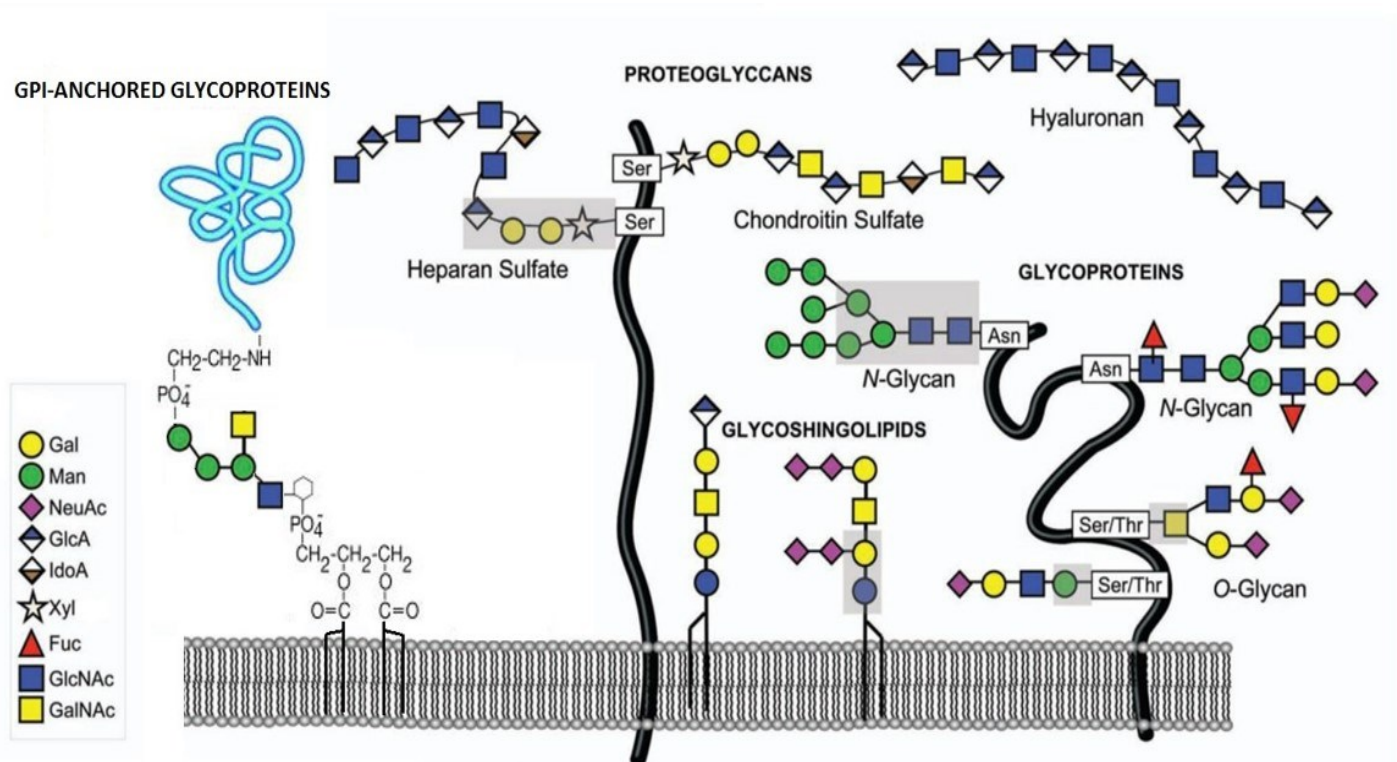


Figure 1.1. Major classes of mammalian glycans. Each class shares the common core structures boxed in grey. Sequences are representative structural variations on mammalian cells. Gal, galactose; Man, mannose; NeuAc, N-Acetylneuraminic acid; GlcA, glucuronic acid; IdoA, iduronic acid; Xyl, xylose; Fuc, fucose; GlcNAc, N-acetylglucosamine; GalNAc, N-acetylgalactosamine; Ser, serine; Thr, threonine; Asn, asparagine. Figure is adapted from [1, 2]. Not shown here are intracellular O-glycosylated (O-GlcNAcylated) proteins.

A glycoprotein is a protein with one or more glycans covalently attached via different residues of the polypeptide chain: N-glycans via asparagine residue; O-glycans via serine (Ser) or threonine (Thr) residue [3].

N-glycans share a pentasaccharide core sequence in common, $\text{Man}\alpha 1-6(\text{Man}\alpha 1-3)\text{Man}\beta 1-4\text{GlcNAc}\beta 1-4\text{GlcNAc}\beta 1-\text{Asn}$, where Man is mannose, GlcNAc is N-acetylglucosamine and Asn is asparagine [4]. They are divided into three types based on different residues attached to the core: oligomannose (high-mannose) type, complex type, and hybrid type (Figure 1.2).

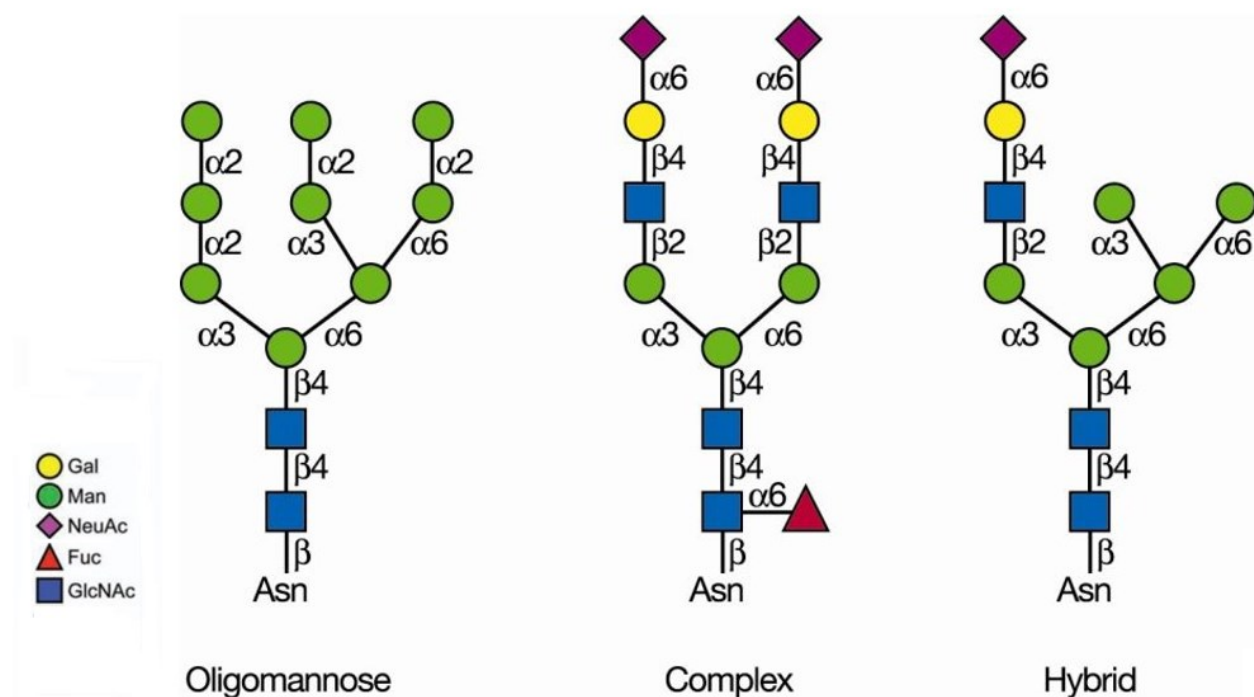


Figure 1.2. Three general types of N-glycans in a mature glycoprotein: oligomannose, complex and hybrid. They contain the common core $\text{Man}_3\text{GlcNAc}_2\text{Asn}$. Figure is taken from [2].

O-glycans are frequently GalNAc α 1-Ser/Thr types although there are several other types such as O-Fuc, O-Xyl, O-Man, O-GlcNAc, O-Gal, and O-Glc linked glycans (GalNAc, N-acetylgalactosamine; Fuc, fucose; Xyl, xylose; Gal, galactose; Glc, glucose) [5]. The O-GalNAc glycans (often named mucin O-glycans) can be extended to form different core regions: four common core structures designated cores 1 to 4 and an additional four designated cores 5 to 8 (Table 1.1) [6]. Only cores 1 to 4 and core 6 have been shown to be extended complex O-glycans. O-GalNAc glycans can terminate with Fuc, Gal, GlcNAc and NeuAc in α -linkages, GalNAc in both α and β linkages and sulphate. Both type 1 (Gal β 1-3GlcNAc) and type 2 (Gal β 1-4GlcNAc) can be repeated several times in poly-N-acetyllactosamine units. The poly-N-acetyllactosamine units and terminal structures may also be found on N-glycans.

Table 1.1. Structural domains of mucin-type O-glycans

Backbones	Core type	
Gal β 1-3GlcNAc β 1-3 (Type 1) Gal β 1-4GlcNAc β 1-3 (Type 2) Gal β 1-4GlcNAc β 1-6 Gal (3,6Gal-branched) Gal β 1-4GlcNAc β 1-3	Gal β 1-3GalNAc α -Ser/Thr	Core 1
	GlcNAc β 1-6 GalNAc α -Ser/Thr Gal β 1-3	Core 2
	GlcNAc β 1-3GalNAc α -Ser/Thr	Core 3
	GlcNAc β 1-6 GalNAc α -Ser/Thr GlcNAc β 1-3	Core 4
	GalNAc α 1-3GalNAc α -Ser/Thr	Core 5
	GlcNAc β 1-6GalNAc α -Ser/Thr	Core 6
	GalNAc β 1-6GalNAc α -Ser/Thr	Core 7
	Gal α 1-3GalNAc α -Ser/Thr	Core 8

The core sequences are listed, and linear and branched extensions are shown. Adapted from [6].

A proteoglycan is a protein carrying one or more glycosaminoglycan (GAG) chains, such as chondroitin sulphates (CSs), heparan sulphate (HS) and heparin, via Xyl residue linked to the Ser residue of the polypeptide chain [7]. Some proteoglycans also carry both GAG and O-/N-glycans. The other member of the GAG family, keratan sulphate (KS), is expressed on O-/N-glycan chains.

A GPI-anchored glycoprotein is a protein attached by a GPI anchor which consists of a glycan bridge between a phosphatidylinositol and a phosphoethanolamine in amide linkage to the carboxyl terminus of the protein.

A glycosphingolipid (often called a glycolipid) is a lipid moiety ceramide carrying a glycan via Glc or Gal to the terminal primary hydroxyl group of the lipid. Major core structures of vertebrate glycosphingolipids include lacto, neolacto, ganglio and globo series (Table 1.2). The glycolipid can be neutral or anionic. A ganglioside is an anionic glycolipid consisting of one or more sialic acid residues.

Table 1.2. Major core and backbone sequences of vertebrate glycosphingolipids

Subfamily series	Sequences
Lacto	GlcNAc β 1-3Gal β 1-4Glc β 1-Cer
	Gal β 1-3GlcNAc β 1-3Gal β 1-4Glc β 1-Cer
Neolacto	Gal β 1-4GlcNAc β 1-3Gal β 1-4Glc β 1-Cer
Ganglio	GalNAc β 1-4Gal β 1-4Glc β 1-Cer
	Gal β 1-3GalNAc β 1-4Gal β 1-4Glc β 1-Cer
Globo	Gal α 1-4Gal β 1-4Glc β 1-Cer
	GalNAc β 1-3Gal α 1-4Gal β 1-4Glc β 1-Cer

Cer, ceramide.

1.1.2 Biosynthesis of glycans

Endoplasmic reticulum (ER)-Golgi-plasma membrane pathway is a universal feature of initiation and maturation of eukaryotic glycoconjugates.

N-glycan biosynthesis (Figure 1.3) begins from extending dolichol phosphate (Dol-P) to Dol-P-P-GlcNAc2Man5 on the cytoplasmic face of the ER membrane [8, 9]. The Dol-P-P-GlcNAc2Man5 is flipped to the luminal side and extended to Dol-P-P-GlcNAc2Man9Glc3, followed by attachment to a protein synthesized and translocated through the ER membrane. The protein-bound N-glycan is subsequently remodelled in the ER and Golgi through the actions of a complex series of membrane-bound glycosidases and glycosyltransferases [10].

In contrast to N-glycosylation, O-GalNAc glycan biosynthesis does not involve lipid-linked intermediates at initial stages. The first step is the transfer of GalNAc from UDP-GalNAc to Ser/Thr residues of proteins, followed by synthesis of core sequences and structures and elongation catalysed by successive actions of a series of glycosyltransferases in the Golgi apparatus (Figure 1.4) [5, 11].

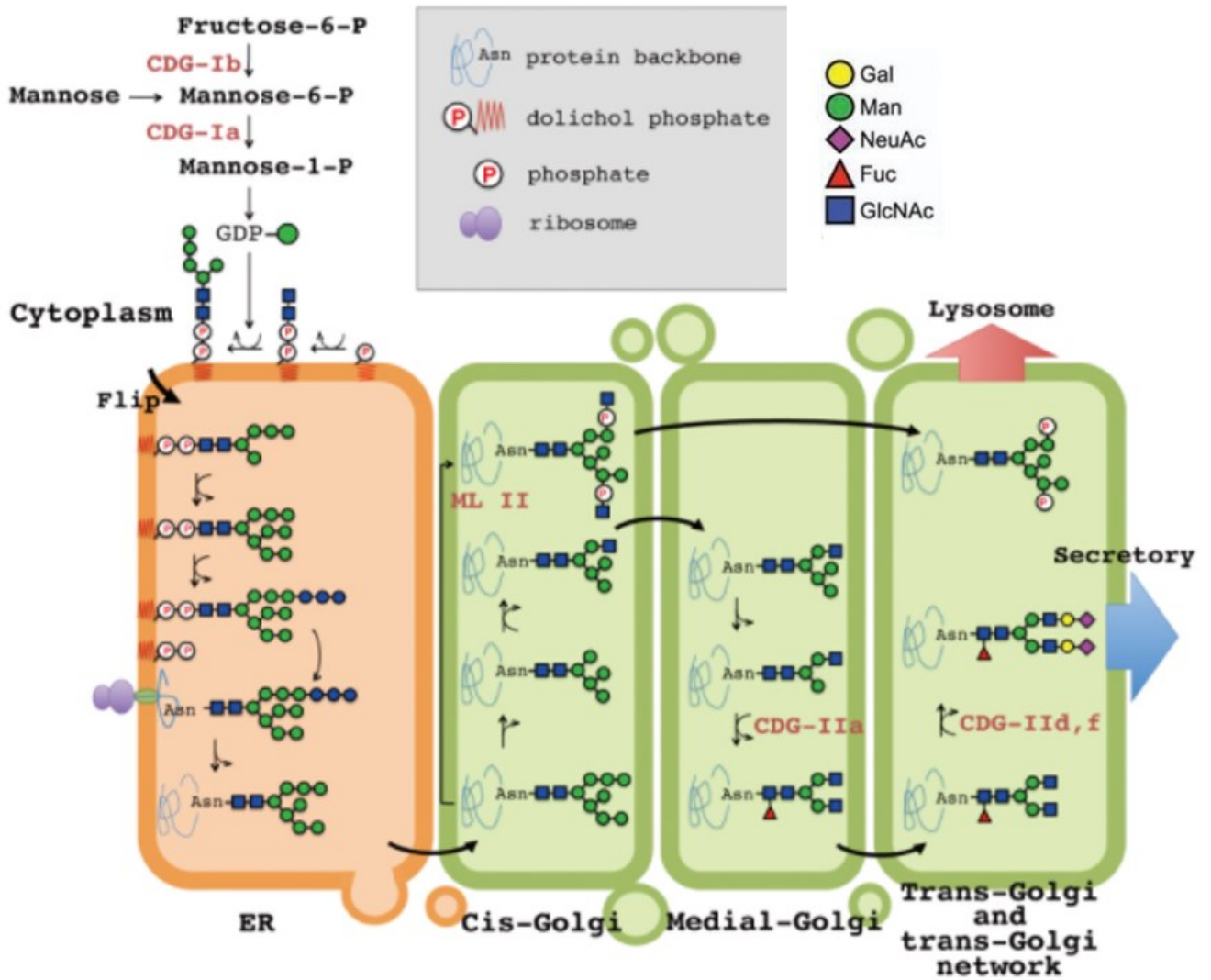


Figure 1.3. Biosynthesis and processing pathway of N-glycans. Figure is adapted from [12].

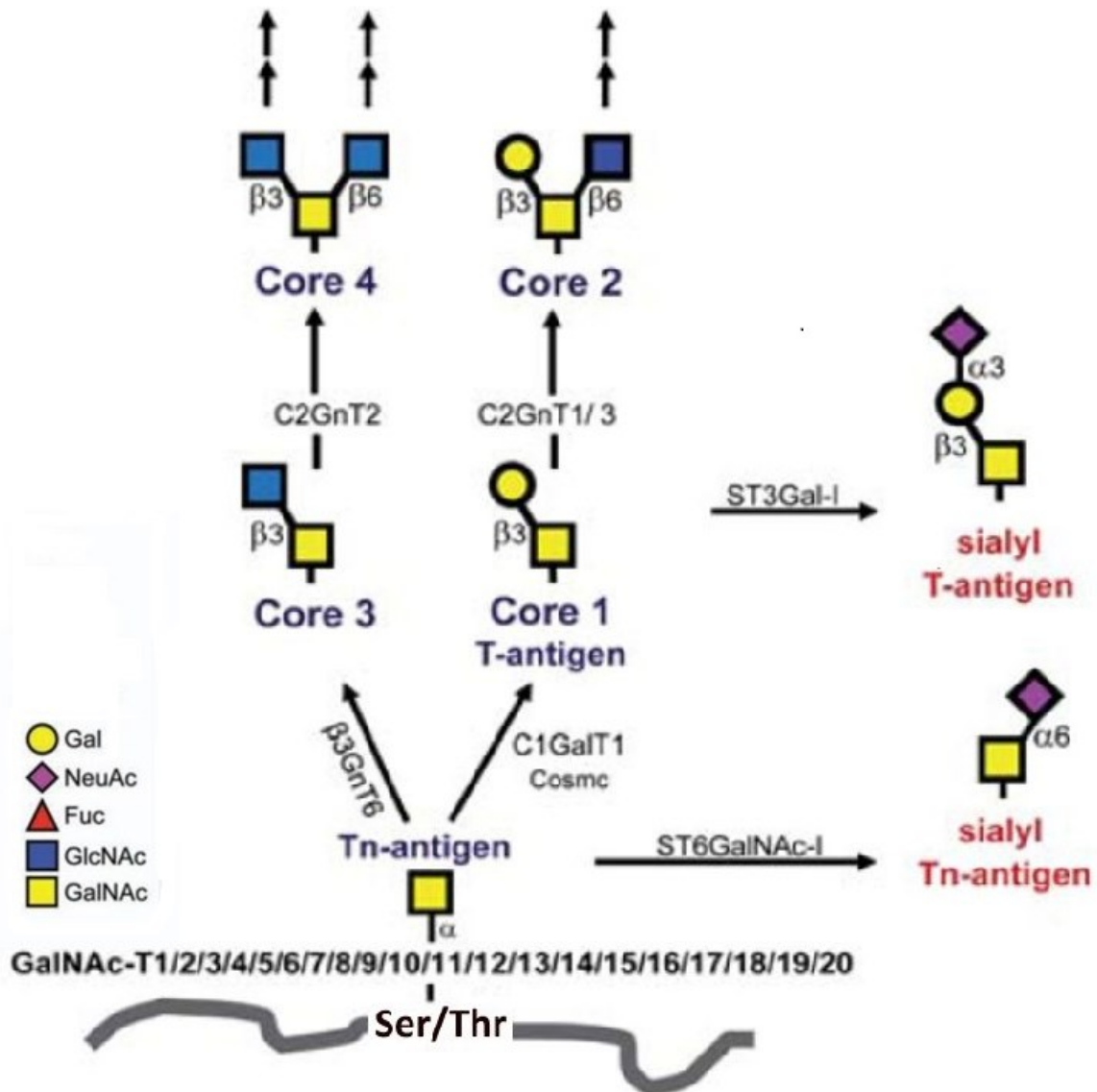


Figure 1.4. Biosynthesis and processing pathway of mucin-type O-glycosylation. The glycosylation is initiated by up to 20 GalNAc transferases forming the Tn motif, followed by elongation through the core 1 synthase, C1Gal-T1, or the core 3 synthase, β 3GnT6, and further branched by the core 2 synthases, C2GnT1/3 or the core 4 synthase, C2GnT2. Figure is adapted from [13].

Biosynthesis of CS, HS and heparin begins with attachment of Xyl to the Ser residue of a protein, followed by addition of GlcA-Gal-Gal- [14, 15]. The tetrasaccharide linkage region can undergo phosphorylation at the C-2 position of Xyl and sulphation of the Gal residues. Chain polymerization and modification reaction including sulphation and epimerization are catalysed by a series of glucosyltransferases in the Golgi apparatus. Details of the GAG chains and biosynthesis are discussed in the following section 1.2.

Biosynthesis of GPI anchors starts from preassembly of a GPI precursor in the ER membrane, followed by attachment of the GPI to newly synthesized protein in the lumen of the ER before lipid remodelling in the ER or carbohydrate side-chain modifications in the Golgi [16, 17]. Glycosphingolipids are initiated by the addition of Glc or Gal to a ceramide (Cer). Glc-Cer is synthesized on the cytoplasmic face of the ER and early Golgi apparatus, whereas Gal-Cer is on the luminal face of the ER [18]. They then flip into the Golgi lumen and are elongated by a series of glycosyltransferases.

1.1.3 Biological roles of glycans

Glycans have critical structural and modulatory roles [7, 19-24]. Glycosylation enables secreted proteins to be soluble, hydrophilic or negatively charged, thus reducing unwanted nonspecific interactions in extracellular spaces and protecting against proteolysis. Glycans of bacteria, archaea and fungi have critical roles in forming cell walls that resist large differences in osmolarity between the cytoplasm and the environment, and give protection from bacteriophages or antibiotics in the environment. The specific recognition of glycans by glycan-binding proteins (GBPs) either from the same organism or a different organism can mediate cell-cell interactions, cell-microbe interactions and molecular mimicry of host glycans by pathogens [21, 23, 24].

1.2 Glycosaminoglycans

GAGs are linear polysaccharides with alternating disaccharides consisting of an amino sugar (GlcNAc or GalNAc) and an uronic acid [glucuronic acid (GlcA) or iduronic acid (IdoA)] or Gal [25]. They include CS, heparin, HS, KS and hyaluronan (HA). The typical sequences of the GAGs are shown in Figures 1.5 and 1.6.

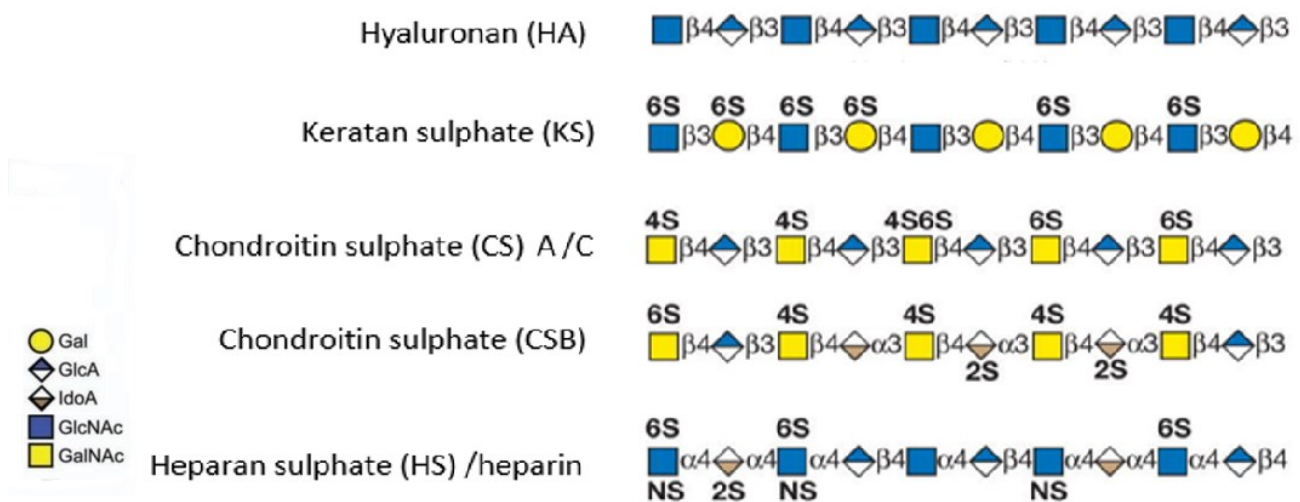


Figure 1.5. Schematic representation of GAGs: HA, KS, CS, and HS and heparin. Figure is adapted from [2].

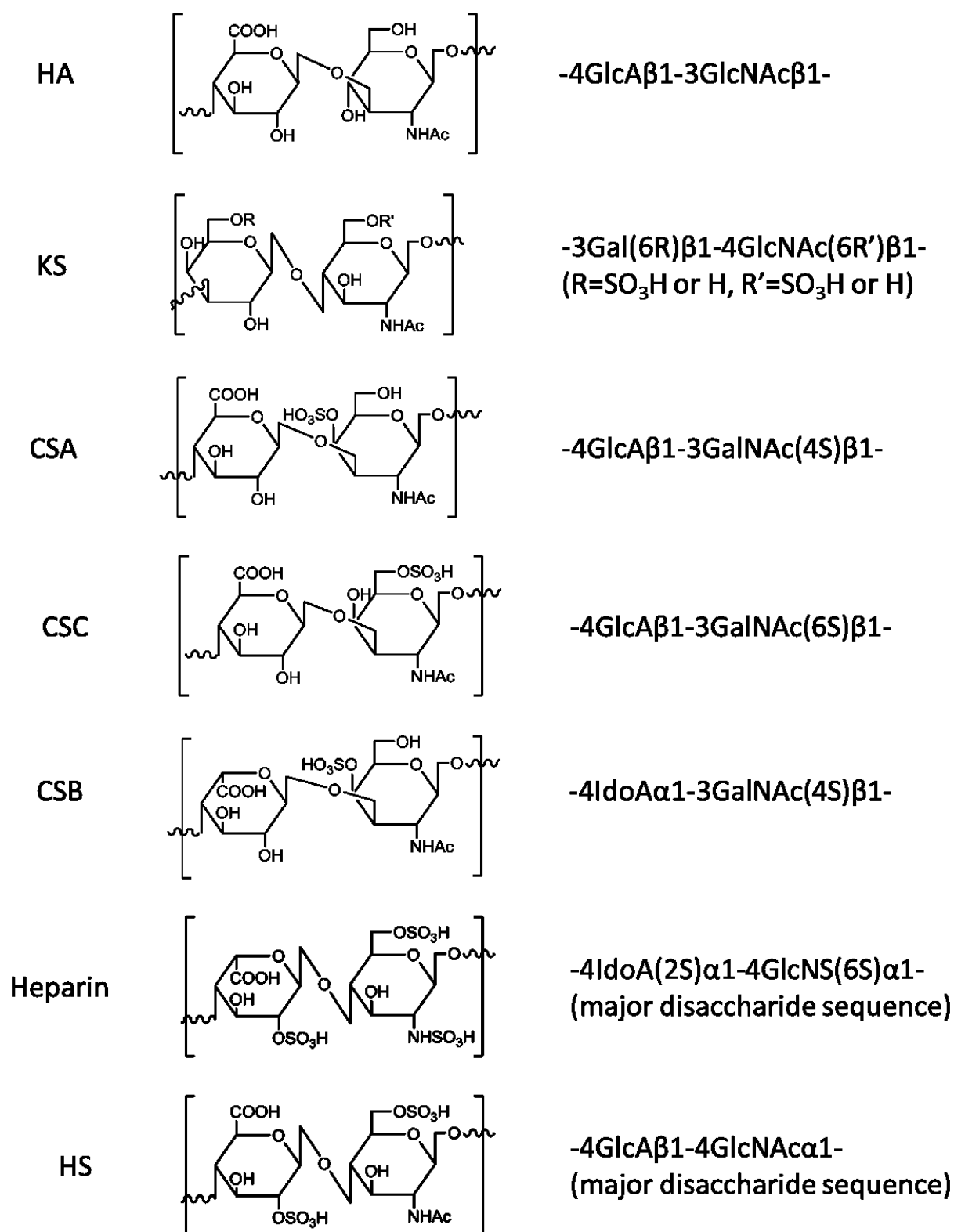


Figure 1.6 Structure of the disaccharide sequences of typical GAGs: HA, KS, CSA, CSC, CSB, heparin and HS [26-28].

GAGs are typically sulphated on either amino or hydroxyl groups and extremely polar, except HA that lacks sulphates. The currently known proteoglycans carrying GAGs can be divided into two major types, cell-surface proteoglycans and extracellular matrix proteoglycans. The former type of proteoglycans includes glypican linked by a GPI anchor and syndecan spanning the plasma membrane [29]. The latter type includes aggrecan, perlecan, decorin and biglycan [30]. Although the proteoglycans also carry O- and N-glycans typically found on glycoproteins, the much greater lengths of the GAG dominate the physico-chemical properties of proteoglycans. Through interactions with GAG-binding proteins, the GAGs participate in many biological processes such as haemostasis, anticoagulation, growth factor control, and cell adhesion, inflammation, pathogen attachment [31-33].

1.2.1 Chondroitin sulphates

Most CSs consist of GlcA β -3 linked to GalNAc as their disaccharide units. The sulphate groups on some hydroxyl groups in the disaccharide units are responsible for the diversity of CS: CSA with GlcA-GalNAc(4S), CSC with GlcA-GalNAc(6S), CSD with GlcA(2S)-GalNAc(6S) and CSE with GlcA-GalNAc(4,6S) [27]. An uncommon structure with 3-O-sulphation of GlcA has been identified, such as CSK with GlcA(3S)-GalNAc(4S) [34]. CSB contains IdoA α -3 linked to GalNAc as the disaccharide units, with sulphate on the 4 position of GalNAc [27].

The biosyntheses of CSs are initiated by formation of a linkage region tetrasaccharide GlcA β 1-3Gal β 1-3Gal β 1-4Xyl-Ser [14]. The CS biosynthesis involves the first addition of GalNAc to the tetrasaccharide by GalNAc transferase I, the polymerization of GalNAc and GlcA units by β 4GalNAc and β 3GlcA transferases, the epimerization of GlcA to IdoA by GlcA C5 epimerase which is specific for CSB biosynthesis, and sulphation of GalNAc

by 4-O- or 6-O- sulfotransferases before sulphation of IdoA residues by 2-O-sulfotransferase) [35, 36], (Figure 1.7).

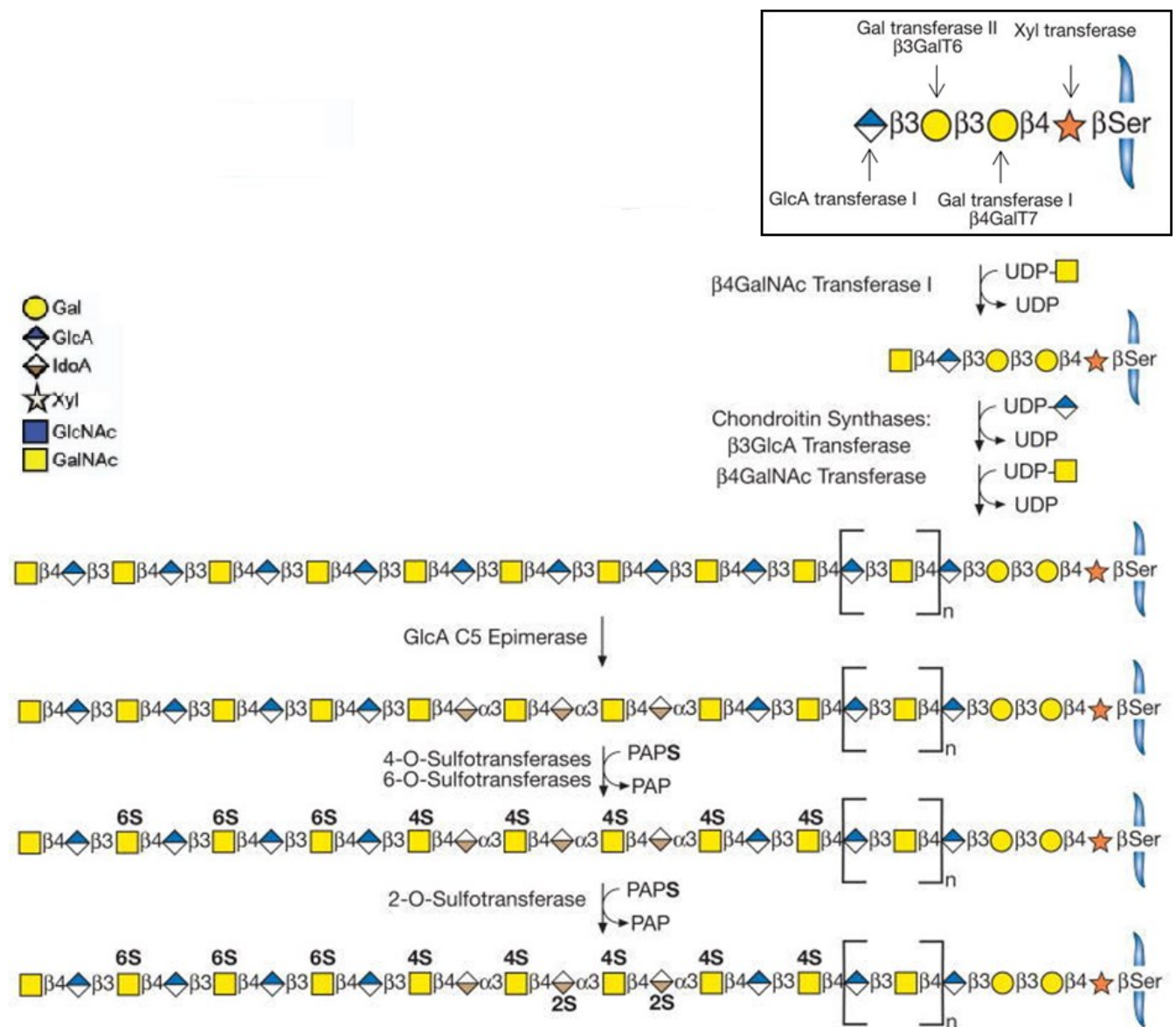


Figure 1.7. Schematic representation of CS biosynthesis. A number of glycosyltransferases participate in the synthesis of the common tetrasaccharide linkage region and repeating disaccharide region characteristic to CS chains. Figure is adapted from [2].

1.2.2 Heparan sulphate and heparin

Most of heparin in the body is stored in the granules of mast cells and the polysaccharide is released only in response to certain stimuli. In contrast to heparin which originates exclusively from the mast cells, HS resides on the plasma membrane of all animal cells studied so far [37-40].

Biosyntheses of HS and heparin also start from the linkage region tetrasaccharide $\text{GlcA}\beta 1\text{-3Gal}\beta 1\text{-3Gal}\beta 1\text{-4Xyl}\beta\text{-Ser}$, followed by the addition of the first GlcNAc catalysed by the enzyme EXTL3. However, the following polymerisation of $\text{GlcA}\beta 1\text{-4GlcNAc}\alpha 1$ is catalysed by the other two enzymes EXT1 and EXT2, prior to a series of modification reactions (Figure 1.8) [37, 41, 42]. GlcNAc residues are deacetylated by GlcNAc N-deacetylase and sulphated by N-sulfotransferases to form GlcNS. GlcA residues adjacent to and toward the reducing side of GlcNS are converted to IdoA by an epimerase that is different from the one involved in CSB synthesis. The IdoA residues are then sulphated by 2-O-sulfotransferase, although certain GlcA units can also undergo the 2-O sulphation. The addition of 2-O sulphate groups to GlcA or IdoA blocks the epimerization reaction. Some glucosamine residues are then sulphated by 6-O-sulphotransferase, followed by 3-O-sulphotransferase-catalyzed sulphation on certain sugar residues.

During biosynthesis, heparin undergoes extensive sulphation and uronic acid epimerization [37]. More than 80% of the GlcNAc residues are N-deacetylated and N-sulphated and more than 70% of the GlcA is converted to IdoA. Therefore, heparin is highly sulphated and mostly contains IdoA and GlcNS. Although HS chains vary among species or tissues, they share some common characteristics. All the HS chains contain both high sulphation (IdoA-GlcNS) and low sulphation (GlcA-GlcNAc) domains. The domain closest to the core protein at the reducing end is generally a GlcA-GlcNAc

domain. The compositions of the HS chains are controlled through the action of different N-deacetylase and N-sulphotransferase isoforms which determine number of N-sulphated groups.

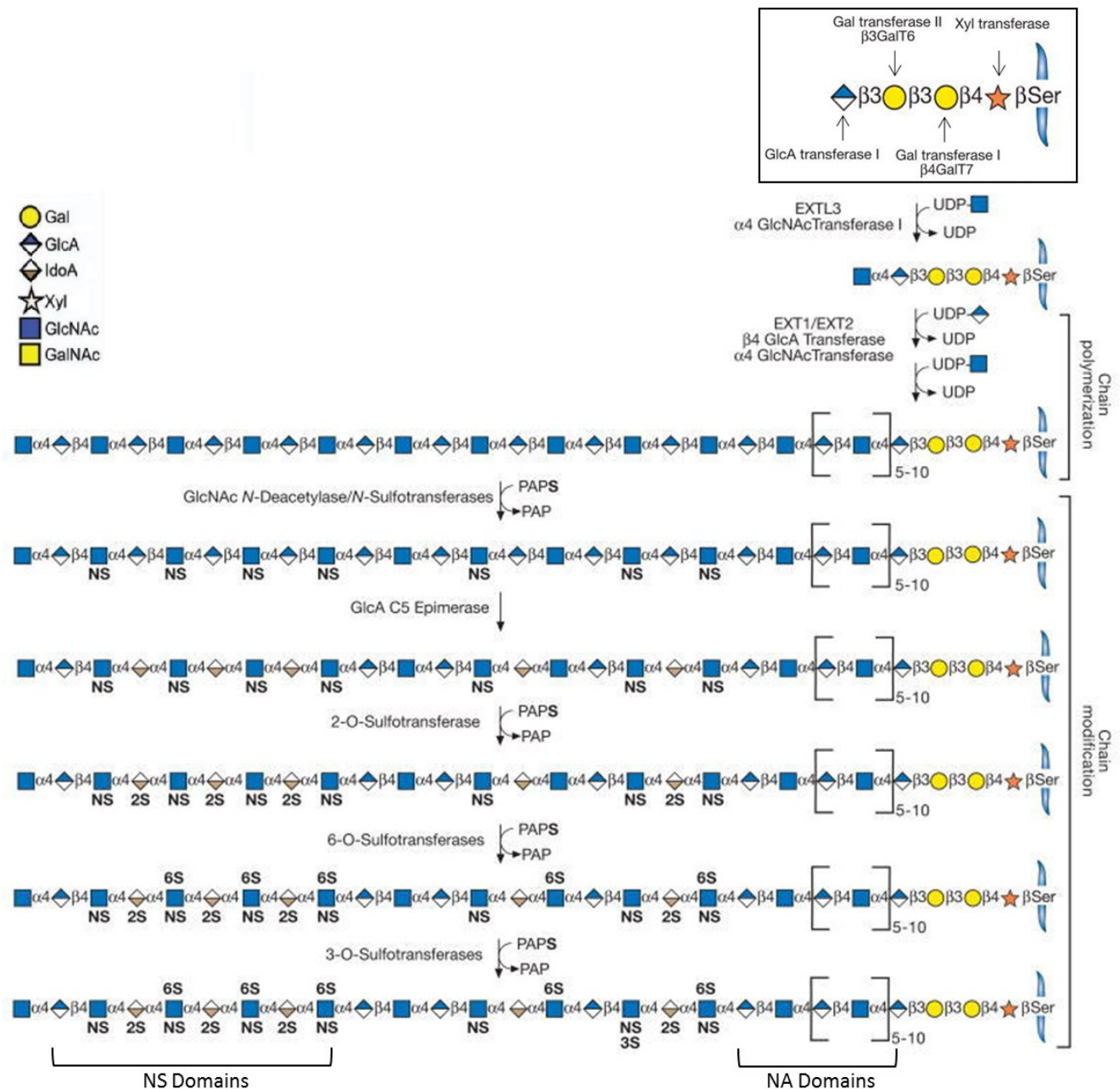


Figure 1.8. Schematic representation of HS and heparin biosynthesis. A number of glycosyltransferases participate in the synthesis of the common tetrasaccharide linkage region and repeating disaccharide region characteristic to HS and heparin chains. Representatives of N-sulphated (NS) domains and N-acetylated (NA) domains are labelled. Figure is adapted from [2].

1.2.3 Keratan sulphate

In contrast to other GAGs, KS is a sulphated poly-N-acetylglucosamine which contains Gal instead of uronic acid, with $\text{-3Gal}\beta\text{1-4GlcNAc}\beta\text{1-}$ residue as its repeating disaccharide unit [26]. KS is attached to an N- or O-glycan core via Asn or Ser/Thr, respectively, rather than to the typical $\text{GlcA}\beta\text{1-3Gal}\beta\text{1-3Gal}\beta\text{1-4Xyl-Ser}$ proteoglycan linkage region. KS proteoglycans are major components of the extracellular matrix of the cornea and cartilage [43, 44]. There are three different types of KS based on their linkage to protein (Figure 1.9).

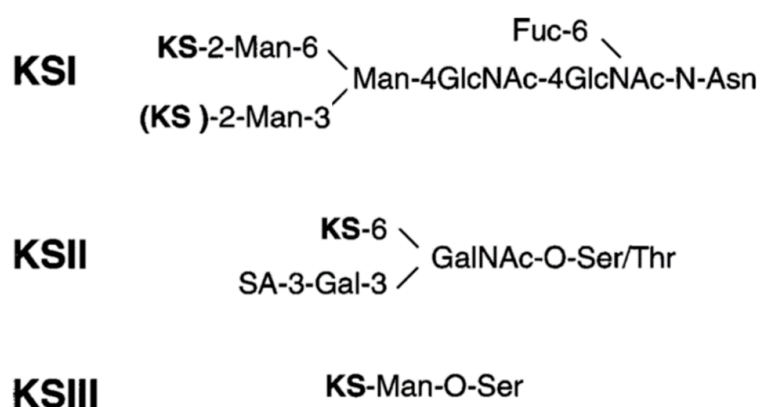


Figure 1.9. Three classes of KS (KSI, KSII and KSIII) defined according to the glycan core regions and linkages to protein. SA represents capping sugars, frequently sialic acid. KS-2 and KS-6 refer to the linkage of KS chains to the glycan core region. (KS) indicates the presence of KS is variable in KSI. Figure is taken from [45, 46].

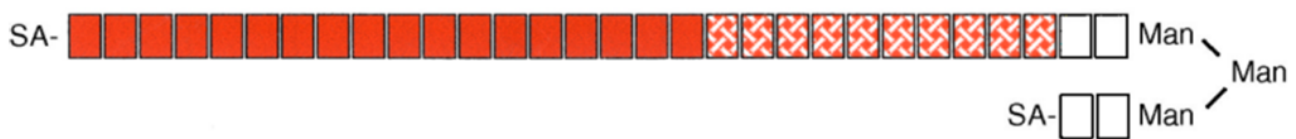
KS I, found in corneal proteins such as lumican, keratocan and mimecan, is linked to the mannose of an N-glycan core structure, which is an abundant source of KS [47]. Porcine corneal KS has been well studied and found to extend only the C-6 branch of the Man-containing core with the C-3 branch terminating with a single lactosamine capped by sialic acid [48]. Extension at the single branch is supported by several studies of corneal KS; evidence for the presence of two-chain (biantennary) extension has also been provided [49]. The KS chain is sulphated in a distinct and non-random pattern. Two non-sulphated lactosamine disaccharides ($\text{Gal}\beta\text{1-4GlcNAc}$) close to the reducing

end are followed by 10-12 mono-sulphated disaccharides [Gal β 1-4GlcNAc(6S)], with the non-reducing end of 8-34 di-sulphated disaccharides [Gal(6S) β 1-4GlcNAc(6S)] (Figure 1.10a) [45]. The capping structures vary; these can be NeuAc α 2-3 or 2-6, N-glycolylneuraminic acid (NeuGc) α 2-3 or 2-6, Gal α 1-3, GalNAc(6S) β 1-3 and GlcNAc(6S) β 1-3 [49, 50]. In bovine cornea, about 70% of the KS chains terminate with sialic acid, and only low levels of Fuc α 1-3 are observed [49]. The recently described chicken egg white KS proteoglycan is N-linked with a much larger molecular weight than that of the bovine corneal KS [51]. The KS is rich in both NeuAc α 2-8 and 2-9, and the sialylation content is much higher than can be accommodated by simple chain-terminating sialic acid residues [51]. This raises the possibility of there being polysialic acid sequences. KS I is not limited to cornea but found in several cartilage proteoglycans such as fibromodulin, PRELP (proline arginine-rich end leucine-rich repeat protein) and osteoadherin [52-54]. The noncorneal KS I differs from that in cornea. KS I in fibromodulin is a biantennary structure, which is relatively short (8-9 disaccharides), more highly sulphated and with a few fucoses (Figure 1.10b) [55, 56].

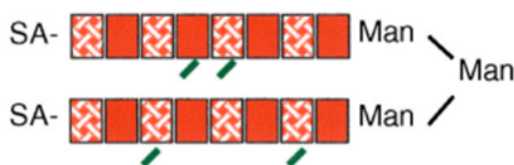
KS II is found on an O-glycan core 2 structure linked to protein through a Ser/Thr residue [57]. It is exclusively linked to the protein aggrecan, the most abundant proteoglycan of cartilage [58]. KS II can be further classified into two types. KS-II-A from articular cartilage contains Fuc α 1-3 and NeuAc α 2-6 residues, which are absent in KS-II-B from non-articular cartilage [59, 60]. KS II from bovine articular cartilage contains 5-11 di-sulphated disaccharides interrupted occasionally by a mono-sulphated disaccharide, with sialic acid at the non-reducing termini, and Fuc throughout the chain but not within four residues of the non-reducing terminus (Figure 1.10c). But KS II from tracheal cartilage is not fucosylated and capped only by NeuAc α 2-3 [61]. KS III was found in brain, which is attached to protein via Man O-linked to Ser [57].

Elongation and sulphation of KS chains is catalysed by several Golgi-localized enzymes (Figure 1.11). The KS is extended by the alternative actions of a β 3GlcNAc transferase and a β 4Gal transferase. Sulphation of GlcNAc must occur before further elongation and may be required for chain elongation, whereas sulphation of Gal can occur after polymerization and appears optional [45, 62]. The sequence of biosynthesis is GlcNAc, C-6 sulphation of a GlcNAc residue exposed at the nonreducing end by GlcNAc6S transferase and galactosylation. After formation of the polysaccharide chain, some Gal residues are sulphated by KSGal6S transferase. Gal in corneal KS is often partially sulphated, whereas GlcNAc is almost completely sulphated. The GlcNAc transferase and Gal transferase are found to be common enzymes with a widespread expression [45].

(a) **KSI in cornea**



(b) **KSI in fibromodulin of articular cartilage**



(c) **KSII in aggrecan of articular cartilage**



Figure 1.10. The range of KS chain structures. Polymer structure is represented graphically for KS samples from several sources. Each box represents a disaccharide with the backbone of [-3Gal β 1-4GlcNAc β 1-]. □ = unsulphated disaccharide, ☒ = disaccharide sulphated on GlcNAc only, ■ = disaccharide sulphated on both Gal and GlcNAc. Angled tails on the boxes indicate fucosylation. SA represents capping sugars, frequently sialic acid. Figure is taken from [45].

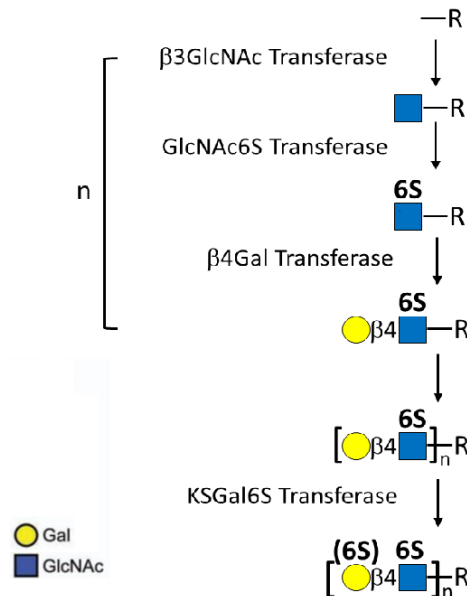


Figure 1.11. Schematic representation of KS biosynthesis. This illustrates the sequence of addition of sugars and sulphate to the growing chain (R). Figure is adapted from [45].

1.2.4 Hyaluronan

HA is a polymer of $\text{-3GlcNAc}\beta 1\text{-4GlcA}\beta 1\text{-}$ without sulphates and exists as a free polysaccharide instead of linking to a core protein as a proteoglycan. HA assembly occurs at the inner surface of the plasma membrane rather than the normal ER-Golgi pathway in eukaryotic cells. HA biosynthesis is catalysed by HA synthases.

1.3 Stem Cells and Glycans Expressed on Stem Cells

1.3.1 Major classes of stem cells

Stem cells, which are undifferentiated cells, can not only reproduce themselves but also generate daughter cells with different functions, such as those of the skin, the blood and the lining of the intestine [63]. A stem cell has two basic properties: self-renewal and pluripotency. In the strictest sense, pluripotency means stem cells can give rise to all types of cells. However, multipotent or unipotent progenitor cells are also sometimes called stem cells. There are two major categories of stem cells.

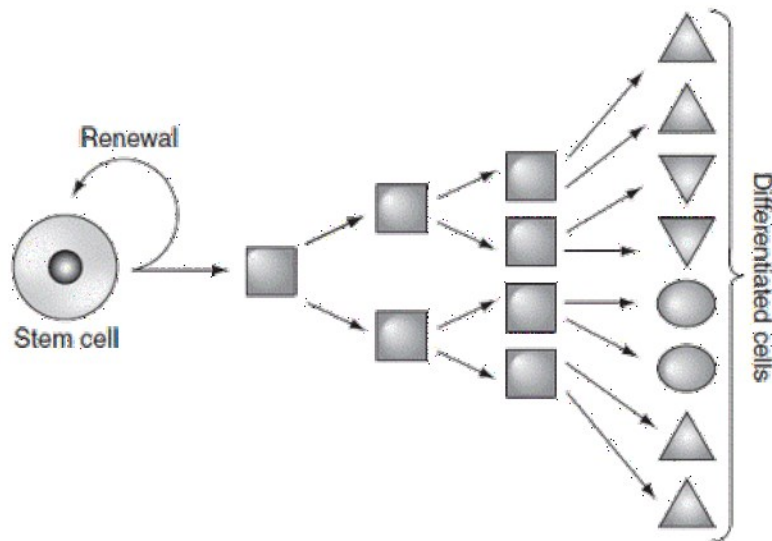


Figure 1.12. Two basic properties of stem cells: self-renewal and potency. Figure is taken from [63]

Embryonic stem cells/ Induced pluripotent stem cells

Embryonic stem (ES) cells were originally prepared from mouse embryos, followed by the discovery of human ES cells. All animal embryos starting life from a fertilized egg will proceed to become blastulas, then develop to three germ layers: ectoderm (later becoming the epidermis and the central nervous system), endoderm (later becoming muscles and connective tissues) and mesoderm (later becoming the epithelial lining of the gut and respiratory system) [63].

Induced pluripotent stem (iPS) cells, which are extremely similar to ES cells, are made by genetically reprogramming the adult cells such as human adult skin tissue and blood. The remarkable technique was invented by Dr Shinya Yamanaka and his colleagues at Kyoto University by using the transcription factors Oct3/4, Sox2, c-Myc and Klf4 on cells from human faces [64]. Since then, the technology of preparing iPS cells has advanced very rapidly and been used in numerous laboratories due to its ability to establish new cell lines from specific individuals and being free from ethical problems.

Somatic stem cells

Somatic stem cells include foetal stem cells and adult stem cells. Based on different sources, there are two types of foetal stem cells: foetal proper stem cells coming from the tissue of the foetus proper, and extraembryonic foetal stem cells from extraembryonic membranes. They are not immortal but multipotent with a high level of cell division [65]. However, due to the ethical issues of using cells from foetuses for disease treatment and the risks of intrauterine procedures to the pregnancy, their clinical usefulness has not been explored extensively [66].

The research on adult stem cells does not pose as many ethical issues as that on foetal stem cells. Adult stem cells, also called tissue-specific stem cells, play an important role in maintaining and repairing the tissues where they are found. Pluripotent adult stem cells can be found in umbilical cord blood and other tissues [67]. The adult stem cells from bone marrow have been used in disease treatment [68, 69]. However, the quantity of bone marrow stem cells declines with age.

1.3.2 Glycans expressed on stem cells

Glycans expressed on cell surfaces are tissue-specific and developmentally regulated. These glycans are therefore ideally suited as markers for identifying and isolating specific cell types, which is a major challenge in cell biology. ES cells and iPS cells are said to have highly similar glycomes [70]. Due to the prominent expression and lineage-specific signatures on stem cells, glycan surface antigens such as the stage-specific embryonic antigens (SSEA-1, SSEA-3, SSEA-4 and SSEA-5) and tumour-rejection antigens (TRA-1-60 and TRA-1-81) have been used as ES/iPS markers [71, 72]. Several markers are shared by tumour stem cells such as embryonal carcinoma (EC) cells derived from germ cell tumours. The overlap of ES/iPS cell markers with tumour stem cells causes uncertainty in the application of these markers in clinical research. The

glycans of stem cells include GAGs, N-glycan-, O-glycan-, glycolipid-associated glycans and free oligosaccharides as described below.

GAGs expressed on stem cells

Investigation of total cellular glycomics of human stem cells has shown that human ES and iPS cells share similar relative amounts of CSs and HS which however differ from the levels in human EC cells [70]. In the human ES/iPS cells, most HS chains are unsulphated and the predominant CS are 4-sulphated at GalNAc residues [70]. This trend is also shared by human EC cells, but the latter have HS that is slightly more sulphated [70]. An undersulphated KS-type chain that is recognized by a monoclonal antibody R-10G has been found to be expressed on human ES/iPS cells, but not on human EC cells [73]. The R-10G antigen therefore can be used to distinguish human ES/iPS cells from EC cells. HS (approximately 80% of the total) and CS (about 20%) are also the main GAG chains produced in mouse ES cells [74].

There is considerable information on the involvement of HS and CS in ES cell differentiation. The differentiation of ES cells results in increase of amount and sulphation of HS and CS chains, and a decrease of non-sulphated HS and an increase of N-sulphated HS are observed [74-76]. ES cells deficient in HS and CS tetrasaccharide linkage synthesis (GlcA transferase $I^{-/-}$) or HS-synthesis (EXT1 $^{-/-}$) retain their self-renewal potential but fail to initiate differentiation [77-79]. The GlcA transferase $I^{-/-}$ ES cells cultured with CSA or CSE restore the proliferative capacity, and the CS is suggested to be bound by a major component of extracellular matrix E-cadherin [77]. This is a calcium-dependent adhesion protein, a class-1 transmembrane protein that is found in epithelial tissues and mediates ES cell differentiation [77]. Wild-type ES cells treated with chondroitinase ABC have been reported to have difficulty in the generation of embryoid bodies that are three-dimensional aggregates of differentiating ES cells [77, 80]. Differentiation of the EXT1 $^{-/-}$ ES cells is restored by addition of HS and heparin; the

N- and 6-O-sulphate groups are essential for the rescue [79]. HS deficiency leads to defects in FGF signalling which triggers the transition of ES cells to lineage commitment [78]. These data suggest that both HS and CS chains are indispensable for differentiation of ES cells.

N-glycans expressed on stem cells

Satoma et al. (2009) first used mass spectrometric and nuclear magnetic resonance (NMR) spectroscopic analysis methods to investigate N-glycan profiles of four human ES lines, embryoid bodies, and stage 3 differentiated cells from the same lines [81]. Five neutral high-mannose sequences (Man5GlcNAc2, Man6GlcNAc2, Man7GlcNAc2, Man8GlcNAc2 and Man9GlcNAc2) and five acidic complex-type sequences (NeuAc1Gal2Man3GlcNAc4, NeuAc1Gal2Man3GlcNAc4Fuc1, NeuAc1Gal2Man3GlcNAc4Fuc2, NeuAc2Gal2Man3GlcNAc4Fuc1, and NeuAc1Gal3Man3GlcNAc5Fuc1) are the most abundant components shared by all the studied cell lines. Expression of other N-glycans is affected by the status of the cell lines. The amount of NeuAc1Gal2Man3GlcNAc4Fuc2 significantly decreased while the NeuAc1Gal2Man3GlcNAc5Fuc1 increased in the differentiated cells. Subsequently, comparison of N-glycans on ES and iPS cells towards differentiated cell lines was extensively studied using various methods [70, 82-85]. Comparable changes are observed in the expression of N-glycans during differentiation of ES and iPS cells [84]. Relative quantities of high Man type do not differ significantly during differentiation [84, 85], whereas non-reducing terminal Fuc types and type 1 (Gal β 1-3GlcNAc) decrease, and the type of linkage of sialic acid changes from NeuAc α 2-6 to NeuAc α 2-3 [82, 85]. High amounts of NeuAc α 2-6 are expressed on ES and iPS cells and they are crucial to the maintenance of the cell pluripotency as neuraminidase treatment induces differentiation [86]. This α 2-6 sialylation is also found to be a marker of the

differentiation potential in stem cells as strong binding with NeuAc α 2-6-specific lectins was specific for the cells with differentiation ability [87].

O-glycans expressed on stem cells

O-glycans extended with core 1 or 2 and terminated with β 1-3Gal were found to be abundant in human ES/iPS cells [70]. Several glycoprotein-associated glycan antigens that are specially expressed on the surface of ES/iPS cells have been used to define these cells (Table 1.3). BC2LCN antigen, which was found on only undifferentiated human ES/iPS cells but not differentiated somatic cells, requires Fuc α 1-2Gal β 1-3GlcNAc/GalNAc containing glycans such as H-type 1 (Fuc α 1-2Gal β 1-3GlcNAc), H type 3 (Fuc α 1-2Gal β 1-3GalNAc) and Lewis b [Fuc α 1-2Gal β 1-3(Fuc α 1-2)GlcNAc] [88, 89]. Alkaline treatment greatly reduced the binding of BC2LCN to the glycoprotein podocalyxin isolated from human ES/iPS cells, indicating that the major BC2LCN antigen-positive glycans are O-glycans [89, 90]. TRA-60 and TRA-1-81 antigens have been extensively used as biomarkers of human ES/iPS cells and they are also on human EC cells [91, 92]. The antigens are expressed on the glycoprotein podocalyxin and their expression decreases upon differentiation [93]. TRA-60 and TRA-1-81 antigens require a poly-N-acetyllactosamine-type tetrasaccharide with a type 1 sequence (Gal β 1-3GlcNAc) but not type 2 sequence (Gal β 1-4GlcNAc) at the non-reducing end [46]. The NeuAc α 2-3 form of the tetrasaccharide was not bound by the TRA-1-60 antibody. R-10G antibody was produced together with R-17F antibody, both of which specifically bound to human ES/iPS cells but exhibited little or no binding to hEC cells [73, 94]. R-10G antigen has been suggested to be KS-type chain on podocalyxin, whereas R-17F antigen is H-type 1 chain (Fuc α 1-2Gal β 1-3GlcNAc) on both podocalyxin and a glycolipid [95]. The R-17F antibody exhibits potent dose-dependent cytotoxicity to human ES/iPS cells [94]. The i antigen (Gal β 1-4GlcNAc β 1-3Gal β 1-4GlcNAc-) has been reported to be expressed on the surfaces of human mesenchymal stem cells but not

in their differentiated cells [96]. Mouse ES cells have been shown to contain large amounts of branched poly-N-acetylactosamine (I antigen) and its content progressively decreases during embryogenesis [97].

Glycolipids expressed on stem cells

Domination of globo and lacto series of glycosphingolipid glycans has been observed from human ES/iPS cells, and SSEA-3, SSEA-4 and SSEA-5 are all observed in the human stem cells [70]. SSEA-3 and -4 antigens are the stage-specific embryonic antigens from a glycolipid originally found on human teratocarcinoma cells [98]. SSEA-3 is the sequence $\text{Gal}\beta 1\text{-3GalNAc}\beta 1\text{-3Gal}\alpha 1$ while SSEA-4 is $\text{NeuAc}\alpha 2\text{-3Gal}\beta 1\text{-3GalNAc}\beta 1$ requiring sialylation of the non-reducing terminus [98]. These antigens diminished during differentiation of human EC cells. Expression of both antigens was also found on human ES/iPS cells and depletion of these antigens on human ES cells by inhibiting sphingolipid and glycosphingolipid biosynthesis did not affect other properties of pluripotency of the cells [99]. SSEA-5 antigen is $\text{Fuc}\alpha 1\text{-2Gal}\beta 1\text{-3GlcNAc}\beta 1\text{-3Gal}\beta 1\text{-4Glc}$, which is only expressed on undifferentiated but not on differentiated human ES cells [100]. SSEA-1 antigen which is Lewis-X, $\text{Gal}\beta 1\text{-4GlcNAc}(\text{Fuc}\alpha 1\text{-3})$, found on glycoproteins and glycolipids is expressed on murine but not human ES/iPS cells and its expression decreases upon differentiation [101-104]. R-17F antigen expressed on the glycoprotein podocalyxin is also on glycolipid [94].

