



U N I V E R S I T Y O F
LIVERPOOL

Institute of Systems, Molecular and Integrative Biology

**Heparin-based Analogues and
The Control of Vascular Proliferation**

Thesis submitted in accordance with the requirements of the
University of Liverpool for the degree of Doctor in the Philosophy
(Ph.D.)

by

Kai-Wen Wang

03/2021

Abstract

Heparin-based Analogues and The Control of Vascular Proliferation

Kai-Wen Wang

Stent insertion into disease-narrowed arteries often damages blood vessels and triggers vascular smooth muscle (VSM) proliferation as a healing response, re-blocking arteries. To combat this, anti-proliferative agents can be incorporated into stents, but these delay healing, inhibit endothelial cell (EC) re-growth and promote thrombosis. This study aims to identify novel heparin-based analogues that inhibit VSM proliferation, promote EC proliferation and retain anti-thrombotic activity. In MTT-assays, incubation with naive heparin complexed with either Na⁺, K⁺, Mg²⁺ or Ca²⁺ ions (10 µg/µl for 4 days in normal growth media, GM) significantly reduced human coronary artery smooth muscle cell (HCASMC) growth compared to incubation in GM alone ($P < 0.01$). The inhibitory effect ranged from $48 \pm 4.5\%$ (Na-heparin) to $28 \pm 13.7\%$ (Mg-heparin). Cation-complexed forms of normal heparin also reduced human coronary artery endothelial cell (HCAEC) number but to a lesser extent: $19 \pm 0.3\%$ (Na-heparin ($P < 0.01$)) to $5.0 \pm 3.5\%$ (Ca-heparin (not significant)). The ratio of HCAEC:HCASMC cell growth thus showed that Na-, K-, Mg- and Ca-heparin have the desired effect of suppressing HCASMC proliferation, while having minimal effect on HCAECs. Partially or fully-desulphated heparin analogues complexed with either Na⁺, K⁺, Mg²⁺ or Ca²⁺ also showed promising activity profiles; partially-desulphated heparin 4 (predominant repeating structure; I₂S A^{60H}NAc) consistently being the best performing analogue across all cations. In transwell migration assays, cationic desulphated heparin analogues significantly delayed HCASMC migration ($P < 0.01$), but had no significant effect on HCAECs. These effects were not due to induction of apoptosis and, importantly, all cationic desulphated heparins retained their differential effects on HCASMC/HCAECs in dual culture systems. Heparins with appropriate activity profiles were screened for their effects on blood coagulation and ability to interact with platelet factor 4 (PF4), a key determinant of heparin-induced thrombocytopenia. As expected, heavily-desulphated cationic heparin analogues lost anti-coagulant activity. Native (non-denatured) gel electrophoresis followed by silver staining was used to visualise protein complexes formed through the interaction of heparin analogues and PF4. All cationic forms of heparin tested formed PF4/heparin complexes. Mechanistically, differential effects of heparin analogues likely result from differences in growth factor (GF) signalling. In RT-PCR screens, transcripts for platelet-derived growth factor receptors, PDGFRA/B, were expressed only in HCASMCs. By growing HCASMCs in different growth factors, however, we determined that signalling via PDGFRs is not the primary cause of the differential heparin effects. Here, the anti-proliferative effects of heparin analogues were maintained, or enhanced, in media containing only fibroblast growth factor (FGF2) or epidermal growth factor (EGF), while anti-proliferative activity was decreased in media supplemented with predominantly PDGF. To elucidate differential GF signalling, heparin analogue-treated HCASMC lysates were immunoblotted with anti-phosphotyrosine antibodies. Here, although immunoreactive band 'fingerprints' associated with EGFR activation were identified, no clear banding pattern differences were observed in cells treated with different heparin analogues. Ultimately, more sophisticated proteomic analysis will be required, but the differential effects on HCASMCs/HCAECs likely represents differences in signalling downstream of receptor activation, with candidate pathways activated by FGFs and EGF. In conclusion, to the best of our knowledge this is the first comprehensive analysis of the effects of heparin compounds on human vascular cells. Results highlight partially-desulphated, Na-heparin 4 and Ca-heparin 4, as potential lead analogues with promising activity profiles that may ultimately form the basis for novel, next-generation stent coats.

Key Words: heparin, human coronary arteries endothelial cells (HCAECs), human coronary arteries smooth muscle cells (HCASMCs)

Acknowledgements

Firstly, I would like to express my gratitude and appreciation for my supervisors, **Prof. Caroline Dart** and **Dr. Ed Yates**, who contributed their support and guidance during this research and without whom I would not have been able to complete my thesis.

I would like to show my sincere thanks to my assessors, **Prof. David Fernig** and **Dr. Bettina Wilm** for their insightful comments and suggestions during my four-year study.

I would also like to extend my special thanks to **Mrs Patricia Procter** from University of Keele for her contribution of coagulation assay.

I am grateful for our **Laboratory Technician Team** who always provided technical supports and advises when I needed them.

I would also like to thank **everyone in the lab** for sharing their experiences and suggestions on experiments.

In addition to professional support, I am deeply grateful to **my family** who contributed their financial support and encourages during my study. Furthermore, I also wish to thank **my friends and flatmates** for their company and emotional support, especially during the Covid-19 pandemic.

At last, I wish to thank **everyone** who inspires or encourages me in every moment.

Table of Contents

Abstract	1
Acknowledgements	3
Table of Contents	4
List of Figures	7
List of Tables	10
List of Abbreviations.....	11
Chapter 1: General Introduction	14
1.1 Arterial Structure	14
1.2 Background of Atherosclerosis.....	15
1.2.1 Mechanism of Atherosclerosis Development	15
1.2.2 Demography and Risk Factors	17
1.2.3 Therapies	19
1.3 Mechanisms of Restenosis	22
1.3.1 Injury and Pro-inflammatory Factors Lead to Restenosis	22
1.3.2 Cell Signalling Involved in Vascular Hyperplasia.....	22
1.3.3 The Interaction Between Heparin, Growth Factors and Growth Factor Receptors Modulates Cell Signalling	23
1.4 Stent Development	24
1.5 The Potential Role of Heparin in Treatment	25
1.6 Aims.....	27
Chapter 2: Materials and Methodology	28
2.1 Cell Culture	28
2.2 Heparin Analogue Preparation	28
2.3 MTT Assay.....	30
2.4 Western Blotting.....	30
2.5 Transwell Migration Assay	32

2.6	Cell Co-cultivation and Cell Sorting	33
2.7	Coagulation Assay	33
2.8	HIT Assay	33
2.9	Polymerase Chain Reaction (PCR)	34
2.10	Statistical Analysis	36
Chapter 3: Differential Activities of Heparin Analogues on hVSMC/hEC proliferation & migration		37
3.1	Introduction.....	37
3.1.1	Heparin Derivatives (Sulphated and Cation Forms) Affects Heparin-Protein Interaction	38
3.1.2	Modifications of Heparin Alter Its Activities.....	39
3.1.3	Apoptosis	41
3.1.4	Aim of This Chapter	44
3.2	Results	44
3.2.1	Desulphated Heparin Analogues Have Differential Effects on HCASMC/HCAEC Proliferation and Migration.....	44
3.2.2	The Effects of Cationic Heparin Analogues on HCASMC/HCAEC Proliferation and Migration	47
3.2.3	Cationic Heparin Analogues Showed Differential Effects on HCASMC/HCAEC Migration	50
3.2.4	Effects of Heparin Analogues in Co-cultures of hVSMC/hECs	51
3.2.5	Effects of Cationic Desulphated Heparin Analogues in VSMC/ECs.....	52
3.3	Discussion	57
Chapter 4: Effects of Heparin Analogues on Blood Coagulation		60
4.1	Introduction	60
4.1.1	Coagulation Cascade	61
4.1.2	Anticoagulant Activity of Heparin	65
4.1.3	The Influence of Modified Heparin Analogues on Antithrombin Binding	67
4.1.4	Evaluation of Coagulation	67
4.1.5	Side Effects of Heparin: Heparin-Induced Thrombocytopenia	68

4.1.6 Aim of This Chapter:	69
4.2 Results.....	70
4.2.1 Coagulant Activity – aPTT Assay	70
4.2.2 Coagulant Activity – PT Assay.....	73
4.2.3 HIT Assay	75
4.3 Discussion	77
Chapter 5: Elucidating Signalling Effects of Heparin Analogues on HCASMCs and HCAECs	84
5.1 Interaction Between Heparin and GF-GFRs.....	85
5.2 Cell Signalling in Proliferation.....	90
5.3 Results.....	96
5.3.1 GFRs in HCASMCs and HCAECs: Differential Expression	96
5.3.2 Proliferative Activity of Heparin is Affected by Different Growth Factors	97
5.3.3 Effect of Heparin Analogues on Signalling Pathways Downstream of GFRs ...	98
5.4 Discussion	100
Chapter 6: General Discussion.....	103
6.1 Summary of Novel Findings	103
6.2 Chemically Modified Forms of Heparin Have Differential Effects on VSMC and EC Proliferation	106
6.3 Modified Forms of Heparin Have Differential Effects on Blood Coagulation	108
6.4 The molecular basis of the differential effects of heparin analogues on HCASMCs/HCAECs	110
6.5 Limitations and Future Directions	111
Chapter 7: References	117
Appendix	135

List of Figures

Figure 1.1. Structure of arteries, veins and capillaries. Image is drawn by PowerPoint.	15
Figure 1.2. The key event in early atherosclerosis is damage to the endothelium.	16
Figure 1.3. Balloon angioplasty and stent implantation.	19
Figure 1.4. Scheme of neointima formation caused by blood vessel wall injury ..	22
Figure 1.5. Schematic of mitogenesis caused by thrombin and PDGF.....	23
Figure 1.6. Difference between heparin and heparan sulphate (HS).	26
Figure 2.1. Scheme of transwell migration assay.	32
Figure 3.1. Structural features of HS and heparin.	38
Figure 3.2. Scheme of the main apoptosis pathways.	42
Figure 3.3. Bid and Bax insert into membrane of mitochondria, resulting in apoptosis	43
Figure 3.4. Desulphated heparin analogues produced by Dr. Ed Yates	45
Figure 3.5. Effect of desulphated forms of heparin on HCASMC and HCAECs cell number.....	46
Figure 3.6. The differential growth index for these analogues was calculated as mean EC numbers divided by mean SMC numbers for each analogue....	46
Figure 3.7. Effect of cation-forms of heparin on HCASMC and HCAECs cell number.....	47
Figure 3.8. The differential of growth index for cation forms of heparin for HCAECs and HCASMCs.....	48
Figure 3.9. Cation forms of heparin do not induce apoptosis in HCASMCs or HCAECs.	49
Figure 3.10. Boxplot diagrams for cell migration.	50
Figure 3.11. The growth ratio of HCAEC/HCASMCs in co-cultivation system.	51
Figure 3.12. Effect of cation-desulphated forms of heparin on HCASMC proliferation	52

Figure 3.13. Effect of cation-desulphated forms of heparin on HCAEC proliferation	53
Figure 3.14. The growth ratio of HCAEC/HCASMCs treated with cationic desulphated heparin analogues.	53
Figure 3.15. The EC/SMC ratio of cationic desulphated heparin analogues in co-cultivation.	54
Figure 3.16. Cation-desulphated heparin analogues significantly delay HCASMC migration but have no effect on HCAECs.	55
Figure 3.17. The cation-desulphated heparin candidates do not induce apoptosis in HCASMCs.	56
Figure 4.1. The key components of haemostasis.	61
Figure 4.2. Scheme of the coagulation cascade.	62
Figure 4.3. Conformational changes of antithrombin bound by heparin.	66
Figure 4.4. HIT is caused by IgG antibodies formed in response to complexes of PF4/heparin.	69
Figure 4.5. The anti-coagulant activity of cation-desulphated heparin analogues in aPTT assay.	73
Figure 4.6. The anti-coagulant activity of cation-desulphated heparin analogues in PT assay.	75
Figure 4.7. Silver stained native gels (A) and the densitometry of gels (B) showing the presence of heparin-PF4 complexes.	76
Figure 4.8. Potential regulatory roles of serine protease-heparin complexes in coagulation	79
Figure 4.9. Relative size of Na ⁺ , K ⁺ , Mg ²⁺ , Ca ²⁺ and their typical coordinations	82
Figure 5.1. The structure of FGFR and the scheme of FGF-FGFR-heparin complexes.	87
Figure 5.2. Scheme of human VEGFA isoforms depicted as monomers.	88
Figure 5.3. Basic structure of the transmembrane form of HB-EGF.	89
Figure 5.4. Signalling pathways in SMC proliferation during neointimal formation	92

Figure 5.5. Scheme of VEGF-VEGFR2-NRP1-HS-induced signalling in ECs	94
Figure 5.6. Scheme of Ang-1 and Ang-2 signalling pathways leading to EC quiescence and proliferation.	94
Figure 5.7. RT-PCR analysis of cDNA isolated from HCASMCs and HCAECs using primers against major growth factor receptors.....	96
Figure 5.8. Addition of different growth factors affects the action of cation forms of heparin on HCASMC proliferation	98
Figure 5.9. Immunoblot analysis using phospho-tyrosine-specific antibodies reveals little change in growth factor-stimulated signalling in HCASMCs in the presence of non-desulphated cation form heparin analogues. The cells were incubated with 10 µg/ml heparin analogues in GM for 10 min after one hour serum starvation.	99
Figure 5.10. Immunoblot analysis using phospho-tyrosine-specific antibodies reveals little change in growth factor-stimulated signalling in HCASMCs in the presence of cation-desulphated heparins.....	100
Figure 6.1. Schematic of Stents with Heparin Coatings.....	113
Appendix 2. Heparin-coated plate data.....	136
Appendix 3. Coated heparin lost in the washing procedure.	137
Appendix 4. The intact image of western blot shown in Figure 3.9	138
Appendix 5. The intact image of western blot shown in Figure 3.17	139
Appendix 6. Conformation of native antithrombin and active antithrombin binding with heparin.	140

List of Tables

Table 1.1. The risk factors of CVDs and the symptoms of hypertension.....	18
Table 1.2. Management before PCI.....	20
Table 1.3. Pharmatherapy after PCI	21
Table 2.1. Desulphated heparin analogues.	29
Table 2.2. The details of antibodies.....	31
Table 2.3. The list of the primers used in this project.....	35
Table 3.1. Summary of the capacity of heparin cation form to prevent neointimal formation.....	57
Table 4.1. Information of clotting factors and proteins	64
Table 4.2. Desulphated heparin analogues	71
Table 4.3. Summary of the results in aPTT and PT assay.....	78
Table 4.4. The interpretation of aPTT/PT results in clinical treatment.....	80
Table 4.5. Different LMWHs show diverse anti-Xa/XIIa ability	81
Table 5.1. The energy of non-covalent binding.....	85
Table 5.2. Essential O-sulphation for FGF binding	86
Table 5.3. Summary of the major roles of growth factor receptors in SMCs and ECs.	95
Table 6.1. The summary results of cell proliferative activity of desulphated heparin	104
Table 6.2. The summary results of cell proliferation, migration, apoptosis and anti-coagulant activity.	105
Table 6.3. List of methods for heparin quantification in collagen scaffolds.....	112
Table 6.4. Commercial heparin coating technologies	114
Appendix 1. The FACS raw data of HCASMC/HCAEC co-cultivation.	135

List of Abbreviations

2D, two-dimension

3D, three-dimension

Ang, angiopoietin

aPTT, activated partial thromboplastin time

Arg, arginine

AT, antithrombin

BSA, bovine serum albumin

cBid, cleaved Bid

CDK, cyclin dependent kinase

CHF, congestive heart failure

CHO, Chinese hamster ovary

CT, computed tomography

CVD, Cardiovascular disease

DES, drug-eluting stent

DR, death receptor

EC, endothelial cell

EGF, epidermal growth factor

EGFR, epidermal growth factor receptor

Eph, ephrin receptor tyrosine kinases

ER, endoplasmic reticulum

FACS, fluorescent activated cell sorting

FADD, FAS-associated death domain

FAK, focal adhesion kinase

FCS, fetal calf serum

FGF, fibroblast growth factor

FGFR, fibroblast growth factor receptor

GAG, glycosaminoglycan

GAPDH, glyceraldehyde 3-phosphate dehydrogenase

GM, growth media

GF, growth factor

GFR, growth factor receptor

GPCR, G protein-coupled receptor

HAEC, human aortic endothelial cell

HB-EGF, epidermal growth factor-like growth factor

HCASMC, human coronary artery smooth muscle cell

HCAEC, human coronary artery endothelial cell

HCII, heparin cofactor II

His, histidine

HIT, heparin-induced thrombocytopenia

HRP, horseradish peroxidase

HSPG, heparan sulphate proteoglycan

HS, heparan sulphate

IGFR, insulin-like growth factor receptor

IL-4, interleukin-4

LDL, low-density lipoprotein

LMWH, low molecular weight heparin

Lys, Lysine

M0, non-supplemented media

mTOR, mammalian target of rapamycin

MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

NICE, National Institute for Health and Care Excellence

NMR, nuclear magnetic resonance

NRP, neuropilin

PAR, protease activated receptor

PBS, phosphate buffered saline

PCI, percutaneous coronary intervention

PCR, polymerase chain reaction

PDGF, platelet derived growth factor

PDGFR, platelet derived growth factor receptor

PF4, platelet factor 4

PI3k, phosphoinositide-3 kinase

PLC γ , phospholipase C γ

PN, protease nexin

PT, prothrombin time

RCL, reactive centre loop

RSL, reactive site loop

RT-PCR, reverse transcription polymerase chain reaction

S.D., standard deviation

SDS, sodium dodecyl sulphate

SDS-PAGE, sodium dodecyl sulphate-polyacrylamide gel electrophoresis

α -SMA, α smooth muscle actin

SRCD, synchrotron radiation circular dichroism

STAT, signal transducer and activator of transcription

tBid, truncated Bid

TBST, Tris-buffered saline with Tween-20

TGF, transforming growth factor beta

TGFR, transforming growth factor beta receptor

Thr, threonine

TNF, tumour necrosis factor

TRADD, TNFR-associated death domain

TT, thrombin time

UFH, unfractionated heparin

VEGF, vascular endothelial growth factor

VEGFR, vascular endothelial growth factor receptor

VSMC vascular smooth muscle

ZPI, Z-dependent protease inhibitor

Chapter 1: General Introduction

1.1 Arterial Structure

The circulatory system is made up of the heart and a network of blood vessels including arteries, veins and capillaries, which deliver nutrients and oxygen to cells and tissues. Arteries comprise a single layer of endothelial cells (ECs) which form the tunica intima, a vascular smooth muscle (VSMC)-rich layer which forms the tunica media and an outer layer of connective tissue known as the tunica adventitia or tunica externa (**Figure 1.1**). In healthy vessels, VSMCs are quiescent, proliferate slowly and regulate blood flow by changing their degree of contraction (Izzard et al., 2002). The contractile activity of VSMCs is determined by autonomic nerve inputs, circulating hormones, local metabolites and haemodynamic forces, all of which act to sustain tissue perfusion and maintain normal blood pressure (Brozovich et al., 2016; Duncker and Bache, 2008). In response to vascular injury, VSMCs down-regulate the expression of genes encoding contractile proteins and up-regulate proteins involved in proliferation and migration (Garg et al., 2011a). This phenotypic plasticity is a normal part of wound healing, but vascular remodelling is also at the heart of the pathogenesis of several diseases, including atherosclerosis, pulmonary hypertension, the failure of bypass vein grafts, and restenosis after angioplasty (Frismantiene et al., 2018; Hedin et al., 2004).

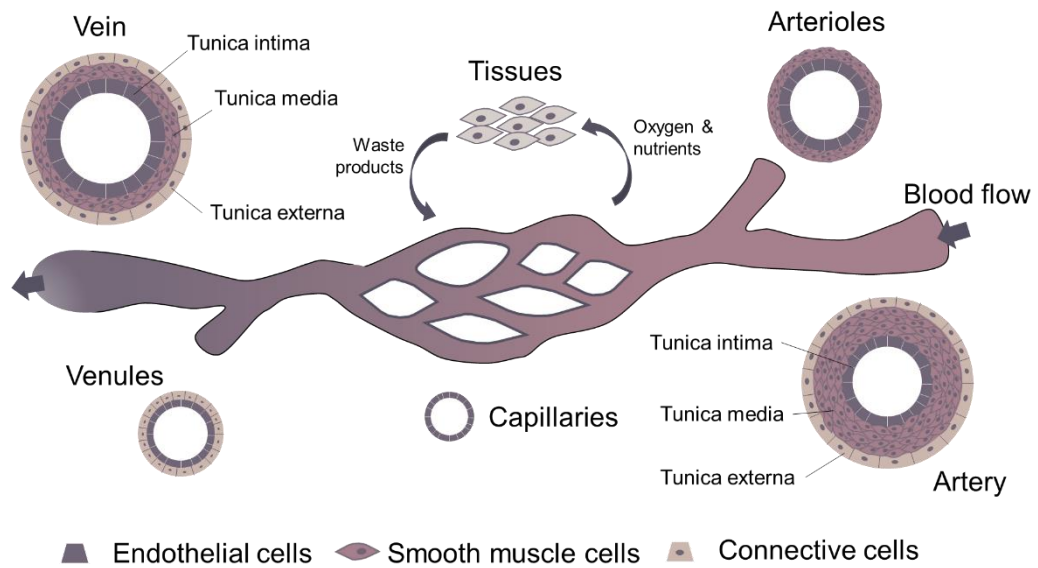


Figure 1.1. Structure of arteries, veins and capillaries. Image drawn with PowerPoint.

1.2 Background of Atherosclerosis

Atherosclerosis is a severe vascular disease that can result in stroke, ischaemic heart disease, and myocardial infarction. It is the leading cause of mortality worldwide (World Health Organisation, 2018).

1.2.1 Mechanism of Atherosclerosis Development

The development of atherosclerosis is slow, involving chronic inflammation and the build-up of lipid-laden plaques in arteries. The disease in its early stages is rarely detected, unless thrombosis caused by the erosion and/or rupture of plaque leads to blood clots and ischaemic tissue damage. An early event in the development of atherosclerosis is damage to the endothelial layer, caused by smoking, hypertension, or natural wear of the endothelial layer caused by blood turbulence, particularly at points where blood vessels branch (Davies, 2009; Kwak et al., 2014). As the endothelium becomes more permeable, it allows low-density lipoprotein (LDL) migration to the underlying layer. LDL particles trapped within the subendothelial space are modified, possibly by oxidation, and induce endothelial expression of adhesion molecules, which attract monocytes (Steinberg, 2009). Monocytes transform

to macrophages and engulf the modified LDL particles through scavenger receptors until the macrophages become overloaded with lipid and turn into 'foam cells'. Accumulations of dying foam cells release lipid that forms pools within the arterial wall, so-called 'fatty streaks' - yellow patches visible on the arterial wall and the first outward sign of atherosclerosis (Gisterå and Hansson, 2017; Park et al., 2009; Skålen et al., 2002). Further damage occurs when surrounding VSMCs and endothelial cells secrete a range of cytokines and growth factors, which induce VSMCs to switch from a contractile to a migratory proliferative phenotype. Migratory VSMCs form a fibrous cap over the lipid pool generating a mature atherosclerotic lesion (Schwartz et al., 2007). This fibrous cap stabilizes the plaque and provides a protective barrier between platelets in the blood stream and prothrombotic materials in plaque. Plaques can remain stable for many years causing no or few clinical symptoms (Virmani et al., 2002). Partial occlusion of arteries feeding the heart muscle can lead to angina pectoris, a condition whereby blood/oxygen delivery is sufficient at rest, but inadequate during moderate exercise, leading to exercised-induced chest pain. However, over-developed atherosclerotic lesions can lead to acute vascular disease. The main characteristics of these 'vulnerable plaques' include high levels of necrosis in the intima, thinning of the fibrous cap and an elevated inflammatory state. This can lead to a breakdown of the cap, resulting in platelet activation and acute luminal thrombosis (Moore and Tabas, 2011). Depending on the location and severity of the arterial occlusion, this can lead to myocardial infarction or stroke.

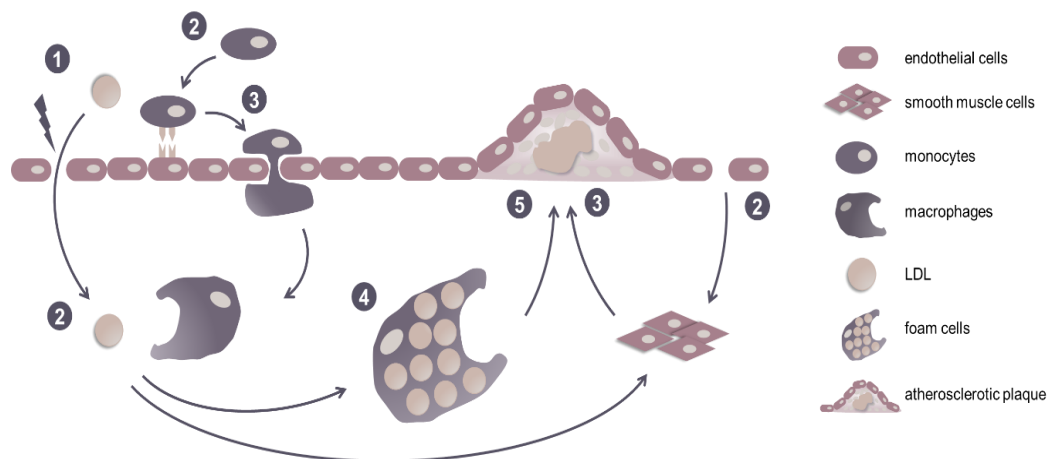


Figure 1.2. The key event in early atherosclerosis is damage to the endothelium. When the endothelium becomes more permeable, it allows LDL migration to the underlying layer. These modified LDL particles induce endothelial expression of adhesion molecules which attract monocytes. Invading monocytes transform to macrophages and ingest modified LDL particles, ultimately becoming overloaded with lipid and turning into 'foam cells'. At the same time, the damage also stimulates surrounding VSMC proliferation and migration. Image drawn with PowerPoint.

1.2.2 Demography and Risk Factors

Cardiovascular diseases (CVDs) are the most common cause of death globally. According to statistics from the World Health Organisation in 2016 (World Health Organisation, 2018), there were nearly 17.9 million deaths from CVDs, representing 31% of all global deaths. Of these, ischaemic heart disease and stroke are the main killers. These diseases have remained the leading cause of death in the last decades (World Health Organisation, 2018). There are a number of risk factors leading to cardiovascular diseases. According to the World Health Organisation, hypertension is the most important risks (**Table 1.1**). The morbidity rate is raised by merging with other risk factors.

Table 1.1. The risk factors of CVDs and the symptoms of hypertension (World Health Organisation, 2018).

Risk Factors of CVDs	Target-organ Damage	Symptoms with Hypertension
I. Classic factors: <u>Hypertension:</u> 55 y men or 65 y women <u>Cigarette smoking:</u> Cholesterol in blood > 6.5 mmol/l (250 mg/dl) <u>Diabetes:</u> Familial vascular diseases II. Other factors: HDL decreases LDL increases Diabetes with proteinuria Glucose intolerance decrease Overweight Long-time-sitting lifestyle Fibrinogen increase	Left ventricular hypertrophy Proteinuria/slight renal failure (Creatinine conc 1.2-2.0 mg/dl) Atherosclerosis discover Entire/partial retinopathy	<u>I. Cerebrovascular disease:</u> Ischemic stroke transient cerebral ischemia Cerebral haemorrhage <u>II. Cardiac disease:</u> Infraction Angina pectoris Coronary bypass surgery Congestive heart failure <u>III. Renal disease:</u> Diabetic nephropathy Renal failure Creatinine conc > 2.0 mg/dl <u>IV. Vascular disease:</u> Aortic aneurysm Symptoms of artery diseases <u>V. Hypertensive retinopathy:</u> Retinal haemorrhage papilledema

1.2.3 Therapies

There are several treatments to combat the symptoms of atherosclerosis. Balloon angioplasty followed by stent implantation (**Figure 1.3**) is used widely to improve the size of the vessel lumen at the site of the lesion. A folding stent is inserted into the vessel with a balloon, followed by inflation of the balloon to unfold the stent. The stent is left within the blood vessel to maintain the lumen size (**Section 1.3**). There are also some drug-combined treatments before/after surgery. Currently, the most commonly used drugs can be divided into anti-ischaemic, anti-thrombotic and lipid-lowering drugs. In anti-ischaemic therapy, carvedilol and Ramipril are used to improve the oxygen supply-demand interaction (Koepfli et al., 2004) by decreasing heart rate and myocardial contractility, and reduce the risk of congestive heart failure (CHF) by suppressing ventricular remodelling (Yousef et al., 2000). Antiplatelet and anticoagulant drugs are used to prevent thrombosis after percutaneous coronary intervention (PCI), such as aspirin and Fondaparinux (the pentasaccharide sequence derived originally from heparin) (Roffi et al., 2016). Lipid-lowering treatments (generally with statins) are applied as a long-term management strategy in order to reduce the patients' low-density-lipoprotein (LDL) cholesterol and thereby decrease the risk of development of further atheromus (Baigent et al., 2011; Cannon et al., 2015).

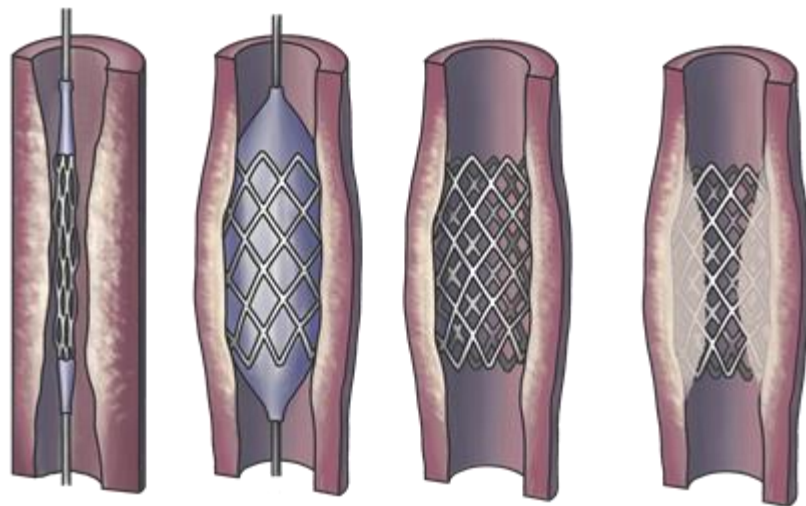


Figure 1.3. Balloon angioplasty and stent implantation. The folding stent is inserted into the vessel with a balloon. The stent is unfolded by the inflation of the balloon and is left at the site to maintain the shape of vessel. This procedure, however, unavoidably injures the inner surface of the blood vessel wall which leads to restenosis. Modified from Nucleus Medical Media 2015 (<http://www.scvc.com.au/coronary-angioplasty-and-stents-via-radial-artery.html>). Images drawn with Procreate.

Table 1.2. Management before PCI (Kasper et al., 2015).

Drug category	Application	Mechanism	Dosage	Limitation
Anti-ischemic therapy				
Nitrates	Administer sublingually Intravenous When symptoms sustained	Reduce myocardial wall tension and oxygen requirement	0.3-0.6 mg/5 min × 3 times (sublingual) 5-10 µg/min continuous infusion	Severe hypotension Avoid sildenafil or other PDE-5 inhibitor
Beta blockers	Unstable angina Stroke volume ↓ Heart rate ↓	Decreases adrenergic-activated heart rate and blood pressure to reduce myocardial oxygen demand	Metoprolol: 5 mg/5 min x 3 times (intravenous) 25-50 mg/6 h (oral)	Asthma 2° or 3° atrioventricular block Low heart rate (< 60 beats/min) Left ventricular failure
Morphine sulphate	Pain relief Symptoms persist after anti-ischemic therapy	Effective analgesic for pain	2-5 mg/ 5 min	Hypotension Bradycardia Respiratory depression
Anti-thrombotic Therapy				
Aspirin	Anti-platelet	Irreversible inhibit platelet activation	Initial 325 mg nonenteric formulation followed by 75-100 mg/d of an enteric or a nonenteric formulation	Intestinal bleeding
Clopidogrel	Anti-platelet	Prevents ADP induced platelet aggregation	Loading dose of 300-600 mg followed by 75 mg/d	Intestinal bleeding (rare)
Heparin	Anti-coagulant	Prevent thrombus formation	weight-adjust manner, Usually 100 IU/kg or around 50-60 IU/kg if GP IIb/IIIa receptor inhibitor is given	Haemorrhage, bruising, thrombocytopenia (rare)
Fondaparinux	Anti-coagulant	Inhibit the activation of factor Xa	2.5 mg/day subcutaneous	
General therapy				
Oxygen	Decrease oxygen demand Prevent hypoxemia			

PDE, phosphodiesterase; ADP, adenosine diphosphate

Table 1.3. Pharmatherapy after PCI (Kasper et al., 2015).

ARB, Angiotensin II receptor blockers; ACEI, angiotensin-converting enzyme inhibitor; EF, ejection fraction; HF, heart failure.

Drug category	Application	Dosage	Limitation
Anti-ischemic therapy			
ACEI	Ventricular remodelling ↓ Risk of CHF ↓ *Additive benefits with beta blockers	Ramipril: 2.5 mg twice daily	Hyperkalemia ACEI intolerant Acute kidney failure Bilateral renal artery stenosis
Beta blockers	Unstable angina	Carvedilol: 6.25 mg twice daily	Asthma Low cardiac output Left ventricular failure
ARB	Ventricular remodelling ↓ Risk of CHF ↓ Cooperation with ACEI		Renal Dysfunction Hyperkalemia
Anti-thrombotic Therapy			
Aspirin		Chewed tablet 81 mg	
Clopidogrel		75 mg/day for 1 year	
Lipid Lowering Drug			
Statin	Liver cell LDL ↓ Liver cell LDL receptor ↑ Blood LDL ↓		

1.3 Mechanisms of Restenosis

1.3.1 Injury and Pro-inflammatory Factors Lead to Restenosis

Balloon angioplasty followed by stent implantation is used clinically to improve the size of the vessel lumen in patients who suffer from coronary artery disease. However, following surgery 30-40% of patients are diagnosed with restenosis, the re-blocking of the artery due to cell overgrowth or, blood clots (thrombosis) at the site of the stent implantation (Alfonso et al., 2003). Thrombosis occurs either early (within days/weeks of surgery) due to surgery-induced arterial injury, or late (>30 days after surgery) due to interaction between the blood and the stent. Restenosis is mainly caused by over-proliferation of vascular smooth muscle cells (VSMCs) triggered by arterial injury.

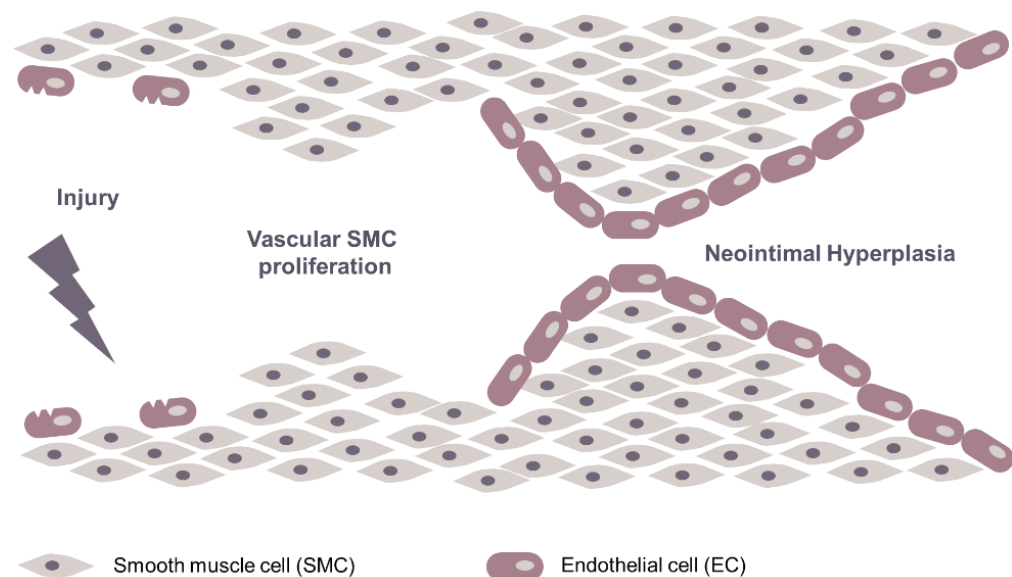


Figure 1.4. Scheme of neointima formation caused by blood vessel wall injury. The injury stimulates vascular smooth muscle cells (VSMCs) proliferation, resulting in neointimal hyperplasia. Modified from (Carpenter and Schoenfisch, 2012). Image drawn with PowerPoint.

1.3.2 Cell Signalling Involved in Vascular Hyperplasia

Tissue injury induced by stent implantation causes platelet activation and aggregation at the site of damage. The formation of neointima and the proliferation of VSMCs is thought to occur due to c-fos expression, a pro-oncogene which promotes cell growth (Indolfi et al., 1995) (**Figure 1.5**). c-fos itself is upregulated in response to thrombin, platelet derived growth factor (PDGF) and fibroblast growth factor -2 (FGF2) (Fager,

1995). Thrombin, which is formed following tissue injury, is involved in the coagulation cascade, and also activates signalling that can change the phenotypes of platelets, ECs and other immune cells, resulting in cell migration, angiogenesis, and haemostasis (Minami et al., 2004; R Isenovic et al., 2010). Thrombin stimulates platelets and VSMCs to produce PDGF (Bitto et al., 2018; Monje et al., 2003; Tsai et al., 2012), and endothelial cells to produce both PDGF and FGF2 (Fager, 1995) by binding to protease activated receptors (PARs). Growth factor stimulated over-growth of VSMCs causes the stent to be buried within the vessel wall and consequently leads to re-narrowing of the lumen of the blood vessel (Farb, 2002; Indolfi et al., 2000).

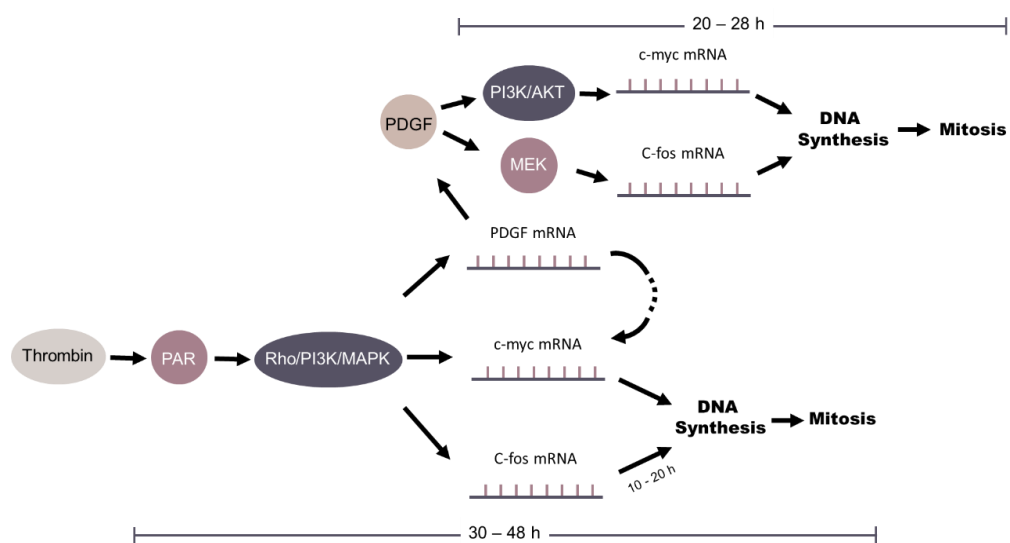


Figure 1.5. Schematic of mitogenesis caused by thrombin and PDGF. PDGF induces cell proliferation. When PDGF is absent, thrombin can promote the expression of endogenous PDGF. (Bitto et al., 2018; Fager, 1995; Monje et al., 2003) by binding to PAR1, PAR2 and PAR4 to activate the downstream signalling. However, heparin can block thrombin-induced PAR4 activation, leading to suppression of cell proliferation (Lin et al., 2019). PAR, protease activated receptors. Image drawn with PowerPoint.

1.3.3 The Interaction Between Heparin, Growth Factors and Growth Factor Receptors Modulates Cell Signalling

The heparin-GFs-GFRs complexes can activate cell signalling to mediate numerous biological events. Some growth factor receptors have been identified that bind to heparin/HS to regulate downstream signalling. FGFR1 and FGFR2, for example, have high affinity with FGFs. The binding of FGFs to FGFR1 or FGFR2 modulates SMC and EC proliferation via activation of the signalling, which can be adjusted by

interaction with heparin or HS (Yu et al., 2014). Therefore, FGF-FGFR-HS/heparin complexes are identified as key regulators in signal transduction (Pellegrini, 2001; Pellegrini et al., 2000; Pomin, 2016; Schlessinger et al., 2000). However, their interaction/affinity can vary as a consequence of different conformations of complexes themselves. Different members of the various FGF families possess different combinations of binding domains, altering their affinities with heparin and leading to distinct complexes (Li et al., 2016; Ori et al., 2008; Xu et al., 2012). In addition, different GAG structures can also induce different GF-GFR complex formation (Guimond et al., 2009). These different FGF-FGFR-HS/heparin complex structures bring to various biological responses – suppression or enhancement of proliferation and differentiation (Ori et al., 2008; Pellegrini, 2001; Pellegrini et al., 2000) (more information in **Chapter 5**). The structure can also be altered by charge distribution of heparin derivatives. For instance, removal of 6-O sulphate groups from the glucosamine residues of heparin interrupts the interactions between heparin and FGF2 (Sugaya et al., 2008), and also, 2-O-desulphated heparin showed low affinity for VEGF (Roy et al., 2011) compared to intact heparin. Some papers report that the anti-proliferative activity is less dependent on the affinity of heparin binding to growth factors (Letourneur et al., 1995), however, heparin binding to SMC surface is required to inhibit SMC proliferation.

1.4 Stent Development

A number of studies have demonstrated that the delivery of anti-proliferative drugs at the arterial injury site inhibits restenosis (Khan et al., 2014; Wykrzykowska et al., 2009). The first generation of drug-eluting stents (DESs) were made up of a mainly stainless steel platform with a slotted-tube appearance. These stents are covered with sirolimus (rapamycin), which inhibits proliferation by blocking the mammalian target of rapamycin (mTOR), and paclitaxel, which prevents disassembly of microtubules and thus disrupts cell division (Khan et al., 2014). Second generation DESs were coated with newer rapamycin derivatives (zotarolimus or everolimus) on a cobalt-chromium platform, which caused less arterial injury due to thinner struts (Akin et al., 2011). Third generation DESs were an extension of the second generation with the platinum-chromium platform covered in biodegradable polymers as the drug/polymer coating was believed to more efficiently control long-term drug release (Kereiakes et al., 2011). The fourth generation DES, a fully erodible stent consisting of bioabsorbable and polymer-free agent, has been developed, however, there is not enough evidence regarding its safety or efficiency (Khan et al., 2014).

Stent coating materials with anticoagulant activity can avoid intrinsic coagulation by inactivating platelets (Ollivier et al., 2016), and the materials with antiproliferative

activity can provide continued suppression of VSMC proliferation in long-term treatment (Khan et al., 2014). Despite DESs significantly decreasing the occurrence of in-stent stenosis, the risks of stent failure are still relatively high because of the delay of re-endothelialisation (Polyak et al., 2016), which leads to thrombosis. When a stent is not covered by endothelial cells, the flow turbulence at the edge of the stent drives thrombus formation. Thus, early thrombus formation, immediately following surgery, is due to vascular injury with fibrin and platelet deposition, whereas the late in-stent thrombosis (months to years after implantation) is due to delayed re-endothelialisation and vessel wall healing caused by coating drugs with anti-proliferative and/or pro-inflammatory activity (Nakazawa et al., 2008). Nonetheless, some studies have shown that drugs, such as the widely used anticoagulant agent heparin and also vitamin K antagonists, can efficiently prevent thrombus formation and reduce thrombosis (Hemker and Beguin, 1991). Indeed in multicentre, randomized human trials (BENESTENT II and MENTOR), heparin-coated stents significantly reduced stent thrombosis, but had no measurable effect on VSMC proliferation or restenosis (Serruys et al., 1998; Vrolix et al., 2000). However, subtle changes in the structure of heparin can significantly modify its activity, for example, increasing/improving its anti-proliferative activity while maintaining or reducing effects on coagulation (Chung et al., 2015a; Duckworth et al., 2015).

1.5 The Potential Role of Heparin in Treatment

The polysaccharide heparin is a generally well-tolerated anti-coagulant that also inhibits VSMC proliferation (Khorana et al., 2003). Heparin, one of the members of the glycosaminoglycan (GAGs) family, is constituted by repeating disaccharide sequences of a uronic acid and a glucosamine (Garg et al., 2003). It is a close relative of heparan sulphate (HS), which is found on the surface of all eukaryotic cells and constitutes a major component of the extracellular matrix (see differences between heparin and HS in **Figure 1.6**). Both heparin and HS play important roles in wound healing process (Olczyk et al., 2015). These GAGs can interact with proteins and numerous binding ligands, such as growth factors, via their sulphated groups and glucuronic acid/iduronic acid residues (**Figure 1.7**) to regulate cell signalling and biological activities.

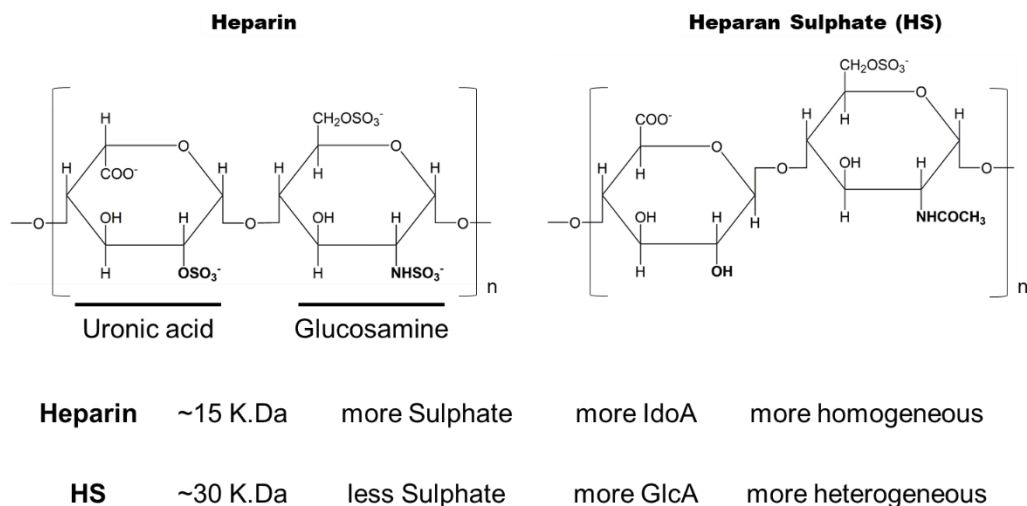


Figure 1.6. Difference between heparin and heparan sulphate (HS). The structure of heparin is greater homogeneous compared to HS. Heparin contains more N-sulphated groups and more IdoA residue than HS (Olczyk et al., 2015). Images are drawn with ChemDraw 18.1.

