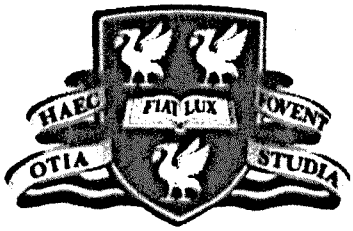

THE UNIVERSITY *of* LIVERPOOL

Heparan Sulphate : Methods development and interaction studies

Thesis submitted in accordance with the requirements of The University of Liverpool for the degree of Doctor in Philosophy.

By

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Dedicated to my mother and father, without whose council and support this would not have been possible.

Abstract

Methods for the sequence determination of the biologically and medically important polysaccharides heparan sulphate / heparin and oligosaccharides derived from them have lagged behind those of nucleic acid and protein analysis. This is principally due to their chemical and structural complexity and, in the case of heparan sulphate, the scarcity of available material.

Improved reducing-end labelling methodologies have been developed which are compatible with the capabilities of LASER-induced detection, permitting use with either standard liquid chromatography (HPLC) or capillary electrophoresis (CE) analysis techniques. This has resulted in sensitivity in the 100 femtomoles - 1 picomole for HPLC detection and 1-5 femtomoles in the case of CE. Improved labelling methods relying on acid catalysed reductive amination employing the highly sensitive fluorescent label BODIPY-FL-hydrazide were established; with the advantage that heating can be avoided, resulting in reduced loss of N-sulphate groups. An alternative catalyst, dibutyltin chloride with phenylsilane as a reducing agent, was also explored and found to effectively promote conjugation while avoiding the use of organic acids.

In an attempt to overcome the sluggish reactivity of reducing end chemistry, an alternative labelling method was explored, exploiting the unsaturated bond which is introduced into the molecule following bacterial lyase digestion of HS/heparin polysaccharides. The double bond was attacked with mercuric ions to give a stable cyclic mercurinium intermediate in high yield. This allows the strong interaction between mercury and thiols to be exploited permitting conjugation or immobilisation to thiol-derivatized labels and gold-surfaces. This will facilitate a wide range of derivatives and labels, including dual-end labelling to be developed as well as novel synthetic chemistry at the non-reducing end.

One example of the biological role of heparan sulphate/heparin saccharides is the mediation of the rosetting phenomenon observed with red blood cells infected with the parasite *Plasmodium falciparum*, the causative agent of severe malaria. The ability of chemically modified heparin poly- and oligosaccharides to disrupt the rosetting phenomenon was explored along with anticoagulation, a likely side-effect of these derivatives. The oligosaccharides of porcine intestinal mucosal heparin were found to be ineffective at disrupting the rosettes formed by the R-29 parasite strain, while the intact polysaccharide was effective (IC_{50} 70 nM). A 6-de-O-sulphated heparin derivative was effective as an inhibitor (IC_{50} 120 nM), while the anticoagulant activity was < 1 % that of heparin. N-acetylated heparin (IC_{50} 690 nM) exhibited around 10 % the activity of heparin in rosette disruption but an anticoagulation activity $<10^{-4}$ that of heparin. Size-defined oligosaccharides from 6-de-O-sulphated polysaccharide at 0.4 $mg.ml^{-1}$ disrupted rosetting at a similar rate of disruption to the polysaccharide, while those from N-acetylated heparin were capable of disrupting rosetting to approximately 70 % of the level observed for heparin.

It is anticipated that the improved analytical methods which will accrue from the above labelling strategies should find widespread application in the study of structure-function relationships of heparan sulphate and heparin in both biological and medical contexts.

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Chapter 1

Introduction

1.1 Glycosaminoglycans

The glycosaminoglycans (GAGs) are a family of linear, anionic carbohydrates which are in the main found attached to proteins and are implicated in various protein interactions. Carbohydrates present within this family possess a backbone disaccharide repeat unit and are classified according to the constituent monosaccharides forming the repeat element. Chain length and substitution pattern differ between glycosaminoglycans leading to a wide range of interactions and functions for this highly diverse class of carbohydrates. The component monosaccharides, linkage type, relative size and general functional relevance of each glycosaminoglycan class members is summarised in table 1. Glycosaminoglycan family members include chondroitin sulphate, keratan sulphate, dermatan sulphate, heparan sulphate, heparin and hyaluronic acid. With the notable exception of hyaluronic acid, which is not synthesised bound to a protein core, glycosaminoglycans are linked to a protein by one of two covalent methods described in section 1.2.1 to form proteoglycans.

1.2 Proteoglycans

Proteoglycans (PG) are a class of proteins to which one or more glycosaminoglycan (GAG) carbohydrate chains are covalently attached. Attachment of the GAG chains to the core protein occurs via a linkage region and the GAG chains are often considered to extend from the protein in a brush like manner [1]. Initially, an O-linked glycosidic bond is formed between a serine residue within the protein and a tetra-saccharide linker, composed of glucuronic acid β 1→ 3 galactose β 1→ 3 galactose β 1→ 4 xylose β 1 – O – Ser[2], the exceptions to this being hyaluronic acid (which is not synthesised on a core protein) and keratan sulphate, class 1, which can attach via an N-asparaginyl bond.

The size of the core protein can vary between ten thousand and six hundred thousand Daltons (Da), and the number and type of the attached GAG chains can also differ. The chemical groups substituting the GAG chains (e.g. sulphate and N-acetyl) also lead to greater diversity[3]. The type of core protein does not appear to dictate the structure of

Table 1: Glycosaminoglycan family members

Glycosaminoglycan	Disaccharide unit	Linkage	Source	Function / Role	Typical size
Chondroitin Sulphate	Glc(±2S) GalNAc(±4S, ±6S)	β (1, 3)	Extra-cellular matrix	Structural component Regulatory role Alternative medicine	12 – 20 kDa
Dermatan Sulphate *(Chondroitin Sulphate B)*	Ido(±2S) GalNAc(±4S, ±6S)	β (1, 3)	Skin Blood vessels Heart (valves) Lungs Tendons	Carcinogenesis Cardiovascular disease Coagulation Fibrosis Infection Wound repair	15 – 40 kDa
Heparan Sulphate	Glc(±2S) GlcNAc(±6S, ±3S) Glc(±2S) GlcNS(±6S, ±3S) Ido(±2S) GlcNAc(±6S, ±3S) Ido(±2S) GlcNS(±6S, ±3S)	α (1, 4)	All animal tissues	Development Regulation Pathogen entry Inflammation Structural role	25 – 100 kDa
Heparin	Ido2S GlcNS(6S)	α (1, 4)	Mast cells	Anticoagulant	5 – 40 kDa
Hyaluronate *(Hyaluronic acid or Hyaluronan)*	GlcA GlcNAc	β (1, 3)	Connective tissue Epithelial tissue Neural tissue	Cell proliferation Cell migration Lubricant Tumour growth	300 – 2000 kDa
Keratan Sulphate	Gal GlcNAc(6S) Gal GalNAc(6S)	β (1, 4)	Cartilage Cornea Skeletal tissue	Structural component	4 – 20 kDa

the GAG which it may carry, i.e. one core protein may have differing GAG structures in different cell lines [4]. The properties of the core protein, as well as the GAG chains carried, will dictate the binding properties of a particular PG.

Proteoglycans are grouped into one of three families depending on the basis of their attachment (or lack of it) to the cell membrane. These groups are (i) trans-membrane PGs (e.g. the syndecan family), (ii) glycosylphosphatidylinositol (GPI) linked PGs (e.g. the glypican family) and (iii) non-attached extra-cellular matrix PGs (e.g. the perlecan family).

1.2.1 Heparan sulphate

Heparan sulphate (HS) is an important member of the GAG family. HS is found on both the cell surface of virtually all mammalian cells and in the extra-cellular matrix [5]. It has the highest degree of variability, due to its sequence diversity, of any of the GAG family members although its fine structure (i.e. sequence) and binding properties can differ from that of the related GAG heparin. The sequence diversity of the HS chains allows specific intra-chain sequences to interact with proteins and regulate their activities. Such proteins are known to include extra-cellular matrix (ECM) proteins, growth factors, enzymes and pathogen surface proteins [6].

There are three types of heparan sulphate proteoglycan:

(i) Syndecan - These are proteins attached to the cell surface via a trans-membrane region. There are four family members known in mammals [7].

(ii) Glypican - These are also proteins attached to the cell surface, but via a glycosylphosphatidylinositol (GPI) anchor as opposed to a trans-membrane domain. There are six mammalian family members.

(iii) ECM - These are not cell surface bound, but are present free in the extra-cellular matrix (ECM) e.g. perlecan.

1.2.2 Heparan sulphate structure

The heparan sulphate polysaccharide chain is a linear co-polymer consisting of repeating hexuronic acid and α-D glucosamine residues forming the backbone disaccharide units. The hexuronic acid unit can be either α-L iduronic acid or its C-5 epimer β-D glucuronic acid (IdoA or GlcA). C-2 of the hexuronic acids and C-6 (and more rarely C-3) of the glucosamine can be O-sulphated; while C-2 of the glucosamine can be N-sulphated (NS) or N-acetylated (NAc). The presence of free amine groups (NH_2) has also been reported [8]. The HS chains have regions of differing sulphation densities creating a domain structure within the polysaccharide chain. Generally these can have extensive modification, possessing the C-5 epimer iduronate along with high levels of sulfation, ("S domains") no or low sulphation ("NA domains") or intermediate sulfation ("NA/NS domains"). The level and variety of sulphation, acetylation and the overall domain organisation is thought to be specific to their cellular origins [9] and is spatially and temporally regulated, although the exact mechanism by which this occurs is not fully understood.

The three domain regions within a GAG chain can therefore be described as follows [10]:

(i) NA domain - most of the disaccharide repeats are GlcA-GlcNAc - spacers between S domains

(ii) S domains (or NS) - heavily sulphated and epimerised - up to approximately 8 disaccharide repeats long

(iii) NA / NS domain - found either side of S domains - repeating N-acetylated/N-sulphated disaccharides

1.2.3 Heparin

Heparin is a structural analogue of HS, sharing a common biosynthetic precursor (heparatosan, GlcA-GlcNAc), consisting of the same disaccharide repeat unit for its

backbone and contains the same sulphate and acetyl modifications as HS albeit in markedly different proportions. Where HS and heparin differ is in their levels of sulphation and their organisation within the polysaccharide chain [11]. Where HS has the NA, NA/NS and S domains, heparin consists predominantly of highly sulphated domains throughout the majority of the chain. In comparison to HS which has only around 30% of the disaccharide repeats sulphated or epimerised [12], heparin has around 80% of its disaccharide content modified. Heparin is also widely available because of its pharmaceutical use and lends itself as a proxy to the S domains of HS, especially in the early stages of method development, and is generally active in assays where HS is also effective but lacks the regulatory capacity of HS. The analogy between heparin and HS should not be pushed too far because of the structural differences between the two polymers.

1.2.4 Biosynthesis of heparan sulphate and heparin

While the un-glycosylated precursor core protein is synthesised in the rough endoplasmic reticulum of the cell, the GAG chain biosynthesis occurs in the Golgi apparatus [13].

The tetra-saccharide linkage region (consisting of Xyl-Gal-Gal-GlcA) is added to a specific serine (Ser) residue on the core protein [14] by the actions of xylosyltransferase and galactosyltransferases I and II respectively. The addition of a GlcA residue by glucuronyltransferase I to the terminal Gal residue completes the linker region. The serine residue is normally flanked on one or both sides by at least two acidic residues and a di-peptide Ser-Gly/Ala motif [7]. It should be noted that not all of the potential attachment sites within the core protein have this linker attached. Occasionally the linker region itself may be modified by 2-O-phosphorylation at the xylose residue [15]. Other modifications such as 4-O and 6-O sulphated Gal residues have also been reported in CS and DS synthesis but to date not for heparin or heparan sulphate [16-18].

A GlcNAc residue is then added to the linker molecule by α1→4N-acetylglucosaminyltransferase I, committing the chain to HS synthesis. HS co-

polymerase (EXT-1 and EXT-2 enzymes) then add subsequent GlcA-GlcNAc residues, from pre-cursor UDP-GlcNAc and UDP-GlcA, to achieve chain extension. This results in a uniform, unmodified, repeating disaccharide unit throughout the entire GAG chain [-4)-D-GlcA-(β1-4)-D-GlcNAc-(α1-] [19] termed heparatosan.

The enzyme N-de-acetylase / N-sulpho-transferase (NDST) then hydrolytically removes the N-acetyl (NAc) group of N-acteylglucosamine (GlcNAc) for some residues (by the action of the N-deacetylase domain of the NDST) and replaces it with a N-sulphate (NS). Phosphoadenosine-phosphosulphate (PAPS) is the sulphate donor for this and all subsequent sulpho-transferase reactions. This does not occur uniformly throughout the chain and only some of the residues become de-N-acetylated and hence N-sulphated [20, 21], although these modifications are essential for subsequent modifications. The possibility that the initial de-N-acetylation reaction occurs but not the subsequent N-sulfation should also be pointed out as a means of introducing free amino groups into the glucosamine residues. This argument is led weight by the knowledge that of the four isozymes of NDST (NDST 1 – 4), NDST 1 and 2 have similar N-deacetylase and N-sulpho-transferase activity while NDST 3 and 4 possess higher N-deacetylase or N-sulpho-transferase activities respectively.

Glucuronosyl C-5 epimerase, a single isoform, also acts on some glucuronic acid (GlcA) residues, in the region of previously N-sulphated residues, irreversibly converting the residue to the C-5 epimer iduronic acid [22-24]. It is thought that the epimerisation modification happens post N-sulfation but before the addition of O-sulphate groups [22]. The presence of iduronate residues within the chain is thought to add flexibility facilitating various protein interactions [25]. The epimerase and NDST give rise to regions of elevated GlcNS and IdoA content which, following further O sulphation, leads to the formation of highly sulphated S domains [26].

Post epimerisation, the sulpho-transferase 2OST (one isoform known to date) can act on iduronic acid (this can also occur, less frequently, in GlcA residues in HS) residues at the C-2 position to add a 2-O-sulphate [27]. The sulpho-transferase 6OST can then act on

GlcNAc or GlcNS at C-6 to add a 6-O-sulphate [28]. Multiple isoforms of this enzyme exist, namely 3 full length and 1 splice variant in humans [29, 30], with murine variants showing differing substrates specificities and activities [29, 31]. Murine 6-O-sulphotransferases have been localised in the Golgi [32]. More rarely, another sulpho-transferase; the 3OST can act on GlcNS residues at C-3 to add a 3-O-sulphate [33]. This modification is important in the binding of HS to antithrombin [34] although several other sequences containing this rare modification are known to exist [35], not all related to antithrombin activity (because they also exist in non antithrombin binding fractions, personal communication Dr E Yates). Seven isoforms of the 3-OST are known to exist in humans, with two of the isoforms being splice variants, with differing sequence specificities and localisation [36-40]. It should be noted that not all substrate sites for these enzymes are modified and not all of the modifications occur on any disaccharide repeat. This reaction set rarely goes to completion and is not template driven as in DNA→mRNA→protein synthesis and hence does not often give the same terminal product repeatedly.

The existence of multiple isoforms of the enzymes NDST, 6OST and 3OST gives rise to questions of differing substrate specificities for the isoforms. In contrast, there is only one isoform of both C-5 epimerase and 2OST known to date. The enzymes involved in biosynthesis are given in table 2. The mechanism outlined above for the biosynthesis of heparin and heparan sulphate is only a proposed model based on current understanding. The order and mode of interaction (and the ability to revisit various stages), the specificities and the overall control of the biosynthetic pathway are poorly understood and most of the knowledge has been obtained only recently. The proposed biosynthesis is based on inferences in model systems and *in vitro* assays and is by no means a definitive account of *in vitro* biosynthesis; in particular, the mechanism by which regulation of these enzymes is achieved is not yet known. Biosynthetic modifications are not definitive, with the potential for post-Golgi processing to occur, for example, modification with endo-sulphatases (sulf family, discussed in 1.3.3.6) and / or the mammalian hydrolase, heparanase. An outline of the hypothetical pathways is shown in figure 1.

Table 2: Enzymes involved in (human) heparin / heparan sulphate biosynthesis

Name	Abbreviation	Amino acids	Chromosome	mRNA expression
Xyl transferase	XylT 1	959	16p12	Ubiquitous
	XylT 2	865	17q21.3-17q22	Ubiquitous
Gal transferase I	GalT I	327	5q35.2-q35.3	Ubiquitous
Gal transferase II	GalT II	329	1p36.33	Ubiquitous
GlcA transferase I	GlcAT I	335	1p36.33	Ubiquitous
GlcA /GlcNAc transferase	EXT 1	746	8q24.11-q24.13	Ubiquitous
	EXT 2	718	11p12-p11	Ubiquitous
GlcNAc transferase I	GlcNAcT I (EXTL2)	330	1p21	Ubiquitous
GlcNAc transferase II	GlcNAcT II (EXTL1)	676	1p36.1	Brain, heart & skeletal muscle
GlcNAc transferase I/II	GlcNAcT I/II (EXTL3)	919	8p21	Ubiquitous
GlcNAc N-deacetylase /	NDST 1	882	5q33.1	Ubiquitous
N-sulphotransferase	NDST 2	883	10q22	Ubiquitous
	NDST 3	873	4q26	Brain, kidney & liver
	NDST 4	872	4q25-q26	-
GlcA C-5 epimerase	Hsepi (GLCE)	618	15q23	Ubiquitous
HS 2-O-sulphotransferase	HS2ST	356	1p31.1-p22.1	Ubiquitous
HS 6-O-sulphotransferase	HS6ST 1	401	2q21	Ubiquitous
	HS6ST 2	499	Xq26.2	Brain
	HS6ST 2S	459	Xq26.2	Fetal kidney, ovary & placenta
	HS6ST 3	471	13q32.1	-
HS 3-O-sulphotransferase	HS3ST 1	307	4p16	Brain, heart, lung & kidney
	HS3ST 2	367	16p12	Brain
	HS3ST 3A	406	17p12-p11.2	Ubiquitous
	HS3ST 3B	390	12p12-p11.2	Ubiquitous
	HS3ST 4	456	16p11.2	Brain
	HS3ST 5	346	6q22.31	Skeletal muscle
	HS3ST 6	311	16p13.3	-

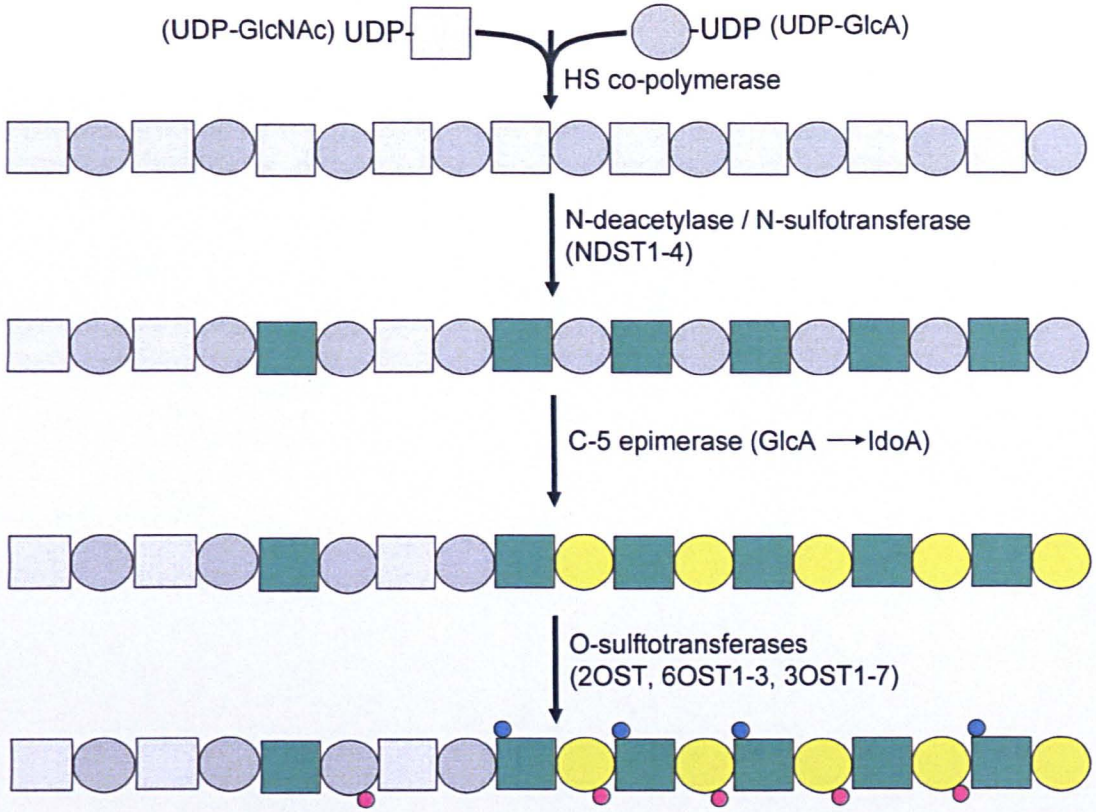

Figure 1 – The biosynthetic pathway shared by heparin and heparan sulphate. Based on a figure by Turnbull *et al*. 2001 (Trends Cell Biol. Feb;11:75-82)

1.2.5 Degradation of heparan sulphate and heparin

The degradation of heparin and heparan sulphate occurs in the endosomal / lysosomal compartments. Heparan sulphate proteoglycans are internalised and transported via clathrin mediated endocystosis to join with the early endosome where protein degradation begins [41]. The process by which heparin is internalised is, to the author's knowledge, still elusive but may involve interactions with other cell surface proteins (personal communication, Dr S Guimond and Prof. J Turnbull).

Initially de-polymerisation of the heparin and heparan sulphate polysaccharides to smaller oligosaccharide (≈ 5kDa) is facilitated by the endo-hydrolytic action of an endo-glucuronidase, heparanase in the endosome [see 1.3.1.2]. After polysaccharide cleavage, a family of lysosomal exo-enzymes attack the small oligosaccharide fragments and sequentially remove the terminal non-reducing end residues (exo-glycosidases) and sulphate groups (exo-sulphatases). This process is known to occur in the lysosome and the enzymes involved are considered to be present as multi-enzyme complexes [42]. Details of the enzymes involved are outlined in table 3. Another enzyme, an N-acetyltransferase, which transfers the acetyl group on to glucosamine residues, is also present in the lysosomal membrane; although strictly speaking this enzyme is not degradative, it is involved in heparin / heparan sulphate degradation [43]. This represents the counterpart to the equivalent N-deacetylase "degradation" step in biosynthesis

Following degradation the monosaccharide building blocks are re-used within the cell or excreted. This is facilitated by lysosomal transporters. The uronic acid monomers, N-acteylglucosamine monomers and inorganic sulphates are specifically transported out of the lysosome [44-46].

Table 3: Lysosomal enzymes implicated in heparin and heparan sulphate degradation

Enzyme	Substrate Specificity [§]
Exo-glycosidases	
Iduronidase	IdoA
Glucuronidase	GlcA
α-N-acetylglucosaminidase	GlcNAc
Exo-sulphatases	
Iduronate-2-sulphatase	IdoA(2S)
Glucosamine-6-sulphatase	GlcNAc(6S), GlcNS(6S)
Sulphamidase (glucosamine N-sulphatase)	GlcNS
Glucuronate-2-sulphatase	GlcA(2S)
Glucosamine-3-sulphatase	GlcNS(3S)
Acetyl-transferase	
Acetyl-CoA:α-glucosaminide N-acetyltransferase	$GlcNH_2$

[§]. The substrate specificities for the exo-enzymes are expressed as the non-reducing end terminal group recognised. Glycosidases cleave the whole non-sulphated monosaccharide, whereas sulphatases remove only the sulphate group. The acetyl-transferase transfers an acyl group to C-2 of the glucosamine replacing the amine.

1.3 Chemical properties of heparin and heparan sulphate

Heparan sulphate has a high degree of sequence variation with a theoretical choice of ≈ 32 different disaccharide subunits to build oligosaccharides and polysaccharides from (without considering the more rare modifications) [47]. Compared with an arsenal of 20 amino acids for peptide / protein synthesis, and 5 common nucleic acids forming the basis of RNA / DNA, heparan sulphate molecules can be considered to be one of the potentially most information-dense molecules that occur in nature.

The cause of much of this variation, the O and N sulphates, lends directly to some of the more difficult chemical properties of heparan sulphate. The polarity and high charge density of HS lends the molecule solubility only in highly polar solvents such as water. These acidic groups are also sensitive to cleavage and other types of rearrangements

leading to structural modifications which, if unintended, are undesirable although they can also be exploited.

The glycosidic linkage between C-1 of the glucosamine residue and C-4 of the uronic acid residue also provides for an efficient de-polymerisation point exploited for degenerative oligosaccharide preparation by both enzymatic and chemical techniques. Furthermore, de-polymerisation yields a free reducing end which can be exploited in attachment and / or conjugation with other molecules to form useful glyco-conjugates.

1.3.1 Heparan sulphate and heparin polysaccharide de-polymerisation

The similarity in the constituent disaccharides of heparin and HS gives rise to methods for de-polymerisation suitable for both polymers. De-polymerisation of heparin and HS falls into two categories; chemical and enzymatic. For chemical degradation, two commonly used strategies are outlined below, while enzyme digestion relies on readily available bacterial eliminases (lyases).

De-polymerisation of the parent HS polysaccharide chain is essential in elucidating structure – function relationships between HS moieties and proteins. The necessity to identify small, relatively low charged species is also driven by the quest to develop pharmaceutical agents based on HS. A single polysaccharide chain of HS may possess several possible sites (of identical or distinct sequence) for protein interactions or multiple, structurally distinct binding sites, within the same sequence. De-polymerisation allows an attempt to de-convolute the many reported (and, as yet, elucidated) interactions although caution must be used with regard to this somewhat simple strategy because polysaccharides and oligosaccharides have been shown in some cases to possess differing activities (personal communication Dr A Powell).

Decreasing the size of the HS molecule under study is also useful from a practical perspective. With current technology, attempting to separate a HS / heparin polysaccharide chain to near purity is an impossible exercise because of the very high

number of structural isomers present. Studying small building blocks allows relatively high sample purity to be achieved (although this is by no means a trivial undertaking). Reducing the size of an oligosaccharide (and hence the amount and density of charge) also opens up the possibility of other chemistries in alternative solvent systems.

The ultimate use of the oligosaccharide molecule dictates the strategy (or strategies) used for de-polymerisation. For example, where immobilisation is required, the researcher may be prepared to sacrifice the fidelity of the resultant oligosaccharide in an attempt to obtain useful immobilisation yields. In contrast, sequence identification requires faithfulness of the HS fragment to that of the original polysaccharide and minimising the loss of sequence information is paramount. A combination of polysaccharide de-polymerisation techniques may be deployed to verify the veracity of any derived sequence information.

Oligosaccharide molecules used to elucidate pharmaceutical pre-cursors could be derived from naturally occurring structures or chemically modified ones. The provenance of these molecules is of high importance.

Outlined below are the common strategies for de-polymerisation of heparin and HS.

1.3.1.1 Nitrous acid cleavage

A rapid and facile method for the cleavage of heparin and heparan sulphate polysaccharides is granted by means of acid hydrolysis using nitrous acid. This method cleaves between both N-unsubstituted and N-sulphated glucosamine and uronic acid residues, but does not cut at N-acetyl glucosamine residues due to the stability of this group to nitrous acid [48]. The specificity of cleavage for this reaction produces oligosaccharides consisting of even number multiples of constituent disaccharide units (di, tetra, hexa etc...).

One distinct advantage of this method, compared to that of free radical or enzyme degradation, is the production of a comparatively reactive reducing end residue. The

glucosamine residue present at the original point of scission is modified in the product to a 2,5-anhydrommanose residue. This molecule bears a reactive aldehyde group, which is not present in unfavourable equilibria, as is the case for the glucosamine residue where there is inter-conversion between the open, reactive (aldehyde) and closed, un-reactive (enol) forms.

Further selectivity can be imparted to the reaction by varying the pH of the nitrous acid solution. pHs in the range of 2.5 – 4.0 tend to skew the reaction to cleave between N-unsubstituted, free amino glucosamine and uronic acid residues, while pHs <2.5 bias the reaction towards the cleavage of the bond between N-sulphated glucosamine and uronic acid residues (although cleavage will still occur at N-unsubstituted residues). This relies on the de N-sulfation reaction, which occurs at low pH [see 1.3.2.2], yielding an un-substituted, free amine group at C-2 of the (previously) N-sulphated glucosamine residue [48, 49]. It should also be noted that the rate of scission can be influenced by neighbouring saccharide residues (personal communication with Drs. Powell and Yates).

A major disadvantage to this de-polymerisation method is the possibility of unwanted modifications arising within the newly generated oligosaccharide (such as de-sulphation). These can not be monitored easily and may produce unnatural oligosaccharide species. This could have serious ramifications for sequencing and the determination of structure – function relationships.

Other disadvantages of this de-polymerisation procedure are the difficulty in determining the sulphation pattern of adjacent residues when sequencing (nitrous acid does not depolymerise at N-acetyl containing glucosamine residues and the lack of a useful chromophore in the resultant oligosaccharides, hindering further purification, separation and manipulation.

1.3.1.2 Enzyme digestion

The enzymatic digestion of nascent polysaccharides to yield oligosaccharide products is possible using three enzymes derived from the gram negative bacterium[50], *Flavobacterium heparinase* found commonly in soil.

The enzymes used are endo-lyases (eliminases) that cleave the parent polysaccharide, with different specificities [51], at the glycosidic linkage present between the uronic acid and the glucosamine residues. The specificity for this linkage region yields products which are multiples of disaccharide subunits, thereby generating even-numbered oligosaccharide fragments (as for nitrous acid degradation).

Oligosaccharides derived from enzymatic cleavage possess an intact and unmodified glucosamine residue containing free reducing ends which can be derivatized or coupled using reductive amination.

One feature of enzymatic cleavage is the creation of an un-saturated carbon bond (C=C) between C-4 and C-5 of the iduronic / glucuronic acid residues. The introduction of this bond creates a Δ-uronic acid residue at the non-reducing end, thereby obscuring the previous identity of the uronic acid residue (both iduronic and glucuronic acid degrade to a common structure). One advantage of this degradation method, however, is the introduction of a chromophore into the molecule, a C=C bond, which absorbs in the ultra-violet region of the spectrum at a wavelength maximum of 232nm. This chromophore is superior to the only other chromophores present, in the C=O bond of N-acetyl groups and carboxylic acid groups, and has a coefficient of extinction of approximately 5,500 M cm^{-1}.

The three commonly used bacterial lyase enzymes differ in their specificities (see table 4) with heparatinase II considered to be the least specific. Heparatinase I generally cleaves within areas of low sulfation, whereas heparatinase III favours regions which are more densely sulphated [51]

The mammalian heparanase enzyme can also de-polymerise heparan sulphate chains [52]. The heparanase enzyme is usually found extra-cellularly in organs of the lymph system or the placenta and intra-cellularly as part of the overall turnover of HS. It is only expressed in small quantities in normal cells [53]. To date, two splice variants of heparanase I are known to exist [54] although BLAST sequence homology has identified another isoform (heparanase 2) [55]. It should be noted that most of the work carried out to characterise this enzyme has been performed using over-expression or tumour cell lines [53]. In contrast to the bacterial lyase enzymes, heparanase is a hydrolase and cleaves in a manner which is different to the bacterial endo-lyases, only at β-glucuronosyl bonds. It is proposed that the mammalian enzyme requires the presence of two separate S-domains, cleaving in between the two. It is thought that recognition is by the overall conformation of the polysaccharide as opposed to a simple binding sequence [56, 57].

1.3.1.3 Free radical degradation

Free radical degradation of polysaccharide chains has not proved to be a common procedure for the generation of oligosaccharide species although it does possess a major advantage over that of nitrous acid or enzyme degradation in that it cleaves to yield not only multiples of disaccharides but also "odd numbered" fragments. Furthermore, unlike enzymatic digestion, the uronic acid remains unmodified as either iduronic or glucuronic acid.

Free radical degradation is commonly mediated by the presence of copper (I) ions and is thought to predominantly cut at the glycosidic linkages of the polysaccharide chain [58]. Cleavage is usually at the glycosidic linkage and there is some specificity for de-polymerisation at 2-sulphated IdoA residues (personal communication, Dr E Yates) Oligosaccharides derived in this manner (although the exact mechanism is poorly understood) would appear to be free from the de-N and de-O sulfation problems which could hamper acid degradation. The degraded polysaccharide fragments also possess a

reducing end terminus which can be conjugated (to fluorophores for example) or attached (to surfaces) by reductive amination.

Table 4: Enzymes available for the modification and / or de-polymerisation of heparan sulphate

Enzyme	Substrate Specificity [§]
Polysaccharide lyases	
Heparitinase I *(Heparinase III)*	GlcNR(± 6S)α1→4GlcA
Heparitinase II *(Heparinase II)*	GlcNR(± 6S)α1→4GlcA/IdoA
Heparitinase III *(Heparinase I)*	GlcNS(± 6S)α1→4IdoA(2S)
Bacterial exoenzymes	
Δ4,5-glycuronate-2-sulphatase	ΔUA(2S)
Δ4,5-glycuronidase	ΔUA
Exoglycosidases	
Iduronidase	IdoA
Glucuronidase	GlcA
α-N-acetylglucosaminidase	GlcNAc
Exosulphatases	
Iduronate-2-sulphatase	IdoA(2S)
Glucosamine-6-sulphatase	GlcNAc(6S), GlcNS(6S)
Sulphamidase (glucosamine N-sulphatase)	GlcNS
Glucuronate-2-sulphatase	GlcA(2S)
Glucosamine-3-sulphatase	GlcNS(3S)

[§] For the bacterial lyase enzymes the substrate specificities are shown as the linkage specificity. The substrate specificities for the exoenzymes are expressed as the non-reducing end terminal group recognised. Glycosidases cleave the whole non-sulphated monosaccharide, whereas sulphatases remove only the sulphate group.

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reducing end terminus which can be conjugated (to fluorophores for example) or attached (to surfaces) by reductive amination.

A more random free radical degradation procedure has also been reported in the literature using iron (II) ions in preference to copper [58].

1.3.2 Chemical sensitivities of heparan sulphate and heparin

An important area of heparin and heparan sulphate chemistry, but one which is frequently overlooked, concerns the sensitivities of these molecules to various chemical conditions, especially extremes of pH. Exposure to such conditions could result in extensive modification of the carbohydrate analyte. The altered structure may introduce artefacts into binding assays and could ultimately result in erroneous sequence identification in structure-function studies. These structural changes could also be made inadvertently, and would therefore, in the majority of cases proceed unnoticed, possibly rendering whole investigations invalid.

It should also be noted that changes to reducing end disaccharides (nearest neighbours) and may influence the reactivity of the reducing end carbonyl group (with wide reaching implications for surface studies and quantification techniques) while inter-chain modifications are liable to influence protein binding and add to the already high heterogeneity of these molecules.

The chemical modifications described in this subsection should draw attention to the care required in the preparation and use of these molecules. It is also included in anticipation that the reader will bear these susceptibilities in mind when interrogating further methodologies and / or results, and also because they form the basis for preparing some of the chemically modified heparin derivatives used later in this work.

1.3.2.1 Sensitivity of heparan sulphate and heparin to basic conditions

Heparan sulphate polysaccharides and oligosaccharides are particularly sensitive to alkaline conditions both at the reducing end terminus and intra-chain.

The rate of reaction of the carbonyl groups present at the reducing end terminus is determined not only by the rate of mutarotation (i.e. the equilibrium between the closed ring form and the open chain aldehyde containing form) but also by their differing tendencies to convert to ene-diols under even mild basic conditions.

One such modification is the conversion of N-acetyl glucosamine to N-acetyl mannosamine proceeding via a 1,2-ene-diol favoured by basic conditions that has been reported to occur at concentrations as low as 0.01mM ammonium hydroxide [59]. The method is analogous with the Lobry de Bruyn-van Eckenstein rearrangement known to occur in monosaccharides possessing a free reducing end [60]. Although this modification occurs only at the reducing end, and is usually not considered for larger oligosaccharides, the result is to add further heterogeneity and this will have a bearing on the reducing end reactivity.

Under more basic conditions attack of C-2 of the iduronic acid residue, which is electron deficient due to the attached electronegative 2-sulphate group, occurs by the neighbouring oxygen present at C-3 (present as an hydroxyl at neutral and acidic pHs, but stripped of its proton at basic pHs). At pH values 9 - 12 (and more markedly with heating) a 2,3 epoxide group is formed with the loss of the sulphate group [49, 61, 62].

Exposure of the newly generated epoxide to acidic conditions yields an α -L-galacturonic residue, with inverted stereochemistry at C-2 and C-3, by opening of the epoxide [61, 62], while exposure to severely basic conditions (pH >12) facilitates nucleophilic attack of the epoxide by hydroxide nucleophile to yield an iduronic acid residue in a stereo-selective manner. This latter reaction is the basis of the selective 2-de-O-sulfation of heparin. It is unknown whether (the rare) 2-O-sulphated glucuronic acid residue

undergoes similar modification. Exposure to such strongly basic conditions may also cause de-polymerisation by β-elimination [63].

The severe basic conditions used to remove the iduronic acid 2-O-sulphate group present within heparin or HS can lead to a further modification of glucosamine residues possessing a sulphate group at C-3 and an N-sulphate at C-2 (the reaction occurs if C-6 of the glucosamine is O-sulphated or unsubstituted, but not if C-2 is un-substituted or N-acetylated). Under these conditions an N-sulphated-aziridine derivative is formed by nucleophilic attack at C-3 of the glucosamine residue by the nitrogen atom (de-protonated) at C-2. This reaction results in inversion of the stereochemistry at C-3 and the loss of the associated O-sulphate group. This reaction requires the presence of both the 3-O and N-sulphates and does not occur under the weaker basic conditions amenable to only epoxide formation [35, 64].

1.3.2.2 Sensitivity of heparan sulphate and heparin to acidic conditions

Weakly acidic conditions (thereby protonating the carbonyl group) are commonly employed to catalyze the reductive amination reaction during labelling or surface attachment at the reducing end (see 3.1.7). At pHs of 4.0 and below, however, de-N-sulphation may occur especially if heating is employed, although the N-sulphate groups may be replaced using established N-sulphation techniques [65]. It should be noted that mutarotation via the open chain ring type (the reactive aldehyde containing form), and hence the inter-conversion between the pyranose forms, appears to occur more slowly in acidic conditions. There is also the possibility of de-N-sulphation artefacts arising from the partial de-polymerisation of the polysaccharide using nitrous acid (but no definitive study of this exists) [63].

Exposure of heparin and heparan sulphate poly- and oligosaccharides to more acidic conditions promotes the further removal of O-sulphate groups. This occurs first with the loss of the O-sulphate attached via C-6 of the glucosamine residue followed by the removal of the O-sulphate present at C-2 of the iduronic acid residue and finally the 3-O-

sulphate group of glucosamine. A variant of this degradation technique is exploited in the manufacture of chemically modified heparin polysaccharides, kinetically controlling the reactivity in the solvolytic de-sulphation of heparin [1.3.3.1].

1.3.3 Chemical and enzymatic modification of heparan sulphate and heparin

The modification of native heparin and heparan sulphate molecules is an important tool which has two main functions. Both of these functions ultimately have the same goal of elucidating the structure – function relationships with regard to interacting partners. The first application is in the creation of polysaccharides and oligosaccharide library fragments thereof [see 1.4.5] in useful amounts which allows the interrogation of binding events. The second facilitates the determination of the precise di-saccharide constituent sequence present within an oligosaccharide. The first approach generally relies on chemical preparations while the latter is more reliant on enzymatic approaches, both of which are outlined below.

Modifications can occur intra chain, at the reducing end (containing a reducing carbonyl group) and / or at the non-reducing end. Intra chain modifications can be made chemically throughout the entire polysaccharide (either the addition or removal of sulphates for example) or selectively, using the endosulphatase enzymes (sulfs) which remove 6-O-sulphates specifically. Modifications can occur at the reducing end facilitated by the presence of the reducing end carbonyl at C-1 of the glucosamine residue allowing for conjugation or immobilisation [see 1.3.4.1 and 1.3.4.2]. Non-reducing end modifications can occur enzymatically by means of a group of exo-enzymes and or chemically by the presence of the C=C bond after bacterial lyase digestion [as detailed in chapter 3].

1.3.3.1 Selective chemical removal of the N-sulphate group

The (near) complete removal of the N-sulphate groups present at C-2 of the polysaccharide chain is achievable as described by Inoue *et al.* [66]. De-N-sulfation is

facilitated by solvolytic attack of the heparin polysaccharide. The heparin polysaccharide is initially converted to its acidic form by proton exchange of the inherent ion paired cations using acid pre-treated ion exchange resin. The acidic heparin polysaccharide (H^+ form) is subsequently neutralised using pyridine to yield the pyridinium heparin polysaccharide which is insoluble in DMSO. Solvolytic attack of the N-S bond present in the N-sulphate group occurs when the heparin polysaccharide is dissolved in DMSO, containing 10% methanol (MeOH), upon heating at 55°C for 30 minutes. After completion the products are isolated by precipitation in cold ethanol. This method of de-N-sulphation yield free amino groups present at C-2 of the glucosamine (as well as free inorganic sulphate) without the side effects of hydrolytic (acid promoted) de-N-sulphation [66].

1.3.3.2 Chemical addition of N-sulphate groups

The addition (or re-addition) of N-sulphate groups to a free amine group present at C-2 of glucosamine residues can be carried out essentially as described by Lloyd *et al.* [65]. Typically, an aqueous solution of heparin polysaccharide is adjusted to pH 9.0 with saturated sodium hydrogen carbonate. Sulphur trioxide complex is added to the reaction mixture and the pH again adjusted to pH 9.0. The reaction mixture is heated to 55°C for 3 hours. After the initial incubation, a further addition of sulphur trioxide complex is made and the pH maintained at 9.0. Upon completion the products are isolated by precipitation in cold ethanol.

1.3.3.3 Chemical *acetylation of free amines*

Free amines present at C-2 of the glucosamine residue (such as created by de-N-sulphation as in section 1.3.3.1) can be N-acetylated as described by Dansihevsky *et al.* [67]. De-N-sulphated heparin is dissolved in saturated sodium hydrogen carbonate and acetic anhydride is added on ice. The solution is stirred continuously at 0°C for 2 hours, with the pH maintained at 9.0 by the addition of sodium hydrogen carbonate. Products are isolated by precipitation in cold ethanol.

1.3.3.4 Selective chemical removal of O-sulphate groups

The O-sulphate group present at C-2 of the iduronic acid, or more rarely glucuronic acid, residue can be removed selectively as described by *Jaseja et al.* [61]. This involves dissolving the initial heparin polysaccharide in a strong base (1M sodium hydroxide), freezing and lyophilising before re-dissolving in water pH adjusted to 7.0 with hydrochloric acid. The products are then isolated by precipitation in cold ethanol followed by dialysis to remove inorganic salts.

The O-sulphate present at C-6 of the glucosamine residue (either N-acetly of N-sulfo) can also be selectively removed by a slight modification of the method outlined by Ayotte & Perlin [68]. This method also concomitantly de N-sulphates the polysaccharide, although re-N-sulfation can be carried out [as in 1.3.3.2]. The products are isolated by precipitation in cold ethanol followed by dialysis to remove inorganic salts.

1.3.3.5 Enzymatic removal of sulphate groups using exo-sulphatases

The enzymatic removal of sulphate groups is possible at the non-reducing end disaccharide using the lysosomal exo-sulphatases. As these enzymes are exo-enzymes and hence only function on a small part of the oligosaccharide or polysaccharide chain they are unlikely to be useful in the modification of polymers for direct binding or immobilisation studies. These enzymes have been used successfully for the sequencing of heparan sulphate oligosaccharide using step wise removal of sulphate groups along the chain (coupled with nitrous acid digestion and exo-glycosidases with either gel-based fluorescence (integral glycans sequencing) or chromatographic radioactively labelled detection systems [69-72].

1.3.3.6 Enzymatic removal of O-sulphate groups using endo-sulphatases

The selective removal of 6-O-sulphate groups is possible enzymatically by a family of enzymes known as the sulphatases or sulfs. To date two sulfs exits, termed sulf 1 and sulf

2, both having specificity for a tri-sulphate motif present in heparan sulphate chains [73, 74]. Due to this specific requirement these enzymes are not of significant use in the modification of polymer chains unless for a precise application. It should be noted that these enzymes are endo-sulphatases and remove 2-O-sulphates within the chain.

1.3.4 The reducing end, equilibrium & mutarotation

Heparan sulphate and heparin oligosaccharides cleaved either chemically or enzymatically both possess an aldehyde group at the terminus of the carbon backbone, and are therefore said to be reducing sugars. Polysaccharide chains may also have a reducing end present but this is dependent on the method of cleavage from the proteoglycans. Reduction can be employed as a final step reducing the aldehyde group to an alcohol. Two types of reducing end mono-saccharides are created dependent on the cleavage mode used. Enzymatic cleavage yields a N-acetyl(or sulpho)glucosamine terminal residue while chemical, nitrous acid cleavage yields an 2,5-anhydrommanose residue. The 2,5-anhydrommanose residue has a more reactive reducing end compared to that of the N-acetyl(or sulpho)glucosamine.

The presence of a free aldehyde group at the terminus of the carbohydrate carbon backbone is an important chemical property. This group provides a suitable point for the attachment of carbohydrates, the detection and quantification of carbohydrates and their conjugation to other molecules (and other carbohydrate monomers / polymers) via chemical and metabolic processes.

The ability of the aldehyde group to reduce metals was the basis for the term 'reducing end' commonly used in the field of carbohydrate chemistry. It should be made clear that not all mono, oligo or poly-saccharides possess a reducing end. The ability of carbohydrates to exist in a non-reactive cyclic conformation in equilibrium with the reactive (and aldehyde containing) open-chain form means that the extent of reaction of the aldehyde group depends on the equilibrium position and the rate of inter-conversion (mutarotation).

The rate of conversion is affected by solvent (a solvent must be amphoteric to allow mutarotation, i.e. be able to donate and receive protons; examples include H_2O, formamide etc.) temperature, pH, the external environment and the level and type of substitutions present not only on the immediate mono-saccharide (although this has the greatest effect) but also on more remote modifications on neighbouring saccharide sub-units (adding yet another level of complexity) [63]. A more detailed description of the finer details of these concepts is given in the introduction to chapter 3. Examples of the differing rate of mutarotation are presented in table 5.

Table 5: Mutarotation of carbohydrates in dimethyl formamide 22°C

Carbohydrate	Equilibrium rotation DMF	Equilibrium rotation H_2O	Reaction t½ Difference	Reaction t½ (hours)
β-L-Arabinose	+38.7	+105.5	-66.8	35.0
β-D-Fructose	-22.4	-92.3	+69.9	2.4
α-D-Galactose	+28.7	+81.0	-52.3	19.5
α-D-Glucose	+63.3	+52.5	+10.8	228.0
β-D-Mannose	+39.9	+14.2	+25.7	6.4
α-L-Sorbose	-51.3	-43.3	-8.0	14.0
D-Ribose	-26.2	-23.7	-2.5	-
α-D-Xylose	+33.1	+19.0	+14.1	176.0

Based on a table originally produced in Isbell *et al.* 1969[75].

1.3.4.1 Reductive amination of glucosamine residues

The reducing end of the oligosaccharide molecule contains a carbonyl group present at C-1 of the glucosamine which provides for a convenient site for conjugation or attachment to other molecules or surfaces. Reductive amination relies on formation of an imine (or Schiff base) between the surface or molecule and the carbohydrate to which derivatisation is required. Imine formation occurs between the carbonyl group of the glucosamine reducing end and an amino containing group (amine, hydrazide, hydroxyl amine etc...) present on the molecules / surface to which conjugation is required [63].

Several competing factors influence the rate and yield of this reaction, which can at best be described as sluggish if the reaction proceeds un-catalyzed with a yield of only a few percent. This is mainly due to the unfavourable equilibrium which exists between the closed and open ring conformation of the glucosamine residue [63]. A more in-depth discussion can be found in 3.1.7. The reaction rate and yield maybe increased by acid catalysis (which promotes protonation of the carbonyl group, the first step in the reaction mechanism), although this can pose problems due to the possibility of de-N-sulfation. Alternative means of catalysing this reaction are currently being sought.

The attachment of carbohydrates to surfaces has been shown to be improved by microwave heating in the amphoteric solvent formamide and it is known that this improvement is also observed in free solution. Another consideration in the yield of this reaction is the use of generally more reactive hydroxyl amines or hydrazides. The imine (Schiff's base) is frequently reduced to an amine in order to stabilize the glyco-conjugate by the addition of a suitable reducing agent.

1.3.4.2 Attachment, immobilisation and labelling

The attachment and / or immobilisation of heparan sulphate and heparin derived oligosaccharides is a useful tool in the glycobiologist's toolkit. Attachment of oligosaccharides by chemical means to column matrices allows the facile creation of affinity columns which can be used to select interacting partners from crude mixtures. The interacting partners can then be eluted by competition or by the disruption of ionic interactions.

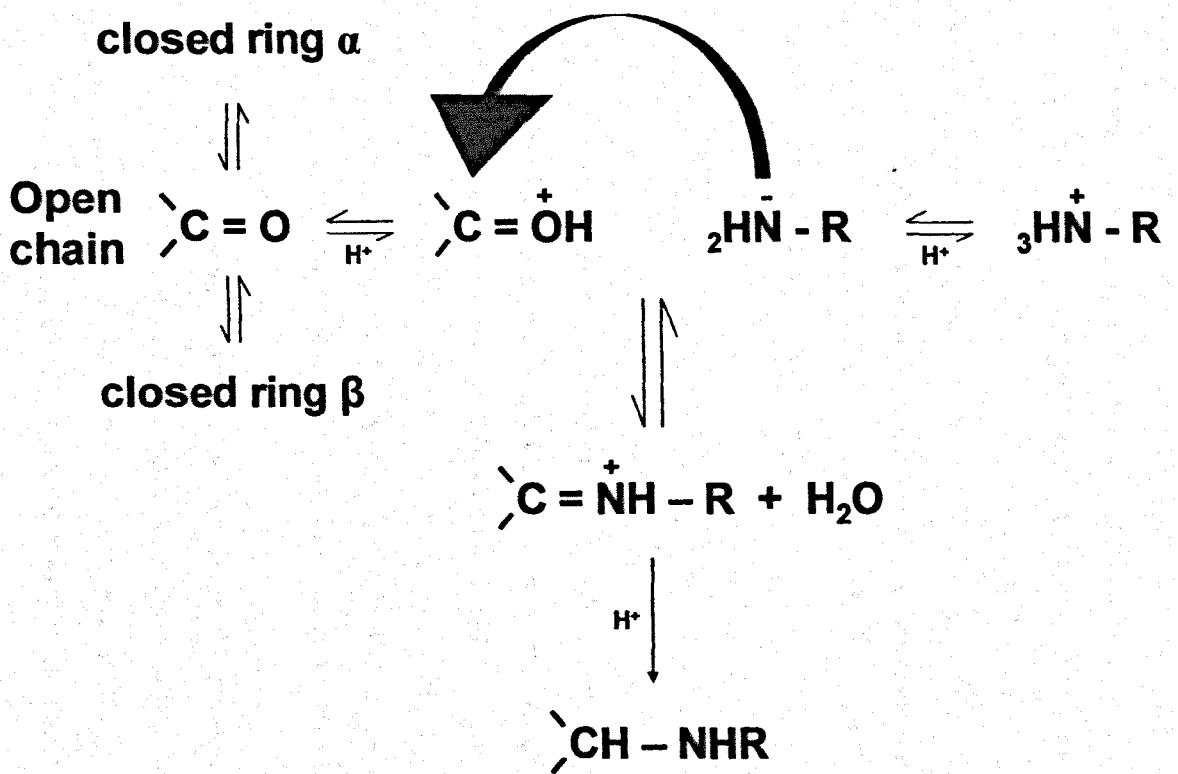


Figure 2 – The mechanism of reductive amination. Three equilibria dictate the reductive amination reaction: (i) the equilibrium between the open chain and closed ring conformations, (ii) the protonation of the carbonyl group at C-1 and (iii) the presence of the amine group in an un-protonated form. The lone pair electrons of the amine nitrogen attack the electron deficient carbonyl carbon atom.

The use of optical biosensors such as the BIACore® or the IAsys® instruments allows for the study of interaction kinetics and binding affinities by the immobilization of carbohydrates to an appropriate surface. Interactions can then be followed using the change in refractive index as a measure of surface attachment and subsequent binding. Attachment for these methods is commonly via the reducing end carbonyl group, but oligosaccharides may also be chemically attached inter chain utilising condensation reactions via the carboxylic acid group present at C-6 of the uronic acid residue.

The use of fluorophores to aid detection also relies on conjugation of the reducing end carbonyl group. Labelling with fluorophores allows increased sensitivity and therefore detection in analytical assays. This coupling may also be used for tracking molecules in cell assays using fluorescence microscopy.

1.4 Obtaining heparan sulphate and heparin

Obtaining heparin and heparan sulphate is commonly a multi-step procedure with many levels of process. These involve isolation, separation and purification strategies, some of which are at the limit of conventional biochemical and chemical techniques. There is a desperate need for techniques which can extract workable amounts HS and heparin efficiently, separate to (near) purity the individual species and isolate them for interrogation in future assays which mimic real *in vivo* conditions as closely as possible.

Generally speaking heparin is used as a model for HS because of the formers ready availability. This is due to its application as a pharmaceutical anticoagulant. Although both polysaccharides share the same biosynthetic pathway, the arrangement of disaccharide constituents is markedly different, with heparin being more similar to that of the S domains of HS. The overall and local conformation of oligosaccharide and polysaccharide heparin derived HS analogues must be fully considered when using heparin as a model of HS. This said, the use of heparin, heparin derived HS analogues and, where possible, HS allows the scientist to probe biological systems in order to obtain

better understanding of the many interactions involving these complex molecules at a structural level.

1.4.1 Isolation of heparin and heparan sulphate

1.4.1.1. Isolation of heparan sulphate

Obtaining pure and workable amounts of heparan sulphate is by no means an easy feat. Heparan sulphate has to be mechanically extracted from tissue sources which already possess HS in small quantities. This problem is further complicated by the tissue specific sulphation pattern.

Heparan sulphate is found in all animals and is expressed by almost all mammalian cells. The amount of heparan sulphate and the sulphation pattern is tissue [76] and cell specific [77, 78] (and can alter in diseases states [79]). Cells can also produce different HS sequences depending on age [80], developmental state [9] and as a response to growth factors [81]. Hence the choice of tissue source is a crucial consideration in obtaining heparan sulphate. Common sources of commercially available heparan sulphate include bovine lung, porcine intestine and bovine kidney. An indication of the tissue specific nature of HS can be seen from table 6 for bovine derived polysaccharides. HS isolation is begun with a mechanical and / or solvent extraction of tissue.

1.4.1.2. Isolation of heparin

Heparin is found exclusively in the granulocytes of mast cells. Consequently tissues which have high concentrations of mast cells, those usually subject to a high incidence of viral or bacterial challenge [82] are good sources for heparin polysaccharide extraction. Commercially used tissues are bovine lung and porcine intestine. Immunological activation of the mast cells stimulates the de-granulation upon which the proteoglycan is cleaved to yield the GAG and a peptidoglycan [83].

Table 6: Bovine heparan sulphate: Tissue specific sulphation pattern

Di-saccharide	Aorta	Intestine	Kidney	Lung
GlcA-Man(NAc/NS)	75.0	58.0	61.0	62.0
IdoA-Man(NAc/NS)	9.6	8.1	8.5	3.3
GlcA(2S)- Man(NAc/NS)	0.3	0.7	0.3	0.5
GlcA-Man(6S, NAc/NS)	3.7	7.7	9.7	12.0
IdoA-Man(6S, NAc/NS)	3.2	5.7	7.8	9.2
IdoA(2S)-Man(NAc/NS)	5.6	15.0	8.2	9.7
IdoA(2S)-Man(6S, NAc/NS)	2.7	6.1	4.9	4.2

Values stated are % of total di-saccharide composition. Figure based on reference [76] by Maccarana *et al.*

Isolation of heparin is performed by mechanical recovery of animal tissues. The tissue samples are at once preserved in an antioxidant (e.g. 1 – 3 % sodium bisulfite) before subsequent purification

1.4.2 Purification of heparin and heparan sulphate polysaccharides from crude extracts

Heparin and heparan sulphate can generally be purified away from cell debris in the same manner. Various methods exist for purification depending on the type of preparation (analytical or preparative) and hence the amounts and the degree of purity desired. In general all of the methods involve a crude separation of hydrophobic, water insoluble matter by dissolution in a non polar solvent. The supernatant (containing the solubilised hydrophobic molecules) is discarded. Further washes can now be performed in various apolar solvents.

The hydrophilic residue is then generally re-suspended in a polar solvent (usually water) and the proteinacious element degraded using one or more proteases. The mixture is then bound to anion exchange resin. Molecules of opposing or no charge are washed away using successive water washes. The bound molecules are then eluted by the introduction of a counter ion to displace them from the matrix. Salt is then removed from the eluent using size exclusion chromatography. The mixture is generally treated at this stage by a cocktail of lyase enzymes to digest (to completion) other (unwanted) glycosaminoglycan

family members. (e.g. chondroitinase ABC to digest chondroitin sulphate). The mixture is once again bound to anion exchange beads / column and a low concentration of counter ion applied to wash away the small di / oligosaccharides created from lyase digestion of the other GAG polysaccharide. The heparin / heparan sulphate polysaccharides remain bound to the matrix due to their higher charge. Heparin / heparan sulphate polysaccharides are then eluted using a high concentration of counter ion prior to final desalting.

For analytical preparations two facile methods exist to extract the heparin / HS polysaccharide. One such method employs a Trizol® : chloroform extraction to remove hydrophobic and proteinacious molecules using phase separation before diethyl-amino ethanol (DEAE) ion exchange purification (personal communication Dr S Guimond) while another binds the cell extract directly onto ion exchange beads, washing with the detergent Triton® to remove hydrophobic content.

1.4.3 Purification of heparin and heparan sulphate oligosaccharides

The further purification of HS and heparin oligosaccharide molecules arguably poses one of the most demanding challenges facing the field. The disaccharide units contain a high degree of structural isomerism and this can lead to oligosaccharides with the same net charge and mass being present in heterogeneous mixtures. The separation of these molecules relies on the utilization of a wide range of techniques currently available in the field of separation science. The principle techniques are outlined below, although it should be noted that no individual technique can separate a mixture to purity and only a limited number of species can be generated which are pure using a combination of these techniques. There is therefore a pressing need to develop techniques with improved resolving power in this field.

1.4.3.1 Size exclusion chromatography

Size exclusion chromatography (SEC), molecular exclusion, gel filtration or gel permeation chromatography, is the separation of a mixture of molecules by their size or more accurately their hydrodynamic volume. This separation is accomplished by the interaction of the various components of the analyte mixture with a porous stationary phase. An array of differentially sized beads composes the stationary phase creating a fractionation range for the stationary phase within which molecules of various sizes can be separated.

Two common separation types are generally encountered when attempting to purify heparin and heparan sulphate poly and / or oligosaccharides. They are the separation of different sized oligosaccharide fragments generated by de-polymerisation of the nascent polysaccharide from each other, or the removal of inorganic salts from the carbohydrate constituents. Both processes utilise the principles of size exclusion chromatography although the fractionation ranges used are quite different. This type of purification is usually followed by further techniques reliant on different separation principles compared to that of size exclusion.

The removal of inorganic salts requires a stationary phase medium possessing comparatively small pore sizes. Generally speaking these matrices will separate molecules smaller in hydrodynamic volume than that of a tetra-saccharide from molecules of such a hydrodynamic volume and above. A suitable matrix for such a separation is Superdex® G25 Superfine using a water mobile phase (see figure 3).