Table 1.3. Surface antigens of human and mouse stem cells

Antigens	Status of stem cells	Human		Mouse		Structure of epitopes	Carrier molecule	References
		ES/iPS	EC	ES/iPS	EC			
TRA-1-60	Un-differentiated	Yes	Yes	N/A	No	Gal β 1-3GlcNAc β 1-3Gal β 1-4GlcNAc-	Podocalyxin	[91, 93, 105, 106]
	Differentiated	↓	↓	N/A	N/A			
TRA-1-81	Un-differentiated	Yes	Yes	Yes	No			
	Differentiated	↓	↓	N/A	N/A			
R-10G	Un-differentiated	Yes	No	Yes	N/A	Undersulphated KS	Podcalyxin	[73, 105]
	Differentiated	N/A	N/A	N/A	N/A			
R-17F	Un-differentiated	Yes	No	N/A	N/A	H-type 1: Fuc α 1-2Gal β 1-3GlcNAc-	Podcalyxin and glycolipid	[94, 95]
	Differentiated	↓	N/A	N/A	N/A			
BC2LCN	Un-differentiated	Yes	Yes	N/A	N/A	Fuc α 1-2Gal β 1-3GlcNAc/GalNAc-	Glycoprotein	[88-90]
SSEA-1	Un-differentiated	No	No	Yes	Yes	Lewis-X: Gal β 1-4GlcNAc- ₃ Fuc α 1	Glycoprotein and glycolipid	[101-104]
	Differentiated	↑	↑	↓	↓			
SSEA-3	Un-differentiated	Yes	Yes	No	No	Gal β 1-3GalNAc β 1-3Gal-	Glycolipid	[98]
	Differentiated	↓	↓	↑	↑			
SSEA-4	Un-differentiated	Yes	Yes	No	No	NeuAc α 2-3Gal β 1-3GalNAc-	Glycolipid	[98]
	Differentiated	↓	↓	↑	↑			
SSEA-5	Un-differentiated	Yes	N/A	N/A	N/A	H-type 1: Fuc α 1-2Gal β 1-3GlcNAc-	N/A	[100]
	Differentiated	↓	N/A	N/A	N/A			
I	Un-differentiated	N/A	N/A	Yes	Yes	Gal β 1-4GlcNAc β 1-6 Gal- Gal β 1-4GlcNAc β 1-3	N/A	[97, 107]
	Differentiated	N/A	N/A	↓	↓			
i	Un-differentiated	N/A	N/A	N/A	N/A	Gal β 1-4GlcNAc β 1-3Gal β 1-4GlcNAc-	N/A	[107]
	Differentiated	N/A	N/A	Yes	Yes			

Yes: The antigen is expressed; No: The antigen is not expressed; ↑: Expression of the antigen increases; ↓: Expression of the antigen decreases; N/A: No evidences have been reported so far.

1.4 Interactions of Glycosaminoglycans with Growth Factors and Morphogens

1.4.1 Fibroblast growth factors (FGFs)

FGF family is comprised of eighteen secreted signalling FGFs which interact with four cognate FGF receptors (FGFRs) and four intracellular non-signalling FGFs that regulate voltage-gated sodium channels [108]. Most FGFs share an internal core region of similarity with 28 highly conserved and six identical amino-acid residues [109]. FGFRs are members of the receptor tyrosine kinase family with several domains including three extracellular immunoglobulin-like domains (I, II and III), a transmembrane domain, and two intracellular tyrosine kinase domains (TK1 and TK2) [110].

Secreted FGFs are expressed in almost all tissues and organs and they signal by interacting with the FGFR to participate in several essential biological processes. At the cellular level, secreted FGFs regulate fundamental cellular processes that include positive and negative regulation of proliferation, survival, migration, differentiation, and metabolism [108]. FGFs regulate differentiation of the inner cell mass into epiblast and primitive endoderm lineages during early development, whereas later in development FGFs have key roles in organogenesis [111-114]. In the adult, FGFs have important roles in response to injury and tissue repair [115, 116].

1.4.2 Morphogens

Morphogens are a class of secreted signalling proteins that act on cells directly to induce distinct cellular responses in a dose-dependent manner [117, 118]. Possible mechanisms of the gradient of intracellular signals include diffusible inhibitor-initiated extracellular regulation, cell-cell contact between signal producing cells and distant

neighbours, and the transportation of signalling molecules by binding proteins like HS proteoglycans [117]. Morphogens are most commonly referred to as Wnts (the int/Wingless family), Hedgehogs (Hhs) and bone morphogenetic proteins (BMPs).

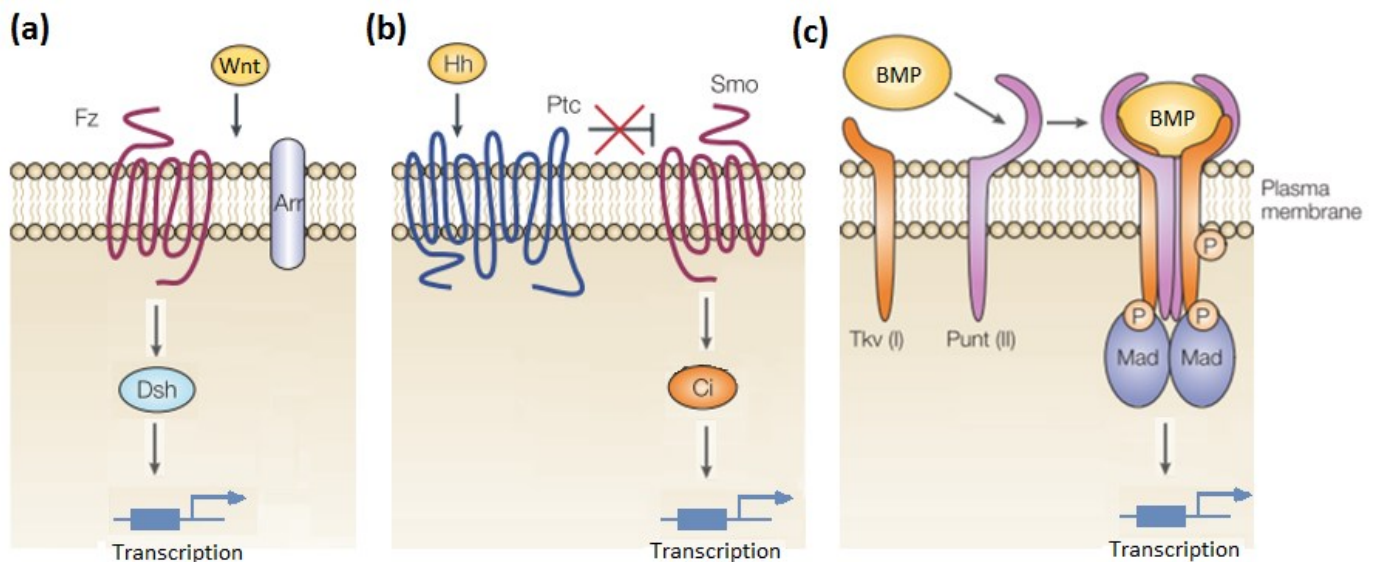


Figure 1.13. Signaling pathways of Wnt, Hh and BMP. Arr, Arrow; Fz, seven-transmembrane-domain protein Frizzled; Dsh, Dishevelled; Ptc, Patched; Smo, Smoothened; Ci, Cubitus interruptus transcriptional effector; Tkv, type I receptor Thick veins; Punt, type II receptor; Mad, transcription factor Mothers against BMP; P, phosphorylation. Figure is adapted from [119].

Wingless family

Wnt proteins are secreted, acylated, highly hydrophobic glycoproteins which are post-translationally modified by O-palmitoylation on a serine residue. The lipid is important for Wnt signalling as enzymatic removal of the palmitate or mutations of the modified cysteine result in loss of activity [120]. Wnt binds to the lipoprotein receptor-related protein 5/6 homologue Arrow (Arr) and the seven-transmembrane-domain protein Frizzled (Fz), which transmits a signal to the cytoplasmic protein Dishevelled (Dsh) to initiate the signalling pathway (Figure 1.13a). Wnt proteins such as Wnt3a are able to maintain the self-renewing fate of haematopoietic stem cells (HSCs). These proliferating cells maintain HSC activity; the donor-derived cells were found in transplanted mice [120]. Wnt signalling is involved in various events in animal

development, including the proliferation of stem cells, the induction of neural crest cells and tumourigenesis [120-123].

Hedgehog family

Hh family are expressed as transmembrane proteins on the surface of cells and play important signalling roles during embryonic development. Members of Hh family consist of Sonic hedgehog (Shh) (which has been extensively studied), Desert hedgehog (Dhh), Indian hedgehog (Ihh), Echidna hedgehog (Echidna hh) and Tiggly-winkle hedgehog (Twhh) [124]. They can act as both short- and long-range signalling molecules [125]. Hh signals can be transmitted between adjacent cells by cell-to-cell contact. Alternatively, a soluble HH generated by NH₂-terminal cleavage of Hh is able to diffuse through the microenvironment and interact with distal cells. A candidate receptor for Hh signalling, Patched (Ptc), has been identified, which is a transmembrane protein capable of forming a physical complex with another transmembrane protein, Smoothened (Smo) [125]. Hh proteins directly interact only with Ptc, but not Smo. Suppression of Ptc by binding to Hh permits Smo activation (Figure 1.13b) [126]. The activated Smo transmits a signal to the Cubitus interruptus (Ci) transcriptional effector, which induces target gene expression. Shh signalling has been reported to regulate the proliferation and differentiation of primitive human blood cells by modulating the morphogenic function of BMP-4, and the proliferative signals of Shh are BMP-4 dependent as Hh antibodies did not affect the downstream of BMP-4-induced proliferation [127]. Only some of these are shown in the Figure 1.12B.

Bone morphogenetic proteins

BMPs are a group of multi-functional growth factors of the transforming growth factor β (TGF β) superfamily. As their name indicates, BMP proteins such as BMP-2, -4, -5, -6, -7 and -9 are important factors regulating chondrogenesis (formation of cartilage) and

skeletogenesis (formation of bones) during normal embryonic development [128]. In addition, BMP proteins are involved in self-renewal of embryonic stem cells through suppression of neural differentiation and in osteoblastic differentiation (differentiation into bone formation cells) of mesenchymal stem cells [129, 130]. BMPs bind to transmembrane serine/threonine kinase called type I receptor (Thick veins, Tkv) and type II receptor (Punt). This results in the formation of a transient tetrameric complex (Figure 1.13c). The type I receptor is activated on phosphorylation by the type II receptor and transduces the signal by phosphorylating the cytoplasmic transcription factor Mothers against BMP (Mad), which then initiates the following target gene expression [131]. Extracellular secreted antagonists, such as Cerbarus, Dan, Gremlin and Noggin, also specifically bind to BMPs to regulate the initiation of the signalling cascade [132]. Treatment of stem cells with low concentrations of BMP-4 induced proliferation and differentiation whereas higher concentrations extended the length of time of maintaining repopulation capacity [133]. In comparison, BMP-2 or -7 at high concentrations inhibited proliferation but maintained the repopulation capacity [133].

1.4.3 Interactions between Glycosaminoglycans and growth factors and morphogens

Fibroblast growth factor signalling

Interaction of HS and FGFs has been extensively investigated. HS regulates the activity of extracellular FGFs involved in critically important cellular activities including angiogenesis, cellular proliferation, cellular motility, differentiation and adhesion. HS is thought to modulate the diffusion of FGFs through extracellular matrix and stabilize FGFs against degradation [134, 135]. The FGFs signal through the membrane-bound signalling tyrosine kinase, FGFR1-4, and an HS co-receptor. The generated FGF-FGFR-HS complex then activates the FGFR intracellular tyrosine kinase domain by

phosphorylation of specific tyrosine residues (The FGF signalling pathway). The activated acceptor is coupled to intracellular signalling pathways including the RAS-MAPK, PI3K-AKT, PLC γ and STAT pathways, which mostly regulate gene expression in nucleus [108].

Pellegrini et al. firstly described an asymmetrical model of FGF1-FGFR2c-heparin 2:2:1 ternary complex by crystallization (Figure 1.14) [136]. They found that a dimeric form of FGFR2c was induced by simultaneous binding to two FGF1 molecules and a heparin decasaccharide. The heparin chain was observed to interact with both FGF1 molecules and promote them into a dimer. The chain also bridges the two FGFR2c, but only binds to one of them. No FGF1 affinity was detected to the FGFR2c in absence of the heparin decasaccharide [137].

Based on crystallization of FGF2-FGFR1c-heparin, Schlessinger et al. proposed a different model of the ternary complex which was a symmetrical 2:2:2 (Figure 1.14) [138]. Here, the heparin chain contacted with both FGF and FGFR via its nonreducing ends and form a 1:1:1 FGF:FGFR:heparin ternary complex. A second complex is then recruited to the first complex via direct FGFR:FGFR contacts, followed by secondary interactions between FGF in one ternary complex and FGFR in the other ternary complex. The two heparin chains therefore stabilized the two FGF-FGFR dimers by augmenting direct FGFR-FGFR and secondary FGF-FGFR interactions.

Kinetic studies of interaction of FGF2, FGFR1c and heparin showed that the strongest binary interaction was the FGF2-heparin interaction, followed by the FGF2-FGFR1c and the relatively weak FGFR1c-heparin interaction [139]. The binding affinity of FGFR1c to FGF2-heparin was much greater than of the FGFR1c-FGF2 interaction. HS chain on the cell surface HSPG thus was proposed to electrostatically attract FGF to the cell surface first and then recruit the FGFRs.

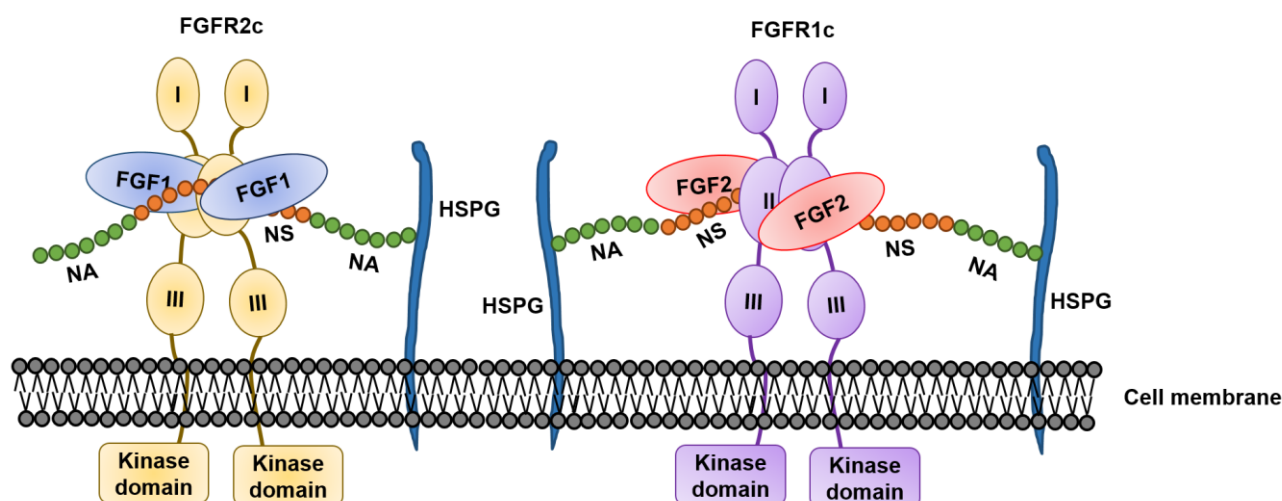


Figure 1.14. Schematic representation of FGF, FGFR and HS ternary complex at the cell surface. FGF1-FGFR2c-HS is an asymmetrical 2:2:1, and FGF2-FGFR1c-HS is a symmetrical 2:2:2. TK1 and TK2, intracellular tyrosine kinase domains; HSPG, heparin sulphate proteoglycan; NA, N-acetylated (NA) domains of HS; NS, N-sulphated (NS) domains of HS.

Heparin chains with contiguous sulphated domains which are *N*-sulphated disaccharide units, the NS domains, at the non-reducing domains are thought to interact with the FGF-FGFR protein complex [140, 141]. Chemo-enzymatically generated *N*-sulphated decasaccharides with differing *O*-sulphation positions were compared for their effects on FGF2-induced cell signalling. The heparin-like chain with 6-*O*-sulphate groups induced markedly enhanced stimulation of cell signalling compared with other decasaccharides with three to five differently distributed *O*-sulphate groups which had only a modest effect [140]. The FGF2 signalling was thought to require at least 4-5 disaccharide repeats of non-reducing NS domains and it was proposed that there is a requirement for non-reducing terminal NS to be flexible [142]. However, it is difficult to explain the lack of FGF2 signalling of another similarly GlcNAc-terminated HS chain with 5 disaccharide repeats of non-reducing GlcNS domains. In comparison, FGF1 signalling was proposed to rely on longer (~13) disaccharide repeats of non-reducing NS domains and on the non-reducing terminal NS [142].

Wingless signalling

HS also serves as a co-receptor at cell surface to enhance Wnt-mediated activation. The HS can stabilize the signalling activity by maintaining the solubility of Wnt proteins and facilitating their diffusion [143]. Gene mutation of glycosyltransferases of HS biosynthesis reduced Wnt signalling and the signalling was also observed to be regulated by a heparin-specific GlcNAc sulfatase [144, 145]. A HS structure containing IdoA2S and GlcNS6S/GlcNS6S3S has been reported to be bound by a Wnt signalling-inhibitory antibody [146].

On the other hand, HS is inhibitory in the presence of Notum that is an extracellular enzyme secreted as the only Wnt feedback inhibitor in the metazoan kingdom from planarians to humans (Figure 1.15) [147]. The shape of the hydrophobic pocket of Notum allows it to accommodate cis-unsaturated fatty acids like palmitoleate. This ability allows Notum to cleave the palmitoleate moiety of Wnt proteins to suppress their signalling activity, however, which requires the binding of Notum to HS as well [147]. Notum has been tested in our lab's GAG microarray and showed much stronger binding to heparin-derived oligosaccharides and relatively weaker binding to oligosaccharides derived from CSA, CSB, CSC and HS [147].

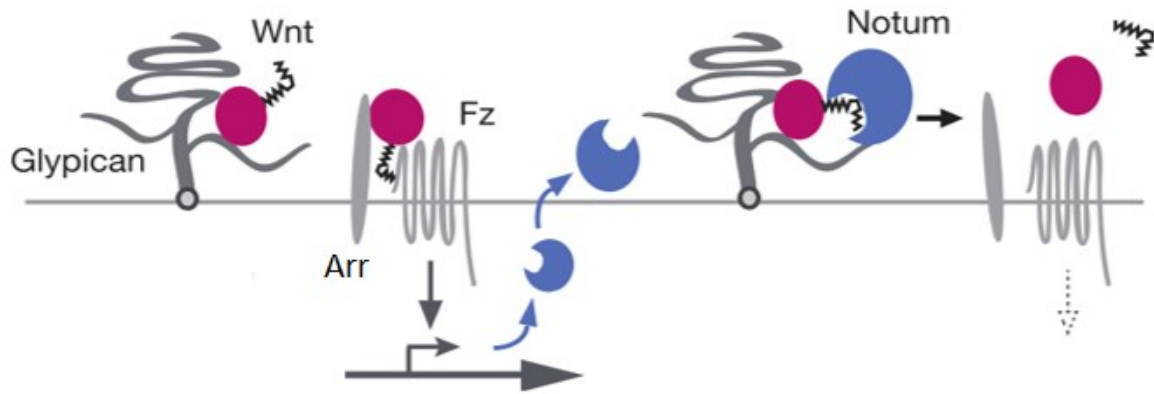


Figure 1.15. Schematic representation of feedback control by Notum. Notum deacylates Wnt in a glypican-assisted fashion. Arr, Arrow; Fz, seven-transmembrane-domain protein Frizzled. Figure is taken from [147].

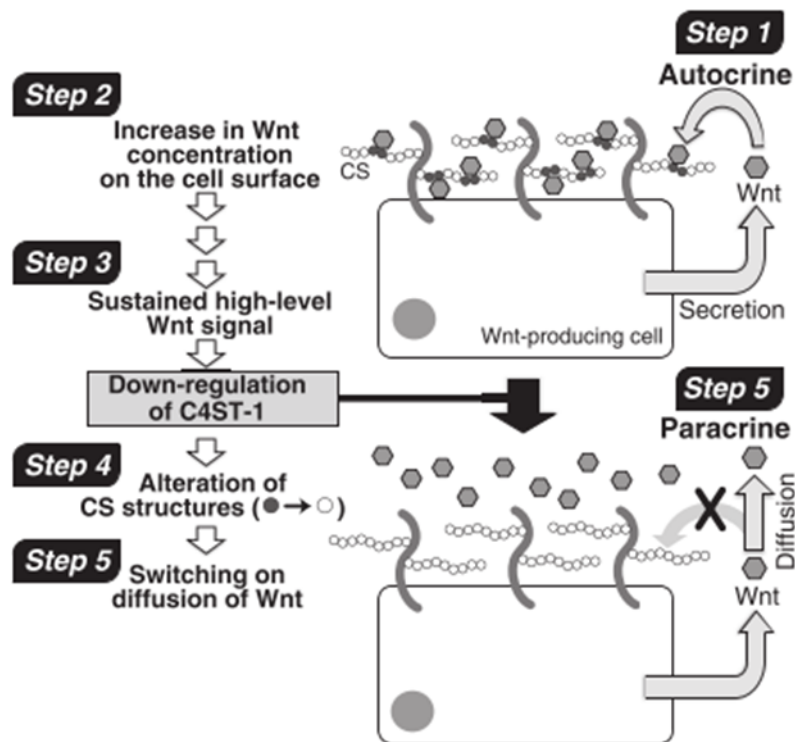


Figure 1.16. A model of CS-mediated regulation of Wnt-3a diffusion from Wnt-producing cells. C4ST-1, chondroitin 4-O-Sulphotransferase-1. Gray circles and white circles in step 4 indicate CS disaccharide structures with a high and low affinity to Wnt-3a, respectively. Figure is taken from [148].

In addition to HS, CS synthesized by 4-O-sulphotransferase-1 (C4ST-1) has been found to be bound by Wnt-3a with high affinity and the transferase expression was negatively regulated by Wnt signalling to trigger release of Wnt molecules from its producing cells (Figure 1.16) [148]. Signalling of Wnt molecules down-regulates 4-O-sulphotransferase-1 gene expression when the Wnt is caught by CS chains expressed on the cell surface or in extracellular matrix. The CS chains with altered sulphation patterns have low affinity toward Wnt proteins so that the diffusion of Wnt molecules is switched on.

Depressed Wnt-3a signalling in mouse L cell fibroblast mutant cells deficient in HS-synthesis (EXT1^{-/-}) and CS 4-O-sulphation (C4ST-1^{-/-}) has been found to be rescued by introduction of the C4ST-1 transferase but not of EXT-1 [149]. CSE but not CSA has been found to cause a reduction in early cardiac progenitors, but to promote later differentiation steps into mature cardiomyocytes through repressing Wnt-initiated β -catenin signalling in EB cultures [150]. CSE has been reported to be involved in a negative regulatory mechanism in breast cancer cell motility through interference with a pro-tumourigenic Wnt signalling [151].

Hedgehog signaling

All morphogens of the Hh family are synthesized as dual-lipidated proteins (a N-terminal signalling domain and a C-terminal cholesterol transferase domain), which results in their firm attachment to the surface of the cell in which they were produced. Secreted glycoprotein Scube2 (signal peptide, cubulin domain, epidermal-growth-factor-like protein 2) is implicated in the release of Shh from the cell membrane into the extracellular space through proteolytic removal of both lipidated peptides (shedding) [152]. HS acts as both positive and negative regulators of Shh function (Figure 1.17) [153]. Shh tethered to the cell membrane is supposed to be assembled into the glypican HS proteoglycan hubs, followed by attracting sheddases or sheddase

activators such Scube 2 [154]. The release depends on the sulphation degree of HS and occurs only when HS does not block access to the Cardin-Weintraub (CW) sheddase target site of Shh [153]. It might be impaired by increased electrostatic Shh-HS interactions.

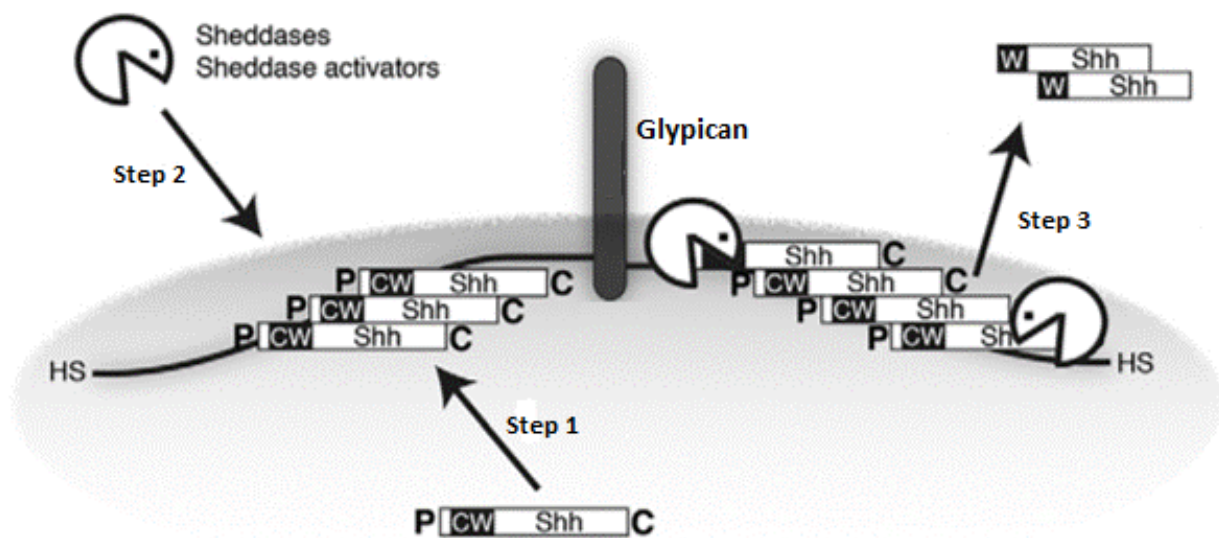


Figure 1.17. A model of Shh release by action of glypican HS proteoglycans. P, palmitate; C, cholesterol; CW, Cardin-Weintraub. Figure is adapted from [153].

Both HS and CS are found to be important in regulating the Hh signalling molecules. For example, deletion of the gene encoding a HS polymerizing enzyme causes abnormal signalling and restricted distribution of Hh in *Drosophila* [155]. Brachymorphic mouse model for undersulphated CS reveal the importance of sulphation on normal CS function in *Ihh* signalling, and the *Ihh* interacts with CS proteoglycan particularly CSA in the direct binding measurement [156]. The HS and CS-binding sites are the basic amino acids in the N-terminal region of the Hh proteins. KS has been also found to be bound by Shh [157].

Bone morphogenetic protein signalling

Both a number of BMPs and their antagonists interact with HS [158]. Action of BMPs is highly restricted to the region within and around the cells that produce them. A

widely established mechanism of restricting the BMPs diffusion is their binding to HS [159]. BMP-2 and BMP-4 have been reported to interact with HS via the basic cores of amino acids in their N-terminal region [159, 160]. HS and heparin but not desulphated heparin is found to enhance the biological activity of BMPs by protecting them from degradation and from inhibition by BMP antagonists, as well as continuously serving the ligands to their receptors on the cell membranes [161-163]. BMP antagonists such as Noggin bind to BMP4 to inhibit its function by preventing interaction with receptors on the cell surface [164]. The BMP antagonists also bind to HS and the HS-binding domain is separate from that which binds BMP [164].

1.5 Micro-methods for Investigating Glycan-protein Interactions

1.5.1 Carbohydrate microarray methods to investigate glycan-protein interactions

Carbohydrate microarrays first described in 2002 are arrays of immobilized glycans, glycoproteins and polysaccharides on solid surfaces for biological research and biomedical application. The microarray-based technologies have been developed as versatile platforms for high throughput analysis of glycan-protein interactions.

Wang et al. presented a method for non-covalently immobilizing polysaccharides and glycoproteins on a nitrocellulose-coated glass slide [165], whereas Willats et al. used black polystyrene-coated slides [166], (Figure 1.18a). The retention of the polysaccharides on the arrayed slides after the repeated washing steps in the analysis was found to differ for different micromolecules and also to be dependant on their molecular weights. Dextran preparations ranging from 20 kDa to 2,000 kDa and inulin of 3.3 kDa were reported to be well retained on nitrocellulose-coated glass slides [165].

Microarray of sequence-defined oligosaccharides originate from the neoglycolipid (NGL) technology introduced by Feizi and colleagues [167], (Figure 1.18b). This was to enable oligosaccharides immobilized on solid surfaces for studying the antigenicities and receptor functions. NGL technology is the core of the first microarray system that was introduced for sequence-defined oligosaccharides isolated from natural sources and chemical synthesis [168]. The lipid-linked glycans which also include natural glycolipids are arrayed in the liposomal formulation in the presence of carrier lipids. Immobilized glycans on the array surface display multivalency (cluster effect) unlike in solution. To some extent, they mimic display on the surface of cells [169].

Other noncovalent immobilization methods have been developed, including a fluororous-based array of C8F17-tagged monosaccharaides on fluorinated solid supports (Figure 1.18c), biotinylated glycans immobilized on a 384-well streptavidin-coated plate via biotin-streptavidin interaction (Figure 1.18d), and GAG polysaccharides printed on poly-lysine-coated slides via electrostatic interaction (Figure 1.18e) [170-172]. Another similar approach already used in protein microarrays was immobilization of oligosaccharides using glycoconjugate molecules that present a DNA sequence for anchoring onto DNA chips through hybridization [173], (Figure 1.18f). An approach based on direct attachment of unmodified oligosaccharides onto a gold surface as a hydrazide-derivatized, self-assembled monolayer has been reported (model not shown) [174].

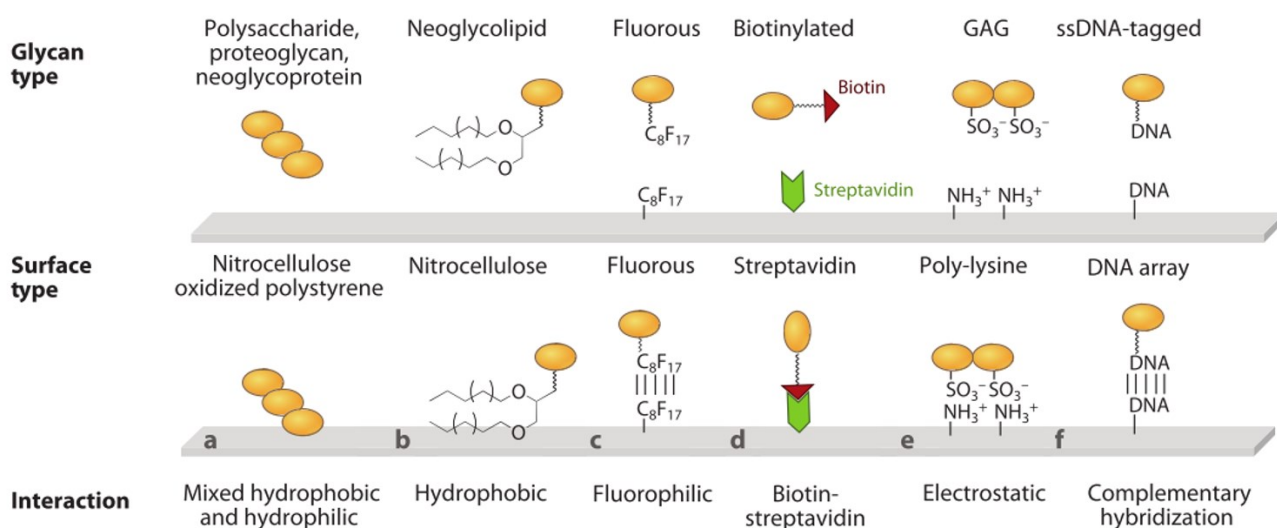


Figure 1.18. Non-covalent immobilization methods for the preparation of carbohydrate microarrays. Figure is adapted from [1].

In addition, several covalent immobilization-based strategies have been reported. Shin's group prepared maleimide conjugates of glycans and attached them to the thiol groups modified-glass slides by hetero-Michael reaction between the maleimide moiety and the thiol group [175, 176], (Figure 1.19-a1). The reverse combination was employed, that synthetic oligosaccharide structures were immobilized covalently on bovine serum albumin-coated glass slides by way of a hydrophilic thiol linker [177], (Figure 1.19-a2). Disulphide bridge reactions were utilized for immobilisation of galactosyl residues onto pulsed plasma deposited polyallylmercaptan [178], (Figure 1.19-a3).

In addition to thiol chemistry, amine chemistry has been extensively employed for arraying glycans. Schwarz et al. have immobilized p-aminophenyl glycosides on glass slides modified with cyanuric chloride and patterned with a hydrophobic Teflon mask [179], (Figure 1.19-b1). Blixt et al. have described a carbohydrate microarray in which amine functionalized glycans are immobilised on the N-hydroxysuccinimide (NHS) ester-derivatized glass slides [180], (Figure 1.19-b2). This is the basis of the Consortium of Functional Glycomics (CFG) microarrays. The immobilization method has been

extended to fluorescently labelled glycans (glycan-2,6-diaminopyridine conjugates) [181]. Cumming's group has then developed a carbohydrate microarray by printing the fluorescently labelled glycans on epoxy-activated glass slides [181, 182], (Figure 1.19-b3). Heparin oligosaccharides with 2,5-anhydromannose unit at the reducing end, which were obtained by nitrous acid treatment were attached to amine-coated slides [183], (Figure 1.19-b4). The reverse combination was employed by immobilisation of CS oligosaccharides sequentially derivatized with allyl after ozonolysis and 1,2-(bisaminooxy) ethane treatment to aldehyde-coated glass slides [184], (Figure 1.19-b5).

Cycloadditions was also described. Mrksich and colleagues have described a microarray in which cyclopentadiene-containing monosaccharides were covalently immobilized on a benzoquinone-coated gold surface by a Diels-Alder reaction [185], (Figure 1.19-c1). Wong's group developed a microarray that includes carbohydrates by using cycloaddition reaction between azide-containing substances and alkynylated lipids covalently attached to microtitre plates [186], (Figure 1.19-c2).

An alternative method for the covalent attachment of underivatized glycans is photoimmobilization such as utilization of photoactivatable dextran-coated analytical platforms (Figure 1.19-d1) and phthalimide-amine monolayers-coated slides (Figure 1.19-d2) [187, 188]. Another example is the recently reported microbial glycan microarray of characterized bacterial polysaccharides on NHS-activated glass slides [189].

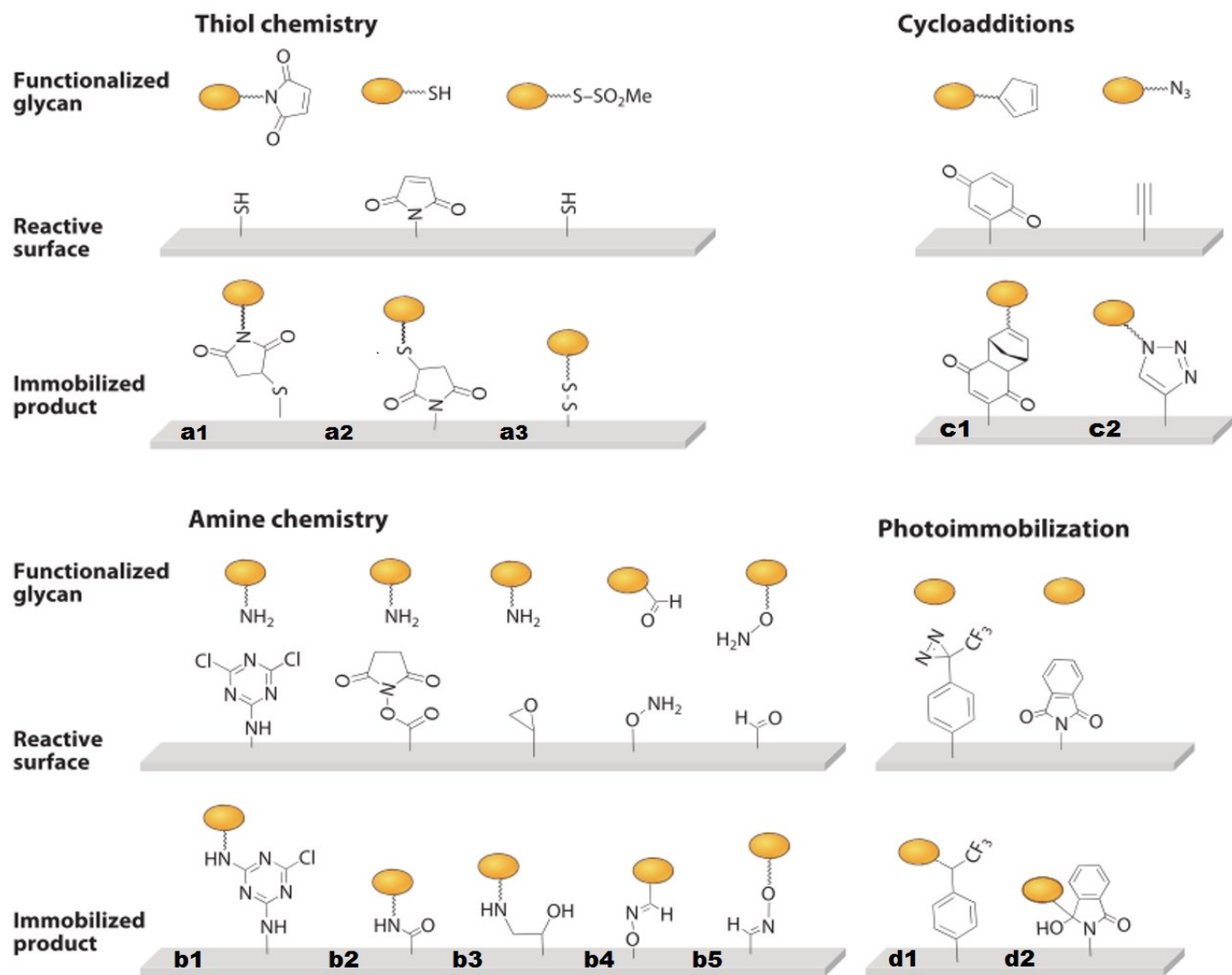


Figure 1.19. Covalent immobilization methods for the preparation of carbohydrate microarrays.
Adapted from [1].

1.5.2 Carbohydrate microarray system based on the neoglycolipid technology

Currently there are two carbohydrate microarray platforms with large coverage of glycan sequences. One is the NGL-based carbohydrate microarray facilities in the Glycoscience Laboratory, the other is that of the CFG built by National Institute of General Medical Sciences in the US.

As mentioned above, NGL technology was developed in 1985 to address the need for a sensitive microtechnique for direct binding analyses with oligosaccharides to study recognition by carbohydrate-recognizing molecules [167]. Inspired by the thin-layer chromatography (TLC)-binding method for glycolipids, an amino phospholipid and reductive amination were selected as the conjugation principle of oligosaccharides to make artificial glycolipids, NGLs [190, 191]. This method was used for reducing oligosaccharides and for reductively released O-glycans after mild periodate oxidative cleavage [192]. The resulting NGLs could be immobilized on matrices in a highly desirable, clustered state or direct-binding analyses. The NGLs in the TLC-binding experiments were found to have excellent ionization properties in MS.

Almost two decades later, the NGL technology became the basis of a microarray platform for assigning oligosaccharide ligands for carbohydrate-binding proteins [193, 194]. The oligosaccharides are converted into NGLs by conjugation with 1,2-dihexadecyl-sn-glycerol-3-phosphoethanolamine (DHPE) or into fluorescent NGLs by N-aminoacetyl-N-(9-anthracenylmethyl)-1,2-dihexadecyl-sn-glycerol-3-phosphoethanolamine (ADHP) via reductive amination (Figure 1.20) [191]. These NGLs are ready for immobilization on nitrocellulose plates through non-covalent hydrophobic interactions. The advantage of the ADHP fluorescent label is that it allows quantification of the amount of retained sugar on the TLC plate by comparing the

fluorescence intensity before and after washing, otherwise the NGLs are detected by staining with primulin.

The strategy for generation of NGLs by reductive amination results in ring-opening of the reducing-end sugar. This may limit applicability to short-chain oligosaccharides and thus may affect their recognition by carbohydrate-binding proteins. The majority of recognition motifs are satisfactorily presented with NGLs of longer oligosaccharides. An attractive alternative to overcoming the ring opening is oxime ligation by conjugating reducing oligosaccharides with an aminooxy-functionalized DHPE (AOPE) [195], (Figure 1.20). This approach was introduced by Dr Yan Liu in the Glycosciences Laboratories. A significant proportion of NGLs produced by oxime ligation have ring closed ends and the yield of conjugates is high for long oligosaccharides and for highly sulphated oligosaccharides such as GAG chains. The NGL-based microarray system of Glycoscience Laboratory currently contains ~830 sequence-defined probes derived from a variety of natural sources or chemically synthesized [196]. These probes include oligosaccharide from N-glycans (high mannose, complex and hybrid), O-glycans (mucin type, O-fucosyl, O-mannosyl), glycolipids (neutral, sialylated and sulphated sequences), blood group antigen-related sequences (A, B, H, Lewis a, b, x and y and their sialylated and sulphated forms), poly-N-acetyllactosamines, GAGs as well as fungal, bacterial and plant polysaccharides. These NGL and glycolipid probes are robotically dispensed onto nitrocellulose-coated glass slides at low fmol levels in a liposome formulation in the presence of carrier lipids. This mode of presentation allows an element of mobility, simulating to some extent the cell surface display of glycans.

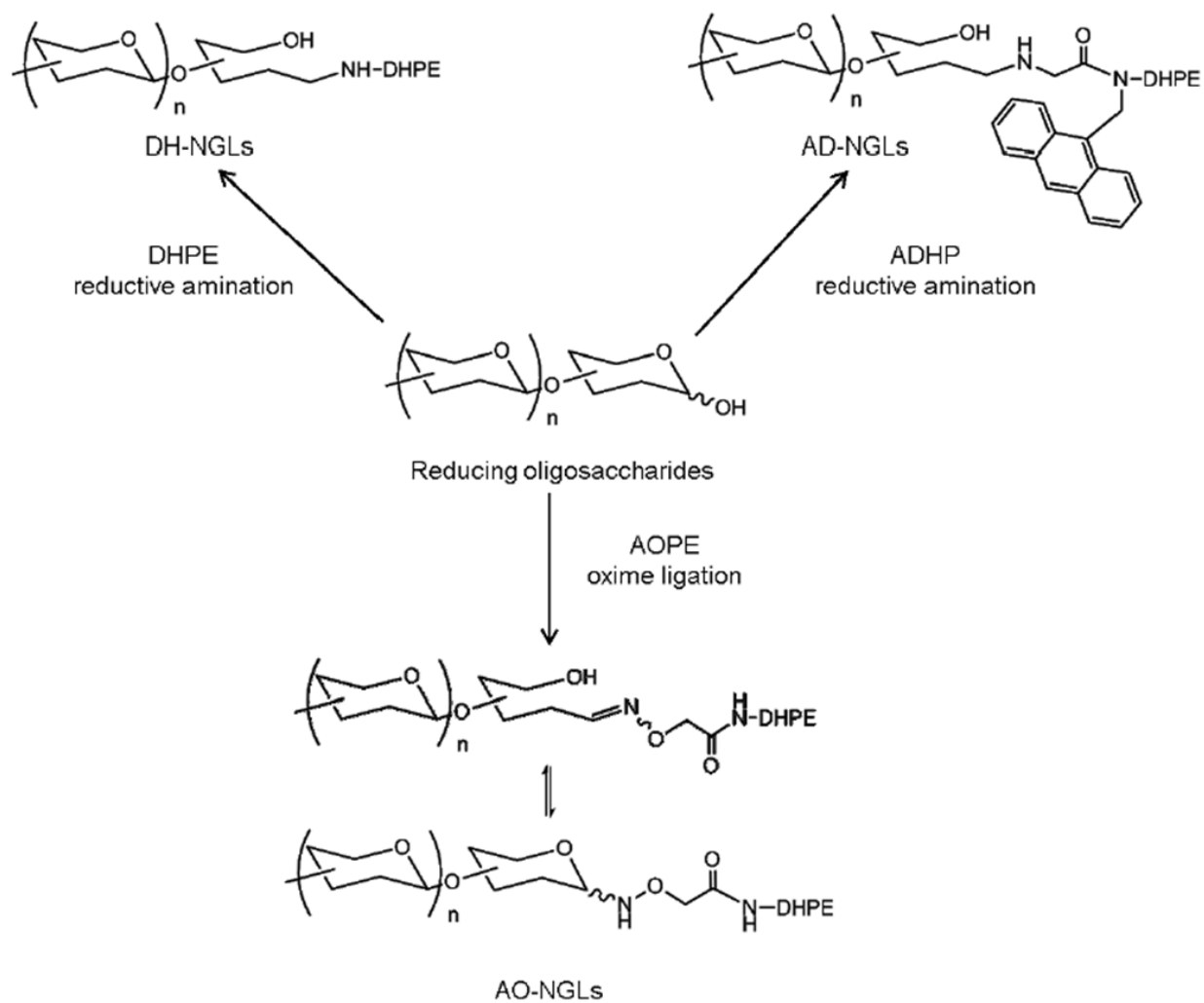


Figure 1.20. Reaction schemes for preparation of NGLs from reducing oligosaccharides. DHPE and ADHP conjugation is by reductive amination and AOPE conjugation is by oxime ligation. Figure is adapted from [195].

An integral component of the microarray system is a database that holds all of the microarray data, the experimental conditions and information on saccharide probes and proteins (Figure 1.21). There is an associated interactive software for presentation of microarray data, filtering, sorting and deep mining of every data point, established by Dr Mark Stoll in our laboratory [197]. The software is being revised to accept a wide range of experimental formats and to be made available to the scientific community.

The NGL technology lends itself well to generating 'designer' microarray approach which is a term used for microarrays of NGLs derived from ligand-bearing glycomes [169, 198, 199]. This is to reveal the harboured oligosaccharide ligands by depolymerization of ligand-positive glycoproteins and polysaccharides, conversion of the oligosaccharide fractions into NGLs, their isolation and structural characterization. The approach is exemplified by the steps in the characterization of a human stem cell antigen R-10G, which is the subject of Chapter 4 in this thesis.

Chapter 2

Materials and Methods

2.1 Materials

All of the chemical reagents used in my thesis were purchased from Sigma unless stated otherwise.

2.1.1 Solvents for high performance thin layer chromatography (HPTLC) plate staining

Table 2.1. Solvents for HPTLC plate staining

Name	Preparation
Orcinol spray reagent	0.9 g orcinol monohydrate was dissolved in 25 mL H ₂ O, added with 375 mL ethanol and 50 mL concentrated H ₂ SO ₄ . The solution was then stirred and cooled to 4°C, and kept at ambient temperature.
Primulin stock solution	Primulin was dissolved in acetone/H ₂ O (v/v), 1:9 to generate a solution with 1 mg/mL. The solution was kept at 4°C.
Primulin spray reagent	The primulin stock solution was diluted to 1:100 in acetone/H ₂ O (v/v), 4:1, and kept at ambient temperature.
Primulin TLC staining reagent	The primulin stock solution was diluted to 1:100 in phosphate buffered solution (PBS) buffer, which was freshly prepared.

2.1.2 Proteins and antibodies

Table 2.2. Proteins

Protein (Abbreviation)	Company or provider	Catalogue No.	Dilution or concentration used
Human factor H	Frederick Gyapon Quast	Not known	50 µg/mL
Recombinant Human Wnt-3a	R&D system	5036-WN	10 µg/mL
Human sonic hedgehog (Shh)	Novusbio	NBP2-35265	10 µg/mL
Human fibroblast growth factor 1 (FGF1)	Sino Biological Inc.; Abcam	10013-HNAE	20-40 µg/mL
Human fibroblast growth factor 2 (FGF2)	Sino Biological Inc.	10014-HNAE	20 µg/mL
Recombinant human/mouse/rat bone morphogenetic protein 2 (BMP-2)	R&D system	355-BM	10 µg/mL
Recombinant human bone morphogenetic protein 4 (BMP-4)	R&D system	314-BP	10 µg/mL

Table 2.3. Antibodies

Antibody	Isotype	Company or provider	Catalogue No.	Dilution or concentration used
R-10G	Mouse IgG	Millipore	MABD151	10-100 µg/mL
MZ15	Mouse IgG	Fiona M. Watt	N/A	10 µg/mL
1B4	Mouse IgG	Bruce Caterson	N/A	0.76 µg/mL
5D4	Mouse IgG		N/A	1/100*
CS56	Mouse IgM	Sigma	C8035	1/200*
Anti-CS Di4S	Mouse IgG	Seikagaku	270432-CS	1/200*
Anti-CS Di6S	Mouse IgM		270433-CS	1/200*
TRA-1-60	Mouse IgM	Santa Cruz Biotechnonology	sc-21705	50 µg/mL
TRA-1-81	Mouse IgM		sc-21706	50 µg/mL
anti-i P1A ELL1	Human IgM	Glycosciences Laboratory	N/A	1/200*
Human/Mouse Wnt-3a antibody	Rat IgG	R & D system	MAB1324	2µg/mL
Human/Mouse Shh N-Terminus antibody	Rat IgG		MAB464	2µg/mL
Human FGF2 antibody	Mouse IgG		MAB233	5 µg/mL
Human FGF1 antibody	Mouse IgG		MAB232	5 µg/mL
Human BMP-2/BMP-4 antibody	Mouse IgG		MAB3552	2 µg/mL
Goat anti-human Factor H	Goat IgG	Frederick Gyapon Quast	Not known	200 µg/mL
Biotin anti-mouse IgG	Not known	Sigma	B7264	2 µg/mL
Biotin anti-mouse IgM	Not known		B9265	5 µg/mL
Biotin anti-goat IgG	Not known		B7014	8 µg/mL
Biotin anti-rat IgG	Not known	Vector	BA-4000	5 µg/mL

Biotin anti-human IgM	Not known		BA-3020	2.5 µg/mL
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* asterisks are five antibodies of which concentrations are not available, but dilutions are recommended.

2.1.3 Glycosidases

Table 2.4. Glycosidases

Glycosidase	EC No.	Source	Company provider or	Catalogue No.
Chondroitinase ABC	4.2.2.4	<i>Proteus vulgaris</i>	Sigma	9024-13-9
Heparinase I	4.2.2.7	<i>Flavobacterium heparinum</i>	IBEX Technologies	9025-39-2
Heparinase III	4.2.2.8			37290-86-1
Keratanase I	3.2.1.103	<i>Pseudomonas sp.</i>	Seikagaku	E60120
Keratanase II	3.2.1	Not known	Robert J. Linhardt	N/A

Please note that to avoid confusion in the names of the two KS specific enzymes, the term 'keratanase I' was used throughout the thesis instead of the commonly used unqualified 'keratanase'.

2.1.4 Polysaccharides

Table 2.5. Polysaccharides

Polysaccharide(Abbreviation)	Source	Company provider or	Catalog No.
Keratan sulphate (KS)	Bovine cornea	Robert J. Linhardt	N/A
	Chicken egg white		N/A
	Bovine articular cartilage	Gavin M. Brown	N/A
Hyaluronan (HA)	Bovine vitreous humour	Sigma	H7630
Heparin (Hep)	Porcine intestinal mucosa	Sigma	H3149
Heparan sulphate (HS)	Bovine kidney	Sigma	H7640
	Porcine intestinal mucosa, Fraction I	Celsus Laboratories	HO-3105 ^a
		Glycosciences Laboratory ^b	N/A
Chondroitin sulphateA (CSA)	Bovine trachea	Sigma	C8529
Chondroitin sulphate B (CSB)	Bovine mucosa	Sigma	C3788
Chondroitin sulphate C (CSC)	Shark cartilage	Sigma	C4384
Dextran T2000 (Dex)	Bacterial product	Pharmacosmos	5510 2000 9007
Dextran sulphate (DexS)	Dextran esterified with sulfuric acid	Sigma	42867

a Four batches of HS from Celsus Laboratories were used in the thesis. The Lot No of the deAc-HS described in Chapter 3 is HO-01214. b HS-1A, -1B and 1C were prepared as described [200].

2.1.5 Oligosaccharides and neoglycolipids

Synthesized and natural source-derived oligosaccharides were used in my thesis (Table 2.6). The latter was prepared by my colleague Dr Frederick Gyapon Quast in Glycosciences Laboratory, including CSB 2-20 mers obtained by gel filtration chromatography of the CSB from bovine mucosa after digestion with chondroitinase ABC as described [201, 202] and heparin 2-20 mers obtained by gel filtration chromatography of the heparin (porcine intestinal mucosa) partially depolymerized with heparinase I as described [203].

NGLs of oligosaccharides of HS and HA were included in my thesis which were prepared by my colleagues, Dr Wengang Chai and Dr Yan Liu, in Glycosciences Laboratory (Table 2.7). HS 4-6 mers were obtained from nitrous acid treatment of HS (porcine intestinal mucosa) as described [204], and were conjugated with AOPC by oxime ligation as described [195]. HA 4-12 mers were obtained from partially hydrolysed bovine vitreous humour HA by hyaluronidase [205], and were converted to NGLs by reductive amination with the lipid DHPE [191].

Table 2.6. Oligosaccharides

Designation	Sequences	Company or provider	Structural features
Tetra-0S	Gal β 1-4GlcNAc β 1-3Gal β 1-4GlcNAc	Tokyo Chemical Industry	poly- <i>N</i> -acetyllactosamine
Tetra-4S	Gal β 1-4GlcNAc β 1-4Gal β 1-4GlcNAc SU-4 SU-4 SU-4 SU-4		
CSB-2*	Δ UA-3GalNAc SU-4	Dr Frederick Gyapon Quast	GAG
CSB-4*	Δ UA-3GalNAc β -4IdoA α -3GalNAc SU-4 SU-4		
CSB-6*	Δ UA-3GalNAc β -4IdoA α -3GalNAc β -4IdoA α -3GalNAc SU-4 SU-4 SU-4		
CSB-8*	Δ UA-3GalNAc β -4IdoA α -3GalNAc β -4IdoA α -3GalNAc β -4IdoA α -3GalNAc SU-4 SU-4 SU-4 SU-4		
CSB-10*	Δ UA-3GalNAc β -4IdoA α -3GalNAc β -4IdoA α -3GalNAc β -4IdoA α -3GalNAc β -4IdoA α -3GalNAc SU-4 SU-4 SU-4 SU-4 SU-4		
CSB-12*	Δ UA-3GalNAc β -4IdoA α -3GalNAc β -4IdoA α -3GalNAc β -4IdoA α -3GalNAc β -4IdoA α -3GalNAc β -4IdoA α -3GalNAc SU-4 SU-4 SU-4 SU-4 SU-4 SU-4		
CSB-14*	Δ UA-3GalNAc β -4IdoA α -3GalNAc β -4IdoA α -3GalNAc β -4IdoA α -3GalNAc β -4IdoA α -3GalNAc β -4IdoA α -3GalNAc β -4IdoA α -3GalNAc SU-4 SU-4 SU-4 SU-4 SU-4 SU-4 SU-4		
CSB-16*	Δ UA-3GalNAc β -4IdoA α -3GalNAc β -4IdoA α -3GalNAc β -4IdoA α -3GalNAc β -4IdoA α -3GalNAc β -4IdoA α -3GalNAc β -4IdoA α -3GalNAc β -4IdoA α -3GalNAc SU-4 SU-4 SU-4 SU-4 SU-4 SU-4 SU-4 SU-4		
CSB-18*	Δ UA-3GalNAc β -4IdoA α -3GalNAc β -4IdoA α -3GalNAc β -4IdoA α -3GalNAc β -4IdoA α -3GalNAc β -4IdoA α -3GalNAc β -4IdoA α -3GalNAc β -4IdoA α -3GalNAc β -4IdoA α -3GalNAc SU-4 SU-4 SU-4 SU-4 SU-4 SU-4 SU-4 SU-4 SU-4		
CSB-20*	Δ UA-3GalNAc β -4IdoA α -3GalNAc β -4IdoA α -3GalNAc β -4IdoA α -3GalNAc β -4IdoA α -3GalNAc β -4IdoA α -3GalNAc β -4IdoA α -3GalNAc β -4IdoA α -3GalNAc β -4IdoA α -3GalNAc SU-4 SU-4 SU-4 SU-4 SU-4 SU-4 SU-4 SU-4 SU-4 SU-4		
Hep-2*	SU-2 Δ UA-4GlcNS SU-6		

Hep-4*	SU-6 ΔUA-4GlcNSα-4IdoAα-4GlcNS SU-6 SU-2 SU-6	Dr Frederick Gyapon Quast	GAG
Hep-6*	SU-2 ΔUA-4GlcNSα-4IdoAα-4GlcNSα-4IdoAα-4GlcNS SU-6 SU-2 SU-6 SU-2 SU-6		
Hep-8*	SU-2 ΔUA-4GlcNSα-4IdoAα-4GlcNSα-4IdoAα-4GlcNSα-4IdoAα-4GlcNS SU-6 SU-2 SU-6 SU-2 SU-6 SU-2 SU-6		
Hep-10*	SU-2 ΔUA-4GlcNSα-4IdoAα-4GlcNSα-4IdoAα-4GlcNSα-4IdoAα-4GlcNSα-4IdoAα-4GlcNS SU-6 SU-2 SU-6 SU-2 SU-6 SU-2 SU-6 SU-2 SU-6		
Hep-12*	SU-2 ΔUA-4GlcNSα-4IdoAα-4GlcNSα-4IdoAα-4GlcNSα-4IdoAα-4GlcNSα-4IdoAα-4GlcNSα-4IdoAα-4GlcNS SU-6 SU-2 SU-6 SU-2 SU-6 SU-2 SU-6 SU-2 SU-6 SU-2 SU-6		
Hep-14*	SU-2 ΔUA-4GlcNSα-4IdoAα-4GlcNSα-4IdoAα-4GlcNSα-4IdoAα-4GlcNSα-4IdoAα-4GlcNSα-4IdoAα-4GlcNSα-4IdoAα-4GlcNS SU-6 SU-2 SU-6 SU-2 SU-6 SU-2 SU-6 SU-2 SU-6 SU-2 SU-6 SU-2 SU-6		
Hep-16*	SU-2 ΔUA-4GlcNSα-4IdoAα-4GlcNSα-4IdoAα-4GlcNSα-4IdoAα-4GlcNSα-4IdoAα-4GlcNSα-4IdoAα-4GlcNSα-4IdoAα-4GlcNS SU-6 SU-2 SU-6 SU-2 SU-6 SU-2 SU-6 SU-2 SU-6 SU-2 SU-6 SU-2 SU-6 SU-2 SU-6		
Hep-18*	SU-2 ΔUA-4GlcNSα-4IdoAα-4GlcNSα-4IdoAα-4GlcNSα-4IdoAα-4GlcNSα-4IdoAα-4GlcNSα-4IdoAα-4GlcNSα-4IdoAα-4GlcNS SU-6 SU-2 SU-6 SU-2 SU-6 SU-2 SU-6 SU-2 SU-6 SU-2 SU-6 SU-2 SU-6 SU-2 SU-6		
Hep-20*	SU-2 ΔUA-4GlcNSα-4IdoAα-4GlcNSα-4IdoAα-4GlcNSα-4IdoAα-4GlcNSα-4IdoAα-4GlcNSα-4IdoAα-4GlcNSα-4IdoAα-4GlcNS SU-6 SU-2 SU-6 SU-2 SU-6 SU-2 SU-6 SU-2 SU-6 SU-2 SU-6 SU-2 SU-6 SU-2 SU-6 SU-2 SU-6		

SU is for sulphate. * Asterisks that follow the names of certain probes indicate that predominant sequences are shown.

Table 2.7. NGLs

Designation	Sequences	Company or provider	Structural features
HA-4-DH	GlcA β -3GlcNAc β -4GlcA β -3GlcNAc-DH	Dr Wengang Chai and Dr Yan Liu	GAG
HA-6-DH	GlcA β -3GlcNAc β -4GlcA β -3GlcNAc β -4GlcA β -3GlcNAc-DH		
HA-8-DH	GlcA β -3GlcNAc β -4GlcA β -3GlcNAc β -4GlcA β -3GlcNAc β -4GlcA β -3GlcNAc-DH		
HA-10-DH	GlcA β -3GlcNAc β -4GlcA β -3GlcNAc β -4GlcA β -3GlcNAc β -4GlcA β -3GlcNAc β -4GlcA β -3GlcNAc-DH		
HA-12-DH	GlcA β -3GlcNAc β -4GlcA β -3GlcNAc β -4GlcA β -3GlcNAc β -4GlcA β -3GlcNAc β -4GlcA β -3GlcNAc β -4GlcA β -3GlcNAc-DH		
HS-4-AO	GlcA β -4GlcNAc α -4GlcA β -4aMan-AO (sulphation at various positions)		
HS-6-AO	GlcA β -4GlcNAc α -4GlcA β -4GlcNAc α -4GlcA β -4aMan-AO (sulphation at various positions)		
HS-8-AO	GlcA β -4GlcNAc α -4GlcA β -4GlcNAc α -4GlcA β -4GlcNAc α -4GlcA β -4aMan-AO (sulphation at various positions)		

AO is for AOPE and DH is for DHPE, which are different lipid tags for NGL preparation.