Cell surface HS interacts with growth factors, particularly fibroblast growth factors (FGFs), regulating binding specificity between FGFs and their receptors (Rifkin and Moscatelli, 1989). The ability of heparin to act as a proxy for HS underlies much of its activity, including its effects on cellular proliferation. Heparin has been reported to exhibit anti-proliferative activity efficiently on VSMCs both *in vitro* (Hoover et al., 1980) and *in vivo* (Guyton et al., 1980). This strong inhibitor of VSMC proliferation, delays the G1 phase through cAMP-induced repression of cyclin D1 and cyclin dependent kinase (CDK)-2 (Vadiveloo et al., 1997). In contrast, heparin promotes human aortic endothelial cell (HAEC) proliferation by modulating the response to vascular endothelial growth factor (VEGF) (Weatherford et al., 1996). Heparin is also a well-established anti-coagulant used in the treatment of thromboembolic diseases, because it can bind proteins of the blood clotting cascade to inhibit coagulation (Sydow-Plum and Tabrizian, 2008). With its anticoagulant properties, heparin has potential to be an attractive candidate for incorporation into stent materials. Indeed, in multicentre, randomized clinical trials, heparin-coated stents significantly reduced stent thrombosis, but showed no measurable effect on VSMC proliferation or restenosis (Babapulle and Eisenberg, 2002; Serruys et al., 1998; Vrolix et al., 2000). However, heparin-based analogues can be modified to minimize unwanted effects and maximize desired activities through chemical alteration (Yates et al., 2004). The structure of heparin can be changed by altering its charge. Heparin carries negatively charged sulphated groups that can readily interplay with those amino acids with positive charge on protein, such as lysine and arginine (Guimond et al., 2009; Rudd et al., 2007). When the negative charge is decreased, removal of sulphated groups

for example, the affinity with protein is also changed, as well as the structure of the complex, followed by the alteration of biological activities (Yates et al., 2004). For instance, the low sulphated heparin derivatives inhibit circulating galectin-3, which promotes cell proliferation and migration, but lose the capacity of anticoagulation (Duckworth et al., 2015). Heparin can sometimes cause severe immune responses, such as heparin-induced thrombocytopenia (HIT) which is a result of the interaction between heparin and platelet factor 4 (PF4) (Aster et al., 2009; Greinacher et al., 1994). This disease is rare, but with high mortality rate that increases the risks after heparin treatment. Therefore, the structure-modified heparin analogues may reduce the risk of side effects as the activity is changed. Several low molecule weight heparins have been developed to avoid HIT with their shortened sequences, which are unable or weakly bind to PF4 forming a huge complex (Rota et al., 2008). The conformational change of heparin can also decrease the risks of other side effects. LMWHs, such as Bemiparin and Nadroparin, are developed to prevent thromboembolism after surgery and blood clotting in haemodialysis (Chapman and Goa, 2003; Shafiq et al., 2006). These LMWH derivatives are commonly applied as the standard of care for the clinical management of venous thromboembolism with their strong inhibition of VSMC and thrombosis, but some of them also have several disadvantages (i.e. bleeding risks and clinical efficacy) (Jeske et al., 2011).

1.6 Aims

Since vascular disease affects 17.9 million people globally each year (World Health Organisation, 2018), it is essential to improve its treatment. Heparin is a widely-used anti-coagulant with anti-proliferative activity on VSMCs. Unfortunately, severe adverse events have been reported in some patients due to their damaged vascular wall or immune responses, resulting in restenosis, thrombosis and high risk of death. It has been established that heparin-based analogues can be modified to minimize unwanted effects and maximize desired activities through chemical alteration. The central aim of this project is to identify novel safe polysaccharides that:

- Inhibit human coronary artery smooth muscle cell (HCASMC) proliferation
- Promote human coronary artery endothelial cell (HCAEC) proliferation
- Have anti-thrombotic activity
- Avoid the side effects, for example, heparin-induced thrombocytopenia (HIT)
- Can be attached to stent materials and retain these activities

Chapter 2: Materials and Methodology

2.1 Cell Culture

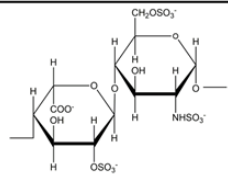
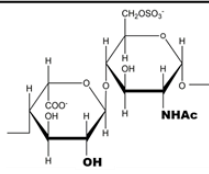
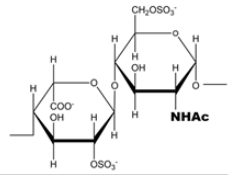
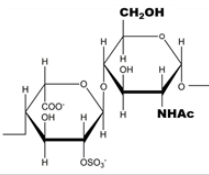
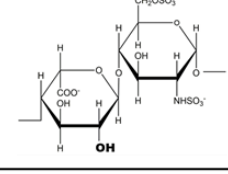
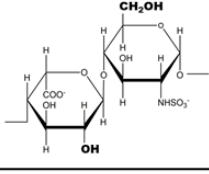
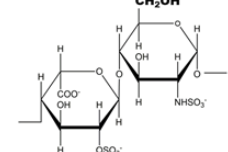
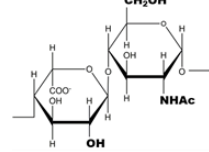
Human coronary artery smooth muscle cells (HCASMCs) and human coronary artery endothelial cells (HCAECs) (PromoCell, Heidelberg, Germany) were grown at 37 °C with 5% (v/v) CO₂. There were two types of media for each cell line: growth media (GM) and non-supplemented media (M0). The GM for HCASMCs (PromoCell) contained 5 % (v/v) fetal calf serum (FBS), 0.5 ng/ml epidermal growth factor, 2 ng/ml fibroblast growth factor-2 and 5 µg/ml insulin. The GM for HCAECs (PromoCell) contained 5% (v/v) FBS, 5 ng/ml epidermal growth factor, 10 ng/ml fibroblast growth factor-2, 20 ng/ml insulin-like growth factor, 0.5 ng/ml vascular endothelial growth factor 165, 1 µg/ml ascorbic acid, and 0.2 µg/ml hydrocortisone. The M0 is a naïve media without the supplements described above. All media included a 1% (v/v) penicillin/streptomycin solution to prevent bacterial contamination. The media was changed every two days during the culture period. When the density of cells reached 65% confluence, cells were resuspended with 0.25% (v/v) trypsin, diluted in fresh media and put into a new 75 cm² flask for further incubation.

2.2 Heparin Analogue Preparation

Porcine intestinal mucosal heparin (Celsus, Cincinnati, Ohio, USA). Although the detailed cation composition of the starting heparin was not provided by the manufacturer, it is reasonable to expect that the behaviour of heparin from different manufacturers will be broadly similar. The preference of various cations to bind heparin (from Sigma) has been studied. For example, using heparin in a common salt form (tris-ammonium), followed by equilibration with a number of salts of common physiological cations, and analysis using atomic absorption spectrometry (Stevic et al., 2011). This enabled the order of preference of the cations for heparin to be determined as: Mn(II) > Cu (II) > Ca > Zn > Co(II) > Na > Mg > Fe (III) > Ni > Al > Sr. These results highlight several important points: (1) it is very likely that heparin prior to treatment with cation exchange resin contained numerous physiological cations; (2) since the preference for Na ions is not as high as for Mn (II), Cu (II), Ca, Zn and Co(II), treating the heparin with sodium ion exchange resin is very likely to have removed a proportion of these cations from the heparin starting material. The starting heparin, although nominally in the sodium form (since its manufacture involves precipitation in sodium buffers) will therefore very likely have a cation composition distinct from that

of the sodium heparin that has been generated in this thesis by treatment with sodium ion exchange resin. In the work of Zhang et al., (Zhang et al., 2014), Celsus heparin was employed and they also demonstrated that different cation forms of this material exhibited distinct binding characteristics with FGF-1 and IL-7. Our analogues were designed and produced by Dr. Ed Yates (Institute of Systems, Molecular and Integrative Biology, University of Liverpool) according to literature methods and conformed to the expected structures by NMR (Yates et al., 1996). These derivatives 1-8 were sulphated or desulphated at different sites on heparin and these polysaccharides were labelled with numbers for blind tests.

Table 2.1. Desulphated heparin analogues.

Non - desulphated	1		Dual Desulphated	5	
	2			6	
Single Desulphated	3			7	
	4			8	
		Fully Desulphated			

To produce different cation forms of heparin, strong ion exchange beads (Alfa Aesar) were exchanged exhaustively with a 1M solution of NaCl, KCl, CaCl₂, MgCl₂, ZnCl₂, MnCl₂ or FeSO₄. The cation-loaded beads were then recovered by filtration, washed extensively with de-ionised water and added to 200 µl of 2 mg/ml heparin solution thereby transferring the cation to heparin. The liquid containing the cation form of heparin was then collected and was dried using a freeze dryer. Before use, the cation form of heparin was re-dissolved in deionized water and stored in the fridge.

2.3 MTT Assay

To investigate the effects of cation forms of heparin on proliferation, cell number was determined by an MTT assay. After a four-day incubation in the presence or absence of heparin analogues, 5 μ l of 5 mg/ml MTT reagent (Sigma-Aldrich) was added into each well in the 96-well plate and incubated at 37 °C in the dark for 4 h. Following the production of a purple precipitate, the 'stop solution' which contained 10% (w/v) sodium dodecyl sulphate (SDS) in 0.01M HCl was added into each well to terminate the MTT reaction. The cells were then left in the incubator overnight. The absorbance of each well was measured at 450 nm using a spectrophotometer.

2.4 Western Blotting

HCASMCs and HCAECs were incubated with growth medium in the presence or absence of heparin analogues (10 μ M final concentration). After 48 h treatment, cells were incubated for 10 minutes on ice with lysis buffer (250 mM NaCl, 3 mM EDTA, 3 mM EGTA and 0.5% (v/v) Triton X100 in 1M Tris-HCl, pH 7.6) containing 1% (v/v) protease inhibitor cocktail (Sigma-Aldrich). The whole cell lysate was centrifuged at 15,000 x g for 10 mins at 4°C and the supernatant was then transferred to a new tube and mixed with 4x SDS-sample buffer (40% (v/v) glycerol, 240 mM Tris/HCl pH 6.8, 8% (w/v) SDS, 0.04% (v/v) bromophenol blue, 5% (v/v) beta-mercaptoethanol). The mixture was heated at 95 °C for 10 mins. The cooled lysate was next loaded at 25 μ g total protein/well onto polyacrylamide gels and proteins within the lysate separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) at 130 V for 2 h. Separated proteins were transferred electrophoretically onto nitrocellulose membranes (Hybond ECL, GE Healthcare) at 350 mA for 1 hr on ice in transfer buffer (25 mM Tris-Base, 192 mM glycine, 20% (v/v) methanol). Subsequently, the membrane was blocked in 10% (w/v) skimmed milk or 5% (w/v) bovine serum albumin (BSA) in Tris-buffered saline with Tween-20 (TBST: 20 mM Tris-HCl, 137 mM NaCl, 0.1% (v/v) Tween 20, pH 7.6) solution at 4 °C overnight or ~2 hrs at room temperature. Following incubation with primary antibodies (anti-caspase-3, anti-cleaved caspase-3, anti-p-tyrosine and anti-GAPDH, see details in **Table 2.2**) in TBST at 4 °C overnight, the membrane was washed 3 times with TBST solution for 10 min each time. This was followed by incubation with horseradish peroxidase (HRP)-conjugated secondary antibody (1:5000, v/v) at room temperature for 1 h. Secondaries used were either anti-mouse IgG (H+L) HRP-conjugated polyclonal

antibody (Strattech Scientific Ltd., Newmarket, U.K) or anti-rabbit IgG (H+L) HRP-conjugated polyclonal antibody (Cell signaling Technology). Membranes were subsequently washed three times for 10 m each time in TBST. Protein bands were visualised by addition of ECL Western Blotting Detection Reagent (GE Healthcare) and exposure to light-sensitive Hyperfilm (GE Healthcare).

Table 2.2. The details of antibodies.

Antibody	Dilution	Description	Manufacturer	Cat. No.
For Western Blotting				
Anti-caspase-3	1:500	Monoclonal	Cell Signaling Technology	#9662
Anti-cleaved caspase-3	1:1000	Monoclonal	Cell Signaling Technology	#9664
Anti-p-Tyrosine	1:1000	Monoclonal (mix)	Cell Signaling Technology	#8954
Anti-GAPDH	1:3000	Monoclonal	Cell Signaling Technology	#5174
Anti-rabbit IgG HRP	1:5000	Polyclonal	Cell Signaling Technology	#7074
Anti-mouse IgG HRP	1:5000	Polyclonal	Strattech Scientific Ltd.	GMIG-001-SSL
For FACS				
Anti-CD31	1:50	Monoclonal	abcam	ab9498
Anti-α-SMA	1:50	Monoclonal	abcam	ab32575
Anti-rabbit IgG (Alexa Fluor® 488)	1:2000	Polyclonal	abcam	ab150077
Anti-mouse IgG (Alexa Fluor® 647)	1:2000	Polyclonal	abcam	ab150107

2.5 Transwell Migration Assay

HCASMCs or HCAECs were grown in the upper chamber of a transwell plate (VWR International) with 300 μ l of serum-free medium (M0 media) (**Figure 2.1**). The lower chamber was filled with supplemented growth media in the presence or absence of 10 μ m/ml of a cation form of heparin. After incubation at 37 °C for 24 h, the upper chambers were separated from the lower chambers and all of the media was removed. The upper chamber was cleaned with cotton buds to remove excess cells that did not migrate through the pores on the membrane. The membrane on the upper chamber was then immersed in solution A supplied by a Quickstain Kit (GE Healthcare Life Sciences). The chamber (membrane) was subsequently immersed into B solution (pink stain) for 3 mins and transferred to C solution (blue stain) for a further 3 mins incubation. Migrated cells were counted on the lower chamber side of the membrane under a phase contrast microscope.

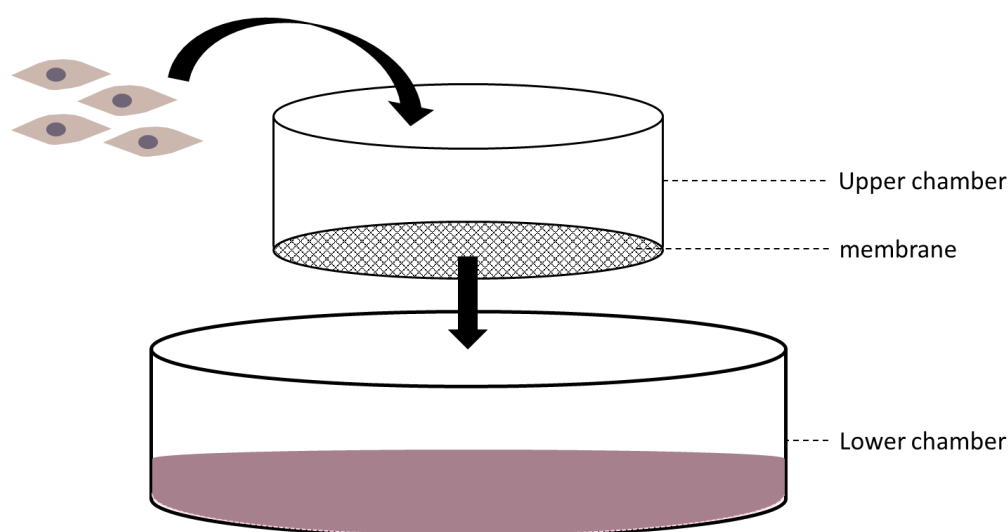


Figure 2.1. Scheme of transwell migration assay. The cells are seeded into the upper chamber in non-serum media while the heparin-containing growth media was placed in the lower chamber. Cells migrated through the membrane separating the two chambers and were counted on the lower chamber-side of the membrane. Image drawn with PowerPoint.

2.6 Cell Co-cultivation and Cell Sorting

HCASMCs and HCAECs were incubated together in 10 cm dish at 37 °C for 24 h. The co-cultivation media was a mix of HCASMC growth media and HCAEC growth media (1:1 v/v). After incubation, the media was replaced by fresh co-cultivation media with 100 µg/ml of heparin analogues and incubated for 5 days. The cells were re-suspended and centrifuged at 300 xg for 3 min to remove the old medium. After washing with phosphate buffered saline (PBS), the cells were re-suspended in 0.5 % (w/v) paraformaldehyde solution in PBS and incubated at room temperature for 20 min in dark. The paraformaldehyde was removed and the cells were washed by 0.05 % (v/v) Tween-20 in PBS with centrifugation 3 times at 300 xg at 4 °C for 5 min. The cells were re-suspended in ice-cold PBS containing 10% (v/v) FCS and 1% (w/v) sodium azide and a 1:50 dilution (v/v) of the primary antibodies (see **Table 2.2**), anti-CD31 (an endothelial cell marker) and anti- α -smooth muscle actin (α -SMA, a smooth muscle marker) at room temperature for 1 h in dark. Subsequently, the cells were washed 3 times by centrifugation at 300 xg at 4 °C for 5 m in ice cold PBS. Cells were then incubated with secondary antibody (1:2000 v/v) (see **Table 2.2**) in 3 % BSA/PBS for 30 m at 4 °C in dark. The cells were washed 3 times again by centrifugation at 300 xg at 4 °C for 5 m in ice-cold PBS. Before analysis, the cells were re-suspended immediately in 1 ml of ice cold PBS with 3 % (w/v) BSA and 1 % (w/v) sodium azide in dark. These samples were analysed by Fluorescent Activated Cell Sorting (FACS) using BD FACSCanto™ II Flow Cytometer with BD FACSDiva™ software.

2.7 Coagulation Assay

The effects of the heparin-based polysaccharides on the intrinsic or extrinsic coagulation pathways was assessed by determining the activated partial thromboplastin time (aPTT) and prothrombin time (PT) respectively. This analysis was carried out by Mrs Patricia Procter at the University of Keele.

2.8 HIT Assay

50 ng of cationic heparin analogues (Na^+ , K^+ , Mg^{2+} and Ca^{2+}) or 50 ng unmodified heparin (positive control) were treated with 250 ng of recombinant platelet factor 4 (PF4) protein (ABCam) at 37°C for 4 hours before electrophoresis. The heparin-PF4

complexes was analysed by native (non-denatured) gel electrophoresis. The native gel was formed by stacking 5%, 10% and 20% (w/v) acrylamide (Sigma-Aldrich) gel, followed by electrophoresis at 30 V (~25 mA) for 2 hours on ice. Following electrophoresis, protein complexes of PF4 were visualised by silver staining using a silver staining kit (Sigma-Aldrich) and following manufacturer's instructions.

2.9 Polymerase Chain Reaction (PCR)

Total RNA was extracted from HCASMCs and HCAECs using an RNeasy Mini Kit (Qiagen) according to manufacturer's protocol. Total RNA was treated with DNase I by incubating 8 µl total RNA, 1 µl 10x DNase I buffer and 1µl DNase I (1 U/µl; Invitrogen) at room temperature for 15 minutes. 1 µl EDTA (25 mM) was then added and the reaction heated at 65°C for 10 minutes. First strand cDNA was synthesized using SuperScript® III reverse transcriptase (Invitrogen) according to the manufacturer's instructions.

Touchdown PCR was carried out using HotStarTaq Master Mix Plus (Qiagen), following manufacturer's instructions. Primers used to amplify growth factor receptors were either from published literature or designed using Primer3Web (<http://primer3.ut.ee/>) which calculated the GC content, melting temperature (T_m) and product size (**Table 2.3**). Each individual primer was then checked for secondary structure using NetPrimer (PremierBiosoft) (<http://www.premierbiosoft.com>). All primers were synthesized by Sigma-Aldrich. The PCR protocol involved an initial denaturation at 95 °C for 5 m, followed by denaturation at 94°C for 30 s, annealing at 69 °C to 50 °C (changing by 1 °C for each reaction cycle) for 30 s, extension at 72 °C for 1 m, and a final extension at 72°C for 10 m. The PCR products were electrophoresed on 3 % (w/v) agarose gels containing Midori Green (1:10,000 v/v; GC Biotech) for 1 h at 80V. Bands were excised under ultraviolet light and products purified using a QIAquick Gel Extraction kit (Qiagen) according to manufacturer's instructions. Products were verified by sequencing (GATC Biotech, Germany).

Table 2.3. The list of the primers used in this project. “Start” corresponds to the number of the base in the human mRNA; “Len” is the length in base pairs.

	<i>T_m</i> (°C)	<i>Sequence</i>	<i>Start</i>	<i>Len</i>	<i>Ref.</i>
<i>FGFR1</i>	57.7	5'- AGAGGACAATGTGATGAAGATA -3' (fwd)	1851	22	(Saucedo et al., 2015)
(128 bp)	64.8	5'- GGTCAAATAATGCCTCGGGT -3' (rev)	1978	20	
<i>FGFR2</i>	64.9	5' - AGGACGCTGGGGAATATACG -3' (fwd)	1183	20	
(219 bp)	64.8	5'- CTGGCTTCTTGGTCGTGTTC -3' (rev)	1401	20	
<i>FGFR3</i>	63.6	5'- AGCAGCTCACCTTCAAGGAC -3' (fwd)	1799	20	
(533 bp)	64.2	5'- CGACAGGTCCAGGTACTCGT -3' (rev)	2331	20	
<i>FGFR4</i>	73.0	5'- CGCGGCGTCCACCACATT -3' (fwd)	1958	18	(Saucedo et al., 2015)
(100 bp)	62.8	5'- GTGTGTACACCCGGTCAAAC -3' (rev)	2057	20	
<i>VEGFR1</i>	63.9	5'- GGAACAAGGCAAGAAACCAA -3' (fwd)	3096	20	(Fiedler et al., 2005)
(216 bp)	63.8	5'- CGATGAATGCACTTTCTGGA -3' (rev)	3311	20	
<i>VEGFR2</i>	63.9	5'- ATCCCTGTGGATCTGAAACG -3' (fwd)	2863	20	(Fiedler et al., 2005)
(196 bp)	63.9	5'- CCAAGAACTCCATGCCCTTA -3' (rev)	3058	20	
<i>VEGFR3</i>	67.5	5'- GCTGCTGGAGGAAAAGTCTG -3' (fwd)	2169	20	
(228 bp)	69.0	5'- AGGACCCAGAAGAAGACAGC -3' (rev)	2396	20	
<i>PDGFRA</i>	63.9	5'- ATCAATCAGCCCAGATGGAC -3' (fwd)	1817	20	(Chong et al., 2013)
(891 bp)	63.5	5'- TTCACGGGCAGAAAGGTACT -3' (rev)	2707	20	
<i>PDGFRB</i>	66.7	5'- GGCAAAAGGGACAAAGAGGG -3' (rev)	4974	20	
(164 bp)	63.3	5'- GCCACCTCTCACATCCTTCT -3' (rev)	5137	20	
<i>Tie1</i>	66.8	5'- GCCATGATCAAGAAGGACGG-3' (fwd)	2602	20	(Uchida et al., 2000)
(243 bp)	64.3	5'- GTTCTCTCCGACCAGCACAT-3' (Rev)	3006	20	

<i>Tie2</i>	51.6	5'- CCTTAGTGACATTCTTCC-3' (fwd)	2047	18	(Zhang et al., 2001)
(243 bp)	61.6	5'- GCAAAAATGTCCACCTGG-3' (Rev)	2289	18	
<i>TGFR1</i>	65.7	5'- CTTTGGACCCAGGAAACAGC -3' (fwd)	1745	20	
(168 bp)	63.7	5'- ATGATCTCCAGCACAGCAGA -3' (rev)	1912	20	
<i>TGFR2</i>	64.8	5'- TCCTTCAAGCAGACCGATGT -3' (fwd)	1656	20	
(241 bp)	61.3	5'- AGCACTCAGTCAACGTCTCA -3' (rev)	1896	20	
<i>TGFR3</i>	62.9	5'- CCTAAGTGTGTGCCTCCTGA -3' (fwd)	2503	20	
(211 bp)	67.3	5'- CAATGCCCATCACGGTTAGG -3' (rev)	2713	20	
<i>EGFR</i>	75.4	5'- GGACGACGTGGTGGATGCCG-3' (fwd)	3207	20	(Chia et al., 1995)
(208 bp)	75.7	5'- GGCGCCTGTGGGGTCTGAGC-3' (rev)	3414	20	
<i>IGF1R</i>	67.8	5'- GAATTCCTTCCGCTCGTGG -3' (fwd)	576	20	
(214 bp)	64.0	5'- CTTCCATGTGTCCCCTGTCT -3' (rev)	789	20	
<i>GAPDH</i>	65.0	5'- GAGTCCACTGGCGTCTTCAC -3' (fwd)	365	20	(Fiedler et al., 2005)
(188 bp)	64.1	5'- GGTGCTAAGCAGTTGGTGGT -3' (rev)	604	20	

2.10 Statistical Analysis

Results are expressed as the mean \pm S.D. Intergroup differences were analysed using repeated measures one-way ANOVA followed by Tukey post-hoc test, for simple comparisons; levels of significance were * $p < 0.05$, § $p < 0.01$.

Chapter 3: Differential Activities of Heparin Analogues on hVSMC/hEC proliferation & migration

3.1 Introduction

Stent insertion into disease-narrowed arteries often damages the blood vessel and triggers VSMC proliferation as a healing response. This vascular hyperplasia, which is caused by increased cell proliferation and migration, re-blocks arteries leading to tunica intimal formation. There are several different cell types involved in hyperplasia, including adventitia-derived stem cell, smooth muscle progenitor cells, endothelial precursor cells and bone marrow-derived cells (Moonen et al., 2015; Saiura et al., 2001; Tanaka et al., 2003). However, VSMCs and ECs play the most important roles (see **Section 1.3**). It is believed that inhibition of VSMC proliferation and migration may be a useful strategy for preventing neointimal formation. To combat hyperplasia, anti-proliferative agents can be incorporated into stents, but these significantly delay healing and inhibit EC re-growth leaving pro-thrombotic stent surfaces exposed. The polysaccharide heparin is a generally well-tolerated anti-coagulant that also inhibits VSMC proliferation (Khorana et al., 2003). It is thus an attractive candidate for incorporation into stent materials. Indeed, in multicentre, randomized clinical trials, heparin-coated stents significantly reduced stent thrombosis, but showed no measurable effect on VSMC proliferation or restenosis (Serruys et al., 1998; Vrolix et al., 2000). However, changing heparin's structure through desulphation and/or complexing heparin with cations is known to alter the molecule's charge distribution, geometry, and conformation and ultimately modifies heparin's biological activity (Rudd et al., 2007). This interaction between heparin and FGFs mainly relies on electrostatics, therefore, alteration of charge distribution on heparin changes the affinity with these binding proteins, as well as the structure of FGF-FGFR-heparin complex. Consequently, this conformational change leads to different biological activity (Guimond et al., 2009; Pellegrini et al., 2000). Therefore, the aim of this chapter was to identify novel heparin-based analogues that had a differential effect on HCASMC and HCAEC proliferation. I also assessed effects on migration and ability to induce apoptosis.

3.1.1 Heparin Derivatives (Sulphated and Cation Forms) Affect Heparin-Protein Interaction

Heparin, as a highly sulphated glycosaminoglycan (GAG), is involved in numerous biological processes, including blood clotting, cell growth, and immunological responses, through its interaction with different proteins (Beamish et al., 2009; Capila and Linhardt, 2002; Gu et al., 2010). It is structurally similar to HS in that it is composed of repeating disaccharide units of uronic acid and D-glucosamine (**Figure 3.1**). However, it has a distinct, more heavily sulphated, and homogenous repeating pattern compared to HS (Garg et al., 2011a). In addition, it is used widely as an experimental proxy for HS because of its underlying structural similarity to HS and relative abundance as a commercially-available anti-coagulant.

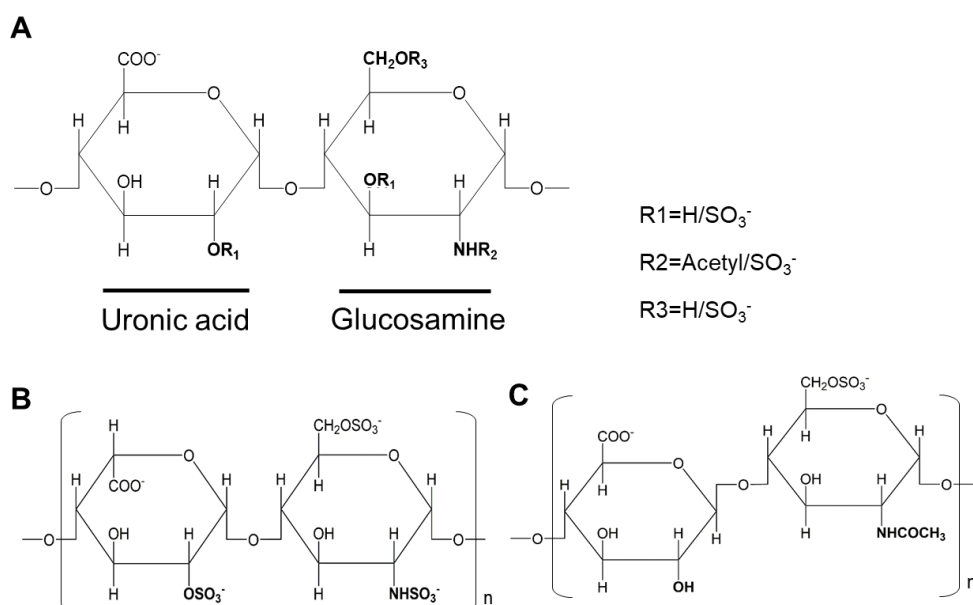


Figure 3.1. Structural features of HS and heparin. (A) Possible substitution patterns in heparin/HS. (B) The major disaccharide unit of heparin, differences exist between sources (C) The major repeating disaccharide unit of HS. Considerable variation in HS composition also occurs between species and tissues (Casu and Lindahl, 2001; Garg et al., 2011a). Images drawn with ChemDraw 18.1.

The structure of heparin relates to the interaction with protein binding

The biological activity of a heparin derivative on a cell type depends upon the interaction between the derivatives, growth factors (GFs) and growth factor receptors (GFRs). For instance in VSM, anti-proliferative activity depends on the interaction between GFs-GFRs-heparin/HS (Garg et al., 2003). Fibroblast growth factors (FGFs)

and their receptors are typical elements involved in FGF-FGFR-heparin complex which regulates many bioactivities. This ternary complex is principally constructed through ionic interactions. Heparin carries strong negative charge due to numerous sulphated groups on glucosamine residues and thereby, it can regulate biological activity by binding to positively charged amino acids on specific proteins (Singh et al., 2019). This binding interaction is widely involved in the regulation of cell growth, anti-coagulant action and other biological events, moreover, it possesses a high degree of specificity (Xu et al., 2012). FGFs from different FGF-subfamilies show distinct secondary structures when complexed with heparin, whereas members from within the same subfamilies present similar secondary structures when complexed with heparin. This is due to the similarity of the type and number of heparin binding sites more closely resembling each other within sub-families than between sub-families (Li et al., 2016; Xu et al., 2012). The diversity of FGFs contributes to the diversification of signaling systems and signaling capacities (Xu et al., 2012).

Cation binding also changes the structure of heparin-protein

Since HS and heparin carry negatively charged sulphate groups, they can readily complex with cations. This can alter the charge distribution and modify heparin's conformation and activity (Guimond et al., 2009; Rudd et al., 2007). For example, the affinity of Na⁺-bound heparin to FGF1 is decreased relative to heparin, while interaction with FGF2 are unaltered (Xu et al., 2012). Furthermore, the acetyl-rich heparin converts its activity from supporting FGF-1/FGFR1c signalling to being suppressing in Baf3 cells when the coordinated cation is changed from sodium to copper (Guimond et al., 2009). In addition, each sulphated group on heparin can only neutralise one of the charge of a divalent cation. Therefore, a divalent cation can bind two sulphates (or a sulphate and a carboxylate) and this can influence the orientation and conformation of adjacent residues and the intervening glycosidic linkages (Rudd et al., 2007). However, it is distinct for different cations with their ionic radii, charge and coordination. The different structures of diverse cationic form heparin analogues exhibit specific NMR spectra (Rudd et al., 2007). More discussion of complex roles played by GFs/GFRs in both VSMCs and ECs can be found in **Chapter 5**.

3.1.2 Modifications of Heparin Alter Its Activities

Structural modifications of heparin can lead to characteristic changes in its biological action: for example totally desulphated heparin loses its anti-proliferative activity (Castellot et al., 1984). The loss of negative charge might be expected decrease the ionic interaction between heparin and its binding proteins. Therefore, the sulphation

of heparin can be expected to be important for interaction with GFs/GFRs to induce anti-proliferative activity in VSM (Castellot et al., 1984). O-sulphation of heparin is required for anti-proliferation and anti-coagulation, as the removal of 6-O-sulphation and the substitution of hexosamine decrease anti-proliferative activity, whereas the over-O-sulphation of heparin/HS enhances SMC anti-proliferation (Danishefsky et al., 1977; Garg et al., 2011b; Garg et al., 2003).

Size-modified heparin also induces different effects on SMC proliferation. One of the low-molecular-weight-heparins (LMWHs), SR 80258A (Sanofi Recherche), blocks serum-induced, but not PDGF-BB-induced, SMC proliferation and migration in culture (Geary et al., 1995). Another similarly modified heparin, Astenose, however, has no effect on SMC proliferation (Wilcox et al., 1994). This size dependent inhibition also relies on specific heparin fragments (Kazi et al., 2002). The hexasaccharides and large fragments retain anti-proliferative activity while tetrasaccharides and disaccharides are inactive (Castellot et al., 1981). Pentasaccharides also retain anti-proliferative activity and here, 6-O-sulphation on the internal glucosamine residue has been held to be essential (Castellot et al., 1986; Garg et al., 2003). The majority of experimental evidence supports the idea that there are several sequences within HS or heparin capable of binding a given protein, but that not all sequences bind. Furthermore, a range of binding affinities exists within these binding structures and some of these can be relatively high ($K_d \sim 10$ nM for fibroblast growth factors; FGFs) (Deepa et al., 2002). Cations are also important for the interaction between heparin and anti-coagulation factors. For example, the activity of heparin-antithrombin binding to thrombin is significantly reduced in the absence of Ca^{2+} (Speight and Griffith, 1983) (See details in **Chapter 4**).

In addition to heparin having anti-proliferative and anti-coagulant activity, it may also have apoptotic effects on several cell lines. For instance, heparin induces apoptosis by Akt suppression in oral squamous cell carcinoma (Ueda et al., 2009). It also leads to apoptosis in lymphoblasts (Erduran et al., 1999) and nasopharyngeal tumour cells (Li et al., 2001). Furthermore, sulphated GAGs are also involved in the modulation of the apoptotic process, and modifications of GAG chains are likely relevant to the sensitivity of tumour cells to apoptosis (Kozlowski and Pavao, 2011). Conversely, heparin prevents Zika virus-induced apoptosis and necrosis in human neural progenitor cells (Ghezzi et al., 2017). It is reported that unfractionated heparin increases apoptosis in human oral squamous cell carcinoma (Ueda et al., 2009). However, low molecular weight heparin (LMWH)-induced apoptosis is controversial (Zhang et al., 2016b). Furthermore, unmodified heparin has been observed to have a protective effect in some cases in which apoptosis is involved, although the mechanism is not clear (Ghezzi et al., 2017). Therefore, given their potential effects on cell

number/proliferation, whether heparin analogues trigger cell apoptosis is an important question.

3.1.3 Apoptosis

Cell death is an event whereby a cell permanently loses its biological function due to stimuli such as damage or death signals. The phenomenon can be roughly divided to programmed and non-programmed cell death. Programmed cell death, such as apoptosis, is a protease cascade-regulated cell death. Non-programmed cell death, as known as necrosis, is generally caused by a traumatic injury. In this section, we only focus on programmed cell death – apoptosis.

Extrinsic and intrinsic pathways

Apoptosis is cell programmed death which is activated via both extrinsic and intrinsic pathways. The extrinsic pathway, or so-called receptor-dependent pathway, is induced by the binding of death signals to cell surface receptors (Güneydaş and Topçul, 2016). Death ligands such as tumour necrosis factor (TNF), Fas ligand, Apo-2 ligand, and TNF-like ligand 1A bind to their respective receptors (the death receptors, DRs), for instance the Fas/Apo-1/CD95 receptor or DR4/5 (Huang et al., 2016; McIlwain et al., 2013; Nikolettou et al., 2013). Ligand binding induces receptor oligomerization and the recruitment of a multi-protein complex (the death-inducing signalling complex or DISC) to intracellular portions of the receptors. The complex forms through interaction between death domains on the receptors and the FAS-associated death domain (FADD) or TNFR-associated death domain (TRADD) on adaptor protein, and leads to the recruitment and activation of caspase-8 (McIlwain et al., 2013; Miao et al., 2011). Active caspase-8 promotes the downstream activation of caspase -3, -6 and -7 thereby inducing apoptosis. Active caspase-8 can also induce the intrinsic pathway.

Typically, the intrinsic pathway is initiated by multiple cellular stress stimuli such as DNA damage, endoplasmic reticulum (ER) stress, hypoxia or metabolic stress. These trigger cytochrome c release from mitochondria, leading to apoptosome formation (McIlwain et al., 2013). The release of cytochrome C from mitochondria is controlled by the Bcl-2 family of proteins (Huang et al., 2016). The BH3-domain-only protein, Bid, is one of Bcl2 family members which is also known as a cytosolic pro-apoptotic factor (Huang et al., 2016). After cleavage by active caspase-8, cleaved Bid (cBid) remains as a complex of two fragments (the p7 fragment and the tBid fragment) due to hydrophobic interactions (**Figure 3.2**) (Shamas-Din et al., 2013). On interaction with

the mitochondrial membrane, these fragments separate, allowing the truncated Bid (tBid) to insert into the mitochondria outer membrane (Lovell et al., 2008). tBid subsequently recruits the pro-apoptotic Bax protein, resulting in mitochondrial membrane permeabilization and cytochrome C release (Billen et al., 2009; Kim et al., 2017; Peixoto et al., 2017).

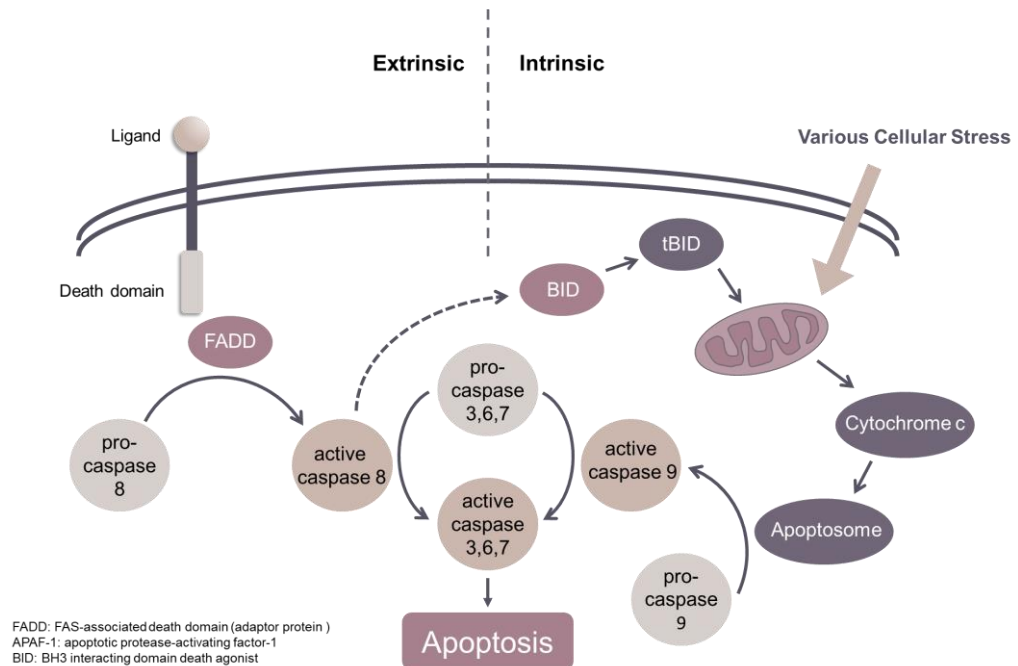


Figure 3.2. Scheme of the main apoptosis pathways. The extrinsic pathway is induced by receptor activation whereas the intrinsic pathway is introduced by various cellular stress stimuli (McIlwain et al., 2013). Both of them activate the downstream caspase cascade, leading to apoptosis. Image drawn with PowerPoint.

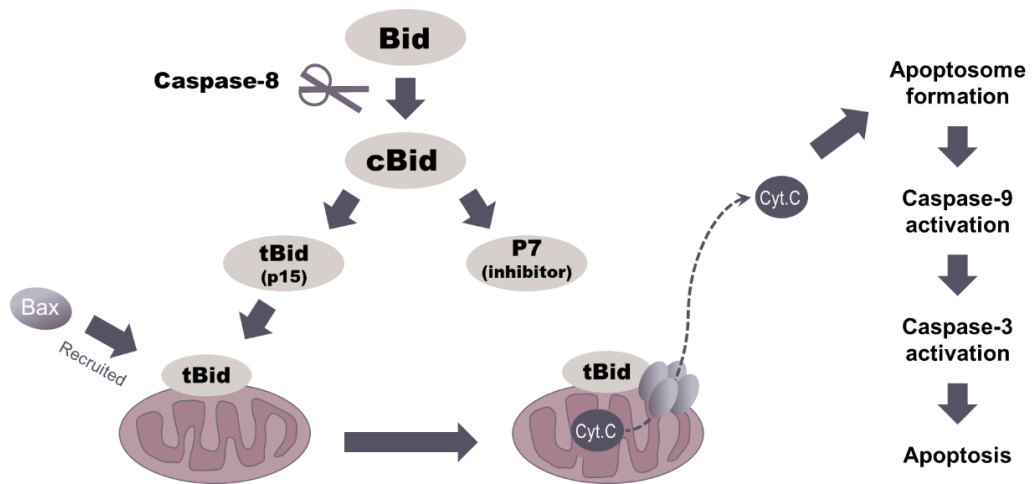


Figure 3.3. Bid and Bax insert into membrane of mitochondria, resulting in apoptosis. After cleavage by caspase-8, cBid remains as a complex until reaching the membrane of mitochondria. The inserted tBid recruits Bax to the mitochondrial membrane results in pore formation and the release of cytochrome C (Billen et al., 2009). Image drawn with PowerPoint.

Released cytochrome c forms the apoptosome with Apaf-1 and pro-caspase-9 (Misiti et al., 2008; Nam et al., 2016), leading to caspase-9 activation. Active caspase-9 subsequently activates caspase-3, -6, -7, resulting in apoptosis (McIlwain et al., 2013). During apoptosis, the dying cells are observed as shrunken, rounded and condensed. The plasma membrane forms blebs and the cytoskeleton and DNA in the nucleus collapse and break into fragments resulting in the formation of apoptotic bodies which can be phagocytosed and degraded (Alberts et al., 2013; Lemasters, 2018). Apoptosis is not inflammatory and there is no adaptive immune response (Miao et al., 2011). However, if these apoptotic bodies are not cleared by macrophages, they can lyse leading to secondary necrosis (Fink and Cookson, 2005).

To date, there is no evidence that heparin may cause apoptosis on SMCs or ECs. However, as mentioned above, heparin may have pro-apoptotic activity on several cell lines (Erduran et al., 1999; Li et al., 2001; Ueda et al., 2009), and some of the modified heparin analogues induce apoptosis in tumour cells (Kozlowski and Pavao, 2011; Ueda et al., 2009). Since the properties of our modified heparins have been changed, they may have the potential to trigger apoptosis. Therefore, we need to investigate whether any heparin analogue-induced reduction in cell number observed in MTT assays is actually caused by apoptosis.

3.1.4 Aim of This Chapter

As the activity of heparin can be altered, the purification of pharmaceutical heparin from crude heparin and modification of that heparin to develop novel heparin analogues is possible. The aim of this chapter is to assess how these different heparin analogues impact on HCASMCs and HCAECs cell number *in vitro*. To ensure that any effects on cell number are through changes in proliferation as opposed to changes in apoptosis, the question of whether some heparin analogues induce apoptosis is also assessed. In addition, the influence of heparin analogues on cell migration, which relates to wound healing, is investigated.

3.2 Results

3.2.1 Desulphated Heparin Analogues Have Differential Effects on HCASMC/HCAEC Proliferation and Migration

Proliferative activity

The desulphated heparin analogues were selected from our in-house library. These polysaccharides were all labelled with numbers 2-8 (**Figure 3.4**). To assess the effect of desulphated heparin analogues on cell proliferation, cells were seeded (a density of 5×10^3 cells per well for HCASMCs and 2.5×10^3 cells per well for HCAECs) in a 96-well plate in normal growth media (GM). Cells were incubated in either GM alone (control) or GM containing heparin or different heparin analogues ($10 \mu\text{g}/\mu\text{l}$) at 37°C for 4 days. An MTT assay was subsequently used to determine cell growth. **Figure 3.5** shows that in HCASMCs, unmodified heparin significantly reduced cell growth against normalized control (reduction on $24.8 \pm 7.3\%$, $p < 0.05$). In contrast, the heparin analogue-3 significantly increased HCASMC proliferation ($113.2 \pm 6.5\%$, $p < 0.05$). All other analogues showed no significant effect. For HCAECs, heparin analogue-5 and -8 significantly reduced cell growth (reduction on $15.8 \pm 4.6\%$ and $11.9 \pm 1.9\%$ against normalized control, respectively, $p < 0.05$).

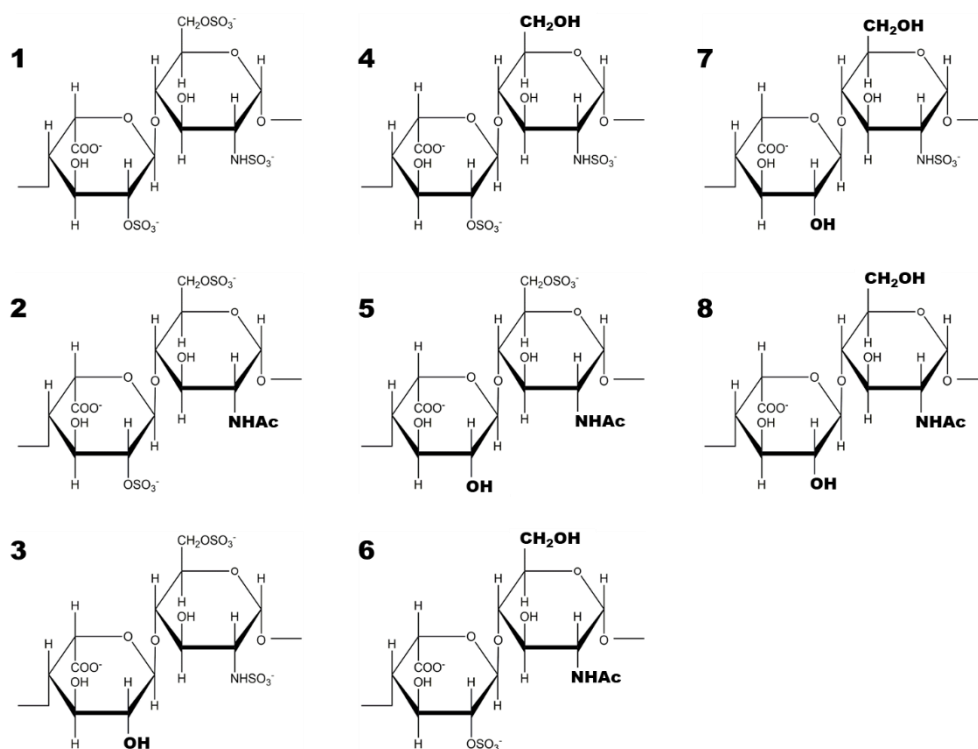


Figure 3.4. Desulphated heparin analogues produced by Dr. Ed Yates (Institute of Systems, Molecular and Integrative Biology, University of Liverpool). Images drawn with ChemDraw 18.1.

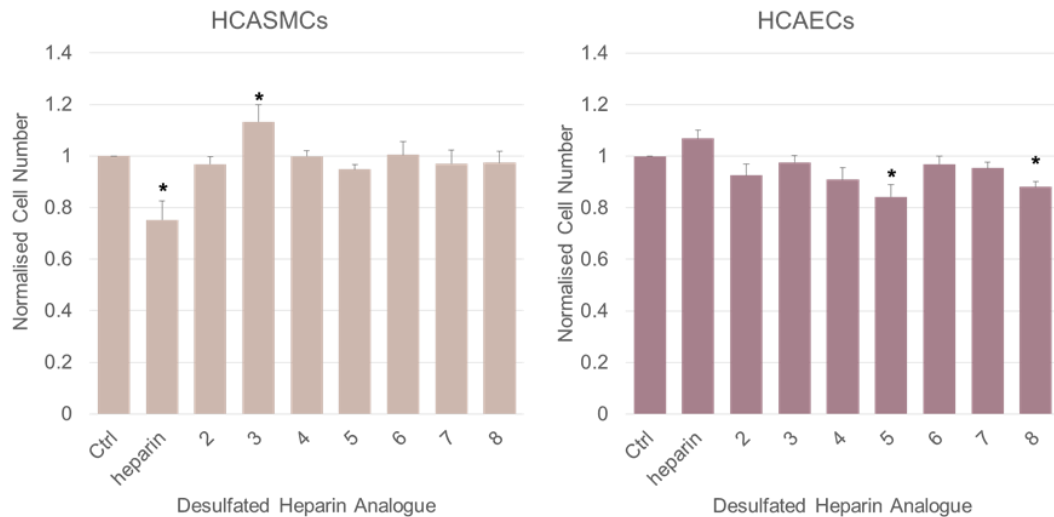


Figure 3.5. Effect of desulphated forms of heparin on HCASMC and HCAECs cell number. Results were expressed as mean \pm S.D. Results from N=3 experimental repeats with n=8 repeats within each experiment. * $P < 0.05$ compared to control (no heparin treatment) with statistical significance determined by a one-way ANOVA followed by Tukey post-hoc test. The cell growth of control group is set at 1.

