THE ROLE OF HEPARAN SULFATE IN POXVIRUS INFECTIONS

MAYANK KHANNA

A thesis submitted for the degree of Doctor of Philosophy at The Australian National University

November 2015
STATEMENT

I certify that this thesis and the research to which it pertains are the product of my own work, except where otherwise stated in the text. The wild type IHD-J strain of VACV was a kind gift from Dr. David Tscharke. All the heparin based sulfated polysaccharides tested in this thesis were a gift from Dr. Craig Freeman. GFP expressing recombinant WR and IHD-J strains of VACV were constructed with assistance from Dr. Ronald Jackson. All the EM images were taken by Ms. Cathy Gillespie. Mr. Cameron Jack assisted in performing all the bioinformatic searches. Protein samples for mass spectrometry analysis were sent to the Australian Proteome Analysis Facility (APAF) at the Macquarie University, where the team of scientist led by Dr. Xiaomin Song performed all the mass spectrometry assays and helped in the interpretation of data generated.

Mayank Khanna
(Author)

Prof. Christopher R. Parish
(Supervisor)
Cancer and Vascular Biology Group
Department of Cancer Biology and Therapeutics

Dr. Charani Ranasinghe
(Supervisor)
Molecular Mucosal Vaccine Immunology
Department Immunology and Infectious Diseases
ACKNOWLEDGEMENTS

My PhD has been a very long and exhausting journey. I could not have completed this journey without the support of some incredible people in my life. First, and foremost, I would like to dedicate this thesis to my grandparents, Sri. Shyam Sunder Khanna (grandpa or dadaji) and Smt. Krishna Devi (grandma or dadi). Thanks for all the blessings and encouragement dada dadi. I could not have dreamt of doing a PhD if it weren’t because of all your sacrifices. I would also like to add a very special thank you to the rest of my family, including my parents (Mr. Anil Khanna and Mrs. Reeta Khanna) and my sister (Miss Jigyasa Khanna), who gave me their unconditional love and support throughout this journey. I am extremely grateful for everything you have done for me. I cannot thank you enough.

From one family to another, I would like to thank my two amazing supervisors, Prof. Chris Parish and Dr. Charani Ranasinghe, not just for helping me finish my PhD, but for helping me grow as a scientist and as an individual. Thank you Chris for all the wisdom, guidance and support over the years. I really appreciate everything you have done for me, from the bottom of my heart. For me you are a walking PubMed database and there hasn’t been a discussion where I haven’t learnt something new from you. You have inspired me to become a person I would never have dreamt of becoming. Thank you for all the time and effort.

Charani, you have been like a second mother to me. I cannot thank you enough for all the support and always being there for me whenever I needed someone.
Thank you for all the academic and non-academic guidance and support. I honestly do not know how I would have completed my thesis without your efforts. So thank you for everything. A special thanks to Dr. Ronald Jackson (who refuses to be referred to as my third supervisor) for all the support over the years. On paper he may be just an advisor, but he held my hand and taught me some invaluable laboratory skills. He has been more than just an advisor to me, and that is the reason why I call him my third supervisor. Thanks Ron.

I would also extend my gratitude towards Dr. Craig Freeman, Dr. Lisa Sedger, Dr. David Tscharke and Mr. Cameron Jack. All the heparin based compounds were a gift from Craig. Thanks David for the IHD-J strain of VACV. Thanks Lisa for all your feedback and advice over the years. Cam helped me with all the bioinformatics work.

I would also like to thank members from both the labs I have been a part of, who have contributed at different stages of my PhD. The long list includes Dr. Ben Quah, David Simon Davis, Dr. Lucy Copland, Anna Browne, Anna Bezos, Dr. Danushka Wijesundra and Dr. Yogesh Jeelall. Thank you for all your help with my experimental work and/or guidance. A lot of my work wouldn’t have been possible if I didn’t have your support. Speaking of support, I would like to thank the MCRF team members Dr. Harpreet Vohra, Mr. Mick Devoy, Ms. Anne Prins and Ms. Cathy Gillespie for their assistance in planning and performing several experiments. Thanks to the animal facility staff, the IT guys, Ms. Karen Edward from multimedia, media wash up team and of course the HDR coordinator Ms. Wendy Riley for treating us students like her family.
Finally, big thank you to all my other lab members who have helped me make this journey a rather enjoyable one. I have made some amazing friends over the years and I will cherish this for the rest of my life. You all made a statistically significant contribution in helping me stay strong and complete this PhD.

As we say, scientific research is a collaborative effort and I am thankful to everyone for their contribution in helping me achieve this milestone. Thank you all from the bottom of my heart.
Abstract

The heparan sulfate (HS) component of heparan sulfate proteoglycans (HSPGs) has been implicated in the initiation of several viral infections, including vaccinia virus (VACV). A cell infected with VACV releases two different forms of VACV, namely the mature virus (MV) released following the death of infected cells and which infects neighbouring cells, and the enveloped virus (EV) ejected from infected cells for long-range dissemination. The relative role of HS in the infectivity of the different forms of VACV is unclear. Furthermore, there is little known about the fine specificity of the VACV-HS interactions. Therefore, in order to develop HS based molecules that could potentially have antiviral properties against HS-dependent viral infection, VACV was used as a prototype virus to understand the structural and functional consequences of the interaction between VACV and HS.

ELISA studies described in Chapter 3 were used to evaluate the specificity of the MV form of VACV for heparin, differentially sulfated HS, chondroitin sulfate (CS) A-D and hyaluronic acid (HA). Lack of appropriate EV specific antibodies meant that similar ELISA studies could not be performed for the EV form of VACV. Nevertheless, the MV form of VACV bound to immobilized heparin and highly sulfated HS (HS^{hi}) with high avidity, compared to lowly sulfated HS (HS^{low}). The MV particles also bound to CS A-D, however, very weakly. Furthermore, the ability of the MV rich Western Reserve (WR) strain of VACV to form plaques in vitro was affected by soluble heparin, WR plaque numbers being reduced 5-fold with an incremental increase in plaque size. The formation of plaques by the EV rich International Health Department-J (IHD-J) strain was
also affected in the presence of heparin, there being a 10-fold reduction in plaque numbers, an incremental increase in plaque size and the disappearance of the trademark ‘comet’ shaped plaques. These data suggest that HS recognition plays a significant role in both MV and EV infectivity, with this recognition being more important for EV infectivity.

To better understand the interaction between heparin/HS and the two forms of VACV, green fluorescent protein (GFP) expressing recombinant strains of VACV were constructed, as described in Chapter 4. Subsequent inhibition of infectivity assays, performed using soluble glycosaminoglycans (GAGs), suggested that sulfated GAGs more easily inhibited EV infections than the MV infections, with heparin and HS being highly potent inhibitors of infection. Furthermore, the ability of the EV form of VACV to establish an infection was significantly reduced in cells treated with the HS-degrading enzyme heparanase and in cells genetically deficient in HS production, compared to the MV form of VACV which appeared largely unaffected. These findings confirmed that recognition of cell surface HS is vital for EV infectivity but less important for the infectivity of the MV form of VACV.

In Chapter 5, the ability of soluble heparin/HS molecules and HS mimetics to inhibit VACV infections was further investigated to identify structural features of these molecules that are responsible for their interaction with VACV particles. The study also aimed to determine whether HS-based molecules could be used as possible antivirals against VACV and potentially against other HS-dependent viral infections. It was observed that the 2-O-sulfate of uronic acid and the 6-O
and N-sulfate groups of glucosamine residues were important for VACV infectivity, with 6-O-sulfate being particularly crucial and EV infections being more dependent on these groups than MV infections. Furthermore, the length of heparin chains did not affect their ability to interact with and inhibit VACV infectivity. However, the linkages of different D-glucose-based HS mimetics had a profound effect on the ability of the sulfated molecule to inhibit VACV infections, with the order of potency being $\beta(1\rightarrow4) > \alpha(1\rightarrow6) > \alpha(1\rightarrow4) > \beta(1\rightarrow3)$. Interestingly, however, a D-mannose-based sulfated oligosaccharide mixture (PI-88, Muparfostat) was identified as the only HS mimetic that was a more potent inhibitor of MV infections than of EV infections, in fact it was a stronger inhibitor of MV infections than unfractioned heparin (UFH). These data suggest that the EV and MV forms of VACV interact with different structural aspects of HS chains and that synthetic HS-based molecules could be designed with the ability to inhibit both EV and MV forms of VACV.

In Chapter 6 studies are described that attempted to identify the proteins on the surface of the EV form of VACV that are responsible for the interaction of the VACV with cell surface HS. Thus, EV and MV outer membrane proteins were solubilised and identified by Western blotting using polyclonal anti-VACV antibodies. Four potential heparin-binding proteins were identified in the EV outer membrane extracts, being 150 kDa, 85 kDa, 60 kDa and 25 kDa proteins. The 150 kDa heparin binding protein was further analysed using 1D nanoLC ESI MS/MS and was found to be a poxvirus DNA directed RNA polymerase, with sequence similarity to the 65 kDa VACV F12 protein, a protein important in EV formation. Bioinformatic searches were also performed to determine
possible HS-binding motifs in VACV proteins and identified the 64 kDa VACV B4R protein and a 78 kDa RNA helicase as likely candidates. Overall, it was concluded that there are multiple HS binding proteins on the outer EV envelope and that it is likely that in many instances the heparin/HS binding site(s) of these EV proteins may not be composed of linear amino acid sequences.

Since both MV and EV forms of VACV bind HS, in Chapter 7 experiments are described that examined the role of heparanase in VACV spread, it being predicted that heparanase may aid spread by releasing VACV from cell surface and extracellular matrix (ECM) HS. Wild type (WT) and heparanase deficient (HPSE -/-) mice were inoculated with the WR strain of VACV via intranasal (i.n.) and intramuscular (i.m.) routes to evaluate the spread of infection in the two groups of mice. The WR strain of VACV was inoculated via the i.n. route when there was a 24 hr delay in weight loss in the HPSE -/- mice compared to the WT mice. Furthermore, this delay in weight loss correlated with a delay in the onset of disease with there being a 24-48 hr delay in the spread of infection from the primary site of inoculation to distant organs like the ovaries. Similarly, when VACV was delivered by the i.m. route, there was a 24-48 hr delay in the infection of the ovaries, although there was a similar delay in infection of the spleen, despite there being no weight loss difference. Overall, the results suggest that VACV depends on host-derived heparanase to aid its spread. Since heparanase mediated degradation of HS aids the infiltration of leukocytes with antiviral activity into sites of infection, the results obtained from the current study are contrary to the prevailing immunological paradigm.
In conclusion, VACV like several other viruses interacts with cell surface HS prior to infecting cells. Furthermore, VACV relies on host-derived heparanase to degrade cell surface and ECM HS to aid its spread. Thus, synthetic HS-based molecules could be designed that could inhibit EV and MV forms of VACV from infecting cells and may simultaneously act as heparanase inhibitors and consequently prevent VACV spread.
### Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>143B</td>
<td>Human osteosarcoma cells</td>
</tr>
<tr>
<td>1D nanoLC ESI</td>
<td>1D nano liquid chromatography electrospray ionization</td>
</tr>
<tr>
<td>MS/MS</td>
<td>tandem mass spectrometry</td>
</tr>
<tr>
<td>ABTS</td>
<td>Peroxidase substrate, 2,2'-azido-bis(3-ethylbenzthiazoline-6-sulphonic acid)</td>
</tr>
<tr>
<td>AEEC</td>
<td>ANU animal experimentation and ethic</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>APAF</td>
<td>Australian proteome analysis facility</td>
</tr>
<tr>
<td>BHK21</td>
<td>Baby hamster kidney-21 cells</td>
</tr>
<tr>
<td>BM</td>
<td>Basement membrane</td>
</tr>
<tr>
<td>bp</td>
<td>Basepair</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>CEV</td>
<td>Cell associated virus</td>
</tr>
<tr>
<td>CHO-K1</td>
<td>Chinese hamster ovary</td>
</tr>
<tr>
<td>CPV</td>
<td>Cowpox virus</td>
</tr>
<tr>
<td>CS</td>
<td>Chondroitin sulfate</td>
</tr>
<tr>
<td>CsCl</td>
<td>Caesium chloride</td>
</tr>
<tr>
<td>ddH2O</td>
<td>Double distilled water</td>
</tr>
<tr>
<td>de2S</td>
<td>Chemically modified heparin lacking 2-O-sulfate</td>
</tr>
<tr>
<td>de6S</td>
<td>Chemically modified heparin lacking 6-Sulfate</td>
</tr>
<tr>
<td>deCarboxyl</td>
<td>Chemically modified heparin lacking carboxyl group</td>
</tr>
<tr>
<td>deNS</td>
<td>Chemically modified heparin lacking N-sulfate</td>
</tr>
<tr>
<td>deS</td>
<td>Chemically modified heparin lacking all sulfate groups</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco's modified eagle medium</td>
</tr>
<tr>
<td>DVT</td>
<td>Deep vein thrombosis</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EEV</td>
<td>Extracellular enveloped virus</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
</tr>
</tbody>
</table>
EM  Electron micrography
EST  Expressed sequence tag
EV   Enveloped virus
FACS Fluorescence-activated cell sorting
FBS  Foetal bovine serum
FGF  Fibroblast growth factor
FIU  Fluorescent intensity unit
FMDV Foot and mouth disease virus
g/mL gram/millilitre
GalNAc Acetylgalactosamine
gB   glycoprotein B
gC   glycoprotein C
GFP  Green fluorescent protein
GFP-\textit{Bsd} Green fluorescent protein - blastcidin resistance
GlcA Glucuronic acid
GlcN Glucosamine
Glu  Glutamine
gp120 glycoprotein 120
HA   Hyaluronic acid
HBV  Hepatitis B virus
HCl  Hydrocholic acid
HexA Hexuronic acid
HIT  Heparin-induced thrombocytopenia
HIV  Human immunodeficiency virus
HPNSE Flavobacterium heparinases
HPSE Human platelet heparanase
HPSE-/- Heparanase knockout
HPV  Human papilloma virus
hr   Hours
HRP  Horseradish peroxidase
HS   Heparan sulfate
HS^{hi} Highly sulfated HS
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>HS&lt;sup&gt;low&lt;/sup&gt;</td>
<td>Lowly sulfated HS</td>
</tr>
<tr>
<td>HSPG</td>
<td>Heparan sulfate proteoglycan</td>
</tr>
<tr>
<td>HSV</td>
<td>Herpes simplex virus</td>
</tr>
<tr>
<td>i.m.</td>
<td>Intramuscular</td>
</tr>
<tr>
<td>i.n.</td>
<td>Intranasal</td>
</tr>
<tr>
<td>IC50</td>
<td>50% Inhibitory concentration</td>
</tr>
<tr>
<td>IdoA</td>
<td>Iduronic acid</td>
</tr>
<tr>
<td>IEV</td>
<td>Intracellular enveloped virus</td>
</tr>
<tr>
<td>IHD-J</td>
<td>International health department strain</td>
</tr>
<tr>
<td>IL</td>
<td>Interlukin</td>
</tr>
<tr>
<td>IMV</td>
<td>Intracellular mature virus</td>
</tr>
<tr>
<td>IV</td>
<td>Immature virus</td>
</tr>
<tr>
<td>JCSMR</td>
<td>John Curtin School of Medical Research</td>
</tr>
<tr>
<td>kb</td>
<td>Kilobase</td>
</tr>
<tr>
<td>KCl</td>
<td>Potassium chloride</td>
</tr>
<tr>
<td>kDa</td>
<td>Kilodaltons</td>
</tr>
<tr>
<td>LMP</td>
<td>Low melting point agarose</td>
</tr>
<tr>
<td>LMWH</td>
<td>Low molecular weight heparin</td>
</tr>
<tr>
<td>LPL</td>
<td>Lipoprotein lipase</td>
</tr>
<tr>
<td>Lys</td>
<td>Lysine</td>
</tr>
<tr>
<td>mAb</td>
<td>Monolconal antibody</td>
</tr>
<tr>
<td>MEM</td>
<td>Minimum essential media</td>
</tr>
<tr>
<td>MEME</td>
<td>Multiple Em for Motif Elicitation</td>
</tr>
<tr>
<td>MFI</td>
<td>Mean fluorescent intensity</td>
</tr>
<tr>
<td>min</td>
<td>Minutes</td>
</tr>
<tr>
<td>mL</td>
<td>Milliliters</td>
</tr>
<tr>
<td>mM</td>
<td>Millimoles</td>
</tr>
<tr>
<td>MOI</td>
<td>Multiplicity of infection</td>
</tr>
<tr>
<td>MTOC</td>
<td>Microtubule-organizing centre</td>
</tr>
<tr>
<td>MV</td>
<td>Mature virus</td>
</tr>
<tr>
<td>MVA</td>
<td>Modified vaccinia ankara</td>
</tr>
<tr>
<td>NaCl</td>
<td>Sodium chloride</td>
</tr>
</tbody>
</table>
nm  Nanometer
ns  Not significant
OPV  Orthopoxvirus
PBS  Phosphate buffer saline
PCR  Polymerase chain reaction
PFU  Plaque forming units
PFU/g  Plaque forming units/gram
pgsA-745  Xylosyltransferase-I-deficient Chinese hamster ovary cells
PVC  Polyvinyl chloride
reNAc  Chemically modified heparin with acetyl group added
RNA  Ribose nucliec acid
RPMI  Roswell park memorial institute media
RRV  Ross river virus
RSB  Research school of Biology
RSV  Respiratory syncytical virus
SAP  Shrimp alkaline phosphatase
SDS-PAGE  Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
sec  Seconds
SEM  Standard error mean
SFV  Semliki virus
SINV  Sindbis virus
SPI  Serine protease inhibitor
ssDNA  Single stranded DNA
TBS  Tris-buffered saline
TGF-β  Transforming growth factor-β
tk  Thymidine Kinase
UFH  Unfractioned heparin
V  Volts
VACV  Vaccinia virus
VEEV  Venezuelan equine encephalitis virus
VEGF  Vascular endothelial growth factor
w/v  Weight/volume
<table>
<thead>
<tr>
<th>WR</th>
<th>Western reserve strain</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>Wild type</td>
</tr>
<tr>
<td>xg</td>
<td>Times g force</td>
</tr>
<tr>
<td>µg</td>
<td>Microgram</td>
</tr>
<tr>
<td>µL</td>
<td>Microlitre</td>
</tr>
</tbody>
</table>
Table of contents

Declaration .................................................................................................................. iii
Acknowledgements ....................................................................................................... iv
Abstract ....................................................................................................................... vii
Abbreviations ............................................................................................................... xii
Table of Contents ...................................................................................................... xvii
List of Figures .............................................................................................................. xxi
List of Tables ............................................................................................................... xxiii

Chapter 1. Literature review
1.1 Introduction to viral infection ............................................................................... 2
1.2 Extracellular matrix (ECM) ................................................................................... 4
1.3 Heparan sulfate proteoglycan (HSPG) ................................................................. 5
  1.3.1 HSPG core proteins ......................................................................................... 5
  1.3.2 Structure of HS ................................................................................................ 7
  1.3.3 Functional role of the HS component of HSPGs .............................................. 9
1.4 Chondroitin sulfate ............................................................................................... 10
1.5 Heparin ................................................................................................................ 11
1.6 Low molecular weight heparin (LMWH) ............................................................. 14
1.7 Heparanase .......................................................................................................... 15
1.8 Role of GAGs in virus infections ......................................................................... 17
1.9 Vaccinia virus (VACV) ......................................................................................... 18
  1.9.1 VACV gene expression and morphogenesis .................................................. 20
  1.9.2 VACV interacts with HS ............................................................................... 23
  1.9.3 Strains of VACV ............................................................................................. 24
1.10 Research question ............................................................................................... 25

Chapter 2. Materials and methods
2.1 Mice and Reagents .............................................................................................. 30
  2.1.1 Mice ............................................................................................................... 30
  2.1.2 Ethics statement ............................................................................................. 30
  2.1.3 Cell lines ........................................................................................................ 30
  2.1.4 Viruses ............................................................................................................ 31
    2.1.4.1 Cloning GFP-\textit{Bsd} in a MVA vector .................................................. 32
    2.1.4.2 Transfection ............................................................................................. 36
    2.1.4.3 Plasmid DNA purification and confirmation of GFP-\textit{Bsd} orientation .................. 36
    2.1.4.4 Transfection of WR-VACV and IHD-J-VACV to express GFP-\textit{Bsd} .................. 37
  2.1.4.5 PCR confirmation of the purity of the recombinant
GFP-Bsd VACV strains .......................... 40
2.1.4.6 Purification of the MV and EV forms of VACV from WR and IHD-J strains respectively .......................... 42
2.1.5 Virus titrations and plaque assays .......................... 44
2.2 In vitro techniques ................................................. 45
  2.2.1 ELISA assays ................................................. 45
  2.2.2 Heparanase treatment ........................................ 46
  2.2.3 FACS assays: HS staining .................................... 47
  2.2.4 FACS assays: VACV infection ................................ 48
  2.2.5 VACV membrane protein extraction ......................... 49
  2.2.6 Interaction of VACV proteins with heparin coupled beads --- 50
  2.2.7 SDS-PAGE .................................................. 51
  2.2.8 Western blotting ............................................ 51
  2.2.9 1D NanoLC ESI MS/MS analysis for protein identification -- 52
2.3 In vivo techniques ................................................. 53
  2.3.1 VACV inoculation ............................................ 53
  2.3.2 In vivo virus spread ........................................ 53

Chapter 3. Affinity of VACV for different glycosaminoglycans
  3.1 Abstract .................................................... 56
  3.2 Introduction and rationale ..................................... 57
  3.3 Results .......................................................... 59
    3.3.1 Analysis of the interaction of MV form of VACV with HS ---- 59
      3.3.1.1 Purified MV preferentially interacts with highly sulfated HS (HS^hi) and heparin .......................... 59
    3.3.2 Interaction of MV form of VACV with CS .................... 62
    3.3.3 Soluble heparin interferes with VACV plaque formation ---- 68
  3.4 Discussion ..................................................... 74

Chapter 4. Use of GFP expressing VACV to probe role of heparin/HS recognition in VACV infections
  4.1 Abstract .................................................... 80
  4.2 Introduction and rationale ..................................... 81
  4.3 Results .......................................................... 83
    4.3.1 Construction of recombinant VACV WR and VACV IHD-J strains expressing GFP .............................. 83
    4.3.2 Kinetics of VACV replication as measured by GFP expression ................................................. 83
    4.3.3 Enzymatic treatment of cells \textit{in vitro} to remove cell surface HS and the effects of this treatment on virus infectivity .......................... 89
    4.3.4 VACV infection of CHO-K1 and HS deficient mutant CHO (pgsA-745) cell lines ............................ 93
    4.3.5 Differences in the infectivity of the MV and EV forms of VACV in the presence of heparin are not due to VACV strain

xviii
Chapter 8. Final discussion

8.1 Introduction ................................................................. 204
8.2 VACV interacts with cell surface HS .................................. 205
8.3 EV and MV-VACV interact differently with cell surface HS ...... 208
8.4 Role of heparanase in VACV spread ................................. 210
8.5 Chemically modified heparins and HS mimetics can inhibit VACV infections .................................................. 213
8.6 Anti-cancer drug PI-88 can inhibit VACV infections .............. 215
8.7 EV surface proteins that interact with HS/heparin ................. 216
8.8 Conclusion and future directions ...................................... 218

References .............................................................................. 220
List of Figures

Figure 1.1: Structure and location of heparan sulfate (HS) within the blood vessel wall. ......................................................... 6
Figure 1.2: Flow chart depicting the monosaccharide modifications that form different combinations of HS disaccharides. ...................... 8
Figure 1.3: Biosynthesis of HS and heparin. ......................................................... 13
Figure 1.4: VACV morphogenesis. ......................................................... 19
Figure 1.5: VACV interacts with cell surface HS prior to infecting cells. ---- 26

Figure 2.1: Cloning of GFP-Bsd cassette into pUC57-MVA vector. .......... 35
Figure 2.2: Confirmation of successful recombination events using PCR. - 41

Figure 3.1: Interaction of the MV form of VACV with immobilised HS\textsuperscript{hi} and HS\textsuperscript{low} in the absence or presence of soluble heparin. --------- 61
Figure 3.2: Interaction of the MV form of VACV with immobilised heparin in the absence or presence of soluble HS\textsuperscript{hi} and HS\textsuperscript{low}. .......................... 63
Figure 3.3: Interaction of the MV form of VACV with immobilised HS\textsuperscript{hi} in the absence or presence of soluble HS\textsuperscript{hi} and HS\textsuperscript{low}. .......................... 64
Figure 3.4: Interaction of MV form of VACV with immobilised HS\textsuperscript{hi} in absence or presence of soluble CS, fucoidan and hyaluronic acid. ----- 67
Figure 3.5: Effects of heparin on plaque formation by the IHD-J (EV) and WR (MV) strains of VACV. ......................................................... 73

Figure 4.1: GFP expression in 143B cells infected with MV-GFP. .......... 86
Figure 4.2: GFP expression in 143B cells infected with EV-GFP. .......... 87
Figure 4.3: Effects of heparin on MV-GFP and EV-GFP \textit{in vitro}. .......... 91
Figure 4.4: Removal of cell surface HS by enzymatic treatment of target cells. .......................................................................... 95
Figure 4.5: Effects of enzymatic treatment of target cells on MV-GFP and EV-GFP infections \textit{in vitro}. ......................................................... 96
Figure 4.6: GFP expression in wild type CHO-K1 and HS deficient CHO-K1 (pgsA-745) cells infected with MV-GFP and EV-GFP. .......... 98
Figure 4.7: Effects of heparin on infectivity of MV-GFP and EV-GFP obtained from different strains of VACV. ................................. 100
Figure 4.8: Effects of differently sulfated HS on MV-GFP and EV-GFP infections \textit{in vitro}. ......................................................... 103
Figure 5.1: Effect of different LMWHs on MV-GFP and EV-GFP infections in vitro.---------------------------------------- 123
Figure 5.2: Effect of different chemically modified heparins on MV-GFP and EV-GFP infections in vitro.----------------------- 127
Figure 5.3: Effect of additional chemically modified heparins on MV-GFP and EV-GFP infections in vitro.---------------------- 131
Figure 5.4: Effects of sulfated malto-saccharides on MV-GFP and EV-GFP infections in vitro.-------------------------------- 136
Figure 5.5: Effects of isomalto- and cello-oligosaccharides on MV-GFP and EV-GFP infections in vitro.----------------------- 140
Figure 5.6: Effects of sulfated laminari-oligosaccharides on MV-GFP and EV-GFP infections in vitro.------------------------ 141
Figure 5.7: Chemical structure of the anti-cancer drug PI-88 and the effect of PI-88 on MV-GFP and EV-GFP infections in vitro.------- 143

Figure 6.1: Flow chart depicting the multi-step process used to obtain EV and MV membrane proteins.-------------------------- 159
Figure 6.2: Visualization of EV and MV membrane proteins using Western blotting.------------------------------------------ 165

Figure 7.1: Weight loss analysis post intranasal inoculation with VACV WR in WT and HPSE -/- C57BL6 mice over a course of 5 days.------ 186
Figure 7.2: Measurement of lung VACV titres in WT and HPSE -/- C57BL6 mice at day 2 post i.n. inoculation of the WR strain of VACV. -- 188
Figure 7.3: Measurement of VACV WR strain spread in WT and HPSE -/- C57BL/6 mice over 5 days post intranasal inoculation.---------- 191
Figure 7.4: Weight loss analysis post intramuscular inoculation with VACV WR in WT and HPSE -/- C57BL6 mice over 5 days.-------- 193
Figure 7.5: Measurement of VACV titres in the quadriceps muscles of WT and HPSE -/- C57BL6 mice at day 2 post intramuscular inoculation of the WR strain of VACV.---------------------------------------- 194
Figure 7.6: Measurement of VACV WR strain spread in WT and HPSE -/- C57BL/6 mice over 5 days post intramuscular inoculation.------ 197
List of Tables

Table 2.1: Primer sequences used for confirmatory PCR reactions. ---- 42
Table 5.1: Comparison of IC50 values for various LMWHs and chemically modified heparins for EV and MV infections in vitro. --------- 125
Table 5.2: Structure of different D-glucose based sulfated saccharides (HS mimetics) used in this study. ----------------------------- 135
Table 5.3: Comparison of IC50 values for various sulfated saccharides (HS mimetics) for EV an MV infections in vitro. --------------- 138
Table 6.1: List of protein hits from 1D nanoLC ESI MS/MS analysis of the 150 kDa unidentified protein. -------------------------- 172
Literature review

Chapter 1:
1.1 Introduction to viral infections

Infectious diseases pose a major threat to public health around the world. It is believed that over 10 million deaths occur annually, accounting for a staggering quarter of all deaths globally (Aquino, Lee, & Park, 2010). Increasing efforts are being made to control and treat infectious diseases, however, the problem of emerging and re-emerging pathogens is still a major concern for medical and public health in the 21st century (Morens, Fooks, & Fauci, 2004). Viral infections are of particular concern as the advances in the field of antiviral drug development have lagged behind those of bacteriocidal drugs and antibiotics, especially in the case of emerging viral pathogens where the development of antiviral therapies has significantly lagged behind the time of initial viral emergence by years, or even decades (McFadden, 2005). A classic example of this situation was with severe acute respiratory syndrome (SARS), this syndrome being induced by a new member of a neglected virus family, namely, the SARS coronavirus, that successfully crossed into humans from unsuspected reservoirs, necessitating the scientific community to rush into first understanding virus-host dynamics before any thought could be given to developing strategies to treat this infection (Finlay, See, & Brunham, 2004).