2.2. Partial Depolymerisation of Glycosaminoglycan Polysaccharides

2.2.1 Chondroitinase ABC digestion of chondroitin sulphate A and C

CSA and CSC were partially depolymerized as described by Suzuki and his colleagues [201, 202]. Briefly, each polysaccharide (225 mg) was incubated with 0.56 units of chondroitinase ABC in 4 mL NaH_2PO_4 buffer (5 mM, 200mM NaCl, pH 7.0) at 30°C. The reactions were monitored by ultraviolet (UV) absorbance at 232 nm for the 4,5-unsaturated uronic acid generated after cleavage of the glycosidic bonds between uronic acid and *N*-acetyl-glucosamine. The reactions were stopped by heating the reaction solutions in a water bath at 100°C for 1 min. The reaction mixtures were freeze-dried for further analyses.

2.2.2 Heparinase III digestion of heparan sulphate

Partial digestion of HS was carried out as described by Rice and Linhardt for heparin [203]. Briefly, the polysaccharide (200 mg) was incubated with 0.4 units of heparin lyase III in 6.5 mL NaH_2PO_4 buffer (5 mM, 200 mM NaCl, pH 7.0) at 30°C. The digestions were monitored by UV absorbance at 232 nm for the 4,5-unsaturated uronic acid. The reactions were stopped by heating the reaction solutions in a water bath at 100 °C for 1 min. The reaction mixtures were freeze-dried for further analyses.

2.2.3 Keratanase I digestion of keratan sulphate

The conditions of KS treatment were as described by Nakazawa et al. [206]. Briefly, KS (10 mg) was incubated with 0.5 units of keratanase I in 0.5 mL Tris-HCl buffer (20 mM, pH 7.4) at 37°C. The reaction was monitored with a Superdex Peptide column. A few μl of reaction solution was injected, and the elution was by NH_4OAc (50 mM) at a flow

rate of 0.3 mL/min and monitored by a RI detector. The reaction was stopped at the indicated times by heating the solution in a water bath at 100°C for 1 min.

2.2.4 Keratanase II digestion of keratan sulphate

Keratanase II digestion of KS was performed as described by Brown et al. [207]. Briefly, KS (20 mg) was incubated with 0.96 units of keratanase II in 2 mL NH₄OAc buffer (50 mM, pH 7.4) at 37°C. The reaction was monitored with a Superdex Peptide column. A few µl of reaction solution was injected, and the elution was by NH₄OAc (50 mM) at a flow rate of 0.3 mL/min and monitored by a RI detector. The reaction was stopped at the indicated times by heating the solution in a water bath at 100°C for 1 min.

2.2.5 Analytical-scale acid hydrolysis of keratan sulphate

The conditions of acid hydrolysis of KS were as described by Dr Wengang Chai [208]. Briefly, the KS (1.4 mg) was incubated with 0.1 M HCl at 80°C. The reaction was monitored with a Superdex Peptide column. A few µl of reaction solution was injected, and the elution was by NH₄OAc (50 mM) at a flow rate of 0.3 mL/min and monitored by a RI detector. The reaction was stopped at the indicated times by adding 0.1 M NaOH to the reaction solution until pH 7.0 was reached.

2.3 Fractionation and Purification of Oligosaccharide Fragments of Glycosaminoglycans

2.3.1 Bio-Gel P6 chromatography

Bio-Gel P-6 column (fractionation range of 1,000 to 6,000 Da, 1.6 × 90 cm, Bio-Rad) was used to fractionate GAG digests by size to obtain oligosaccharide fractions with different chain lengths. Each freeze-dried GAG digest was dissolved in 1 mL of 200 mM NH₄Cl and applied to the column. Elution was carried out by 200 mM NH₄Cl, at a flow

rate of 15 mL/h. Eluates of CSA, CSC and HS were monitored sequentially by UV 232 nm (for the 4,5-unsaturated uronic acid) and RI detectors, whereas eluates of KS was monitored only by RI detector. The recorder speed was 0.5 mm/min and the collector speed was 6 min/tube. Fractions were pooled as indicated and freeze-dried.

2.3.2 Medium pressure liquid chromatography (MPLC)

MPLC with Superdex Peptide column (fractionation range of 100 to 7,000 Da, 1 × 30cm, GE) was used to monitor the progress of polysaccharide digestion and to fractionate GAG digests obtained in small-scale, and also for desalting. Briefly, freeze-dried material was dissolved in 200 µl of 50 mM NH₄OAc and applied to Superdex Peptide column. Elution was carried out by 50 mM NH₄OAc, at a flow rate of 0.3 mL/min, and monitored by RI. The recorder speed was 2 mm/min and the collector speed was 1 min/tube. Fractions were analysed by ES-MS and fractions were pooled as indicated. The pooled fractions were co-evaporated repeatedly with H₂O by freeze-drying to remove the volatile buffer salt NH₄OAc.

2.3.3 Desalting by Sephadex G10 chromatography

Sephadex G10 column (1 x 35 cm, GE) was used for desalting of fractions with large amount of material, e.g. those collected from Bio-Gel P6 chromatography. Each freeze-dried fraction was dissolved in 1.5 mL H₂O and applied to the column. H₂O was used for elution at a flow rate of 20 mL/h. Eluate was monitored by a RI detector. The recorder speed was 0.5 mm/min and the collector speed was 4.5 min/tube. Fractions were pooled as indicated and freeze-dried.

2.4 Quantification of Glycosaminoglycans

2.4.1 Carbazole assay

Carbazole reagent was used to detect uronic acid residues in CSA, CSC and HS poly- and oligosaccharides using D-glucurono-6,3-lactone as standard [209]. Briefly, each freeze-dried oligosaccharide fraction was diluted in H₂O to generate a solution between 0.4-16 nmol/ μ L for quantification use. Screw-capped glass tubes were added with 1.5 mL of tetraborate-H₂SO₄ solution (0.96 g of sodium tetraboratedecahydrate dissolved in 100 mL of concentrated H₂SO₄, kept at room temperature) and cooled on ice. The tubes were tilted and 5 μ L of the oligosaccharide fraction solutions and D-glucurono-6,3-lactone standard solutions (D-glucurono-6,3-lactone dissolved in H₂O to generate solutions of 0.4, 1, 4, 8 and 16 nmol/ μ L) were carefully added on the surface of the tetraborate-H₂SO₄. The tubes were then placed back on ice to allow the solutions to diffuse for 10 min and gently rotated on ice for 30 sec to mix both layers. All the tubes were vortexed briefly, heated for 10 min at 102°C and placed immediately on ice. 50 μ L of cold (4°C) carbazole solutions (125 mg of carbazole dissolved in 100 mL of 100% ethanol, stored in the dark, N₂-protected and well-sealed) were added to the tubes followed by brief vortexing and heating for 15 min at 102°C. All the tubes were then cooled on ice. The absorbance at 525 nm of the resulting solution was measured using a UV-1800 spectrophotometer (Shimadzu).

Uronic acid content in the oligosaccharide fractions was determined against the calibration curve recorded by the standards. The molar quantities of the oligosaccharide fractions were estimated based on the uronic acid content and molecular mass determined by MS analysis.

2.4.2 Hexose assay

Quantification of KS oligosaccharide fractions was carried out by dot orcinol-sulphuric acid method on TLC plates [192], using Gal as standard.

Each freeze-dried oligosaccharide fraction was dissolved in H₂O to generate a solution containing Gal between 0.05-1 µg/µl for quantification use. 1 µl of the oligosaccharide fraction solutions and Gal standard solutions (Gal dissolved in H₂O to generate solutions of 0.05, 0.1, 0.2, 0.5 and 1.0 µg/µl) were dotted on a HPTLC plate. The plate was sprayed with orcinol reagent. The developed colour of the oligosaccharide fractions and Gal standard solutions was measured at 550 nm by a CS-9000 flying-spot scanner (Shimadzu).

Gal content in the oligosaccharide fractions was determined against the calibration curve recorded by the standards. The molar quantities of the oligosaccharide fractions were estimated based on the content and molecular mass determined by MS analyses.

2.5 High Performance Liquid Chromatography (HPLC)

GAG oligosaccharide fractions were further fractionated by HPLC (Gilson system) to isolate the antigen-positive oligosaccharide component. Strong anion-exchange, normal-phase (amide and amine columns) and porous graphitized carbon (PGC) column HPLC were used for detailed fractionation.

Amide column (4.6 x 250 mm, Waters), amine column (4.6 x 250 mm, Waters) and maltose column (4.6 x 250 mm, Waters) were performed with a gradient of ACN/H₂O (70:30) with 7.5 mM KH₂PO₄ (solvent A) to ACN/H₂O (20:80) with 7.5 mM KH₂PO₄ (solvent B) in 40 min at a flow rate of 0.5 mL/min. Strong anion-exchange (SAX) column (4.6 x 250 mm, Waters) and HiTrap ion exchange column (7 x 25 mm, GE Healthcare) was used with a gradient of 10 mM LiClO₄ (solvent A) to 500 mM LiClO₄ (solvent B) in

40 min at a flow rate of 1 mL/min. Porous graphitized carbon (PGC) Hypercarb column (30 x 4.6 mm, Hypersil) were performed with a gradient of H₂O with 0.05% trifluoroacetic acid (TFA) (solvent A) to ACN/H₂O (80:20) with 0.05% TFA (solvent B) in 40 min at a flow rate of 0.5 mL/min. Detection was by UV at 206 nm. Details of the gradient conditions were shown in HPLC legends of specific KS oligosaccharide fractions. Fractions were pooled according to the chromatogram.

The fractions obtained from SAX column and HiTrap column were freeze-dried, whereas the fractions from amide, amine and maltose columns were dried under N₂ stream. All of them were then desalted by Superdex Peptide column and lyophilized. The fractions obtained from PGC Hypercarb column were neutralized with 50 mM NH₄HCO₃ and dried under N₂ stream, followed by multiple time-lyophilization to remove remaining salt.

2.6 Chemical Desulphation of Oligosaccharides Obtained from Keratan Sulphate

Desulphation of KS oligosaccharides was performed as described [210]. After several preliminary experiments, the conditions I selected included: freeze-dried P6 hexasaccharide fraction (150 nmol) was added to 125 µl of 20 mM methanolic HCl for incubation at room temperature for 48 h; freeze-dried tri-sulphated hexasaccharide fraction (100 nmol) was added to 40 µl of 20 mM methanolic HCl for incubation at room temperature for 15 h. The reactions were stopped by neutralizing with 50 µl of 50 mM NH₄HCO₃. The reaction product was then desalted by Superdex Peptide column, followed by multiple time-lyophilization.

2.7 Preparation of Neoglycolipids

2.7.1 Preparation of lipid reagent *N*-aminooxyacetyl-1,2-dihexadecyl-*sn*-glycero-3-phosphoethanolamine (AOPE)

The lipid reagent AOPE was prepared in three steps as described [195]. The first was to prepare Boc-AOPE. Typically, 100 μmol of 1,2-dihexadecyl-*sn*-glycero-3-phosphoethanolamine (DHPE) and 300 μmol of Boc - aminooxyacetic acid were dissolved in 10 mL of chloroform in a glass tube. The tube was placed at 0°C on ice and slowly added with 2 mL of freshly prepared 1-ethyl-3-(3-dimethylamino-propyl) carbodiimide-HCl (EDC) solution (20 $\mu\text{g}/\mu\text{l}$, CH_3Cl). The reaction mixture in the tube was stirred for 1 hr at 0°C followed by 4 hrs at ambient temperature. The reaction was monitored by HPTLC (developed with $\text{CH}_3\text{Cl}/\text{EtOH}/\text{H}_2\text{O}$, 50:50:1) of an aliquot of the reaction mixture. After the reaction finished, the reaction mixture was washed twice with 10 mL of water to remove excess EDC. The organic layer containing Boc-AOPE was collected and evaporated under a N_2 stream.

The dried Boc-AOPE was dissolved in 1 mL of CH_2Cl_2 for purification use. Silica cartridge (12cc, x5, Phenomenex) was pre-washed and equilibrated with 15 mL MeOH, 15 mL $\text{CH}_2\text{Cl}_2/\text{MeOH}/25\% \text{NH}_3$ solution, 100:10:1 and 15 mL MeOH. Each column was added with 200 μl Boc-AOPE solution, washed with 4 mL $\text{CH}_2\text{Cl}_2/\text{MeOH}/25\% \text{NH}_3$ solution, 100:10:1 for 5 times, and eluted with 4 mL $\text{CH}_2\text{Cl}_2/\text{MeOH}/25\% \text{NH}_3$ solution, 100:30:1 for 5 times. Both washes and elutes were collected and labelled fraction 1 to 10, respectively. An aliquot of each fraction was checked for the purified Boc-AOPE by HPTLC (developed with $\text{CH}_2\text{Cl}_2/\text{MeOH}/25\% \text{NH}_3$ solution, 100:10:1). The result was further confirmed by matrix - assisted laser desorption/ionization mass spectrometry (MALDI - MS) analysis.

Second, the purified Boc-AOPE was de-protected to AOPE. Briefly, 10 μ mol of Boc-AOPE dissolved in 500 μ l of CH_2Cl_2 was added with 50 μ l of TFA in a glass tube at 0°C on ice. The reaction mixture in the tube was stirred for 5 hrs at 0°C on ice. The reaction was stopped by adding 50 μ l of toluene and drying under N_2 stream. The evaporation was repeated for 5 times to completely remove the TFA. An aliquot of the reaction mixture dissolved in $\text{CH}_2\text{Cl}_2/\text{MeOH}$, 1:1 was checked for AOPE by HPTLC (developed with $\text{CHCl}_3/\text{EtOH}/\text{H}_2\text{O}$, 50:50:1). The result was further confirmed by MALDI-MS analysis. The prepared AOPE solution was stored at -20°C.

Finally, quality of the AOPE was checked by incubating 20 μ l of the AOPE (~10 nmol/ μ l) and 200 nmol lyophilized lactose in 30 μ l $\text{CHCl}_3/\text{MeOH}$, 1:1 for 16 hrs at room temperature. The starting material lactose should disappear when monitored by HPTLC (developed with $\text{CHCl}_3/\text{MeOH}/\text{H}_2\text{O}$, 130:50:9) of the reaction mixture (~1 nmol of lactose).

2.7.2 Lipid conjugation of oligosaccharides to prepare neoglycolipids

NGLs of shorter and longer oligosaccharides were prepared using AOPE prepared above, essentially as described [195]. In brief, lyophilized reducing oligosaccharides (100 nmol) were dissolved in 10 μ l of HPLC H_2O in a glass vial. 5 equivalent AOPE (500nmol, 10 nmol/ μ l in $\text{CHCl}_3/\text{MeOH}$, 1:1) solution were added to oligosaccharides with short chain lengths (di- to octa-saccharides), whereas 10 equivalent AOPE were added to those longer than octasaccharides. The vials were sealed and kept at 37°C for 16h (shorter oligosaccharides) or 32 h (longer oligosaccharides). The screw caps of vials were then loosened and the vials were placed at 60°C for 1 to 2 hours to evaporate most solvents. The vials were dried under N_2 stream and added with 100 μ l of $\text{CHCl}_3/\text{MeOH}/\text{H}_2\text{O}$, 25:25:8. The reaction was monitored by HPTLC of an aliquot of the reaction mixture. $\text{CHCl}_3/\text{MeOH}/\text{H}_2\text{O}$, 60:35:8 was used for HPTLC development of

NGLs of shorter chain lengths such as di- to octa- saccharides. Whereas, CHCl₃/MeOH/H₂O, 25:25:8 was for those longer than octasaccharides as these more highly charged chains needed stronger solvent for moving. The prepared NGL and excess AOPE were visualized under long-wave UV light after primulin staining.

2.8 Purification of Neoglycolipid Products

In order to remove the excess AOPE in the conjugation products, two purification methods were mainly used, including silica cartridge and semi-prep HPTLC.

2.8.1 Purification of neoglycolipids using silica cartridge

The cartridge purification was performed as described [191], by using normal-phase silica cartridges to remove excess lipids (AOPE).

Briefly, each N₂ stream-dried conjugation product was reconstituted in 100 µl of CHCl₃/MeOH/H₂O, 130:50:9. The solution in vial was added to a silica cartridge (1cc, Waters Sep-Pak) that was prewashed and equilibrated sequentially with 4mL MeOH, 4mL H₂O, 6mL 0.2M NH₄OAc, 12mL H₂O, 4mL MeOH and 6mL CHCl₃. The cartridge was washed with 300 µl of CHCl₃/MeOH/H₂O, 130:50:9 for 6 times, eluted with 300 µl of CHCl₃/MeOH/H₂O, 60:35:8 for 4 times and 300 µl of CHCl₃/MeOH/H₂O, 25:25:8 for 4 times. All the washes and eluates were collected as fraction 1 to 14. An aliquot (1-2 µl) of each fraction was checked by HPTLC (developed with CHCl₃/MeOH/H₂O, 60:35:8 or 25:25:8) to identify the fractions containing NGLs but not excess AOPE. The NGL-containing fractions were then combined and dried under N₂ stream. The silica cartridge-purified NGLs were stored in CHCl₃/MeOH/H₂O, 25:25:8 at -20°C. NGLs of di- to octasaccharides obtained from silica cartridge purification were analysed by ES-MS, whereas the complexity of NGLs of longer chains limited their analysis by ES-MS.

2.8.2 Purification of neoglycolipids by semi-preparative HPTLC

Semi-preparative HPTLC was performed in my thesis for the conjugation products of short oligosaccharide chains and with limited amount of materials. The N₂ stream-dried conjugation products were reconstituted in CHCl₃/MeOH/H₂O, 25:25:8 and applied as long strips on HPTLC plates. The plates were developed in CHCl₃/MeOH/H₂O, 60:35:8. Two vertical edges (1mm) were then cut from the plate, stuck together and stained with primulin. The NGL and excess AOPE were visualized under long-wave UV light. The NGL bands on the main plates were localized by the two edges and cut out. The silica gel of each NGL band was scraped off the plates and packed for a mini-column. The column was eluted with CHCl₃/MeOH/H₂O, 25:25:8. The eluted NGL was stored at -20°C before ES-MS analysis.

2.9 Quantification of Neoglycolipids

Most of the purified NGLs described in my thesis were quantified following a modified densitometry method with maltopentaose conjugated to DHPE (Malto-DHPE) as standard [191].

400, 200, 100, 50 pmol of Malto-DHPE standards (50 picomol/μl, CHCl₃/MeOH/H₂O, 25:25:8) were applied on HPTLC plate as 4 mm-wide bands, followed by development in CHCl₃/MeOH/H₂O, 60:35:8. NGL samples (50 to 400 picmol) were then applied as 4 mm-wide bands on the same plate. The plate was sequentially immersed in PBS buffer, PBS with 1% primulin, and PBS buffer for 10, 20 and 10 min, respectively. The NGLs were measured by densitometer in linear reflectance fluorescence mode with excitation at 370 nm. The quantities of the NGLs in each sample were calibrated by factor ½ after calculation by the calibration curve with the standard Malto-DHPE.

Small amounts of NGLs with short chain lengths were quantified by applying together with the Malto-DHPE standards on the HPTLC plate before development in $\text{CHCl}_3/\text{MeOH}/\text{H}_2\text{O}$, 60:35:8. Later steps were the same as the above mentioned. The quantities of the NGLs in each sample were directly calculated by the calibration curve with the standard Malto-DHPE. All the quantified NGLs were then adjusted to 50-100 pmol/ μl as stock solution and stored at -20°C .

2.10 Mass Spectrometry (MS) Analysis

MS analysis was carried out by Dr Yibing Zhang. MALDI-MS was performed on an AXIMA Assurance instrument (Shimadzu). Laser energy was set at attenuation scale 80. For analysis, the oligosaccharides were dissolved in H_2O and the NGLs in $\text{CHCl}_3/\text{MeOH}/\text{H}_2\text{O}$, 25:25:8. Each sample was applied on the sample target together with 1 μl of matrix of 2',4',6'-trihydroxyacetophenone. ES-MS and CID-MS/MS were carried out on a Waters (Manchester, UK) Q-TOF-type mass spectrometer SYNAPT-G2. Cone voltage was at 80 eV and capillary voltage at 3 kV. Source temperature was at 80°C and the desolvation temperature at 150°C . Product-ion spectra were obtained using argon as the collision gas at a pressure of 1.7 bar. The collision energy was adjusted between 12 and 20 eV for optimal fragmentation. A scan rate of 1.5 sec/scan was used for both ES-MS and MS/MS experiments and the acquired spectra were summed for presentation. For analysis, oligosaccharides were dissolved in H_2O and NGLs in $\text{CHCl}_3/\text{MeOH}/\text{H}_2\text{O}$, 25:25:8 typically at a concentration of 10-20 pmol/ μl , of which 1 μl was injected via a HPLC injector. Solvent (ACN/2mM NH_4HCO_3 1:1) was delivered by a HPLC pump (Waters) at a flow rate of 10 $\mu\text{l}/\text{min}$.

2.11 NMR Spectroscopy

NMR spectra were recorded by Prof Barbara Mulloy. ^1H -NMR, as well as 2D ^{13}C - ^1H HSQC and TOCSY NMR spectra of oligosaccharides were recorded at 700 MHz using a

BruckerAvance spectrometer equipped with cryoprobe, in D₂O solution at 30°C. 1D and 2D pulse sequences were supplied by the equipment manufacturer. The observed ¹H chemical shifts were relative to internal acetone (2.225ppm). Each sample (~240 µg) was taken up in D₂O 99.9% (Apollo Scientific, Stockport, UK) exchanged by lyophilization, then re-dissolved in 300 µl D₂O and transferred to a Shigemi NMR microtube (Sigma).

2.12 Generation of Microarrays of Polysaccharides and Oligosaccharide Neoglycolipids

2.12.1 Polysaccharide microarrays

The polysaccharides were arrayed in duplicate at 0.3µg/µl and 1 µg/µl in H₂O (90 and 300 pg/spot, respectively) on 16-pad nitrocellulose-coated glass slides using a non-contact arrayer (Nano-plotter 2.1, Gesim, Grosserkmannsdorf, Germany) together with 200 ng/mL Cy3 solution as the tracer to monitor the quality of arraying.

2.12.2 Oligosaccharide neoglycolipid microarrays

Unlike the direct printing of polysaccharides on the slides, liposome preparation was needed for NGL immobilization. NGL stock solutions were used to prepare 5 pmol/µl working solutions in CHCl₃/MeOH/H₂O 25:25:8 for liposome preparation use.

Briefly, 10 µl and 30 µl of each NGL working solution were added to two tubes, respectively, which contained 5 µl of phosphatidycholine (200 pmol/µl) and 5 µl of cholesterol (200 pmol/µl). The tubes were open and kept in oven at 37°C for 16h to evaporate the solvent.

To each tube 10 µl of Cy3 solution (2 ng/mL, H₂O) was added to reconstitute the NGL-lipid mixture. The NGL in duplicate (2 and 5 fmol/spot, respectively) were arrayed on

16-pad nitrocellulose-coated glass slides using the non-contact arrayer. The Cy3 dye contained was the tracer to monitor the quality of arraying.

2.13 Microarray Analysis

Microarray analyses were performed essentially as described [211]. In my thesis, blockers included 3%(w/v) bovine serum albumin (BSA) or 1% (w/v) BSA in PBS, 1% (w/v) BSA and 0.33% (w/v) casein in PBS, or 3% (w/v) BSA and 5 mM CaCl₂ in Hepes-buffered saline (HBS); diluents included 1% (w/v) BSA or 1% (w/v) BSA and 0.33% (w/v) casein in PBS, or 3% (w/v) BSA and 5 mM CaCl₂ in HBS.

The arrayed slides were placed in a 16-pad silicon gasket and inserted into a FAST frame. Each pad of the slide was added with a blocker for 1 h blocking. The blocker was then removed. Proteins or primary antibodies dissolved in diluents were added to each pad and incubated for 1.5 h. The binding of the proteins or primary antibodies to the polysaccharides or NGLs on the slide was detected using their corresponding biotinylated secondary antibodies followed by an Alexa Fluro-647-labelled streptavidin (1 µg/mL in dilutes). The slides were scanned at 633 nm using a Genepix scanner (Molecular Devices, USA). The binding of all the biotinylated secondary antibodies to the polysaccharides or NGLs were analysed as negative controls of their corresponding proteins or primary antibodies. All the microarray binding experiments were performed at ambient temperature except for the anti-i at 4°C.

Both precomplexing and non-precomplexing conditions were examined in my thesis. Pads under non-precomplexing conditions were analysed following the above procedures. In terms of the other pads under precomplexing conditions 30 min incubation of the proteins or primary antibodies with their secondary antibodies was needed before overlay. Binding was detected with Alexa Fluor-647-labeled streptavidin as above. Data analysis and presentation was performed using the dedicated

microarray software established by Dr Mark Stoll in our laboratory [197].

Chapter 3

Microarrays of Glycosaminoglycan Polysaccharides and Oligosaccharide Neoglycolipids of Chondroitin Sulphates, Heparan Sulphates and Heparin

3.1 Introduction

GAGs are highly polar and linear polysaccharides with alternating disaccharide units of N-acetylhexosamine (HexNAc) and uronic acid (UA) residues, and they include CSA, CSB, CSC, HS, heparin, and HA (Figure 1.5). Although KS does not contain UA, it is traditionally considered as a GAG because it is highly sulphated. GAG-protein interactions can exert profound physiological effects on processes such as haemostasis, anticoagulation, growth factor control, cell adhesion, inflammation, and attachment of pathogens to host cells [212]. Carbohydrate microarrays are well suited to revealing the GAG-protein interaction that are involved in these biological processes.

Although carbohydrate microarrays have been used extensively to characterize glycan-protein interactions, the construction and application of GAG microarrays have lagged behind due mainly to the difficulty of isolation and preparation of GAG oligosaccharides and immobilization of these highly polar molecules [213-216]. Much of the earlier part of my work has been to generate GAG polysaccharides and oligosaccharide NGL microarrays in which the polysaccharides and derived oligosaccharide NGLs are printed and non-covalently immobilized (adsorption) on the nitrocellulose-coated glass slides [165]. This was for initial screening analysis to detect if and which GAG polysaccharides and derived oligosaccharides can be bound by mAb R-10G which has been shown to be expressed on human ES/iPS cells but not EC cells [73].

When I joined the Glycosciences Laboratory, there were already some microarrays of GAG polysaccharides and oligosaccharide NGLs but the repertoire was limited and needed to be increased for my project [168, 196]. This Chapter describes the generation of microarrays of GAG polysaccharides (CSA, CSB, CSC, HS, heparin, KS and HA) and oligosaccharide NGLs derived from them. Although there have been three

types of lipids: DHPE, ADHP and AOPE used for the NGL preparations, I selected AOPE for linking to the reducing-end monosaccharides by oxime ligation as a significant proportion of the monosaccharides linked to the lipid are ring closed [195].

3.2 Generation of Glycosaminoglycan Polysaccharide Microarray

A GAG polysaccharide microarray designated Set 2 was generated including HA, CSA, CSB, CSC, heparin, HS and KS (Table 3.1). Dextran reference components, neutral (Dex) and highly sulphated dextran (DexS), were included as well.

Table 3.1. Polysaccharides included in GAG polysaccharide microarray

Position ^a	Polysaccharide (abbreviation)	Source
1	Hyaluronic acid(HA)	Bovine vitreous humour
2	Keratan sulphate (KS)	Bovine cornea
3	Keratan sulphate (KS)	Bovine articular cartilage
4	Keratan sulphate (KS)	Chicken egg white
5	Chondroitin sulphate A (CSA)	Bovine trachea
6	Chondroitin sulphate (CSB)	Bovine mucosa
7	Chondroitin sulphate (CSC)	Shark cartilage
8	Heparin (HEP)	Porcine intestinal mucosa
9	Heparan sulphate (HS)	Porcine intestinal mucosa, Fraction I
10	Heparan sulphate (HS)	Bovine kidney
11	Dextran sulphate (DexS)	Dextran esterificated with sulfuric acid (included as a control)
12	Dextran T2000 (Dex)	Bacterial product (included as a control)

^a Position, probe position in the microarray.

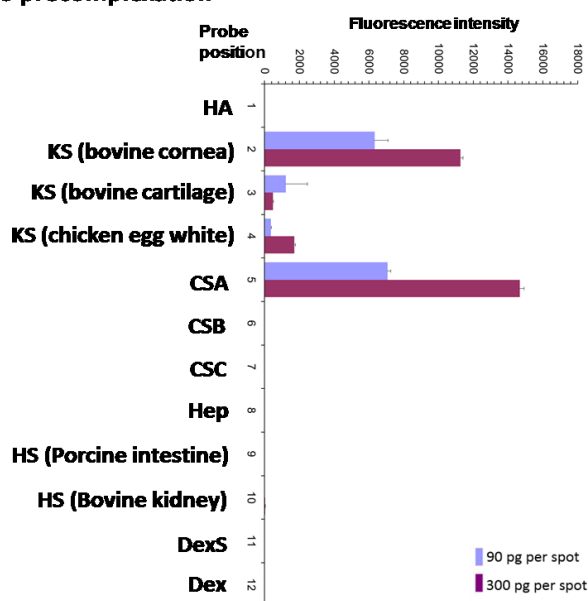
Quality of the generated microarray was examined by anti-GAG antibodies with known specificities, such as MZ15, 5D4, 1B4 and CS-56. The mAbs MZ15, 5D4 and 1B4 was originally prepared to specifically bind KS [217, 218], and mAb CS-56 has been reported to bind both CSA and CSC [219]. However, there were no related antibodies or proteins available for validation of the arrayed heparin and HS at the time of analysis. This issue has been addressed in the microarray analyses using several growth factors and

morphogens which showed binding to the arrayed heparin or HS (described in Chapter 6).

As Figure 3.1 shows, 1B4 bound to the three KS preparations arrayed in the GAG polysaccharide microarray, which is consistent with the reported specificity [217]. The antibody showed strong binding to bovine corneal KS and much weaker binding to bovine articular cartilage and chicken egg white KSs (Figure 3.1). The KS binding by MZ15 and 5D4 is described in Chapter 5 (Figure 5.1). However, these antibodies were found to also bind to the preparation of CSA with the intensity similar to that to bovine corneal KS (Figures 3.1 and 5.1). The anti-CSA and CSC antibody, CS56, showed strong binding to the arrayed CSA and CSC (Figure 3.1), which is consistent with published data [219].

Therefore, the GAG polysaccharide microarray constructed was considered validated and ready for subsequent binding analysis of the stem cell antibody R-10G.

mAb 1B4 (IgG, 0.76 ug/ml)
No precomplexation



mAb CS56 (IgM, 1/200)
No precomplexation

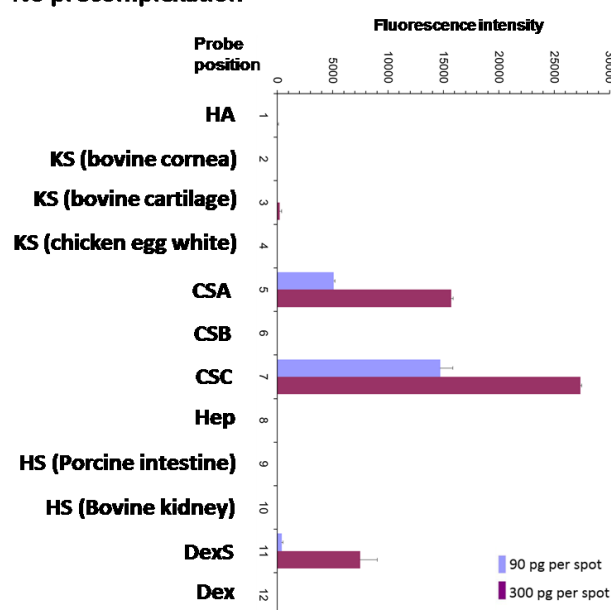


Figure 3.1. GAG polysaccharide microarray analysis of mAbs 1B4 (IgG) and CS56 (IgM). Probe abbreviations are defined in Table 3.1. Blocking solution was PBS with 3% (w/v) BSA, and diluent was PBS with 1% (w/v) BSA. Binding was detected using biotinylated anti-mouse IgG or IgM followed by Alexa Fluor-647-labeled streptavidin. Results are means of fluorescence intensities of duplicate spots, printed at 90 and 300 pg per spot, with error bars representing half of the difference between the two values. 1B4 was analysed only once; the results shown here for CS56 are representative of three analyses using this microarray.

3.3 Generation of Oligosaccharide Neoglycolipids of Chondroitin Sulphate A, B, and C, Heparin and Deacetylated Heparan sulphate

In contrast to the polysaccharide microarray, generation of oligosaccharide NGL microarrays are not as straightforward, simply because the polar GAG oligosaccharide cannot be immobilized directly on the slide surface (e.g. nitrocellulose surface used in this study). The oligosaccharides need to be converted into NGLs before construction of microarrays. For preparation of the NGLs, GAG oligosaccharides need to be produced by partial depolymerisation of the polysaccharides.

My work in this section includes preparation of oligosaccharides from CSA, CSC and deAc-HS (3.3.1), and preparation, purification and characterization of NGLs from CSA, CSC, deAc-HS, CSB, and heparin oligosaccharides (3.3.2). Other oligosaccharides and/or NGLs are available in the laboratory, e.g HA oligosaccharide NGLs, and they can be used in this study as further preparation is not necessary.

3.3.1 Preparation of oligosaccharides of chondroitin sulphate A, B, and C, heparin and deacetylated heparan sulphate

GAG degrading enzymes contain hydrolases and lyases: the former includes heparin hydrolase, keratanase, hyaluronic acid hydrolase and various exoglycosidases; the latter are mainly of bacterial origin, including heparinases, chondroitinases and hyaluronidases [220, 221]. GAG lyases have been purified and extensively utilized for analytical and industrial applications. The lyases are characteristic of eliminative cleavage by acting on the non-reducing side of uronic acids and forming an unsaturated C4-C5 bond (Figure 3.2), and the unsaturated bond can be simply measured on the 232nm absorbance [222]. However, the degradation products from hydrolysis of GAGs do not absorb UV light which increases difficulties in detection and analysis. Therefore, GAG lyases, chondroitinase ABC, heparinase I and III, were selected for degrading GAG polysaccharides: chondroitinase ABC acts on polysaccharides containing β 1-4-D-hexosaminy and β 1-3-D-glucuronosyl (in the case of CSA and CSC) or α 1-3-L-iduronosyl linkages (in the case of CSB) [201]; heparinase I acts primarily on heparin and heparinase III is active only on HS [28].

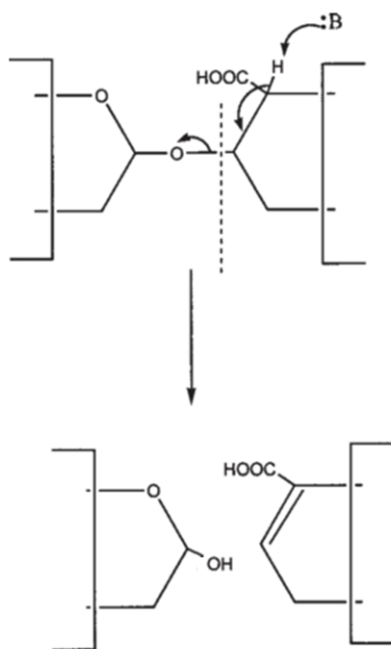


Figure 3.2. Eliminative cleavages of GAGs by lyases. Taken from [28].

Preparation of chondroitin sulphate A and C oligosaccharides

Treatment of CSA and CSC with chondroitinase ABC was monitored by UV 232 nm for the amount of unsaturated uronic acids generated to achieve 50% digestions and evenly distributed oligomers were produced. Each digestion product was fractionated by gel filtration chromatography on a Bio-Gel P6 column to obtain ten fractions (Figure 3.3).

Fractions 1-3 of CSA and CSC were analysed by ES-MS to confirm the chain lengths of these pooled fractions (Table 3.2). Fraction 1 of CSA was indeed the disaccharide with mainly one sulphate, and Fraction 2, tetrasaccharides with one or two sulphates and Fraction 3, hexasaccharides with two or three sulphates. These are consistent with the cleavage specificity of chondroitinase ABC and all the oligosaccharides generated by the lyase digestion are even-numbered. The chain lengths of Fractions 4 to 10 can therefore be deduced based on the available MS data of Fractions 1-3 (Table 3.2) and the specificity of the chondroitinase ABC. The CSC oligosaccharide fractions were

similarly assigned (Table 3.2). All these size-defined oligosaccharide fractions were quantified by uronic assay and their deduced sequences are shown in Appendix 1.

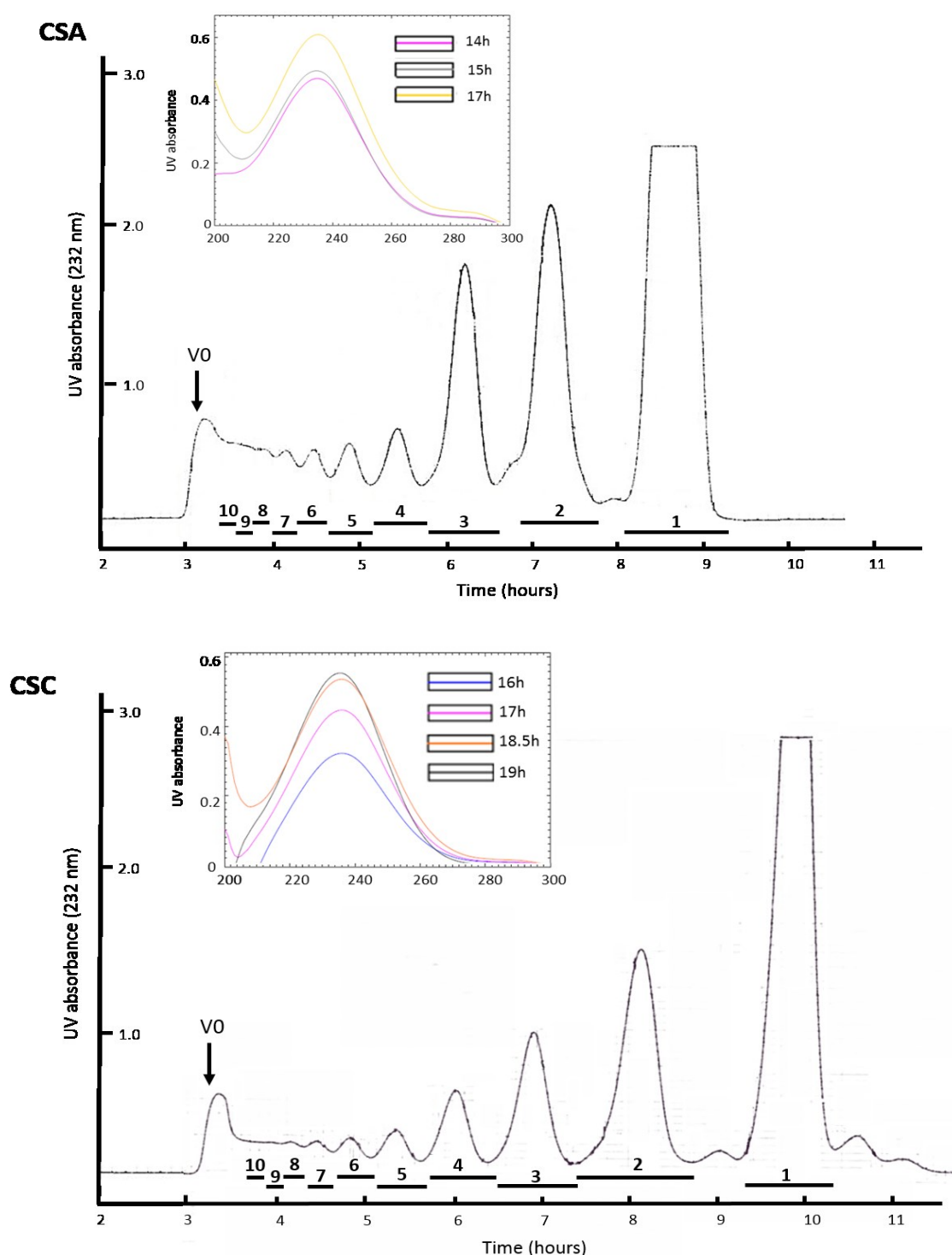


Figure 3.3. Gel filtration on Bio-Gel P6 of the oligosaccharides derived from CSA and CSC after partial digestion for 17 h and 19 h, respectively with chondroitinase ABC. In each case, ten fractions (1-10) were pooled as shown. Insets are UV absorbance profiles after the incubation times indicated. V₀ (void volume) is shown.

Table 3.2. Negative-ion ES-MS analysis of oligosaccharides in selected Bio-Gel P4 fractions of CSA and CSC digests and deduced compositions

Fractions		Oligosaccharides		Assignment
		Detected major ions (m/z)*	Deduced compositions	
CSA	F1	458 (z=1)	Δ UA1.GalNAc1.S1	2mer
	F2	458 (z=2) 837 (z=1)	Δ UA1.GlcA1.GalNAc2.S2 Δ UA1.GlcA1.GalNAc2.S1	4mer
	F3	647 (z=2) 698 (z=2)	Δ UA1.GlcA2.GalNAc3.S2 Δ UA1.GlcA2.GalNAc3.S3.Na	6mer
CSC	F1	458 (z=1) 560 (z=1)	Δ UA1.GalNAc1.S1 Δ UA1.GalNAc1.S2.Na	2mer
	F2	458 (z=2) 837 (z=1)	Δ UA1.GlcA1.GalNAc2.S2 Δ UA1.GlcA1.GalNAc2.S1	4mer
	F3	709 (z=2)	Δ UA1.GlcA2.GalNAc3.S3.Na2	6mer

*m/z is mass to charge ratio.

Δ UA, 4,5-unsaturated uronic acid; GlcA, glucuronic acid; GalNAc, N-Acetyl-galactosamine; S, sulphate.

The numbers in the deduced compositions refer to monosaccharides, sulphates and sodium (Na) per molecule of oligosaccharide.

Preparation of deacetylated heparan sulphate oligosaccharides

As the two HS preparations arrayed on the GAG polysaccharide microarray were of limited amount, a new batch of HS (HO-01214) derived from porcine intestinal mucosa was obtained from Celsus for HS oligosaccharide preparation.

Partial digestion of the HS by heparinase III was achieved similarly as for CS by monitoring of UV 232 nm, specific absorption of the generated unsaturated uronic acid residue. The digestion product was fractionated by gel filtration chromatography on Bio-Gel P6 and five fractions, Fractions 1 to 5, were collected. For Fraction 2, the splitting peaks were collected separately as Fractions 2a and 2b (Figure 3.4). The profile of the gel filtration chromatography was different than those of CSA and CSC, reflecting the heterogeneity of the HS sequence.

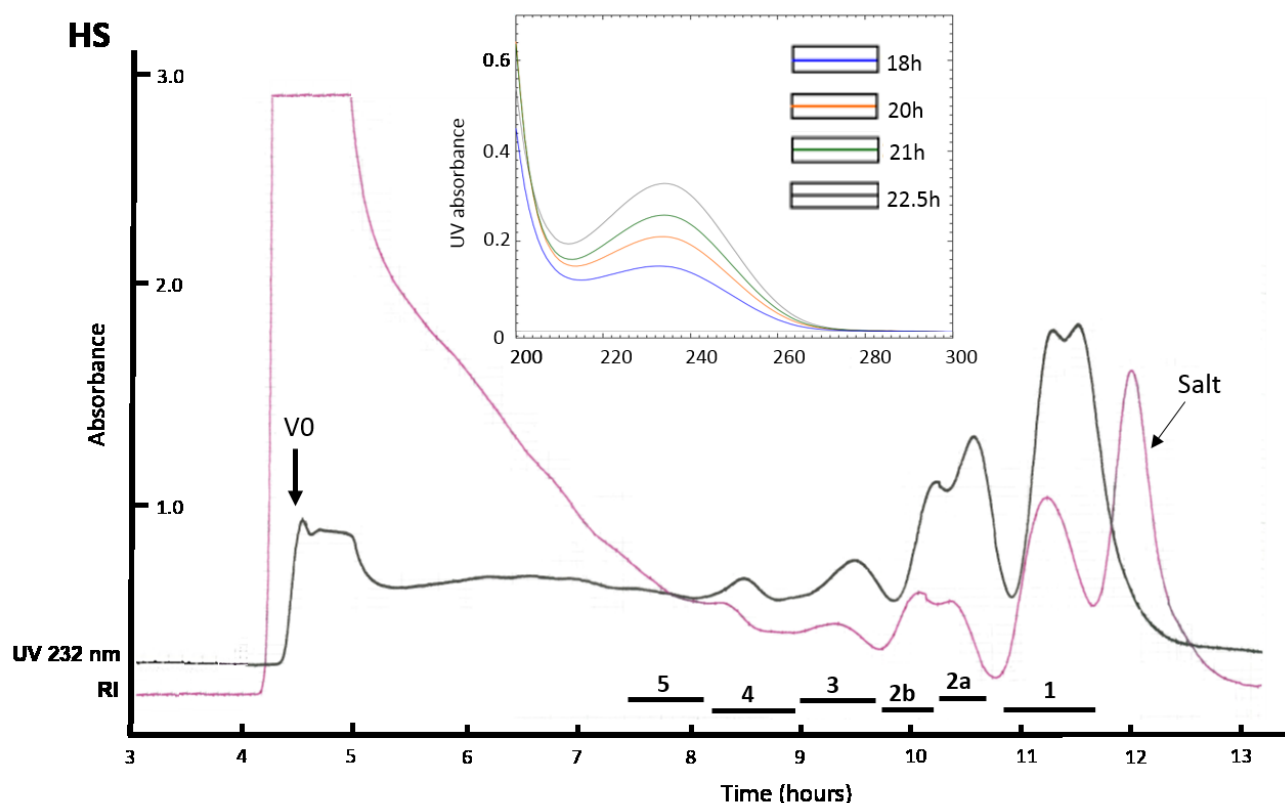


Figure 3.4. Gel filtration on Bio-Gel P6 of the oligosaccharides derived from HS after partial digestion for 22.5 h with heparinase III. Six fractions (Fractions 1 to 5) were pooled as shown. Insets are UV absorbance profiles after the incubation times indicated. V₀ (void volume) and salt peak are shown.

These oligosaccharide fragments were analysed by ES-MS and the derived compositions are listed in Table 3.3. Oligosaccharide fraction Fraction 1 consists of the disaccharide Δ UA-GlcNS as the main component. Both Fractions 2a and 2b are tetrasaccharides but the main components are different; Fraction 2a contains mainly Δ UA1-UA1.GlcN2 and Fraction 2b contains Δ UA1-UA1.GlcN1.GlcNS1 and Δ UA1-UA1.GlcNS2. Fractions 3, 4 and 5 were assigned as hexa-, octa-, and decasaccharides, respectively, based on the deduced compositions. To our surprise, there is a very high content of free GlcN-containing components and these were found in all fractions, e.g. there are two GlcN residues contained in the tetrasaccharide Fraction 2a, up to three GlcN are in a hexasaccharide and an octasaccharide and four GlcN in a decasaccharide sequence (Table 3.3).

Table 3.3. Negative-ion ES-MS analysis of oligosaccharides in selected Bio-Gel P4 fractions of heparan sulphate digests and deduced compositions

Fractions	Oligosaccharides	
	Detected major ions (m/z)	Deduced compositions
F1	416 (z=1)	Δ UA1.GlcNS1
F2a	673 (z=1)	Δ UA1.UA1.GlcN2
F2b	753 (z=1)	Δ UA1.UA1.GlcN1.GlcNS1
	673 (z=1)	Δ UA1.UA1.GlcN2
	833 (z=1)	Δ UA1.UA1.GlcNS2
F3	715 (z=1)	Δ UA1.UA1.GlcN1.GlcNAc1
	544.6 (z=2)	Δ UA1.UA2.GlcN2.GlcNS1
	1010 (z=1)	Δ UA1.UA2.GlcN3
	584.6 (z=2)	Δ UA1.UA2.GlcN1.GlcNS2
	1090 (z=1)	
F4	512.6 (z=2)	Δ UA1.UA3.GlcN3.GlcNS1
	713.2 (z=2)	Δ UA1.UA3.GlcN2.GlcNS2
	1026 (z=1)	
	753.2 (z=2)	
F5	681.2 (z=2)	Δ UA1.UA4.GlcN4.GlcNS1
	881.7 (z=2)	Δ UA1.UA4.GlcN3.GlcNS2
	921.7 (z=2)	Δ UA1.UA4.GlcN2.GlcNS3
	961.7 (z=2)	
	1363 (z=1)	

Δ UA, 4,5-unsaturated uronic acid; UA, uronic acid; GlcN, N-glucosamine; GlcNAc, N-Acetyl-glucosamine; S, sulphate. Adapted from [223].

To further investigate the unusual features of the HS from Celsus (HO-01214), its ^1H NMR spectrum was compared with spectra of other HS preparations, including HS from bovine kidney (Sigma) and samples of porcine mucosal HS 1 from our own laboratory, HS-1B and HS-1A (Figure 3.5). The most obvious difference between them lies in the methyl signal of the acetyl group at 2.04 ppm. In contrast to the high intensity of this peak in the spectra of other three HS preparations (Figure 3.5a, c, d), a very weak peak was observed in the spectrum of HO-01214. This suggests that the HO-01214 has much lower degree of N-acetylation on the amino sugar compared with the other two porcine HS samples.

^1H NMR spectra for three other HS preparations, porcine mucosal HS-1C prepared as described by Casu et al. [200], and two very old batches of Celsus, HO-10697 and HO-10095 are also shown in Figure 3.6. All of the three HSs contained the acetyl methyl signal at 2.04 ppm normally characteristic of HS.

HS polysaccharide is known to consist of repeating disaccharide units of 50%-75% -4GlcNAc α 1-4GlcA β 1- and smaller amounts of -4GlcNAc(6S) α 1-4GlcA β 1- and -4GlcNS α 1-4GlcA β 1- [28]. The unusual residue GlcN in HS had been recognized in the 1990s and the amount varies depending on the source, 0.7 to 4% of total glucosamine in HS or 1 to 2 residue in each HS chain [224-227]. The presence of the large amount of GlcN in the HS from Celsus (HO-01214) was clearly abnormal. Our findings have been published [223] for the attention of the scientific community.

This preparation of HS was thereafter named as deacetylated-HS (deAc-HS) and the derived oligosaccharides fractions were used in the study alongside HS oligosaccharides with the correct structures prepared in this laboratory.

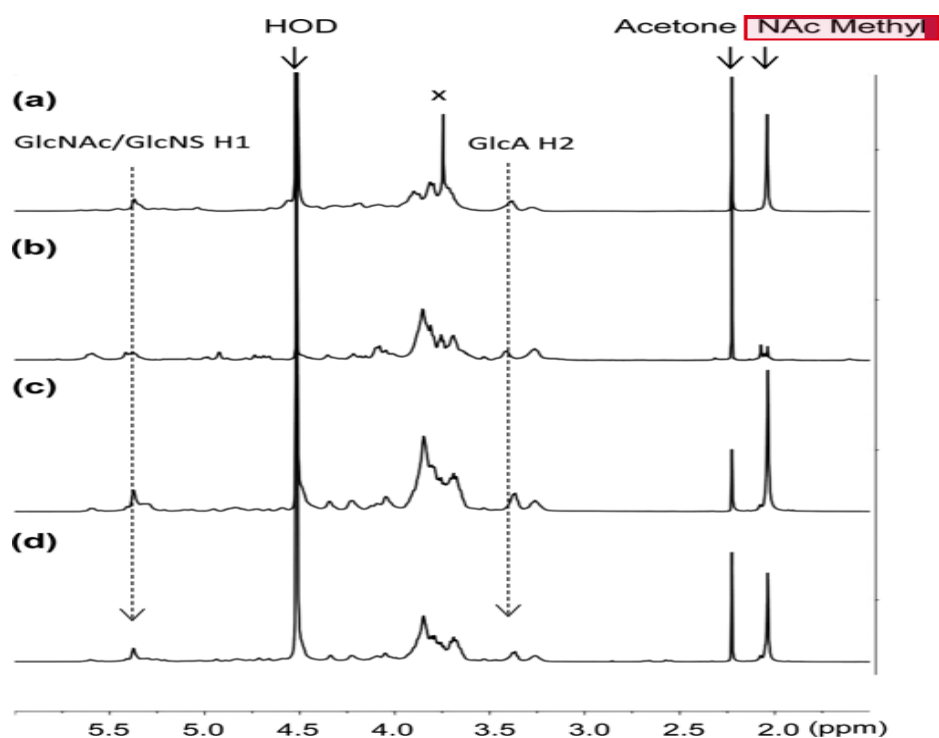


Figure 3.5. ^1H NMR spectra of different HS preparations. (a) bovine kidney HS obtained from Sigma; (b) porcine intestinal HS, HO-01214, obtained from Celsus; (c) porcine intestinal HS-1B; and (d) porcine intestinal HS-1A. X, signal from an unidentified impurity in this sample. Taken from [223].

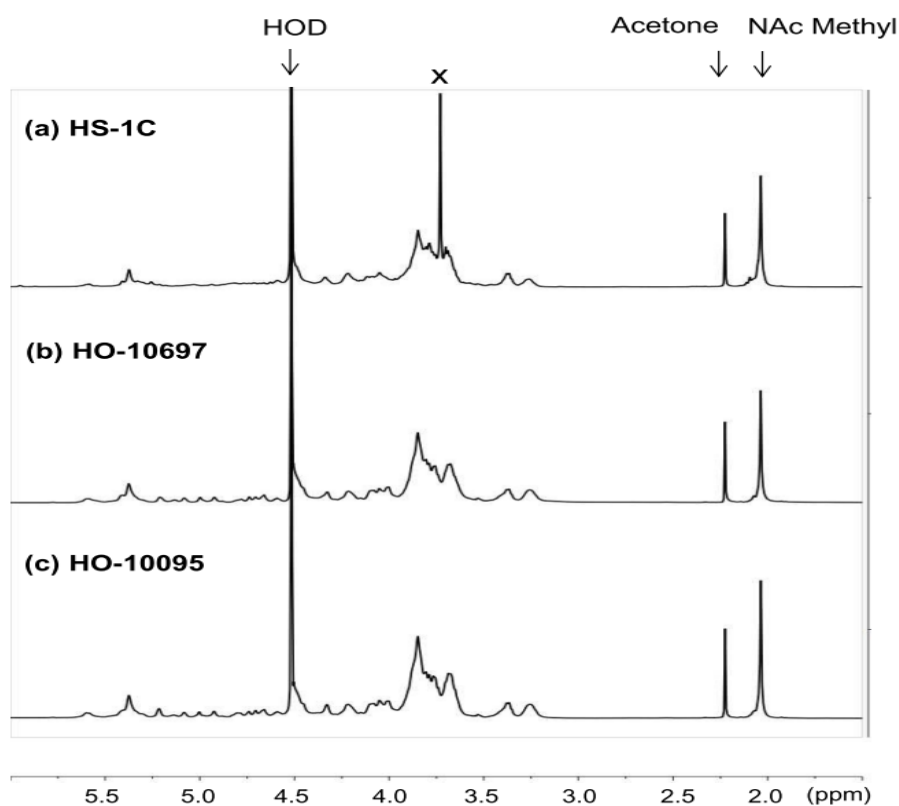


Figure 3.6. ^1H NMR spectra of different HS preparations. (a) porcine intestinal HS-1C; (b) porcine intestinal HS, HO-10697, obtained from Celsus; (c) porcine intestinal HS, HO-10095, obtained from Celsus. X, signal from an unidentified impurity in this sample. Taken from [223].

3.3.2 Preparation of neoglycolipids derived from chondroitin sulphate A, B, and C, heparin and deacetylated heparan sulphate oligosaccharides

For generation of oligosaccharide NGL microarrays, the obtained oligosaccharides of CSA, CSB, CSC, heparin and deAc-HS were conjugated to APOE via oxime ligation to be NGLs. Conjugation mixtures of CSA, CSB, CSC and heparin oligosaccharide fractions were resolved by HPTLC and stained with primulin (Figure 3.7). Data of NGLs derived from the deAc-HS oligosaccharides were not shown here.

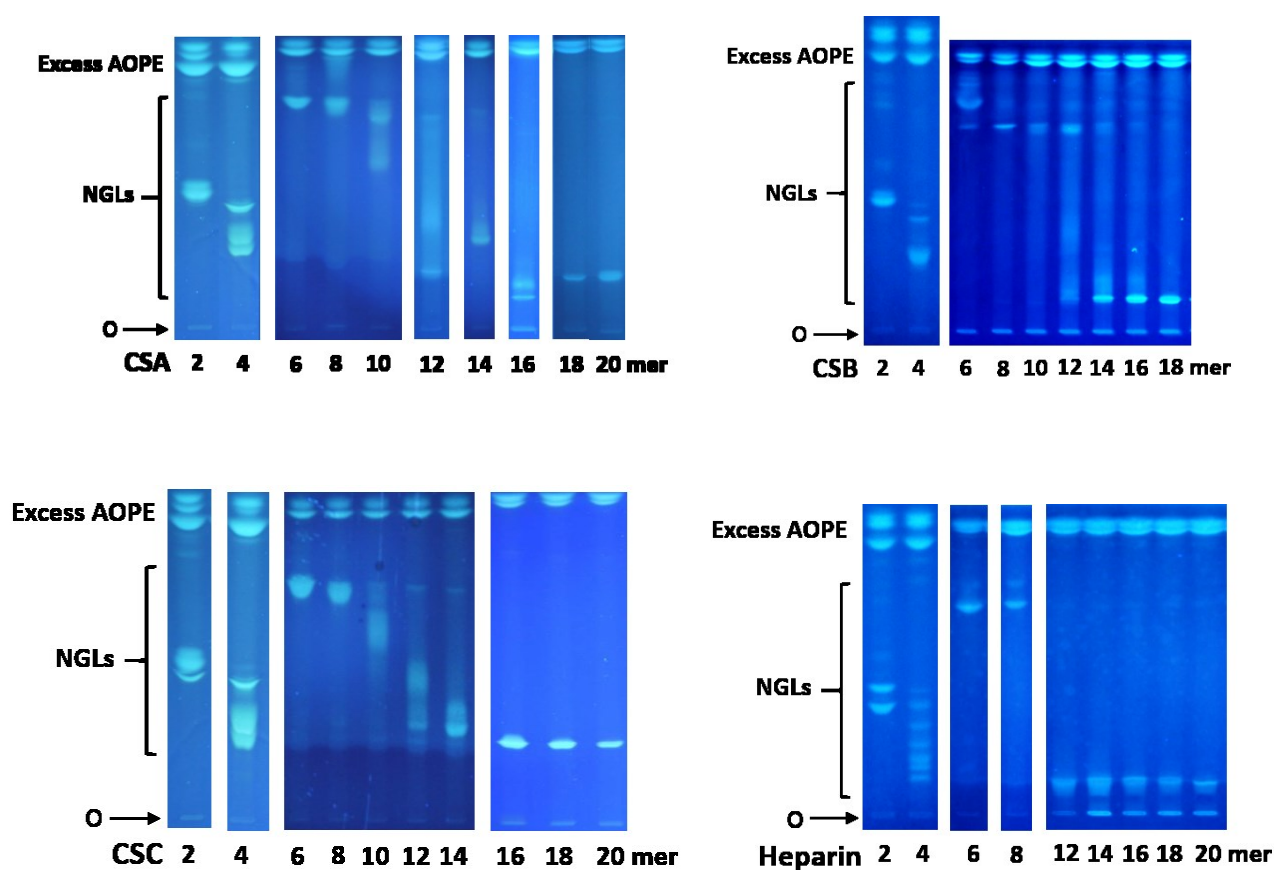


Figure 3.7. HPTLC and primulin staining of NGL reaction mixtures of CSA, CSB, CSC and heparin oligosaccharide fractions. Starting amount of each fraction for NGL generation was 100 nmol. NGLs were revealed under UV light with long wavelength after staining with primulin. Chromatography of 2 to 4 mers was in solvent $\text{CHCl}_3:\text{MeOH}:\text{H}_2\text{O}$ 60/35/8 (v/v/v) and 6 to 20 mers in 25/25/8 (v/v/v). O indicates the origin (position of application). The spaces between figures mean that they are separate chromatographs.

The top bands on the silica gel HPTLC plates resolved with each mixture were excess AOPE, and the bands below AOPE were NGLs. The NGLs derived from oligosaccharides of different chain lengths had different mobilities when chromatographed in the same solvent. The strength of the NGL bands revealed under UV light varied: NGLs of longer chains was weaker than those shorter-chains. This was due to the difficulty raised by higher charge of longer chains in conjugation with AOPE.