Oligosaccharide fragments created by partial de-polymerisation can be separated from a mixture using a matrix of Sephadex® G30 (Amersham Biosciences) allowing facile resolution over a fractionation range of hydrodynamic volumes comparable to that of disaccharides through to hexadecasaccharides. Separation is achieved using ammonium bicarbonate as the mobile phase, thereby ion paring with negatively charged groups and

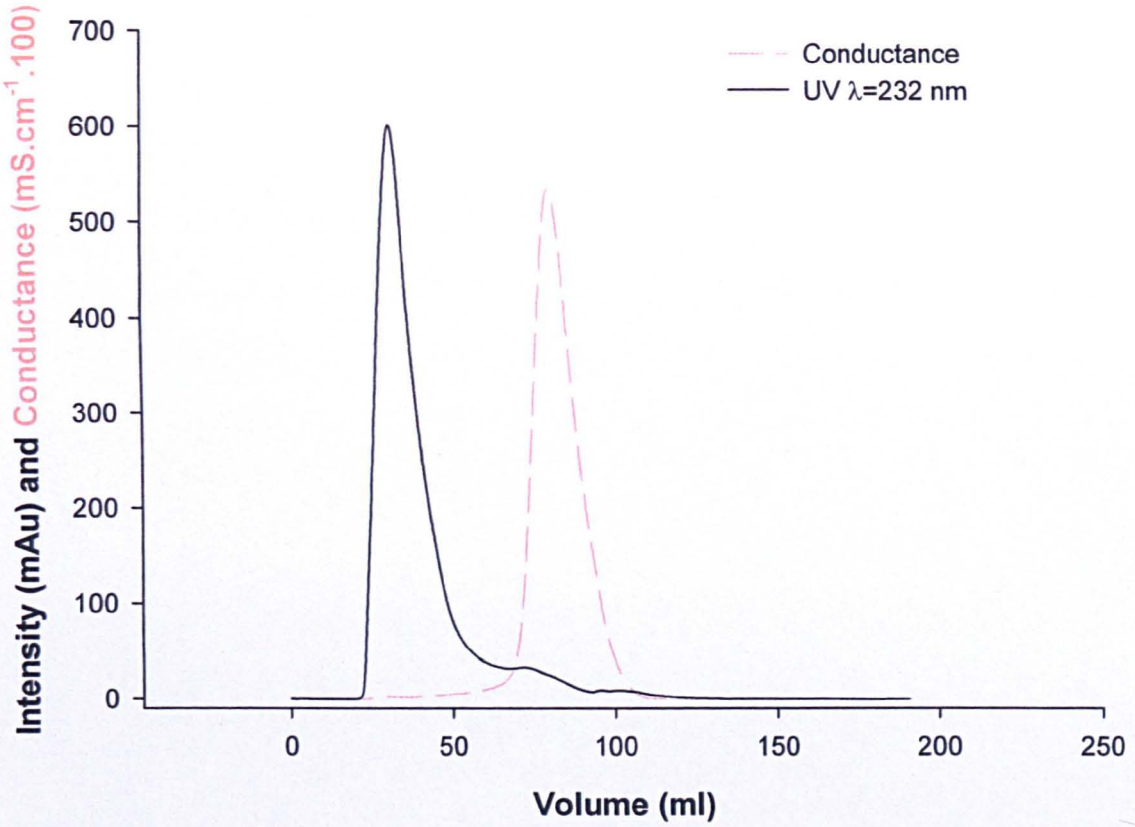


Figure 3 - The size exclusion chromatography elution profile of a fraction corresponding to the retention time for disaccharide standard 3 [ΔUA-GlcNSO$_3$] after strong anion exchange chromatography separation. Separation was carried out on two HiPrep ® 26/10 desalting columns (Amersham). A 38 minute elution was carried out at a flow rate of 5 ml.min^{-1} in HPLC grade water. The disaccharide standard elutes first (between 20 and 60 ml volume) as detected by UV absorbance (λ=232 nm) while the second peak detected by electrical conductance is that of the contaminating sodium chloride salt (between 65 and 110 ml volume) used to elute in the strong anion exchange preparation.

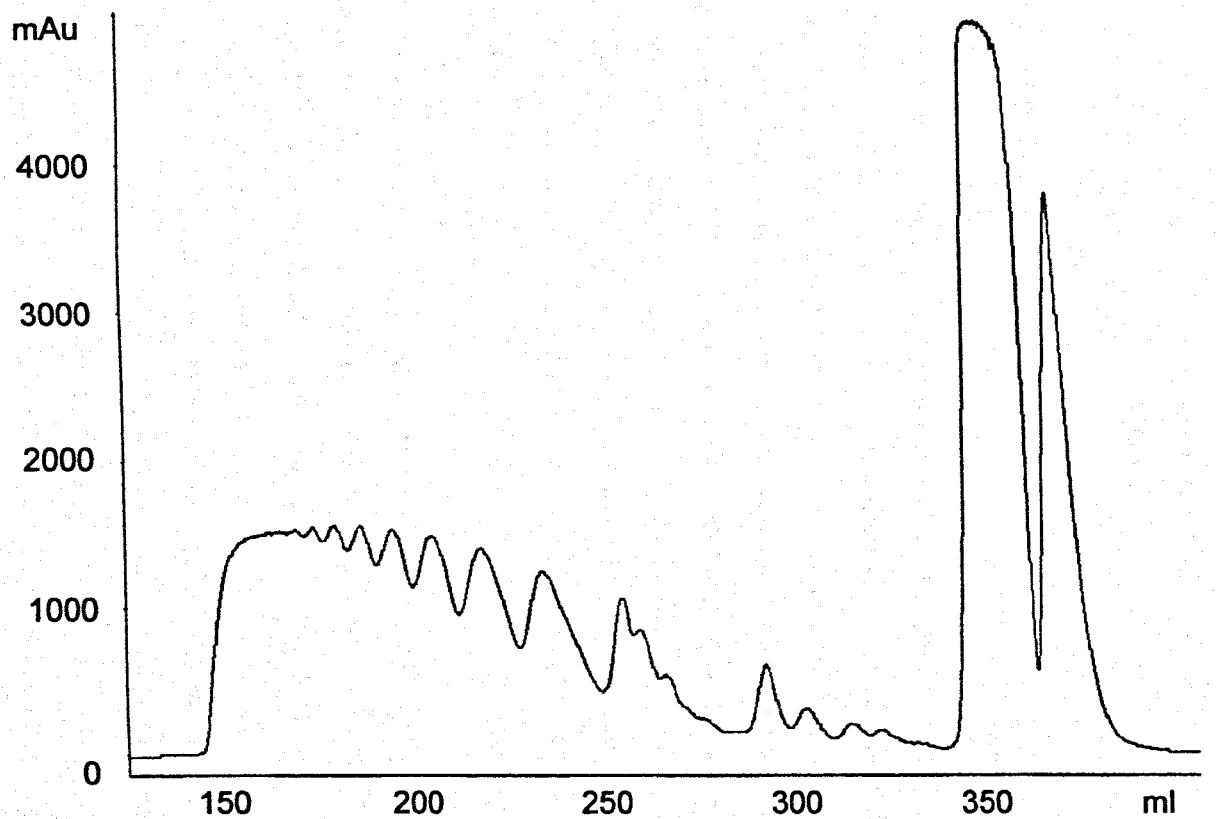


Figure 4 – The size exclusion chromatography elution profile of a partial nitrous acid digestion of porcine mucosal derived heparan sulphate (PMHS). Separation was carried out on two Amersham XK-16 columns (15mm x 1m) connected in series and packed with Superdex ® 30 matrix. A 0 – 1000 minute elution was carried out at a flow rate of 0.5 $ml.min^{-1}$ in 0.5 M ammonium hydrogen carbonate. Detection was performed in the UV (λ=215 nm) with the peak furthest to the right corresponding to contaminating salts. The peaks then group (right to left) into disaccharides, tetrasaccharides, hexasaccharides etc…

minimising interactions with the matrix based on charge. Ammonium bicarbonate is volatile and can be removed by lyophilisation.

Separation becomes increasingly difficult as the oligosaccharide size increases. For the larger oligosaccharides (hexadecasaccharide and above), overlap between peaks is inevitable and oligosaccharide pools are highly likely to contain a heterogeneous mixtures of oligosaccharides (see figure 4).

1.4.3.2 Gel Electrophoresis

Electrophoresis separates analyte mixtures based on their charge, size, conformation and hydrodynamic properties. Separation occurs by means of interactions between the gel matrix and charged analyte molecules which experience a migratory force towards electrodes possessing opposite charge. The interaction of an analyte with the matrix causes a frictional force (which differs in magnitude for each analyte) and this is proportional to the distance migrated by a given analyte in a constant electric field. Charged carbohydrates are generally separated using agarose or more commonly poly-acrylamide (with the proportion of acrylamide being in excess of 30%) gel based matrices.

The creation of a poly-acrylamide gel matrix requires the polymerisation of acrylamide monomers with N,N'-methlylenebisacrylamide cross linkers. The size of the pores present within the gel are dependant on both the amount of cross linker present and the size of the acrylamide monomer. The polymerisation reaction is usually stabilised by the presence of N,N,N',N'-tetramethylenediamine (TEMED) and ammonium persulphate is used as a source of free radicals. Oxygen inhibits the reaction and therefore polymerisation is conventionally carried out between two thin glass plates with the top of the gel overlaid with water saturated butanol or similar to provide a clean horizontal boundary.

Oligosaccharide analysis is routinely performed using 33% polyacrylamide gels with a 19:1 acrylamide:bisacrylamide ratio. Gradient gels can be used but these prove problematic to cast with reproducibility a real cause for concern. In contrast to protein electrophoresis, these carbohydrates are run in their native form and therefore a direct size to migration relationship cannot be elucidated.

Detection is usually by staining with a dye such as Azure A with detection limits in the order of a few micrograms [51]. Detection can also be achieved using fluorescent tags [69] or radiolabels [84]. Preparative scale purification is also possible but remains cumbersome with carbohydrates having to be manually extracted from the gel and duplicate gels must be used to identify band positions as staining modifies the original saccharides. This strategy relies on the ability to reproduce gels accurately, which can be difficult. Furthermore, separation using poly-acrylamide gels yields only reasonable resolution, and is not a significant improvement compared to that of size exclusion chromatography coupled to anion exchange or reverse phase chromatographies.

Resolution is significantly improved using capillary electrophoresis (discussed in chapter 2) although this is not possible routinely on a preparative scale owing to the minuscule sample loads and physical constrains of the machine. These constraints effectively relegate capillary electrophoresis as a separation strategy to purely an analytical role.

1.4.3.3 Reverse phase chromatography

Reverse phase high performance liquid chromatography separates molecules based on their hydrophobicity. This technique does not therefore immediately seem viable for the separation of molecules which are highly polar. To overcome this problem, the negatively charged carboxylic acid group present within every disaccharide repeat (and any sulphate groups) is masked using an ion paring reagent. This is accomplished by the formation of organic salts, usually composed of a hydrophobic ammonium salt, such as tetrabutylammonium [85] hydroxide to neutralise the acidic form of the oligosaccharide.

Separation occurs by the binding of the analyte molecule to the hydrophobic stationary phase, which usually composed of silica derivatized with alkyl chains, in a moderately polar mobile phase. Molecules are differentially eluted using a gradient of the initial polar mobile phase to that of an apolar organic solvent. Compounds are separated on account of their different hydrophobicities. Detection is usually achieved in line using UV detection of the C=C bond at λ=232nm generated by bacterial lyase digestion or at λ=215nm if using nitrous acid separated oligosaccharides.

Reverse phase ion paired separation has, to date, only been used to separate relatively small oligosaccharides, generally not exceeding an octasaccharide. In similarity to strong anion exchange chromatography, the removal of organic salts remains a problem (although to a lesser extent because lower concentrations are used in reverse phase than in strong anion exchange).

One attractive advantage of this separation technique is the possibility of coupling liquid chromatography separations directly to electro-spray mass spectrometry (due to the volatile solvents employed) to aid detection [86]. Fragmentation techniques (such as MS/MS) may also be used allowing the identification and classification of structural isomers.

1.4.3.4 Strong anion exchange chromatography

The basis for this mode of chromatography is the separation of a mixture of analytes on the basis of their differing overall charge and its distribution and arrangement within the molecule.

Anion exchange chromatography provides a matrix which has been modified to yield a positively charged head group by the derivatisation of strong base onto a stable support medium. These positively charged groups interact with the negative charges present on the analyte and hence bind them from the mobile phase. The column is designed to remain positively charged over a wide range of mobile phase pH values, i.e. 1 – 14.

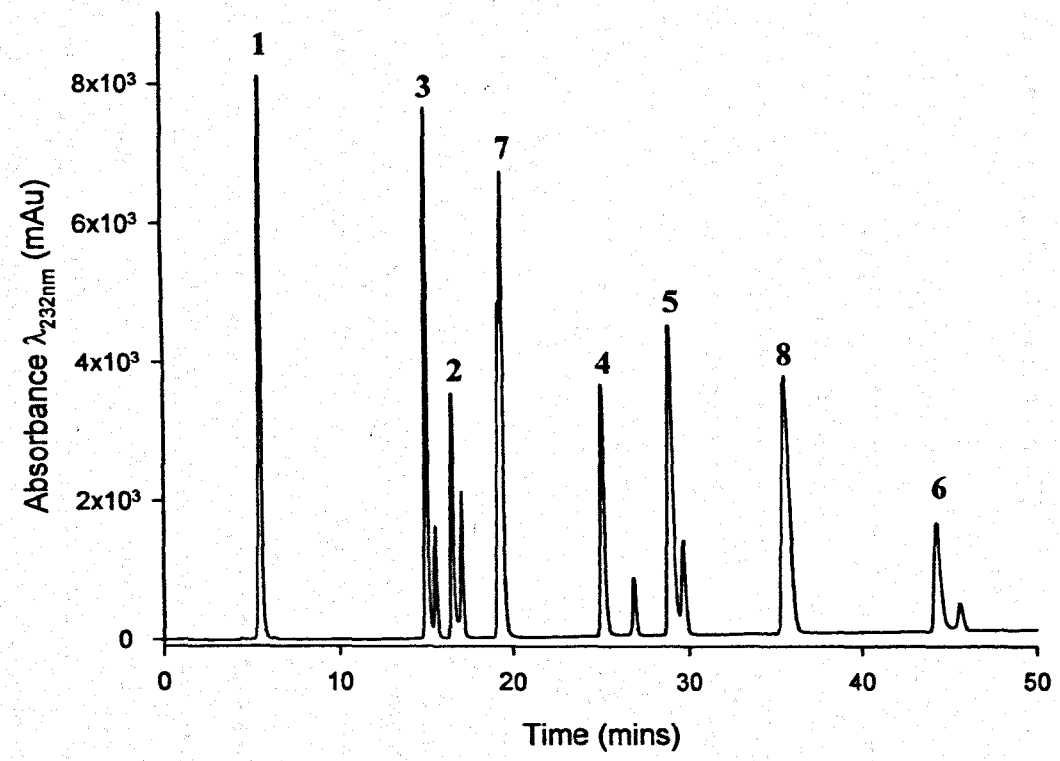


Figure 5 – Disaccharide profile for eight common naturally occurring disaccharides derived from heparan sulphate. Separation was achieved using high performance strong anion exchange chromatography on a cetyl-triethylammonium ion derivatized C18 silica based column (C18 Hypersil) with a 0 – 90 minutes, 0 to 2 M ammonium methane sulphonate linear gradient, pH 3.5; flow rate 0.22ml.min^{-1}. Disaccharide peaks: 1, ΔUA-GlcNAc; 2, ΔUA-GlcNAc(6S); 3, ΔUA-GlcNSO$_3$; 4, ΔUA(2S)-GlcNAc; 5, ΔUA-GlcNSO$_3$(6S); 6, ΔUA(2S)-GlcNSO$_3$; 7, ΔUA(2S)-GlcNAc(6S); 8, ΔUA(2S)-GlcNSO$_3$(6S).

Analytes are introduced on to the column in the desired mobile phase and bind ionically to the matrix. The strength of each interaction is dependent on the amount of charge present on the analyte, its conformation, the arrangement of charge and the environmental pH of the mobile phase. For binding to occur the pH of the mobile phase must be above the pKa of the acidic groups present within the molecules. If the opposite situation arises, the analytes will not bind to the matrix and instead pass through the column to waste. However the low pKas of the groups present within the heparin and heparan sulphates molecules make this an unlikely scenario [72].

Oligosaccharides are selectively eluted from the column in a manner that allows for their separation based on charge. A suitable counter ion must be introduced into the mobile phase which acts as a competitor to the analyte for the charged column matrix. The choice of counter ion is important, and the type of anion exchange column used and the analytes charge state of the analyte need to be considered. Detection is conventionally performed using an in-line UV detector to detect either the C=O or the C=C bond as for reverse-phase ion pairing chromatography as in section 1.4.3.3

A common technique in heparin or heparan sulphate structure determination is disaccharide analysis. This involves the de-polymerisation of the glycosaminoglycan chains into their constituent disaccharide components, usually by a cocktail of all three common bacterial lyase enzymes (heparatinases).

The resolution of all eight Δ-disaccharide standards can be performed with relative ease [87] (see figure 5), although this is heavily dependant on the choice of column employed for the separation (see 3.1.4). The separation of disaccharides derived from chemical degradation using nitrous acid cleavage poses more of a challenge although it does possess the advantage of preserving the hexuronic acid residue identity. Detection can be achieved by reducing end labelling with radioactivity (3H-borohydride) to overcome the lack of a sensitivity of the chromophore. Both the polymer based Propac and the C18 CTA column method can also be used to separate larger saccharides [87, 88].

1.4.4 Synthesis of heparan sulphate and heparin

Initially, the possibility of chemically creating oligosaccharides from simple carbohydrate pre-cursors to yield oligosaccharide structures would seem an attractive prospect. One drawback to such an approach is the absence of defined binding structures or even common patterns / motifs to which chemical synthesis could be applied. Where such sequence data does exist, especially if of pharmaceutical relevance, successful attempts have been made to synthesis chemical homologues of naturally occurring oligosaccharides [89-91]. Examples of synthetically engineered polysaccharides / oligosaccharides include interactions / binding with platelets [92], herpes simplex virus (HSV) [93], the fibroblast growth factor (FGF) family [94-100], and antithrombin III (AT-III) [89-91, 101-103].

The synthesis of a highly complex heparan sulphate or heparin related molecule is not a trivial undertaking [104], requiring many chemical preparations, modification (which are stereo-selective) and purification steps. The solvent incompatibilities and susceptibility of this class of carbohydrates to many common reaction conditions necessitates an array of protecting groups to be employed during synthesis. The leads to what is generally a limited, arduous and tedious process for the creation of usually comparatively small oligosaccharides species, frequently in low yields. The interested reader is directed to the book chapter by Noti *et al.* [105] and the review by Polleti and Lay [104] as a starting point for reaction and mechanistic details.

These physico-chemical restrictions, coupled to poor availability of interacting sequence structures, prohibit the practical use of chemical synthesis as a tool in the creation of test sequences / structures in order to probe for highly specific interactions (in a library – shotgun approach) due to time and cost constraints. As a consequence, the use of degradative strategies to generate oligosaccharides is normally employed using heparin as a starting material [see 1.4.5]. This approach is not without limitations. The large amounts of starting polysaccharide required, the cost of de-polymerisation and the

subsequent modifications, separations and purifications leads to only small amounts of workable pure sequences, but scale-up is possible if desired.

It is foreseen that a combination of both the degradative and synthetic strategies will be utilised in the future, the former to probe and elucidate interacting structures and the latter to generate worthwhile amounts of saccharide for binding / activity assays and potential administration as pharmaceuticals (where the cost of production is offset by the future applications).

1.4.5 Synthetic analogue libraries for heparan sulphate and heparin

In an attempt to combat the overlying problem of the lack of workable amounts of pure oligosaccharides derived from heparin and heparan sulphate, semi-synthetic chemically modified analogue libraries were created by Yates *et al.* [106]. The primary polysaccharide library consists of intact heparin and 7 chemically modified polysaccharides, each with a single modification carried out to (near) completion. A more detailed explanation of the creation of these libraries and their structures is given in 5.2.5 where they are directly applied. It should be noted that these libraries work in a complementary manner to that of synthesis, i.e. an already highly functionalized polymer chain e.g. heparin is the starting material and this is selectively simplified by the removal of its constituent groups to yield products which can be used to interrogate interactions and start to address the necessary structural motifs related to a particular activity.

These chemical libraries provide a tool to deconstruct the structure of binding motifs. Although this can provide useful detail with regard to the need for the presence or absence of particular groups it must be considered at all times that these are modified heparins and not HS polysaccharides. Hence the domain structure is not present as in HS and the overall sequence pattern within a polysaccharide will represent that of a minimalised heparin not HS. Furthermore the chemical modifications may or may not provide conformations or even sequences which are found occurring naturally in HS. This may not be of concern in the elucidation of new pharmaceuticals. Therefore the

ablation of an interaction or subsequent activity in a chemically modified heparin may be due to overall conformation as much as sequence. The possibility of domains interacting to facilitate binding in HS by overall conformation is a real possibility and one which should not be ignored due to its complexity. A single modification within a polysaccharide chain may have serious conformation implications throughout the polymer.

1.5 Thesis aims

The aims of this section of the thesis are to attempt the improvement of reducing end labelling by investigating labelling conditions, coupling chemistries, separation techniques and improved fluorescent tags.

The thesis will also look at an alternative strategy for labelling of this class of carbohydrate molecules by attacking the saccharide fragments in a way previously exploited with the desire to improve the efficiency of labelling and therefore the yield. Samples of chemically modified heparins will be interrogated in both the polysaccharide and de-polymerised, oligosaccharide form, in an attempt to disrupt a medically relevant interaction which is though to occur between red blood cells and *Plasmodium falciparum* infected erythrocytes which can be fatal in malaria.

Chapter 2

Methods and materials

2.1 Materials

Acetic acid; glacial 99.997%. Aldrich, UK. Catalogue No. 338826

Acetic anhydride; $^{13}C_2$. Sigma, UK. Catalogue No. 486848

Acrodisc syringe filters; PVDF 0.2um. Sigma, UK. Catalogue No. Z260304

2-amino acridone (AMAC). Fluka, UK. Catalogue No. 06627

Ammonium hydrogen carbonate; Reagent plus. Sigma, UK. Catalogue No. A6141

Bodipy ® FL hydrazide, 4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene-3-propionic acid, hydrazide. Invitrogen, UK. Catalgoue No. D2371

Borate buffer, 50 mM pH 8.0. Fluka, UK. Catalogue No. 82633

Bradykinin fragment 1-7; ≥97% (HPLC). Sigma, UK. Catalogue No. B1651

Calcium acetate; laboratory reagent grade. Fisher Scientific. Catalogue No. C/0920/53

Calcium acetate, ReagentPlus®, ≥99% (titration), powder. Sigma, UK. Catalogue No. C1000.

Chloroauric acid; 99.999%. Aldrich, UK. Catalogue No. 254169

Chondroitin sulphate disaccharide; Δdi-0S sodium salt. Sigma, UK. Catalogue No. C3920

Compressed gas; 1 % O_2, 3 % CO_2 and 96 % N_2, BOC, UK.

Deuterium oxide (D_2O); 99.9% atom D. Aldrich, UK. Catalogue No. 151882

Dibutyltin dichloride. Sigma, UK. Catalogue No. 20,549-4

Dimethyl sulphoxide (DMSO); anhydrous 99.9%. Aldrich, UK. Catalogue No. 27,685-5

Dionex Propac-PA1; 4 x 250 mm analytical column. Dionex, UK. Catalogue No. 039658

Dionex Propac-PA1; 9 x 250 mm analytical column. Dionex, UK. Catalogue No. 040137

Di-sodium hydrogen phosphate; ≥98%, powder, cell culture tested, insect cell culture tested. Sigma, UK. Catalogue No. S5136

DY-495 hydroxyl amine. Dyomics, GmbH Germany. Catalogue No. DY495aminooxy

Ethanol; anhydrous. Aldrich, UK. Catalogue No. 277649

Ethidium bromide; 10 mg/mL, for molecular biology, aqueous solution. Sigma, UK. Catalogue No. E1510

Ethylenediaminetetraacetic acid disodium salt dihydrate (EDTA.Na_2). Sigma, UK. Catalogue No. E1644

Fibroblast growth factor II (FGF-2); carrier free. R&D Systems, UK. Catalogue No. 233-FB-025/CF

Formamide; spectrophotometric grade, >99%. Sigma, UK. Catalogue No. 295876

Gentamicin sulphate; liquid, sterile-filtered, cell culture tested . Sigma, UK. Catalogue No. G1397

Gentisic acid; sodium salt. Sigma, UK. Catalogue No. G5129

Giemsa stain; for staining. Fluka, UK. Catalogue No. 48900

Glucose; powder, ≥99.5%, insect cell culture tested. Sigma, UK. Catalogue No. G7021

L-glutamine; ≥99%, from non-animal source, cell culture tested. Sigma, UK. Catalogue No. G8549

Glycerol. cell culture tested, insect cell culture tested, ~99% (GC). Sigma, UK. Catalogue No. G2025

HEPES buffer; 1M in H_2O. Fluka, UK. Catalogue No. 83264

HBS-P buffer; 6 x 200ml. Biacore AB, UK. Catalogue No. BR-1003-68

Heparatinase I; in 5% sucrose. Ibex, Canada. Catalogue No. 50-012

Heparatinase II; in 5% sucrose. Ibex, Canada. Catalogue No. 50-011

Heparatinase I; in 5% sucrose. Ibex, Canada. Catalogue No. 50-010

2-O-desulphated heparin. Modified from porcine mucosal heparin.

2-O-desulphated N-acetyl heparin. Modified from porcine mucosal heparin.

6-O-desulphated heparin. Modified from porcine mucosal heparin.

6-O-desulphated N-acetyl heparin. Modified from porcine mucosal heparin.

2-O and 6-O-desulphated heparin. Modified from porcine mucosal heparin.

2-O and 6-O-desulphated N-acetyl heparin. Modified from porcine mucosal heparin.

Heparin; bovine lung, sodium form. Merck Biosciences, UK. Catalogue No. 375093

Heparin; porcine intestinal mucosal, sodium form, 201IU.mg^{-1}. Celsus Inc., USA. Catalogue No. PH-03004

Heparan sulphate; porcine intestinal mucosal, sodium salt. Sigma, UK. Catalogue No. H9902.

HiPrep ® 26/10 desalting column. Amersham Pharmacia, Sweden. Catalogue No. 17-5087-01 (stored in 20% ethanol, 4° C)

Histopaque®-1077; density: 1.077 g/ml at 25 °C, aseptically filled. Sigma, UK. Catalogue No. 10771

Human Serum; pooled. Sera labs, UK. Catalogue No. S-123-H

Hyaluronic acid disaccharide; ≥98%. Sigma, UK. Catalogue No. H9649

Hydrochloric acid; 37%. Aldrich, UK. Catalogue No. 25814,8

Mercuric acetate; puriss. p.a., ACS reagent, ≥99.0%. Fluka, UK. Catalogue No. 83352

Methanol, Chromanorm HPLC gradient grade. VWR, UK. Catalogue No. 2086.320

Methionine; puriss., ≥99.0%. Fluka, UK. Catalogue No. 64340

Nalgene® SFCA filter unit; 0.2 um pore, 50 mm membrane diameter, 500 ml. VWR, UK Catalogue No. 156-4020

N-Ethyl-*N*'-(3-dimethylaminopropyl)carbodiimide (EDC); purum, 97%. Fluka, UK. Catalogue No. 39391

Nitrogen, compressed liquid. BOC, UK.

1,8 Octanedithiol; ≥97%. Aldrich, UK. Catalogue No. O3605

Pd-10 Size exclusion column; de-salting. GE Healthcare, UK. Catalogue No. 17-0851-01

Phenylsilane; purum >97%. Sigma, UK. Catalogue No. 79174

Phosphoric acid; 99.999%. Sigma, UK. Catalogue No. 466132

Plasmagel ®; Laboratoire Roger Bellon, France.

Potassium chloride; ReagentPlus®, ≥99.0%. Sigma, UK. Catalogue No. P4504

Potassium chloride. cell culture tested, insect cell culture tested, ≥99%, powder. Sigma, UK. Catalogue No. P5405

Potassium di-hydrogen phosphate; ACS reagent, ≥99.0%. Sigma, UK. Catalogue No. P0662

RPMI 1640 medium; with sodium bicarbonate, without L-glutamine. Sigma, UK. Catalogue No. R0883

SIA Kit Au; Biacore sensor chips. Biacore AB, UK. Catalogue No. BR-1004-05

Silica capillary, bare fused, 50 um. Microsolv, USA. Catalogue No. 04051-C25

Slide-A-Lyser ® dialysis cassette; Pierce, UK. Catalogue No. 66107

Sodium acetate; biochemical grade. BDH, UK. Catalogue No. 4438941C

Sodium acetate; powder, ≥99%, cell culture tested, insect cell culture tested. Sigma, UK. Catalogue No. S5636

Sodium borohydride; powder 98%. Aldrich, UK. Catalogue No. 45,288-2

Sodium citrate; Sigma, UK. Catalogue No. S1804

Sodium di-hydrogen phosphate; ACS reagent, ≥99.0%. Sigma, UK. Catalogue No. S9763

Sodium chloride; HPLC grade. Fisher Scientific, UK. Catalogue No. S/3165/53

Sodium cyanoborohydride; >95% purum. Fluka, UK. Catalogue No. 71435

Sodium hydrogen carbonate; SigmaUltra, ≥99.5%. Sigma, UK. Catalogue No. S6297

Sodium hydroxide; 1N for HPCE. Fluka, UK. Catalogue No. 72082

Sodium hydroxide; 1N volumetric standard. Sigma, UK. Catalogue No. S2567

Sodium hydroxide; 10N in H_2O. Aldrich, UK. Catalogue No. 656054

Sodium hydrogen phosphate; 99.995%. Sigma, UK. Catalogue No. 255743

Sodium lactate; puriss., ≥99.0%. Fluka, UK. Catalogue No. 71720

Sodium phosphate; 96%. Aldrich, UK. Catalogue No. 342483

Sodium triacetoxyborohydride; 95%. Aldrich, UK. Catalogue No. 31,639-9

Sorbitol; cell culture tested. Sigma, UK. Catalogue No. S3889

Superdex-30 ® resin; preparatory grade. Amersham Pharmacia, Sweden. Catalogue No. 17-0905-03 (stored in 20% ethanol, 4° C)

TSK-GEL G3000PW_{XL} size exclusion column; 6 um pore, 7.8 mm ID & 30 cm length; Supelco, UK. Catalogue No. 808021

Tetrahydrofuran; Chromasolv® Plus, for HPLC, ≥99.9%, inhibitor-free. Sigma, UK. Catalogue No. 34865

Water; HPCE grade. Fluka, UK. Catalogue No. 95283

Water; Chromanorm HPLC grade. VWR, UK. Catalogue No. 23596.320

Whole blood; type O^+; Oxford blood supplies, UK.

XK 16 size exclusion column; GE Healthcare, UK. Catalogue No. 18-8776-01

2.2 Methods

2.2.1 The creation of polysaccharides for subsequent digestion to Δ-disaccharides

The eight parental chemically modified polysaccharides were supplied by Dr E Yates. They were prepared as described in Yates *et al.* [107]. Table 14 [section 5.2.1] outlines the parent chemically modified polysaccharides used and the predominant repeat unit which is contained within each polysaccharide chain. The chemically modified polysaccharides contain all eight common naturally occurring disaccharide subunits.

2.2.2 Production of Δ-disaccharides by enzymatic digestion of parent polysaccharides

A mixture containing 5mg of all eight parent chemically modified polysaccharides was made up to a final volume of 80 µl. 20 µl of lyase digestion buffer (1.2 M sodium acetate, 12.5 mM calcium acetate, pH 7.0) was added to the mixture along with the addition of 100 mU of heparatinase I, 100 mU of heparatinase II and 100 mU of heparatinase III. The reaction mixture was incubated for 24 hours at 27°C to allow for total digestion to occur. The sample was heated to 100°C for 5 minutes to denature the three enzymes present at the end of the overnight incubation.

2.2.3 Size separation of oligosaccharides created by lyase digestion of polysaccharides

Oligosaccharides and disaccharides created by lyase digestion were separated into individual size pools by size exclusion chromatography. Separation was carried out on an Amersham Biosciences Akta purifier FPLC machine (as per the equipment section) fitted with two XK-16 columns in series (30 mm x 200 cm), pre-packed with Superdex® 30 resin. The sample was made up to a volume of 1 ml with HPLC water and introduced onto the column using an appropriate sized loop at a flow rate of 0.5 $ml.min^{-1}$ in 0.5 M ammonium hydrogen carbonate solution. The run was carried out isocratically at the same flow rate in 0.5 M ammonium hydrogen carbonate buffer for 1 column volume (500 ml). 2 ml fractions were collected between elution volumes of 100 ml to 400 ml.

Detection was carried out in line using a UV detector set at λ=232 nm in order to detect the C=C double bond introduced by bacterial lyase digestion. Collected fractions were isolated and pooled into size separated pools (compared to that of known chromatograms defined by polyacrylamide gel electrophoresis of mass calibrated size standards).

2.2.4 Salt removal by serial lyophilisation

Ammonium hydrogen carbonate salt present within the running buffer used for size exclusion chromatographic separation of sized oligosaccharides was removed using serial lyophilisation. Fractions collected from the XK-16 columns were frozen at -80°C and lyophilized until the sample was present as a dry powder. The lyophilized sample was re-suspended in 5 ml of HPLC water and lyophilized to dryness. The sample was again re-suspended and lyophilized.

2.2.5 Separation of eight disaccharide standards by strong anion exchange chromatography

The size separated disaccharides were further separated into individual species using strong anion exchange chromatography performed on a Schimadzu SCL-10AVP system equipped with a Dionex Propac-PA1 preparative SAX column (9 x 250 mm). The column was pre-washed in 2 M sodium chloride for 30 minutes at a flow rate of 1 $ml.min^{-1}$ before equilibration for 1 hour in HPLC water at the same flow rate. The sample was made up to a final volume of 1ml and injected onto the column in water. The flow was held constant isocratic in HPLC water for 1 minute allowing unbound material to pass through the column to waste. A 0 – 1.5 M sodium chloride linear gradient was setup over a period of 67.5 minutes at a flow rate of 1 $ml.min^{-1}$ with the column maintained at 40°C. Detection was performed in line using a UV detector set at λ=232 nm in order to detect the C=C double bond present between C-4 and C-5 of the uronic acid residue introduced by lyase digestion. Fractions were collected at 15 second intervals (250 µl) using an automated fraction collector calibrated previously to a flow rate of $1ml.min^{-1}$.

The column was washed post-run by increasing the gradient from 1.5 M to 2 M over 2 minutes, followed by an isocratic wash step in 2 M sodium chloride for 90 seconds. The column was returned to flow in (isocratic) HPLC water and allowed to equilibrate for 5 minutes at a flow rate of 1 $ml.min^{-1}$. The elution profile for the digest was compared to that of a mixture of known disaccharide standards, pre-run under the same conditions. Disaccharides were identified based on their retention time compared to that of the reference chromatogram, and the relevant collected fractions pooled for subsequent lyophilisation.

2.2.6 Salt removal using size exclusion chromatography FPLC

Two HiPrep ® 26/10 columns (packed with G-25 fine; 25 mm O/D, 100 mm length per column) were connected in series on an Amersham Biosciences AKTA Purifier machine. The columns were flushed through with HPLC grade H_2O for 20 minutes at a flow rate of 5 $ml.min^{-1}$ in order to equilibrate the columns. Separation from small inorganic molecules (such as salts) was achieved using isocratic water flow at a rate of 5 $ml.min^{-1}$ using ultraviolet detection, in line at λ=215, 232 and 280 nm over a period of 38 minutes. 2ml fractions were collected throughout the run and the disaccharide molecules were generally contained in fractions 10 to 30 corresponding to molecules whose hydrodynamic volume is greater than that of the exclusion limit of the column. Small inorganic molecules whose size was within the fractionation range of the column, or smaller, eluted after 65 ml.

2.2.7 Reducing end labelling of Δ-disaccharides with the fluorophore 2-amino acridone

Unsaturated Δ-disaccharides were labelled as detailed below, as originally specified by Militsopolou *et al.* (2002) [108] 10 nanomoles of each Δ-disaccharide was lyophilized to dryness. The resulting powder was re-suspended in 5 µl of a 0.1 M AMAC solution in DMSO:glacial acetic acid (17:3 v/v) with sodium cyanoborohydride (1M) [or sodium triacetoxy borohydride or $NaBH_4$]. The reaction mixture was heated at 45°C for 4 hours (as per the original procedure), 8 hours or overnight. The samples were frozen and

lyophilized to dryness. The samples were re-suspended in a mixture of 50:50 v/v DMSO:H_2O and diluted as required in HPCE / HPLC water.

2.2.8 Reducing end labelling of Δ-disaccharides with the D-495 hydroxylamine

Unsaturated Δ-disaccharides were labelled with the novel fluorophore D-495 hydroxylamine. The chemical structure can be found in section 2.3.4. 10 nanomoles of each Δ-disaccharide was lyophilized to dryness. The resulting powder was re-suspended in 5 µl of D-495 hydroxylamine (in methanol, 1mg.ml^{-1}) and the solvent removed by evaporation using a vacuum centrifuge heated to 36°C. The resulting powder was re-suspended in 5 µl of DMSO : glacial acetic acid 17:3 v/v (or methanol or formamide) and incubated at 45°C for 2 hours, 4 hours, 6 hours or overnight. Post incubation, 5 µl of a 1 M sodium borohydride solution (aqueous) was added to the reaction mixture and incubated at room temperature for 30 minutes. The reaction mixture was flash frozen in liquid nitrogen and lyophilised until dry and the resulting powder re-suspended in a mixture of 50:50 v/v DMSO:H_2O and diluted as required in HPCE / HPLC water.

2.2.9 Reducing end labelling of Δ-disaccharides with the fluorophore Bodipy hydrazide

Unsaturated Δ-disaccharides were labelled with the fluorophore Bodipy Fl hydrazide. 10 nanomoles of each Δ-disaccharide was lyophilized to dryness. The resulting powder was re-suspended in 5 µl of Bodipy FL hydrazide (in methanol, 1.25 mg.ml^{-1}) and evaporated to dryness using a vacuum centrifuge heated to 36°C. The resulting powder was re-suspended in 5 µl of DMSO : glacial acetic acid 17:3 v/v (or methanol or formamide) and incubated at room temperature for 4 hours, 6 hours or overnight. Post incubation, 5 µl of a 1 M sodium borohydride solution (aqueous) was added to the reaction mixture and incubated at room temperature for 30 minutes. The reaction mixture was flash frozen in liquid nitrogen and lyophilised until dry and the resulting powder re-suspended in a mixture of 50:50 v/v DMSO:H_2O and diluted as required in HPCE / HPLC water.

2.2.10 Analysis of fluorophore purity by normal mode and reverse mode capillary electrophoresis

A 50 μm un-fused bare silica capillary (50 cm effective length) was washed successively for three minutes in 1 M sodium hydroxide, three minutes in HPCE water and 5 minutes in either 50 mM borate running buffer, pH 8.0, (normal mode) or 50 mM phosphate buffer, pH 3.5 (reverse mode) prior to use. A solution of each fluorophore (1 $mg.ml^{-1}$) was diluted 1 in 1000 in HPCE water and the analytes injected (individually) on to the capillary at the left hand electrode using pressure injection for 15 seconds at 0.5 p.s.i. Capillary electrophoresis was performed at 22 kV in normal mode for 25 minutes or at 30 kV in reverse mode for 40 minutes. After 25 and 40 minutes respectively, the voltage was increased to 30 kV and a pressure of 0.5 p.s.i. imposed over the capillary from the left hand electrode to the right hand electrode in order to wash off the capillary any remaining analyte. Detection was performed in-line using laser-induced fluorescence with excitation at λ=488nm and detection at λ=520nm. The capillary was washed, as above, at the end of each run prior to subsequent use.

2.2.11 Detection of eight individual Bodipy labelled Δ-disaccharides by high performance reverse mode capillary electrophoresis

A 50 μm un-fused bare silica capillary (50 cm effective length) was washed successfully in three minutes in 1 M sodium hydroxide, three minutes in HPCE water and 5 minutes in 50 M phosphate running buffer, pH 3.5, prior to use. A solution of each fluorophore labelled Δ-disaccharide glyco-conjugate (made up in DMSO:H_2O 50:50 v/v prior to use) (the analyte) was injected (individually) on to the capillary at the (-ve) left hand electrode using pressure injection for 15 seconds at 0.5 p.s.i. Capillary electrophoresis was performed at 30 kV in 50 mM phosphate buffer, pH3.5 using reverse mode for 30 minutes. After 30 minutes the voltage was increased to 30 kV and a pressure of 0.5 p.s.i. imposed over the capillary from the left hand (-ve) electrode to the right hand (+ve) electrode, in order to wash off the capillary any remaining analyte, for 8 minutes. Detection was performed in-line using laser-induced fluorescence with excitation at

λ=488nm and detection at λ=520nm. The capillary was washed, as above, at the end of each run prior to subsequent use.

2.2.12 Determining the detection limit of Bodipy labelled disaccharides by high performance reverse mode capillary electrophoresis

Individual disaccharides (from 2.2.11) were serially diluted in HPCE water to determine the level of detection. Dilutions were carried out to give, 1:100,000, 1:200,000, 1:1,000,000 and 1:2,000,000 dilutions. In order to minimize run times, consumables and maximise laser life, run times were adjusted to finish after the migration time expected for the disaccharide (this can be estimated from previous runs and based on the net charge of the disaccharide). The high voltage over-imposed pressure gradient was performed to ensure no residual analytes were present on the capillary. The following run times were used for the various fluorescent Δ-disaccharide glyco-conjugates: standard 6 : electrophoresis run time of 16 minutes; standards 4, 5 and 8 : electrophoresis run time of 16 minutes; standards 2, 3 and 7 : electrophoresis run time of 20 minutes and standard 1 : electrophoresis run time of 30 minutes.

2.2.13 Determining the linearity range with varying concentration ranges of Bodipy labelled disaccharides for high performance reverse mode capillary electrophoresis

In order to determine the linearity range with varying concentrations of Bodipy, serial dilutions were performed as above and the peak area recorded to allow for the relationship between area and amount to be determined.

2.2.14 Separation of a mixture of eight Bodipy labelled Δ-disaccharides by high performance reverse mode capillary electrophoresis

A 50 μm un-fused bare silica capillary (50 cm or 1 m effective length) was washed successfully in three minutes in 1 M sodium hydroxide, three minutes in HPCE water and 5 minutes in appropriate running buffer prior to use. A solution containing a mixture of

each fluorophore labelled Δ-disaccharide glyco-conjugate (made up in DMSO:H_2O 50:50 v/v prior to use) (the analyte) was injected (individually) on to the capillary at the (-ve) left hand electrode using pressure injection for 15 seconds at 0.5 p.s.i. Capillary electrophoresis was performed at various voltages (between 11 and 30 kV), concentrations of phosphate buffer (10 – 90 mM) and pH ranges (both equal and non-equal in the electrode buffer vials) using reverse mode for time periods up to 200 minutes. After the separation time had been reached the voltage was increased to 30 kV and a pressure of 0.5 p.s.i. imposed over the capillary from the left hand (-ve) electrode to the right hand (+ve) electrode, in order to wash off the capillary any remaining analyte. Detection was performed in-line using laser-induced fluorescence with excitation at λ=488nm and detection at λ=520nm. The capillary was washed, as above, at the end of each run prior to subsequent use.

2.2.15 Detection of eight individual Bodipy labelled Δ-disaccharides by high performance strong anion exchange chromatography

Detection of each of the eight fluorescently labelled Δ-disaccharides, and separation from free Bodipy hydrazide tag, was possible using a linear sodium chloride gradient imposed over an isocratic mobile phase of sodium hydroxide solution. In order to perform the detection, a Propac PA-1 column was regenerated by successive washes of 1 M hydrochloric acid for 60 minutes, HPLC water for 20 minutes, 1N sodium hydroxide for 60 minutes and HPLC water for 30 minutes, all at a flow rate of 1 $ml.min^{-1}$. The column was equilibrated in 150 mM sodium hydroxide solution for 20 minutes at a flow rate of 2 $ml.min^{-1}$ prior to use. Each fluorescently labelled disaccharide was made up to 1ml and injected on to the column in 150mM sodium hydroxide solution held isocratic for 11 minutes at a flow rate of 2 $ml.min^{-1}$ which allowed the free tag to elute. The fluorescently labelled disaccharide(s) was / were eluted with a linear gradient of sodium chloride elution solution (0 – 1 M over 30 minutes), with 150 mM of sodium hydroxide isocratic, at a flow rate of 2 $ml.min^{-1}$. Fluorescent detection was performed inline using a fluorimeter set at λ_{exc} = 488nm, λ_{emm} = 520nm, Following elution, the column was washed in 2 M sodium chloride, 300 mM sodium hydroxide solution for 3 minutes at a

flow rate of 2 $ml.min^{-1}$ before re-equilibration was performed prior to the next column run.

2.2.16 Labelling and separation of a mixture of eight Bodipy labelled Δ-disaccharides by high performance strong anion exchange chromatography

A mixture of all eight Δ-disaccharides (10 nanomoles each) was labelled as described previously [2.2.9] with 80 µl of Bodipy label (1.25 $mg.ml^{-1}$ in methanol) being used instead of 10 µl as per the original method. The mixture was re-suspended in 1 ml and separation and detection carried out as detailed in 2.2.15.

2.2.17 Determining the detection limit of Bodipy labelled disaccharides by high performance strong anion exchange chromatography

The limit of detection for the Bodipy labelled disaccharide mixture was determined by serial diluting the labelled mixture by 1:1,000, 1:10,000, 1:100,000, and 1:1,000,000. 1ml of each sample was injected onto the column as per the method detailed in 2.2.16.

2.2.18 Fluorescent labelling of porcine mucosal heparin and porcine mucosal heparan sulphate derived Δ-disaccharides

10ug of porcine mucosal heparin and porcine mucosal heparan sulphate were digested and labelled as per the method detailed in 2.2.9 except for the use of 80 µl of Bodipy hydrazide in methanol (1 $mg.ml^{-1}$) instead of 10 µl. Each sample was diluted and approximately 100 picomoles of the totally digested and labelled sample injected onto the column and separated and detected as for the mixture of fluorescently labelled disaccharide in 2.2.16.

2.2.19 Investigating the effect of acidic reaction conditions on the de-N-sulphation of heparin using ^{13}C N-acetylation and NMR

100 mg of porcine mucosal heparin was dissolved in 50 % acetic acid solution (v/v). The reaction mixture was incubated at room temperature or at 65°C for 4 hours or 2 hours respectively. Following reaction, 150 µl of saturated sodium hydrogen carbonate solution was added to each sample to neutralise the acid. Samples were lyophilised and re-suspended in 500 µl of HPLC grade water prior to desalting as detailed in section 3.2.9. The samples were lyophilised and re-suspended in saturated sodium hydrogen carbonate on ice. A 100 µl aliquot of ^{13}C labelled acetic anhydride was added to the mixture and incubated on ice for 2 hours. A further aliquot of acetic anhydride was added and the reaction mixture incubated for a further 2 hours. The mixture was then lyophilised and made up to 500 µl in HPLC water before de-salting as in section 2.2.25. The lyophilised sample was re-suspended in an appropriate volume of D_2O for NMR analysis.

2.2.20 The use of metal catalysis in the reducing end labelling of Δ-disaccharides with the fluorophore Bodipy hydrazide

Unsaturated Δ-disaccharides were labelled with the fluorophore Bodipy hydrazide using dibutyltin chloride as a catalyst as opposed to glacial acetic acid. 10 nanomoles of each Δ-disaccharide was lyophilized to dryness. The resulting powder was re-suspended in 5 µl of Bodipy FL hydrazide (1.25 mg ml^{-1} in methanol) and evaporated to dryness using a vacuum centrifuge heated to 36°C. The resulting powder was re-suspended in 5 µl of DMSO containing 8 nmoles of dibutyltin chloride and 16 nmoles of the reducing agent phenylsilane. The reaction mixture was incubated at room temperature for 4 hours. Following incubation, the reaction mixture was flash frozen in liquid nitrogen and lyophilised until dry and the resulting powder re-suspended in a mixture of 50:50 v/v DMSO:H_2O and diluted as required in HPCE / HPLC water.

2.2.21 The generation of UA-GlcNAc Δ-disaccharide (standard 1) polysaccharide precursor

The Δ-disaccharide UA-GlcNAc was generated from the parental chemically modified polysaccharide supplied by Dr E Yates which has all of the O-sulphates and the N-sulphates chemically removed. The polysaccharide chain was then completely N-acetylated. This chemically modified polysaccharide was prepared and characterised as described [107]

2.2.22 Complete enzymatic digestion of the chemically modified parent polysaccharide

5 mg of 2,6 O and N-de-sulphated chemically modified polysaccharide was made up to a final volume of 80 µl. 20 µl of lyase digestion buffer (1.2 M sodium acetate, 12.5 mM calcium acetate, pH 7.0) was added to the mixture along with the addition of 15 mU of heparatinase I and 15 mU of heparatinase II. The reaction mixture was incubated for 24 hours at 37° C to allow for total digestion to occur. The sample was heated to 100° C for 5 minutes to denature the two enzymes present at the end of the overnight incubation.

2.2.23 Separation of disaccharide standard 1 by strong anion exchange chromatography

Disaccharides generated in 2.2.22 were size separated as in 2.2.3 with the fraction corresponding to disaccharides collected. The fraction was then serially lyophilised as in 2.2.4 to remove the salt. The size separated disaccharides were further separated into individual pure species using strong anion exchange chromatography performed on a Schimadzu 10AVP system equipped with a Dionex Propac-PA1 preparative SAX column (9 x 250 mm) as described in section 2.2.5. The sodium chloride present after gradient elution was removed using two HiPrep 26/10 columns connected in series on an Amersham Biosciences AKTA Purifier machine as described previously in section 2.2.6.

2.2.24 De-polymerisation of bovine lung heparin polysaccharide

The bovine lung heparin polysaccharide was lyophilized and re-suspended in an appropriate volume of water to give a 100 mg ml^{-1} solution. An aliquot of lyase buffer was added to the heparin solution accordingly to yield a 1 : 5 dilution of the lyase buffer (1.2 M sodium acetate, 12.5 mM calcium acetate, pH 7.0). Heparatinase II enzyme was added to the solution in a ratio of 2.5 mU per mg of initial polysaccharide. The mixture was incubated at 37° C for 6 hours. Digestion was terminated by incubation at 100° C for 5 minutes post incubation. Samples were then size separated as in 2.2.3 and removed from inorganic salts present within the digestion buffer using serial lyophilisation (2.2.4) and size exclusion chromatography (see 2.2.25). Collected fractions were compared to chromatograms defined by polyacrylamide gel electrophoresis of mass calibrated size standards. Samples were lyophilized and stored frozen at -20°C until required.

2.2.25 Removal of organic salts from oligosaccharides fragments

A set of PD-10 (GE healthcare) size exclusion chromatography columns were equilibrated prior to use with 30 ml of H_2O under gravity flow. Each of the oligosaccharide sized pools were loaded individually onto the filter present at the top of the column, in a final volume of 500 µl. The 500 µl was allowed to enter the column before the addition of 1.5 ml of water. The breakthrough was allowed to pass to waste under gravity flow. A further 3 ml of water were added to the column and the eluent collected under gravity flow until elution was complete. This contained molecules excluded from the column matrix, generally comparable in size to that of a tetra-saccharide or larger. The bovine lung heparin oligosaccharides were present in this fraction and were lyophilised. Small molecules, such as inorganic salts are eluted from the column by the subsequent addition of 1ml aliquots of water (under gravity flow) up to 10 ml.

2.2.26 Oxymercuration of Δ-disaccharide standard 1

The model disaccharide standard 1, ΔUA-GlcNAc (10 μmoles), derived by heparatinase enzyme digestion and bearing an unsaturated 4,5 uronic acid at the non-reducing end was reacted in a mixture of water and THF (1:1 v/v) at 40° C for 2 hours in a solution containing 0.1 millimoles of mercuric acetate.

2.2.27 Removal of free mercuric acetate salts from the reaction mixture

Following reaction, the unspent mercuric salts were removed from the product using a PD-10 size exclusion column as in 2.2.25. The reaction mixture was loaded onto the filter present at the top of the column, in a final volume of 500 μl. The 500 μl was allowed to enter the column before the addition of 1.5 ml of water. The breakthrough was allowed to pass to waste under gravity flow. A further 3 ml of water were added to the column and the eluent collected under gravity flow until elution was complete. The eluate collected was lyophilised and made up to a total volume of 500 μl in a solution of 100 mM EDTA.Na_2 in order to remove any free mercuric acetate. The above procedure was repeated using 100 mM EDTA.Na_2 as opposed to water twice with lyophilisation in between. Finally the sample was made up to 500 μl with HPLC grade $H_2O$and the procedure repeated as above again to buffer exchange the mercury labelled disaccharide into water.

2.2.28 Reduction of standard 1

An aliquot of standard 1 derived from section 2.2.21-23 (10 μmoles) was added to an equal volume of 1 M sodium borohydride and incubated at 37° C for 30 minutes. The resulting solution was neutralized with an excess of sodium hydrogen carbonate (saturated) and de-salted as described in section 2.2.6. The final product was lyophilized and stored at – 20° C prior to oxymercuration.

2.2.29 Oxymercuration of bovine lung heparin oligosaccharides

A model oligosaccharide from bovine lung heparin of size octadecasaccharide, with a predominant repeat unit of ΔUA(2S)-GlcNS(6S) (10 μmoles), derived by heparatinase enzyme digestion and bearing an unsaturated 4,5 uronic acid at the non-reducing end was reacted in a mixture of water and THF (1:1 v/v in 1 ml) at 40° C for 2 hours in a solution containing 0.1 mmoles of mercuric acetate. Following reaction, the unspent mercuric salts were removed from the product using a PD-10 size exclusion column as detailed in section 2.2.27. The salt free product was lyophilised and stored at -20° C until required

2.2.30 MALDI-MS of reduced and non reduced model disaccharide ΔUA-GlcNAc

The Hg labelled model disaccharide standard [Hg(OAc)-UA-GlcNAc] glycoconjugate (10 μmoles) was re-suspended in 10 μl of dH_2O. 1 μl of the labelled disaccharide was diluted with 1ul of gentisic acid and spotted on to a silver coated MALDI-MS target. The remaining mixture was allowed to dry under an air stream A separate, identical Hg labelled disaccharide was reduced with $NaBH_4$, neutralised with $NaHCO_3$, desalted on a PD-10 column to reduce the carbonyl reducing end group of the Hg(OAc)-UA-GlcNAc and lyophilised prior to spotting. MALDI-MS was carried out in positive ion mode with 60-75% laser power on a Bruker MALDI-TOF MS machine. The MALDI-MS machine was mass calibrated prior to use using a reference standard suitable for the predicted mass range (bradykinin fragment 1-7, Mr = 756.85)

2.2.31 ^1H 1D, 2D COSY & TOCSY NMR of Hg-(OAc)-UA-GlcNAc and ^1H 1D NMR of Hg(OAc)-HA & Hg(OAc)-CS disaccharides.

Disaccharides of HS (ΔUA-GlcNAc), chondroitin sulphate and hyaluronic acid were labelled as in section 2.2.26, desalted as per section 2.2.27 and lyophilised prior to use. Unlabelled samples were also prepared as controls. The lyophilised samples were re-suspended in D_2O prior to NMR analysis. NMR experiments were carried out on a

Bruker 400 MHz instrument at 313 K. Chemical shifts were reported downstream from TSP as an external reference standard (0 ppm).

2.2.32 Conjugation of Hg-labelled HS oligosaccharides to Au-thiol derivatized surfaces (in collaboration with Dr S J Patey)

Plain gold "SIA Au" sensor chip surfaces were incubated with 5 mM 1,8-octanedithiol in 100% ethanol for 20 hours in order to derivatized the surface with an alkly chain presenting a terminal thiol group. The sensor chips were then washed in 100% ethanol to remove unbound material. The sensor chips were assembled as per the manufacturer's instructions and docked accordingly into a BIAcore 2000 machine. All experiments were performed in a HBS-P buffer at a flow rate of 30 $\mu l.min^{-1}$. 100 nM FGF-2 was injected and allowed to pass over the surface while binding was monitored. *Ex situ*, 2 $mg.ml^{-1}$ mercury-labelled dp16 oligosaccharide derived from bovine lung heparin was allowed to bind to the freshly derivatized 1,8-octanedithiol surface for 3 hours. This surface was docked into the BIAcore and flushed with 3mM $EDTA.Na_2$. 100 nM and 50 nM FGF-2 were injected across the surface and specific binding to the Hg-HS derivatized surface was monitored. 100 nM FGF-2 was then incubated with 0.1 $mg.ml^{-1}$ heparin for 15 minutes prior to injection over the dp-16 surface. Further controls of the 1,8-octanedithiol surface with unlabelled heparin fragments or mercury acetate were also performed.

2.2.33 Conjugation of Hg-labelled HS oligosaccharides to Au nanoparticles (in collaboration with Dr NTK Thanh)

A stock solution of the peptide CALNN (cysteine-alaninie-leucine-asparagine-asparagine) was prepared by dissolving in phosphate buffer (160 mM NaCl, 3 mM KCl, 8 mM Na2HPO4, 1 mM KH2PO4). Concentrated NaOH was used to adjust the pH to 7.2 and the resulting 1.9 $mM.ml^{-1}$ solution was filtered through a 0.2 mm filter and stored in aliquots at -80° C. Gold nanoparticles (12.3 nm) were prepared by citrate reduction of chloroauric acid ($HAuCl_4$) [109]. A solution of $HAuCl_4$ (aqueous, 100 mL, 1 mM) was

refluxed for 5-10 min, and a warm (50-60° C) aqueous solution of sodium citrate (10 mL, 38.8 mM) was added quickly to the $HAuCl_4$. Reflux was continued for another 30 minutes until the solution was observed to become deep-red in colour. The solution was then filtered through a 0.45 μm Millipore ® syringe filter to remove any precipitated matter and the pH was adjusted to 7.0 using a dilute NaOH solution. The filtrate was stored at room temperature. CALNN-capped gold nanoparticles were prepared by mixing 12.3 nm citrate gold nanoparticles with a peptide stock solution in a volume ratio of 10 to 1. The excess peptide was removed by size exclusion chromatography using a G25 gel matrix running in 120 mM phosphate buffer at pH 7.4.

50 μl of 1 M EDC was added to 400 μl of Au-CALNN (OD~0.32) while vortexing, and the reaction tube was left to stand for 15 minutes. To the reaction mixture 50 μl of 0.33 M methionine was added and incubated at room temperature for 1 hour. Excess reagents were removed by dialysis in a Slide-A-Lyser ® dialysis cassette (Pierce) over night in 1l of 120 mM phosphate buffer. A model oligosaccharide of size dp16 (16-mer, predominant repeat ΔUA-GlcNAc, 0.25 mg, 0.63 μmoles) was derived from bovine lung HS by heparatinase enzyme digestion, as for heparin in section 2.2.24 with heparatinase I (2.5 $mU.mg^{-1}$) being used as opposed to heparatinase II or III. The oligosaccharide was isolated using size exclusion chromatography as per section 2.2.3 and the resultant size separated fractions pooled and desalted as in section 2.2.25. The salt free product was lyophilised and stored at -20° C prior to use. The dp16 fragment was labelled with mercury as for the heparin fragment in section 2.2.29. 1 μl of dp16-Hg was added to 100 μl of Au-CALNN-Met the reaction was monitored using a scanning UV/Vis spectrophotometer over the range λ= 350 - 900 nm after a reaction time of 30 minutes.