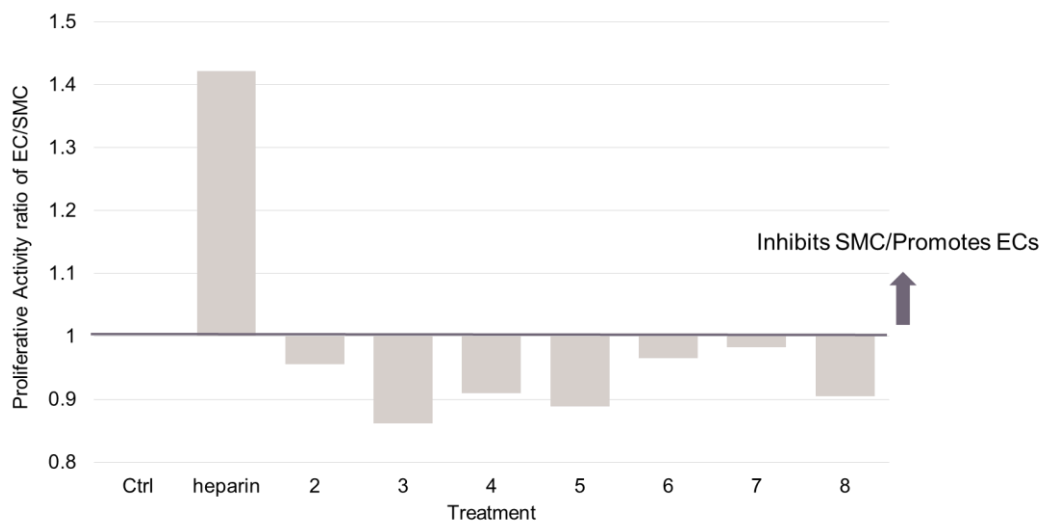


Figure 3.6. The differential growth index for these analogues was calculated as mean EC numbers divided by mean SMC numbers for each analogue (HCAEC/HCASMC). Here a desirable activity profile would show values > 1 (i.e. suppresses VSMC proliferation relative to EC proliferation). These data indicate that only unmodified heparin has this effect.

3.2.2 The Effects of Cationic Heparin Analogues on HCASMC/HCAEC Proliferation and Migration

Cation forms of heparin have differential effects on HCASMC/HCAEC proliferation

Since none of the desulphated heparin analogues produced a desirable differential activity profile, we next assessed the effects of different cation forms of normal heparin. Again, cells were seeded in a 96-well plate and incubated with either GM alone (control) or GM containing heparin or different cation forms of heparin (10 µg/ml) at 37 °C for 4 days. An MTT assay was again used to determine cell number. The results show that all the cation forms of heparin significantly reduced HCASMC number compared to control (**Figure 3.7**; $P < 0.05$). In HCAECs, these analogues also significantly suppressed cell proliferation except for Ca-heparin (**Figure 3.7**). The anti-proliferative activity of Na-, K-, Mg- and Ca-forms of heparin on HCAECs was not as marked as the effects on HCASMCs. This is made clearer when the ratio of HCAEC:HCASMC cell number is calculated (**Figure 3.8**). This growth index shows that Na-, K-, Mg- and Ca- heparin have the desired effect of suppressing HCASMC proliferation, while having less effect on HCAEC proliferation.

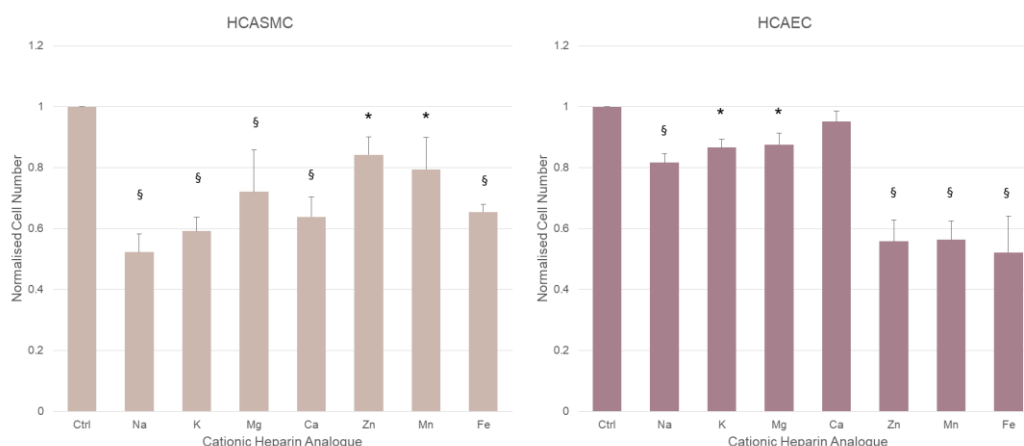


Figure 3.7. Effect of cation-forms of heparin on HCASMC and HCAECs cell number. Results were expressed as mean \pm S.D. Results from N=3 experimental repeats with n=8 repeats within each experiment. * $P < 0.05$ and § $P < 0.001$ compared to control (no heparin treatment) with statistical significance determined by a one-way ANOVA followed by Tukey post-hoc test. Ctrl, control (normalized to 1.0); Na, sodium-heparin; K, potassium-heparin; Mg, magnesium-heparin; Ca, calcium-heparin; Zn, zinc-hep

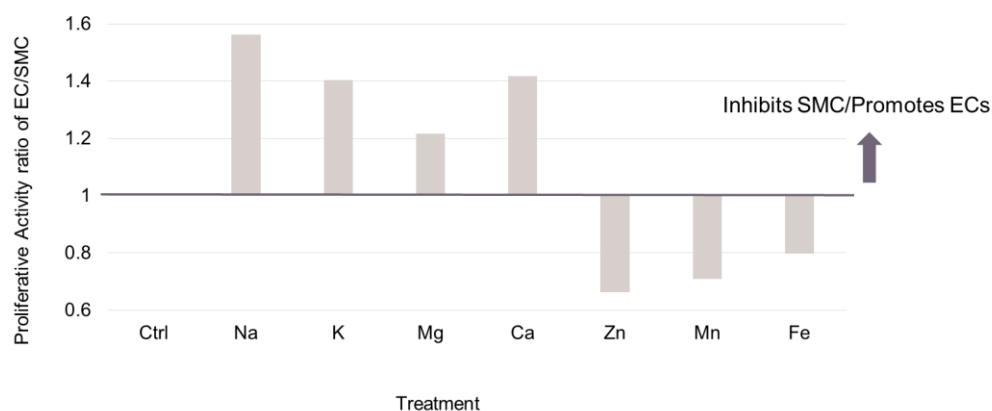


Figure 3.8. The differential of growth index for cation forms of heparin for HCAECs and HCASMCs. Values over 1.0 indicate potentially interesting compounds that inhibit HCASMC proliferation while having less effect on HCAECs. Ctrl, control; Na, sodium-heparin; K, potassium-heparin; Mg, magnesium-heparin; Ca, calcium-heparin; Zn, zinc-heparin; Mn, manganese-heparin; Fe, iron-heparin.

The anti-proliferative activity of cationic heparin is not caused by apoptosis

To determine whether the reduction in cell number seen in MTT assays was due to the induction of apoptosis by the cation forms of heparin, lysates of HCASMCs and HCAECs were immunoblotted with antibodies against caspase 3, an important inducer of apoptosis (Güneydaş and Topçul, 2016, Mohamed et al., 2010). Activation (cleavage) of caspase 3 to smaller fragments (17 and 19 KDa) indicates the induction of apoptosis and antibodies against caspase-3 and cleaved caspase-3 were used. Staurosporine, a protein kinase inhibitor that can quickly induce cell apoptosis, was used as a positive control (Zheng et al., 2000). **Figure 3.9** shows that none of the cation forms of heparin (10 µg/ml for 2 days) induced caspase 3 cleavage in HCASMCs and HCAECs.

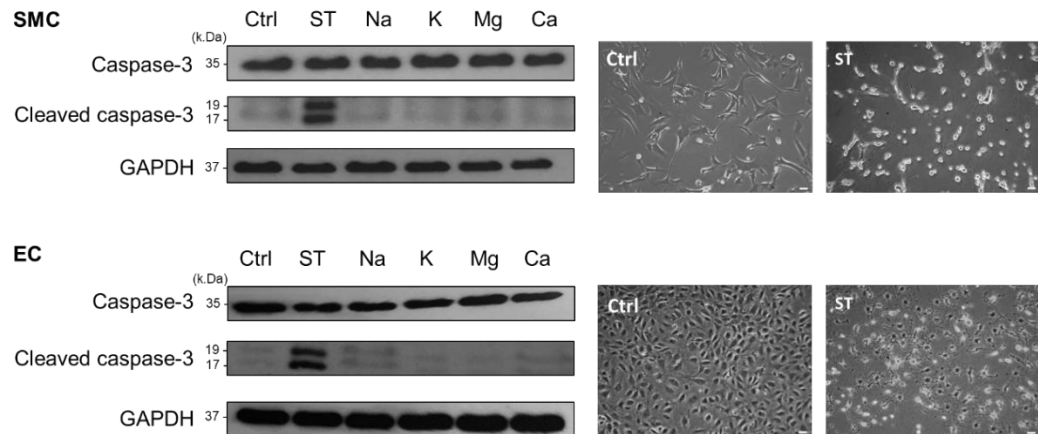


Figure 3.9. Cation forms of heparin do not induce apoptosis in HCASMCs or HCAECs. HCASMCs (A) or HCAECs (B) were grown in (10 μ g/ml) heparin analogues for 4 days before being lysis and proteins within the lysates separated by SDS-PAGE. Separated proteins were immunoblotted for caspase 3 and cleaved (activated) caspase 3, an indication of the induction of apoptosis. Cells were incubated with 1 μ M of staurosporine for 3 hr as a positive control. Ctrl, control; ST, staurosporine; Na, sodium-heparin; K, potassium-heparin; Mg, magnesium-heparin; Ca, calcium-heparin. Bar, 10 μ m. Images of whole blots are in **Appendix 4**.

3.2.3 Cationic Heparin Analogues Showed Differential Effects on HCASMC/HCAEC Migration

Vascular cell migration plays a key role in restenosis. After vascular wall injury caused by stent implantation, the proliferation and migration of SMC leads to neointima formation which underlies restenosis. A migration assay was used to assess the effects of the cation forms of heparin on both HCASMCs and HCAECs. Cells were seeded into the upper chamber of the transwell plate and treated with different cation forms of heparin (10 µg/ml) for 24 hr at 37 °C. All the cells migrating through the membrane to the lower chamber were counted under a phase contrast microscope. The results show that Na-, Mg- and Ca- heparin significantly suppressed HCASMC migration (**Figure 3.10**; One-way ANOVA: $F_{5,36} = 4.56$, $P < 0.05$, $n=7$) and K-heparin had no effect. There was no marked difference between different cation-heparin treatments on HCAECs except for Mg-heparin (One-way ANOVA: $F_{5,36} = 1.37$, $P = 0.26$). There was however significant variation within the results.

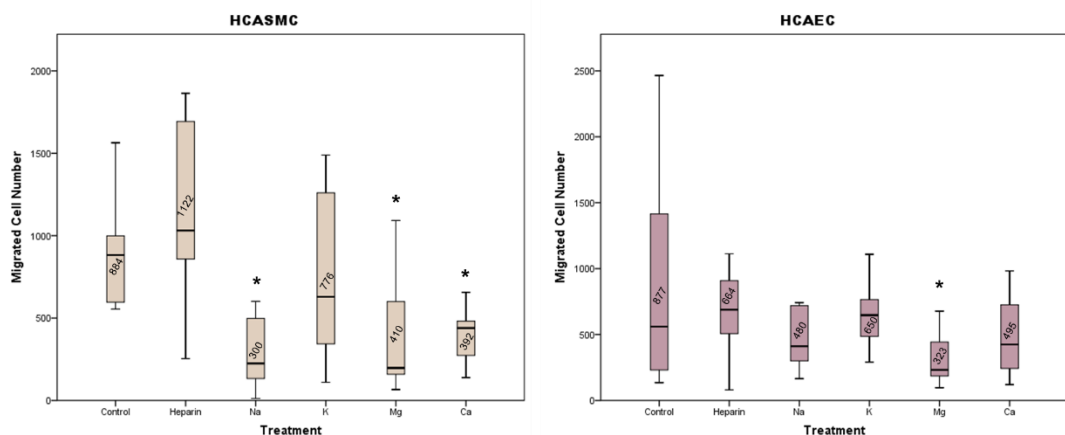


Figure 3.10. Boxplot diagrams for cell migration. The Na-, and Ca-heparin significantly reduced HCASMC migration. All the cells passing through the transwell membrane were counted. Results from N=2 experimental repeats with n=3 technical repeats. * $P < 0.05$, $n=7$. Means are represented by the value. Ctrl, control; Na, sodium-heparin; K, potassium-heparin; Mg, magnesium-heparin; Ca, calcium-heparin.

3.2.4 Effects of Heparin Analogues in Co-cultures of hVSMC/hECs

Within intact arteries *in vivo*, ECs continuously communicate with SMCs via hetero-cellular junctions and signalling molecules to mediate contractile state, metabolism, proliferation and differentiation of SMCs (Parkington, 2008; William, 2005). In addition, EC/SMC interaction is vital for maintaining the appropriate microenvironment of blood vessels including ECM composition, pericyte function, and the response to shear stress induced by blood flow (Davies, 1988; Hartmann, 2007; Wang, 2009). To generate a more physiological assessment of the effects of the heparin analogues, we used fluorescence-activated cell sorting (FACS) to determine cell numbers following incubation with the analogues when SMCs and ECs were co-cultured together. **Figure 3.11** shows the results of these experiments. Although the Na-, K- and Ca-heparin analogues showed promising activity profiles on HCASMCs and HCAECs in the single-culture system, Na- and Ca-heparin analogues had no significant effect compared to control in co-culture. The K-heparin showed a weaker activity profile in the co-culture system, but Mg-heparin showed a similar activity to heparin. Thus, co-cultivation appears to identify active compounds missed in single cultivation screens.

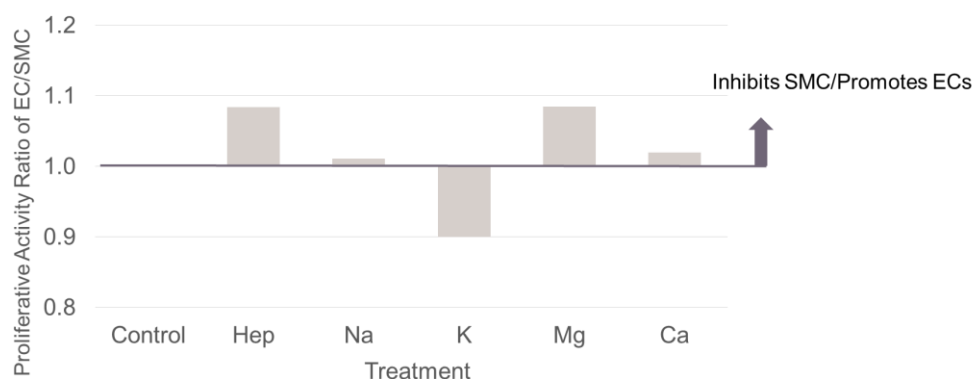


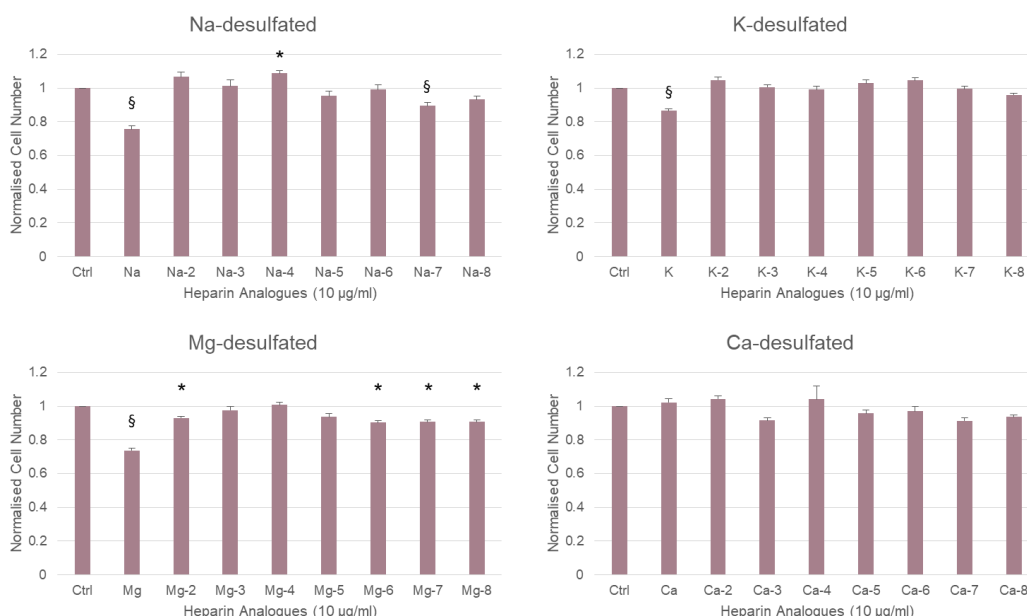
Figure 3.11. The growth ratio of HCAEC/HCASMCs in co-cultivation system. Values over 1.0 indicate potentially interesting compounds that inhibit HCASMC proliferation while having less effect on HCAECs.

3.2.5 Effects of Cationic Desulphated Heparin Analogues in VSMC/ECs

Since the results of the SMC/EC co-culture experiments contradicted the possible utility of the cation forms of heparin, the above experiments were repeated using a new set of analogues where cations (Na^+ , K^+ , Mg^{2+} , Ca^{2+}) were complexed to the desulphated forms of heparin.

Proliferative activity of cation-desulphated heparin analogues

HCASMCs and HCAECs are grown separately in 96-well plate and treated with 10 $\mu\text{g/ml}$ heparin analogues for 4 days. The results of the subsequent MTT assays are



shown in **Figures 3.12 and 3.13**.

Figure 3.12. Effect of cation-desulphated forms of heparin on HCASMC proliferation. Results were expressed as mean \pm S.D. Results from N=3 experimental repeats with n=8 technical repeats. * $P < 0.05$ and § $P < 0.001$ compared to control (no heparin treatment) with statistical significance determined by a one-way ANOVA followed by Tukey post-hoc test. Ctrl, control (normalized to 1.0). Ctrl, control (normalized to 1.0); Na, sodium; K, potassium; Mg, magnesium; Ca, calcium; 1-8, different levels of desulphated heparin analogues.

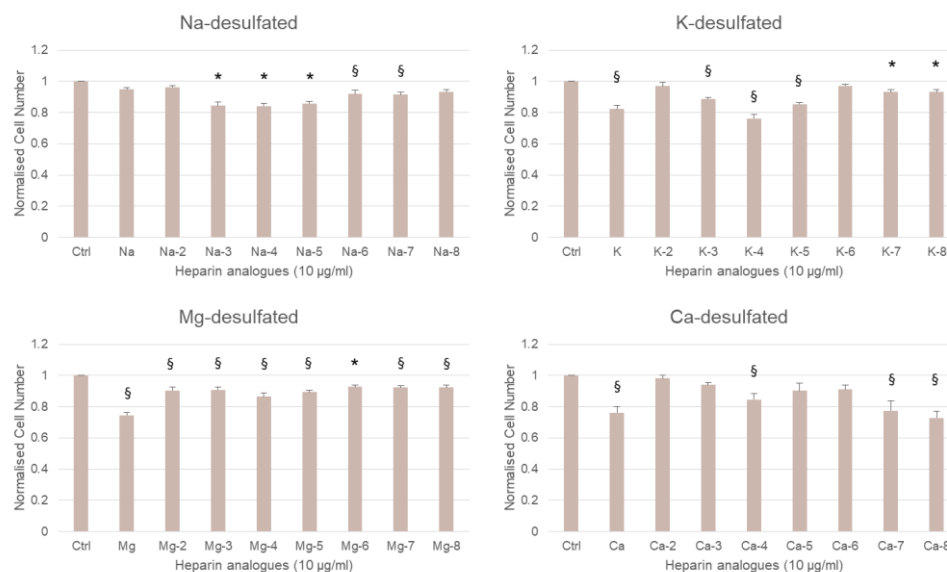


Figure 3.13. Effect of cation-desulphated forms of heparin on HCAEC proliferation. Results were expressed as mean \pm S.D. Results from N=3 experimental repeats with n=8 repeats within each experiment. * P<0.05 and § P<0.001 compared to control (no heparin treatment) with statistical significance determined by a one-way ANOVA followed by Tukey post-hoc test. Ctrl, control (normalized to 1.0). Na, sodium; K, potassium; Mg, magnesium; Ca, calcium; 1-8, different levels of desulphated heparin analogues.

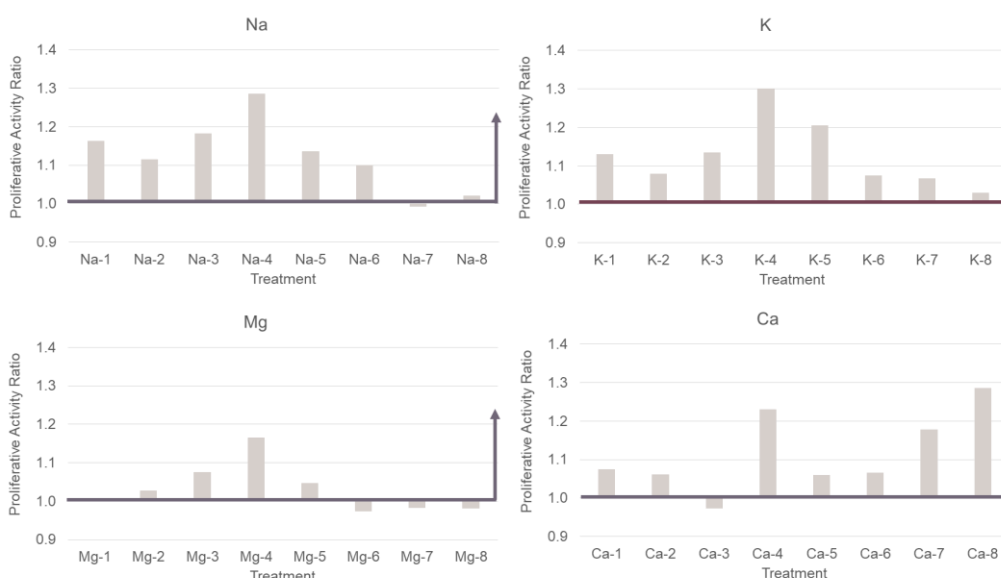


Figure 3.14. The growth ratio of HCAEC/HCASMCs treated with cationic desulphated heparin analogues. Values >1 indicate potentially interesting compounds that inhibit HCASMC proliferation while having less effect on HCAECs.

Proliferative activity of cation-desulphated heparin analogues in co-cultivation system

Since some of the cationic desulphated heparins showed promising activity profiles in terms of inhibiting SMC proliferation while promoting EC proliferation, eight high-performing analogues (Na-3, Na-4, K-3, K-4, Mg-3, Mg-4, Ca-3 and Ca-4) were selected to be assessed in the co-culture system. These analogues were incubated with co-cultured HCASMCs and HCAECs for 4 days. Cells were then separated using a primary antibody (anti- α -SMA antibody for HCASMCs and anti-CD31 antibody for HCAECs) and conjugated secondary antibody label (Alexa Fluor® 488 for anti- α -SMA antibody and Alexa Fluor® 647 for anti-CD31 antibody), followed by analysis with FACS. The growth index shows that all of these analogues retained their differential effects on SMC and EC growth when SMC and ECs were cultured together (**Figure 3.15**).

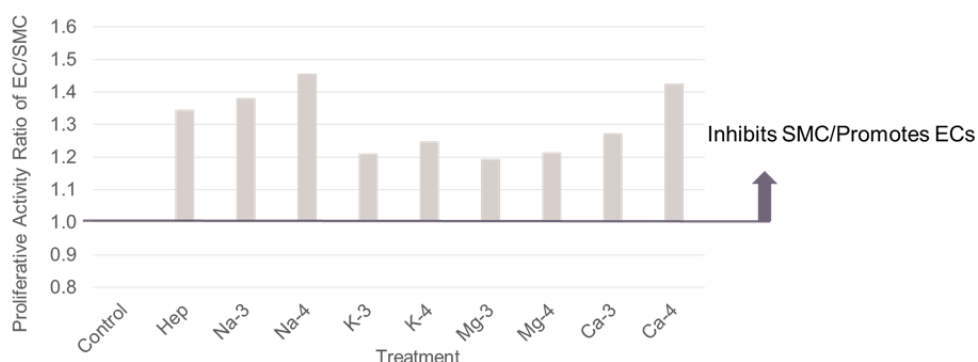


Figure 3.15. The EC/SMC ratio of cationic desulphated heparin analogues in co-cultivation. Values >1 indicate potentially interesting compounds that inhibit HCASMC proliferation while having less effect on HCAECs. Ctrl, control; Hep, heparin; Na, sodium; K, potassium; Mg, magnesium; Ca, calcium; 3,4, different levels of desulphated heparin analogues.

Migration activity of HCASMCs and HCAECs treated with cation-desulphated heparin analogues

The effects of these new cationic desulphated heparins analogues on cell migration were also assessed. As previously, HCASMCs or HCAECs were seeded into the upper chamber of the transwell plate. Cells were then treated with different cation forms of desulphated heparin (10 µg/ml) for 24 h at 37 °C. Cells migrating through the membrane to the lower chamber were counted under a phase contrast microscope. The results reveal that all of these analogues significantly delay HCASMC migration, but have no significant effect on HCAECs (**Figure 3.16**; One-way ANOVA: $F_{11,24} = 18.971$, $P < 0.01$, $n=3$).

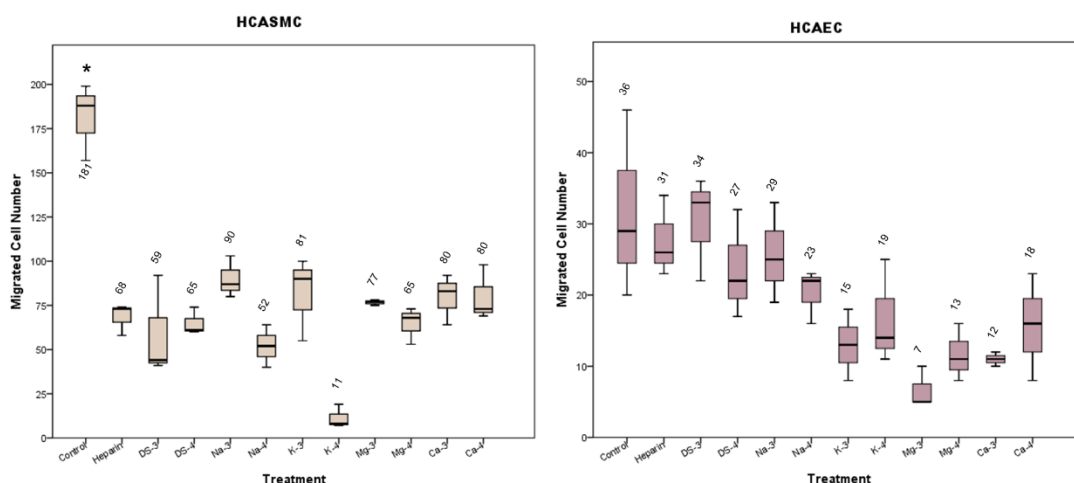


Figure 3.16. Cation-desulphated heparin analogues significantly delay HCASMC migration but have no effect on HCAECs. The Na-, and Ca-heparin significantly reduced HCASMC migration. All the cells passing through the transwell membrane were counted. Results from N=3 experimental repeats with n=3 technical repeats. * $P < 0.05$, $n=3$. Means represented by the value. Ctrl, control; Na, sodium-heparin; K, potassium-heparin; Mg, magnesium-heparin; Ca, calcium-heparin.

The anti-proliferative activity of cationic desulphated heparins is not caused by apoptosis

The cationic desulphated heparin analogues significantly reduce HCASMC number in MTT assays both in isolated single culture and in SMC/EC co-culture systems. To determine whether this reduction in cell number is caused by suppression of proliferation as opposed to an increase in apoptosis, treated lysates of HCASMCs and HCAECs were immunoblotted with antibodies against caspase 3. The apoptosis-inducer, staurosporine (1 μ M) was again used as a positive control. The results demonstrate that that none of the cation forms of desulphated heparin (10 μ g/ml for 2 day) induced caspase 3 cleavage in HCASMCs and HCAECs (**Figure 3.17**).

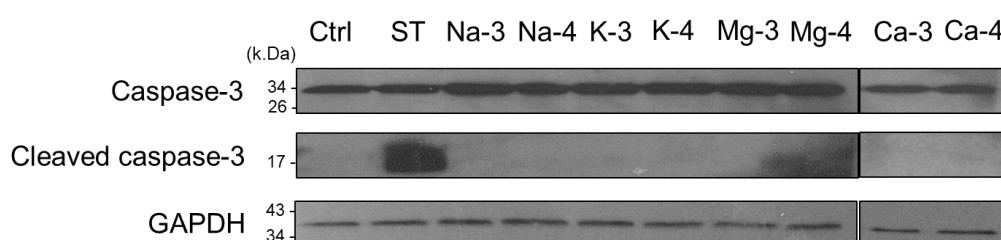


Figure 3.17. The cation-desulphated heparin candidates do not induce apoptosis in HCASMCs. The lysates of HCASMCs were analysed by SDS-PAGE. Separated proteins were immunoblotted for caspase 3 and cleaved caspase 3. The cells were incubated with 1 μ M of staurosporine for 3 h as a positive control. Ctrl, control; ST, staurosporine; Na, sodium-heparin; K, potassium-heparin; Mg, magnesium-heparin; Ca, calcium-heparin; The numbers -3 and -4, refer to the heparin analogue used (see detail in **Figure 3.4**). Images of whole blots are in **Appendix 5**.

3.3 Discussion

The results of this chapter show that chemically modified forms of heparin have differential effects on VSMC and EC proliferation. For instance, Ca-heparin has no effect on HCAEC proliferation whereas zinc-heparin inhibits HCAEC growth.

Several previous studies have reported that chemical or enzymatic modification of heparin can change not only the structure and molecular weight of heparin, but also its properties and characteristics (Duckworth et al., 2015; Garg et al., 2011b). Our desulphated heparin derivatives alter the anti-proliferative capacity of HCASMCs and the pro-proliferative activity of HCAECs, but none of the analogues demonstrated the desired effect of repressing SMC growth and enhancing EC growth. However, they may exhibit other desirable activities such as cell migration or anti-thrombotic activity. For instance, 2-O, 3-O-desulphated heparin is known to inhibit P-selectin-mediated cell adhesion (Wei et al., 2004). Next, the effects of fully-sulphated heparin complexed with different cations (Guimond et al., 2009, Rudd et al., 2007) were tested. The data show that Na⁺, K⁺ and Ca²⁺ -complexed forms of heparin have promising activity profiles in terms of differential effects on HCASMC/HCAEC proliferation/migration (summarised in **Table 3.1**). These effects are not due to the induction of cell death and may relate to the expression of different growth factor receptors on the cell surface.

Table 3.1. Summary of the capacity of heparin cation form to prevent neointimal formation. ↓, inhibition; ↓↓, high inhibition; -, no significant effect; /, no testing.

	HCASMCs							HCAECs						
	Na	K	Mg	Ca	Zn	Mn	Fe	Na	K	Mg	Ca	Zn	Mn	Fe
Proliferation	↓↓	↓↓	↓↓	↓↓	↓	↓	↓↓	↓↓	↓	↓	-	↓↓	↓↓	↓↓
Migration	↓	-	↓	↓	/	/	/	-	-	↓	-	/	/	/
Apoptosis	-	-	-	-	/	/	/	-	-	-	-	/	/	/

The biological activities of heparin such as anti-proliferation and anticoagulation rely on their interaction with proteins. This interaction can be affected by metal ion binding (Seo et al., 2011). For instance, the interaction between FGFs and their receptors is enhanced by heparin/HS which are required for the formation of functional FGF-FGFR signalling complexes (see more details in **Chapter 5**). The binding selectively is modulated by a combination of charge density, charge distribution and conformational change. Higher levels of sulphation (higher negative charge density) on heparin leads to stronger binding to FGFs via multivalent interactions (Minsky et al., 2017). Cationic ions with higher charge density contribute a stronger interaction as divalent cations are able to bind two sulphates on the polysaccharide (Rudd et al., 2007). However, some polysaccharides with similar charge densities but different sulphation patterns interact differently with FGFs (Li et al., 2016). In addition, different charge densities sometimes drive similar conformations – Fe^{3+} and Mn^{2+} -bound heparins had similar structures when studied using synchrotron radiation circular dichroism (SRCD) (Rudd et al., 2007), which may be due to their similar effective ionic radii (0.61–0.77 nm and 0.67–0.82 nm, respectively) (Bell, 1977).

The alteration of heparin-protein interactions also mediate its anti-coagulant activity

Heparin is widely used as an anticoagulant drug due to its interaction with antithrombin III and suppression of blood coagulation factors (i.e. thrombin and factor Xa) (Rubin et al., 1996). Generally, heparin electrostatically binds to positively-charged residues on coagulation-related heparin binding proteins, although hydrogen bonds and van der Waals interactions also contribute to heparin-protein interactions (Bolten et al., 2018) (see details in **Chapter 4**). These electrostatic interactions can be changed via removal of sulphation or addition of cations. N-sulphation is required for anticoagulation. N-desulphated heparin is completely unable to interact with antithrombin-III (AT-III), resulting in inactive thrombin and antithrombin, which are two of the key coagulant factors (Danishefsky et al., 1977; Garg et al., 2011b), whereas 6-O-desulphated heparin loses the activity of factor Xa inhibition (Atha et al., 1987). Factor XIIa can interact with a plethora of negatively-charged substrates (i.e. heparin), thus depletion of sulphation on GAGs reduces XIIa binding (Wujak et al., 2015). The affinity of heparin binding to coagulant factors can not only be changed by charge distribution but also by the size of heparin. Fondaparinux, a synthetic pentasaccharide drug, can bind to factor Xa, but has no activity against thrombin because the fragment (1.7 kDa) is too short to bridge antithrombin to thrombin (Holmer et al., 1981; Oosta et al., 1981). Although both anticoagulant and non-anticoagulant fragments of heparin can inhibit VSMC proliferation *in vivo* (Clowes, et al., 1985), anti-proliferative activity is unrelated to anticoagulant activity (Azizkhan et al., 1980). It has been argued that the biological activities of HS and of heparin reside in specific sequences, although

there is little experimental evidence for this (Meneghetti et al., 2015). Indeed, even the supposedly highly-specific interaction between the heparin pentasaccharide sequence (Hook et al., 1976; Lam et al., 1976) and anti-thrombin is now thought to be more somewhat more relaxed (see more details in **Chapter 4**). The effects of the same heparin analogue on HCASMCs and HCAECs can also be different. For instance, Ca-heparin inhibits HCASMC proliferation, whereas it has no effect on HCAEC proliferation. These different effects on two cell types may be caused by the distribution of growth factor receptors which activate diverse signals and results in various biological responses (see details in **chapter 5**).

Some of the chemically modified heparin analogues provide the desired activities, however, these promising activities are changed in the co-cultivation system. The value of K-heparin in the growth ratio of HCAEC/HCASMC is 1.4 in the single cell type cultivation system but it drops to 0.9 in the co-cultivation system. Interestingly, most of the cationic heparin analogues have no significant effect on HCAECs while others enhance the anti-SMC proliferative activity. Communication between VSMCs and VECs is fundamental to normal behaviour in vascular tissue and supported by considerable *in vitro* and *in vivo* evidence. Aside from paracrine signaling from ECs that controls the contractile state of SMC (for example nitric oxide signaling), other pathways control SMC growth and development, for instance, ephrin receptor tyrosine kinases (Eph), which are a large family of transmembrane proteins activated by ephrin ligand binding. This eph-ephrin axis induces endothelial-smooth muscle cell communication during vessel formation (Li et al., 2018). In addition, EC-expressed Jagged1 interacts with NOTCH3 on adjacent SMCs, followed by activation of NTOCH signalling and enhancement of NTOCH3 expression in the SMCs (Liu et al., 2009). It is also revealed in rats that damaged VSMCs switch to a synthetic phenotype and activate PKC δ and STAT3 to recruit ECs to the injury site for re-endothelialisation (Gomez and Owens, 2012; Ren et al., 2019). Furthermore, HS mediates ephrin/Eph receptor signalling by binding to ephrin-A3 in Chinese hamster ovary (CHO) cells (Irie et al., 2008) and heparin downregulates NOTCH signalling in human stromal cells (ref Laner-Plamberger, 2019). Therefore, the co-cultivation system is a better model to mimic *in vivo* circumstances.

In this chapter, we have conducted preliminary screens of the anti-proliferation and anti-migration activities of various heparin analogues on HCASMCs and HCAECs. Those analogues with promising activities, such as Na-desulphated, K-desulphated, and Ca-desulphated heparins, will be examined for their anti-thrombotic activity and coagulant activity in the following chapter.

Chapter 4: Effects of Heparin Analogues on Blood Coagulation

4.1 Introduction

Among heparin's physiologically relevant activities is the ability to influence the activity of proteins within the coagulation cascade and reduce blood clotting. Heparin is thus well-established as the major anti-coagulant in the treatment of thromboembolic diseases. Until recently, its anti-coagulant activity and the possibility (in a small, but significant proportion of users) of heparin-induced thrombocytopenia, a decrease in platelets which arises from an immune response to heparin usage (Aster et al., 2009), has tended to limit its use in other areas, including as an anti-proliferative agent. In this chapter we assess the effects of heparin analogues on blood coagulation and their potential to induce thrombocytopenia.

Damage to blood vessels triggers the process of haemostasis to stop bleeding. The process involves three distinct phases: 1) vasoconstriction of the damaged vessel to limit blood flow and loss; 2) formation of a platelet 'plug' (primary haemostasis); and 3) coagulation or blood clot formation (so-called secondary haemostasis, although primary and secondary haemostasis occur simultaneously) (Austin, 2017; Gale, 2011). A platelet plug, also known as haemostatic plug, is an aggregation of platelets in early haemostasis in response to injury. When platelets are recruited to the damage site, they start to accumulate and adhere to each other, resulting in platelet plug formation to stop bleeding (Gale, 2011). The formation of a platelet plug occurs before fibrin clot formation, which is a more permanent resolution to injury. Thus, this process is considered as primary haemostasis. Meanwhile, during coagulation, activation of coagulant factors and thrombin cause the blood to change from a liquid into a gel through the conversion of fibrinogen to fibrin which strengthens the platelet plug. These coagulant factors are named by Roman numerals according to the date of their discovery, additionally, using "-a" to indicate the activated form.

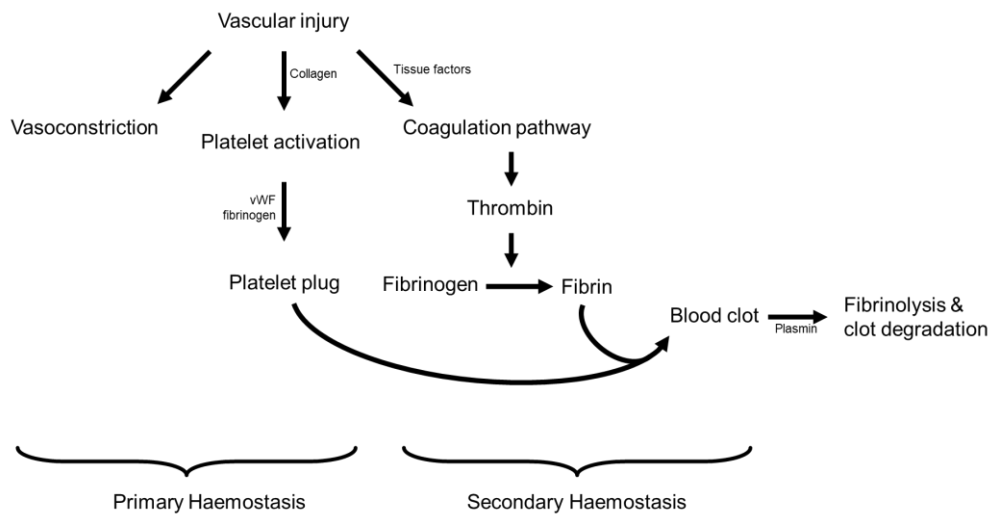


Figure 4.1. The key components of haemostasis. Haemostasis consists of four main steps: 1) vasoconstriction refers to blood vessel narrowing; 2) primary haemostasis refers to the activity of platelets that helps clot formation; 3) secondary haemostasis refers to the activity of coagulant proteins that interact with platelets to help blood clotting; 4) clot dissolution refers to the process of wound healing and degradation of clots (fibrinolysis) (Austin, 2017; Gale, 2011). Image drawn with PowerPoint.

4.1.1 Coagulation Cascade

The coagulant cascade of secondary haemostasis can be divided into the *contact activation pathway* (also known as the intrinsic pathway) and the *tissue factor pathway* (also called the extrinsic pathway). Both feed into a common pathway ending in the thrombin-dependent formation of fibrin strands (**Figure 4.2**).

Contact activation pathway

The contact activation pathway is a parallel pathway for factor XII-induced thrombin activation. This activation is mainly mediated by factor XIIa binding to collagen. The damaged vascular endothelium induces exposure of haemostatic components, such as tissue factor and collagen (van der Meijden et al., 2009). Collagen fibrils, with negative charge, are the main proteins of the extracellular matrix (Bailey and Paul, 1999). When factor XII binds to collagen, it triggers auto-activation of XII (van der Meijden et al., 2009; Wu, 2015). Meanwhile, the exposure of collagen also leads to adhesion and activation of platelets (Nieswandt and Watson, 2003), resulting in the release of Ca^{2+} from platelets (van der Meijden et al., 2009). GAGs, such as HS and

heparin, also provide negative charge that triggers factor XII activation, which is reviewed in Renne, 2012 (Renné et al., 2012; Samuel et al., 1992). The active form of factor XII activates factor XI which in turn activates factor IX (Achneck et al., 2010; Palta et al., 2014). Active factor IX (IXa) interacts with its cofactor factor VIIIa to form a tenase complex on the phospholipid surface of activated platelets, following by activation of factor X in the presence of Ca^{2+} (Achneck et al., 2010; Hemker et al., 1983; Mann, 2003). Factor X leads into the common pathway.

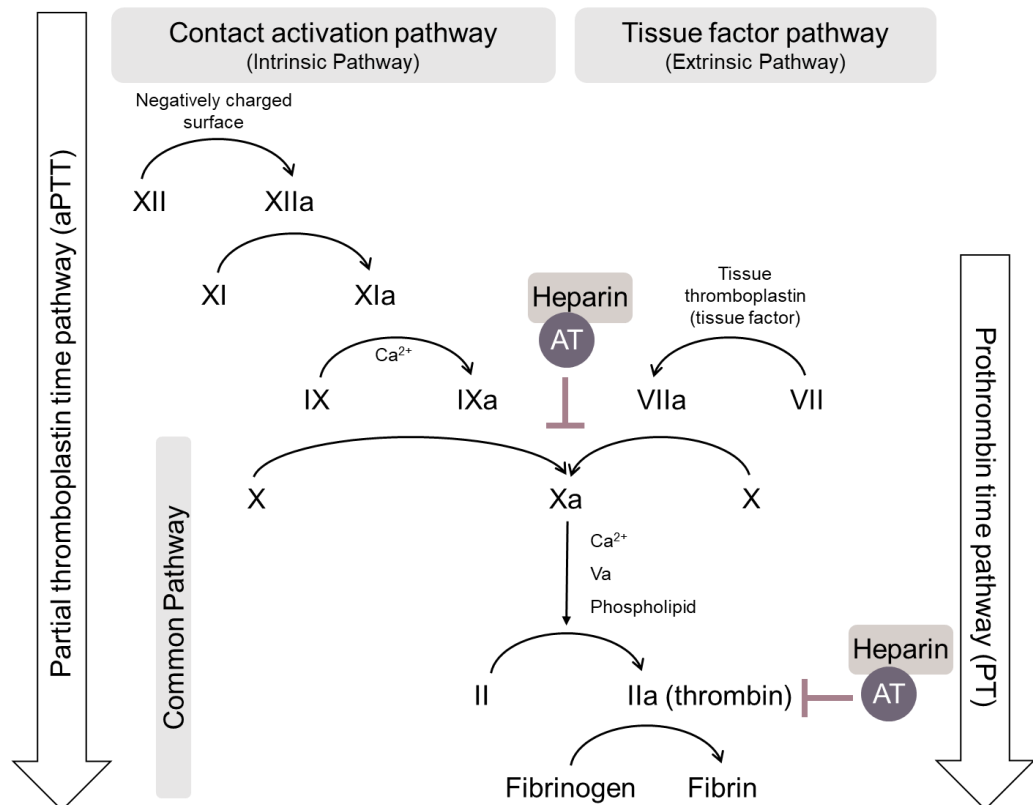


Figure 4.2. Scheme of the coagulation cascade. Coagulation can be divided into the contact activation pathway and the tissue factor pathway, both leading to the common pathway. Thrombin and factor Xa are the most sensitive to the inhibitory effect of the antithrombin/heparin complex. The inhibitory activity of antithrombin can be enhanced by heparin binding. AT, antithrombin (Oduah et al., 2016). Image drawn with PowerPoint.

Tissue factor pathway

The tissue factor pathway is considered to be the first step in plasma-mediated coagulation activated by tissue factors presented in the subendothelial tissue (Lasne et al., 2006). When the blood vessel endothelium is damaged, factor VII leaves the circulation and comes into contact with tissue factor expressed on the surface of stromal fibroblasts and leukocytes. This forms an activated tissue factor-factor VIIa complex which converts factor X to Xa (Owens III and Mackman, 2010). Factor Xa can be inhibited by linking to tissue factor inhibitor, followed by generation of tissue-factor-inhibitor/Xa complexes. These complexes can subsequently bind to tissue-factor/VIIa, leading to a larger complex (tissue-factor-inhibitor/Xa/ tissue-factor/VIIa) that simultaneously suppresses both factor VIIa and Xa (Dahm et al., 2008). In addition, protein S can enhance this interaction in the presence of calcium and phospholipid (Ezihe-Ejiofor and Hutchinson, 2013).

Common pathway

The final common pathway begins with the activation of factor X. This can be via factor IXa from the contact activation pathway or VIIa from the tissue factor pathway. Xa and its cofactors (factor Va, tissue phospholipids, platelet phospholipids and Ca^{2+}) forms prothrombinase which converts prothrombin (factor II) to thrombin (IIa) (Kumar et al., 2014; Palta et al., 2014). Thrombin, a serine protease, converts fibrinogen into insoluble fibrin and activates factor XIII, which crosslinks fibrin polymers to generate a network for clot stabilisation (Kumar et al., 2014). Antithrombin, a serine protease inhibitor (serpin), functions to inactivate thrombin and factor X. Its activity is substantially increased through interaction with heparin (Palta et al., 2014).

Table 4.1. Information of clotting factors and proteins (Palta et al., 2014).