All the mixtures were then purified by silica cartridges to remove excess AOPE. HPTLC of fractions eluted from the purification of selected conjugation mixture were shown in Figure 3.8. The lipid AOPE was mainly eluted earlier in Fractions 2 and 3 by $\text{CHCl}_3/\text{MeOH}/\text{H}_2\text{O}$ 130:50:9, followed by elution of CSA and CSC NGLs. NGLs of shorter CSA and CSC oligosaccharides (2 to 8 mers) were eluted in fraction 4-10. Whereas NGLs of the longer oligosaccharides showed higher affinity with the silica cartridges and had to be eluted in Fractions 11 to 14 with stronger solvent 25:25:8.

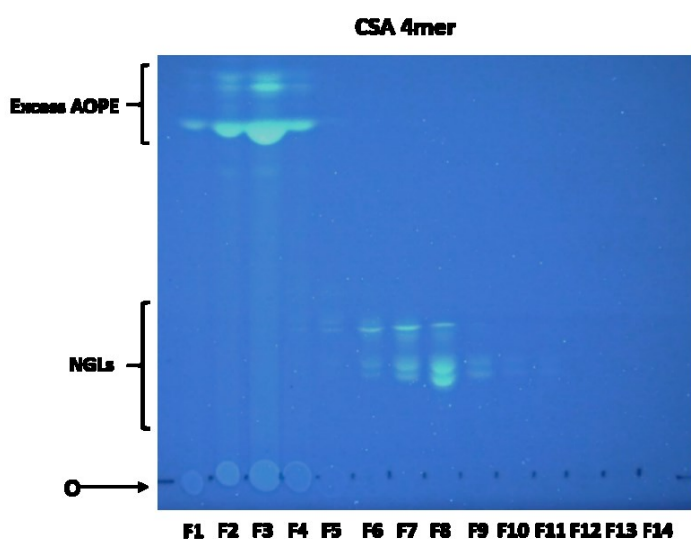
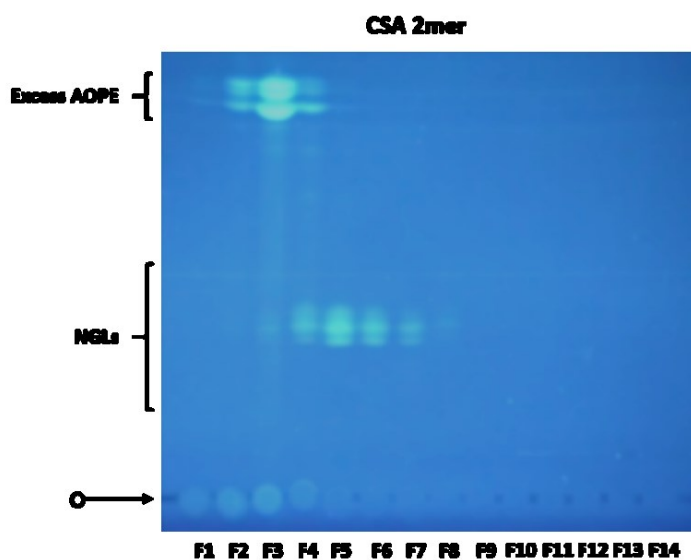
NGLs derived from di- to hexa- saccharides of the CSA, CSC, CSB, heparin and deAc-HS were analysed by ES-MS. CSA and CSC 2 to 6 mers were taken examples and their deduced compositions are shown, which are consistent with their oligosaccharide compositions (Table 3.4). All the prepared NGLs were quantified using a modified densitometry method in which the Malto-DH standards were chromatographed with solvent after application on HPTLC plates while the NGL samples were not (Figure 3.9). The yields of NGLs derived from the oligosaccharide fractions of CSA, CSC, CSB, heparin and deAc-HS ranges from 20% to 40% of starting oligosaccharide, which was as expected. The sequences of the prepared NGL probes are shown in Appendix 2.

All the NGLs were then arrayed as GAG oligosaccharide NGL microarray (Set 6) together with the already available NGLs of HA oligosaccharides 4 to 18 mers and HS oligosaccharides 4 to 8 mers.

Table 3.4. Negative-ion ES-MS analysis of NGLs of di- to hexa- oligosaccharides of CSA and CSC

Oligosaccharides		NGLs	
		Detected major ions (m/z)	Deduced compositions
CSA	2mer	-	-
	4mer	544 (z=3) 777 (z=2)	Δ UA1.GlcA1.GalNAc2.S2-AO Δ UA1.GlcA1.GalNAc2.S1-AO
	6mer	1006 (z=2) 1057 (z=2)	Δ UA1.GlcA2.GalNAc3.S2-AO Δ UA1.GlcA2.GalNAc3.S3-AO
CSC	2mer	-	-
	4mer	817 (z=2) 777 (z=2)	Δ UA1.GlcA1.GalNAc2.S2-AO Δ UA1.GlcA1.GalNAc2.S1-AO
	6mer	697 (z=2)	Δ UA1.GlcA2.GalNAc3.S3-AO

Δ UA, 4,5-unsaturated uronic acid; GlcA, glucuronic acid; GalNAc, N-Acetyl-galactosamine; S, sulphate; AO is for AOPE conjugation.



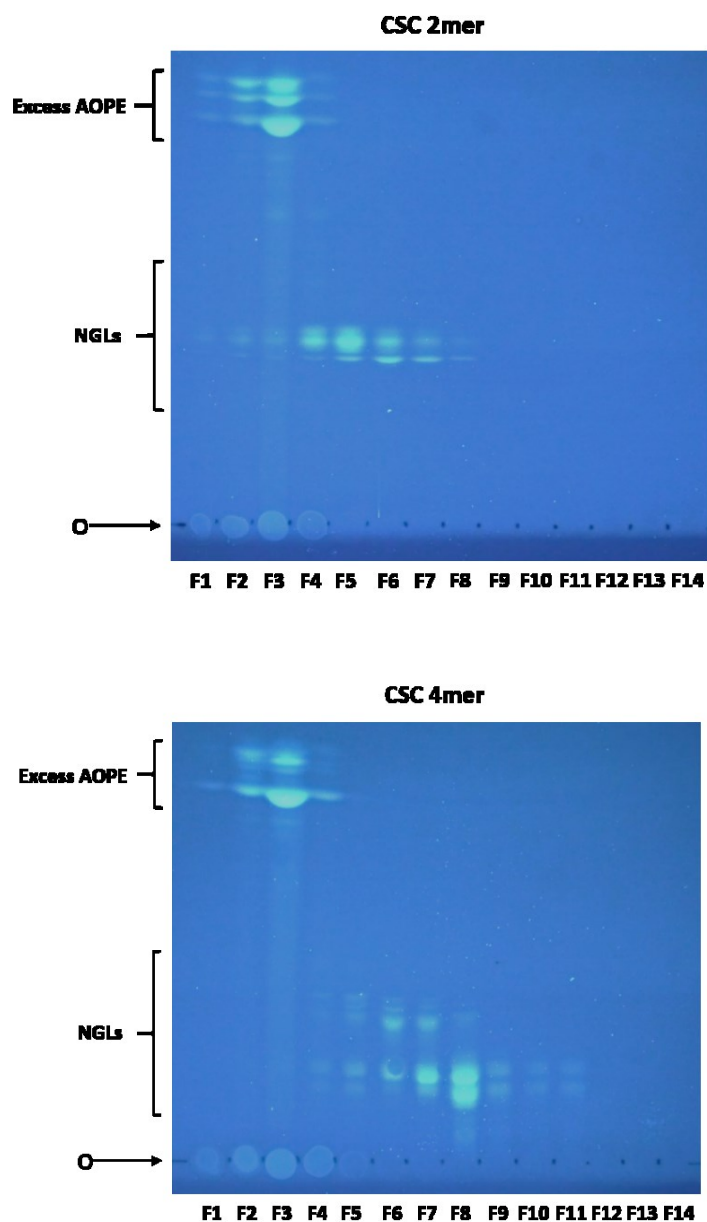


Figure 3.8. HPTLC and primulin staining of aliquot of eluted fractions from silica purification of NGL reaction mixtures of CSA and CSC 2 to 4 mers. NGLs were revealed under UV light with long wavelength after staining with primulin. Chromatography was in solvent $\text{CHCl}_3:\text{MeOH}:\text{H}_2\text{O}$ 60/35/8 (v/v/v). O indicates the origin (position of application).

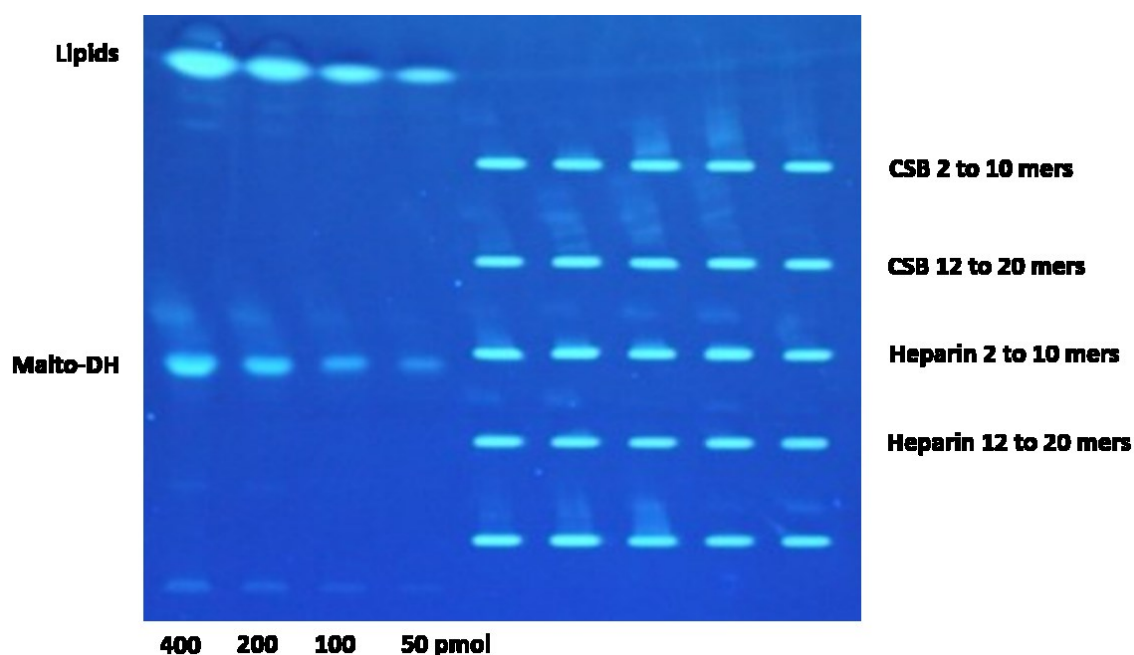


Figure 3.9. HPTLC and primulin staining of non-chromatographed CSA and CSC 2 to 20 mers quantified by chromatographed Malto-DH standards. Chromatography of Malto-DH standards were in solvent $\text{CHCl}_3:\text{MeOH}:\text{H}_2\text{O}$ 60/35/8 (v/v/v). NGLs were revealed under UV light with long wavelength after staining with primulin.

3.3.3 Validation of the generated glycosaminoglycan oligosaccharide neoglycolipid microarray

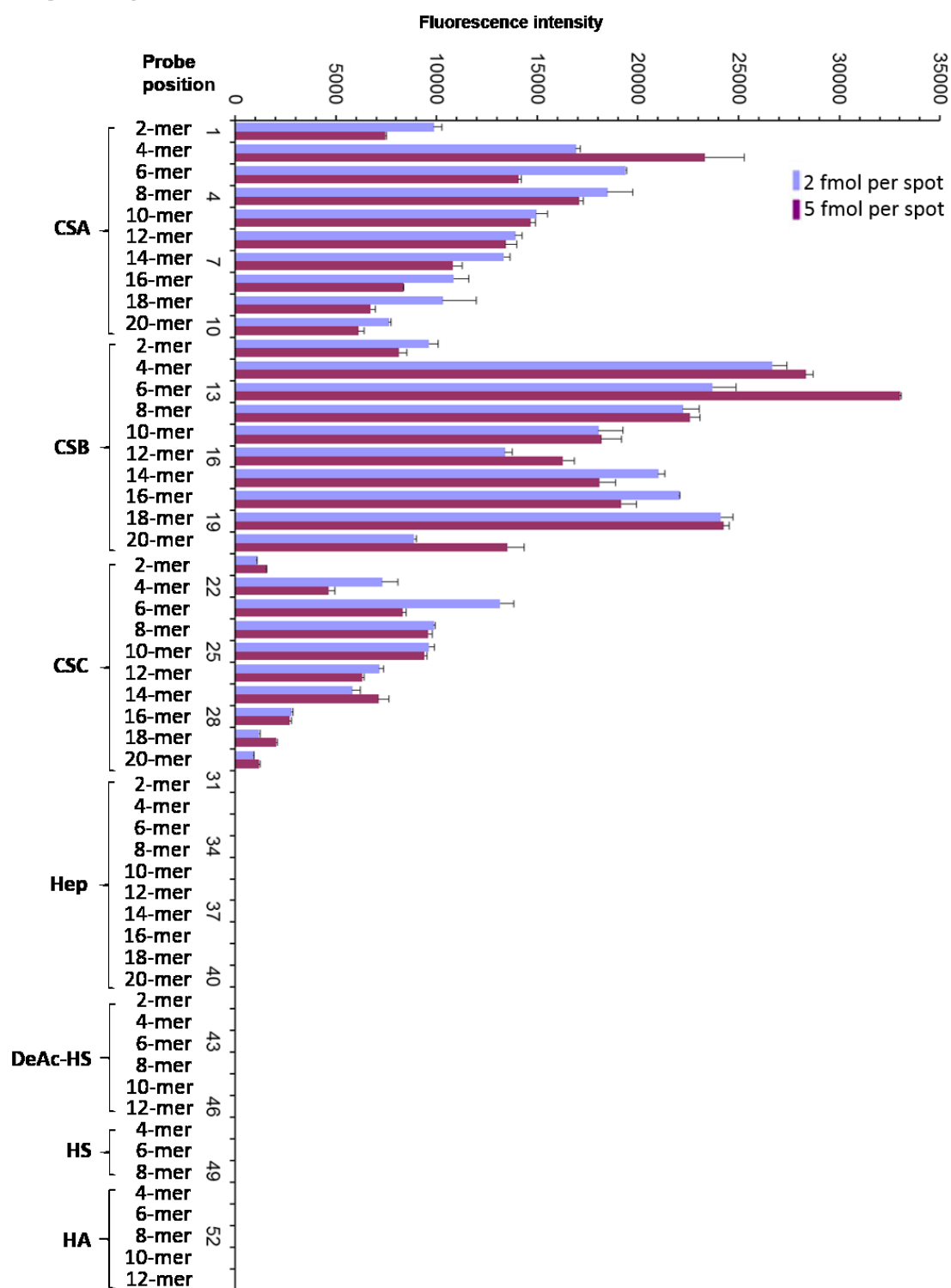
Quality of the GAG oligosaccharide NGL microarray Set 6 was checked with anti-CS Di4S, anti-CS Di6S, and human factor H. Anti-CS Di4S, anti-CS Di6S have been reported to bind to oligosaccharides obtained from chondroitinase ABC digestion of CSA, CSB and CSC. The former showed binding preference to oligosaccharides with sulphates on 4 position of uronic acid, whereas the later preferred those with sulphates on 6 position of uronic acid [228]. Human factor H has been reported to bind heparin, CS and HA [229-232].

The anti-CS Di4S and anti-CS Di6S bound to NGLs of 2-20mers of CSA, CSB and CSC, but not to NGLs of oligosaccharides from heparin, deAc-HS, HS and HA (Figure 3.10). The binding signals given by anti-CS Di4S were stronger overall with the arrayed CSA and CSB NGLs. The binding of anti-CS Di6S was stronger with CSC NGLs. The cross reactions

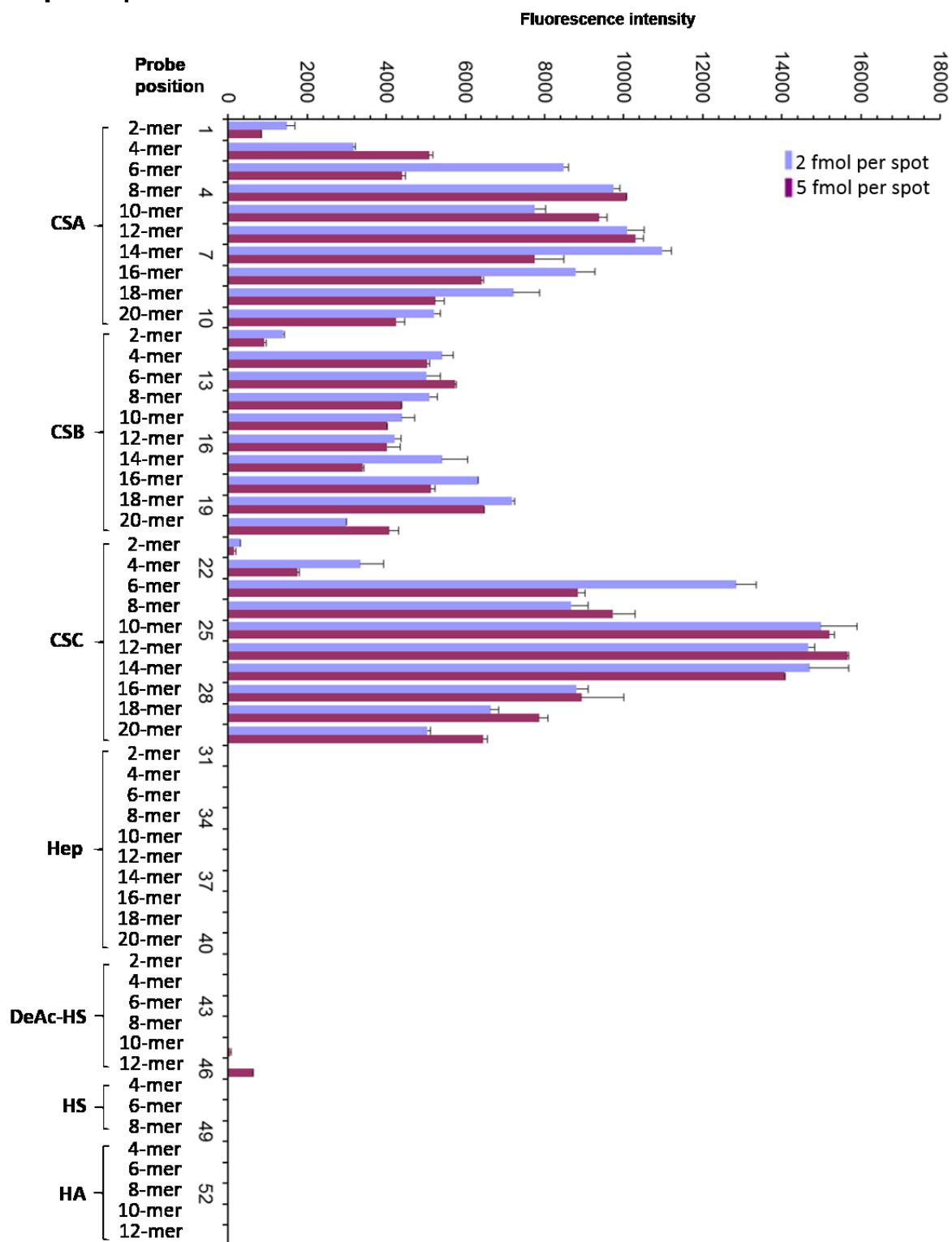
are explained by the inevitable composition overlaps of commercially available chondroitin sulphate polysaccharides; those for CSA and CSC have been documented previously [233]. Human factor H showed overall binding to all the arrayed NGLs. It bound strongly to NGLs of heparin oligosaccharides and less strongly to NGLs of CSB, CSC, CSA and HS oligosaccharides. Very little binding to NGLs of oligosaccharides from deAc-HS and HA was observed (Figure 3.10).

Therefore, the microarray Set 6 is considered as being validated for subsequent microarray binding analyses.

mAb anti-CS Di4S (IgG, 1/200)
No precomplexation



mAb anti-CS Di6S (IgM, 1/200)
No precomplexation



**Human factor H (50 µg/mL)
No precomplexation**

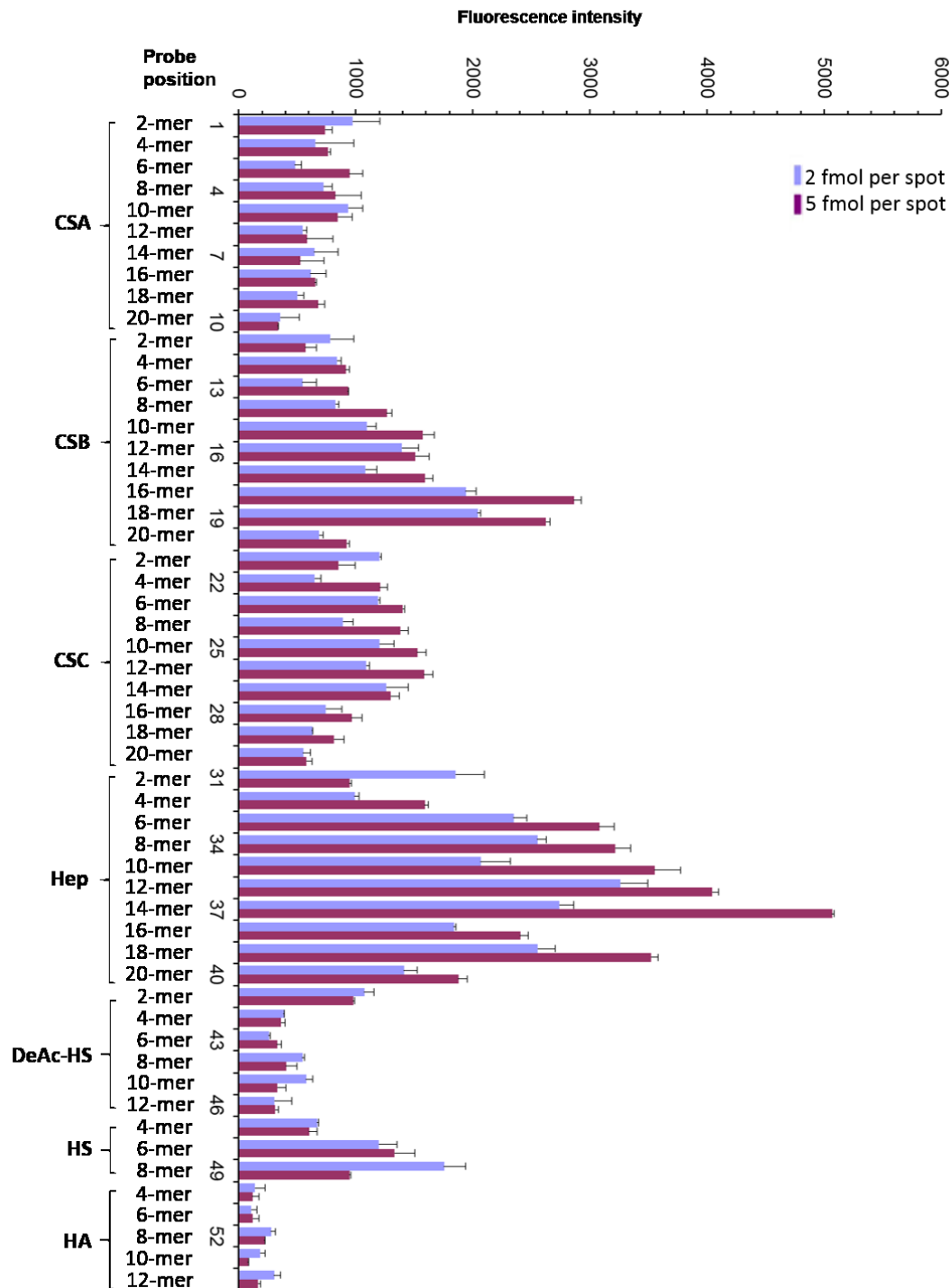


Figure 3.10. Microarray analysis of mAbs anti-CS Di4S (IgG) and anti-CS Di6S (IgM), and human factor H using GAG oligosaccharide NGL microarray Set 6. Blocking solution was PBS with 3% (w/v) BSA, and diluent was PBS with 1% (w/v) BSA. Binding of antibodies was detected using biotinylated anti-mouse IgG or IgM followed by Alexa Fluor-647-labeled streptavidin; binding of human factor H was detected using Goat anti-human Factor H antisera and anti-goat IgG followed by Alexa Fluor-647-labeled streptavidin. Results are means of fluorescence intensities of duplicate spots, printed at 2 and 5 fmol per spot, with error bars representing half of the difference between the two values. The results shown here are representative of three analyses with anti-CS Di4S and anti-CS Di6S and four analyses with human factor H using this microarray.

3.4 Discussion

GAGs are linear sulphated polysaccharides (HA without sulphates). The structural heterogeneity and complexity of the GAGs raise the difficulties both in their purification from natural sources and precise assignments of the sequences. The oligosaccharides are arrayed as NGLs of size-defined fractions, which are monitored by MS. This enables us to study GAG-protein interaction in vitro with small amount of GAG samples. Selected antigen-positive fractions can be subfractionated as necessary and analysed by MS and NMR. The importance of monitoring by MS and NMR was highlighted by the discovery of the deacetylated status of a commercial sample of HS (HO-01214) from Celsus.

During digestion of the HS (HO-01214) from Celsus, the very high content of GlcN and low content of GlcNAc were found, indicating a N-deacetylation and/or N-desulfation procedure had taken place. The HS was very different from all other HS preparation analysed and not as defined by Celsus in the product information of comprising primarily aGlcNAcb1-4GlcA (75%).

In my thesis, the major application of the quality-checked GlycosaminoGlycome microarrays has been for characterization of the carbohydrate antigen recognized by anti-stem cell antibody R-10G. This paved a way to the generation of 'designer' microarray from a selected polysaccharide (bovine corneal KS) to characterize the antigenic determinant for this antibody. The microarrays have also been used in initial investigation of the GAG-binding preferences of several growth factors and morphogens.

Chapter 4

Characterization of the Human Stem Cell Antigen R-10G with Keratan Sulphate Designer Microarrays

4.1 Introduction

My work described in this Chapter is characterization of a human iPS/ES cell marker which is a GAG-type glycan antigen recognized by the mAb R-10G. This antibody (IgG) was produced by Kawasaki and colleagues [73]. The antibody was screened from 960 hybridomas produced by immunizing C57BL/6 mice with the human iPS cell line Tic. Hybridomas screenings showed that R-10G exhibited strong reactivity toward Tic cells but no or very weak reactivity toward the human EC cell line 2102Ep, which was confirmed in the follow-up immunocytochemistry experiment using three human iPS, two human ES and one human EC cell lines [73].

The antigen recognized by R-10G, therefore, is distinct from other human stem cell antigens such as TRA-1-60, TRA-1-81, SSEA-3 and SSEA-4 which are expressed on both human iPS/ES and EC cells [91, 92, 98, 99]. The R-10G antigen thus can act as a marker to distinguish between normal and malignant human stem cells. The carrier of the R-10G antigen, podocalyxin, is a sialomucin glycoprotein, heavily O-glycosylated with five potential N-glycan and four potential GAG glycosylation sites [73, 234]. Interestingly, the glycoprotein is also the carrier of the TRA-1-60 and -81 antigens. It has been shown that the R-10G antigen-positive component (~250 kDa) isolated from the human iPS cell line Tic with an R-10G-affinity column was sensitive to endo- β -galactosidase and keratanase II (the latter is KS-specific) [73]. R-10G was shown to bind to bovine corneal KS and the binding was more strongly inhibited by bovine corneal KS than by the more highly-sulphated shark cartilage [73]. The authors suggested that the R-10G antigen is expressed on undersulphated KS. However, the precise sequence of the R-10G antigen was not determined. While my thesis work was in progress, Kawasaki's group has investigated the specificity of mAb R-10G using chemically synthesised tetrasaccharides [235]. I refer to their data later in the Discussion Chapter (7).

To elucidate the carbohydrate structure of the R-10G antigen, the 'designer' microarray approach was used [198], which involves:

- i) initial GAG polysaccharide microarray screening analysis to detect polysaccharide binding and select the antigen-positive polysaccharide for further detailed analysis;
- ii) generate GAG oligosaccharide fractions of different chain lengths by partial depolymerisation and convert these into NGL probes;
- iii) construct NGL microarrays of GAG oligosaccharide fractions to identify those that are positive for the R-10G antigen;
- iv) select an antigen-positive fraction to subfractionate by multiple chromatographies to determine the sequence (e.g. the degree and pattern of sulphation) of the R-10G antigen by MS and NMR.

4.2 Bovine Corneal Keratan Sulphate Identified as R-10G

Antigen-Positive Polysaccharide using a Glycosaminoglycan Polysaccharide Microarray

The GAG polysaccharide microarray described in Chapter 3, containing CSA, CSB, CSC, HEP, HS, HA and three KS preparations isolated from bovine cornea, bovine articular cartilage and chicken egg white (Table 3.1 in Chapter 3), was used to assess initially the binding of R-10G to GAG polysaccharides.

R-10G showed strong binding to the bovine corneal KS (Figure 4.1) whereas there was very weak binding to the KS isolated from chicken egg white and almost no binding to the KS from bovine articular cartilage. There was no binding observed to all the other types of GAG polysaccharides except for the very weak binding to CSA (Figure 4.1). The

bovine corneal KS was then selected as R-10G antigen-positive polysaccharide for further studies.

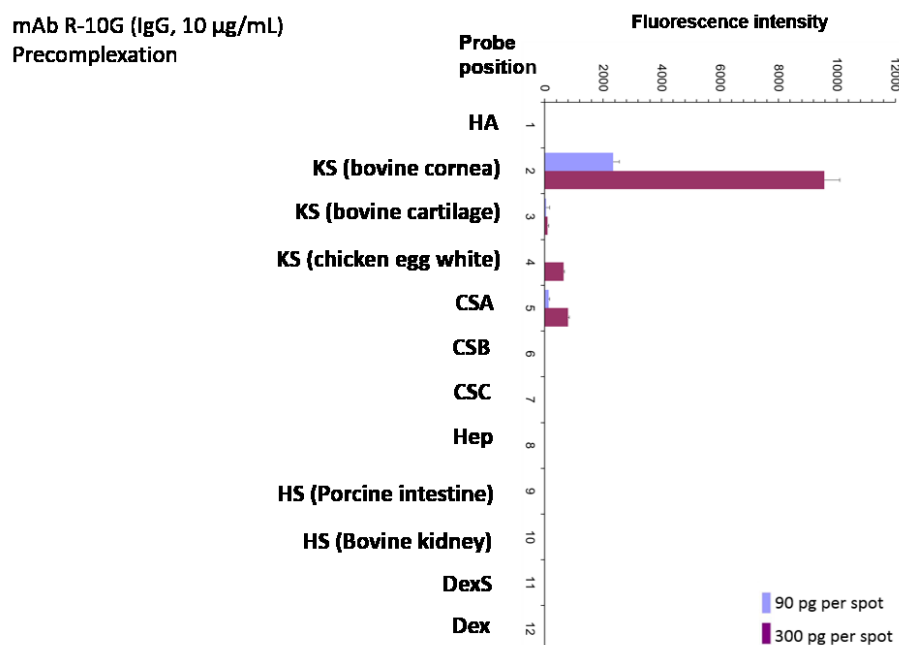


Figure 4.1. GAG polysaccharide microarray analysis of mAb R-10G (IgG). Probe abbreviations are defined in Table 3.1. Blocking solution and diluent were PBS containing 1% (w/v) BSA and 0.33% (w/v) casein. Binding was detected using biotinylated anti-mouse IgG followed by Alexa Fluor-647-labeled streptavidin. Results are means of fluorescence intensities of duplicate spots, printed at 90 and 300 pg per spot, with error bars representing half of the difference between the two values. The result shown here is representative of numerous analyses with R-10G using this microarray.

4.3 Partial Depolymerization of Bovine Corneal Keratan Sulphate using Different Methods to Prepare Diverse Oligosaccharides

The bovine corneal KS polysaccharide was partially depolymerised to obtain oligosaccharides of different chain lengths. KS has been known to consist of repeating β 3Gal β 1-4GlcNAc β 1- disaccharide units with sulphates on various positions of these residues. Both enzymatic (two KS specific glycosidases, keratanase I and II) and chemical (mild acid) methods were used for treatment of the bovine corneal KS to obtain diverse oligosaccharides.

The cleavage principles of the two enzymes for KS chains are different (Figure 4.2). Keratanase I from *Pseudomonas* sp. acts on nonsulphated β 1-4 galactosidic linkages with neighbouring sulphated GlcNAcs, thus yielding oligosaccharides with GlcNAc at non-reducing ends [206]. Keratanase II, an endo- β -N-acetylglucosaminidase, acts on 6-O-sulphated β 1-3 acetylglucosaminidic linkages, producing oligosaccharides with Gal at the non-reducing ends [207]. The random nature of mild acid hydrolysis results in cleavage of KS at both β 1-4 galactosidic and β 1-3 N-acetylglucosaminic linkages regardless of sulphate substitution. The KS oligosaccharide fragments generated by acid hydrolysis are therefore more heterogeneous and might include sequences different from those produced after the enzyme digestions. Please note that to avoid confusion in the names of the two KS specific enzymes, the term ‘keratanase I’ was used throughout the thesis instead of the commonly used unqualified ‘keratanase’.

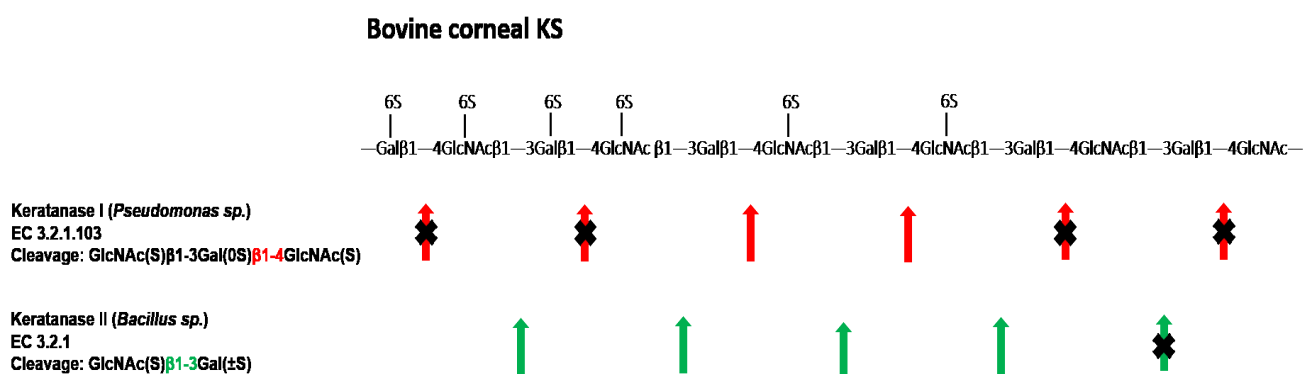


Figure 4.2. Schematic representation of the cleavage specificities of keratanase I and keratanase II for bovine corneal KS. The cleavage sites on an idealized KS chain from bovine cornea are indicated by arrows. Arrows with crosses represent sites that are not cleaved.

4.3.1 Partial digestion of bovine corneal keratan sulphate with keratanase I to obtain oligosaccharides with GlcNAc at the non-reducing ends

The bovine corneal KS was treated with keratanase I and the reaction was monitored at 5, 8 and 22h by Superdex Peptide chromatography of an aliquot of each reaction mixture (Figure 4.3) and stopped at 23h to achieve partial digestion. The final reaction mixture was chromatographed by Bio-Gel P6 and ten fractions were collected as indicated (Figure 4.4).

The compositions of Fractions 1-3 could be deduced by ES-MS (Table 4.1). Fraction 1 contained a disaccharide with one sulphate (Gal1.GlcNAc1.S1) as the major component. Fraction 2 contained tetrasaccharides with two or three sulphates, Gal2.GlcNAc2.S2 and Gal2.GlcNAc2.S3. There were minor ions assigned as tetrasaccharides with one sulphate (Gal2.GlcNAc2.S1). Fraction 3 consisted of hexasaccharides with two (Gal3.GlcNAc3.S2), three (Gal3.GlcNAc3.S3), four (Gal3.GlcNAc3.S4) or five (Gal3.GlcNAc3.S5) sulphates.

In accord with the specificity of keratanase I, Fractions 1 to 3 obtained after the enzyme digestion contained even-numbered monosaccharides. The backbone sizes of major oligosaccharides under peaks 4 to 10 could thus be extrapolated. They were assigned as various sulphated 8 to 20 mers.

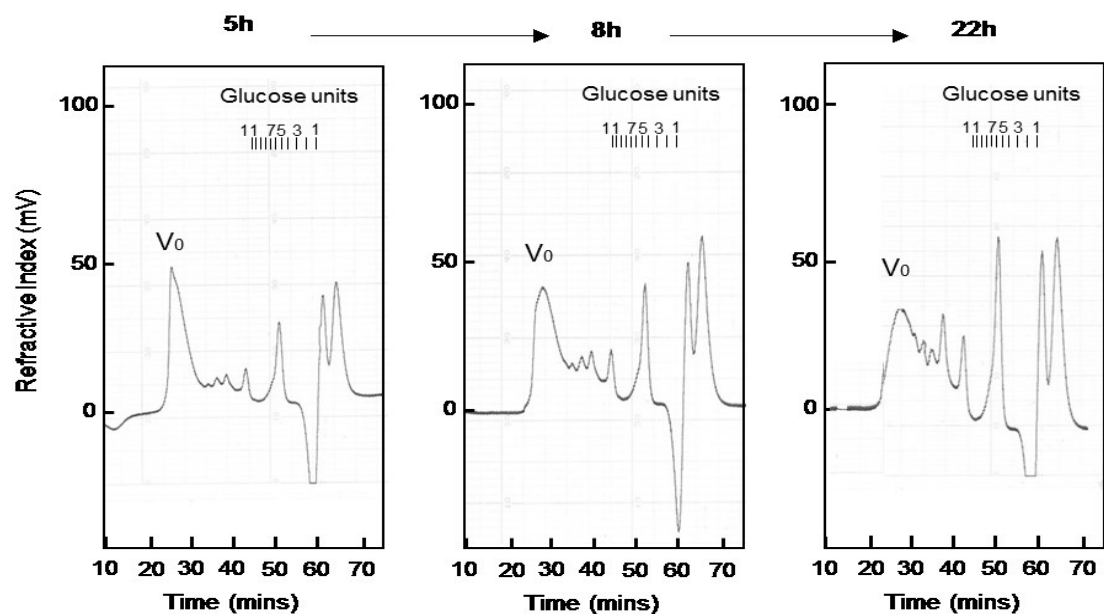


Figure 4.3. Chromatography on Superdex Peptide column of aliquots of bovine corneal KS digested with keratanase I at 5, 8 and 22h. Positions of elution of glucose oligomers (glucose units, degrees of polymerization 1-11) and V_0 (void volume) are shown.

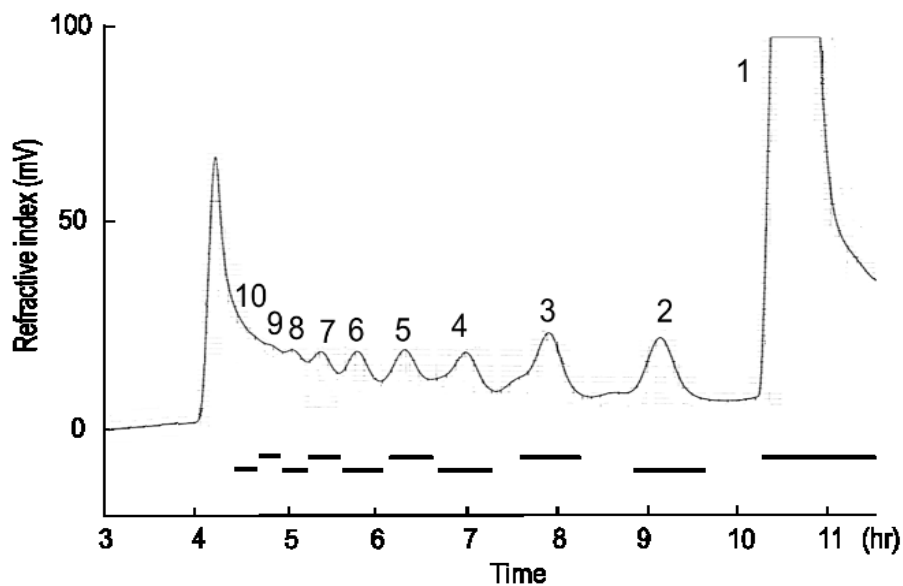


Figure 4.4. Chromatography on Bio-Gel P6 of the oligosaccharides derived from bovine corneal KS after partial digestion (23h) with keratanase I. Ten fractions were pooled after as shown.

Table 4.1. Negative-ion ES-MS analysis of oligosaccharides in Bio-Gel P6 fractions 1-3 of bovine corneal KS digests with keratanase I

Fractions	Oligosaccharides		Fractions	Oligosaccharides	
	Detected ions (m/z)*	Deduced compositions		Detected ions (m/z)	Deduced compositions
F1	462 (z=1)	Gal1.GlcNAc1.S1	F3	635.7 (z=2)	Gal3.GlcNAc3.S2
F2	453.1 (z=2)	Gal2.GlcNAc2.S2		450.1 (z=3)	Gal3.GlcNAc3.S3
	504.1 (z=2)	Gal2.GlcNAc2.S3.Na1		686.6 (z=2)	Gal3.GlcNAc3.S3.Na1
	328.4 (z=2)	Gal2.GlcNAc2.S3		734.6 (z=2)	Gal3.GlcNAc3.S4.K
	827.2 (z=1)	Gal2.GlcNAc2.S1		785.6 (z=2)	Gal3.GlcNAc3.S5.Na1.K1

*m/z is mass to charge ratio.

Gal, Galactose; GlcNAc, N-acetylglucosamine; S, sulphate.

The numbers in the deduced compositions refer to monosaccharides, sulphates, sodium (Na) and potassium (K) per molecule of oligosaccharide.

4.3.2 Partial digestion of bovine corneal keratan sulphate with keratanase II to obtain oligosaccharides with Gal at the non-reducing ends

The keratanase II was used to partially digest the bovine corneal KS for preparation of oligosaccharides terminating with Gal (with or without sulphate) at the non-reducing ends. The reaction was monitored at 2, 5 and 6.5h by Superdex Peptide chromatography of an aliquot of each reaction mixture (Figure 4.5) and stopped at 7.5h to achieve partial digestion. The final reaction mixture was chromatographed by Bio-Gel P6 and nine fractions were collected as indicated (Figure 4.6).

Compositions of Fractions 1-3 were deduced by their ES-MS analyses (Table 4.2). Di-sulphated disaccharide (Gal1.GlcNAc1.S2) was the major component of Fraction 1. Fraction 2 mainly contained tetrasaccharides with three, four or two sulphates, Gal2.GlcNAc2.S3, Gal2.GlcNAc2.S4 and Gal2.GlcNAc2.S2 (in order of the abundance). Fraction 3 consisted of hexasaccharides with three (Gal3.GlcNAc3.S3), four (Gal3.GlcNAc3.S4) or two (Gal3.GlcNAc3.S2) sulphates.

Backbones of the oligosaccharides contained in the fractions 1 to 3 were even-

numbered, corresponding to the cleavage specificity of keratanase II. The backbone sizes of major oligosaccharides in other fractions giving peaks in the chromatogram therefore were extrapolated. Fractions 4 to 9 are assigned as various sulphated 8 to 18 mers.

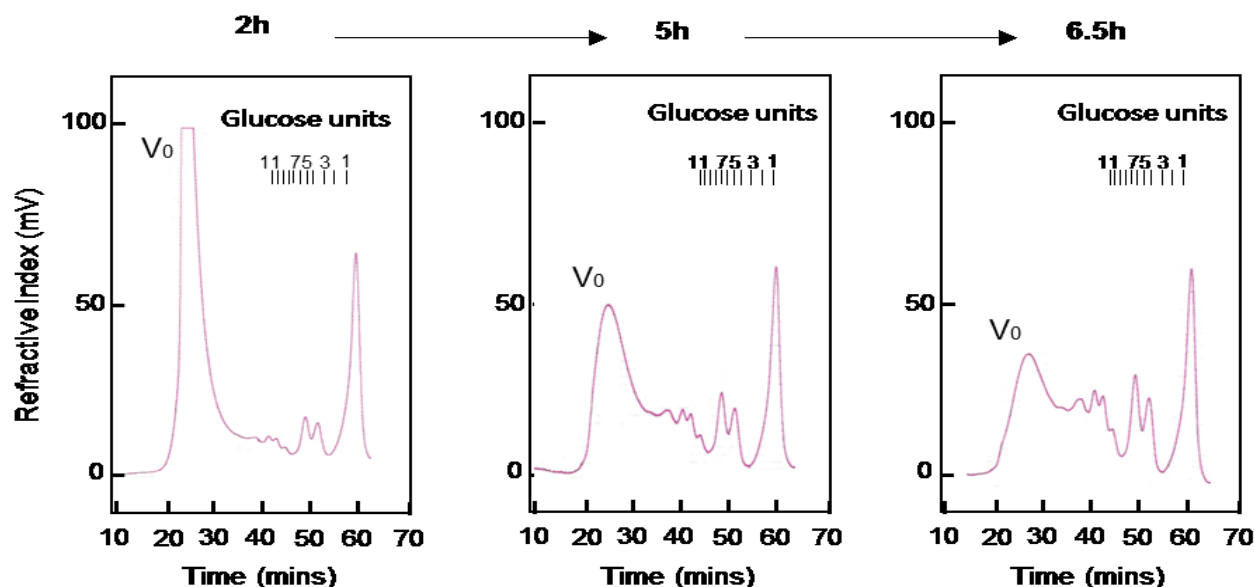


Figure 4.5. Chromatography on Superdex Peptide of aliquots of bovine corneal keratan sulphate digested with keratanase II at 2, 5 and 6.5h. Positions of elution of glucose oligomers (glucose units, degrees of polymerization 1-11) and V_0 (void volume) are shown.

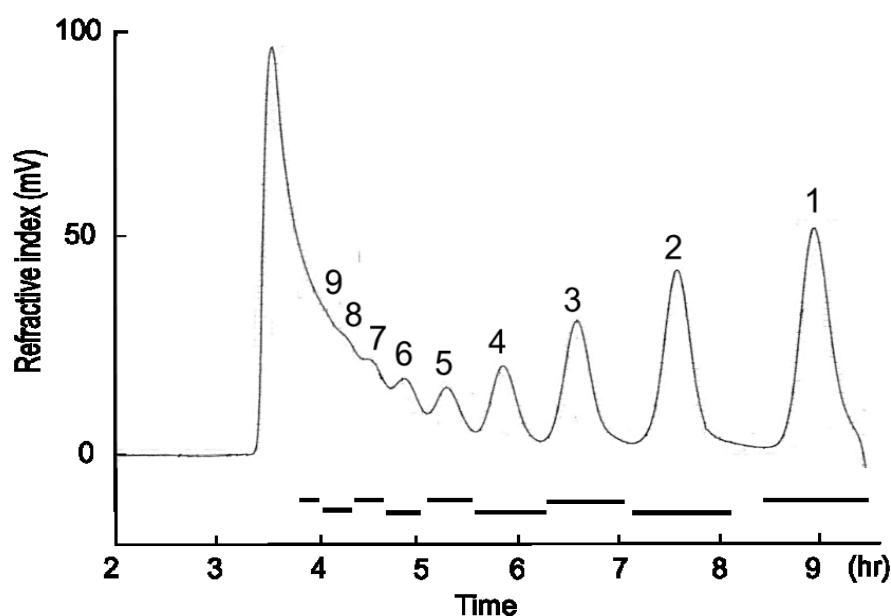


Figure 4.6. Chromatography on Bio-Gel P6 of the oligosaccharides derived from bovine corneal KS after partial digestion (7.5h) with keratanase II. Nine fractions were pooled after as shown.

Table 4.2. Negative-ion ES-MS analysis of oligosaccharides in Bio-Gel P6 Fractions 1-3 of bovine corneal KS digests with keratanase II

Fractions	Oligosaccharides		Fractions	Oligosaccharides	
	Detected ions (m/z)	Deduced compositions		Detected ions (m/z)	Deduced compositions
F1	419.6 (z=3)	Gal1.GlcNAc1.S2	F3	450.1 (z=3)	Gal3.GlcNAc3.S3
	629.2 (z=2)	Gal1.GlcNAc1.S2		686.7 (z=2)	Gal3.GlcNAc3.S3.Na1
	640.9 (z=2)	Gal1.GlcNAc1.S2.Na1		357.3 (z=4)	Gal3.GlcNAc3.S4
F2	328.4 (z=3)	Gal2.GlcNAc2.S3		484.1 (z=3)	Gal3.GlcNAc3.S4.Na1
	504.1 (z=2)	Gal2.GlcNAc2.S3.Na1		737.7 (z=2)	Gal3.GlcNAc3.S4.Na2
	266 (z=4)	Gal2.GlcNAc2.S4		635.7 (z=2)	Gal3.GlcNAc3.S2
	453.1 (z=2)	Gal2.GlcNAc2.S2			

To corroborate the products of keratanase I and keratanase II digestion of KS and identify the reducing and non-reducing end residues of the derived products, NMR spectra of the tetrasaccharide fractions from the two digestions were compared. Published results for high-field ^1H and ^{13}C NMR spectroscopy of KS oligosaccharides are available [207, 236-239]. However, the oligosaccharides analysed are reduced rather than reducing, so exact comparisons of reducing end residues are not possible. ^1H NMR spectra indicate that both tetrasaccharides are mixtures (Figure 4.7), and the spectra cannot offer any scope for comparison with literature values. Signals from non-reducing end residues and internal residues overlap and are not easily distinguished.

Complete analysis by 2D NOESY and HMBC would give unambiguous confirmation of sequence but would be difficult without purification of single molecular species. Fortunately, $^{13}\text{C}/^1\text{H}$ HSQC gives us a way in to the problem (Figure 4.8). Literature values for ^1H and ^{13}C chemical shifts of internal and non-reducing end residues help identify the reducing end β -anomeric signals [207, 236-239]. Carbon-proton β -anomeric cross-peaks from the reducing end monosaccharide residue have similar proton chemical shifts to their internal and non-reducing end equivalents, but are shifted upfield in the carbon dimension, as indicated in Figure 4.8.

The keratanase I digestion-derived tetrasaccharide is then established as

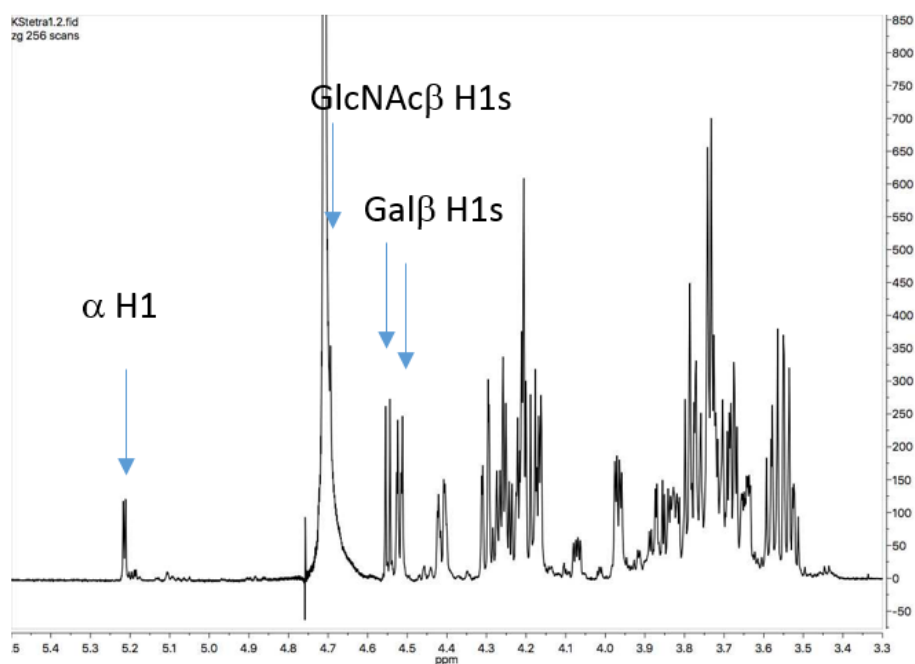


whereas the keratanase II digestion-derived tetrasaccharide is



where R_1 is expected to be sulphate, and R_2 is sulphate or OH.

Tetra I



Tetra II

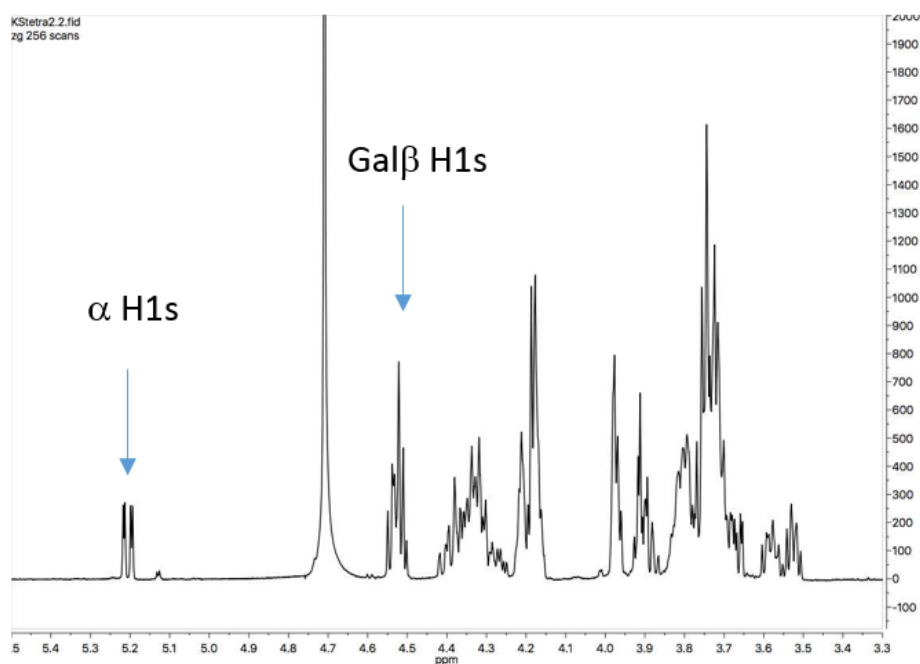
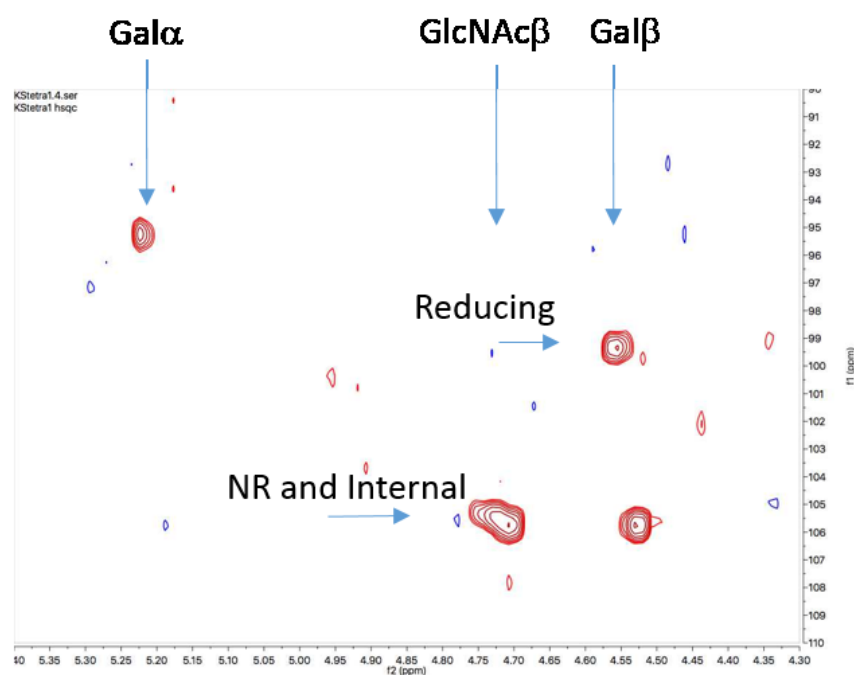


Figure 4.7. ¹H NMR spectra of tetrasaccharide fractions obtained from depolymerisation of bovine corneal KS with keratanase I and keratanase II. Tetra I indicates keratanase I digestion-derived tetrasaccharide while tetra II indicates keratanase II digestion-derived tetrasaccharide. 's' is to show a plural, for example, in Tetra II there are two α H₁.

Tetra I



Tetra II

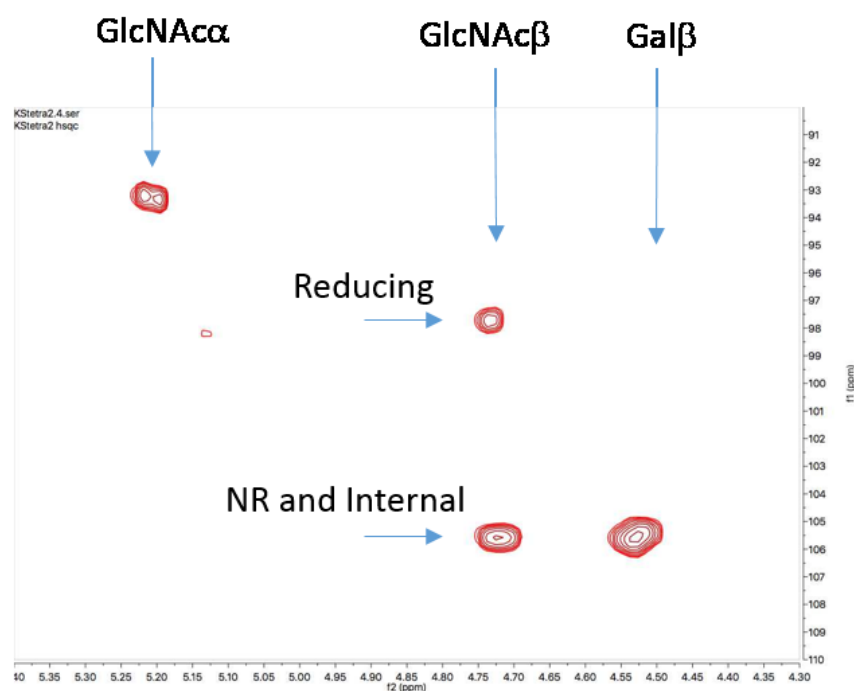


Figure 4.8. Anomeric region of the HSQC NMR spectra of tetrasaccharide fractions obtained from depolymerisation of bovine corneal KS with keratanase I and keratanase II. Tetra I indicates keratanase I digestion-derived tetrasaccharide while tetra II indicates keratanase II digestion-derived tetrasaccharide. NR and internal means non-reducing and internal residues. Proton chemical shifts of the cross-peaks indicate that Tetra I has Gal at its reducing end whereas Tetra II has GlcNAc at its reducing end.

4.3.3 Mild acid hydrolysis of bovine corneal keratan sulphate

In an exploratory study, KS was treated with 0.1 M HCl and the Superdex Peptide chromatography profiles were observed at 2h and 4h of treatment. The reaction was stopped at 4h when the peak at void volume almost disappeared (Figure 4.9). Three fractions were collected as shown in each run. They were pooled and analysed by ES-MS (Figure 4.10, Table 4.3).

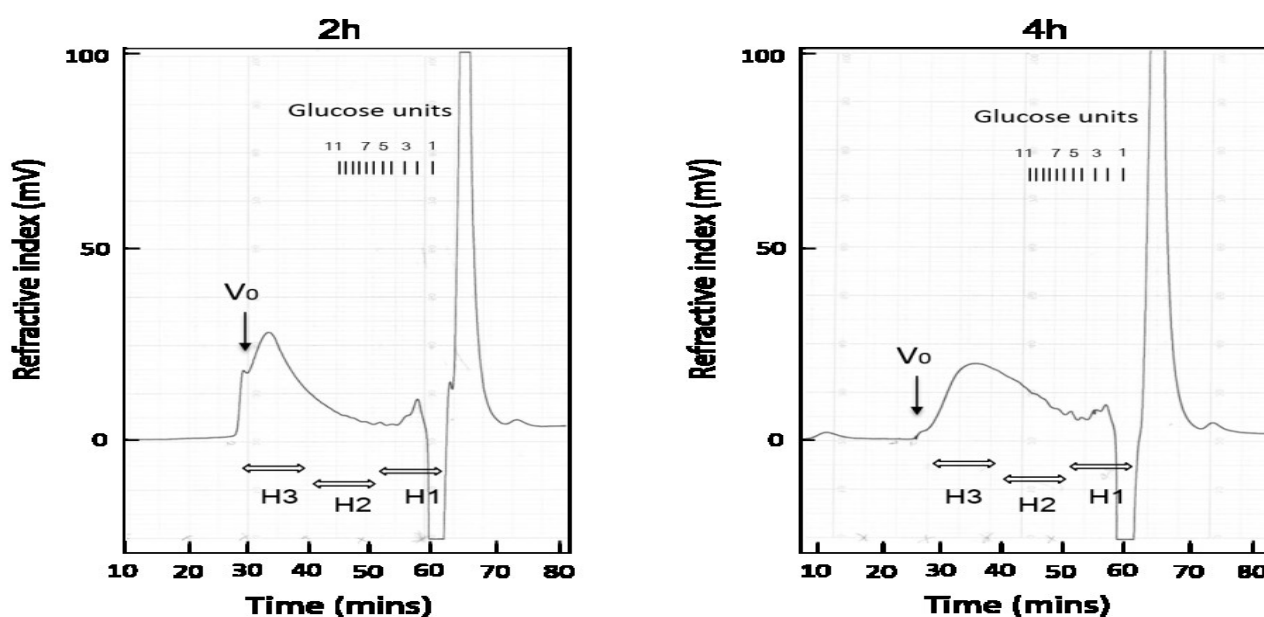


Figure 4.9. Chromatography on Superdex Peptide of oligosaccharides derived from bovine corneal KS after 2 h and 4 h acid hydrolysis. Three fractions (H1-H3) in each case were pooled as shown. Positions of elution of glucose oligomers (glucose units, degrees of polymerization 1-11) and V0 (void volume) are shown.