Variola virus, a member of the poxvirus family and the causative agent of the now eradicated smallpox, has killed more members of the human population over the span of recorded history than all of the other infectious diseases combined (McFadden, 2005). In 1980, thanks to a worldwide vaccination campaign, the World Health Organization (WHO) was able to declare the complete eradication of smallpox from the human community, however, a considerable fear still remains
that variola virus, or other pathogens from the poxvirus family such as monkeypox, could re-emerge and cause a pandemic in the human population before effective vaccination strategies could be put in place. A threat of smallpox being used as a potential weapon of bioterrorism has also maintained active interest in mechanisms of infection and pathogenesis of variola virus (Hughes, Irausquin, & Friedman, 2010). A key lesson learnt from past pandemics was the need to constantly develop techniques to understand viral tropism, an understanding crucial for the development of novel antiviral therapies to counter new infectious diseases.

In order to establish an infection, viral pathogens adopt various strategies to survive within the host, in particular, the overpowering of the host immune response. Detailed studies understanding the strategies that infecting viral pathogens adopt to evade the immune system, particularly viruses with a large genome such as poxviruses, revealed that viruses are often packed with several virulence factors which are deployed to invade host cells, damage host tissue, diseminate the virus and cause secondary viral infections (Aquino et al., 2010). Viruses have been shown to exploit a wide variety of attachment molecules on the surface of cells, and use them as receptors to invade cells and establish an infection (Rostand & Esko, 1997). Virus entry, spread and pathogenesis can be better understood by identifying the cell surface receptor(s) a particular virus targets (Shukla & Spear, 2001). Virus entry is mainly a two-step process, with the first step being attachment of the virus particle to a primary receptor, often ubiquitous cell surface-associated carbohydrate moieties, followed by interaction with a co-receptor that is usually a cell specific transmembrane protein belonging to a well
defined receptor superfamily (Shukla et al., 1999; Summerford & Samulski, 1998). Understanding the interaction between proteins on the surface of virus particles, and the cell surface receptors exploited by the virus particles to enter cells, are essential to understand viral tropism, which further helps in designing effective antiviral therapies. The following sections of this review will provide a general overview of the polysaccharide, heparan sulfate (HS), a key component of the extracellular matrix (ECM) and an important cell attachment receptor for many viruses.

1.2 Extracellular matrix (ECM)

The ECM is a dense latticework of macromolecules that not only occupies the extracellular space in tissue, but also provides a molecular scaffolding for cells to interact within different organs (Parish, Freeman, & Hulett, 2001). Basement membranes (BM), a specialized class of ECM, are produced as thin but rigid sheet-like structures by endothelial, epithelial and many mesenchymal cells (Parish et al., 2001; Yurchenco & Schittny, 1990). The BM plays a pivotal role in providing a concrete support for different cell layers within a tissue and, thereby, provide a surface on which cells can migrate, proliferate and differentiate (Parish et al., 2001; Yurchenco & Schittny, 1990). Due to their dense structure BMs can also act as a molecular sieve that hinders cell migration and even the diffusion of macromolecules (Jalali-Heravi, Asadollahi-Baboh, & Shahbazikhah, 2008; Yurchenco & Schittny, 1990). As a result, there are specific mechanisms in place that license the migration of certain cell types, for example inflammatory leukocytes and metastatic tumor cells, by focally degrading BMs to allow cells to cross the barrier (Parish, 2006; Yurchenco & Schittny, 1990).
1.3 Heparan sulfate proteoglycan (HSPG)

Extensive research has shown that a number of bacteria, parasites and viruses use the heparan sulfate (HS) component of cell surface heparan sulfate proteoglycans (HSPGs) as an initial receptor to attach to cells (Liu & Thorp, 2002; Rostand & Esko, 1997; Wadstrom & Ljungh, 1999; Zhu, Li, & Liang, 2011). HSPGs are also one of the most abundant constituents of BM, along with type IV collagen and laminin (Figure 1.1) (Levy-Adam, Ilan, & Vlodavsky, 2010; Parish et al., 2001; Yurchenco & Schittny, 1990; Zcharia et al., 2009).

1.3.1 HSPG core proteins

HSPGs are glyco-conjugated macromolecules composed of a carbohydrate polymers (HS component) covalently attached to protein cores (Li & Vlodavsky, 2009; Sasisekharan & Venkataraman, 2000; Sugahara & Kitagawa, 2002). The HS chains present on cell surfaces have been shown to be attached to two main classes of core proteins, namely, syndecans and glypicans (Figure 1.1) (Bernfield et al., 1999; Li & Vlodavsky, 2009). These two types of core proteins not only allow HS chains to be anchored to the cell surfaces, but also determine where, when and to what extent these HS chains are expressed (Bernfield et al., 1999). The syndecan core proteins are a family of transmembrane proteins with four isoforms that carry HS chains near their extracellular tips (Bernfield et al., 1999). They have also been shown to occasionally carry chondroitin sulfate (CS) chains linked closely to the cell surface (Li & Vlodavsky, 2009; Rostand & Esko, 1997). The glypican core proteins, on the other hand, exist as six different isoforms that carry their HS chains near the plasma membrane, with occasional HS chains being expressed near the tip of their ectodomains (Bernfield et al., 1999; Kjellen & Lindahl, 1991).
Figure 1.1: Structure and location of heparan sulfate (HS) within the blood vessel wall. In vessel walls, HS is expressed as either cell surface associated heparan sulfate proteoglycan (HSPG), such as syndecan or glypican, or as HSPGs in the subendothelial basement membrane (BM) as perlecan, agrin (not shown) and type XVIII collagen (not shown), associated with other BM components, such as laminin, type IV collagen (not shown) and entactin (not shown). HS side chains are polysaccharides composed of alternating N-acetylg glucosamine and D-glucuronic acid/D-iduronic acid residues that are sulfated at various positions along the length of the chain (Adapted from Parish, 2006).
A feature of glypicans is that they do not penetrate the lipid bilayer of plasma membranes. Instead, they are linked to membranes via glycosylphosphatidylinositol (GPI) anchors attached to their protein cores (Fransson et al., 2004; Rostand & Esko, 1997).

The HSPGs present in the ECM are covalently attached to three main classes of core proteins – agrin, collagen XVIII and perlecan (Figure 1.1) (Li & Vlodavsky, 2009). Agrin core proteins are synthesised by motor neurons and bind to specific signalling receptors on the muscle cell surface (Denzer, Gesemann, Schumacher, & Ruegg, 1995). Collagen XVIII and perlecan, on the other hand, are distributed widely in tissue ECMs and BMs and have a complex modular structure that allows them to interact with a wide range of other ECM components (Iozzo, 1998).

**1.3.2 Structure of HS**

The HS glycosaminoglycan (GAG) of HSPGs is composed of repeating disaccharides of glucosamine and hexuronic acid, joined in alternating sequences by 1,4-glycosidic linkages, which gives these carbohydrate chains their flexibility to bind to different proteins (Figure 1.1 and 1.2) (Li & Vlodavsky, 2009; Lindahl, 1990; Lindahl et al., 1979). The hexuronic acid may exist in two forms, namely, as β-D-glucuronic acid (GlcA) or α-L-iduronic acid (IdoA) (Coombe & Kett, 2005). The monosaccharide constituents of the HS chains are sequentially modified by a series of reactions involving N- and O-sulfation and epimerization of the glucuronic acid residues to iduronic acid (Figure 1.2) (Lindahl, 1990). The modification reactions during HS biosynthesis are highly regulated, resulting in heterogenous sulfation and epimerization patterns that could be tissue and cell-type specific.
Figure 1.2: Flow chart depicting the monosaccharide modifications that form different combinations of HS disaccharides. The HS disaccharides are made up of a hexuronic (HexA) and a D-glucosamine (GlcN) joined in alternating sequences by α1,4-glycosidic linkages, giving the polysaccharide its flexibility. The D-glucosamine and hexuronic acid residues are joined by β1,4-glycosidic linkages (not shown). Hexuronic acids can exist as either β-D-glucuronic acid (GlcA) or α-L-iduronic acid (IdoA), both of which can exist as an unsulfated, or as a 2-O-sulfated monosaccharide. D-glucosamine, however, can be N-sulfated, N-acetylated or exist as free amine. Furthermore, glucosamine residues can also exist as unsulfated, 3-O- or 6-O sulfated monosaccharides, or any combination of N- and/or O-sulfated monosaccharides.
Furthermore, the sulfation and epimerization of HS chains is not evenly distributed along the HS chains but occurs in ‘hot spots’ of extensive modification linked by areas of relatively little modification (Parish, 2006).

1.3.3 Functional role of the HS component of HSPGs

The role of the HS component of HSPGs is not just restricted to the formation of ECM and BM. HS has also been shown to bind a myriad of proteins, thereby regulating their availability and hence their function (Coombe & Kett, 2005; Rapraeger, 1993). As described previously, the linear HS chains exhibit phenomenal structural diversity, which is made possible by differential patterns of sulfation and uronic acid epimerization. These structural features of HS are responsible for promoting high affinity binding of several growth factors like fibroblast growth factor (FGF) (Lin & Bissell, 1993), transforming growth factor-β (TGF-β) (McCaffrey, Falcone, & Du, 1992) and vascular endothelial growth factor (VEGF) (Park, Keller, & Ferrara, 1993), as well as other HS binding proteins. Growth factors can also bind to HS via accessory proteins (Taipale & Keski-Oja, 1997). Chemokines and cytokines, such as interleukin (IL)-3 (Roberts et al., 1988) and IL-4 (Jones, Williams, Finlayjones, & Hart, 1995), also bind to HS with a very high affinity. Due to the heterogeneous nature of HS, it is able to interact with different types of chemokines that bind to subsets of HS structures, thereby raising the possibility that HS is involved in determining the specificity of leukocyte migration and recruitment in vivo (Li & Vlodavsky, 2009). Enzymes, notably lipoprotein lipase (LPL), have also been shown to bind to HS (Wang et al., 2010). Such interactions provide the cell with highly accessible ‘depots’ of growth factors,
cytokines, chemokines and other proteins, hence averting the need to synthesize these factors *de novo* when required.

An increasing number of studies have shown that HSPG play a crucial role in facilitating the interaction of cells with components of the microenvironment that control functions such as cell adhesion, proliferation, shape and differentiation (Bernfield et al., 1999; David, 1993; Wight, Kinsella, & Qwarnstrom, 1992). Furthermore, the trans-membrane and membrane anchored HSPG may also act as co-receptors, which together with other cell surface molecules, form receptor complexes that bind ligands and mediate their action (Zcharia et al., 2001). In addition to its co-receptor properties, syndecan-4 has also been shown to be a key molecule in numerous cellular processes. Several studies have shown that clustering of syndecan-4 directly initiates a signaling cascade that activates PKCα and Rac1, which are essential for cell adhesion and directional cell migration (Levy-Adam et al., 2010).

### 1.4 Chondroitin sulfates (CS)

Like HS, the chondroitin sulfates (CS) are another family of GAGs that are covalently attached to proteoglycans and can occasionally play a similar role as HS in being a cell surface receptor for viruses (Hsiao, Chung, & Chang, 1999). The CS GAGs consist of a repeating disaccharide composed of N-acetylgalactosamine (GalNAc) and either glucuronic acid (GlcA) or iduronic acid (IdoA) residues (Roden, 1980; Silbert & Sugumaran, 2002). However, unlike HS, the residues of CS are sulfated in highly predictive and uniform positions to yield the four types of CS chains, namely, CS-A [chondroitin 4-sulfate], CS-B [also known as DS (Dermatan
sulfate) (Trowbridge & Gallo, 2002)], CS-C [chondroitin 6-sulfate] and CS-D [chondroitin 2,6-sulfate (Silbert & Sugumaran, 2002)]. In the case of CS-A, the GalNAc residues are 4-O-sulfated, with CS-C being 6-O-sulfated and CS-D being 2 and 6-O-sulfated. DS are a modified form of CS in which a portion of D-glucuronic acid residues are epimerized to L-iduronic acid residues (Silbert & Sugumaran, 2002). Furthermore, similar to HS, the CS chains are attached to serine residues in their core proteins via glycosidic linkages. In vertebrates, CS proteoglycans are a major component of connective tissue, particularly cartilages, and are also found on cell surfaces and in BMs (Iozzo, 1998; Kjellen & Lindahl, 1991).

1.5 Heparin

Like HS, heparin is another linear GAG that is expressed in the form of a proteoglycan (Li & Vlodavsky, 2009; Sasisekharan & Venkataraman, 2000). Heparin and HS share a great deal of resemblance in terms of their molecular structure (repeating disaccharide units of glucosamine and hexuronic acids), however, heparin contains more iduronic acid residues and is more evenly and extensively sulfated than HS (Figure 1.3) (Feyzi, Trybala, Bergstrom, Lindahl, & Spillmann, 1997; Li & Vlodavsky, 2009). Furthermore, the core protein of heparin, serglycin, compared to those of HS, shows differential expression patterns (Li & Vlodavsky, 2009). Heparin has been shown to exhibit biological functions including regulation of cellular behaviour and, more importantly, anticoagulation (Yurchenco & Schittny, 1990). Connective tissue mast cells are the only known cell type that produces heparin (Feyzi et al., 1997). Heparin is produced as a serglycin proteoglycan, to which 10-15 heparin chains can be attached (Li & Vlodavsky, 2009), with each nascent heparin chain averaging 60-100 kDa (Horner, 1971).
which when bound to the core protein, results in a proteoglycan of approximately 1000 kDa (Li & Vlodavsky, 2009). There is much greater glucuronic acid epimerization and sulfation of heparin than of HS, in fact, studies have shown that 80-85% of glucosamine residues in heparin are N-sulfated, compared to 40-60% in HS (Rostand & Esko, 1997). Naturally occurring heparin, which is released from mast cells as 10-15 kDa heparin chains, is often referred to as unfractioned heparin (UFH).

The anticoagulation activity of heparin can be attributed to a unique pentasaccharide sequence in the polymer (Lindahl et al., 1979). Antithrombin III, a protease inhibitor, binds specifically to a pentasaccharide sequence of heparin, an interaction that enhances the ability of antithrombin III to inhibit the coagulation proteases thrombin and Factors IXa and Xa (Li & Vlodavsky, 2009). Mast cell derived heparin can also exhibit anti-inflammatory activity by interfering with the recruitment of leukocytes to inflammatory sites by interacting with members of the selectin family of adhesion receptors, namely, E-, P- and L-selectin (Wang, Brown, Varki, & Esko, 2002). Studies have also shown that heparin can have additional effects *in vivo* on selectin function, such as causing leukocytosis (Sasaki, 1967) and inhibiting tumour metastasis by blocking the P-selectin mediated interaction of platelets with tumour cells (Borsig et al., 2001).

In addition to the anticoagulant properties of heparin, there are several pharmacokinetic limitations due to its high negative charge causing unwanted binding to several plasma proteins (e.g., histidine-rich glycoprotein, vitronectin, lipoproteins, fibronectin and fibrinogen), proteins secreted by platelets (e.g,
**Figure 1.3: Biosynthesis of HS and heparin.** Both HS and heparin are linear polysaccharides consisting of alternating \(N\)-acetylglucosamine (GlcNAc) and \(D\)-glucuronic acid (GlcA) residues. The first step in the biosynthesis of HS and heparin chains is the formation of a polysaccharide-protein linkage region, involving the attachment of four sugar units (glucuronyl-galactosyl-galactosyl-xylosyl) to a serine residue of the core protein. The tetrasaccharide sequence is extended by addition of GlcNAc and GlcA residues, which is accompanied by modifications including \(N\)-deacetylation/\(N\)-sulfation of the GlcNAc, C5-epimerization of GlcA to L-iduronic acid (IdoA), \(O\)-sulfation of IdoA at C2, and C6 sulfation of GlcNS residues. The GlcNS can also be \(O\)-sulfated at C3, a hallmark modification for formation of the antithrombin III (AT)-binding pentasaccharide sequence (Adapted from Li & Vlodavsky, 2009).
platelet factor 4 and high-molecular-weight von Willebrand factor) and endothelial cells (Hirsh, 1991; Young, Prins, Levine, & Hirsh, 1992).

Furthermore, similar to other anticoagulants, excessive use of heparin can also cause complications of bleeding and induce heparin-induced thrombocytopenia (HIT) and osteoporosis (Hirsh, 1998). These limitations prompted the development of a new class of heparin-derived molecules with the same anticoagulant properties of heparin, but with generally reduced unwanted side effects.

1.6 Low molecular weight heparin (LMWH)

Chemical or enzymatic depolymerization of traditional heparin yields small molecules approximately one-third the size of heparin (i.e., 4-5 kDa). These small molecules, termed low molecular weight heparins (LMWH), are more homogeneous anticoagulants than traditional UFH. Furthermore, LMWHs have been shown to have a smaller risk of bleeding, a lower binding affinity for several plasma proteins, a lower chance of HIT, and have a more predictable behavior, all of which makes them safer drugs compared to heparin (Franze, Gennari, Minghetti, & Cilurzo, 2015; Hirsh, 1998; Ingle & Agarwal, 2014; TenCate et al., 1997). This is why LMWHs started to replace heparin as an antithrombotic drug in the prevention and treatment of DVT in the mid 1980s (Franze et al., 2015; Mousa, 2002). Despite this remarkable reduction in side effects, the anticoagulant activity of both heparin and LMWH is exerted via the same unique pentasaccharide sequence (Weitz, 1997).
1.7 Heparanase

An important regulator of HS function is the HS degrading enzyme, heparanase. There is increasing evidence that degradation of the ECM and BM is a prerequisite for metastatic tumour cells and leukocytes to invade tissues (Parish et al., 2001). Indeed these invading cells deploy a battery of degradative enzymes, which enables them to remodel and traverse the ECM and BM barriers. Proteases belonging to the serine, matrix metalloproteinase, cysteine and aspartic protease families, have been shown to disassemble ECM barriers (Duffy, 1996). Invading cells have also been shown to express another ECM degrading enzyme, heparanase, which attacks HS in the ECM and BMs. Heparanase is an endo-β-glucuronidase that was first reported 40 years ago (Höök, Wasteson, & Oldberg, 1975). It plays a crucial role in cell invasion by cleaving HS side chains at a limited number of sites, and hence releasing saccharide products of 4-7 kDa (Levy-Adam et al., 2010; Parish et al., 2001). Indeed several separate studies have demonstrated the increased expression of heparanase mRNA in metastatic rat and human breast cancer cell lines, as opposed to the low or lack of expression in non-metastatic variants (Hulett et al., 1999; Vlodavsky et al., 2000). Furthermore, tumours of larger size have been shown to be associated with heparanase upregulation in head, neck, tongue, breast, hepatocellular and gastric carcinomas, indicating that the role of heparanase is not only limited to tumour metastasis but also in accelerated growth of primary tumours (Doweck et al., 2006; El-Assal, Yamanoi, Ono, Kohno, & Nagasue, 2001; Levy-Adam et al., 2010). It has been previously reported that human platelets are another major source of mammalian heparanase, which could be released by activated platelets at sites of tissue injury, inflammation and tumour growth/metastasis. It should be noted, however, that
there is only one heparanase encoding gene in the mammalian genome, making heparanase an excellent drug target. In fact, my supervisor’s laboratory has developed PI-88 (Muparfostat), a heparanase inhibitor that has reached a Phase III clinical trial in hepatocellular carcinoma patients.

Today it is widely accepted that heparanase activity can be correlated with cell invasion associated with metastatic tumours (Levy-Adam et al., 2010; Li & Vlodavsky, 2009). Such degradation of HS in the ECM by heparanase also liberates ECM bound factors, such as HS-binding growth factors, cytokines, chemokines, and enzymes, that bind to HS with high affinity and are made available for physiological processes like angiogenesis and wound healing (Hulett et al., 1999; Parish et al., 2001; Szymczak, Kuzniar, & Klinger, 2010). Heparanase has also been shown to have an impact on smooth muscle proliferation. Post injury, heparanase degrades inhibitory HS in the ECM of blood vessel walls, and this permits smooth muscle cell proliferation (Campbell, Rennick, Kalevitch, & Campbell, 1992). Interestingly, there are some reports suggesting that heparanase activity is affected by the physiological status of the local environment (Gilat et al., 1995). Changes in the pH of the local environment, for example, can cause heparanase to either function as an enzyme or as an adhesion molecule (Gilat et al., 1995; Levy-Adam et al., 2010), with the maximal ECM degrading activity of heparanase being observed to be at pH 6.2-6.8 (Gilat et al., 1995). It should be emphasized, however, that although heparanase has been implicated in cell invasion by numerous studies, the enzyme could potentially deplete cells of cell surface HS and affect cell adhesion and the ability of viruses to both infect and escape from infected cells, as
demonstrated in a recently published study using herpes simplex virus 1 (HSV-1) (Hadigal et al., 2015).

1.8 Role of GAGs in virus infections

GAGs expressed on cell surfaces serve as low affinity receptors that facilitate the interaction of pathogens with their respective secondary internalization receptors (Aquino et al., 2010). Since HS is ubiquitously expressed on the surface of cells in both vertebrate and invertebrate species, a number of viruses from different families have been shown to have HS dependent entry mechanisms including, herpes simplex virus (HSV) (Wudunn & Spear, 1989), human papillomavirus (HPV) (Giroglou, Florin, Schafer, Streeck, & Sapp, 2001), hepatitis B virus (HBV) (Cooper, Tal, Lider, & Shaul, 2005), respiratory syncytial virus (RSV) (Hallak, Collins, Knudson, & Peeples, 2000), foot-and-mouth disease virus (FMDV) (Jackson et al., 1996), human immunodeficiency virus type 1 (HIV-1) (Roderiquez et al., 1995) and dengue virus (Chen et al., 1997). Furthermore, alphaviruses like sindbis virus (SINV) (Zhu et al., 2010), Ross River virus (RRV) (Heil, Albee, Strauss, & Kuhn, 2001), Venezuelan equine encephalitis virus (VEEV) (Bernard, Klimstra, & Johnston, 2000) and semliki forest virus (SFV) (Smit et al., 2002) have also been found to use cell surface HS as a receptor. Of particular relevance to this thesis are the poxviruses that have also been shown to interact with cell surface HS (Chung, Hsiao, Chang, & Chang, 1998; Lin, Chung, Heine, & Chang, 2000; Moss, 2006; Schmidt, Bleck, & Mercer, 2012). An overview of vaccinia virus (VACV), the poxvirus investigated in this thesis, is presented below.
1.9 Vaccinia Virus (VACV)

Vaccinia virus (VACV), a large double stranded DNA virus, is a member of the *Orthopoxvirus* (OPV) genus of the *Poxviridae* family (Roberts & Smith, 2008).

Poxviruses are known to have large, ovoid or brick-shaped virions that replicate in the cytoplasm of the host cell (Moss, 2006; Sodeik & Krijnse-Locker, 2002). VACV is often used as a laboratory prototype virus to study poxviruses (Condit, Moussatche, & Traktman, 2006). This prototype poxvirus contains over 100 polypeptides and a genomic DNA molecule that encodes more than 200 genes (Geada, Galindo, Lorenzo, Perdiguer, & Blasco, 2001).

VACV has a complex morphogenesis that leads to the formation of two distinct forms of infectious virus particles, the intracellular mature virus (IMV) and the extracellular enveloped virus (EEV) (Roberts & Smith, 2008). The IMV form of VACV was renamed as mature virus (MV) and the EEV form as extracellular virus (EV) (Moss, 2006). The two forms differ by the number of membranes that surround the virus (Figure 1.4). MV is the simplest form of the virus surrounded by a single membrane and, as the name suggests, remains inside the cell until cell lysis (Payne & Kristensson, 1985; Sodeik & Krijnse-Locker, 2002). MV particles contain a biconcave, DNA-containing core, flanked by lateral bodies which fill the concavity of the core (Condit et al., 2006). The EV form of VACV, on the other hand, is essentially an MV wrapped in a second membrane that allows the virus to be released from infected cells before virus-induced cell death (Condit et al., 2006; Roberts & Smith, 2008). Although the MV form makes up the bulk of the infectious virion progeny, they are not able to mediate cell-to-cell spread of the virus (Carter et al., 2003). The MV form of VACV is the cytopathic version of VACV, as the MV
**Figure 1.4: VACV morphogenesis.** In a cell infected with vaccinia virus (VACV), the naked viral core is moved on the microtubule network to the virus factories where the core uncoats and the replication process initiates to produce the immature virion (IV). The culmination of a replication cycle results in intracellular mature virus (MV) particles that further move on the microtubule network to the Golgi apparatus where they are wrapped by membranes derived from early endosomal or **trans**-Golgi cisterna and become intracellular enveloped virus (IEV) particles. Finally, the IEVs move to the plasma membrane of the cell via the microtubule network where the outer IEV membrane fuses with the plasma membrane to form cell-associated enveloped virus (CEV). Actin tail formation pushes the CEV away from the infected cell, this VACV now being the extracellular enveloped virus (EV) particle (Adapter from Roberts and Smith, 2008).
particles are only released upon death of an infected cell. The EV form of VACV, on the other hand, is released from a live cell. The EV form of VACV is also known as cell-associated enveloped virus (CEV), if it is retained on the cell surface (Condit et al., 2006). Both CEV and EV are important for viral dissemination. EVs have been shown to mediate long-range dissemination in vitro, whereas the CEVs are known to infect neighbouring uninfected cells. Interestingly, the intracellular and extracellular forms of VACV contain different viral proteins which are found within either outer membranes, which makes MV and EV structurally, functionally and antigenically different (Roberts & Smith, 2008).

1.9.1 VACV gene expression and morphogenesis

VACV particles have been shown to be composed of four different structural elements, namely, the core, lateral bodies, outer membrane and the envelope which is a characteristic feature of only EV (Sodeik & Krijnse-Locker, 2002). The proteinaceous core membrane surrounds the core, which contains the viral genome, DNA associated proteins, viral structural proteins, and other necessary transcriptional enzymes that are required to initiate replication (Condit et al., 2006; Roberts & Smith, 2008; Sodeik & Krijnse-Locker, 2002). Poxviruses differ from other classes of DNA viruses in that the virus remains in the cytoplasm of the cell for the entire infectious cycle, indicating that the poxviruses have evolved to be highly independent of the host cell, especially for the processes that involve DNA replication (Broyles, 2003).

To initiate infection, the single membrane of MV fuses with the cell membrane, and the remainder of the infectious virion, the naked core, enters the cell cytoplasm.
The core is then transported deeper within the cell on microtubules to the perinuclear region where viral replication takes place (Carter et al., 2003). It has been shown that heparin causes rupture of the outer EV envelope, thereby releasing MV encapsulated within the outer membrane (Law, Carter, Roberts, Hollinshead, & Smith, 2006). The first step in viral replication is the transcription of the viral genome into early mRNA by the viral associated enzymes packaged within the genome core. The proteins translated from these early mRNAs are not only involved in replication of viral DNA, but also in the modification of the host cell to advantage the virus by aiding its escape from the innate immune system (Broyles, 2003; Roberts & Smith, 2008). As the DNA replicates, transcription of intermediate genes commences. These intermediate genes encode a number of regulatory proteins that induce transcription of the late genes. The transcriptional products of the late genes are viral proteins that make up new virus particles and other enzymes, which are packaged into virions to initiate the next round of transcription in another infected cell (Broyles, 2003; Roberts & Smith, 2008).