Number	Clotting factor name	Function
I	Fibrinogen	Clot formation
II	Prothrombin	Activation of I, V, VII, VIII, XI, XIII, protein C, platelets
III	Tissue factor	Co-factor of VIIa
IV	Calcium	Facilitates coagulation factor binding to phospholipids
V	Proacclerin, labile factor	Co-factor of X-prothrombinase complex
VI	-	-
VII	Stable factor, proconvertin	Activates IX, X
VII	Antihaemophilic factor A	Co-factor of IX-tenase complex
IX	Antihaemophilic factor B	Activates X by interaction with VIII
X	Stuart-Prower factor	Prothrombinase complex with V to activate thrombin
XI	Plasma thromboplastin antecedent	Activates IX
XII	Hageman factor	Activates XI, VII and prekallikrein
XIII	Fibrin-stabilising factor	Crosslinks fibrin
XIV	Prekallikrein	Serine protease zymogen
XV	High molecule weight kininogen	Co-factor
XVI	vWF	Binds to VIII, mediates platelet adhesion
XVII	Antithrombin III	Inhibits IIa, Xa and other proteases
XVIII	Heparin co-factor	Inhibits IIa
XIX	Protein C	Inactivates Va and VIIIa
XX	Protein S	Cofactor for activated protein C

4.1.2 Anticoagulant Activity of Heparin

Antithrombin suppresses the activation of serine proteases, factor IXa, Xa, TF-VIIa and thrombin, by forming an antithrombin-protease complex that blocks substrate access to the protease's active sites (Elmisbah and Aiderous, 2018). Formation of the inhibitory complex requires interaction between the protease and an exposed reactive site loop (RSL) or reactive centre loop (RCL), on the surface of the antithrombin molecule. This inhibitory activity is increased in the presence of heparin or heparan sulphate through both the formation of stable heparin-antithrombin-protease complexes (Li et al., 2004) and through conformational changes induced in the RSL (Whisstock et al., 2000). The conformational changes involve amino acids P14 (Ser380) and P15 (Gly379) within the N-terminal region of RSL, which is called the hinge region (Desai, 2004). This conformational change in the hinge region caused by heparin binding leads to expulsion of P14 and P15 from the body of antithrombin (Whisstock et al., 2000). This allosteric system has been held to promote the activity of antithrombin to inactivate factor IXa and factor Xa. However, in the absence of heparin, the amino acids P14 and P15 on RSL are embedded in the top of beta sheet A within antithrombin (Izaguirre et al., 2014). Antithrombin can still inactivate factor Xa and IXa without heparin, but heparin enhances the inhibitory activity of antithrombin several thousand-fold (Izaguirre et al., 2014). The strongest activation of antithrombin by heparin occurs through unusual pentasaccharide sequences, based on GlcNAc6S-GlcA-GlcNS3S6S-IdoA2S-GlcNS6S- containing a scarce, central GlcNS,3S,6S residue. The conformational change of antithrombin binding to pentasaccharide is shown in **Figure 4.3** (Capila and Linhardt, 2002; Olson et al., 2002; Skinner et al., 1997).

Heparin binding to antithrombin causes a conformational change of antithrombin, leading to serpin-protease interaction (i.e. antithrombin to factor Xa) by protease binding to the extend helix D (Dementiev et al., 2013; Li et al., 2004; Olson and Björk, 1991). Specific residues on antithrombin found to be important for the interaction are the basic residues Lys 114, Lys 125 and Arg129 (Garg et al., 2011a) (Rashid et al., 2014). In addition, crystal structures of a ternary complex between antithrombin, thrombin and heparin show antithrombin and thrombin bound to the same linear heparin chain (Izaguirre et al., 2014; Olson and Björk, 1991; Petitou et al., 2003). This enables close contact between the two proteins and stabilises extensive interactions, thereby increasing antithrombin's inhibitory activity (Li et al., 2004; Olson et al., 2004). However, thermal stabilization experiments, linked to anticoagulation assays and protein secondary structure measurements using circular dichroism spectroscopy, showed that those polysaccharides with high anti-Xa activity did not necessarily induce the same conformational change in AT as heparin, rather, thermal stabilization of the

AT-polysaccharide complex correlated with anticoagulant activity (Lima et al., 2013). This indicates that conformational change is less important to anti-coagulant activity.

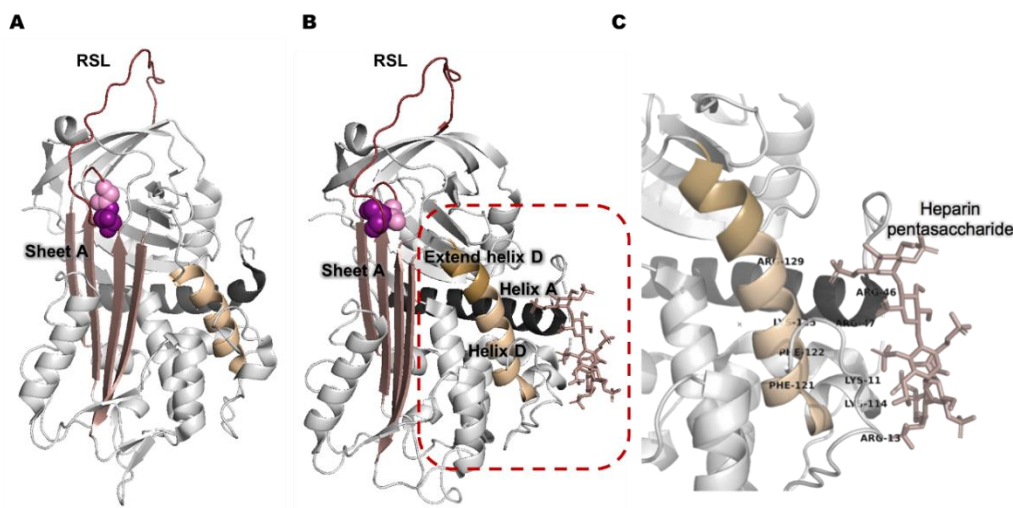


Figure 4.3. Conformational changes of antithrombin bound by heparin. Structures of (A) native (unbound) antithrombin (1E05) and (B) antithrombin in complex with heparin pentasaccharide (PDB: 1E03) are depicted in diagrammatic form. The heparin binding sites on antithrombin (C) are Lys-11 and Arg-13 in the N-terminus of antithrombin; Arg-46 and Arg-47 in the helix A (dark grey); and Lys-114, Phe-121, Phe-122, Lys-125 and Arg-129 in the region of the helix D (Beige). Heparin binding to antithrombin triggers expulsion of P14 (purple) and P15 (pink) from sheet A, enhancing the inhibitory activity of antithrombin to factor IXa and Xa (Izaguirre et al., 2014; Rashid et al., 2014). More perspectives of antithrombin structure are in **Appendix 6**. Images are drawn with PyMOL.

Although heparin is a well-known anticoagulant drug, it is found naturally in the body in secretory granules in mast cells, which regulate inflammation and immunity (Forsberg et al., 1999). It is worth noting that physiologically, the heparin produced by mast cells is not the principal activator of antithrombin. Instead, heparan sulphate, which is found extensively on the surface of vascular cells, has been proposed as the physiological mediator of the above effects (Casu and Lindahl, 2001).

4.1.3 The Influence of Modified Heparin Analogues on Antithrombin Binding

The molecular weight of the heparin chains affects its interaction with antithrombin (Cosmi, 1997). To form a ternary complex with antithrombin and thrombin, oligosaccharides of more than 18 units are required (Hirsh et al., 2001). Unfractionated heparin (UFH) and LMWH have weak ability to inactivate thrombin due to their short fragments, however, LMWH is effectively enriched with high affinity pentasaccharides able to interact with Xa (Hirsh et al., 2001). This high affinity pentasaccharide is often considered as the primary antithrombin binding site due to its direct electrostatic and hydrogen-bonding for the interaction between 3-O-sulphated groups and antithrombin (Desai et al., 1998). Furthermore, several reports revealed that net charge plays an important role in the interaction between heparin and antithrombin (Chavante et al., 2014; Lima et al., 2013; Seyrek et al., 2007). For instance, heparin-like glycosaminoglycan with higher proportions of sulphated disaccharide units show subtle differences in conformational change compared to heparin when binding to antithrombin in NMR (Meneghetti et al., 2015). The inhibitory effect of antithrombin-heparin on thrombin is dose-dependently enhanced by monovalent cations, such as Li^+ , Na^+ and K^+ (Griffith et al., 1980). In the absence of heparin, these cations can also enhance the inactivation of thrombin with antithrombin. However, with the exception of Li^+ , which has no effect, these monovalent cations may also influence the interaction of heparin and antithrombin (Griffith et al., 1980).

4.1.4 Evaluation of Coagulation

Several methods are available to assess the function of the coagulation cascade. These include measurement of activated partial prothromboplastin time (aPTT), prothrombin time (PT), thrombin time (TT), as well as the quantitative determination of fibrinogen levels. Of these, aPTT and PT assays are commonly used as a coagulant screen in clinical diagnosis (Goel and Ness, 2016). The aPTT test measures the activity of the contact activation and common pathways, and the PT test the activity of the tissue factor pathway (**Figure 4.2**). Other evaluation methods of coagulation, take ultra-specific thrombin generation tests for example, are more sensitive and can reflect the real hemostasis state of the individual patient much better, allowing an earlier diagnosis of abnormal coagulation. However, aPTT and PT assays are well established and they provide means of screening for abnormalities in intrinsic coagulation pathway factors IX, XI, XII, extrinsic pathway factors VII and common pathway factors II, V, X and fibrinogen (Oduah et al., 2016; Thomas et al., 2015).

Prothrombin time, PT: measuring activity of the tissue factor pathway

In this assay phospholipid and CaCl_2 are added to citrated plasma in the presence of tissue factor and thrombin to stimulate coagulation (Goel and Ness, 2016; Jenny et al., 2006). The time measured is from the addition of Ca^{2+} to clot formation. This is normally 11 - 14 seconds.

Activated partial prothromboplastin time, aPTT: measuring activity of the contact activation pathway

The aPTT assay measures activity of the contact activation pathway (intrinsic pathway), which is initiated by blood interaction with negatively charged surfaces. Here, phospholipid and CaCl_2 are added to citrated plasma in the presence of activators of the contact activation pathway such as micronized silica and ellagic acid or kaolin (Ignjatovic, 2013). In general, time between Ca^{2+} addition and clot formation is normally around 30 - 40 seconds.

4.1.5 Side Effects of Heparin: Heparin-Induced Thrombocytopenia

A potential side effect of prolonged heparin usage is heparin-induced thrombocytopenia (HIT), a decrease in platelets which arises from an immune response to the complex formed between platelet factor 4 (PF4) and heparin (Aster et al., 2009). It is rare (5 -15 % of patients) and transient, but it raises the mortality rate in post-surgery patients. HIT is defined as a syndrome that causes a decrease in platelet number of more than 50% and/or thrombotic complications detected 5 -10 days after vascular surgery. After surgery or infection, the activation of platelets induces PF4 release from α -granules (**Figure 4.4**). PF4 can bind to polyanions, such as heparin, or polyanions on the surface of bacteria (Greinacher, 2015), forming PF4-polyanion-complexes (Kreimann et al., 2014). PF4 binds to negative-charge-rich heparin with high affinity but it is size-dependent since small heparin fragments (less than 6 saccharides) are ineffective at bridging PF4 tetramers (Rauova et al., 2005) although a previous study claimed the smallest heparin fragment for PF4 binding is 20 saccharides (Stringer and Gallagher, 1997). In response to these complexes, activated B lymphocytes generate anti-PF4-polyanion IgGs (so called HIT antibodies), which recognise the neoepitopes on the positively-charged PF4 within PF4-polyanion-complexes (Brandt et al., 2014; Zheng et al., 2013). The Fc portion of these IgGs can also bind to Fc RIIa receptors on platelets and induce Fc receptors clustering, resulting in severe platelet activation and aggregation (Arman and Krauel, 2015; Kasthuri et al., 2012). Moreover, these immune complexes can cross-link to Fc RI receptors on

monocytes to activate them, followed by tissue factor expression on active monocytes (Cines et al., 1987). These activated platelets and monocytes stimulate thrombin formation which acts upon endothelial cells (ECs). Activated ECs then express tissue factors which enhance thrombin generation (Cines et al., 1987). Furthermore, the high levels of intravascular platelet activation and aggregation leads to a sharp decrease of platelet number and an increase in the production of platelet-derived microparticles, which accelerates thrombin formation (Greinacher, 2015).

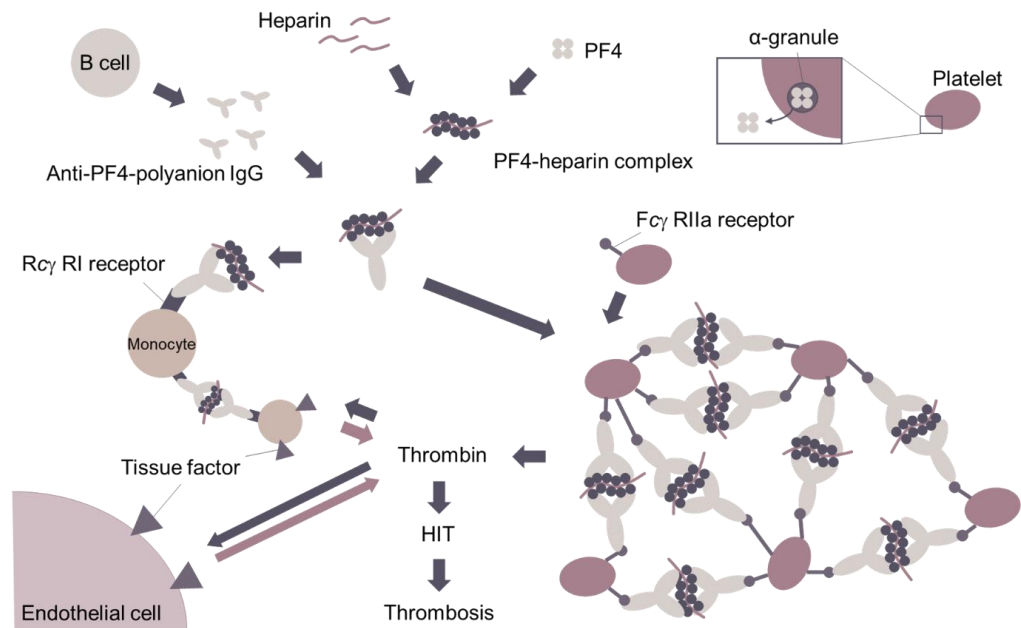


Figure 4.4. HIT is caused by IgG antibodies formed in response to complexes of PF4/heparin. It results in platelet aggregation and thromboembolic events. PF4, platelet factor 4; HIT, heparin-induced thrombocytopenia (Greinacher, 2015). Image drawn with PowerPoint.

4.1.6 Aim of This Chapter:

The aim of this chapter was to use aPTT and PT assays to assess the effects of our novel heparin analogues on blood coagulation. We also investigated the ability of the heparin analogues to form complexes with PF4 as a means of assessing their potential to induce thrombocytopenia.

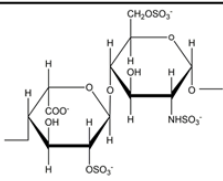
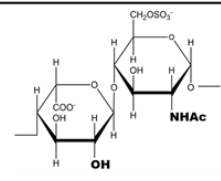
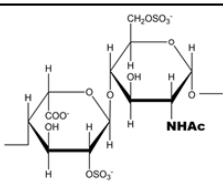
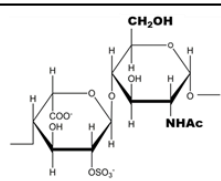
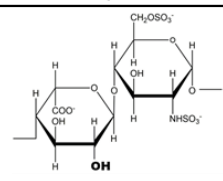
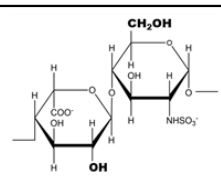
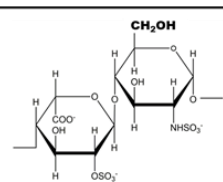
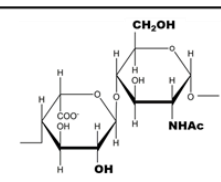
4.2 Results

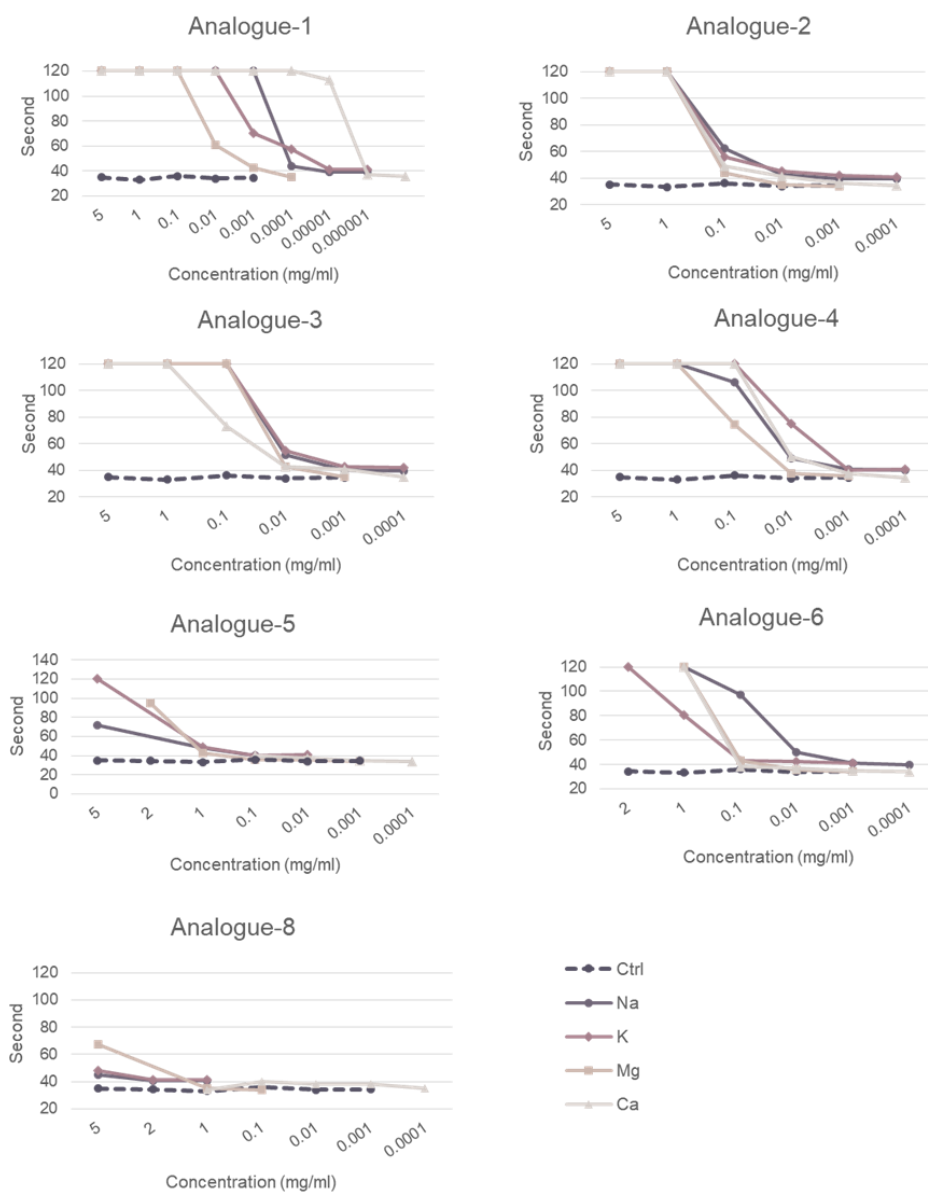
Since structural modifications to heparin result in changes in the way it interacts with growth factors/growth factor receptors (**Chapter 3**), we reasoned that it may also alter the interaction with antithrombin. Therefore, to assess whether anticoagulant activity is preserved in our heparin analogues, we used human plasma in the aPTT assay to measure the activity of the contact activation and common pathways, and the PT assay to measure activity of the tissue factor pathway. aPTT and PT assays were conducted in collaboration with Patricia Procter at the University of Keele, UK.

4.2.1 Coagulant Activity – aPTT Assay

The aPTT assay was conducted in the presence of 1mg/ml of the cation-forms of (fully-sulphated) heparin and cation-desulphated forms of heparin (see **Table 4.2**). Time to coagulation for each of the analogues and for unmodified heparin is shown in **Figure 4.5** below. A time of 32 seconds was taken as normal blood coagulation time (i.e. in the absence of heparin) (Hernaningsih and Butarbutar, 2019). The maximum assay time was set at 120 seconds. The cationic heparin analogues and cation-desulphated heparin analogues numbers 2-4 retained their anticoagulant activity, as well as the Mg-6 (cation form of the derivative number 6 (see **Figure 4.5B**) and calcium-6 desulphated forms. The anticoagulant activity of the remaining cationic forms of derivatives 5-8 was significantly reduced ($P < 0.01$). Interestingly, the analogue 1 (untreated heparin, Section 2.2) is normally in the sodium form. Thus, the results of untreated heparin and Na-1 heparin should be similar. However, our data showed the different effect between untreated heparin and Na-1 heparin in **Figure 4.5 A**. The data may indicate that the untreated heparin is somehow different from Na-1 heparin, and this needs further investigation.

Table 4.2. Desulphated heparin analogues. Images are drawn with ChemDraw 18.1.

Non - desulphated	1 		5 
Slightly Desulphated	2 	Heavily Desulphated	6 
	3 		7 
	4 	Fully Desulphated	8 

A

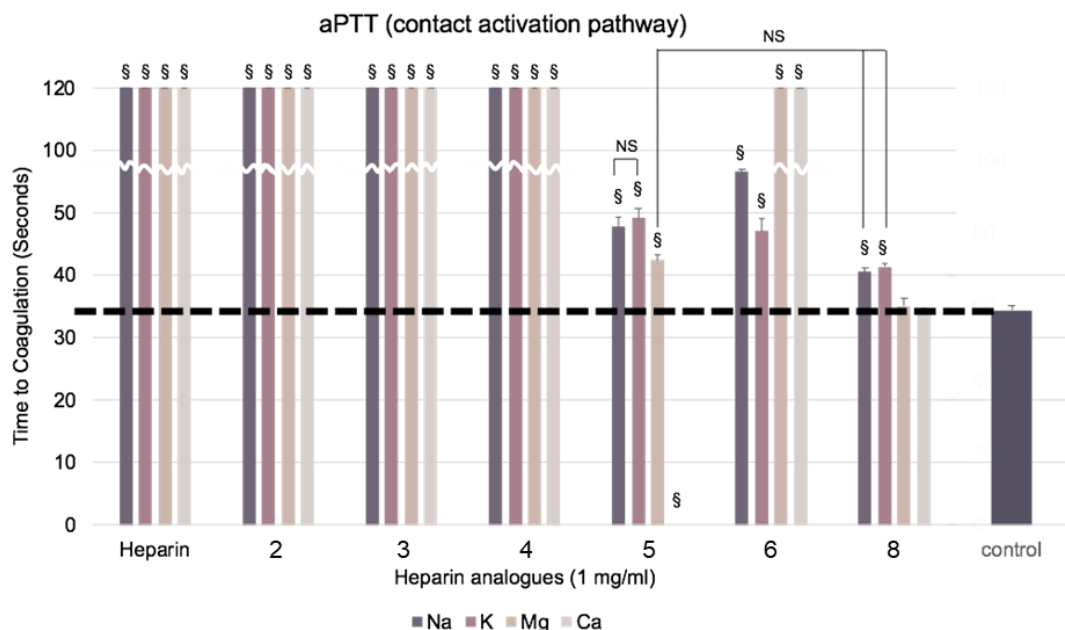
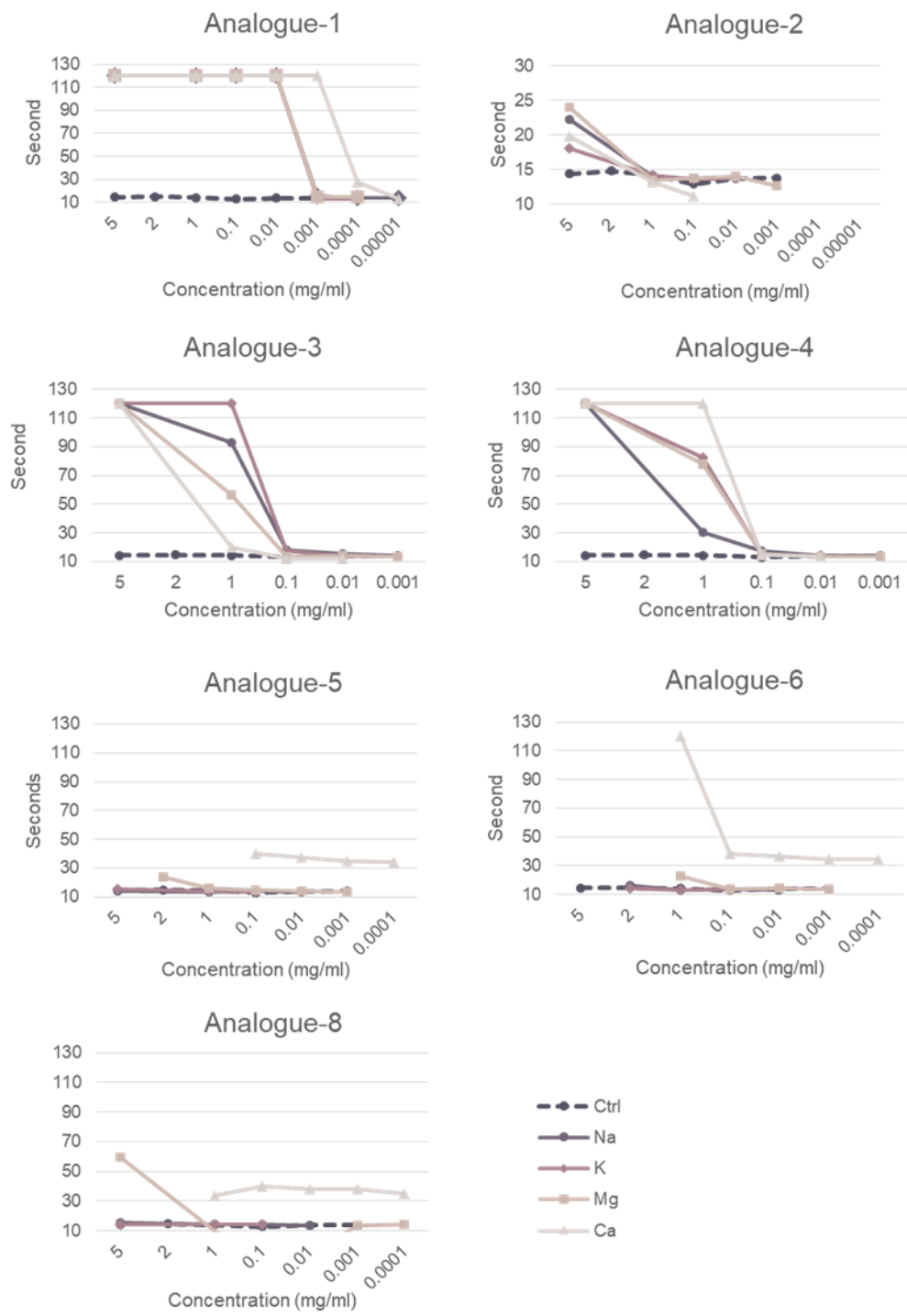
B

Figure 4.5. The anti-coagulant activity of cation-desulphated heparin analogues in aPTT assay. (A) Raw data of dose response curve for the analogues. (B) The summary histogram of the anti-coagulant activity of 1 mg/ml heparin analogues. Results were expressed as mean \pm S.D. Results from $n=3$ experimental repeats within an experiment. § $P < 0.001$ compared to control (untreated plasma) with statistical significance determined by a one-way ANOVA followed by Tukey post-hoc test. NS, no significant, $P > 0.05$. Na, sodium-binding; K, potassium-binding; Mg, magnesium-binding; Ca, calcium-binding.

4.2.2 Coagulant Activity – PT Assay

To determine effects on the tissue factor pathway, time to coagulation was also measured for the same analogues using PT assays (**Figure 4.6**). A time of 15 seconds was taken as normal blood coagulation time (i.e. in the absence of heparin). Here, anticoagulant activity was absent or significantly reduced in cation-exchanged analogues 2, 5, 6 and 8. Cation forms of naive heparin retained anticoagulant activity. Cation-exchanged analogues 3 and 4, K-3 and Ca-4, retained anticoagulant activity, while all other cation forms of these analogues showed a significant reduction in activity.

A

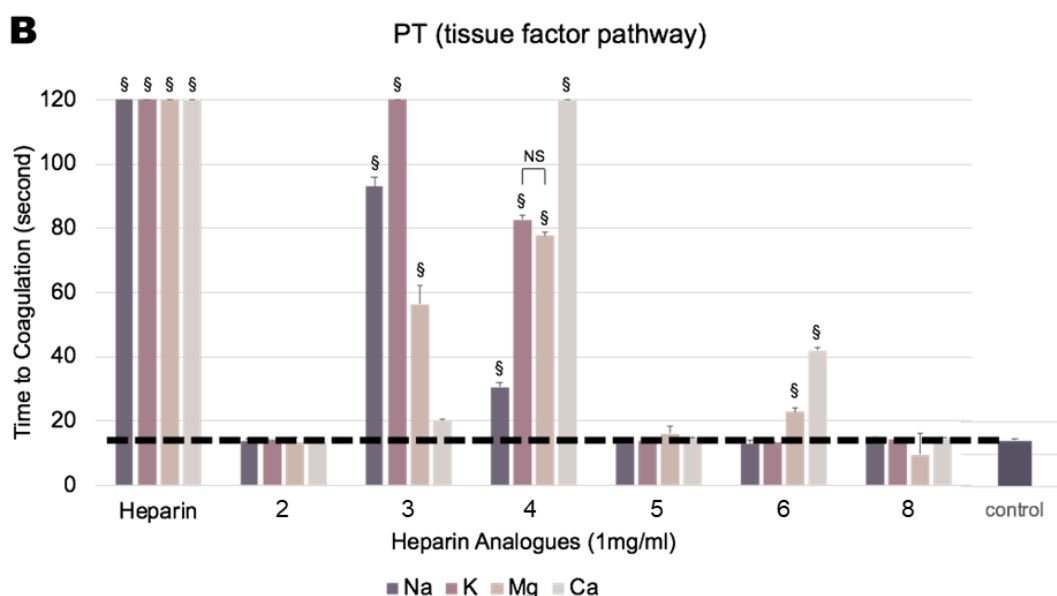


Figure 4.6. The anti-coagulant activity of cation-desulphated heparin analogues in PT assay. **(A)** Raw data of dose response curve for the analogues. **(B)** The summary histogram of the anti-coagulant activity of 1 mg/ml heparin analogues. Results were expressed as mean \pm S.D. § $P < 0.001$ compared to control (untreated plasma) with statistical significance determined by a one-way ANOVA followed by Tukey post-hoc test, $n=3$. NS, no significant, $P > 0.05$. Na, sodium-binding; K, potassium-binding; Mg, magnesium-binding; Ca, calcium-binding.

4.2.3 HIT Assay

To assess the potential of the heparin analogues to form complexes with PF4, we used native (non-denatured) gel electrophoresis to measure the size of heparin-PF4 complexes. 50 ng of heparin analogues or 50 ng unmodified heparin (positive control) were treated with 250 ng of recombinant PF4 protein at 37°C for 4 hours before electrophoresis. The native gel was constructed by stacking 5%, 10% and 20% (w/v) acrylamide gels, following by electrophoresis at 30 V for 2 hours on ice. Following electrophoresis, protein complexes of PF4 were visualised by silver staining. All the cationic forms of heparin showed the formation of PF4/heparin complexes. Na-heparin seemed to form less robust structures compared to heparin and K-, Ca-forms, whilst Mg-heparin seemed to form a large complex trapped at the top of gel.

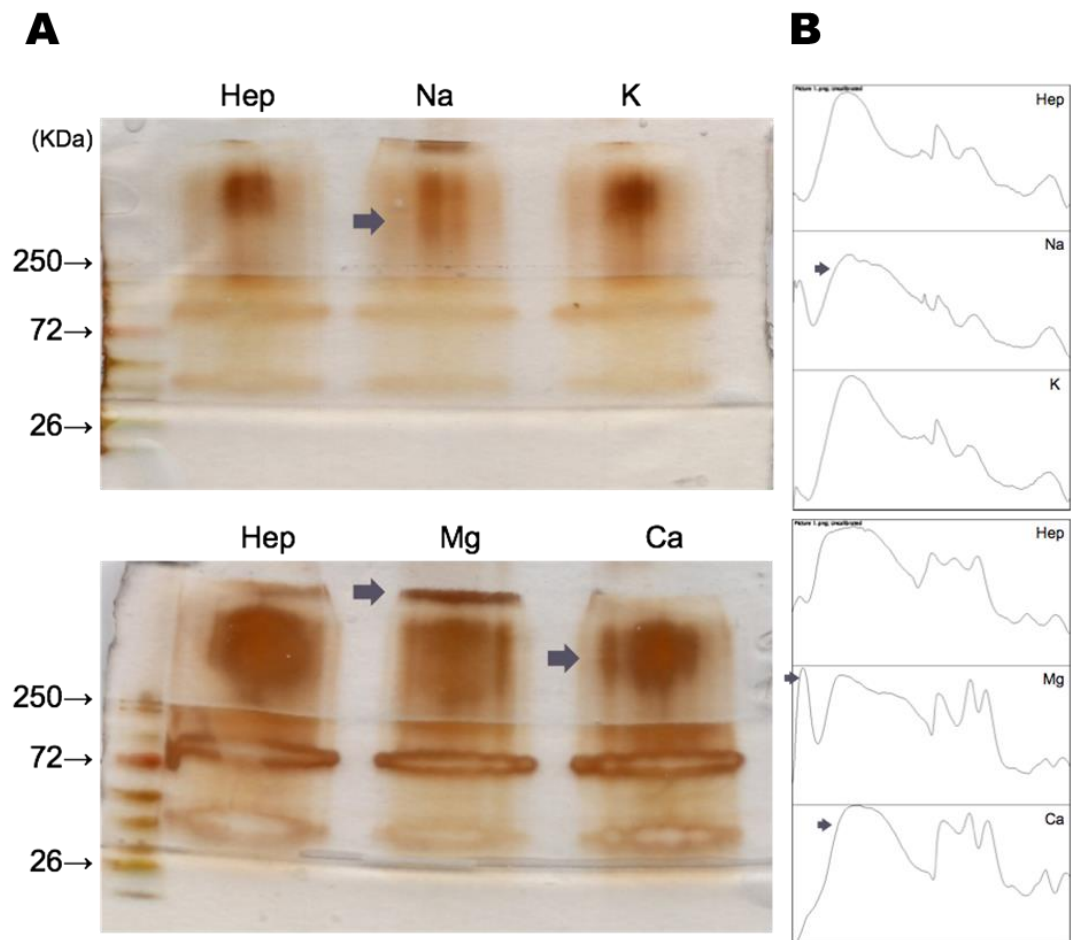
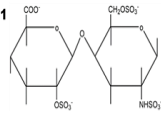
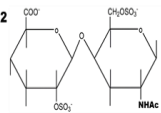
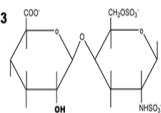
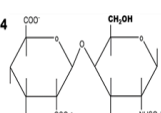
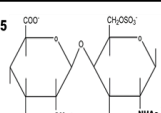
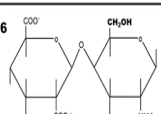
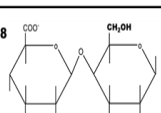


Figure 4.7. Silver stained native gels **(A)** and the densitometry of gels **(B)** showing the presence of heparin-PF4 complexes. Recombinant PF4 protein were incubated with 50 ng of heparin analogues to form PF4/heparin complexes. Arrows show the difference compared to naive heparin.

4.3 Discussion

The results in this chapter reveal that chemically modified heparin analogues have different effects on coagulation (summarised in **Table 4.3**). For example, in the aPTT assay, the fully-desulphated analogues lose their activity, whilst most of the slightly desulphated analogues retain activity which is similar to unmodified heparin. Several studies have reported that net charge affects the interaction between antithrombin and heparin which are the key mediators of anti-coagulation (Chavante et al., 2014; Lima et al., 2013; Seyrek et al., 2007). Moreover, the stabilisation of heparin-antithrombin is essential for anti-coagulant activity (Lima et al., 2013). Our PT assay results show that the anti-coagulant activity of slightly desulphated heparins are generally reduced, except for the analogue K-3 and analogue Ca-4. The aPTT assay measures effects on the contact activation pathway and common pathway, whereas PT assay measures effects on the tissue factor pathway and common pathway (see **Figure 4.2**). It may be surmised that desulphation probably affects interactions with several coagulation-relevant proteins and to different extents. Many heparin-binding serpins have been identified, such as antithrombin, protein C inhibitor, protease nexin (PN) - 1, protein Z-dependent protease inhibitor (ZPI) and heparin cofactor II (HCII) (**Figure 4.8**) (Whisstock et al., 2000). These serpins are also regulators in the coagulant cascade (Bhakuni et al., 2016). Heparin interacts with the positive residues on these serpins to activate their anti-coagulant activity. Similar to antithrombin, the region for target protease binding on serpins is on their RSL which is extended and unfolded by heparin binding (Khan et al., 2011; Whisstock et al., 2000). However, these serpins can inhibit other pro-coagulation proteases. Take PN-1 for example, its efficient inhibitory activity towards thrombin, factor Xa and factor XIa is dependent on heparin (Bhakuni et al., 2016; Knauer et al., 2000). Protein C inhibitor can act as a pro-coagulant or an anti-coagulant serpin. It regulates anti-coagulant activity by forming a ternary complex with heparin and proteases such as thrombin and factor Xa (Bhakuni et al., 2016; Neese et al., 1998). Nevertheless, heparin enhances the inhibitory effect of protein C inhibitor on factor Xa only in the presence of Ca^{2+} . This is because the exosite of factor Xa is exposed by Ca^{2+} binding to the negatively-charged domain of factor Xa where it forms a ternary complex with heparin and serpins (Bhakuni et al., 2016). Since the structures of these enzymatic and/or chemical modified analogues are changed, as well as their charge contribution (Rudd et al., 2007), their interaction with other serpins may also be altered, as well as their inhibitory activity to pro-coagulant factors.

Table 4.3. Summary of the results in aPTT and PT assay.

		aPTT (s)		PT (s)				aPTT (s)		PT (s)	
		Monovalent Cation				Divalent Cation					
Non - desulphated	<div>1</div>	Na	120±0	120±0	Mg	120±0	120±0	120±0			
		K	120±0	120±0	Ca	120±0	120±0				
Single Desulphated	<div>2</div>	Na	120±0	14±0.3	Mg	120±0	13.5±1.4				
		K	120±0	13.7±0.4	Ca	120±0	13.2±0.9				
	<div>3</div>	Na	120±0	93±2.8	Mg	120±0	56.4±5.9				
		K	120±0	120±0	Ca	120±0	20±0.4				
	<div>4</div>	Na	120±0	30.5±1.4	Mg	120±0	77.7±0.9				
		K	120±0	82.7±1.5	Ca	120±0	120±0				
Dual Desulphated	<div>5</div>	Na	47.7±1.5	13.1±0.3	Mg	42.4±0.8	16.1±2.4				
		K	49.1±1.6	13.6±0.5	Ca	0	14.5±0.4				
	<div>6</div>	Na	96.8±0.7	13.1±1.0	Mg	120±0	22.9±1.4				
		K	80.5±3.5	13.2±0.3	Ca	120±0	41.9±1.2				
Fully Desulphated	<div>8</div>	Na	40.5±0.7	14.6±0.2	Mg	35.03±1.2	9.6±6.5				
		K	41.3±0.6	14.1±0.4	Ca	33.9±0.7	14.6±0.4				
					Untreated plasma	34.5±1.0	14.4±0.7				

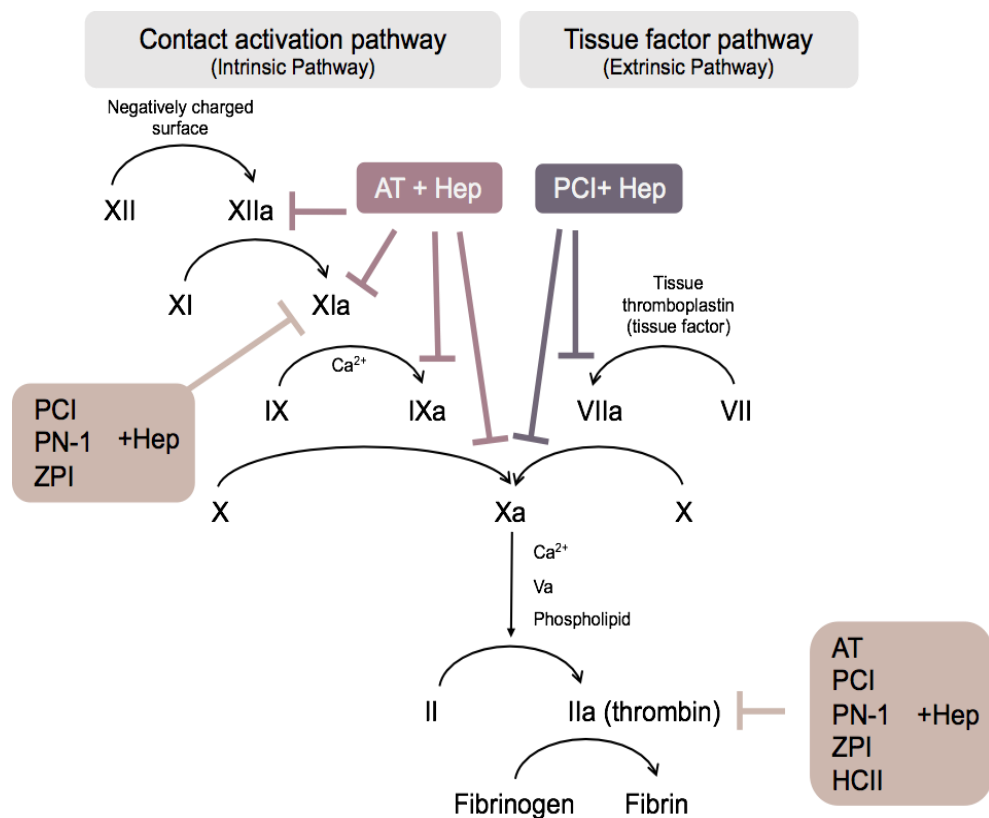


Figure 4.8. Potential regulatory roles of serine protease-heparin complexes in coagulation. AT, antithrombin; PCI, protein C inhibitor; PN-1, protease nexin -1; ZPI, protein Z-dependent protease inhibitor; HCII, heparin-cofactor II (Bhakuni et al., 2016). Image is drawn with PowerPoint.

The affinity of heparin for antithrombin may influence the inhibitory activity to factor Xa and thrombin. Heparin binds to antithrombin in a two-step process: (1) formation of a heparin-antithrombin complex with weak interaction ($K_d \sim 20 \mu\text{M}$); (2) conformational change of antithrombin to a compact and high-affinity complex with heparin ($K_d \sim 20 \text{nM}$) (Bhakuni et al., 2016; Schreuder et al., 1994). The interaction between heparin and antithrombin is regulated by ionic and non-ionic residues in helix A and helix D of antithrombin, as well as residues in the N-terminal regions (see **Figure 4.3**) (Bhakuni et al., 2016; Desai, 2004). In principle, the important heparin binding sites on antithrombin are Lys-11, Arg-13 in N terminal, Arg46, Arg47 in helix A, Lys 114, Lys 115, Arg 129 in helix D, which are positively-charged regions (Bhakuni et al., 2016; Rashid et al., 2014). The 2-O, 3-O desulphated heparin derived from UFH shows low affinity for antithrombin, resulting in low inhibitory activity to factor Xa and factor IIa (Rao et al., 2010). Therefore, it is considered that the charge of heparin is an important factor in determining its affinity to antithrombin. However, the key is complementarity between the surfaces of the protein and sugar that engage. This is

affected by the sulphation of the sugar and but its coordination of cations. Thus, it can be assumed that the affinity of our cationic and/or desulphated heparin analogues to antithrombin may also altered not only due to the presence of less negative charge, but also due to changes in the shape of the sugar surface and the position in space of groups that engage the protein.

In general, heparin mainly influences blood coagulation through interaction with antithrombin, resulting in the inactivation of pro-coagulant factors. There is some evidence showing that the antithrombin-heparin complex interacts and inhibits other factors in the coagulant cascade, such as factor Xa, factor IXa, factor XIa and factor XIIa (Onishi et al., 2016; Stead et al., 1976). Thus, although the administration of heparin interferes predominantly with the activation of factor Xa in the common pathway, it still mediates the activation of proteases in the contact activation pathway (Croizat et al., 2000). For this reason, the aPTT assay is primarily used clinically to monitor heparin therapy, although in cases of heparin overdose the tissue factor pathway may also be affected (Ichikawa et al., 2017). Clinically, the aPTT/PT test results are used to indicate deficiencies in specific coagulant factors (**Table 4.4**). Our aPTT/PT data may provide a strategy to identify which coagulant factors are affected by our analogues. In addition, it is unclear how chemical or enzymatically-modified heparin analogues interact with serine proteases other than antithrombin to inactivate coagulant factors such as factor VIIa, IXa, XIa and XIIa (Bhakuni et al., 2016). However, our data suggests that the heparin analogues have distinct effects on anti-coagulation which may be caused by the alteration of affinity to various serpins that suppress different coagulant factors.

Table 4.4. The interpretation of aPTT/PT results in clinical treatment (Thomas et al., 2015).

Description		
PT ↑	aPTT –	Deficiency of factor VII
PT –	aPTT ↑	Deficiency of VIII, IX, XI, XII; in the presence of heparin
PT ↑	aPTT ↑	Inhibition of factor Xa, prothrombin; overdose of heparin
PT ↓	aPTT ↓	Risk of thrombin formation ↑

The molecular weight (chain length) of heparin analogues affects the anti-coagulant activity via interactions with coagulant factors other than antithrombin. For instance, Fondaparinux, a synthetic heparin pentasaccharide, has been developed for clinical

treatment as a highly selective anticoagulant. It inhibits activation of factor Xa, yet has no activity against thrombin (factor IIa) because its sequence is too short to bridge antithrombin and thrombin (Cheng, 2002; Holmer et al., 1981; Oosta et al., 1981; Thomas et al., 2015). In addition, different molecular weight LMWHs exhibited different levels of factor-Xa/factor-IIa inhibitory ratios (Gerotziafas et al., 2007; Thomas et al., 2015).

Table 4.5. Different LMWHs show diverse anti-Xa/XIIa ability (Thomas et al., 2015).

<i>Agent</i>	<i>Molecular weight (k.Da.)</i>	<i>Anti-Xa/IIa (anti-Xa IU/mg)</i>	<i>Ratio</i>
<i>Unfractionated heparin (UFH)</i>	15	193/193	1
<i>Tinzaparin</i>	6.8	90/45	2
<i>Dalteparin</i>	6.0	130/52	2.5
<i>Enoxaparin</i>	4.2	100/25	3.9
<i>Fondaparinux</i>	1.7	930/0	-

Heparin can also interact with PF4, causing a severe immune reaction. Our data (**Figure 4.7**) indicates the formation of PF4-heparin complexes with four cationic heparin analogues, Na⁺, K⁺, Ca²⁺ and Mg²⁺. Native gel electrophoresis suggest that Na-heparin in particular caused slightly less aggregation than unmodified heparin. Since the heparin binding site on PF4 contains positively charged Arg-20, Arg-22, His-23, Thr-25, Lys-46 and Arg-49, which regulate the electrostatic interaction between heparin and PF4 (Mayo et al., 1995; Rao et al., 2010), it is considered that the affinity of PF4 binding to heparin depends largely on how “negatively charged” the saccharide is. In addition, heparin binding to PF4 also depends on the size of heparin. The shorter heparin chains (less than 6 saccharides) are insufficient to bridge PF4 tetramers (Rauova et al., 2005; Stringer and Gallagher, 1997). PF4 presents high affinity for negatively charged molecules and can also interact with low-sulphated glycosaminoglycans, such as HS, with low affinity (Nader, 1991; Rauova et al., 2006). Therefore simplistically, the generally lower levels of formation of PF4/heparin complexes in our Na-heparin and K-heparin data may be because the monovalent cations on heparin analogues neutralise some of the negative charge from original heparin, decreasing the interaction with PF4. Unexpectedly however, the Ca-heparin analogue, which carries less negative charge and should thereby lead to less

aggregation compared to monovalent cationic heparin analogues, had a similar effect on PF4-heparin accumulation as unmodified heparin. It may indicate the occurrence of the cation-induced conformational change of PF4-heparin complex because of the charge distribution on heparin altered by cation binding. Moreover, the other divalent cationic heparin – Mg-bound heparin, exhibits a larger PF4-heparin complex compared to the other heparin analogues. The divalent cations – Mg^{2+} and Ca^{2+} exhibit stronger coordination with the heparin as the negative charged sulphation or carboxylation groups from heparin can replace two of these oxygens from water when they bind the ion (Seo et al., 2011). In addition, hydration also leads to geometry changes, especially for carboxylate and sulphate groups. Divalent cations have a greater impact on saccharide dehydration than monovalent ones (Teychené et al., 2018). As a consequence, the interaction between divalent cations and heparin causes an overall compaction of structure (**Figure 4.9**). Since cation-bound heparin displays a relatively compact sugar chain, it may affect PF4 binding. Some reports observed that amino acids Thr-25 and Asn 47 on PF4 provide hydrogen bonds to heparin (Mayo et al., 1995). Hydrogen bonds are proposed between the sulphonate ($NHSO_3^-$) proton and the 3-O-sulphated group of the 3,6-O-sulphated N-sulfoglucosamine residue, and an additional hydrogen bond in the C3-OH groups of glucuronic acid and 2-O-sulphation of iduronic acid residues in the pentasaccharide (Beecher et al., 2014; Mayo et al., 1995). Furthermore, 2-O sulphation of iduronic acid of heparin is required for PF4 binding (Rao et al., 2010). As a result of these studies, it is speculated that hydrogen bonding may be involved in divalent cationic interaction with PF4. However, there is not enough information to explain the interaction between PF4 and the divalent cationic heparins.