Fraction H1 contained both even- and odd- numbered oligosaccharides, variously sulphated. The backbones of the even-numbered oligosaccharides included di- and tetra-saccharides; there are two possible backbone sequences for each oligosaccharide as MS alone cannot provide information on their sequences. The backbone sequence of the odd-numbered oligosaccharides could be readily deduced from their molecular masses. The even-numbered oligosaccharides could have the same backbones as those existing in keratanase I digests of the KS, whose reducing termini were Gal (Structures 1 and 3), as well as those in keratanase II digests of KS, whose reducing termini were

GlcNAc (Structures 2 and 4). The odd-numbered trisaccharide (Structure 5 and 6) would be distinctive products of acid hydrolysis.

Structure 1	GlcNAc β 1-3Gal
Structure 2	Gal β 1-4GlcNAc
Structure 3	GlcNAc β 1-3Gal β 1-4GlcNAc β 1-3Gal
Structure 4	Gal β 1-4GlcNAc β 1-3Gal β 1-4GlcNAc
Structure 5	GlcNAc β 1-3Gal β 1-4GlcNAc
Structure 6	Gal β 1-4GlcNAc β 1-3Gal

Similarly, Fraction H2 contained both even- and odd- numbered oligosaccharides with longer chain lengths than those in Fraction H1, ranging from tetra- to hepta-saccharides. The ES-MS spectrum of H3 was uninformative: no ions for KS-related oligosaccharide were detected in this fraction. As the exclusion limit of the superdex peptide column used for chromatography in my research is 20,000 Da, the size of KS oligosaccharides in Fraction H3 could range from 15 to 80 mer. These heterogeneous long chains are difficult to be analysed by ES-MS.

In sum, the oligosaccharides in Fractions H1 and H2 have even- and odd- numbered molecules with differing sulphate substituents as the depicted below. The odd-numbered oligosaccharides contribute to the diversity of oligosaccharide probes in the microarray.

- 2mer** 2a **GlcNAc** β 1-3Gal + (S_{0-2})
 2b **Gal** β 1-4GlcNAc + (S_{0-2})
- 3mer** 3a **GlcNAc** β 1-3Gal β 1-4GlcNAc + (S_{0-3})
 3b **Gal** β 1-4GlcNAc β 1-3Gal + (S_{0-3})
- 4mer** 4a **GlcNAc** β 1-3Gal β 1-4GlcNAc β 1-3Gal + (S_{0-4})
 4b **Gal** β 1-4GlcNAc β 1-3Gal β 1-4GlcNAc + (S_{0-4})
- 5mer** 5a **GlcNAc** β 1-3Gal β 1-4GlcNAc β 1-3Gal β 1-4GlcNAc + (S_{0-5})
 5b **Gal** β 1-4GlcNAc β 1-3Gal β 1-4GlcNAc β 1-3Gal + (S_{0-5})
- 6mer** 6a **GlcNAc** β 1-3Gal β 1-4GlcNAc β 1-3Gal β 1-4GlcNAc β 1-3Gal + (S_{0-6})
 6b **Gal** β 1-4GlcNAc β 1-3Gal β 1-4GlcNAc β 1-3Gal β 1-4GlcNAc + (S_{0-6})
- 7mer** 7a **GlcNAc** β 1-3Gal β 1-4GlcNAc β 1-3Gal β 1-4GlcNAc β 1-3Gal β 1-4GlcNAc + (S_{0-7})
 7b **Gal** β 1-4GlcNAc β 1-3Gal β 1-4GlcNAc β 1-3Gal β 1-4GlcNAc β 1-3Gal + (S_{0-7})

Table 4.3. Negative ES-MS analysis of oligosaccharide in Superdex Fractions, H1 and H2 of acid hydrolysate of bovine corneal KS

Fractions	Oligosaccharides		Fractions	Oligosaccharides	
	Detected ions (m/z)	Deduced compositions		Detected ions (m/z)	Deduced compositions
H1	462 (z=1)	Gal1.GlcNAc1.S1	H2	827 (z=1)	Gal2.GlcNAc2.S1
	564 (z=1)	Gal1.GlcNAc1.S2.Na1		453.1 (z=2)	Gal2.GlcNAc2.S2
	624 (z=1)	Gal2.GlcNAc1.S1		929 (z=1)	Gal2.GlcNAc2.S2.Na1
	665 (z=1)	Gal1.GlcNAc2.S21		534.2 (z=2)	Gal3.GlcNAc2.S2
	767 (z=1)	Gal1.GlcNAc2.S2		382.4 (z=3)	Gal3.GlcNAc2.S3
	865 (z=1)	Gal2.GlcNAc1.S3.Na1. H ₂ O		396.1 (z=3)	Gal2.GlcNAc3.S3
	351 (z=2)	Gal2.GlcNAc1.S2		635.7 (z=2)	Gal3.GlcNAc3.S2
	827 (z=1)	Gal2.GlcNAc2.S1		716.7 (z=2)	Gal4.GlcNAc3.S2
	929 (z=1)	Gal2.GlcNAc2.S2.Na1		767.7 (z=2)	Gal4.GlcNAc3.S3.Na1
				504.2 (z=3)	Gal4.GlcNAc3.S3
				397.9 (z=3)	Gal4.GlcNAc3.S4
				736.7 (z=2)	Gal3.GlcNAc4.S2
				788.2 (z=2)	Gal3.GlcNAc4.S3.Na1

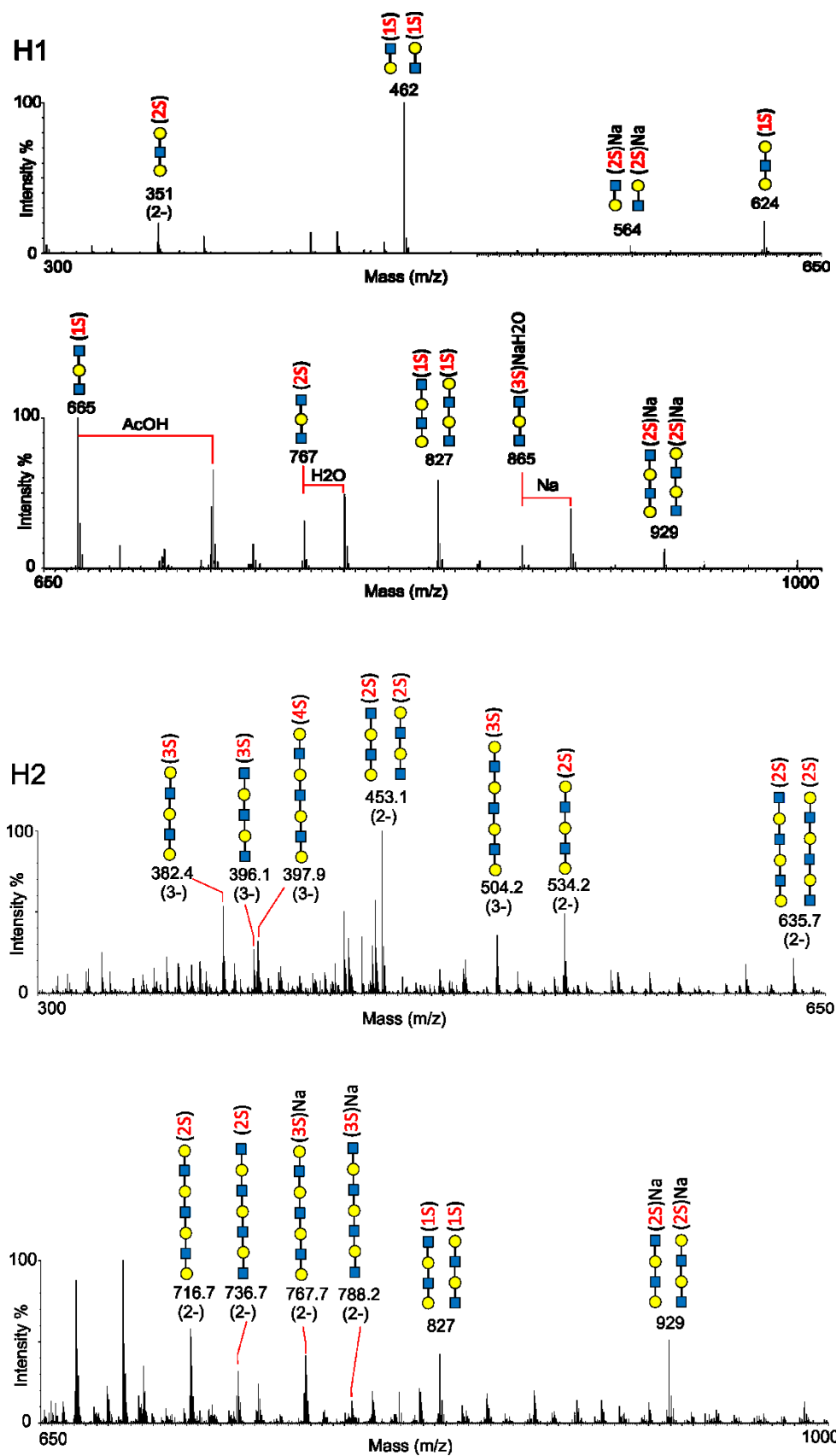
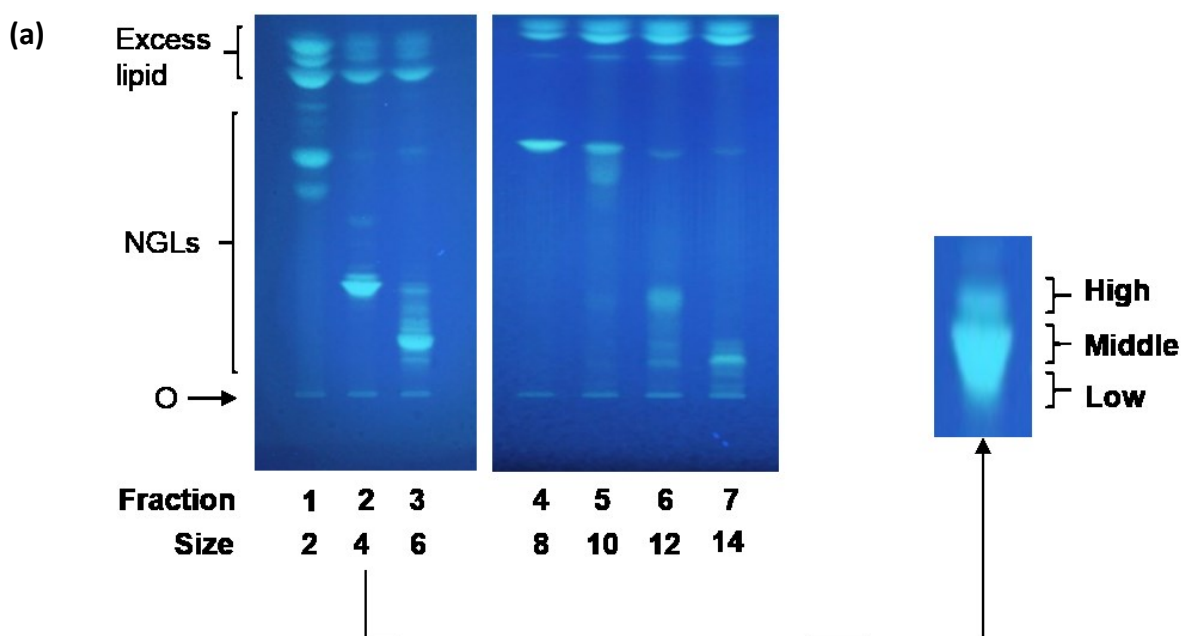


Figure 4.10. Negative-ion ES-MS spectra and suggested sequences of H1 and H2 fractions obtained after acid hydrolysis of bovine corneal KS.

4.4 Preparation of Neoglycolipid Probes of Bovine Corneal Keratan Sulphate Oligosaccharides for Arraying

KS oligosaccharides obtained after keratanase I and keratanase II digestions and acid hydrolysis were converted to NGLs by conjugating to the lipid APOE by oxime ligation. As the composition of the acid hydrolysis Fraction H3 was difficult to be identified by ES-MS, only the Fraction H1 and H2 which had been clearly analysed were converted to NGLs. Examples of HPTLC figures of the prepared NGLs were shown in Figure 4.11.

The mobility of NGLs on TLC depended on charge. NGLs of KS fractions that are shorter or less sulphated move faster than those longer or highly sulphated. The NGLs derived from fractions containing shorter chains were analysed by ES-MS. Their deduced compositions are almost consistent with the MS data of their correspondent oligosaccharides.



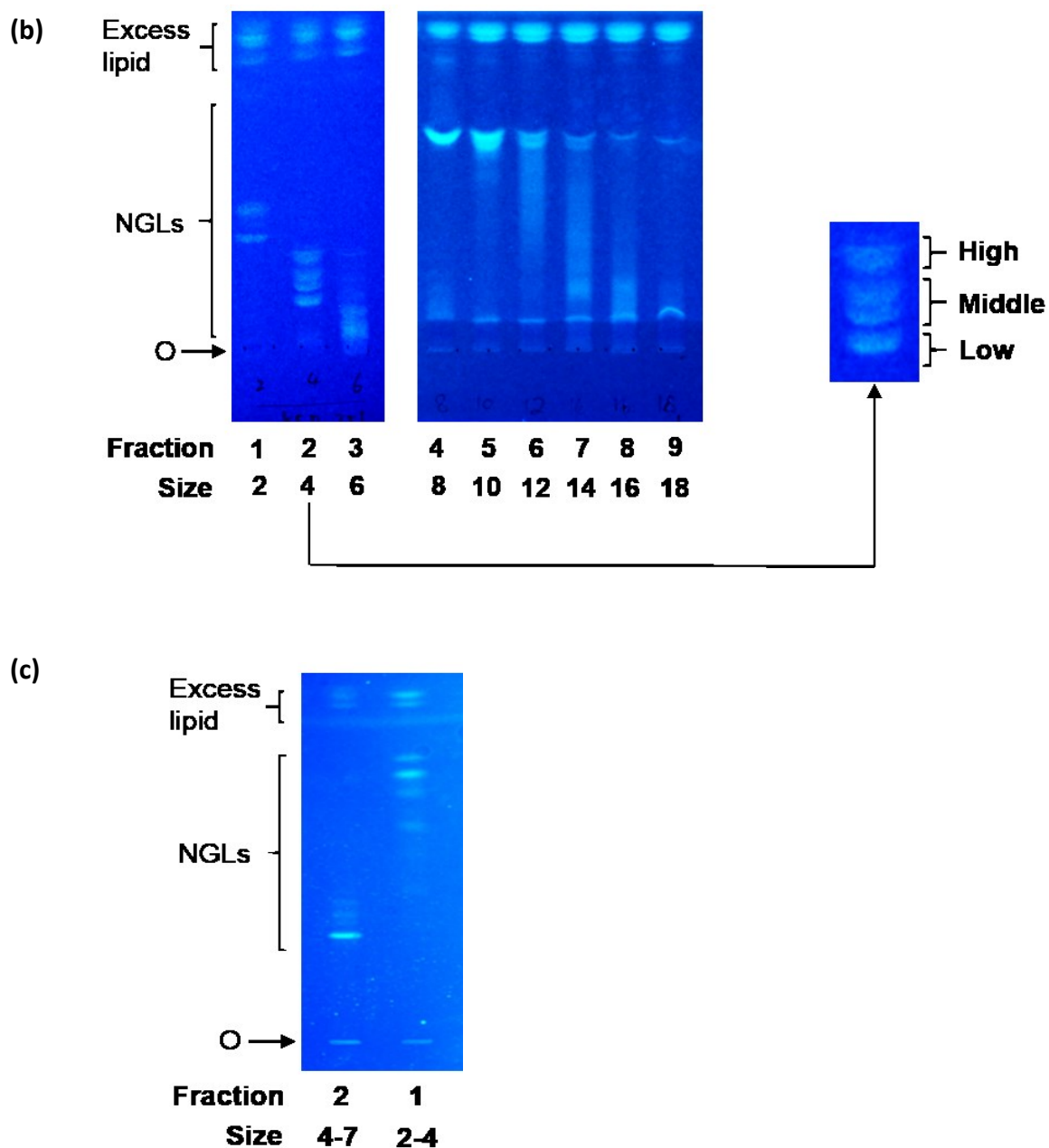


Figure 4.11. Examples of HPTLC and primulin staining of NGLs of KS oligosaccharide fractions. (a) From partial keratanase I digestion, (b) From partial keratanase II digestion, (c) From acid hydrolysis. NGLs were revealed under UV light with long wavelength after staining with primulin. Chromatography was in solvent $\text{CHCl}_3:\text{MeOH}:\text{H}_2\text{O}$ 60/35/8 (v/v/v). O indicates the origin. (position of application).

4.5 Keratan Sulphate Hexasaccharide Identified as the Minimum Chain Length Expressing R-10G Antigen using Keratan Sulphate Oligosaccharide Neoglycolipid Microarrays

NGLs of KS oligosaccharides derived from bovine corneal KS following partial keratanase I, II or acid depolymerisation were arrayed as KS oligosaccharide NGL microarray designated as set 1. These included NGLs of keratanase I digestion-derived 2 to 20 mers and keratanase II digestion-derived 2 to 18mers; subfractions of NGL of keratanase I and keratanase II digestion-derived tetrasaccharides designated as low, middle and high migrating on HPTLC (Figure 4.11 a and b); NGLs of two synthetic tetrasaccharides with the sequences Gal β 1-4GlcNAc β 1-3Gal β 1-4GlcNAc and Gal(6S) β 1-4GlcNAc(6S) β 1-3Gal(6S) β 1-4GlcNAc(6S) (these are designated OS and 4S respectively) (Figure 4.12).

R-10G strongly bound to NGLs of KS oligosaccharide fractions obtained after keratanase II partial digestion. By comparison, the binding to keratanase I oligosaccharide fractions was very weak. The minimum chain length among keratanase II fractions bound by R-10G was hexasaccharide fraction. No binding was observed to NGLs of keratanase II tetrasaccharide fraction and to the derived subfractions. R-10G showed strong binding to Fraction H2 obtained after acid hydrolysis which contained a variety of KS oligosaccharides such as tetra-, penta-, hexa and hepta-saccharides.

A separate analysis was carried out to determine if the R-10G is exclusively specific for the KS oligosaccharides using the GAG oligosaccharide NGL microarray described in Chapter 3 that include NGLs of oligosaccharides derived from CSA, CSB, CSC, heparin, deAc-HS, HS and HA. There were no significant binding signals given by R-10G (Figure 4.13).

Thus, R-10G has exclusive specificity for oligosaccharide sequences on KS rather than on other GAGs. The keratanase II digestion-derived oligosaccharides terminating with non-reducing Gal rather than those terminating with non-reducing GlcNAc from keratanase I digestion express R-10G antigen. The minimum R-10G antigen-positive oligosaccharide presented as NGLs is hexasaccharide.

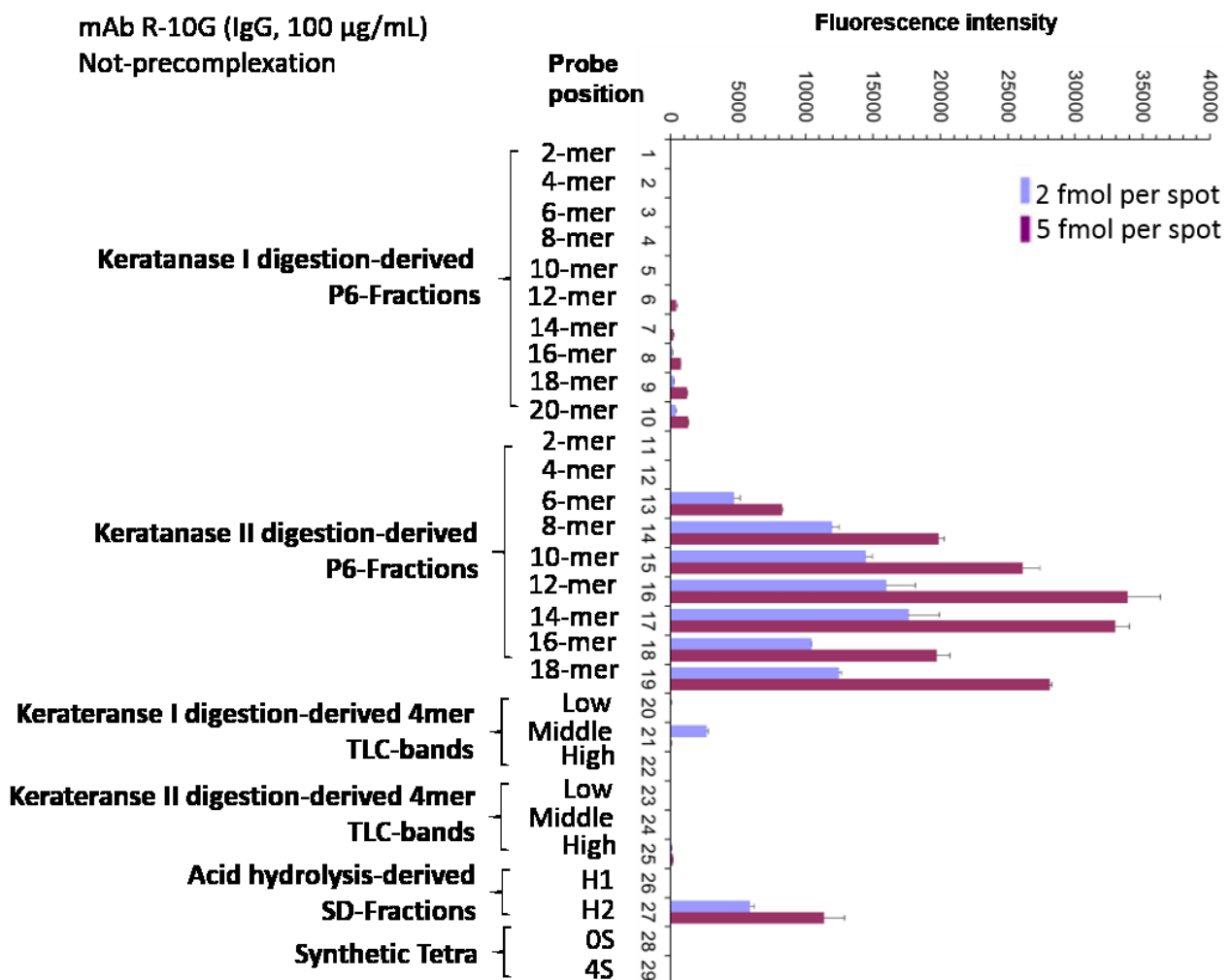


Figure 4.12. Microarray analysis of mAb R-10G (IgG) using KS oligosaccharide NGL microarray set 1. Blocking solution and diluent were PBS containing 1% (w/v) BSA and 0.33% (w/v) casein. Detection of binding was as described in Figure 4.1. Results are means of fluorescence intensities of duplicate spots, printed at 2 and 5 fmol per spot, with error bars representing half of the difference between the two values. The result shown here is representative of numerous analyses with R-10G using this microarray.

mAb R-10G (IgG, 100 µg/mL)
Not-precomplexation

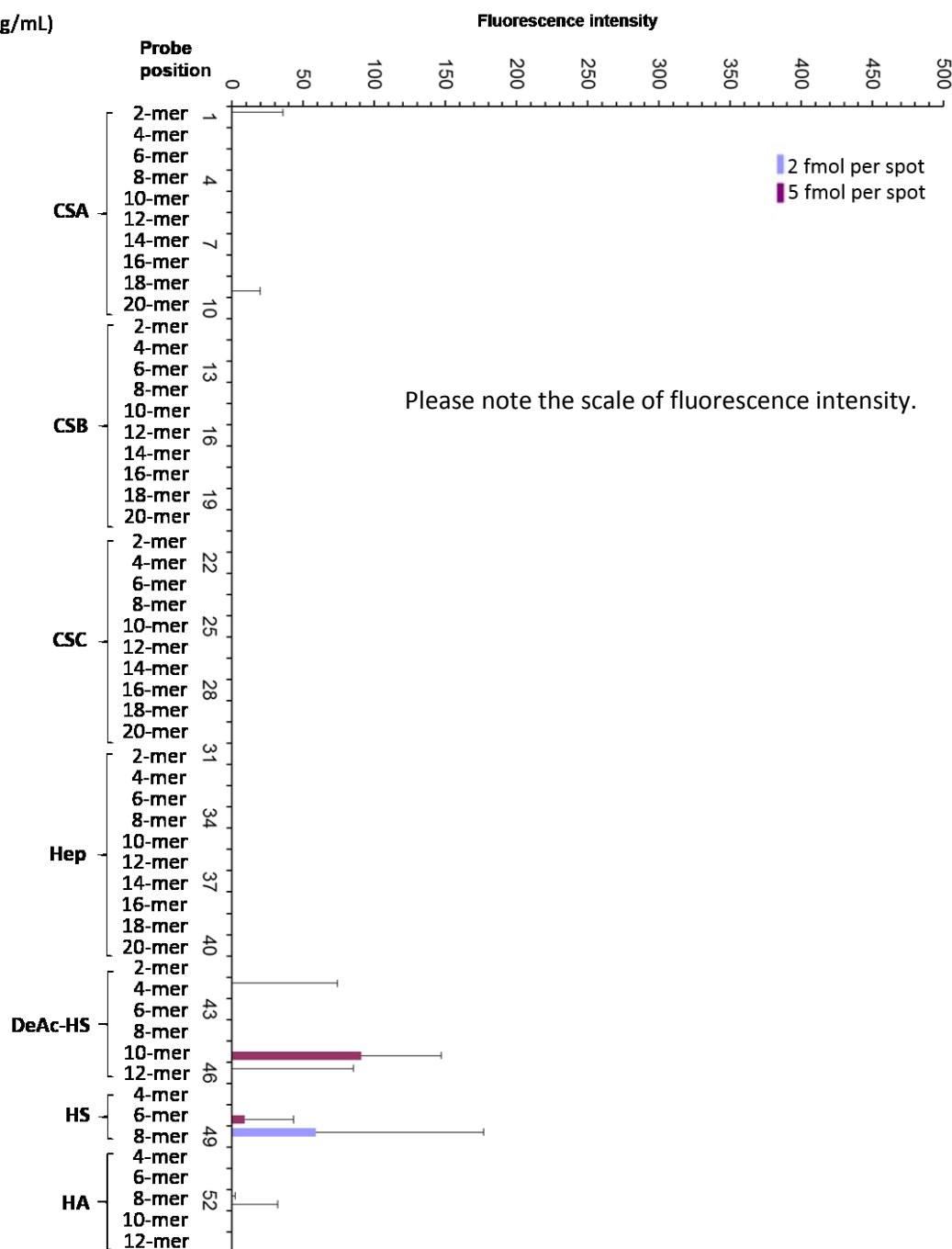


Figure 4.13. Microarray analysis of mAb R-10G (IgG) using GAG oligosaccharide NGL microarray Set 6. Blocking solution and diluent were PBS containing 1% (w/v) BSA and 0.33% (w/v) casein. Detection of binding was as described in Figure 4.1. Results are means of fluorescence intensities of duplicate spots, printed at 2 and 5 fmol per spot, with error bars representing half of the difference between the two values. The result shown here is representative of one analysis with R-10G using this microarray and another not shown using a different microarray with the same composition.

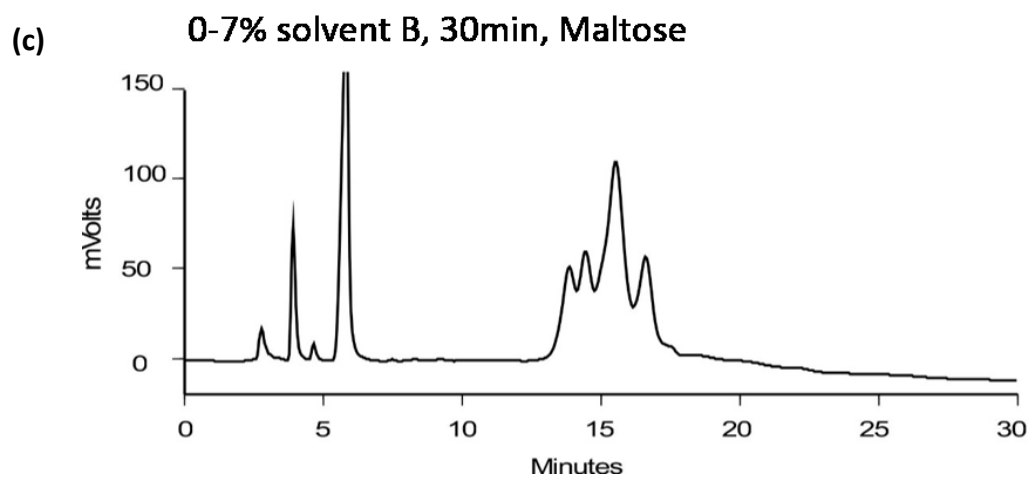
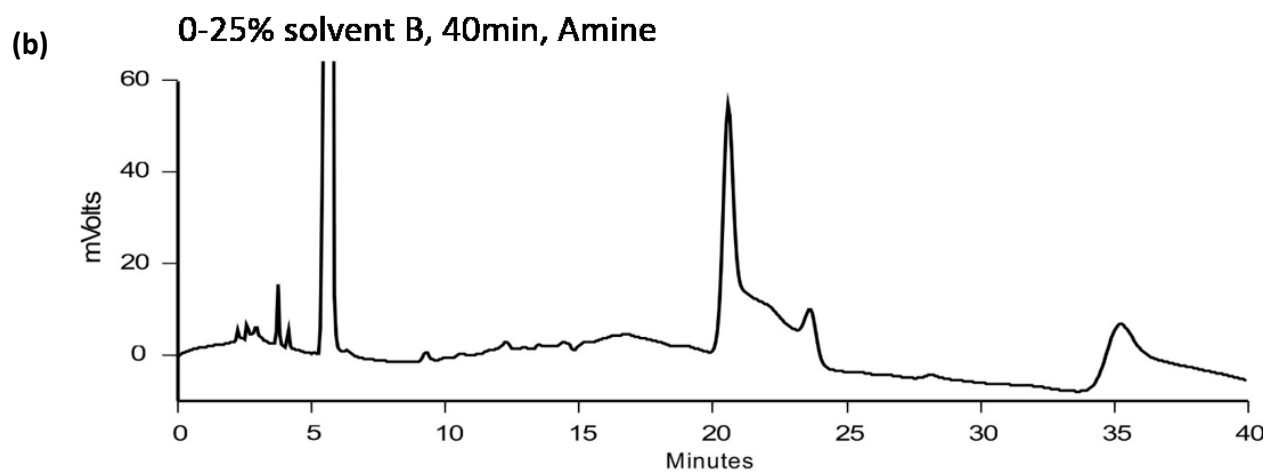
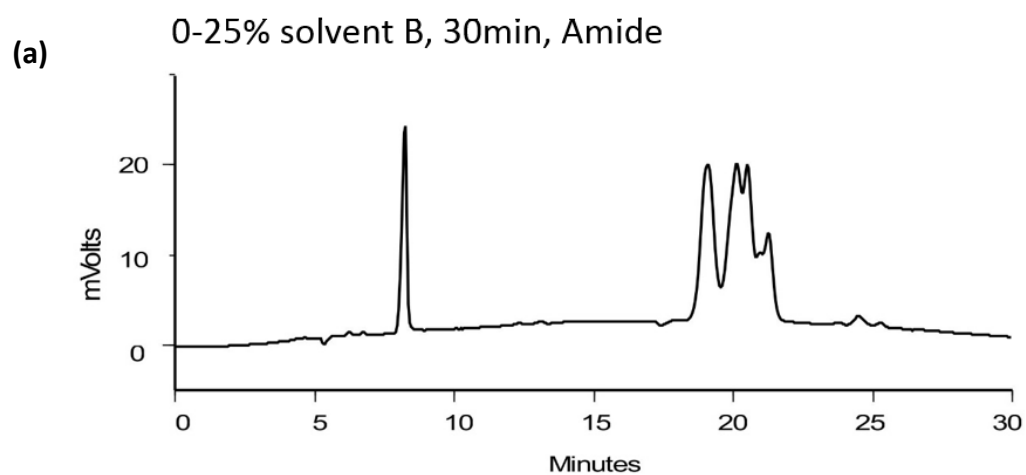
4.6 Sulphation Requirement for the R-10G Antigen

The shortest KS oligosaccharide fractions bound by R-10G were keratanase II digestion-derived P6 hexasaccharide fraction and Fraction H2 from acid hydrolysis which was more heterogeneous containing tetra- to hepta-saccharides. The keratanase II digestion-derived P6 hexasaccharide fraction was therefore selected for fractionation by HPLC and analysis of the fractions for binding by R-10G.

4.6.1 HPLC of the R-10G antigen-positive hexasaccharide fraction and preparation of neoglycolipids of the subfractions

In order to explore HPLC conditions for fractionation of KS oligosaccharides, the relatively abundant keratanase II digestion-derived tetrasaccharide fraction was used. The columns included Amide, Amine, Maltose and SAX columns. For each column and solvent, several gradients were evaluated (not shown). The respective optimum gradients are shown in Figure 4.14 a, b, c and d.

Performance of the SAX column which separates saccharides by charge was the most satisfactory. The four major subfractions of the keratanase II digestion-derived tetrasaccharide fraction were collected (Figure 4.14d), followed by ES-MS analysis. Fraction 1 was di-sulphated, both Fractions 2 and 3 were tri-sulphated, and Fraction 4 was tetra-sulphated. The obtained four fractions were then conjugated to AOPE (Figure 4.15) to include in the array.



(d) 30-60% solvent B, 40min, SAX

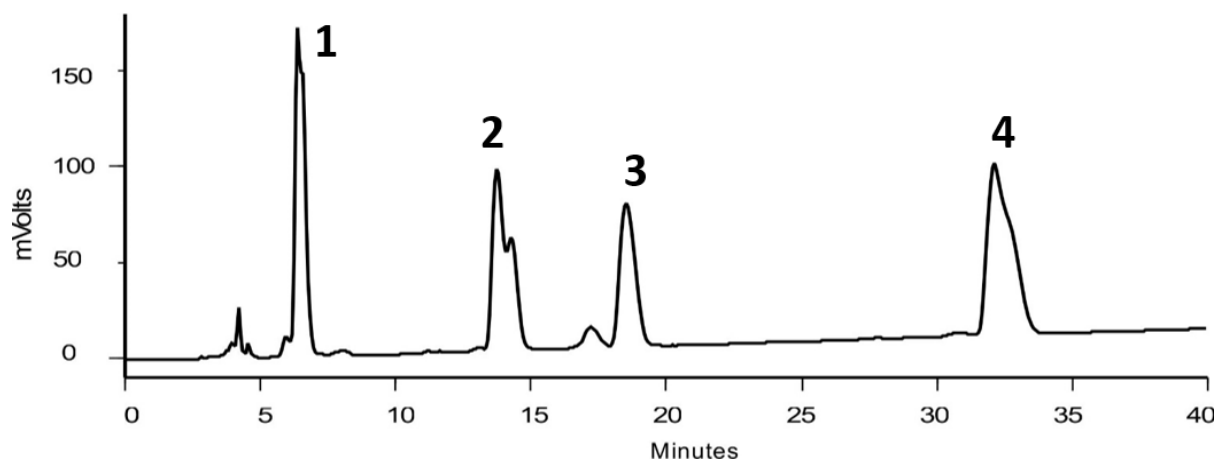


Figure 4.14. HPLC of keratanase II digestion-derived tetrasaccharide with Amide, Amine, Maltose and SAX columns. (a), (b) and (c) were performed with a gradient of ACN/H₂O (70:30) with 7.5 mM KH₂PO₄ (solvent A) to ACN/H₂O (20:80) with 7.5 mM KH₂PO₄ (solvent B) at a flow rate of 0.5 ml/min, whereas (d) was performed with a gradient of 10 mM LiClO₄ (solvent A) to 500 mM LiClO₄ (solvent B) at a flow rate of 1 ml/min. Details of the gradient and runing period are labelled in each panel. Four fractions were collected as indicated in (d).

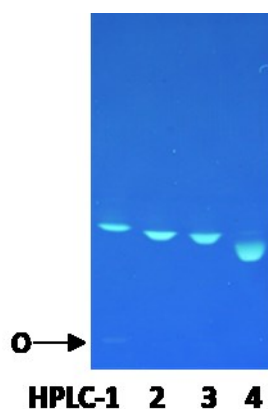


Figure 4.15. HPTLC of purified NGLs derived from the SAX-HPLC fractions of keratanase II digestion-derived P6 tetrasaccharide fraction. NGLs were revealed under UV light with long wavelength after staining with primulin. Solvent CHCl₃:MeOH:H₂O 60/35/8 (v/v/v) was used.

The SAX column was used for chromatography of R-10G antigen-positive hexasaccharide fraction. Nine subfractions were collected (Figure 4.16) and converted to NGLs (Figure 4.16). Subfractions 1 to 5 designated HPLC-1 to 5 and NGLs of HPLC-1 to 9 gave clear ES-MS data (Table 4.4). Taken together the ES-MS data of oligosaccharides and NGLs were interpreted as follows with respect to the main components. In the subfractions, HPLC-1 was a tri-sulphated hexasaccharide; HPLC-2 and 3 were tetra-sulphated hexasaccharides, HPLC-4, 5 and 7 contained penta-sulphated hexasaccharides; HPLC-9 contained hexa-sulphated hexasaccharides. The other two small peaks, HPLC-6 and 8 fractions consisted of tri- and tetra-sulphated tetrasaccharides and penta-sulphated pentasaccharides, respectively.

The MS-deduced components of the HPLC hexasaccharide fractions were consistent with the HPTLC of their NGLs (Figure 4.17). The NGL band derived from each HPLC hexasacchride fraction was more compact than those derived from the original P6 hexasaccharide fraction (Figure 4.11b).

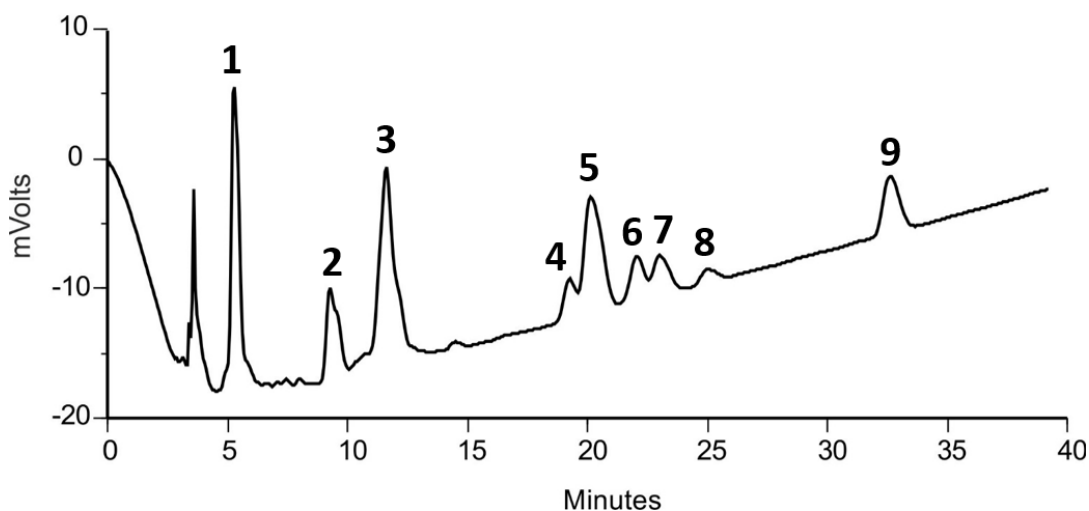


Figure 4.16. HPLC of keratanase II degestion-derived P6 hexasaccharide fraction with SAX column. It was performed with a gradient of 10 mM LiClO₄ (solvent A) to 500 mM LiClO₄ (solvent B) at a flow rate of 1 ml/min. The gradient of 30-60% solvent B was used with the runing period of 40min. Nine fractions were collected as indicated.

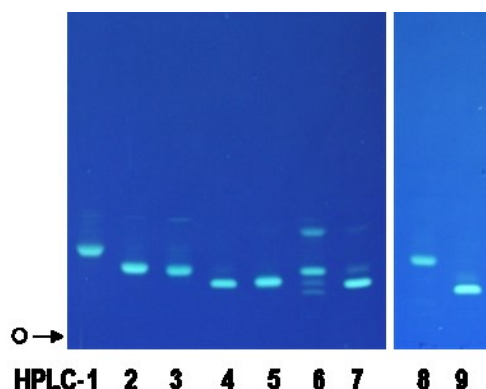


Figure 4.17. HPTLC of purified NGLs derived from the SAX-HPLC fractions of keratanase II digestion-derived P6 hexasaccharide fraction. NGLs were revealed under UV light with long wavelength after staining with primulin. Solvent $\text{CHCl}_3\text{:MeOH:H}_2\text{O}$ 60/35/8 (v/v/v) was used. The spaces between figures mean that they are separate chromatographs.

Table 4.4. Negative-ion ES-MS analysis of oligosaccharides in SAX-HPLC fractions of keratanase II digestion-derived P6 hexasaccharide fraction and derived NGLs

Fractions	Oligosaccharides		NGLs		Assignment of major component
	Detected ions (m/z)	Deduced compositions	Detected ions (m/z)	Deduced compositions	
HPLC-1	450.1 (z=3) 686.6 (z=2)	Gal3.GlcNAc3.S3 Gal3.GlcNAc3.S3.Na	516.9 (z=4) 696.9 (z=3)	Gal3.GlcNAc3.S3.AO Gal3.GlcNAc3.S3.Na1.AO	KS-II-6(3S)
HPLC-2	357.4 (z=4) 450.2 (z=3) 484.2 (z=3)	Gal3.GlcNAc3.S4 Gal3.GlcNAc3.S3 Gal3.GlcNAc3.S4.Na1	429.3 (z=5) 542.4 (z=4) 536.9 (z=4)	Gal3.GlcNAc3.S4.AO Gal3.GlcNAc3.S4.Na1.AO Gal3.GlcNAc3.S4.AO	KS-II-6(4S)
HPLC-3	357.4 (z=4) 450.2 (z=3) 484.2 (z=3)	Gal3.GlcNAc3.S4 Gal3.GlcNAc3.S3 Gal3.GlcNAc3.S4.Na1	429.3 (z=5) 542.4 (z=4) 536.9 (z=4)	Gal3.GlcNAc3.S4.AO Gal3.GlcNAc3.S4.Na1.AO Gal3.GlcNAc3.S4.AO	KS-II-6(4S)
HPLC-4	301.7 (z=5) 357.4 (z=4) 382.9 (z=4)	Gal3.GlcNAc3.S5 Gal3.GlcNAc3.S4 Gal3.GlcNAc3.S5.Na1	370.9 (z=6) 429.3 (z=5) 449.9 (z=5)	Gal3.GlcNAc3.S5.AO Gal3.GlcNAc3.S4.AO Gal3.GlcNAc3.S5.Na1.AO	KS-II-6(5S)
HPLC-5	301.7 (z=5) 382.9 (z=4) 357.4 (z=4)	Gal3.GlcNAc3.S5 Gal3.GlcNAc3.S5.Na1 Gal3.GlcNAc3.S4	371.1 (z=6) 429.3 (z=5) 449.9 (z=5) 567.9 (z=4)	Gal3.GlcNAc3.S5.AO Gal3.GlcNAc3.S4.AO Gal3.GlcNAc3.S5.Na1.AO Gal3.GlcNAc3.S5.Na2.AO	KS-II-6(5S)
HPLC-6	-	-	425.7 (z=4) 356.3 (z=5) 445.6 (z=4) 370.9 (z=6)	Gal2.GlcNAc2.S3.AO Gal2.GlcNAc2.S4.AO Gal2.GlcNAc2.S4.AO Gal3.GlcNAc3.S5.AO	KS-II-4(3S) KS-II-4(4S)
HPLC-7	-	-	371.0 (z=6) 429.3 (z=5) 449.7 (z=5) 562.4 (z=4)	Gal3.GlcNAc3.S5.AO Gal3.GlcNAc3.S4.AO Gal3.GlcNAc3.S5.AO Gal3.GlcNAc3.S5.AO	KS-II-6(5S)
HPLC-8	-	-	343.9 (z=6) 396.9 (z=5)	Gal2.GlcNAc3.S5.AO Gal2.GlcNAc3.S4.AO	KS-II-5(5S)

			417.3 (z=5) 521.9 (z=4) 527.4 (z=4)	Gal2.GlcNAc3.S5.Na1.AO Gal2.GlcNAc3.S5.Na1.AO Gal2.GlcNAc3.S5.Na2.AO	
HPLC-9	-	-	329.2 (z=2) 371.0 (z=6) 387.9 (z=6) 449.7 (z=5)	Gal3.GlcNAc3.S6.AO Gal3.GlcNAc3.S5.AO Gal3.GlcNAc3.S6.Na1.AO Gal3.GlcNAc3.S5.Na1.AO	KS-II-6(6S)

Gal, Galactose; GlcNAc, N-Acetyl-Glucosamine; S, sulphate. KS-II-6(3S) means keratanase II digestion-derived hexasaccharide with three sulphates. Other abbreviations such KS-II-4(3S) and KS-II-5(5S) can be deduced like the KS-II-6(3S).

4.6.2 The tri-sulphated hexasaccharide (HPLC-1) identified as being R-10G antigen-positive

A second KS oligosaccharide NGL microarray of 44 NGLs designated as Set 2a was generated (Figure 4.18). This included NGLs of the four HPLC subfractions (HPLC-1 to 4) of keratanase II digestion-derived P6 tetrasaccharide fraction and NGLs of the nine HPLC subfractions (HPLC-1 to 9) of keratanase II digestion-derived P6 hexasaccharide fraction. These were arrayed together with the NGLs of keratanase I and keratanase II digestion-derived P6 fractions and NGLs of the two synthetic tetrasaccharides which were arrayed in set 1.

R-10G was found to bind strongly to the NGL of the tri-sulphated hexasaccharide subfraction HPLC-1; the binding strength was stronger than to the parent keratanase II digestion-derived P6 hexasaccharide fraction in the same array (Figure 4.18). Little or no binding was observed with the NGLs of the other subfractions HPLC-2 to 9 which contained tetra-, penta- and hexa-sulphated hexasaccharides. The tetrasaccharide subfractions derived from the P6 tetrasaccharide fractions elicited no binding with R-10G. Therefore, the tri-sulphated hexasaccharide was proposed to constitute the R-10G antigen.

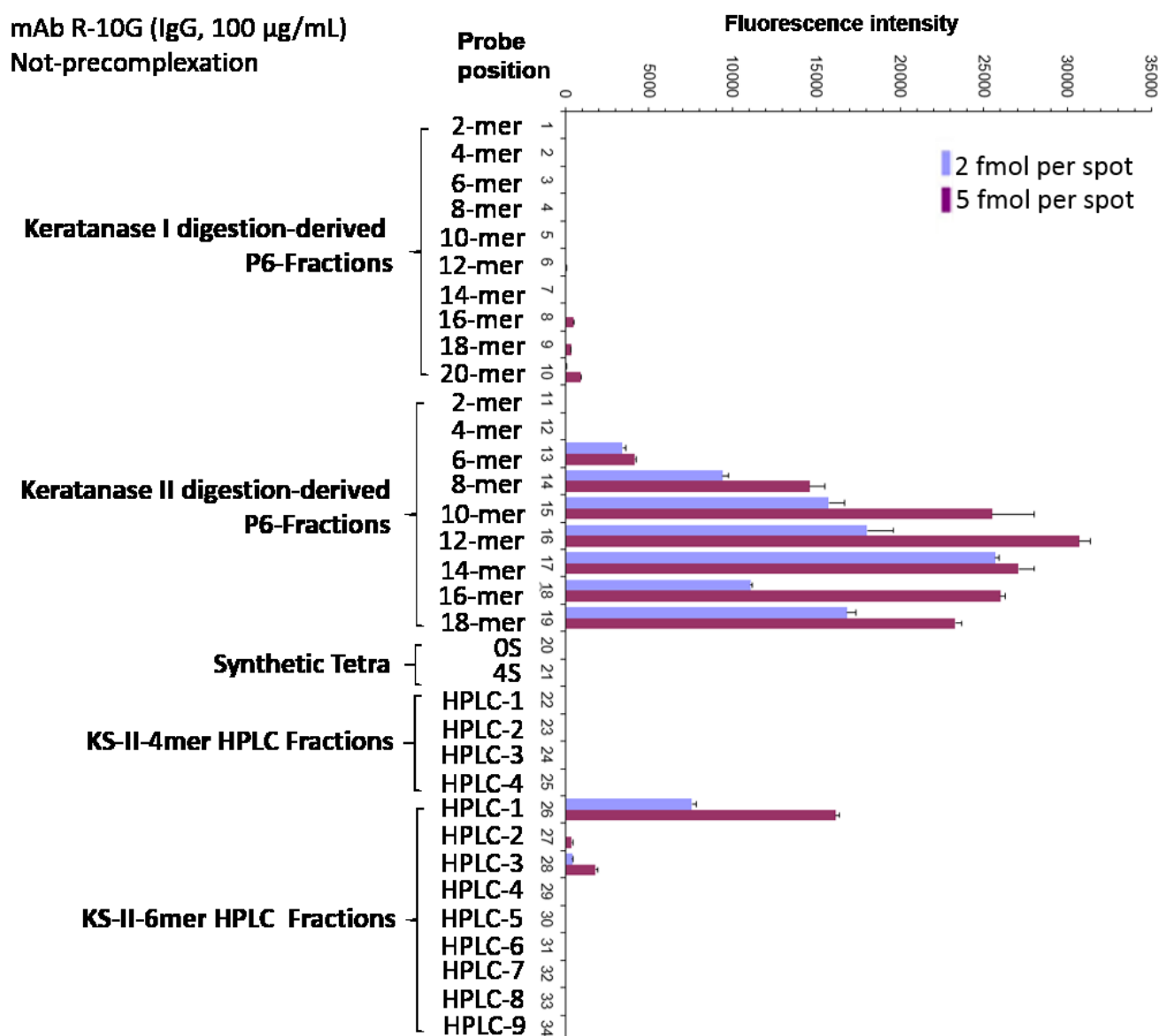


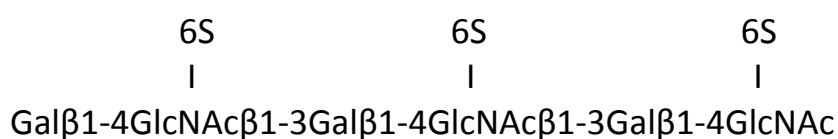
Figure 4.18. Microarray analysis of mAb R-10G (IgG) using KS oligosaccharide NGL microarray Set 2a. Blocking solution and diluent were PBS containing 1% (w/v) BSA and 0.33% (w/v) casein. Detection of binding was as described in Figure 4.1. Results are means of fluorescence intensities of duplicate spots, printed at 2 and 5 fmol per spot, with error bars representing half of the difference between the two values. KS-II-4mer means keratanase II digestion-derived P6 tetrasaccharide fraction, and KS-II-6mer means keratanase II digestion-derived P6 hexasaccharide fraction. The result shown here is representative of numerous analyses with R-10G using this microarray.

4.6.3 Assignment of the sequence of the tri-sulphated hexasaccharide (HPLC-1)

ES-MS data on the oligosaccharide and NGL of the R-10G antigen-positive HPLC-1 are shown in Figure 4.19. These indicated that the composition was Gal3.GlcNAc3.S3. Known specificity of keratanase II for digestion of KS deduced that the reducing end is sulphated GlcNAc and the non-reducing end is Gal with or without sulphate.

The positions of the sulphate groups and the glycosidic linkages of the hexasaccharide were established by 700-MHz ^1H NMR and two-dimensional correlated spectroscopy (Figure 4.20). By comparison with literature values for ^1H and ^{13}C chemical shifts of internal and non-reducing end residues of KS oligosaccharides [236, 238, 240, 241], the proton and carbon spectra of the hexasaccharide analysed were assigned as summarised in Table 4.5. These assignments were then checked against correlations in the HSQC and TOCSY spectra (Figures 4.20b and c). Signals in the anomeric proton region (5.3-4.5 ppm) and the N-acetamido methyl region (2.1-2.0 ppm, data not shown) are consistent with the presence of a reducing-end GlcNAc, internal GlcNAc β and Gal β residues. The chemical shifts of the H6 and H6' signals of the GlcNAc residues in the low field region, 4.3-4.4 ppm, indicates that the sulphate groups are located on C6 of these residues. Non-sulphation at C6 of the Gal residues is established by the presence of H6 and H6' signals at higher field (3.8-3.7 ppm).

The sequence of the R-10G antigen-positive tri-sulphated hexasaccharide is therefore established to be



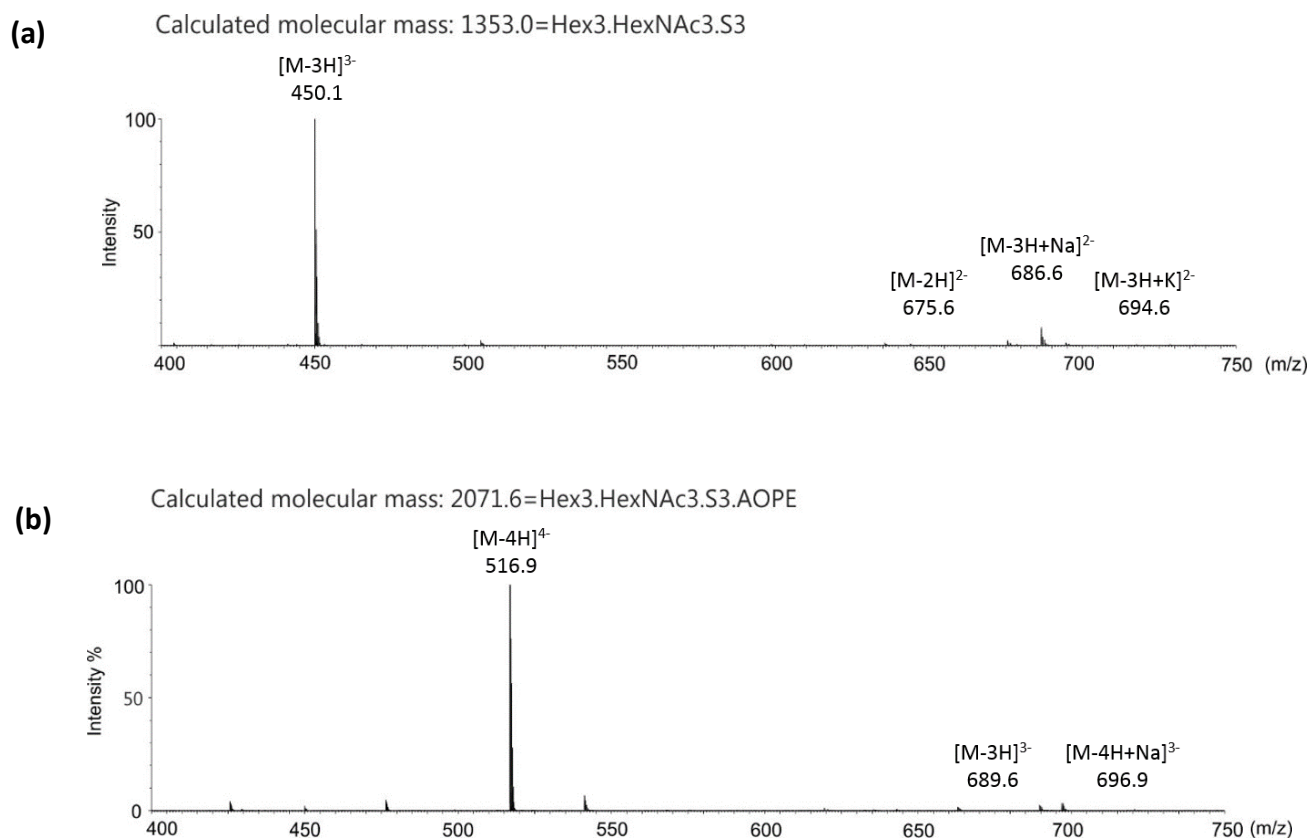
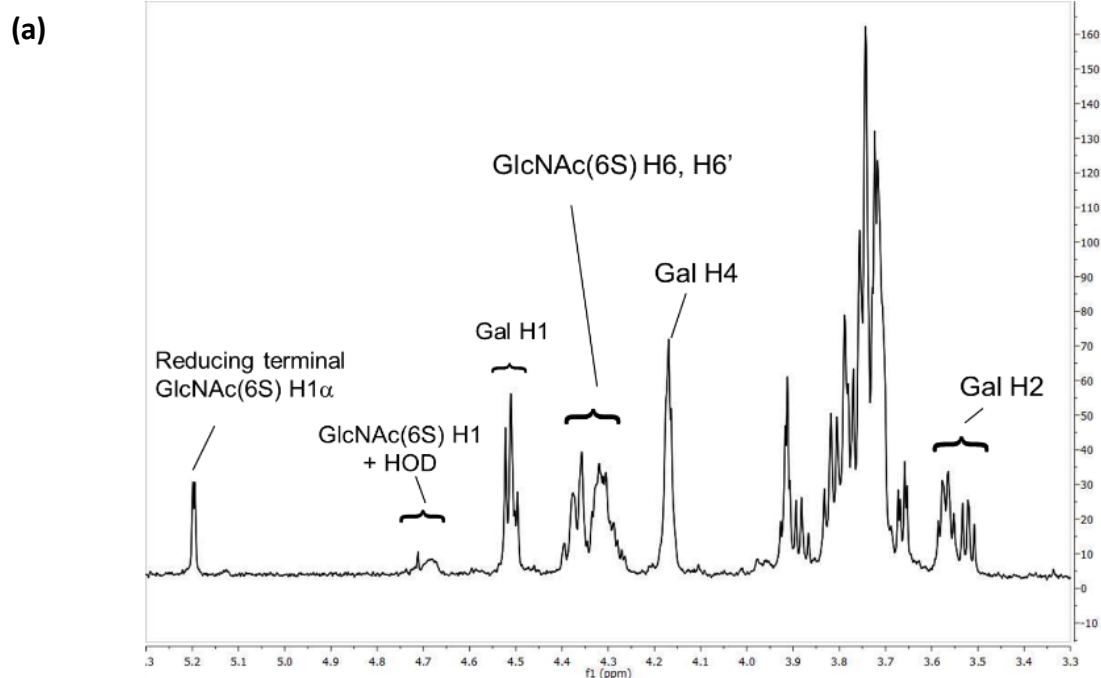
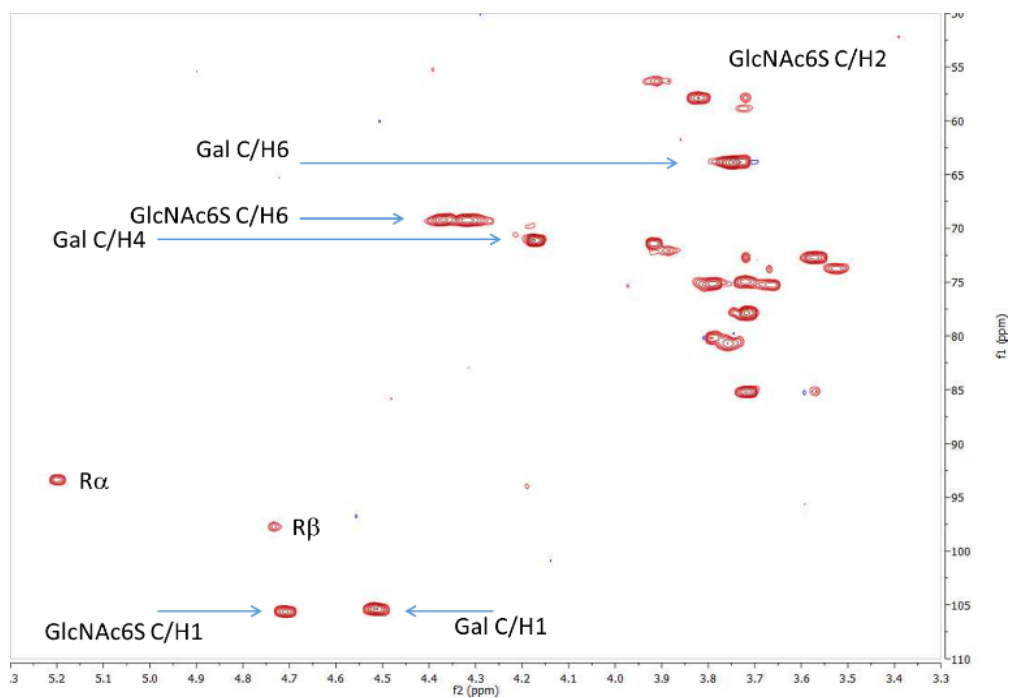


Figure 4.19. Negative-ion ES-MS of the HPLC-1 strongly bound by R-10G. (a) oligosaccharide and (b) its corresponding NGL.