The cytoplasmic sites where viral replication takes place are known as viral/DNA factories (Figure 1.4). These sites contain few if any cellular organelles (Carter et al., 2003; Condit et al., 2006; Roberts & Smith, 2008). A crescent-shaped arrangement is the first visible structure that appears in the virus factories. It contains proteins and lipids, and grows to form an oval structure. This structure engulfs the virus core components and is termed an immature virion (IV) (Figure 1.4). As the viral genome is packaged into IV, the action of several proteolytic enzymes modifies the core proteins. These modifications transform the virus into
the brick-shaped intracellular MV particles (Condit et al., 2006; Roberts & Smith, 2008).

A few of these newly formed MVs are transported away from the viral factories to a site near the microtubule-organizing centre (MTOC) where they become enveloped by a double cellular membrane to form intracellular enveloped virus (IEV) particles (Carter et al., 2003; Roberts & Smith, 2008). The double membrane is derived from endosomal or trans-Golgi cisternae (Roberts & Smith, 2008). The IEV are then transported to the periphery of the infected cell, where the outer IEV membranes fuse with the plasma membrane and produce cell-associated enveloped virus (CEV) on the cell surface (Figure 1.4). In order to infect the neighboring cells, CEVs need to detach from the infected cells. CEVs induce actin polymerization beneath the plasma membrane in order to push the virions away from the cell. Once detached from cells, these CEVs are called extracellular enveloped virus (EV) (Carter et al., 2003).

CEV and EV forms of VACV are easily able to evade host antibody and complement by being wrapped in a host-derived outer membrane (Roberts & Smith, 2008). The outer membrane of the MV form of VACV is highly resistant to breakage, which makes it an ideal viral form to cause inter-host transmission (Chahroudi et al., 2005; Sodeik & Krijnse-Locker, 2002). EVs, on the other hand, are known to mediate cell-to-cell spread of the virus as they have acquired an extra membrane from the trans-Golgi network (Chahroudi et al., 2005; Vanderplasschen, Hollinshead, & Smith, 1998).
1.9.2 VACV interacts with HS

Previous studies have shown that there are at least three GAG-binding receptors on the surface of MV (Chung et al., 1998; Ho et al., 2005; Lin et al., 2000). To cause an infection, virions attach to cell surface GAGs and fuse with the plasma membrane to enter a cell. The receptors required for plasma membrane fusion are still unknown. Envelope proteins H3L and A27L have been shown to bind HS, whereas D8L has been shown to interact with chondroitin sulfate (Ho et al., 2005; Lin et al., 2000). Previous studies have also shown that B5R and A34R proteins present on the outer envelope of the EV form of VACV, when in contact with cell surface HS, initiate the dissolution of the outer EV envelope, which is followed by the fusion of inner viral membrane with the plasma membrane of cells (Law et al., 2006).

Envelope protein A27L has been shown to bind cell surface HS moieties, an interaction required for fusion of the virus with the target cell plasma membrane (Lin et al., 2000). Extensive studies performed on A27L show that the protein can be divided into four functional domains, namely, a signal peptide for protein processing (Takahashi, Oie, & Ichihashi, 1994), a cell surface GAG binding region rich in lysine and arginine residues (Hsiao, Chung, & Chang, 1998), a coiled-coil domain that is involved in self assembly (Ho et al., 2005), and a C-terminal sequence that has been shown to interact with A17L, another vaccinia viral protein, and is vital for Golgi membrane wrapping of the MV during the formation of the intracellular and extracellular enveloped forms of the virus (Rodriguez, Esteban, & Rodriguez, 1995). Studies performed using A27L mutants showed a
phenotype where the plaque size was greatly reduced indicating that A27L is essential for viral spread (Rodriguez & Esteban, 1987).

The H3L gene encodes p35 protein, a HS binding molecule, which is an immunodominant antigen on the surface of MV. Studies have shown that the H3L mutant virus is not only structurally different to wild type MV, but also has greatly reduced infectivity compared to wild type MV (Lin et al., 2000).

1.9.3 Strains of VACV

Intensive disease surveillance, combined with a comprehensive vaccination strategy using VACV, was required to successfully eradicate smallpox. However, there is little historical evidence of the biological origin of VACV, the relationship between different strains of VACV used to eradicate smallpox, or how they have evolved from a common ancestral strain (Qin, Favis, Famulski, & Evans, 2015). Western Reserve (WR) and International Health Department-J (IHD-J) are two of the most commonly used laboratory-passaged strains of VACV and were the strains of VACV used in the assays described in this thesis. These two strains of VACV are known to plaque differently, with the IHD-J plaques being ‘comet’ shaped and the majority of WR plaques, on the other hand, being round with only a few plaques being comet-like in appearance (Figure 3.5A). Both the WR and IHD-J strains of VACV produce similar amounts of MV, IEV and CEV, however, about 40% more EV is released by the IHD-J strain (Blasco & Moss, 1992). This makes the IHD-J strain the more infectious of the two strains. Since more EV is released by the IHD-J strain, the spread of virus to distant cells is more efficient. This results in the majority of IHD-J plaques appearing as comets.
Most of the previous studies performed with VACV have been with the MV form of VACV as this form is easily purified to high concentrations and is easy to work with. Most purification protocols used to isolate EV particles involve high-speed centrifugation, which inevitably damages the outer-membranes of the EV particle and, therefore, results in the release of the enclosed MV particle (Figure 3.5E and F). Since the MV and EV forms of VACV differ markedly in the make up of their outer membranes, the role of cell surface HS in the infectivity of the two forms of the virus may differ substantially.

1.10 Research question

A steady increase in the numbers of drug resistant viruses has become a reason for concern in the scientific community. This has resulted in increasing efforts to develop new classes of antiviral drugs that are less intrusive, less toxic and more resistant to virus evasion strategies compared to the traditional treatments. One approach is to target virus entry into cells by developing compounds that block virus particles from interacting with their cell surface receptors and, thereby, prevent virus particles from establishing an infection. Since it is known that many viruses and other pathogens use cell surface HS as an initial docking site prior to their interaction with their specific cell surface receptors, which then allows them to enter and establish an infection, a detailed understanding of the interaction between viruses and cell surface HS would be vital for the development of novel HS based antiviral drugs.
Figure 1.5: VACV interacts with cell surface HS prior to infecting cells. A diagrammatic representation of VACV interacting with cell surface HS. VACV bound to cell surface HS is then able to more efficiently interact with more specific cell surface receptors (not shown).

Using VACV as a prototype virus, the underlying hypothesis is that VACV uses cell surface HS as an initial docking site prior to interacting with its cell surface receptors (Figure 1.5). Heparanase mediated cleavage of cell surface HS, or the use of cells genetically deficient in cell surface HS expression, would hamper the ability of VACV to cause an infection. Understanding the interaction between VACV and cell surface HS at a structural level in 3D space would allow development of small molecules based on the parent HS structure, which could interfere and inhibit VACV interacting with cell surface HS.

Based on the hypothesis outlined above, the research described in this thesis attempted to address the following research aims.
**Aim 1:** Establish heparin, heparan sulfate and chondroitin sulfate binding specificities of VACV mature virus and determine effects of heparin on the Western Reserve and the International Health Department-J strains of VACV.

**Aim 2:** Construct green fluorescent protein expressing Western Reserve and International Health Department-J strains of VACV to determine the role of cell surface heparan sulfate in VACV infections *in vitro*.

**Aim 3:** Determine the structural features of heparin (and heparan sulfate) that are required for the molecule to interact with VACV.

**Aim 4:** Identify protein(s) on the surface of outer envelope of the extracellular enveloped virus that interact with heparan sulfate.

**Aim 5:** Evaluate the role of heparanase in VACV infections *in vivo*. 
Material and methods

Chapter 2:
2.1 Mice and Reagents

2.1.1 Mice
Pathogen free female C57BL/6 and heparanase knockout (HPSE-/-) (5-8 weeks old) mice were obtained from the Australian Phenomics Facility at ANU, and maintained under the ANU Animal Experimentation and Ethics Committee (AEEC) guidelines.

2.1.2 Ethics statement
The mice used for all the experiments for the purpose of this PhD thesis, were maintained in accordance with AEEC approved guidelines, protocol number A2011/018. All the experiments performed were in accordance with the Australian National Health and Medical Research Council guidelines within the Australian Code and Practice for the Care and Use of Animals for Scientific Purposes. For every mouse used in every experiment performed, a health score sheet was maintained over the course of the experiment. Mice were monitored daily post infection and were scored for signs of distress, illness and weight loss. All the mice were ethically sacrificed by the method of cervical dislocation in accordance with the above AEEC approved protocol.

2.1.3 Cell lines
Human osteosarcoma 143B cells (obtained from ATCC) were grown in MEM (GIBCO-Invitrogen, Grand Island, NY) supplemented with 5% heat inactivated foetal bovine serum (FBS - GE Healthcare, Pittsburg, PA), 50 μg/mL penicillin, 50 μg/mL streptomycin and 50 μg/mL neomycin (all provided by the media unit, John Curtin School of Medical Research - JCSMR).
Baby hamster kidney BHK-21 cells (obtained from ATCC), Chinese hamster ovary
CHO-K1 cells (provided by Hulett/Hogarth, The Austin Research Institute,
Melbourne, Australia), and xylosyltransferase-I-deficient Chinese hamster ovary
pgsA-745 cells (provided by Dr. Eva Lee, JCSMR, Canberra, Australia), were all
grown in RPMI-1640 (GIBCO-Invitrogen) supplemented with 5% FBS.

All the cell lines were incubated at 37°C in a Hepa-Filtered IR Incubator (Forma
Scientific Inc., Marietta, OH) in humidified atmosphere containing 5% CO₂. Cells
were passaged every two days, or when 100% confluency (~ 1 x 10⁶ cells/mL) was
reached, depending on the rate of cell growth. Confluent cells were subcultured at
a cell concentration of 2 x 10⁵ cells/mL and allowed to grow to the maximum
concentration of 1 x 10⁶ cells/mL.

2.1.4 Viruses

Two different strains of VACV were used for the purpose of this thesis, namely, the
International Health Department strain (IHD-J) obtained with thanks from Dr.
David Tscharke (RSB, Australian National University, Canberra, Australia) and the
Western Reserve strain (WR). Recombinant forms of both strains of VACV were
constructed by inserting green fluorescent protein – blasticidin resistance (GFP-
Bsd) genes in their genomes. The blasticidin resistance gene Bsd, when fused with
GFP gene, has previously been shown to be an ideal bi-functional selection marker
for engineering recombinant poxviruses, due to its small size and the ability to
allow drug selection and visual screening (Wong, Lin, Melo-Silva, Smith, &
Tscharke, 2011). The GFP-Bsd cassette used in this thesis is based on the cassette
used previously, but has been modified by Dr. Ronald Jackson, JCSMR, such that the
GFP molecule fluoresces brighter at 470 nm, is more stable at 37°C, and has its own strong promoter, all of which enhance GFP expression compared to the original GFP-\textit{Bsd} sequence.

\textbf{2.1.4.1 Cloning GFP-\textit{Bsd} in a MVA vector}

As mentioned above, the GFP-\textit{Bsd} cassette used in this thesis is a modified version of the cassette that has previously been published. To clone the GFP-\textit{Bsd} cassette into a Modified Vaccinia Ankara (MVA) vector, both pUC57 (GFP-\textit{Bsd} carrier vector: Figure 2.1A) and pUC57-MVAF DNA vectors were first digested using the restriction enzyme \textit{FseI} (New England BioLabs, Ipswich, MA). A restriction digest mix was prepared was composed of 1 μL of \textit{FseI} restriction enzyme (2 units), 1 μL of 10X NEBuffer 4 (supplied with \textit{FseI}), 1 μL of 100X bovine serum albumin (BSA) (supplied with \textit{FseI}) and 6 μL of double distilled water (ddH₂O) (Media Unit, JCSMR). The respective vector DNAs (1 μL) were added to the reaction mixture and incubated at 37°C for 1 hr. To denature the restriction enzyme post digestion, the mixture was heated at 65°C for 20 min.

The second step in cloning the GFP-\textit{Bsd} gene cassette into the MVAF vector was the dephosphorylation of 5’ phosphate of the digested MVAF vector. Shrimp alkaline phosphatase (SAP) (Promega, Madison, WI) was used to catalyze the reaction. Initially 10 μL of ddH₂O was added to both digested DNA vectors, with the digested pUC57 being left on ice, whereas, the pUC57-MVAF DNA vector was further processed prior to treatment with SAP. A mixture containing 2 μL of 3 M sodium acetate (Sigma-Aldrich, St. Louis, MO) and 60 μL of absolute ethanol was added and the samples incubated at -20°C for 30 min. The pUC57-MVAF DNA vector was
then centrifuged at 30,100 g for 25 min to allow the precipitated DNA to pellet. The supernatant was removed and the DNA containing pellet left to air dry. Once dried, 16 μL of ddH2O, 2 μL of SAP buffer (supplied with SAP enzyme) and 2 μL SAP enzyme (2 units) added and the mixture incubated at 37°C for 15 min. After phosphatase treatment, SAP was denatured by incubating the tube at 70°C for 15 min.

Both the pUC57 GFP carrier and MVAF vectors were loaded and run on a 1% agarose gel (Sigma-Aldrich) containing 3 μL of SYBR safe DNA gel stain (ThermoFisher Scientific, Marietta, OH). Both DNA samples (10 μL) were mixed with 10X BlueJuice gel loading buffer (2 μL) (ThermoFisher Scientific), loaded onto the gel along with 1 kb+ DNA ladder (ThermoFisher Scientific), a mixture of 20 highly purified double-stranded DNA bands ranging from 100 bp to 12,000 bp, to estimate the molecular weight of digested DNA fragments, and run at 80 V for 45 min. Figure 2.1B shows the restriction digest products. Lane 1 contains the 3.9 kb empty MVAF vector and lane 3 shows the 1 kb+ DNA ladder. Lane 4 contains two bands, a smaller 1.1 kb band and a larger 2.7 kb band. The 1.1 kb band is the GFP-\textit{Bsd} cassette resulting from the restriction digest, whereas, the 2.7 kb band represents the remaining vector (Figure 2.1A).

Both the 3.9 kb band in lane 1 (empty pUC57-MVAF vector) and the smaller band in lane 4 (GFP-\textit{Bsd} cassette) were excised and put in the same tube for purification using the MiniElute gel extraction kit (Qiagen, Valencia, CA). To perform the ligation of the two DNA plasmids, 4 μL of T4 DNA ligase (Promega) and 5 μL of 10X ligase buffer (provided with DNA ligase) were added to 10 μL of a purified plasmid
Figure 2.1: Cloning of GFP-\textit{Bsd} cassette into pUC57-MVA vector. (A) Diagrammatic representation of pUC57 carrier vector which contains a 1.1 kb GFP-\textit{Bsd} construct. (B) Agarose gel (1\%) showing restriction digest of both pUC57 (GFP-\textit{Bsd}) carrier (lane 4) and empty pUC57-MVA vectors (lane 1). Both empty pUC57-MVA vectors were digested using the restriction enzyme FseI. Lane 2 was left empty and lane 3 contains a 1 kb+ DNA ladder. (C) pUC57-MVA gene map containing the GFP-Bsd gene fragment in correct orientation. (D) Another 1\% agarose gel showing restriction digest of products after a transformation assay. Four different colonies were treated with the restriction enzymes EcoRI and BstEI. Lane 1 contains a 1 kb+ DNA ladder. Lanes 2, 3 and 5 show two bands, a 3.5 kb and a smaller 1.6 kb fragments, indicating that the GFP-\textit{Bsd} cassette is inserted in the correct orientation. Lane 4 also has two bands, a 4.2 kb and a smaller 0.8 kb, indicating that the GFP-\textit{Bsd} cassette is inserted in the incorrect orientation. (E) WR-GFP plaques and (F) IHD-J-GFP plaques forming on 143B monolayers. Images were taken using an Olympus IX81 fluorescence microscope at 400X magnification.
preparation and incubated overnight at 16 °C. The GFP-\textit{Bsd} cassette can ligate with the empty pUC57-MVAF vector in two different orientations. Figure 2.1C shows the correct orientation of ligation in the direction of the F7 region. GFP-\textit{Bsd} ligation towards the F8 region would be the incorrect, and would require the cloning process to be repeated from the beginning.

\textbf{2.1.4.2 Transfection}

To perform transfections, 10 μL of plasmid DNA from the cloning step was added to 50 μL of DH5α (\textit{endA1}) \textit{E. coli} strain (ThermoFisher Scientific) and incubated on ice for 20 min. Heat shock treatment of the transfection mixture was performed by incubating the mixture at 42°C for 30 sec, with the heat shock treatment forming small holes in the bacterial membranes allowing plasmid DNA to enter the bacteria. The mixture was put on ice for 2 min to allow the bacterial cell membranes to reseal. Liquid broth (500 μL) (Media Unit, JCSMR) added and the bacteria incubated at 37°C for 1 hr. The transfected DH5α bacteria were inoculated on a liquid broth agar plate supplemented with 50 μg/mL ampicillin and grown overnight. Five different single colonies were randomly picked from the agar plate using a sterile toothpick, inoculated into five different 5 mL liquid broth tubes supplemented with 50 μg/mL of ampicillin and incubated overnight in a 37°C shaking incubator.

\textbf{2.1.4.3 Plasmid DNA purification and confirmation of GFP-\textit{Bsd} orientation}

To purify amplified plasmid DNA, the PureYield Plasmid Miniprep system kit (Promega) was used. Purified plasmid DNA from all 4 colonies was digested to confirm the orientation of the GFP-\textit{Bsd} cassette in the pUC57-MVAF-GFP vector.
Figure 2.1C shows the pUC57-MVAF vector map with the GFP-\textit{Bsd} sequence inserted in the correct orientation, between the F7 and F8 region. Restriction enzymes \textit{EcoRI} and \textit{BstEII} (New England BioLabs) were used to digest all five pUC57-MVAF-GFP vectors. A 50 μL reaction mixture was prepared containing 5 μL of 10X NEBuffer (supplied with both restriction enzymes), 2 μL of \textit{EcoRI}, 2 μL of \textit{BstEII}, 5 μL of 10X BSA (supplied with both restriction enzymes) and 35 μL of ddH$_2$O. The reaction mixture was divided into 4 tubes, each with 10 μL of the reaction mix, followed by addition of 2 μL of purified plasmid DNA to the respective tubes with the tubes being incubated at 37°C for 1 hr for digestion. Once digested, the products were run on a 1% agarose gel as described earlier in section 2.1.4.1. Figure 2.1D shows the digested products of all five colonies tested. Lanes 2, 3 and 5 have two bands, a 3.5 kb and a smaller 1.6 kb fragment, showing the digestion products expected from the insertion of the GFP-\textit{Bsd} cassette in the correct orientation in pUC57-MVAF vector (Figure 2.1C). Lane 4, however, has a 4.2 kb fragment and a 0.8 kb fragment, indicating that the GFP-\textit{Bsd} cassette is inserted in the incorrect orientation in the pUC57-MVAF-GFP plasmid.

2.1.4.4 Transfection of WR-VACV and IHD-J-VACV to express GFP-\textit{Bsd}

Lipofectamine 2000 transfection reagent (ThermoFisher Scientific) was used to aid DNA uptake and recombination between the WR-VACV/IHD-J-VACV genomes and the pUC57-MVAF-GFP vectors, which if successful, would result in insertion of the GFP-\textit{Bsd} cassette in the WR-VACV and IHD-J-VACV genomes. Lipofectamine reagent (40 μL) was added to serum free MEM (1 mL) and, in a separate tube, pUC57-MVAF-GFP plasmid (40 μL) added to serum free MEM (1 mL), with both
tubes being incubated for 20 min at room temperature, prior to being mixed together.

BHK-21 cell monolayers were grown in two T25 tissue culture flasks until 80 % confluent. Medium from both flasks was removed gently, washed once with sterile phosphate buffer saline (PBS) (Sigma-Aldrich) and replaced by 0.5 mL of fresh MEM (serum free). Both flasks were incubated in a 37°C incubator for 1 hr prior to the addition of a further 4.5 mL of MEM (serum free) to both flasks along with 1 mL lipofectamine - MVAF-GFP plasmid mix and 100 μL of 10² PFU of either WR or IHD-J strains of VACV. The flasks were then incubated for 72 hr in a 37°C incubator.

Cell monolayers from the flasks infected with the WR and IHD-J strains of VACV, along with the lipofectamine-plasmid mix were subsequently released by scraping. Cells were sonicated to release VACV particles within which were used to infect fresh flasks of 80% confluent BHK-21 cells under blasticidin (ThermoFisher Scientific) selection (10 μg/mL) for 72 hr in a 37°C incubator. After blasticidin selection, cell monolayers were released by scraping, sonicated to release virus as before and, serial dilutions (1:10) of released WR-VACV and IHD-J-VACV infected monolayers made, which were then used to infect 100% confluent BHK-21 cell monolayers cultured in 6-well plates in the absence of any blasticidin. VACV was allowed to infect cells for 1 hr at 37°C and then replaced with 2 mL of 1% warm low-melting-point (LMP) agarose (Thermo Fisher Scientific) mixed with an equal volume of 1X DMEM (Media unit, JCSMR) and incubated for 48 hr in a 37°C
incubator. Following the incubation, green plaques were visualized under an Olympus IX81 fluorescence microscope at 400X magnification.

Green plaques from both WR-VACV and IHD-J-VACV infected wells, which potentially were derived from GFP expressing VACV, were scraped using sterile toothpicks, transferred to tubes containing 100 μL of MEM (serum free) and freeze thawed once to release VACV particles. The released GFP expressing WR-VACV and IHD-J-VACV particles were put through a second round of blasticidin selection.

Fresh monolayers of 100% confluent BHK-21 cells were infected with the released GFP expressing WR-VACV and VACV IHD-J in separate 12-well plates (Thermo Fisher Scientific) and incubated for 1 hr in a 37°C incubator. Unbound VACV particles were removed from the cell monolayers and replaced with fresh RPMI (supplemented with 5% FBS and 10 μg/mL blasticidin). The infected monolayers were incubated for further 48 hr in a 37°C incubator. The culture medium was subsequently removed from the cell monolayers, replaced with 100 μL of fresh RPMI (supplemented with 5% FBS) and a syringe plunger used to scrape the infected cells off the bottom of each wells. The infected cells were then freeze-thawed once to release VACV particles and serial dilutions made which were then used to infect confluent monolayers of BHK-21 cells grown in 6-well plates in the absence of any blasticidin selection under 1% LMP agarose as described earlier. This process was repeated twice to ensure WR-VACV and IHD-J-VACV genomes had undergone successful recombination events with the pUC57-MVAF-GFP plasmid and were expressing the GFP-\textit{Bsd} cassette without any contaminating wild type WR-VACV and IHD-J-VACV in the respective recombinant cultures.
2.1.4.5 PCR confirmation of the purity of the recombinant GFP-Bsd VACV strains

To confirm that there were no contaminating wild type WR-VACV and IHD-J-VACV in the recombinant WR-VACV and recombinant IHD-J-VACV preparations respectively, polymerase chain reactions (PCR) was performed on DNA isolated from green plaques using primers to amplify any MVAF plasmid containing the GFP-Bsd cassette and, therefore, identifying presence of wild type WR-VACV or IHD-J-VACV in green plaques. InstaGene Matrix (Bio-Rad, Hercules, CA) was used to prepare DNA from mammalian cells by following the protocol supplied by the manufacturer. HotStarTaq (Qiagen) master mix was used with the MVAF forward and reverse primers (Table 2.1) to amplify the products. For every template to be amplified, 12.5 μL of mastermix, 1 μL of forward primers, 1 μL of reverse primers, 8.5 μL of ddH₂O and 2 μL of template was used such that the total reaction volume for each template tested was 25 μL. The cycling conditions are summarized in Figure 2.2A. Following the amplification, PCR products were run on 1% agarose gels as described earlier in section 2.1.4.1. A 400 bp PCR product is indicative of the presence of intact pUC57-MVAF-GFP plasmid along side wild type VACV strains. Amplified PCR products from cells obtained from supposedly recombinant WR-VACV and IHD-J-VACV plaques are shown in Figures 2.2B and 2.2C respectively. Lane 1 in both gels contains a 1 kb + DNA ladder, PCR products from pUC57-MVAF-GFP DNA plasmid (positive control) in lane 3 and the amplified products from WR-VACV and IHD-J-VACV plaques, respectively, in lane 5.
Figure 2.2: Confirmation of successful recombination events using PCR. (A) Standard PCR cycle conditions used to amplify pUC57-MVAF-GFP plasmid DNA. Samples were incubated at 95°C for 15 min, and then 35 cycles of 94°C for 1 min, 55°C for 30 sec and 72°C for 1 min. After all 35 cycles, samples were incubated at 72°C for 10 min before bringing the temperature down to 15°C. Products from a PCR amplification of DNA purified from WR-VACV (B) and IHD-J-VACV (C) infected cell monolayers. Lane 1 contains the 1 kb+ DNA ladder, lane 3 has pUC57-MVAF-GFP (positive control) and lane 4 contains amplified products from the DNA purified from a single plaque from WR-VACV (B) and IHD-J (C).
Since there are no products formed from the PCR amplification of the WR-VACV and IHD-J-VACV plaques, it can be concluded that both genomes contain the GFP-Bsd cassette. Figure 2.1E and 2.1F show plaques formed on 143B cell monolayers when infected with recombinant WR-VACV and recombinant IHD-J-VACV, respectively.

Table 2.1: Primer sequences used for confirmatory PCR reactions

<table>
<thead>
<tr>
<th>Oligo name</th>
<th>Length (bp)</th>
<th>Sequence (5’-3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MVAF Forward</td>
<td>25</td>
<td>GAGCAGCCTCGTCCACGTACACCGC</td>
</tr>
<tr>
<td>MVAF Reverse</td>
<td>31</td>
<td>GGTTCCTATAATTGTAACATCCTTTCTCTCC</td>
</tr>
</tbody>
</table>

2.1.4.6 Purification of the MV and EV forms of VACV from WR and IHD-J strains respectively

To obtain purified GFP expressing mature virus (MV-GFP) stocks from recombinant WR-VACV, BHK-21 cell monolayers were grown in 6 T175 flasks until 80% confluent. Cells were then infected with recombinant WR-VACV at a multiplicity of infection (MOI) of 0.1 for 1 hr at 37°C. Cell monolayers were washed once with PBS, 25 mL of fresh RPMI medium (serum free) added and the monolayers incubated for a further 48 hr at 37°C. Infected cell monolayers were scraped off the flask surface and pelleted by centrifugation at 1800xg for 5 min. The supernatant was discarded and each cell pellet resuspended in 3 mL of 10 mM
Tris-HCl buffer, pH 9.0 and pooled together prior to homogenizing with 40 strokes of a glass dounce homogeniser. The cells were centrifuged again for 5 min at 300xg to remove nuclei and other cell debris, the supernatant retrieved and split in 3 mL aliquots. All aliquots were sonicated at intensity 3 for 30 sec in an ice-cold cup sonicator, the sonicated preparations were pooled again and layered onto a 17 mL cushion of 36% (weight/volume - w/v) sucrose (Sigma-Aldrich) in a clear ultracentrifuge tube and centrifuged at 32,000xg for 1 hr at 4°C in a SW 28 rotor under vacuum to pellet MV-GFP particles. Supernatant was discarded, the MV-GFP pellet resuspended in 500 μL of a 1 mM Tris-HCl buffer at pH 9, with the purified MV-GFP being stored at -80°C. To obtain MV rich stocks for enzyme-linked immunosorbent assays (ELISAs) and plaque assays, wild type WR strain was used instead of GFP expressing recombinant WR strain. For Western blots, detergent treatment of MV-GFP particles was performed as described in section 2.2.5.