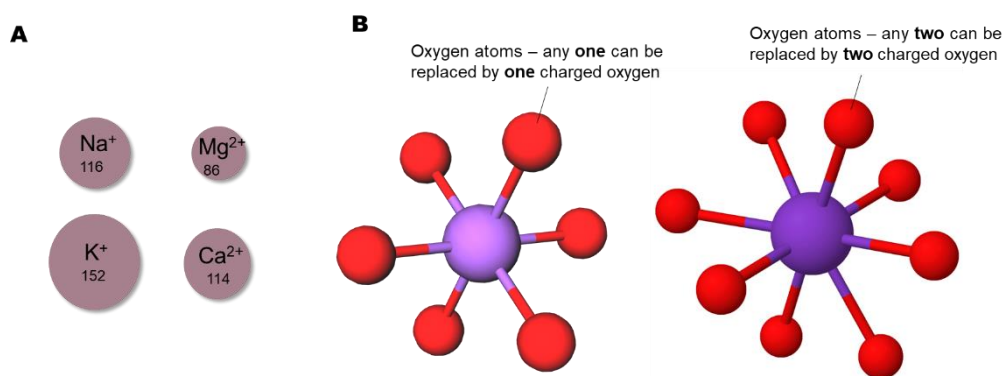


Figure 4.9. Relative size of Na^+ , K^+ , Mg^{2+} , Ca^{2+} and their typical coordinations. (A) Sizes of atoms and ions in pm. (B) Typically, monovalent cation shows six-fold coordination (left) and eight-fold coordination for divalent cations (right). Each cation is typically coordinated by either six oxygen atoms (usually from water) for Na^+ and K^+ , or eight oxygen atoms for Mg^{2+} and Ca^{2+} . Images are drawn with MolView (<http://molview.org/>).

PF4 is a tetrameric chemokine with four cysteines which forms two disulphide bonds. Both X-ray and NMR studies reveal that all of the monomeric subunits in tetrameric PF4 consist of a region of extended loops (N-terminal regions) followed by three-stranded antiparallel β -sheet domains with α -helix laying across (Huynh et al., 2019; Zhang et al., 1994). The tetrameric structure of PF4 (assembled by monomers A, B, C and D) is stabilised by a salt bridge within the AB/CD interface and by the antiparallel β -sheet-type hydrogen interaction between AC/BD dimers (Zhang et al., 1994). There are three interactions involved in this energy stabilisation: electrostatic interaction, hydrogen bonding, and van der Waals forces. The dominating energetic forces are provided by electrostatic interaction and hydrogen bonding (Zhang et al., 1994). In addition, the tetrameric structure of PF4 is essential for formation of the large heparin-PF4 complexes that HIT antibodies recognise. However, the anti-PF4/heparin antibodies can also identify PF4 in complex with polyanions (Greinacher, 2015). When PF4 binds to heparin, the structure is changed, exposing the neoepitopes which leads to anti-PF4/heparin antibody production (Kreimann et al., 2014). In addition, the conformational change of PF4 binding to heparin requires energy, which is provided by two PF4 tetramers clustering together (Huynh et al., 2019). PF4 has strong positive charge and the repulsive force can be neutralised by the negative charge of long chain heparin (Greinacher and Delcea, 2015; Kreimann et al., 2014). When two PF4 molecules approach each other, two clouds of positive charges fuse into one cloud. This process releases energy for conformational alteration of PF4, resulting in HIT antigen exposure (Sauerborn et al., 2013). In summary, charge alteration may mediate the affinity of heparin to other proteins and influences the interaction within heparin-activated complexes. This conformational change then leads to different biological responses.

In this chapter, we have accessed the anti-coagulant and anti-thrombotic activities of various heparin analogues. Our results suggest that the structural modification of heparin changes their activities in biological events and it is conceivable that these alterations will associate with cell signalling. Therefore, we will investigate the expression of growth factor receptors and assess how these modified analogues influence cell signalling in the next chapter.

Chapter 5: Elucidating Signalling Effects of Heparin Analogues on HCASMCs and HCAECs

Heparan sulphate (HS) belongs to the glycosaminoglycan (GAG) family and is found on the cell surface and in the extracellular matrix attached to proteins to form heparan sulphate proteoglycans (HSPGs). HS regulates cell signalling by interacting with a large number of extracellular proteins, including growth factors and growth factor receptors, known collectively as the heparan sulphate interactome (Beamish et al., 2009; Gu et al., 2010; Ori et al., 2008). Heparin and its analogues, which are more sulphated compared to HS, act as a proxy for HS and interact with proteins by binding to specific amino-acid sequences via both ionic interaction and non-ionic interaction (Garg et al., 2011a; Olczyk et al., 2015; Thompson et al., 1994a). In general, heparin-binding proteins comprise regions of positively-charged basic residues, such as lysine, arginine and histidine, whereas heparin contains a highly negative charge density. Many studies have revealed that these basic residues determine the properties of heparin binding, with arginine showing the highest affinity for heparin and histidine the lowest (Rudd et al., 2017). In addition to basic residues, hydrophobic residues, which are usually next to individual basic amino acids, are also essential for heparin-protein stabilisation because they can contribute non-electrostatic interactions (such as hydrogen bonding and van der Waals bonds) (Bolten et al., 2018) and therefore provide binding energy (see energy of binding in **Table 5.1**), for example in the case of heparin-thrombin and heparin-antithrombin (Fromm et al., 1995; Rudd et al., 2017). In the case of GF-GFR-heparin complexes, the negatively-charged regions on heparin can bind to cationic sites on proteins, resulting in conformational change (i.e. FGF-FGFR-heparin) and modulation of downstream signal transduction (Beamish et al., 2009; Rifkin and Moscatelli, 1989; Zhang, 2010). For the formation of heparin-serpin complexes, non-ionic interactions such as hydrogen bonding, hydrophobic interactions and van der Waals provide additional binding force in addition to ionic interactions (Bolten et al., 2018; Thompson et al., 1994a). Since it has been reported in FGF2-heparin binding, non-electrostatic interactions may also be involved in the formation of other GF-GFR-heparin complexes (Raman et al., 2003). In the above analyses, the energetic contribution of the non-covalent interactions of protein and sugar have not been separated from that of conformational change in the protein induced by sugar binding. The latter will account for most of the free energy change associated with hydrophobic bonding and likely for a good proportion of the hydrogen bonding.

Table 5.1. The energy of non-covalent binding (Campbell and Farrell, 2008).

Type of bond	Energy	
	(kJ mol ⁻¹)	(kcal mol ⁻¹)
<i>Ionic interaction</i>	> 85	> 20
<i>Hydrogen bond</i>	20	5
<i>Hydrophobic interaction</i>	4-12	1-3
<i>Van der Waals interaction</i>	4	1

5.1 Interaction between Heparin and GF-GFRs

Several growth factors and their receptors interact with heparin/HS to regulate the downstream signalling, resulting in different biological responses.

Fibroblast growth factors (FGFs)

FGFs represent a family of 23 heparin-binding growth factors that activate cell proliferation by binding to their receptor and HS. The HS/heparin interaction is required for FGF-FGFR complex stabilizing to modulate biological function (Guglieri et al., 2008). Specific patterns along the HS chains have been identified as important in the formation of FGF-FGFR-HS/heparin complexes: 6-O-sulphation is required for interaction with FGF1, FGF4, FGF7, FGF10 and PDGFs (El Masri et al., 2017), 2-O-sulphation has high affinity with FGF2, whereas both 2-O- and 6-O-sulphation are required for FGF4 and FGF7 binding (Ashikari-Hada et al., 2004; El Masri et al., 2017). However, later research using an assay that doesn't depend on electrostatic interactions showed that FGF4 has a preference for 2-O sulphation and N-sulphation and less so for 2-O or N-sulfation paired with 6-O sulphation (Li et al., 2016). FGF8 has weak binding with two types of octasaccharide, which are an oligosaccharide composed of HexA(2S)-GlcNSO₃(6S) and an oligosaccharide composed of HexA-GlcNSO₃ (Ashikari-Hada et al., 2004). In addition, the sequence domain between β -strand 10 and 12 of FGFs encoded in exon 3 is important for the interaction of FGFs with the heparin-FGFR complex (Luo et al., 1998). The nine residues between β -strand 10 and 12 represent a XBXXB pattern (X, any residue; B, basic residue; G, glycine) which are called the "glycine box" (Ashikari-Hada et al., 2004; Luo et al., 1998). The glycine box (positive charge) commonly exists in the FGF family (Ashikari-Hada et al., 2004) (Table 5.2), but can only be considered to contribute in part to the canonical, primary, heparin binding sites of the FGF family (li al al 2016)

Table 5.2. Essential O-sulphation for FGF binding. The red square represents the

O-sulphation requirement	GF	Heparin binding regions															
2-O-sulphate	FGF2	118	L	K	R	T	G	Q	Y	K	L	G	S	K	T	G	P
6-O-sulphate	FGF10	180	L	N	G	K	G	A	P	R	R	G	Q	K	T	R	R
2-O-sulphate or 6-O-sulphate	FGF18	153	F	T	K	K	G	R	P	R	K	G	P	K	T	R	E
2-O-sulphate and 6-O-sulphate	FGF4	181	L	S	K	N	G	K	T	K	K	G	N	R	V	S	P
	FGF7	167	L	N	Q	K	G	I	P	V	R	G	K	K	T	K	K
	FGF1	111	L	K	K	N	G	S	C	K	R	G	P	R	T	H	Y
	FGF8	171	F	T	R	K	G	R	P	R	K	G	S	K	T	R	Q

glycine box, which is defined as a dominant sequence (XBXGXXBBG) for FGF binding to the heparin-FGFR complex (Ashikari-Hada et al., 2004; Luo et al., 1998).

FGFRs are canonical receptor tyrosine kinases consisted an extracellular ligand binding domain (with three subunits – domain 1 (D1), domain 2 (D2) and domain 3 (D3)), a transmembrane helix and an intracellular domain (Johnson and Williams, 1992; Pomin, 2016). Mechanistically, the conformation and charge distribution of cation forms of heparin affects the affinity and/or the structure of FGF-FGFR-heparin complexes (Guimond et al., 2009; Rudd et al., 2007). With different subtypes of FGF ligand and different FGFRs, there are diverse conformations of FGF-FGFR-heparin complexes and, FGF1-FGFR2-heparin and FGF2-FGFR1-heparin are widely known to have a 2:2:1 (asymmetric) and 2:2:2 (symmetric) stoichiometry, respectively (Pellegrini, 2001; Pellegrini et al., 2000) (**Figure 5.1**). Oligosaccharide sequences with a certain sulphate group pattern can also form a temporary 1:1:1 complex by cross-linking to the ligand and receptor (Pellegrini, 2001), followed by formation of 2:2:2 complex. Thus, the FGF-FGFR-heparin complexes produced by different ligands, different receptors and different polysaccharides (HS or heparin) lead to different conformations which can therefore alter the biological outcome. In the symmetric FGF-FGFR model, the dimers are regulated by the protein-protein contacts between two adjacent FGF-FGFR complexes, and interaction is enhanced through the interaction between heparin/HS and FGF-FGFR, and the FGF interaction from D2 of the other FGF-FGFR half. Moreover, the individual heparin binding domains on these two FGFs and FGFRs are associated into one heparin binding region where two heparins unite, enhancing the affinity of heparin with FGF-FGFR (Mohammadi et al., 2005; Pellegrini, 2001; Pellegrini et al., 2000). On the other hand, there is no direct protein-protein contact between the two FGF-FGFR complexes in the asymmetric model. The dimer

is formed solely by the ability of heparin to dimerise the FGFs (Pellegrini et al., 2000). However, the difference of biological functions between these two structures remains unknown.

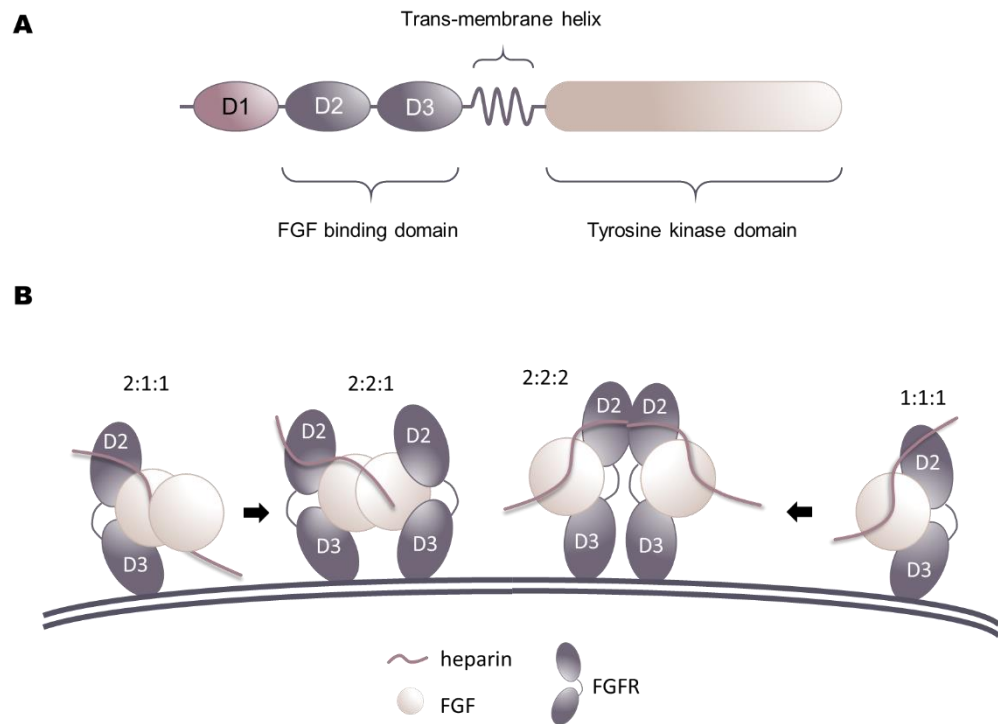


Figure 5.1. The structure of FGFR and the scheme of FGF-FGFR-heparin complexes. **(A)** A FGFR consists a ligand binding domain, a transmembrane and a tyrosine kinase domain. **(B)** There are two ternary FGF-FGFR-heparin models. FGF dimerization produces a stable 2:1:1 complex prior to receptor dimerization. Heparin induces FGF-FGF dimerization and leads to an asymmetric 2:2:1 complex. An 1:1:1 complex in the symmetric model recruits another 1:1:1 ternary complex via receptor dimerization, resulting in a biologically active symmetric 2:2:2 complex that activates FGF-dependent signalling (Pomin, 2016). Images are drawn with PowerPoint.

Vascular endothelial growth factors (VEGFs)

HS/heparin also influences the receptor binding of VEGFs. There are four isoforms of VEGFA, VEGF₁₂₁, VEGF₁₆₅, VEGF₁₈₉ and VEGF₂₀₆, sharing the same receptor binding region. VEGFA binding to HS, as for all heparin-binding proteins, controls its diffusion and, in the case of VEGF, provide spatially restricted stimuli which trigger vascular formation (Ruhrberg et al., 2002). VEGF₁₆₅, VEGF₁₈₉ and VEGF₂₀₆ bind with increasing affinity to heparin, which means they form increasingly steep gradients from their source cell. Binding to heparin augments the affinity for their receptors, forming, analogously to FGFs, a VEGFA-VEGFR2-heparin ternary complex, which enhances

VEGF signal transduction in endothelial cells (Jakobsson et al., 2006; Keck et al., 1997). In addition, to heparin enhancing the interaction of VEGF₁₆₅ with VEGFR2 it also binds to VEGFR1, neuropilin (NRP)-1 and NRP-2, which are considered as co-receptors that increase the stability of the VEGF-VEGFR complex (Xu et al., 2011). The VEGF dimer requires more than just sulphation on heparin/HS, such as sulphation on 2-O, 6-O and N position to support VEGF binding (Ferrerias et al., 2012). Thus, N-sulphation and 6-O-sulphation on heparin are required for VEGF₁₆₅ to interact with VEGFR2 while 6-O-sulphation and 2-O-sulphation are required for heparin to bind to VEGFR1 (Teran and Nugent, 2015). However, VEGF₁₆₅ is unable to bind to 2-O-sulphated or 6-O-sulphated groups on octasaccharides although it can bind to these groups on native heparin (Ashikari-Hada et al., 2004). This result indicates that a longer binding domain is required for VEGF₁₆₅ binding, consistent with a single sugar chain engaging both heparin binding sites in the VEGF dimer. While VEGF₁₂₁ is unable to interact with heparin due to the lack of a heparin binding domain (Keck et al., 1997), HS and heparin can still regulate the interaction between VEGF₁₂₁ and VEGFR1 by binding to the receptor without binding to the ligand (Cohen et al., 1995; Jakobsson et al., 2006). Nevertheless, VEGF₁₂₁ and VEGF₁₆₅ binding to VEGFR1 is reduced in the presence of exogenous heparin (Krilleke et al., 2009). Furthermore, both HS and heparin are unable to mediate the interaction between VEGF₁₂₁ and VEGFR2 (Gitay-Goren et al., 1996; Teran and Nugent, 2015). The heparin-binding VEGFA complexes have additional functions beyond the endothelium, supporting endothelial cell survival and proliferation (Krilleke et al., 2009).

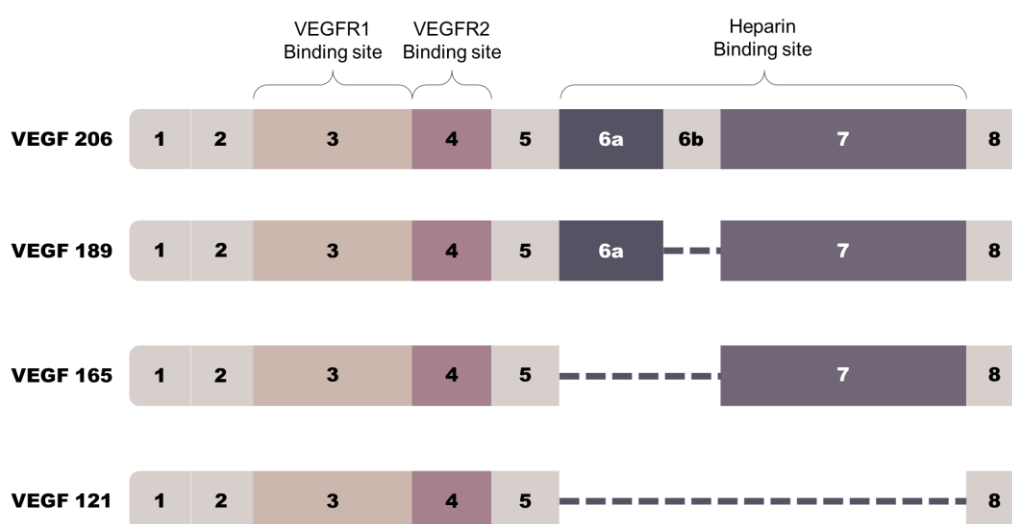


Figure 5.2. Scheme of human VEGFA isoforms depicted as monomers. VEGF₁₂₁, without heparin binding domain, is unable to bind to heparin. Exon domains are not drawn to scale (Keck et al., 1997; Krilleke et al., 2009). Images drawn with PowerPoint.

Epidermal growth factors (EGFs) and EGF-like growth factors (HB-EGFs)

The binding of heparin with epidermal growth factor (EGF)-like growth factors (HB-EGFs) and EGF receptors (EGFRs) is found on smooth muscle cells. Binding of ligands, EGFs and HB-EGFs, to EGFRs cause receptor homo- or hetero- dimerization and auto-phosphorylation (Schreier et al., 2013; Tzahar et al., 1996). The proliferative activity of EGFs and HB-EGFs promotes SMC and macrophage proliferation and migration and atherosclerotic plaque formation (Lamb et al., 2004; Miyagawa et al., 1995), moreover, HB-EGF has high affinity for EGFRs and enhances cell division and migration (Vinante and Rigo, 2013). Unlike EGF and α TGF, HB-EGF, like the majority of the EGF family, is a heparin-binding protein. The heparin binding site on HB-EGF is primarily located at the N-terminal of the EGF-like domain which contains three sequences for heparin recognition that do not match the XBBBXXBX and XBBXXBX sequences often used to predict such sites, that were derived from collagen (Besner et al., 1992; Thompson et al., 1994b). Ligands from the EGF family share amino acid sequences (around 40 – 45 residues) containing six cysteine residues, which are considered the EGF-like domain, leading to formation of a CX₇CX₄CX₁₀CXCX₈C consensus sequence (Raab and Klagsbrun, 1997). In addition to the 21-amino acid extension from amino acid 93 to 113 in the heparin binding domain, the sequence KRKKK₉₃₋₉₇, KKR₁₀₃₋₁₀₅ and RKYK₁₁₀₋₁₁₃ are the most able to interact with heparin (Takazaki et al., 2004). The HSPG on the cell surface can regulate the activity and the binding of HB-EGF. When the amount of HSPGs on SMCs is reduced, the activity of HB-EGF is decreased to the level of EGF and TGF α (Raab and Klagsbrun, 1997). HB-EGF activates EGFR1 and EGFR4 with binding to HSPGs on the cell surface, however, some reports show that it also activates EGFR2 via receptor transactivation (Holbro and Hynes, 2004). The EGFR signalling transduction can also be activated by cross-talk with non-tyrosine kinase receptors such as G protein-coupled receptors (GPCRs), which trigger HB-EGF binding to EGFRs (Liebmann, 2011; Schreier et al., 2013).

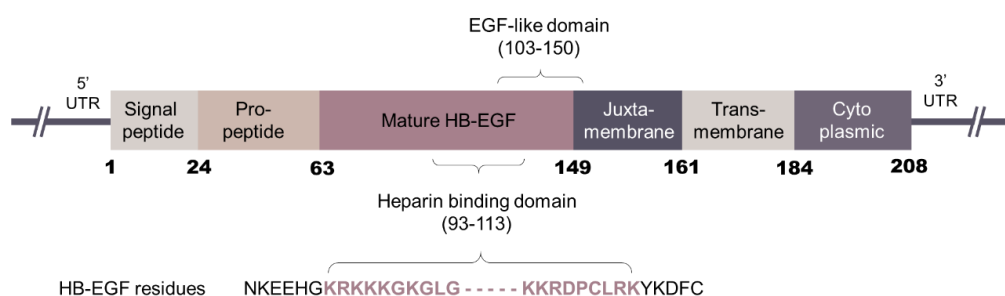


Figure 5.3. Basic structure of the transmembrane form of HB-EGF. The mature region contains a hydrophilic domain and an EGF-like domain. The hydrophilic domain holds the cationic (basic) sequences, involved in interactions with heparin and HSPGs. The

heparin-binding domain of HB-EGF (in bold and coloured) is rich in basic amino acid residues - arginine (R) and lysine (K). Exon domains are not drawn to scale (Paizis et al., 1998; Verstovsek et al., 2017; Vinante and Rigo, 2013). Image drawn with PowerPoint.

5.2 Cell Signalling in Proliferation

Ultimately, the effect of a heparin derivative on the proliferation of a cell type will depend upon the interaction between the derivative, growth factors (GFs) and growth factor receptors (GFRs). The affinity of protein binding to heparin affects subsequent signal transduction. This depends on the charge and conformation of heparin derivatives, which can be altered by chemically modified desulphation or cation binding. In the context of this project it is therefore important to understand the complex roles played by GFs-GFRs in both smooth muscle cells (SMC) and endothelial cells (EC).

Growth factor receptors associated with vascular hyperplasia

SMC proliferation and migration plays a key role in atherosclerosis and/or restenosis. The VSMC switch from quiescent contractile smooth muscle to the proliferative (synthetic) phenotype, which is associated with plaque stability, is mediated by several growth factor receptors, including fibroblast growth factors receptors (FGFRs), platelet-derived growth factor receptor β (PDGFR β) and insulin-like growth factor-1 receptor (IGF1R) (Alexander and Owens, 2012; Cai et al., 2015; Chen et al., 2016a; Jan et al., 2011; Owens et al., 2004). In contrast, activation of transforming growth factor beta receptors (TGF β R) can switch synthetic cells back to contractile state to inhibit hyperplasia (Chen et al., 2016a). The situation is complicated by cross-talk between the signalling pathways (**Figure 5.4**).

Platelet-derived growth factor receptor B (PDGFRB)

Both PDGF-BB and PDGFRB are essential in neointimal hyperplasia as they regulate VSMC migration and proliferation (Andrae et al., 2008; Raines, 2004). The binding of PDGF-BB to PDGFRB induces VSMC migration through phosphorylation of Src and activation of the focal adhesion kinase (FAK) pathway (Son et al., 2014), and stimulates VSMC proliferation via Erk1/2 MAPK and Akt/mTOR pathways (Choudhury et al., 1997; Choudhury et al., 2006; Silvestre-Roig et al., 2013). However, overexpression of FAK inhibits PDGF-BB-induced VSMC proliferation and migration (Taylor et al., 2001). PDGFRB pathway activation is followed by leukocyte

accumulation and advanced plaque formation in mice (He et al., 2015). Many growth factors and cytokines are released by invading inflammatory cells. PDGFs are secreted from those inflammatory cells and also endothelial cells at the site of injury, attracting VSMC migration to the intima and proliferation (Andrae et al., 2008). PDGF-BB facilitates VSMC proliferation, in contrast, blocking the PDGFR pathway causes significant inhibition of VSMC proliferation. The downstream signalling of PDGFRs includes Ras/Erk, Src, phosphoinositide-3 kinase (PI3K)/Akt, phosphatases C γ and JAK/STATs which are important for VSMC proliferation. Heparin inhibits VSMC proliferation via PDGF-induced, but not EGF-induced Erk activation (Pukac et al., 1998). It is suggested that other heparin-binding growth factors may be involved in the response to PDGFs (Millette et al., 2005; Pukac et al., 1998). FGF2 and FGFR1 mediate the proliferative activity of PDGF-BB. It is observed that early Erk activation induced by PDGF-BB is independent of FGF2, but continued Erk activation relies on FGF2-FGFR1, whereas FGF2 and FGFR1 are not involved in PDGF-BB-induced PI3K activation (Millette et al., 2005). Heparin can also inhibit SMC proliferation through suppression of protein kinase C α -isoenzyme activity, which correlates to SMC proliferation and differentiation (Herbert et al., 1996).

Similar to PDGFs, FGF2 promotes SMC proliferation and migration, as well as the SMC phenotypic switch (Chen et al., 2016a).

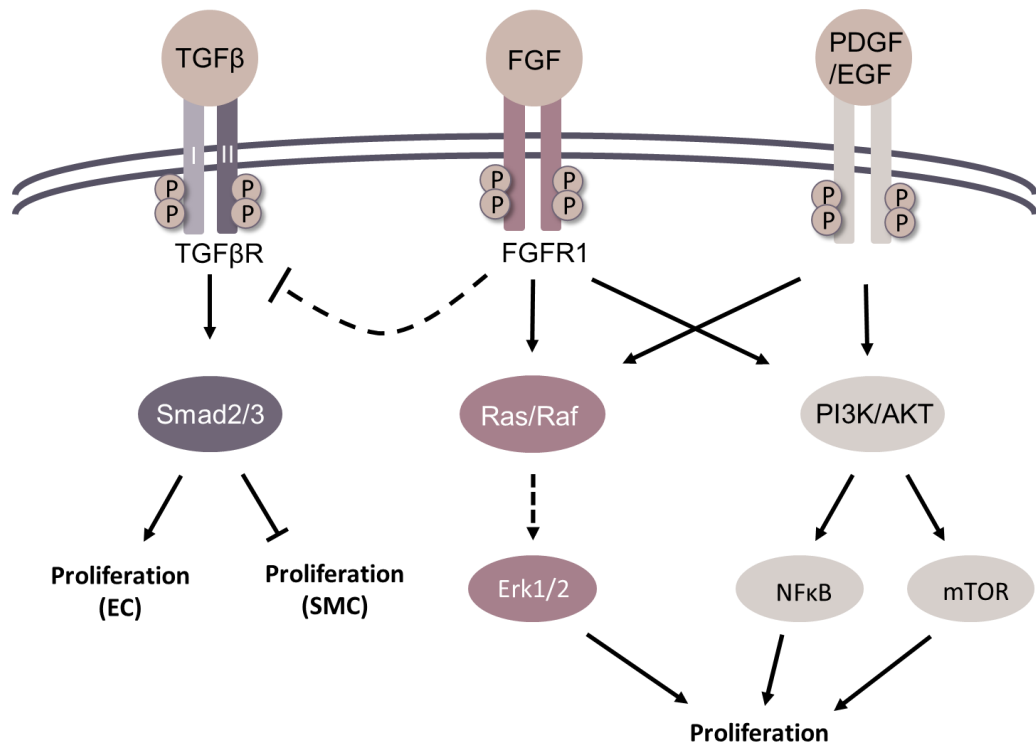


Figure 5.4. Signalling pathways in SMC proliferation during neointimal formation. Ligands binding induces phosphorylation of tyrosine kinase on receptors and activate downstream signalling. FGF signalling regulates the TGF β signalling in smooth muscle cells and endothelial cells: FGFR inhibition improves TGF β /TGFR signal transduction, leading to the SMC phenotypic switch (synthetic to contractile) and suppression of SMC proliferation. In endothelial cells, the TGF β /TGFR signalling transduction enhances neointima formation (Chen et al., 2016b). PDGF- and EGF-dependent regulation also involves neointimal hyperplasia. The Ras/Raf pathway and the PI3K/Akt pathway activated by growth factors (i.e. PDGF, bFGF, EGF) are the vital pathways in neointimal formation. In addition, NF κ B pathway also links to the PI3K/Akt pathway, leading to cell division and inflammatory gene expression (Koeppel and Schober, 2016). Image drawn with PowerPoint.

EC proliferation is equally important in vascular repair. Interaction between vascular endothelial growth factor (VEGF) and its receptor (VEGFR) can promote endothelial progenitor cells to repair the arterial damage (Yang et al., 2015). Although early VEGF activity is linked to EC restoration, its long-term activity promotes SMC proliferation and plaque formation/restenosis which causes failure of coronary artery bypass grafting (Kusumanto et al., 2006; Podemska-Jedrzejczak et al., 2018). Specific structures within heparin/HS chains are required for VEGF binding: octasaccharides (oligosaccharides with a degree of polymerization (dp)8) are the minimum size for VEGF binding and tetradecasaccharides (oligosaccharides with dp14) present the most effective binding with VEGF₁₆₅, an VEGFA isoform, compared to unfractionated

heparin (Zhao et al., 2012). VEGFA, a dominant mediator of angiogenesis, can bind to both VEGFR1 and VEGFR2, but the main signal transduces through VEGFR2 (Jakobsson et al., 2006; Peach et al., 2018), leading to endothelial cell proliferation, survival and migration. These studies also indicated that the affinity of VEGF is based on the conformation and the level of sulphate of heparin/HS (effective nuclear charge roughly -7.1 ± 0.40 Zeff for native heparin) (Kenrick, 2019). HSPGs and NRP-1 are also identified as co-receptors which enhance the affinity and stability of heparin/HS binding to the VEGF₁₆₅-VEGFR complex (Jakobsson et al., 2006; Peach et al., 2018; Teran and Nugent, 2015; Zhao et al., 2012) (**Figure 5.5**). Recently, PDGF binding to VEGFR2 has been discovered on ECs. The PDGF cross-family binding augments VEGFR2 activation and amplifies the signalling when the concentration of PDGFs is at least 25-fold greater than VEGFs (Mamer et al., 2017). However, the mechanism of PDGF-VEGFR cross-family signalling in the regulation of vascular hyperplasia is unclear.

The transmembrane tyrosine kinase receptors, Tie1 and Tie2, are also important in angiogenesis, proliferation and migration of endothelial cells (Fiedler and Augustin, 2006). The interaction between the ligand, angiopoietin (Ang), and Tie2 regulates the proliferative state of the endothelium. For instance, Ang-1-mediated Tie2 activation leads to endothelial quiescence, whereas activation of Tie2 by Ang-2 results in inflammation and EC proliferation (Fiedler and Augustin, 2006; Fujikawa et al., 1999) (**Figure 5.6**).

The major role of VEGF-VEGFRs in SMCs and ECs is summarized in **Table 5.3**.

In this chapter we explore which growth factor signalling pathways are impacted in SMCs and ECs with a view to explaining the differential effect of heparin analogues on VSMC and EC proliferation.

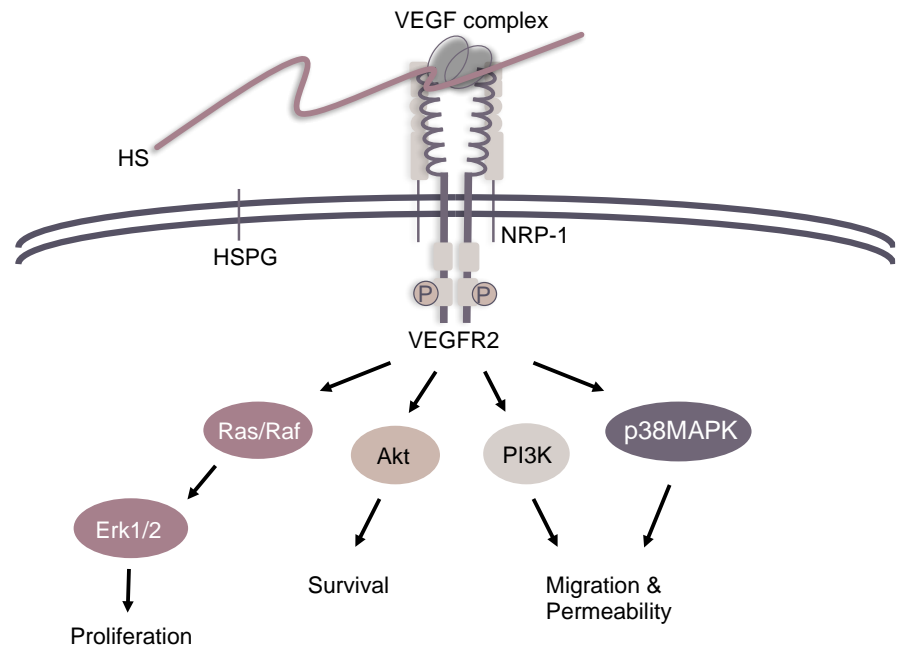


Figure 5.5. Scheme of VEGF-VEGFR2-NRP1-HS-induced signalling in ECs. The synergistic complex composed of VEGF, VEGFR2, neuropilin-1 (NRP-1), heparin and heparan sulphate proteoglycan (HSPG) regulates proliferation, survival, migration and permeability in ECs (Jakobsson et al., 2006; Teran and Nugent, 2015). Image drawn with PowerPoint.

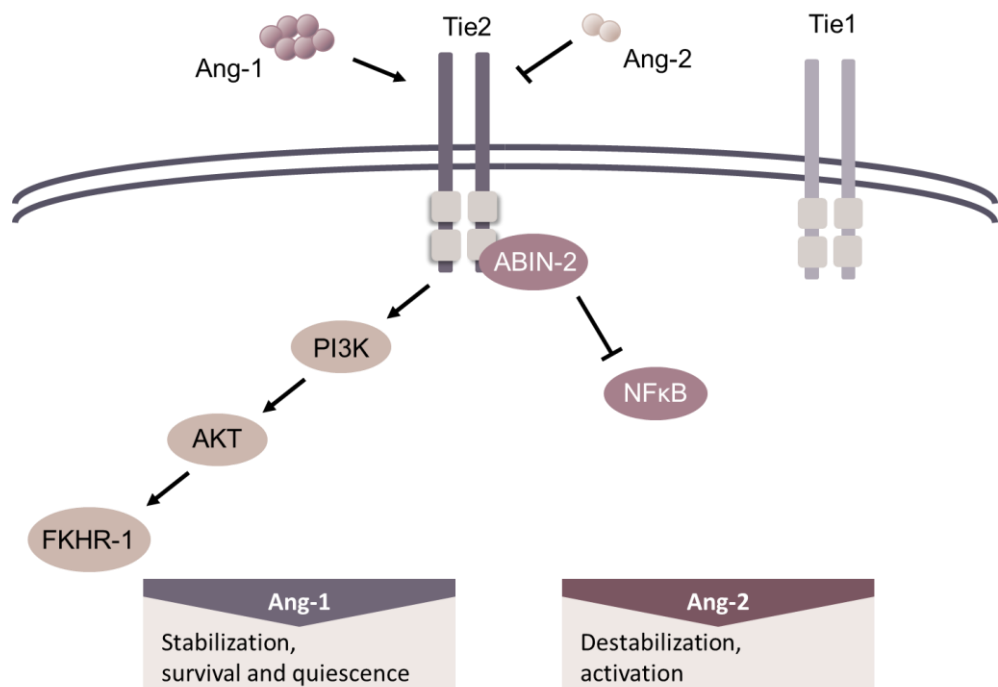


Figure 5.6. Scheme of Ang-1 and Ang-2 signalling pathways leading to EC quiescence and proliferation. EC proliferation is regulated by Ang-1 (activation) or Ang-2 (inhibition) stimuli through Tie2 signalling (Fiedler and Augustin, 2006). Image drawn with PowerPoint.

Table 5.3. Summary of the major roles of growth factor receptors in SMCs and ECs.

GFRs	Event Involved	Ref.
FGFRs	SMC migration and proliferation +	(Chen et al., 2016a)
	Plaque formation +	
	SMC switch (contractile to synthetic)	
	Interaction with heparin and HS	(Wujak et al., 2015; Yu et al., 2014)
VEGFRs	SMC proliferation +	(Kusumanto et al., 2006; Podemska-Jedrejczak et al., 2018; Yang et al., 2015)
	EC proliferation +	
	EC migration	(Jakobsson et al., 2006; Krilleke et al., 2009)
	Interaction with heparin	
PDGFRβ	SMC migration and proliferation +	(Andrae et al., 2008)
	Plaque formation +	
	Leukocyte accumulation +	(He et al., 2015)
	SMC phenotypic switch (contractile to synthetic)	(Chen et al., 2016a)
IGF1Rs	SMC phenotypic switch (contractile to synthetic)	(Cai et al., 2015; Jan et al., 2011)
	SMC proliferation +	(Zhang et al., 2017)
	SMC migration +	
EGFRs	SMC proliferation +	(George et al., 2013; Yogi et al., 2011);)
	inflammation	
	Interaction with heparin	
TGFRs	Smads 2 and 3 activation (angiogenesis)	(Mack, 2011; Shi and Massague, 2003)
	SMC phenotypic switch (synthetic to contractile)	(Chen et al., 2016a)
	Plaque formation (on SMCs) -	(Chen et al., 2016a)
Tie2	Inflammation	(Fiedler and Augustin, 2006; Fujikawa et al., 1999)
	EC phenotypic switch	

+, positive correlation; -, negative correlation

5.3 Results

The effect of heparin and its analogues on proliferation/migration of a particular cell type will likely reflect the types of growth factor receptor expressed on the cell surface. As a starting point, we used reverse transcription PCR (RT-PCR) to identify transcripts of growth factor receptors in HCASMCs and HCAECs.

5.3.1 GFRs in HCASMCs and HCAECs: Differential Expression

The differential effects of the cation forms of heparin on HCASMC and HCAEC proliferation/migration may be due to the differential expression of growth factor receptors (GFRs) between these two cell types. To investigate this, a RT-PCR screen was undertaken (**Figure 5.7**). These cells express a range of GFRs. Key differences were that PCR products for VEGFR1, 2, 3 were obtained from HCAECs while products for only VEGFR1, 2 were obtained from HCASMCs. In contrast PCR products for both PDGFRA and PDGFRB were obtained in HCASMCs, but not from HCAECs.

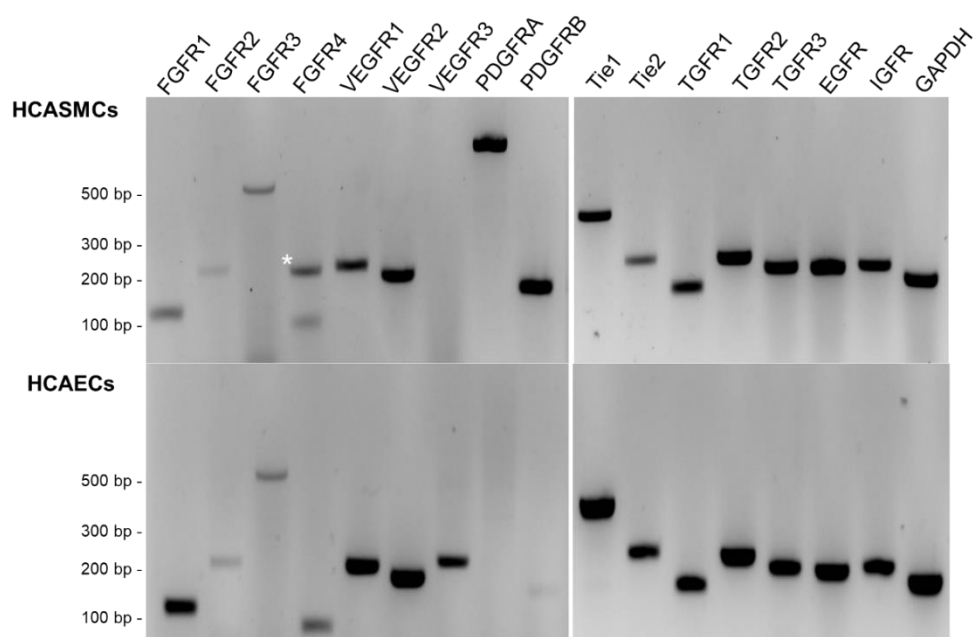


Figure 5.7. RT-PCR analysis of cDNA isolated from HCASMCs and HCAECs using primers against major growth factor receptors. All PCR products were purified and confirmed by sequencing. * indicates a non-specific amplification product.

5.3.2 Proliferative Activity of Heparin is affected by Different Growth Factors

A key difference between HCASMCs and HCAECs in terms of transcript levels is the presence of PDGFRs in SMCs. Activation of PDGFRs in SMCs mediates proliferation (Andrae et al., 2008) and thus these receptors may represent the target of the heparin analogues that induce differential proliferation in SMCs compared to ECs. To investigate this further, HCASMCs were grown in normal fully supplemented growth media, or basal media (no growth factors) supplemented with either FCS (~40 ng/ml PDGF) or FGF2 (10 ng/ml) or EGF (10 ng/ml) in the presence of heparin and the different cation forms of heparin (Na^+ , K^+ , Mg^{2+} , Ca^{2+} , 100 $\mu\text{g/ml}$) (Kwon et al., 2016). Following four days incubation, HCASMC number was assessed using MTT assays. Data were normalized relative to cell number for HCASMCs grown in fully supplemented GM in the absence of heparin (**Figure 5.8**). It was hypothesised that if PDGFRs mediate the differential effect, the anti-proliferative activity of the cation forms of heparin should be maintained in fully supplemented GM and in basal media supplemented with FCS (PDGF), but lost in basal media containing only FGF2 or EGF.

For unmodified heparin, HCASMC proliferation was significantly higher in basal media containing only FGF2 or EGF compared to fully supplemented GM (**Figure 5.8**). This is consistent with the idea that the differential effect is mediated via the activation of PDGFRs. Interestingly, for the cation forms of heparin, the anti-proliferative activity was maintained or enhanced in basal media containing only FGF2 or EGF, while anti-proliferative activity was decreased for Mg^{2+} and Ca^{2+} forms in basal media supplemented with FCS (predominantly PDGF). One of the most striking effects was the significant enhancement of anti-proliferative activity in EGF-containing media for the Ca^{2+} forms of heparin.

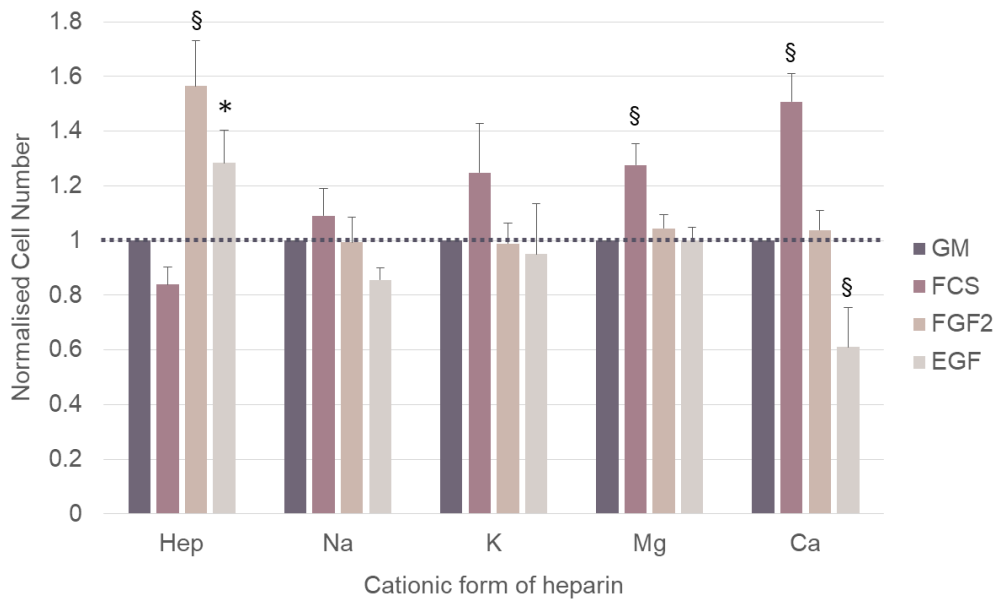


Figure 5.8. Addition of different growth factors affects the action of cation forms of heparin on HCASMC proliferation. Cell number in the control group (grown in fully supplemented GM without heparin) is set at 1.0. GM, fully supplemented growth media; FCS, basal media with fetal calf serum (mainly PDGFs); FGF2, basal media with 10 ng/ml of fibroblast growth factor-2 (FGF2); EGF, basal media with 10 ng/ml of epidermal growth factor. * $P < 0.05$, § $P < 0.001$ compare to GM in each group; $n=8$; Tukey test.

5.3.3 Effect of Heparin Analogues on Signalling Pathways Downstream of GFRs

Activation of GFRs can be monitored by immunoblotting cell lysates with antibodies against phosphorylated tyrosine. We thus exposed HCASMCs in fully supplemented GM or fully supplemented GM containing 10 μ g/mL of various heparin analogues. HCASMCs were subsequently lysed, proteins within the lysates separated by SDS-PAGE and immunoblotted with antibodies against phospho-tyrosine. Immunoreactive banding patterns were analysed to determine which analogues altered the banding patterns, indicating potential changes in GFR signalling.

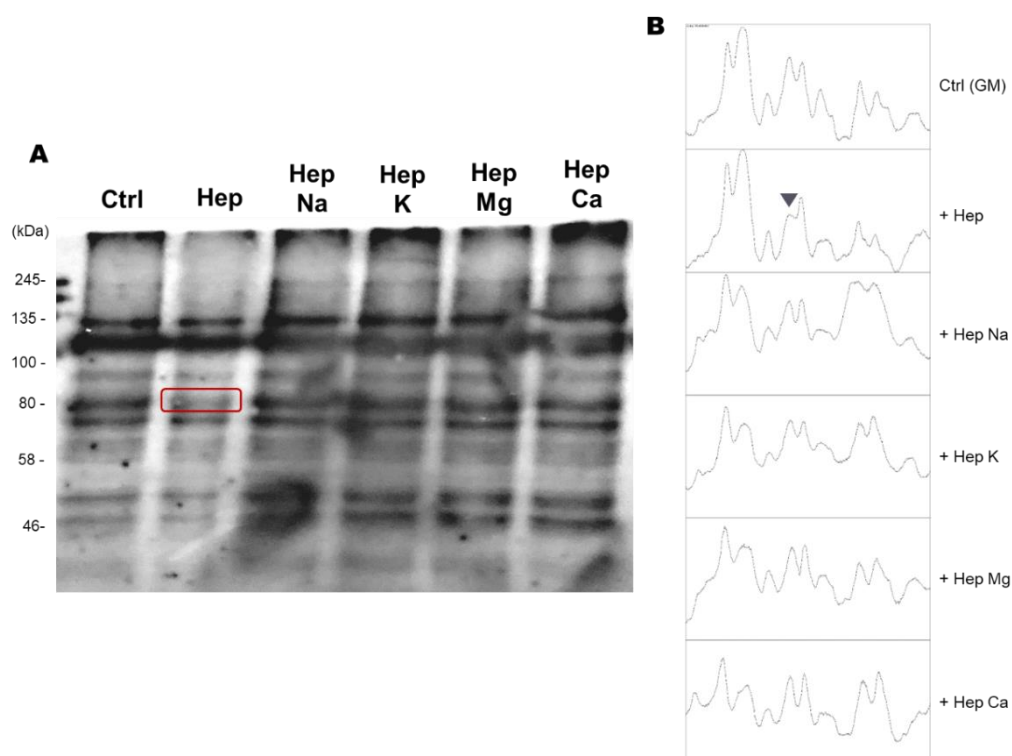


Figure 5.9. Immunoblot analysis using phospho-tyrosine-specific antibodies reveals little change in growth factor-stimulated signalling in HCASMCs in the presence of non-desulphated cation form heparin analogues. The cells were incubated with 10 μ g/ml heparin analogues in GM for 10 min after one hour serum starvation. The lysates of HCASMCs were analysed by 10% SDS-PAGE gel. Squares and arrows in (A) immunoblot and (B) densitometry show the difference compared to control (incubation in fully supplemented growth media (GM)).