(b)



(c)

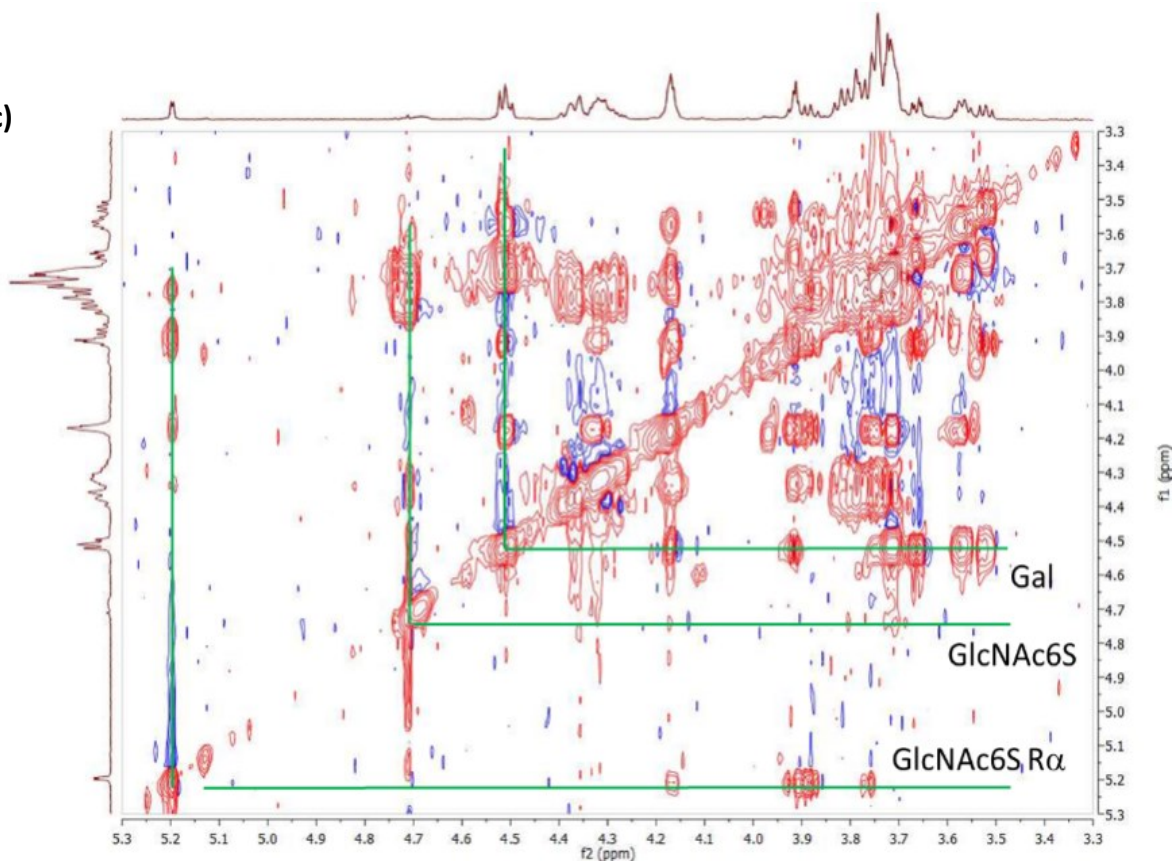


Figure 4.20. NMR spectra of the HPLC-1 strongly bound by R-10G. (a) ^1H spectrum at 700 MHz, 30 $^{\circ}\text{C}$, (b) HSQC spectrum, and (c) TOCSY spectrum.

Table 4.5. The chemical shifts of the R-10G antigen-positive tri-sulphated hexasaccharide HPLC-1

Terminal Gal		Internal Gal		Internal GlcNAc(6S)		Reducing terminal GlcNAc(6S)	α	β
C(1)	105.4	C(1)	105.4	C(1)	105.6	C(1)	93.4	97.7
C(2)	73.8	C(2)	72.7	C(2)	57.9	C(2)	56.3	ND
C(3)	75.3	C(3)	85.2	C(3)	74.9	C(3)	ND	ND
C(4)	71.4	C(4)	71.1	C(4)	80.7	C(4)	ND	ND
C(5)	ND	C(5)	77.9	C(5)	75.2	C(5)	ND	ND
C(6)	ND	C(6)	63.9	C(6)	69.2	C(6)	ND	ND
				C(NAc)	24.9			
H(1)	4.51	H(1)	4.52	H(1)	4.71	H(1)	5.2	4.73
H(2)	3.52	H(2)	3.57	H(2)	3.82	H(2)	3.91	ND
H(3)	3.67	H(3)	3.71	H(3)	3.72	H(3)	ND	ND
H(4)	3.91	H(4)	4.17	H(4)	3.76	H(4)	ND	ND
H(5)	ND	H(5)	3.71	H(5)	3.79	H(5)	ND	ND
H(6)	ND	H(6)	3.72-3.74	H(6, 6')	4.37, 4.32	H(6)	ND	ND
				H(NAc)	2.03			

ND means not determined.

4.7 Minimum Sulphation Requirement on the hexasaccharide for R-10G Antigenicity

4.7.1 Subfractionation of the under-sulphated hexasaccharides and analyses of their R-10G antigenicities

I attempted to investigate further the degree and pattern of sulphation required for R-10G antigenicity. For this, as the naturally-occurring KS hexasaccharides with lower sulphation (e.g. containing less than 3 sulphates) are of limited amount, three approaches were made for this purpose:

- (a) direct isolation from the keratanase II digestion-derived P6 hexasaccharide fraction (designated F3 in Table 4.2 and Figure 4.6);
- (b) chemical desulphation of the above mentioned keratanase II digestion-derived P6 hexasaccharide fraction;

(c) chemical desulphation of the R-10G antigen-positive tri-sulphated hexasaccharide (designated HPLC-1 in Table 4.4 and Figure 4.16).

Details of the procedures of subfractionation are in the Appendix 3. The compositions of the subfractions are listed in Table 4.6. One non-sulphated, threKERe mono-sulphated and two di-sulphated hexasaccharide fractions were obtained. They were converted to NGLs and added to the microarray Set 2a, the composite array is designated Set 2b.

The non-sulphated hexasaccharide was not bound by R-10G; the mono-sulphated fractions were bound but less strongly than the di-sulphated fractions (Figure 4.21). The naturally mono- and di-sulphated hexasaccharide [source (a)] elicited stronger R-10G binding than those from the desulphated hexasaccharide [source (b) and (c)]. These data indicate that it is not essential to have three sulphates on a KS hexasaccharide for the R-10G antigenicity.

Table 4.6. Negative-ion ES-MS analysis of hexasaccharides with 0 to 2 sulphates in SAX-HPLC fractions of source (a), (b) and (c) and derived NGLs

Source	Subfraction*	Oligosaccharides		NGLs		Designation and sulphate content
		Detected ions (m/z)	Deduced compositions	Detected ions (m/z)	Deduced compositions	
(a) keratanase II digestion-derived P6-6mer fraction	F1.2	595.6 (z=2) 1192.3 (z=1)	Gal3.GlcNAc3.S1	955.4 (z=2)	Gal3.GlcNAc3.S1.AO	KS-II-6(1S)
	F1.3					
	F1.4	635.7 (z=2)	Gal3.GlcNAc3.S2	995.0 (z=2) 663.0 (z=3) 2012.9 (z=1)	Gal3.GlcNAc3.S2.AO Gal3.GlcNAc3.S2.Na1.AO	KS-II-6(2S)
(b) keratanase II digestion-derived P6-6mer fraction (chemically desulphated)	F1	1112.4 (z=1)	Gal3.GlcNAc3	914.9 (z=2)	Gal3.GlcNAc3.AO	KS-II-6(0S)
	F2	595.7 (z=2) 1192.3 (z=1)	Gal3.GlcNAc3.S1	954.9 (z=2) 636.6 (z=3)	Gal3.GlcNAc3.S1.AO	KS-II-6(1S)
	F3					
	F4					
(c) R-10G antigen-positive tri-sulphated hexasaccharide (chemically desulphated)	F1	595.6 (z=2) 1192.3 (z=1)	Gal3.GlcNAc3.S1	954.9 (z=2) 636.6 (z=3)	Gal3.GlcNAc3.S1.AO	KS-II-6(1S)
	F4	635.7 (z=2)	Gal3.GlcNAc3.S2	662.9 (z=3) 994.9 (z=2)	Gal3.GlcNAc3.S2.AO	KS-II-6(2S)

*Also see Appendix 3.

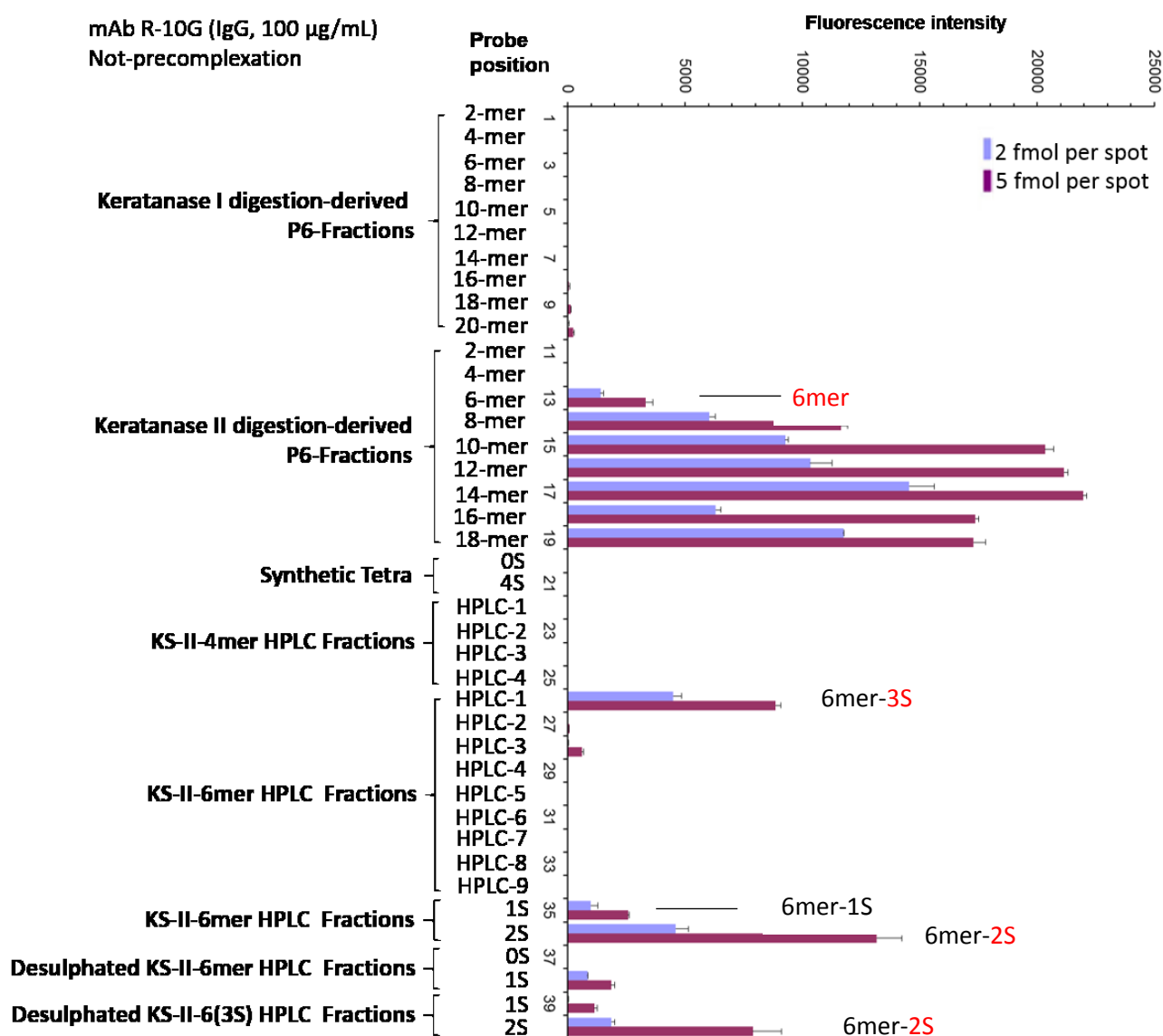


Figure 4.21. Microarray analysis of mAb R-10G (IgG) using KS oligosaccharide NGL microarray Set 2b. Blocking solution and diluent were PBS containing 1% (w/v) BSA and 0.33% (w/v) casein. Detection of binding was as described in Figure 4.1. Results are means of fluorescence intensities of duplicate spots, printed at 2 and 5 fmol per spot, with error bars representing half of the difference between the two values. KS-II-6(3S) is the HPLC-1 of KS-II-6mer, which is the R-10G antigen-positive tri-sulphated hexasaccharide. The result shown here is representative of numerous analyses with R-10G using this microarray.

4.7.2 Further subfractionation of mono- and di-sulphated hexasaccharide fractions and analyses of their R-10G antigenicities

Each mono- and each di-sulphated hexasaccharide fraction was then further fractionated by HPLC with PGC Hypercarb column (Figures 4.22 and 4.23). As the amounts of the oligosaccharides isolated from each of the preparation are limited, the main peaks with identical retention times were pooled.

Multiple fractions were obtained after HPLC of the mono-sulphated hexasaccharides and an example is in Figure 4.22. Each fraction was analysed by ES-MS analysis for chain length and degree of sulphation. The two larger peaks (Fractions 2 and 5 in Figure 4.22) were selected for NGL preparation and arraying in a small focused array which included keratanase II digestion-derived hexasaccharide and octasaccharide fractions as positive controls. However, there was no R-10G binding detected to either of the two mono-sulphated fractions (data not shown).

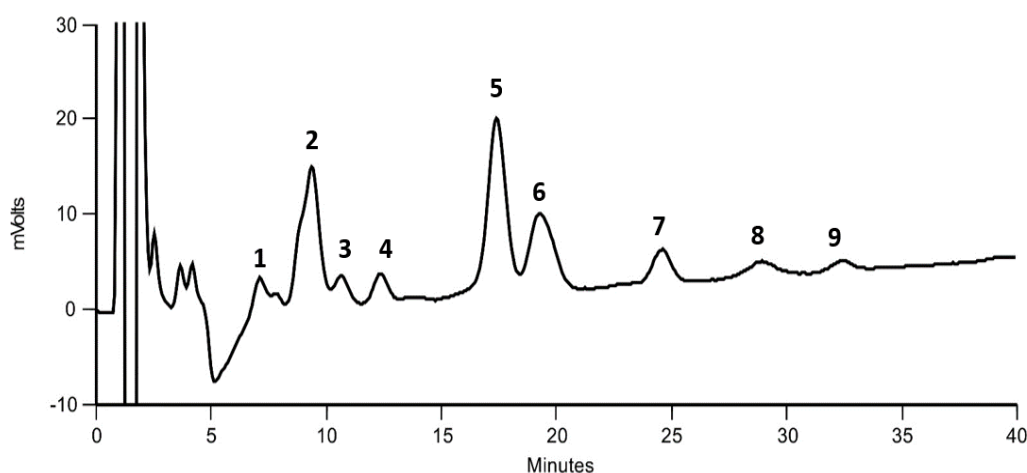


Figure 4.22. HPLC of mono-sulphated hexasaccharides with PGC Hypercarb column. It was performed with a gradient of H₂O with 0.05% TFA (solvent A) to ACN/H₂O (80:20) with 0.05% TFA (solvent B) at a flow rate of 0.5 ml/min. The gradient of 25-35% solvent B was used with the running period of 40min. Nine fractions were collected as indicated.

The di-sulphated hexasaccharide fraction from the natural source (a) was fractionated by HPLC and seven fractions were obtained (Figure 4.23). Four major peaks (Fractions 1, 2, 3 and 5) were selected for NGL preparation and arraying after ES-MS analyses confirming their chain length and sulphate content. The four NGLs were added to the microarray Set 2b, to constitute microarray Set 2c.

Interestingly, NGLs derived from two of the four HPLC fractions were strongly bound by R-10G with the signal of Fraction 5 being slightly higher than that of Fraction 2 (Figure 4.24). Both of the signal intensities were considerably higher than those elicited by their parent di-sulphated hexasaccharide fraction and the tri-sulphated hexasaccharide.

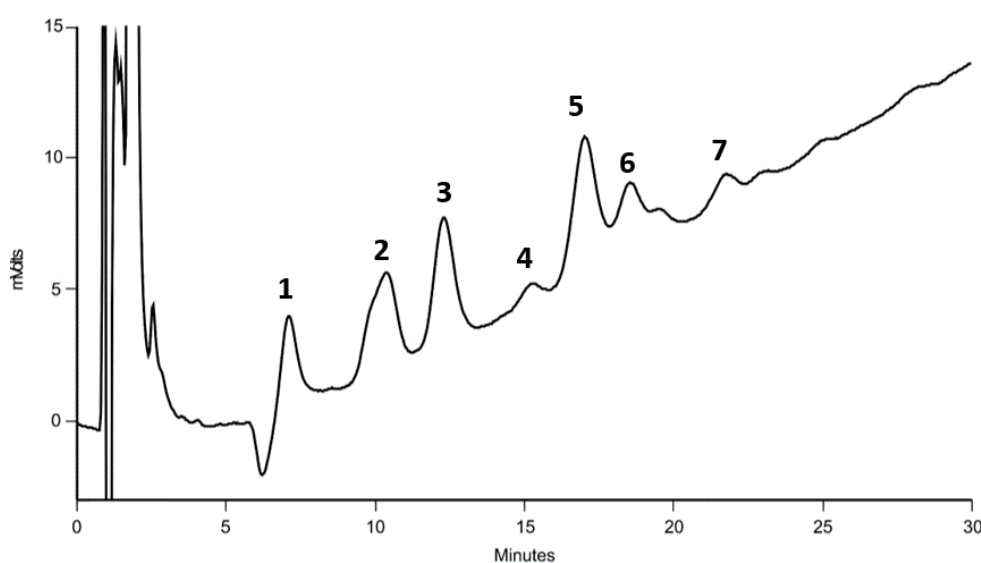


Figure 4.23. HPLC of di-sulphated hexasaccharides with PGC Hypercarb column. The solvents and gradient were the same as those in Figure 4.22. Seven fractions were collected as indicated.

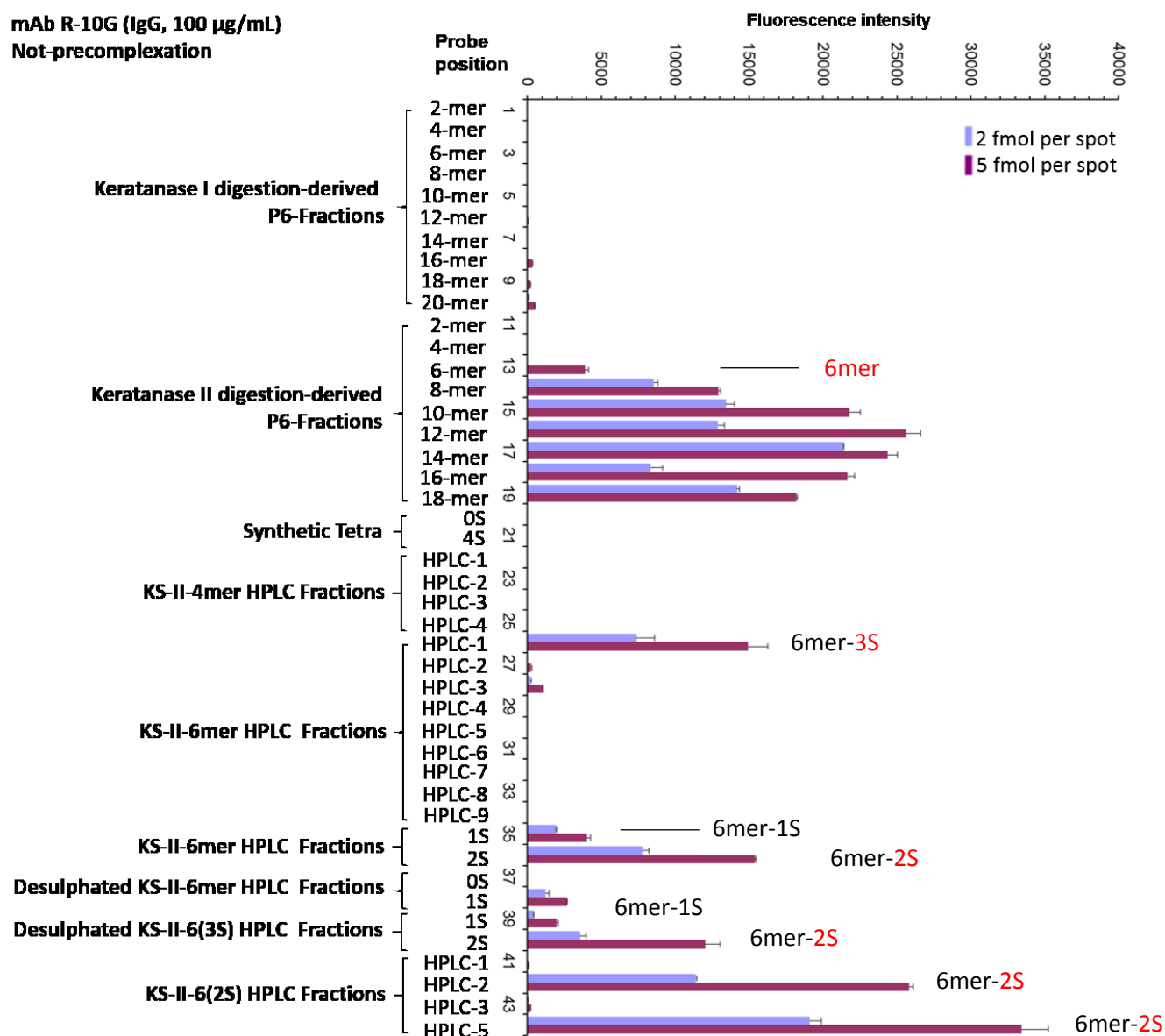


Figure 4.24. Microarray analysis of mAb R-10G (IgG) using KS oligosaccharide NGL microarray Set 2c. Blocking solution and diluent were PBS containing 1% (w/v) BSA and 0.33% (w/v) casein. Binding was detected using biotinylated anti-mouse IgG followed by Alexa Fluor-647-labeled streptavidin. Results are means of fluorescence intensities of duplicate spots, printed at 2 and 5 fmol per spot, with error bars representing half of the difference between the two values. The result shown here is representative of numerous analyses with R-10G using this microarray.

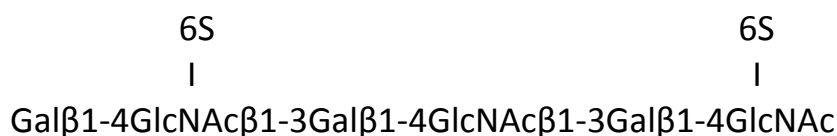
4.7.3 Sequence determination of the R-10G antigen-positive di-sulphated hexasaccharide subfractions

It is striking that the four di-sulphated hexasaccharide subfractions have different R-10G antigenicities; two (HPLC-2 and -5) were very active and two (HPLC-1 and -3) inactive. These findings are highly indicative of a requirement for a particular pattern of sulphation for R-10G antigen expression. For sequence determination the NGLs of the four subfractions were analysed by ES-CID-MS/MS.

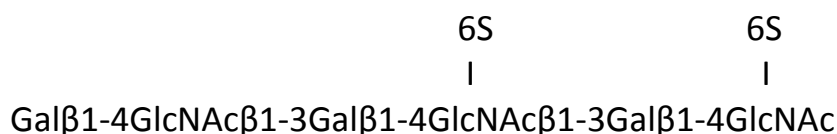
The product-ion spectra of the two active NGLs of subfractions HPLC-2 and -5 are very similar (Figure 4.25); these differed from the spectra of the two inactive subfractions HPLC-1 and -3 (Figure 4.26). The similarity of their product-ion spectra and the antigenic activities suggested that these two pairs may be anomeric isomers resolved by the high resolution PGC Hypercarb column as previously indicated [242]. To confirm this assumption, two standard GlcNAc-terminating tetrasaccharides were subjected to PGC Hypercarb HPLC. Indeed, using the same elution solvent and similar gradient, the isomers with α and β configurations can be as far as 10 minutes apart in retention time (data not shown), suggesting that HPLC-1/-3, and HPLC-2/-5 are the same in sequence.

Although extensive desulphation can take place for multiply sulphated sugars, sequence of the active di-sulphated hexasaccharide (HPLC-2/5) can still be deduced from the observed fragment ions using only $[M-2H]^{2-}$ as the precursors (Figure 4.25). One sulphate at the reducing terminal GlcNAc is apparent with the sulphated Z_1 ion (m/z 1018) and further corroborated by the Z_2 (m/z 1180). The latter ion also confirmed the absence of the sulphate on the Gal residue. The other sulphate can also be unambiguously assigned at the non-reducing

terminal GlcNAc with the unique sulphated B₂ ion at m/z 444. Thus, a sequence of active di-sulphated hexasaccharide can be deduced as:



The spectra of the two inactive NGLs from HPLC-1/-3 (Figure 4.26) were different from those of the active HPLC-2/-5. The presence of Z₁ (m/z 1018) and Z₂ (m/z 1180) ions similarly confirmed the location of the sulphate at the reducing terminal GlcNAc. The lack of a sulphate at the non-reducing terminal GlcNAc is evidenced by the absence of the B₂ ion at m/z 444. The second sulphate is therefore deduced to be at the internal GlcNAc and a hexasaccharide sequence for the inactive fraction can be deduced as:



Due to the limited materials that can be isolated, full assignment of the proposed structures made by NMR is not feasible. ES-CID-MS product-ion spectrum of the HPLC-5 free sugar (Figure 4.27) before NGL conjugation can be used to ascertain the assignment of the active sequence as some additional fragment ions were present, although the spectrum is more complex due to the sulphate-related fragmentation. One sulphate at the non-reducing terminal GlcNAc again can be deduced from the B₂ (m/z 444) and C₂ (m/z 462) ions and the other sulphate at the reducing terminal GlcNAc by B₅ (m/z 971) and C₅ (m/z 989) ions. The lack of a sulphate at either internal or reducing terminal Gal residues can be evidenced by the B₃/C₃ (m/z 606/624) and B₅/C₅ (m/z 971/989) ions. The ^{2,4}A₆ ion at m/z 1031 can even confirm the 6-O-position of the sulphate.

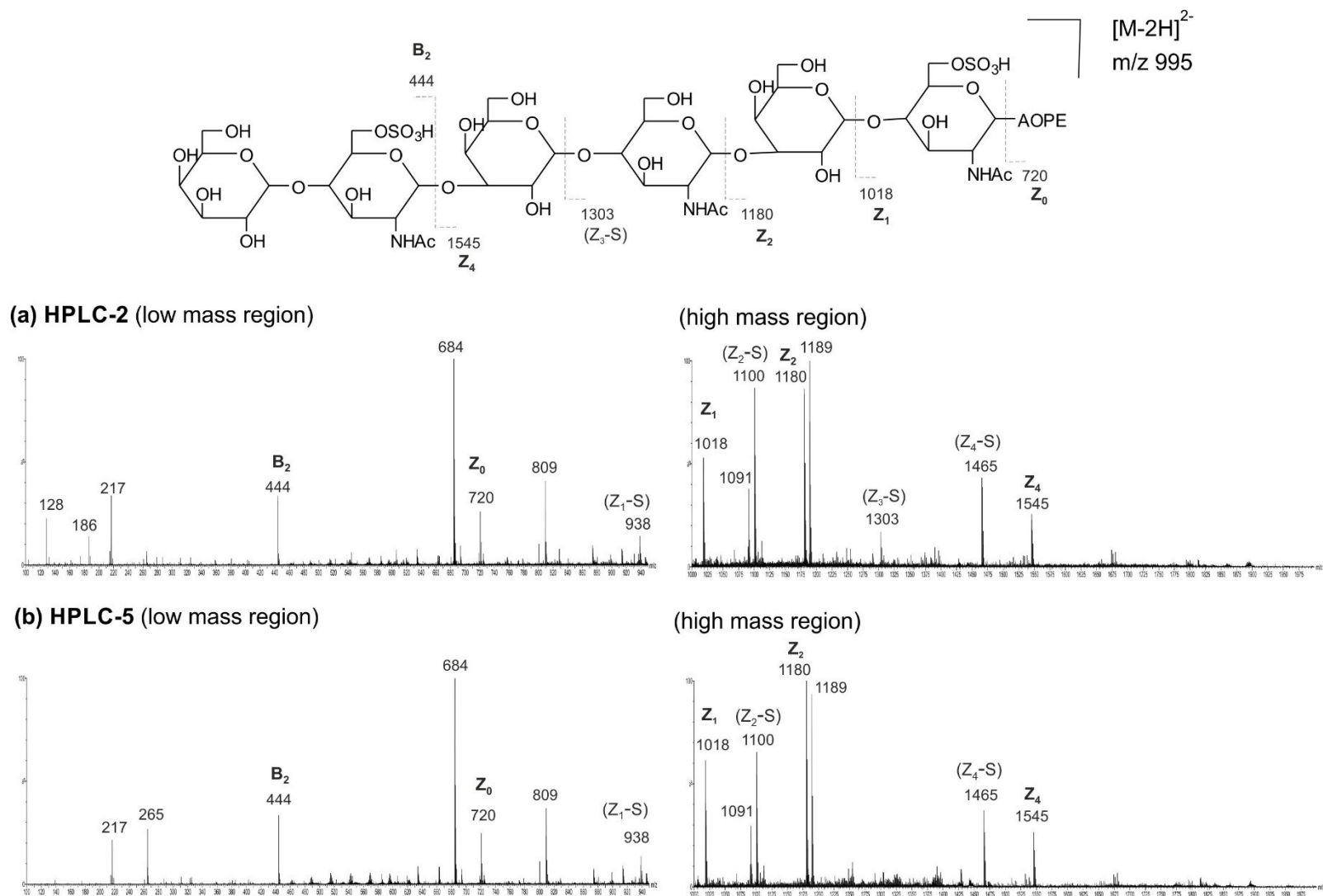


Figure 4.25. ES-CID-MS/MS product-ion spectra of the R-10G antigen-positive di-sulphated hexasaccharides HPLC-2 (a) and -5 (b) as NGLs. Two mass regions (low and high) are shown here. Fragmentation is shown above the spectra for interpretation of the spectra.

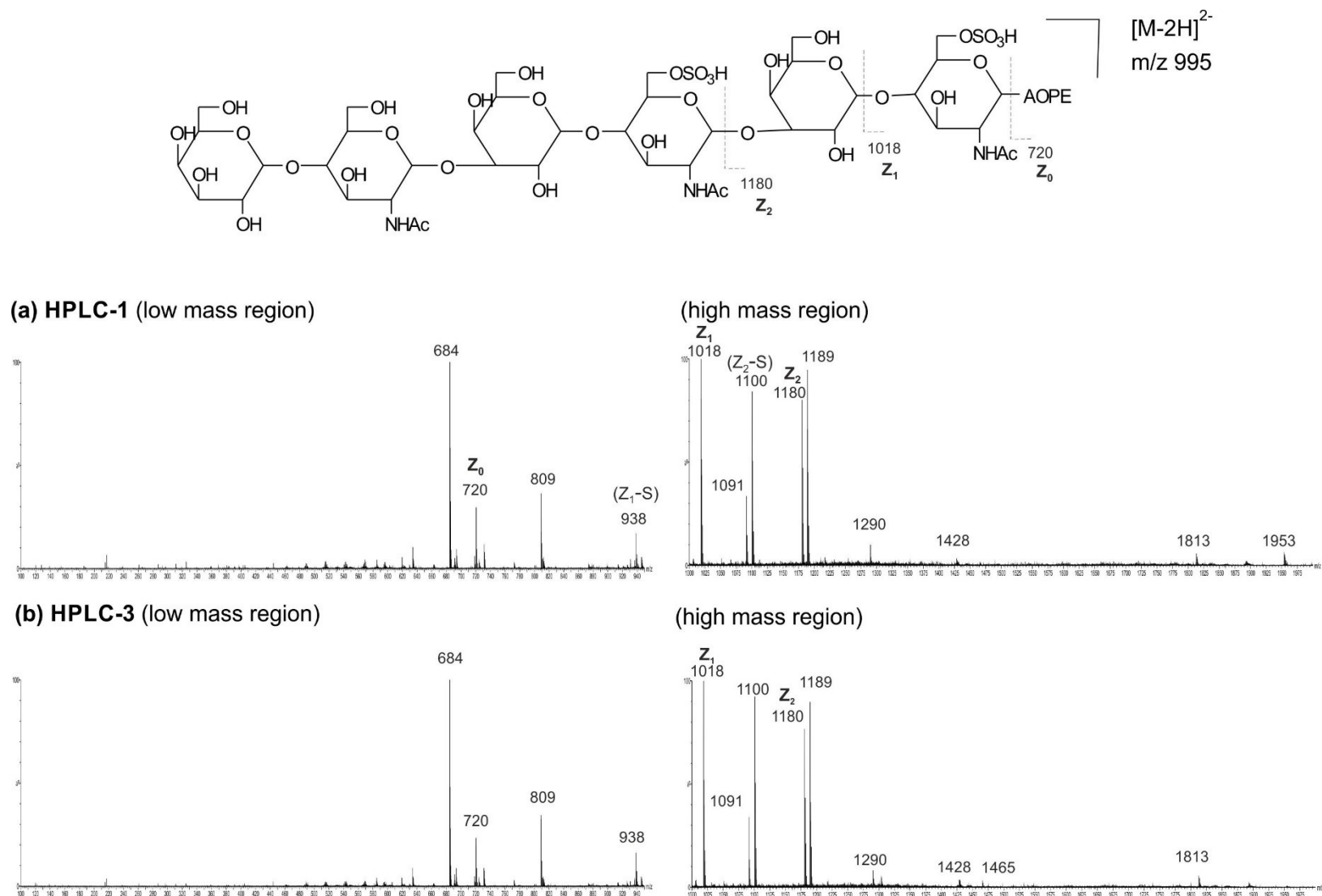


Figure 4.26. ES-CID-MS/MS product-ion spectra of the R-10G antigen-negative di-sulphated hexasaccharides HPLC-1 (a) and -3 (b) as NGLs. Two mass regions (low and high) are shown here. Fragmentation is shown above the spectra for interpretation of the spectra.

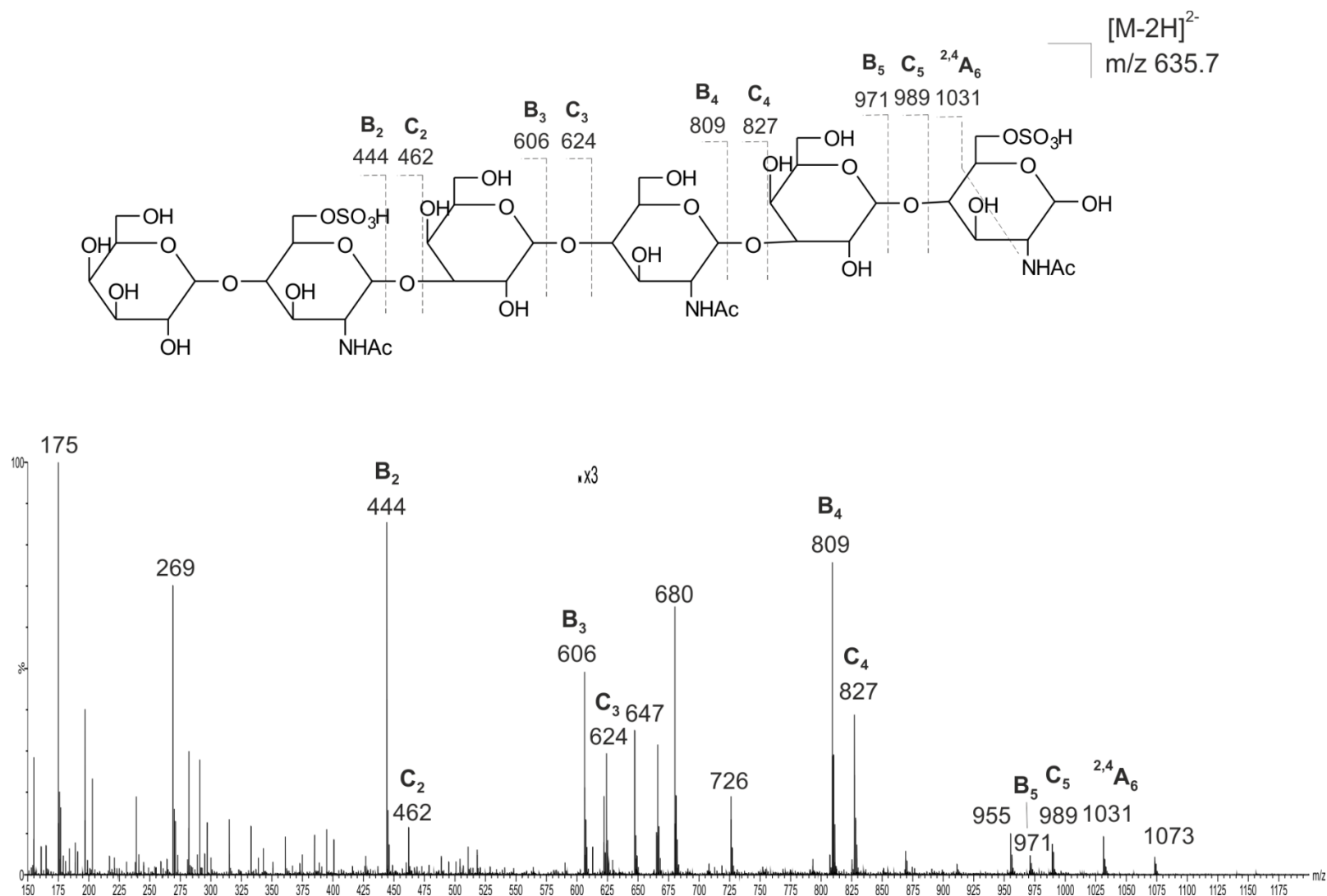


Figure 4.27. ES-CID-MS/MS product-ion spectrum of the R-10G antigen-positive di-sulphated hexasaccharides HPLC-5. Fragmentation is shown above the spectrum for interpretation of the spectra.

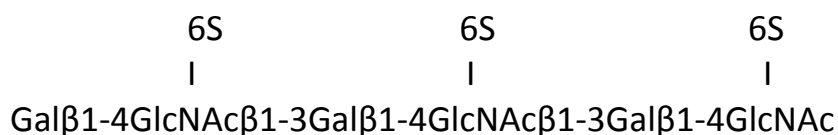
4. 9 Discussion

Generation of a more comprehensive GlycosaminoGlycome microarray, than those previously described [170, 183, 184, 214-216, 243-245], and designer KS arrays, were important for characterization of the antigen recognized by mAb R-10G.

Using a microarray of a variety of GAG polysaccharides mAb R-10G was shown to bind almost exclusively to bovine corneal KS. The KS preparations from chicken egg white and bovine articular cartilage gave little or no binding to R-10G. Compared with bovine corneal KS, chicken egg white KS is more highly sialylated. The sialic acids are in the form of both α 2,8 and α 2,9 linkages, which differ from the α 2,3 and α 2,6 in bovine corneal KS [51]. It is likely that the capping sialic acids on the chicken egg white KS have masked the effect on R-10G antigenicity. Bovine articular cartilage KS is highly sulphated and also fucosylated [46, 207]. The weak R-10G binding to this KS is consistent with the requirement of the undersulphation for expression of R-10G antigen [73].

Clearly, the presence of terminal non-reducing end Gal is important for expression of R-10G antigen. Keratanase II rather than keratanase I digestion generates such Gal-terminating oligosaccharides [206, 246]. Although the keratanase II digestion-derived disaccharide and tetrasaccharide fractions terminate with Gal, no binding of mAb R-10G was observed to these. The minimum chain length bound by the antibody was hexasaccharide.

The keratanase II digestion-derived P6 hexasaccharide fraction contained oligosaccharides with 1 to 6 sulphates. The antigen-positive hexasaccharide that could be isolated in sufficient quantity for full characterization including NMR was the tri-sulphated form.



Among the hexasaccharide fractions obtained from sources (a), (b) and (c), the non-sulphated form was not bound and the mono-sulphated forms showed little or no binding. Among the di-sulphated hexasaccharides, it is striking that two were bound very strongly (probe positions 42 and 44 in Figure 4.24); these are the same glycan (Structure 1, below) simply different at the reducing termini of α and β . Whereas the other two (probe positions 41 and 43 in Figure 4.24) were not bound; these are the same glycan (Structure 2, below).

Taken together, from the designer approach that included antigenic analyses in conjunction with MS and NMR sequencing of the hexasaccharides, I have further concluded that the hexasaccharide (Structure 1, overleaf) with sulphate on the non-reducing and reducing GlcNAc is active whereas the hexasaccharide (Structure 2) with sulphate on the internal and reducing GlcNA is negative. This suggests that the sulphate at the non-reducing terminal GlcNAc residue is the key element and the sulphate at the internal GlcNAc residue is not required. It raises a possibility that the mono-sulphated hexasaccharide with sulphate at the non-reducing GlcNAc (Structure 4) constitutes the antigen. Work is underway to isolate the mono-sulphated hexasaccharide (Structure 4) and the di-sulphated hexasaccharides (Structure 3).

$ \begin{array}{c} 6S \\ \\ \text{Gal}\beta 1-4\text{GlcNAc}\beta 1-3\text{Gal}\beta 1-4\text{GlcNAc}\beta 1-3\text{Gal}\beta 1-4\text{GlcNAc} \end{array} $	(1) Active
$ \begin{array}{c} 6S \qquad \qquad 6S \\ \qquad \qquad \\ \text{Gal}\beta 1-4\text{GlcNAc}\beta 1-3\text{Gal}\beta 1-4\text{GlcNAc}\beta 1-3\text{Gal}\beta 1-4\text{GlcNAc} \end{array} $	(2) Inactive
$ \begin{array}{c} 6S \qquad \qquad 6S \\ \qquad \qquad \\ \text{Gal}\beta 1-4\text{GlcNAc}\beta 1-3\text{Gal}\beta 1-4\text{GlcNAc}\beta 1-3\text{Gal}\beta 1-4\text{GlcNAc} \end{array} $	(3) Not tested
$ \begin{array}{c} 6S \\ \\ \text{Gal}\beta 1-4\text{GlcNAc}\beta 1-3\text{Gal}\beta 1-4\text{GlcNAc}\beta 1-3\text{Gal}\beta 1-4\text{GlcNAc} \end{array} $	(4) Not tested

Chapter 5

Comparison of the Specificities of mAbs MZ15, 5D4, anti-i P1A ELL1, TRA-1-60 and TRA-1-81 with mAb R-10G

5.1 Introduction

The purpose of this chapter has been to apply the GlycosaminoGlycome microarrays to extend knowledge on the specificities of two anti-KS antibodies, MZ15 and 5D4, and anti-i P1A ELL1 antibody known to recognize backbones of KS, and two anti-human stem cell antibodies, TRA-1-60 and TRA-1-81.

MZ15 was raised by Bruce Caterson and colleagues against whole chondrocytes [217], whereas 5D4 (IgG) was raised by Fiona Watt and colleagues against proteoglycan isolated from chondroitinase ABC-treated human articular cartilage proteoglycan monomer [218]. Competitive inhibition radioimmunoassay analyses of the two antibodies showed that the binding of the two antibodies was inhibited by both corneal and skeletal KS glycopeptides [217, 218]. Desulphation of these KS glycopeptides reduced their antigenicity [217, 218]. Ten Feizi and colleagues carried out inhibition of binding assays using a series of structurally characterized oligosaccharides obtained after endo- β -galactosidase digestion of bovine corneal KS. They observed that the shortest oligosaccharide that was able to inhibit the binding of the MZ15 and 5D4 was a linear penta-sulphated hexasaccharide [247].

Anti-i P1A ELL1 was from a patient with cold agglutinin disease [248]. The i antigen is a prominent erythrocyte antigen existing in the human foetus and diminishes after birth and during the first year of life, and it is expressed on the linear nonsulphated poly-N-acetyllactosamine backbone of the blood group antigens and of KS [107].

The other two commonly used anti-human stem cell antibodies, TRA-1-60 and TRA-1-81 (IgM), were included to compare with R-10G and their specificities were evaluated using our microarrays. The two antibodies were produced by hyperimmunizing a male mouse with 2012Ep human EC cells [91]. They were suggested to bind to a KS proteoglycan on human EC cells, and there was evidence that the proteoglycan bound

by TRA-1-60 was sialylated [249]. Following binding analyses in a CFG glycan array it was shown that the two antibodies bound to two glycans (Structures 1 and 2) of poly-N-acetylactosamine type with a type 1 sequence (Gal β 1-3GlcNAc) at the non-reducing end [106].

Structure 1 Gal β 1-3GlcNAc β 1-3Gal β 1-4GlcNAc

Structure 2

Galβ1-3GlcNAcβ1-3Galβ1-4GlcNAcβ-1
|
Galβ1-4Glc
|
Galβ1-3GlcNAcβ-1

Except for two KS disaccharides there were no other KS chains included in the CFG array and these were not bound by the two antibodies. As TRA-1-60 and TRA-1-81 epitopes are important human stem cell markers, unambiguous characterization of these antibodies is a priority, especially whether they bind to KS. I have addressed in the course of elucidating of R-10G antigen by GlycosaminoGlycome microarray.

5.2 Comparison of Glycosaminoglycan-Binding Properties of MZ15, 5D4 and Anti-i P1A ELL1 with R-10G

The anti-KS antibodies, MZ15 and 5D4, were tested on the GAG polysaccharide microarray; their binding patterns were similar (Figure 5.1). They bound strongly to bovine corneal KS and weakly to the KS preparations from bovine articular cartilage and chicken egg white, which is similar to the pattern of the binding of R-10G (Figure 4.1). The strong binding of MZ15 and 5D4 to the preparation of CSA has not been reported previously and is unexplained. 5D4 bound to the dextran sulphate whereas MZ15 and R-10G did not. Although anti-i P1A ELL1 antibody was not analysed on the same microarray, our data using another microarray that contained bovine corneal KS

polysaccharide show that the antibody did not bind to the KS polysaccharide (Data not shown).

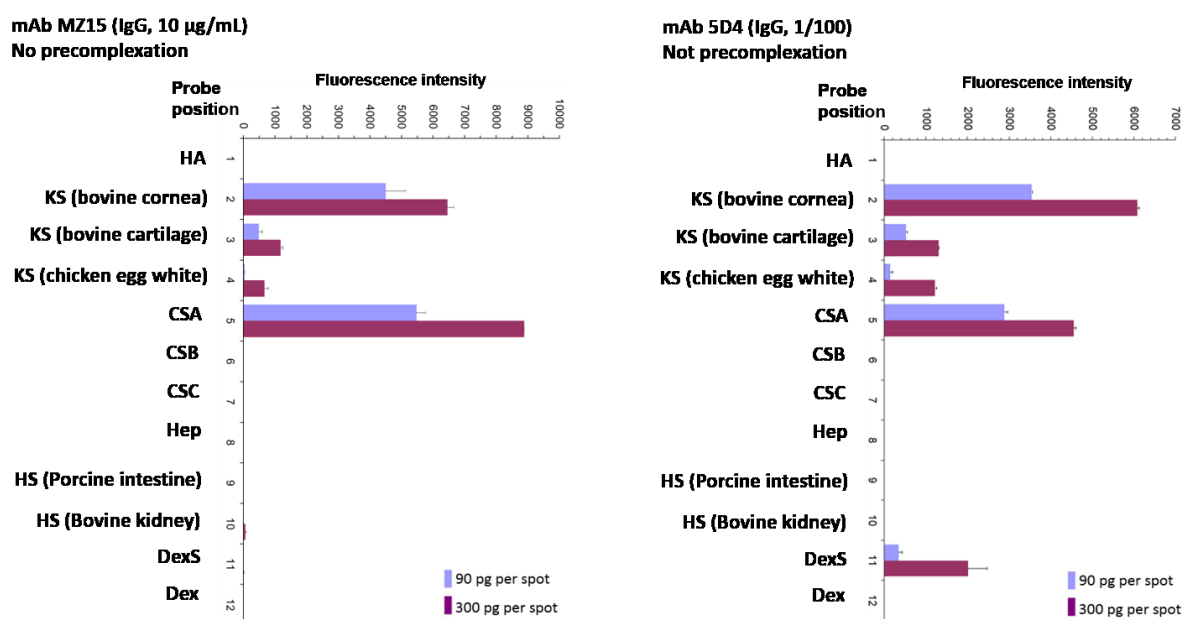


Figure 5.1. GAG polysaccharide microarray analysis of mAbs MZ15 and 5D4 (IgG). Probe abbreviations are defined in Table 3.1. Blocking solution was PBS with 3% (w/v) BSA, and diluent was PBS with 1% (w/v) BSA. Binding was detected using biotinylated anti-mouse IgG followed by Alexa Fluor-647-labeled streptavidin. Results are means of fluorescence intensities of duplicate spots, printed at 90 and 300 pg per spot, with error bars representing half of the difference between the two values. The results shown here for MZ15 are representative of numerous analyses using this microarray; 5D4 was analysed only once using this microarray.

In terms of the binding to the NGLs of KS oligosaccharides, MZ15 and 5D4 bound to NGLs of both keratanase I and II digestion-derived oligosaccharides with higher binding signal to the keratanase I digestion-derived terminating with GlcNAc at the non-reducing end (Figures 5.2, 5.3 and 5.5). This is completely different from R-10G which bound strongly to the Gal-terminating keratanase II digestion-derived oligosaccharides rather than to the GlcNAc-terminating oligosaccharides (Figures 4.24 and 5.5). The minimum KS chain lengths bound by the MZ15 and 5D4 are keratanase I digestion-derived hexasaccharide. The two antibodies did not bind to the R-10G antigen-positive di and tri-sulphated hexasaccharides. The focus of my research being on undersulphated KS oligosaccharides, we did not isolate oversulphated

oligosaccharides longer than hexasaccharide which the MZ15 and 5D4 would be predicted to bind.

The anti-i P1A ELL1 antibody consistent with its recognition of Gal terminating linear poly-N-acetyllactosamine sequences [248] showed relatively weak binding to unfractionated keratanase II digestion-derived fractions (Figures 5.4 and 5.5). Strong binding was to the non- and mono-sulphated hexasaccharide fractions and certain di-sulphated hexasaccharide fractions (Figures 5.4 and 5.5). The antibody did not bind to the hexasaccharides with three to six sulphates. Interestingly, among the four HPLC subfractions isolated from the di-sulphated hexasaccharide, anti-i P1A ELL1 bound only to the two fractions which were not bound by R-10G.

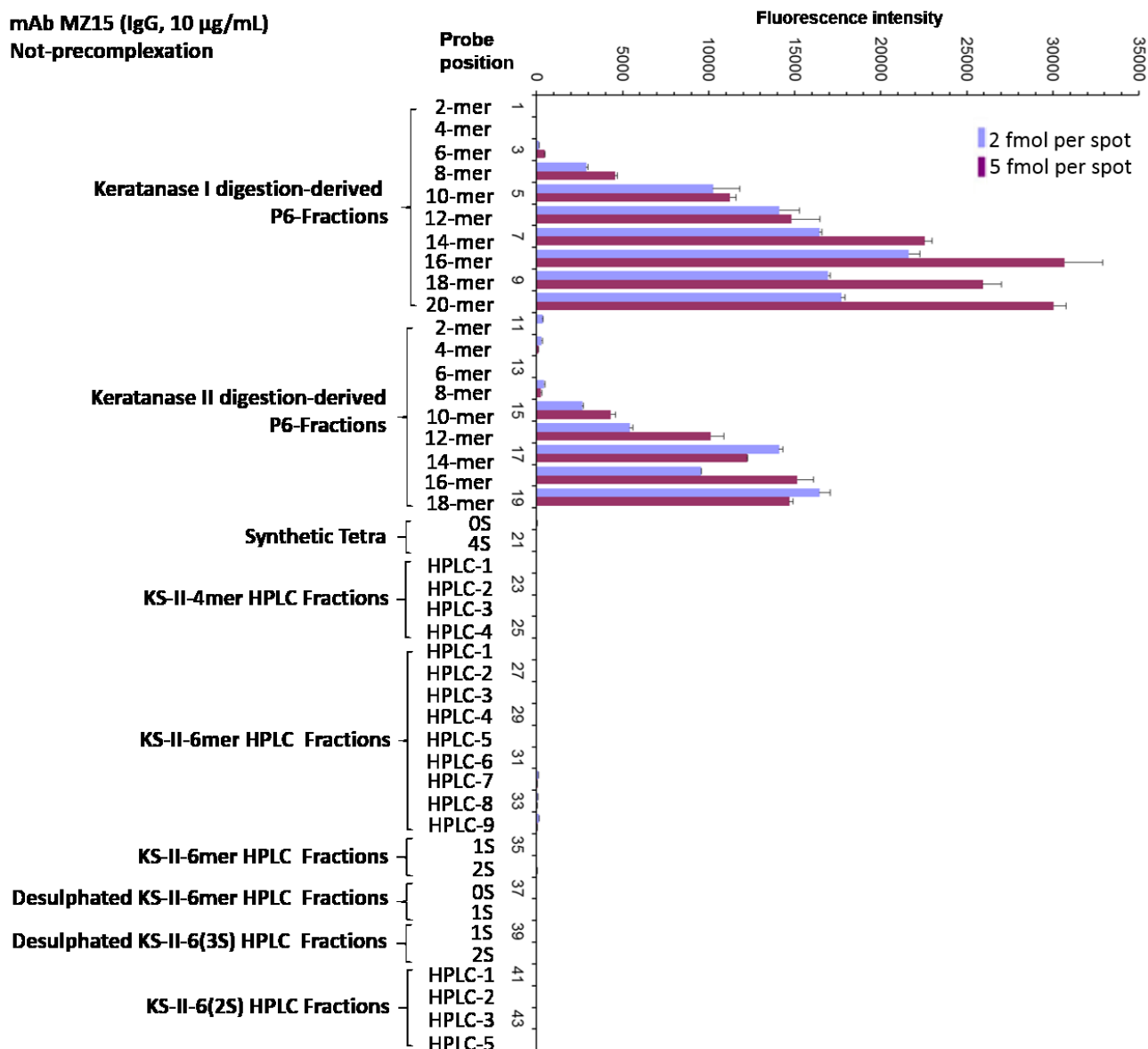


Figure 5.2. Microarray analysis of mAb MZ15 (IgG) using KS oligosaccharide NGL microarray Set 2c. Blocking solution was PBS with 3% (w/v) BSA, and diluent was PBS with 1% (w/v) BSA. Detection of binding was as described in Figure 5.1. Results are means of fluorescence intensities of duplicate spots, printed at 2 and 5 fmol per spot, with error bars representing half of the difference between the two values. The result shown here is representative of numerous analyses with MZ15 using this microarray.

mAb 5D4 (IgG, 1/50)
Not-precomplexation

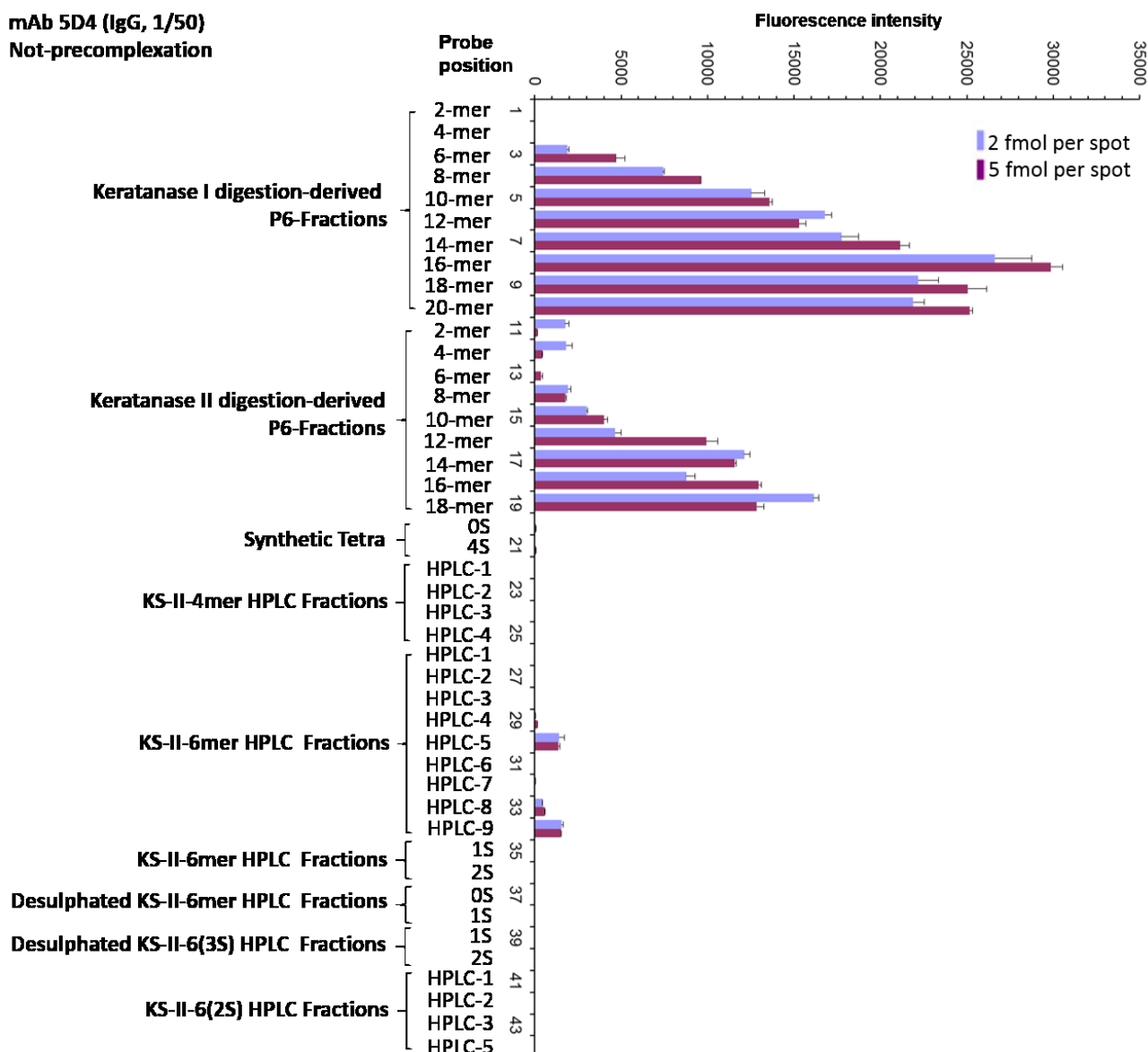


Figure 5.3. Microarray analysis of mAb 5D4 (IgG) using KS oligosaccharide NGL microarray Set 2c. Blocking solution was PBS with 3% (w/v) BSA, and diluent was PBS with 1% (w/v) BSA. Detection of binding was as described in Figure 5.1. Results are means of fluorescence intensities of duplicate spots, printed at 2 and 5 fmol per spot, with error bars representing half of the difference between the two values. The result shown here is representative of numerous analyses with 5D4 using this microarray.