2.2.34 Culture medium

The following two media were used throughout parasite culture:

Complete medium – "Yellow"

18.75 ml of 1 M HEPES buffer, 5 ml of 20 % (w/v) glucose, 3 ml of 1 M sodium hydroxide, 5 ml of 200 mM L-glutamine and 250 µl of gentamicin sulphate (all pre-filtered through a 0.22 um filter) were added to 500 ml of RPMI-1640 medium with pre-added sodium bicarbonate. The solution was mixed thoroughly and again filtered using a Nalgene® SFCA 0.2 um pore, 50 mm membrane diameter, 500 ml filter unit. The solution was stored for up to 1 week at 4° C.

Growth medium – "Blue"

15 ml of 1 M HEPES buffer, 4 ml of 20 % (w/v) glucose, 2.4 ml of 1 M sodium hydroxide, 4 ml of 200 mM L-glutamine and 200 µl of gentamicin sulphate (all pre filtered through a 0.22 um filter) were added to 400 ml of RPMI-1640 medium with pre-added sodium bicarbonate. 40 ml of pooled human serum was added. The solution was mixed thoroughly and again filtered using a Nalgene® SFCA 0.2 um pore, 50 mm membrane diameter, 500 ml filter unit. The solution was stored for up to 1 week at 4° C.

2.2.35 Giemsa staining

In order to inspect the condition of the parasite culture and / or access the level of parasitaemia, cells were stained using Giemsa solution. Giemsa stain is a differential stain which is used to stain protozoan parasites such as *Plasmodium* a different colour to that of the red blood cells. Red blood cells stain pink while the parasites are stained purple. The stain is composed of a mixture of methylene azure, eosin, methanol and glycerol and has found widespread use in the diagnosis of malaria. Phosphate buffer was prepared by the addition of 1 g of di-sodium hydrogen phosphate and 0.7 g of potassium di-hydrogen phosphate to 1 l of double distilled milliQ® water. The solution was adjusted to pH 7.2 by the addition of 2 % (w/v) di-sodium hydrogen phosphate (to raise the pH) or 2 % potassium di-hydrogen phosphate (to lower the pH). The phosphate buffer solution was

filtered through a 0.2 um filter and stored in a sealed container out of direct sunlight at room temperature. Giemsa staining solution was prepared by the addition of 10 ml of stock solution to 90 ml of phosphate buffer (prepared as above). The solution was thoroughly mixed and filtered using a 0.4 um syringe filter.

Using a 2 ml aspirating pipette, an aliquot of parasite culture was removed by scraping the pipette over the lower surface of the tissue culture flask. The aliquot was spotted on to a microscope slide and a thin film smear made by carefully dragging a second microscope slide at a 45 ° across the surface. The slides were allowed to dry before an excess of methanol was pipetted over the surface of the slide and allowed to stand for 30 seconds in order to fix the red blood cells. Methanol was then removed by evaporation by waving in a warm air stream. The fixed cells upon the slide were then overlaid with an excess of 10 % Giemsa stain and allowed to stand for 15 minutes at room temperature. The excess Giemsa solution was then washed off the slide surface with water. The slide was blotted, on both the reverse side and bottom edge, to remove excess water. The slide was dried under a warm air stream before analysis. Analysis of the stained slide was carried out using oil immersion light microscopy under an x100 objective lens. Parasitaemia were obtained by counting, in triplicate between, 200 – 250 red blood cells.

2.2.36 Reconstitution of frozen P. falciparum culture stabilates

Parasites required for continuous culture were obtained from stabilates which had been stored in liquid nitrogen. When required, the stabilates were removed and allowed to warm to 37° C in a suitable incubator. Upon thawing the stabilates were transferred to a 50 ml centrifuge tube. A volume equal to 1/5 th of the total stabilate volume of 12 % (w/v) sodium chloride was added to the stabilate, in 10 µl aliquots, drop wise with continuous agitation. Post addition, the mixture was allowed to stand for 5 minutes at room temperature. 5 ml of 1.8 % (w/v) sodium chloride was added to the mixture in 100 µl aliquots over a 5 minute time period with constant, gentle agitation. The suspension was allowed to stand for a further 5 minutes at room temperature. 5 ml of 0.9 % (w/v) sodium chloride containing 0.2 % (w/v) glucose was added to the mixture in 100 µl

aliquots over a 5 minute time period with constant, gentle agitation. The suspension was again allowed to stand for a further 5 minutes at room temperature.

After equilibration, the erythrocytes were pelleted by centrifugation at 1800 rpm for 5 minutes in a bench top centrifuge. The supernatant was removed using an aspirator and the pellet gently re-suspended in 25 ml of pre-warmed (37° C using a water bath) serum free culture medium (yellow). The suspension was inverted several times to wash the red blood cells. The erythrocytes were re-pelleted at 1800 rpm for 5 minutes and the supernatant removed again using an aspirator. The red blood cells were re-suspended in a suitable volume (see 2.2.38) of pre-warmed (37° C) growth medium and transferred to a clean and sterile tissue culture flask. Gas (1 % O_2, 3 % CO_2 and 96 % N_2) was bubbled through the medium using a sterile pipette for approximately 20 seconds. The flask was immediately sealed and transferred to a 37° C incubator. 24 hours after the onset of incubation, the parasite culture was removed from incubation and a thin smear of culture medium analysed (see 2.2.35) in order to assess the degree of parasitaemia. The culture was then washed and diluted in an appropriate volume of washed uninfected red blood cells and growth medium as per parasite culture conditions (see 2.2.38).

2.2.37 Human red blood cell preparation

Human plasma reduced whole blood containing type O^+ red blood cells were obtained pre-packed in an anticoagulant solution. The red blood cells were then subject to a washing procedure to separate erythrocytes away from anticoagulant (NB control experiments without added heparins were also conducted (see 5.2.4, 5.2.5, 5.2.7 & 5.2.9), white blood cells and other undesired components. Blood was stored for up to four weeks in the unwashed form at 4° C. 5 ml of complete (serum free, "yellow") medium was added to 5 ml of human plasma reduced whole blood. The solution was agitated well to mix. This mixture was carefully overlaid on top of 5 ml of Histopaque®-1077 solution to prevent mixing. The preparation was centrifuged for 15 minutes at 3000 rpm in order to remove the white blood cells. The supernatant was carefully aspirated in order to not disturb the pelleted erythrocytes. 20 ml of complete medium (serum free) was added to

the erythrocyte pellet. The pellet was carefully re-suspended and agitated to wash the cells. The suspension was then centrifuged for 3 minutes at 3000 rpm. The supernatant was carefully aspirated off. The whole of this wash step was then repeated again using 10 ml of complete medium. Finally, the erythrocyte pellet was re-suspended in an equal volume of complete (serum free) medium to yield a 50 % red blood cell solution. This was stored at 4° C for a period not in excess of 10 days.

2.2.38 Continuous culture of Plasmodium falciparum

Parasites were cultured throughout at 37° C under a low oxygen tension. Temperatures above 37° C lead to the production of heat-shock proteins while the parasites are killed at temperatures in excess of 40° C. The parasites required regular changes of growth media every 24 hours in order to provide a continual supply of the required nutrients. The parasites have a life cycle of approximately 48 hours when present in the erythrocytic cycle stage of their development. Therefore the parasites are present in the late trophozoite stage every 2 days after which they form schizonts laden with infectious daughter cells. The rupturing of these schizonts and the invasions of fresh red blood cells by daughter merozoites causes the culture to expand.

The multiplication rate of the culture *in vivo* is thought to be approximately 5 fold with each growth cycle. The parasites had to be regularly assessed for their stage and the parasitaemia level of the culture was determined regularly (see 2.2.35). Furthermore they were diluted every 48 hours and given fresh washed red blood cells before the formed schizonts had ruptured. This maintained a steady parasitaemia and thus healthy parasites. Generally optimal culture conditions were considered to be a culture containing 10 – 25 µl of packed red blood cells per unit culture (1 – 2 % haematocrit) with a parasitaemia between 5 – 10 % (50 – 100 µl of packed parasitized red blood cells per ml of total packed blood cells). Usually 1 µl of packed red blood cells is considered to contain approximately 1 x 10^7 cells. The volume of fresh media required during culture was calculated using the formula:

Medium volume (ml) = Parasitaemia x packed total red blood cell volume (ml) x 10

In order to maintain the culture at a given parasitaemia or expand the culture for splitting, the culture was given fresh washed red blood cells every 48 hours. The culture replicates on average 5 fold every cycle so, by maintaining the correct ratio of blood from the previous culture cycle to that of fresh blood added, the parasitaemia and packed red blood cell volume can be controlled. The size of flask (non vented screw cap) used to grow the culture depended on the packed red blood cell volume and the amount of medium present. The following guide-lines were observed:

- <= 200 µl of packed red blood cells in 10 – 20 ml of medium – 25 cm^2 tissue culture flask
- 500 µl – 1000 µl of packed red blood cells in 25 – 45 ml of medium – 75 cm^2 tissue culture flask
- >= 1000 µl of packed red blood cells in 50 – 70 ml of medium – 175 cm^2 tissue culture flask

In order to change the medium, the spent culture was transferred to a sterile 50 ml centrifuge tube and spun at room temperature, 1800 rpm for 5 minutes. The supernatant was aspirated and discarded. The resultant pellet was re-suspended and agitated to wash in a 10 fold excess of serum free "yellow" culture medium (pre-warmed at 37° C) and spun again at room temperature, 1800 rpm for 5 minutes. The supernatant was aspirated and discarded. The pelleted red blood cells were re-suspended in the appropriate amount of growth "blue" medium (pre-warmed at 37° C) and fresh washed red blood cells (pre-warmed at 37° C). The suspension was transferred to a tissue culture flask of appropriate size before gas was applied to the flask using a sterile pipette for 20 seconds. The flask were sealed securely and returned to incubate at 37° C overnight.

2.2.39 Synchronization of Plasmodium falciparum cultures

Cultures which are allowed to grow unrestrained (so called "free wheeling") will rapidly become a mixture of differing life cycle stages. In order to counter this problem, two techniques were used to re-synchronize cultures.

2.2.39.1 Ring developmental stage enrichment by sorbitol lysis

The first technique employed, sorbitol lysis, relies on the parasite being present in a metabolically active state (this usually occurs 20 hours post invasion) and therefore being able to actively uptake molecules from the extra cellular matrix into the cell cytoplasm. This process is facilitated by embedded parasite proteins present in the infected erythrocyte's membrane. The presence of this mode of uptake makes the selection of differing stage parasites possible by the permeability of parasite infected erythrocytes to sorbitol solution. This method was employed to select for parasite infected red blood cells at the ring stage of development. The culture was transferred to a clean and sterile 50 ml centrifuge tube and centrifuged in a bench top centrifuge at room temperature, 1800 rpm for 5 minutes. The supernatant was removed by aspiration and the cells were re-suspended in a ten fold excess (by volume) of 5 % sorbitol solution. The mixture was incubated at 37° C for 20 minutes. After incubation the mixture was subject to centrifugation at room temperature, 1800 rpm for 5 minutes. The supernatant was removed by aspiration and the pelleted cells were re-suspended in serum free complete "yellow" medium and gently agitated in order to wash the cells and remove traces of sorbitol. The cells were pelleted using centrifugation at room temperature, 1800 rpm for 5 minutes. The supernatant was removed by aspiration and the cells were re-suspended in an appropriate volume of growth "blue" medium and transferred to an appropriate tissue culture flask as specified in the continuous culture methods. The culture was gassed, sealed and incubated overnight at 37 $^{\circ}$C.

2.2.39.2 Trophozoite developmental stage enrichment by gel flotation

The other method of culture synchronization that was used was flotation using gelatin based gel solutions. This method enriches the culture for trophozoite and schizont developmental stage parasite infected red blood cells. The parasite culture was transferred to a clean and sterile centrifuge tube. The red blood cells were pelleted by centrifugation at room temperature, 1800 rpm for 5 minutes. The supernatant was removed by aspiration. The red blood cell pellet was re-suspended in a mixture which comprised of a total volume 5 times that of the red blood cell pellet volume. The final solution contained red blood cells, serum free complete "yellow" medium and Plasmagel® present in the following ratio 0.2 : 0.3 : 0.5 accordingly. The mixture was transferred to a sterile and clean 15 ml centrifuge tube and incubated at 37° C for 20 minutes.

After incubation the supernatant was removed and transferred to a clean and sterile 15ml centrifuge tube for centrifugation at room temperature, 1500 rpm for 5 minutes. The supernatant was discarded and the pellet re-suspended in 10 ml of serum free complete "yellow" medium. The mixture was gently agitated to wash. The red-blood cells were re-pelleted by centrifugation at room temperature, 1500 rpm for 5 minutes. The parasitaemia was then assessed (see Giemsa staining) and the parasites returned to culture in an appropriate volume of washed red blood cells and growth "blue" medium as detailed in the continuous culture section. The culture was gassed, sealed and incubated overnight at $37^{\circ}C$.

2.2.40 Assay to determine the extent of rosette disruption

Cultured parasites required for the assay were screened using Giemsa staining prior to use for both the level of parasitaemia and general well being of the culture. Only parasite cultures where the parasitaemia was at least 5 % and below 8 % were used for the rosetting assay. The parasite culture selected for use in the assay was transferred to a clean and sterile 50 ml centrifuge tube. The red blood cells were pelleted by centrifugation at room temperature, 1500 rpm for 5 minutes. The spent culture medium

was removed carefully by aspiration. The parasites were carefully re-suspended and washed in 25 ml of pre-warmed (37° C) serum free complete "yellow" medium. The parasites were re-pelleted by centrifugation at room temperature, 1500 rpm for 5 minutes and the supernatant removed by aspiration.

The parasites were diluted (if necessary) with washed uninfected red blood cells (pre warmed to 37° C) to yield a total parasitaemia of 5 %. The cells were then gently re-suspended in a volume of growth "blue" medium (pre-warmed to 37° C) to ensure that the cells were present at a density of 100 µl of pelleted red blood cells per 1 ml of growth "blue" medium. Ethidium bromide was added to the cell suspension (2 µl of a 10 $mg.ml^{-1}$ solution per ml of cell suspension) in order to stain the parasite DNA and allow for the facile detection of infected red blood cells. The mixture was inverted several times to allow the suspension and stain to mix. For each experimental condition or sample to be tested, an aliquot of 150 µl of the suspension was transferred in to an individual Bijou tube. Three control tubes were also similarly setup and labelled 0 minutes, 15 minutes and 30 minutes. The 0 minutes tube was at this stage set aside for counting as an initial control. The remaining Bijou tubes were transferred to an appropriate holder and placed upon a 3 axis rocker which allowed for gentle mixing in a circular motion at the bottom of the Bijou tubes of the suspension for 15 minutes. After this step the 15 minute control was removed.

To the remaining tubes (except the 30 minute control) the test sample (heparin or chemically modified heparin poly / oligosaccharides) was added at an appropriate concentration. (see methods for individual assays). The samples were again mixed on the 3 axis rocker for a further 15 minutes. After the agitation period, an 8 µl aliquot of each tube was transferred onto a glass microscope slide and covered with a glass cover-slip prior to counting. The level of rosetting present within each sample was determined using oil immersion fluorescence microscopy (100x objective lens). Parasite infected red blood cells were counted in to two categories based on an assessment of their rosetting status (i.e. whether they were present as a rosette or as a single cell). A rosette was scored as an infected red blood cell surrounded by two or more uninfected red blood cells.

Experiments were conducted in triplicate with between 200 and 250 cells counted per replicate.

2.2.41 Cryo-preservation of Plasmodium falciparum

Throughout the duration of the experiments aliquots of culture were frozen for long term storage. This was a precautionary measure which allowed for the preservation of this particular lab strain of parasite in case of contamination or the need to stop experiments and restart at a later date (continuous culture for long periods of time proves expensive and time consuming). Parasites could only be frozen and stored using liquid nitrogen when they were present at the ring development stage. This involves the addition to the media of the parasite infected red blood cells of a preservative agent to protect the integrity of the parasites during freezing. 142.5 g of glycerol, 4 g of sodium lactate, 30 mg of potassium chloride and 3.9 g of sodium di-hydrogen orthophosphate were mixed with water and made up to 250 ml to create the cryo-preservative solution. The pH of the solution was raised to 6.8 with 5 M sodium hydroxide. The solution was passed through a 0.2 um filter before use. The parasite culture was transferred to a clean and sterile centrifuge tube and centrifuged at room temperature, 1800 rpm for 5 minutes. The supernatant was removed by aspiration and the pellet gently re-suspended in the residual volume. The volume of cryo-preservative needed was 1.67x the volume of the pelleted red blood cells. The cryo-preservative was added in aliquots of 1/5 th of the required volume. The first aliquot was added slowly over 1 minute. The solution was then allowed to equilibrate at room temperature for 5 minutes. The remaining aliquots were further added over a period of 5 minutes. The mixture of cryo-preservative and parasite infected red blood cells were transferred to 1 ml sterile freezing vials and were frozen using a freezer for 24 hours at -80° C. The vials were then transferred into liquid nitrogen for long term storage.

2.2.42 Size exclusion chromatographic determination of polysaccharide purity

All seven chemically modified polysaccharides and heparin were analyzed using high performance size exclusion chromatography in order to determine the level of purity of the polysaccharides and check for de-polymerisation caused by the chemical treatments. The experiments were carried out using a Dionex Bio-LC system as specified in the equipment sub section. Purified polysaccharides were lyophilized and re-suspended in an appropriate volume of HPLC grade water to give a 5 $mg.ml^{-1}$ solution. An aliquot of 200 µl of each polysaccharide solution was loaded onto at 250 µl loop and the sample injected on to a TSK-gel column and eluted isocratically in HPLC water at a flow rate of 1 $ml.min^{-1}$ over a total run time of 30 minutes. In-line detection of the polysaccharides was effected by the presence of the polysaccharides acetate group which absorbs in the UV at λ=200 nm along with majority of other molecules, in particular inorganic salts. Detection was also performed concurrently at λ=280 nm and λ=232 nm to identify any possible protein contaminants or digestion products respectively. Control samples of a sodium chloride dilution were performed at concentrations of 1 M sodium chloride. Also HP-SEC of sodium acetate at a concentration of 0.1 M was carried out.

2.2.43 Strong anion exchange chromatographic determination of polysaccharide purity

In order to determine the level of purity within the polysaccharide, the carbohydrate chain was digested using bacterial lyase enzymes to completion. A common analysis technique, disaccharide composition analysis, was then carried out upon the digested samples which allowed for the determination of the constituent disaccharides present within the original polysaccharide sample. This allowed for verification and quantification of the chemical modifications carried out. Polysaccharide samples were lyophilized and re-suspended in HPLC water to yield a final concentration of 1mg in 800 µl. A 200 µl aliquot of lyase buffer (1.2 M sodium acetate, 12.5 mM calcium acetate, pH 7.0) was then added to each of the samples. 2.5 mU / mg each of heparatinase I, II and III were added to the mixture prior to incubation at 36° C for 24 hours. Digestion was terminated by heating the samples to 100° C for 5 minutes in order to denature the heparatinase enzymes. The

enzymes were lyophilized and re-suspended in 200 µl of HPLC water prior to analysis. 200 µl of the test polysaccharide sample was loaded on to a 250 µl loop prior to injection. The sample was subject to strong anion exchange chromatography using a Dionex Bio-LC system equipped with a Dionex Propac-PA1 analytical SAX column (4 x 250 mm). The sample was injected onto the column in water and the flow held constant isocratic (in HPLC water) allowing unbound material to pass through the column to waste. A 0 – 1.5 M sodium chloride linear gradient was setup over a period of 67.5 minutes at a flow rate of 1 ml min-1. Detection was performed in line using a UV detector set at λ=232 nm in order to detect the C=C double bond present between C-4 and C-5 of the uronic acid residue introduced by lyase digestion. The profile was compared to that of a mixture of known disaccharide standards run under the same conditions and the relative amounts present calculated based on their representative areas.

2.2.44 Determining the anticoagulation activity of chemically modified heparin polysaccharide and oligosaccharide fragments.

Stock solutions of 100 $\mu g.ml^{-1}$ solutions of each polysaccharide and oligosaccharide sample were made in 1x assay buffer (Coatest® heparin kit). 3-fold serial dilutions were made across a 96 well plate leaving a final volume of 12.5 µl in each well. 1x buffer was used as a control well. To each well 37.5 µl of antithrombin III (AT-III, 2 $IU.ml^{-1}$) was added and the plate incubated for 2 minutes at 37° C. Following incubation, 37.5 µl of Factor Xa (12 $nkat.ml^{-1}$) was added to each well and the plate gently agitated before incubation for 1 minute at 37° C. The chromogenic substrate (S-222) was added to each well (37.5 µl of 7.5 $\mu g.ml^{-1}$) and the plate incubated for 10 minutes at 37° C. The plate was allowed to stand at room temperature for 15 minutes before 37.5 µl of 20 % acetic acid was added to each well. The plate was immediately read using a plate reader at λ= 405 nm. This method was a modification the kit manufactures instructions to allow for the use of multi-well plate formats.

Chapter 3

Improved reducing end labelling and detection exploiting the superior fluorophore 4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-*s*-indacene-3-propionic acid hydrazide (BODIPY ® FL hydrazide)

3.1 Introduction

In order to understand the many complex interactions that both heparin and heparan sulphate are involved in, it is essential to have background knowledge of the carbohydrate moieties contained within the carbohydrate chain. The constituent disaccharide sub-units form a base from which the overall structure is imparted. Although no regulatory process has to date been elucidated for the ordered creation of heparin and HS fine structure (in a template driven manner akin to proteins), it is generally accepted within the field, that such complexity and heterogeneity is not random and controlling mechanisms must exist.

The protein analogy is especially relevant when considering the overall conformation and spatial distribution of the polysaccharides. While it is imperative to have knowledge of the primary building blocks (the carbohydrate's primary structure) it would be foolish to consider the polymer as solely a sum of these components. The potential for further degrees of order to exist (such as the conformation of the chain in a local and global manner) via intra-chain interactions (via hydrophobic interactions, hydrogen bonding and charge repulsion / attraction), inter-chain (hydrogen bonding and hydrophobic interactions), with the carbohydrates environment (metal cations, pH) and possibly with the core protein to which the carbohydrate chain is attached, combine to make this molecule one of the most complex to occur in nature.

The use of affinity methods to identify heparin / HS – protein binding partners (from both natural and chemically derived oligosaccharides) [110] combined with bioassays to determine their regulatory potential in biological processes [111] makes the isolation and determination of the primary structure of heparin and heparan sulphate polysaccharide chains of fundamental importance. The sequence information and structural requirements obtained by these techniques may lead to the isolation (or creation) of novel pharmaceutical candidates.

3.1.1 Towards improved separation and detection

Currently the isolation and sequence determination of heparin / HS is hampered by two main problems; the lack of starting material (HS polysaccharide is scarce and difficult to both isolate and purify) due to the tissue specific nature of HS, and the pressing need for improved methodologies in line with those that are common place for nucleic acid or protein research. Method development in the GAG field has progressed relatively slowly compared to that of other bio-polymers, hampered by the difficult chemistries of this class of molecules (heterogeneity and isomerism being the principle ones), although extensive studies of GAG biochemistry are relatively recent. The processes involved, from the initial isolation, subsequent separation and ultimate purification of a pure specie (before any sequencing procedure can be embarked upon), are often multi-stage, laborious tasks using substantial amounts of starting materials with low yields and considerable losses throughout [72].

To compensate for the lack of starting materials available, two complementary strategies are currently in place (while ongoing research continues into the isolation of workable amounts of HS). The first approach relies on the creation of starting materials using the commercially available, pharmaceutical heparin as a template, as discussed in section 1.4.5 [106]. This method has the advantage of being easily scaleable to produce large quantities of material although the downside is the creation of some non-naturally occurring motifs, which may cause problems if looking for naturally occurring sequences. This apparent disadvantage may be turned to advantage, however, if probing for potential pharmaceuticals. The development of improved separation strategies to overcome the problems of isomerism and heterogeneity is crucial to advance the field.

The second strategy attempts to tackle the problem of low sample quantities by improving the detection method(s) employed. Heparin and heparan sulphate polysaccharides possess only weak chromophores in the N-acetyl and carboxylic acid groups. These absorb at λ=215 nm. The introduction of another chromophore into the molecule is possible by lyase digestion (de-polymerisation) of the parent polysaccharide

chains by the creation of a C=C bond between C-4 and C-5 of the uronic acid residue. This chromophore absorbs more strongly than the N-acetyl group with an extinction coefficient of approximately 5,500 $cm^{-1}.M^{-1}$ at λ=232 nm [112].

To overcome this detection problem, the incorporation of radioactive isotopes, both metabolically and chemically, or the conjugation with fluorescent molecules is commonplace. Other methods of detection include the use of stains (for PAGE, such as Azure A) or indirect detection using solvents possessing native chromophores which have their absorbance either quenched or amplified [113].

3.1.2 Determining the sequence of heparin and heparan sulphate

The sequencing of heparin and heparan sulphate oligo / polysaccharide chains is significantly hampered by the lack of starting materials and difficulties of purification. All of the current strategies need some form of sample manipulation ranging from salt removal to derivatisation, and losses to often miniscule sample amounts, quickly escalate. This problem is further compounded by the necessity of current methodologies to start with near pure samples; a feat which if feasible, is extremely arduous and labour intensive, requiring many different separation strategies (SEC, SAX, RP-HPLC). To date, no single strategy possesses the ability to completely sequence a mixture of oligosaccharides and purification remains a major challenge.

In general current sequencing strategies fall into three categories [72]:

(a) Nuclear magnetic resonance (NMR); obtaining direct sequence information without the need for labelling.

(b) Mass-spectrometric (MS); analysis after treatment with an arsenal of modification enzymes.

(c) Separation of partially de-polymerised, end-labelled saccharides (radioactive or chemical) either post-enzyme treatment, or without, followed by the detection of resultant band shifts or mass changes depending on the nature of the particular enzyme.

With the exception of NMR, which is currently fundamentally limited by the magnetic field strength to small oligosaccharides (and is inherently insensitive, while being non-destructive), all of the other strategies rely on temperamental modification enzymes and the ability to resolve oligosaccharides of stepwise increasing size. The ability to resolve oligosaccharides decreases with increased chain length and separation of large oligosaccharides can prove problematic with many overlapping bands or peaks (dependant on gel based or chromatographic separation). A breakdown of sequencing strategies detailing the advantages and disadvantages for each can be found in table 7.

3.1.3 Disaccharide component analysis of heparin and HS

A quick and facile analysis of the components forming a heparin or heparan sulphate polysaccharide chain can be obtained by a technique known as disaccharide analysis. This is of particular use when profiling changes in the sulphation patterns on a global scale in different tissues, or during the development or diseased states. This simple technique can also elucidate crude empirical sequence data.

Heparin or heparan sulphate polysaccharide chains are exhaustively digested to completion, yielding the constituent disaccharide sub-units using a cocktail of three bacterially derived lyase enzymes [see section 2.2.2]. The resulting disaccharides are separated and identified by comparison to a reference disaccharide set. The relative amounts are then compared for each disaccharide to build up a characteristic profile for each source of polysaccharide.

Table 7: The merits and weaknesses of the common sequencing strategies

Technique	Advantages	Disadvantages
IGS	Uses widespread PAGE technique	Loss of sensitivity caused by acid digestion
	Small sample amounts	Reducing-end fluorophore in low yield
	Enzyme activity is vital	
Radiolabel (IGS/Step)	Small sample amounts	Radioactivity handling & disposal
	Increased detection levels	Disposal of labeled samples
	Metabolic labeling possible	
MALDI-MS	Accurate mass data	Indirect detection
	Mass database	Peptide coupling problems
	Small sample amounts	Ionization problems
ES-MS	Very small sample amounts	Ion pairing problems
	Tandem MS/MS	Ionization problems
	Accurate mass data	
	Mass database	
NMR	Isomer information present	Substantial sample amount needed
	Gives structural information	Spectrum very complex for large oligosaccharides
	Non destructive	
	No labels required	Pure saccharides needed

Analysis of the disaccharide components following digest can be performed via a number of techniques. These include mass spectrometry, high performance liquid chromatography and capillary electrophoresis. An overview of these techniques is given within this section along with the limits of detection and other relevant details.

3.1.4 High performance strong anion exchange analysis of disaccharides

Column chromatography relies on the presence of two distinct phases, the stationary phase or column matrix, and the mobile phase or solvent. The sample (mixture) is generally introduced on to the matrix in the mobile phase whereupon it interacts and becomes immobilised and hence withdrawn from the through flow. The nature of this interaction dictates the level, and ultimately the success of the separation. The bound

molecules are then eluted from the matrix in a manner which separates them based on differential intrinsic properties, such as charge, hydrophobic nature or hydrodynamic volume. The use of inorganic solvents or salt counter ions is common place and depends on the nature of the column used. Detection of the molecules is usually performed in line using conductance, UV/Vis or diode array being the most common. The separation is detailed on a chromatogram which provides a record of the peak intensity against elution time [114]. Different modes of separation exist depending on the column and the solvent system(s) employed. Commonly these are normal mode, reverse phase, ion-exchange and size exclusion. Since size exclusion separates on the basis of hydrodynamic volume, and the fact that some of the disaccharide standards present are structural isomers, this method is not of particular use in their resolution.

Detection is commonly carried out at λ=232 nm or 215 nm, although more sensitive detection of the analytes can be obtained by the use of radioactivity to label the analytes or by conjugation to fluorophores (see section 2.1.6). Generally, approximately 20 – 50 picomoles of sample can be detected on common HPLC systems using UV detection.

Strong anion exchange chromatography involves the use of a column matrix possessing a positive charge throughout a wide a range of pHs by the derivatisation of a strong base onto the matrix in order to separate molecules on the basis of their intrinsic charges. Analytes within the sample mixture bind to the positively charged matrix ionically. The strength of the interaction is determined by the number of charges present on the molecules and also their spatial arrangement and dissociation constants (thereby making the interaction strength dependant on the environmental pH). If the environmental pH of the mobile phase is below that of the dissociation constant of the acidic groups they will become protonated and hence will not interact with the column, thereby passing through in the column breakthrough. Elution from the column occurs in a charge dependent manner (higher charges eluting later in a gradient) by the disruption of the analyte – matrix interaction by competitor ions introduced into the mobile phase. The selection of the ion type is dependent on the analytes in question and the column used.

For the separation of heparin and heparan sulphate oligosaccharides and disaccharides, two different groups of column matrices exist. These are C18 silica based matrices (post-derivatisation with a suitably cationic group for subsequent ion exchange) or the alternative polymer based matrices. While the initial C18 based columns (pre-derivatisation) are usually cheaper, they are not as stable as their polymer counterparts, with peak broadening, incoherent retention times and short working lives being common problems [72].

Good robust polymer columns for anion exchange are Propac-PA1 columns, which generally give highly reproducible and consistent elution times which are essential for performing the analytical separations required for disaccharides analyses. This type of column is widely used and produces reproducible results (peak shape and retention times are highly consistent) over many runs. Sample loads of up to a mg of oligosaccharide / disaccharides material can be loaded onto the column from within the mobile phase per individual run on a 250mm long x 4mm diameter column without overloading. For preparative separations a 9mm diameter column is also available giving similar resolution with maximum sample loads of 4 – 5 mg [88].

Separation of disaccharide standards and analytes with the Propac PA1 column can be performed at pH values between 3.5 and 6.0, although superior baseline resolution is obtained at pH 3.5 where the matrix is totally positively charged. The carboxylic acid group of HS saccharides may also be protonated at this pH allowing separation to occur solely on the charge present from the sulphate groups (decreased overall charge allows for increased resolution). A linear sodium chloride competitor ion gradient between 0 – 1.5 M over a 68 minutes time period completely separates all eight common disaccharide standards at a flow rate of 1 $ml.min^{-1}$. Sample loading is also carried out in pH 3.5 water as the mobile phase. Generally speaking separations require a minimum workable amount of starting material in the order of 1 – 10 micrograms of oligosaccharide / disaccharide [72].

A cheaper alternative to the polymer based Propac-PA1 column is the C18 Hypersil silica based column. This reverse phase column is converted into a strong anion exchange column by the reversible derivatisation (typically overnight) with a quaternary ammonium compound, cetyltrimethylammonium (CTA) hydroxide. This is introduced as described by Mourrier *et al.* [87] with the amount of derivatisation controlled by the level of methanol present in the derivatisation solvent (MeOH:H2O). This column does not possess the longevity of its polymer counterparts and appears to require re-derivatisation after 3 - 4 months due to unstable retention times, decreased resolution and evidence of peak broadening.

The resulting C18 CTA derivatized column gives better resolution of the 8 unsaturated disaccharide standards than the Propac PA-1 column separating some of the disaccharides in to their α and β anomers. This column is again run at an acidic pH with a mobile phase of HPLC lowered to pH 3.0 with methane sulphonic acid. The competing ion used for this column is (ammonium) methane sulphonate with a 0 – 2M linear gradient being deployed over 74 minutes, at a flow rate of 0.22 $ml.min^{-1}$. The mobile phase is also less UV absorbent compared to that of sodium chloride used on the Propac-PA1 polymer column and the choice of competitor ion also permits interface with electro-spray mass spectrometry.

A SAX Partisil column is sometimes employed for the separation of nitrous acid derived disaccharides, although better separation has been achieved by using 2 Propac-PA1 columns in series with a narrower sodium chloride counter ion gradient, with sharper peaks being observed or by the use of a dihydrogen phosphate gradient for the singly sulphated disaccharides [114]. Other attempts to further improve the level of detection have involved the use of radioactivity and fluorophore conjugation [71, 115, 116].

Both the C18 CTA and the Propac-PA1 columns can be used to separate larger oligosaccharides by using increased concentrations of sodium chloride and longer run times. It should be noted that the resolving power decreases with increasing

oligosaccharide size and resolution becomes increasingly difficult with more and more unresolved peaks.

The use of reverse-phase methods to separate molecules on the basis of their differing hydrophobic nature can also be applied to disaccharides. This method requires the use of an ion pairing agent (counter ion) to mask the acidic charged groups present within all eight of the unsaturated disaccharides. Interactions between the hydrophobic alkyl chains (derivatized on to the silica) present with the column and the analyte are broken by the introduction into the mobile phase of decreasingly polar solvent mixtures. The stronger the hydrophobic interaction the higher the concentration of non-polar solvent required to perturb the bond and elute the analyte. In contrast to the strong anion exchange methods used to separate larger oligosaccharides, reverse phase has only been used to separate comparatively small oligosaccharides. Reverse phase methods suffer from the increased preparation required utilising organic salts such as tetrabutylammonium hydroxide to ion pair. In contrast, SAX suffers from the need to remove salt following separation. Reverse phase methodologies do allow for the relatively facile coupling to electro-spray mass spectrometers.

3.1.5 Capillary electrophoresis

Conventional polyacrylamide gel electrophoresis is not suitable for the analysis of disaccharides because the technique does not possess sufficient resolving power, with disaccharides generally migrating as a single band. Generally speaking, the application of higher voltages improves resolution (up to the point where heating becomes problematic) but even at high voltages, PAGE cannot resolve disaccharides. Furthermore, detection using conventional stains (such as Azure A) is not possible owing to the small size of the molecule (leaching readily out of gels) and relatively low charge (compared to that of oligosaccharides).

In an attempt to overcome these problems, a relatively new technique in carbohydrate analysis, known as capillary electrophoresis, has been employed. This overcomes the

Joule heating problem which prohibits the use of very high voltages in conventional (slab) electrophoresis, by exhibiting a high surface to volume ratio allowing efficient cooling. Detection can also be performed both directly or indirectly using fixed UV/Vis or diode array UV/Vis detectors by means of a small window present in the capillary, thereby overcoming the detection problems of PAGE. The use of high voltages (≈30kV) also significantly reduces run times compared to PAGE.

Capillary electrophoresis takes place in a small, narrow bore capillary usually made of fused silica. The capillary can be gel filled, as in PAGE, or can contain free solution. One distinct advantage of employing such a small bore capillary for electrophoretic separation is the minimisation of consumable costs (after the sizeable initial outlay) and also the use of only a minute amount of sample, in the order of 10 - 100 nl [117] although an initial volume of approximately 5 μl is required for sample loading. This presents CE as an attractive possibility for sample analysis but prohibits its use (without modification) preparatively. The sample can be in introduced onto the capillary, at the end furthest from the detector window, using two different modes; electrokinetic (using voltage to drive the charged molecules into the capillary) or hydrodynamically (using a pressure difference across the capillary). The latter method gives a quantitative analysis while the former does not (more highly charged molecules will enter at a faster rate).

Electrophoresis occurs in the capillary, which is situated between two buffer vials containing platinum electrodes, which have a high potential difference applied across them. Analytes will migrate depending on their charge, the environmental conditions (buffer and its pH) and the polarity of the potential difference. After completion of a run, the capillary can be re-used after flushing any remaining material from inside and applying regenerating solutions before the buffer solution required for the next run.

The presence of the silanol groups on the capillary wall in this mode of electrophoresis does however present one nuance, that of endo-osmotic flow (EOF). This occurs when the environmental pH is greater than 2. The silanol groups become ionised and dissociate, losing protons. The negative silanol moiety can now ion pair with positively charged

ionic species present in the buffer, thereby creating an electric double layer. Positive ionic species present within the buffer trap water molecules during their migration towards the negative electrode leading to a flow condition termed endo-osmotic flow [118]. The use of low pH buffers can minimise the dissociation of the silanol coated capillary wall thereby reducing EOF. Derivatisation of the silanol groups (with for example, methyl cellulose) can also be employed to avoid the formation of the electric double layer.

A number of different CE modes exist. These include capillary zone electrophoresis, capillary gel electrophoresis, capillary iso-electric focussing, capillary isotachophoresis and micellular electrokinetic capillary chromatography. The polarity of the machine can also be reversed leading to normal or reverse modes for the above separation techniques. The use of micellular techniques has been demonstrated for CS and DS [119] but not so far, for the more heterogeneous HS, and this coupled with the non-amphoteric nature of HS, precludes both micellular and iso-electric focussing as techniques for HS separation.

Capillary zone electrophoresis, occurring in free solution, is the most common mode employed to separate heparin and heparan sulphate saccharides. Both normal mode and reverse mode separations have been described on HS derived saccharides [120]. In normal mode separations, the buffer is usually close to neutral pH, allowing separation of the molecules by both endo-osmotic and electrophoretic migration, while in reverse mode the buffer is commonly at a low pH thereby avoiding endo-osmotic flow.

The detection of heparin and heparan sulphate saccharides is often achieved through the C=C (λ=232 nm) chromophore introduced upon bacterial lyase digestion. Detection as low as a few micromoles has been reported by Ruiz-Calero *et al.* [121] using direct UV detection for HS disaccharide standards. Indirect detection can also be carried out using chromogenic buffer systems where either quenching or amplification is promoted by the presence of the analyte [113]. The use of fluorophores conjugated to the HS derived analytes is also possible permitting lower detection thresholds especially when coupled with laser induced fluorescence (LIF) detection [see next section].

3.1.6 Fluorescence conjugation and detection

The coupling of carbohydrate molecules with fluorescent molecules to form glyco-conjugates is an attractive prospect for improving the level of detection for column and electrophoretic based separations. Strategies exist for the coupling of fluorescent tags to disaccharide molecules in order to improve the limit of detection and also the amount of starting material (heparin of HS) required. This provides an important tool in disaccharide analysis during structural composition studies and is especially pertinent for HS where pure starting material is often scarce.

The choice of fluorophore is critical. Solubility issues must be overcome and the excitation / emission spectra carefully considered for each application. Furthermore, the fluorophore must contain a suitable chemical group for attachment to the carbohydrate, usually an amine containing moiety is required for reductive amination [see 1.1.6 and 1.3.4]. Examples of such groups are amines, hydrazides and hydroxyl amines (also known as amino-oxy) which possess a lone-pair of electrons allowing for nucleophilic attack of the carbohydrate (anomeric) carbonyl group.

Previously, the fluorophores 2-aminobenzamide (2AB) [116] and 2-aminobenzoic acid [122] were coupled to HS derived disaccharide for disaccharide analysis although the methods required cumbersome clean up steps involving paper chromatography. The separation of all standards was not possible for HS using reverse phase methodologies although this has been achieved for CS using SAX performed on a Carbopac PA-1 column at high pH. Detection is usually achieved with a fluorimeter with variable excitation and emission wavelengths.

Analysis using HPLC has been reported using the uncharged fluorophore 2-aminoacridone (AMAC) with a detection level 10 times greater than that of UV alone [123], and somewhat more successfully using capillary electrophoresis [108]. This fluorophore possesses excitation / emission characteristics which are suitable for detection using an Argon ion source laser with a laser induced fluorescence detection

module. This provided for increased sensitivity compared to a standard bulb based fluorimeter. The tag also provides a chromophore with improved UV absorbance characteristics at λ=255nm. Detection of 12 known HS standards (8 common and 4 rare free amine containing standards) has been reported with a separation time of just 30 minutes using AMAC derivatisation and reverse mode CE in acidic phosphate buffer [108]. This method claims to carry out detection at the femto – attomolar level [124].

3.1.7 The reducing end revisited

Conjugation of disaccharide molecules (and oligosaccharides) is performed conventionally by reductive amination with the amine group present on the fluorophore molecule. The reducing end carbonyl forms an imine (or Schiff's base) via a condensation reaction [see 1.1.6 and 1.3.4] with the amine present on the fluorophore. This reaction is sluggish mainly due to the poor reactivity of the saccharide molecule. This poor reactivity can be attributed to the rate of mutarotation between the open (and reactive aldehyde containing) and closed (pyranose ring) forms of the carbohydrate. Mutarotation is promoted by the use of amphoteric solvents [see 1.1.6].

The reductive amination reaction can be catalysed by the use of mildly acidic conditions (and the rate improved by heating). It should be noted that there remains the possibility of de-N-sulphation, especially where heat is applied to increase the rate. The labelling conditions used in all of the fluorophore conjugations mentioned above [3.1.6] rely on the use of acid catalysis with heat.

The basis for acid catalysis relies on the relative positions of two equilibria, that of the carbonyl and that of the amine. These equilibria are outlined below. Carbonyl reactivity occurs in the protonated form, promoted by acidic conditions, while amine reactivity occurs when the equilibrium is shifted away from the protonated form. These two situations are mutually incompatible. To accommodate this apparent anomaly, the reaction is usually carried out in acid (favouring a protonated carbonyl) with a huge excess of amine containing fluorophore which will have a very small (but significant, due

to the huge molar excess) proportion of amine groups present in a non-protonated condition. If this reaction is carried out under conditions in which an excess of amine is not possible, (e.g. attachment of a sugar to an amino surface) the equilibrium will be at a different position (more basic conditions).

It can be clearly seen from the above and the other section outlining the principles of this reaction [see 1.1.6 and 1.3.4] that the reaction is not simple and relies on many competing and non-complementary conditions, unfavourable reaction kinetics and can result in comparatively low (compared to other carbohydrates) yields. Evidence also exists that the structure of the disaccharide is implicated in the labelling efficiency leading to non uniform labelling (see section 3.2.13 and personal communication Dr E Yates).

3.1.8 Aims

The aim of this chapter is to attempt to label with fluorescent molecules, and detect with high sensitivity, Δ-disaccharide fragments derived from lyase digestion of parental polysaccharides. This will aid in determining the composition of the original polysaccharide and provides information with regard to structure – function relationships.

The use of novel fluorophores will be investigated which are compatible with laser induced fluorescence detection as routinely employed in capillary electrophoresis. Various labelling conditions will be attempted and separation and detection will be carried out on two differing platforms, that of capillary electrophoresis (CE) and that of high performance strong anion exchange column chromatography (HPSAX).

3.2 Results

3.2.1 Chemically modified heparin polysaccharides

Heparin polysaccharides were characterised by NMR (previously) and disaccharide analysis and assayed for purity by SEC. The results for the NMR of each of the polysaccharides are attached in the appendices, while the SAX data is found in 5.2.1.

3.2.2 Production of Δ-disaccharide standards by enzymatic digestion of the parent polysaccharide

Each of the seven chemically modified polysaccharides from section 2.2.1 were mixed together equimolar proportions along with porcine mucosal heparin and digested to completion using a cocktail of bacterially derived lyase enzymes as described in section 2.2.2. This exhaustive digestion yields predominantly disaccharide products with minor contributions from other oligosaccharide sizes. The resultant mixture of fragments were separated from salt into size defined pools using size exclusion chromatography employing two serial XK-16 Superdex ® 30 columns connected in series as per section 2.2.3 (see figure 6). The size pools and, more importantly, the disaccharide pools (A,B and C combined) were further desalted using serial lyophilisation (see 2.2.4), three times to remove the volatile ammonium hydrogen carbonate running buffer. After salt removal the de-salted disaccharide pool was loaded onto a preparative Propac-PA1 column and the mixture separated using strong anion exchange chromatography as detailed in section 2.2.5. Fractions were collected containing 250 µl in volume and peaks pooled after cross reference with the corresponding chromatogram. 10 µl of 1 $mg.ml^{-1}$ disaccharide reference standards was mixed and also subjected to identical analysis without fraction collection to obtain a reference chromatogram (see figures 7 and 8). Peaks which were collected from the chemically modified heparin digestion

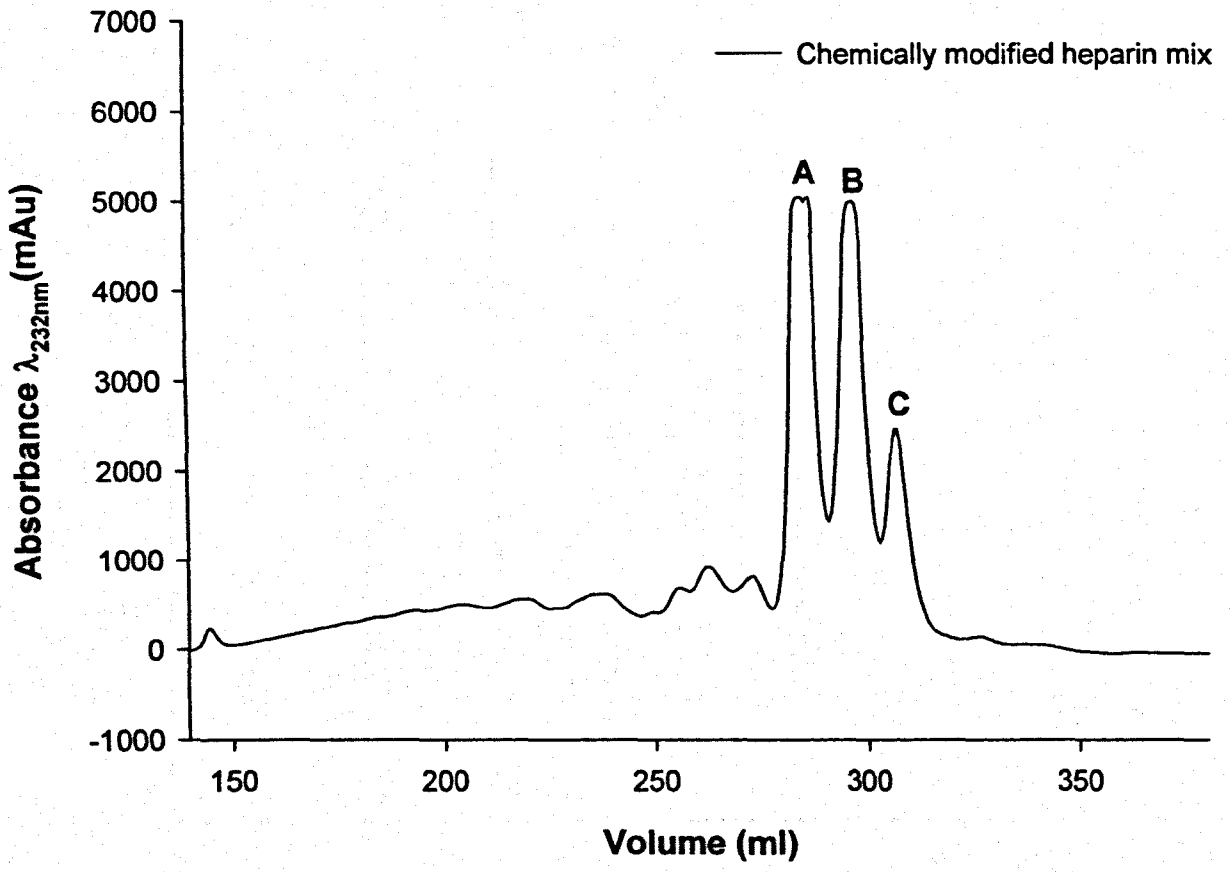


Figure 6 - The size exclusion chromatography elution profile of a total heparatinase (I, II and III) enzyme digestion of a mixture of all seven chemically modified heparin polysaccharides and porcine mucosal heparin. Separation was carried out on two Amersham XK-16 columns (15mm x 1m) connected in series and packed with Superdex ® 30 matrix. A 0 – 1000 minute elution was carried out at a flow rate of 0.5 $ml.min^{-1}$ in 0.5 M ammonium hydrogen carbonate. Detection was performed in the UV (λ=232 nm). Peaks labelled A, B and C contain disaccharides

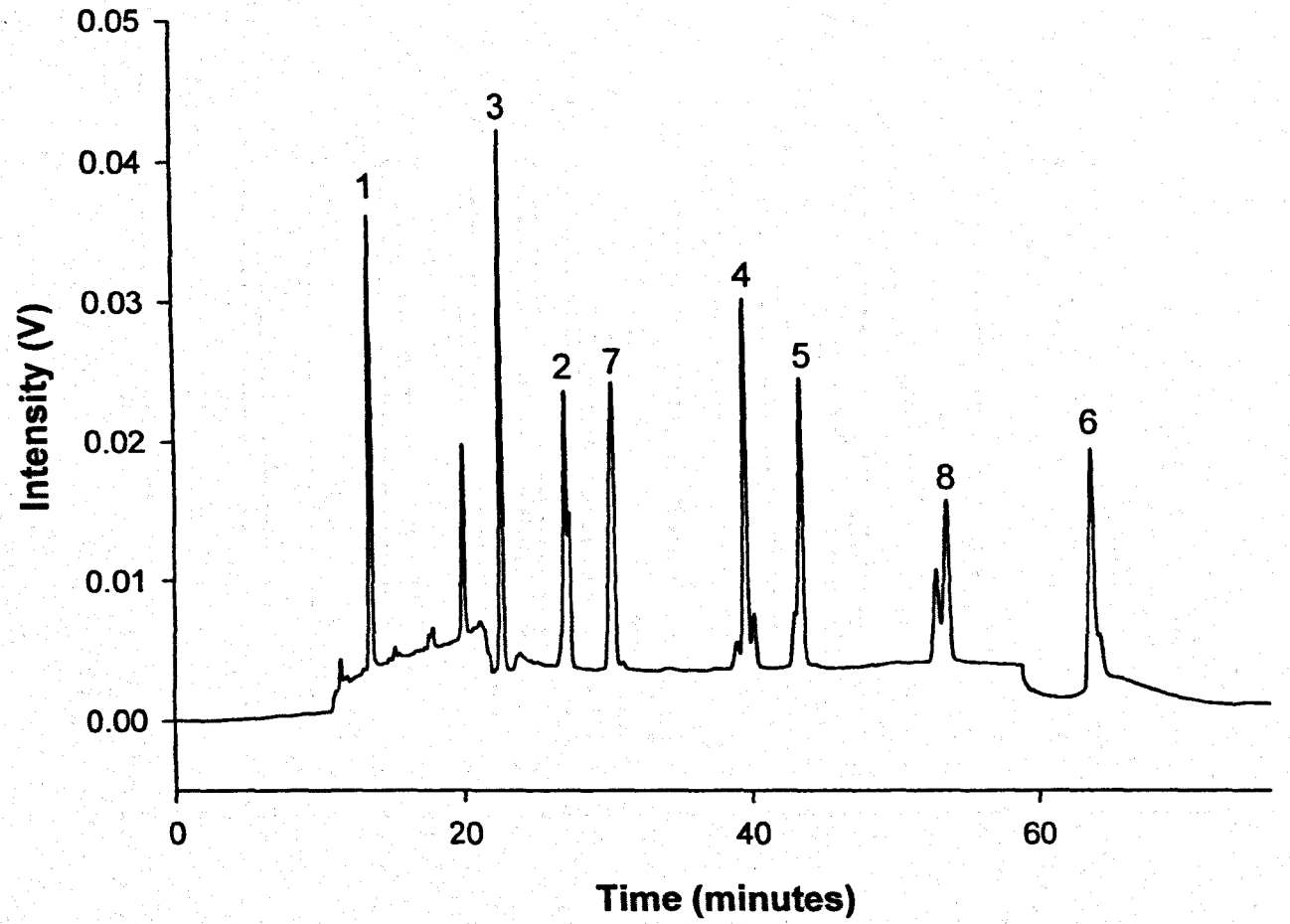


Figure 7 - Disaccharide profile for eight common naturally occurring disaccharides obtained from Dextra laboratories, UK (10ul, 1mg.ml^{-1} of each standard). Separation was achieved using high performance strong anion exchange chromatography on a polymer based Propac-PA1 analytical column (Dionex) with a 0 – 68 minutes, 0 to 1.5 M sodium chloride linear gradient at a flow rate of 1 ml.min^{-1}. Disaccharide peaks: 1, ΔUA-GlcNAc; 2, ΔUA-GlcNAc(6S); 3, ΔUA-GlcNSO$_3$; 4, ΔUA(2S)-GlcNAc; 5, ΔUA-GlcNSO$_3$(6S); 6, ΔUA(2S)-GlcNSO$_3$; 7, ΔUA(2S)-GlcNAc(6S); 8, ΔUA(2S)-GlcNSO$_3$(6S).

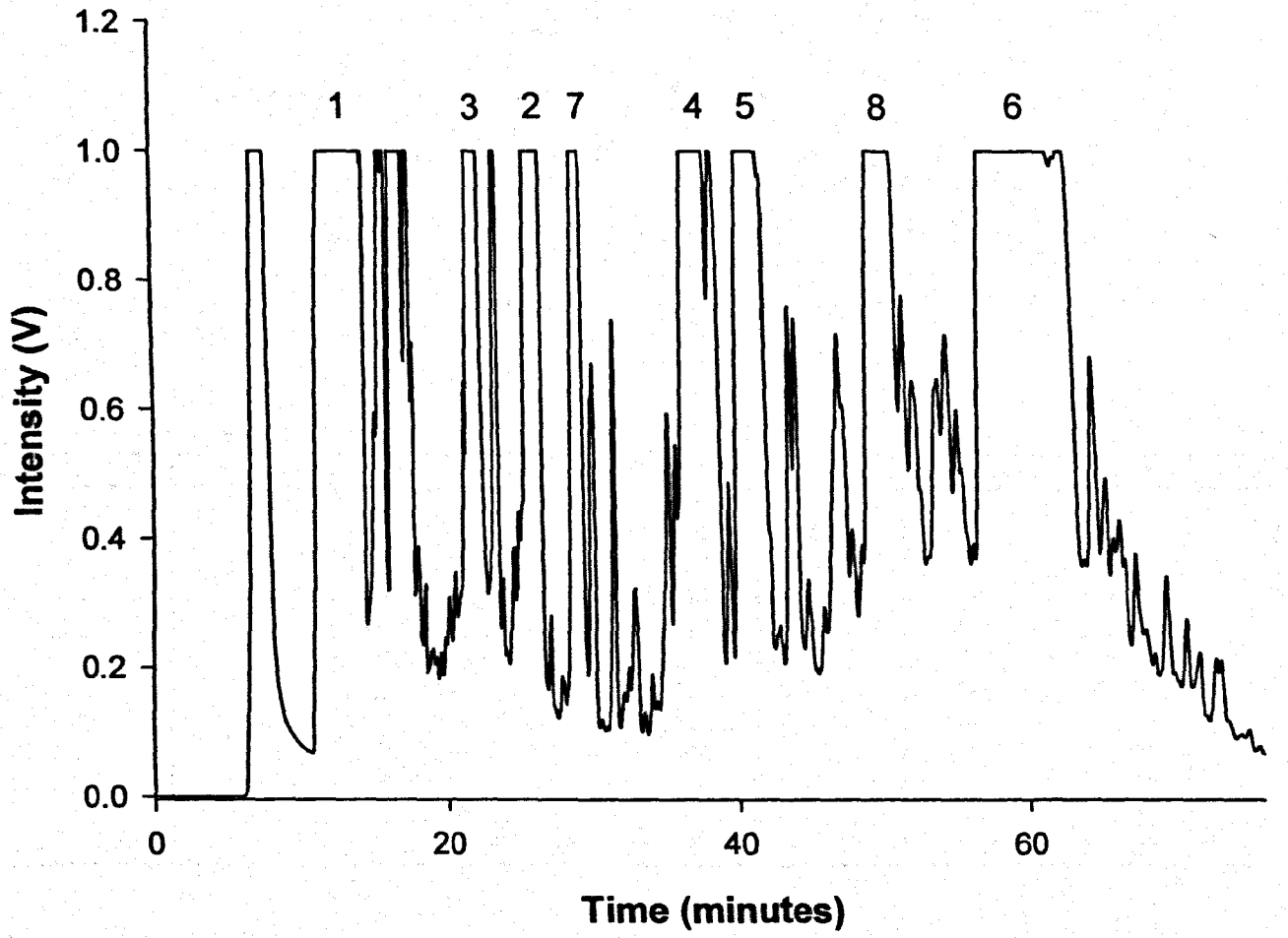
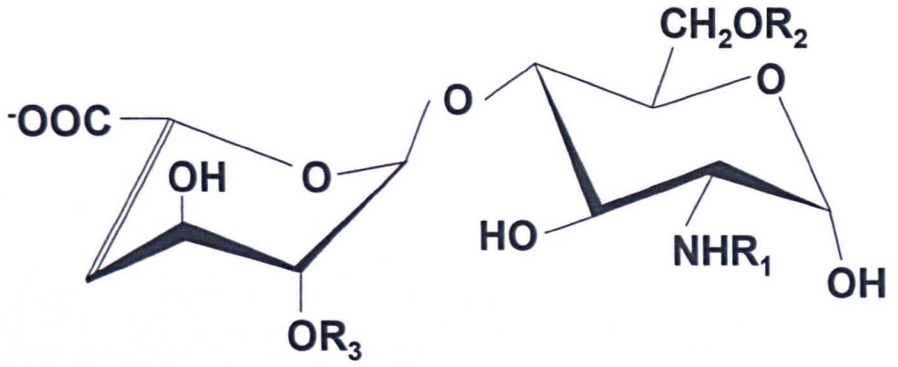


Figure 8 – Separation profile for the disaccharides obtained from the enzymatic digestion of a mixture of chemically modified heparin polysaccharide precursors. Separation was achieved using high performance strong anion exchange chromatography on a polymer based Propac-PA1 analytical column (Dionex) with a 0 – 68 minutes, 0 to 1.5 M sodium chloride linear gradient at a flow rate of 1 $ml.min^{-1}$. Disaccharide peaks (by comparison to figure 10): 1, ΔUA-GlcNAc; 2, ΔUA-GlcNAc(6S); 3, ΔUA-$GlcNSO_3$; 4, ΔUA(2S)-GlcNAc; 5, ΔUA-$GlcNSO_3$(6S); 6, ΔUA(2S)-$GlcNSO_3$; 7, ΔUA(2S)-GlcNAc(6S); 8, ΔUA(2S)-$GlcNSO_3$(6S).