To obtain a purified stock of GFP expressing enveloped virus (EV-GFP), the recombinant IHD-J-VACV was used to infect 80% confluent monolayers of BHK-21 cells in T175 flasks. Cell monolayers were infected at a MOI of 0.1 for 1 hr at 37°C, washed once with PBS and prior to adding 12 mL of fresh RPMI medium (serum free) and incubated for further 48 hr at 37°C. To collect EV-GFP particles, supernatants from the infected cells were removed without disturbing the cell monolayers and centrifuged at 230xg for 10 min to remove any cell debris. The supernatant was then used as the EV rich stock to perform all fluorescence-activated cell sorting (FACS) based assays. To obtain EV rich stocks for plaque assays, wild type IHD-J strain was used instead of GFP expressing recombinant IHD-J strain. Low speed centrifugation was performed to prevent any damage to
the EV outer membrane and, hence, avoid any MV contamination of EV preparations. The EV-GFP preparations were stored at 4°C for up to 2 weeks. To obtain EV particles for Western blots, EV containing supernatants were further centrifuged at 18,000xg for 1 hr to pellet EV particles, which were resuspended in 500 μL of a 1 mM Tris-HCl buffer at pH 9. The detergent treatment of EV particles performed as described in section 2.2.5.

2.1.5 Virus titrations and plaque assays

For the purpose of VACV titrations, 143B cells were used. Cells were seeded into 6-well plates and incubated overnight until the cells reached 100% confluency. Serial dilutions (1:10) of virus stocks were made in MEM (supplemented with 5% FBS), culture medium removed from the 143Bs containing wells and replaced with 100 μL of each dilution. The cells were then incubated at 37°C for up to 1 hr ensuring cells did not dry out. The virus dilutions removed from the cells and replaced with 2 mL of MEM (supplemented with 5% FBS). Cells were then incubated for a further 48 hr period. Following this incubation period, the culture medium was removed from each well and the cells stained with 0.1% crystal violet (Sigma-Aldrich) in 20% ethanol to visualize plaques. The resulting plaques were then counted to determine virus titers. Plaque diameters were measured using ImageJ particle analysis plugin with settings (Size pixel^0.05-5; Circularity 0.0-1.0) under Olympus IX81 fluorescence microscope.
2.2 In vitro techniques

2.2.1 ELISA assays

To perform ELISA assays, 96 U-well PVC microtitre plastic plates (Dynex Technologies Inc., Chantilly, VA) were used. Plate wells were coated with BSA (Sigma-Aldrich) conjugated with heparin, highly sulfated heparan sulfate (HS\textsuperscript{hi}) or lowly sulfated heparan sulfate (HS\textsuperscript{lw}), at 10 μg/mL (25 μL/well) in PBS overnight at 4°C. Unbound BSA conjugate was removed next day by gently flicking the plate. Non-specific binding to ELISA wells was blocked with PBS/1%BSA (200 μL/well) for 2 hr at 37°C. Blocking buffer was then removed by gently flicking the plate and the wells were washed three times (200 μL/well) with PBS/0.05% Tween-20 (Sigma-Aldrich). Purified MV particles from the WR strain of VACV were diluted to 1 x 10\textsuperscript{5} PFU/mL in PBS/1%BSA, added to the ELISA wells (50 μL/well) and incubated for 2 hr at 4°C. Unbound MV particles were removed by flicking the plate. Wells were washed three times (200 μL/well) with PBS/0.05% Tween-20.

A biotin-conjugated polyclonal rabbit-anti-VACV antibody (Mybiosource, San Diego, CA) was used at 4.5 μg/mL final concentration in PBS/1%BSA and added (50 μL/well) for 2 hr at 4°C. Unbound antibody was removed by flicking and the wells were washed three times as described earlier. Detection of antibodies bound to MV particles in ELISA wells was achieved by using a secondary antibody, streptavidin-conjugated horseradish peroxidase (HRP) (GE Healthcare), used at 0.1% final concentration in PBS/1%BSA and added (50 μL/well) for 1 hr at 4°C. Unbound antibody was removed and wells washed as described above. Peroxidase substrate, 2,2'-azido-bis(3-ethylbenzthiazoline-6-sulphonic acid) (ABTS) (Kirkegaard and Perry Laboratories Inc., Gaithersburg, MD), was used as a
colorimetric substance to detect peroxidase conjugated antibodies bound to the wells, with ABTS being added (100 μL/well) for 30 min at 37°C. The absorbance readings of the enzymatic product formed post substrate addition was read at 405 nm on a Tecan Infinite® M200Pro microplate reader. Histogram plots from analysed data were constructed using GraphPad Prism® (version 5.0f).

2.2.2 Heparanase treatment

To investigate the effect of heparan sulfate (HS) removal from 143B cells on VACV infections, cell surface HS on 143B cells was digested using both Flavobacterium heparinases (HPNSE) (Sigma-Aldrich) and human platelet heparanase (HPSE) (gift from Dr. Craig Freeman, John Curtin School of Medical Research, Canberra, Australia). There are three different HPNSEs available, each targeting different HS sequences. For the purpose of the assays performed in this thesis, each HPNSE (HPNSE I, II and III) was used either individually, or as a cocktail of all three to digest cell surface HS and evaluate the role of cell surface HS in VACV infection.

All three HPNSEs were prepared in 50% glycerol (Sigma-Aldrich) at a stock concentration of 0.25 units/μL. 143B cells were grown in 48-well flat bottom plates and incubated overnight to reach 100% confluency. Culture medium was aspirated off the confluent 143B cells monolayers and the wells washed with sterile PBS. Cells were then treated for 1 hr at 37°C with each HPNSE either individually, or as a cocktail of all three enzymes, at a final concentration of 0.25 units/mL (100 μL/well) in pH 5.5 serum free MEM. After incubation, the HPNSEs were aspirated from each well and the cell monolayers were washed with PBS prior to VACV infection.
HPSE was prepared at a stock concentration of 4 μg/μL and confluent monolayers of 143B cells grown in 48-well plates treated with platelet heparanase at a final concentration of 4 μg/mL (100 μL/well) in pH 5.5 serum free MEM for 1 hr at 37°C. After the incubation, cell monolayers were washed with sterile PBS and treated cells either detached from the monolayer and stained for cell surface HS expression (see 2.2.3 below), or kept as monolayers and infected with VACV to evaluate the role of HS in VACV infection.

2.2.3 FACS assays: HS staining

To confirm that the HPNSE and HPSE treatments removed cell surface HS from 143B cell monolayers, treated and untreated (control) cells were stained with a HS-specific antibody and run on a BD LSR bench top flow cytometer. The untreated and treated 143B cells were initially released from monolayers by incubation with 0.25% trypsin-EDTA (GIBCO-Invitrogen) (100 μL/well) for 5 min at 37°C, and transferred to V-bottom 96-well plates (Thermo Fisher scientific). The cells were washed once with FACS buffer (PBS supplemented with 0.1% BSA) and potential Fc receptors blocked prior to HS staining by incubation with Fc block (clone: 2.4G2; BD Pharmingen, Franklin Lakes, NJ) at 2.5 μg/mL final concentration in FACS buffer and added to cells (25 μL/well) for 10 min on ice. Cells were then washed twice with FACS buffer and cell surface HS detected by incubation with the HS-specific monoclonal antibody (mAB) 10E4 (Amsbio, Cambridge, MA) at a concentration of 4 μg/mL in FACS buffer (25 μL/well) for 30 min on ice. It should be emphasized, however, that the 10E4 HS-binding mAb interacts with a HS epitope not easily removed by HPSE or HPNSE, and probably binds to HS stubs remaining after digestions (Prof. C. Parish, personal communication).
Mouse IgM κ-myeloma (clone: C48-6; BD Pharmingen) at 20 μg/mL in FACS buffer (25 μL/well) was used as an isotype control being incubated for 30 min on ice. Also, FACS buffer (25 μL/well) was added to cells not exposed to antibodies, which served as negative controls for HS staining. After incubation with the primary 10E4 antibody or an isotype control antibody, cells were washed twice with FACS buffer, and a secondary goat F(ab’)2 anti-human IgM-PE (SouthernBiotech, Birmingham, AL) was added to detect surface bound antibodies. The secondary antibody was used at a concentration of 2.5 μg/mL in FACS buffer, added to cells (25 μL/well) and incubated for 30 min on ice. The secondary antibody was not added to control cells not exposed to primary antibodies, and instead only FACS buffer (25 μL/well) being added. After this incubation, cells were washed twice with FACS buffer and run on an LSR benchtop flow cytometer to detect PE binding to cells.

### 2.2.4 FACS assays: VACV infection

Purified MV-GFP (obtained from the WR-GFP strain) and EV-GFP (obtained from the IHD-J-GFP strain) were used for FACS based assays of VACV infection based on GFP fluorescence. 143B cells were seeded in 48-well plates and incubated overnight until the cells reached 100% confluency. Doubling dilutions of heparin and HS mimetics, starting from 100 μg/mL down to 0.09 μg/mL, were made in serum free MEM. Each dilution (350 μL) of porcine mucosa heparin (or HS mimetics) was incubated with virus MOI of 1 for 1 hr at 4°C prior to addition (100 μL/well) to 143B cell monolayers, from which overnight culture medium had been aspirated, and incubated for 1 hr at 37°C. Virus preparations without added heparin/HS mimetics served as positive controls, whereas wells receiving no virus
served as negative controls. When 143B cell monolayers were treated with HPNSE and HPSE, or when the VACV infection used CHO-K1 cells and xylosyltransferase I deficient pgsA-745 cells, both purified MV-GFP and purified EV-GFP were used for infection without any pre-incubation with heparin (or HS mimetics).

Following the 1 hr incubation at 37°C, virus mixtures were aspirated, the monolayers washed with sterile PBS, fresh MEM (supplemented with 5% FBS) added (500 μL/well) and the cells incubated for another 5 hr at 37°C. All culture medium was then aspirated, 0.25% trypsin-EDTA added (100 μL/well) and wells incubated for 5 min at 37°C to detach cells from the surface of wells. Once detached, cells were fixed in 0.5% paraformaldehyde (100 μL/well), transferred to cluster tubes (Sigma-Aldrich), and run on a BD FACS Calibur, with 30,000 gated events being collected. Data collection and analysis were performed using FLOWJO software and GraphPad Prism was used to plot histograms from analyzed data.

2.2.5 VACV membrane protein extraction
To detect proteins on the surface of VACV particles that interact with HS on cell surfaces, MV (from WR strain) and EV (from IHD-J strain) were purified and concentrated as described in section 2.1.4.6. A detergent cocktail containing 2% Brij®-58 (Sigma-Aldrich), 50 mM β-mercaptoethanol (Sigma-Aldrich) and 1% final concentration of complete protease inhibitor (4-(2-Aminoethyl) benzenesulfonyl Fluoride Hydrochloride) (Roche, Indianapolis, IN), was prepared in 50 mM Tris-HCl buffer at pH 7.5. Equal volumes of concentrated VACVs and detergent cocktails were mixed and incubated at room temperature for 30 min. To separate membrane proteins from VACV cores, the complete detergent-virus mixture was
loaded onto a caesium chloride (CsCl) (Sigma-Aldrich) gradient (in PBS) made of 1.30 g/mL (3 mL), 1.25 g/mL (4 mL) and 1.20 g/mL (5 mL) of CsCl, in clear ultracentrifuge tubes. Tubes were then centrifuged at 154,000xg for 30 min under vacuum and without any brakes in a Beckman-Coulter LE-80K ultracentrifuge. The cloudy layer of separated proteins, formed above the CsCl gradient, was carefully removed using a 29-gauge needle and syringe, by making a hole in ultracentrifuge tube wall from outside. The protein mixture was then added to a 10 kDa cutoff Amicon ultrafiltration device (Milipore, Billerica, MA) to filter out CsCl from the protein mixture by centrifuging at 3200xg in a Beckman-Coulter Allegra x-15r bench-top centrifuge for 10 min, with the concentrated protein mixture being resuspended in 500 μL of PBS.

2.2.6 Interaction of VACV proteins with heparin coupled beads

The membrane protein mixture extracted from MV and EV particles were incubated with heparin-Sepharose 6 fast flow beads (GE Healthcare) to separate heparin-binding proteins from other VACV membrane proteins. This was performed by placing an aliquot (500 μL) of heparin beads in a 1.5 mL tube, centrifuging briefly to settle the beads and washing the beads thrice with sterile PBS. Settled beads were then resuspended in membrane proteins mixture (100 μL) extracted from MVs and EVs, and incubated overnight on a small tube rotator at 30 rpm at 4°C. Tubes were then centrifuged to settle the Sepharose beads and supernatants containing unbound proteins removed. To release heparin-bead bound proteins, settled Sepharose beads were resuspended in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer (100 μL), and boiled at 100°C for 5 min, the process releasing proteins bound to the heparin-
beads. The Sepharose beads were then settled again by centrifugation and supernatant, containing heparin-binding proteins removed.

2.2.7 SDS-PAGE

To visualize heparin-binding VACV membrane proteins and other VACV membrane proteins, samples from 2.2.6 were first run on SDS-PAGE. Initially all samples were mixed with an equal volume of SDS-PAGE sample buffer and boiled at 100°C for 5 min. Heparin-binding VACV membrane proteins, non-heparin binding VACV membrane proteins and the complete mixture of VACV membrane proteins, were then loaded onto 4-20% Tris-HEPES mini-gels (NuSep, Bogart, GA) and run at 80 V for up to 1 hr. Proteins were then transferred to a membrane by Western blotting (see below) for specific detection using anti-VACV antibodies.

2.2.8 Western blotting

Western blotting was performed to visualize the proteins present in all the VACV membrane protein preparations. Proteins were transferred from SDS-PAGE gels to Hybond-C-Super nitrocellulose membranes (GE Healthcare) by electroblotting in transfer buffer (25 mM Tris, 192 mM glycine in 10% methanol), at 100 V for 1 hr. Membranes were then blocked with 5% BSA in Tris-buffered saline (TBS, 25 mM Tris, 137 mM NaCl, 2.7 mM KCl, pH 7.4) at 4°C overnight on a platform rocker. The following day blocking buffer was removed and the membranes were washed extensively twice for 10 min with TBS/0.1% tween-20 (Sigma-Aldrich). Membranes were then incubated with either a biotin-conjugated polyclonal rabbit-anti-VACV antibody (Mybiosources), or a mouse-anti-VACV A56 IgG1 mAb (Immune
Technology Corp., New York, NY), diluted in TBS/0.1% Tween-20, at 4°C overnight on a platform rocker.

The membranes were then washed extensively thrice for 20 min with TBS/0.1% Tween-20. Membranes incubated with biotin-conjugated polyclonal primary antibodies were incubated at 4°C for 1 hr on a platform rocker with streptavidin-conjugated HRP as the secondary detection reagent, diluted in TBS/0.1% tween-20. Conversely, membranes incubated at 4°C for 1 hr on a platform rocker with the mouse anti-VACV A56 IgG1 mAb were incubated with a HRP-conjugated rabbit anti-mouse Ig antibody (Dako, Carpinteria, CA), diluted in TBS/0.1% Tween-20. The membranes were then washed extensively thrice for 10 min with TBS/0.1% Tween-20. Finally membranes were developed using ECL Western blotting reagents (GE Healthcare) for 2 min, membranes being imaged using an ImageQuant LAS 4000 imager with 2 min exposure time.

### 2.2.9 1D NanoLC ESI MS/MS analysis for protein identification

The protein bands on the SDS-PAGE gel were excised and sent to Australian Proteome Analysis Facility (APAF) at Macquarie University, NSW, for identification. A team of scientists led by Dr. Xiaomin Song performed 1D nano liquid chromatography electrospray ionization tandem mass spectrometry (1D nanoLC ESI MS/MS), on the protein samples. Briefly, the samples were digested using trypsin-EDTA and loaded onto a Capillary LC system that is coupled to an MS/MS instrument. A reversed phase C18 column was used to separate peptides, which were directly eluted into the MS. The ions entering MS were continuously sampled and upon detection of a peptide, there was an automated switching from
MS to MS/MS mode and the peptides were fragmented. Upon the completion of the run, computer software (e.g., Mascot) interrogated proteins, DNA or EST databases to identify the protein(s). Blast searching and de-novo sequencing was performed on the peptides that could not be identified from the initial database search.

2.3 In vivo techniques

2.3.1 VACV inoculation

Prior to inoculation, all VACV stocks were sonicated (2 x 15 sec, 100 W, Branson Sonifier B12) and diluted in sterile PBS.

For intranasal (i.n.) inoculation, mice were lightly anaesthetized using methoxyfluorane. Once under anesthesia, 10 μL of virus was delivered to each nostril (20 μL total) using a pipette.

For intramuscular (i.m.) inoculation, mice were fully anaesthetized using methoxyfluorane and 50 μL of virus was injected in each quadriceps (100 μL total) using a 29-gauge needle and syringe.

2.4.2 In vivo virus spread

To determine the spread of VACV at different time points post infection, animals were euthanized ethically, and organs including ovaries, spleen, quadriceps muscles and lungs were removed. All the organs were stored at -20°C. Prior to organ virus titrations, organs were thawed on ice in 200 μL of MEM (supplemented with 5% FBS). All the organs were then homogenized (using a PRO200 Micro-homogeniser, PRO Scientific Inc., Oxford, CT), and the total volume was made to 1
mL with MEM (supplemented with 5% FBS). The homogenate was then sonicated 
(2 x 30 sec) and serial dilutions (1:10) were made, which were then plated on 80% 
confluent 143B cells as described in section 2.1.6.
Affinity of VACV for different glycosaminoglycans

Chapter 3:
3.1 Abstract

Vaccinia virus (VACV) has previously been shown to interact with the heparan sulfate (HS) component of heparan sulfate proteoglycans (HSPGs), using known receptors on the MV form of VACV. In this Chapter, the specificity of the MV form of VACV for heparin, differentially sulfated HS, chondroitin sulfates (CS) A-D and hyaluronic acid (HA) was evaluated using an enzyme-linked immunosorbent assay (ELISA). Overall, the MV form of VACV exhibited a high binding specificity for heparin and the highly sulfated version of HS (HS\text{hi}), but only weakly reacted with lowly sulfated HS. The MV form of VACV also bound to CS A-D, however, with much lower affinity. Unfortunately, an ELISA could not be used to investigate the GAG binding specificity of the EV form of VACV due to a lack of appropriate EV specific antibodies. Nevertheless, plaque forming assays with the MV rich WR and EV rich IHD-J strains of VACV revealed that heparin had a strain-specific effect on plaque formation. Upon co-incubation with heparin, the IHD-J strain of VACV lost the trademark 'comet' plaques. Furthermore, heparin co-incubation resulted in an incremental increase in WR and IHD-J plaque diameters and a reduction in plaque counts by 5- and 10-folds respectively. Collectively, these data suggest that HS recognition plays a significant role in the infectivity of the MV and EV forms of VACV, with this recognition process being more important for the EV form.
3.2 Introduction and rationale

Up until the early 1990s the interaction of viruses with cell surfaces was thought to be a simple single step process, virus particles attaching to a single cell surface molecule by a single virus attachment protein (Haywood, 1994). With the development of sophisticated new biochemical and genetic techniques it became evident, however, that virus entry into cells involves a multi-step process. In fact, it is now well established that multiple interactions between virus attachment proteins and cellular co-receptors is required for viruses to successfully enter a cell (Schneider-Schaulies, 2000).

HSPGs are ubiquitously expressed on cell surfaces, in the extracellular matrix (ECM) and in basement membranes (BM), which is why it is not surprising that many viruses, including herpes simplex virus (Wudunn & Spear, 1989), hepatitis B virus (Cooper et al., 2005), human papillomavirus (Giroglou et al., 2001), dengue virus (Chen et al., 1997) and hepatitis C virus (Barth et al., 2003) use the HS component of HSPG as an initial docking site for infection (Zhu et al., 2011). Despite the knowledge that a large number of viruses use HS as a receptor or a co-receptor for infection, the role of virus-HS interactions in viral pathogenicity remains rather poorly understood. Therefore, a detailed understanding of how HS plays a role in virus attachment could give vital cues on the molecular mechanisms of virus tropism and, therefore, aid in the development of novel therapeutics that target HS.

VACV has at least three known glycosaminoglycan (GAG) binding proteins on the surface of its MV form. VACV proteins A27L and H3L bind HS on target cells (Chung
et al., 1998; Lin et al., 2000), whereas D8L has been shown to interact with cell surface chondroitin sulfate (CS) (Hsiao et al., 1999). These studies have also shown that soluble heparin could inhibit VACV binding to cells and can reduce VACV plaque formation.

In contrast to the MV form of VACV, little is known about the role of cell surface HS in the infectivity of the EV form of VACV. This is because the outer envelope of the EV form of VACV is extremely fragile, making it incredibly difficult to purify intact EV particles to study. Most purification systems result in damaging the outer EV envelope, resulting in contamination with the enclosed MV. However, there are at least two proteins on the outer envelope of the EV form of VACV that have been shown to interact with HS, namely the 24-28 kDa A34R and the 42 kDa B5R proteins (Law et al., 2006). Since EV is the form of virus required for long-range dissemination (Blasco, Sisler, & Moss, 1993b; Roberts & Smith, 2008), it would be important to understand the interaction between the EV form of VACV and cell surface HS during the course of an infection. A protocol is used in this Chapter that was developed to obtain EV rich preparations from the IHD-J strain of VACV, a strain of virus that produces large amounts of the EV form. Hence, the specific aims of this Chapter were to establish, based on ELISAs, the binding specificity of the MV form of VACV for heparin, HS and CS, and evaluate the specific effects of heparin on the ability of the MV rich WR strain and the EV rich IHD-J strain of VACV to form plaques.
3.3 Results

3.3.1 Analysis of the interaction of the MV form of VACV with HS

VACV A27L is a MV membrane protein that interacts with cell surface HS and has been shown to be crucial for fusion of virus-infected cells (Chung et al., 1998; Gong, Lai, & Esteban, 1990; Hsiao et al., 1998; Rodriguez, Rodriguez, & Esteban, 1993). A27L has also been shown to associate and form a stable complex with A17L, which is crucial for wrapping of MV in Golgi derived membranes (Rodriguez & Smith, 1990; Rodriguez, Risco, Carrascosa, Esteban, & Rodriguez, 1997). Deletion of A27L results in a small plaque phenotype (Dallo, Rodriguez, & Esteban, 1987; Paez, Dallo, & Esteban, 1987). The H3L gene product, p35, is the other known HS binding protein present on the surface of MV. Deletion of H3L was shown to attenuate VACV virulence in vivo and in vitro compared to the wild type (WT) VACV (Lin et al., 2000). These findings were observed using plaque assays as the primary method of detection. The research described in this thesis focuses on studying the early interaction between cell surface HS and the two forms of VACV, with the ultimate aim of finding HS mimetics that could potentially be used as inhibitors of VACV infection. Thus, initial studies presented in this Chapter used ELISAs to validate the HS binding specificity of MV particles. Unfortunately, the HS binding specificity of the EV form of VACV could not be investigated in this study as no EV-specific antibodies were available that were suitable for ELISAs.

3.3.1.1 Purified MV preferentially interacts with highly sulfated HS (HS\textsuperscript{hi}) and heparin

The WR strain of VACV was used to infect baby hamster kidney-21 (BHK-21) cell monolayers to obtain MV particles, which were then purified on a sucrose cushion
as described in section 2.1.4.6 of this thesis. ELISA plates were coated with 10 μg/mL of either bovine serum albumin (BSA) conjugated highly sulfated HS (HS\textsuperscript{hi}) or BSA conjugated lowly sulfated HS (HS\textsuperscript{low}) overnight. The purified MV particles were then incubated in HS\textsuperscript{hi} or HS\textsuperscript{low} coated ELISA wells in the presence or absence of soluble heparin at varying concentrations. Bound MV particles were then detected using a sandwich ELISAs as described in section 2.2.1 of this thesis. The results obtained demonstrated that MV particles bound to immobilised HS\textsuperscript{hi} more efficiently than to immobilised HS\textsuperscript{low} (Figure 3.1A & B). Furthermore, as little as 10 μg/mL of soluble heparin was able to inhibit MV particles from binding to immobilised HS in the assay wells (p < 0.001). In fact, MV binding to HS\textsuperscript{hi} was reduced to below background levels when incubated in the presence of soluble heparin (Figure 3.1A). Since heparin, like HS\textsuperscript{hi}, is a highly sulfated form of HS, these results indicate that MV particles preferentially interact with highly sulfated versions of HS.

Additional ELISAs were performed using immobilised heparin (BSA-heparin) to confirm the previously obtained results, using HS\textsuperscript{hi} or HS\textsuperscript{low} as soluble competitive inhibitors of MV binding to immobilised heparin. In these assays, ELISA wells were coated with BSA-heparin overnight prior to adding purified MV particles in the presence or absence of either HS\textsuperscript{hi} or HS\textsuperscript{low}. As expected, MV particles bound to immobilised heparin at similar levels compared to immobilised HS\textsuperscript{hi} (compare Figures 3.1A and 3.2). However, as much as 50 μg/mL of soluble HS\textsuperscript{hi} was required to significantly inhibit MV particles from attaching to immobilised heparin (P < 0.001) (Figure 3.2A). Conversely, soluble HS\textsuperscript{low} could not inhibit MV attachment to immobilised heparin even at a concentration as high as 100 μg/mL (Figure 3.2B).
Figure 3.1: Interaction of the MV form of VACV with immobilised HS\textsuperscript{hi} and HS\textsuperscript{low} in the absence or presence of soluble heparin. HS\textsuperscript{hi} and HS\textsuperscript{low} were immobilised in assay wells as BSA conjugates and MV (10\textsuperscript{6} PFU) binding to the wells determined by ELISA using polyclonal rabbit anti-VACV antibodies. The ability of soluble heparin (10-100 μg/mL) to inhibit MV binding to immobilised HS\textsuperscript{hi} (A) and HS\textsuperscript{low} (B) is presented. Dashed lines indicate background binding of MV to BSA coated wells. Control bars represent MV binding to immobilised HS\textsuperscript{hi} and HS\textsuperscript{low} in the absence of soluble heparin. Data presented as mean ± SEM (n=8) and representative of three independent experiments. Statistical analysis was performed using one-way ANOVA with Dunnett’s multiple comparisons. *** - p < 0.001.
Nevertheless, results obtained from these assays were consistent with previous findings presented in Figure 3.1 suggesting that purified MV particles interact with highly sulfated versions of HS (heparin and HS\textsuperscript{hi}) more efficiently compared to lowly sulfated versions (HS\textsuperscript{low}).

Since it is HS that is present in the ECM and BMs, and not heparin, the subsequent assays were designed to understand the interaction between MV particles and soluble forms of both HS\textsuperscript{hi} and HS\textsuperscript{low}. Purified MV particles were added to ELISA wells pre-coated with BSA-HS\textsuperscript{hi} at a concentration of 10 μg/mL, in the presence or absence of soluble HS\textsuperscript{hi} or HS\textsuperscript{low}. Since MV particles have a weaker affinity for HS compared to heparin, the highest concentration of the two soluble HS molecules used as competitive inhibitors was increased to 800 μg/mL. Results obtained from this assay indicate that soluble HS\textsuperscript{hi} was able to significantly inhibit virus attachment to immobilised HS\textsuperscript{hi} at concentrations of 200 μg/mL and above (p < 0.001) (Figure 3.3A). HS\textsuperscript{low}, on the other hand, was a much weaker inhibitor of MV attachment compared to HS\textsuperscript{hi}, as a statistically significant inhibition (p < 0.05) of MV binding to ELISA wells was only observed at a concentration of 800 μg/mL (Figure 3.3B). Results obtained from these ELISAs were consistent with the results observed in Figures 3.1 and 3.2, collectively suggesting that MV particles have a high affinity for highly sulfated versions of the HS molecule.