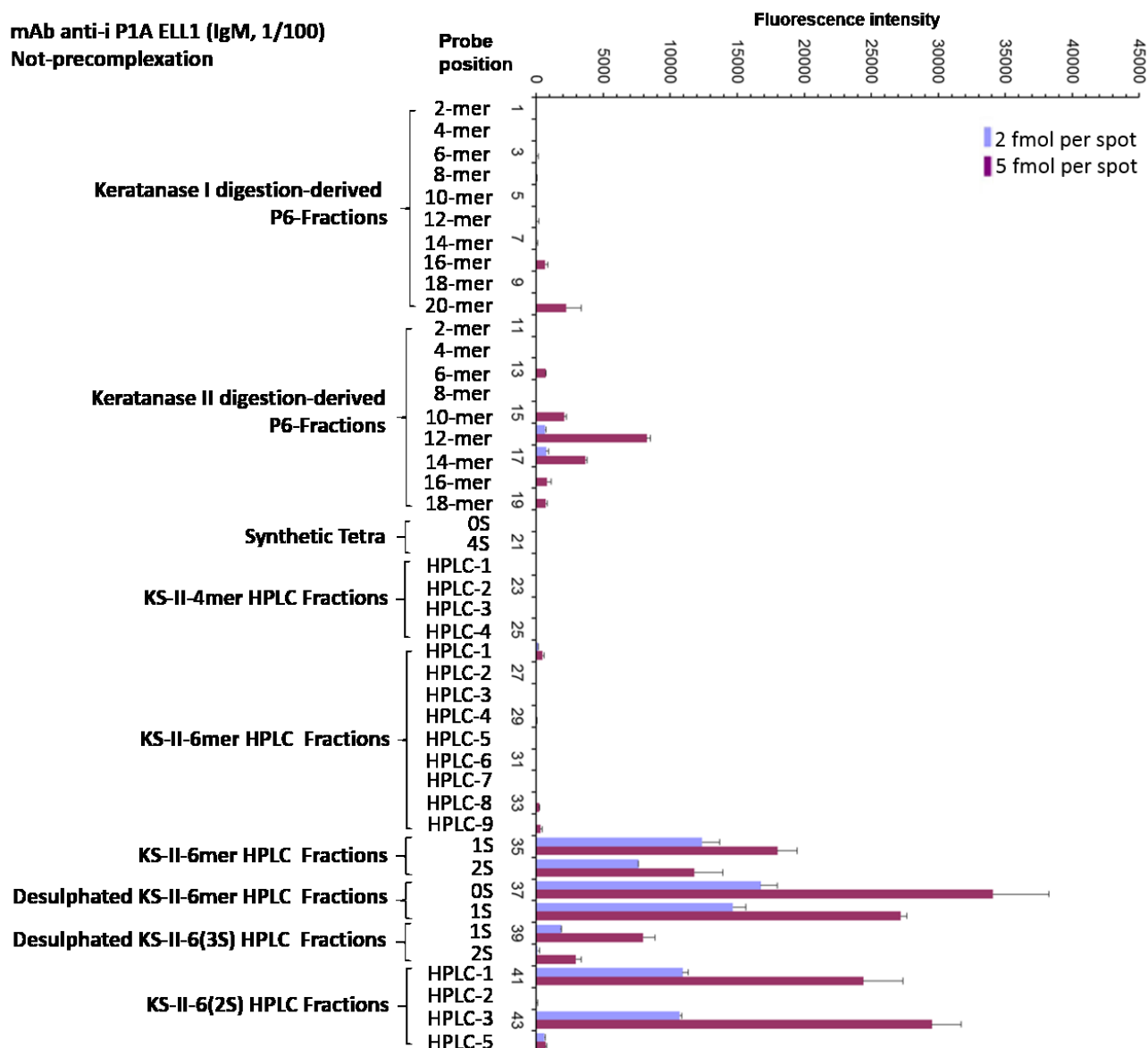


Figure 5.4. Microarray analysis of mAb anti-i P1A ELL1 (IgM) using KS oligosaccharide NGL Set 2c. Blocking and diluent solution was HBS containing 3% (w/v) BSA and 5 mM CaCl₂. Binding was detected using biotinylated anti-human IgM followed by Alexa Fluor-647-labeled streptavidin. Results are means of fluorescence intensities of duplicate spots, printed at 2 and 5 fmol per spot, with error bars representing half of the difference between the two values. Anti-i was analysed only once using this microarray.

Probe		Position	MZ15	5D4	R-10G	anti-i P1A ELL1
Keratanase I digestion-derived P6 Fractions	2 mer	1				
	4 mer	2				
	6 mer	3				
	8 mer	4				
	10 mer	5				
	12 mer	6				
	14 mer	7				
	16 mer	8				
	18 mer	9				
	20 mer	10				
Keratanase II digestion-derived P6 Fractions	2 mer	11				
	4 mer	12				
	6 mer	13				
	8 mer	14				
	10 mer	15				
	12 mer	16				
	14 mer	17				
	16 mer	18				
Synthetic tetrasaccharides	0S	20				
	4S	21				
KS-II-4mer HPLC fraction	HPLC-1	22				
	HPLC-2	23				
	HPLC-3	24				
	HPLC-4	25				
KS-II-6mer HPLC fraction	HPLC-1	26				
	HPLC-2	27				
	HPLC-3	28				
	HPLC-4	29				
	HPLC-5	30				
	HPLC-6	31				
	HPLC-7	32				
	HPLC-8	33				
	HPLC-9	34				
	1S	35				
Desulphated KS-II-6mer HPLC fractions	0S	37				
	1S	38				
Desulphated KS-II-6(3S) HPLC fractions	1S	39				
	2S	40				
KS-II-6(2S) HPLC fractions	HPLC-1	41				
	HPLC-2	42				
	HPLC-3	43				
	HPLC-5	44				

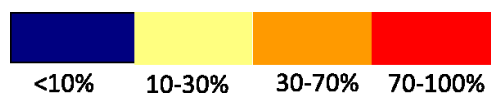


Figure 5.5. Relative intensities of binding of R-10G, MZ15, 5D4 and anti-i P1A ELL1 to probes in KS oligosaccharide NGL microarray Set 2c.

5.3 Investigation of TRA-1-60 and TRA-1-81 Binding to Glycosaminoglycan and Poly-N-acetyllactosamine-containing Microarrays

TRA-1-60 and TRA-1-81 were tested on GlycosaminoGlycome microarray including GAG polysaccharides and NGLs of GAG oligosaccharides. They did not bind to any KS polysaccharides or derived NGLs, and nor to any other arrayed GAG probes (data not shown).

However, in agreement with earlier report [106], TRA-1-60 and TRA-1-81 gave strong binding to three structures at positions 12, 13 and 26 in the microarray (designated CLL array in Appendix 4) (Figures 5.6 and 5.7). The common feature was the presence of non-reducing type-1 followed by internal type-2 as a part of linear and branch glycans. A difference was revealed between these two antibodies in that TRA-1-60 gave binding albeit weaker to the structure at position 63 with a fucose 3-linked to the inner GlcNAc (Figures 5.6 and 5.7). The binding was abolished in the presence of 2-linked fucose at the non-reducing end (position 64).

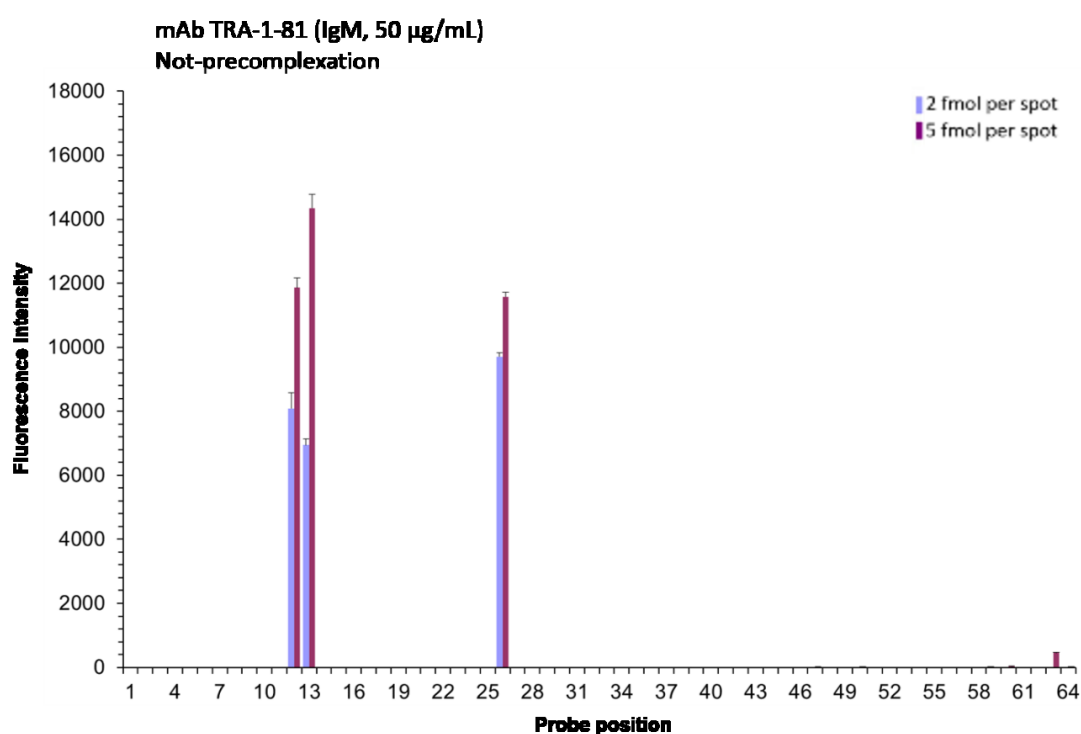
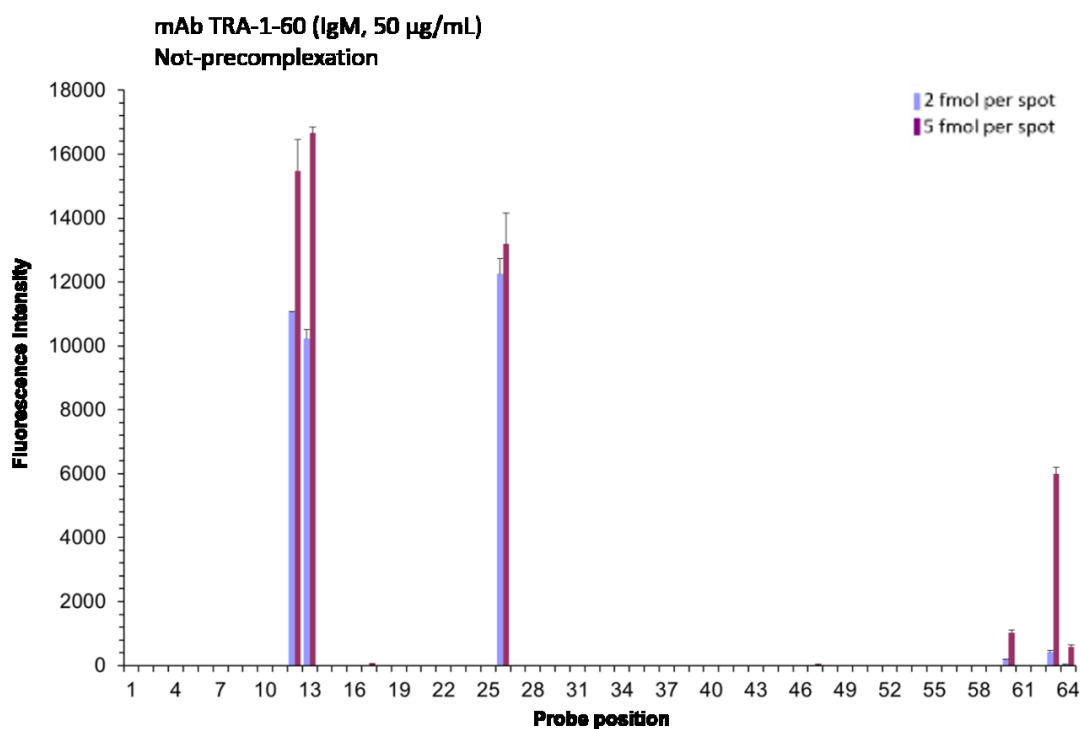


Figure 5.6. Microarray analysis of mAbs TRA-1-60 and TRA-1-81 (IgM) using CLL microarray set. Probe names and structures are in Appendix 4. Blocking solution was PBS with 3% (w/v) BSA, and diluent was PBS with 1% (w/v) BSA. Binding was detected using biotinylated anti-mouse IgM followed by Alexa Fluor-647-labeled streptavidin. Results are means of fluorescence intensities of duplicate spots, printed at 2 and 5 fmol per spot, with error bars representing half of the difference between the two values. The results shown here are representative of three analyses with TRA-1-60 and TRA-1-81 using this microarray.

Probe Position	TRA-1-60	TRA-1-81	Structure
12			$\text{Gal}\beta\text{-3GlcNAc}\beta\text{-3Gal}\beta\text{-4GlcNAc}\beta\text{-6}$ $\text{Gal}\beta\text{-4Glc-DH}$ $\text{Gal}\beta\text{-3GlcNAc}\beta\text{-3}$
13			$\text{Gal}\beta\text{-4GlcNAc}\beta\text{-6}$ $\text{Gal}\beta\text{-4GlcNAc}\beta\text{-6}$ $\text{Gal}\beta\text{-3GlcNAc}\beta\text{-3}$ $\text{Gal}\beta\text{-4Glc-DH}$ $\text{Gal}\beta\text{-3GlcNAc}\beta\text{-3}$
15			$\text{Gal}\beta\text{-4GlcNAc}\beta\text{-6}$ $\text{Gal}\beta\text{-4GlcNAc}\beta\text{-3Gal}\beta\text{-4Glc}\beta\text{-Cer}$ $\text{Gal}\beta\text{-4GlcNAc}\beta\text{-3}$
26			$\text{Gal}\beta\text{-3GlcNAc}\beta\text{-3Gal}\beta\text{-4GlcNAc}\beta\text{-3Gal}\beta\text{-4Glc-DH}$
27			$\text{Gal}\beta\text{-4GlcNAc}\beta\text{-3Gal}\beta\text{-4GlcNAc}\beta\text{-3Gal}\beta\text{-4Glc-DH}$
62			$\text{Gal}\beta\text{-3GlcNAc}\beta\text{-3Gal}\beta\text{-4GlcNAc}\beta\text{-6}$ $\text{Fuc}\alpha\text{-4}$ $\text{Fuc}\alpha\text{-3}$ $\text{Gal}\beta\text{-4Glc-DH}$ $\text{Gal}\beta\text{-3GlcNAc}\beta\text{-3}$ $\text{Fuc}\alpha\text{-4}$
63			$\text{Gal}\beta\text{-3GlcNAc}\beta\text{-3Gal}\beta\text{-4GlcNAc}\beta\text{-6}$ $\text{Fuc}\alpha\text{-3}$ $\text{Gal}\beta\text{-4Glc-DH}$ $\text{Fuc}\alpha\text{-2Gal}\beta\text{-3GlcNAc}\beta\text{-3}$
64			$\text{Fuc}\alpha\text{-2Gal}\beta\text{-3GlcNAc}\beta\text{-3Gal}\beta\text{-4GlcNAc}\beta\text{-6}$ $\text{Fuc}\alpha\text{-3}$ $\text{Gal}\beta\text{-4Glc-DH}$ $\text{Fuc}\alpha\text{-2Gal}\beta\text{-3GlcNAc}\beta\text{-3}$

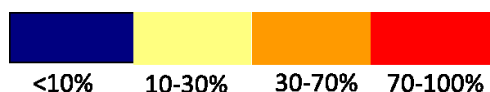


Figure 5.7. Relative intensities of binding of TRA-1-60 and TRA-1-81 to selected probes in CLL microarray set. Full probe list is shown in Appendix 4.

5.4 Discussion

The GlycosaminoGlycome microarray generated in the previous sections for the characterisation of R-10G antigens were used for comparison of the antigen recognized by mAb R-10G with those recognized by other antibodies directed to KS and by anti-human stem cell mAbs TRA-1-60 and TRA-1-81.

The mAbs MZ15 and 5D4 differ from R-10G in their binding profiles. MZ15 and 5D4 bound both to the keratanase I digestion-derived oligosaccharides terminating with GlcNAc and the keratanase II digestion-derived oligosaccharides terminating with Gal at the non-reducing end albeit less intensely to the latter. These observations suggest that MZ15 and 5D4 may be 'groove-type' antibodies that are able to interact with non-terminal sequences of KS. Oversulphated oligosaccharides of the type known to be recognized by the two antibodies were not isolated in the present investigation with the exception of the hexasaccharides from keratanase II digestion which gave little or no binding (Figures 5.2, 5.3 and 5.5). The octa- and longer over-sulphated analogs were not among the oligosaccharides isolated.

Our microarray data with anti-i P1A ELL1 extend knowledge on oligosaccharide recognition by this antibody. An earlier report shows that fully sulphated KS chains are not bound by anti-i [210]. Here we have shown that this anti-i can tolerate the presence of one or two sulphates on the KS hexasaccharides. Indeed, there was reciprocal binding activity of R-10G and anti-i with the probes at positions 41 to 44 (Figures 5.4 and 5.5).

Contrary to suggestions from earlier studies [249], the antigens recognized by TRA-1-60 and TRA-1-18 are not expressed on KS. The non-sulphated hexasaccharide sequence with type-2-type-2 backbone is moreover not bound by the two antibodies, confirming

that the i antigen is not cross-active with the antigens recognized by TRA-1-60 and TRA-1-81. Our results using the CLL microarray are consistent with those of the CFG [106], namely that the non-reducing type-1 followed by internal type-2 is bound by TRA-1-60 and TRA-81. A new finding here is that the two antibodies differ in their ability to bind to the 3-fucosylated inner GlcNAc residue.

In conclusion, we have shown that on the one hand, the antigenicities of R-10G and MZ15/5D4 are distinct and on the other hand, TRA-1-60/TRA-1-81 and i antigens are distinct.

Chapter 6

Investigation of Glycosaminoglycan-binding Preferences of Growth Factors and Morphogens using 'GlycosaminoGlycome' Microarrays

6.1 Introduction

FGFs are a family of proteins important in regulation of cell proliferation and differentiation during embryonic development through their binding to FGFRs [108]. Morphogens are molecules that specify different cell fates in a concentration-dependent manner [117]. They have been reported to interact with GAGs and participate in various biological processes such as self-renewal and differentiation of stem cells. The growth factors, FGF1 and FGF2, and morphogens, such as Wnt-3a, Shh, BMP-2 and BMP-4, were selected for analysis using the GlycosaminoGlycome microarrays. This was (a) to increase knowledge on their specificities as the microarray is more comprehensive than those previously reported [170, 183, 184, 214-216, 243-245]; (b) to address my special area interest, namely whether any of these proteins can interact with KS, thus in aiding future work on the role of KS on stem cells.

As R-10G is a predominant antigen in human ES/iPS cells, the growth factors and morphogens investigated were those of human origin. In the preliminary studies described in this chapter they were analysed using the GlycosaminoGlycome microarrays described in Chapters 3 and 4, which included GAG polysaccharides (CSA, CSB, CSC, heparin, HS, HA and KS) and oligosaccharide NGLs derived from them.

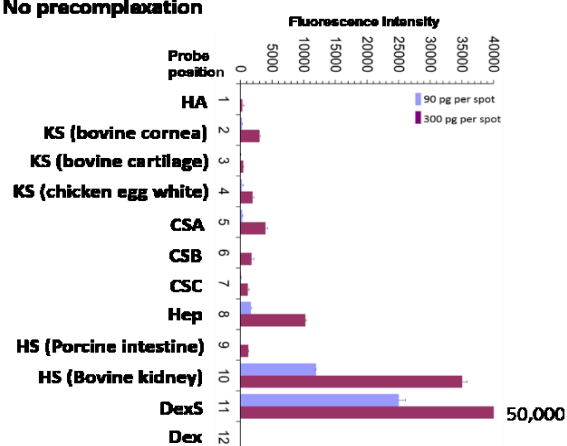
6.2 Initial Screening of Glycosaminoglycan-Binding Activities of the Growth Factors and Morphogens using the Glycosaminoglycan Polysaccharide Microarray

At the polysaccharide level, all six proteins gave binding signals with dextran sulphate consistent with sulphate being an important motif in their recognition structures (Figure 6.1). The maximum binding signals reached with dextran sulphate are indicated in the Figure 6.1. The binding signals with the GAG polysaccharides were considerably

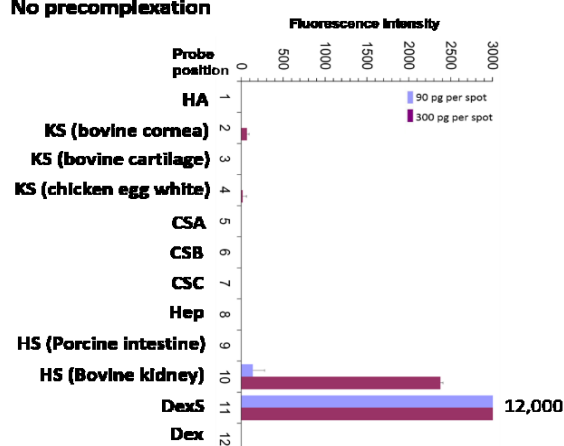
lower than with dextran sulphate. This may be a reflection of the more efficient immobilization of the dextran sulphate than the GAG polysaccharides.

Only four of the proteins investigated (FGF2, Wnt-3a, BMP-2 and BMP-4) gave detectable binding signals with the GAG polysaccharides. A common feature among them was binding signals with the HS from bovine kidney. With these four proteins, binding signals elicited with the arrayed heparin were of unexpectedly lower intensity than with the HS and with Wnt-3a, heparin binding was undetectable. Here also we should consider the issue of relative immobilization efficiency of the very hydrophilic heparin relative to HS which has a lower content of sulphates. Concerns about the immobilization efficiency applied also to the CSs and KS in the array. The antibody binding data to CSA, CSC and KS in Chapter 3 (Figure 3.1), Chapter 4 (Figures 4.1) and Chapter 5 (Figure 5.1 and 5.2) implies that the retention of these polysaccharides is sufficient to elicit substantial binding signals. With FGF2, BMP-2 and BMP-4, binding intensities equal to or lower than with HS were observed with CSA, CSB, CSC and KS.

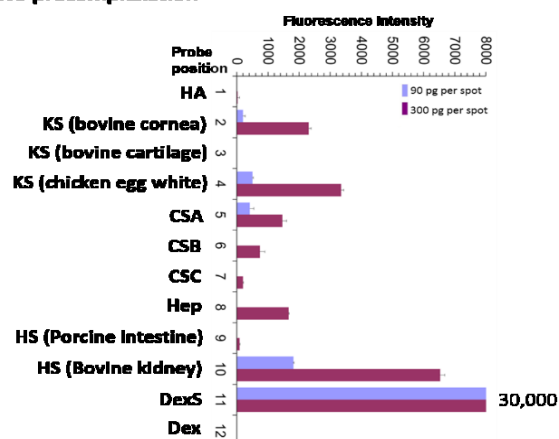
Human FGF2 (20 µg/mL)
No precomplexation



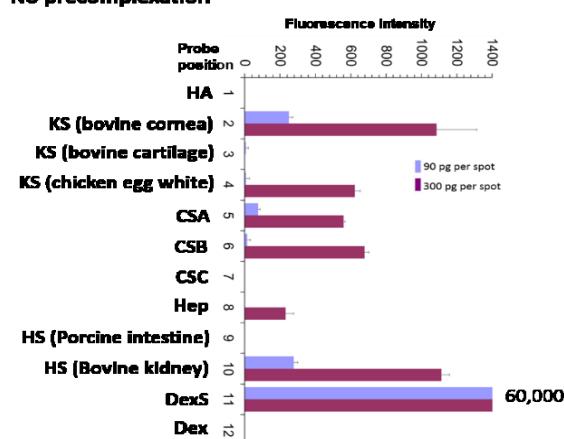
Recombinant human Wnt-3a (10 µg/mL)
No precomplexation



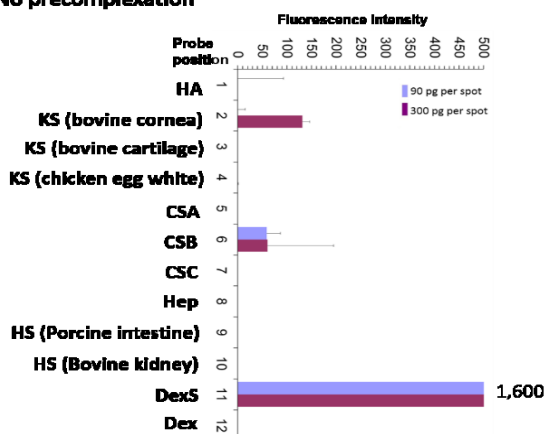
Recombinant human/mouse/rat BMP-2 (10 µg/mL)
No precomplexation



Recombinant human BMP-4 (10 µg/mL)
No precomplexation



Human FGF1 (20 µg/mL)
No precomplexation



Human Shh (10 µg/mL)
No precomplexation

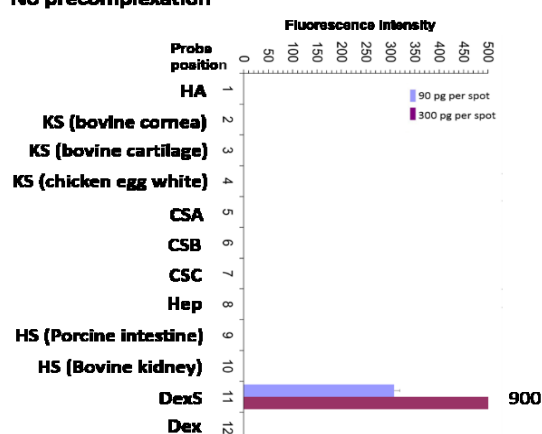


Figure 6.1. GAG polysaccharide microarray analysis of the growth factors and morphogens. Probe abbreviations are defined in Table 3.1. Blocking solution was PBS with 3% (w/v) BSA, and diluent was PBS with 1% (w/v) BSA. Binding was detected using respective human FGF2 antibody, human/mouse Wnt-3a antibody, human BMP-2/BMP-4 antibody, biotinylated anti-mouse IgG and biotinylated anti-rat IgG followed by Alexa Fluor-647-labeled streptavidin. Results are means of fluorescence intensities of duplicate spots, printed at 90 and 300 pg per spot, with error bars representing half of the difference between the two values. The results shown here are representative of three analyses with the proteins using this microarray.

6.3 Screening of the Growth Factors and Morphogens for Binding to Glycosaminoglycan Oligosaccharide Neoglycolipid Microarrays

The GAG oligosaccharide NGL microarrays of CSA, CSB, CSC and heparin (2 to 20 mers), HS (4 to 8 mers), deAc-HS (2 to 12 mers), HA (4 to 12 mers) and KS (2 to 20 mers) were next used in analysis of the growth factors and morphogens. These NGLs are in GAG oligosaccharide NGL microarray Set 6 in Chapter 3 and in KS oligosaccharide NGL microarray Set 2b in Chapter 4 and are depicted in the figures in this chapter.

Binding signals were observed with FGF2, BMP-2, BMP-4 and Shh. The first pattern of binding was FGF2 and BMP-2 which was similar overall (Figures 6.2, 6.3, 6.7 and 6.8). The two proteins gave binding signals with all the sulphated GAG oligosaccharide NGLs in GAG oligosaccharide NGL microarray Set 6 and no binding was detected with the HA derived NGLs which are not sulphated (Figures 6.2a, 6.3a and 6.7). Heparin oligosaccharide NGLs were the most strongly bound, from the disaccharide upwards. HS and deAc-HS NGLs were also bound. Binding signals were observed with the CS NGLs; those from CSB and CSC were stronger but there was diminished binding with CSA-derived longer oligomer NGLs.

In the KS oligosaccharide NGL microarray Set 2b, FGF2 and BMP-2 showed binding to NGLs of both of the keratanase I and keratanase II digestion-derived oligosaccharides (Figures 6.2b, 6.3b and 6.8). The intensity of binding to the keratanase II digestion-derived oligosaccharides diminished with the increase of chain length of the oligosaccharides. The hexasaccharide fraction obtained from the keratanase II digestion was subfractionated in the course of investigation of the recognition by R-10G described in Chapter 4. FGF2 and BMP-2 gave binding to some but not all of the subfractions. Notably, there was little or no binding to the undersulphated R-10G antigen-positive hexasaccharides at probe positions 26, 35, 36 and 38 (Figures 6.2b,

6.3b and 6.8). Thus the binding preferences of FGF2 and BMP-2 were to more highly sulphated oligosaccharides.

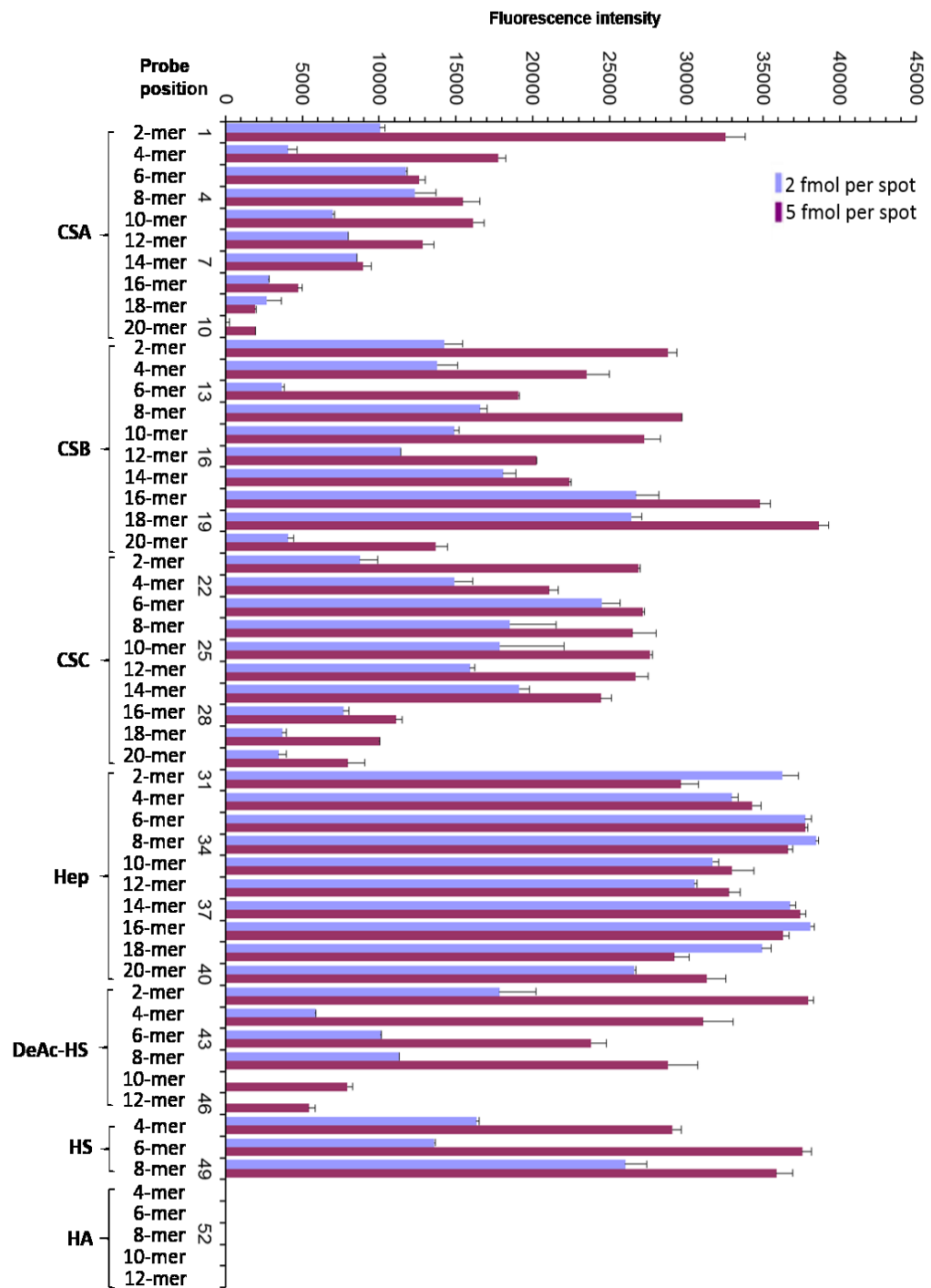
The second pattern of binding to NGLs was with BMP-4 and Shh. Both of these proteins showed predominant binding to heparin oligosaccharide NGLs (Figures 6.4, 6.5, 6.7 and 6.8). The lack of a binding signal at the 5 fmol spot of heparin 2-mer is unexplained; Shh binding was recorded in a different array set (not shown). Shh also bound to certain of the short oligomers of HS, CSA, CSB, and CSC. Neither protein gave binding signals with the KS derived oligosaccharide NGLs, KS oligosaccharide NGL microarray set 2b (data not shown).

With Wnt-3a, no binding signals were detected using GAG oligosaccharide NGL microarray Set 6 and KS oligosaccharide NGL microarray Set 2b (data not shown). As there was a lack of availability of microarray Set 6 for a repeat analysis, the Wnt-3a was analysed on a composite microarray which consisted of the original GAG polysaccharide microarray Set 2 and some selected GAG oligosaccharide NGLs (designated as GAG polysaccharide microarray Set 2'). There was strong binding to heparin oligosaccharide NGLs included (6 and 14 mers) (Figure 6.6). Analysis with this latter microarray has been performed three times and the results were reproducible.

FGF1 did not give binding signals when analysed using the GAG oligosaccharide NGL microarray Set 6, the KS oligosaccharide NGL microarray Set 2b and the GAG polysaccharide microarray Set 2' (data not shown). A fresh batch of the protein was purchased on the account of the known instability of FGF1; again binding was not detected.

(a)

Human FGF2 (20 µg/mL)
No precomplexation



(b) Human FGF2 (20 µg/mL)
No precomplexation

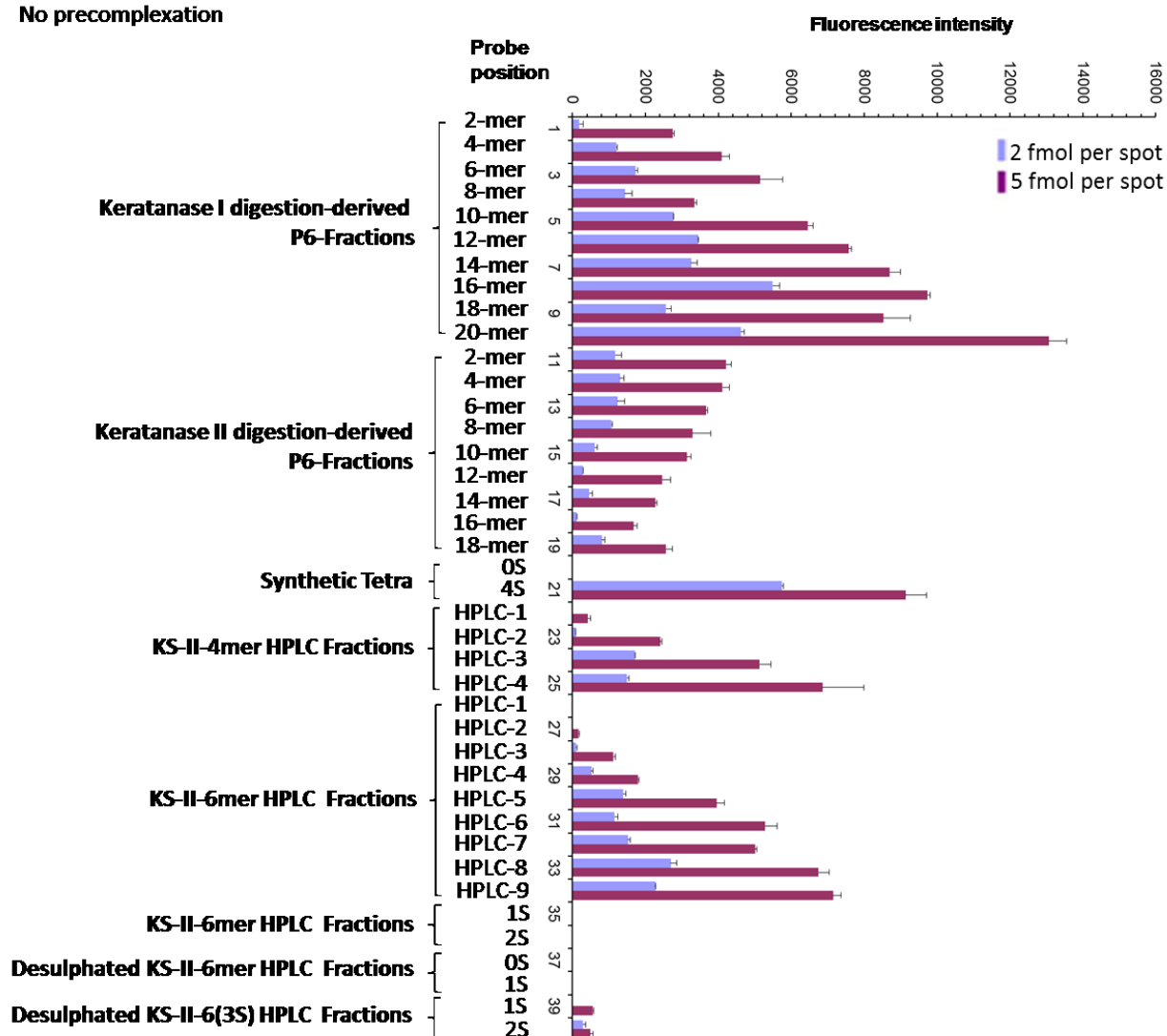
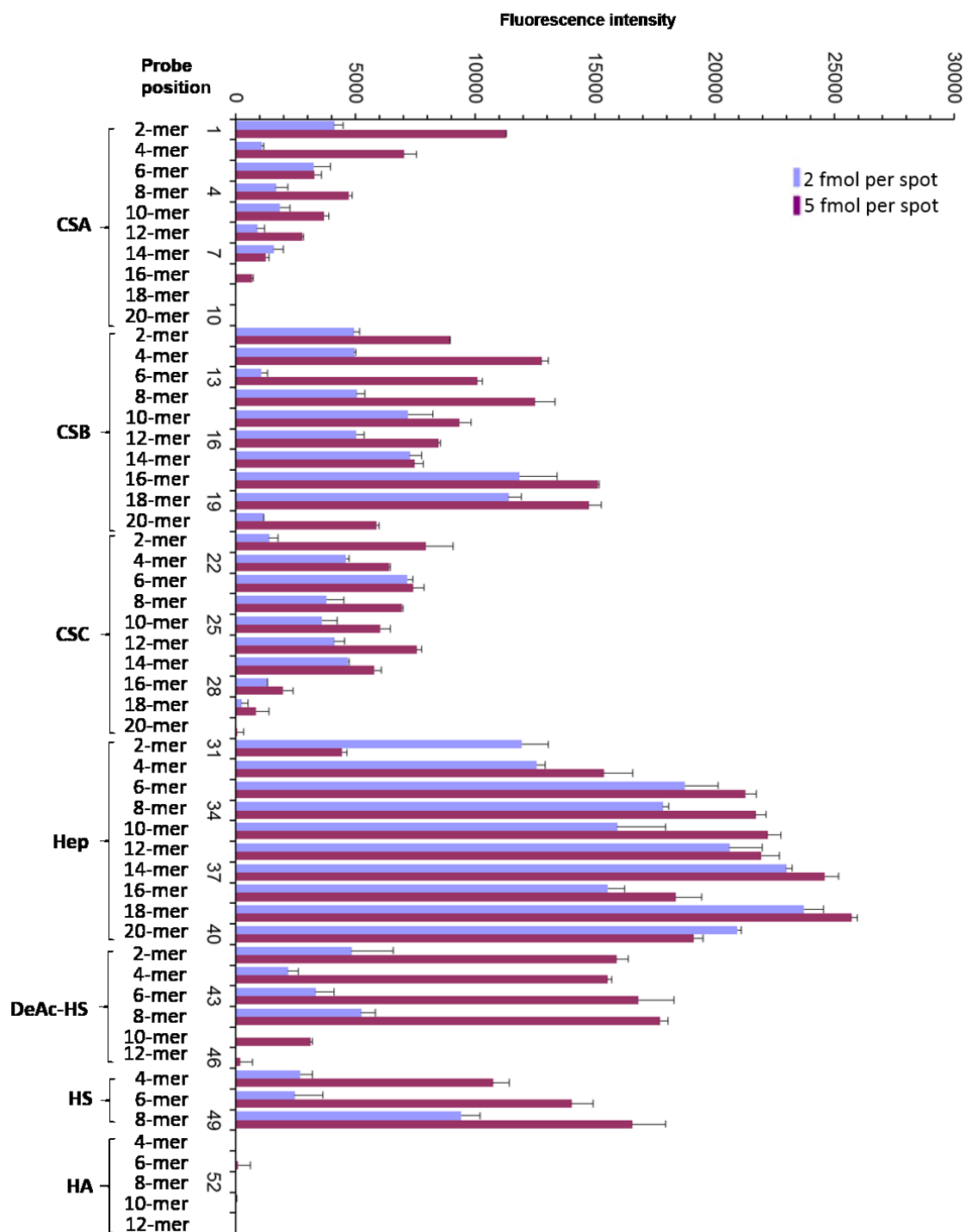


Figure 6.2. Microarray analyses of recombinant human FGF2 using GAG oligosaccharide NGL microarray Set 6 (a) and KS oligosaccharide NGL microarray Set 2b (b). Blocking solution was PBS with 3% (w/v) BSA, and diluent was PBS with 1% (w/v) BSA. Binding was detected using human FGF2 antibody and biotinylated anti-mouse IgG followed by Alexa Fluor-647-labeled streptavidin. Results are means of fluorescence intensities of duplicate spots, printed at 2 and 5 fmol per spot, with error bars representing half of the difference between the two values. The result shown here is representative of one analysis with FGF2 using these microarrays and another not shown using a different microarray with the same composition.

- (a) Recombinant human/mouse/rat BMP-2 (10 $\mu\text{g/mL}$)
No precomplexation



(b) Recombinant human/mouse/rat BMP-2 (10 µg/mL)
No precomplexation

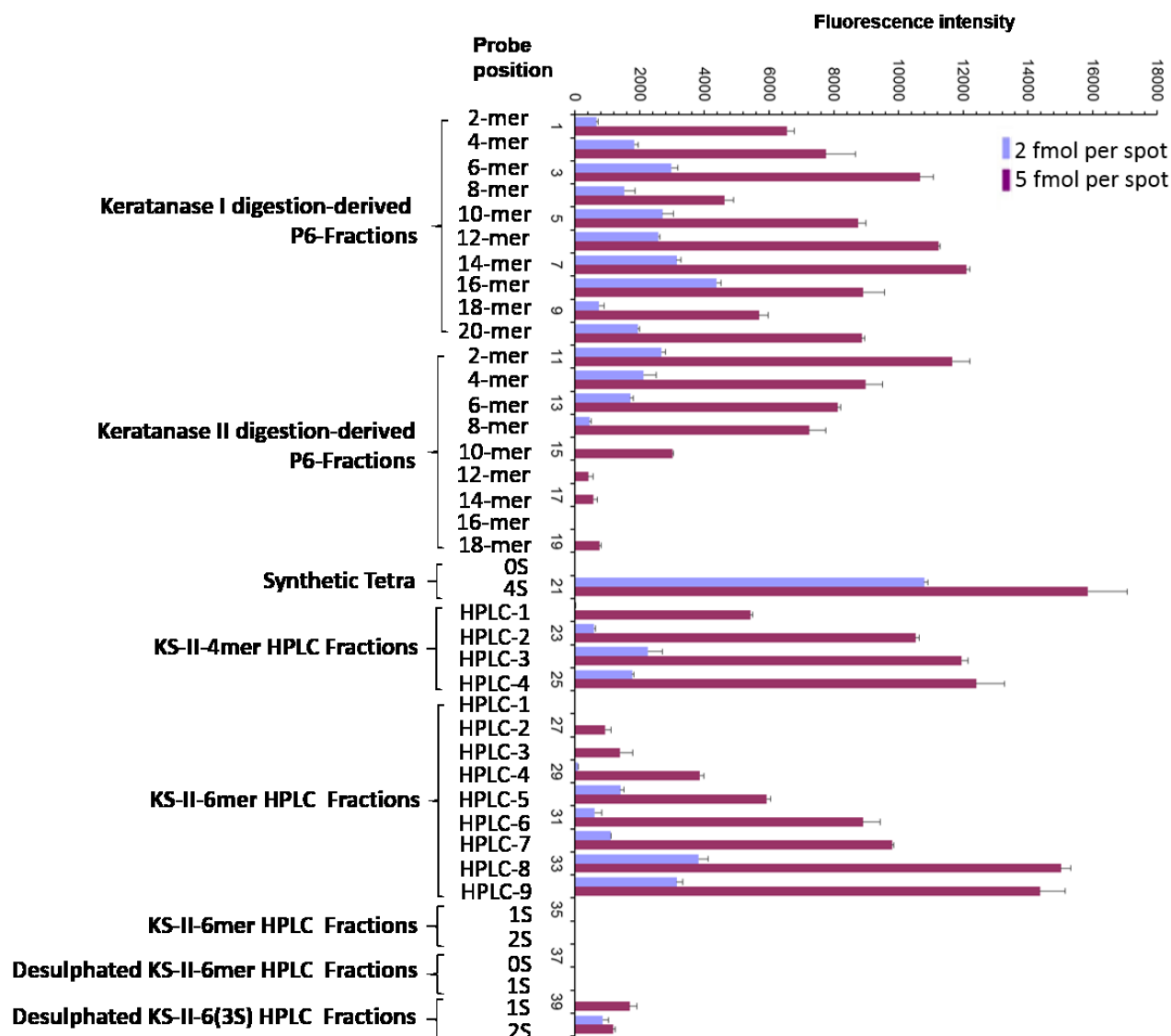


Figure 6.3. Microarray analyses of recombinant human/mouse/rat BMP-2 using GAG oligosaccharide NGL microarray Set 6 (a) and KS oligosaccharide NGL microarray Set 2b (b). Blocking solution was PBS with 3% (w/v) BSA, and diluent was PBS with 1% (w/v) BSA. Binding was detected using human BMP-2/BMP-4 antibody and biotinylated anti-mouse IgG followed by Alexa Fluor-647-labeled streptavidin. Results are means of fluorescence intensities of duplicate spots, printed at 2 and 5 fmol per spot, with error bars representing half of the difference between the two values. The result shown here is representative of one analysis with BMP2 using these microarrays and another not shown using a different microarray with the same composition.

Recombinant human BMP-4 (10 µg/mL)
No precomplexation

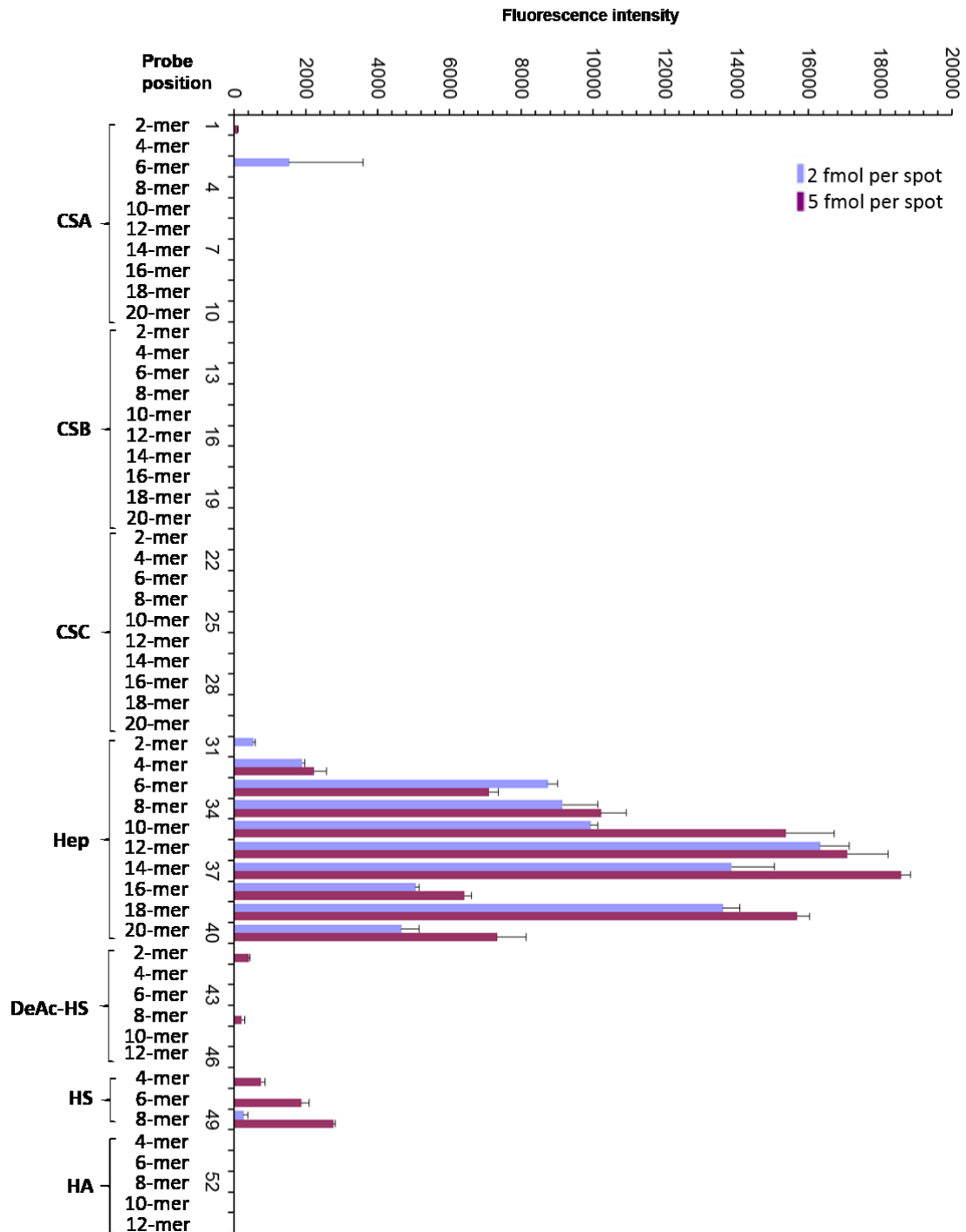


Figure 6.4. Microarray analysis of recombinant human BMP-4 using GAG oligosaccharide NGL microarray Set 6. Blocking solution was PBS with 3% (w/v) BSA, and diluent was PBS with 1% (w/v) BSA. Binding was detected using human BMP-2/BMP-4 antibody and biotinylated anti-mouse IgG followed by Alexa Fluor-647-labeled streptavidin. Results are means of fluorescence intensities of duplicate spots, printed at 2 and 5 fmol per spot, with error bars representing half of the difference between the two values. The result shown here is representative of one analysis with BMP-4 using this microarray and another not shown using a different microarray with the same composition.

Human Shh (10 µg/mL)
No precomplexation

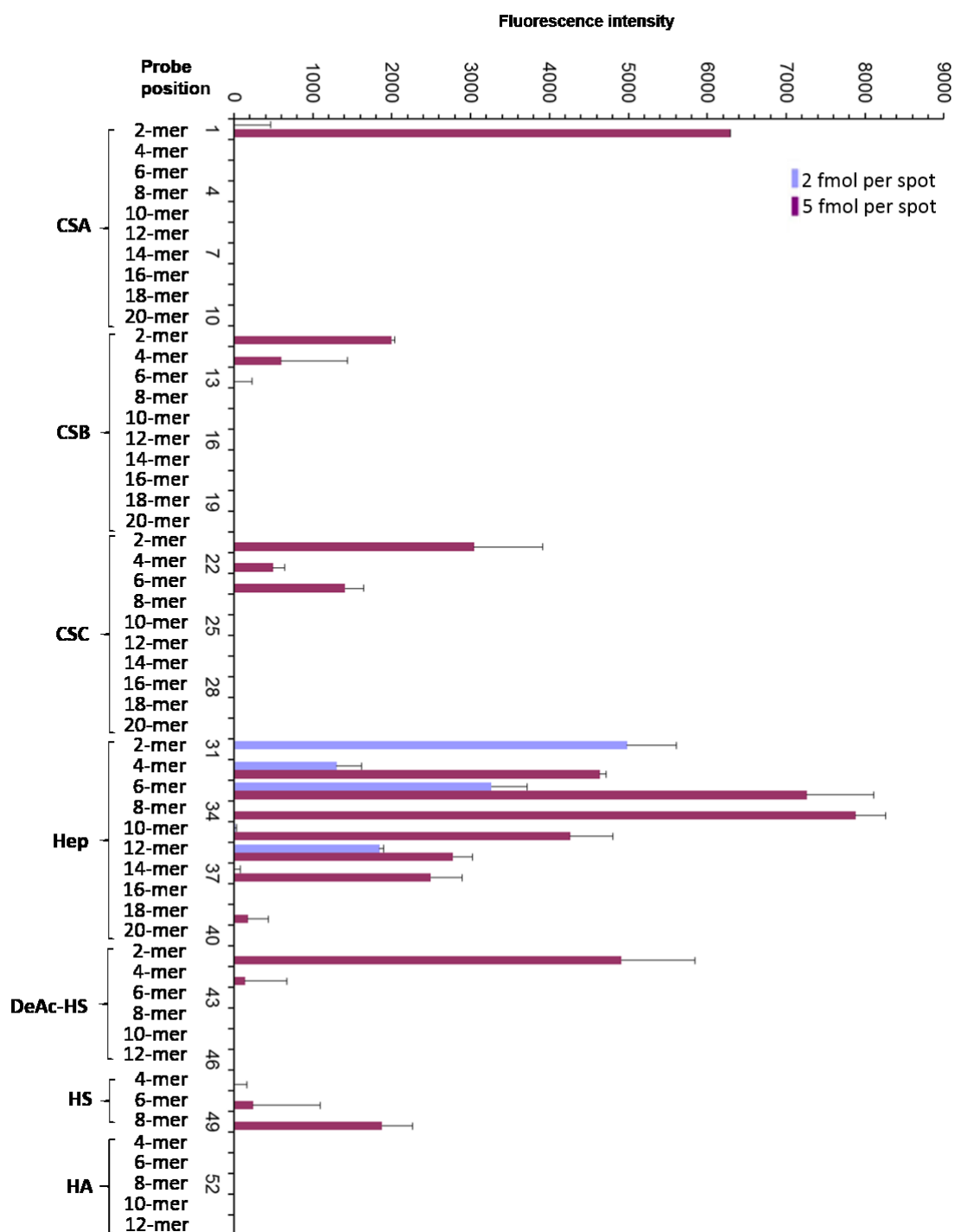


Figure 6.5. Microarray analysis of recombinant human Shh using GAG oligosaccharide NGL microarray Set 6. Blocking solution was PBS with 3% (w/v) BSA, and diluent was PBS with 1% (w/v) BSA. Binding was detected using human/mouse Shh N-Terminus antibody and biotinylated anti-rat IgG followed by Alexa Fluor-647-labeled streptavidin. Results are means of fluorescence intensities of duplicate spots, printed at 2 and 5 fmol per spot, with error bars representing half of the difference between the two values. The result shown here is representative of one analysis with Shh using this microarray and another not shown using a different microarray with the same composition.

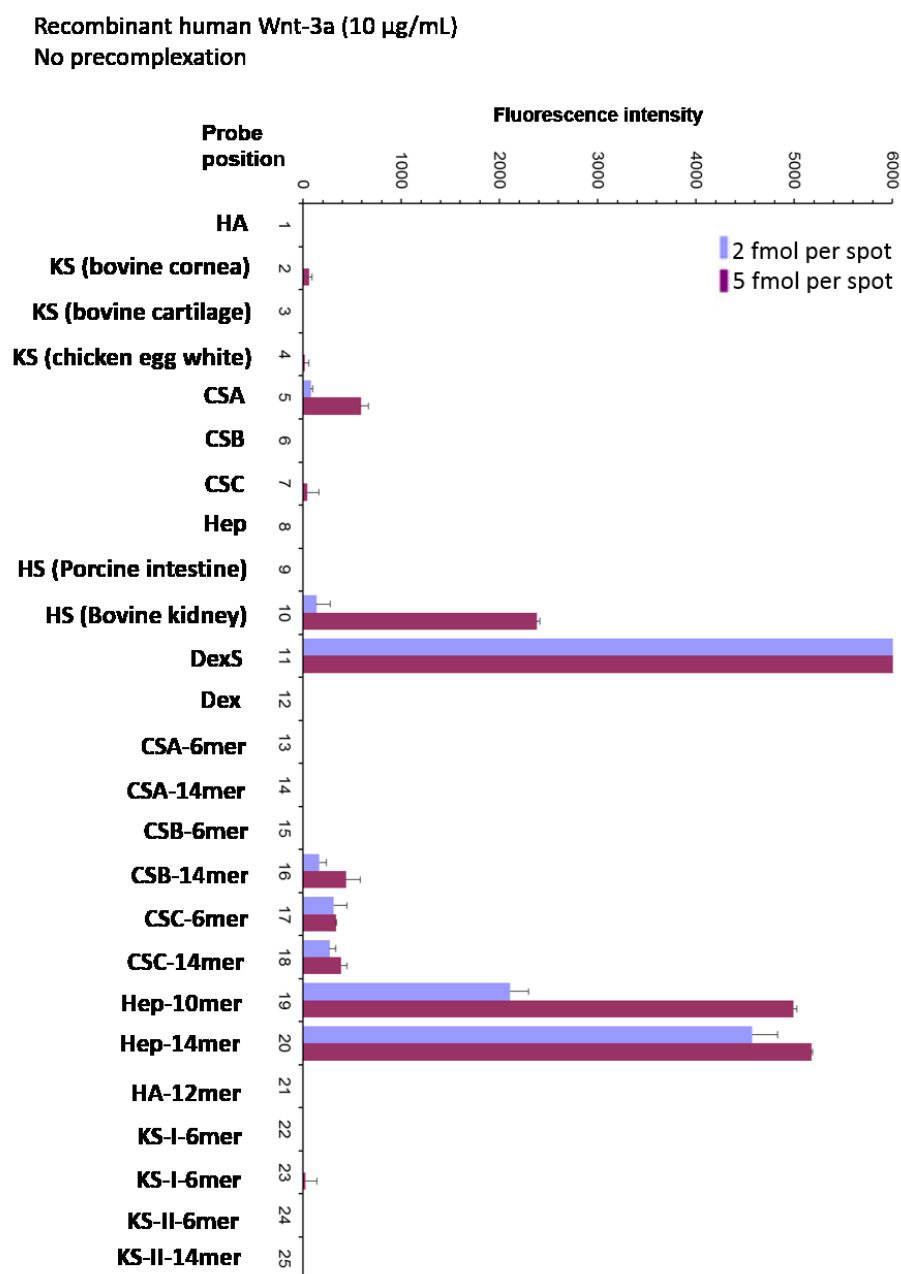


Figure 6.6. Microarray analysis of recombinant human Wnt-3a using GAG polysaccharide microarray Set 2' to which GAG polysaccharides and selected oligosaccharide NGLs had been added. Blocking solution was PBS with 3% (w/v) BSA, and diluent was PBS with 1% (w/v) BSA. Binding was detected using human/mouse Wnt-3a antibody and biotinylated anti-rat IgG followed by Alexa Fluor-647-labeled streptavidin. Results are means of fluorescence intensities of duplicate spots, printed at 90 and 300 pg per spot for polysaccharides, and printed at 2 and 5 fmol per spot for NGLs, with error bars representing half of the difference between the two values. The result shown here is representative of three-time analyses with Wnt-3a using this microarray.

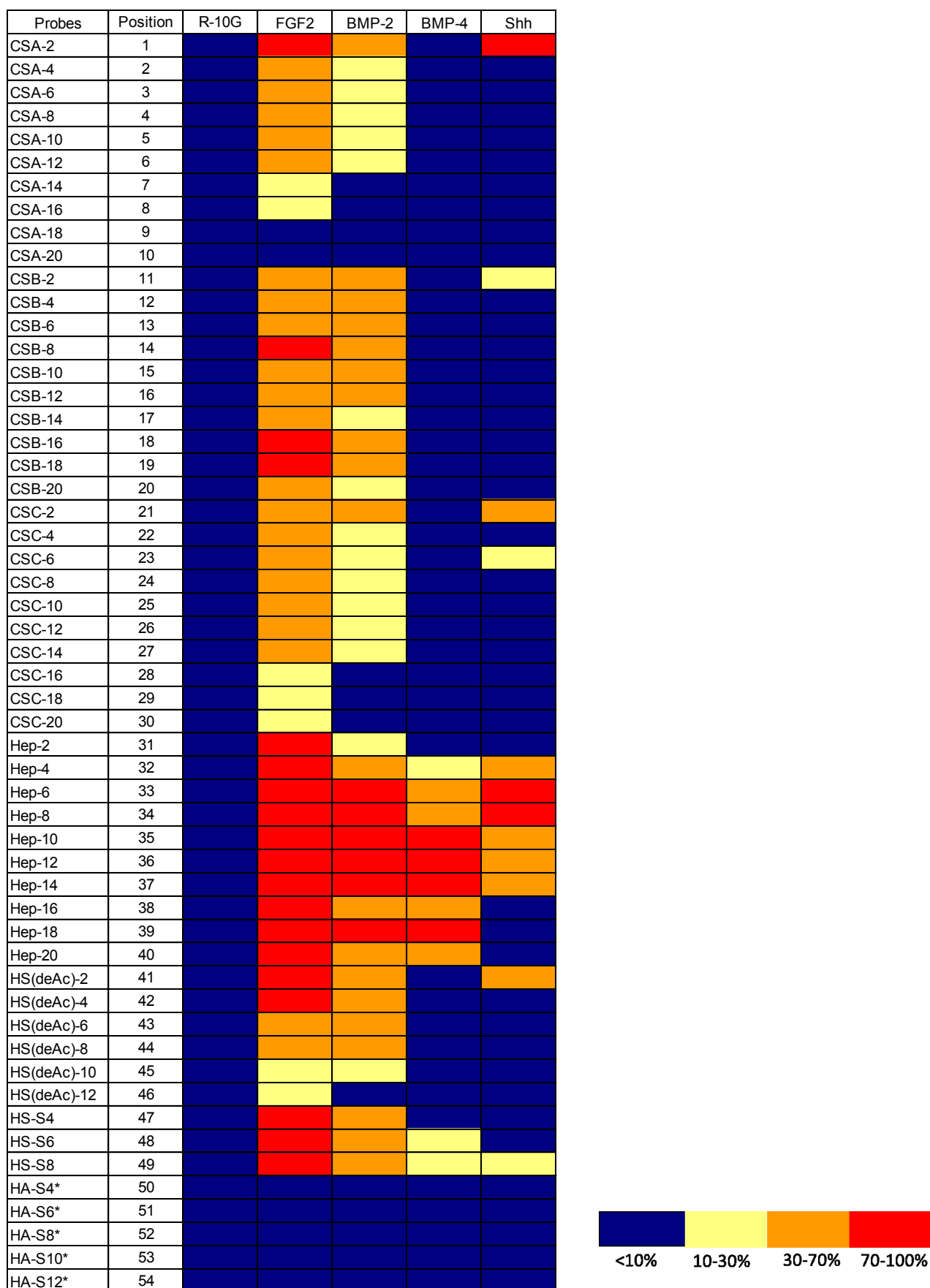


Figure 6.7. Relative intensities of binding of R-10G, FGF2, BMP-2, BMP-4 and Shh to probes in GAG oligosaccharide NGL microarray set 6. *DH-NGLs.