Standard No.	Sigma Ref.	Unit Formula	R1 (GlcC2)	R2 (GlcC6)	R3 (UAC2)	FW (+Na)	Net Charge
1	IV-A	Δ-UA-GlcNAc	Ac	H	H	401.3	-1
2	II-A	Δ-UA-GlcNAc(6S)	Ac	Sulf	H	503.34	-2
3	IV-S	Δ-UA-GlcNS	Sulf	H	H	461.3	-2
4	II-S	Δ-UA-GlcNS(6S)	Sulf	Sulf	H	563.34	-3
5	III-S	Δ-UA(2S)-GlcNS	Sulf	H	Sulf	563.34	-3
6	I-S	Δ-UA(2S)-GlcNS(6S)	Sulf	Sulf	Sulf	665.36	-4
7	III-A	Δ-UA(2S)-GlcNAc	Ac	H	Sulf	503.34	-2
8	I-A	Δ-UA(2S)-GlcNAc(6S)	Ac	Sulf	Sulf	605.38	-3
9	IV-H	Δ-UA-GlcNH	H	H	H	360.28	0
10	II-H	Δ-UA-GlcNH(6S)	H	Sulf	H	462.32	-1
11	III-H	Δ-UA(2S)-GlcNH	H	H	Sulf	462.32	-1
12	I-H	Δ-UA(2S)-GlcNH(6S)	H	Sulf	Sulf	564.37	-2

Figure 9 – Reference diagram and table for all eight naturally occurring Δ-disaccharides and the four rare, free amine containing disaccharides. The laboratory notation (as used in this thesis), Sigma reference, abbreviated structure, structural moieties, mass and net charge are all described. Abbreviations used: Ac, Acetyl; GlcNAc, N-acetyl glucosamine; GlcNS, N-sulpho glucosamine; H, proton; Sulf, O-sulphate (if present at R_1 or R_2) or N-sulphate (R_3); ΔUA, unsaturated (C-4 & C-5) uronic acid.

corresponding to the 8 common disaccharides were identified (by reference to authentic standards) and pooled into individual standards.

Sodium chloride used to elute the disaccharides was removed from the fractions using FPLC size exclusion chromatography. This was carried out as detailed in 2.2.6 using two HiPrep Sephadex G-50 columns connected in series. Fractions were collected corresponding to 2 ml in volume thorough the run. The disaccharide and salt peaks were identified on the reference chromatogram (see 1.4.3.1) and relevant fractions pooled and lyophilised to yield pure individual disaccharides. An aliquot from each pure disaccharide was re-run using SAX as outlined above to confirm the presence of a single peak indicating purity. All eight disaccharide standards were obtained as single entities. An outline of the structure of all eight disaccharides can be found in Figure 9.

3.2.3 Reducing end labelling of Δ-disaccharides with the fluorophore 2-aminoacridone (AMAC)

Eight Δ-disaccharides were labelled in DMSO:glacial acetic acid with heat (at 45°C as described previously by Militsopolou *et al.* [108]. The samples were prepared as outlined in section 2.2.7 and were subjected to reverse mode CE analysis in 50 mM phosphate buffer at pH 3.5. Separation was carried out as described in section 2.2.11 modified (as in Milisouplou *et al.* [108, 124]) to operate at both 18 kV and 30 kV respectively with detection at 255 nm using diode array detector and using LIF excitation : detection at λ= 488:520 nm respectively. The experiments were initially conducted using sodium cyanoborohydride present in the reaction mixture as per the original method. These experiments yielded no apparent signal using UV or LIF detection. The experiment was repeated using the reducing agents triacetoxyborohydride and sodium borohydride present at the same concentration as that of the sodium cyanoborohydride. Sodium borohydride was not added to the acidified reaction mixture but added after the heated incubation period and left to react for 1 hr (sodium borohydride will reduce the anomeric carbonyl unlike sodium cyanoborohydride

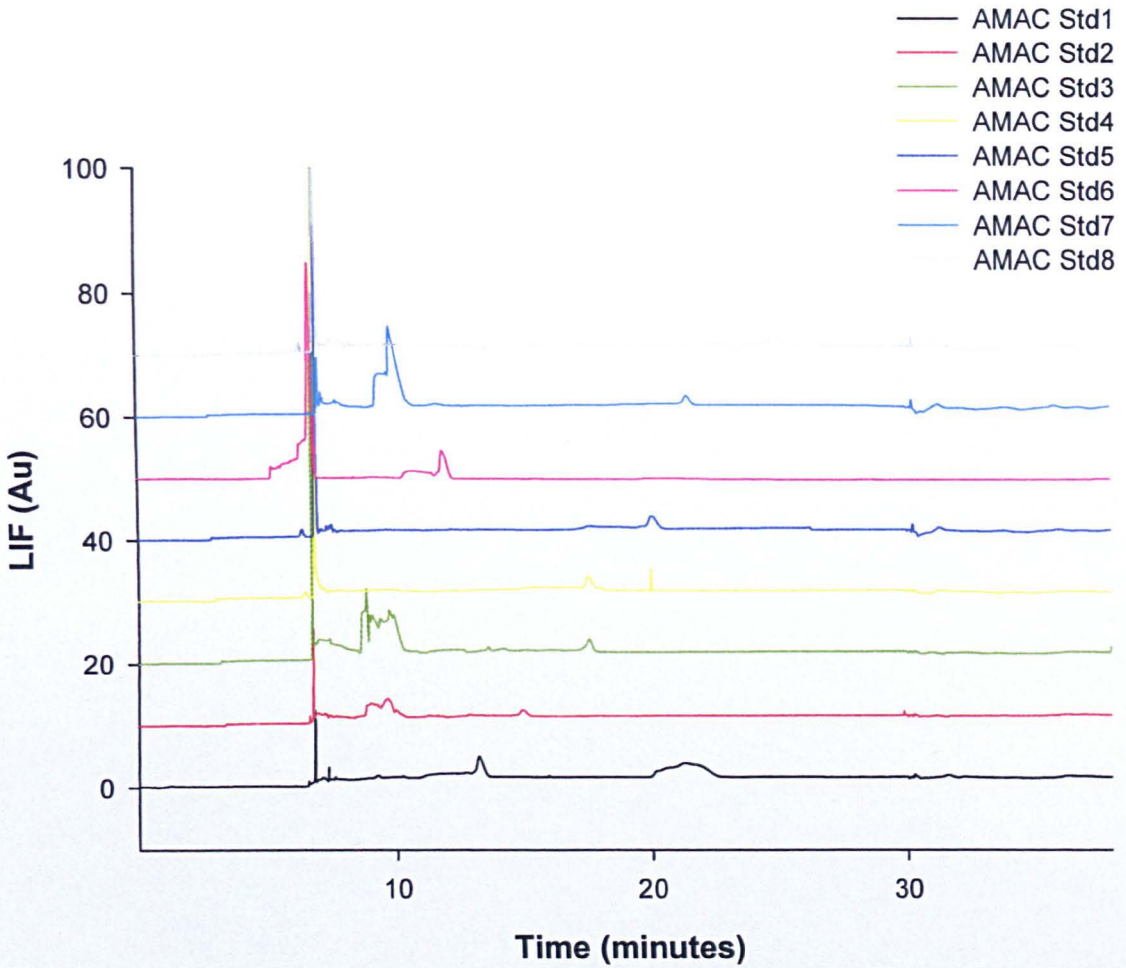


Figure 10 – High performance capillary electrophoresis chromatogram showing the separation of eight individual, 2-aminoacridone (AMAC) labelled disaccharides. Electrophoresis was performed in an un-fused bare silica capillary of 50 cm effective length and 50 µm internal diameter, at 30 kV in reverse mode after a 30 second injection at 0.5 p.s.i. for 30 minutes. Separation was carried out in 50 mM phosphate buffer, pH 3.5. An imposed pressure of 0.5 p.s.i (30 kV, reverse mode) was applied after 30 minutes for a duration of 8 minutes to flush the capillary.

or triacetoxyborohydride). No evidence of labelled disaccharide was obtained when triacetoxyborohydride was used although very weak peaks are present when the reducing agent sodium borohydride is used and LIF detection is employed (see figure 10). These peaks seem to overlap substantially and bear no resemblance to those published [108].

3.2.4 Analysis of fluorophore purity using normal mode and reverse mode capillary electrophoresis

Due to the failure to label efficiently unsaturated disaccharides with AMAC, other fluorophores were considered which appeared to fit into the constraints of the Laser present on the LIF module for CE. Potential candidates include Alexafluor 488 hydrazide, Bodipy FL hydrazide and D-495 hydroxyl amine (a custom made fluor derivatised with a hydrazide group from Dyomics). The structure of these fluorophores is shown in figure 11 while the relevant physicochemical properties are outlined in table 8. Each of the fluorophores above were dissolved in an appropriate solvent (see table 8) at a concentration of 1 $mg.ml^{-1}$ and then diluted 1:1000 into HPCE grade water. Normal mode and reverse mode CE analysis of each of the fluorophores was carried out as described in section 2.2.10 using LIF detection. The resultant chromatograms are shown in figure 12 and 13.

It can be seen from the chromatograms that only Bodipy hydrazide exists as a single predominant peak. All of the other fluorophores possess shoulders or multiple contaminating peaks. 10 µl of each of the fluorophores were loaded onto a gel and run for 24 hours at 85 V. Evidence for multiple bands can also be seen by PAGE gel electrophoresis (data not shown).

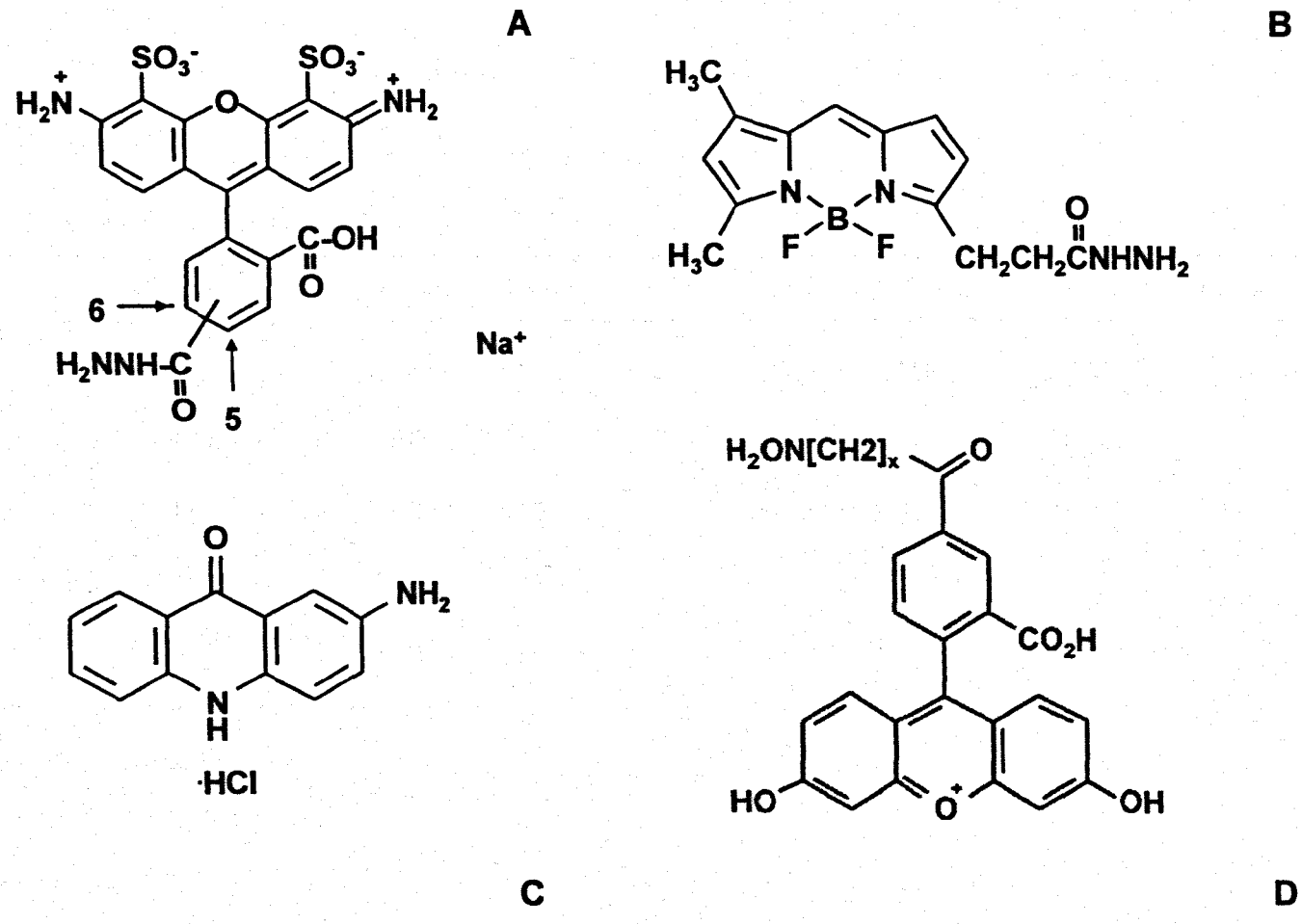


Figure 11 – The chemical structure of four fluorophores with excitation and emission properties suitable for use with Beckman LIF detector module ($\lambda_{excitation}$ = 488nm and $\lambda_{emmision}$ = 520nm). The four fluorophores are: A – Alexafluor 488 ® hydrazide, B – Bodipy ® Fl hydrazide, C – 2-amino acridone (AMAC) and D – DY495 hydroxylamine.

Table 8: Comparison of fluorophores compatible with LIF detection (λ_{exc} = 488 nm, λ_{emm} = 520 nm)

Fluorophore	Abbreviation	λexc	λemm [∞]	Solubility	Extinction coefficient	Mr.
2-aminoacridone	AMAC	423	521	DMSO, DMF	5,200	246.70
Alexafluor 488 hydrazide	AF488	492	517	H2O	71,000	570.28
Bodipy FL hydrazide	Bodipy	503	510	MeOH, DMSO	71,000	306.12
DY-495-X5 hydroxylamine	D495	493	521	MeOH, DMSO	70,000	604.26
Fluorescein	-	490	514	H2O	93,000	376.28

[∞] Excitation and emission data are quoted as the λmax.
All data was obtained from the suppliers Invitrogen and Dyomics

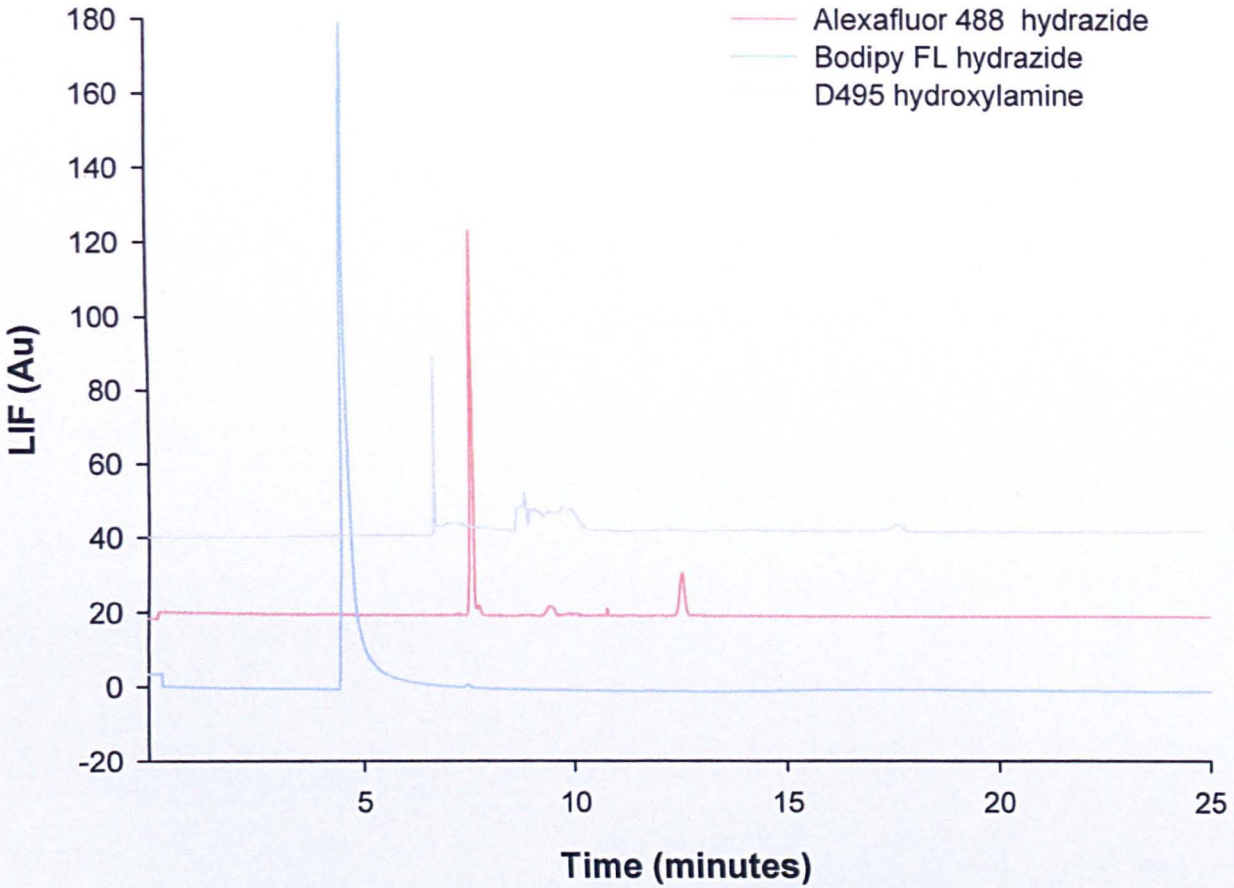


Figure 12 - High performance capillary electrophoresis chromatogram showing the separation of three fluorophores (1 mg.ml^{-1}) with excitation and emission spectra suitable for use with the Beckman LIF module. Electrophoresis was performed in an un-fused bare silica capillary of 50 cm effective length and 50 µm internal diameter, at 22 kV in normal mode after a 5 second injection at 0.5 p.s.i. for 25 minutes. Separation was carried out in 50 mM borate buffer, pH 8.0.

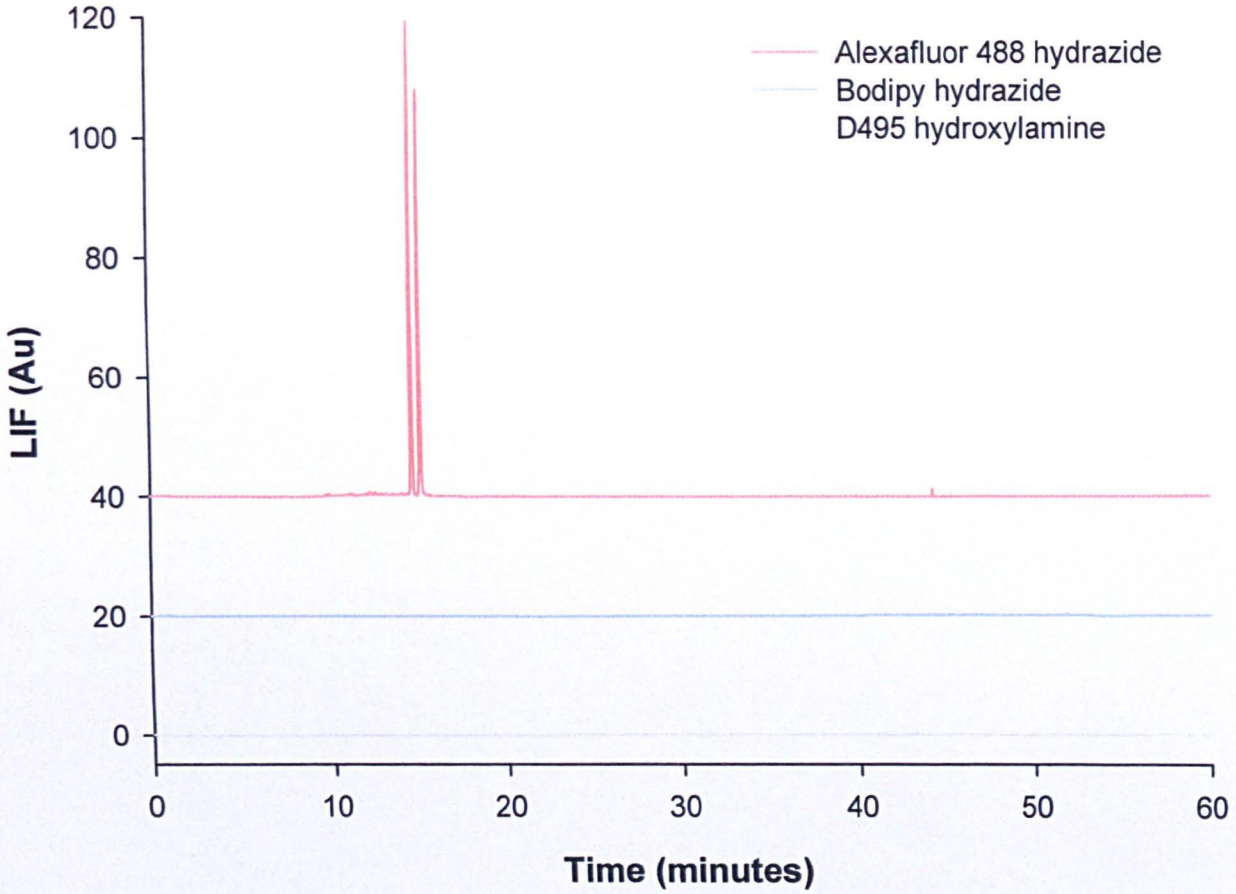


Figure 13 - High performance capillary electrophoresis chromatogram showing the separation of three fluorophores (1 mg.ml^{-1}) with excitation and emission spectra suitable for use with the Beckman LIF module. Electrophoresis was performed in an un-fused bare silica capillary of 50 cm effective length and 50 µm internal diameter, at 30 kV in reverse mode after a 5 second injection at 0.5 p.s.i. for 40 minutes. Separation was carried out in 50 mM phosphate buffer, pH 3.5. An imposed pressure of 0.5 p.s.i (30 kV, reverse mode) was applied after 40 minutes for a duration of 20 minutes to flush the capillary.

3.2.5 D-495 hydroxyl amine conjugation of unsaturated Δ-disaccharides.

From the fluorophore results observed in section 3.2.4 it is clear that two candidate fluorophores stand out. These are D-495 hydroxyl amine and Bodipy Fl hydrazide. Experiments were briefly conducted out with Alexafluor 488® although spectra were unclean with many peaks and no clear evidence of labelling (data not shown).

Attempts to label two representative unsaturated disaccharides with differing charges (Std 1 & 6) were carried out. The label was soluble in only organic solvents while the disaccharide standards are insoluble in methanol but soluble in water and DMSO. Therefore initial experiments were carried out in methanol water (50:50 v/v), methanol with glacial acetic acid (17:3 v/v), Bodipy and in Bodipy with glacial acetic acid (17:3 v/v). Standards were reduced with either sodium triacetoxyborohydride *in situ* or post reaction with sodium borohydride as carried out in section 2.2.7. Detection was performed in the UV at both 232nm and at 488nm (the wavelength with which the laser excites) and also using LIF 488:520 nm.

Initial data suggested evidence for labelling of both disaccharide standards in methanol and standard 6 in DMSO with acid. DMSO gave cleaner spectra when compared to that of methanol for standard 6 which contained many spurious peaks. The peaks absorbed at both 232 and 488nm but only the DMSO labelled standard 6 emitted weakly at 520nm when excited by the 488nm laser of the LIF detection module. (Figure 14) Therefore research on D-495 was discontinued as it appeared that labelling was not successful for all disaccharides and fluorescence was very weak. Either the labelling yield was very low or the fluorophore degraded and failed to fluoresce following labelling.

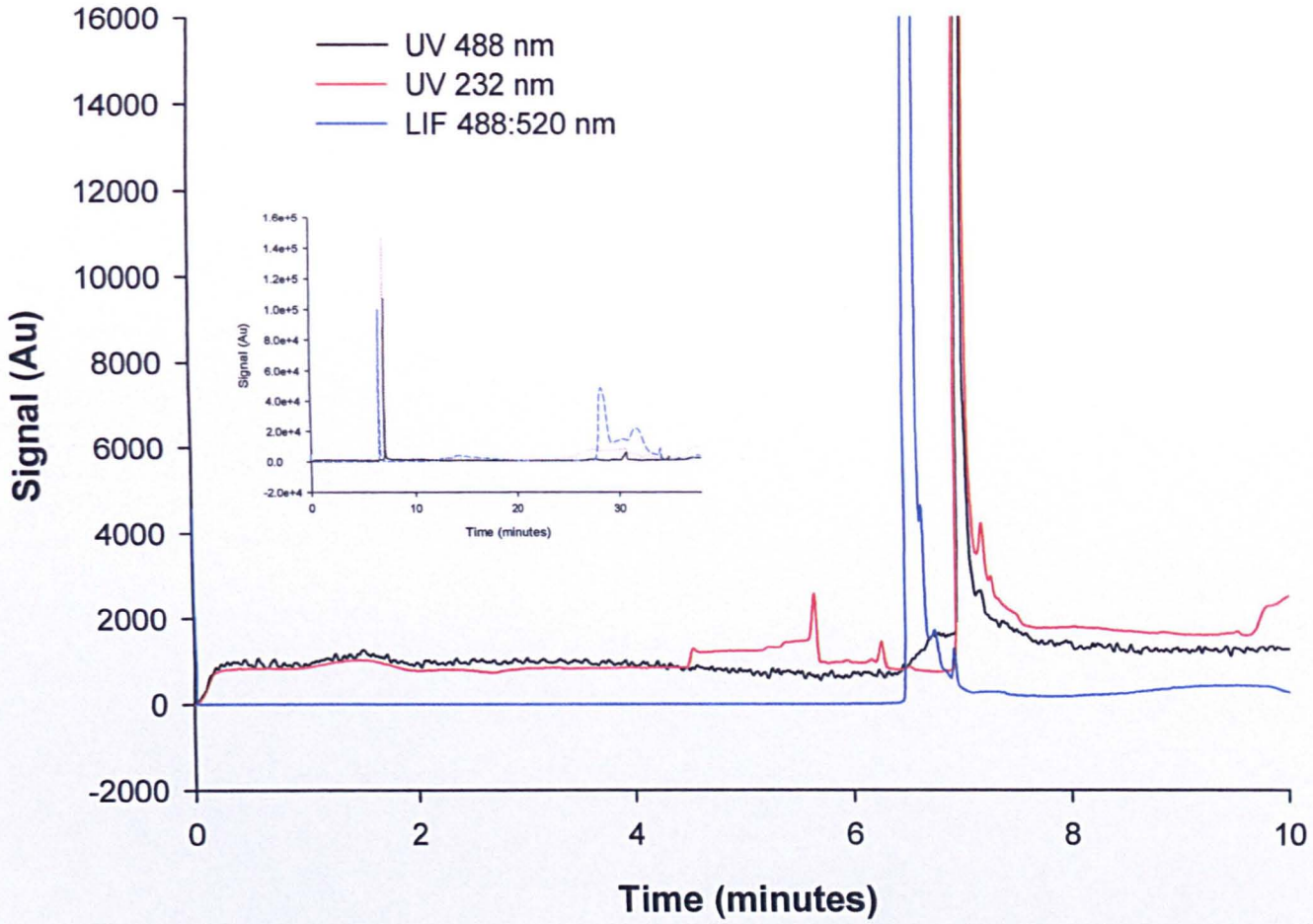


Figure 14 - High performance capillary electrophoresis chromatogram of standard 6 after conjugation with the fluorophore DY495 – hydroxylamine. Electrophoresis was performed in an un-fused bare silica capillary of 50 cm effective length and 50 µm internal diameter, at 30 kV in reverse mode after a 30 second injection at 0.5 p.s.i. for 30 minutes. Separation was carried out in 50 mM phosphate buffer, pH 3.5. An imposed pressure of 0.5 p.s.i (30 kV, reverse mode) was applied after 30 minutes for a duration of 8 minutes to flush the capillary. Detection was carried out using a diode array detector set at λ =232 nm (red), λ =488 nm (black) and using LIF detection, λ_{exc} =488 nm and λ_{emm} =520 nm (blue).

3.2.6 Bodipy FL conjugation of unsaturated Δ-disaccharide

The second potential fluorophore candidate investigated was that of Bodipy-FL hydrazide. Relatively insoluble in water, the fluorophore is highly soluble in DMSO, methanol and formamide. Conjugation of the two model disaccharides (standard 1 and 6, as in section 3.2.8) was performed on the molecule in different solvents present within the reaction mixture. Experiments were carried out in the following reaction mixtures : DMSO water (17:3), DMSO glacial acetic acid (17:3 v/v) and in methanol (19:1 v/v). For each condition incubations were allowed to progress for 4 hours, 8 hours or 12 hours. It should be noted that the experiments were conducted without the application of heat to the reaction mixture.

Labelling of the two disaccharides did not appear to occur appreciably in methanol or DMSO in water at either 4, 8 or 12 hours of incubation (data not shown). It would appear that labelling occurred after 4 hours in DMSO in the presence of acid catalysis, although after 8 hours the chromatogram showed evidence of degradation products (data not shown).

The intensity of the peaks observed was outside of the range of the LIF detector and so the samples were diluted 10 fold and a 1000 fold. Conjugation of the all eight naturally occurring disaccharide standards was carried out with Bodipy FL hydrazide using the method detailed in section 2.2.9. Each disaccharide was individually run on the CE using LIF detection after a 1 in 1000 dilution in water. The chromatograms for each sample showed a clean single peak for all eight individually Bodipy conjugated disaccharide standards. This is illustrated by example chromatograms for each charge class (i.e. un, mono di or tri-sulphated) in Figure 15.

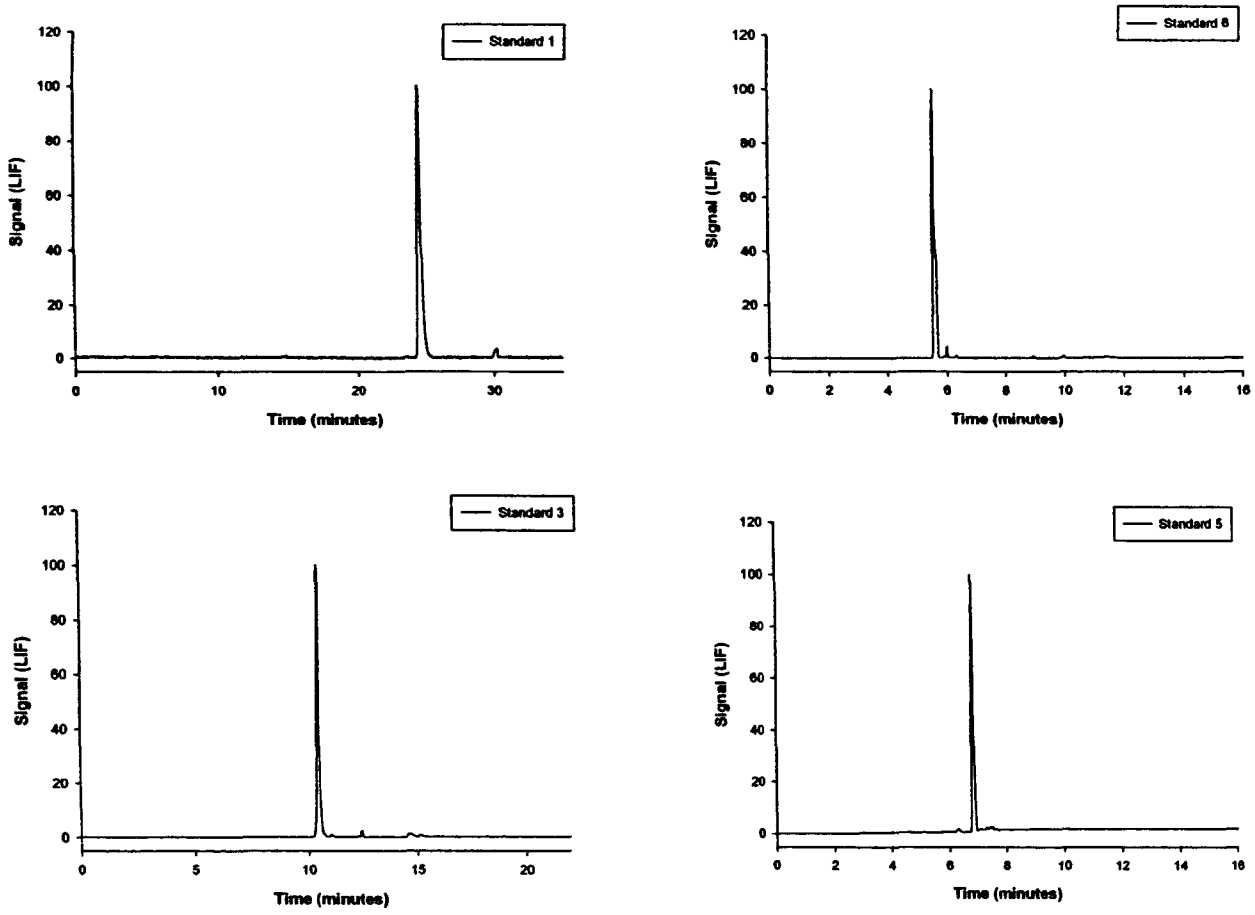


Figure 15 - High performance capillary electrophoresis chromatogram of four representative disaccharide standards (non, mono di and tri-sulphated) after conjugation with the fluorophore Bodipy ® Fl hydrazide (10 picomoles) and 1 in 1000 dilution. Electrophoresis was performed in an un-fused bare silica capillary of 50 cm effective length and 50 μm internal diameter, at 30 kV in reverse mode after a 30 second injection at 0.5 p.s.i. for 30 minutes. Separation was carried out in 50 mM phosphate buffer, pH 3.5. An imposed pressure of 0.5 p.s.i (30 kV, reverse mode) was applied after 30 minutes for a duration of 8 minutes to flush the capillary. Detection was carried out using LIF detection, λ_{exc} =488 nm and λ_{emm} =520 nm. The signals were normalised to highlight the presence of only individual peaks and the purity of the labelling procedure.

3.2.7 Determining the level of detection for Bodipy FL conjugated unsaturated Δ-disaccharides

In a quest to establish the sensitivity of the Bodipy fluorophore when conjugated with the eight naturally occurring disaccharide standards, 10 nmoles of each standard was labelled as in 3.2.6. Individual labelled standards were then serially diluted by a factor of 1,000, 100,000, 200,000, 2,000,000, and 10,000,000 before CE analysis as before. The height and area of each peak was calculated using Beckman 32 Karat software and recorded. The limits of detection for each labelled disaccharide are given in table 9 along with representative chromatograms.

Table 9: The limit of detection for Bodipy labelled disaccharides using CE LIF detection. (n=5)

Disaccharide standard	**Limit of detection (moles)**	**Average height (RFU x 10 000) [3 sf]**
1	1×10^{-15}	212
2	5×10^{-15}	331
3	5×10^{-15}	1110
4	5×10^{-15}	828
5	5×10^{-15}	1030
6	5×10^{-15}	562
7	5×10^{-15}	1470
8	1×10^{-15}	1330

Each of the eight disaccharide standards labelled with Bodipy could be seen comfortably after a 2,000,000 dilution into water was carried out, with some labelled disaccharides still detectable after a 10,000,000 fold dilution which represents an effective amount of 1 femtomole present in the vial.

3.2.8 Determining the linearity of detection for Bodipy FL conjugated unsaturated Δ-disaccharides

The linearity of detection with regard to the sample amount was calculated using the area values obtained from the experiments conducted in 3.2.7 to determine the level of detection. The linearity range of the eight disaccharides is given in table 10 and plotted in figure 16.

3.2.9 Separation of a mixture containing all eight Bodipy FL conjugated unsaturated Δ-disaccharides

The ability of CE to separate and resolve all eight Bodipy fluorescently labelled disaccharide standards was investigated. This would be needed for the routine use of this fluorescent technique in the disaccharide sequence analysis. A mixture of all eight Bodipy labelled disaccharides was made using the 1000 fold dilution of each labelled standards.

Experiments to separate the labelled disaccharides standards were conducted using two lengths of capillary, 50 cm or 1 m to the detector window. The voltage across the capillary was also varied along with the concentration and the pH of the buffer solution used. Formic acid or formic acid brought to the correct pH by the presence of γ-amino butyric acid (GABA) was also attempted in order to resolve the fluorescent standards. At the time of writing, no set of conditions had yet to be found giving base line resolution of all eight disaccharide standards. Standards 1 and 6, the two extremes of charge, are readily resolved, but the Δ-disulphated and Δ-monosulphated standards remain a problem, possibly due to the presence of structural isomers and the two groups of identical net charge. The effects of different voltages on resolution are shown in figure 17 using a 1 m, 50 μm internal diameter bare silica capillary. It was noted however, that when repeat runs were carried out, the resolution improved as the buffer depleted, as can be seen in figure 18.

Table 10: The average peak area for diluted Bodipy labelled disaccharide standards with CE LIF

Dp2 standard	Dilution factor	Average area (AU)	Deviation[ζ] (%)
1	x 1,000	25,247,000	5.65
	x 100,000	355,800	7.49
	x 200,000	122,500	4.27
	x 2,000,000	13,980	16.9
	x 10,000,000	3,327	23.3
2	x 1,000	6,717,000	1.34
	x 100,000	64,560	2.98
	x 200,000	28,260	4.78
	x 2,000,000	2,320	23.8
	x 10,000,000	N/A	N/A
3	x 1,000	15,160,000	0.92
	x 100,000	151,000	5.45
	x 200,000	62,890	4.56
	x 2,000,000	6,398	12.0
	x 10,000,000	N/A	N/A
4	x 1,000	9,749,000	2.32
	x 100,000	56,910	0.39
	x 200,000	33,000	11.9
	x 2,000,000	3,731	8.9
	x 10,000,000	N/A	N/A
5	x 1,000	10,229,000	2.59
	x 100,000	68,240	5.76
	x 200,000	36,600	5.71
	x 2,000,000	3,754	2.71
	x 10,000,000	N/A	N/A
6	x 1,000	6,140,000	2.31
	x 100,000	54,280	2.62
	x 200,000	24,540	16.5
	x 2,000,000	2,508	11.5
	x 10,000,000	N/A	N/A
7	x 1,000	12,050,000	4.00
	x 100,000	136,700	1.09
	x 200,000	58,910	4.55
	x 2,000,000	6,691	2.83
	x 10,000,000	N/A	N/A
8	x 1,000	7,297,000	10.3
	x 100,000	61,190	3.9
	x 200,000	26,830	0.81
	x 2,000,000	4736	1.11

[ζ] Deviation is standard from the mean expressed as a percentage of the average area

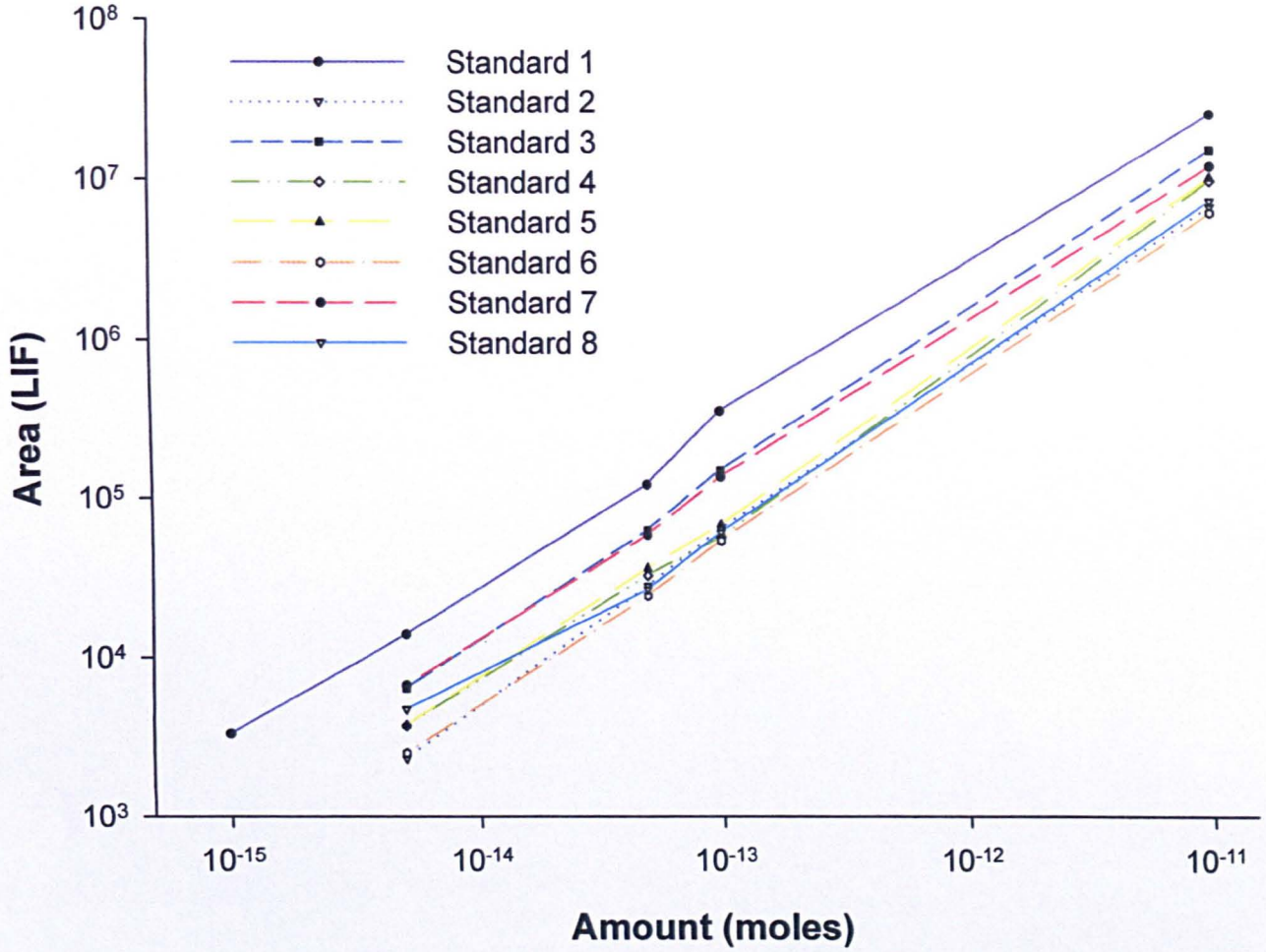


Figure 16 – Linearity plot for each of eight BODIPY labelled Δ-disaccharides. The average area for each disaccharide conjugate present within a CE run (triplicate CE runs) is plotted against the amount of sample present within a peak.

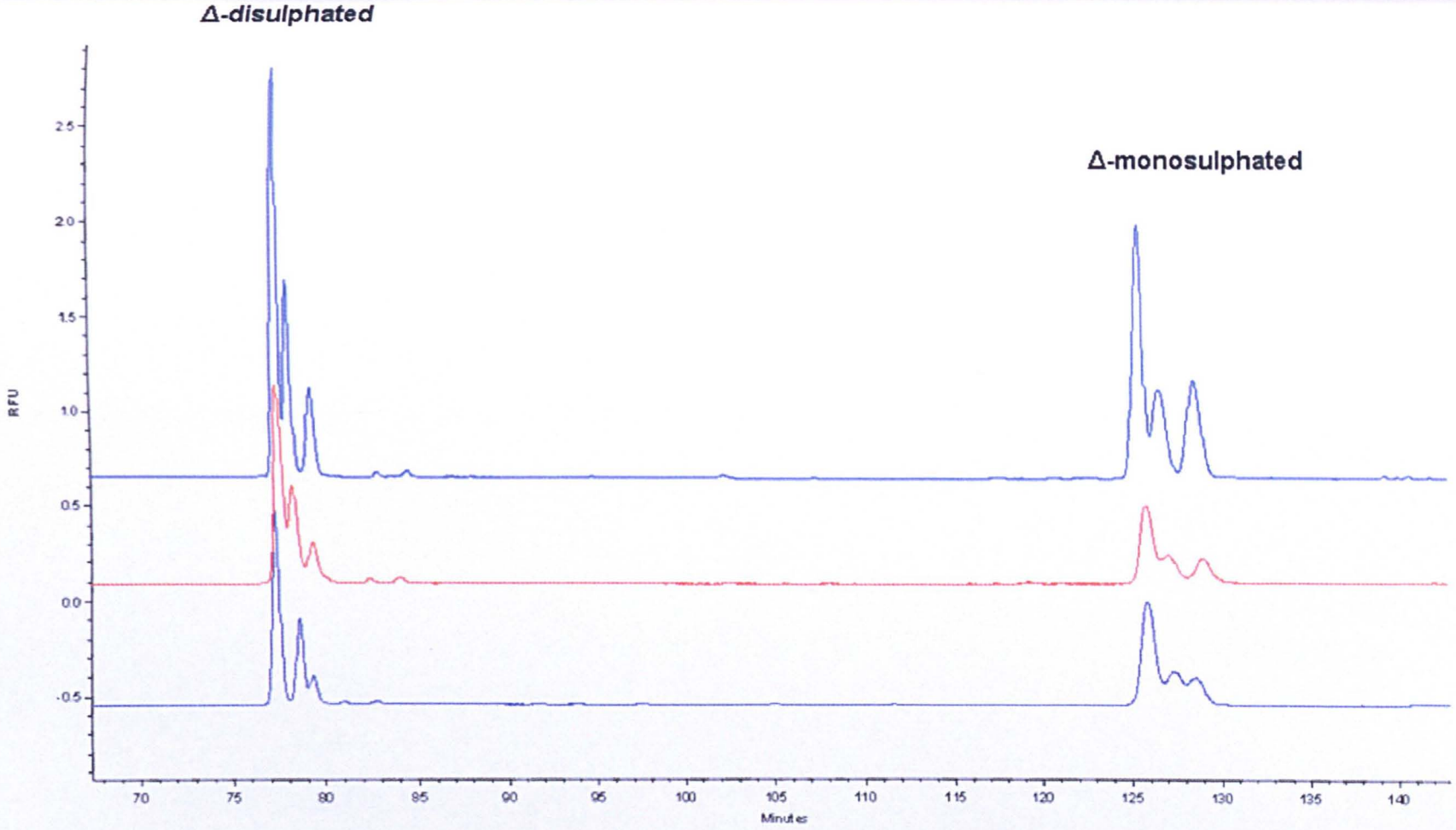


Figure 17 – High performance capillary electrophoresis chromatogram section showing the separation of Δ-disulphated (standards 4, 5 & 8) and Δ-monosulphated (standards 2, 3, & 7). Electrophoresis was performed in 50 mM phosphate running buffer, pH 3.5, on a 1 m, 50 μm internal diameter, un-fused, bare silica capillary. Injection was carried out under pressure (0.5 p.s.i) for 30 seconds. Separation was achieved using a voltage of 11 kV (black), 13 kV (red) and 16 kV in reverse mode. The 13 kV and 16 kV chromatograms were normalised in reference to the 11 kV chromatogram, allowing for easy comparison of the resolutions.

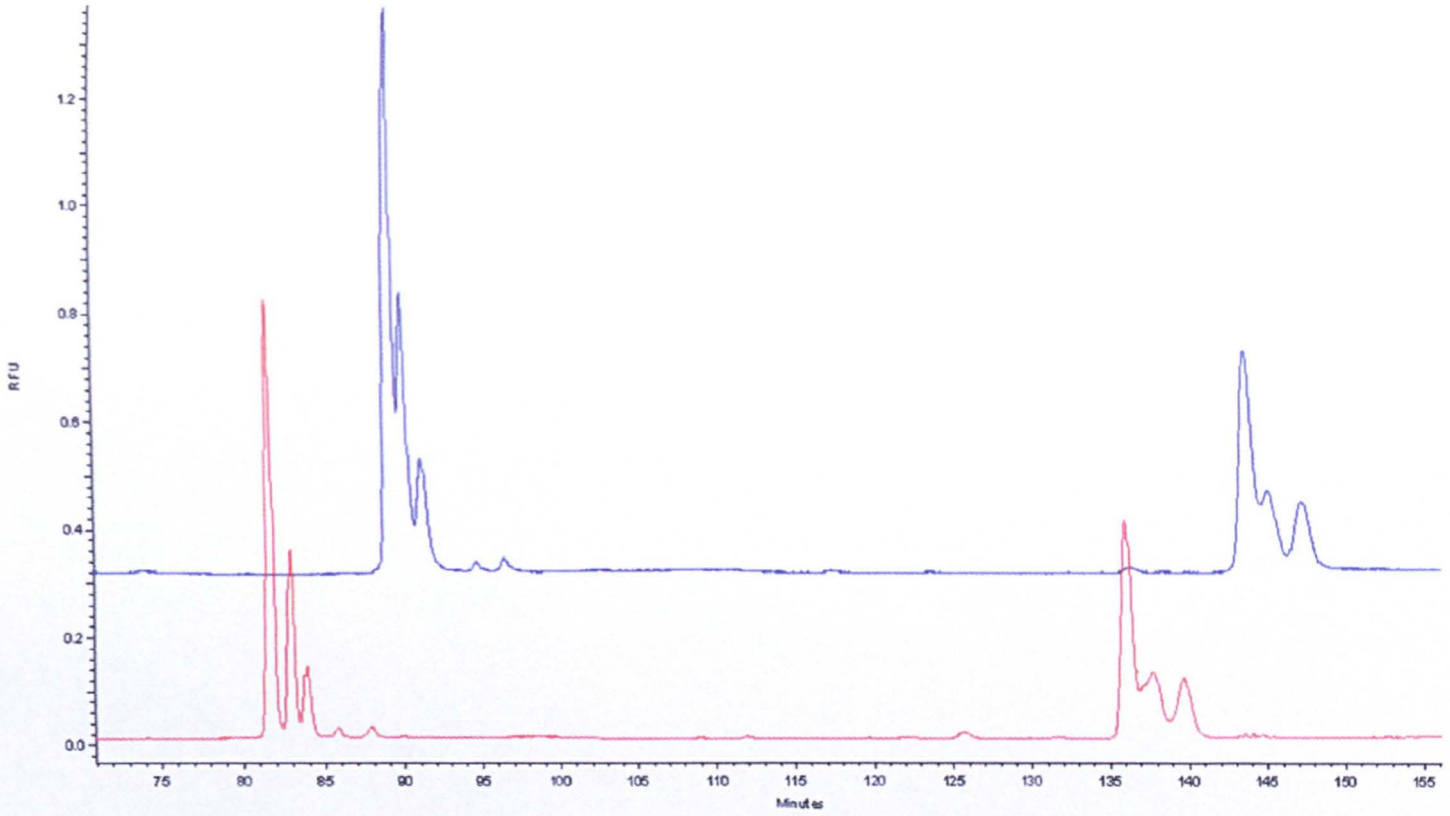


Figure 18 – High performance capillary electrophoresis chromatogram section showing the separation of Δ-disulphated (standards 4, 5 & 8) and Δ-monosulphated (standards 2, 3, & 7). Electrophoresis was performed in 50 mM phosphate running buffer, pH 3.5, on a 1 m, 50 μm internal diameter, un-fused, bare silica capillary. Injection was carried out under pressure (0.5 p.s.i) for 30 seconds. Separation was achieved using a voltage of 13 kV in reverse mode. The HPCE separation was repeated five times without replenishing the electrode buffer vials. The first run (blue) and the last run (red) are plotted after normalisation to the last run chromatogram, allowing for easy comparison of the resolutions.

3.2.10 The use of non-equivalent pH buffers present in the electrode vials for the separation of a mixture containing all eight Bodipy FL conjugated unsaturated Δ-disaccharides

It became clear from figure 18 that repeating a CE run under the same conditions in un-replenished electrode buffer vials improved the separation. The buffer in the vials depletes as the run progresses by electrolysis. In order to try and mimic this depletion the pH of the electrode buffer vials was measured after a run where reasonably good separation had been achieved (cathode, pH ≈ 5.0; anode pH ≈ 3.0). Phosphate buffers of differing pHs for each vial (left or right of the capillary cartridge) were made over a range near to that measured. The effect of starting in non-equivalent pH (at a fixed concentration) environments was investigated and the resulting chromatograms shown in figure 19. Although differences in the resolution were achieved by varying the pH, no set of conditions was found to give complete baseline resolution of all eight standards (and more importantly the Δ-disulphated [standards 4, 5 & 8] and Δ-monosulphated [standards 2, 3, & 7] which almost co-migrate, within charge (net) defined groups).

3.2.11 The use of non-equivalent buffer concentrations present in the electrode vials for the separation of a mixture containing all eight Bodipy FL conjugated unsaturated Δ-disaccharides

It became clear from figure 19 that the improvement in separation seen in 3.2.10 after repeat runs was more than just a pH effect. Therefore the pHs measured initially in 3.2.10 were kept constant for buffer solution, left and right accordingly, and the concentration at that buffer solution varied in order to try and mimic this depletion of the electrode buffer vials. The effect of starting in non equivalent pH and concentration environments were investigated and the resulting chromatograms shown in figure 20.

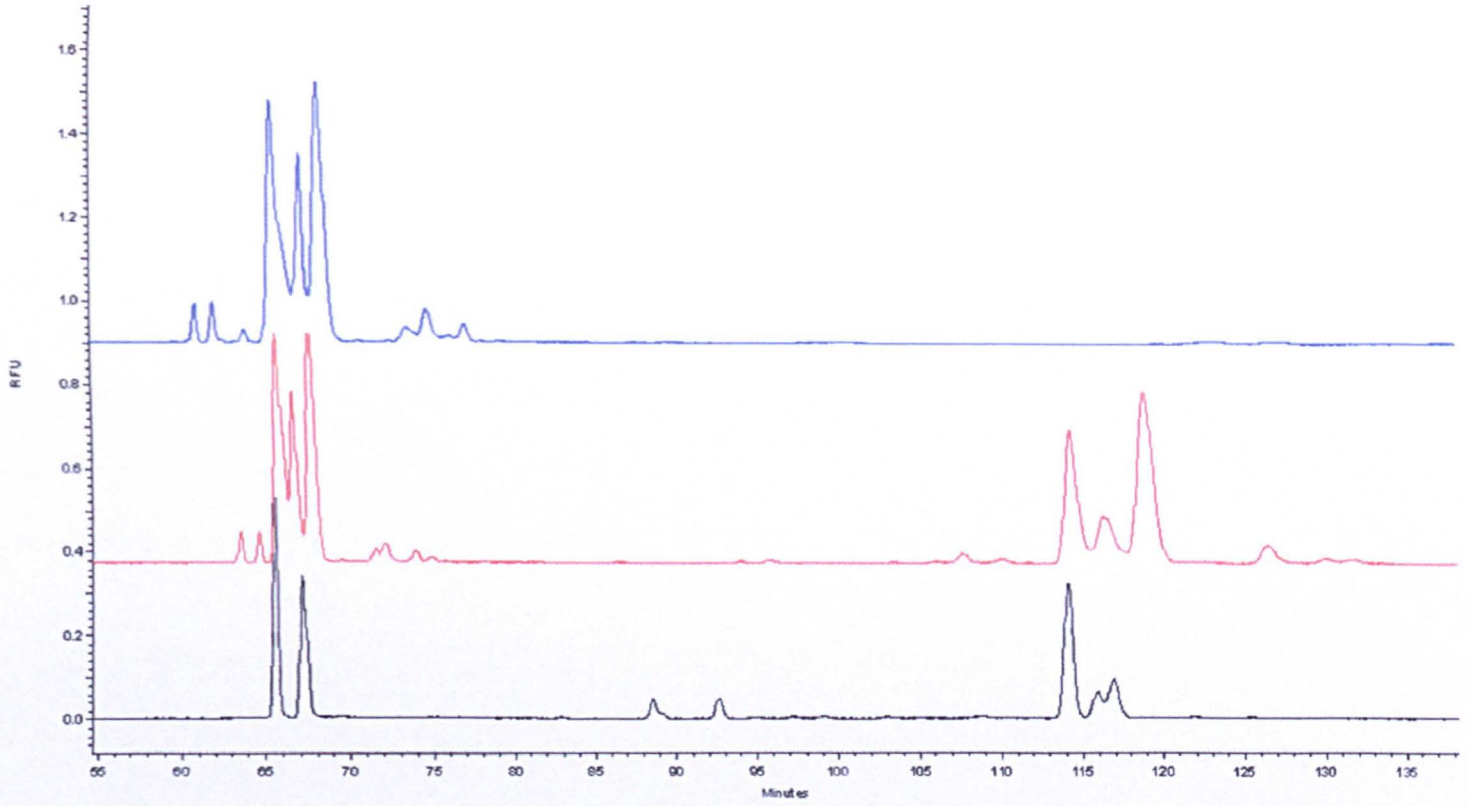


Figure 19 – High performance capillary electrophoresis chromatogram section showing the separation of Δ-disulphated (standards 4, 5 & 8) and Δ-monosulphated (standards 2, 3, & 7). Electrophoresis was performed in 50 mM phosphate running buffer, on a 1 m, 50 μm internal diameter, un-fused, bare silica capillary. Injection was carried out under pressure (0.5 p.s.i) for 30 seconds. Separation was achieved using a voltage of 16 kV in reverse mode with phosphate buffer present in non-equivalent pHs. The electrode buffer pHs used were (cathode then anode) pH 5.0 & 3.0 (black), pH 5.0 & 4.5 (red) and pH 5.0 & 5.5 (blue).

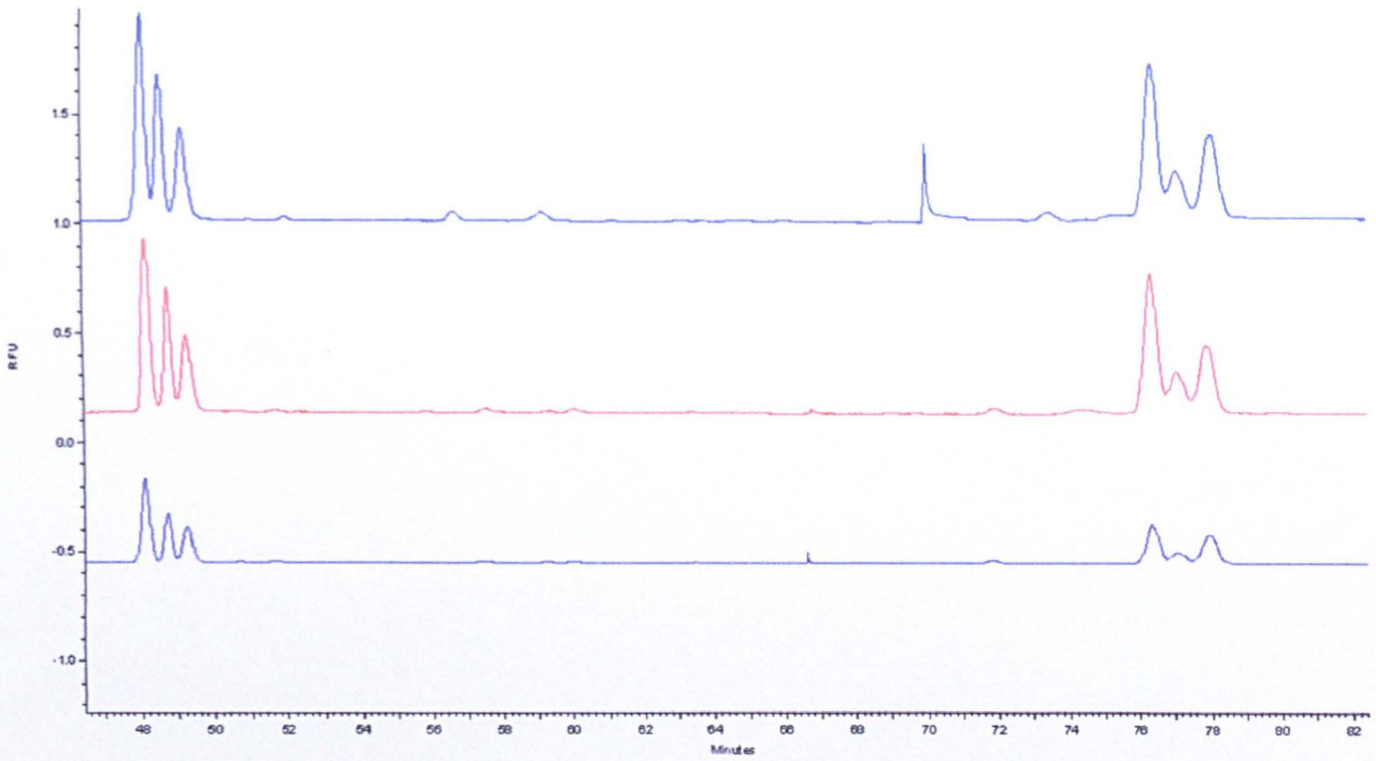


Figure 20 - High performance capillary electrophoresis chromatogram section showing the separation of Δ-disulphated (standards 4, 5 & 8) and Δ-monosulphated (standards 2, 3, & 7). Electrophoresis was performed in phosphate running buffer, on a 1 m, 50 μm internal diameter, un-fused, bare silica capillary. The cathode running buffer was fixed at pH 5.0 while the anode buffer was fixed at pH 3.0. Injection was carried out under pressure (0.5 p.s.i) for 30 seconds. Separation was achieved using a voltage of 16 kV in reverse mode with phosphate buffer present in non-equivalent concentrations. The electrode buffer concentrations used were (cathode then anode) 40 & 50 mM (black), 30 & 50 mM (red) and 30 & 60 mM (blue).