3.3.2 Interaction of MV form of VACV with chondroitin sulfates (CS)

The MV form of VACV has a protein, D8L, on its outer membrane that has previously been shown to bind to cell surface CS (Hsiao et al., 1999). Studies using
Figure 3.2: Interaction of the MV form of VACV with immobilised heparin in the absence or presence of soluble HS$^{hi}$ and HS$^{low}$. Soluble heparin was immobilised in assay wells as a BSA conjugate and MV binding ($10^6$ PFU) to wells determined by ELISA using polyclonal rabbit anti-VACV antibodies. The ability of soluble HS$^{hi}$ (A) and HS$^{low}$ (B) (10 μg/mL) to inhibit MV binding to immobilised heparin is presented. Dashed lines indicate background binding of MV to BSA coated wells. Control bars represent MV binding to immobilised heparin in the absence of soluble HS$^{hi}$ or HS$^{low}$. Data presented as mean ± SEM (n=8) and representative of three independent experiments. Statistical analysis was performed using one-way ANOVA with Dunnett’s multiple comparisons. *** - p < 0.001; ns – not significant – p > 0.05.
Figure 3.3: Interaction of the MV form of VACV with immobilised HS$^\text{hi}$ in the absence or presence of soluble HS$^\text{hi}$ and HS$^\text{low}$. HS$^\text{hi}$ was immobilised in assay wells as a BSA conjugate and MV binding ($10^6$ PFU) to wells determined by ELISA using polyclonal rabbit anti-VACV antibodies. The ability of soluble HS$^\text{hi}$ (A) and HS$^\text{low}$ (B) (10 µg/mL) to inhibit MV binding to immobilised HS$^\text{hi}$ is presented. Dashed lines indicate background binding of MV to BSA coated wells. Control bars represent MV binding to immobilised HS$^\text{hi}$ in the absence of soluble HS$^\text{hi}$ or HS$^\text{low}$. Data presented as mean ± SEM (n=8) and representative of three independent experiments. Statistical analysis was performed using one-way ANOVA with Dunnett’s multiple comparisons. * - p < 0.05; *** - p < 0.001; ns – not significant – p > 0.05.
mutant viruses lacking either A27L or D8L, or double mutants lacking both proteins, indicate the importance of D8L in VACV infectivity (Hsiao et al., 1999; Lai, Gong, & Esteban, 1991; Maa, Rodriguez, & Esteban, 1990). These studies have also shown that soluble CS, but not heparin, could compete with the soluble VACV D8L protein for binding to cells, suggesting that CS on the surface of cells could acts as a receptor for VACV D8L. Also, lack of functional D8L was shown to results in low virus titres and reduced infectivity (Hsiao et al., 1999). However, these studies also showed that soluble CS could not inhibit MV infectivity (Chung et al., 1998). A more detailed study showed that soluble CS-A, CS-B and CS-C had no effect on MV infectivity (Carter, Law, Hollinshead, & Smith, 2005). Since most of the virus infectivity studies in vitro focus on viral plaque assays, the final outcome of an infection, the early interaction of virus proteins with cell surface receptors is often overlooked. Therefore, it was important to determine if soluble CS could act as a competitive inhibitor to virus binding to immobilised HS\textsuperscript{hi}. Since the current experimental approach involves using an ELISA, this study would provide a highly sensitive indication of whether CS can inhibit the binding of MV to HS and, therefore, provide an estimate of MV affinity for CS compared to HS.

There are four types of CS chains, namely, CS-A [chondroitin 4-sulfate], CS-B [also known as DS (Dermatan sulfate) (Trowbridge & Gallo, 2002)], CS-C [chondroitin 6-sulfate] and CS-D [chondroitin 2,6-sulfate (Silbert & Sugumaran, 2002)]. Along with all four CS, fucoidan and hyaluronic acid (HA) were also used in this study as positive and negative controls respectively. Fucoidan is a highly sulfated, heparin like, polysaccharide that is derived from brown seaweeds (Hsu et al., 2013). Previously, fucoidan has been shown to be a potent inhibitor of VACV infectivity.
Figure 3.4: Interaction of the MV form of VACV with immobilised HS$^{hi}$ in the absence or presence of soluble CS, fucoidan and hyaluronic acid. HS$^{hi}$ was immobilised in assay wells as a BSA conjugate and MV binding ($10^6$ PFU) to wells determined by ELISA using polyclonal rabbit anti-VACV antibodies. The ability of soluble CS-A (A), CS-B (B), CS-C (C), CS-D (D), fucoidan (E) and hyaluronic acid (F) to inhibit MV binding to immobilised HS$^{hi}$ is presented. Dashed lines indicate background binding of MV to BSA coated wells. MV control bars represent background binding of MV to immobilised HS$^{hi}$ in the absence of any inhibitory compound. Heparin control (Hep control) bars represent binding of MV to immobilised HS$^{hi}$ in the presence of 10 μg/mL of soluble heparin. Data presented as mean ± SEM (n=8) and representative of three independent experiments. Statistical analysis was performed using one-way ANOVA with Dunnett’s multiple comparisons. * - p < 0.05; ** - p < 0.01; *** - p < 0.001; ns – not significant – p > 0.05.
HA is a non-sulfated GAG that is upregulated during tissue injury and wound healing, and regulates several aspects of tissue repair including activation of inflammatory cells to enhance immune responses (Papakonstantinou, Roth, & Karakiulakis, 2012). To determine the interaction of MV particles with different CS molecules, BSA-HS<sub>hi</sub> (10 μg/mL) was immobilised in ELISA wells overnight. Purified MV particles were added to the wells with or without a soluble competitive inhibitor. Results obtained suggest that high concentrations (100 μg/mL) of all four CS could partially inhibit MV from interacting with immobilised HS<sub>hi</sub>, although at slightly different levels (Figure 3.4). Importantly, high concentrations of CS-A (Figure 3.4A), CS-B (Figure 3.4B) and CS-C (Figure 3.4C) showed statistically significant (p < 0.001) inhibition of MV attachment to immobilised HS<sub>hi</sub>. Out of the four CS molecules examined, CS-D appeared to be the weakest inhibitor of MV attachment to immobilised HS<sub>hi</sub> (Figure 3.4D). Interestingly, the CS inhibitory activity, with the exception of CS-A, tended to not increase with increasing concentrations of the respective CS, suggesting that only a subset of HS<sub>hi</sub> binding molecules could be blocked by CS. HA did not inhibit MV particles from attaching to immobilised HS<sub>hi</sub> (Figure 3.4F), whereas fucoidan (Figure 3.4E) was just as effective as heparin. Results obtained from these assays further confirm that the MV form of VACV tends to interact with GAGs that have a high sulfate content.

3.3.3 Soluble heparin interferes with VACV plaque formation

Viruses lacking certain proteins associated with their dissemination have a reduced plaque forming ability. For example, VACV strains lacking the GAG binding proteins A27L, H3L or D8L have been shown to have a reduced plaque count, as
well as a reduced plaque size (Chung et al., 1998; Hsiao et al., 1999; Lin et al., 2000; Roberts et al., 2009). Since it has been reported previously that MV surface proteins A27L and H3L interact with cell surface HS, the effect of soluble heparin on plaque formation by the MV form of VACV was examined next. In addition, the effect of soluble heparin on EV plaque formation was investigated, particularly as some studies have suggested that heparin affects EV entry but not MV entry into cells (Bengali, Satheshkumar, & Moss, 2012).

It is known that the WR and IHD-J strains of VACV produce similar numbers of cell associated virus (CEV) (Figure 1.4), however, a significantly higher proportion of the EV form is shed from cells infected with the IHD-J strain of VACV (Blasco & Moss, 1992), thus making the IHD-J strain the more infectious of the two strains. As more EV particles are released by the IHD-J strain of VACV, the infection of distal cells is more efficient, resulting in ‘comet’ shaped plaques. In contrast, the majority of WR plaques are round, with no or minimal plaques being comet-like in appearance. Since plaque morphology of a particular strain of VACV is dependent on the number of EV particles released from an infected cell, it was of interest to determine the effects of soluble heparin in the culture medium on the release of EV (and MV) from infected cells and, therefore, the effects on the plaque morphology of the two VACV strains.

As described in section 2.1.4.6 of this thesis, the IHD-J strain of virus was used to infect BHK-21 cell monolayers in serum free conditions to obtain an EV rich virus preparation. During this process, the EV particles were not subjected to high-speed centrifugation in order to prevent any damage to the integrity of the outer EV
envelope. The MV particles used in plaque assays were obtained from the WR strain of VACV.

Confluent monolayers of 143B osteosarcoma cells were infected with either VACV pre-incubated with heparin (virus + heparin) or without any pre-incubation (virus alone). The cell monolayers were then washed to remove any traces of free VACV and covered with fresh culture medium, with or without soluble heparin, such that the monolayers that were infected with the virus + heparin mixture were overlaid by culture medium containing heparin.

Infected monolayers were incubated for 48 hr at 37°C prior to staining. Overall, data obtained from the plaque assay analysis indicated that heparin can induce a significant change in the plaque morphology of the IHD-J strain, but not the WR strain (Figure 3.5A). The ‘comet’ shaped plaques, which are a hallmark of the IHD-J strain (EV rich), disappeared when soluble heparin was added to the culture medium. In fact, in the presence of heparin there was > 90% reduction (Figure 3.5B) in the number of IHD-J strain plaques forming comets ($p < 0.0001, n = 275$ plaques/treatment). Since comets result from the release of the EV form of VACV into the culture medium, this finding suggests that EV particles interact with heparin more efficiently than MV particles. Furthermore, a detailed analysis of the plaque morphology revealed that the addition of heparin significantly increased the diameter of both WR-VACV and IHD-J-VACV plaques ($p < 0.0001, n = 30$ plaques/treatment) (Figure 3.5C). This is a rather interesting finding since any interference with virus infectivity usually causes a reduction in plaque size. Another important finding from this study was that there was a ten-fold reduction
(Figure 3.5D) in IHD-J-VACV plaque formation upon addition of heparin (p = 0.0024), whereas only a five-fold reduction in WR-VACV plaque formation was observed in the presence of heparin (p = 0.0218). Photos of the two VACV strains used in this study were taken using an electron microscope (EM). Cell monolayers infected with the IHD-J strain of VACV (Figure 3.5E) produced a high proportion of EV particles, whereas cells infected with the WR strain (Figure 3.5F) produced mostly MV particles.
Figure 3.5: Effects of heparin on plaque formation by the IHD-J (EV) and WR (MV) strains of VACV. Both EV (obtained from IHD-J-VACV) and MV (obtained from WR-VACV) were pre-incubated with heparin prior to infection. Both strains of VACV were then added to a confluent monolayer of 143B osteosarcoma cells in the presence (virus + heparin) or absence (virus alone) of heparin (10 μg/mL). (A) Representative plaque assay plates depicting the effects of heparin (10 μg/mL) on plaque formation by the IHD-J and WR strains. The trademark 'comet' shaped EV plaques disappeared in the presence of heparin. (B-D) Quantification of effects of heparin (10 μg/mL) on (B) percentage of IHD-J strain plaques developing comets, (C) IHD-J and WR plaque diameters and (D) plaque numbers. (E-F) EM photos of the two strains of viruses used in this study (50,000X magnification) with the IHD-J strain (E) having many more virus particles with an outer envelope (red arrows) than the WR strain (F). In (B) data presented as mean ± SEM (n=6) of percentage of 275 plaques forming comets in heparin treated and untreated cell monolayers infected with the IHD-J strain of VACV, the data presented being from one representative assay. Plaque diameters in (C) obtained for 30 plaques for each treatment and plaque numbers (D) from 6 replicates. Data presented as mean ± SEM with a Mann-Whitney t-test being used for the statistical analysis. Assays measuring number of comets formed by IHD-J (B), plaque diameters (C) and plaque numbers (D) were repeated thrice with plots shown in this figure being of the data obtained from one representative experiment.
3.4 Discussion

This study aimed to investigate the dependence of VACV infections on recognition of cell surface HS and, ultimately, whether HS recognition could be targeted to develop antivirals. Since the interaction of the MV form of VACV with cell surface HS is one of the first events that takes place in VACV infection of cells, it was important to develop assays that enabled one to study this early interaction. Therefore, an ELISA was initially used to evaluate the interaction of the MV form of VACV with differentially sulfated HS, CS and heparin, the latter being a heterogeneous polysaccharide, which is distinguished from HS by its higher extent of sulfation.

Immobilizing either HS\textsuperscript{hi} or HS\textsuperscript{low} in assay wells as BSA conjugates and inhibiting MV attachment to the immobilised HS using heparin showed that the MV form of VACV preferentially interacts with heparin (Figure 3.1A and 3.1B). In fact, a heparin concentration as low as 10 μg/mL was able to significantly inhibit MV attachment to both immobilised HS\textsuperscript{hi} and HS\textsuperscript{low}. The strong affinity of MV particles for highly sulfated polysaccharides was also evident when comparing the attachment of MV to immobilised HS\textsuperscript{hi} and HS\textsuperscript{low} in the absence of heparin (control bars in Figures 3.1A and 3.1B), with the MV form of VACV binding much more strongly to HS\textsuperscript{hi} compared to HS\textsuperscript{low}. These results were consistent with the previous findings suggesting that the MV form of VACV has strong affinity for highly sulfated HS molecules (Chung et al., 1998).

Furthermore, similar results were obtained when the assay setup was reversed, i.e., heparin was immobilised in the wells (Figure 3.2A and 3.2B). It was clear that
MV bound to highly sulfated polysaccharides with much higher affinity than to lesser-sulfated polysaccharides. Thus, a very high concentration of HS\textsuperscript{hi} (100 μg/mL) was required to significantly inhibit MV binding to immobilised heparin (Figure 3.2A), whereas HS\textsuperscript{low} was unable to inhibit MV attachment to immobilised heparin at the concentration range tested (Figure 3.2B). A similar conclusion was drawn when HS\textsuperscript{hi} and HS\textsuperscript{low} were examined for their ability to inhibit binding of MV to immobilised HS\textsuperscript{hi} (Figure 3.3). Unfortunately, such comparative ELISAs could not be performed with the EV form of VACV due to the unavailability of appropriate EV specific antibodies.

Previous studies have shown that the D8L protein from the MV form of VACV binds CS, with mutants MV particles lacking D8L having a reduced infectivity (Hsiao et al., 1999). Consistent with this observation it was found that at high concentrations, all four CS types could partially inhibit MV attachment to immobilised HS\textsuperscript{hi}. However, this inhibition of HS\textsuperscript{hi} binding could be purely due to steric hindrance. Thus, soluble CS bound to VACV D8L on the surface of MVs could be indirectly interfering with other proteins on the surface of MV from interacting with immobilised HS\textsuperscript{hi}. Since MV interacts with HS/heparin and CS via different surface proteins, a convincing conclusion could not be drawn from these assays. Unfortunately, none of the four CS types were available as BSA conjugates, therefore, inhibition assays involving immobilised CS that would resolve this issue could not be performed.

Data presented in this Chapter shows by ELISAs that MV particles can bind with high avidity to immobilised heparin and HS\textsuperscript{hi}, with soluble heparin having the
ability to inhibit this interaction. Consistent with this observation, subsequent studies revealed that soluble heparin significantly inhibited MV rich WR plaque numbers with an incremental increase in plaque size. However, the effects observed with soluble heparin were more dramatic for the EV rich IHD-J strain of VACV. The trademark ‘comets’ formed by the IHD-J strain plaques disappeared in the presence of heparin along with a 10-fold reduction in plaque numbers and again there being an incremental increase in plaque diameters. A possible explanation for this phenomenon is that heparin is able to inhibit freshly released EV particles from infected cells and, therefore, rendering them unable to infect distant cells. The more dramatic effect of heparin on EV rich IHD-J than MV rich WR strain plaque formation is consistent with previous studies that have shown that soluble heparin affects EV cellular entry more than MV entry, in vitro (Bengali et al., 2012).

The incremental increase in plaque size in the presence of heparin could be attributed to the mechanisms VACV particles use to infect cells. VACV particles use two major mechanisms to spread from cell-to-cell. Progeny MV particles from an infected cell can directly be pushed into neighboring cells, thereby minimizing the events associated with forming EV particles (Marsh & Helenius, 2006). MVs can also be transported to the plasma membrane where they can be released from the cell as EVs, which then infect neighboring cells (Doceul, Hollinshead, van der Linden, & Smith, 2010; Marsh & Helenius, 2006). It is likely that the inability of freshly released EV particles to infect distal cells in the presence of heparin promotes the punching mechanism as the predominant process used to infect neighboring cells. This could explain why addition of heparin results in plaques
with increased diameters. Furthermore, there is also evidence that suggests that the VACV serine proteinase inhibitor (K2) contains the heparin-binding site of antithrombin III (Brum, Turner, Devick, Baquero, & Moyer, 2003; Turner & Moyer, 2006). The K2 protein forms a complex with VACV A56, also known as VACV haemagglutinin (HA), with the complex being expressed on the surface of infected cells (DeHaven, Gupta, & Isaacs, 2011; Moss, 2006). The A56/K2 complex has been shown to inhibit syncytia formation (Moss, 2006). Furthermore, studies have also shown that antibodies blocking K2 or A56 promote syncytia formation (DeHaven et al., 2011). Therefore, it is also likely that heparin, like antibodies against K2, may have similar effect on K2 function and, therefore, promote syncytia formation. This possibility would again explain why an incremental increase in plaque size was observed when cells were infected with WR or IHD-J strains of VACV in the presence of heparin.

In conclusion, it is likely that at an individual plaque level, heparin may assist the rate at which a primary plaque grows in size. However, an overall reduction in plaque numbers was also observed for both strains of VACV when used to infect cells in the presence of heparin, suggesting that heparin interferes with both EV and MV uptake by cells. It is likely that heparin blocks HS-binding receptors on the surface of the MV particles. Since the addition of heparin does not completely inhibit the infectivity of MV particles, this would indicate the presence of alternative mechanisms of MV entry into cells. In contrast, comet formation resulting from the release of the EV form of VACV appears to be completely dependent on HS recognition. Collectively, these data suggest that both EV and MV
forms of VACV recognise and interact with HS/heparin, with this recognition process being more important for the EV form.
Use of GFP expressing VACV to probe role of heparin/HS recognition in VACV infections

Chapter 4:
4.1 Abstract

There are two infectious forms of VACV, namely the MV and the EV form, both being structurally, chemically and antigenically different. To better understand the role of heparin and cell surface HS in both forms VACV infection, GFP expressing strains of VACV were constructed by inserting a GFP-\textit{Bsd} gene sequence in the genomes of both WR (MV rich) and IHD-J (EV rich) strains of VACV, the GFP component of the bi-functional sequence functioning as a fluorescent marker for infectivity. Overall, the results from VACV inhibition assays with the MV-GFP and EV-GFP forms indicate that heparin and HS\textsuperscript{hi} inhibit the infectivity of both the MV and EV forms of VACV, with the EV form being much more easily inhibited.

Infection assays in which cells were pre-treated with enzymes to degrade cell surface HS and in cells genetically deficient in HS production, suggested a role for cell surface HS as a co-receptor for infection, especially for the EV form of VACV. Therefore, based on these studies, soluble GAGs have potential to act as inhibitors of EV infections.
4.2 Introduction and rationale

VACV, like many other viruses, has been shown to interact with cell surface GAGs. In fact, several studies have established that sulfated GAGs play an important role in VACV infections (Carter et al., 2005). The MV form of VACV has two known HS binding proteins on its outer membrane (Chung et al., 1998; Lin et al., 2000), whereas the EV form of VACV has two proteins on its outer envelope, which upon contact with cell surface HS, initiate dissolution of the outer EV envelope (Law et al., 2006). Indeed, ELISA assays reported in Chapter 3 showed that purified MV particles bind to highly sulfated immobilised heparin and HS. Also, these strong interactions translated into soluble heparin inhibiting MV infections in vitro, WR (MV rich) plaque numbers being reduced 5-fold and the diameter of plaques being increased in the presence of heparin. However, heparin had an even greater effect on IHD-J (EV rich) plaques reducing their number 10-fold, increasing their diameters and totally preventing formation of the characteristic ‘comet’ shaped plaques. ELISA based assays on the EV form of VACV, however, could not be performed due to the unavailability of EV specific antibodies.

The difference in the effects of soluble heparin on plaque formation by the two strains of VACV could result from the fact that the EV form of VACV differs from the MV form by the presence of at least one extra outer membrane (Ichihashi, 1996). Due to the extra membrane(s), EV and MV particles, however, not only differ structurally, but also chemically and antigenically (Condit et al., 2006). Therefore, a fundamental understanding of the mechanisms by which MV and EV forms of VACV infect cells was required to explain the plaque assay discrepancies. A deeper
understanding of VACV-heparin/HS interactions would also help determine whether such interactions can be exploited to develop novel GAG-based antivirals.

The EV form of VACV has to interact with cellular receptors on uninfected cells in order to enter and cause an infection. The MV form of VACV, however, can enter in neighboring cells by directly ‘punching’ through the plasma membranes (Mercer et al., 2010; Schmidt et al., 2012). Such ‘punching’ into neighboring cells bypasses the need for the infectious VACV particle to be released from the infected cell into the culture medium and re-enter an uninfected cell. Therefore, to study the effects of heparin/HS on VACV infections, it was essential to target the early stages of an infection. Hence the major aim of this Chapter was to construct green fluorescent protein (GFP) expressing WR and IHD-J strains of VACV to be used in a highly sensitive novel assay that would allow virus infections to be monitored by flow cytometry. The second aim of this chapter was to establish the role of cell surface HS in VACV infections using GFP expressing MV and EV forms of VACV to quantify infectivity.
4.3 Results

4.3.1 Construction of recombinant VACV WR and VACV IHD- strains expressing GFP.

ELISA studies described in Chapter 3 showed that the MV form of VACV binds to immobilized heparin and HS[hi]. Plaque assays on the other hand, have shown that soluble heparin more efficiently interferes with the plaquing, *in vitro*, of the EV rich IHD-J, than the MV rich WR strain of VACV. In light of these observations and along with prior knowledge that many viruses have heparin/HS binding proteins on their surface, studies were undertaken to understand the interaction between MV and EV forms of VACV with heparin and other HS mimetics and, therefore, establish the importance of cell surface HS in VACV infections and, whether heparin (or other sulfated HS mimetics) can act as possible antivirals. Prior to understanding the effects of heparin/HS mimetics on the infectivity of the two forms of VACV, it was important to develop assays that could be used to study early stages of VACV infection. Kinetics studies of VACV infections indicate that new VACV virions start forming 5 to 6 hr after initiation of an infection (Payne & Kristenson, 1979). Therefore, to evaluate the role of highly sulfated HS/heparin in VACV infectivity, assays were needed to be designed that would enable quantification of the first replication cycle. A flow cytometry based assay was, therefore, developed with GFP, under the control of an early-late VACV promoter, being used as a fluorescent marker to evaluate the infectivity of VACV *in vitro* in the presence of heparin/HS mimetics. In order to perform these assays, recombinant VACV WR and VACV IHD-J strains expressing GFP were first engineered as described in section 2.1.4. Briefly, for the construction of recombinant VACV, the GFP-*Bsd* gene was initially cloned from the pUC57 vector.
and inserted in the MVAF vector to create the pUC57-MVAF vector. Dr. Ron Jackson designed the GFP-\textit{Bsd} gene used in this thesis. This particular GFP molecule fluoresces brighter at 470nm, is more stable at 37°C, and has its own strong promoter, all of which enhance GFP expression. BHK-21 cells were infected with the parental strain VACV WR and, subsequently, transfected with the GFP-\textit{Bsd} containing pUC57-MVAF vector. Recombinant virus particles generated were selected using 4 rounds of blasticidin selection. To confirm that there was no contamination of parental VACV particles in the recombinant VACV preparations, DNA was extracted from infected cells and a PCR was performed using MVAF primers. GFP expressing recombinant VACV IHD-J was also constructed using the same protocol as for recombinant VACV WR.

\textbf{4.3.2 Kinetics of VACV replication as measured by GFP expression}

Previous studies on the kinetics of VACV replication have reported that new virions form 5 to 6 hr post infection (Payne & Kristenson, 1979). In order to develop inhibition assays using GFP expressing VACV and heparin/HS mimetics, optimal infection conditions were required to be established. A time-course assay was first undertaken to establish the time taken by the GFP expressing VACV strains to infect cells \textit{in vitro} and produce new progeny virions, as measured by flow cytometry of GFP fluorescence.

Confluent monolayers of 143B human osteosarcoma cells were infected with purified MV-GFP (obtained from the WR-GFP strain) and EV-GFP (obtained from the IHD-J-GFP strain) for 1 hr. Cells were then washed to remove unbound VACV, covered with fresh culture medium and incubated further. The infection was
stopped and the adherent cells released by trypsin treatment at different time points (2, 4, 6, 8 and 12 hr) post infection, the cells fixed using 0.5% paraformaldehyde and GFP expression measured by flow cytometry, with data being expressed as fluorescence intensity units (FIU) (Figure 4.1 and 4.2). Since an early-late promoter drives GFP expression in VACV infected cells, a shift in the population of cells expressing GFP can be seen as early as 2 hr post infection, with 20% of the cells being GFP positive in MV-GFP infected wells (Figure 4.1), and approximately 7% being GFP positive in EV-GFP infected wells (Figure 4.2). The proportion of GFP positive cells at 6 hr post MV-GFP infection was approximately 60% and 40% post EV-GFP infection, 6 hr being how long it takes for the first progeny virions to be generated. Over 90% of the cells were infected with the virus by 12 hr post infection regardless of the form of VACV used. Since a substantial proportion of cells became GFP positive within 6 hr post infection, it was then important to assess the effects of heparin on MV-GFP and EV-GFP infections, as detected by GFP expression, at this early time point.

To evaluate the effects of heparin on the kinetics of virus infectivity, a more detailed kinetic assay was performed. 143B cell monolayers were infected with purified forms of both MV-GFP (obtained from the WR-GFP strain) and EV-GFP (obtained from the IHD-J-GFP strain) in the absence (virus alone) or in the presence of heparin (1, 10 and 100 μg/mL). As before, the infection was stopped at different time points (2, 4, 6, 8, 12 and 24 hr) post infection, and the adherent cells released by trypsin treatment, fixed and analyzed for GFP expression by flow cytometry (Figure 4.3).
Figure 4.1: GFP expression in 143B cells infected with MV-GFP. 143B cells were infected with purified MV-GFP virus at MOI of 1. Adherent cells were then released by treatment with trypsin at different time points post infection (0, 2, 4, 6 and 12 hr), fixed and analyzed using flow cytometry for GFP expression in fluorescent intensity units (FIU). Representative contour plots show GFP expression within cells at different time points. Values associated with the GFP gate of each plot represent the % of GFP positive cells.
Figure 4.2: GFP expression in 143B cells infected with EV-GFP. 143B cells were infected with purified EV-GFP virus at MOI of 1. Adherent cells were then released by treatment with trypsin at different time points post infection (0, 2, 4, 6 and 12 hr), fixed and analyzed using flow cytometry for GFP expression in fluorescent intensity units (FIU). Representative contour plots show GFP expression within cells at different time points. Values associated with the GFP gate of each plot represent the % of GFP positive cells.
Overall heparin caused a significant inhibition of both EV-GFP and MV-GFP virus infections of adherent 143B cell monolayers. In fact, all the three concentrations of heparin tested (1, 10 and 100 μg/mL) had an apparent impact on VACV infectivity. A direct comparison between EV-GFP and MV-GFP infectivity of 143B cell monolayers in the presence of heparin, however, indicated that heparin is more effective at inhibiting EV-GFP infections than MV-GFP infections. This is clearly evident from the results obtained from the time-course inhibition assay where at the 24 hr time point as little as 1 μg/mL of heparin could significantly inhibit EV-GFP infections, whereas the highest concentration of heparin (100 μg/mL) failed to significantly inhibit MV-GFP infectivity.