The result of this experiment suggests no clear change in immunoreactive banding patterns among the non-desulphated cationic heparin analogues compared to control (fully supplemented GM) and only minor differences between unmodified heparin and control compared to fully supplemented GM (**Figure 5.9**). Our proliferative assays (**Figure 5.8**) suggest that EGF-mediated signalling pathways may be responsible for the anti-proliferative response. In an attempt to identify which band/s in the fully supplemented GM come from activation of EGFRs we also exposed HCASMCs to EGF (10 ng/ml) alone before lysing the cells and immunoblotting the lysates. Results showed an enhanced immunoreactive band at ~180 kDa in the presence of EGF alone compared to fully supplemented GM, suggesting that this band indicates activation of the EGFR. There was no clear evidence of any changes in intensity of this band in the presence of either sulphated (**Figure 5.9**) or desulphated (**Figure 5.10**) cationic heparin analogues.

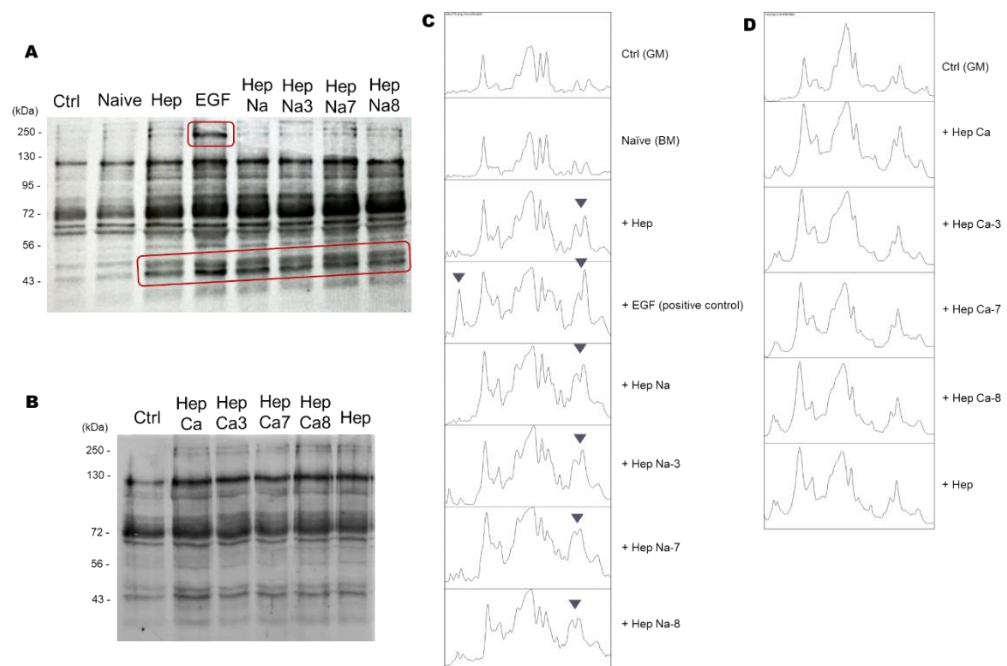


Figure 5.10. Immunoblot analysis using phospho-tyrosine-specific antibodies reveals little change in growth factor-stimulated signalling in HCASMCs in the presence of cation-desulphated heparins. HCASMCs were activated by cation-desulphated heparin analogues for 10 minutes in growth media (GM) after one hour serum starvation. The cell lysates were analysed by 10% SDS-PAGE gel. Squares and arrows show the differences compared to control (GM).

5.4 Discussion

The differential effects of heparin analogues on HCASMC and HCAEC proliferation likely results from differences in signalling through growth factor-mediated pathways between these two cell types. Our PCR results indicate that HCASMCs and HCAECs have a number of GFRs in common, but there are some differences, notably: (1) transcripts for VEGFR3 were only found in HCAECs; (2) transcripts for PDGFRA and PDGFRB were expressed on HCASMCs but not on HCAECs. Differences in the presence of cell surface receptors is however not the whole story since there is also clear evidence of cell-specific signalling downstream of receptor activation. For example, while both HCASMCs and HCAECs express EGFRs, the same stimuli to these receptors results in different outcomes. EGF-heparin interacts with EGFRs and HSPGs on the SMC surface, leading to cell growth and hyperplasia (Raab and Klagsbrun, 1997), however, heparin inhibits SMC proliferation and migration via

suppression of HB-EGF-induced EGFR activation (Kalmes et al., 2000) while heparin shows no effect to the same pathway on EC (Arkonac et al., 1998).

Mechanistically, it seems likely that the shape of the different cation forms heparin will affect the affinity and/or the structure of the GF-GFR-heparin complex. Alteration of charge distribution on heparin has been shown to affect heparin's conformation and its affinity to FGFs. The different conformations of different cationic heparins were studied by NMR (Rudd et al., 2007) and these different conformations influence FGF binding (Xu et al., 2012) and brings about different biological outcomes (Rudd et al., 2007). Despite the fact that heparin-induced anti-VSMC proliferation has been studied for more than three decades, the full mechanism of how heparin suppresses VSMCs remains unknown (Gilotti et al., 2014). To date, it has been reported that heparin inhibits VSMC proliferation by suppression of mitogenic protein kinase C (PKC)-dependent c-fos induction and mitogen-activated protein kinase (MAPK) pathway activation (Hedin et al., 1998). In addition, heparin also reduces the activity of cyclin dependent kinase (CDK)-2 by increasing levels of protein p27^{kip1} to prevent cell cycle transition from G1 phase to S phase (Fasciano et al., 2005; Sakakibara et al., 2005). Moreover, p27^{kip1} is negatively regulated by PDGF-BB in VSMCs via the ERK-dependent pathway (Sakakibara et al., 2005).

The expression of PDGFRs in HCASMCs, but not HCAECs, makes these receptors possible candidates for the differential anti-proliferative effects we see with our heparin analogues. A chemically modified heparin called LHT7, which is a low-molecular weight heparin conjugated with seven taurocholates, has been shown to inhibit PDGF-BB-induced angiogenesis by suppression of the tyrosine phosphorylation of PDGFR β , leading to an effective suppression of tumour growth. In addition, this suppression of proliferation is effectively inhibited by combination of PDGF β and FGF2 (Chung et al., 2015b). However, our data obtained by growing HCASMCs in different growth factors (**Figure 5.5**) suggest that signalling via PDGFRs is not the primary cause of the differential heparin proliferation effects. Here the anti-proliferative effects of the cation forms were maintained or enhanced in basal media containing only FGF2 or EGF, while anti-proliferative activity was generally decreased in basal media supplemented with FCS (predominantly PDGF). The clearest effects in these experiments was observed for the Ca²⁺ forms of heparin where there was the significant reduction in anti-proliferative activity in HCASMCs grown in media supplemented only with FCS (predominantly PDGFs) and a significant enhancement in anti-proliferative activity for cells grown in media supplemented with EGF alone. Previous research revealed that heparin inhibits VSMC proliferation through suppression of PDGF-induced Erk activation, but not EGF-induced Erk activation (Pukac et al., 1998). This may indicate that the Ca²⁺ forms of heparin mediate their effects through the Erk/MAPK pathway. Since HCASMCs and HCAECs both express EGFRs this is likely to reflect differences in signal transduction once these receptors are activated. In VSMCs, G-protein-

coupled receptors (GPCRs) are activated by thrombin, leading to cleavage of pro-HB-EGF (George et al., 2013; Prenzel et al., 1999). The active HB-EGFs stimulate EGFR phosphorylation by interaction with HSPGs and receptors, resulting in activation of downstream signalling such as the MAPK pathway, phosphoinositide-3 kinase (PI3K) and phospholipase C γ (PLC γ) (Holbro and Hynes, 2004; Makki et al., 2013). However, HB-EGF-induced EGFR activation is blocked by heparin binding (George et al., 2013). In endothelial cells, EGF binding and EGFR phosphorylation activates ERK1/2, leading to cell proliferation. In addition to this, the interaction between EGFs and PDGFRs induces phosphorylation of tyrosine residues on their receptors, creating binding sites for phospholipase C which associates and produces the factors for protein kinase C activation (Margolis et al., 1989; Monteiro et al., 2018; Nishizuka, 1992). Furthermore, VEGF expression also depends on the activation of EGFRs, especially after hypoxia stimuli (Monteiro et al., 2018). Hypoxia provokes angiogenesis by signalling through hypoxia-inducible transcription factors, which regulate many angiogenic genes, of which the induction of VEGF is the most significant (reviewed in Carmeliet, 2003).

We also attempted to elucidate differential activation and modification of GFRs in HCASMCs by immunoblotting treated cell lysates with anti-phosphotyrosine antibodies. Here, although we identified immunoreactive band 'fingerprints' associated with the activation of the EGFR, we saw no clear differences in banding patterns in cells treated with different heparin analogues. Thus, ultimately more sophisticated techniques such as global phospho-proteomics will be required to determine the intracellular signalling pathways through which heparin analogues suppress HCASMC proliferation. Although we did not show how these cationic heparin analogues influence GF-GFR signalling, our immunoblots (**Figure 5.10**) show some differences in the patterns of tyrosine phosphorylation in response to cationic heparin analogues. This may support this idea that these cations not only alter the shape of heparin but also alter (1) the affinity to GFs and GFRs, and (2) the shape of GF-GFR-heparin complexes, resulting in different signalling activation. Since GF signalling is complex within these cell types, with considerable cross-over between pathways, differences in transcripts for GFRs between HCASMCs and HCAECs may also govern the proliferative differences we see for the same heparin treatment.

Chapter 6: General Discussion

6.1 Summary of Novel Findings

The aim of this project was to identify heparin-based analogues that could ultimately be used as novel biocompatible stent coating materials. Ideal compounds would be ones that maintain anti-thrombotic activity and minimize vascular smooth muscle cell growth while having enhancing, or having no effect, on endothelial cell proliferation. To this end we have assessed the effect of a range of desulphated and cation-complexed forms of heparin on human coronary artery smooth muscle cell (HCASMC) and human coronary artery endothelial cell (HCAEC) proliferation and migration. To the best of our knowledge this is the first comprehensive analysis of the differential effects of these compounds on these important cell types (Summarised in **Table 6.1 and 6.2**). Candidate compounds with the appropriate activity profile in HCASMC/HCAECs were screened for their effects on blood coagulation pathways and their ability to interact with platelet factor 4 (PF4), a key determinant of heparin-induced thrombocytopenia. We also explored the molecular basis of the differential effects of heparin analogues on HCASMC/HCAECs signalling. The novel findings are that:

- Naive heparin complexed with either Na^+ , K^+ , Mg^{2+} or Ca^{2+} suppresses HCASMC proliferation while having minimal effects on HCAEC activity;
- None of the desulphated heparin analogues demonstrated this desired activity profile on HCASMCs/HCAECs;
- A range of desulphated heparins complexed with either Na^+ , K^+ , Mg^{2+} or Ca^{2+} showed promising activity profiles (Na1-6; K1-8; Mg2-5; Ca1-2 and Ca4-8); desulphated heparin 4 (predominant repeating structure; $\text{I}_{2\text{S}}\text{A}^{6\text{OH}}\text{NAc}$) consistently being the best performing analogue in terms of its differential proliferation effects across all cations. All cationic desulphated heparins tested retained their differential effects on HCASMC/ HCAEC proliferation in dual culture systems;
- Candidate compounds K-3 and Ca-4 maintained anti-thrombotic activity;
- The differential effects of the heparin analogues on HCASMC and HCAEC proliferation likely represents differences in signalling downstream of GFRs. Key candidate signalling cascades are those activated by FGFs and EGF.

Table 6.1. The summary results of cell proliferative activity of desulphated heparin. ↓, inhibition ($P<0.05$); ↓↓, strong inhibition ($P<0.01$); NS, no significant effect.

Analogues	Proliferation	
	SMC	EC
HEP	↓↓	NS
DS-1	NS	NS
DS-2	NS	NS
DS-3	NS	NS
DS-4	NS	NS
DS-5	NS	↓
DS-6	NS	NS
DS-7	NS	NS
DS-8	NS	↓

Table 6.2. The summary results of cell proliferation, migration, apoptosis and anti-coagulant activity. ↑, enhance (P<0.05); ↑↑, strong enhance (P<0.01); ↓, inhibition (P<0.05); ↓↓, strong inhibition (P<0.01); NS, no significant effect; blank, no testing.

Analogues	Proliferation		Migration		Co-cultured proliferation		Apoptosis		Anti-coagulation	
	SMC	EC	SMC	EC	SMC	EC	SMC	EC	aPTT	PT
Hep	↓↓	NS			↓	NS			↑↑	↑↑
Na	↓↓	↓↓	↓		NS	NS	NS	NS	↑↑	↑↑
K	↓↓	↓			NS	↓	NS	NS	↑↑	↑↑
Mg	↓↓	↓	↓	↓	NS	NS	NS	NS	↑↑	↑↑
Ca	↓↓	NS	↓		NS	NS	NS	NS	↑↑	↑↑
Zn	↓	↓↓								
Mn	↓	↓↓								
Fe	↓↓	↓↓								
Na-2	NS	NS							↑↑	NS
Na-3	↓↓	NS	↓↓	NS	↓↓	↑↑	NS		↑↑	↑↑
Na-4	↓↓	↑	↓↓	NS	↓↓	↑↑	NS		↑↑	↑↑
Na-5	↓↓	NS							↑↑	NS
Na-6	↓	NS							↑↑	NS
Na-7	↓	↓↓								
Na-8	NS	NS							↑↑	NS
K-2	NS	NS							↑↑	NS
K-3	↓↓	NS	↓↓	NS	↓↓	↑	NS		↑↑	↑↑
K-4	↓↓	NS	↓↓	NS	↓↓	↑	NS		↑↑	↑↑
K-5	↓↓	NS							↑↑	NS
K-6	NS	NS							↑↑	NS
K-7	↓	NS								
K-8	↓	NS							↑↑	NS
Mg-2	↓↓	↓							↑↑	NS
Mg-3	↓↓	NS	↓↓	NS	↓↓	NS	NS		↑↑	↑↑
Mg-4	↓↓	NS	↓↓	NS	↓↓	NS	NS		↑↑	↑↑
Mg-5	↓↓	NS							↑↑	NS
Mg-6	↓	↓							↑↑	↑↑
Mg-7	↓↓	↓								
Mg-8	↓↓	↓							NS	NS
Ca-2	NS	NS							↑↑	NS
Ca-3	NS	NS	↓↓	NS	↓↓	NS	NS		↑↑	↑↑
Ca-4	↓↓	NS	↓↓	NS	↓↓	↑↑	NS		↑↑	↑↑
Ca-5	NS	NS							↑↑	NS
Ca-6	NS	NS							↑↑	↑↑
Ca-7	↓↓	NS								
Ca-8	↓↓	NS							NS	NS

6.2 Chemically Modified Forms of Heparin Have Differential Effects on VSMC and EC Proliferation

The repeating disaccharide units of uronic acid and D-glucosamine in the heparan sulphate (HS) chain mediate binding with numerous proteins at the cell surface – the so-called ‘HS interactome’ (Gómez Toledo et al., 2021; Olsen et al., 2003; Ori et al., 2008; Reizes et al., 2001; Witt and Lander, 1994). This interaction usually occurs between positively-charged amino acids on target proteins, such as growth factors, and negatively-charged sulphated groups in HS. Heparin is structurally similar to HS, but has a distinct, more heavily sulphated, and homogenous repeat pattern. Because ionic interaction plays a key role between protein and heparin/HS, alteration of sulphated groups in heparin/HS affects its affinity for growth factors and other proteins (Greinacher and Delcea, 2015; Krauel et al., 2012; Xu and Esko, 2014). The structures of these chemically modified heparin analogues are also changed by alteration of charge distribution (Guimond et al., 2009; Rudd et al., 2007). In addition, the negative charge of these analogues, and thus structure and function, is further altered by complexing with cations (Remko and von der Lieth, 2006; Seo et al., 2011). The ionic radii and ionic valence of each metal ion plays important roles in the conformational change of heparin, the number of metal ion adducts also contributes to the overall structure (Rudd et al., 2007; Seo et al., 2012). However, the structure-function relationship of these macromolecular structures needs to be further investigated.

In addition to electrostatic interactions between heparin and proteins, non-ionic interactions, such as hydrogen bonding and van der Waals contacts, contribute additional energy to stabilise the heparin-protein complex (Raman et al., 2003). The heparin binding sites on proteins mainly consist of positively-charged basic residues, which provide ionic interaction (Rudd et al., 2017). However, biophysical analysis of the interaction of FGF2 with heparin demonstrate that pure ionic interactions contribute only 30 % of binding energy associated with complex formation (Thompson et al., 1994a). While H-bonding will at least in part be directly consequent on the bonds formed in binding, hydrophobic interactions and at least some of the H-bonding will occur due to changes in the protein conformation, that results from binding.

Our results show that normal heparin complexed with Na⁺, K⁺, Mg²⁺ and Ca²⁺ have promising activity profiles in terms of differential effects on HCASM/C/HCAEC proliferation/migration (**Chapter 3**). These initial findings were obtained in a single culture system (i.e., each cell type grown alone) and when HCASMCs and HCAECs were cultured together to more closely mimic the physiological conditions within an artery, these differential effects were only retained for the Mg²⁺ analogue (see detail

in **Appendix 1**). A caveat to these results is that analysis of these early co-culture experiments were performed by imaging of HCASMCs and HCAECs, and it is difficult to differentiate between the cells. In later co-culture experiments, cells were separated and analysed by fluorescent activated cell sorting (FACS), which is likely to increase the accuracy of the data. We also conducted a comprehensive screen of a range of desulphated heparins complexed with Na^+ , K^+ , Mg^{2+} and Ca^{2+} . Interestingly in single culture systems, desulphated heparin 4 ($\text{I}_{25}\text{A}^{60\text{H}}\text{NAc}$), which had no significant effect on HCASMC or HCAEC proliferation, was consistently the best performing analogue in terms of its differential effects when complexed with Na^+ , K^+ , Mg^{2+} and Ca^{2+} . Importantly, in co-cultures, all cationic desulphated heparins tested retained their differential effects on HCASMC/ HCAEC proliferation. It should be noted that under physiological conditions “uncomplexed heparin” is, in fact, usually complexed with mostly Na^+ and some Ca^{2+} or other cations (personal communication E.A. Yates), thereby might be expected to have similar activities with Na-heparin. However, our data show different effects on SMC migration and EC proliferation between Na-heparin and uncomplexed heparin. This may be due to the conformational differences between the pure Na^+ form and the mixed-cation form heparin caused by coordination difference, although this needs to be further investigated.

Metal ions play important roles within biological systems. For instance, divalent Mg^{2+} and Ca^{2+} regulate cell growth and enzyme-catalysed processes through interaction with numerous proteins/enzymes (Da Silva and Williams, 2001; Kahl and Means, 2003; Pasternak et al., 2010). In addition, heparin has high affinity with a range of metal ions, which have significant effects on heparin/HS interactions with proteins. FGF1 binding to heparin/HS is reduced in the presence of 10 μM K^+ , Mg^{2+} and Ca^{2+} but unaffected by the presence of Zn^{2+} in SMCs (Zhang et al., 2014), though this is not the same as using cation coordinated forms of the sugar (Guimond et al., 2009; Rudd et al., 2008; Rudd et al., 2007). In **Figure 3.7** K-, Mg- and Ca-heparin are shown to have greater inhibitory effect on HCASMC proliferation compared to Zn-heparin. This would indicate that conformational change induced by Zn coordination is insufficient or geometrically incorrect to elicit a large effect. Divalent cations, such as Mg^{2+} and Ca^{2+} , are important in many heparin-protein interactions and also in the affinity and stability of the complex. The carboxylate group in the iduronate residue of heparin is required for Ca^{2+} specific binding (Chevalier et al., 2004; Rabenstein et al., 1995). Since cation binding triggers conformational change of heparin (Rudd et al., 2007), the affinity, specificity and stability of modified heparin-protein complexes are also changed, resulting in different signalling (Chevalier et al., 2004; Srinivasan et al., 1975). Yet the signalling outcomes induced by metal ion association with heparin/HS remains unclear. Furthermore, NMR, FTIR and EPR spectroscopies indicate that copper preferentially binds between the iduronate residue and the adjacent 6-O-

sulphated glucosamine (Rudd et al., 2008). This may also suggest that the position of sulphated group could affect the binding preference of cations.

6.3 Modified Forms of Heparin Have Differential Effects on Blood Coagulation

The charge alteration of heparin can also affect the affinity to other proteins, for example, antithrombin (AT). In blood coagulation, heparin binds to antithrombin forming a heparin-antithrombin complex. The heparin-binding sites on antithrombin are Lys-11 and Arg-13 in the N-terminus; Arg-46 and Arg-47 in the helix A; and Lys-114, Phe-121, Phe-122, Lys-125 and Arg-129 in the region of the helix D (Jin et al., 1997). Moreover, heparin interacts with thrombin mainly via electrostatic binding, as well as with antithrombin. While it has been claimed that heparin binds to antithrombin-III mainly through non-electrostatic interactions (Heuck et al., 1985; Nahain et al., 2018), this may confound primary points of interaction, the sugar-protein interface and new non-covalent bonds formed within the protein due to the substantial conformational change it undergoes upon heparin binding. Thus, non-ionic interactions contribute to the stability of the heparin-protein complex. In the case of heparin-thrombin interaction, 86 % of heparin binding energy is from ionic interactions and only 14 % from non-ionic interactions, consistent with thrombin undergoing more modest conformational change than antithrombin III. In contrast, in the heparin-antithrombin-III interaction only 40 % of the binding energy is from ionic interactions and non-ionic interactions with non-polar residues are therefore considered to play a key role in heparin binding (Olson and Björk, 1991). Many of these will come from the considerable conformational change that occurs in antithrombin III upon heparin binding (**Fig. 4.3**). The heparin binding site on proteins usually consists of cationic basic residues and nearby-non-polar hydrophobic residues. These hydrophobic residues induce the contact of phenyl rings to non-polar stems of cationic heparin-binding residues, resulting in extensive and specific ionic and non-ionic interaction for pentasaccharide binding (Jairajpuri et al., 2003). Thus, changes in charge distribution and conformation of the heparin analogues affect anti-coagulation properties of the molecules.

Promising candidates were assessed for potential side-effects particularly their anti-coagulant activity, and ability to interact with platelet factor 4 (PF4) which can lead to an immune response and heparin-induced thrombocytopenia (HIT) (**Chapter 4**).

In aPTT assays, which measures the activity of the contact activation and common pathways in the blood coagulation cascade, Na⁺, K⁺, Mg²⁺ and Ca²⁺-complexed desulphated heparin analogues 2, 3 and 4 retained their anticoagulant activity, as well as the Mg²⁺ and Ca²⁺-complexed desulphated heparin analogue 6. The anticoagulant activity of Na⁺, K⁺, Mg²⁺ and Ca²⁺-complexed desulphated heparins 5 and 8 was significantly reduced compared to unmodified heparin. In PT assays (tissue factor pathway), anticoagulant activity was absent or significantly reduced in Na⁺, K⁺, Mg²⁺ and Ca²⁺-desulphated heparin analogues 2, 5, 6 and 8. These results indicate that heavy desulphated heparins may lose their inhibitory activity on Xa or thrombin, resulting non-coagulation. Furthermore, desulphated heparin analogues 3 and 4 in PT assay showed a mixed response dependent upon which cation they were complexed with. K-3 and Ca-4 retained anticoagulant activity, while all other cation forms of these analogues showed a significant reduction in anticoagulant activity compared to unmodified heparin. Thus, candidate compounds K-3 and Ca-4 appear to have the best activity profiles in terms of differential effects on HCASMC/ HCAEC proliferation while maintaining anti-thrombotic activity. These findings are novel. It may also be advantageous under certain conditions to utilize heparin derivatives with reduced anti-coagulant activity, and here the differential effects of the cation-complexed desulphated heparins on the coagulation cascade may be useful in tailoring a given response. Our data regarding interaction with platelet factor 4 (PF4) is inconclusive. We had hoped that by using native (non-denatured) gel electrophoresis we might see a clear difference in the formation of high molecular weight complexes when the heparin analogues were incubated with recombinant PF4. Here we were only able to assess cation-complexed non-desulphated heparin, but no substantive difference could be detected in protein banding patterns on silver-stained gels. It is of note that the sulphated domains of heparin, in both glucosamine and uronic acid residues, are associated with high affinity binding to PF4 (Stringer and Gallagher, 1997). In addition, PF4 binds to heparin more strongly than 2-O, 3-O desulphated heparin (partially desulphated heparin) because of a combination of charge and conformational effects. A minimum heparin chain length of 6 monosaccharides is required for PF4-heparin interaction (Maccarana and Lindahl, 1993; Rauova et al., 2005) and the chain length of ~12 saccharide units is required to form PF4-polyanion complexes for anti-PF4-heparin antibodies recognition and binding (Visentin et al., 2001). Furthermore, the binding efficiency of PF4 to heparin is increased by chain length and the extent of sulphation (Krauel et al., 2012; Zucker and Katz, 1991). Thus desulphated heparin analogues may pose less risk in terms of HIT than unmodified heparin.

6.4 The molecular basis of the differential effects of heparin analogues on HCASMCs/HCAECs

The effect that a given heparin derivative has on a cell proliferation will depend upon the interaction between the derivative, growth factors (GFs) and the cell's growth factor receptors (GFRs). This will depend on the conformation of the derivative, which can be altered by changes in sulphation patterns and/or complexing with different cations (Rudd et al., 2007), the availability of different GFs and the presence and downstream signalling capabilities of different classes of GFRs. In many cases it is likely that the heparin derivatives disrupt native binding between heparan sulphate (HS) and GF-GFRs. Although the FGF-FGFR-heparin complex is more stable than the complex with HS, presumably because of higher sulphated domains (negative charge) on heparin (Xu and Esko, 2014), our modified heparin analogues may alter their affinity to FGF-FGFR complex, thereby, native binding of HS may affect the results. There are three basic approaches to avoid this interference: (1) using BaF3 cells which are naturally devoid of HS, (2) using heparanase-treated cells or, (3) employing an inhibitor of sulphation, such as chlorate, in the cell culture medium to inhibit HS biosynthesis.

To explore how heparin analogues might affect signalling, we examined the differential expression of GFR transcripts between HCASMCs and HCAECs in **Chapter 5**. Key differences are that PCR products for VEGFR3 were obtained only from HCAECs, while PCR products for both PDGFRA and PDGFRB were obtained only in HCASMCs (See **Figure 5.7**). Since PDGFRs couple via Ras/Raf pathway and PI3K/Akt pathways to cell proliferation (Choudhury et al., 1997; Choudhury et al., 2006; Silvestre-Roig et al., 2013), block of signalling at these receptors may explain the anti-proliferative effects of heparin analogues on HCASMCs. However, the anti-proliferative effect of the cation-complexed heparin analogues (particularly Ca-heparin) was significantly reduced if HCASMCs were grown in basal media containing fetal calf serum (FCS, which contains mainly PDGFs – roughly 40ng/ml (Kwon et al., 2016)) compared to fully supplemented growth media (containing FCS, bFGF, EGF and insulin). This result suggests that the anti-proliferative effects of the heparin analogues are not associated with PDGFs. Anti-proliferative effects were maintained if HCASMCs were grown media supplemented with FGF2 or EGF alone, suggesting that the presence of these factors is in some way important for maintaining the inhibitory effect of the heparin analogues. Activation of both FGFRs and EGFRs couple to proliferation in SMCs and FGFRs have been shown to interact with heparin and HS (Pellegrini, 2001; Vinante and Rigo, 2013). Although we detected no difference in the presence of transcripts for FGFRs1-4 or EGFR between HCASMCs and HCAECs, it should be remembered that due to differences in downstream coupling (**Section 5.2**), activation

of a GFR can trigger different biological responses in ECs and SMCs. For instance, due to FGFR/TGF β R crosstalk, FGF/FGFR signalling leads to TGF β /TGF β R-dependent atherogenesis in ECs, but decreases atherogenesis in SMCs (Chen et al., 2016b).

We went on to screen which signalling pathways were impacted by the heparin analogues. We chose to use EGF as a positive control. Here, we stimulated HCASMCs with heparin analogues prior to lysing cells and separating proteins within the lysate with SDS-PAGE. We then immunoblotted treated lysates with antibodies against phosphorylated tyrosine to produce a distinct immunoreactive banding pattern ('fingerprint') associated with activation of the EGFR. This was used as a reference pattern against which we could compare HCASMCs that had been serum-starved, grown in normal fully supplemented growth media (control), or grown in normal growth media containing different heparin analogues. Perhaps not surprisingly this approach produced a ladder multiple phosphorylated proteins with no clear differences between control and heparin-treated cells. More sophisticated phosphoproteomic approaches will be required to define the signalling pathways in HCASMCs impacted by heparin treatment.

6.5 Limitations and Future Directions

Heparin coating

Since the ultimate goal is to attach heparin analogues onto stent materials, preliminary experiments were conducted to assess whether heparin analogues maintain their activity when physically attached to surfaces. We tried to attach heparin to the surfaces by incubating different concentrations of heparin (negative charge) with plastic culture plates pre-coated with either poly-D-lysine or poly-L-lysine (positive charge) or with commercially available amine-coated plates (from VWR). The preliminary heparin-coated plate data (see **Appendix 2**) showed these cationic heparins may be able to keep their anti-HCASMCMC-proliferative activity after attachment to a surface. However, the quantity of heparin attached to the surface was variable due to the abundance of heparin lost in the washing procedure (see **Appendix 3**) and additionally, the inability to quantify the amount of heparin attached to the plate surface. Recently, several methods for polysaccharide measurement have been developed, such as the Periodic acid-Schiff (PAS) stain and fluorescent probe assays, although their accuracy is controversial (Firshman et al., 2006; Hui et al., 2017). Furthermore, polysaccharides often possesses multiple binding sites for a single protein, and proteins have preferences for specific positions of sulphate groups and sugars. Therefore, to keep this "biofunctional surface" for interaction with protein

is a complicated issue (Powell et al., 2004). Because of these reasons, we are currently seeking appropriate ways for heparin coating, as well as quantification of the amount of heparin attached to surfaces.

Table 6.3. List of methods for heparin quantification in collagen scaffolds (Lammers et al., 2011).

METHOD	MECHANISM	SENSITIVITY	LIMITATION
FACTOR Xa ASSAY	Surplus factor Xa hydrolyses to chromogenic substrate and releases chromophore p-nitroaniline (absorbance measured at 405 nm) after heparin-AT-III-induced inactivation of Xa.	ng	Anti-coagulant activity is required
FARNDALE ASSAY	Absorbance changed of dimethylmethylene blue in the presence of multiple sulphated groups (absorbance measured at 525 nm)	µg	Long procedure (2 days), polysulphated groups are required
HEXOSAMINE ASSAY	Glucosamine is converted to pyrroles by acetyl acetone and pyrroles are condensed to a coloured product. (absorbance measured at 525 nm)	µg	Long procedure (3 days), robust test
URONIC ACID ASSAY	Acid hydrolysis of uronic acid generates coloured hydroxybiphenyl (absorbance measured at 450 nm)	ng	Collagen interference, hazardous chemicals (hot 80% H ₂ SO ₄)

With reference to National Institute for Health and Care Excellence (NICE) guidelines, acute myocardial infarction patients should be offered an ACE inhibitor, dual antiplatelet therapy (i.e. aspirin with a second antiplatelet agent, such as clopidogrel or ticagrelor), beta blocker and statins (National Institute for Health and Care Excellence, 2020). Heparin can also be offered after cardiac surgery for several days to prevent thrombosis of the coronary artery during the recovery period (National Institute for Health and Care Excellence, 2020). Therefore, the biocompatibility of heparin analogues and these prescription medicines also needs to be further assessed.

Moreover, several stents have been developed with biomaterial coatings (scheme of heparin coating on stent is shown in **Figure 6.1** and commercial heparin coating techniques are shown in **Table 6.4**). The first generation of drug-eluting stents (DESs), for example, were coated with rapamycin to inhibit VSMC proliferation (Khan et al., 2014) and the second generation of DESs are covered with newer rapamycin derivatives to reduce restenosis (Akin et al., 2011). The incidence rate of restenosis

with drug-coated stents is lower than bare stents, however, these patients still need to be offered antiplatelet therapy for more than 6 months after surgery to prevent clotting. Taking into consideration the efficiency of anti-stenosis and clotting prevention, these coating stents are developed to release specific chemical stably and continually (6-12 months, or more) (Akin et al., 2011; Khan et al., 2014).

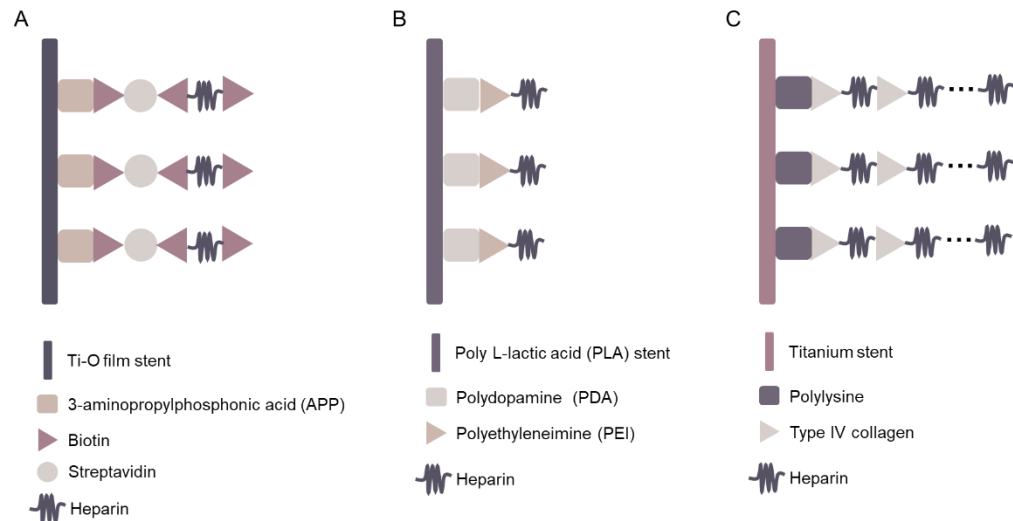


Figure 6.1. Schematic of Stents with Heparin Coatings. (A) The Ti-O/APP stent is coated with biotin and streptavidin, followed by heparin/biotin incubation to generate poly layers of heparin/biotin/ streptavidin (Weng et al., 2012); (B) The 3D-printed bioresorbable PLA stent is coated with PDA and immobilised with PEI, followed by heparin grafting (Lee et al., 2019); (C) Titanium stent is activated by NaOH to create a hydrophilic surface followed by polylysine incubation for an amino surface. The stent is immersed in heparin and type IV collagen repeatedly to generate poly layers (Zhang et al., 2016a). Image drawn with PowerPoint.

Table 6.4. Commercial heparin coating technologies (Biran and Pond, 2017).

Name	Company	Technique
BioInteractions ASTUTE® Advanced Heparin Coating/Medtronic Trillium® Biosurface	BioInteractions Ltd	Heparin, polyethylene oxide chains, and sulphonate groups covalently bonded to a hydrophilic priming layer
AMC THROMBOSHIELD® Treatment	Edwards Lifesciences, LLC	Heparin ionically bonded with benzalkonium chloride
Atrium HYDRAGLIDE®	Atrium Medical (Maquet Getinge Group)	Covalently bonded heparin complex
CARMEDA® BioActive Surface (CBAS® Heparin Surface)	Carmeda AB (Carmeda AB is a wholly owned subsidiary of W.L. Gore & Associates, Inc.)	Heparin covalently bonded by endpoint attachment to a base matrix
CORLINE® Heparin Surface (CHS™)	Corline Systems AB (Swedish company)	Macromolecular complex of heparin with polyamines. Layers of positively charged polyethyleneimine (PEI) and negatively charged dextran sulphate (DS) polymers comprise the base matrix.
DURAFLO II®	Edwards Lifesciences, LLC	Heparin ionically bonded with benzalkonium chloride
Flowline BIPORE® Heparin Surface	Jotec GmbH	Heparin covalently and ionically bonded
Surmodics Photolink® Heparin Coating	Surmodics, Inc.	Heparin covalently bonded by light activated chemistry
Perouse POLYMAILLE® Flow Plus Heparin	Perouse Medical (French company)	Heparin covalently bonded
Maquet BIOLINE® Coating	Maquet Cardiovascular, LLC	Heparin ionically and covalently bonded to an albumin priming layer
Medtronic Hepamed™ Heparin Coating	Medtronic plc	Heparin covalently bonded to a matrix

Development of bioresorbable stents

Despite their many benefits, DESs exhibit several disadvantages, such as late stent thrombosis, challenges on adaptive/expansive vascular remodelling and influence on multi-slice computed tomography (CT) imaging (Amabile et al., 2014). To combat these drawbacks, some companies are seeking the development of bioresorbable stents. Similar to metal stents, bioresorbable stents are able to restore blood flow, but then are gradually resorbed and eliminated afterwards, resulting in natural reconstruction of vascular wall and functional restoration (Yee et al., 2020). The key healing period of blood vessels is in the first 3 – 9 months after cardiovascular intervention (Yee et al., 2020). Therefore, bioresorbable stents are expected to support vessels during this period and then be removed from of body when they are no longer required.

Bioresorbable stents can be classified broadly into metal-based and polymer-based. Magnesium-based scaffold, as one of the metal-based bioresorbable stents, has been approved in several countries. It consists of magnesium alloy and 95% can be degraded within one year (Li and Zheng, 2013). In spite of harmless degradation, the functional degradation of magnesium-based scaffold is around 30 days, which is much shorter than the healing period (3 – 9 months) year (Li and Zheng, 2013; Yee et al., 2020). Therefore, the following studies pay more attention to alloying or coating to reduce the corrosion rate of bioresorbable stents. Some of our heparin analogues can facilitate wound healing by enhancing HCAEC proliferation. For example, Na-3, Na-4 and Ca-4 showed pro-HCAEC-proliferative activity in co-cultivation systems. They may assist vascular wound healing and decrease the healing period to improve the functionality of short-lived bioresorbable stents.

Polymer-based bioresorbable stents, primarily based on poly-L-lactide, are also approved in several countries. These can maintain a stable scaffold and degrade into lactic acid, a natural by-product of metabolism (Gogas et al., 2012). However, these have been reported to incur severe problems, especially regarding safety performance, including a higher risk of thrombosis (Montone et al., 2017). Conversely, these polymers combined with heparin analogues which can reduce the incidence of restenosis may enhance the safety of polymer-based bioresorbable stents.

Three-dimensional (3D) cell cultivation

Traditional two-dimensional (2D) cell culture is not entirely representative of the natural cell environment, as it does not allow cells to grow in all directions *in vitro*. As cells can only grow in a flat monolayer on a plate (Lovitt et al., 2014). In order to mimic *in vivo* conditions, several cell co-cultivation methods have been developed. For example, three-dimensional (3D) cultivation is believed to mimic a physiologic environment. The 3D cultivation can be classified into scaffold 3D culture and scaffold-free 3D culture. Scaffold 3D culture provides a supporting scaffold to allow cell growth in all directions, such as hydrogels and inert matrices, imitating the *in vivo* environment (Sadat-Shojai, 2018). The former is a polymeric material comprising a network of cross-linked polymers, whereas the latter is a sponge-like membrane made of polystyrene (Sadat-Shojai, 2018). ECM is generally used as a scaffold in scaffold 3D cell culture, allowing cells to differentiate into spatial 3D structures (Geckil et al., 2010; Haycock, 2011; Jensen and Teng, 2020). Scaffold-free 3D culture mainly relies on cell self-assembly into clusters on low adhesion plates, hanging drop plates and micropatterned surfaces (Alghuwainem et al., 2019; Napolitano et al., 2007). These techniques have been utilised widely for tumour cells, osseous tissue, neuronal cells and cardiomyocytes (Haycock, 2011; Lovitt et al., 2014). Compared to 2D cultures, 3D cell cultures much more accurately predict the efficacy or toxicity of drug treatment (Lovitt et al., 2014).

In conclusion, the polysaccharide heparin and heparin-related compounds have long been known to have a profound inhibitory effect on VSMC proliferation (Clowes and Karnowsky, 1977; Hedin et al., 2004). This coupled with its activity as major anti-coagulant makes it an attractive candidate for incorporation into stent materials. Indeed, in the multicentre, randomized human trials, BENESTENT II and MENTOR, heparin-coated stents significantly reduced stent thrombosis, but disappointingly, showed no measurable effect on VSMC proliferation or restenosis (Serruys et al., 1998; Vrolix et al., 2000). We show here that changes in the complex structure of heparin, by chemical modification and/or by forming complexes with various cations, modify its activity with regard to HCASMCS and HCAECs. Further investigation of lead heparin analogues that suppress vascular smooth muscle cell growth, while having no effect on endothelial cell proliferation, may ultimately yield novel next-generation stent coats.

Chapter 7: References

- Achneck, H. E., B. Sileshi, A. Parikh, C. A. Milano, I. J. Welsby and J. H. Lawson. 2010. Pathophysiology of bleeding and clotting in the cardiac surgery patient: from vascular endothelium to circulatory assist device surface. *Circulation*, 122, 2068-2077.
- Akin, I., H. Schneider, H. Ince, S. Kische, T. Rehders, T. Chatterjee and C. Nienaber. 2011. Second-and third-generation drug-eluting coronary stents. *Herz*, 36, 190-197.
- Alberts, B., D. Bray, K. Hopkin, A. Johnson, J. Lewis, M. Raff, K. Roberts and P. Walter. 2013. *Essential cell biology*. Garland Science.
- Alexander, M. R. and G. K. Owens. 2012. Epigenetic control of smooth muscle cell differentiation and phenotypic switching in vascular development and disease. *Annu Rev Physiol*, 74, 13-40.
- Alfonso, F., J. Zueco, A. Cequier, R. Mantilla, A. Bethencourt, J. R. López-Minguez, J. Angel, J. M. Augé, M. Gómez-Recio, C. Morís, R. Seabra-Gomes, M. J. Perez-Vizcayno and C. Macaya. 2003. A randomized comparison of repeat stenting with balloon angioplasty in patients with in-stent restenosis. *Journal of the American College of Cardiology*, 42, 796-805.
- Alghuwainem, A., A. T. Alshareeda and B. Alsowayan. 2019. Scaffold-free 3-D cell sheet technique bridges the gap between 2-D cell culture and animal models. *International journal of molecular sciences*, 20, 4926.
- Amabile, N., G. Souteyrand, S. Ghostine, N. Combaret, M. S. Slama, N. Barber-Chamoux, P. Motreff and C. Caussin. 2014. Very late stent thrombosis related to incomplete neointimal coverage or neoatherosclerotic plaque rupture identified by optical coherence tomography imaging. *European Heart Journal–Cardiovascular Imaging*, 15, 24-31.
- Andrae, J., R. Gallini and C. Betsholtz. 2008. Role of platelet-derived growth factors in physiology and medicine. *Genes & development*, 22, 1276-1312.
- Arkonac, B. M., L. C. Foster, N. E. Sibinga, C. Patterson, K. Lai, J.-C. Tsai, M.-E. Lee, M. A. Perrella and E. Haber. 1998. Vascular endothelial growth factor induces heparin-binding epidermal growth factor-like growth factor in vascular endothelial cells. *Journal of Biological Chemistry*, 273, 4400-4405.
- Arman, M. and K. Krauel. 2015. Human platelet IgG Fc receptor Fcγ RIIA in immunity and thrombosis. *Journal of Thrombosis and Haemostasis*, 13, 893-908.
- Ashikari-Hada, S., H. Habuchi, Y. Kariya, N. Itoh, A. H. Reddi and K. Kimata. 2004. Characterization of growth factor-binding structures in heparin/heparan sulfate using an octasaccharide library. *Journal of Biological Chemistry*, 279, 12346-12354.
- Aster, R. H., B. R. Curtis, J. G. McFarland and D. W. Bougie. 2009. Drug-induced immune thrombocytopenia: pathogenesis, diagnosis, and management. *Journal of Thrombosis and Haemostasis*, 7, 911-918.
- Atha, D. H., J. C. Lormeau, M. Petitou, R. D. Rosenberg and J. Choay. 1987. Contribution of 3-O- and 6-O-sulfated glucosamine residues in the heparin-induced conformational change in antithrombin III. *Biochemistry*, 26, 6454-6461.
- Austin, S. K. 2017. Haemostasis. *Medicine*, 45, 204-208.
- Azizkhan, R., J. C. Azizkhan, B. R. Zetter and J. Folkman. 1980. Mast cell heparin stimulates migration of capillary endothelial cells in vitro. *The Journal of experimental medicine*, 152, 931-944.
- Babapulle, M. N. and M. J. Eisenberg. 2002. Coated stents for the prevention of restenosis: Part II. *Circulation*, 106, 2859-66.
- Baigent, C., M. J. Landray, C. Reith, J. Emberson, D. C. Wheeler, C. Tomson, C. Wanner, V. Krane, A. Cass and J. Craig. 2011. The effects of lowering LDL cholesterol with simvastatin plus ezetimibe in patients with chronic kidney disease (Study of Heart and Renal Protection): a randomised placebo-controlled trial. *The Lancet*, 377, 2181-2192.
- Bailey, A. J. and R. G. Paul. 1999. The mechanisms and consequences of the maturation and ageing of collagen. *Proceedings of the Indian Academy of Sciences-Chemical Sciences*. Springer.
- Beamish, J. A., L. C. Geyer, N. A. Haq-Siddiqi, K. Kottke-Marchant and R. E. Marchant. 2009. The effects of heparin releasing hydrogels on vascular smooth muscle cell phenotype. *Biomaterials*, 30, 6286-6294.