The use of phosphate buffer at non-equivalent concentrations and pHs did appear to affect the resolution favourably with separations similar to that achieved after buffer depletion being mimicked in an initial HPCE run, although compete baseline resolution is still elusive.

3.2.12 Separation and fluorescence detection of eight individually Bodipy labelled Δ-disaccharides using high performance strong anion exchange chromatography

10 nmoles of each of the eight disaccharide standards was labelled as described in section 2.2.9 with Bodipy FL hydrazide. Each disaccharide (1 nmole) was then separated using a Propac-PA1 ion exchange column in a mobile phase containing 150 mM sodium hydroxide and sequentially eluted with a linear salt gradient of 0 – 1 M sodium chloride counter ions. Detection was carried out using a fluorimeter as detailed in section 2.2.15. The separation of all eight individually labelled disaccharide standards was achieved in just over 40 minutes, with single peaks being observed for each labelled standard (data not shown).

3.2.13 Separation and fluorescence detection of a mixture of all eight Bodipy labelled Δ-disaccharides using high performance strong anion exchange chromatography

A mixture containing 10 nmoles of each disaccharide separated and purified in section 2.2.5) was labelled as described in section 2.2.16. 1 nmole of the mixture was injected on to the column and elution was performed as in 2.2.16. The peaks present were identified compared to that of the individual standards and their retention times in 3.2.11 and complete resolution of all eight Bodipy labelled Δ-disaccharides was observed [see Figure 21]. It should be noted that some variation in retention time can occur after fresh mobile phase, elution and regeneration solutions are made due to the propensity of CO_2 to dissolve in the sodium hydroxide solution and form carbonate ions, which can act as a strong competitor ion for this type of SAX column. The relative labelling efficiency for each disaccharide standard was calculated from repeat runs (n=8) and is represented as a correction factor in table 11.

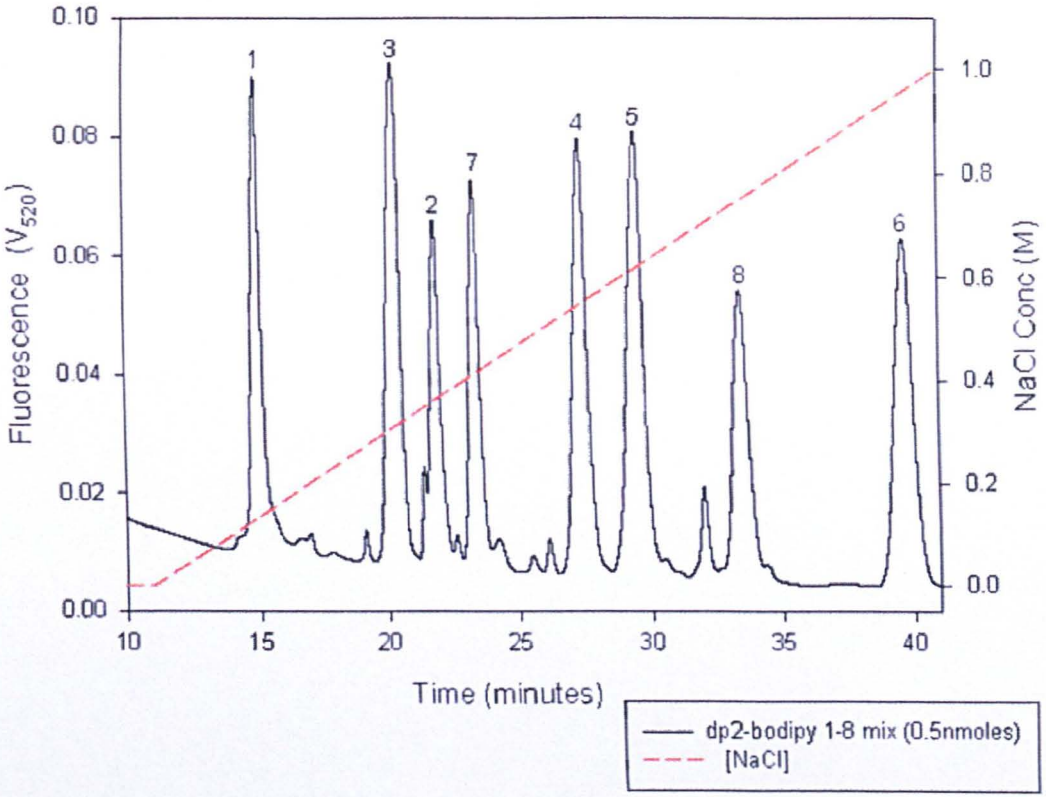


Figure 21 - Separation of heparin / heparan sulphate disaccharides conjugated with Bodipy ® Fl hydrazide (0.5 nanomoles) on a Propac PA1 column. The profile shows separation of the eight major conjugated disaccharide subunit components of the parental polysaccharide following bacterial lyase enzyme digestion. The linear sodium chloride gradient (0 – 1 M over 30 minutes, beginning at 11 minutes) is represented on the chromatogram by the red hashed line. The structures of the Bodipy labelled standards resolved were: 1 – ΔUA-GlcNAc, 2 – ΔUA-GlcNS, 3 - ΔUA-GlcNAc(6S), 4 – ΔUA(2S)-GlcNAc, 5 - ΔUA-GlcNS(6S), 6 – ΔUA(2S)-GlcNS, 7 – ΔUA(2S)-GlcNAc(6S), 8 – ΔUA(2S)-GlcNS(6S). Abbreviations: ΔUA – unsaturated uronic acid residue produced at the non-reducing end post eliminative lyase cleavage.; GlcNAc – N-acetyl glucosamine; GlcNS – N-sulpho glucosamine; 2S and 6S – 2-O-sulphate and 6-O-sulphate respectively.

Table 11: Relative labelling efficiency and correction factor for 8 Δ-disaccharide standards from heparan sulphate/heparin. (n=8)

Δ-Disaccharide standard [∞]	Relative efficiency (%)	Correction factor
1	77.4	1.3
2	58.8	1.7
3	84.5	1.2
4	84.5	1.2
5	100.0	1.0
6	94.5	1.1
7	70.8	1.4
8	92.8	1.1

[∞] Δ-Disaccharide structures are detailed in figure 24.

The Propac PA-1 column was selected for its ability to bind all eight fluorescently labelled Bodipy hydrazide disaccharide standards. Other columns tried were the AS4-C column (used previously for fluorescent separations of CS, DS and keratan sulphates) in basic pH and the C18 derivatized CTA column prepared as by Mourrier *et al.* [87] It was found that the AS4A-SC column bound seven of the eight standards, but standard 1 (ΔUA-GlcNac) eluted in the breakthrough slightly after the free tag which elutes due to its uncharged nature first (although the exact position depends on the amount of dissolved CO_2 in the mobile phase and eluent solutions) . The C18-CTA derived column bound both the free tag and the labelled disaccharides and elution could not be carried out using 2 M ammonium methane sulphonate. The column had to be regenerated by removal of the CTA using pure methanol and acetonitrile washes; therefore no separation was achieved and this column is considered incompatible for use with the Bodipy hydrazide tag.

3.2.14 Determination of the level of detection of a mixture of all eight Bodipy labelled Δ-disaccharides using high performance strong anion exchange chromatography

Dilutions of the mixture used in 3.2.13 were made using water and the experiment conducted in 3.2.12 repeated with dilutions corresponding to 1 picomole (10,000 fold) and 100 femtomoles (100,000 fold) carried out. All eight disaccharide standards are still visible in the chromatogram for sample containing 1 picomole of each standard while standards 1-3, 5 and 7 are detectable at 100 femtomole (see figure 22).

3.2.15 Disaccharide analysis of Bodipy labelled disaccharides from the exhaustive digestion of porcine intestine mucosal heparin (PMH) and porcine intestine mucosal heparan sulphate (PMHS)

100 picomoles of PMH and PMHS were exhaustively digested to yield disaccharides as described in section 2.2.2. The resulting disaccharides were labelled with Bodipy FL hydrazide (2.2.18) and the resulting chromatograms compared to known Bodipy labelled disaccharide standards as created in 3.2.12 and 3.2.13. The peak area was analysed and the correction factor calculated in 3.2.13 applied to give a comparison between the disaccharide constituents present in both porcine intestinal mucosa heparin and heparan sulphate see table 12 and figure 22.

3.2.16 Determination of the extent of de-N-sulphation of porcine mucosal heparin when heated in acidic conditions

Experiments were conducted to determine the relative level of de-N-sulphation which could occur when samples are heated in the presence of acids (see section 1.3.2.2). In order to achieve this, porcine mucosal heparin (100 mg) was exposed to acid and heat before the level of de-N-sulphation was observed using ^{13}C NMR. Prior to NMR, samples were re-N-acetylated with isotopically labelled ^{13}C acetic anhydride as described in

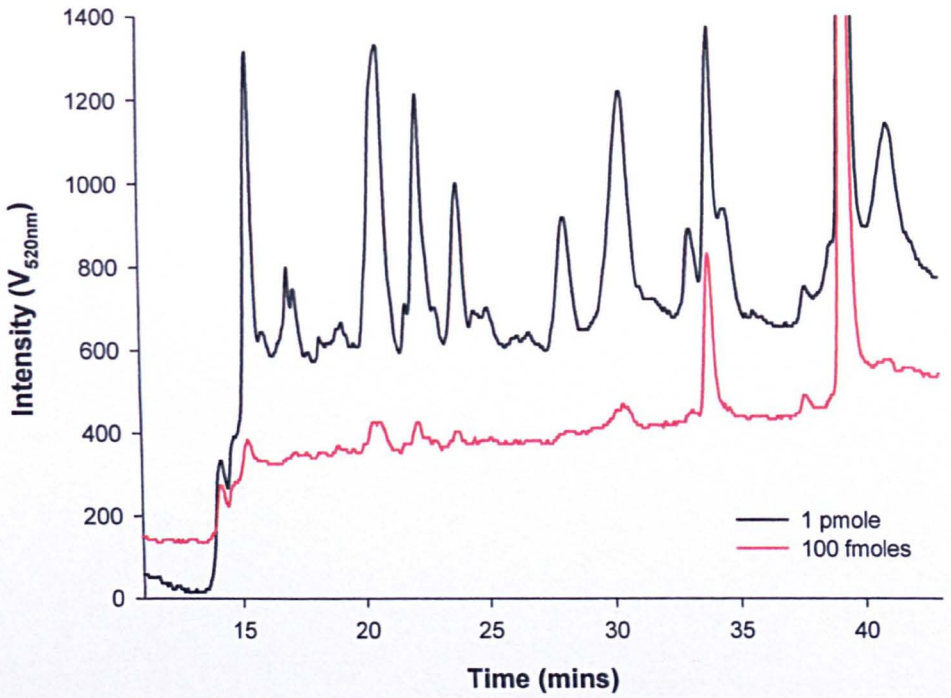


Figure 22 - Separation of 1 picomole and 100 femtomoles (each, following serial dilution) of eight disaccharides after conjugation with Bodipy ® Fl hydrazide on a Propac PA1 column. The profile shows separation and detection of all eight common conjugated Δ-disaccharides at a level of detection of 1 picomole with some peaks still being visible at 100 femtomoles. A linear sodium chloride gradient of 0 – 1 M over 30 minutes after an initial wash step of 11 minutes isocratic 150 mM sodium hydroxide was employed at a flow rate of 2 $ml.min^{-1}$ to separate the analytes.

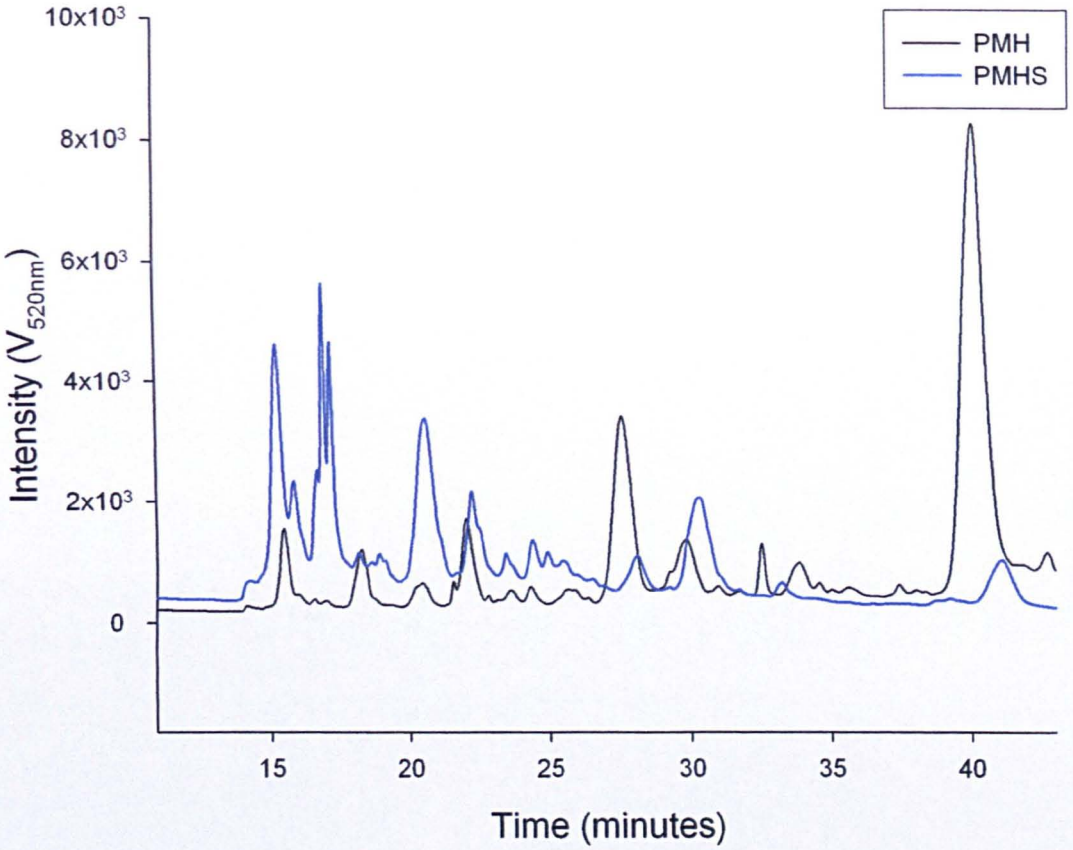


Figure 23 - Separation of porcine intestine mucosal heparin / heparan sulphate disaccharides following total enzymatic digestion and conjugation with Bodipy ® Fl hydrazide on a Propac PA1 column. The profile shows separation of the eight major conjugated disaccharide subunit components of the parental polysaccharides folowing exhaustive bacterial lyase enzyme digestion. A linear sodium chloride gradient of 0 – 1 M over 30 minutes after an initial wash step of 11 minutes isocratic 150 mM sodium hydroxide was initiated at a flow rate of 2 ml.min^{-1} in order to separate the analytes. Porcine mucosal heparin derived labelled disaccharides are shown in black while those from porcine mucosal heparan sulphate are shown in blue.

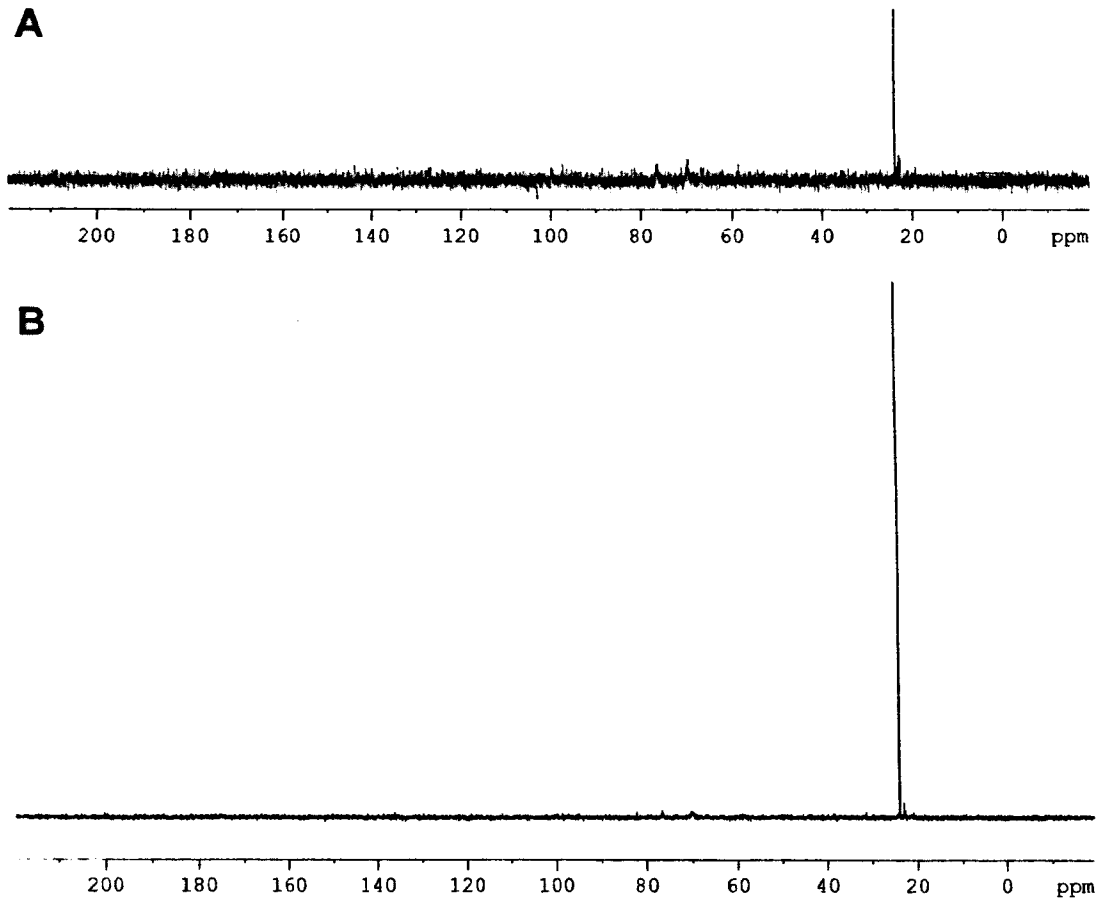


Figure 24 – 1D ^{13}C NMR spectra for heparin which has been dissolved in 50% acetic acid and either [A] incubated at room temperature for 4 hours, or [B] incubated at 65° C for 2 hours. The extent of de-N-sulphation was measured quantitatively by N-acetylation (N-acetyl groups are attached where previous N-sulphate groups resided) with the carbon isotope ^{13}C in acetic anhydride.

section 2.2.19. The relative levels of de-N-sulphation are shown in Figure 24. De-N-sulphation can be seen to occur appreciably more when heat is applied to heparin present in acidic conditions.

Table 12: Corrected disaccharide composition analysis of porcine mucosal heparin and heparan sulphate. (n=3)

Δ-Disaccharide standard [∞]	Heparin (%)	Heparan sulphate (%)
1	5.8	39.3
2	8.4	15.3
3	2.1	28.7
4	22.9	3.2
5	9.0	5.4
6	49.5	6.8
7	0.6	0.5
8	1.7	0.9

[∞] Δ-Disaccharide structures are detailed in figure 12.

3.2.17 The use of the metal catalyst di-butyltin chloride with the reducing agent phenylsilane (instead of acid catalysis) in the fluorescent labelling of a mixture containing all eight fluorescently labelled Bodipy disaccharides.

10 nanomoles of each disaccharide was labelled with Bodipy hydrazide as described in section 2.2.20 using dibutyltin chloride as the catalyst as opposed to acetic acid and phenylsilane as the reducing agent (*in situ*) as opposed to sodium borohydride. 100 picomoles of the resulting labelled mixture was analysed as for 2.3.13. The resulting chromatogram (figure 25) shows the labelling of all eight disaccharides, although the extent of labelling is lower than that of acid catalysis.

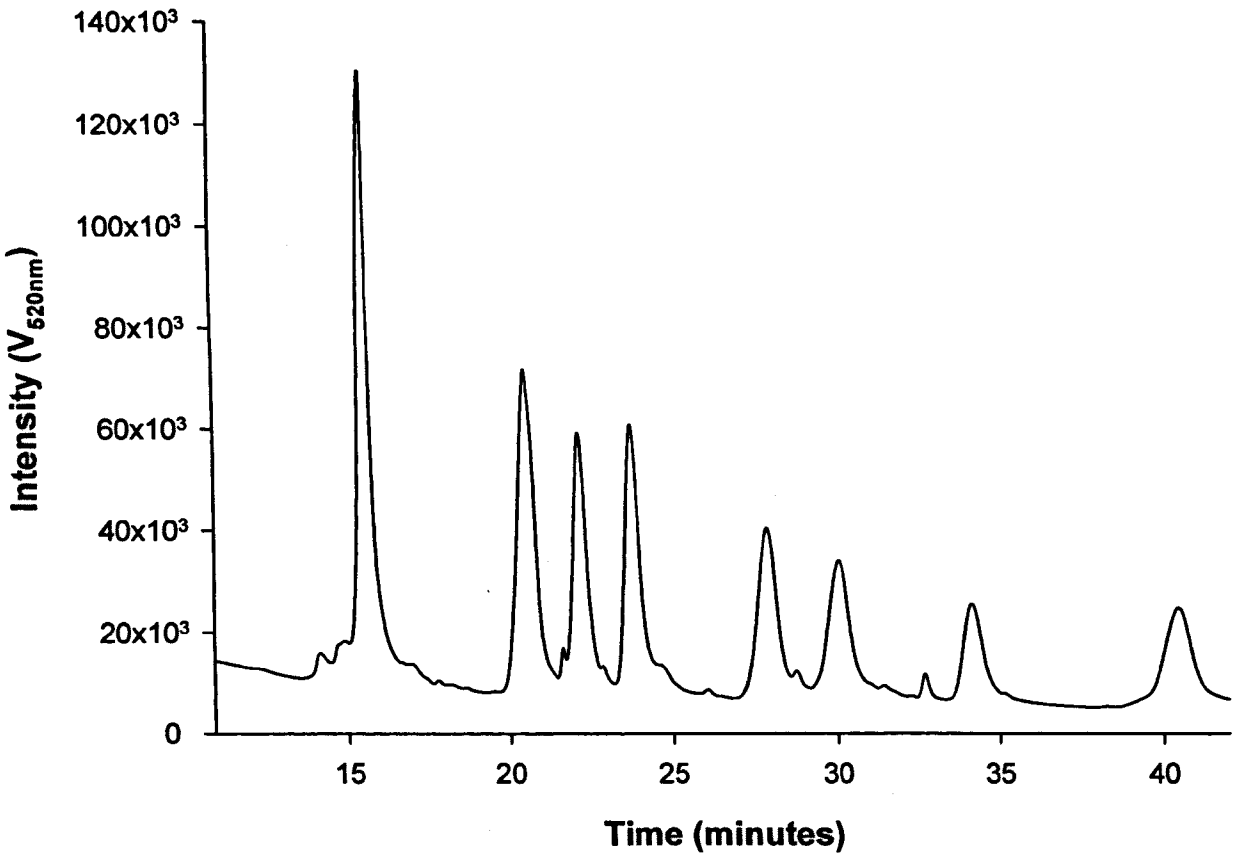


Figure 25 – Separation of eight heparin / heparan sulphate disaccharides conjugated with Bodipy ® Fl hydrazide (1 nanomole of each), using di-butyltin chloride as a metal catalyst, on a Propac PA-1 column. The profile shows separation of the eight major conjugated Δ-disaccharides using a linear sodium chloride gradient, 0 – 1 M over 30 minutes, following an 11 minute wash in isocratic 150 mM sodium hydroxide.

3.3 Discussion

3.3.1 Utilising the reducing end labelling of heparin and heparan sulphate derived oligosaccharides for conjugation / immobilisation

The reducing end carbonyl group provides a point for conjugation and immobilisation for heparin and heparan sulphate derived oligosaccharides. This conjugation chemistry has been exploited for labelling of the oligosaccharides for structural analysis and in experiments to investigate the binding properties of these carbohydrates. Reducing end coupling changes only the monosaccharide residue with which conjugation is achieved. The assumption therefore, is that reducing end labelled oligosaccharides molecules are considered unchanged by this association, although other considerations such as surface effects may need to be taken into account.

This single point mode of attachment proves useful for suitable orientation of the molecules upon a surface, whereas intra-chain labelling methods (e.g. exploitation of carbonyl moieties via EDC activation) could cause the molecule to be orientated in too close proximity to the surface (i.e. lying down) while it is also conceivable that modification could occur in binding sites present within the chain. The possibility of unfavourable orientation needs to be considered in terms of the charged groups regularly interspersed within the carbohydrate, which could orient along a polar surface (in a mode akin to ion pairing). Whereas the nature of these interactions is complex, it should not be disregarded as being unimportant or biologically irrelevant as the majority of proteins expressing HS or interacting with it are also present attached to biological surfaces, i.e. cell membranes, which posses far more complex fluid systems and motions (lipid rafts etc).

Attachment to surfaces adds further complexity to reducing end equilibria because the equilibria cannot be skewed to favour the product by adding excess amine to overcome the problems of amine protonation, as it can during labelling in solution. The amine surface possesses a finite density of amine nucleophiles and consequently, condensation

reactions which occur for immobilised heparin or heparan sulphate oligosaccharides onto the surface generally exhibit lower yields compared to those present in free solution and the optimum pH will probably be higher. It is apparent from these limitations that alternative strategies need to be developed for increasing the efficiency of this mode of attachment. The use of metal catalysis and conjugation via the non-reducing end discussed below provides an attractive possibility to overcome these limitations.

Coupling of oligosaccharides of heparin and heparan sulphate to other molecules in free solution yields glyco-conjugates in higher efficiency although these are still extraordinarily low, usually in the region of a few percent. This is primarily due to low rates of mutarotation (which only occurs in amphoteric solvents) compounded by limitations resulting from amine and carbonyl equilibria. Acid catalysis has generally been used to increase the yield although this is still poor and comes with its own problems of de-N-sulphation. An extensive study with regard to de-N-sulphation is desperately needed (after the findings in section 3.2.16 on the full length polysaccharide) to explore such factors such as solvent, pH, temperature and whether the presence of other moieties within a disaccharide / oligosaccharide affords protection from this solvolytic cleavage. Such a study is currently under way. This side-effect of reducing end labelling catalysis is frequently overlooked whether by ignorance or due to its complexity.

The reactivity of a saccharide reducing end is also highly likely to be sequence dependent. With regard to disaccharide molecules, this is likely to be quite pronounced. When one considers that a change of stereochemistry at a single carbon atom can facilitate different physico-chemical properties within a mono- or disaccharide, it is highly likely that the presence of bulky charged groups such as the strongly electron withdrawing sulphate group, hydrophobic groups or even the ever present carboxylic acid group of the uronic acid, could influence the reactivity of the reducing end. It is surmised that modifications further away from the reducing end in oligosaccharides of increasing length will be less significant. Labelling with Bodipy hydrazide, as described in chapter 3, appears to show variation within (at the extreme) one order of magnitude although this

is only for one type of fluorophore. It should be noted here that what applies for the disaccharide / oligosaccharides is almost certainly true for the molecules to which the carbohydrate is to be conjugated; i.e. small structural changes in the fluorophore will have consequences on the propensity of the nitrogen lone pair in the amine containing moiety to carry out nucleophilic attack. When the two molecules exist as a conjugate their properties will be some combination of the properties of the two molecules if they are of similar size (i.e. a disaccharide and a simple fluorophore), this could be quite different to the individual molecules if one is water insoluble before (i.e. hydrophobic) and the other is polar. Increasing the size of the carbohydrate fragment compared to that of the molecule to be attached will minimise the properties imposed on the product by the coupling molecule. It is necessary in all binding or biological assays to bear in mind the modifications which have been made prior to the assay and any ramifications of this derivatisation for subsequent interactions.

3.3.2 Bodipy hydrazide allows for the highly sensitive detection of heparin and heparan sulphate

The incorporation of radioactive isotopes or the coupling of molecules to aid detection (such as fluorescent entities or molecules possessing highly absorbent chromophores) is an important step when attempting to perform disaccharide compositional analysis on heparin or HS from scarce sources and / or sequence elucidation. The weak chromophore of the C=C bond introduced during bacterial lyase digestion will only allow detection down to a few micrograms. The limitations imposed by this quantity are quite significant when the multi-step isolation and purification procedures are considered together with their inherent losses.

Labelling of the molecule can also be performed using radioactivity, either by metabolic labelling or by reduction with a tritium carrying reducing agent. The use of radioactivity is undesirable as it comes with safety concerns, problematic handling and requires training and restrictive administration. Metabolic labelling of animals is not possible due to health constraints (and cost) and where cells can be cultured in the presence of

radioactive isotopes (usually radio-labelled PAPS) this is costly. Radioactive methods have been applied previously by Merry *et al.* [71] and Vives *et al.* [125] using a step wise sequencing strategy, analogous to the fluorescence based integral glycans sequencing (IGS) strategy by Turnbull *et al.* [69] (modified by Drummond *et al.* [126]) utilising HPLC detection. This also required the containment of all radioactivities within the machine work area, the use of scintillation counting and costly disposal.

To date a number of attempts have been made to improve the detection of HS disaccharides and oligosaccharides. These include mass spectrometry, coupled techniques such as MS-MS and LC-MS [127-129] and fluorescent conjugation. Mass spectrometric techniques require high cost equipment, skilled operators and are time consuming. As of yet, they have failed to significantly improve detection due to the difficulties of ionising sulphated carbohydrates [72].

To increase sensitivity and reduce the limit of detection, fluorescent techniques have been relatively successfully employed. Glycosaminoglycan family members such as HA, KS, CS and DS have been labelled successfully with fluorescent tags employing PAGE electrophoresis [130], HPLC [130] and CE [131] separation. Fluorescent tags used include 2-aminoacridone (AMAC), 8-aminonaphthalene-1,3,6-trisulfonic acid (ANTS), amino-pyridine, and 2-amino benzoic acid (2-AA) [116, 123, 131-134] amongst others. The level of detection is usually around the order of picomoles to high femtomoles. Owing to the poor reactivity of the reducing end of heparin and heparan sulphate derived di / oligosaccharides (in general and compared to other GAGS), efficient labelling methods are in short supply.

The most useful fluorophores used to derivatise heparin and heparan sulphate oligosaccharides to date employ either 2-aminoacridone or 2-aminobenzamide (2-AB). Both of these reactions take place in DMSO and are acid catalysed using acetic acid. When employed with liquid chromatography techniques, AMAC was found to offer only a 10-fold improvement in the level of detection compared to that of conventional UV detection at λ=232 nm [116]. Derivatisation with 2-AB afforded better sensitivity

compared to AMAC, although complete resolution of all the derivatized disaccharide standard peaks was not possible and an additional clean up step involving paper chromatography was necessary during purification[116].

The use of acid catalysis and elevated temperatures is also unfavourable because of the possibility of de-N-sulphation. While this can be corrected for in disaccharide analyses (assuming free amines do not occur naturally), it becomes more of a serious problem when labelling oligosaccharides for sequencing. The implementation of a post-column derivatisation process which attaches the fluorophore to the analyte after separation has been successfully demonstrated with detection limits in the order of 10 picomoles [135, 136] although this method involves serious modifications and re-configuration of the HPLC set-up and specialised equipment is necessary, such as the use of a 10 m post-column reaction coil which needs to be heated to 110°C.

With regard to fluorophore choice for use in capillary electrophoresis, AMAC and 2-amino benzoic acid (2-AB) have both been used successfully. 2-AB detection is performed in the UV and as such is limited in its sensitivity. The separation and detection of AMAC disaccharide derivatives has been reported in the UV at λ=255 nm with a 10-fold increase in sensitivity compared to UV detection at λ=232 nm and at least a 100-fold increase when using the more sensitive laser induced fluorescence detection. Unfortunately, the limit of detection quoted here could be misleading, as the attomole detection threshold quoted refers to the amount of material on the capillary. Unfortunately, the volume stated represents a volume of 140 nl on the capillary; it is not mentioned in the literature that at least 8 µl must be present in the vial to achieve this detection and represents a physical constraint for CE. The values quoted are therefore only hypothetical limits of detection.

The use of AMAC as a fluorophore has proved problematic, with no evidence of labelling when the strategies outlined in the literature have been followed (using CE detection). Evidence of weak labelling can be obtained if sodium borohydride is used as a reducing agent instead of reduction with sodium cyanoborohydride. It has been reported that this

coupling can prove to be unstable and this can be considered a likely explanation. Owing to this, AMAC has not proven to be an effective and efficient fluorophore, especially when highly reproducible data is needed for sequencing.

To address this problem, other fluorophores have been considered which fit the constrains of the LIF module for the CE, namely excitation close to that of the laser's operating wavelength, λ=488 nm and emission near to that of the detectors sensitivity maximum, i.e. λ=520 nm.

Of these fluorophores, Alexaflour 488 hydrazide was found to be too impure, possibly due to oligomerisation (personal communication, Dr A Powell and Dr M Brille) and the high degree of negative charge of the fluorophore complicates the matter further by increasing the overall net charge of the glyco-conjugate rendering the analytes more similar to each other. This is something that needs to be avoided especially when separating stereoisomers such as some heparin and heparan sulphate derived disaccharides.

Another fluorophore based on fluorescein (which unfortunately is too pH sensitive to be practicable) was synthesised for use with an amine containing hydroxylamine label. This fluorophore, although seemingly pure, appeared to only label very weakly and seemed to be selective for the tri-sulphated (standard 6) disaccharide but showed evidence of only weak labelling. Another explanation could be the loss of fluorescence upon conjugation of the molecule.

The fluorophore which gave the best results to date, was that of the fluorescent label Bodipy FL hydrazide. The increased yield (compared to previously studied fluorophores) could be due to the improved reactivity of the hydrazide group present on the fluorophore. This fluorophore offers a greatly enhanced co-efficient of extinction compared to that of AMAC and possesses excitation and emission characteristics compatible with the LIF module laser (see table 7). The molecule is also uncharged, which alleviates the problem of increasing the net charge of the conjugate, makes the

molecules more amenable to separation and less able to bind to oppositely charged groups on separation columns employed in HPLC.

Other advantages of Bodipy hydrazide are its hydrophobic nature (only slightly soluble in water) which allows for the possibility of phase separation, should clean up steps be necessary. This hydrophobicity is also extremely useful when strong anion exchange separation is used to separate labelled disaccharides on polymer based columns as the free tag passes through in the breakthrough. The downside to this is that silica-based derivatized SAX columns such as CTA are sensitive to the free tag (it binds strongly and can only be removed by column regeneration), and in the case of the C18-CTA column, the binding was reversible and required regeneration. Furthermore, the labelling of large oligosaccharide fragments may require the use of ion pairing reagents to allow solubility in applicable solvents. The polymer based column, coupled to the lack of interaction of the free tag with the matrix, obviates the need for any time consuming clean up steps, which would undoubtedly cause sample loss prior to disaccharide analysis. The lack of negative charge also prevents migration of the free tag in reverse mode CE, which could complicate the spectra due to peaks co-migrating.

The work carried out in this thesis has shown the separation, using fluorescence detection (fluorimeter), of all eight naturally occurring Bodipy labelled disaccharide standards to baseline utilising strong anion exchange chromatography. Using this separation technique, the labelled disaccharides can be detected down to 1 pmole for all eight and as low as 100 fmoles for some of the standards. The method also does not require any clean up steps, labelling can be carried out in the presence of salt (e.g. lyase buffer), lyase enzymes and BSA carrier protein. This is a significant improvement to the field of HS composition analysis compared to that already achieved using column chromatography. The necessity for basic conditions during the run (150 mM NaOH) is probably to maintain the labelled disaccharides in a negatively charged state (ionising hydroxide OH groups to O^-) so they interact with the matrix, after conjugation with the relatively more hydrophobic fluorophore.

Separation of the eight disaccharides using CE is not as successful as for HPLC and further experiments need to be conducted using different buffer systems, concentrations, pHs and or different CE modes in order to separate, reproducibly to baseline. The use of pre-derivatized capillaries to either abolish or control EOF may permit better separation to be carried out at higher environmental pH ranges. Although separation of different charge groups (i.e. 0, -1, -2 etc..) is possible, baseline resolution of stereoisomers is not achievable, although significant progress has been made.

The advantage of CE is the low volumes required and the use of LIF detection, which permits sensitivity of between 1 and 5 femtomoles present within the sample vial. The hypothetical level of detection would therefore be 3.75 attomoles on the capillary, based on the 30 nl injection currently performed (calculated using CE Expert Lite, Beckman Coulter). This is a huge improvement and once the separation issues have been resolved, this labelling procedure will provide the most sensitive of all the analytical techniques available for HS detection to date. Further improvements in the level of detection could be made by injecting more sample volume onto a larger bore capillary. This will further improve detection sensitivity but some resolving power will be lost (which is the more important issue at present).

Another merit of Bodipy is that the reaction mixture is not heated, which limits the possibility of de-N-sulphation. The removal of the N-sulphate group has been demonstrated in this thesis in polysaccharide chains under various acidic conditions and is particularly severe when heating is employed. The author is not aware of any definitive studies in regard to this phenomenon for oligosaccharides or disaccharides and it is not known whether disaccharides suffer from this phenomenon. This is an area which will continue to be researched by the author beyond the timescale of this thesis.

Another development which will provide advancement to the field is the demonstration of metal catalysis for the first time for this class of molecules. This simple metal catalysis provides a serious alternative to acid catalysis and should decrease the possibility of de-N-sulphation. The yield is at the moment inferior to that of acid catalysis but ongoing

work to develop reaction conditions and elucidate other candidate metal catalysis is being conducted. Further work will also need to be conducted to elucidate the mechanism(s) by which catalysis is achieved. The method could potentially be used with other carbohydrate molecules and fluorophores which previously did not label with an appreciable rate or yield.

To conclude, the improvements concerning reducing end labelling reported in this chapter allow for improved labelling of disaccharide molecules with a novel fluorophore (in this field); Bodipy FL hydrazide. Although the initial outlay made maybe slightly increased for this fluorophore compared to others commercially available, this is significantly outweighed by the increase in the level of detection. The level of detection for both HPLC SAX and CE is superior to that previously used and for the CE is currently at the cutting edge of the HS field. The level of detection for HPLC SAX is currently limited by the detection method although coupling of a LIF detector is being actively researched. The improved sensitivity and threshold of detection, coupled to the possible use of metal catalysis, which can be employed when the sensitivities of acid catalysis are to be avoided, presents an enormous step towards the realistic possibility of routine sequencing of HS from tissue samples. This should allow for a better understanding of the HS structure / function relationship to be obtained. The labelling method should also be readily transferable to other glycosaminoglycans such as CS and DS as well as larger oligosaccharide fragments (work in progress). Labelled fragments could be used in cell imaging (to show HS localisation) as well as for improved sensitivities in sequencing strategies such as IGS. Such is the speed at which the fluorophore market improves and expands, the labelling efficiency of new fluorophores should be regularly investigated to try and improve detection even further.

Chapter 4

A novel method for labelling of the non-reducing end of GAG Δ-oligosaccharides via the formation of mercury adducts.

4.1 Introduction

In an attempt to overcome the problems encountered in reducing-end labelling, i.e. that of low yield and the potential for chemical modification (especially de-N-sulphation, see 1.3.2.2) of the polysaccharide when acid catalysis is employed, other possibilities for derivatisation and / or attachment were investigated. The efficient coupling of GAG oligosaccharides to labels allowing increased sensitivity / detection and helping in the determination of oligosaccharide sequence, as well as their immobilisation on surfaces to form arrays and biosensor surfaces, would seem an attractive prospect. This novel mode of attachment would also permit the possibility of dual-end labelling at both the reducing and non-reducing. Such developments would assist in the drive to elucidate structure-activity relationships and in the discovery of binding partners within this structurally complex and biologically important class of carbohydrates.

Currently, labelling or surface attachment occurs via the condensation reaction between reducing end carbonyl groups (aldehyde) of the sugars and the amine groups of tags and / or surfaces to form Schiff bases or imines. Examples include the conjugation to fluorophores and tags to further aid detection in biological systems [137] or sequencing methodologies [69, 126] and as a means of coupling to derivatised glass surfaces. However, the efficiency of this reaction can vary, and it is also dependent on the structure of the reducing end sugar monomer [126]. The reaction can be acid catalysed but this cannot be utilised for some GAGs, including the medically and biologically important heparan sulphate (HS), because of their inherent sensitivity to acid [66, 138].

One potential site of attack is provided by the carbon – carbon double bond (C=C) between C-4 and C-5 of the unsaturated uronic acid residue (ΔUA) which is introduced upon digestion with bacterially derived lyase enzymes which depolymerise heparin and heparan sulphate polysaccharides (see section 1.3.1.2). This alkene group provides an alternative site for the conjugation of GAG-derived oligosaccharides.

The reaction of the carbon – carbon double bond with mercuric acetate is a well established reaction [139] in which the π electrons of the double bond attack the electrophilic Hg^{2+} atom present in the mercuric acetate thereby displacing an acetate group and forming a cyclic mercurinium intermediate [140] (oxymercuration). This initial step has been employed previously at slightly acidic pHs (a pH of 5.5) to remove the terminal non-reducing end monosaccharide by the treatment of the intermediate with acid ion exchange resin [141] (see figure 26).

The formation of mercurinium intermediates paves the way for other conjugation possibilities including the avid chelation by sulfhydryl groups of mercury [142]. This interaction can be exploited to produce glyco-conjugates attached via the saccharide's non-reducing end. This permits for the creation of versatile glyco-conjugates, such as GAGs coupled to gold nanoparticle conjugates (AuNPs) (see figure 27) and their attachment biosensor surfaces (see figure 28).

4.1.1 Olefins and mercury salts

The use of metal salts in addition reactions to unsaturated C=C bonds (alkene) present within a carbon chain (known as olefins) have been demonstrated previously. The involvement of metals such had platinum, copper, silver, iron, mercury and palladium has been reported [143]. The alkene containing molecule is thought to form coordination complexes by their involvement in the coordination sphere in a mode analogous to that involving a lone pair of electrons [139, 143].

Of the plethora of metallic salts which are known to interact with olefins, mercury salts provide stable products and possess the most variety with regard to possible reactions. The nature of the various interactions are in themselves controversial, being considered to be quasi-complexes, due to the existence of both addition-like and coordinative

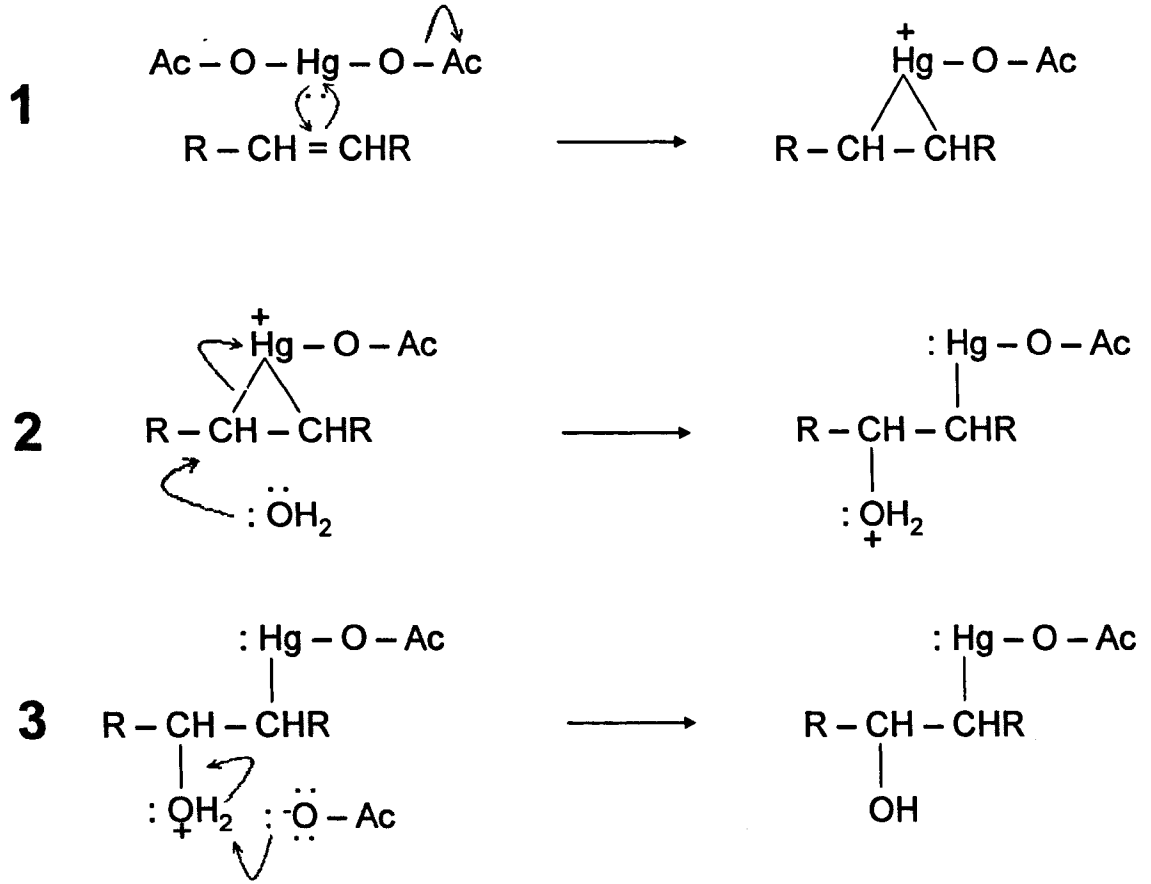


Figure 26 – Reaction mechanism for the oxymercuration of an alkene containing molecule with mercuric acetate in the presence of water and tetrahydrofuran. The initial step involves the formation of a cyclic mercurinium ion. The mercurinium ion is subject to attack by water forming the oxymercurial.

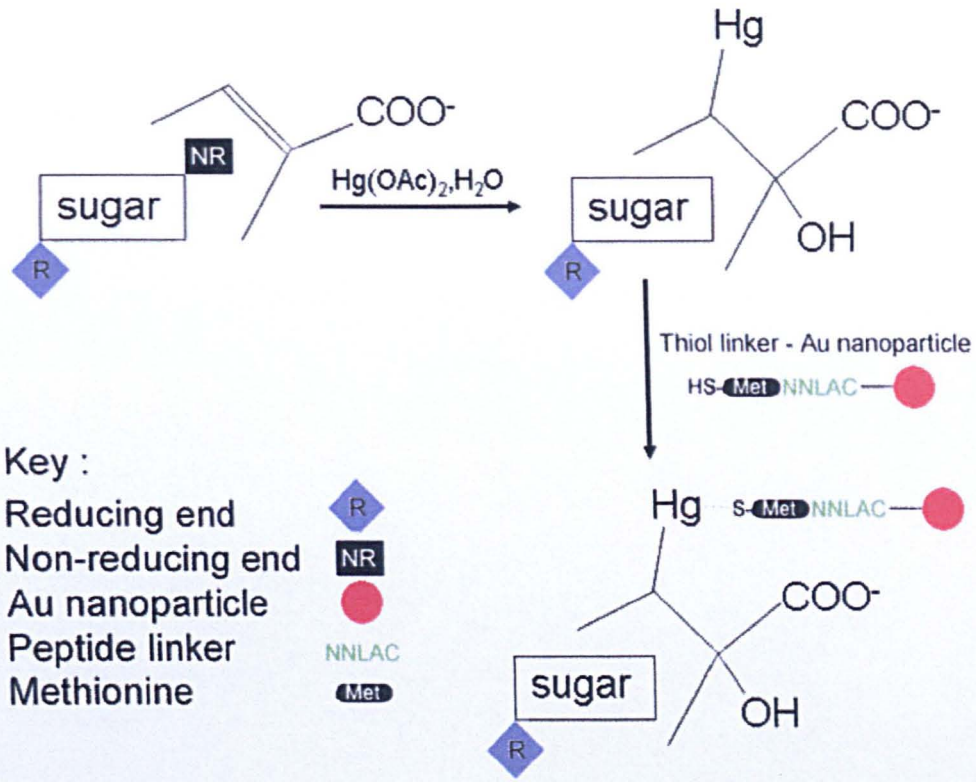
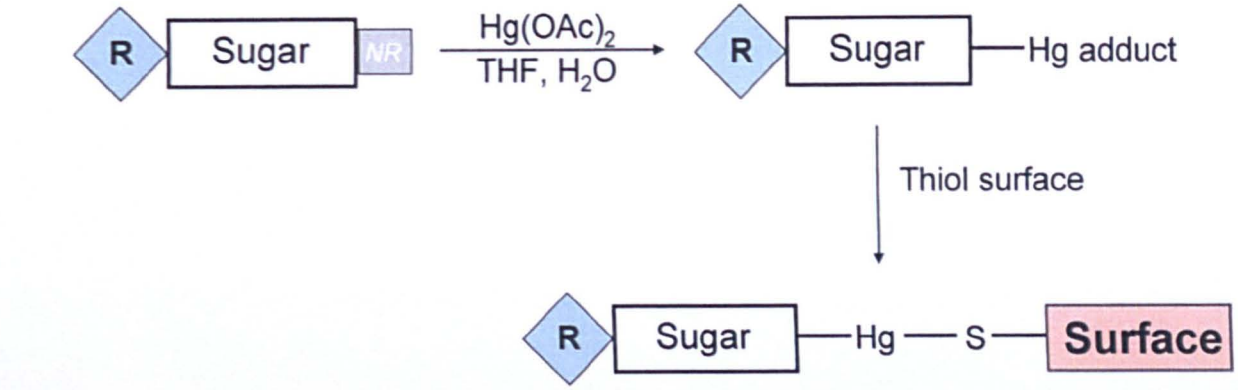


Figure 27 – Conjugation of glycosaminoglycan sugar to gold nanoparticles utilising non reducing end mercury labelling of the carbohydrate. Gold nanoparticles are coupled to a peptide containing a terminal thiol (CALNN-methionine) which is known to form a strong interaction with mercury containing species.



Key:

Glycosaminoglycan Sugar

Reducing end R

Non-reducing end

Figure 28 – Immobilisation of glycosaminoglycan sugar to gold surfaces utilising non reducing end mercury labelling of the carbohydrate. Gold surfaces are with a dual ended thiol which is known to form a strong interaction with both gold and mercury containing entities, thereby cross linking the non-reducing end labelled sugar with the surface.

properties [139]. Reactions with mercuric salts could potentially involve addition, oxidation or substitution reactions and it was not until the turn of the 20th century that the conditions for addition were described [139].

Kinetic studies [144, 145] showed that the reaction was complex, reversible and could not be considered as just an addition reaction or coordination product. The discovery of long lived, reversible intermediate products provided an explanation for the complex reaction towards the addition products, with resonance thought to be the reason for complex stability [144]. Predicting the actual product formed from the use of mercuric salts in aqueous solution is not trivial and is highly dependent on the olefin structure, pH of the reaction mixture, the acid of the mercuric salt, the reaction temperature and the concentrations involved. The solvent system employed affects the rate of reaction and can also take part in the reaction itself [139].

The type of mercuric salt used in oxymercuration dictates the final outcome of the reaction and influences the rate. Mercuric salts of mineral acids are not known to react with C=C bonds whereas mercuric salts of organic acids do. Mercuric salts which contain a mixture of mineral and organic acids also do not react. Acetate and nitrate salts of mercury are conventionally used because of their propensity to yield simple addition products with a workable yield. The presence of strong acidic conditions can skew the reaction equilibria towards the reactants, especially for mercuric nitrate, making neutralisation important but this is not the case for mercuric acetate which is not a sufficiently strong acid [139].

4.1.2 The mercurinium ion

The process of oxymercuration is initiated by electrophilic addition of the mercuric salt to the C=C of the olefin. This step leads to the production of a cyclic intermediate known as the mercurinium ion. The electron deficient mercurinium ion is then attacked by a nucleophile, usually present within the solvent or by the solvent itself. The initial reaction which creates the mercurinium ion is a rapid and reversible stage, while the second step is

rate determining (see figure 29). The product created by nucleophilic attack is known as an oxy-mercurial [140]. This product can be reduced to remove the mercuric group in a process known as demercuration.

The presence of the mercurinium ion as an intermediate has been shown to exist by NMR (^{1}H, ^{13}C and ^{199}Hg), gas phase detection and by kinetic studies [140, 146-150], although the data for NMR was obtained in acidic conditions and remains somewhat controversial as to whether it exists in reaction conditions. The nature of the intermediate is thought to be dependent on the degree and type of functionalisation present on the carbons atoms involved; with both open or cyclic (both symmetrical and asymmetrical) intermediates thought to exist [140, 151, 152]. The presence of functional groups which can donate electrons is thought to promote the formation of non-cyclic intermediates [152].

4.1.3 Overview of mercuric acetate mediated oxymercuration

A common method for the addition across a double bond employs the acetate salt of mercury. Oxymercuration generally occurs in a solvent system of tetrahydrofuran (THF) and water (50:50 v/v) and involves the use of mercuric acetate [$Hg(OAc)_2$] to attack the C=C double bond. This method forms a cyclic mercurinium ion / intermediate which is attacked by water molecules to yield an alcohol present within the alkyl (C-C) carbon chain along with the presence of an acetoxymercuric group.

The attack of the cyclic mercurinium ion / intermediate by water occurs at the carbon which is most substituted / functionalised. The nucleophilic attack releases the electrons previously tied into the cyclic intermediate which neutralises the electron deficient Hg atom and attaches - $^{+}OH_2$ to the most functionalised carbon. The electron deficient

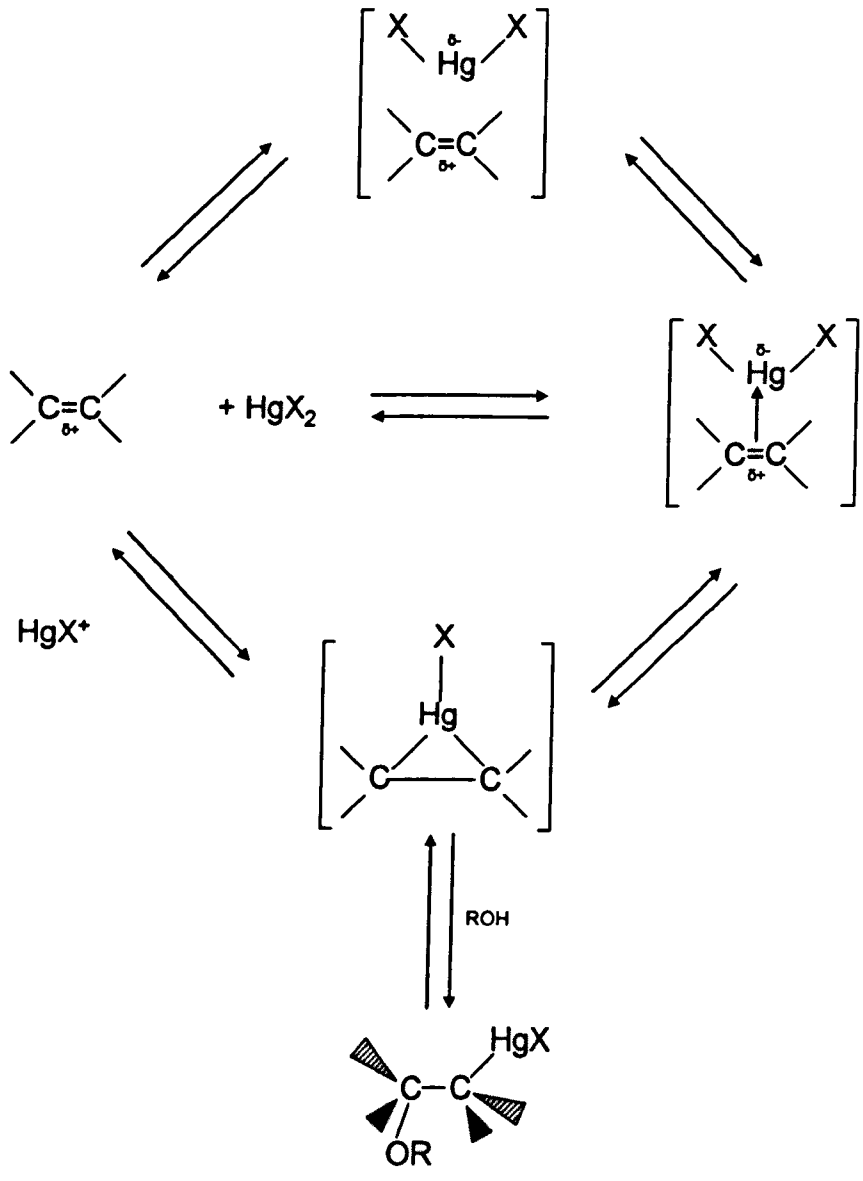


Figure 29 – Oxymercuration reaction mechanism. The formation of an oxymercurial via a cyclic mercurinium ion following the attack of the mercuric salt by the π bond electrons of the C=C bond.

oxygen atom loses one of its attached hydrogen atoms by attack from the acetoxy ion ($^{-}$OAc) which was released upon the initial attack of the C=C double bond by mercuric acetate. Attachment to the double bond occurs in a *trans* configuration and is therefore stereo-specific (anti-addition). This stereo-specificity is caused by the presence of the cyclic mercuric intermediate which dictates the arrangement following the attack by water. The reaction follows the Markovnikov rule. Conventionally this mechanism includes a subsequent reduction step (e.g. sodium borohydride) which removes the acetoxymercuric group replacing it with a proton. This reduction step removes any stereochemistry present as the addition of the proton can occur in a *cis* or *trans* manner.

The simplified outline of the oxymercuration reaction outlined above does not however describe some of the finer mechanistic details which are important in understanding the final product(s) and the possibility of intermediates when the reaction is applied to unsaturated uronate sugars.

4.1.4 Aims

The aim of this chapter was to investigate the potential of the non-reducing end unsaturated carbon – carbon (C=C) double bond as a new point of attachment. The use of mercury salts, specifically mercuric acetate, will be employed to attack the double bond.

Analysis of any potential mercury labelled products will be performed using analytical techniques such as mass spectrometry and NMR.

The attachment of the non-reducing end mercury labelled glyco-conjugates will be attempted utilising the known mercury-thiol interaction. This reaction will be probed (in collaboration with Drs Patey and Thanh) for attachment and potential biological activity.

4.2 Results

4.2.1 Oxymercuration of Δ-oligosaccharide fragments

The oxymercuration reaction is a standard organic chemistry reaction which yields a bridged cyclic mercurinium intermediate. An outline of the proposed structure can be seen in figure 30. The C=C double bond created during lyase digestion absorbs at $\lambda=232_{nm}$. Therefore the reaction can be monitored by the loss of absorbance at this wavelength (data not shown) although this has to be performed after clean up as the acetate ion also absorbs at this wavelength. Some residual absorbance usually remains because the presence of acetate groups (absorbing at $\lambda=215_{nm}$) and the level of absorption remaining depends on the amount of starting material. The intermediate described in figure 30 is possibly stabilised by the presence of the uronic acid carboxylic acid group, which is protonated by the low pH of the mercuric acetate (which also catalyses the initial π bond electrons of the C=C bond to attack the mercuric acetate), and it may be this that prevents conventional rearrangement. The reduction step (demercuration) which classically follows oxymercuration is omitted because this leads to the destruction of the cyclic mercurinium intermediate and removal of the Hg atom.