At 6 hr post infection, the time point of most interest, over 60% of cells infected with MV-GFP and just under 50% of cells infected with EV-GFP, became positive for GFP expression. Heparin caused a statistically significant inhibition of both MV and EV infections, compared to the respective virus alone controls, even at its lowest concentration (1 μg/mL) at the 6 hr time point. Therefore, the 6 hr time point was used for all inhibition assays performed henceforth. A direct comparison between the MV-heparin and EV-heparin interactions indicate that on average, heparin more efficiently inhibits infection by the EV form of VACV, suggesting that EV has a higher avidity for heparin and/or cell surface HS plays a much greater role in EV infections than MV infections. These observations are consistent with the plaque assay findings (Figure 3.5A).
4.3.3 Enzymatic treatment of cells in vitro to remove cell surface HS and the effects of this treatment on virus infectivity

The data presented in earlier section 4.3.2 of this Chapter indicates that heparin can inhibit the infectivity of both the EV and MV forms of VACV. The simplest interpretation of these findings is that cell surface HS represents a co-receptor for both EV and MV. To directly test this conclusions, HS degrading enzymes were used to deplete cell surface HS from target cells. There are two types of HS degrading enzymes available, being mammalian and bacterial in origin. Since it has been established that EV particles interact more avidly with cell surface HS than MV particles, and that this interaction is crucial for EV infectivity, we hypothesized that the treatment of cell monolayers with HS degrading enzymes in vitro to remove cell surface HS, would impact on the infectivity of purified EV-GFP, and possibly of MV-GFP. Three different Flavobacterium heparinases (HPNSE - Sigma-Aldrich) are commercially available, all capable of degrading heparin and HS, but with different substrate specificities. All three HPNSEs were used to treat cell monolayers, either individually (HPNSE 1, HPNSE 2 or HPNSE 3), or as a cocktail of all three enzymes (HPNSE 1+2+3). Human platelet heparanase (HPSE - gift from Dr. Craig Freeman, John Curtin School of Medical Research, Canberra, Australia) was also used to remove cell surface HS from target cells in vitro. Adherent 143B monolayers were treated with the different HPNSEs and HPSE for 1 hr and the presence of cell surface HS determined by flow cytometry using a HS-specific mAb (10E4) (Figure 4.4). HPNSE 1 was the least effective of the three commercially available HPNSE enzymes, resulting in only a small (~30% depletion) but significant reduction in HS expression following HPNSE 1 treatment. The other
Figure 4.3: Effects of heparin on infectivity of MV-GFP and EV-GFP in vitro.
143B cells were infected with either purified MV-GFP or purified EV-GFP, both in the absence (virus alone) or presence of heparin (1, 10 and 100 μg/mL) at MOI of 1. Adherent cells were then released by treatment with trypsin at different time points post infection, fixed and analyzed using flow cytometry for GFP expression. Data presented as histograms shows mean percentage of GFP positive cells ± SEM (n=3) in the absence or presence of heparin (1, 10 and 100 μg/mL). For both viruses, statistically significant differences between GFP expression at different time points in cells infected with virus alone, and in cells infected in the presence of heparin, was determined using two-way ANOVA with Bonferroni multiple comparisons. The data presented are representative of at least two independent experiments. * - p < 0.05; ** - p < 0.01; **** - p < 0.0001; ns – not significant – p > 0.05.
two HPNSEs were more effective at removing HS (~60% depletion), although combining all three enzymes resulted in no improvement in HS removal (Figure 4.4 A and B). However, the most effective removal of HS was observed following HPSE treatment (~80% depletion). One-way ANOVA analysis of the results confirmed that all the HPNSE and HPSE tested in these experiments significantly reduced cell surface HS expression. HPSE, however, was the most effective at cell surface HS removal, of all the enzymes studied. It is worth noting, however, that the 10 E4 HS-binding mAb interacts with a HS epitope not easily removed by HPSE or HPNSE treatments, and probably binds to HS stubs remaining after enzymatic digestions (Prof. C. Parish, personal communication), thus, complete removal of cell surface HS was not seen using any of the four HS degrading enzymes.

Enzymatic treatment of cells in vitro using HPNSE and HPSE removes cell surface HS. Since many viruses, including both MV and EV forms of VACV, probably use cell surface HS as an initial docking ligand to infect cells, it is important to understand the role of HS in VACV infections. Cell monolayers treated with all the three HPNSE individually or as a cocktail of all three, along with the monolayers treated with HPSE, were infected with MV-GFP and EV-GFP and incubated for 6 hr as previously. Untreated cell monolayers infected with the respective viruses were used as 100% positive controls. Treatment of the cell monolayers with all four enzymes significantly reduced both MV-GFP and EV-GFP infectivity, although the effects were generally modest, being only 15-20% for MV-GFP and 15-50% for EV-GFP.
As expected, among the enzymes tested, HPSE treatment of cell monolayers had the greatest effect on the infectivity of both MV-GFP and EV-GFP, with HPSE treatment of cells reducing MV-GFP infectivity by about 20%, whereas the same treatment reducing EV-GFP infectivity by 50% (Figure 4.5). These results are consistent with the previous findings and build on the knowledge that both MV and EV forms of VACV interact with cell surface HS which contributes to their infectivity, with the EV form of VACV being more dependent on this interaction compared to the MV form of VACV. Nevertheless, these studies may be underestimating the role of HS in MV and EV infectivity as the different enzymatic treatments may have only partially removed HS from the target cells. Therefore, to evaluate the role of HS in MV and EV infections, a cell line deficient in cell surface HS was used in the following study.

4.3.4 VACV infection of CHO-K1 and HS deficient mutant CHO-K1 (pgsA-745) cell lines

VACV is known to have a very broad host range, with it being able to replicate in cell lines from mammalian to avian in origin. Chinese hamster ovary (CHO) cells, however, are known to be non-permissive for VACV replication (Drillien, Spehner, & Kirn, 1978; Franke, Roseman, & Hruby, 1985). Nonetheless, a cowpox virus (CPV) gene (CHO-hr) encoding a 77 kDa proteins (CP77), when engineered into the VACV genome at the thymidine kinase (tk) locus, allows VACV replication in CHO cells (Ramseyewing & Moss, 1995; Sphener, Gillard, Drillien, & Kirn, 1988). Previous studies have shown CHO hr is in fact disrupted in VACV WR (Kotwal & Moss, 1988).
Figure 4.4: Removal of cell surface HS by enzymatic treatment of target cells. Confluent monolayers of 143B cells were treated with HPNSE 1, HPNSE 2, HPNSE 3, a mixture of HPNSE 1, 2 and 3, or HPSE, at pH 5.5 for 1 hr, and then examined for cell surface expression of HS by immunofluorescent flow cytometry using a HS-specific mAb (clone 10E4). (A) Representative histograms (n = 6) showing cell surface HS remaining following different HPNSE or HPSE treatments. (B) Histogram (n = 6) summarizing cell surface HS removal (staining for HS expression) following different HPNSE or HPSE treatments, data being presented as mean fluorescence intensity (MFI). One-way ANOVA (Dunnett’s Multiple Comparison) was used to determine the statistical significance (* - p < 0.05; *** - p < 0.001) of cell surface HS remaining following enzymatic treatment, relative to HS on untreated cells. Data presented are representative of 3 independent experiments.
Figure 4.5: Effects of enzymatic treatment of target cells on MV-GFP and EV-GFP infections in vitro. Confluent monolayers of 143B cells were treated with HPNSE 1, HPNSE 2, HPNSE 3, HPNSE 1+2+3 and HPSE, at pH 5.5 for 1 hr. Cells were then infected with either purified MV-GFP or EV-GFP at a MOI of 1. Adherent cells were released by treatment with trypsin at 6 hr post infection, fixed and analyzed using flow cytometry for GFP expression. Representative histograms show mean percentage of GFP positive cells ± SEM (n=6) relative to control 143B cells infected with the respective viruses but not put through any enzymatic treated (100% positive control). One-way ANOVA (Dunnett’s Multiple Comparison) was used to determine statistical significance (* - p < 0.05; ** - p < 0.01; *** - p < 0.001) of the effects of enzymatic treatment on EV and MV infections relative to the respective ‘untreated’ controls. Data presented are representative of 3 independent assays.
Nevertheless, VACV is able to infect CHO cells and initiate the virus replication cycle, but the cycle is aborted at the intermediate gene expression stage.

Expression of early VACV genes, however, remains unperturbed (Ramseyewing & Moss, 1995). The parent CHO cell line produces high levels of cell surface HS and chondroitin sulfate (CS). However, pgsA-745, a mutant CHO cell line, has a defect in its xylosyltransferase, the first sugar transfer reaction in GAG formation (Esko, Stewart, & Taylor, 1985), with this mutation leading to the disruption of the synthesis of cell surface HS and CS in pgsA-745 cells.

Although replication of VACV is abortive in CHO cell lines, early VACV genes are still translated. Since an early-late promoter drives transcription of the GFP-bsd cassette in both recombinant VACV WR and VACV IHD-J constructs, VACV cell entry assays can potentially be performed in these cell lines. Furthermore, the property of the parent cell line to produce high levels of cell surface HS and CS, and a mutation in the same cell line inhibiting the synthesis of cell surface GAGs, provides an ideal opportunity to test the dependence of the MV and EV forms of VACV on cell surface GAGs to infect the cells in vitro.

CHO and pgsA-745 cell monolayers were infected with a low (1) and a high (10) MOI of both MV-GFP (obtained from the WR-GFP strain) and EV-GFP (obtained from the IHD-J-GFP strain). As before, the infection was stopped and the adherent cells released by trypsin treatment at 6 hr post infection. A relatively low percentage of both cell types were infected by MV-GFP (18%) and EV-GFP (12%). However, the GFP expression in the MV-GFP infected CHO and pgsA-745 cell monolayers, measured as the mean fluorescent intensity, is almost identical at
Figure 4.6: GFP expression in wild type CHO-K1 and HS deficient CHO-K1 (pgsA-745) cells infected with MV-GFP and EV-GFP. CHO-K1 and pgsA-745 cell lines were infected with purified MV-GFP or EV-GFP viruses at two different MOIs (1 and 10). Adherent cells were then released by treatment with trypsin 6 hr post infection, fixed and analyzed using flow cytometry for GFP expression.

Representative histograms of GFP expression by both CHO-K1 and pgsA-745 cells based on the mean fluorescence intensity (MFI) ± SEM (n=3). The data presented are representative of at least four independent experiments, with statistical analysis performed using an unpaired Student’s t-test.
both concentrations of VACV used (Figure 4.6). This indicates that MV entry into cells is not heavily dependent on the presence of cell surface GAGs. However, GFP expression in EV-GFP infected pgsA-745 cell monolayers, compared to the expression in CHO cell monolayers, is significantly reduced. These results highlight the importance of cell surface GAGs for EV infection of cells. The MV form of VACV, however, is not heavily dependent on GAGs to infect cells and, consequently, must have alternative mechanisms of cell entry.

**4.3.5 Differences in the infectivity of the MV and EV forms of VACV in the presence of heparin are not due to VACV strain differences**

Infectious VACV particles exist in two different forms, namely the EV and the MV forms. The EV form is essentially the MV form wrapped in at least one additional membrane (Figure 1.4). The presence of one or more membranes around the EV makes this form of VACV structurally, chemically and antigenically different from the MV form of VACV (Condit et al., 2006). It has been reported that the VACV IHD-J strain releases 40 times more EV compared to the VACV WR strain, although the size of the primary plaques are similar in the two strains (Law et al., 2006; Law, Hollinshead, & Smith, 2002; Smith, Vanderplasschen, & Law, 2002). Both the WR and IHD-J strains of VACV produce equal amounts of intracellular enveloped virus (IEV) and cell-associated enveloped virus (CEV) particles. A single codon mutation (Lys-151→Glu), located in the putative carbohydrate recognition domain of the A34R protein, has been identified to be the cause of more EV release from the IHD-J strain compared to the WR strain. At the nucleotide level, the VACV A34R gene sequence of the VACV WR strain differs from that of the VACV IHD-J strain at six different positions, four of which are silent (Blasco, Sisler, & Moss, 1993a). There
Figure 4.7: Effects of heparin on infectivity of MV-GFP and EV-GFP obtained from different strains of VACV. 143B cell monolayers were infected, at MOI of 1 and in the presence of doubling dilutions of heparin, with MV-GFP or EV-GFP isolated from either VACV WR or VACV IHD-J strains. Adherent cells were released by treatment with trypsin at 6 hr post infection, fixed and analyzed using flow cytometry for % GFP positive cells relative to control cells infected with respective viruses in the absence of inhibitors (100% positive control). Representative plots showing mean percentage of GFP positive cells relative to control cells ± SEM (n=3). The data presented are representative of at least three independent experiments.
have been no other reports of the two strains of VACV behaving differently in other ways.

Since the VACV WR strain produces much less of the EV form of the virus than the VACV IHD-J strain, the VACV IHD-J was used as a source of the EV form of VACV for all the studies reported in this thesis. Initial studies already described in this thesis suggest that the EV form of VACV interacts with heparin much more strongly than does the MV form. However, since the MV and EV forms of VACV were isolated from different VACV strains, namely the VACV WR and VACV IHD-J strains respectively, it was essential to rule out the possibility that the differences seen in the interactions of the EV and MV forms of VACV with heparin is due to strain differences rather than being an intrinsic property of the two virus forms. Thus, the MV and EV forms of VACV were isolated from cultures of GFP expressing recombinant VACV WR and VACV IHD-J strains. Both MV-GFP and EV-GFP were purified using protocols described earlier in this thesis. Adherent 143B cells were then infected with purified MV-GFP and EV-GFP forms of VACV in the absence or presence of heparin. A wide concentration range (0.09 μg/mL to 100 μg/mL) of heparin was used in this assay to determine the heparin IC50 for the MV and EV forms of VACV isolated from the two different strains.

Overall, heparin inhibited the infectivity of the EV form of VACV much more effectively than the MV form, regardless of the VACV strain from which it was obtained (Figure 4.7). In fact, the heparin inhibition curves for the EV form of VACV obtained from both strains of VACV virtually overlap each other. Likewise, the heparin inhibition curves for the MV form of VACV isolated from the two
different VACV strains also overlap each other. The IC50 for heparin inhibition of
the EV infections was 0.19 μg/mL, whereas the IC50 for the MV infections was 4.7
μg/mL, which translates to over a 16-fold difference in the IC50 values for the two
forms of VACV. These results indicate that the differences seen between the EV-
heparin and MV-heparin interactions are due to the two different forms of VACV,
and not due to the VACV strain differences. Therefore, for all subsequent assays,
purified EV-GFP was obtained from the recombinant VACV IHD-J strain and
purified MV-GFP from the recombinant VACV WR strain. As seen in this study,
titration curves for the inhibition of MV-GFP and EV-GFP infectivity by heparin are
highly reproducible, regardless of the strain of origin of the two virus forms.
Therefore, in all subsequent inhibition assays of VACV infectivity by HS mimetics,
heparin was used as a reference compound to evaluate the relative potency of
other inhibitors.

4.3.6 Analysis of the interaction of MV-GFP and EV-GFP with differently
sulfated HS
ELISA assays described in Chapter 3 examining the interaction of the MV form of
VACV with immobilized heparin, HS\textsuperscript{hi} and HS\textsuperscript{low} revealed that the interaction
between HS and purified MV was highly dependent on the level of sulfation of the
HS chains. Thus, heparin, a highly sulfated form of HS, showed the strongest
interaction with the purified MV particles. HS\textsuperscript{low} on the other hand, which was the
least sulfate HS tested, showed the weakest interaction with purified MV particles
(Figures 3.1, 3.2 and 3.3). The unavailability of EV specific antibodies meant a
similar ELISA could not be performed with the purified EV particles.
Figure 4.8: Effects of differently sulfated HS on MV-GFP and EV-GFP infections in vitro. 143B cells were infected with purified MV-GFP (obtained from VACV WR) or EV-GFP (obtained from VACV IHD-J), at a MOI of 1, in the presence of doubling dilutions of HS$^{hi}$ or HS$^{low}$ starting from 100 μg/mL. Adherent cells were released by treatment with trypsin at 6 hr post infection, fixed and analyzed using flow cytometry for % GFP positive cells relative to control cells infected with the respective viruses in the absence of inhibitors (100% positive control). Representative plots showing mean percentage of GFP positive cells relative to control cells ± SEM (n=3). The data presented are representative of at least three independent experiments.
With the development of the new FACS based infection assays using GFP producing VACV described in section 4.3.2, both purified MV-GFP and EV-GFP particles were used to examine the effect of differently sulfated HS on their infectivity. Thus, adherent cells were infected with the purified forms of MV-GFP and EV-GFP in the absence or presence of HS\textsuperscript{hi} and HS\textsuperscript{low} (Figure 4.8). As mentioned in section 4.3.5, a wide concentration range (0.09 µg/mL to 100 µg/mL) of the differently sulfated HS preparations were used in this assay to estimate the IC\textsubscript{50} of HS\textsuperscript{hi} and HS\textsuperscript{low} for the MV and EV infections.

The results obtained from this study were as expected, the interaction of EV with HS\textsuperscript{hi} being much stronger than the interactions with HS\textsuperscript{low}, with the IC\textsubscript{50} of HS\textsuperscript{hi} for the EV-GFP infection being 0.39 µg/mL compared with 12.5 µg/mL for HS\textsuperscript{low}, a 32-fold difference. Likewise, the interaction of MV-GFP with HS\textsuperscript{hi} was much stronger than with HS\textsuperscript{low}, with the IC\textsubscript{50} being 12.5 µg/mL compared with an IC\textsubscript{50} of >100 µg/mL for HS\textsuperscript{low}. A comparison of these findings with the heparin inhibition data reported in section 4.3.5 shows that the EV-GFP and MV-GFP interact most avidly with heparin, with HS\textsuperscript{hi} being a 2-fold less effective inhibitor based on IC\textsubscript{50} values. These studies with HS\textsuperscript{hi} and HS\textsuperscript{low} also confirmed that EV-GFP interacts with HS with a much greater avidity than MV-GFP. Thus, highly sulfated GAGs, such as heparin and HS\textsuperscript{hi}, are much more potent inhibitors of EV infections than MV infections.
4.4 Discussion

To increase the sensitivity of infection assays, recombinant variants of both the WR and IHD-J strains of VACV expressing GFP were constructed by inserting a GFP-\textit{Bsd} sequence driven by an early-late poxvirus promoter at an inter-genic site. Blasticidin, first discovered over 50 years ago, is produced by \textit{Streptomyces griseochromogenes} (Takeuchi, Hirayama, Ueda, Sakai, & Yonehara, 1958). An aminoacylnucleoside antibiotic, blasticidin acts as a protein synthesis blocker by interfering with the peptidyl transferase reaction (Tamura, Kimura, & Yamaguchi, 1995). The use of the \textit{Bsd} gene sequence as a blasticidin resistance gene has been demonstrated to be a more powerful selection marker than neomycin/G418 resistance, to select for transfected mammalian cell lines (Kimura, Kamakura, Tao, & Yamaguchi, 1994; Kimura, Takatsuki, & Yamaguchi, 1994). Along with blasticidin being a better selection marker, it is also a smaller gene and allows easy visual screening. These properties make the \textit{Bsd} gene an ideal selection marker. The fusion between GFP-\textit{Bsd} not only enables selection of recombinant VACV particles expressing GFP, but also allows us to study very accurately early stages of a VACV infection.

Kinetic studies on VACV replication performed using time-course electron microscopy and plaque assays have shown that the first round of progeny virions is produced by 5-6 hr post infection (Doceul et al., 2010; Payne & Kristenson, 1979). In order to understand the early events of virus entry into cells, it was important to study early time points during an infection. Since an early-late promoter drives the expression of GFP in virus-infected cells, this allowed us to detect virus infections at an early stage. A time-course assay was initially
performed to measure GFP expression in infected cells over time. This allowed us to select a time point where approximately 50% of the cells would be expressing GFP, for future inhibition assays. Since a substantial proportion of cells infected using either MV-GFP or EV-GFP, were infected by 6 hr post infection, and the knowledge that 6 hr is about the time it takes for the first round of viral replication to complete, the 6 hr time point was used for subsequent inhibition assays (Figure 4.1 and 4.2). Selecting an optimum time point for such inhibition assays is especially important as VACV uses multiple mechanisms of cell entry, including the infection of cells by ‘punching’ MV particles from infected cells to neighboring uninfected cells, which bypasses the need for VACV particles to interact with HS and other cellular receptors (Mercer et al., 2010; Schmidt et al., 2012). To evaluate the role of cell surface components in virus attachment and entry, it is essential to develop assay conditions that would eliminate non-HS dependent virus entry mechanisms.

The initial ELISA findings revealed that purified MV particles bound strongly to the highly sulfated GAGs, heparin and HSβ, immobilized on assay wells (Figure 3.1, 3.2 and 3.3) and can reduce MV plaquing by 5-fold (Figure 3.5D). A time-course of heparin inhibition of the MV-GFP and EV-GFP infections revealed that heparin can inhibit the infectivity of both viruses early during an infection (Figure 4.3). Thus, even the lowest concentration of heparin tested (1 μg/mL) had a significant effect on MV-GFP infectivity at 12 hr post infection, but by 24 hr even the highest concentration of heparin used (100 μg/mL) failed to have any effect on MV-GFP infectivity. This finding contrasts with the EV-GFP infectivity, which was still substantially inhibited by heparin 24 hr after culture initiation. The results
obtained with the GFP producing VACV strains confirm and expand upon the ELISA and plaque assay results, with both forms of VACV interacting with heparin but the EV form of VACV interacting much more strongly. This difference was seen whether the MV and EV forms of VACV were obtained from either the VACV WR or VACV IHD-J strains of VACV (Figure 4.6).

As discussed earlier, this difference in heparin sensitivity could partially be explained, at least at the later time points, by the ability of MV particles to enter uninfected neighboring cells by punching through the plasma membrane of the neighboring cells, without having the need to be released into the culture medium, and hence bypassing the MV-heparin interaction. On the other hand, heparin has a much greater effect on EV infectivity at both early and late time points. Thus, even at 24 hr post infection, 1 μg/mL of heparin was able to inhibit EV infections by 50%. This is a remarkable finding since the EV form only differs from the MV form of VACV by the presence of at least one extra outer membrane (Ichihashi, 1996; Smith et al., 2002). Also, the effects of heparin on EV infectivity go beyond the 24 hr post infection, as seen with the plaque assay results where the trademark ‘comets’ did not form even by 48 hr post infection (Figure 3.5A). These findings highlight that heparin, and presumably cell surface HS, play a far greater role in EV infectivity than in MV infectivity.

The simplest interpretation of the heparin inhibition experiments is that HS is a coreceptor for VACV infections, with soluble heparin simply blocking the HS binding sites on VACV particles. It is likely that HS binding receptors on the surface of MV particles bind to cell surface HS with a lower avidity compared to cell surface
receptors on EV particles. Two approaches were used to directly test this hypothesis, namely removal of cell surface HS by enzymatic treatment prior to infection, or to try and infect cells that are genetically HS deficient.

Three bacterial enzymes that degrade HS (HPNSE 1, 2 and 3) and mammalian heparanase (HPSE) were used, with varying success, to remove cell surface HS. However, treatment of cell monolayers with all of these enzymes caused a statistically significant, but modest, reduction in both MV-GFP and EV-GFP infectivity (Figure 4.5), although these experiments could be underestimating the role of cell surface HS in VACV infectivity due to the partial removal of cell surface HS. An alternative approach to understand the significance of cell surface HS in VACV infections was, therefore, embarked upon by infecting cells genetically deficient in producing cell surface HS. CHO-K1 cells produce normal levels of cell surface HS, whereas the pgsA-745, mutant CHO-K1 cells, are HS deficient (Esko et al., 1985). However, the lack of CHO-hr gene in VACV makes these adherent cells non-permissive for VACV infections. VACV plaque assays, therefore, cannot be performed on CHO-K1 and pgsA-745 adherent cells. Fortunately, since an early-late promoter drives the GFP-Bsd gene expression in recombinant strains of both VACV WR and VACV IHD-J, a flow cytometry based infection assay to detect GFP fluorescence in infected cells, could still be undertaken. The results obtained from this assay were consistent with the previous findings that HS is a more important co-receptor for the EV form of VACV than the MV form.

VACV infection of 143B cells identified cell populations with varying GFP expression, especially by 8-12 hr post infection. Since an early-late poxvirus
promoter drives the transcription of the GFP-bsd reporter gene used in the current study, this allows quantification of GFP expression in infected cells soon after the primary infection, even before the first set of progeny virus particles are produced. In fact, kinetic studies on VACV have reported that progeny VACV are formed by 5 to 6 hr post infection (Payne & Kristenson, 1979) whereas GFP expression can be seen within 2 hr of infection in both MV-GFP and EV-GFP infected cells (Figure 4.1 and 4.2), indicating that only primary virus infection is being observed at this time point. As time progresses and one full replication cycle of VACV is completed, newly formed virus particles are able to infect neighboring cells at around 6 hr post initial infection, some of which may not have been infected by the initial infection at 0 hr. It is likely that a second round of replication may also start in the primary infected cell and, therefore, results in the expression of even higher amounts of GFP, whereas the neighbouring cells may only just start to produce GFP as a result of a delayed infection. Furthermore, not every virus particle may enter cells at the same time. These discrepancies in the timing of infection result in populations of cells that are infected early during infection and, therefore, are highly GFP expressing by 12 hr, along with populations of cells that are infected by the newly formed virions from the primary infection and, thus, are low GFP expressing at 8-12 hr.

Since VACV replication in CHO and pgsA-745 cells is abortive, only the cells infected by the primary infection express GFP. Virus particles once inside the primary cell would undergo early gene transcription and, thus, express GFP, however, since no progeny virus would form within the primary cell, no secondary infection of neighbouring cells would take place. This is the reason why only a very
small proportion of CHO and pgsA-745 cells could be infected and, therefore, unlike the 143B cells, high and low GFP expressing cell populations could not be observed. This prompted the use of mean fluorescence intensity (MFI) rather than % GFP positive cells as a measure of infection in these non-permissive cells.

A previous report suggests that the outer membrane of EV lyses when in contact with sulfated GAGs, resulting in the release of the inner MV particle, which then causes the infection (Law et al., 2006). Furthermore, results obtained from the enzymatic removal of cell surface HS using HPNSE and HPSE, along with infection studies in cells genetically deficient in producing cell surface HS performed in this thesis confirm the previous findings that cell surface HS is particularly important for EV infections. However, use of time course inhibition assays in the current study also suggests that both MV and EV forms of VACV have quite distinct infection profiles, when used to infect cells in presence of heparin and other sulfated GAGs. Our work also suggests that this disruption of the outer EV envelope must be extremely slow as even at 24 hr post infection, the EV-GFP particles incubated with heparin are unable to infect cells monolayers efficiently. In fact, this heparin-EV interaction slows down the VACV infection, consistent with heparin blocking a HS binding sites on EV that recognizes cell surface HS and aids EV entry into cells. These results, in conjunction with previously published work, imply that cell surface HS is crucial for EV infections, however, more research needs to be undertaken to identify all the HS binding proteins on the surface of the EV form of VACV.
Heparin and HS share a very similar molecular structure (Li & Vlodavsky, 2009). Connective tissue mast cells are the only known cell type that produce heparin (Li & Vlodavsky, 2009; Yurchenco & Schittny, 1990). However, unlike HS, heparin is not a component of the ECM. Therefore, it was essential to understand the interaction of purified MV-GFP and EV-GFP particles with HS, measured using the highly sensitive flow cytometry detection of GFP fluorescence. A broad concentration range of differently sulfated HS (HS\textsuperscript{hi} and HS\textsuperscript{low}) was used to inhibit MV-GFP and EV-GFP infections \textit{in vitro}. As expected, HS\textsuperscript{hi} (IC50 = 0.39 μg/mL) was more efficient at inhibiting EV-GFP infections compared to HS\textsuperscript{low} (IC50 = 12.5 μg/mL), this difference translating into a 32-fold difference in inhibitory activity. Similarly, HS\textsuperscript{low} was much less effective than HS\textsuperscript{hi} at inhibiting MV-GFP infectivity, although the fold differences based on comparison of IC50 values, could not be calculated (Figure 4.8). In contrast, HS\textsuperscript{hi} was almost as effective as heparin at inhibiting both MV-GFP and EV-GFP infections, there only being a 2-3 fold difference based on IC50 values, indicating that heparin provides a good representation of the HS motifs recognized by the MV and EV forms of VACV.