- Beecher, C. N., R. P. Young, D. J. Langeslay, L. J. Mueller and C. K. Larive. 2014. Hydroxyl-proton hydrogen bonding in the heparin oligosaccharide Arixtra in aqueous solution. *The journal of physical chemistry B*, 118, 482-491.
- Bell, C. F. 1977. *Principles and applications of metal chelation*. Oxford University Press.
- Besner, G. E., D. Whelton, M. A. Crissman-Combs, C. L. Steffen, G. Y. Kim and D. R. Brigstock. 1992. Interaction of heparin-binding EGF-like growth factor (HB-EGF) with the epidermal growth factor receptor: modulation by heparin, heparinase, or synthetic heparin-binding HB-EGF fragments. *Growth Factors*, 7, 289-296.
- Bhakuni, T., M. F. Ali, I. Ahmad, S. Bano, S. Ansari and M. A. Jairajpuri. 2016. Role of heparin and non heparin binding serpins in coagulation and angiogenesis: a complex interplay. *Archives of biochemistry and biophysics*, 604, 128-142.
- Billen, L. P., A. Shamas-Din and D. W. Andrews. 2009. Bid: a Bax-like BH3 protein. *Oncogene*, 27 Suppl 1, S93-104.
- Biran, R. and D. Pond. 2017. Heparin coatings for improving blood compatibility of medical devices. *Advanced drug delivery reviews*, 112, 12-23.
- Bitto, N., E. Liguori and V. L. Mura. 2018. Coagulation, microenvironment and liver fibrosis. *Cells*, 7, 85.
- Bolten, S. N., U. Rinas and T. Scheper. 2018. Heparin: role in protein purification and substitution with animal-component free material. *Applied microbiology and biotechnology*, 102, 8647-8660.
- Brandt, S., K. Krauel, K. E. Gottschalk, T. Renné, C. A. Helm, A. Greinacher and S. Block. 2014. Characterisation of the conformational changes in platelet factor 4 induced by polyanions: towards in vitro prediction of antigenicity. *Thrombosis and haemostasis*, 112, 53-64.
- Brozovich, F., C. Nicholson, C. Degen, Y. Z. Gao, M. Aggarwal and K. G. Morgan. 2016. Mechanisms of vascular smooth muscle contraction and the basis for pharmacologic treatment of smooth muscle disorders. *Pharmacological reviews*, 68, 476-532.
- Cai, Y., D. J. Nagel, Q. Zhou, K. D. Cygnar, H. Zhao, F. Li, X. Pi, P. A. Knight and C. Yan. 2015. Role of cAMP-phosphodiesterase 1C signaling in regulating growth factor receptor stability, vascular smooth muscle cell growth, migration, and neointimal hyperplasia. *Circ Res*, 116, 1120-32.
- Campbell, M. K. and S. O. Farrell. 2008. Biochemistry. Brooks. *Cole, Belmont, CA*.
- Cannon, C. P., M. A. Blazing, R. P. Giugliano, A. McCagg, J. A. White, P. Theroux, H. Darius, B. S. Lewis, T. O. Ophuis, J. W. Jukema, G. M. De Ferrari, W. Ruzyllo, P. De Lucca, K. Im, E. A. Bohula, C. Reist, S. D. Wiviott, A. M. Tershakovec, T. A. Musliner, E. Braunwald, R. M. Califf and I.-I. Investigators. 2015. Ezetimibe Added to Statin Therapy after Acute Coronary Syndromes. *N Engl J Med*, 372, 2387-97.
- Capila, I. and R. J. Linhardt. 2002. Heparin-protein interactions. *Angewandte Chemie International Edition*, 41, 390-412.
- Carpenter, A. W. and M. H. Schoenfisch. 2012. Nitric oxide release: Part II. Therapeutic applications. *Chemical Society Reviews*, 41, 3742-3752.
- Castellot, J. J., M. L. Addonizio, R. Rosenberg and M. J. Karnovsky. 1981. Cultured endothelial cells produce heparinlike inhibitor of smooth muscle cell growth. *The Journal of cell biology*, 90, 372-379.
- Castellot, J. J., Jr., D. L. Beeler, R. D. Rosenberg and M. J. Karnovsky. 1984. Structural determinants of the capacity of heparin to inhibit the proliferation of vascular smooth muscle cells. *J Cell Physiol*, 120, 315-20.
- Castellot, J. J., Jr., J. Choay, J. C. Lormeau, M. Petitou, E. Sache and M. J. Karnovsky. 1986. Structural determinants of the capacity of heparin to inhibit the proliferation of vascular smooth muscle cells. II. Evidence for a pentasaccharide sequence that contains a 3-O-sulfate group. *J Cell Biol*, 102, 1979-84.
- Casu, B. and U. Lindahl. 2001. Structure and biological interactions of heparin and heparan sulfate.
- Chapman, T. M. and K. L. Goa. 2003. Bemiparin. *Drugs*, 63, 2357-2377.
- Chavante, S. F., A. S. Brito, M. Lima, E. Yates, H. Nader, M. Guerrini, G. Torri and A. Bisio. 2014. A heparin-like glycosaminoglycan from shrimp containing high levels of 3-O-sulfated D-glucosamine groups in an unusual trisaccharide sequence. *Carbohydrate research*, 390, 59-66.

- Chen, P.-Y., L. Qin, G. Li, G. Tellides and M. Simons. 2016a. Fibroblast growth factor (FGF) signaling regulates transforming growth factor beta (TGF β)-dependent smooth muscle cell phenotype modulation. *Scientific reports*, 6, 33407.
- Chen, P. Y., L. Qin, G. Li, G. Tellides and M. Simons. 2016b. Smooth muscle FGF/TGF β cross talk regulates atherosclerosis progression. *EMBO molecular medicine*, e201506181.
- Cheng, J. W. 2002. Fondaparinux: a new antithrombotic agent. *Clinical therapeutics*, 24, 1757-1769.
- Chevalier, F., R. Lucas, J. Angulo, M. Martin-Lomas and P. M. Nieto. 2004. The heparin–Ca²⁺ interaction: the influence of the O-sulfation pattern on binding. *Carbohydrate research*, 339, 975-983.
- Chia, C. M., R. Winston and A. H. Handyside. 1995. EGF, TGF- α and EGFR expression in human preimplantation embryos. *Development*, 121, 299-307.
- Chong, J. J., H. Reinecke, M. Iwata, B. Torok-Storb, A. Stempien-Otero and C. E. Murry. 2013. Progenitor cells identified by PDGFR- α expression in the developing and diseased human heart. *Stem cells and development*, 22, 1932-1943.
- Choudhury, G. G., C. Karamitsos, J. Hernandez, A. Gentilini, J. Bardgett and H. E. Abboud. 1997. PI-3-kinase and MAPK regulate mesangial cell proliferation and migration in response to PDGF. *American Journal of Physiology-Renal Physiology*, 273, F931-F938.
- Choudhury, G. G., L. Mahimainathan, F. Das, B. Venkatesan and N. Ghosh-Choudhury. 2006. c-Src couples PI 3 kinase/Akt and MAPK signaling to PDGF-induced DNA synthesis in mesangial cells. *Cellular signalling*, 18, 1854-1864.
- Chung, F.-T., H.-C. Chen and C.-L. Chou. 2015a. A higher restenosis rate of benign endobronchial mass after cryotherapy in endobronchial tuberculosis. *Journal of Internal Medicine of Taiwan*, 26, 99-106.
- Chung, S. W., S. M. Bae, M. Lee, T. A. Al-Hilal, C. K. Lee, J. K. Kim, I.-S. Kim, S. Y. Kim and Y. Byun. 2015b. LHT7, a chemically modified heparin, inhibits multiple stages of angiogenesis by blocking VEGF, FGF2 and PDGF-B signaling pathways. *Biomaterials*, 37, 271-278.
- Cines, D. B., A. Tomaski and S. Tannenbaum. 1987. Immune endothelial-cell injury in heparin-associated thrombocytopenia. *N Engl J Med*, 316, 581-9.
- Clowes, A. W. and M. J. Karnowsky. 1977. Suppression by heparin of smooth muscle cell proliferation in injured arteries. *Nature*, 265, 625-6.
- Cohen, T., H. Gitay-Goren, R. Sharon, M. Shibuya, R. Halaban, B.-Z. Levi and G. Neufeld. 1995. VEGF₁₂₁, a vascular endothelial growth factor (VEGF) isoform lacking heparin binding ability, requires cell-surface heparan sulfates for efficient binding to the VEGF receptors of human melanoma cells. *Journal of Biological Chemistry*, 270, 11322-11326.
- Croizat, H., L. Ponchio, F. Nicolini, R. Nagel and C. Eaves. 2000. Problems in laboratory monitoring of heparin dosage. *British journal of haematology*, 111, 397-406.
- Da Silva, J. F. and R. J. P. Williams. 2001. *The biological chemistry of the elements: the inorganic chemistry of life*. Oxford University Press.
- Dahm, A., P. Sandset and F. Rosendaal. 2008. The association between protein S levels and anticoagulant activity of tissue factor pathway inhibitor type 1. *Journal of Thrombosis and Haemostasis*, 6, 393-395.
- Danishefsky, I., M. Ahrens and S. Klein. 1977. Effect of heparin modification on its activity in enhancing the inhibition of thrombin by antithrombin III. *Biochimica et Biophysica Acta (BBA)-General Subjects*, 498, 215-222.
- Davies, P. F. 2009. Hemodynamic shear stress and the endothelium in cardiovascular pathophysiology. *Nature clinical practice Cardiovascular medicine*, 6, 16-26.
- Deepa, S. S., Y. Umehara, S. Higashiyama, N. Itoh and K. Sugahara. 2002. Specific molecular interactions of oversulfated chondroitin sulfate E with various heparin-binding growth factors: implications as a physiological binding partner in the brain and other tissues. *Journal of Biological Chemistry*.
- Dementiev, A., R. Swanson, R. Roth, G. Isetti, G. Izaguirre, S. T. Olson and P. G. Gettins. 2013. The allosteric mechanism of activation of antithrombin as an inhibitor of factor IXa and factor Xa: heparin-independent full activation through mutations adjacent to helix D. *Journal of Biological Chemistry*, 288, 33611-33619.
- Desai, U. R. 2004. New antithrombin-based anticoagulants. *Medicinal Research Reviews*, 24, 151-181.

- Desai, U. R., M. Petitou, I. Björk and S. T. Olson. 1998. Mechanism of heparin activation of antithrombin Role of individual residues of the pentasaccharide activating sequence in the recognition of native and activated states of antithrombin. *Journal of Biological Chemistry*, 273, 7478-7487.
- Duckworth, C. A., S. E. Guimond, P. Sindrewicz, A. J. Hughes, N. S. French, L. Y. Lian, E. A. Yates, D. M. Pritchard, J. M. Rhodes, J. E. Turnbull and L. G. Yu. 2015. Chemically modified, non-anticoagulant heparin derivatives are potent galectin-3 binding inhibitors and inhibit circulating galectin-3-promoted metastasis. *Oncotarget*, 6, 23671-87.
- Duncker, D. J. and R. J. Bache. 2008. Regulation of coronary blood flow during exercise. *Physiological reviews*, 88, 1009-1086.
- El Masri, R., A. Seffouh, H. Lortat-Jacob and R. R. Vivès. 2017. The “in and out” of glucosamine 6-O-sulfation: the 6th sense of heparan sulfate. *Glycoconjugate journal*, 34, 285-298.
- Elmisbah, T. E. and M. Aiderous. 2018. LEVELS OF TROPNIN AND CREATINE KINASE MB IN MYOCARDIAL INFARCTION PATIENTS. *Int J Med Lab Res*, 3, 18-22.
- Erduran, E., Y. Tekelioğlu, Y. Gedik and A. Yıldıran. 1999. Apoptotic effects of heparin on lymphoblasts, neutrophils, and mononuclear cells: results of a preliminary in vitro study. *American journal of hematology*, 61, 90-93.
- Ezihe-Ejiofor, J. A. and N. Hutchinson. 2013. Anticlotting mechanisms 1: physiology and pathology. *Continuing Education in Anaesthesia, Critical Care & Pain*, 13, 87-92.
- Fager, G. 1995. Thrombin and proliferation of vascular smooth muscle cells. *Circulation research*, 77, 645-650.
- Farb, A. 2002. Morphological Predictors of Restenosis After Coronary Stenting in Humans. *Circulation*, 105, 2974-2980.
- Fasciano, S., R. C. Patel, I. Handy and C. V. Patel. 2005. Regulation of vascular smooth muscle proliferation by heparin: inhibition of cyclin-dependent kinase 2 activity by p27kip1. *Journal of Biological Chemistry*, 280, 15682-15689.
- Ferreras, C., G. Rushton, C. L. Cole, M. Babur, B. A. Telfer, T. H. van Kuppevelt, J. M. Gardiner, K. J. Williams, G. C. Jayson and E. Avizienyte. 2012. Endothelial heparan sulfate 6-O-sulfation levels regulate angiogenic responses of endothelial cells to fibroblast growth factor 2 and vascular endothelial growth factor. *Journal of Biological Chemistry*, 287, 36132-36146.
- Fiedler, J., F. Leucht, J. Waltenberger, C. Dehio and R. E. Brenner. 2005. VEGF-A and PlGF-1 stimulate chemotactic migration of human mesenchymal progenitor cells. *Biochemical and biophysical research communications*, 334, 561-568.
- Fiedler, U. and H. G. Augustin. 2006. Angiopoietins: a link between angiogenesis and inflammation. *Trends in immunology*, 27, 552-558.
- Fink, S. L. and B. T. Cookson. 2005. Apoptosis, pyroptosis, and necrosis: mechanistic description of dead and dying eukaryotic cells. *Infect Immun*, 73, 1907-16.
- Frishman, A. M., S. J. Valberg, J. B. Bender, E. Annandale and D. Hayden. 2006. Comparison of histopathologic criteria and skeletal muscle fixation techniques for the diagnosis of polysaccharide storage myopathy in horses. *Veterinary Pathology*, 43, 257-269.
- Forsberg, E., G. Pejler, M. Ringvall, C. Lunderius, B. Tomasini-Johansson, M. Kusche-Gullberg, I. Eriksson, J. Ledin, L. Hellman and L. Kjellén. 1999. Abnormal mast cells in mice deficient in a heparin-synthesizing enzyme. *Nature*, 400, 773-776.
- Frismantiene, A., M. Philippova, P. Erne and T. J. Resink. 2018. Smooth muscle cell-driven vascular diseases and molecular mechanisms of VSMC plasticity. *Cellular signalling*, 52, 48-64.
- Fromm, J., R. Hileman, E. Caldwell, J. Weiler and R. Linhardt. 1995. Differences in the interaction of heparin with arginine and lysine and the importance of these basic amino acids in the binding of heparin to acidic fibroblast growth factor. *Archives of biochemistry and biophysics*, 323, 279-287.
- Fujikawa, K., E. Presman, J. M. Isner and L. Varticovski. 1999. Expression of tie1 and tie2 proteins during reendothelialization in balloon-injured rat carotid artery. *J Vasc Res*, 36, 272-81.
- Gale, A. J. 2011. Continuing education course# 2: current understanding of hemostasis. *Toxicologic pathology*, 39, 273-280.
- Garg, H. G., R. J. Linhardt and C. A. Hales. 2011a. *Chemistry and biology of heparin and heparan sulfate*. Elsevier.

- Garg, H. G., H. Mrabat, L. Yu, C. A. Hales, B. Li, C. N. Moore, F. Zhang and R. J. Linhardt. 2011b. Anti-proliferative effects of O-acyl-low-molecular-weight heparin derivatives on bovine pulmonary artery smooth muscle cells. *Glycoconj J*, 28, 419-26.
- Garg, H. G., L. Yu, C. A. Hales, T. Toida, T. Islam and R. J. Linhardt. 2003. Sulfation patterns in heparin and heparan sulfate: effects on the proliferation of bovine pulmonary artery smooth muscle cells. *Biochimica et Biophysica Acta (BBA)-Molecular Basis of Disease*, 1639, 225-231.
- Geary, R. L., N. Koyama, T. W. Wang, S. Vergel and A. W. Clowes. 1995. Failure of heparin to inhibit intimal hyperplasia in injured baboon arteries: the role of heparin-sensitive and-insensitive pathways in the stimulation of smooth muscle cell migration and proliferation. *Circulation*, 91, 2972-2981.
- Geckil, H., F. Xu, X. Zhang, S. Moon and U. Demirci. 2010. Engineering hydrogels as extracellular matrix mimics. *Nanomedicine*, 5, 469-484.
- George, A. J., R. D. Hannan and W. G. Thomas. 2013. Unravelling the molecular complexity of GPCR-mediated EGFR transactivation using functional genomics approaches. *FEBS J*, 280, 5258-68.
- Gerotziakas, G., A. Petropoulou, E. Verdy, M. Samama and I. Elalamy. 2007. Effect of the anti-factor Xa and anti-factor IIa activities of low-molecular-weight heparins upon the phases of thrombin generation. *Journal of Thrombosis and Haemostasis*, 5, 955-962.
- Ghezzi, S., L. Cooper, A. Rubio, I. Pagani, M. R. Capobianchi, G. Ippolito, J. Pelletier, M. C. Z. Meneghetti, M. A. Lima and M. A. Skidmore. 2017. Heparin prevents Zika virus induced-cytopathic effects in human neural progenitor cells. *Antiviral research*, 140, 13-17.
- Gilotti, A. C., W. Nimlamool, R. Pugh, J. B. Slee, T. C. Barthol, E. A. Miller and L. J. Lowe-Krentz. 2014. Heparin Responses in Vascular Smooth Muscle Cells Involve cGMP-Dependent Protein Kinase (PKG). *Journal of cellular physiology*, 229, 2142-2152.
- Gisterå, A. and G. K. Hansson. 2017. The immunology of atherosclerosis. *Nature Reviews Nephrology*, 13, 368.
- Gitay-Goren, H., T. Cohen, S. Tessler, S. Soker, S. Gengrinovitch, P. Rockwell, M. Klagsbrun, B.-Z. Levi and G. Neufeld. 1996. Selective binding of VEGF121 to one of the three vascular endothelial growth factor receptors of vascular endothelial cells. *Journal of Biological Chemistry*, 271, 5519-5523.
- Goel, R. and P. M. Ness. 2016. Prothrombin and Partial Thromboplastin Time. *Trauma Induced Coagulopathy*. Springer.
- Gogas, B. D., M. Radu, Y. Onuma, L. Perkins, J. C. Powers, J. Gomez-Lara, V. Farooq, H. M. Garcia-Garcia, R. Diletti and R. Rapoza. 2012. Evaluation with in vivo optical coherence tomography and histology of the vascular effects of the everolimus-eluting bioresorbable vascular scaffold at two years following implantation in a healthy porcine coronary artery model: implications of pilot results for future pre-clinical studies. *The international journal of cardiovascular imaging*, 28, 499-511.
- Gomez, D. and G. K. Owens. 2012. Smooth muscle cell phenotypic switching in atherosclerosis. *Cardiovascular research*, 95, 156-164.
- Gómez Toledo, A., J. T. Sorrentino, D. R. Sandoval, J. Malmström, N. E. Lewis and J. D. Esko. 2021. A Systems View of the Heparan Sulfate Interactome. *Journal of Histochemistry & Cytochemistry*, 69, 105-119.
- Greinacher, A. 2015. Heparin-Induced Thrombocytopenia. *N Engl J Med*, 373, 252-61.
- Greinacher, A. and M. Delcea. 2015. Another surprising finding in heparin-induced thrombocytopenia—eat big. *Journal of Thrombosis and Haemostasis*, 13, 1414-1415.
- Greinacher, A., B. Pötzsch, J. Amiral, V. Dummel, A. Eichner and C. Mueller-Eckhardt. 1994. Heparin-associated thrombocytopenia: isolation of the antibody and characterization of a multimolecular PF4-heparin complex as the major antigen. *Thrombosis and haemostasis*, 71, 247-251.
- Griffith, M. J., G. Beavers, H. S. Kingdon and R. L. Lundblad. 1980. Effect of monovalent cations on the heparin-enhanced antithrombin III/thrombin reaction. *Thrombosis research*, 17, 29-39.
- Gu, Z., B. E. Rolfe, Z. P. Xu, A. C. Thomas, J. H. Campbell and G. Q. Lu. 2010. Enhanced effects of low molecular weight heparin intercalated with layered double hydroxide nanoparticles on rat vascular smooth muscle cells. *Biomaterials*, 31, 5455-5462.
- Guglieri, S., M. Hricovini, R. Raman, L. Polito, G. Torri, B. Casu, R. Sasisekharan and M. Guerrini. 2008. Minimum FGF2 binding structural requirements of heparin and

- heparan sulfate oligosaccharides as determined by NMR spectroscopy. *Biochemistry*, 47, 13862-13869.
- Guimond, S. E., T. R. Rudd, M. A. Skidmore, A. Ori, D. Gaudesi, C. Cosentino, M. Guerrini, R. Edge, D. Collison and E. McInnes. 2009. Cations Modulate Polysaccharide Structure To Determine FGF- FGFR Signaling: A Comparison of Signaling and Inhibitory Polysaccharide Interactions with FGF-1 in Solution. *Biochemistry*, 48, 4772-4779.
- Güneydaş, G. and M. Topçul. 2016. *Which Way do You Prefer to Death ? Cell Death and Beyond*. OMICS International.
- Guyton, J. R., R. D. Rosenberg, A. W. Clowes and M. J. Karnovsky. 1980. Inhibition of rat arterial smooth muscle cell proliferation by heparin. In vivo studies with anticoagulant and nonanticoagulant heparin. *Circulation Research*, 46, 625-634.
- Haycock, J. W. 2011. 3D cell culture: a review of current approaches and techniques. *3D cell culture*, 1-15.
- He, C., S. C. Medley, T. Hu, M. E. Hinsdale, F. Lupu, R. Virmani and L. E. Olson. 2015. PDGFR β signalling regulates local inflammation and synergizes with hypercholesterolaemia to promote atherosclerosis. *Nature communications*, 6, 7770.
- Hedin, U., G. Daum and A. W. Clowes. 1998. Heparin inhibits thrombin-induced mitogen-activated protein kinase signaling in arterial smooth muscle cells. *Journal of vascular surgery*, 27, 512-520.
- Hedin, U., J. Roy and P.-K. Tran. 2004. Control of smooth muscle cell proliferation in vascular disease. *Current opinion in lipidology*, 15, 559-565.
- Hemker, H. and S. Beguin. 1991. Thrombosis and thrombin. *Annales pharmaceutiques francaises*.
- Hemker, H., J. Van Rijn, J. Rosing, G. Van Dieijen, E. Bevers and R. Zwaal. 1983. Platelet membrane involvement in blood coagulation. *Blood cells*, 9, 303-317.
- Herbert, J.-M., M. Clowes, H. J. Lea, M. Pascal and A. W. Clowes. 1996. Protein kinase C α expression is required for heparin inhibition of rat smooth muscle cell proliferation in vitro and in vivo. *Journal of Biological Chemistry*, 271, 25928-25935.
- Hernaningsih, Y. and T. V. Butarbutar. 2019. The Effects of Plasma Prothrombine Time and Activated Partial Thromboplastin Time Based on Different Instruments and Methods. *Journal of Clinical and Diagnostic Research*, 13, 21-25.
- Heuck, C., U. Schiele, D. Horn, D. Fronda and E. Ritz. 1985. The role of surface charge on the accelerating action of heparin on the antithrombin III-inhibited activity of alpha-thrombin. *Journal of Biological Chemistry*, 260, 4598-4603.
- Hirsh, J., T. E. Warkentin, S. G. Shaughnessy, S. S. Anand, J. L. Halperin, R. Raschke, C. Granger, E. M. Ohman and J. E. Dalen. 2001. Heparin and low-molecular-weight heparin mechanisms of action, pharmacokinetics, dosing, monitoring, efficacy, and safety. *Chest*, 119, 64S-94S.
- Holbro, T. and N. E. Hynes. 2004. ErbB receptors: directing key signaling networks throughout life. *Annu. Rev. Pharmacol. Toxicol.*, 44, 195-217.
- Holmer, E., K. Kurachi and G. SÖDERSTRÖM. 1981. The molecular-weight dependence of the rate-enhancing effect of heparin on the inhibition of thrombin, factor X a, factor IX a, factor XI a, factor XII a and kallikrein by antithrombin. *Biochemical Journal*, 193, 395-400.
- Hook, M., I. Bjork, J. Hopwood and U. Lindahl. 1976. Anticoagulant activity of heparin: separation of high-activity and low-activity heparin species by affinity chromatography on immobilized antithrombin. *FEBS Lett*, 66, 90-3.
- Hoover, R. L., R. Rosenberg, W. Haering and M. J. Karnovsky. 1980. Inhibition of Rat Arterial Smooth Muscle Cell Proliferation by Heparin II - in vitro studies. *Circulation Research*, 47, 578-58.
- Huang, K., J. Zhang, K. L. O'Neill, C. B. Gurumurthy, R. M. Quadros, Y. Tu and X. Luo. 2016. Cleavage by Caspase 8 and Mitochondrial Membrane Association Activate the BH3-only Protein Bid during TRAIL-induced Apoptosis. *J Biol Chem*, 291, 11843-51.
- Hui, H., W. Ma, J. Cui, M. Gong, Y. Wang, Y. Zhang, T. He, Y. Bi and Y. He. 2017. Periodic acid-Schiff staining method for function detection of liver cells is affected by 2% horse serum in induction medium. *Molecular medicine reports*, 16, 8062-8068.
- Huynh, A., D. M. Arnold, J. G. Kelton, J. W. Smith, P. Horsewood, R. Clare, A. Guarné and I. Nazy. 2019. Characterization of platelet factor 4 amino acids that bind pathogenic

- antibodies in heparin-induced thrombocytopenia. *Journal of Thrombosis and Haemostasis*, 17, 389-399.
- Ichikawa, J., M. Kodaka, K. Nishiyama and M. Komori. 2017. Influence of Heparin on the Fibrinogen Level Measured by the Prothrombin Time-Derived Method During Cardiac Surgery With Cardiopulmonary Bypass. *Journal of cardiothoracic and vascular anesthesia*, 31, e48-e50.
- Ignjatovic, V. 2013. Activated Partial Thromboplastin Time. In: Monagle, P. (ed.) *Haemostasis: Methods and Protocols*. Humana Press, Totowa, NJ.
- Indolfi, C., A. Cioppa, E. Stabile, E. Di Lorenzo, G. Esposito, A. Pisani, A. Leccia, L. Cavuto, A. M. Stingone and A. Chieffo. 2000. Effects of hydroxymethylglutaryl coenzyme A reductase inhibitor simvastatin on smooth muscle cell proliferation in vitro and neointimal formation in vivo after vascular injury. *Journal of the American College of Cardiology*, 35, 214-221.
- Indolfi, C., G. Esposito, E. Di Lorenzo, A. Rapacciuolo, A. Feliciello, A. Porcellini, V. E. Avvedimento, M. Condorelli and M. Chiariello. 1995. Smooth muscle cell proliferation is proportional to the degree of balloon injury in a rat model of angioplasty. *Circulation*, 92, 1230-1235.
- Irie, F., M. Okuno, K. Matsumoto, E. B. Pasquale and Y. Yamaguchi. 2008. Heparan sulfate regulates ephrin-A3/EphA receptor signaling. *Proceedings of the National Academy of Sciences*, 105, 12307-12312.
- Izaguirre, G., S. Aguila, L. Qi, R. Swanson, R. Roth, A. R. Rezaie, P. G. Gettins and S. T. Olson. 2014. Conformational activation of antithrombin by heparin involves an altered exosite interaction with protease. *Journal of Biological Chemistry*, 289, 34049-34064.
- Izzard, T. D., C. Taylor, S. D. Birkett, C. L. Jackson and A. C. Newby. 2002. Mechanisms underlying maintenance of smooth muscle cell quiescence in rat aorta: role of the cyclin dependent kinases and their inhibitors. *Cardiovascular Research*, 53, 242-252.
- Jairajpuri, M. A., A. Lu, U. Desai, S. T. Olson, I. Bjork and S. C. Bock. 2003. Antithrombin III phenylalanines 122 and 121 contribute to its high affinity for heparin and its conformational activation. *Journal of Biological Chemistry*, 278, 15941-15950.
- Jakobsson, L., J. Kreuger, K. Holmborn, L. Lundin, I. Eriksson, L. Kjellen and L. Claesson-Welsh. 2006. Heparan sulfate in trans potentiates VEGFR-mediated angiogenesis. *Dev Cell*, 10, 625-34.
- Jan, H., K. S. Borensztajn, S. Moimas, S. van Heiningen, P. Teeling, T. J. van Berkel and E. A. Biessen. 2011. IGF-1 has plaque-stabilizing effects in atherosclerosis by altering vascular smooth muscle cell phenotype. *The American journal of pathology*, 178, 924-934.
- Jenny, R., P. E. Haley and B. Earp. 2006. Method for manufacturing a tissue factor-based prothrombin time reagent. Google Patents.
- Jensen, C. and Y. Teng. 2020. Is it time to start transitioning from 2D to 3D cell culture? *Frontiers in molecular biosciences*, 7, 33.
- Jeske, W. P., D. Hoppensteadt, A. Gray, J. M. Walenga, J. Cunanan, L. Myers, J. Fareed, A. Bayol, H. Rigal and C. Viskov. 2011. A common standard is inappropriate for determining the potency of ultra low molecular weight heparins such as semuloparin and bemiparin. *Thrombosis research*, 128, 361-367.
- Jin, L., J. P. Abrahams, R. Skinner, M. Petitou, R. N. Pike and R. W. Carrell. 1997. The anticoagulant activation of antithrombin by heparin. *Proceedings of the National Academy of Sciences*, 94, 14683-14688.
- Johnson, D. E. and L. T. Williams. 1992. Structural and functional diversity in the FGF receptor multigene family. *Advances in cancer research*, 60, 1-41.
- Kahl, C. R. and A. R. Means. 2003. Regulation of cell cycle progression by calcium/calmodulin-dependent pathways. *Endocrine reviews*, 24, 719-736.
- Kalmes, A., B. R. Vesti, G. Daum, J. A. Abraham and A. W. Clowes. 2000. Heparin blockade of thrombin-induced smooth muscle cell migration involves inhibition of epidermal growth factor (EGF) receptor transactivation by heparin-binding EGF-like growth factor. *Circulation Research*, 87, 92-98.
- Kasper, D., A. Fauci, S. Hauser, D. Longo, J. Jameson and J. Loscalzo. 2015. *Harrison's Principles of Internal Medicine 19/E (Vol. 1 & Vol. 2)*, 19 edn. McGraw-Hill Education, U.S.A.
- Kasthuri, R. S., S. L. Glover, W. Jonas, T. McEachron, R. Pawlinski, G. M. Arepally, N. S. Key and N. Mackman. 2012. PF4/heparin-antibody complex induces monocyte tissue

- factor expression and release of tissue factor positive microparticles by activation of FcγRI. *Blood, The Journal of the American Society of Hematology*, 119, 5285-5293.
- Kazi, M., K. Lundmark, P. Religa, I. Gouda, O. Larm, A. Ray, J. Swedenborg and U. Hedin. 2002. Inhibition of rat smooth muscle cell adhesion and proliferation by non-anticoagulant heparins. *Journal of cellular physiology*, 193, 365-372.
- Keck, R. G., L. Berleau, R. Harris and B. A. Keyt. 1997. Disulfide structure of the heparin binding domain in vascular endothelial growth factor: characterization of posttranslational modifications in VEGF. *Archives of biochemistry and biophysics*, 344, 103-113.
- Kenrick, S. W. T. C. 2019. AN6301: Discriminating heparin from chondroitin sulfate by charge:mass ratio
- Kereiakes, D. J., L. A. Cannon, J. A. Ormiston, M. A. Turco, T. Mann, G. J. Mishkel, T. McGarry, H. Wang, P. Underwood and K. D. Dawkins. 2011. Propensity-matched patient-level comparison of the TAXUS Liberté and TAXUS element (ION) paclitaxel-eluting stents. *The American journal of cardiology*, 108, 828-837.
- Khan, M. S., P. Singh, A. Azhar, A. Naseem, Q. Rashid, M. A. Kabir and M. A. Jairajpuri. 2011. Serpin inhibition mechanism: a delicate balance between native metastable state and polymerization. *Journal of amino acids*, 2011.
- Khan, W., R. Thipparaboina, S. Farah, J. Z. Weinberger and A. J. Domb. 2014. Drug-Eluting Stents. *Focal Controlled Drug Delivery*. Springer.
- Khorana, A. A., A. Sahni, O. D. Altland and C. W. Francis. 2003. Heparin inhibition of endothelial cell proliferation and organization is dependent on molecular weight. *Arterioscler Thromb Vasc Biol*, 23, 2110-5.
- Kim, W. S., K. S. Lee, J. H. Kim, C. K. Kim, G. Lee, J. Choe, M. H. Won, T. H. Kim, D. Jeoung, H. Lee, J. Y. Kim, M. Ae Jeong, K. S. Ha, Y. G. Kwon and Y. M. Kim. 2017. The caspase-8/Bid/cytochrome c axis links signals from death receptors to mitochondrial reactive oxygen species production. *Free Radic Biol Med*, 112, 567-577.
- Knauer, D. J., D. Majumdar, P.-C. Fong and M. F. Knauer. 2000. SERPIN regulation of factor X_{ia}: the novel observation that protease nexin 1 in the presence of heparin is a more potent inhibitor of factor X_{la} than C1 inhibitor. *Journal of Biological Chemistry*, 275, 37340-37346.
- Koepfli, P., C. A. Wyss, M. Namdar, M. Klainguti, G. K. von Schulthess, T. F. Lüscher and P. A. Kaufmann. 2004. B-adrenergic blockade and myocardial perfusion in coronary artery disease: differential effects in stenotic versus remote myocardial segments. *Journal of Nuclear Medicine*, 45, 1626-1631.
- Koeppel, T. and A. Schober. 2016. Restenosis in vascular surgical practice-molecular biology and strategies for prevention. *Oxford Textbook of Vascular Surgery*. Oxford University Press, Oxford.
- Kozlowski, E. O. and M. Pavao. 2011. Effect of sulfated glycosaminoglycans on tumor invasion and metastasis. *Front. Biosci*, 3, 1541-1551.
- Krauel, K., C. Hackbarth, B. Fürll and A. Greinacher. 2012. Heparin-induced thrombocytopenia: in vitro studies on the interaction of dabigatran, rivaroxaban, and low-sulfated heparin, with platelet factor 4 and anti-PF4/heparin antibodies. *Blood, The Journal of the American Society of Hematology*, 119, 1248-1255.
- Kreimann, M., S. Brandt, K. Krauel, S. Block, C. A. Helm, W. Weitschies, A. Greinacher and M. Delcea. 2014. Binding of anti-platelet factor 4/heparin antibodies depends on the thermodynamics of conformational changes in platelet factor 4. *Blood, The Journal of the American Society of Hematology*, 124, 2442-2449.
- Krilleke, D., Y.-S. E. Ng and D. T. Shima. 2009. The heparin-binding domain confers diverse functions of VEGF-A in development and disease: a structure–function study. Portland Press Limited.
- Kumar, V., A. K. Abbas, N. Fausto and J. C. Aster. 2014. *Robbins and Cotran pathologic basis of disease, professional edition e-book*. elsevier health sciences.
- Kusumanto, Y. H., R. A. Tio, B. G. Loef, W. J. Sluiter, N. H. Mulder and G. A. Hospers. 2006. Systemic VEGF levels after coronary artery bypass graft surgery reflects the extent of inflammatory response. *Acute Card Care*, 8, 41-5.
- Kwak, B. R., M. Back, M. L. Bochaton-Piallat, G. Caligiuri, M. J. Daemen, P. F. Davies, I. E. Hoefer, P. Holvoet, H. Jo, R. Krams, S. Lehoux, C. Monaco, S. Steffens, R. Virmani, C. Weber, J. J. Wentzel and P. C. Evans. 2014. Biomechanical factors in

- atherosclerosis: mechanisms and clinical implications. *Eur Heart J*, 35, 3013-20, 3020a-3020d.
- Kwon, D., J.-S. Kim, B.-H. Cha, K.-S. Park, I. Han, K.-S. Park, H. Bae, M.-K. Han, K.-S. Kim and S.-H. Lee. 2016. The effect of fetal bovine serum (FBS) on efficacy of cellular reprogramming for induced pluripotent stem cell (iPSC) generation. *Cell transplantation*, 25, 1025-1042.
- Lam, L. H., J. E. Silbert and R. D. Rosenberg. 1976. The separation of active and inactive forms of heparin. *Biochem Biophys Res Commun*, 69, 570-7.
- Lamb, D. J., H. Modjtahedi, N. J. Plant and G. A. Ferns. 2004. EGF mediates monocyte chemotaxis and macrophage proliferation and EGF receptor is expressed in atherosclerotic plaques. *Atherosclerosis*, 176, 21-26.
- Lammers, G., E. M. Van de Westerlo, E. M. Versteeg, T. H. van Kuppevelt and W. F. Daamen. 2011. A comparison of seven methods to analyze heparin in biomaterials: quantification, location, and anticoagulant activity. *Tissue Engineering Part C: Methods*, 17, 669-676.
- Lasne, D., B. Jude and S. Susen. 2006. From normal to pathological hemostasis. *Canadian journal of anaesthesia*, 53, S2-S11.
- Lee, S. J., H. H. Jo, K. S. Lim, D. Lim, S. Lee, J. H. Lee, W. D. Kim, M. H. Jeong, J. Y. Lim and I. K. Kwon. 2019. Heparin coating on 3D printed poly (l-lactic acid) biodegradable cardiovascular stent via mild surface modification approach for coronary artery implantation. *Chemical Engineering Journal*, 378, 122116.
- Lemasters, J. J. 2018. Molecular Mechanisms of Cell Death. *Molecular Pathology (Second Edition)*. Elsevier.
- Letourneur, D., B. L. Caleb and J. J. Castellot Jr. 1995. Heparin binding, internalization, and metabolism in vascular smooth muscle cells: I. Upregulation of heparin binding correlates with antiproliferative activity. *Journal of cellular physiology*, 165, 676-686.
- Li, H. L., K. H. Ye, H. W. Zhang, Y. R. Luo, X. Da Ren, A. H. Xiong and S. Rui. 2001. Effect of heparin on apoptosis in human nasopharyngeal carcinoma CNE2 cells. *Cell research*, 11, 311-315.
- Li, N. and Y. Zheng. 2013. Novel magnesium alloys developed for biomedical application: a review. *Journal of Materials Science & Technology*, 29, 489-502.
- Li, W., D. J. Johnson, C. T. Esmon and J. A. Huntington. 2004. Structure of the antithrombin–thrombin–heparin ternary complex reveals the antithrombotic mechanism of heparin. *Nature structural & molecular biology*, 11, 857-862.
- Li, Y., L. Li, Z. Qian, B. Lin, J. Chen, Y. Luo, J. Qu, J. U. Raj and D. Gou. 2018. Phosphatidylinositol 3-Kinase-DNA Methyltransferase 1-miR-1281-Histone Deacetylase 4 Regulatory Axis Mediates Platelet-Derived Growth Factor-Induced Proliferation and Migration of Pulmonary Artery Smooth Muscle Cells. *J Am Heart Assoc*, 7.
- Li, Y., C. Sun, E. A. Yates, C. Jiang, M. C. Wilkinson and D. G. Fernig. 2016. Heparin binding preference and structures in the fibroblast growth factor family parallel their evolutionary diversification. *Open biology*, 6, 150275.
- Liebmann, C. 2011. EGF receptor activation by GPCRs: an universal pathway reveals different versions. *Molecular and cellular endocrinology*, 331, 222-231.
- Lima, M. A., A. J. Hughes, N. Veraldi, T. R. Rudd, R. Hussain, A. S. Brito, S. F. Chavante, I. I. Tersariol, G. Siligardi, H. B. Nader and E. A. Yates. 2013. Antithrombin stabilisation by sulfated carbohydrates correlates with anticoagulant activity. *MedChemComm*, 4.
- Lin, Y.-C., Y.-C. Ko, S.-C. Hung, Y.-T. Lin, J.-H. Lee, J.-Y. Tsai, P.-H. Kung, M.-C. Tsai, Y.-F. Chen and C.-C. Wu. 2019. Selective inhibition of PAR4 (protease-activated receptor 4)-mediated platelet activation by a synthetic nonanticoagulant heparin analog. *Arteriosclerosis, thrombosis, and vascular biology*, 39, 694-703.
- Liu, H., S. Kennard and B. Lilly. 2009. NOTCH3 expression is induced in mural cells through an autoregulatory loop that requires endothelial-expressed JAGGED1. *Circulation research*, 104, 466-475.
- Lovell, J. F., L. P. Billen, S. Bindner, A. Shamas-Din, C. Fradin, B. Leber and D. W. Andrews. 2008. Membrane binding by tBid initiates an ordered series of events culminating in membrane permeabilization by Bax. *Cell*, 135, 1074-84.
- Lovitt, C. J., T. B. Shelper and V. M. Avery. 2014. Advanced cell culture techniques for cancer drug discovery. *Biology*, 3, 345-367.

- Luo, Y., W. Lu, K. A. Mohamedali, J.-H. Jang, R. B. Jones, J. L. Gabriel, M. Kan and W. L. McKeehan. 1998. The glycine box: a determinant of specificity for fibroblast growth factor. *Biochemistry*, 37, 16506-16515.
- Maccarana, M. and U. Lindahl. 1993. Mode of interaction between platelet factor 4 and heparin. *Glycobiology*, 3, 271-277.
- Mack, C. P. 2011. Signaling Mechanisms That Regulate Smooth Muscle Cell Differentiation.
- Makki, N., K. W. Thiel and F. J. Miller. 2013. The epidermal growth factor receptor and its ligands in cardiovascular disease. *International journal of molecular sciences*, 14, 20597-20613.
- Mamer, S. B., S. Chen, J. C. Weddell, A. Palasz, A. Wittenkeller, M. Kumar and P. Imoukhuede. 2017. Discovery of high-affinity PDGF-VEGFR interactions: redefining RTK dynamics. *Scientific reports*, 7, 1-14.
- Mann, K. G. 2003. Thrombin formation. *Chest*, 124, 4S-10S.
- Margolis, B., S. Rhee, S. Felder, M. Mervic, R. Lyall, A. Levitzki, A. Ullrich, A. Zilberstein and J. Schlessinger. 1989. EGF induces tyrosine phosphorylation of phospholipase C-II: a potential mechanism for EGF receptor signaling. *Cell*, 57, 1101-1107.
- Mayo, K., E. Ilyina, V. Roongta, M. Dundas, J. Joseph, C. Lai, T. Maione and T. Daly. 1995. Heparin binding to platelet factor-4. An NMR and site-directed mutagenesis study: arginine residues are crucial for binding. *Biochemical Journal*, 312, 357-365.
- McIlwain, D. R., T. Berger and T. W. Mak. 2013. Caspase functions in cell death and disease. *Cold Spring Harb Perspect Biol*, 5, a008656.
- Meneghetti, M. C., A. J. Hughes, T. R. Rudd, H. B. Nader, A. K. Powell, E. A. Yates and M. A. Lima. 2015. Heparan sulfate and heparin interactions with proteins. *Journal of the Royal Society Interface*, 12, 20150589.
- Miao, E. A., J. V. Rajan and A. Aderem. 2011. Caspase-1-induced pyroptotic cell death. *Immunol Rev*, 243, 206-14.
- Millette, E., B. H. Rauch, O. Defawe, R. D. Kenagy, G. Daum and A. W. Clowes. 2005. Platelet-derived growth factor-BB-induced human smooth muscle cell proliferation depends on basic FGF release and FGFR-1 activation. *Circ Res*, 96, 172-9.
- Minami, T., A. Sugiyama, S. Q. Wu, R. Abid, T. Kodama and W. C. Aird. 2004. Thrombin and phenotypic modulation of the endothelium. *Arterioscler Thromb Vasc Biol*, 24, 41-53.
- Minsky, B. B., P. L. Dubin and I. A. Kaltashov. 2017. Electrostatic forces as dominant interactions between proteins and polyanions: an ESI MS study of fibroblast growth factor binding to heparin oligomers. *Journal of the American Society for Mass Spectrometry*, 28, 758-767.
- Misiti, F., F. Orsini, M. E. Clementi, W. Lattanzi, B. Giardina and F. Michetti. 2008. Mitochondrial oxygen consumption inhibition importance for TMT-dependent cell death in undifferentiated PC12 cells. *Neurochem Int*, 52, 1092-9.
- Miyagawa, J., S. Higashiyama, S. Kawata, Y. Inui, S. Tamura, K. Yamamoto, M. Nishida, T. Nakamura, S. Yamashita, Y. Matsuzawa and et al. 1995. Localization of heparin-binding EGF-like growth factor in the smooth muscle cells and macrophages of human atherosclerotic plaques. *J Clin Invest*, 95, 404-11.
- Mohammadi, M., S. K. Olsen and R. Goetz. 2005. A protein canyon in the FGF-FGF receptor dimer selects from an a la carte menu of heparan sulfate motifs. *Current opinion in structural biology*, 15, 506-516.
- Monje, P., M. J. Marinissen and J. S. Gutkind. 2003. Phosphorylation of the carboxyl-terminal transactivation domain of c-Fos by extracellular signal-regulated kinase mediates the transcriptional activation of AP-1 and cellular transformation induced by platelet-derived growth factor. *Molecular and cellular biology*, 23, 7030-7043.
- Monteiro, H. P., M. T. O. Albuquerque, C. J. R. Oliveira and M. F. Curcio. 2018. Signal Transduction Pathways in Endothelial Cells: Implications for Angiogenesis. *Endothelium and Cardiovascular Diseases*. Elsevier.
- Montone, R. A., G. Niccoli, F. De Marco, S. Minelli, F. D'Ascenzo, L. Testa, F. Bedogni and F. Crea. 2017. Temporal trends in adverse events after everolimus-eluting bioresorbable vascular scaffold versus everolimus-eluting metallic stent implantation: a meta-analysis of randomized controlled trials. *Circulation*, 135, 2145-2154.
- Moonen, J.-R. A., E. S. Lee, M. Schmidt, M. Maleszewska, J. A. Koerts, L. A. Brouwer, T. G. Van Kooten, M. J. Van Luyn, C. J. Zeebregts and G. Krenning. 2015. Endothelial-to-mesenchymal transition contributes to fibro-proliferative vascular disease and is modulated by fluid shear stress. *Cardiovascular research*, 108, 377-386.

- Moore, K. J. and I. Tabas. 2011. Macrophages in the pathogenesis of atherosclerosis. *Cell*, 145, 341-355.
- Nader, H. B. 1991. Characterization of a heparan sulfate and a peculiar chondroitin 4-sulfate proteoglycan from platelets. Inhibition of the aggregation process by platelet chondroitin sulfate proteoglycan. *Journal of Biological Chemistry*, 266, 10518-10523.
- Nahain, A. A., V. Ignjatovic, P. Monagle, J. Tsanaktsidis and V. Ferro. 2018. Heparin mimetics with anticoagulant activity. *Medicinal research reviews*, 38, 1582-1613.
- Nakazawa, G., E. Ladich, A. V. Finn and R. Virmani. 2008. Pathophysiology of vascular healing and stent mediated arterial injury. *EuroIntervention: journal of EuroPCR in collaboration with the Working Group on Interventional Cardiology of the European Society of Cardiology*, 4, C7-10.
- Nam, Y. J., A. Kim, M. S. Lee, Y. K. Shin, D. S. Sohn and C. S. Lee. 2016. Lamotrigine Attenuates Proteasome Inhibition-Induced Apoptosis by Suppressing the Activation of the Mitochondrial Pathway and the Caspase-8- and Bid-Dependent Pathways. *Neurochem Res*, 41, 2503-2516.
- Napolitano, A. P., D. M. Dean, A. J. Man, J. Youssef, D. N. Ho, A. P. Rago, M. P. Lech and J. R. Morgan. 2007. Scaffold-free three-dimensional cell culture utilizing micromolded nonadhesive hydrogels. *Biotechniques*, 43, 494-500.
- National Institute for Health and Care Excellence. 2020. NG185 - Acute coronary syndromes *NICE guideline*
- Neese, L. L., C. A. Wolfe and F. C. Church. 1998. Contribution of basic residues of the D and H helices in heparin binding to protein C inhibitor. *Archives of biochemistry and biophysics*, 355, 101-108.
- Nieswandt, B. and S. P. Watson. 2003. Platelet-collagen interaction: is GPVI the central receptor? *Blood*, 102, 449-461.
- Nikoletopoulou, V., M. Markaki, K. Palikaras and N. Tavernarakis. 2013. Crosstalk between apoptosis, necrosis and autophagy. *Biochim Biophys Acta*, 1833, 3448-3459.
- Nishizuka, Y. 1992. Intracellular signaling by hydrolysis of phospholipids and activation of protein kinase C. *Science*, 258, 607-614.
- Oduah, E. I., R. J. Linhardt and S. T. Sharfstein. 2016. Heparin: past, present, and future. *Pharmaceuticals*, 9, 38.
- Olczyk, P., Ł. Mencner and K. Komosinska-Vassev. 2015. Diverse roles of heparan sulfate and heparin in wound repair. *BioMed research international*, 2015.
- Ollivier, V., C. Roques, N. Receveur, M. Gratz, L. Feldman, D. Letourneur, C. Gachet, P. H. Mangin and M. Jandrot-Perrus. 2016. Bioreactivity of stent material: Activation of platelets, coagulation, leukocytes and endothelial cell dysfunction in vitro. *Platelets*, 1-11.
- Olsen, S. K., M. Garbi, N. Zampieri, A. V. Eliseenkova, D. M. Ornitz, M. Goldfarb and M. Mohammadi. 2003. Fibroblast growth factor (FGF) homologous factors share structural but not functional homology with FGFs. *Journal of Biological Chemistry*, 278, 34226-34236.
- Olson, S. and I. Björk. 1991. Predominant contribution of surface approximation to the mechanism of heparin acceleration of the antithrombin-thrombin reaction. Elucidation from salt concentration effects. *Journal of Biological Chemistry*, 266, 6353-6364.
- Olson, S. T., I. Björk and S. C. Bock. 2002. Identification of critical molecular interactions mediating heparin activation of antithrombin: implications for the design of improved heparin anticoagulants. *Trends in cardiovascular medicine*, 12, 198-205.
- Olson, S. T., R. Swanson, E. Raub-Segall, T. Bedsted, M. Sadri, M. Petitou, J.-P. Héroult, J.-M. Herbert and I. Björk. 2004. Accelerating ability of synthetic oligosaccharides on antithrombin inhibition of proteinases of the clotting and fibrinolytic systems Comparison with heparin and low-molecular-weight heparin. *Thrombosis and haemostasis*, 92, 929-939.
- Onishi, A., K. St Ange, J. S. Dordick and R. J. Linhardt. 2016. Heparin and anticoagulation. *Front Biosci (Landmark Ed)*, 21, 1372-1392.
- Oosta, G. M., W. T. Gardner, D. L. Beeler and R. D. Rosenberg. 1981. Multiple functional domains of the heparin molecule. *Proceedings of the National Academy of Sciences*, 78, 829-833.
- Ori, A., M. C. Wilkinson and D. G. Fernig. 2008. The heparanome and regulation of cell function: structures, functions and challenges. *Front Biosci*, 13, 4309-4338.