In order to confirm the presence of the cyclic mercurinium intermediate, matrix assisted laser desorbed ionisation mass spectrometry (MALDI-MS) was carried out using gentisic acid as a matrix (known to aid the ionisation of [sulphated] carbohydrates, personal communication with Dr E Yates). The ability of the Hg to bind to thiol groups was exploited to illustrate how Hg^{+} labelled oligosaccharides derived from heparin and HS could be coupled to surfaces and also shows coupling to both an optical biosensor and conjugation to nanoparticles.

4.2.2 MALDI-MS of reduced and non reduced model disaccharide ΔUA-GlcNAc

The MALDI-MS equipped with a time of flight (TOF) detector was used in the negative ion mode. The calculated mass of the oxymercurated ΔUA-GlcNAc standard present in

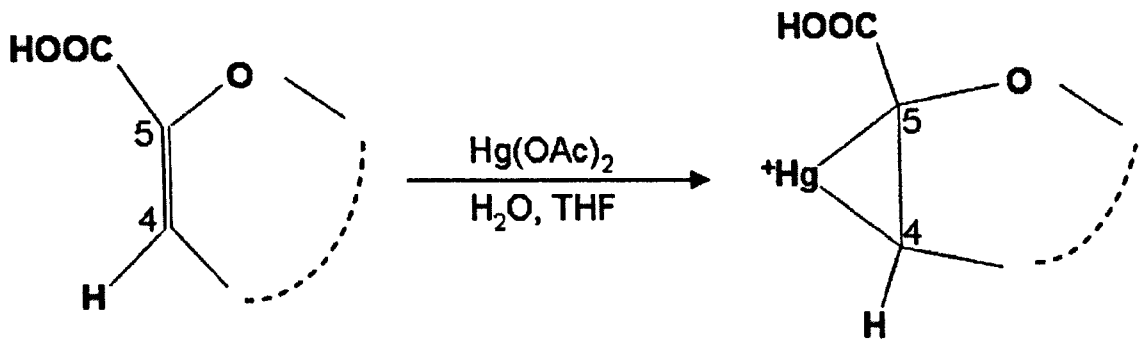


Figure 30 – Proposed stable mercurinium intermediate formed by oxymercuration of the C=C bond between C-4 and C-5 of an uronic acid introduced during bacterial lyase digestion. The formation of the intermediate is carried out in tetrahydrofuran and water (1:1 v/v) at 40°C for 120 minutes.

the cyclic mercurinium ion intermediate would be expected to have a calculated, theoretical mass of 638.9 m/z. The reaction mixture gave a spectrum (Figure 31) which shows a peak present at m/z 640. This peak corroborates with that expected if the machine error is taken into account. The mass spectrometer was calibrated with a bradykinin 1-7 fragment, Mr 756.8, which is commonly used as a MS reference standard. A peak was observed at m/z.757.76 [$M+H^+$] for the calibration standard. Peaks at m/z 685 and 692 are as yet unassigned, but it would appear that they originate from an interaction of the mercuric acetate and the matrix because they are present in controls lacking the mercury labelled sugar. Reduced ΔUA-GlcNAc disaccharides which were also subject to the same mercuric acetate labelling conditions showed a similar profile. The peak at m/z 668 (Δ=28) is as yet unassigned from the reaction mixture.

4.2.3 ^{1}H 1D and 2D COSY & TOCSY NMR of Hg-(OAc)-UA-GlcNAc and ^{1}H 1D NMR of Hg(OAc)-CS disaccharides.

Full assignment of the mercurinium intermediate formed by oxymercuration and verified by MALDI-TOF MS was performed on the COSY spectrum of Hg-(OAc)-UA-GlcNAc. This shows the presence of both the α and β reducing end anomers which confirms the existence of the intact disaccharide. In addition, the chemical shift of the uronic acid residue H-4 is evidence of the reaction of the C=C double bond with the mercuric acetate yielding a C-C bond which would be expected by the oxymercuration process (see figures 32 and 33).

The TOCSY spectrum also shows the presence and association of only 4 carbon associated protons and not 5 which would be the case if the cyclic mercurinium ion had opened to yield the addition product, with 2 protons being present on C-4 of the N-acetlyglucosamine residue. The TOCSY spectra shows the differences in the 4 carbon associated protons before and after oxymercuration where the changes in chemical shift (and hence their environment) are dramatically evident.

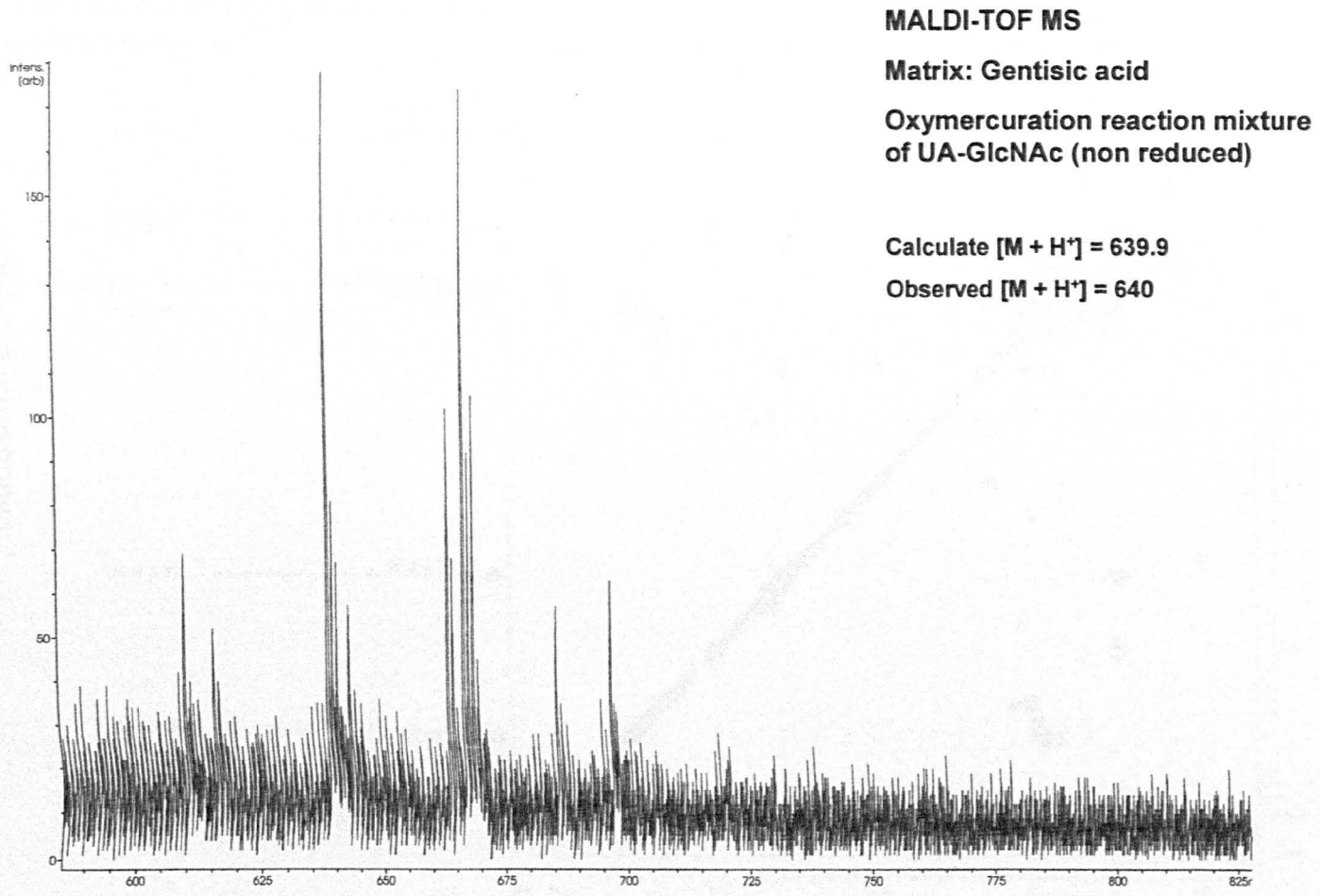


Figure 31 – Mass spectrogram of the reaction mixture following labelling of Δ-disaccharide standard 1 [ΔUA-GlcNAc] with mercuric acetate in tetrahydrofuran and water (1:1 v/v) at 40° C for 120 minutes. An aliquot of the reaction mixture was mixed with the matrix gentisic acid and spotted onto a silver target plate. Mass spectrometry was performed on a Bruker MALDI-TOF MS machine in negative ion mode. The machine was calibrated using bradykinin fragment 1 – 7.

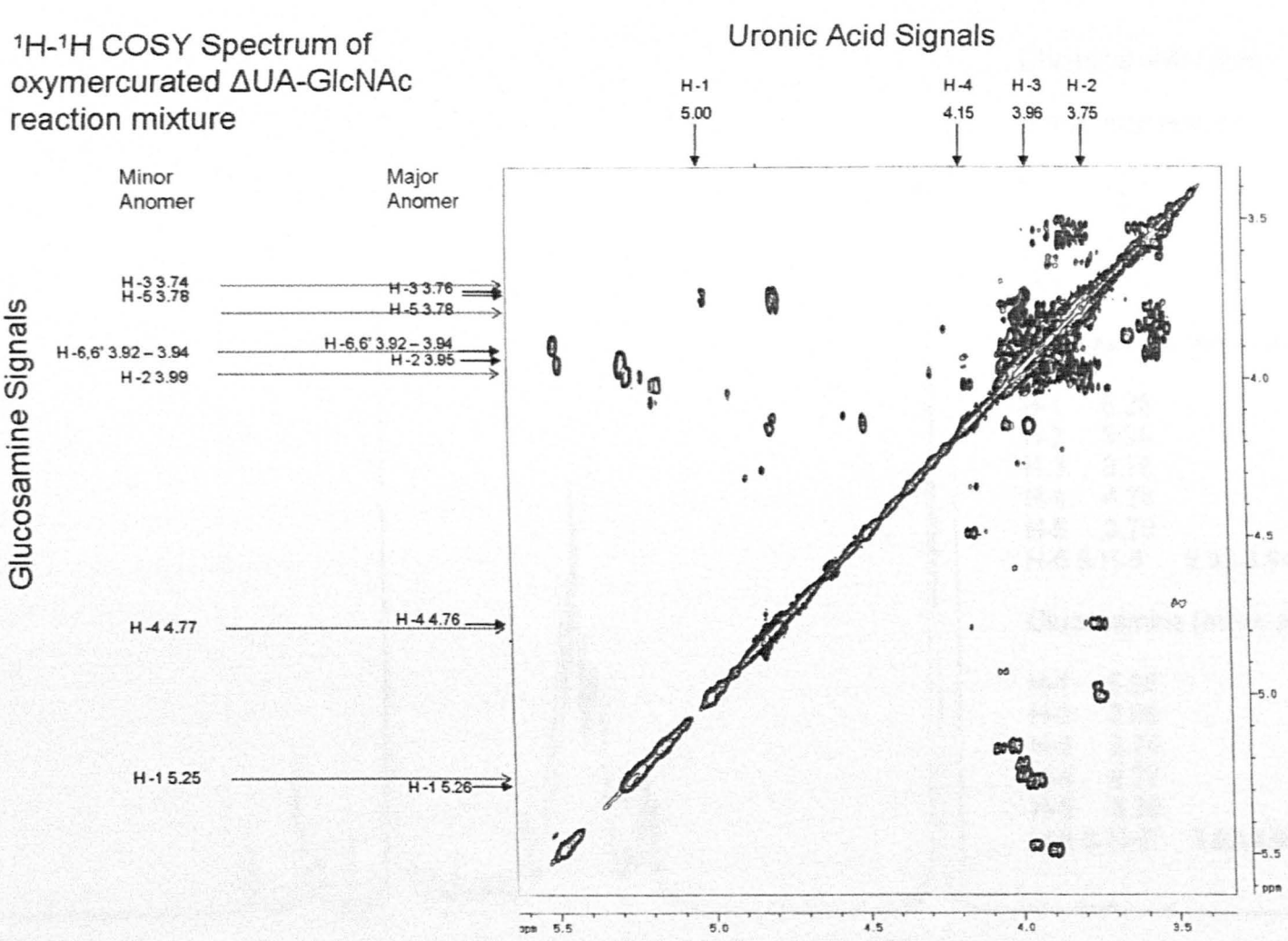


Figure 32 – ^{1}H-^{1}H COSY NMR spectrum of the oxymercurated ΔUA-GlcNAc reaction mixture following labelling of Δ-disaccharide standard 1 [ΔUA-GlcNAc] with mercuric acetate in tetrahydrofuran and water (1:1 v/v) at 40° C for 120 minutes. Assignments for both the minor and major anomers of the glucosamine residue are labelled along with those for the uronic acid residue.

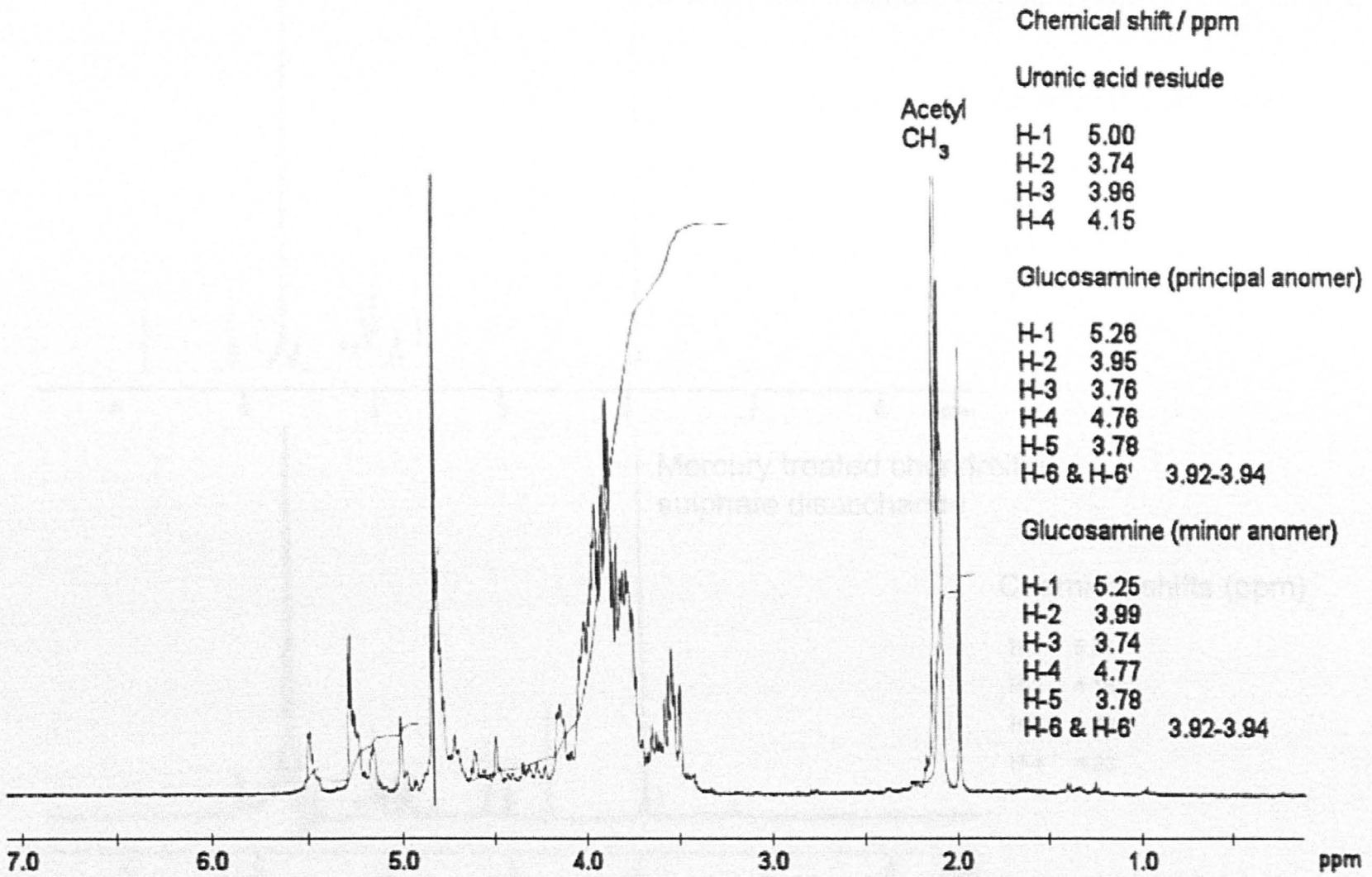


Figure 33 - ^{1}H 1D NMR spectrum of the oxymercurated ΔUA-GlcNAc reaction mixture following labelling of Δ-disaccharide standard 1 [ΔUA-GlcNAc] with mercuric acetate in tetrahydrofuran and water (1:1 v/v) at 40° C for 120 minutes. Assignments for both the minor and major anomers of the glucosamine residue are labelled along with those for the uronic acid residue.

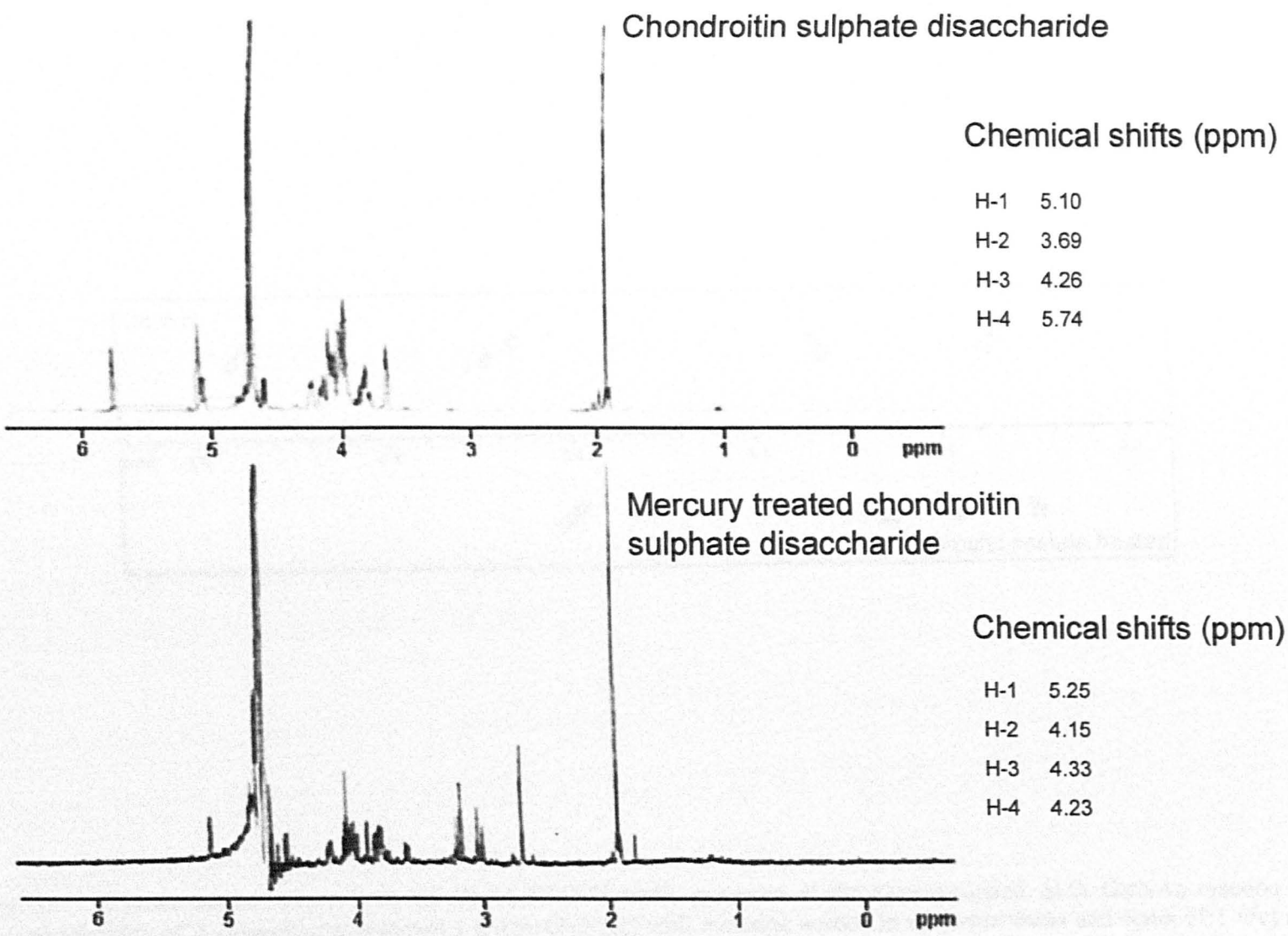


Figure 34 - ^{1}H 1D NMR spectra of the oxymercurated ΔUA-GalNAc reaction mixture following labelling of Δ-disaccharide from chondroitin sulphate [ΔUA-GalNAc] with mercuric acetate in tetrahydrofuran and water (1:1 v/v) at 40° C for 120 minutes and the original starting Δ-disaccharide. Assignments for the uronic acid residue carbon associate protons are made both pre and post oxymercuration.

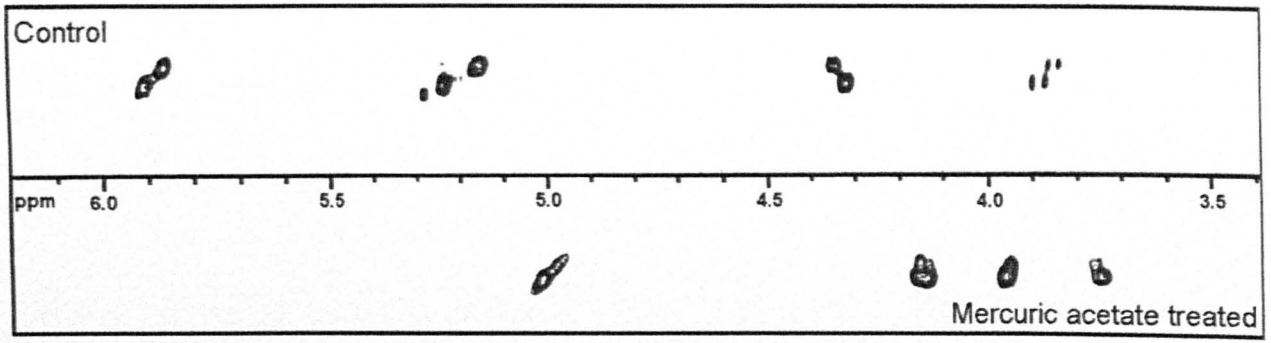


Figure 35 – A cross section taken from the ^{1}H-^{1}H TOCSY NMR spectrum of the oxymercurated ΔUA-GlcNAc reaction mixture following labelling of Δ-disaccharide standard 1 [ΔUA-GlcNAc] with mercuric acetate in tetrahydrofuran and water (1:1 v/v) at 40° C for 120 minutes and the original starting Δ-disaccharide. The change in the chemical shifts of the protons attached to the four uronic acid carbons can be seen after mercuric acetate treatment.

The 1D ^{1}H protons spectrums below show the differing spectra after oxymercuration for both Hg-(OAc)-UA-GlcNAc and Hg-(OAc)-CS where again the chemical shift for H-4 is markedly different following oxymercuration. This shows that the oxymercuration technique is not just limited to heparin and heparan sulphate disaccharides but to other glycosaminoglycans as well (see figure 34).

The NMR data suggests that the reaction products contain a disaccharide which is still intact (confirmed by MALDI-MS) and has not been subject to any rearrangement by the presence of 4 carbon associated protons and the α and β reducing end isomers. The NMR spectra also show a near quantitative reaction yield from the changes in chemical shifts of the protons and the absence of former peaks compared to the un-reacted spectra (see TOCSY, Figure 35).

4.2.4 Conjugation of Hg-labelled HS oligosaccharides to Au-thiol derivatized surfaces (in collaboration with Dr S J Patey)

Binding of fibroblast growth factor-2 to a heparin hexadecasaccharide immobilised by mercury labelling at the non-reducing end was shown [in figure 36] using a BIAcore optical biosensor. The instrument works by measuring subtle changes in the refractive index of the surface, which are related to the addition (or removal) of molecules to (or from) the surface. The change in refractive index is assumed to be proportional to the mass attached at the surface. Control experiments were conducted to eliminate the possibility of FGF-2 binding to the un-derivatized Au surface or the thiol-derivatized Au surface alone (see figure 37) and to exclude the involvement of free Hg^{2+} ions bridging from surface thiols to anionic groups on the oligosaccharide (incubated with excess $Hg(OAc)_2$ then FGF-2, no binding observed). Furthermore, the binding of FGF2 to the thiol-Au surface coated with Hg-derivatized HS oligosaccharides was prevented by the addition of heparin (see Figure 38).

The data shows that the Hg-labelled sugar binds to the 1,8 octanedithiol surface, by indirect observation using the known heparin ligand FGF2. The interaction between a

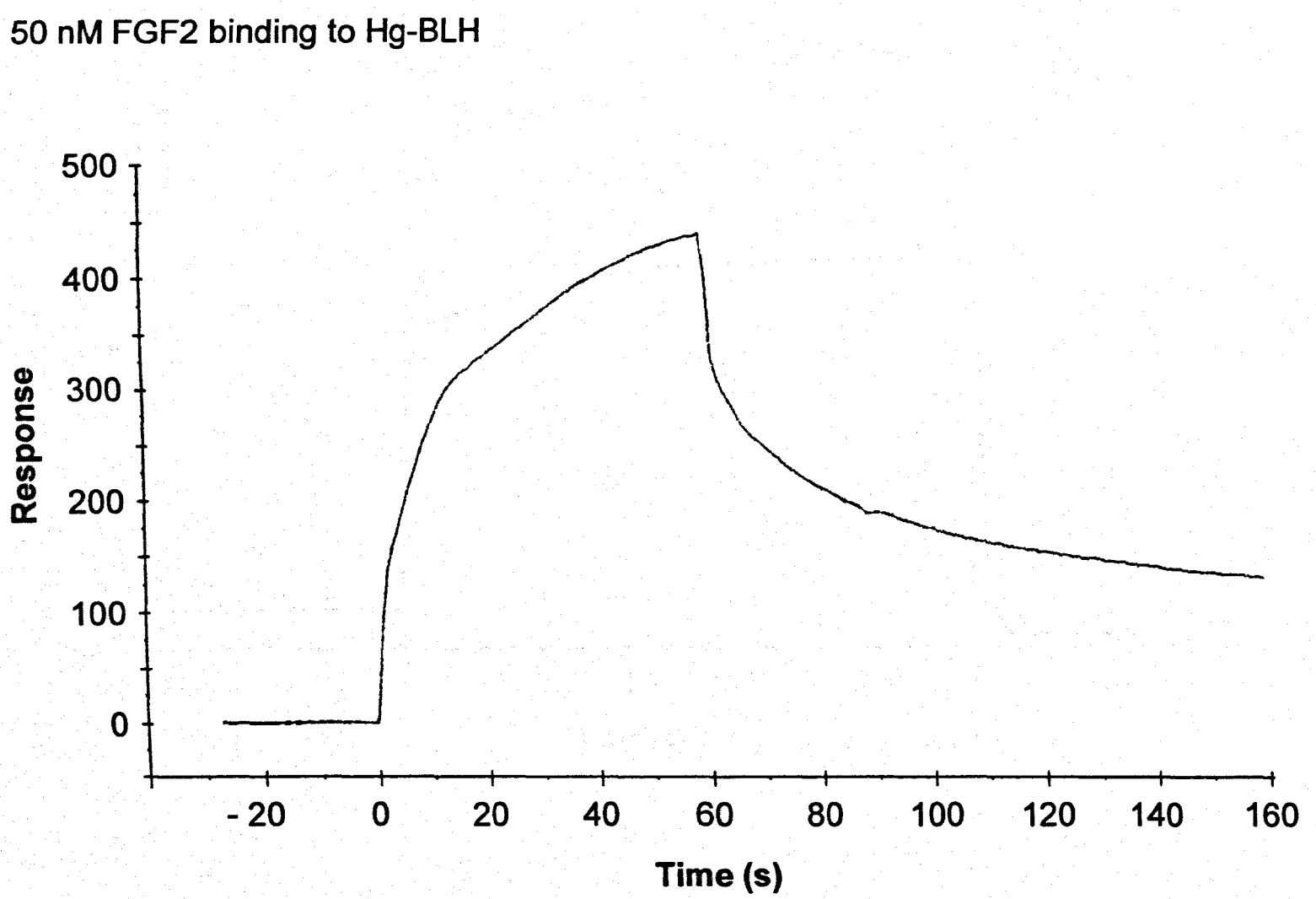


Figure 36 – BIAcore sensorgram of the fibroblast growth factor II (FGF2) ligand binding to heparin oligosaccharides immobilised via their non-reducing end on the surface of the sensor chip. The sensor chip was initially derivatized with 1,8 octanedithiol and the non-reducing end mercury labelled oligosaccharide attached via the thiol – gold interaction. After the bulk shift observed the line increases as

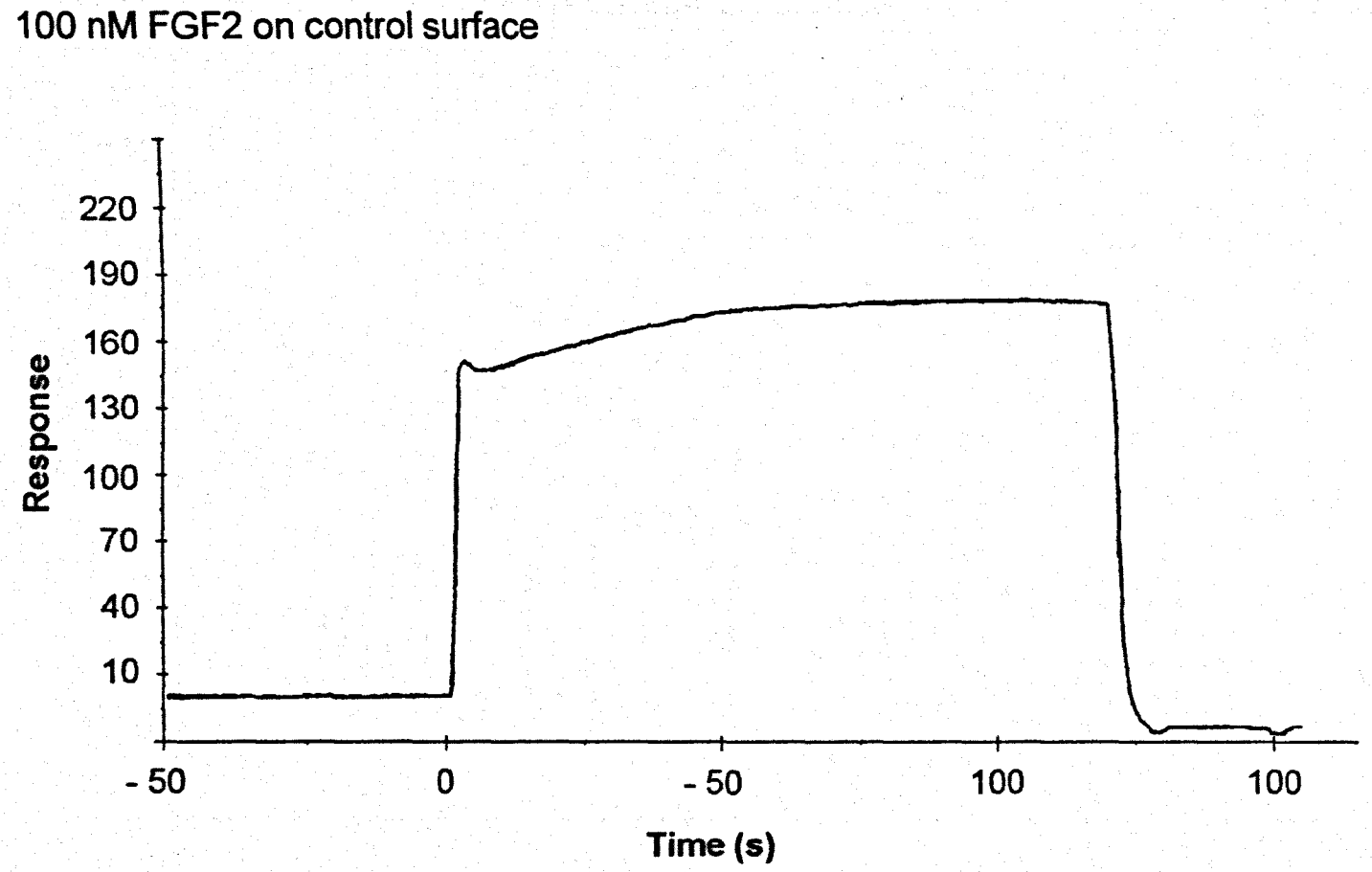


Figure 37 – BIAcore sensorgram of the fibroblast growth factor II (FGF2) ligand being washed over a control surface of the sensor chip initially derivatized with 1,8 octanedithiol. An initial bulk shift is observed but the line stabilises and shown only a minor change in response.

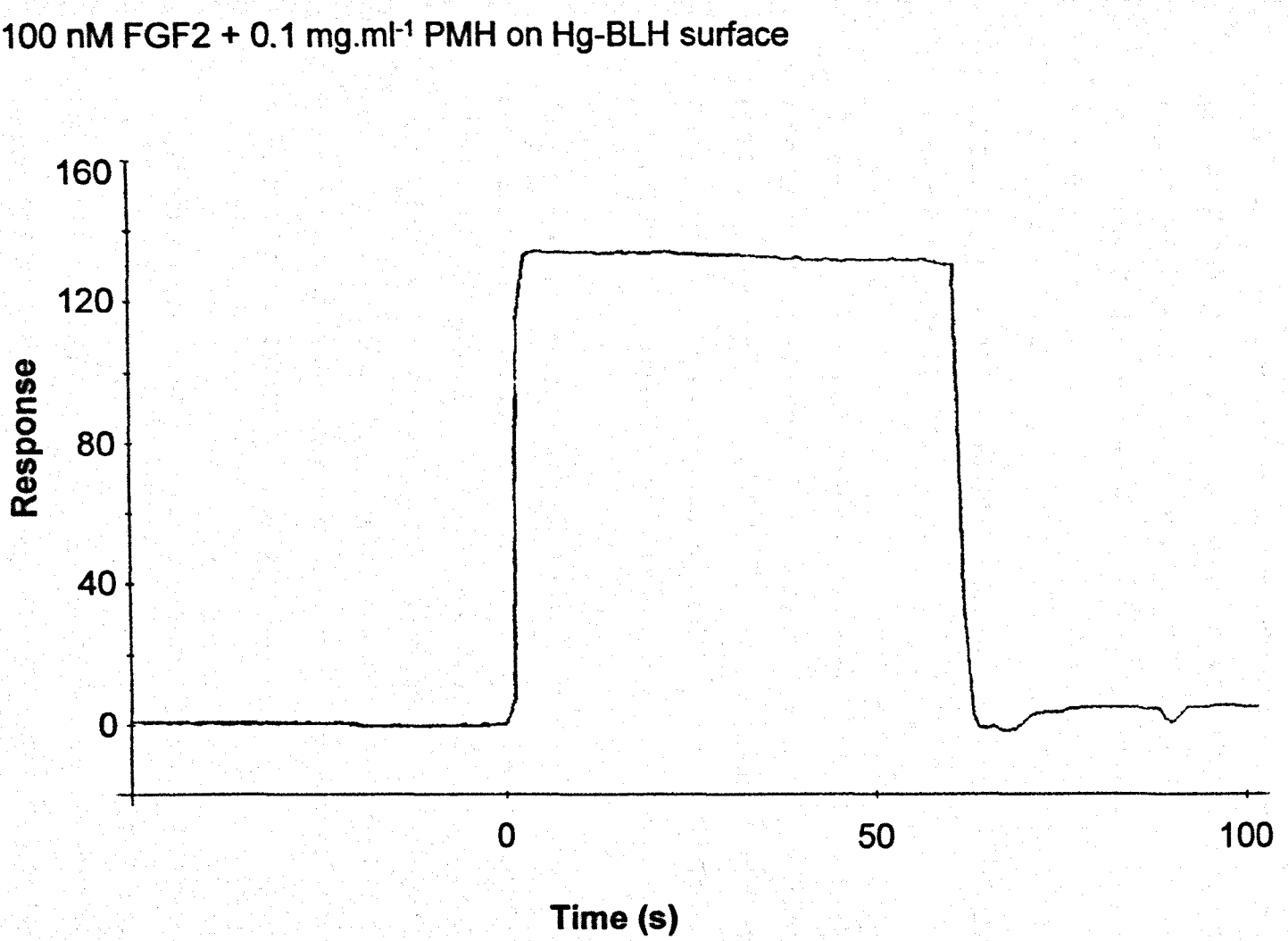


Figure 38 - BIAcore sensorgram of the fibroblast growth factor II (FGF2) ligand and heparin polysaccharide being washed over a surface containing heparin oligosaccharides immobilised via their non-reducing end on the surface of the sensor chip. The sensor chip was initially derivatized with 1,8 octanedithiol and the non-reducing end mercury labelled oligosaccharide attached via the thiol – gold interaction. An initial bulk shift is observed but the line stabilises and shows no increase in response as the heparin in free solution competes successfully against the immobilised surface for the FGF2.

thiol and Hg containing molecules is well known and accounts for its toxicity in nature. The interaction between FGF2 and heparin oligosaccharides is also well known and used here to detect the presence of heparin immobilised on the surface. The surface is pre-derivatized with 1,8 octanedithiol which interacts with gold surface present on the native chip (Au-thiol interaction). These results (figure 36) further illustrate the veracity of this method of labelling (via the non reducing end) and demonstrates its application for larger oligosaccharide fragments.

4.2.5 Conjugation of Hg-labelled HS oligosaccharides to Au nanoparticles (in collaboration with Dr NTK Thanh)

The conjugation of mercury labelled heparan sulphate (dp16) oligosaccharide to gold nano-particles was shown utilising the reaction of Hg to sulphur containing species. The methionine residue of the Au-CALNN-Met nanoparticle-peptide conjugate contains a methyl thiol which also reacts with the Hg-OAc moiety of the cyclic mercurinium intermediate in a mode analogous to that used for the BIAcore biosensor.

Attachment of the Hg-labelled oligosaccharide fragment was demonstrated by a change in the absorbance spectra which is indicative of the attachment of molecule to the surface of the nanoparticle (personal communication Dr N Thanh). A change in the absorbance spectra can be seen when the Hg-labelled disaccharide is added to the peptide linker containing nanoparticle (see figure 39). Control experiments suggest that this is due to the presence solely of the mercury present in the mercurinium intermediate of the labelled bovine lung heparin oligosaccharide (see table 12).

Binding of heparin to the nanoparticles without oxymercuration treatment was also observed although this was attributed to ionic bridging to between heparin, mercuric acetate and the Au-CALNN-Met nanoparticle. This interaction was readily disrupted by the presence of EDTA.Na_2 (100 mM). The conjugation of the Au-CALNN-Met nanoparticle and the Hg-sugar was not disrupted by the presence of EDTA and the difference in the nanoparticle before and after the addition of mercury labelled HS

oligosaccharide can be seen. The attachment or conjugation was monitored by the presence of a shift in the plasmon resonance of the gold nanoparticle glyco-conjugate.

This experiment and the relevant controls (see table 13) show the attachment of the non-reducing end Hg-labelled sugar to the AuNP.

Table 13: Conjugation of Au nanoparticles - reaction conditions

Reaction mixture	**Change observed in spectrum**
Au-CALNN-Met & sucrose (non reducing)	No
Au-CALNN-Met, sucrose & $Hg(OAc)_2$	No
Au-CALNN-Met & PMH	No
Au-CALNN-Met, PMH & $Hg(OAc)_2$	*Yes* [∞]
Au-CALNN-Met & BLH fragment (dp16)	No
Au-CALNN-Met & Hg-labelled BLH fragment (dp16)	**Yes**

[∞] The interaction between Au-CALNN-Met, porcine mucosal heparin (PMH) and mercuric acetate [$Hg(OAc)_2$] is considered as ionic as the spectral changes can be reversed by the addition of EDTA.Na_2.

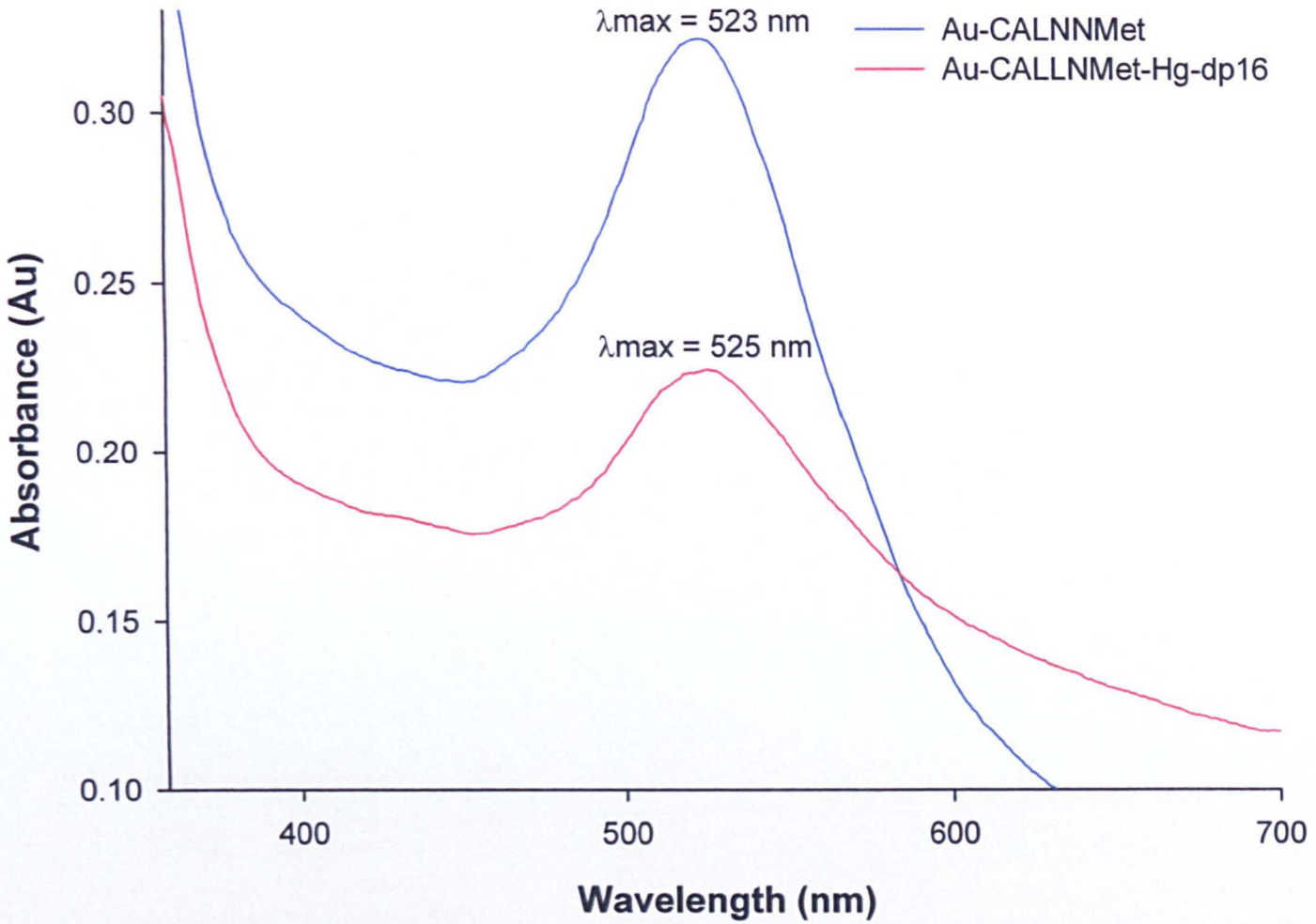


Figure 39 – Absorbance spectra for both gold nanoparticles conjugate with a CALNN-methionine peptide and gold nanoparticles conjugate with a CALNN-methionine peptide with a heparan sulphate oligosaccharide attached (dp16, 16-mer) to the peptide via the non-reducing end mercury – thiol (Met) interaction. A shift in the absorbance maximum of the plasmon resonance can be observed from λ=523 nm to λ=525nm indicative of an attachment at the nanoparticle surface.

4.3 Discussion

4.3.1 Non-reducing end labelling of heparin and heparan sulphate derived oligosaccharides

The reaction of a heparin disaccharide with mercury acetate has been shown to yield what appears to be a stable mercurinium ion. MALDI-TOF MS confirms the presence of an ion within the mixture which corresponds to the calculated m/z value for the mercurinium ion expected to be formed by the attack of the C=C bond present between C-4 and C-5 of the uronic acid residue. The use of NMR also shows that the molecule remains intact, by the presence of the α and β reducing end anomers. The chemical shifts present from the protons in the ring system also suggest that there has been a change in the structure of the molecule, with some what unsurprisingly the position corresponding to the proton at C-4 of the uronic acid showing the greatest change.

Conventionally the cyclic mercurinium ion is thought to be attacked by the solvent, i.e. water, leading to the opening of the cyclic intermediate and the addition of an hydroxyl to one of the original alkene carbons. This does not seem to be the case from the MALDI-MS obtained. Furthermore, the cleavage of the uronic acid monomer from the rest of the disaccharide has been shown by Ludwigs *et al.* [141] although this occurred at a near neutral pH and with the presence of acidic ion exchange resin. This reaction was monitored by paper chromatography and the loss of the C=C bond (which would occur even if there was rearrangement leading to the cleavage of the disaccharide). It is postulated that this event either occurs under very specific conditions, (as we see no evidence for it) or that the ion exchange resin either contributes to such an arrangement or contaminates the reaction mixture leading to a case of mistaken identity (for free GlcNAc). Data exists for the attack of the mercury double bond without this cleavage in the form of a follow-on project from this study conducted by Drs T Rudd and A Powell, whereby an attempt was made to directly attack to double bond with a mercury derivatized surface containing an acetyl moiety. The opening of the double bond and the formation of the cyclic intermediate was shown to occur, without the loss of the acetyl

signal which was being directly measured using PMIRAS. This immobilised reaction variant of Rudd and Powell [153] did not possess the same efficiency and yield as that of the free solution method reported here, which would seem to be near quantitative based on the change in chemical shift signals between the reacted and un-reacted forms. This reaction method corroborates with the data provided here to suggest that re-arrangement and subsequent cleavage does not occur when the reaction is carried out in an un-buffered water based system.

The stability of mercurinium intermediates is known to be structurally dependent with cyclic forms being favoured in the absence of strongly electron donating neighbouring groups [140, 152]. The stability of the mercurinium intermediate for the heparin / HS derived disaccharide standard 1 could be explained by the observation that the rate constant for the nucleophilic attack of the cyclic mercurinium ion intermediate is considerably reduced (and hence the equilibrium skewed to the reactants) when the alkene is present with many differing groups attached (i.e. a tertiary alkene) [154], such as is the case for standard 1. Therefore the forward reaction, the nucleophilic attack of water, would be un-favoured (due to the poor rate constant and equilibrium) leaving the cyclic mercurinium ion prevailing.

NMR studies conducted originally to try and elucidate the structure of the mercurinium ion were carried out in acidic conditions (not highly acidic as the reverse reaction leading to the original alkene / olefin is favoured) where the mercurinium ion was found to be most stable [148, 149]. This may account (not only for the results of Ludwig *et al.* [141], although there are more non-elucidated complicating factors than just acidity) for the increased stability of the mercurinium ion in the standard 1 glyco-conjugate because the disaccharide standards are acidic owing the presence of the carboxylic acid group and sulphate groups (for standards 2-8). Pasto *et al.* [155] have also mooted their beliefs that the stability of the mercurinium intermediate (and even the finer details of the exact thermodynamics of the mechanism) are highly dependent on the associated functional groups [155] of the original alkenes.

Further evidence for the formation of the mercurinium intermediate (or at least a non ionic mercury atom present within the molecules) is indirectly indicated by the immobilisation of oligosaccharides which have been subject to oxymercuration on both gold surfaces of nanoparticles and a biosensor. A change in the plasmon resonance of the nanoparticle indicated binding of a molecule to the surface. A similar oligosaccharide post mercuric acetate treatment does not bind. Heparin itself binds in the presence of mercuric acetate, but this binding is believed to be only ionic as it can be disrupted by the presence of EDTA (which is known to chelate divalent cations such as that formed by mercury). This is not the case for the mercury labelled oligosaccharides which remain present on the surface (indicated by the change in the plasmon resonance) with EDTA present.

Of more significance (due to its biological application) is the data obtained from the BIAcore biosensor. This data not only shows that a thiol surface (created by the derivatisation of the gold with a double ended thiol using the known interaction of gold and sulphur) binds to the sugar molecule but also the molecule is still biologically active as seen by the interaction of the BLH fragment with FGF2; again a well-documented interaction. This occurs in the presence of EDTA (thereby negating the possibility of mercury bridging and ionic problems) and can also be competitively disrupted using heparin. This further suggests that this novel mode of attachment is transparent and non-invasive in the case of biological bindings. Further studies need to be conducted to see if the oligosaccharides still maintain activity, in an assay such as the BAF3 assay where cell differentiation is related to FGF:FGFR:HS complex formation and signalling.

One major advantage of this mode of attachment is the ability to circumvent the problems of carbohydrate mutarotation, carbonyl protonation (and hence activation) and Schiff's base formation equilibria. This method provides for a new mode of attachment where by default there are only two possible types of uronic acid monosaccharide present, i.e. with or without a C-2 O-sulphate. (The uronic acid epimer is lost by the creation of the C=C bond) This method does however lead to the destruction of the C=C chromophores introduced during lyase digestion, and only lyase digested fragments can be labelled.

However, the author sees this as a small price to pay compared to the huge benefits which can be provided for by non-reducing end mediated attachment.

The exact mechanism for the oxymercuration for this class of molecules needs further investigation and the dependency of structure on yield need to be determined (e.g. effect of 2-O-sulphate group). This, however, is time consuming, requiring a high degree of chemical competence and considerable amount of starting material (and therefore high cost). Investigations are also needed into reactivity of larger molecules such as oligosaccharides and larger molecules derived from other GAG family members.

4.3.2 Conjugation via the non-reducing end

The presence of the Hg atom at the non-reducing end of oligosaccharides allows for further conjugation as seen in the results by the attachment to gold surfaces either via a peptide linker of via a di-thiol linker The exploitation of the high affinity interaction formed between sulphur containing moieties such as thiols and mercury [142] allows for the possibility of fluorophore conjugation in order to improve the level of detection for this class of molecules. This thesis has shown improved detection using the fluorophore Bodipy hydrazide at the reducing end and it is envisaged that this fluorophore could be coupled to the non-reducing end. This coupling mechanism may lead to better yields as eluded to by the initial NMR data shown in this thesis (near quantitative yield). Attempts to couple the non reducing end of the GAG molecules to fluorophores have failed due to incompatible solvent systems and impurities from the reaction mixture effecting fluorescence but it is believed that this could easily be overcome with more research into the field. This would greatly improve detection of HS and provide two ends for the attachment / conjugation of the molecule which could assist sequencing (see section 4.3.5).

The possibility exists of utilising the non-reducing end and reducing end chemistries in order to synthesise oligomers from disaccharides and smaller oligosaccharides. Further

work will need to be undertaken to understand the chemistry of the non reducing end and its implications.

4.3.3 Immobilisation using the non-reducing end

The non-reducing end handle obtained by the presence of mercury also provides a convenient point for the attachment or immobilisation of oligosaccharides. This is especially true for biosensors such as Biacore, IASys, PMIRAS and the Quartz crystal microbalance (QCM). These machines measure a change in the surface properties when a binding event occurs. This can be via a change in the frequency or a change in the optical properties etc. The ability to derivatise gold surfaces using either a peptide linker or a di-thiol containing molecules allows binding studies to be carried out upon HS molecules and potentially other GAG family members. From these studies kinetics and affinities can be obtained for various interactions involving these carbohydrates. Typically poor reducing end chemistries have made required sample contents demanding and this, coupled to differential binding of the different structures, has hampered progress. The non-reducing end may provide a solution to this problem.

The non-reducing end could also be used for the attachment of oligosaccharides to micro-array media. Currently immobilisation on micro-array surfaces is problematic, involving complex chemistries and is again hampered by differing and poor reaction yields. The non-reducing end could provide an alternative means of attachment which has more uniform reactivity and improved yield. Furthermore, it allows for quantification, as the reducing end remains available for derivatisation. Conversely, the non-reducing end could be used to quantify currently used reducing end immobilised micro-arrays. Improvements in this field of HS application could allow for high throughput screening of HS structures with regard to protein binding and allow for the determination of sequence requirements.

The use of nanoparticles also provides for improved levels of detection. It is envisaged that single nanoparticle detection will soon be a reality (personal communication, Prof. D

Fernig) and the ability to couple oligosaccharides to these entities will greatly improve detection. The molecules could also be used for targeting, where a specific HS structure targets the nano-particle to an interacting protein. This could be used for identification or for therapeutics uses where the metallic physico-chemical properties of the nanoparticle are exploited for instance, hypothetically, to kill a cancer cell targeted by HS by heat generated by microwave source. The same possibility could be used to treat parasitic diseases where known interactions with HS are used for targeting (see chapter 5).

This mode of attachment could be used for more conventional attachment, e.g. in heparin column, replacing the less efficient reducing end. Potentially wherever reducing end labelling has been used, non-reducing end coupling could also be deployed.

4.3.4 Utilising the oxymercuration reaction

The formation of a cyclic intermediate is conventionally followed by the nucleophilic attack of the solvent. In the case of water:THF the mercurinium intermediate appears to be quite stable for HS derived oligosaccharides and disaccharides.

Alternative solvent systems and reaction conditions could be investigated in an attempt to exploit this nucleophilic attack as a means of attachment of other desirable groups into the HS or potentially other GAG molecules. Classically, where alcohols are used as solvents for mercuration, the alkyl chain is incorporated in to the molecule in an ultimately Markovnikof style addition. This could potentially be utilised to incorporate alkyl chains onto the oligosaccharide, thereby creating "lipid" like molecules, which could presumably be incorporated into membranes. The possibility to introduce other groups should be investigated especially ones suitable for high yield further attachment or conjugation. This could also provide for a useful tool in the synthesis of HS oligosaccharides if coupling could be investigated between the reducing end and the non-reducing ends.

4.3.5 Dual-end sequencing strategy

The developments to reducing end and non-reducing end labelling described within this thesis lend themselves to the creation of a new hypothetical model for sequencing. Such a strategy, known as the "dual-end sequencing strategy" is outlined below and is so called because it utilises both termini of the oligosaccharide to elucidate the sequence. This method if followed to fruition should provide for a rapid, facile methodology which allows sequence determination form previously un-workable amounts of material. It also minimises the use of enzymes to the robust and dependable bacterial lyases. The strategy proposed is outlined below and represented graphically in figure 40.

Oligosaccharides are isolated enzymatically using bacterial lyase enzymes. The resultant oligosaccharide contains a free reducing end and a double bond between C-4 and C-5 of the uronic acid reside present at the non-reducing end. The non-reducing end of the oligosaccharide is subject to attack by mercury acetate as described in chapter 3, yielding what is often termed a (stable) cyclic mercurinium intermediate. The mercury conjugated oligosaccharide is then attached via the Hg atom to a thiol containing moiety (exploiting the known interaction between Hg and thiols) present on derivatized beads. A spacer is likely to be employed between the bead and the thiol group to prevent problems of steric hindrance in later enzymatic digestion steps. The non-reducing end immobilized oligosaccharide is labelled via its reducing end *in situ* (i.e. while still attached to the beads) with BODIPY FL hydrazide as outlined in chapter 3. The reducing end labelled oligosaccharide, which is immobilized on the beads via its non-reducing end is then subject to partial enzymatic cleavage using the fluorescence of the oligosaccharide containing beads as a guide to the extent of cleavage, whereby the amount of saccharide cleaved is indicated by the loss of fluorescence and the total fluorescence present in the eluent. This step allows the level of de-polymerisation to be controlled to give an optimal partial digestion. This step has proved problematic in other sequencing strategies such as IGS.

The carbohydrate fragments which are released from the column during de-polymerisation are collected, The saccharides now possess a common reducing end reference frame (i.e. the Bodipy fluorophore) and can be fed into a conventional IGS style sequencing strategy. The fragments available at this stage permit for the first classical sequencing opportunity and detection could be improved by using LIF detection CE coupled to IGS.

The remaining oligosaccharides bound to the beads are labelled again and reduced to stabilize the fluorophore at the reducing end. This will concomitantly release the remaining oligosaccharides from the column. The resultant oligosaccharides are a sized ladder of BODIPY labelled oligosaccharides possessing a common non-reducing end. These are size separated using size exclusion chromatography with LIF detection and each size pool is collected, desalted, and exhaustively enzymatically cleaved to release a fluorescently labelled disaccharide (originating from sequential positions in the chain) which is then identified by LIF-HPLC SAX or LIF-CE. This is the second novel opportunity to sequence and should provide data which is complementary to the first.

This strategy coupled together with the advancements made within this thesis with regard to the non-reducing end mercury labelling of lyase derived oligosaccharides and the use of improved reducing end fluorescent labelling using BODIPY hydrazide. This combined with LIF detection should give unprecedented sensitivity due to the vast improvement in the level of detection. The method also allows control of the partial enzyme de-polymerisation of the HS oligosaccharides through the loss of fluorescence due to the elution of fluorescently labelled oligosaccharide fragments from the column.

Furthermore, this strategy provides two opportunities to obtain sequence information from either a conventional reducing end IGS based, reducing end labelled oligosaccharide ladder or a sequentially labelled non-reducing end oligosaccharide ladder. These are complementary and allow confirmation and overlap sequencing. For example, if the enzymes are hindered from cutting at the non-reducing end attached to the column to leave a tetrasaccharide stub.

The method may allow for the quick, facile identification of the primary sequence present within the oligosaccharide. This could be further used to develop high throughput screening of oligosaccharide fragments. It is also envisaged that this strategy could allow the sequencing of simple mixtures. The intensities of each of the peaks present on the chromatograms should allow the individual components of the mixture to be identified and sequenced. This will prove more problematic if two non-isomeric oligosaccharides are present in similar proportions.

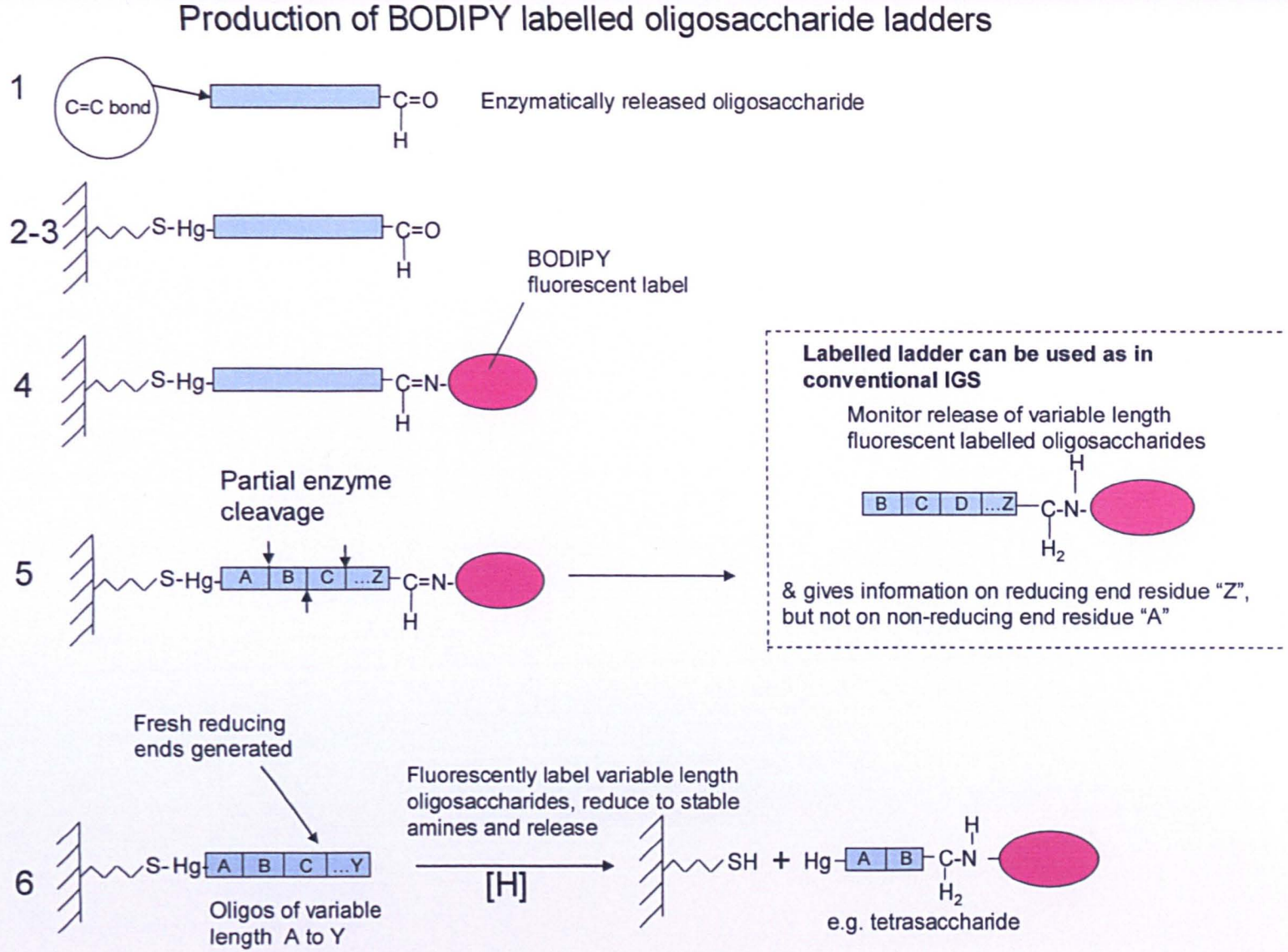


Figure 40a – Schematic representation of the dual-end labelling strategy.