In conclusion, we have successfully constructed GFP GFP-\textit{Bsd} expressing VACV WR and VACV IHD-J strains of VACV and used the two strains to demonstrate that both the MV and EV forms of VACV interact strongly with heparin. Degradation of cell surface HS using different HPNSEs and HPSE resulted in partial inhibition of EV and MV infectivity of cells. This was probably due to the inability of HS degrading enzymes to completely degrade cell-surface HS. The residual infection observed in treated cells could be attributed to the small number of HS binding sites still present on the surface of cells. Results obtained from infection of cells genetically
deficient in cell surface HS confirm the interpretation of the results obtained with the HS degradation assays. Together, results from both these assays substantiate the finding that interaction with cell surface HS is very important for EV infections. In contrast, the MV form of VACV is not heavily dependent on HS interactions for infection. Similarly, recognition of HS$^{hi}$ but not HS$^{low}$ is required for the EV form of VACV to infect cells, indicating that EV infection of cells is dependent on the degree of sulfation of cell surface GAGs. These findings are a significant first step towards novel drug development for poxvirus infections.
Interaction of chemically modified heparin and HS mimetics with EV and MV forms of VACV

Chapter 5:
5.1 Abstract

It was demonstrated in Chapter 4 of this thesis that cell surface HS can potentially act as a co-receptor for VACV entry into cells. It was also demonstrated that heparin is able to inhibit EV infectivity more effectively than MV infectivity. To take these findings further and develop effective and safe HS/heparin-based antivirals, it was first important to understand the structural features of HS/heparin that are required for its interaction with the EV and MV forms of VACV. In this Chapter it is shown that the 2-O-sulfate of uronic acid and the 6-O- and N-sulfation of glucosamine residues are all important for VACV infectivity, with the 6-O-sulfate of glucosamine being the most crucial. It was also shown that the size of the heparin chains does not affect the ability of the molecule to inhibit VACV infections. In fact, heparin chains as small as 1.7 kDa (i.e., a pentasaccharide) are able to inhibit VACV infections just as well as 5-30 kDa unfractioned heparin. These findings are extremely significant as they highlight the structural requirements for HS/heparin chains to act as efficient antivirals. Subsequent screening of various sulfated di- and oligo-saccharides (HS mimetics) confirmed that sulfated pentasaccharides are generally the most effective inhibitors of both MV and EV infections. However, the linkages of different D-glucose-based sulfated saccharides has a profound effect on the ability of the sulfated saccharides to inhibit VACV infections, with the order of potency being $\beta(1\rightarrow4) > \alpha(1\rightarrow6) > \alpha(1\rightarrow4) > \beta(1\rightarrow3)$. Furthermore, although none of the sulfated D-glucose-based saccharides tested could inhibit VACV infections as effectively as heparin, the D-mannose-based sulfated oligosaccharide mixture PI-88 (Muparfostat) inhibited MV infections more effectively than heparin but was a weak inhibitor of EV infections. Collectively, these data suggest that the MV and EV forms of VACV interact with distinct but structurally related HS motifs on target
cells, with the position of negatively charged sulfate groups in 3D space determining the specificity of the interactions.
5.2 Introduction and rationale

Heparin and HS are linear GAG chains that are synthesized as proteoglycans, i.e.,
glyco-conjugated macromolecules composed of carbohydrate polymers covalently
attached to a protein core (Li & Vlodavsky, 2009; Sasisekharan & Venkataraman,
2000; Sugahara & Kitagawa, 2002). The HS and heparin chains share a great deal
of structural similarity, however, the protein cores of these GAG chains show
distinct expression patterns, the heparin bearing proteoglycan, for example, being
largely restricted to mast cells (Li & Vlodavsky, 2009). The HS and heparin chains
are composed of repeating disaccharides of glucosamine and hexuronic acid,
joined in alternating sequences by 1,4-glycosidic linkages, which gives these
carbohydrate chains their flexibility to bind to different proteins (Li & Vlodavsky,
2009; Lindahl, 1990; Lindahl et al., 1979). The carbohydrate chains of both heparin
and HS are sequentially modified by a series of reactions involving N- and O-
sulfation and epimerization of the glucuronic residues to iduronic acid (Lindahl,
1990). The modification reactions during HS biosynthesis are highly regulated,
resulting in heterogenous sulfation and epimerization patterns that could be tissue
and cell-type specific (Esko & Lindahl, 2001; Ledin et al., 2004). It is believed that
the selective modifications allow regulated interactions between HS and various
HS binding proteins (Kreuger, Spillmann, Li, & Lindahl, 2006). However, the
modification reactions in heparin biosynthesis are more extensive resulting in a
relatively homogenous structure (Esko & Lindahl, 2001). The size of nascent
heparin chains is 60-100 kDa, whereas heparin prepared from animal tissue is a
mixture of saccharide fragments of 5-30 kDa (Horner, 1971).
Naturally occurring unfractioned heparin (UFH), commonly referred to as just 'heparin', has several pharmacokinetic limitations due to its high negative charge, which causes binding to several plasma proteins (e.g., histidine-rich glycoprotein, vitronectin, lipoproteins, fibronectin and fibrinogen), proteins secreted by platelets (e.g., platelet factor 4 and high-molecular-weight von Willebrand factor) and endothelial cells (Hirsh, 1991; Young et al., 1992). Along with pharmacokinetic limitations, UFH, like several other anticoagulants, can cause complications of bleeding and produce thrombocytopenia and osteoporosis (Hirsh, 1998).

Chemical or enzymatic depolymerization of traditional heparin yields low molecular weight heparins (LMWH), which are a more homogeneous anticoagulant than heparin and approximately one third the size of heparin (i.e., 4-5 kDa). LMWHs have been widely shown to have the ability to overcome some of the limitations of UFH, and to be more effective in the treatment of deep vein thrombosis (DVT) (Hirsh, 1998).

Results shown in this thesis so far have demonstrated the ability of highly sulfated HS and heparin to inhibit VACV infectivity, with the two molecules being more effective against EV than MV infections. However, in order to develop GAG based antivirals with minimal off-target effects, it was essential to further understand the specific modifications/structures of the GAG chains that interact with the two forms of VACV and, therefore, affect their infectivity. Hence, the specific aims of this Chapter were to understand, based on FACS assays, the key structural features of heparin (and HS) required to efficiently inhibit VACV infectivity. In order to determine the structural requirements, a systematic approach of using LMWHs as
possible inhibitors of VACV infectivity, was initially undertaken. This was followed by understanding the role of different heparin modifications on anti-VACV activity along with testing several synthetic sulfated oligosaccharides (HS mimetics) as possible inhibitors of VACV infectivity.
5.3 Results

5.3.1 Differences in VACV infectivity in the presence of different low molecular weight heparins (LMWH)

LMWH started to replace heparin as an antithrombotic drug in the prevention and treatment of DVT in the mid-1980s (Franze et al., 2015; Mousa, 2002). The adaptation of LMWH was due to their reduced side effects, such as smaller risk of bleeding, lower binding affinity for several plasma proteins, lower chance of heparin-induced thrombocytopenia (HIT) and more predictable behavior, all of which made them a safer drug compared to heparin (Franze et al., 2015; Hirsh, 1998; Ingle & Agarwal, 2014; TenCate et al., 1997). It should be noted, however, that both heparin and LMWH exert their anticoagulant activity by the same unique pentasaccharide sequence (Weitz, 1997).

Today, most LMWHs available on the market are derived from heparin using four different depolymerisation strategies, namely, oxidative cleavage, deaminative cleavage and chemical or enzymatic β-elimination (Franze et al., 2015). The size of LMWHs, their molecular weight distribution and their degree of sulfation is dependent on the specific cleavage reactions used for their generation, the resultant cleavage points along the length of the UFH chains producing LMWH preparations with distinct physical, chemical and biological properties (Minghetti, Cilurzo, Franze, Musazzi, & Itri, 2013). Enoxaparin is one such LMWH, derived from the β-elimination cleavage of UFH chains. Previous studies have shown enoxaparin to be one of the most suitable antithrombotic agent which can be delivered intra-dermally (Franze et al., 2015).
Fondaparinux, a new class of antithrombotic agent, is a totally synthetic pentasaccharide sequence that is structurally based on the antithrombin III binding site of heparin (Bauer, Eriksson, Lassen, & Turpie, 2001; Chang et al., 2014). Unlike heparin, which interacts with several plasma proteins involved in coagulation, fondaparinux exhibits only factor Xa inhibitory activity via binding to antithrombin III and consequently inhibiting thrombin generation (Bauer et al., 2001; Petitou et al., 2002). Furthermore, fondaparinux has been shown to be safer, and have superior efficacy and pharmacological properties compared to heparin, however, the drug is much more expensive than naturally available heparin (Walenga et al., 2002). Nevertheless, in order to identify safe GAG based antivirals, it was important to determine the ability of fondaparinux, along with other commercially available LMWHs, to inhibit MV and EV infections. The LMWHs used in the inhibition assays described in this thesis include 1.7 kDa fondaparinux, 3 kDa enoxaparin, 4–6 kDa LMWH (Fluka-Sigma Aldrich), and a 5 kDa LMWH, all of which were gifts from Dr. Craig Freeman, John Curtin School of Medical Research.

All LMWHs tested had a similar inhibitory effect on the EV and MV forms of VACV, indicating that chain length is not a factor that determines the inhibitory capacity of different heparin chains (Figure 5.1). In fact, the fondaparinux pentasaccharide appeared to be the most potent inhibitor of all the LMWH examined, particularly with MV infections, with the inhibition curves almost overlapping with that of heparin (dashed lines) for the respective VACV forms (Figure 5.1A), the IC50 values of fondaparinux for MV and EV infections being, respectively, only 2-fold and 3-fold higher than that of heparin (Table 5.1). Enoxaparin, a 3 kDa LMWH, was also able to inhibit both MV and EV infectivity, with the IC50 values for MV and EV
infectivity being 4-fold more than heparin for both VACV forms (Figure 5.1B, Table 5.1). The larger LMWHs, including the 4-6 kDa Fluka LMWH and the 5 kDa LMWH, were also able to inhibit VACV infections efficiently, the IC50 values for both being identical (Figure 5.1C and D), with values for the IC50 for EV infections being 2-fold and for MV infections being 5-fold higher than that of heparin (Table 5.1).

5.3.2 Differences in VACV infectivity in the presence of chemically modified heparins

Several studies have demonstrated that heparin can act as an inhibitor of heparanase activity *in vitro*, inhibiting the degradation of cell surface and ECM HS and consequently inhibiting tumour metastasis and inflammatory responses (Barner et al., 1987; Irimura, Nakajima, & Nicolson, 1986; Parish, Coombe, Jakobsen, Bennett, & Underwood, 1987). Heparin, however, is a potent anticoagulant and can induce hemorrhagic complications when administrated in high concentrations. This led to the development of safer chemically modified derivatives of heparin with low anticoagulant potency, but similar heparanase inhibitory activity as heparin (Barner et al., 1987). Chemically modified heparins are essentially heparin chains that have been modified by addition or removal of specific groups, such as sulfate, acetyl or carboxyl groups, across the length of the heparin chains. In order to develop antiviral drugs that were also safe to be administered it was, therefore, important to evaluate the effectiveness of chemically modified heparins as inhibitors of VACV infections. Use of such molecules would also help determine the heparin modifications that enable or inhibit infectivity. The chemically modified heparins used in this Chapter were all a
gift from Dr. Craig Freeman, and were developed and tested in the Parish laboratory at the John Curtin School of Medical Research, Canberra, Australia.

Preliminary results from ELISA assays described in Chapter 3 of this thesis, using HS preparations with different levels of sulfation (i.e., \( \text{HS}^{\text{hi}} \) and \( \text{HS}^{\text{low}} \)), revealed that sulfation density has an impact on the ability of MV to interact with immobilized HS. Furthermore, infection assays performed using the differently sulfated HS preparations as inhibitors of VACV infections, described in Chapter 4 of this thesis, suggest that the level of sulfation also plays an important role in the ability of HS to inhibit EV and MV infections. Previously published studies on Herpes simplex viruses (HSV) using chemically modified heparins and heparanase treatment of target cells have shown that glycoproteins gB and gC on the surface of the HSV-1 and HSV-2 strains recognize different structural features of cell surface HS, which provides an initial docking site for HSV entry into cells (Shukla & Spear, 2001). The presence of 2,3-\( \text{O} \)-sulfate and 6-\( \text{O} \)-sulfate groups on heparin has been shown to be critical for the ability of heparin to bind to HSV-1 gB/gC, but not for heparin-HSV-2 glycoprotein interactions (Feyzi et al., 1997; Trybala, Liljeqvist, Svennerholm, & Bergstrom, 2000). Thus the interaction of viral glycoproteins with cell surface HS is crucial for HSV-1 entry in cells, however, such interactions are relatively less important for HSV-2 infections (Herold, Gerber, Belval, Siston, & Shulman, 1996; Shukla et al., 1999; Shukla & Spear, 2001). Therefore, in order to understand the interaction of VACV with cell surface HS to be able to design antivirals, it was essential to first understand the role of individual sulfate groups of heparin on the ability of heparin to inhibit VACV infectivity, similar to the HSV studies previously published.
**Figure 5.1: Effect of different LMWHs on MV-GFP and EV-GFP infections in vitro.** 143B cells were infected with purified MV-GFP or EV-GFP viruses at a MOI of 1, in the presence of doubling dilutions of different LMWHs, namely fondaparinux pentasaccharide 1.7 kDa (A), enoxaparin 3 kDa (B), LMWH (Fluka) 4-6 kDa (C) LMWH 5 kDa* (D), at concentrations ranging from 0.09 μg/mL to 100 μg/mL. Adherent 143B cells were released with trypsin at 6 hr post infection, fixed, and analyzed using flow cytometry for GFP expression. Representative line plots show mean (± SEM; n=3) percentage of GFP positive cells relative to control cells infected with the viruses in the absence of inhibitors (100% positive control). The ability of heparin to inhibit MV-GFP and EV-GFP infections (dashed lines) is included in each graph for comparison (see Figure 4.7). The data presented are representative of three independent experiments.

*The 5 kDa LMWH was prepared by chemical depolymerization of heparin by Dr. Craig Freeman*
To perform these assays, chemically de-sulfated heparin molecules lacking 2-\(\text{-O-}\)sulfate (de2S), 6-\(\text{-O-}\)sulfate (de6S) or \(\text{N-}\)sulfate (deNS) groups or being completely desulfated (deS), were used to inhibit EV and MV infections. In these assays, adherent 143B cells were infected with purified MV-GFP and EV-GFP forms of VACV in the absence or presence of the different chemically modified heparins. A wide concentration range (0.09 \(\mu\)g/mL to 100 \(\mu\)g/mL) of heparin variants was used in this assay to determine the IC50 of each variant for both the MV and EV forms of VACV (Figure 5.2). For comparison, plots of heparin inhibition of MV and EV infectivity, from section 4.3.5, are displayed as dashed lines in each Figure depicted in Figure 5.2. A diagrammatic representation of each chemically modified heparin, showing the sulfate groups lacking from the heparin chains, is presented next to the respective line plots.

Overall, the chemically modified heparins tested had a greater inhibitory effect on the EV form of VACV, than the MV form, indicating that EV interacts with the sulfate groups of heparin (or HS) with a higher avidity than MV. Some sulfate groups, however, are not crucial for inhibition of MV or EV infectivity, with the 2-\(\text{-O-}\)sulfate of the iduronic/glucuronic acid residues being the least important (Figure 5.2A). Thus heparin molecules deficient in the 2-\(\text{-O-}\)sulfate group were still quite effective inhibitors of VACV infections, exhibiting IC50 values for EV and MV infections 3-fold and 5-fold higher than heparins, respectively (Table 5.1). In contrast, the 6-\(\text{-O-}\)sulfate and \(\text{N-}\)sulfate groups were crucial for inhibition of VACV infectivity, removing either of these two sulfate groups having a very significant impact on the ability of heparin to inhibit EV and MV infections.
Table 5.1: Comparison of IC50 values for various LMWHs and chemically modified heparins for EV and MV infections *in vitro*.

<table>
<thead>
<tr>
<th>Heparin Species†</th>
<th>IC50 (μg/mL) [Fold change]§</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>EV</td>
</tr>
<tr>
<td>Heparin</td>
<td>0.19</td>
</tr>
<tr>
<td>Fondaparinux - 1.7 kDa</td>
<td>0.58 [3x]</td>
</tr>
<tr>
<td>Enoxaparin - 3 kDa</td>
<td>0.78 [4x]</td>
</tr>
<tr>
<td>LMWH (Fluka) - 4-6 kDa</td>
<td>0.39 [2x]</td>
</tr>
<tr>
<td>LMWH - 5 kDa</td>
<td>0.39 [2x]</td>
</tr>
<tr>
<td>de2S</td>
<td>0.58 [3x]</td>
</tr>
<tr>
<td>de6S</td>
<td>12.5 [65x]</td>
</tr>
<tr>
<td>deNS</td>
<td>18.75 [98x]</td>
</tr>
<tr>
<td>deS</td>
<td>&gt; 100 [&gt; 500x]</td>
</tr>
<tr>
<td>deCarboxyl</td>
<td>0.29 [1.5x]</td>
</tr>
<tr>
<td>deNS, reNAc</td>
<td>0.39 [2x]</td>
</tr>
<tr>
<td>de2S, deNS/reNAc</td>
<td>1.17 [6x]</td>
</tr>
<tr>
<td>deS, reNS</td>
<td>37.5 [198x]</td>
</tr>
</tbody>
</table>

† **LMWH**: low molecular weight heparin; **de2S**: de-2-0-sulfate; **de6S**: de-6-0-sulfate; **deNS**: de-N-sulfate; **deS**: de-sulfate; **deCarboxyl**: de-carboxylate; **reNAc**: re-N-acetyl; **reNS**: re-N-sulfate.

§ Fold change ratios calculated by dividing IC50 values of each heparin species for each virus form by the IC50 value of heparin for each virus form.
**Figure 5.2: Effect of different chemically modified heparins on MV-GFP and EV-GFP infections in vitro.** 143B cells were infected with purified MV-GFP or EV-GFP viruses at a MOI of 1, in the presence of doubling dilutions of different chemically modified heparins, namely de-2-O-sulfate (de2S) (A), de-6-O-sulfate (de6S) (B), de-N-sulfate (deNS) (C) and de-sulfated (deS) (D) at concentrations ranging from 0.09 μg/mL to 100 μg/mL. Adherent 143B cells were released with trypsin at 6 hr post infection, fixed, and analyzed using flow cytometry for GFP expression. Diagrammatic representation of different chemically modified heparins is shown next to line plots for respective modified heparins*. Representative line plots show mean (± SEM; n=3) percentage of GFP positive cells relative to control cells infected with the viruses in the absence of inhibitors (100% positive control). The ability of unmodified heparin to inhibit MV-GFP and EV-GFP infections (dashed lines) is included in each graph for comparison (see Figure 4.7). The data presented are representative of three independent experiments.

*For simplicity, iduronic acid is depicted as the hexuronic acid in the heparin disaccharide, but it equally could be glucuronic acid.
A

B

de6S

C

deNS

D

deS

EV - de2S
EV - Heparin
MV - de2S
MV - Heparin

D-glucosamine
L-iduronic acid

EV - de6S
EV - Heparin
MV - de6S
MV - Heparin

D-glucosamine
L-iduronic acid

EV - deNS
EV - Heparin
MV - deNS
MV - Heparin

D-glucosamine
L-iduronic acid

EV - deS
EV - Heparin
MV - deS
MV - Heparin

D-glucosamine
L-iduronic acid

GFP Positive Cells (% Control)

Inhibitor concentration (µg/mL)
Thus for EV infections, the IC50 value for heparin lacking 6-O-sulfate groups on glucosamine residues was 65-fold higher than that of untreated heparin (Figure 5.2B, Table 5.1). Similarly, heparin molecules lacking N-sulfate groups on their glucosamine residues were 98-fold less effective than heparin at inhibiting EV infections (Figure 5.2C, Table 5.1). Furthermore, heparin lacking either 6-O-sulfate or N-sulfate groups was much less effective at inhibiting MV infections, although IC50 values could not be obtained as they were not reached at the highest modified heparin concentration tested. As expected, removing all sulfate groups from heparin chains completely abolished the ability of heparin to have any inhibitory effect on VACV infectivity (Figure 5.2D). The finding that 6-O and N-sulfate groups along the length of the heparin chain are more important for inhibiting VACV infections, than 2-O sulfate groups, lays the ground work for determining the features of cell surface HS important for both EV and MV infectivity.

The next investigation examined the role of non-sulfate groups of heparin on the ability of the molecule to inhibit VACV infections. Therefore, the next set of chemically modified heparins examined the role of the carboxyl group of the iduronic/glucuronic acid residues (deCarboxyl) and the effect of N-acetylation of glucosamine residues on the ability of heparin to inhibit VACV infections. As described earlier, adherent 143B cells were infected with purified MV-GFP and EV-GFP forms of VACV in the absence or presence of different modified heparins. A wide concentration range (0.09 μg/mL to 100 μg/mL) of modified heparins was used in this assay to determine the IC50 values for all heparin variants for both the MV and EV forms of VACV (Figure 5.3). Removal of the carboxyl group from the iduronic/glucuronic acid residues of heparin had a minimal effect on the ability of
heparin to inhibit both MV and EV infections (Figure 5.3A), with the IC50 values for EV and MV infections being, respectively, only 1.5-fold and 3-fold higher than for unmodified heparin. As seen previously in Figure 5.2C, removal of the N-sulfate group from glucosamine residues dramatically reduced the ability of heparin to inhibit VACV infections, compared to the removal of the 2-O-sulfate group, which had little effect on inhibitory activity, suggesting that N-sulfate groups play an important role in the interactions of VACV with heparin/HS, whereas the 2-O-sulfate groups play only a minor role in this interaction. However, the generation of a modified heparin with N-acetyl groups substituting for N-sulfate groups on glucosamine residues, almost completely restored the ability of N-desulfated heparin to inhibit MV and EV infections (Figure 5.3B), with the IC50 values for EV and MV infections being, respectively, 2-fold and 4-fold higher than that for untreated heparin (Table 5.1). However, if the 2-O-sulfate group of N-desulfated/N-reacetylated heparin is also deleted, the ability of heparin to inhibit MV and EV infections is further hampered (Figure 5.3C). This suggests that the interaction of VACV with the 2-O-sulfate groups of iduronic/glucuronic acid may not be crucial for heparin/HS binding, however, it is an important structural component of the heparin molecule, important for the inhibitory activity of heparin as a whole. Thus, the presence of N-acetyl groups on the glucosamine residues of heparin are unable to compensate for the loss of 2-O-sulfate groups from the heparin molecule. As a result the IC50 values for de2S, deNS/reNAc heparin are 6-fold and 11-fold higher than for heparin for EV and MV infections, respectively.
Figure 5.3: Effect of additional chemically modified heparins on MV-GFP and EV-GFP infections in vitro. 143B cells were infected with purified MV-GFP or EV-GFP viruses at a MOI of 1, in the presence of doubling dilutions of different chemically modified heparins, namely de-carboxylated (deCarboxyl) (A), de-N-sulfate, re-N-acetylated (deNS, reNAc) (B), de-2-O-sulfate, de-N-sulfate and re-N-acetylated (de2S, deNS/reNAc) (C) and de-sulfate, re-N-acetylated (deS, reNS) (D), at concentrations ranging from 0.09 μg/mL to 100 μg/mL. Adherent 143B cells were released with trypsin at 6 hr post infection, fixed, and analyzed using flow cytometry for GFP expression. Diagrammatic representation of different chemically modified heparins is shown next to line plots for respective modified heparins*. Representative line plots show mean (± SEM; n=3) percentage of GFP positive cells relative to control cells infected with the viruses in the absence of inhibitors (100% positive control). The ability of unmodified heparin to inhibit MV-GFP and EV-GFP infections (dashed lines) is included in each graph for comparison (see Figure 4.7). The data presented are representative of three independent experiments.

*For simplicity, iduronic acid is depicted as the hexuronic acid in the heparin disaccharide, but it equally could be glucuronic acid.
Finally, although chemical treatment of heparin to remove all 3 types of sulfate groups from the polysaccharide chain resulted in the total loss of inhibitory activity (Figure 5.2D), re-N-sulfation of desulfated heparin resulted in only a slight restoration of inhibitory activity, based on IC50 values, deS, reNS heparins being 200-fold less effective than heparin at inhibiting EV infections (Table 5.1). Furthermore, re-N-sulfation had no detectable effect on the inability of desulfated heparin to inhibit MV infections (Figure 5.3D). Collectively, these data suggest that the 6-O-sulfates on glucosamine residues are the most important negatively charged groups on heparin chains that interact with VACV.

### 5.3.3 Differences in VACV infectivity in the presence of synthetic sulfated oligosaccharides (HS mimetics)

Heparin and HS have been studied extensively and have been shown to interact with a wide range of structurally diverse proteins, such as growth factors, proteases, lipases, cell adhesion molecules and cytokines (Bernfield et al., 1999; Capila & Linhardt, 2002; Esko & Selleck, 2002; Gallagher, 2001; Perrimon & Bernfield, 2000; Turnbull, Powell, & Guimond, 2001). This plethora of interactions, as discussed previously, results in structurally diverse heparin having many undesirable side effects in vivo. There is enough evidence, however, to suggest that heparin/HS binding proteins interact with unique saccharide sequences in heparin/HS (Esko & Selleck, 2002; Gallagher, 2001; Turnbull et al., 2001). In fact, several HS mimetics have been developed which have anti-angiogenic and anti-inflammatory activity like heparin, but lack the undesirable interactions of heparin with other HS binding proteins (Freeman et al., 2005). The most important finding from these HS mimetic studies was that the interaction of many proteins with
heparin/HS is critically dependent on the relative position of negatively charged sulfate groups in heparin/HS molecules (Freeman et al., 2005).

Many viruses have been shown to use HS as a co-receptor for infecting cells (Zhu et al., 2011). In fact, studies have shown that HS is the second largest group of carbohydrate-based receptors for many human viruses (Olofsson & Bergstrom, 2005). As a result, a number of small soluble polysulfated compounds mimicking cell surface HS and, thus, having a broad spectrum of antiviral activity, have been developed. Most of these compounds were first reported many years ago, however, the mechanisms underlying their antiviral activity are still unclear (Baba et al., 1988; Ito et al., 1987).

In order to identify synthetic heparins that could be used as antivirals for VACV infections, sulfated di- and oligo-saccharides that are easy to synthesize, and have been shown previously to have certain biological activities including anti-tumour (Kuda, Yano, Matsuda, & Nishizawa, 2005), anti-apoptotic (Kim, Kim, Kim, Lee, & Lee, 2006), anti-inflammatory (Neyrinck, Mouson, & Delzenne, 2007; Nishimura et al., 2010) and anti-coagulant (Miao, Ishaimichaeli, Peretz, & Vlodavsky, 1995) activities, were examined for their effects on VACV infections in vitro. In fact, four D-glucose-based saccharides were used with differing linkages (i.e., α(1→4), α(1→6), β(1→4) and β(1→3)), which are depicted in Table 5.2.

Previous studies have shown that sulfated maltotetraose (an oligosaccharide based on maltose), but not sulfate maltose, to be a potent inhibitor of angiogenesis (Parish, Freeman, Brown, Francis, & Cowden, 1999). Isomaltose is another D-
glucose-based disaccharide similar to maltose, however, it has an α(1→6)-linkage instead of an α(1→4)-linkage (Table 5.2) (Gaenzle & Follador, 2012). Sulfated forms of both disaccharides, and other longer chain sulfated oligosaccharides based on maltose and isomaltose molecules, were used as inhibitors of VACV to determine if these simple sulfated sugars can act as antivirals. Among the sulfated malto-saccharides tested, maltopentaose appeared to be the most potent inhibitor of EV infectivity with an IC50 value only 4-fold higher than that of heparin (Figure 5.4D, Table 5.3). Maltotriose sulfate was not as potent inhibitor of EV infection as maltopentaose sulfate, but was more effective than maltose and maltotetraose sulfate (Figure 5.4A, B, C and D, Table 5.3), maltotriose sulfate being 16-fold and maltose and maltotetraose sulfate being 50-fold less effective than heparin at inhibiting EV infections (Table 5.3). None of the sulfated malto-saccharides achieved IC50 values for MV infections, although the three sulfated malto-oligosaccharides were partially inhibitory, with sulfated maltopentaose being the most active (Figure 5.4).

Among the three sulfated isomalto-oligosaccharides available for use as inhibitors of VACV infection, isomaltopentaose sulfate appeared to be the most effective inhibitor of EV and MV infections, with the IC50 values for EV and MV infections being, respectively, 8-fold and 5-fold higher than for heparin (Figure 5.5B). Both the other two sulfated isomalto-oligosaccharides used, sulfated isomaltotetraose and isomaltohexaose, were much less effective inhibitors of EV infections (Figure 5.5A and C), being 49-fold less active than heparin (Table 5.3). Similarly, isomaltotetraose and isomaltohexaose sulfate were weak inhibitors of MV infections, barely achieving IC50 values (Figure 5.5 A and C, Table 5.3). Overall,
Table 5.2: Structure of different D-glucose based sulfated saccharides (HS mimetics) used in this study.