- Owens, G. K., M. S. Kumar and B. R. Wamhoff. 2004. Molecular regulation of vascular smooth muscle cell differentiation in development and disease. *Physiol Rev*, 84, 767-801.
- Owens III, A. P. and N. Mackman. 2010. Tissue factor and thrombosis: The clot starts here. *Thrombosis and haemostasis*, 104, 432.
- Paizis, K., G. Kirkland, M. Polihronis, M. Katerelos, J. Kanellis and D. A. Power. 1998. Heparin-binding epidermal growth factor-like growth factor in experimental models of membranous and minimal change nephropathy. *Kidney international*, 53, 1162-1171.
- Palta, S., R. Saroa and A. Palta. 2014. Overview of the coagulation system. *Indian journal of anaesthesia*, 58, 515.
- Park, Y. M., M. Febbraio and R. L. Silverstein. 2009. CD36 modulates migration of mouse and human macrophages in response to oxidized LDL and may contribute to macrophage trapping in the arterial intima. *The Journal of clinical investigation*, 119, 136-145.
- Pasternak, K., J. Kocot and A. Horecka. 2010. Biochemistry of magnesium. *Journal of Elementology*, 15, 601-616.
- Peach, C. J., V. W. Mignone, M. A. Arruda, D. C. Alcobia, S. J. Hill, L. E. Kilpatrick and J. Woolard. 2018. Molecular pharmacology of VEGF-A isoforms: binding and signalling at VEGFR2. *International journal of molecular sciences*, 19, 1264.
- Peixoto, P. M., O. Teijido, O. Mirzalieva, L. M. Dejean, E. V. Pavlov, B. Antonsson and K. W. Kinnally. 2017. MAC inhibitors antagonize the pro-apoptotic effects of tBid and disassemble Bax / Bak oligomers. *J Bioenerg Biomembr*, 49, 65-74.
- Pellegrini, L. 2001. Role of heparan sulfate in fibroblast growth factor signalling: a structural view. *Curr Opin Struct Biol*, 11, 629-34.
- Pellegrini, L., D. F. Burke, F. von Delft, B. Mulloy and T. L. Blundell. 2000. Crystal structure of fibroblast growth factor receptor ectodomain bound to ligand and heparin. *Nature*, 407, 1029-1034.
- Petitou, M., B. Casu and U. Lindahl. 2003. 1976–1983, a critical period in the history of heparin: the discovery of the antithrombin binding site. *Biochimie*, 85, 83-89.
- Podemska-Jedrzejczak, Z., A. Malinska, P. Sujka-Kordowska, M. Nowicki, M. Puslecki, M. Jemielity and B. Perek. 2018. Vascular restenosis in coronary artery bypass grafting might be associated with VEGF-C/VEGFR-3 signaling pathway. *Heart and vessels*, 1-15.
- Polyak, B., M. Medved, N. Lazareva, L. Steele, T. Patel, A. Rai, M. Y. Rotenberg, K. Wasko, A. R. Kohut, R. Sensenig and G. Friedman. 2016. Magnetic Nanoparticle-Mediated Targeting of Cell Therapy Reduces In-Stent Stenosis in Injured Arteries. *ACS Nano*.
- Pomin, V. H. 2016. Paradigms in the structural biology of the mitogenic ternary complex FGF:FGFR:heparin. *Biochimie*, 127, 214-26.
- Powell, A. K., E. A. Yates, D. G. Fernig and J. E. Turnbull. 2004. Interactions of heparin/heparan sulfate with proteins: appraisal of structural factors and experimental approaches. *Glycobiology*, 14, 17R-30R.
- Prenzel, N., E. Zwick, H. Daub, M. Leserer, R. Abraham, C. Wallasch and A. Ullrich. 1999. EGF receptor transactivation by G-protein-coupled receptors requires metalloproteinase cleavage of proHB-EGF. *Nature*, 402, 884-888.
- Pukac, L., J. Huangpu and M. J. Karnovsky. 1998. Platelet-derived growth factor-BB, insulin-like growth factor-I, and phorbol ester activate different signaling pathways for stimulation of vascular smooth muscle cell migration. *Experimental cell research*, 242, 548-560.
- R Isenovic, E., S. Soskic, A. Trpkovic, B. Dobutovic, M. Popovic, Z. Gluvic, B. Putnikovic and P. Marche. 2010. Insulin, thrombin, ERK1/2 kinase and vascular smooth muscle cells proliferation. *Current pharmaceutical design*, 16, 3895-3902.
- Raab, G. and M. Klagsbrun. 1997. Heparin-binding EGF-like growth factor. *Biochim Biophys Acta*, 1333, F179-99.
- Rabenstein, D. L., J. M. Robert and J. Peng. 1995. Multinuclear magnetic resonance studies of the interaction of inorganic cations with heparin. *Carbohydrate research*, 278, 239-256.
- Raines, E. W. 2004. PDGF and cardiovascular disease. *Cytokine & growth factor reviews*, 15, 237-254.
- Raman, R., G. Venkataraman, S. Ernst, V. Sasisekharan and R. Sasisekharan. 2003. Structural specificity of heparin binding in the fibroblast growth factor family of proteins. *Proceedings of the National Academy of Sciences*, 100, 2357-2362.

- Rao, N. V., B. Argyle, X. Xu, P. R. Reynolds, J. M. Walenga, M. Prechel, G. D. Prestwich, R. B. MacArthur, B. B. Walters and J. R. Hoidal. 2010. Low anticoagulant heparin targets multiple sites of inflammation, suppresses heparin-induced thrombocytopenia, and inhibits interaction of RAGE with its ligands. *American Journal of Physiology-Cell Physiology*, 299, C97-C110.
- Rashid, Q., M. Abid and M. A. Jairajpuri. 2014. Elucidating the specificity of non-heparin-based conformational activators of antithrombin for factor Xa inhibition. *Journal of natural science, biology, and medicine*, 5, 36.
- Rauova, L., M. Poncz, S. E. McKenzie, M. P. Reilly, G. Arepally, J. W. Weisel, C. Nagaswami, D. B. Cines and B. S. Sachais. 2005. Ultralarge complexes of PF4 and heparin are central to the pathogenesis of heparin-induced thrombocytopenia. *Blood*, 105, 131-138.
- Rauova, L., L. Zhai, M. A. Kowalska, G. M. Arepally, D. B. Cines and M. Poncz. 2006. Role of platelet surface PF4 antigenic complexes in heparin-induced thrombocytopenia pathogenesis: diagnostic and therapeutic implications. *Blood*, 107, 2346-2353.
- Reizes, O., J. Lincecum, Z. Wang, O. Goldberger, L. Huang, M. Kaksonen, R. Ahima, M. T. Hinkes, G. S. Barsh and H. Rauvala. 2001. Transgenic expression of syndecan-1 uncovers a physiological control of feeding behavior by syndecan-3. *Cell*, 106, 105-116.
- Remko, M. and C.-W. von der Lieth. 2006. Gas-phase and solution conformations of selected dimeric structural units of heparin. *Journal of chemical information and modeling*, 46, 1687-1694.
- Ren, J., T. Zhou, V. S. S. Pilli, N. Phan, Q. Wang, K. Gupta, Z. Liu, N. Sheibani and B. Liu. 2019. Novel paracrine functions of smooth muscle cells in supporting endothelial regeneration following arterial injury. *Circulation research*, 124, 1253-1265.
- Renné, T., A. H. Schmaier, K. F. Nickel, M. Blombäck and C. Maas. 2012. In vivo roles of factor XII. *Blood, The Journal of the American Society of Hematology*, 120, 4296-4303.
- Rifkin, D. B. and D. Moscatelli. 1989. Recent developments in the cell biology of basic fibroblast growth factor. *J Cell Biol*, 109, 1-6.
- Roffi, M., C. Patrono, J.-P. Collet, C. Mueller, M. Valgimigli, F. Andreotti, J. J. Bax, M. A. Borger, C. Brotons and D. P. Chew. 2016. 2015 ESC Guidelines for the management of acute coronary syndromes in patients presenting without persistent ST-segment elevation: Task Force for the Management of Acute Coronary Syndromes in Patients Presenting without Persistent ST-Segment Elevation of the European Society of Cardiology (ESC). *European heart journal*, 37, 267-315.
- Rota, E., M. Bazzan and G. Fantino. 2008. Fondaparinux-related thrombocytopenia in a previous low-molecular-weight heparin (LMWH)-induced heparin-induced thrombocytopenia (HIT). *Thrombosis and haemostasis*, 99, 779-781.
- Roy, S., H. Lai, R. Zouaoui, J. Duffner, H. Zhou, L. P. Jayaraman, G. Zhao, T. Ganguly, T. K. Kishimoto and G. Venkataraman. 2011. Bioactivity screening of partially desulfated low-molecular-weight heparins: a structure/activity relationship study. *Glycobiology*, 21, 1194-1205.
- Rubin, B. G., B. Toursarkissian, D. Petrinec, L. Y. Yang, P. R. Eisenberg and D. R. Abendschein. 1996. Preincubation of Dacron grafts with recombinant tissue factor pathway inhibitor decreases their thrombogenicity in vivo. *Journal of vascular surgery*, 24, 865-870.
- Rudd, T., M. Skidmore, S. Guimond, M. Guerrini, C. Cosentino, R. Edge, A. Brown, D. Clarke, G. Torri and J. Turnbull. 2008. Site-specific interactions of copper (II) ions with heparin revealed with complementary (SRCD, NMR, FTIR and EPR) spectroscopic techniques. *Carbohydrate research*, 343, 2184-2193.
- Rudd, T. R., S. E. Guimond, M. A. Skidmore, L. Duchesne, M. Guerrini, G. Torri, C. Cosentino, A. Brown, D. T. Clarke, J. E. Turnbull, D. G. Fernig and E. A. Yates. 2007. Influence of substitution pattern and cation binding on conformation and activity in heparin derivatives. *Glycobiology*, 17, 983-93.
- Rudd, T. R., M. D. Preston and E. A. Yates. 2017. The nature of the conserved basic amino acid sequences found among 437 heparin binding proteins determined by network analysis. *Molecular BioSystems*, 13, 852-865.
- Ruhrberg, C., H. Gerhardt, M. Golding, R. Watson, S. Ioannidou, H. Fujisawa, C. Betsholtz and D. T. Shima. 2002. Spatially restricted patterning cues provided by heparin-

- binding VEGF-A control blood vessel branching morphogenesis. *Genes & development*, 16, 2684-2698.
- Sadat-Shojai, M. 2018. Controlled pattern of cell growth in modulated protein nanocomplexes: regulating cells spreading in three dimensions. *Materials Today*, 21, 686-688.
- Saiura, A., M. Sata, Y. Hirata, R. Nagai and M. Makuuchi. 2001. Circulating smooth muscle progenitor cells contribute to atherosclerosis. *Nature medicine*, 7, 382-383.
- Sakakibara, K., K. Kubota, B. Worku, E. J. Ryer, J. P. Miller, A. Koff, K. C. Kent and B. Liu. 2005. PDGF-BB Regulates p27 Expression through ERK-dependent RNA Turn-over in Vascular Smooth Muscle Cells. *Journal of Biological Chemistry*, 280, 25470-25477.
- Samuel, M., R. Pixley, M. Villanueva, R. Colman and G. Villanueva. 1992. Human factor XII (Hageman factor) autoactivation by dextran sulfate. Circular dichroism, fluorescence, and ultraviolet difference spectroscopic studies. *Journal of Biological Chemistry*, 267, 19691-19697.
- Saucedo, L., G. N. Buffa, M. Rosso, T. Guillardoy, A. D. Gongora, M. J. Munuce, M. H. Vazquez and C. I. Marin Briggiler. 2015. Fibroblast Growth Factor Receptors (FGFRs) in human male germ cells: evidence for their expression, functionality and involvement in sperm motility regulation.
- Sauerborn, M., M. M. van Beers, W. Jiskoot, G. M. Kijanka, L. Boon, H. Schellekens and V. Brinks. 2013. Antibody response against Betaferon® in immune tolerant mice: involvement of marginal zone B-cells and CD4+ T-cells and apparent lack of immunological memory. *Journal of clinical immunology*, 33, 255-263.
- Schlessinger, J., A. N. Plotnikov, O. A. Ibrahimi, A. V. Eliseenkova, B. K. Yeh, A. Yayon, R. J. Linhardt and M. Mohammadi. 2000. Crystal structure of a ternary FGF-FGFR-heparin complex reveals a dual role for heparin in FGFR binding and dimerization. *Molecular cell*, 6, 743-750.
- Schreier, B., S. Rabe, B. Schneider, M. Bretschneider, S. Rupp, S. Ruhs, J. Neumann, U. Rueckschloss, M. Sibilia, M. Gotthardt, C. Grossmann and M. Gekle. 2013. Loss of epidermal growth factor receptor in vascular smooth muscle cells and cardiomyocytes causes arterial hypotension and cardiac hypertrophy. *Hypertension*, 61, 333-40.
- Schreuder, H. A., B. de Boer, R. Dijkema, J. Mulders, H. J. Theunissen, P. D. Grootenhuys and W. G. Hol. 1994. The intact and cleaved human antithrombin III complex as a model for serpin-proteinase interactions. *Nature structural biology*, 1, 48-54.
- Schwartz, S. M., Z. S. Galis, M. E. Rosenfeld and E. Falk. 2007. Plaque rupture in humans and mice. *Arteriosclerosis, thrombosis, and vascular biology*, 27, 705-713.
- Seo, Y., A. Andaya and J. A. Leary. 2012. Preparation, separation, and conformational analysis of differentially sulfated heparin octasaccharide isomers using ion mobility mass spectrometry. *Analytical chemistry*, 84, 2416-2423.
- Seo, Y., M. R. Schenauer and J. A. Leary. 2011. Biologically relevant metal-cation binding induces conformational changes in heparin oligosaccharides as measured by ion mobility mass spectrometry. *International journal of mass spectrometry*, 303, 191-198.
- Serruys, P. W., B. van Hout, H. Bonnier, V. Legrand, E. Garcia, C. Macaya, E. Sousa, W. van der Giessen, A. Colombo, R. Seabra-Gomes, F. Kiemeneij, P. Ruygrok, J. Ormiston, H. Emanuelsson, J. Fajadet, M. Haude, S. Klugmann and M. A. Morel. 1998. Randomised comparison of implantation of heparin-coated stents with balloon angioplasty in selected patients with coronary artery disease (Benestent II). *The Lancet*, 352, 673-81.
- Seyrek, E., P. L. Dubin and J. Henriksen. 2007. Nonspecific electrostatic binding characteristics of the heparin-antithrombin interaction. *Biopolymers: Original Research on Biomolecules*, 86, 249-259.
- Shafiq, N., S. Malhotra, P. Pandhi, N. Sharma, A. Bhalla and A. Grover. 2006. A randomized controlled clinical trial to evaluate the efficacy, safety, cost-effectiveness and effect on PAI-1 levels of the three low-molecular-weight heparins—enoxaparin, nadroparin and dalteparin. *Pharmacology*, 78, 136-143.
- Shamas-Din, A., S. Bindner, W. Zhu, Y. Zaltsman, C. Campbell, A. Gross, B. Leber, D. W. Andrews and C. Fradin. 2013. tBid undergoes multiple conformational changes at the membrane required for Bax activation. *J Biol Chem*, 288, 22111-27.
- Shi, Y. and J. Massague. 2003. Mechanisms of TGF-beta signaling from cell membrane to the nucleus. *Cell*, 113, 685-700.
- Silvestre-Roig, C., P. Fernández, V. Esteban, Ó. M. Pello, C. Indolfi, C. Rodríguez, R. Rodríguez-Calvo, M. D. López-Maderuelo, G. Bauriedel and R. Hutter. 2013.

- Inactivation of nuclear factor- κ B inhibits vascular smooth muscle cell proliferation and neointima formation. *Arteriosclerosis, thrombosis, and vascular biology*, 33, 1036-1045.
- Singh, A. K., A. S. Bhadauria, P. Kumar, H. Bera and S. Saha. 2019. Bioactive and drug-delivery potentials of polysaccharides and their derivatives. *Polysaccharide Carriers for Drug Delivery*. Elsevier.
- Skålén, K., M. Gustafsson, E. K. Rydberg, L. M. Hultén, O. Wiklund, T. L. Innerarity and J. Borén. 2002. Subendothelial retention of atherogenic lipoproteins in early atherosclerosis. *Nature*, 417, 750-754.
- Skinner, R., J.-P. Abrahams, J. C. Whisstock, A. M. Lesk, R. W. Carrell and M. R. Wardell. 1997. The 2.6 Å structure of antithrombin indicates a conformational change at the heparin binding site. *Journal of molecular biology*, 266, 601-609.
- Son, J. E., H. Jeong, H. Kim, Y. A. Kim, E. Lee, H. J. Lee and K. W. Lee. 2014. Pelargonidin attenuates PDGF-BB-induced aortic smooth muscle cell proliferation and migration by direct inhibition of focal adhesion kinase. *Biochemical pharmacology*, 89, 236-245.
- Speight, M. O. N. and M. J. Griffith. 1983. Calcium inhibits the heparin-catalyzed antithrombin III/thrombin reaction by decreasing the apparent binding affinity of heparin for thrombin. *Archives of biochemistry and biophysics*, 225, 958-963.
- Srinivasan, S. R., B. Radhakrishnamurthy and G. S. Berenson. 1975. Studies on the interaction of heparin with serum lipoproteins in the presence of Ca^{2+} , Mg^{2+} , and Mn^{2+} . *Archives of biochemistry and biophysics*, 170, 334-340.
- Stead, N., A. Kaplan and R. Rosenberg. 1976. Inhibition of activated factor XII by antithrombin-heparin cofactor. *Journal of Biological Chemistry*, 251, 6481-6488.
- Steinberg, D. 2009. The LDL modification hypothesis of atherogenesis: an update. *Journal of lipid research*, 50, S376-S381.
- Stevic, I., N. Parmar, N. Paredes, L. R. Berry and A. K. Chan. 2011. Binding of heparin to metals. *Cell biochemistry and biophysics*, 59, 171-178.
- Stringer, S. E. and J. T. Gallagher. 1997. Specific binding of the chemokine platelet factor 4 to heparan sulfate. *J Biol Chem*, 272, 20508-14.
- Sugaya, N., H. Habuchi, N. Nagai, S. Ashikari-Hada and K. Kimata. 2008. 6-O-sulfation of heparan sulfate differentially regulates various fibroblast growth factor-dependent signalings in culture. *Journal of Biological Chemistry*, 283, 10366-10376.
- Sydow-Plum, G. and M. Tabrizian. 2008. Review of stent coating strategies: clinical insights. *Materials Science and Technology*, 24, 1127-1143.
- Takazaki, R., Y. Shishido, R. Iwamoto and E. Mekada. 2004. Suppression of the biological activities of the epidermal growth factor (EGF)-like domain by the heparin-binding domain of heparin-binding EGF-like growth factor. *Journal of Biological Chemistry*, 279, 47335-47343.
- Tanaka, K., M. Sata, Y. Hirata and R. Nagai. 2003. Diverse contribution of bone marrow cells to neointimal hyperplasia after mechanical vascular injuries. *Circulation research*, 93, 783-790.
- Taylor, J. M., C. P. Mack, K. Nolan, C. P. Regan, G. K. Owens and J. T. Parsons. 2001. Selective expression of an endogenous inhibitor of FAK regulates proliferation and migration of vascular smooth muscle cells. *Molecular and cellular biology*, 21, 1565-1572.
- Teran, M. and M. A. Nugent. 2015. Synergistic binding of vascular endothelial growth factor-A and its receptors to heparin selectively modulates complex affinity. *Journal of Biological Chemistry*, 290, 16451-16462.
- Teychené, J., H. I. n. R.-d. Balmann, L. Maron and S. Galier. 2018. Why are saccharides dehydrated in the presence of electrolytes? Insights from molecular modeling and thermodynamic measurements. *ACS central science*, 4, 1531-1536.
- Thomas, O., E. Lybeck, K. Strandberg, N. Tynngård and U. Schött. 2015. Monitoring low molecular weight heparins at therapeutic levels: dose-responses of, and correlations and differences between aPTT, anti-factor Xa and thrombin generation assays. *PLoS One*, 10, e0116835.
- Thompson, L. D., M. W. Pantoliano and B. A. Springer. 1994a. Energetic characterization of the basic fibroblast growth factor-heparin interaction: identification of the heparin binding domain. *Biochemistry*, 33, 3831-3840.
- Thompson, S. A., S. Higashiyama, K. Wood, N. S. Pollitt, D. Damm, G. McEnroe, B. Garrick, N. Ashton, K. Lau and N. Hancock. 1994b. Characterization of sequences within

- heparin-binding EGF-like growth factor that mediate interaction with heparin. *Journal of Biological Chemistry*, 269, 2541-2549.
- Tsai, W.-B., I. Aiba, Y. Long, H.-K. Lin, L. Feun, N. Savaraj and M. T. Kuo. 2012. Activation of Ras/PI3K/ERK pathway induces c-Myc stabilization to upregulate argininosuccinate synthetase, leading to arginine deiminase resistance in melanoma cells. *Cancer research*, 72, 2622-2633.
- Tzahar, E., H. Waterman, X. Chen, G. Levkowitz, D. Karunagaran, S. Lavi, B. J. Ratzkin and Y. Yarden. 1996. A hierarchical network of interreceptor interactions determines signal transduction by Neu differentiation factor/neuregulin and epidermal growth factor. *Molecular and cellular biology*, 16, 5276-5287.
- Uchida, T., M. Nakashima, Y. Hirota, Y. Miyazaki, T. Tsukazaki and H. Shindo. 2000. Immunohistochemical localisation of protein tyrosine kinase receptors Tie-1 and Tie-2 in synovial tissue of rheumatoid arthritis: correlation with angiogenesis and synovial proliferation. *Annals of the rheumatic diseases*, 59, 607-614.
- Ueda, K., S. Inoue, Y. Zhang, T. Kutsuna, S. Inoue, K. Noto, N. Arai and M. Noguchi. 2009. Heparin induces apoptosis through suppression of AKt in oral squamous cell carcinoma cells. *Anticancer research*, 29, 1079-1088.
- Vadiveloo, P. K., E. L. Filonzi, H. R. Stanton and J. A. Hamilton. 1997. G1 phase arrest of human smooth muscle cells by heparin, IL-4 and cAMP is linked to repression of cyclin D1 and cdk2. *Atherosclerosis*, 133, 61-69.
- van der Meijden, P. E., I. C. Munnix, J. M. Auger, J. W. Govers-Riemslog, J. M. Cosemans, M. J. Kuijpers, H. M. Spronk, S. P. Watson, T. Renné and J. W. Heemskerk. 2009. Dual role of collagen in factor XII-dependent thrombus formation. *Blood*, 114, 881-890.
- Verstovsek, S., R. A. Mesa, J. Gotlib, V. Gupta, J. F. DiPersio, J. V. Catalano, M. W. Deininger, C. B. Miller, R. T. Silver, M. Talpaz, E. F. Winton, J. H. Harvey, Jr., M. O. Arcasoy, E. O. Hexner, R. M. Lyons, R. Paquette, A. Raza, M. Jones, D. Kornacki, K. Sun, H. Kantarjian and C.-I. investigators. 2017. Long-term treatment with ruxolitinib for patients with myelofibrosis: 5-year update from the randomized, double-blind, placebo-controlled, phase 3 COMFORT-I trial. *J Hematol Oncol*, 10, 55.
- Vinante, F. and A. Rigo. 2013. Heparin-binding epidermal growth factor-like growth factor/diphtheria toxin receptor in normal and neoplastic hematopoiesis. *Toxins (Basel)*, 5, 1180-1201.
- Virmani, R., A. P. Burke, F. D. Kolodgie and A. Farb. 2002. Vulnerable plaque: the pathology of unstable coronary lesions. *Journal of interventional cardiology*, 15, 439-446.
- Visentin, G. P., M. Moghaddam, S. E. Beery, J. G. McFarland and R. H. Aster. 2001. Heparin is not required for detection of antibodies associated with heparin-induced thrombocytopenia/thrombosis. *Journal of Laboratory and Clinical Medicine*, 138, 22-31.
- Vrolix, M. C., V. M. Legrand, J. H. Reiber, G. Grollier, M. J. Schali, P. Brunel, L. Martinez-Elbal, M. Gomez-Recio, F. W. Bar, M. E. Bertrand, A. Colombo and J. Brachman. 2000. Heparin-coated Wiktor stents in human coronary arteries (MENTOR trial). MENTOR Trial Investigators. *The American Journal of Cardiology*, 86, 385-9.
- Weatherford, D. A., J. E. Sackman, T. T. Reddick, M. B. Freeman, S. L. Stevens and M. H. Goldman. 1996. Vascular endothelial growth factor and heparin in a biologic glue promotes human aortic endothelial cell proliferation with aortic smooth muscle cell inhibition. *Surgery*, 120, 433-439.
- Wei, M., G. Tai, Y. Gao, N. Li, B. Huang, Y. Zhou, S. Hao and X. Zeng. 2004. Modified heparin inhibits P-selectin-mediated cell adhesion of human colon carcinoma cells to immobilized platelets under dynamic flow conditions. *Journal of Biological Chemistry*, 279, 29202-29210.
- Weng, Y., F. Jing, J. Chen and N. Huang. 2012. Construction of heparinylated multilayer films on TiO via streptavidin/biotin interaction. *Applied Surface Science*, 258, 5947-5954.
- Whisstock, J. C., R. N. Pike, L. Jin, R. Skinner, X. Y. Pei, R. W. Carrell and A. M. Lesk. 2000. Conformational changes in serpins: II. The mechanism of activation of antithrombin by heparin. *Journal of molecular biology*, 301, 1287-1305.
- Wilcox, J., L. Harker, A. Kelly, A. Lumsden, K. Carey, J. Ollerenshaw, A. Willis and S. Hanson. 1994. INHIBITION OF VASCULAR LESION FORMATION IN THE NONHUMAN PRIMATE USING A CHEMICALLY-MODIFIED HEPARIN DERIVATIVE, ASTENOSE

- (TM). *Circulation*. AMER HEART ASSOC 7272 GREENVILLE AVENUE, DALLAS, TX 75231-4596.
- Witt, D. P. and A. D. Lander. 1994. Differential binding of chemokines to glycosaminoglycan subpopulations. *Current Biology*, 4, 394-400.
- World Health Organisation. 2018. Non Communicable Diseases Country Profiles 2018.
- Wu, Y. 2015. Contact pathway of coagulation and inflammation. *Thrombosis journal*, 13, 17.
- Wujak, L., M. Didiasova, D. Zakrzewicz, H. Frey, L. Schaefer and M. Wygrecka. 2015. Heparan sulfate proteoglycans mediate factor XIIa binding to the cell surface. *J Biol Chem*, 290, 7027-39.
- Wykrzykowska, J. J., Y. Onuma and P. W. Serruys. 2009. Advances in stent drug delivery: the future is in bioabsorbable stents. *Expert opinion on drug delivery*, 6, 113-126.
- Xu, D. and J. D. Esko. 2014. Demystifying heparan sulfate–protein interactions. *Annual review of biochemistry*, 83, 129-157.
- Xu, D., M. M. Fuster, R. Lawrence and J. D. Esko. 2011. Heparan sulfate regulates VEGF165- and VEGF121-mediated vascular hyperpermeability. *Journal of Biological Chemistry*, 286, 737-745.
- Xu, R., A. Ori, T. R. Rudd, K. A. Uniewicz, Y. A. Ahmed, S. E. Guimond, M. A. Skidmore, G. Siligardi, E. A. Yates and D. G. Fernig. 2012. Diversification of the structural determinants of fibroblast growth factor-heparin interactions: implications for binding specificity. *Journal of Biological Chemistry*, 287, 40061-40073.
- Yang, Z., C. Wang, S. Yang, T. Hong, F. Wang, L. Xia and C. Wang. 2015. Endothelial progenitor cells induce transplant arteriosclerosis via VEGFR-1/2 activity. *Atherosclerosis*, 238, 26-32.
- Yates, E. A., S. E. Guimond and J. E. Turnbull. 2004. Highly diverse heparan sulfate analogue libraries: providing access to expanded areas of sequence space for bioactivity screening. *Journal of Medicinal Chemistry*, 47, 277-280.
- Yates, E. A., F. Santini, M. Guerrini, A. Naggi, G. Torri and B. Casu. 1996. ¹H and ¹³C NMR spectral assignments of the major sequences of twelve systematically modified heparin derivatives. *Carbohydr Res*, 294, 15-27.
- Yee, D. T. W., J. N. C. Koon, Y. Huang, P. W. E. Hou, H. L. Leo, S. S. Venkatraman and H. Y. Ang. 2020. Bioresorbable metals in cardiovascular stents: material insights and progress. *Materialia*, 100727.
- Yogi, A., G. E. Callera, A. B. Aranha, T. T. Antunes, D. Graham, M. McBride, A. Dominiczak and R. M. Touyz. 2011. Sphingosine-1-phosphate-induced inflammation involves receptor tyrosine kinase transactivation in vascular cells: upregulation in hypertension. *Hypertension*, 57, 809-18.
- Yousef, Z., S. Redwood and M. Marber. 2000. Postinfarction left ventricular remodelling: where are the theories and trials leading us? *Heart*, 83, 76-80.
- Yu, D., X. Rui and S. He. 2014. Effect of heparin-derived oligosaccharide on bFGFR1 and bFGFR2 in vascular smooth muscle cells. *Vasc Endovascular Surg*, 48, 289-96.
- Zhang, E. G., S. K. Smith, P. N. Baker and D. S. Charnock-Jones. 2001. The regulation and localization of angiopoietin-1,-2, and their receptor Tie2 in normal and pathologic human placentae. *Molecular Medicine*, 7, 624.
- Zhang, F., X. Liang, J. M. Beaudet, Y. Lee and R. J. Linhardt. 2014. The effects of metal ions on heparin/heparin sulfate-protein interactions. *Journal of biomedical technology and research*, 1.
- Zhang, K., J.-y. Chen, W. Qin, J.-a. Li, F.-x. Guan and N. Huang. 2016a. Constructing bio-layer of heparin and type IV collagen on titanium surface for improving its endothelialization and blood compatibility. *Journal of Materials Science: Materials in Medicine*, 27, 81.
- Zhang, L. 2010. Glycosaminoglycan (GAG) biosynthesis and GAG-binding proteins. *Progress in molecular biology and translational science*, 93, 1-17.
- Zhang, N., W. Lou, F. Ji, L. Qiu, B. K. Tsang and W. Di. 2016b. Low molecular weight heparin and cancer survival: clinical trials and experimental mechanisms. *Journal of cancer research and clinical oncology*, 142, 1807-1816.
- Zhang, X., L. Chen, D. P. Bancroft, C. Lai and T. E. Maione. 1994. Crystal structure of recombinant human platelet factor 4. *Biochemistry*, 33, 8361-8366.
- Zhang, Z. W., R. W. Guo, J. L. Lv, X. M. Wang, J. S. Ye, N. H. Lu, X. Liang and L. X. Yang. 2017. MicroRNA-99a inhibits insulin-induced proliferation, migration, dedifferentiation, and rapamycin resistance of vascular smooth muscle cells by inhibiting insulin-like

- growth factor-1 receptor and mammalian target of rapamycin. *Biochem Biophys Res Commun*, 486, 414-422.
- Zhao, W., S. A. McCallum, Z. Xiao, F. Zhang and R. J. Linhardt. 2012. Binding affinities of vascular endothelial growth factor (VEGF) for heparin-derived oligosaccharides. *Bioscience reports*, 32, 71-81.
- Zheng, Y., M. Yu, A. Podd, L. Yuan, D. K. Newman, R. Wen, G. Arepally and D. Wang. 2013. Critical role for mouse marginal zone B cells in PF4/heparin antibody production. *Blood, The Journal of the American Society of Hematology*, 121, 3484-3492.
- Zucker, M. B. and I. R. Katz. 1991. Platelet factor 4: production, structure, and physiologic and immunologic action. *Proceedings of the Society for Experimental Biology and Medicine*, 198, 693-702.

Appendix

Appendix 1. The FACS raw data of HCASMC/HCAEC co-cultivation.

EC

	Ctrl	Na	K	Mg	Ca
	5806	6360	5333	4095	3957
	7465	6449	6206	6926	6474
	6579	7587	6454	6758	6432
Mean	6616.67	6798.67	5997.67	5926.33	5621.00
S.D.	830.14	684.17	588.82	1588.20	1441.22

SMC

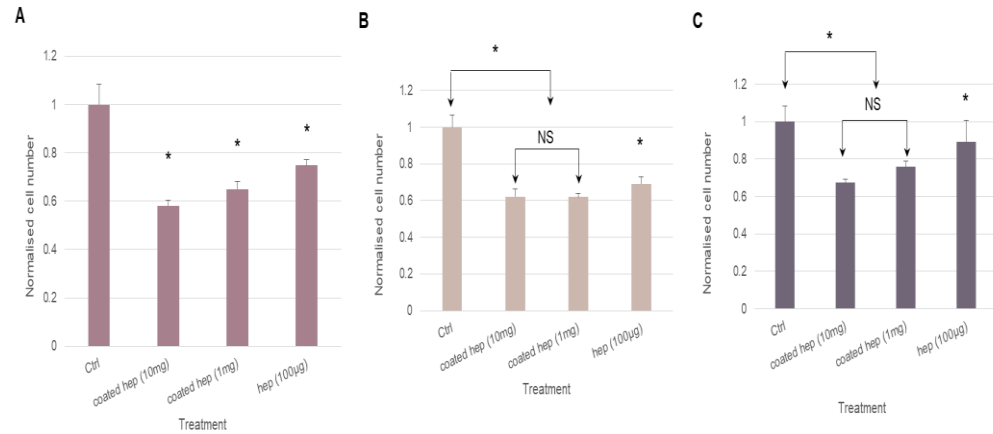
	Ctrl	Na	K	Mg	Ca
	4982	5125	5478	3030	2983
	5861	4716	5481	5088	5155
	4833	6770	5563	4845	5059
Mean	5225.33	5537.00	5507.33	4321.00	4399.00
S.D.	555.52	1087.22	48.23	1124.62	1227.23

EC/SMC

	Ctrl	Na	K	Mg	Ca
	1.17	1.24	0.97	1.35	1.33
	1.27	1.37	1.13	1.36	1.26
	1.36	1.12	1.16	1.39	1.27
Normalised cell number					
	1.00	1.06	0.84	1.16	1.14
	1.00	1.07	0.89	1.07	0.99
	1.00	0.82	0.91	1.02	0.93
Mean	1.00	0.99	0.88	1.08	1.02
S.D.	0.00	0.14	0.04	0.07	0.11

Appendix 2. Heparin-coated plate data.

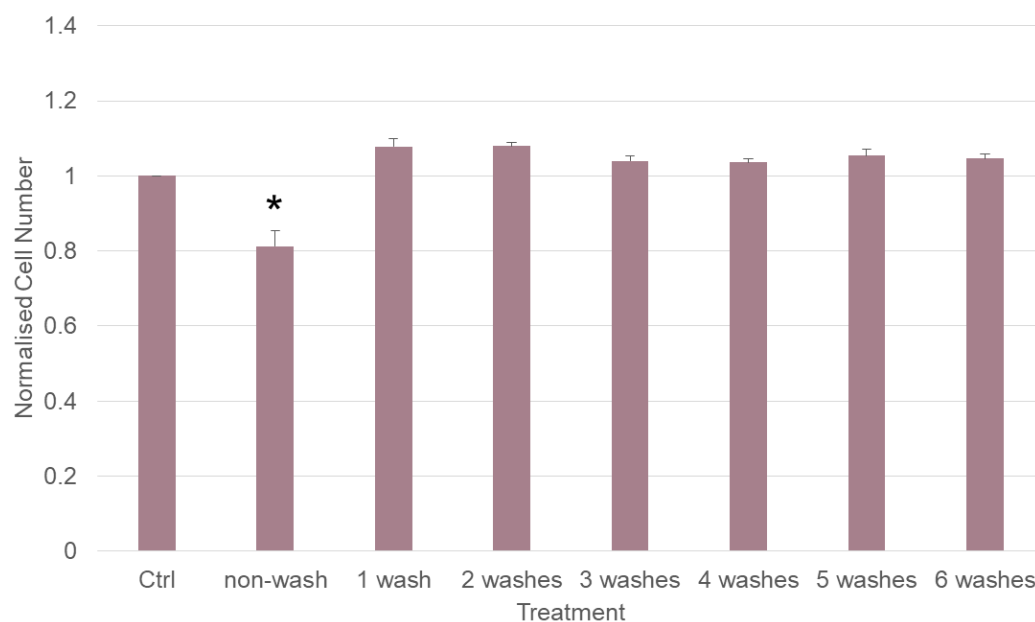
Heparin attached to plate surfaces retains its anti-proliferative activity on HCASMCs. 96-well plates were treated in the presence or absence (C) of poly-D-lysine (A) or poly-



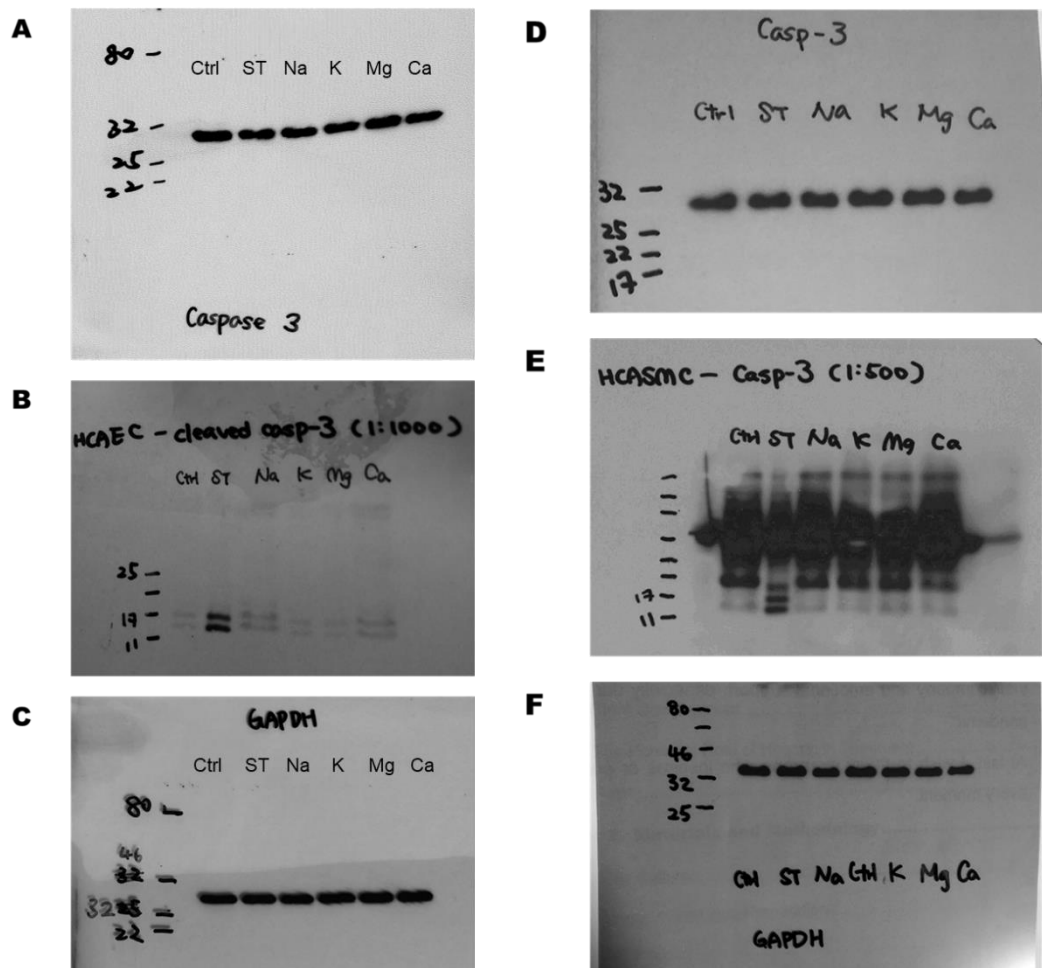
L-lysine (B), incubating overnight until dry, followed treatment with heparin solution at indicated concentration. HCASMCs were then added to plates and allowed to grow for 3 days. HCASMCs were then quantified by MTT assays. * $P < 0.05$; NS, no significance; $n=8$; Tukey test.

Appendix 3. Coated heparin lost in the washing procedure.

Heparin was coated to plate surfaces overnight. The surface was washed using sterilised water, followed by incubating HCASMCs for 3 days. HCASMCs were then quantified by MTT assays. * $P < 0.05$; $n = 8$; Tukey test.

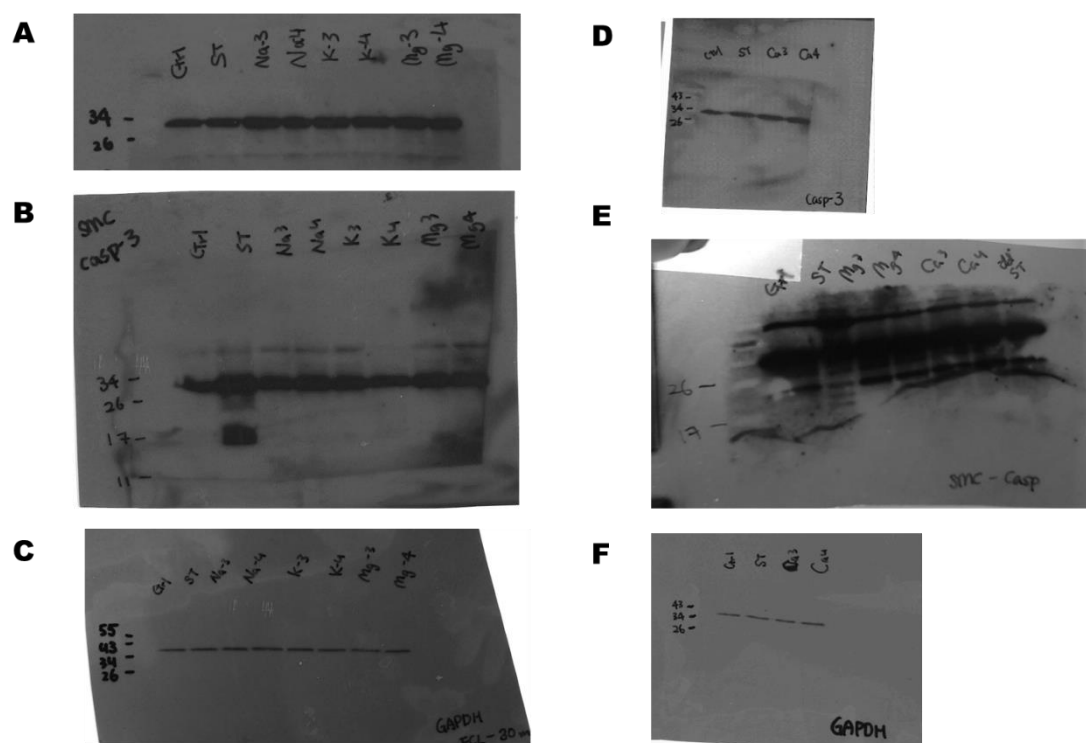


Appendix 4. The intact image of western blot shown in **Figure 3.9**



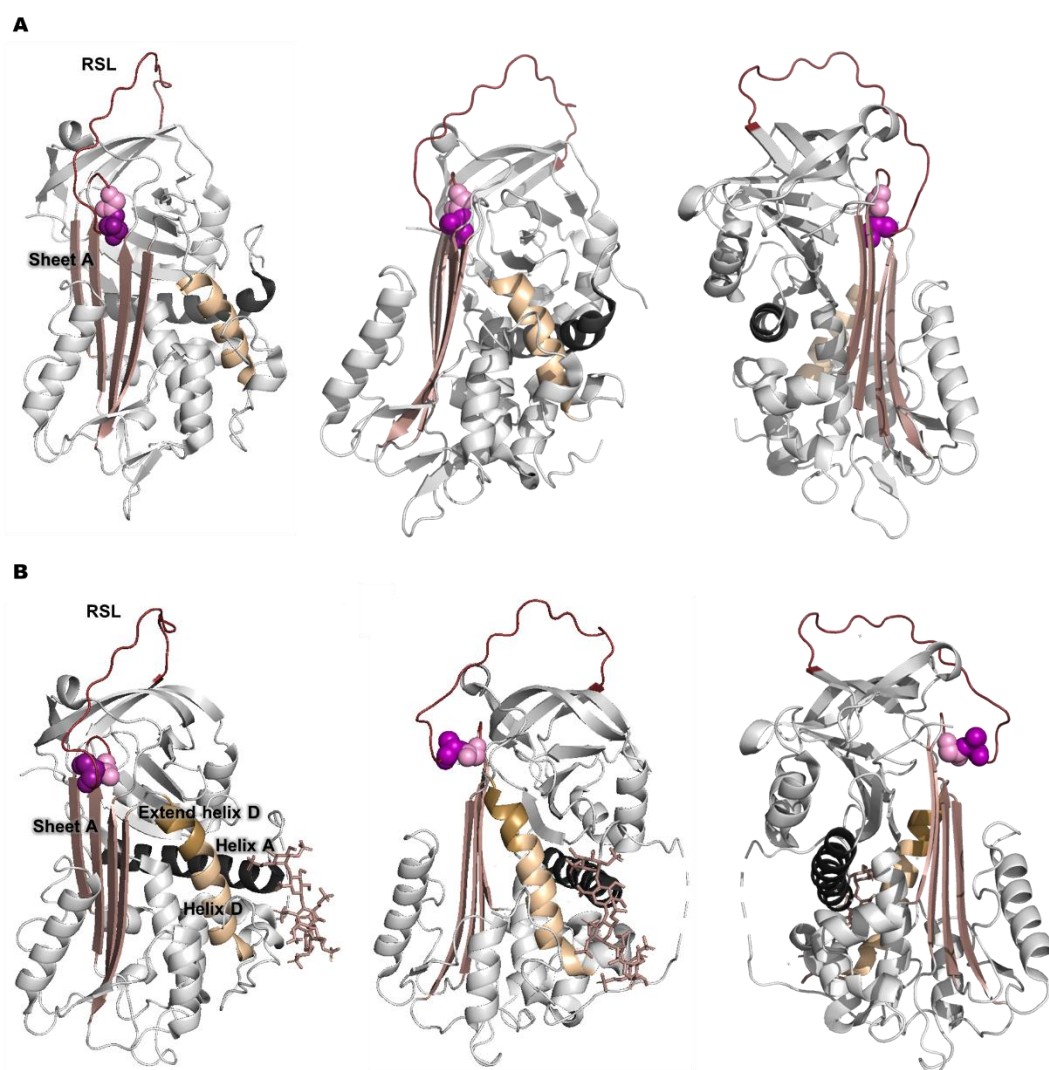
The whole image of western blot for HCAECs (**A-C**) and HCASMCs (**D-F**). Separated protein were immunoblotted for caspase-3 (**A,D,E**),cleaved caspase-3 (**B**) and GAPDH (**C,F**).

Appendix 5. The intact image of western blot shown in **Figure 3.17**



The whole image of western blot for HCASMCs treated with heparin analogues. Separated protein were immunoblotted for caspase-3 (**A,B,D,E**) and GAPDH (**C,F**).

Appendix 6. Conformation of native antithrombin and active antithrombin binding with heparin.



The different perspectives of **(A)** native (unbound) antithrombin (1E05) and **(B)** antithrombin in complex with heparin pentasaccharide (1E03).