7 Sequential identification of BODIPY labelled disaccharides

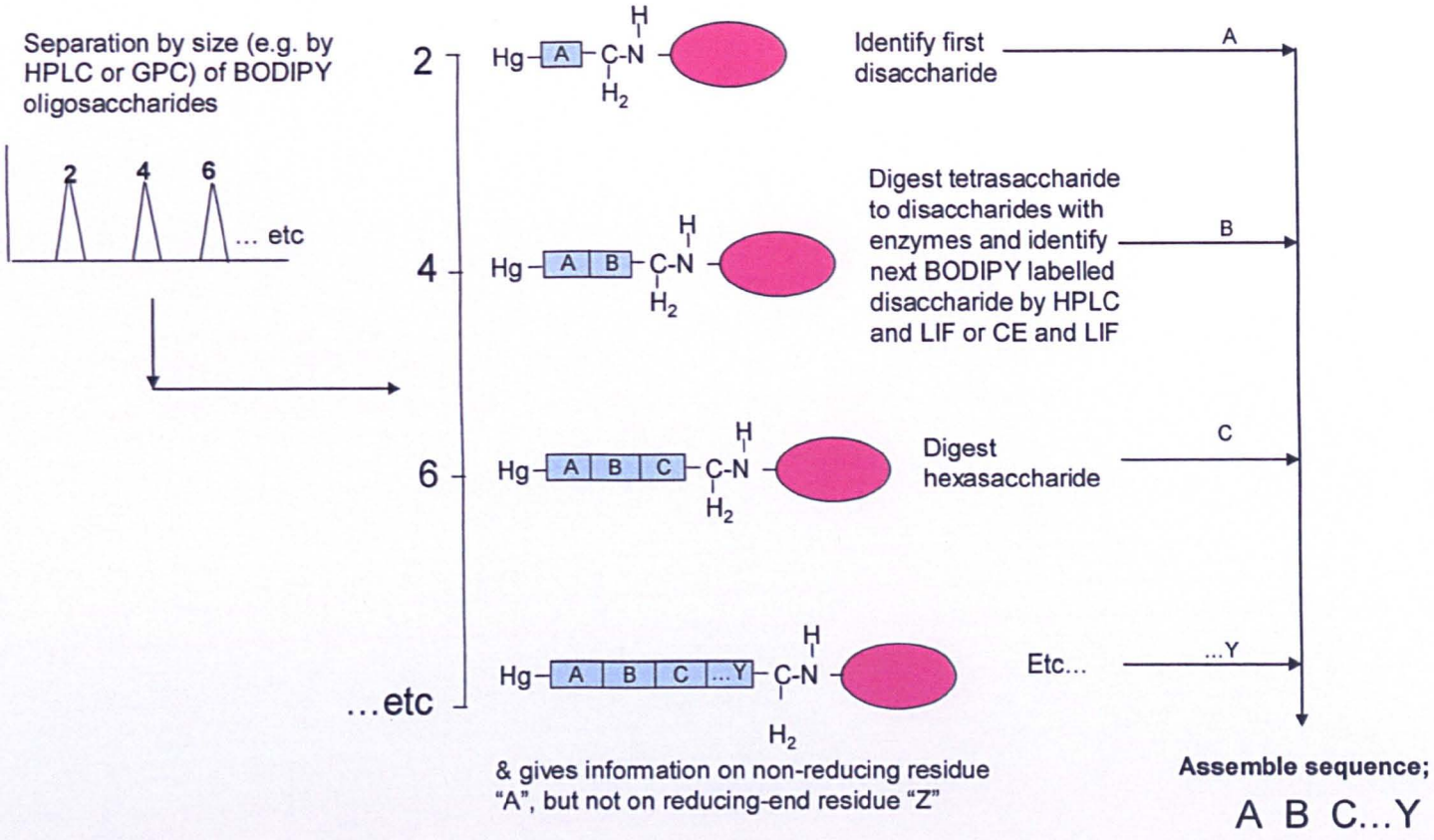


Figure 40b - Schematic representation of the dual-end labelling strategy.

Chapter 5

Heparin / heparan sulphate malaria interactions

5.1 Introduction

5.1.1 Malaria

Malaria is an infectious disease, identified over 100 years ago by Alphonse Laveran [156, 157], as being caused by the infection with species [158] of the highly successful protozoan parasites of the genus *Plasmodium. Plasmodium* are obligate parasites of the red blood cells (erythrocytes) [159]. Infection can occur over a wide range of target animals as diverse as birds, mammals (including humans) and even lizards [160]. Currently four species of *Plasmodium* are known to infect humans causing substantial morbidity in endemic areas. These are *Plasmodium falciparum, Plasmodium malariae, Plasmodium ovale* [161] *and Plasmodium vivax. Plasmodium malariae* has a three day erythrocytic cycle (quartan) while the other human infective strains have only a two day (tertian) succession. Infection occurs by the bite of the female insect vector, the *Anopheles* mosquito [162], of which between 30 and 40 out of approximately 430 known species can diffuse the parasite [163]. In humans the most deadly variety of *Plasmodium* is the prevalent species *Plasmodium falciparum;* partly due to its ability to mediate cerebral malaria, which if untreated is frequently fatal. It is estimated that one third of the world's population is at risk from *Plasmodium falciparum* mediated malaria with approximately 2 million deaths per year and clinical infections in excess of 300 million [164].

5.1.2 Spread of disease

Malaria is established only in areas where transmission is possible, and this is usually mediated by climatic considerations (humidity, rainfall and temperature). Two criteria must be satisfied to ensure successful transmission; (i) the survival and multiplication of the insect vector, the *Anopheles* mosquito and (ii) the survival and development of the parasite (see figure 41). These constraints lead to transmission principally in tropical and sub-tropical areas although areas which experience cooler seasons, have high altitude, include deserts, or possesses other physical barriers to the mosquito's survival will not

support transmission. *Plasmodium falciparum*, can only survive in temperatures above 20°C. This is because the parasite requires an elevated temperature to carry out its life cycle when present in the *Anopheles* mosquito vector. In cooler regions transmission may be seasonal and of a lower intensity. *Plasmodium vivax* may prevail under these conditions because it is more tolerant to lower ambient temperatures. Transmission is usually high in regions which are warm and close to the equator such as South Asia and South America. The highest transmission of malaria is found south of the Sahara in Africa where malaria is transmitted continuously throughout the year and *Plasmodium falciparum* prevails. In areas such as the United States of America and Europe, malaria has been successfully eradicated through public health initiatives driven by their economic development. It should be noted that in some of these areas *Anopheles* mosquitoes capable of transmission are still present and there is still potential for the re-establishment of the disease.

5.1.3 Symptoms of malarial infection

The symptoms associated with malaria are quite broad and range from no symptoms at all to severe disease states and ultimately death. Symptoms are also dependent on the age and state of health of the individual concerned. Symptoms indicative of malaria usually appear after the incubation period, generally between 7 and 30 days post infection. This time period is dependent on the infecting species of *Plasmodium*, with *malariae* having the longest incubation times while

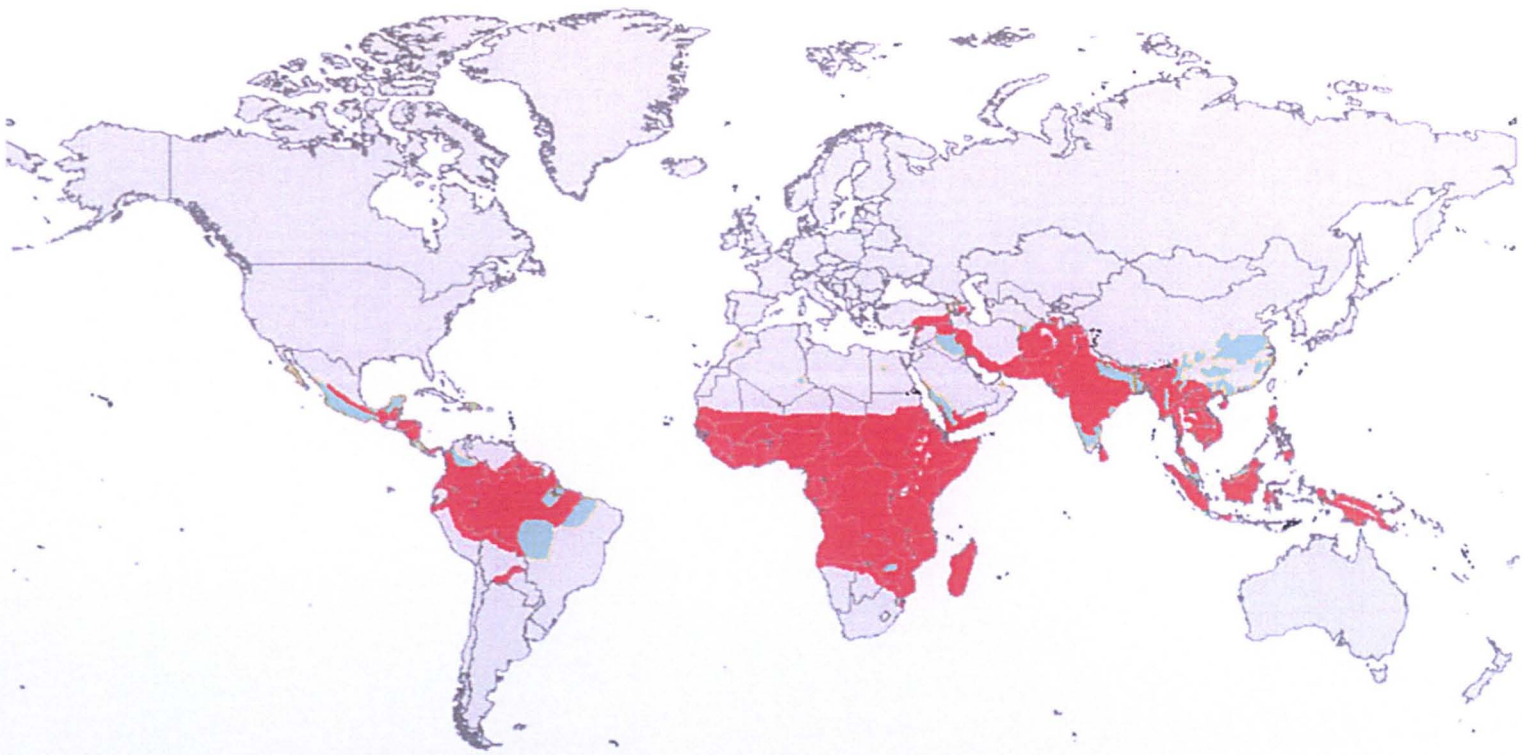


Figure 41 – Global distribution of malaria. Areas highlighted in red are endemic, whereas areas highlighted in cyan witness sporadic outbreaks of infection (based on a figure by the World Health Organisation).

falciparum exhibits the shortest. The onset of symptoms can be delayed (as long as a few months) if the individual has been taking anti-malarial tablets as a preventative, especially in the case of *ovale* and *vivax* infections where dormant liver stage parasites known as hypnozoites can exist.

The symptoms of malaria tend to manifest themselves in two distinct categories, uncomplicated malaria and complicated malaria. In uncomplicated malaria attacks are classically thought to last between 6 – 10 hours. Such attacks consist of a period where the individual feels cold (shivers) followed by a hot stage usually accompanied by headaches, vomiting and high fever (seizure in infants). The individual then experiences a phase where the temperature decreases to normal and experiences tiredness and sweating. Other physical symptoms include elevated body temperature, weakness and enlarged spleen. In the case of *Plasmodium falciparum* infections further symptoms of mild jaundice, an increase in the respiratory rate and the enlargement of the liver may be present. Diagnosis is usually made by analysis of a differentially stained blood smear using a microscope although other indicators of *falciparum* infection commonly include thrombocytopenia (decrease in blood platelet levels), mild anaemia, the elevation of amino-transferases and bilirubin, and or the presence of aberrant bodies in the urine. Severe malaria (which is generally considered a medical emergency) is the complication of a *Plasmodium falciparum* infection by metabolic malfunctions, blood abnormalities and or major organ failures. These complications manifest themselves in the form of haemolysis, pulmonary oedema, acute respiratory distress syndrome (ARDS), blood coagulation abnormalities, thrombocytopoenia, cardiovascular collapse and or cerebral malaria (which may lead to ataxia, speech difficulties, palsies, deafness and blindness).

5.1.4 Treatment of malaria

Treatment of the more benign forms of malaria, i.e. malaria caused by *Plasmodium malariae*, *ovale* and *vivax*, is generally by administration of the drug chloroquine (a quinine derivative) [165]. Chloroquine is probably the most well known malarial preventative (used since the 1940s world wide) and it is also used for malaria treatment at

higher doses; it is generally well tolerated (side effects are blurred vision and gastro-intestinal problems). Chloroquine works by inhibiting the polymerisation of toxic haem monomers (a by product of haemoglobin breakdown, which is essential for the production of crucial amino acids by the parasite) to the non-toxic haemozoin, by sequestering haemozoin and preventing further polymerization, thereby producing increasingly high concentrations of toxic haem [166].

The treatment of the more severe variant, *Plasmodium falciparum*, has been hampered by the emergence, some 20 years post chloroquine introduction, of resistant strains concurrently in both Southeast Asia and South America. Further cases were identified in New Guinea and East Africa by the 1970s [167, 168]. As a result of chloroquine resistance, treatment of *Plasmodium falciparum* is now effected by either administration of amodiaquine (for low grade resistance areas) [169] or the use of a cocktail comprising of both pyrimethamine and sulphadoxine for high resistance regions [170]. Some regions unfortunately demonstrate resistance to all of the above treatments and such multi-drug resistance areas are increasingly using combinatorial therapies based on the compound artemisinin [171]). Somewhat worryingly (although no serious toxicity has been reported in humans [172] some artemisinin derivatives have shown an unusual but consistent, selective pattern of damage to brain-stem nuclei in animal models. Furthermore resistance has also been observed for this class of prophylactics [173].

5.1.5 Cerebral malaria

The most severe complication of malaria, which if left untreated often prove fatal, is that of cerebral malaria (CM). Children under the age of ten are specifically at risk in malaria endemic regions. The symptoms of CM usually involve changes in mental status, fever, convulsions and unconsciousness with coma lasting between 24 and 72 hours. As the coma progresses the subject moves from a rousable to a non-rousable state. Blood samples / smears of the patient commonly show *Plasmodium falciparum* infected erythrocytes in the parasite asexual developmental stages, in the absence of any bacterial

of viral contributors [174]. The presence of ring-like lesions in the brain of an infected individual is also a major attribute of CM.

The instigation of CM is thought to be caused by the sequestration of parasite infected red blood cells to capillaries and venules present within the brain, and the incidence of this complication is directly related to the level of sequestration. The exact mechanism of how parasitized erythrocytes induce coma is poorly understood to date although ideas include the involvement of pro-inflammatory cytokines stimulated by the presence of malaria toxins such as tumour necrosis factor (TNF) [175, 176] and / or hypoxia caused by the mechanical blockage of blood vessels [177, 178].

The involvement of nitric oxide (NO) and an assortment of cytokines in CM led to the proposal of the cytokine model for coma induction in CM by Clark *et al.* [179]. This hypothesis postulated that coma was induced by nitric oxide crossing the blood brain barrier, disturbing the usual NO signalling mechanism used by the neurons. The nitric oxide production was thought to occur at the walls of the blood vessels near to the site of parasitized erythrocyte sequestration. This increase in NO is speculated to cause coma in a manner similar to that of general anaesthetics and ethanol [180, 181].

One alternative hypothesis for coma induction is that mechanical occlusion of the blood vessels causes hypoxia leading to CM. This phenomenon is due to cell – cell adhesion facilitated by interactions of parasite expressed proteins with hyaluronic acid, chondroitin sulphate, CD-36, E-selectin, PECAM-1, ICAM-1, VCAM-1 and thrombospondin [182] [see table 2]. The binding of parasitized erythrocytes to these receptors facilitates occurrences such as cyto-adherance (the binding of infected red blood cells to the endothelial cells of the blood vessel wall), auto-agglutination (parasitized red blood cells binding other infected blood cells) and rosetting (adhesion of parasitized erythrocyte to one or more uninfected red blood cells). It is known that the parasite expressed and erythrocyte membrane bound protein, *Plasmodium falciparum* erythrocyte membrane protein I (PfEMP-1), is involved in these phenomena along with other parasite expressed proteins such as CLAG, Rifin, STEVOR, modified band 3, RSP ½ and sequestrin [183].

Apoptosis of endothelial cells post-binding to parasite infected erythrocytes has also been shown by Pino *et al.* [184].

5.1.6 Rosette formation in Plasmodium falciparum

Rosetting, originally defined in 1988 by David et al. as "the agglutination of uninfected erythrocytes around parasitized erythrocytes" [185], was first described in the malaria parasite *Plasmodium fragile*. Initial experiments confirmed that rosetting is developmentally regulated and is dependent on a factor present on the parasite infected erythrocyte surface (shown by protease treatment). Involvement of the spleen was further implicated [185]. The formation of rosettes was confirmed to occur in the human infective form, *Plasmodium falciparum*, and again shown to require a protein containing entity [186, 187]. It is thought that the rosetting phenomenon may aid the parasite (infected erythrocyte) in host cell immune system evasion and also in transmission by the close proximity generated between the parasitized erythrocyte and uninfected red blood cells present in the rosette.

Correlation between the rosetting phenomenon and the cerebral malaria phenomenon has been reported in various studies in The Gambia [188, 189], Kenya [190], Thailand [191] and Madagascar [192] although some studies report findings to the contrary [193, 194]. Although the parasite strains may express geographically different distribution of the ligands required for rosetting and / or the hosts may also possess differing ligand and concentrations thereof. Furthermore the immune systems response may also differ region and strain specifically [193]. [See figure 42]

The ligand responsible for the rosetting phenomenon was elucidate in 1998 by Chen *et al.* [195] as being the *Plasmodium falciparum* erythrocyte membrane protein 1 (PfEMP-1) protein expressed by the parasite and located upon the infected erythrocyte's membrane. Heparin and / or heparan sulphate was suggested as the receptor [195]. The PfEMP-1 protein is also involved in host cell immune system evasion via modulation of its encoding *var* genes responsible for the antigenic diversity of the protein [196-198].

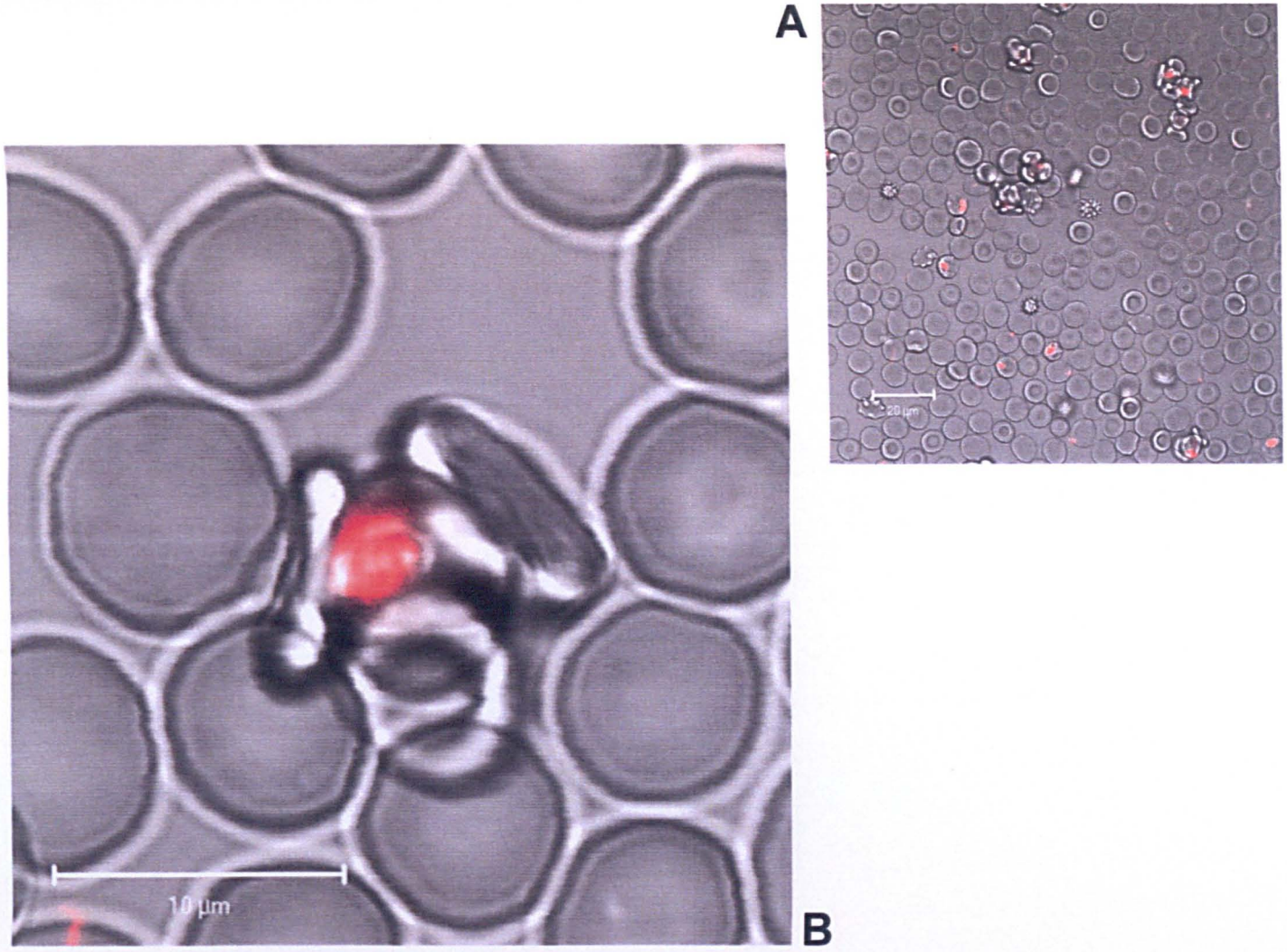


Figure 42 – Example of a *plasmodium falciparum*, strain R29 mediated rosetting. The parasite DNA is stained red with ethidium bromide. In [**A**] a handful of rosettes can be seen in a suspension of red blood cells in RPMI 1640 medium and human serum. In [**B**] a rosette can be observed which contains a parasite infected red blood cell bound to at least three uninfected red blood cells.

Treutiger *et al.* showed that for some *falciparum* strains serum proteins were also essential for rosette formation [199, 200] while Somner proposed that serum components acted as bridging molecules [201]. Other studies have shown that the ABO blood group antigens [202], CD-35 [203] and CD-36 [204] are also possible rosetting receptors. Complement receptor I is thought to play an important role in the PfEMP-1 mediated rosetting [203].

5.1.7 The disruption of rosettes by heparin and heparan sulphate

The use of carbohydrate species in host cell recognition has been shown previously [205] and furthermore heparin and heparan sulphate are known to be implicated in the invasion of host cells by a plethora of micro-organisms [206]. With regard to *Plasmodium* infection, sulphated glycosaminoglycans have been shown previously to be implicated at varying parasite developmental stages. The binding of schizonts to heparan sulphate chains present upon hepatocyte cells during initial infection has been reported as a targeting mechanism [207], while the sequestration of parasite infected red blood cells to the placenta during pregnancy [208] and the endothelial lining has been shown to involve chondroitin sulphate A [209, 210]. Inhibition of merozoite invasion as also been demonstrated in an *in vivo* assay using sulphated carbohydrate conjugates [211, 212].

Heparin polymers have also been shown to disrupt rosettes formed between infected and uninfected erythrocytes [213-215] and the discovery that the rosette receptor ligand, PfEMP-1, possesses a Duffy like domain with the capacity to bind heparin and (hence by deduction) heparan sulphate with the minimal required oligosaccharide chain length being a dp12 (12mer) [216] further supports a possible role for heparin or heparan sulphate molecules in a therapeutic role for the treatment of cerebral malaria. The generation of antibodies to the Duffy binding domain (heparin binding region) ablates rosette formation and prevents sequestration further supporting PfEMP-1 as the rosetting receptor and adding weight to the argument that heparin / heparan sulphate (present on endothelial [217] or erythrocyte cells) could be involved as the receptor [218].

5.1.8 Chemically modified heparin polysaccharides

The use of chemically modified polysaccharides to disrupt rosette formation in *Plasmodium falciparum* infections proves an attractive idea for two main reasons. The first is that the use of polysaccharides, which have been selectively modified, allows attempts at de-convoluting the sequence motifs required for heparin / heparan sulphate binding by the Duffy domain of the PfEMP-1 rosetting receptor to be made. Sequential chemical modifications produce polysaccharides which differ by the removal (or addition) of each functional group present within a disaccharide subunit. It should be noted that modifications are made throughout the chain using an arsenal of techniques. These techniques include:

(i) De-N-sulphation :-
(ii) N-Acetylation :-
(iii) Selective de-2-O-sulphation
(iv) Selective de-6-O-sulphation

The second reason for the use of modified (as opposed to unmodified) heparin polysaccharides are the two main complications which could occur if heparin or heparan sulphate were ever to be used clinically. These complications include the anticoagulation potential of heparin polymers and heparin induced thrombocytopoenia (HIT; a reduction in the platelet count caused by platelet removal at a rate which is faster than they can be replaced). The heparin pentasaccharide binding motif required for the interaction of the polysaccharide with antithrombin III (AT-III) could be ablated by chemical modification thereby alleviating this undesired side-effect.

Previously, heparin which has been sub-fractionated to yield low anti-coagulant containing fractions has been successfully used to disrupt the rosetting phenomenon suggesting that the requirements for rosette disruption and those for anti-thrombotic activity were distinct [213].

A study carried out by Barragan *et al.* [215] showed that certain chemically modified polysaccharides of heparin could inhibit rosetting in the *Plasmodium Falciparum* strain FCR3S1 for which the presence of an N-sulphate group at C-2 of the glucosamine was shown to be a critical structural requirement although the study stopped short of determining the anti-coagulant activity of the modified heparins. This study also alluded to these findings by the use of disaccharide structures (Δ-disaccharide standards, as used in chapter 3). The finding of Barragan *et al.* and the implications thereof to this thesis and the field are discussed in depth in the discussion.

The potential of chemically modified heparins to interact with platelet factor IV (PF-4) and thereby lead to the generation of antibodies to the complex causing heparin induced thrombocytopenia is actively being researched by the author, although it is thought that if the binding is specific, the ablation of binding motifs by chemical modification could lead to anti-rosetting candidates that possess minimal anti-coagulant and anti-thrombocytopoenic activity.

5.1.9 Chemically modified heparin oligosaccharides

The use of full chain heparin or modified heparin polymers as a pharmaceutical could prove problematic due to the size of the molecule and the inherent charge possessed. Modern therapeutics generally have the requirements of being small, relatively uncharged for further progression into clinical trials. Therefore determining the minimal oligosaccharide size which could maintain rosette disruption activity would be desirable. This would also minimise potential side-effects which could occur if full chain modified heparin polymers were prescribed. To date the only study of oligosaccharide size correlated with activity has been carried out by Barragan *et al.* [216] on unmodified bovine lung heparin (BLH) in which a dp12 (12mer) of BLH was shown to be the minimal unit required for rosette disruption.

5.1.10 Aims

It is clear that understanding the interactions between the parasite and its human host will lead to better pharmaceutical design in the creation of novel preventatives, vaccines, therapeutics and ultimately treatment by elucidating weak spots in the invasion process and developmental stages. The ability of heparin and or heparan sulphate oligo / polysaccharides to disrupt the rosetting process which is thought to be a contributing factor in the serious and often fatal condition of cerebral malaria will be investigated using the highly rosetting *Plasmodium falciparum* lab strain R29. Heparin polysaccharides (porcine mucosal heparin, PMH; possessing no known transmissible spongiform encephalopathy risk in contrast to bovine lung heparin) will be used initially to probe the ability of un-derivatized heparin to disrupt R29 rosettes. Furthermore an arsenal of chemically modified versions of PMH will be used to confirm the structural requirements which are relevant for rosette disruption in the strain R29. These initial experiments could confirm or refute the use of heparin as a potential therapeutic in the treatment of CM.

Chemically modified polysaccharides which possess the ability to disrupt rosettes will be assayed to determine the dose response, the concentration at which 50% inhibition occurs (IC_{50}) and their values compared to that of heparin. Modified heparin polysaccharides which disrupt rosettes will be digested and size separated oligosaccharide pools screened by activity in the disruption assay. From the polysaccharide dose response, an oligosaccharide fragment of smallest size will be assayed by dose response for its disruption potential. All of the polysaccharide and oligosaccharides used in the following experiments will be assayed for their anticoagulation activity and the data compared to that of the anti-coagulation power of the pharmaceutical heparin.

5.2 Results

5.2.1 The structural composition of chemically modified polysaccharides

The structural composition of each of the modified polysaccharide polymers along with un-modified porcine mucosal heparin was obtained by the exhaustive de-polymerisation of the polysaccharides using the bacterial derived lyase enzymes as detailed in section 2.2.43. The resultant data conforms with the NMR (see below) that the modification reactions had been performed to near completion. Table 14 shows the relative levels of the disaccharide components present within the polysaccharide chain.

5.2.2 Purity of chemically modified polysaccharides

The purity of all the chemically modified heparins was ensured by size exclusion chromatographic separation from small contaminant molecules with a size less than or equal to 5 kDa. This was achieved as is described in section 2.2.42. Samples were then exchanged to the Na^+ ion form (Dowex W:50, H^+, ion-exchange resin) and subject to NMR for purity analysis by Dr E Yates [see appendix]

Size-exclusion chromatography analysis of the 8 polysaccharides (including PMH) was also carried out using a TSK gel G2000SWXL column as detailed in section 2.2.42. All of the chemically modified polysaccharide samples exhibited a single major peak with retention times varying between 6.03 and 6.17 minutes (mean=6.07 σ_{n-1}=0.05). For all of the modified polysaccharides there were no contaminants at a greater level than 5 % (data not shown).

5.2.3 The disruptive effects of heparin polysaccharide on Plasmodium falciparum mediated rosetting

Experiments were performed to confirm the ability of heparin to disrupt rosettes formed between parasitized (*Plasmodium* infected) red blood cells and uninfected red blood cells.

Table 14: Disaccharide composition of chemically modified heparin polysaccharides

Parent polysaccharide	Disaccharide unit (%)	1	2	3	4	5	6	7	8	Mr (Da.) [∞]
Porcine mucosal heparin		6.8	-	3.4	13.4	7.0	67.4	-	2.0	30,923
N-acetyl heparin		14.2	7.0	-	-	-	3.5	-	75.3	28,568
De-2-sulphated heparin		7.1	-	13.7	79.2	-	-	-	-	26,893
De-2-sulphated N-acetyl heparin		14.4	62.9	3.4	19.3	-	-	-	-	24,940
De-6-sulphated heparin		15.5	43.2	7.0	34.3	-	-	-	-	25,258
De-6-sulphated N-acetyl heparin		55.8	7.7	-	-	-	-	34.8	1.7	22,407
De-2, de-6-sulphate heparin		19.1	-	79.1	-	-	1.8	-	-	22,676
De-2, de-6-sulphate N-acetyl heparin		99.0	-	-	-	-	-	-	1.0	20,167

[∞] Calculated molecular mass of a polysaccharide chain assuming a length of 50 disaccharide subunits and the composition described within this table.

Standards: 1, ΔUA-GlcNAc; 2, ΔUA-GlcNAc(6S); 3, ΔUA-GlcNS; 4, ΔUA-GlcNS(6S); 5, ΔUA(2S)-GlcNS; 6, ΔUA(2S)-GlcNS(6S); 7, ΔUA(2S)-GlcNAc and 8, ΔUA(2S)-GlcNAc(6S).

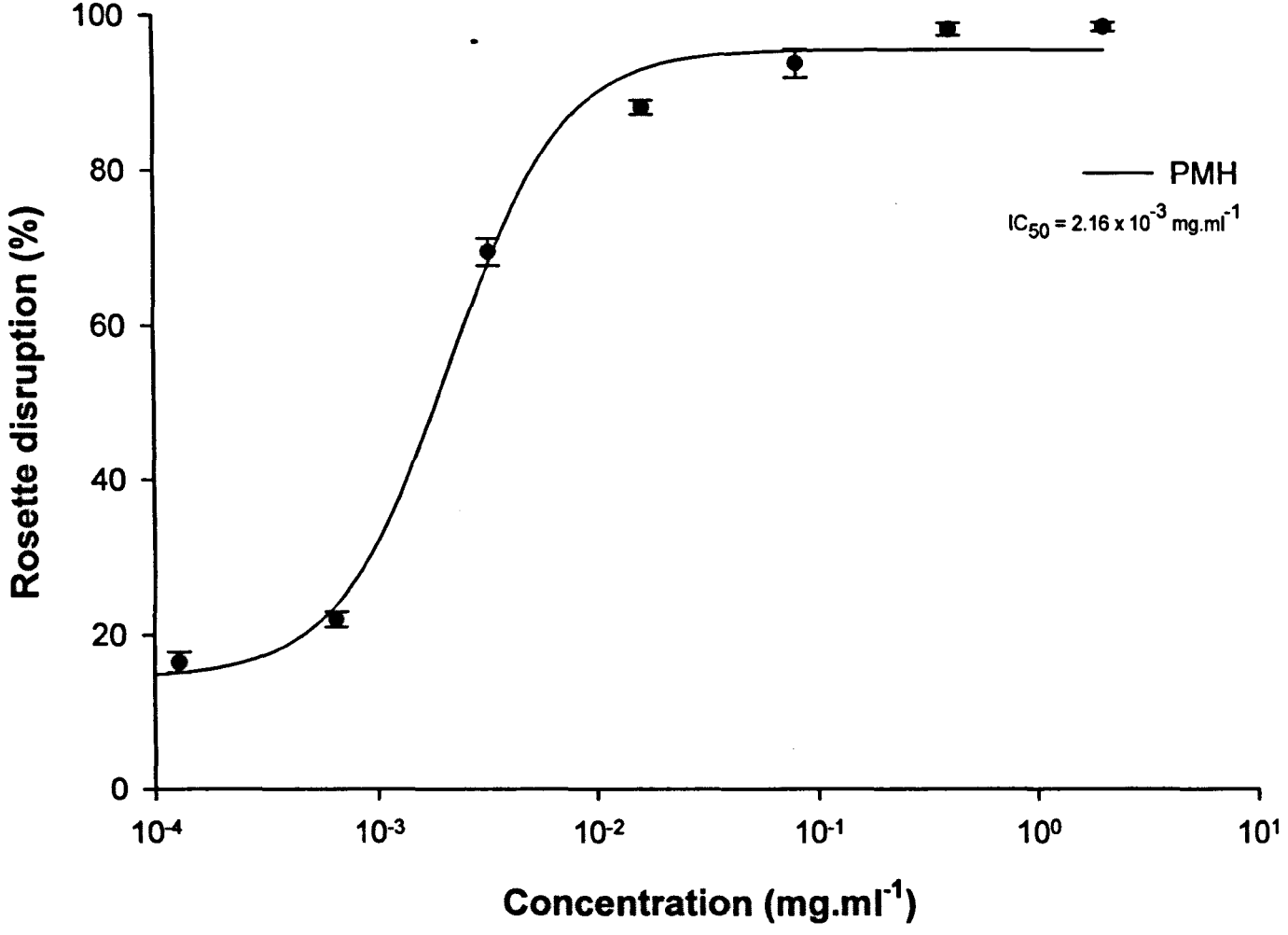


Figure 43- Disruption of *Plasmodium falciparum* mediated rosettes by porcine mucosal heparin (n=3). The level of rosette disruption (percent of parasite infected cells which are non-rosetting) expressed against the concentration of porcine mucosal heparin added.

Parasites from the lab strain R29 (a highly rosetting lab strain implicated in CM; personal communication, Dr A. Craig) were cultured *in vitro* and allowed to form rosettes before differing concentrations of porcine mucosal heparin were added to the rosetting test mixture as detailed in section 2.2.40. The disruptive effects of the added heparin were determined manually by the scoring of parasitized cells forming rosettes compared to those free in solution by microscopy.

The natural level of rosetting for the assay culture was determined and this used as the reference level for rosette formation. The level of disruption was quantified as a percentage of the number of parasitized non-rosetting red blood cells compared to (divided by) the total number of parasitized red blood cells counted (present either free or in a rosette). Porcine mucosal heparin concentrations assayed were in the range of 2 $mg.ml^{-1}$ to 128 $ng.ml^{-1}$ with 5 fold serial dilution performed within this scope. Results are presented as the average per replication and are plotted graphically against heparin concentration on figure 43.

The results clearly suggest that porcine mucosal heparin disrupts rosetting in a concentration dependant manner. The IC_{50} value (i.e. the concentration at which 50% of rosettes are disruptive) for porcine mucosal heparin was determined as 2.16×10^{-3} $mg.ml^{-1}$ (approx. $\approx$ 70 nM) for *Plasmodium falciparum* strain R29 red blood cell rosettes.

The anticoagulation activity of porcine mucosal heparin in this application is an obvious side effect. The ability of a panel of chemically modified polysaccharides to disrupt rosetting was therefore investigated. These polysaccharides exhibit greatly reduced anticoagulant activity compared to that of unmodified heparin [section 5.2.10].

5.2.4 The disruptive effects of size separated oligosaccharides derived from depolymerised porcine mucosal heparin on Plasmodium falciparum mediated rosetting

The ability of smaller oligosaccharide fragments derived from the original heparin polysaccharide to disrupt rosettes was investigated. Porcine intestinal mucosal heparin

was depolymerised using only the bacterial heparatinase hIII as outlined in section 2.2.24. The depolymerised heparin fragments were separated using size exclusion chromatography as described in 2.2.3 and fractions of similar size pooled prior to de-salting. Salt was removed using serial lyophilisation followed by final removal using a PD-10 column [section 2.2.4 and 2.2.25].

Experiments were performed to confirm the ability of heparin oligosaccharides to disrupt rosettes, formed between parasitized (*Plasmodium* infected) red blood cells and uninfected red blood cells [as in section 2.2.40], from R29 strain parasites cultured *in vitro*. Rosettes were allowed to form before different sized oligosaccharide fractions of porcine mucosal heparin were added to the rosetting test mixture as for the full length polysaccharide in section 5.2.3.

The disruptive effects of the added heparin were determined manually by the scoring of parasitized cells forming rosettes compared to those free in solution by microscopy. The natural level of rosetting for the assay culture was determined and this used as the reference level for rosette formation. The level of disruption was quantified as a percentage of the number of parasitized non rosetting red blood cells compared to (divided by) the total number of parasitized red blood cells counted (present either free or in a rosette). Porcine mucosal heparin oligosaccharides were assayed at an overall concentration of 0.4 $mg.ml^{-1}$. Results are presented as the average per replication and are plotted graphically against heparin concentration in figure 44.

In order to confirm the presence of oligosaccharide (as opposed to other UV absorbing material of a similar hydrodynamic volume; in light of the negative results obtained in the rosetting disruption assay) within a sample of dp12 sized oligosaccharide from the heparin digest was digested completely and the resulting profile compared to that of known disaccharide standards. The profile shows the presence of peaks which correlate with known disaccharide standards, and specifically the tri-sulphated disaccharide as would be expected for the main constituent of heparin (data not shown).

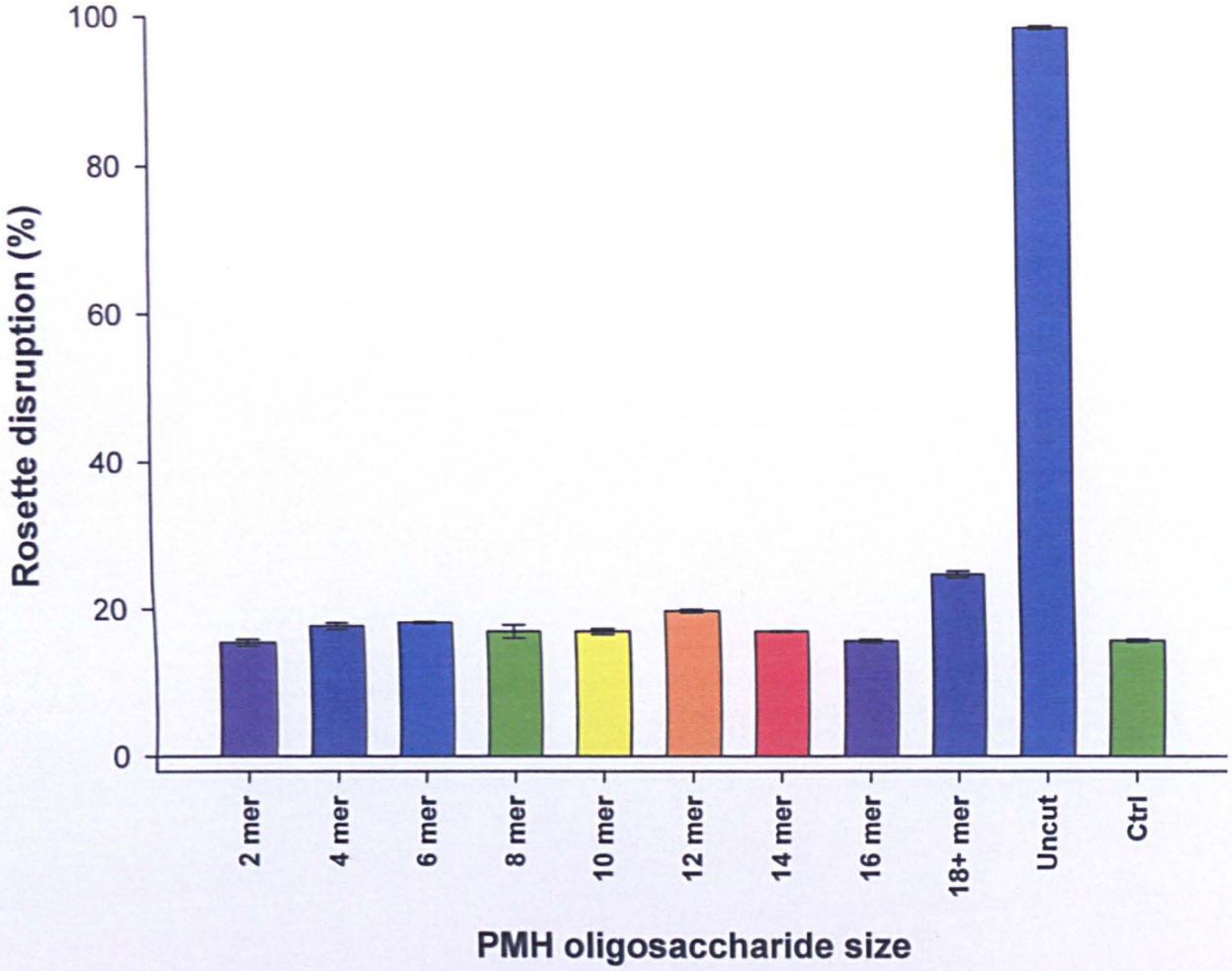


Figure 44 – Disruption of *Plasmodium falciparum* mediated rosettes by porcine mucosal heparin oligosaccharide fragments. The level of rosette disruption (percent of parasite infected cells which are non-rosetting) was scored at a fixed concentration of 0.4 $mg.ml^{-1}$ for sized fractions of porcine mucosal heparin oligosaccharides. The control is the addition of water.

Further evidence for the presence of oligosaccharide was confirmed by the use of the digested material in other unrelated experiments within the laboratory (personal communication, Mr Y Ahmed and Dr A Powell) with expected results ensuing and the presence of characteristic scanning UV profiles over wavelengths λ= 190 – 500 nm. Anticoagulant activity for this digestion is also reported in section 5.2.10.

5.2.5 The disruptive effects of chemically modified heparin polysaccharides on Plasmodium falciparum mediated rosetting

In an attempt to investigate the structural requirements of heparin that are necessary for the disruption of parasitized red blood cell mediated rosetting an investigation was carried out using seven other differentially modified polysaccharide chains, all of which were created initially from porcine mucosal heparin. It should be noted that due to the functional groups removed or added during modification, the molecular mass (Mr) of the polysaccharides will differ. This has been corrected for in subsequent experiments using the composition data based on an average chain length of 50 disaccharide repeat units.

Porcine mucosal heparin is highly sulphated with O-sulphates present on C2 of the iduronic acid, C6 of the glucosamine and an N-sulphate present on C2 of the glucosamine residue and is often used as a model (or proxy) of the S domains of HS, one of the naturally occurring ligands thought to be involved in rosetting. Modified polysaccharides have been sequentially chemically modified to remove these groups allowing for interactions to be interrogated for the presence of essential sulphate modifications. An outline schematic of the modification procedure and its product polysaccharides is shown in Figure 45.

Parasites from the lab strain R29 were cultured *in vitro* and allowed to form rosettes before sample polysaccharide from each of the seven chemically modified heparin derivatives were added to the rosetting test mixture as detailed in section 2.2.40. Porcine intestinal mucosal heparin polysaccharide was also assayed as a control. The disruptive effects of the added heparin were determined manually by the scoring of parasitized cells

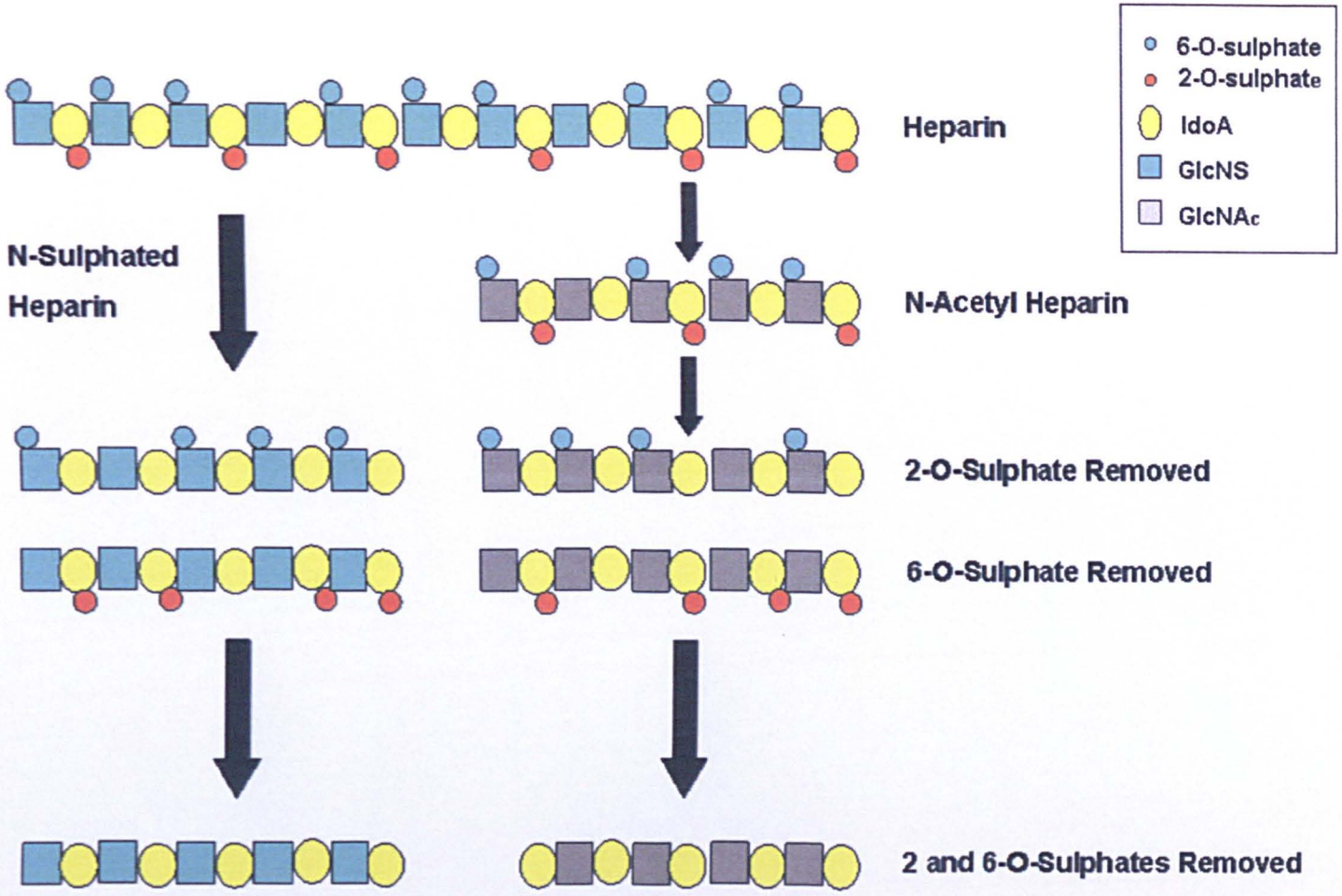


Figure 45 – Chemically modified heparin polysaccharides. Porcine mucosal heparin polysaccharide is de-N-sulphated and re-N-acetylated to yield a totally N-acetylated variant. These two polysaccharides (PMH and N-acetyl PMH) can then undergo selective modification to remove either 2, or 6 –O-sulphate groups, or have both of the modifications carried out. This gives a total of eight polysaccharide variants including the original parent polysaccharide. (Adapted from a figure by Dr S Guimond)

forming rosettes compared to those free in solution by microscopy. The natural level of rosetting for the assay culture was determined and this was used as the reference level for rosette formation. The level of disruption was represented as in section 5.2.3.

Chemically modified polysaccharides were added at a concentration of 0.4 $mg.ml^{-1}$ (this concentration gave maximal inhibition when used with unmodified heparin and was considered to be a workable quantity) thereby assaying 60 μg of each polysaccharide in each sample. Results are presented as the average rosetting disruption per replica and the overall sample average for each modified polysaccharide plotted in Figure 46. The results show that conversion of normal heparin to a completely N-acetylated derivative at C-2 of the glucosamine does not ablate the disruption activity of the polysaccharide significantly.

It should not be concluded that the presence of an N-sulphate or an N-acetyl groups is insignificant for rosette disruption as removal of the 6-Sulphate group alone does not ablate activity but removal of the 6-sulphate and subsequent conversion of N-sulphate groups to N-acetyl renders the polysaccharide all but devoid of disruption activity.The removal of the O-linked sulphate group present at C-2 of the iduronic acid completely destroys the potency of the polysaccharide in the disruption of rosetting. This suggests that this sulphate is essential for rosette disruption. This hypothesis is further substantiated by the lack of potency of polysaccharides removed of both 2 and 6 O-linked sulphate irrespective of the sulphation / acetylation state of C-2 of the glucosamine.

Rosette disruption appears to occur if either of three predominant disaccharide repeat sulphation patterns is present, namely:

(i) -IdoA(2S)-GlcNS(6S)-

- or -

(ii) -IdoA(2S)-GlcNS-

- or -

(ii) -IdoA(2S)-GlcNAc(6S)-

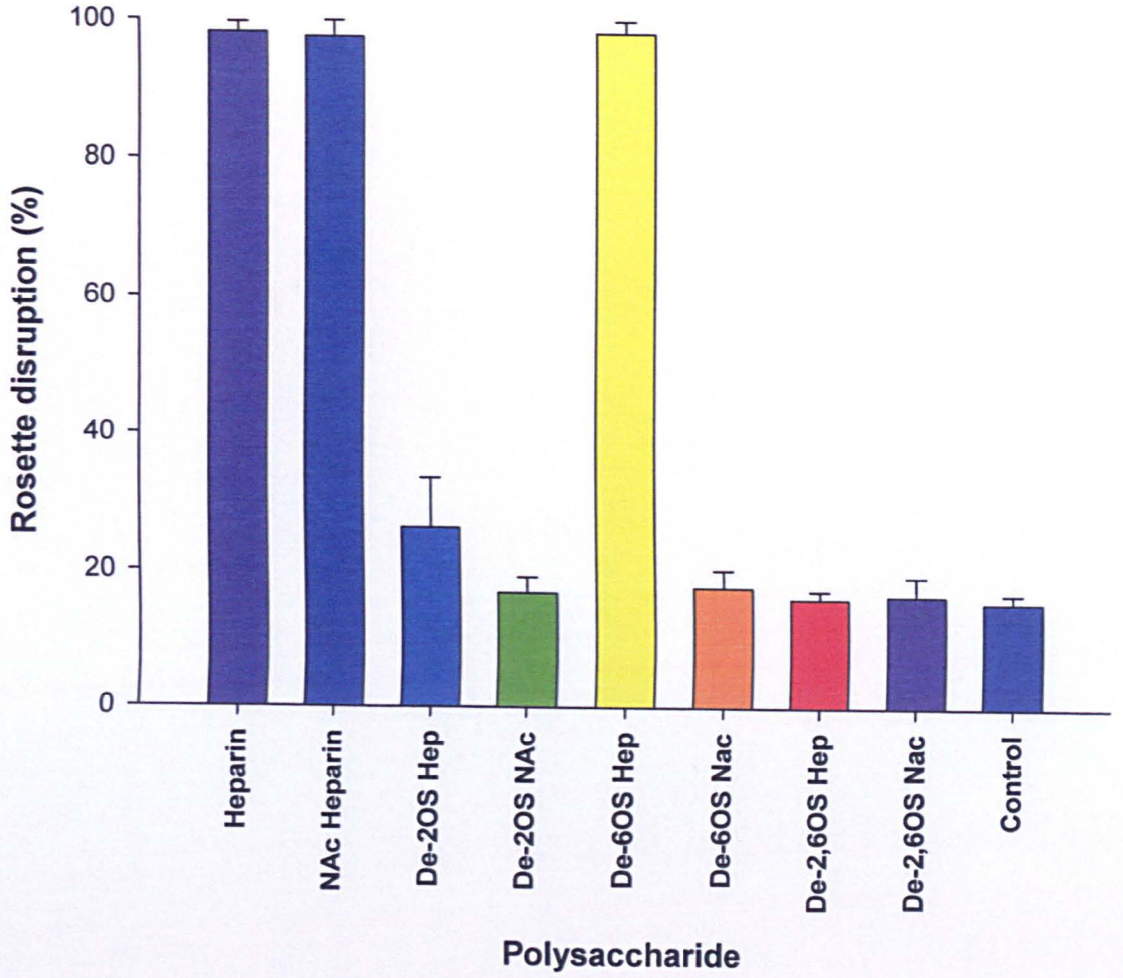


Figure 46 - Disruption of *Plasmodium falciparum* mediated rosettes by chemically modified heparin polysaccharides. The level of rosette disruption (percent of parasite infected cells which are non-rosetting) was scored at a fixed concentration of 0.4 $mg.ml^{-1}$ for each of the chemically modified polysaccharides. The control is the addition of water.

5.2.6 The disruptive effects of a chemically modified polysaccharide containing the predominant repeat unit of IdoA(2S)-GlcNAc(6S) on Plasmodium falciparum mediated rosetting

Chemically modified heparin, which had been totally de-N-sulphated at C2 of the glucosamine residue prior to re-N-sulfation at the same position, to yield a polysaccharide whose major repeat consisted of - IdoA(2S)-GlcNAc(6S)- was shown previously in section 5.2.4 to possesses the ability to disrupt parasite infected red blood cell mediate rosettes with a similar efficacy to that of porcine mucosal heparin at a concentration of 0.4 $mg.ml^{-1}$. Experiments were carried out to determine if the modified polysaccharide possessed a similar disruption capacity to that of porcine mucosal heparin at other concentrations and if the IC_{50} was similar to that of porcine mucosal heparin.

Parasites from the lab strain R29 were cultured *in vitro* and allowed to form rosettes before differing concentrations of porcine mucosal heparin were added to the rosetting test mixture as detailed in section 2.2.40. The disruptive effects of the added heparin were determined manually by the scoring of parasitized cells forming rosettes compared to those free in solution by microscopy. The natural level of rosetting for the assay culture was determined and this was used as the reference level for rosette formation. The level of disruption was represented as in section 5.2.3 previously. Porcine mucosal heparin concentrations assayed were in the range of 2 $mg.ml^{-1}$ to 128 $ng.ml^{-1}$ with 5 fold serial dilution performed within this range. Results are presented as the average per replication and they are plotted against the heparin concentration (Figure 47).

It is clear from the graph that the overall efficacy of chemically modified polysaccharide whose major repeat unit consists of -IdoA(2S)-GlcNAc(6S)- with regard to rosette disruption was inferior to that of porcine mucosal heparin in the lower concentration regions. The IC_{50} for this N-acetylated heparin derivative was 1.97×10^{-2} $mg.ml^{-1}$ ($\approx$ 70 nM) compared to that of 2.16×10^{-3} $mg.ml^{-1}$ ($\approx$ 690 nM) for unmodified porcine mucosal heparin.