Probe		Position	R-10G	FGF2	BMP-2
Keratanase I digestion-derived P6 Fractions	2 mer	1			
	4 mer	2			
	6 mer	3			
	8 mer	4			
	10 mer	5			
	12 mer	6			
	14 mer	7			
	16 mer	8			
	18 mer	9			
	20 mer	10			
Keratanase II digestion-derived P6 Fractions	2 mer	11			
	4 mer	12			
	6 mer	13			
	8 mer	14			
	10 mer	15			
	12 mer	16			
	14 mer	17			
	16 mer	18			
	18 mer	19			
Synthetic tetrasaccharides	0S	20			
	4S	21			
KS-II-4mer HPLC fraction	HPLC-1	22			
	HPLC-2	23			
	HPLC-3	24			
	HPLC-4	25			
KS-II-6mer HPLC fraction	HPLC-1	26			
	HPLC-2	27			
	HPLC-3	28			
	HPLC-4	29			
	HPLC-5	30			
	HPLC-6	31			
	HPLC-7	32			
	HPLC-8	33			
	HPLC-9	34			
	1S	35			
	2S	36			
Desulphated KS-II-6mer HPLC fractions	0S	37			
	1S	38			
Desulphated KS-II-6(3S) HPLC fractions	1S	39			
	2S	40			

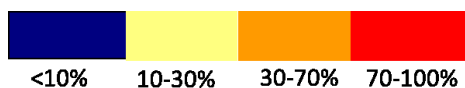


Figure 6.8. Relative intensities of binding of R-10G, FGF2 and BMP-2 to probes in KS oligosaccharide NGL microarray set 2b.

6.4 Discussion

The highly charged nature and heterogeneities of GAGs pose special problems which are highlighted in these exploratory studies *in vitro*. The amounts of polysaccharides retained on the arrayed slide are influenced not only by their charge but also their molecular weights. Quantitative methods need to be developed to determine the amounts retained. What can be said here is that the intensity of binding signals is likely to be a reflection of both of the amounts of GAGs retained on the array and the relative affinities of carbohydrate-binding proteins. It should be mentioned here that with oligosaccharide NGLs there is a tendency to have diminished binding signals with high oligomers. This phenomenon is protein-dependent and was manifest with, for example, anti-CS Di4S and anti-CS Di6S in Chapter 3. This may be due to solubilization of ligand-protein complex at high ligand levels (analogous to prozone effects with antigen-antibody interactions).

The main objective of my analyses of the growth factors and morphogens was to examine their binding to KS derived oligosaccharides. My demonstration of robust binding of BMP-2 to oligosaccharides derived from KS is novel. The similarly robust binding of the FGF2 to the KS oligosaccharides corroborates the binding previously documented by surface plasmon resonance [157].

Although the KS oligosaccharide binding was detected for FGF2 and BMP-2, there was no binding to R-10G antigen-positive di- and tri-sulphated hexasaccharides. I conclude that FGF2 and BMP-2 require four or more sulphates per KS hexasaccharide.

Among the analysed growth factors and morphogens, FGF2 and BMP-2 showed a broad binding profile to oligosaccharide NGLs derived from the sulphated GAGs. This contrasts with the more restricted GAG binding shown by BMP-4, Shh and Wnt-3a. GAG family are highly charged especially because of the high degree of sulphation. The

binding observed with FGF2 and BMP-2 may be a reflection of non-specific ionic interactions. This should be addressed in future studies. Addition of NaCl to the buffer used in microarray binding analysis of the growth factors and morphogens should be considered to decrease the anionic effect of the highly charged state of the GAG probes. Also, the pH of the buffer may influence of the strength of protein-GAG binding.

Examination of the contribution of pH to modulating chemokine CLL5-heparin interactions has shown that at lower pH heparin tetrasaccharides showed minimal movement within the groove of CLL5 whereas at neutral pH the interaction areas could be extended to the outside of the groove [250]. In addition, the traditional competitive inhibition method could be used to examine the binding data from our microarray analysis. Various concentrations of GAG NGL probes could also be examined to compare the binding curves of different types of GAGs by the growth factors and morphogens. This may reveal greater differences in the relative binding strengths. It is also desirable to compare the binding to the same type of GAG but with various degrees and patterns of sulphation. A heparin polysaccharide microarray including heparin and variously modified heparins (e.g. de-6-O-sulphated, de-2-O-sulphated, fully de-O-sulphated and de-N-sulphated/re-acetylated heparins) has been reported for investigation of FGF-2 binding [170]. Three binding patterns were observed: strongest binding to typical heparin and de-6-O-sulphated heparin; relatively weaker binding to de-2-O-sulphated and de-N-sulphated/re-acetylated heparins; very weak binding to fully de-O-sulphated heparin [170]. Sulphation of de-N-acetylated heparin, which is more highly sulphated than typical heparin, could be also included for comparison.

I review below literature data in relation to my work on the interactions of the growth factors and morphogens with GAGs.

Previous binding analyses using a microarray of GAG polysaccharides printed on poly-DL-lysine-coated glass surfaces has suggested FGF1 and FGF2 bind strongly to heparin, HS and CSE polysaccharides in the absence or presence of FGFR1 [170, 215]. In analyses using another microarray of GAGs covalently linked to the NH₂ on a solid surface show FGF-1 binds to heparin and CSE but not to HS, whereas with FGF-2 there was binding to heparin, HS, CSE and CSB [216]. In the 96-well plate proliferation assay, FGFs were added to the wells of cells expressing FGFR3c, followed by the addition of GAG polysaccharides. Both FGF-1 and FGF-2 was found to bind to heparin, HS, CSB and CSE [251]. Taken together, FGF-1 shows binding to heparin and CSE whereas FGF-2 binds to heparin and HS.

The binding analysis of FGF-2 in my GAG polysaccharide microarray is consistent with the reported data. My analyses suggest that FGF-2 also binds to the oligosaccharide NGLs derived from other sulphated GAGs. However, lack of FGF-1 binding to any arrayed GAG polysaccharides and oligosaccharides is unexpected. FGF-1 purchased from two different sources, SinoBiologicals and Abcam, have been tested but no binding to GAGs was observed. It is possible that the presentation of GAGs in the form of NGLs is not appropriate for binding of FGF1. We have also considered the possibility of the lack of stability of FGF1. This is required to be investigated.

Unlike the FGF-1 and FGF-2, studies on the GAG chains bound by Wnt-3a, Shh, BMP-2 and BMP-4 are rather limited. Kinetic assays for the interaction of Wnt-3a with biotinylated GAG polysaccharides immobilized on a streptavidin-coated sensor chip have suggested that Wnt-3a binds to heparin and CSE with high affinity, but not to other GAGs[149]. In a competitive ELISA assay using a Wnt signalling-inhibitory antibody HS20 and a panel of synthetic HS oligosaccharides, a minimum Wnt-binding chain has been suggested to be a HS octasaccharide containing IdoA2S and GlcNS6S, and moreover, 3-O-sulphation in the form of GlcNS6S3S could significantly enhance

the binding [146]. The Wnt-3a binding data in this study using the GAG 'polysaccharide' microarray Set 2 are in agreement with the above-mentioned observation.

Previous binding assay by surface plasmon resonance investigated the interaction of Shh with HS and KS polysaccharides immobilized on a streptavidin-coated sensor chip; Shh bound strongly HS and very weakly to KS [157]. Although there was no binding detected with Shh in my GAG polysaccharide microarray, Shh was found to bind strongly to heparin oligosaccharides NGLs, which is in overall agreement with the reported data indicating that Shh interacts with HS. However, binding to KS oligosaccharide NGLs was not detected.

Dot-blot assay to determine the binding of biotinylated GAG polysaccharides with BMP-2 immobilized onto a nitrocellulose membrane showed BMP-2 bound to heparin and HS [163]. The heparin and HS but not de-sulphated heparin have been reported to enhance the biological activity of BMP-2 and BMP-4 [161]. Strong binding of BMP-2 and BMP-4 to HS and heparin in my GAG polysaccharide microarray analyses was detected, and there was also binding to KS preparations from bovine cornea and chicken egg white. Their binding to GAG oligosaccharides as NGLs is different. BMP-2 bound to the oligosaccharides derived from all the sulphated GAGs, whereas BMP-4 strongly bound only to heparin oligosaccharides and weakly bound to HS oligosaccharides.

In conclusion, the binding of the selected growth factors and morphogens to GAG polysaccharides and oligosaccharides as NGLs in the Glycosaminoglycome microarray is likely to be dependent on the degree of sulphation. There are KS oligosaccharide sequences that satisfy this requirement for FGF2 and BMP-2 recognition. Notably, these features are not those recognized by mAb R-10G which requires undersulphation

of KS. This raises a question how FGF2 and BMP-2 function in human ES/iPS cells growth and differentiation.

Chapter 7

Discussion

The main conclusions from my research are that

- i) R-10G antigen is expressed on KS hexasaccharides terminating with Gal at the non-reducing end and having sulphate residues on all three GlcNAc residues or having sulphate residues on non-reducing and reducing GlcNAc residues. This suggests that the sulphate at the internal GlcNAc residue is not required and the sulphate at the non-reducing terminal GlcNAc residue is the key element.
- ii) TRA-1-60 and TRA-1-81 antigen(s) are not expressed on KS. The antigens are non-reducing type-1 followed by internal type-2. Fucosylation on the inter GlcNAc of the type-1-type-2 chain decreases TRA-1-60 antigenicity but abolishes TRA-1-80 antigenicity.
- iii) FGF2 and BMP-2 have similar binding activities to GAG probes with strong binding to oligosaccharide NGLs derived from sulphated GAGs but not to HA oligosaccharides that are not sulphated; BMP-4 most binds strongly to heparin oligosaccharide NGLs and weakly to HS oligosaccharide NGLs, but not to other arrayed GAG probes; Shh binds to heparin oligosaccharide NGLs and binds only to the shorter chains of other sulphated GAGs; Wnt-3a binds strongly to NGLs of heparin oligosaccharides.
- iv) FGF2 and BMP-2 in contrast to other tested growth factors and morphogens bound to KS.

7.1 Poly-N-acetyllactosamine Type Antigens Expressed on Human ES/iPS Cells

Our laboratory's previous work has made significant contributions to knowledge on antigens expressed on stem cells, using several monoclonal antibodies, including human anti-I and anti-i antibodies, the mouse hybridoma antibodies anti-SSEA-1 raised against F9 cells and VEP8 and VEP9 raised against myeloid HL60 cell line, as well as

FC10.2 raised against human EC cells, LICR LON HT 39/7 [101, 107]. The I, i, SSEA-1, VEP8 and VEP9 antigens have been found to be expressed in undifferentiated and differentiated mouse teratocarcinoma cell lines by immunofluorescence and their principal carriers are glycoproteins [252]. FC10.2 antigen has been reported to be expressed on human embryonic endoderm and embryonal carcinoma cells and its carrier is also glycoprotein [253, 254]. The oligosaccharide sequences recognized by these antibodies are summarized in Table 7.1. The i antigen has recently been reported to be expressed on the surfaces of human mesenchymal stem cells but not in their differentiated progeny [96]. Mouse ES cells were found to contain large amounts of branched poly-N-acetyllactosamine (I antigen) and its content progressively decreases during embryogenesis [97].

My data also suggest that the other two human stem cell markers TRA-1-60 and TRA-1-81 antigens are not KS and their antigens contain non-reducing type-1 followed by internal type-2 which is a poly-N-acetyllactosamine type chain (Table 7.1). The carbohydrate antigens recognized by the two antibodies resembles the FC10.2 antigen [254].

H-type 1, the fucosylated type-1 chain, is recognized by anti-human stem cell antibodies R-17F, BC2LCN and SSEA-5 (Table 7.1) [88, 94, 95, 100]. As KS is sulphated poly-N-acetyllactosamine chain, we suspect that the human stem cells express large amount of poly-N-acetyllactosamine type chains especially non-sulphated and the undersulphated. Studies are underway in collaboration with the Stem Cell Centre led by Dr Catherine Merry at Nottingham University to investigate the expression of R-10G, 5D4, i and I antigens on human stem cells.

Knowledge of the expression of these various carbohydrate antigens on human stem cells will be important in determining whether they can serve as markers of the undifferentiated state and be used in studies of cell differentiation and to isolate

specific stem cell lineages. Expression of the now characterized R-10G antigen on human ES/iPS cells but not EC cells helps us to better understand the difference between normal and malignant stem cells [73]. However, the biological roles of all these stem cell glycan markers have not yet been determined.

Table 7.1. Glycan antigens of stem cells recognized by monoclonal antibodies

Antibody	Oligosaccharide sequences recognized	References
Anti-i	Gal β 1-4GlcNAc β 1-3Gal β 1-4GlcNAc β 1-	[107]
Anti-I	$ \begin{array}{c} \text{Gal}\beta\text{-4GlcNAc}\beta\text{-6} \\ \\ \text{Gal}\beta\text{1-.....} \\ \\ \text{Gal}\beta\text{-4GlcNAc}\beta\text{-3} \end{array} $	[107]
Anti-SSEA-1, VEP8, VEP9	$ \begin{array}{c} \text{Gal}\beta\text{-4GlcNAc-.....} \\ 3 \\ \text{Fuc}\alpha\text{1} \end{array} $	[101, 102, 255]
FC10.2	Gal β 1-3GlcNAc β 1-3Gal β 1-4Glc	[254]
R-10G	Gal β 1-4GlcNAc β 1-3Gal β 1-4GlcNAc β 1-3Gal β 1-4GlcNAc (with 2 sulphates)	Chapter 4
TRA-1-60, TRA-1-81	Gal β 1-3GlcNAc β 1-3Gal β 1-4GlcNAc-	[106] and Chapter 5
R-17F, BC2LCN, SSEA-5	Fuc β 1-2Gal β 1-3GlcNAc-	[88, 94, 95]

7.2 Potential Role of Keratan Sulphate on Human Stem Cells

Several growth factors and morphogens such as FGF1, FGF2, Wnt-3a, Shh, BMP-2 and BMP-4 were analysed using the GlycosaminoGlycome microarray as described in Chapter 6. Heparin elicited very strong binding by these proteins which is consistent with previous reports [143, 153, 160, 256, 257]. A novel finding is the binding of KS by FGF2 and BMP-2, which opens the way into searching for a role of KS on human stem cells.

FGF2 signals by interacting with its membrane-bound receptors FGFR1-4 to participate in embryonic development, during organogenesis, and in the adult where it functions as a homeostatic factor for cortical neurons and vascular smooth muscle; and regulates blood pressure, skeletal development and wound healing [108, 258-264]. HS has been found to be a co-receptor in the signalling process [108]. The ternary complex of FGF2-FGFR1c-heparin has been extensively investigated, and the kinetic studies show that the strongest binary interaction was the FGF2-heparin interaction, followed by the FGF2-FGFR1c and the relatively weak FGFR1c-heparin interaction [138, 139]. Whether KS is similarly involved in the FGF2 signalling requires investigation. For example, it will be interesting to study KS binding by FGFR1-4 using the GlycosaminoGlycome microarray and cell signalling assay by including FGF2, FGFR1-4 and KS.

BMP-2 signals by binding to its transmembrane receptors Tkv and Punt [131]. HS has been reported to interact with a basic core of only three amino acids in the N-terminal region of BMP-2 so that the BMP-2 diffusion is restricted for action within and around the cells producing BMP-2 [159, 160]. HS is also found to enhance the biological activity of BMPs by protecting them from degradation and from inhibition by BMP antagonists [161-163]. Further studies are required to investigate the involvement of KS in BMP-2 signalling, such as binding analyses of BMP antagonists such as Cerbarus, Dan, Gremlin and Noggin, using GlycosaminoGlycome microarray and examination of influence of KS on BMP-2 bioactivity. Recently there has been an examination of HS involvement in BMP-2 signalling by deletion of the gene of enzyme responsible for HS polymerization (EXT1) [265]. To my knowledge there have not been any such studies on the role of KS in cell signalling. The KS-specific sulpho transferase KSGal6S [62] would be an interesting target for such a study.

It is suggested that HS with low levels of sulphate on mouse ES cells maintains their pluripotency by suppressing signalling of FGFs and BMPs, whereas high levels of

sulphation observed in differentiated cell population renders them responsive to FGFs and BMPs [76, 266]. This suggests that the degree of sulphation on GAGs may be one of major differences between undifferentiated and differentiated stem cells. It will be interesting therefore to examine the role of under- and over-sulphation of KS on signaling by FGF2 and BMP-2, respectively.

7.3 Podocalyxin Expressing Stem Cell Antigens

An interesting finding in the recent report from Kawabe's group [73] that the R-10G antigen-positive glycoprotein isolated from human iPS cells is also bound by TRA-1-60 and TRA-1-81. The antigen-positive glycoprotein was identified as podocalyxin which is a heavily glycosylated type-1 transmembrane protein belonging to the CD34 family of sialomucins [73]. This conclusion is similar to that from another study with TRA-1-60 and TRA-1-81 published by Schopperle et al.: the antigen(s) recognized by the two antibodies were found to be expressed on podocalyxin in human EC cells [93]. Considering that R-10G recognizes KS whereas TRA-1-60 and TRA-1-81 recognize type-1 followed by internal type-2 chains, the possible reason for the binding of the three antibodies to podocalyxin is that the carbohydrates they recognize are expressed on this glycoprotein. In other words, podocalyxin carries both KS which is type-2 based as well as type-1-based sequences. According to Kawabe's paper [73], EC cells are not bound by mAb R-10G. It would be interesting to know whether these cells contain glycoforms of podocalyxin that are lacking the R-10G epitope, and by inference lacking KS.

It is difficult to explain the result in Kawabe's paper [73] that the Western-blotting experiments of binding of TRA-1-81 to an R-10G antigen-positive glycoprotein diminished after treatment of the glycoprotein with high concentrations of keratanase II. One possible reason for this is that the keratanase II they used was contaminated

with endo- β -galactosidase which has the ability to digest poly-N-acetyllactosamine sequences either with type 1 termini or without sulphates [267]. Recent data [235] show that a synthetic tetrasaccharide containing type-1 followed by internal type-2 with sulphate on each GlcNAc is weakly bound by TRA-1-60, TRA-1-81 and R-10G. A possible reason, therefore, could be the podocalyxin on the stem cells expressed this type of chain in addition to KS chains.

R-10G has recently been found to bind to a synthetic KS-like (type2-type2) di-sulphated tetrasaccharide with sulphate on each GlcNAc after testing with several biotinylated tetrasaccharides including type1-type2 and type2-type2 with or without sulphates [235]. A possible reason to explain the difference from our data that the minimum KS chain length bound by R-10G is hexasaccharide is the possible effective presentation of the di-sulphated tetrasaccharide by the biotinylated tail but that a longer KS sequence such as the hexasaccharide is required for eliciting binding on a natural KS as an NGL. The research of Leteux et al. in our group [268] investigated the efficiency of presentation of biotinylated oligosaccharides on streptavidin-coated microwells using three monoclonal antibodies. Her studies showed that the biotinyl tag spacer length is critical in the strength of the binding signal when the biotinylated oligosaccharide incorporates the entire antigenic determinant and additional saccharide length is not included. The length of the spacer was less important when the oligosaccharide chain included a stretch beyond the determinant [268].

Podocalyxin contains a mucin domain, a globular domain, a stalk region, a transmembrane domain followed by a cytoplasmic tail in the order from extracellular to intracellular areas [269]. Podocalyxin is thought to be involved in both anti-adhesion and adhesion of cell-cell or cell-molecule [270]. The action of podocalyxin can be like an 'umbrella' preventing the function of adhesion molecules by steric hindrance of its mucin-like ectodomain [270]. For example, podocalyxin prevents nonspecific adhesion

and enhances cell mobility in the hematopoietic system [271, 272]. On the other hand, an extracellular soluble ligand or a counter receptor is said to bind to the mucin-like ectodomain of podocalyxin, which triggers adhesion molecules for activation [270]. For example, podocalyxin carries adhesive glycan ligands recognized by the L-selectin on circulating lymphocytes and thereby regulate cell trafficking in high endothelial venules [273, 274]. High levels of podocalyxin lead to either insufficient basolateral surface areas or to microvillus formation to disrupt integrin-mediated adhesion [269]. Low levels of podocalyxin increase the avidity of integrins (transmembrane receptors that are the bridges for cell-cell and cell-extracellular matrix interactions) to enhance cell adhesion through driving integrins to the basolateral surface of cells [269]. As large amounts of glycans are expressed on the mucin domain of podocalyxin, including the R-10G, TRA-1-60 and TRA-1-81 antigens, it would be interesting to investigate if any glycans or glycan antigens are directly involved in the adhesion or anti-adhesion functions of podocalyxin on human stem cells, and whether there are endogenous glycan-binding proteins (lectins) that are involved in the adhesion.

Galectins are a family of animal β -galactoside-binding lectins that are distributed widely in metazoan organisms [275]. Secreted galectins are able to interact with appropriately glycosylated proteins on the cell surface or in the extracellular matrix [276]. The receptors for the galectins include cell-adhesion molecules such as integrins, and matrix glycoproteins such as fibronectin and laminin isoforms [276]. Previous researches have shown that Galectin-1 and -2 bind to poly-N-acetyllactosamine [277-280]. A combined approach of glycan microarrays and cell surface binding experiments has revealed that Galectin-1 can bind to type-1 chains whereas Galectin-2 can bind strongly to blood group H-type 1 chains [281]. As discussed above, the type-1 chains (TRA-1-60 and TRA-1-81 antigens) and the H-type 1 chain (R-10F, BC2LCN and SSEA-5 antigens) are expressed on podocalyxin of human stem cells [88, 94, 95, 106]. Thus

Galectin-1 and Galectin-2 are candidate receptors for the glycans on podocalyxin involved in the stem cell adhesion.

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Appendices

1. Size-defined GAG oligosaccharides prepared in this study

1	CSA-2*	Δ UA-3GalNAc SU-4
2	CSA-4*	Δ UA-3GalNAc β -4GlcA β -3GalNAc SU-4 SU-4
3	CSA-6*	Δ UA-3GalNAc β -4GlcA α -3GalNAc β -4GlcA α -3GalNAc SU-4 SU-4 SU-4
4	CSA-8*	Δ UA-3GalNAc β -4GlcA β -3GalNAc β -4GlcA β -3GalNAc β -4GlcA β -3GalNAc SU-4 SU-4 SU-4 SU-4
5	CSA-10*	Δ UA-3GalNAc β -4GlcA β -3GalNAc β -4GlcA β -3GalNAc β -4GlcA β -3GalNAc β -4GlcA β -3GalNAc SU-4 SU-4 SU-4 SU-4 SU-4
6	CSA-12*	Δ UA-3GalNAc β -4GlcA β -3GalNAc β -4GlcA β -3GalNAc β -4GlcA β -3GalNAc β -4GlcA β -3GalNAc β -4GlcA β -3GalNAc SU-4 SU-4 SU-4 SU-4 SU-4 SU-4
7	CSA-14*	Δ UA-3GalNAc β -4GlcA β -3GalNAc β -4GlcA β -3GalNAc β -4GlcA β -3GalNAc β -4GlcA β -3GalNAc β -4GlcA β -3GalNAc β -4GlcA β -3GalNAc SU-4 SU-4 SU-4 SU-4 SU-4 SU-4 SU-4
8	CSA-16*	Δ UA-3GalNAc β -4GlcA β -3GalNAc β -4GlcA β -3GalNAc β -4GlcA β -3GalNAc β -4GlcA β -3GalNAc β -4GlcA β -3GalNAc β -4GlcA β -3GalNAc β -4GlcA β -3GalNAc SU-4 SU-4 SU-4 SU-4 SU-4 SU-4 SU-4 SU-4
9	CSA-18*	Δ UA-3GalNAc β -4GlcA β -3GalNAc β -4GlcA β -3GalNAc β -4GlcA β -3GalNAc β -4GlcA β -3GalNAc β -4GlcA β -3GalNAc β -4GlcA β -3GalNAc β -4GlcA β -3GalNAc β -4GlcA β -3GalNAc SU-4 SU-4 SU-4 SU-4 SU-4 SU-4 SU-4 SU-4 SU-4
10	CSA-20*	Δ UA-3GalNAc β -4GlcA β -3GalNAc β -4GlcA β -3GalNAc β -4GlcA β -3GalNAc β -4GlcA β -3GalNAc β -4GlcA β -3GalNAc β -4GlcA β -3GalNAc β -4GlcA β -3GalNAc β -4GlcA β -3GalNAc β -4GlcA β -3GalNAc SU-4 SU-4 SU-4 SU-4 SU-4 SU-4 SU-4 SU-4 SU-4 SU-4
11	CSC-2*	Δ UA-3GalNAc SU-6
12	CSC-4*	Δ UA-3GalNAc β -4GlcA β -3GalNAc SU-6 SU-6
13	CSC-6*	Δ UA-3GalNAc β -4GlcA β -3GalNAc β -4GlcA β -3GalNAc SU-6 SU-6 SU-6
14	CSC-8*	Δ UA-3GalNAc β -4GlcA β -3GalNAc β -4GlcA β -3GalNAc β -4GlcA β -3GalNAc SU-6 SU-6 SU-6 SU-6

32	Hep-4-AO*	SU-6 ΔUA-4GlcNSα-4IdoAα-4GlcNS-AO SU-6 SU-2 SU-6
33	Hep-6-AO*	SU-2 ΔUA-4GlcNSα-4IdoAα-4GlcNSα-4IdoAα-4GlcNS-AO SU-6 SU-2 SU-6 SU-2 SU-6
34	Hep-8-AO*	SU-2 ΔUA-4GlcNSα-4IdoAα-4GlcNSα-4IdoAα-4GlcNSα-4IdoAα-4GlcNS-AO SU-6 SU-2 SU-6 SU-2 SU-6 SU-2 SU-6
35	Hep-10-AO*	SU-2 ΔUA-4GlcNSα-4IdoAα-4GlcNSα-4IdoAα-4GlcNSα-4IdoAα-4GlcNSα-4IdoAα-4GlcNS-AO SU-6 SU-2 SU-6 SU-2 SU-6 SU-2 SU-6 SU-2 SU-6
36	Hep-12-AO*	SU-2 ΔUA-4GlcNSα-4IdoAα-4GlcNSα-4IdoAα-4GlcNSα-4IdoAα-4GlcNSα-4IdoAα-4GlcNSα-4IdoAα-4GlcNS-AO SU-6 SU-2 SU-6 SU-2 SU-6 SU-2 SU-6 SU-2 SU-6 SU-2 SU-6
37	Hep-14-AO*	SU-2 ΔUA-4GlcNSα-4IdoAα-4GlcNSα-4IdoAα-4GlcNSα-4IdoAα-4GlcNSα-4IdoAα-4GlcNSα-4IdoAα-4GlcNSα-4IdoAα-4GlcNS-AO SU-6 SU-2 SU-6 SU-2 SU-6 SU-2 SU-6 SU-2 SU-6 SU-2 SU-6 SU-2 SU-6
38	Hep-16-AO*	SU-2 ΔUA-4GlcNSα-4IdoAα-4GlcNSα-4IdoAα-4GlcNSα-4IdoAα-4GlcNSα-4IdoAα-4GlcNSα-4IdoAα-4GlcNSα-4IdoAα-4GlcNSα-4IdoAα-4GlcNS-AO SU-6 SU-2 SU-6 SU-2 SU-6 SU-2 SU-6 SU-2 SU-6 SU-2 SU-6 SU-2 SU-6 SU-2 SU-6
39	Hep-18-AO*	SU-2 ΔUA-4GlcNSα-4IdoAα-4GlcNSα-4IdoAα-4GlcNSα-4IdoAα-4GlcNSα-4IdoAα-4GlcNSα-4IdoAα-4GlcNSα-4IdoAα-4GlcNSα-4IdoAα-4GlcNS-AO SU-6 SU-2 SU-6 SU-2 SU-6 SU-2 SU-6 SU-2 SU-6 SU-2 SU-6 SU-2 SU-6 SU-2 SU-6 SU-2 SU-6
40	Hep-20-AO*	SU-2 ΔUA-4GlcNSα-4IdoAα-4GlcNSα-4IdoAα-4GlcNSα-4IdoAα-4GlcNSα-4IdoAα-4GlcNSα-4IdoAα-4GlcNSα-4IdoAα-4GlcNSα-4IdoAα-4GlcNSα-4IdoAα-4GlcNS-AO SU-6 SU-2 SU-6 SU-2 SU-6 SU-2 SU-6 SU-2 SU-6 SU-2 SU-6 SU-2 SU-6 SU-2 SU-6 SU-2 SU-6 SU-2 SU-6
41	HS(deAc)-2-AO	high content of GlcN contained (see table 3.3)
42	HS(deAc)-4-AO	high content of GlcN contained (see table 3.3)
43	HS(deAc)-6-AO	high content of GlcN contained (see table 3.3)
44	HS(deAc)-8-AO	high content of GlcN contained (see table 3.3)
45	HS(deAc)-10-AO	high content of GlcN contained (see table 3.3)
46	HS(deAc)-12-AO	high content of GlcN contained (see table 3.3)

47	KS-I-2-AO	GlcNAc β -3Gal-AO (Variously 6S on Gal and GlcNAc)
48	KS-I-4-AO	GlcNAc β -3Gal β -4GlcNAc β -3Gal-AO (Variously 6S on Gal and GlcNAc)
49	KS-I-6-AO	GlcNAc β -3Gal β -4GlcNAc β -3Gal β -4GlcNAc β -3Gal-AO (Variously 6S on Gal and GlcNAc)
50	KS-I-8-AO	GlcNAc β -3Gal β -4GlcNAc β -3Gal β -4GlcNAc β -3Gal β -4GlcNAc β -3Gal-AO (Variously 6S on Gal and GlcNAc)
51	KS-I-10-AO	GlcNAc β -3Gal β -4GlcNAc β -3Gal β -4GlcNAc β -3Gal β -4GlcNAc β -3Gal β -4GlcNAc β -3Gal-AO (Variously 6S on Gal and GlcNAc)
52	KS-I-12-AO	GlcNAc β -3Gal β -4GlcNAc β -3Gal β -4GlcNAc β -3Gal β -4GlcNAc β -3Gal β -4GlcNAc β -3Gal β -4GlcNAc β -3Gal-AO (Variously 6S on Gal and GlcNAc)
53	KS-I-14-AO	GlcNAc β -3Gal β -4GlcNAc β -3Gal β -4GlcNAc β -3Gal β -4GlcNAc β -3Gal β -4GlcNAc β -3Gal β -4GlcNAc β -3Gal-AO (Variously 6S on Gal and GlcNAc)
54	KS-I-16-AO	GlcNAc β -3Gal β -4GlcNAc β -3Gal β -4GlcNAc β -3Gal β -4GlcNAc β -3Gal β -4GlcNAc β -3Gal β -4GlcNAc β -3Gal β -4GlcNAc β -3Gal-AO (Variously 6S on Gal and GlcNAc)
55	KS-I-18-AO	GlcNAc β -3Gal β -4GlcNAc β -3Gal β -4GlcNAc β -3Gal β -4GlcNAc β -3Gal β -4GlcNAc β -3Gal β -4GlcNAc β -3Gal β -4GlcNAc β -3Gal-AO (Variously 6S on Gal and GlcNAc)
56	KS-I-20-AO	GlcNAc β -3Gal β -4GlcNAc β -3Gal β -4GlcNAc β -3Gal β -4GlcNAc β -3Gal β -4GlcNAc β -3Gal β -4GlcNAc β -3Gal β -4GlcNAc β -3Gal β -4GlcNAc β -3Gal-AO (Variously 6S on Gal and GlcNAc)
57	KS-II-2-AO	Gal β -4GlcNAc-AO (Variously 6S on Gal and GlcNAc)
58	KS-II-4-AO	Gal β -4GlcNAc β -3Gal β -4GlcNAc-AO (Variously 6S on Gal and GlcNAc)
59	KS-II-6-AO	Gal β -4GlcNAc β -3Gal β -4GlcNAc β -3Gal β -4GlcNAc-AO (Variously 6S on Gal and GlcNAc)
60	KS-II-8-AO	Gal β -4GlcNAc β -3Gal β -4GlcNAc β -3Gal β -4GlcNAc β -3Gal β -4GlcNAc-AO (Variously 6S on Gal and GlcNAc)
61	KS-II-10-AO	Gal β -4GlcNAc β -3Gal β -4GlcNAc β -3Gal β -4GlcNAc β -3Gal β -4GlcNAc β -3Gal β -4GlcNAc-AO (Variously 6S on Gal and GlcNAc)
62	KS-II-12-AO	Gal β -4GlcNAc β -3Gal β -4GlcNAc β -3Gal β -4GlcNAc β -3Gal β -4GlcNAc β -3Gal β -4GlcNAc β -3Gal β -4GlcNAc-AO (Variously 6S on Gal and GlcNAc)
63	KS-II-14-AO	Gal β -4GlcNAc β -3Gal β -4GlcNAc β -3Gal β -4GlcNAc β -3Gal β -4GlcNAc β -3Gal β -4GlcNAc β -3Gal β -4GlcNAc-AO (Variously 6S on Gal and GlcNAc)
64	KS-II-16-AO	Gal β -4GlcNAc β -3Gal β -4GlcNAc β -3Gal β -4GlcNAc β -3Gal β -4GlcNAc β -3Gal β -4GlcNAc β -3Gal β -4GlcNAc-AO (Variously 6S on Gal and GlcNAc)
65	KS-II-18-AO	Gal β -4GlcNAc β -3Gal β -4GlcNAc β -3Gal β -4GlcNAc β -3Gal β -4GlcNAc β -3Gal β -4GlcNAc β -3Gal β -4GlcNAc β -3Gal β -4GlcNAc-AO (Variously 6S on Gal and GlcNAc)

SU is for sulphate.

AO is for AOPE.

* Asterisks that follow the names of certain probes indicate that predominant sequences are shown.

3. Preparation of KS hexasaccharides from source (a), (b) and (c) and the NGLs derived from them which have been described in Table 4.6 and

Figure 4.21

Source (a): direct isolation from keratanase II digestion-derived P6 hexasaccharide fraction

To prepare R-10G antigen-positive tri-sulphated hexasaccharide, a large amount (700 nmol) of keratanase II digestion-derived P6 hexasaccharide fraction was fractionated by HiTrap ion exchange column. HiTrap is based on an ion exchange principle like SAX column but it has a higher capacity and the elution process is quicker albeit with poorer resolution. There were six peaks collected in total (Figure S1a), followed by ES-MS analysis. The Fraction 1 was a mixture of mono- and di-sulphated hexasaccharides, whereas Fraction 2 contained di- and tri-sulphated hexasaccharides. Fractions 3 to 6 were tri-, tetra-, penta- and hexa-sulphated hexasaccharides, respectively.

Fractions 1 and 2 were then fractionated by SAX to isolate exclusive mono- and di-sulphated hexasaccharides (Figure S1b and c). ES-MS analyses of the obtained peaks showed that both Fractions 1.2 and 1.3 were mono-sulphated, Fractions 1.4 were di-sulphated (Figure S1b), and Fraction 2.2 was di-sulphated and Fraction 2.3 was tri-sulphated (Figure S1c).

However, the amounts of the mono- and di-sulphated hexasaccharides that were isolated from keratanase II digestion-derived P6 hexasaccharide fraction were very small, roughly 10 to 20 nmol in total from 700 nmol of the parent hexasaccharide fraction. The small amounts might be the reason why they were missed in the previous HPLC (Figure 4.16 and Table 4.4). Both naturally obtained mono- (pooled Fractions 1-

2 and 1-3) and di-sulphated (pooled Fractions 1-4 and 2-2) hexasaccharides were then converted to NGLs for arraying.

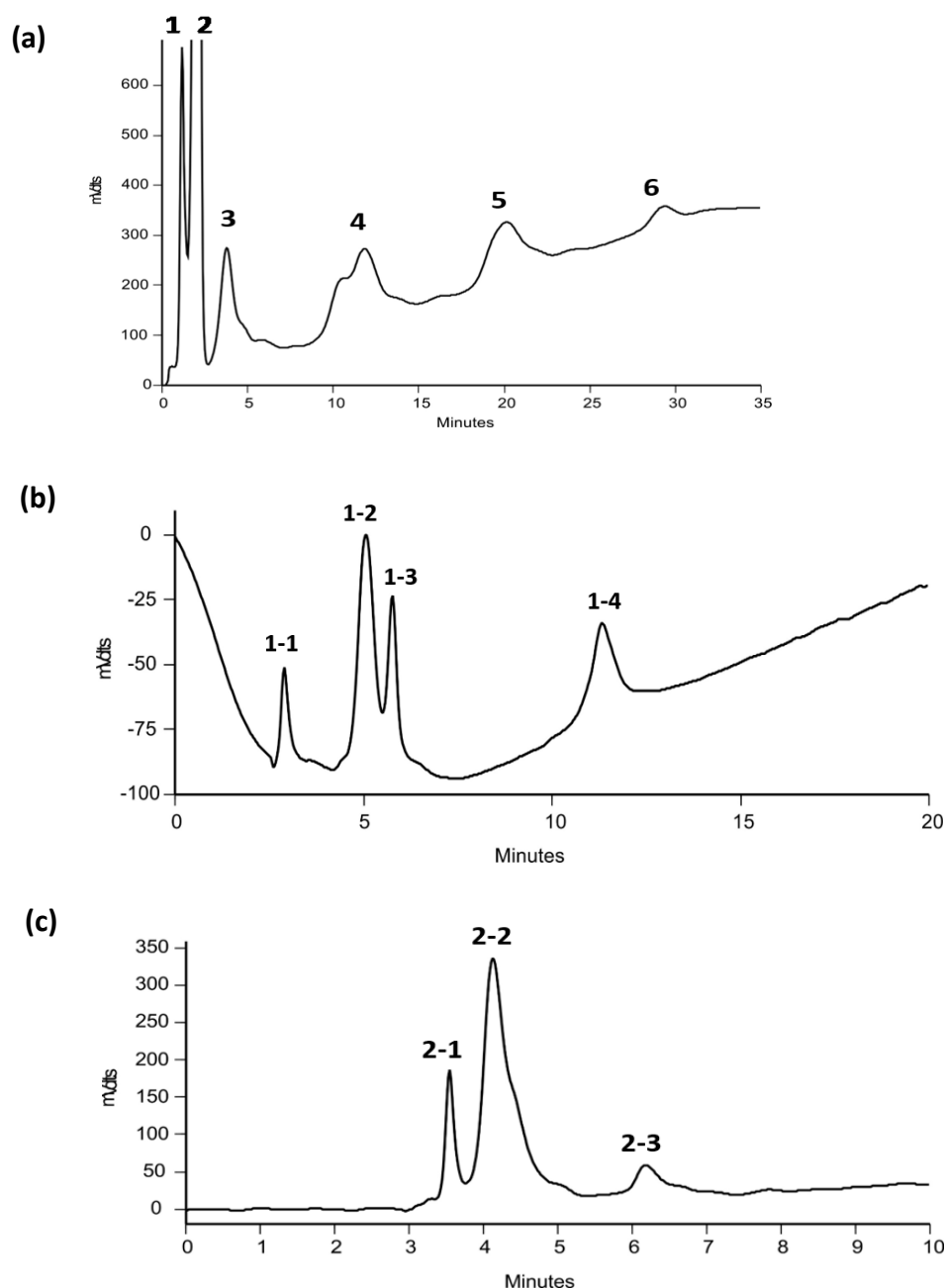


Figure S1. HPLC of keratanase II digestion-derived P6 hexasaccharide fraction. (a) HPLC of keratanase II digestion-derived P6 hexasaccharide fraction with HiTrap ion exchange column. The solvents were the same as those in Figure 4.16. The gradient of 10-40% solvent B was used with the running period of 40min. Six fractions were collected as indicated. (b) HPLC of the fraction 1 obtained in (a) with SAX column. The solvents were the same as those in Figure 4.16. The gradient of 0-20% solvent B was used with the running period of 40min. Four fractions were collected as indicated. (c) HPLC of the fraction 2 obtained in (a) with SAX column. The solvents were the same as those in Figure 4.16. The gradient of 10-30% solvent B was used with the running period of 40min. Three fractions were collected as indicated.

Source (b): Desulphation of keratanase II digestion-derived P6 hexasaccharide fraction

The keratanase II digestion-derived P6 hexasaccharide fraction was a mixture of oligosaccharides with three to six sulphates (Table 4.2). This fraction was selected for desulphation in order to obtain mono- and di-sulphated hexasaccharides with a variety of sulphation patterns such as sulphation on Gal. A large peak (Fraction 1) representing non-sulphated hexasaccharide was obtained together with small amounts of mono-sulphated hexasaccharides (Fraction 2 to 4) (Figure S2). Only little di-sulphated hexasaccharide was detected perhaps due to the overtime desulphation treatment. NGL of the non-sulphated (Fraction 1) and mono-sulphated (pooled Fractions 2 to 4) hexasaccharides were then prepared for arraying.

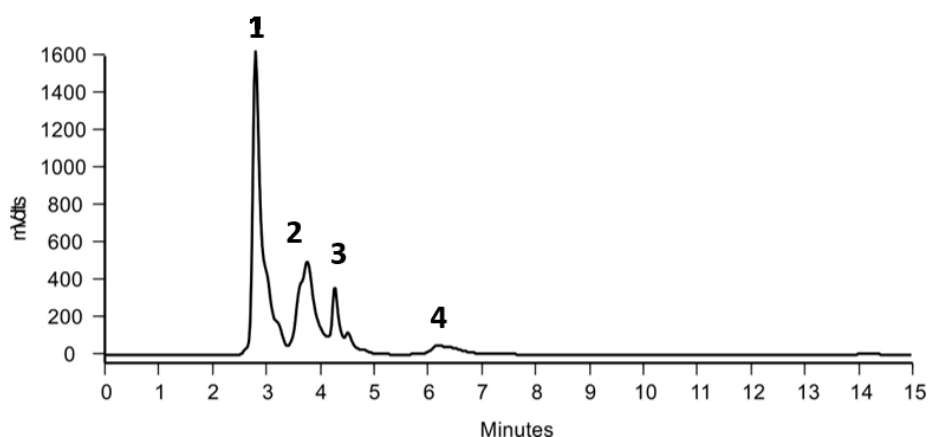


Figure S2. HPLC of desulphation product of keratanase II digestion-derived P6 hexasaccharide fraction with SAX column. The solvents were the same as those in Figure 4.16. The gradient of 5-10% solvent B was used with the running period of 40min. Four fractions were collected as indicated.

Source (c): Desulphation of the R-10G antigen-positive tri-sulphated hexasaccharide

In comparison to the heterogeneity of the product after desulphation of the parental hexasaccharide fraction, the position of sulphates on the desulphated product of R-10G antigen-positive tri-sulphated hexasaccharide (designated HPLC-1 in Table 4.4 and Figure 4.18) is clearly on the GlcNAc residue. Five fractions were obtained after HPLC of the desulphation product (Figure S3). Fraction 1 was determined to be mono-sulphated, Fraction 4 was di-sulphated and Fraction 5 was the apparent tri-sulphated hexasaccharide by ES-MS. Mono- (Fraction 1) and di-sulphated (Fraction 4) hexasaccharides with sulphate only on GlcNAc were then converted to NGLs.

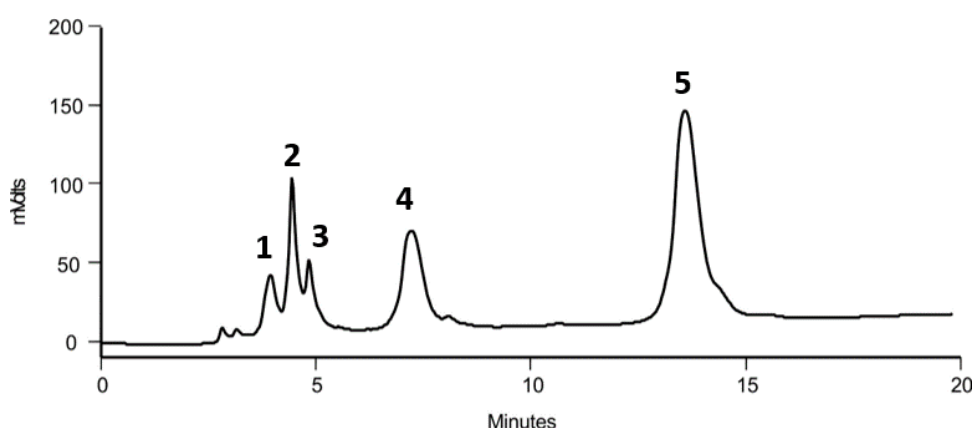


Figure S3. HPLC of desulphation products of the R-10G antigen-positive tri-sulphated hexasaccharide using SAX column. The solvents were the same as those in Figure 4.15. The gradient of 5-30% solvent B was used with the running period of 40min. Five fractions were collected as indicated.

4. Relative intensities of binding of TRA-1-60 and TRA-1-81 to the probes in CLL microarray set which has been described in Figures 5.6 and 5.7.

Probe position	TRA-1-60	TRA-1-81	Structure
1			Gal β -4GlcNAc β -6Gal-AO
2			Gal β -4GlcNAc β -3Gal-AO
3			Gal β -3GlcNAc β -6Gal-AO
4			Gal β -3GlcNAc β -3Gal-AO
5			$\begin{array}{c} \text{GlcNAc}\beta\text{-6} \\ \\ \text{Gal-AO} \\ \\ \text{GlcNAc}\beta\text{-3} \end{array}$
6			$\begin{array}{c} \text{GlcNAc}\beta\text{-6} \\ \\ \text{Gal-AO} \\ \\ \text{Gal}\beta\text{-3GlcNAc}\beta\text{-3} \end{array}$
7			$\begin{array}{c} \text{Gal}\beta\text{-4GlcNAc}\beta\text{-6} \\ \\ \text{Gal-AO} \\ \\ \text{Gal}\beta\text{-3GlcNAc}\beta\text{-3} \end{array}$
8			$\begin{array}{c} \text{Gal}\beta\text{-4GlcNAc}\beta\text{-6} \\ \\ \text{Gal-AO} \\ \\ \text{Gal}\beta\text{-4GlcNAc}\beta\text{-3} \end{array}$
9			$\begin{array}{c} \text{NeuAc}\alpha\text{-6Gal}\beta\text{-4GlcNAc}\beta\text{-6} \\ \\ \text{Gal}\beta\text{-4Glc-DH} \\ \\ \text{Gal}\beta\text{-3GlcNAc}\beta\text{-3} \end{array}$

10			$ \begin{array}{c} \text{Gal}\beta\text{-4GlcNAc}\beta\text{-6} \\ \\ \text{Gal}\beta\text{-4Glc-DH} \\ \\ \text{NeuAc}\alpha\text{-6Gal}\beta\text{-3GlcNAc}\beta\text{-3} \end{array} $
11			$ \begin{array}{c} \text{NeuAc}\alpha\text{-6Gal}\beta\text{-4GlcNAc}\beta\text{-6} \\ \\ \text{Gal}\beta\text{-4Glc-DH} \\ \\ \text{NeuAc}\alpha\text{-6Gal}\beta\text{-4GlcNAc}\beta\text{-3} \end{array} $
12			$ \begin{array}{c} \text{Gal}\beta\text{-3GlcNAc}\beta\text{-3Gal}\beta\text{-4GlcNAc}\beta\text{-6} \\ \\ \text{Gal}\beta\text{-4Glc-DH} \\ \\ \text{Gal}\beta\text{-3GlcNAc}\beta\text{-3} \end{array} $
13			$ \begin{array}{c} \text{Gal}\beta\text{-4GlcNAc}\beta\text{-6} \\ \\ \text{Gal}\beta\text{-4GlcNAc}\beta\text{-6} \\ \qquad \qquad \\ \text{Gal}\beta\text{-3GlcNAc}\beta\text{-3} \qquad \text{Gal}\beta\text{-4Glc-DH} \\ \\ \text{Gal}\beta\text{-3GlcNAc}\beta\text{-3} \end{array} $
14			$ \begin{array}{c} \text{GlcNAc}\beta\text{-6} \\ \\ \text{GlcNAc}\beta\text{-6} \qquad \qquad \text{Gal}\beta\text{-4GlcNAc}\beta\text{-3Gal}\beta\text{-4Glc}\beta\text{-Cer} \\ \qquad \qquad \\ \text{Gal}\beta\text{-4GlcNAc}\beta\text{-3} \\ \\ \text{GlcNAc}\beta\text{-3} \end{array} $
15			$ \begin{array}{c} \text{Gal}\beta\text{-4GlcNAc}\beta\text{-6} \\ \\ \text{Gal}\beta\text{-4GlcNAc}\beta\text{-3Gal}\beta\text{-4Glc}\beta\text{-Cer} \\ \\ \text{Gal}\beta\text{-4GlcNAc}\beta\text{-3} \end{array} $

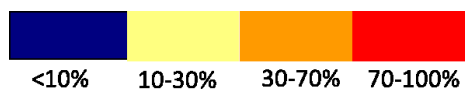
16			$ \begin{array}{c} \text{Gal}\beta\text{-4GlcNAc}\beta\text{-6} \\ \\ \text{Gal}\beta\text{-4GlcNAc}\beta\text{-6} \qquad \text{Gal}\beta\text{-4GlcNAc}\beta\text{-3Gal}\beta\text{-4Glc}\beta\text{-Cer} \\ \qquad \qquad \qquad \\ \text{Gal}\beta\text{-4GlcNAc}\beta\text{-3} \qquad \text{Gal}\beta\text{-4GlcNAc}\beta\text{-3} \\ \\ \text{Gal}\beta\text{-4GlcNAc}\beta\text{-3} \end{array} $
17			$ \begin{array}{c} \text{Gal}\beta\text{-4GlcNAc}\beta\text{-6} \\ \\ \text{Gal}\beta\text{-4Glc-DH} \\ \\ \text{Gal}\beta\text{-3GlcNAc}\beta\text{-3} \end{array} $
18			$ \begin{array}{c} \text{Gal}\beta\text{-4GlcNAc}\beta\text{-6} \\ \\ \text{Gal}\beta\text{-4Glc-DH} \\ \\ \text{Gal}\beta\text{-4GlcNAc}\beta\text{-3} \end{array} $
19			$ \begin{array}{c} \text{Gal}\alpha\text{-3Gal}\beta\text{-4GlcNAc}\beta\text{-6} \\ \\ \text{Gal}\beta\text{-4GlcNAc}\beta\text{-3Gal}\beta\text{-4Glc}\beta\text{-Cer} \\ \\ \text{Gal}\alpha\text{-3Gal}\beta\text{-4GlcNAc}\beta\text{-3} \end{array} $
20			$ \begin{array}{c} \text{Gal}\alpha\text{-3Gal}\beta\text{-4GlcNAc}\beta\text{-6} \\ \\ \text{Gal}\alpha\text{-3Gal}\beta\text{-4GlcNAc}\beta\text{-6} \qquad \text{Gal}\beta\text{-4GlcNAc}\beta\text{-3Gal}\beta\text{-4Glc}\beta\text{-Cer} \\ \qquad \qquad \qquad \\ \text{Gal}\beta\text{-4GlcNAc}\beta\text{-3} \qquad \text{Gal}\beta\text{-4GlcNAc}\beta\text{-3} \\ \\ \text{Gal}\alpha\text{-3Gal}\beta\text{-4GlcNAc}\beta\text{-3} \end{array} $
21			$\text{Gal}\beta\text{-3GlcNAc}\beta\text{-3Gal}\beta\text{-4Glc-DH}$
22			$\text{Gal}\beta\text{-4GlcNAc}\beta\text{-3Gal}\beta\text{-4Glc-DH}$
23			$\text{NeuAc}\alpha\text{-3Gal}\beta\text{-3GlcNAc}\beta\text{-3Gal}\beta\text{-4Glc-DH}$
24			$\text{NeuAc}\alpha\text{-6Gal}\beta\text{-4GlcNAc}\beta\text{-3Gal}\beta\text{-4Glc-DH}$
25			$\text{NeuAc}\alpha\text{-3Gal}\beta\text{-4GlcNAc}\beta\text{-3Gal}\beta\text{-4Glc-DH}$
26			$\text{Gal}\beta\text{-3GlcNAc}\beta\text{-3Gal}\beta\text{-4GlcNAc}\beta\text{-3Gal}\beta\text{-4Glc-DH}$
27			$\text{Gal}\beta\text{-4GlcNAc}\beta\text{-3Gal}\beta\text{-4GlcNAc}\beta\text{-3Gal}\beta\text{-4Glc-DH}$

28			Gal α -4Gal β -4GlcNAc-DH
29			GalNAc β -3Gal α -4Gal β -4Glc β -Cer
30			GalNAc α -3Gal β -4Glc-DH
31			Gal α -3Gal β -4GlcNAc β -3Gal β -4Glc β -Cer
32			Gal β -3Gal β -4GlcNAc β -3Gal β -4Glc β -Cer
33			Gal α -4Gal β -4Glc-AO
34			GalNAc α -3GalNAc β -3Gal α -4Gal β -4Glc β -Cer
35			Gal β -4GlcNAc β -3Gal β -4GlcNAc β -3Gal β -4GlcNAc β -3Gal β -4Glc-DH
36			NeuAc α -3Gal β -4GlcNAc β -3Gal β -4GlcNAc β -3Gal β -4GlcNAc β -3Gal β -4Glc-DH
37			NeuAc α -6Gal β -4GlcNAc β -3Gal β -4GlcNAc β -3Gal β -4GlcNAc β -3Gal β -4Glc-DH
38			NeuAc α -6Gal β -4GlcNAc β -3Gal β -4GlcNAc β -3Gal β -4GlcNAc β -3Gal β -4Glc-DH
39			
40			GlcNAc β -6Gal β -4GlcNAc β -3Gal β -4Glc-DH
41			Gal β -4GlcNAc β -6Gal β -4GlcNAc β -3Gal β -4Glc-DH
42			Fuc α -2Gal β -4GlcNAc β -6Gal β -4GlcNAc β -3Gal β -4Glc-DH
43			Fuc α -2Gal β -4GlcNAc β -6 Gal β -4GlcNAc β -3Gal β -4Glc-DH GlcNAc β -3
44			Fuc α -2Gal β -4GlcNAc β -6 Gal β -4GlcNAc β -3Gal β -4Glc-DH Gal β -4GlcNAc β -3
45			Fuc α -2Gal β -4GlcNAc β -6 Gal β -4GlcNAc β -3Gal β -4Glc-AO Gal β -4GlcNAc β -3

46			$ \begin{array}{c} \text{Fuc}\alpha\text{-2Gal}\beta\text{-4GlcNAc}\beta\text{-6} \\ \\ \text{Gal}\beta\text{-4GlcNAc}\beta\text{-OY} \\ \\ \text{GlcNAc}\beta\text{-3} \end{array} $
47			$\text{Fuc}\alpha\text{-2Gal}\beta\text{-4GlcNAc}\beta\text{-3Gal}\beta\text{-4GlcNAc}\beta\text{-3Gal}\beta\text{-4Glc}\beta\text{-Cer}$
48			$ \begin{array}{c} \text{Fuc}\alpha\text{-2Gal}\beta\text{-4GlcNAc}\beta\text{-6} \\ \\ \text{Gal}\beta\text{-4GlcNAc}\beta\text{-3Gal}\beta\text{-4Glc}\text{-Cer} \\ \\ \text{Fuc}\alpha\text{-2Gal}\beta\text{-4GlcNAc}\beta\text{-3} \end{array} $
49			$ \begin{array}{c} \text{GalNAc}\alpha\text{-3Gal}\beta\text{-4GlcNAc}\beta\text{-3Gal}\beta\text{-4GlcNAc}\beta\text{-3Gal}\beta\text{-4Glc}\beta\text{-Cer} \\ \\ \text{Fuc}\alpha\text{-2} \end{array} $
50			$ \begin{array}{c} \text{GalNAc}\alpha\text{-3Gal}\beta\text{-4GlcNAc}\beta\text{-6} \\ \qquad \qquad \qquad \\ \text{Fuc}\alpha\text{-2} \qquad \qquad \text{Gal}\beta\text{-4GlcNAc}\beta\text{-3Gal}\beta\text{-4Glc}\beta\text{-Cer} \\ \text{GalNAc}\alpha\text{-3Gal}\beta\text{-4GlcNAc}\beta\text{-3Gal}\beta\text{-4GlcNAc}\beta\text{-3} \\ \\ \text{Fuc}\alpha\text{-2} \end{array} $
51			$ \begin{array}{c} \text{Gal}\alpha\text{-3Gal}\beta\text{-4GlcNAc}\beta\text{-6} \\ \qquad \qquad \qquad \\ \text{Fuc}\alpha\text{-2} \qquad \qquad \text{Gal}\beta\text{-4GlcNAc}\beta\text{-3Gal}\beta\text{-4Glc}\beta\text{-Cer} \\ \\ \text{Gal}\alpha\text{-3Gal}\beta\text{-4GlcNAc}\beta\text{-3} \\ \\ \text{Fuc}\alpha\text{-2} \end{array} $

52			$ \begin{array}{c} \text{Gal}\alpha\text{-3Gal}\beta\text{-4GlcNAc}\beta\text{-6} \\ \\ \text{Fuca}\alpha\text{-2} \qquad \qquad \text{Gal}\beta\text{-4GlcNAc}\beta\text{-3Gal}\beta\text{-4Glc}\beta\text{-Cer} \\ \\ \text{Gal}\alpha\text{-3Gal}\beta\text{-4GlcNAc}\beta\text{-3Gal}\beta\text{-4GlcNAc}\beta\text{-3} \\ \\ \text{Fuca}\alpha\text{-2} \end{array} $
53			$ \begin{array}{c} \text{Gal}\beta\text{-3GlcNAc}\beta\text{-3Gal}\beta\text{-4Glc-DH} \\ \\ \text{Fuca}\alpha\text{-4} \end{array} $
54			$ \begin{array}{c} \text{Fuca}\alpha\text{-2Gal}\beta\text{-3GlcNAc}\beta\text{-3Gal}\beta\text{-4Glc-DH} \\ \\ \text{Fuca}\alpha\text{-4} \end{array} $
55			$ \begin{array}{c} \text{Gal}\beta\text{-4GlcNAc}\beta\text{-3Gal}\beta\text{-4Glc-DH} \\ \\ \text{Fuca}\alpha\text{-3} \end{array} $
56			$ \begin{array}{c} \text{Fuca}\alpha\text{-2Gal}\beta\text{-4GlcNAc}\beta\text{-3Gal}\beta\text{-4Glc-DH} \\ \\ \text{Fuca}\alpha\text{-3} \end{array} $
57			$ \begin{array}{c} \text{Gal}\beta\text{-4GlcNAc}\beta\text{-6} \\ \qquad \qquad \\ \text{Fuca}\alpha\text{-3} \qquad \text{Gal}\beta\text{-4Glc-DH} \\ \\ \text{Gal}\beta\text{-3GlcNAc}\beta\text{-3} \\ \\ \text{Fuca}\alpha\text{-4} \end{array} $
58			$ \begin{array}{c} \text{Gal}\beta\text{-4GlcNAc}\beta\text{-6} \\ \qquad \qquad \\ \text{Fuca}\alpha\text{-3} \qquad \text{Gal}\beta\text{-4Glc-DH} \\ \\ \text{Fuca}\alpha\text{-2Gal}\beta\text{-3GlcNAc}\beta\text{-3} \\ \\ \text{Fuca}\alpha\text{-4} \end{array} $

59			<pre> Galβ-4GlcNAcβ-6 Fucaα-3 Galβ-4Glc-DH Galβ-4GlcNAcβ-3 Fucaα-3 </pre>
60			<pre> Fucaα-2Galβ-4GlcNAcβ-6 Fucaα-3 Galβ-4Glc-AO NeuAcα-6Galβ-4GlcNAcβ-3 </pre>
61			<pre> Galβ-3GlcNAcβ-3Galβ-4GlcNAcβ-6 Fucaα-3 Galβ-4Glc-DH Galβ-3GlcNAcβ-3 </pre>
62			<pre> Galβ-3GlcNAcβ-3Galβ-4GlcNAcβ-6 Fucaα-4 Fucaα-3 Galβ-4Glc-DH Galβ-3GlcNAcβ-3 Fucaα-4 </pre>
63			<pre> Galβ-3GlcNAcβ-3Galβ-4GlcNAcβ-6 Fucaα-3 Galβ-4Glc-DH Fucaα-2Galβ-3GlcNAcβ-3 </pre>
64			<pre> Fucaα-2Galβ-3GlcNAcβ-3Galβ-4GlcNAcβ-6 Fucaα-3 Galβ-4Glc-DH Fucaα-2Galβ-3GlcNAcβ-3 </pre>



a Position, probe position in the microarray.

AO is for APOE and DH is for DHPE, which are different lipid tags for NGL preparation.

Cer is for ceramide.