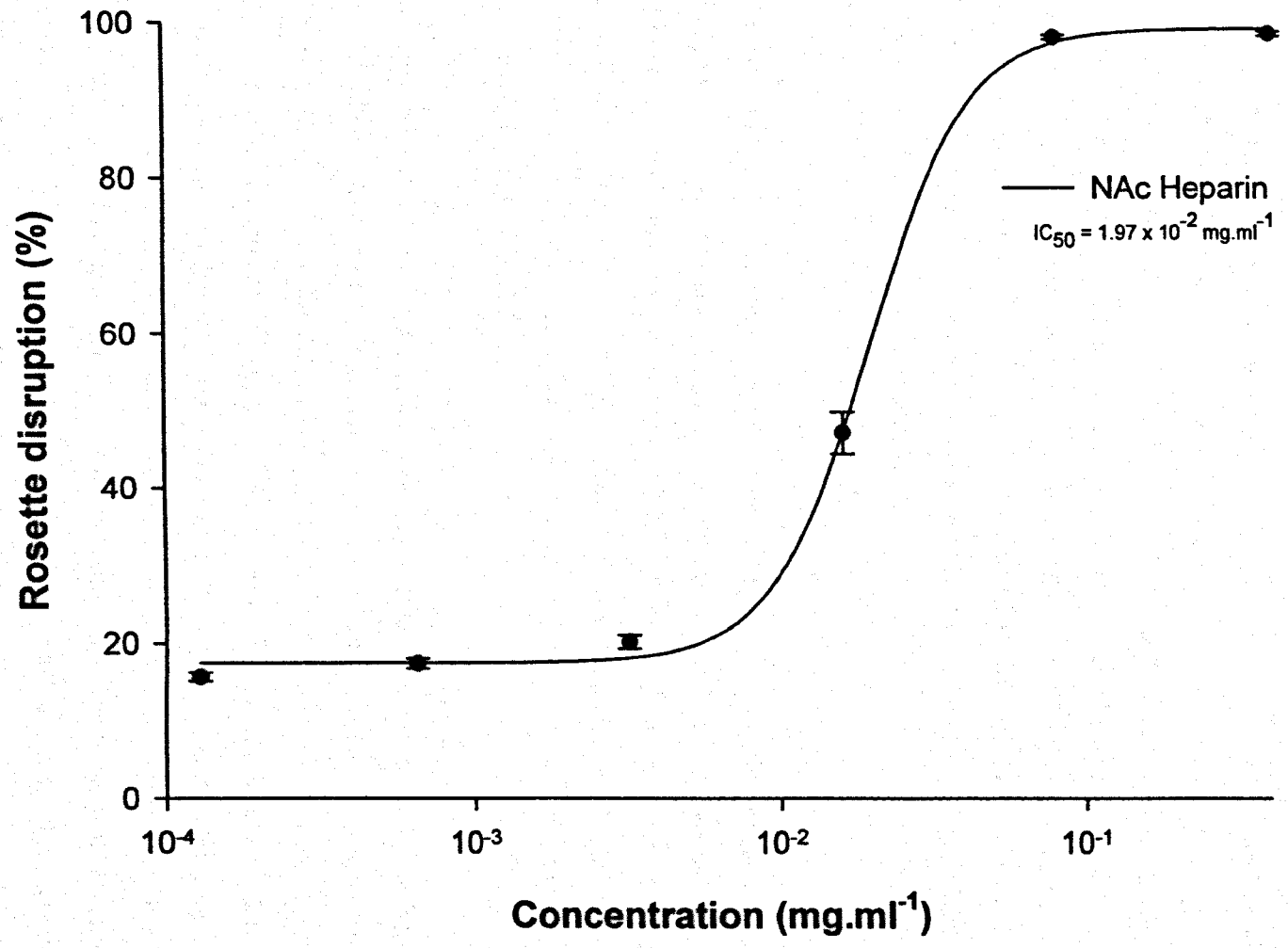


Figure 47 - Disruption of *Plasmodium falciparum* mediated rosettes by a chemically modified variant of porcine mucosal heparin, N-acetyl heparin (n=3). The polysaccharide has all of the N-sulphates removed and replaced by n-acetyl groups. The level of rosette disruption (percent of parasite infected cells which are non-rosetting) expressed against the concentration of the N-acetyl heparin added.

5.2.7 The disruptive effects of size separated oligosaccharides derived from a chemically modified polysaccharide containing the predominant repeat unit of UA(2S)-GlcNAc(6S) on Plasmodium falciparum mediated rosetting

Sized separated oligosaccharides were generated from the parental polysaccharide used in 4.3.6. N-acetylated heparin was depolymerised using the bacterial heparatinase enzyme hII as outlined in section 2.2.24. The depolymerised chemically modified heparin fragments were separated using size exclusion chromatography as described in 2.2.3. and fractions of similar size pooled prior to de-salting. Salt was removed using serial lyophilisation followed by final removal using a PD-10 column [section 2.2.4 and 2.2.25]. Oligosaccharides were assayed to find the smallest oligosaccharide which could possess disruptive activity on rosettes while minimising the level of overall charge possessed by the fragment.

Experiments were performed to confirm the ability of the modified oligosaccharides to disrupt rosettes, formed between parasitized (Plasmodium infected) red blood cells and uninfected red blood cells [as in section 2.2.40], from R29 strain parasites cultured *in vitro*. Rosettes were allowed to form before different sized oligosaccharide fractions of porcine mucosal heparin were added to the rosetting test mixture as for the full length polysaccharide in section 5.2.3.

The disruptive effects of the added heparin were determined manually by the scoring of parasitized cells forming rosettes compared to those free in solution by microscopy. The natural level of rosetting for the assay culture was determined and this was used as the reference level for rosette formation. The level of disruption was quantified as a percentage of the number of parasitized non-rosetting red blood cells compared to (divided by) the total number of parasitized red blood cells counted (present either free or in a rosette). Porcine mucosal heparin oligosaccharides were assayed at an overall concentration of 0.4 $mg.ml^{-1}$. Results are presented as the average per replica and they are plotted against heparin concentration in Figure 48. Disruption of rosettes can be seen at a value of approximately 70% for oligosaccharides of size 12-mer and above, with the 18+-

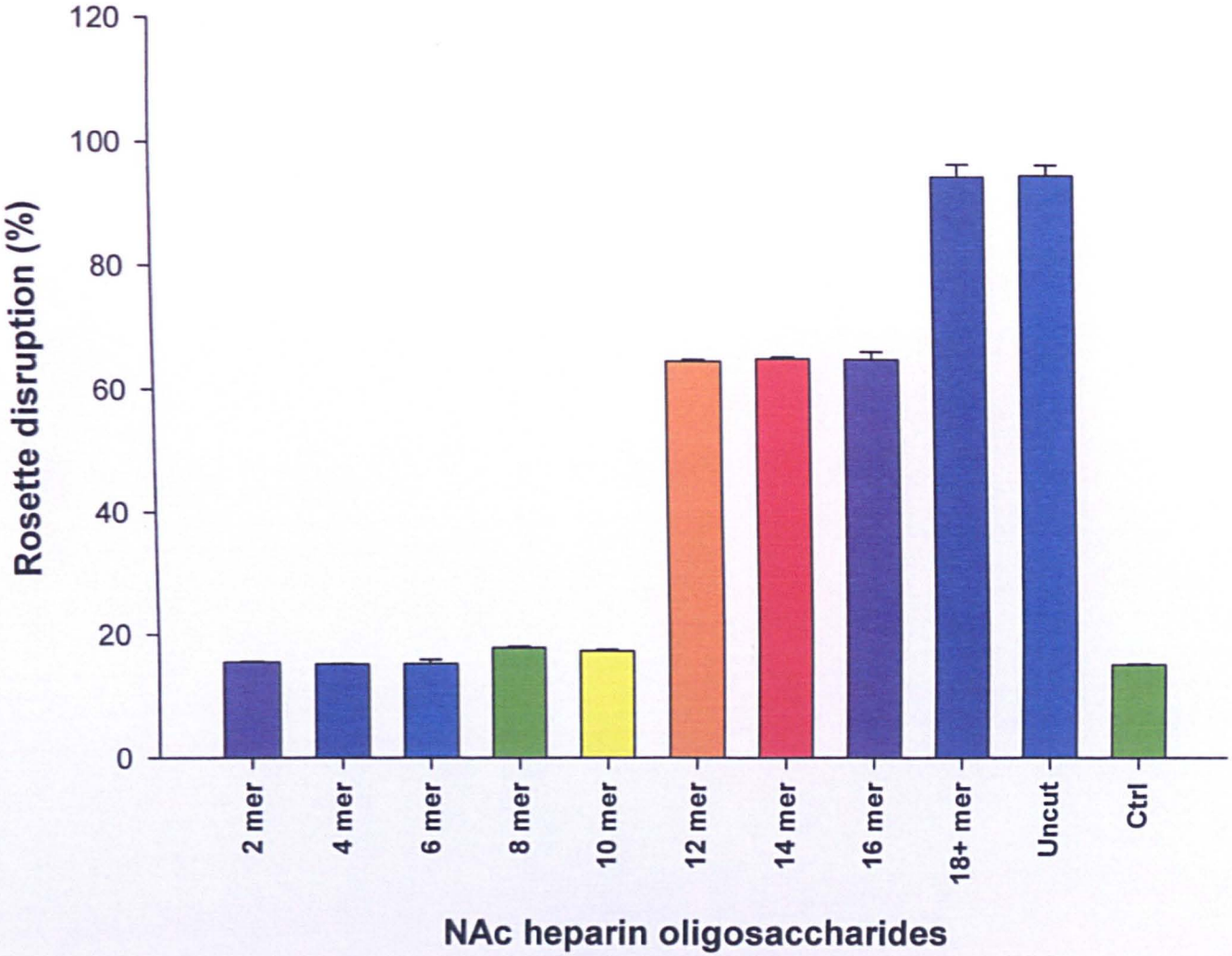


Figure 48 - Disruption of *Plasmodium falciparum* mediated rosettes by N-acetyl heparin oligosaccharides. The level of rosette disruption (percent of parasite infected cells which are non-rosetting) was scored at a fixed concentration of 0.4 $mg.ml^{-1}$ for sized fractions of N-acetyl heparin oligosaccharides. The control is the addition of water.

mer fraction size showing similar disruption potential to that of the full length polysaccharide. Oligosaccharides of size 10-mer and smaller failed to disrupt rosette formation significantly.

5.2.8 The disruptive effects of a chemically modified polysaccharide containing the predominant repeat unit of UA(2S)-GlcNS on Plasmodium falciparum mediated rosetting

The chemically modified polysaccharide containing the predominant repeat unit – IdoA(2S)-GlcNS- was also assayed in a concentration dependent manner, as this polysaccharide was also shown to mediate the disruption of rosettes with a similar efficacy to that of porcine mucosal heparin at a concentration of 0.4 $mg.ml^{-1}$. Experiments were carried out to determine whether the modified polysaccharide possessed a similar disruption capacity to that of porcine mucosal heparin at other concentrations and whether the IC_{50} was similar to that of porcine mucosal heparin.

Parasites from the lab strain R29 were cultured *in vitro* and allowed to form rosettes before differing concentrations of porcine mucosal heparin were added to the rosetting test mixture as detailed in section 2.2.40. The disruptive effects of the added heparin were determined manually by the scoring of parasitized cells forming rosette compared to those free in solution by microscopy. The natural level of rosetting for the assay culture was determined and this was used as the reference level for rosette formation. The level of disruption was represented as in section 5.2.3 previously. Porcine mucosal heparin concentrations assayed were in the range of 2 $mg.ml^{-1}$ to 128 $ng.ml^{-1}$ with 5 fold serial dilution performed within this range. Results are presented as the average per replica and are plotted against the heparin concentration in Figure 49.

The overall efficacy of the 6-de-sulphated polysaccharide appears to be significantly improved compared to that of the N-acetyl heparin polysaccharide and similar to the original porcine mucosal heparin polysaccharide. The IC_{50} of the 6-de sulphated heparin

is 3.05 x 10^{-3} mg.ml^{-1} (≈ 120 nm) compared to that of 1.97 x 10^{-2} mg.ml^{-1} (≈ 70 nM) for the N-acetyl heparin polysaccharide and 2.16 x 10^{-3} mg.ml^{-1} (≈ 690 nM) for the unmodified heparin.

5.2.9 *The disruptive effects of size separated oligosaccharides derived from a chemically modified polysaccharide containing the predominant repeat unit of UA(2S)-GlcNS on Plasmodium falciparum mediated rosetting*

Sized separated oligosaccharides were generated from the parental polysaccharide used in 4.3.8. Briefly, de-6-O-sulphated heparin was depolymerised using the bacterial heparatinase hII as outlined in section 2.2.24. The depolymerised chemically modified heparin fragments were separated using size exclusion chromatography as described in 2.2.3. and fractions of similar size pooled prior to de-salting. Salt was removed using serial lyophilisation followed by final removal using a PD-10 column [section 2.2.4 and 2.2.25]. Oligosaccharides were assayed to find the smallest oligosaccharide which possessed disruptive activity on rosettes while minimising the level of overall charge possessed by the fragment. Experiments were performed to confirm the ability of the modified oligosaccharides to disrupt rosettes as described in section 2.2.40. All of the oligosaccharides were assayed at a concentration of 0.4 mg.ml^{-1}. Results are presented as the average per replica and are plotted against heparin concentration in Figure 50.

5.2.10 *Anti-coagulation activity of the heparin and chemically modified heparin polysaccharides and oligosaccharides used for the rosetting assays.*

The anti-coagulation activity of all the saccharides used in the above rosette disruption experiments were assayed for their potential to interaction with anti-thrombin III and thereby inhibit factor Xa activity coagulation activity. A chromogenic peptide substrate was used to determine the activity of factor Xa within a given sample.

Samples were assayed using a Coatex® heparin test kit with modified methodology [see 4.2.] allowing the use in a 96 well plate format instead of the 1 ml cuvette form specified

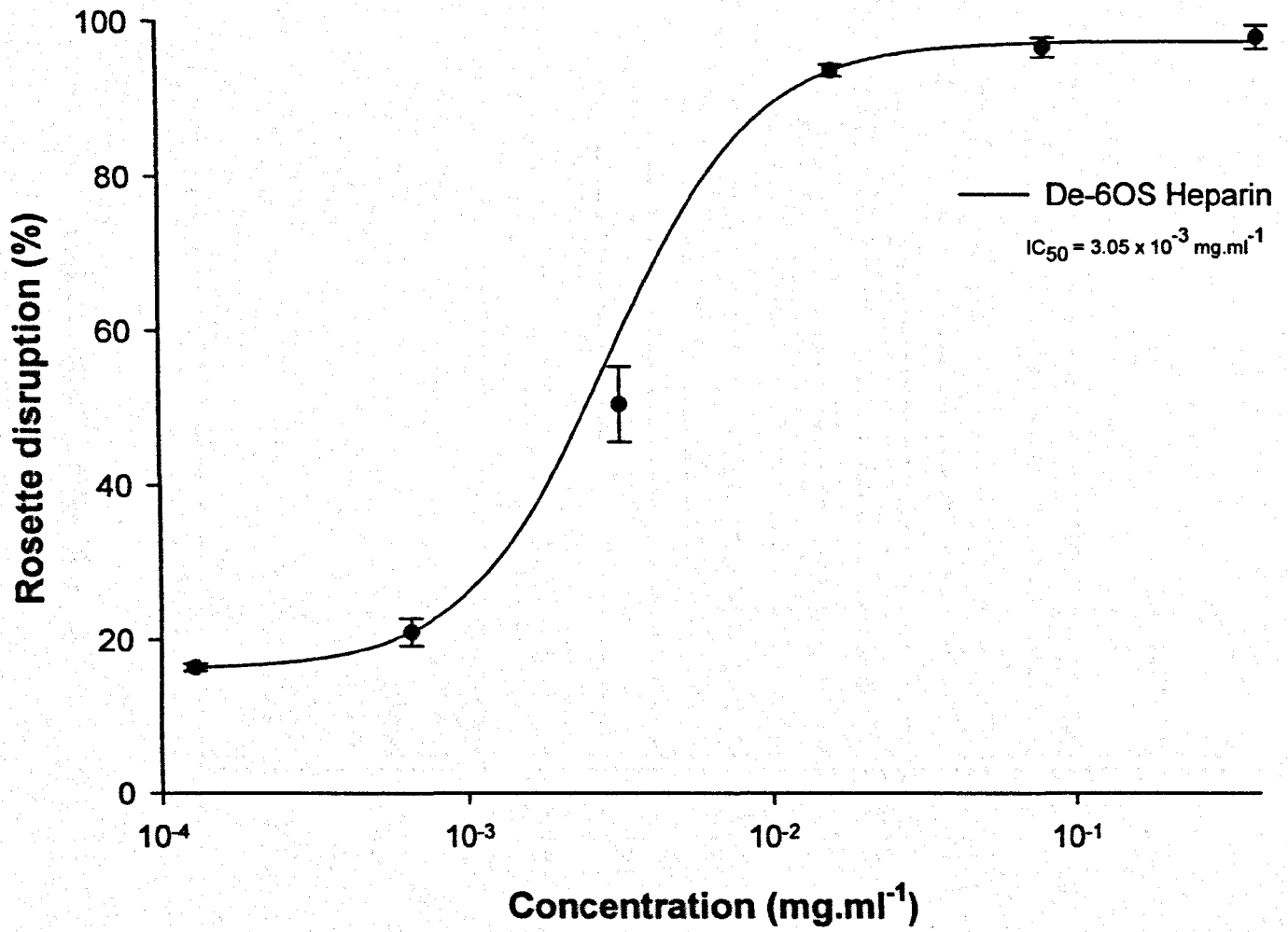


Figure 49 - Disruption of *Plasmodium falciparum* mediated rosettes by a chemically modified variant of porcine mucosal heparin, 6-desulphated heparin (n=3). The polysaccharide has all of the 6-O-sulphate groups removed. The level of rosette disruption (percent of parasite infected cells which are non-rosetting) expressed against the concentration of the 6-desulphated heparin added.

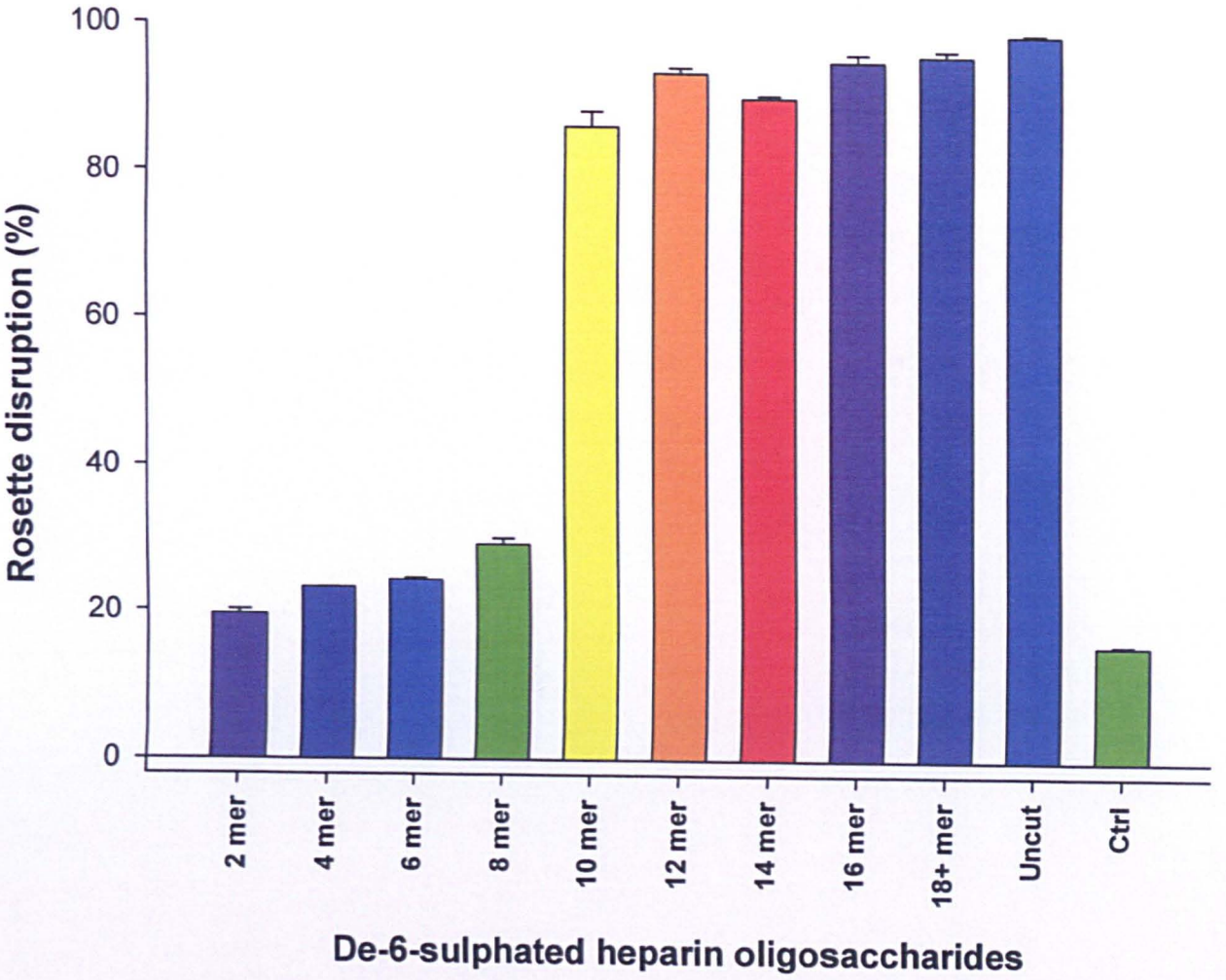


Figure 50 - Disruption of *Plasmodium falciparum* mediated rosettes by 6-desulphated heparin oligosaccharides. The level of rosette disruption (percent of parasite infected cells which are non-rosetting) was scored at a fixed concentration of 0.4 $mg.ml^{-1}$ for sized fractions 6-desulphated heparin oligosaccharides. The control is the addition of water.

in the manufacturer's instructions. Absorbance readings were obtained at λ=405 nm using a plate reader.

The anticoagulant activity of each oligosaccharide / polysaccharide compared to that of porcine mucosal heparin (205 IU mg^{-1}, which is defined as 100% in value) are shown in figures 51 to 53.

With the exception of the dp-18 (18-mer) fraction derived from the chemically modified 6-Desulphated heparin polysaccharide, which contains approximately 12 % of the anticoagulant activity of heparin, all of the other polysaccharide and oligosaccharide fragments posses less than 2% of the anticoagulant activity of porcine mucosal heparin.

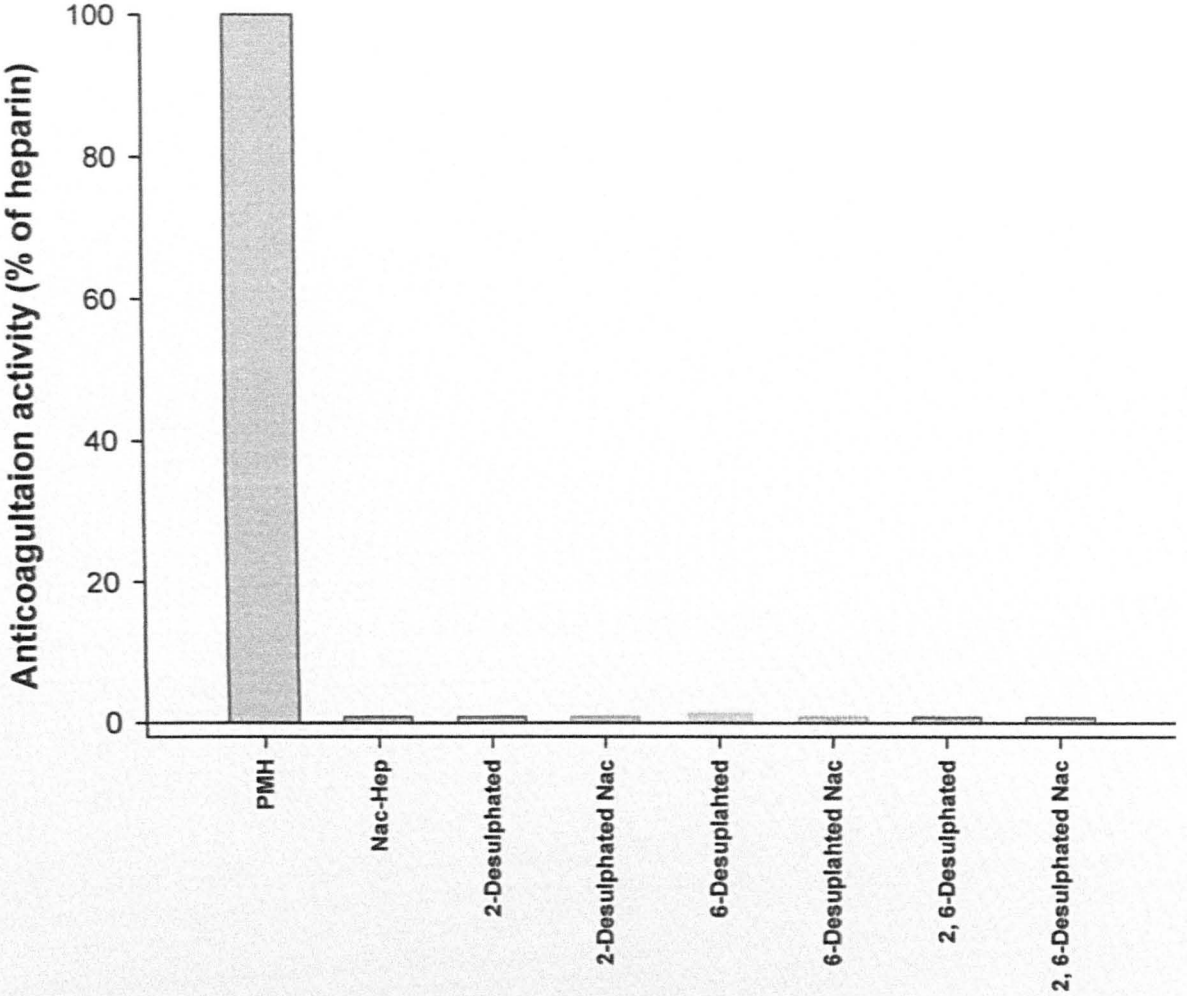


Figure 51 – Anticoagulation activity of chemically modified heparin polysaccharides. The anticoagulation activity of all eight polysaccharide variants (including unmodified PMH) was determined using a plate assay. The assay relies on the cleavage of a chromogenic substrate by Factor Xa. Factor Xa is inhibited by antithrombin III (AT-III) when it is activated by associated with heparin. The anticoagulation activity (IC_{50}) is expressed as a percentage of porcine mucosal heparin.

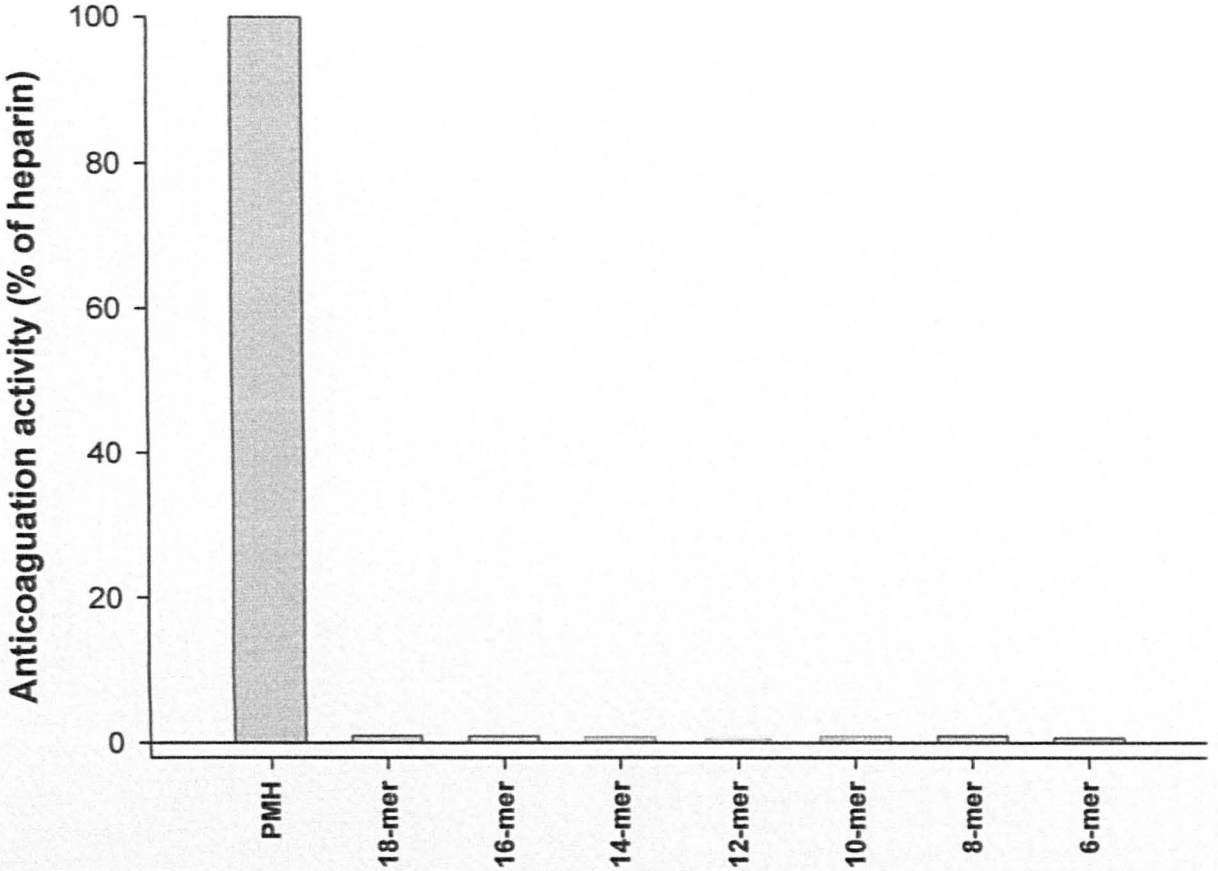


Figure 52 - Anticoagulation activity of chemically modified, N-acetyl heparin size defined oligosaccharides. The anticoagulation activity of all eight polysaccharide variants (including unmodified PMH) was determined using a plate assay. The assay relies on the cleavage of a chromogenic substrate by Factor Xa. Factor Xa is inhibited by antithrombin III (AT-III) when it is activated by associated with heparin. The anticoagulation activity (IC_{50}) is expressed as a percentage of porcine mucosal heparin.

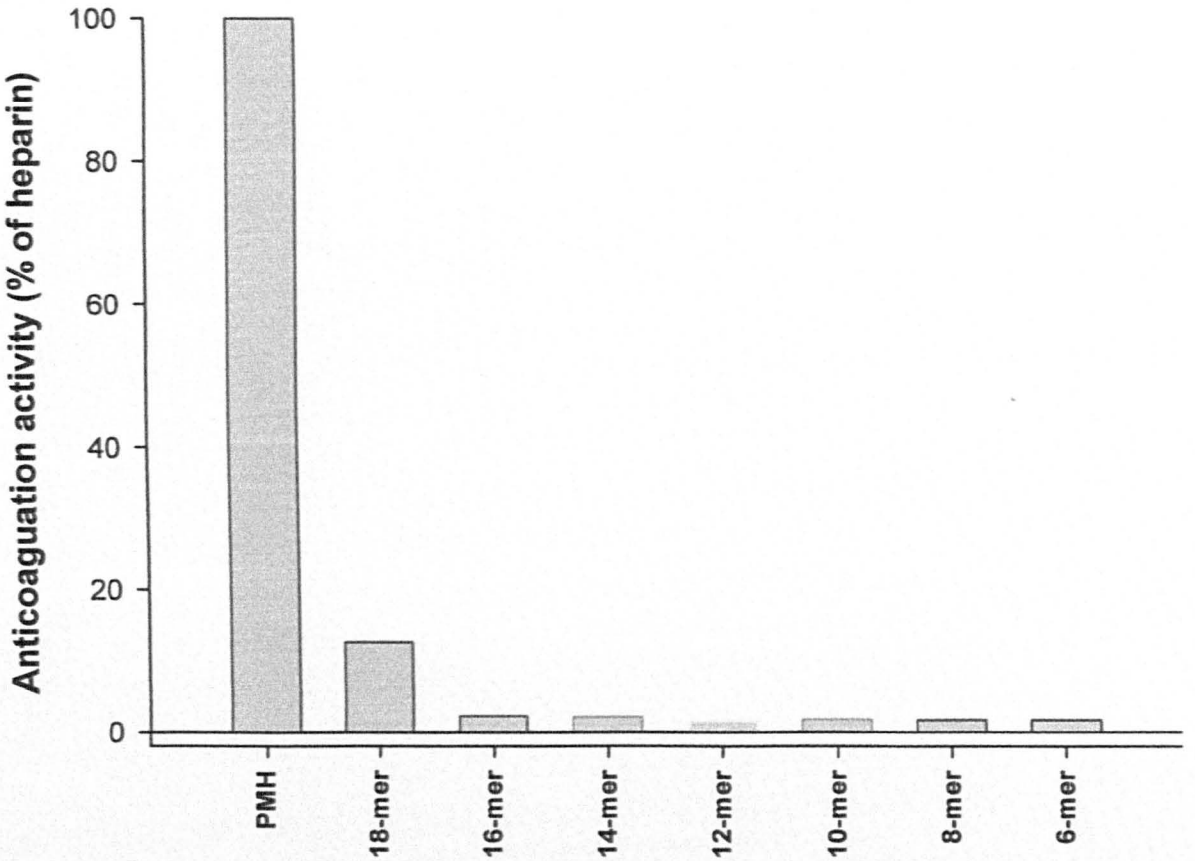


Figure 53 - Anticoagulation activity of chemically modified, 6-desulphated heparin size defined oligosaccharides. The anticoagulation activity of all eight polysaccharide variants (including unmodified PMH) was determined using a plate assay. The assay relies on the cleavage of a chromogenic substrate by Factor Xa. Factor Xa is inhibited by antithrombin III (AT-III) when it is activated by associated with heparin. The anticoagulation activity (IC_{50}) is expressed as a percentage of porcine mucosal heparin.

5.3 Discussion

5.3.1 Rosetting and cerebral malaria

It is thought that three factors mediate cerebral malaria (CM); rosetting, sequestration of parasitized blood cells to the endothelium and parasite stimulated host immune responses. While the deposition to the endothelial surface, mediated by *Plasmodium falciparum* erythrocyte membrane protein 1 (PfEMP-1) and intercellular adhesion molecule 1 (ICAM-1) is widely accepted as playing a leading role, the tasks played by rosetting and inflammation are points for debate. It is thought that inflammatory responses (and therefore cytokines) are promoted by tumour necrosis factor (TNF) α, production of which is thought to be stimulated by the parasite. There is evidence amassing which correlates CM with the rosetting phenomenon and this outweighs those few cases in which no correlation has been seen. These studies could be affected by sample size, statistical analysis and / or likely variations within the *Plasmodium falciparum* strain studied.

The rosetting phenomenon and its exact role in cerebral malaria (CM) is still poorly understood. However, it seems clear that the cross-linking of infected red blood cells to non infected, thereby forming rosettes, must cause health problems. Infected red blood cells can also sequester other infected cells forming rosettes.
The ability to disrupt rosettes formed in *Plasmodium* infection would therefore be highly desirable. Chemical entities which achieve this could form the basis of novel therapeutics for this complication of a very severe and widespread disease.

5.3.2 The use of heparin compounds to disrupt rosetting

The propensity of heparin polysaccharides to disrupt plasmodium falciparum mediated rosettes has been shown previously. These studies show that bovine lung heparin (BLH) polysaccharides possess the ability to disrupt rosettes in a wide variety of plasmodium falciparum strains over a concentration range of 1 to 1000 ug.ml^{-1}. The rosetting receptor

has been reported to be the plasmodium falciparum erythrocyte membrane protein 1 (PfEMP-1) which is expressed by the parasite and translocated to the external face of the red blood cell membrane. In this thesis, porcine intestinal mucosal heparin (PMH) is used which has the advantage of being free form any known transmissible spongiform encephalopathy, which would be of great significance were the carbohydrate to be used in a modified form as a therapeutic. The PMH polysaccharides used here also exhibited rosette disruption over a similar concentration range as previously reported for BLH polysaccharides with an IC_{50} of approximately 70 nM.

Upon isolation of the PfEMP-1 receptor, Barragan *et al.* also reported the minimal heparan sulphate (although BLH was used for the experiment) oligosaccharide size required to disrupt rosette was a 12 mer (or dp12) [216], and this was also the minimal size required for binding to PfEMP-1 (although their data suggests a 14 mer or dp14 is required for saturated binding) [215]. In contrast to the results of Barragan *et al.*, no evidence of rosette disruption was seen for de-polymerised oligosaccharides derived from PMH up to a size of an octadecasaccharide, i.e. dp18. The use of larger size defined fragments is not possible due to incomplete resolution from contaminating full length polysaccharide in size exclusion chromatography. The method of de-polymerisation used here was digestion with the bacterial enzyme heparatinase hIII. The method used by Barragan *et al.* remains unclear as it is re-referenced from an earlier paper [219] in which multiple digestions of both heparin, modified heparin and HS are performed using, at some stage, all three heparatinase enzymes. It is assumed that they have used solely heparatinase hIII (which cuts within highly sulphated regions and would be appropriate for use with heparin), as here, but this remains ambiguous and the possibility remains that other enzymes, possibly heparatinase hII, could have been used. It is not thought that this should account for the differences in the results observed, although this would need further investigation using both enzymes and both heparin sources. It is also highly unlikely, although possible, that this could result from the differences between BLH and PMH starting polysaccharides. Another possibility is the use of different strains of *Plasmodium falciparum*; in this thesis the highly rosetting strain R29 from the IT lineage

was used while the study of Barragan *et al.* employed the FCR3 strain [215], which is distinct (personal communication Dr A. Craig).

The possibility that the inability of heparin sized fractions to inhibit rosetting was caused by an inadvertent absence of material was discounted for the following reasons; exhaustive digestion of an oligosaccharide fragment (dp12) from the same batch used in this assay followed by HPLC SAX disaccharide compositional analysis showed peaks which corresponded to commercially available reference disaccharide reference standards with the predominant standard being [UA(2S)-GlcNS(6S)] as expected for digested PMH. The oligosaccharide also showed a characteristic absorption spectra between 190 nm and 600 nm, absorbing as per PMH except for the presence of a maxima due to the C=C double bond at 232 nm as expected. These oligosaccharides were also used in other (unrelated) activity experiments within the laboratory and the expected (positive) outcomes were seen.

The lack of inhibitory activity for the PMH oligosaccharide fragments suggests the existence of a higher degree of structure which is present in the polysaccharide but not in the derived oligosaccharides. This may be the presence of a conformation which can only be adopted in the polysaccharide and not oligosaccharide fragments (smaller than dp20). The possibility of intra chain interactions which could have been broken (hydrogen bonds, hydrophobic interactions, electrostatic repulsions etc) or interactions with the environment (solvent, hydrogen bonding, the presence of various metal cations) could also explain these findings.

The results of this experiment add further weight to the idea that heparin polysaccharides cannot be considered to be merely a sum of their parts as in a "beads on a string" model. While the primary structure is important, the overall conformation and interactions with its environment are equally as important. The idea that oligosaccharides behave in a manner distinct from polysaccharides and is not just dependent on the length needed to fulfil the requirements of what may loosely be termed a binding site, is one which merits further investigation and serious consideration,

In an attempt to try and elucidate the structural requirements needed for rosette disruption within the polysaccharide chain, the structural motifs required for rosette disruption were probed using chemically modified heparin available to the laboratory. These consist of heparin polysaccharides which have selectively had groups removed or added in a stepwise manner to allow binding, or in this case inhibition, to be studied.

The use of these seven chemically modified PMH polysaccharides in this study showed the requirement of an O-linked sulphate group present at C-2 of the iduronic acid residue. The presence of this group was essential for rosette disruption. The ability to disrupt rosette formation also appeared to require the presence of at least one other sulphate group, either an N-sulphate linked to C-2 of the glucosamine or a 6-O-sulphate group (or the presence of all three as is present in PMH). This suggests that although at least 2 sulphate groups are required for rosette disruption, the interaction possesses more specificity and is not merely a charge (or ionic) mediated interaction alone.

One explanation for this data lies in the altered glycosidic linkage geometry which has been observed for both the 6-de-sulphated and the N-de-sulphated, re-N-acetylated forms of heparin [220]. The outcome of this change in linkage geometry may account for changes in the conformation of the modified polysaccharide molecule allowing for its interaction with PfEMP-1 and for the potential to disrupt rosettes. Further work will need to be carried out to confirm or refute this hypothesis.

The presence of the Ido(2S) residue may also have a bearing on the ability to disrupt rosettes in a manner which is more complex than that of just possessing a sulphate group. The Ido(2S) residue is known by NMR studies [220] to be more flexible than that of iduronate or glucuronate residues and the fact that all but one Ido(2S) containing chemically modified polysaccharides (out of a total of four) is potent in rosette disruption adds further weight to the idea that the conformation, and not just the primary sequence structure of the polysaccharide is important. The Ido(2S) residues may introduce this conformational flexibility into the polysaccharide chain. Again, further work will need to

carried out to confirm or refute this hypothesis and explain why the Ido(2S)-GlcNAc repeat cannot impart these conformational requirements.

Similar experiments using chemically modified polysaccharides have been carried out by Barrigan *et al.* [215] using modified derivatives of BLH on the variant plasmodium strain FCR3S1. The results of these assays suggest that an N-sulphate group attached via C-2 of the glucosamine residue is critical for rosette disruption. These results are in contrast to the requirements found in our assay and it is highly likely that this is due to variation between *Plasmodium falciparum* strains. Barragan *et al.* [215] do however, report that the 2-O-sulphate groups had a significant effect on rosette disruption although were not strictly essential, as they were for the R29 strain used in this thesis. Future work will be carried out to identify other rosetting strains which can be disrupted by heparin and its derivatives and for the elucidation of structural requirements.

The use of the modified polysaccharides derived from heparin highlighted two polysaccharides with the ability to disrupt rosettes at a concentration of 0.4 mg.ml-1. These were de-N-sulphated heparin which had been chemically re-N-acetylated and 6-de-sulphated heparin. Both of these polysaccharides were assayed for their ability to disrupt rosettes dependent on concentration. The de-N-sulphated heparin which had been chemically re-N-acetylated did not possess the same capacity to disrupt as PMH over the concentration range tested (1.97×10^{-2} compared to 2.16×10^{-3} $mg.ml^{-1}$, IC_{50} of approximately 690 nM and 70 nM respectively). The 6-de-sulphated polysaccharide faired better in comparison to PMH and possessed an IC_{50} of ≈120 nM (3.05×10^{-3} $mg.ml^{-1}$).

Oligosaccharides from both de-N-sulphated, re-N-acetylated heparin and the 6-de-sulphated polysaccharide (NAc-hep) were assayed to determine the minimum size requirement for rosette disruption. In contrast to heparin oligosaccharides, which could not disrupt when size defined, de-polymerised fractions up to a dp18 were assayed; the two chemically modified polysaccharides could both disrupt rosette formation, with the NAc-heparin requiring at least an oligosaccharide of length dp12 and 6-de-sulpated

heparin a dp10. The NAc-heparin fragments could only disrupt rosette formation at a level of approximately 65% compared to the full length polysaccharide (95%), with the exception of the dp18 fragment which was probably contaminated by larger fragments (resolution becomes increasingly difficult for SEC separation with size). The 6-de-sulphated fragments all possessed approximately the same level of disruption which was comparable to that of the polysaccharide (86-98%). Therefore, the 6-de-sulphated fragments would probably provide the best option for future use as a therapeutic, where the minimization of charge and size is of paramount importance.

One explanation for the ability of oligosaccharides derived from chemically modified parent polysaccharides to inhibit rosetting may be that removing sulphate groups permits certain conformation to occur, even in oligosaccharides, where these conformations would only be present in limited amounts in full chain heparin. Therefore, upon de-polymerisation, the oligosaccharide fragments derived from heparin no longer posses the ability to adopt such a conformation in sufficient numbers to inhibit binding.

5.3.3 Side effects of heparin polysaccharides

Ultimately, the goal of these studies may be to elucidate structures which could potentially be used as candidatures for novel therapeutics. The use of heparin presents two main problems both of which would be classed as common side effects of heparin administration. As mentioned previously, heparin is already used pharmaceutically as an anticoagulant. This would be an undesirable side-effect if administered in the treatment of malaria. Furthermore, a common side-effect of heparin administration results from heparin binding to platelet factor 4 and the induction of antibodies to the complex. This causes and immune response which leads to a condition known as heparin induced thrombocytopoenia. In this condition the platelet count of an individual is decreased to a critical level. Antibodies which bind to the heparin-platelet factor 4 (PF4) complex have recently been obtained and research is currently underway to design an enzyme linked immmuno assay (ELISA) whereby the potential of chemically modified heparin molecules to associate with PF4 can be screened.

Each of the oligosaccharides and polysaccharides used in the rosetting assays conducted within this thesis were tested for their ability to inhibit coagulation by their ability to associate with antithrombin and suppress factor Xa. This assay is a routinely used to measure the amount of heparin present in the plasma after administration in health care situations. It was found that none of the chemically modified oligosaccharides possessed significant anticoagulant activity compared to that of heparin. This was expected, as all the chemical modifications destroy the anti-thrombin pentasaccharide binding motif essential for anticoagulation activity. The removal of one of the major side-effects associated with heparin administration further enhances the credentials of this class of molecules as potential drug candidates.

5.3.4 Other factors implicated in causing cerebral malaria.

Another attractive property which needs to be investigated further is the ability of heparin and chemically modified derivatives thereof to inhibit or alleviate other symptoms which cause cerebral malaria. These are thought to be the deposition of parasitized red blood cells upon the endothelium and the presence of elevated levels of nitric oxide which is produced as an inflammatory response.

Initial experiments carried out to measure the level of infected red blood cell adhesion to ICAM-1, the protein which interacts with PfEMP-1 have shown that the level of deposition can be reduced to approximately 60% of the uninhibited sequestration by the presence of heparin (initial experiments carried out in collaboration with Dr A Craig). Further experiments are ongoing to see whether chemically modified polysaccharides and oligosaccharides can also reduce the ability of infected red blood cells to attach to the endothelial cell surface.

Another contributory factor to cerebral malaria is thought to be the production of nitric oxide by TNF α (produced in response to parasite antigens) [221] which is thought to cause coma (although some researchers originally thought this was a mechanism for the

destruction of the parasite, it is now thought the parasite is shielded from this by its presence within the red blood cell). The amount of nitric oxide present (which is rapidly converted to nitrite) can be assayed chromogenically, and the background level removed by subtraction. Furthermore, nitric oxide production can be stimulated by the addition of both TNF α and interferon γ to the endothelial cells [222]. The level of nitric oxide production can be assayed after stimulation with these two molecules in the presence and absence of heparin and chemically modified derivatives, in order to assay for a potential role of heparin as an anti-inflammatory agent in this context. Heparin has previously been reported to possess anti-inflammatory behaviour [223, 224].

If it can be demonstrated that heparin based molecules can further reduce deposition and reduce inflammation thereby leading to lower amounts of nitric oxide, coupled with anti-rosetting potential whilst overcoming the major side effects of heparin induced thrombocytopoenia and anticoagulation, this molecule could provide leads in the design and applicant of novel therapeutics. Further work in all of these areas is envisaged.

Chapter 6

General discussion and future direction

6.1 Reducing end labelling of glycosaminoglycan fragments with the highly sensitive fluorophore, Bodipy ® Fl hydrazide

The ability to label heparin and heparan sulphate oligosaccharide fragments with a highly sensitive fluorophore is a major advance in this field. Previous attempts have utilised fluorescent entities with low coefficients of extinction and poor coupling chemistries. This is further hampered by the poor stabilities of the newly created glyco-conjugates, arduous, labour intensive clean up steps and the inability to separate and resolve the many structural isomers present, even, in some cases at the disaccharide level. The separation of disaccharides can be considered to be the most facile of situations, while oligosaccharides pose a greater challenge due to their higher degree of structural isomerism.

The use of Bodipy hydrazide presents an alternative to the currently applied arsenal of fluorophores, with a significantly better co-efficient of extinction. Other fluorophores exist which could potentially be coupled to heparin or HS derived oligosaccharides, but these generally contain highly charged (even sulphated) structures which hamper separation, by adding to the already high net charge of the molecules, and also tend to migrate or elute at similar times to the carbohydrate species under analysis. In contrast, Bodipy hydrazide has no overall charge and is hydrophobic (being only slightly soluble in water). Unbound tag does not migrate towards the anode in capillary electrophoresis, nor does it interact with the positively charged matrix in strong anion exchange chromatography (passing through the column to waste. The hydrophobic nature of this fluorophore avoids unwanted clean up steps in the methods used within this thesis.

The more polar nature of the fluorophore glyco-conjugate (due to the attached carbohydrate moiety) may allow phase separation to be used as a rapid and facile clean up step, should the removal of free tag be necessary. This should aid methodologies which analyse the structure of oligosaccharide components (such as the constituent disaccharide analysis) by virtue of removing tiresome clean up steps where sample losses can quickly compound. The potential for labelling crude tissue extracts following

enzymatic digestion exists and this would significantly assist structural analysis. Furthermore, the labelling procedure seems to be unaffected by the presence of physiological concentrations of salt, lyase digestion enzymes, or carrier proteins and non reducing sugars.

The use of Bodipy hydrazide needs to be extended to encompass other glycosaminoglycan family members and studies are currently underway to determine the optimum labelling conditions, respective yields and the separations strategies which give the highest level of resolution for these glyco-conjugates.

Another distinct advantage of the coupling of Bodipy to HS and heparin derived disaccharides is the comparatively 'soft' labelling conditions which are utilised. The potential for de-N-sulphation in acid conditions has been discussed within this thesis and remains a concern when compositional analyses are performed. Qualitative evidence for increased de-N-sulphation has also been reported here when heat is applied to drive the reaction in acid catalysed conditions and further investigations are underway to characterise this effect. The labelling of disaccharides with Bodipy hydrazide occurs at room temperature and therefore limits the potential for de-N-sulphation, although this reaction is also acid catalysed.

One major step forward may come in the use of metals as catalysts thereby replacing the acidic conditions currently used and the problems inherent with this form of catalysis. The first example of metal catalysis in the labelling of glycosaminoglycan derived saccharides is reported here utilising dibutyltin chloride as a metal catalyst and phenylsilane as the reducing agent in the reductive amination reaction between the sugar and Bodipy fluorophore. It is hoped, and further studies are underway to confirm this, that this method overcomes the de-N-sulphation problem and should lead to more authentic structure and sequence data from heparin and HS oligo- and polysaccharides. The study of metal catalysis is to be extended to encompass other metal catalysts, carbohydrates, fluorophores and to determine the best conditions for conjugation. This form of catalysis will also be studied with regard to reductive amination reaction used in

the attachment of oligosaccharides to surfaces, such as micro-arrays and biosensors. The application of this technique could be widespread, potentially in any situation where reducing end reductive amination is used.

The level of sensitivity afforded by Bodipy conjugation is a significant improvement on that previously available to the field with at least an order of magnitude increase when strong anion exchange chromatography is employed, and at least one hundred fold when capillary electrophoresis is used. The limiting factor here is the resolution for capillary electrophoresis and the level of detection for strong anion exchange chromatography. It is envisaged that comparable levels of detection could be obtained for SAX chromatography if laser induced fluorescence were used in preference to a standard (bulb) fluorimeter and could be applied to state of the art nano-HPLC systems. It is also foreseen that the resolution problem experienced when HPCE separation is employed is only a temporary obstacle (which could be overcome with further studies into the conditions of separation. This will be greatly aided by the many new separation modes being documented and the now routine use of derivatized capillaries to modify the flow conditions. The separation of structural isomers (especially chiral molecules) is becoming a rapidly advancing and well funded field of research

It is envisaged that the work encompassed in this chapter of the thesis will find significant use within the field in a wide range of applications. The chapter not only demonstrates improved coupling to a novel (in the GAG field) fluorophore, but also improved coupling conditions limiting chemical modifications and a new mode of catalysis for the generic reaction used at the reducing end.

6.2 Non -reducing end labelling of glycosaminoglycan fragments via the formation of mercury adducts

The ability to label the other terminus of the oligosaccharide has been demonstrated here and provides an alternative to reducing end labelling and the problems encountered with the sluggish carbonyl chemistry.

The formation of mercury adducts by the attack of carbon – carbon double bond (C=C) introduced via lyase digestion and evidence for the presence of a seemingly stable mercurinium ion has also been described within the thesis. The attachment of the mercury to the C=C double does not appear to cause the rearrangements suggested by Ludwigs *et al.* [141] which would lead to the degradation of the molecule. Controls and NMR studies both confirm that the molecule still possesses a reducing end carbonyl group which can be exploited for further reactions.

The ability to couple via the non-reducing end opens up many possibilities to the field. One such possibility is that of immobilisation via the non-reducing end utilising the known interaction of mercury with thiols. This has been illustrated by the immobilisation of heparin and heparan sulphate fragments to gold surfaces which have been pre-derivatized to contain a thiol over-layer. Binding to a heparin-binding protein, FGF2 has also been shown, which substantiates the immobilisation technique.

The use of gold surfaces is common place in the analytical sciences and this new method of labelling could allow for the attachment to many analytical instruments probing biological functions and binding events. The coupling of oligosaccharides to nanoparticles has also been outlined within this thesis and an ongoing collaboration with Dr Thanh is hoped to facilitate sub-attomole detection of these conjugates. The ability to use oligosaccharides to target nanoparticles also remains an attractive possibility.

The use of non-reducing end chemistries in high throughout analysis is also an attractive prospect. Micro arrays are currently being investigated to screen heparan sulphate oligosaccharide libraries for binding events against known proteins. The ability to couple via the reducing end might provide a higher yield and allow sample dependencies to be reduced. Attachment via this means also leaves a free reducing end which could be used to quantify the amount of saccharide deposited on the surface allowing for quality control and quantification, to ensure that fair screening of libraries is performed.

The attachment of the non-reducing end to fluorophores is also an attractive prospect as this could provide for higher yields. The NMR data suggests near quantitative labelling which would be a significant improvement. One problem which needs to be overcome with further investigation is that of the terminal mercury attacking the fluorophore's conjugated ring system hence leading to loss of fluorescence.

The nature of the oxymercuration reaction also need further investigation as it is hypothesised that this could be used as a means of introducing other functional groups onto the non-reducing end of the molecule. This could allow for combinatorial chemistry strategies to be employed which could lead to the creation of synthetic heparin / heparan sulphate analogues and libraries. These could have potential therapeutic use as well as being a tool for probing biological interactions. The ability to introduce alkyl chains at the reducing end (as can be done in classical oxymercuration) could be useful as this could permit the incorporation of carbohydrate chains artificially into membrane bi-layers.

The combination of both the reducing end and non-reducing end chemistries also opens up the possibility of dual-end labelling. Improvements in reducing end chemistry (chapter 3) and the development of non-reducing end techniques (chapter 4) has led to the proposal of a novel sequencing strategy (as outlined in the chapter discussion, 4.3.5) which incorporates both of these advances. The strategy could lead to a fast and routine approach to obtain sequence information for different heparin and heparan sulphate structures. The ability to obtain sequence information will allow the field to progress by enabling a deeper understanding of HS – protein interactions at the structure function level. The composition of HS can be investigated in different tissues, disease states, developmental states, organisms and in a response to many other stimuli to obtain a better understating of heparin and heparan sulphate function.

6.3 The inhibition of the rosetting phenomenon, implicated in cerebral malaria following *Plasmodium falciparum* infection, by heparin derivatives

The use of chemically modified heparin derivatives as potential therapeutics in the treatment of cerebral malaria caused by *Plasmodium falciparum* infection has been investigated. Heparin has been shown previously to inhibit rosetting and in this study the more favourable porcine mucosal form (which does not possess any of the known encephalopathy risks associated with bovine derivatives) is also shown to inhibit in the variant parasite strain R29. Furthermore, two chemically derived polysaccharides, and their oligosaccharides de-polymerisation products, have been shown to inhibit rosetting. Both of these chemically modified heparins possess minimal anticoagulant activity compared to heparin, (which is excluded as a drug candidate for this reason) and the 6-de-sulphated variety also possess a similar IC_{50} value in the rosetting assay ($\approx$ 120 nM) to that of heparin. These results were again obtained for the R29 strain and are in contrast to the results of Barragan *et al.* [215] performed on a different strain variant.

The results presented in this thesis imply that the ability to disrupt rosettes is both strain and heparin / heparan sulphate structure specific. Further investigations need to be carried out on more *Plasmodium falciparum* rosetting strains (both laboratory and field) to obtain the structural requirements required for disruption. This would be a major undertaking and could not feasibly be done in a reasonable time period by counting cells (by eye) under a fluorescent microscope.

One solution could be the use of fluorescence assisted cell sorting (FACS) analysis which could separate unbound infected erythrocytes from rosettes based on size using a fluorescent nuclear stain. This, if possible due to safety considerations, could allow for the high throughput screening of various *Plasmodium falciparum* strains.

While the structural requirements needed in the development of any potential therapeutic are essential, it should also be remembered that the primary structure and the overall conformation are important in the disruption potential, as can be seen from the heparin

polysaccharide and oligosaccharide. Further studies need to investigate other factors affecting the conformation of the molecule such as cation interactions, hydrogen bonding, steric restrictions, hydrophobic interactions and interactions with neighbouring proteins (which could alter the saccharide conformation). The use of NMR, synchrotron source and infrared circular dichroism (SCCD and IRCD) and Fourier-transform infrared (FTIR) spectroscopies are all currently being investigated as tools for probing conformational changes during protein – carbohydrate interactions. These methods will in the future be useful to observe the heparin (or chemically modified polysaccharide) – PfEMP-1 interactions.

The ability of heparin and chemically modified heparin saccharides to prevent the deposition of infected red blood cells to the endothelium is also actively being studied along with any anti-inflammatory role in TNF α and interferon δ mediated nitric oxide production. Again, these could provide supporting roles in their application and strengthen the case for the use of this class of molecules as novel therapeutics.

The method developments made within this thesis could aid in the attachment of heparin and chemically modified heparin saccharides to surfaces allowing for the interaction with PfEMP-1 to be probed using optical biosensors. The proposed dual end sequencing strategy could also be used to elucidate the sequence which is required to give the saccharide moiety its overall confirmation thereby dictating binding. Saccharides could be screen for interactions with PfEMP-1 by their selection using a heparin affinity column produced by non-reducing end matrix attachment.

Chapter 7

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Appendices

The polysaccharides were characterised by ^{1}H and ^{13}C NMR to confirm their structure as previously reported by *Yates et al.* NMR spectra were recorded in D_2O at 40 °C on a 400 MHz instrument. Assignment was facilitated using a combination of COSY, TOCSY, HMBC two-dimensional spectra. 150mg of polysaccharide sample were used in all ^{13}C spectra. Chemical shift values were recorded relative to the NMR standard trimethylsilyl propionate at 40 °C.

^{1}H and ^{13}C NMR chemical shift values for heparin derived polysaccharides.

	Glucosamine						Iduronate				
Polysaccharide	**A-1**	**A-2**	**A-3**	**A-4**	**A-5**	**A-6**	**I-1**	**I-2**	**I-3**	**I-4**	**I-5**
PMH	99.5	60.7	72.5	78.8	72.0	69.2	102.1	78.9	72.1	79.0	72.3
	5.42	3.31	3.69	3.79	4.05	4.30-4.42	5.23	4.37	4.22	4.14	4.82
Hep-NAc	96.6	56.2	73.0	79.3	72.3	69.6	102.2	76.8	67.3	74.2	70.8
	5.15	4.03	3.76	3.78	4.04	4.31-4.37	5.20	4.37	4.31	4.08	4.91
2-OH	98.1	60.3	72.4	80.1	71.5	68.7	104.6	71.1	70.4	77.2	71.2
	5.34	3.24	3.65	3.71	4.02	4.36 4.23	5.04	3.78	4.12	4.08	4.84
2-OH NAc	97.1	56.2	72.5	79.6	71.8	68.8	104.6	72.0	71.4	77.0	71.9
	5.18	4.00	3.78	3.79	4.08	4.37-4.26	5.01	3.75	3.42	4.10	4.78
6-OH	100.0	60.8	72.4	80.5	73.8	62.6	102.0	77.6	70.7	78.7	71.4
	5.31	3.27	3.71	3.70	3.89	3.86-3.88	5.26	4.35	4.25	4.06	4.84
6-OH NAc	96.8	56.6	72.9	80.6	74.2	62.9	102.3	76.6	67.1	74.1	70.6
	5.14	4.03	3.79	3.76	3.91	3.87-3.92	5.26	4.37	4.28	4.07	4.91
2 & 6-OH	98.2	60.5	72.5	80.2	73.5	62.4	104.3	72.2	71.5	77.8	72.2
	5.39	3.26	3.67	3.72	3.87	3.84-3.88	4.95	3.74	4.11	4.08	4.77
2 & 6-OH NAc	97.1	56.2	72.3	79.6	73.7	62.3	104.3	72.5	72.2	77.3	72.6
	5.18	3.97	3.76	3.74	3.89	3.85-3.88	4.92	3.69	3.89	4.07	4.73

The ^{1}H chemical shift values quoted for position-6 of glucosamine residues (A-6) are intervals. Signals from the carbonyl group of iduronate and acetyl CH_3 groups of *N*-acetylated glucosamine derivatives are not shown.

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