<table>
<thead>
<tr>
<th>Sulfated Saccharide Series</th>
<th>Linkages</th>
<th>Chemical Structure</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maltose</td>
<td>α(1→4)</td>
<td><img src="image" alt="Maltose" /></td>
</tr>
<tr>
<td>Isomaltose</td>
<td>α(1→6)</td>
<td><img src="image" alt="Isomaltose" /></td>
</tr>
<tr>
<td>Cellobiose</td>
<td>β(1→4)</td>
<td><img src="image" alt="Cellobiose" /></td>
</tr>
<tr>
<td>Laminaribiose</td>
<td>β(1→3)</td>
<td><img src="image" alt="Laminaribiose" /></td>
</tr>
</tbody>
</table>
Figure 5.4: Effect of sulfated malto-saccharides on MV-GFP and EV-GFP infections in vitro. 143B cells were infected with purified MV-GFP or EV-GFP viruses at a MOI of 1, in the presence of doubling dilutions of different sulfated malto-saccharides, namely maltose (1155 kDa) (A), maltotriose (1625 kDa) (B), maltotetraose (2090 kDa) (C), and maltopentaose (2562 kDa) (D), at concentrations ranging from 0.09 μg/mL to 100 μg/mL. Adherent 143B cells were released with trypsin at 6 hr post infection, fixed, and analyzed using flow cytometry for GFP expression. Representative line plots show mean (± SEM; n=3) percentage of GFP positive cells relative to control cells infected with the viruses in the absence of inhibitors (100% positive control). The ability of unmodified heparin to inhibit MV-GFP and EV-GFP infections (dashed lines) is included in each graph for comparison (see Figure 4.7). The data presented are representative of three independent experiments.
none of the sulfated malto- and isomalto-oligosaccharides tested could inhibit VACV infections as effectively as some of the LMWHs and chemically modified heparins described in sections 5.3.1 and 5.3.2 of this thesis.

Cellobiose, derived from cellulose, is another D-glucose based saccharide with, in this case, a β(1→4) linkage (Table 5.2). Sulfated cello-oligosaccharides have been extensively tested for their anticoagulant activity, with sulfated cellotetraose being the shortest sulfated cello-oligosaccharide exhibiting significant anticoagulant activity (Wall, Douglas, Ferro, Cowden, & Parish, 2001). The three sulfated oligosaccharides derived from cellulose available for testing, namely cellotetraose, cellopentaose and cellohexaose sulfate (Figure 5.5D, E and F), overall appeared to be better inhibitors of VACV infections compared to the sulfated malto- and isomalto-oligosaccharides used previously in this Chapter. In fact, all three cello-oligosaccharides exhibited comparable antiviral activity against both the EV and MV infections, being ~8-16-fold less effective than heparin (Table 5.3).

The last set of oligosaccharides tested for their ability to inhibit VACV infections were the laminari-saccharides, derived from the storage polysaccharide laminarin present in brown algae. Sulfated laminari-saccharides are β(1→3) linked glucose molecules (Table 5.2), which have previously been shown to exhibit low anticoagulant activity compared to sulfated malto-, isomalto- and cello-saccharides (Wall et al., 2001). Four sulfated laminari-saccharides including, laminaribiose, laminaritriose, laminaritetraose and laminaripentaose, were tested for their ability to inhibit VACV infections in vitro (Figure 5.6). All four sulfated laminari-saccharides had little or no effect on VACV infectivity, being 100-500-fold less
Table 5.3: Comparison of IC50 values of various sulfated saccharides (HS mimetics) for EV and MV infections *in vitro*.

<table>
<thead>
<tr>
<th>Sulfated saccharide</th>
<th>IC50 (μg/mL)</th>
<th>EV</th>
<th>[Fold change]§</th>
<th>MV</th>
<th>[Fold change]§</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heparin</td>
<td>0.19</td>
<td>4.68</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Maltose</td>
<td>50 [263x]</td>
<td>&gt; 100 [&gt; 21x]</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Maltotriose</td>
<td>3.125 [16x]</td>
<td>&gt; 100 [&gt; 21x]</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Maltotetraose</td>
<td>50 [263x]</td>
<td>&gt; 100 [&gt; 21x]</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Maltpentaose</td>
<td>0.78 [4x]</td>
<td>&gt; 100 [&gt; 21x]</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Isomaltoolactose</td>
<td>9.38 [49x]</td>
<td>&gt; 100 [&gt; 21x]</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Isomaltopentaose</td>
<td>1.56 [8x]</td>
<td>25 [5x]</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Isomaltohexaose</td>
<td>9.38 [49x]</td>
<td>100 [21x]</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cellotetraose</td>
<td>1.56 [8x]</td>
<td>50 [11x]</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cellopentaose</td>
<td>1.56 [8x]</td>
<td>37.5 [8x]</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cellohexaose</td>
<td>3.125 [16x]</td>
<td>50 [11x]</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Laminaribiose</td>
<td>75 [395x]</td>
<td>&gt; 100 [&gt; 21x]</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Laminaritriose</td>
<td>100 [526x]</td>
<td>&gt; 100 [&gt; 21x]</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Laminartetraose</td>
<td>25 [132x]</td>
<td>&gt; 100 [&gt; 21x]</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>laminarpentaose</td>
<td>75 [395x]</td>
<td>&gt; 100 [&gt; 21x]</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PI-88 (Muparfostat)</td>
<td>3.125 [16x]</td>
<td>1.17 [0.25x]</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

§ Fold change ratios calculated by dividing IC50 values of each sulfated saccharide for each virus form by the IC50 value of heparin for each virus form.
effective than heparin against EV and exhibiting no detectable inhibitory activity against MV (Figure 5.6, Table 5.3).

5.3.4 Differences in VACV infectivity in the presence of the anti-cancer drug PI-88 (Muparfostat)

PI-88 is a highly sulfated synthetic oligosaccharide composed of predominantly penta- and tetra- saccharides derived from the extracellular phosphomannan produced by the yeast *Pichia holstii* (Khachigian & Parish, 2004; McKenzie, 2007). Mild acid-catalyzed hydrolysis of the phosphomannan liberates a number of oligosaccharides which, upon exhaustive sulfation, result in a highly sulfated mixture of oligosaccharides, referred to as PI-88 or Muparfostat (Khachigian & Parish, 2004). These sulfated oligosaccharides are structurally and functionally different from small heparin-like-polyaromatic anionic-type compounds and other synthetic HS mimetics (Benezra et al., 1994; Parish et al., 1999). Furthermore, PI-88 is a multi-component mixture, composed predominantly of phosphomannopentaose and phosphomannotetraose sulfates (Figure 5.7A and B), which accounts for over 90% of the mixture (Yu et al., 2002). However, small quantities of the di-, tri-, and hexa-phosphomannosulfates and a phosphotetrasaccharylamine sulfate are also present in the PI-88 mixture (Ferro, Fewings, Palermo, & Li, 2001).

PI-88 is an effective inhibitor of the HS degrading endoglycosidase HPSE and is the only HPSE inhibitor to date that has reached advanced clinical trials testing, currently being in a Phase III clinical trial as a monotherapy in post-resection hepatocellular carcinoma patients (McKenzie, 2007). Apart from preventing
Figure 5.5: Effect of isomalto- and cello-oligosaccharides on MV-GFP and EV-GFP infections in vitro. 143B cells were infected with purified MV-GFP or EV-GFP viruses at a MOI of 1, in the presence of doubling dilutions of different isomaltose and cellobiose based sulfated oligosaccharides, namely isomaltitetraose (2090 kDa) (A), isomaltopentaose (2562 kDa) (B), isomaltohexaose (3020 kDa) (C), cellotetraose (2090 kDa) (D), cellopentaose (2562 kDa) (E), and cellohexaose (3020 kDa) (F), at concentrations ranging from 0.09 μg/mL to 100 μg/mL. Adherent 143B cells were released with trypsin at 6 hr post infection, fixed, and analyzed using flow cytometry for GFP expression. Representative line plots show mean (± SEM; n=3) percentage of GFP positive cells relative to control cells infected with the viruses in the absence of inhibitors (100% positive control). The ability of unmodified heparin to inhibit MV-GFP and EV-GFP infections (dashed lines) is included in each graph for comparison (see Figure 4.7). The data presented are representative of three independent experiments.
Figure 5.6: Effect of sulfated laminari-oligosaccharides on MV-GFP and EV-GFP infections *in vitro*. 143B cells were infected with purified MV-GFP or EV-GFP viruses at a MOI of 1, in the presence of doubling dilutions of different laminari-oligosaccharides, namely laminaribiose (1125 kDa) (A), laminaritriose (1626 kDa) (B), laminaritetraose (2090 kDa) (C) and laminaripentaose (2562 kDa) (D), at concentrations ranging from 0.09 μg/mL to 100 μg/mL. Adherent 143B cells were released with trypsin at 6 hr post infection, fixed, and analyzed using flow cytometry for GFP expression. Representative line plots show mean (± SEM; n=3) percentage of GFP positive cells relative to control cells infected with the viruses in the absence of inhibitors (100% positive control). The ability of unmodified heparin to inhibit MV-GFP and EV-GFP infections (dashed lines) is included in each graph for comparison (see Figure 4.7). The data presented are representative of three independent experiments.
**Figure 5.7: Chemical structure of the anti-cancer drug PI-88 (Muparfostat) and the effect of PI-88 on MV-GFP and EV-GFP infections in vitro.**

Diagrammatic representation of the chemical structure of PI-88, adapted from Khachigian & Parish (2004). PI-88 is a mixture predominantly (~90%) composed of (A) phosphomannopentaose and (B) phosphomannotetraose sulfates, with the pentasaccharide to tetrasaccharide ratios ranging from approximately 2:1 to 3:2. The remaining 10% of the PI-88 mixture is composed of di-, tri-, and hexa-phosphomannosulfates, and a phosphotetrasaccharyl amine. (C) 143B cells were infected with purified MV-GFP or EV-GFP viruses at a MOI of 1, in the presence of doubling dilutions of PI-88 at concentrations ranging from 0.09 μg/mL to 100 μg/mL. Adherent 143B cells were released with trypsin at 6 hr post infection, fixed, and analyzed using flow cytometry for GFP expression. Representative line plots show mean (± SEM; n=3) percentage of GFP positive cells relative to control cells infected with the viruses in the absence of inhibitors (100% positive control). The ability of unmodified heparin to inhibit MV-GFP and EV-GFP infections (dashed lines) is included in each graph for comparison (see Figure 4.7). The data presented are representative of three independent experiments.
A

B

R = SO₃Na or H

C

GFP Positive Cells (% Control)

PI-88 concentration (µg/mL)
metastasis of tumours by inhibiting HPSE, PI-88 also reduces tumour growth by inhibiting angiogenesis via blocking the action of several HS-binding growth factors (Khachigian & Parish, 2004). It also has some anticoagulant activity as it is able to enhance heparin cofactor II inhibition of thrombin (Wall et al., 2001). Moreover, additional studies have demonstrated that PI-88 can act as an inhibitor of arterial restenosis following balloon angioplasty (Francis et al., 2003). Since PI-88 is a well-characterized sulfated oligosaccharide mixture that has been used extensively in vivo, we examined the effect of this compound on VACV infections. Adherent 143B cells were infected with purified MV-GFP and EV-GFP forms of VACV in the absence or presence of PI-88 as described earlier for other sulfated oligosaccharides. The inhibition curves generated from the assay were rather unexpected (Figure 5.7C). PI-88 was found to be the only sulfated oligosaccharide identified so far that is able to inhibit MV infections more efficiently than EV infections, with IC50 values for MV infections being 1.17 μg/mL compared to 3.125 μg/mL for EV infections. Moreover, PI-88 was a 4-fold better inhibitor of MV infections than heparin. On the other hand, with EV infections, heparin was a better inhibitor (i.e., 16-fold) than PI-88 (Figure 5.7C).
5.4 Discussion

The HS chains of HSPGs are extraordinarily diverse structures, with differences in positions of the sulfate groups along the length of the chain, and the epimerization of glucoronic acid residues to iduronic acid, giving rise to over 45 different disaccharides combinations that could potentially occur in any given HS chain (Parish, 2006). There is also much evidence to support the view that unique structural motifs in HS, mainly composed of negatively charged carboxyl and sulfate groups, interact and bind to different HS-binding proteins (Esko & Selleck, 2002; Gallagher, 2001; Lindahl & Kjellen, 2013; Turnbull et al., 2001). In fact, HS polysaccharides are capable of binding a large number of proteins and can, therefore, act as regulators of a variety of biological processes (Casu & Lindahl, 2001). One of the very well characterized protein binding sites in HS chains is the antithrombin III binding pentasaccharide sequence of heparin (Bauer et al., 2001). The antithrombin binding pentasaccharide contains four sulfate groups, all of which are essential for antithrombin III binding, thereby resulting in blood anticoagulation activity (Casu & Lindahl, 2001).

However, there are several limitations on the clinical use of heparin based on its pharmacokinetic, biophysical and anticoagulant properties (Hirsh, 1995, 1998). One of the major limitations of heparin is its ability to bind to several proteins and cells non-specifically due to its high negative charge (Barzu, Molho, Tobelem, Petitou, & Caen, 1985; Hirsh, 1991). Heparin is also unable to inactivate surface-bound thrombin-factor Xa complexes and, therefore, has limited efficacy in unstable angina, high-risk coronary angioplasty, and coronary thrombolysis (Hirsh, 1995). Apart from these pharmacokinetic and biophysical limitations, there
are other biological limitations associated with the use of heparin. Platelet activation due to heparin binding has been shown to stimulate the formation of antibodies against heparin-platelet factor 4 complexes that cause heparin-induced thrombocytopenia (Kelton et al., 1994). Moreover, long-term heparin treatment has also been associated with osteoporosis (Barbour et al., 1994; Monreal, Lafoz, Olive, Delrio, & Vedia, 1994).

These limitations prompted the development of a new class of anticoagulants, called the low-molecular-weight heparins (LMWHs) that are usually derived from naturally occurring unfractionated heparin. LMWHs are much smaller than heparin chains, which reduces their affinity for many plasma proteins and, therefore, produces more predictable results when they are administered to patients compared to heparin. Use of LMWH, therefore, reduces the side effects associated with the long-term use of heparin-based antithrombotic drugs, which is why LMWHs started to replace heparin for the prevention of DVT in the mid-1980s (Franze et al., 2015; Mousa, 2002).

In Chapters 3 and 4 of this thesis, it was established that cell surface HS can act as a potential co-receptor for MV and EV entry into cells in vitro. It has been demonstrated in the current study that heparin can inhibit both MV and EV infections, however, with different efficiencies (Figure 4.7). Despite the inhibitory effects of heparin on VACV infectivity, previously published studies summarized above have reported the unwanted side effects associated with prolonged heparin administration in patients. However, the fact that several attempts have been made in the field of glycobiology to develop safe carbohydrate-based drugs, and
the knowledge that VACV, like many other viruses, interacts with cell surface GAGs (Zhu et al., 2011), resulted in studies reported in this thesis to determine whether LMWHs, chemically modified heparins and other biologically active HS mimetics with superior safety profiles to heparin, can inhibit VACV infections by interfering with the VACV-HS interaction. Thus, a highly systematic approach was undertaken to identify potentially new and safe-carbohydrate-based antivirals.

Initially, commonly used LMWHs were used in VACV inhibition assays, as performed previously in section 4.3.6. Fondaparinux, a synthetic pentasaccharide sequence structurally based on the heparin antithrombin III binding domain, was the most effective of all the LMWHs used as possible inhibitors of VACV infections. In fact, fondaparinux was able to inhibit MV and EV infections almost as efficiently as untreated heparin. Furthermore, enoxaparin and two other LMWHs (4-6 kDa Fluka and 5 kDa LMWH) were also able to inhibit both MV and EV infections efficiently, although not as well as heparin or fondaparinux. This finding suggests that the length of the GAG chain is irrelevant when considering inhibition of the VACV-HS interaction, as the 1.7 kDa fondaparinux pentasaccharide is able to effectively inhibit VACV infections in vitro. This finding is very encouraging as it clearly indicates that the VACV-HS interaction is highly specific and not dependent on weak multivalent binding by a long chain, negatively charged HS molecule. In fact, the strong inhibitory activity of fondaparinux for both forms of VACV strongly suggests that both MV and EV forms of VACV recognize and interact with a small pentasaccharide sequence in HS/heparin. Furthermore, since fondaparinux is based on the heparin antithrombin III binding sequence, it is likely that the same sequence is essential for VACV infections as well (Figure 5.1A). Our findings, along
with other studies determining rare sulfation sequences along the length of HS chains, raises the notion of a so called 'sulfation code’, which entails sulfated sequences along the length of HS chains that permit selective, high avidity, protein binding patterns (Lindahl & Kjellen, 2013).

To validate the ‘sulfation code’ hypothesis, it was important to understand how individual modifications of HS chains impact on the ability of HS to interact with VACV. Previous detailed studies investigating HSV attachment and infection of cells in vitro revealed that 2,3-O-sulfate and 6-O-sulfate groups are critical for heparin to interact with the gB and gC glycoproteins on the surface of HSV-1 (Feyzi et al., 1997; Trybala et al., 2000). Sulfate groups on heparin are important for inhibition of both MV and EV infectivity, with the removal of 6-O-sulfate groups from heparin chains having a detrimental effect on the ability of heparin to inhibit VACV infections. However, when all the inhibition results observed with the different desulfated heparins (Figures 5.2 and 5.3) are collated, it becomes evident that all 3 sulfation sites on heparin chains contribute to inhibition of VACV infections. For example, despite removal of 2-O-sulfate groups alone only having a modest effect on the ability of heparin to inhibit VACV infections, when a second sulfate group is removed from heparin (i.e., N-desulfation, N-reacetylation), the effects were dramatic with the doubly desulfated heparin being unable to inhibit VACV infections. Previous reports have shown that the 6-O-sulfate group is the most critical group on the glucosamine residues required for heparin’s anti-inflammatory effects, which are mediated by selectin inhibition (Wang et al., 2002). Results presented in Figure 5.2B also highlight the key role of 6-O-sulfate groups in aiding inhibition of VACV infectivity.
Acetylation of all glucosamine residues along the heparin chains did not markedly influence the inhibitory ability of heparin for VACV infections, nor did removal of the carboxyl groups of the iduronic/glucuronic acid residues of heparin. This is an important finding as it defines the structural features of heparin/HS that are not required for antiviral activity. Such a detailed understanding of heparin/HS chain structure is crucial for the development of antivirals that are not only potent, but also safe to be administered. Several studies published previously have established that HS is the second largest group of carbohydrate-based cell surface receptors for human viruses (Olofsson & Bergstrom, 2005). This has led to the development of HS mimetics as antiviral compounds (Pourianfar, Poh, Fecondo, & Grollo, 2012). The findings reported in this thesis represent one of the few attempts made at understanding the specificity of virus-HS interactions, with VACV being the model virus studied.

As mentioned earlier in this Chapter, heparin has been shown to be an excellent anticoagulant, however, prolonged use of heparin in vivo results in several undesirable side effects due to the ability of heparin to interact with a range of structurally diverse proteins and growth factors (Bernfield et al., 1999; Capila & Linhardt, 2002; Esko & Selleck, 2002; Gallagher, 2001; Perrimon & Bernfield, 2000; Turnbull et al., 2001). This led to the development of sulfated oligosaccharides (HS mimetics) using naturally available saccharides which, like heparin, possess the anti-angiogenic and anti-inflammatory properties of heparin, but lack many undesirable side effects (Freeman et al., 2005). Since several previously published studies, and the initial work described in this thesis, have highlighted the role of cell surface HS as a co-receptor for VACV entry, and shown
that heparin is able to inhibit VACV infections \emph{in vitro}, several sulfated HS mimetics with known anti-coagulant, anti-inflammatory and/or anti-angiogenic, properties were screened to determine if they can also act as antivirals against VACV infections. Sulfated malto-, isomalto-, cello- and laminari-saccharides were used in the assays presented in the current chapter.

In general, many of the sulfated di- and oligo-saccharides tested could not efficiently inhibit MV or EV infections when compared to heparin. Only sulfated maltopentaose from the malto-oligosaccharide series, and sulfated isomaltopentaose from the isomalto-oligosaccharide series had inhibitory activity approaching that of heparin. It was also shown that fondaparinux, also a pentasaccharide, was the most effective LMWH (Figure 5.1A). Both these findings suggest that an ideal carbohydrate based inhibitor of VACV infectivity would be a sulfated pentasaccharide sequence. In terms of the saccharide backbones used, overall the cellulose derived sulfated saccharides were the most effective VACV inhibitors used in this study. In fact, the data obtained indicate that the linkage of different D-glucose-based sulfated saccharides has a profound effect on the ability of the sulfated saccharides to inhibit VACV infections, with the order of potency being $\beta(1\rightarrow4) > \alpha(1\rightarrow6) > \alpha(1\rightarrow4) > \beta(1\rightarrow3)$, and sulfated pentasaccharides being usually the most effective. Such data support the hypothesis that the position of negatively charged sulfate groups in 3D space determines the antiviral activity of these molecules and, therefore, builds on the notion of a ‘sulfation code’.

One of the most interesting results obtained from the VACV inhibition assays using HS mimetics, however, was obtained when PI-88 was used an inhibitor of MV and
EV infections. PI-88, a highly sulfated mixture of D-mannose-based saccharides, is structurally and functionally different from heparin and the other HS mimetics tested in this study (Benezra et al., 1994; Parish et al., 1999). VACV infection assays performed using PI-88 as a potential inhibitor of infection revealed that PI-88, unlike all the LMWHs, modified heparins and HS mimetics tested, acts as a better inhibitor of MV infections than of EV infections (Figure 5.7). In fact, PI-88 was able to inhibit MV infectivity 4-fold more efficiently than heparin, a finding not seen with any of the LMWHs, modified heparins or other HS mimetic assayed. On the other hand, PI-88 was 16-fold less efficient than heparin at inhibiting EV infections.

To understand these results, it is essential to understand the composition of the PI-88 mixture. Firstly, PI-88 is based on the monosaccharide D-mannose, whereas all the other HS mimetics described in this Chapter are D-glucose-based saccharides. Secondly, PI-88 is composed of $\alpha(1 \rightarrow 3)$ and $\alpha(1 \rightarrow 2)$ linked D-mannose residues (Yu et al., 2002), which differs from the linkages of the D-glucose-based HS mimetics examined in this Chapter. Therefore, the remarkable effectiveness of PI-88 against MV infections but not EV infections, could be attributed to the $\alpha(1 \rightarrow 3)$ and $\alpha(1 \rightarrow 2)$ linkage of mannose residues resulting in an orientation of sulfate groups that blocks selectively the interactions of MV particles with cell surface HS.

A third feature that distinguishes PI-88 from the other GAG-based molecules used in this study is its structural heterogeneity. PI-88 is mainly composed of pentasaccharide and tetrasaccharide sulfates, but the mixture also contains small quantities of di-, tri- and hexa-phosphomannosulfates and a phosphotetrasaccharylamine sulfate (Ferro et al., 2001). Such a heterogeneous mixture perhaps targets multiple HS binding sites on the surface of MV particles and, therefore, blocks the particles from interacting with cell surface HS. In fact, PI-
88 has previously been demonstrated to have a significant beneficial effect on disease outcome in dengue virus and flaviviral encephalitis mouse models, such that the mortality rate in PI-88 treated mice was reduced to 39% compared to 84% in saline treated control mice (Lee, Pavy, Young, Freeman, & Lobigs, 2006). Furthermore, results obtained from PI-88 inhibition of VACV infections also support the hypothesis that cell surface HS receptors for MV and EV are structurally different.

Overall, the data presented in this Chapter support the concept that GAG-based antiviral drugs can be developed. Furthermore, a detailed understanding of the structural features of heparin that allow it to inhibit VACV infections has provided valuable insights into the groups/modifications of cell surface HS chains that aid VACV infections. In addition, the sulfated di- and oligo-saccharide-based HS mimetics used in this study support the view that the specificity of the HS-VACV interaction differs markedly between the MV and EV forms of VACV. Finally, the major heparin-/HS-related bioactivity targeted clinically in the past has been antithrombotic activity, however, the data presented in this Chapter suggest that other clinical uses of modified heparins/HS mimetics, notably as antivirals, are feasible.
Studies of the interaction between EV and MV surface proteins and HS/heparin

Chapter 6:
6.1 Abstract

Since several viruses and other pathogens use HS as initial attachment receptors, identifying EV and MV surface proteins interacting with cell surface HS would be vital for the development of novel HS based antiviral drugs. Membrane proteins from purified EV and MV particles were obtained using mild detergent treatment and were pre-incubated with heparin-coupled beads, followed by detection via Western blotting using polyclonal VACV-specific antibodies. Four potential heparin-binding proteins were identified with molecular weights of 150 kDa, 85 kDa, 60 kDa and 25 kDa, with the 85 kDa protein being distinct from the 85 kDa VACV haemagglutinin A56R. Bioinformatic searches performed to identify VACV proteins with HS binding domains identified the 65 kDa B4R protein and the 78 kDa RNA helicase DExH-NPH-II of VACV as possible candidate proteins. Using 1D nanoLC ESI MS/MS analysis of the 150 kDa protein detected by Western blotting identified it to be a poxvirus DNA directed RNA polymerase with sequence similarity to VACV F12, a 65 kDa protein important in the formation of EV. It was concluded that the heparin/HS-binding site of many VACV EV proteins is not composed of linear amino acid sequences and that there may be multiple HS binding proteins on the surface of EV, all of which contribute towards infectivity.
6.2 Introduction and rationale

To understand the tropism of any viral infection it is important to first understand the interactions between the proteins on the surface of viruses and their cell surface receptors. Understanding and targeting such interactions could have implications for antiviral drug development. This is the reason why studying such interactions has lately gained considerable interest from the pharmaceutical and scientific communities. Developing strategies that simply block viruses from interacting with their cell surface receptors would likely be less toxic and intrusive compared to currently used treatments like chemotherapy (Liu & Thorp, 2002). Furthermore, there is increasing concern for the selection of drug-resistant strains of viruses due to prolonged use of antivirals, with immunocompromised patient populations being at the highest risk (Strasfeld & Chou, 2010). To overcome these issues, increasing efforts are being made to develop compounds that block virus particles from binding and entering target cells during early stages of infection and, therefore, possibly preventing infections caused by drug-resistant virus strains. Indeed, a series of compounds have been identified that bind gp120 glycoproteins on the envelope of HIV-1 and block the entry of HIV-1 virus particles (Si et al., 2004).

The role of HS as an adhesion receptor for several bacteria, parasites and viruses has been studied for more than two decades (Rostand & Esko, 1997; Wadstrom & Ljungh, 1999). Extensive research carried out over the years has established a role for cell surface HS in the infections of at least 19 different viruses (Liu & Thorp, 2002; Zhu et al., 2011). A large body of data suggests that viruses interact with HS to facilitate their binding to specific cell surface receptors (Chung et al., 1998;
Hsiao et al., 1999; Lin et al., 2000; Shieh, Wudunn, Montgomery, Esko, & Spear, 1992; Shukla et al., 1999; Tyagi, Rusnati, Presta, & Giacca, 2001). Furthermore, the foot and mouth disease and HSV-1 viruses have been shown to infect target cells via interaction with cell surface HS, even in the absence of known protein receptors on the target cells (Jackson et al., 1996; Spear, Eisenberg, & Cohen, 2000). All these findings, including the results presented in this thesis thus far, collectively indicate that there is an intimate relationship between the structure of HS polysaccharides and their activity in promoting viral infections.

So far, the research focus of this thesis has been to develop an understanding of the key structural features of cell surface HS required for VACV infections. Results presented in Chapters 4 and 5 convincingly show that small molecules, based on the HS polysaccharide structure, are able to inhibit VACV infections, the EV form of VACV being particularly susceptible to inhibition. However, to fully understand the process of heparin/HS mediated VACV inhibition, it was important to identify the proteins(s) on the surface of VACV responsible for such interactions. Therefore, the specific aim of this Chapter was to identify the protein(s) in the outer EV envelope responsible for the interaction with cell surface HS. In order to achieve this, EV particles were first purified and the VACV membrane proteins extracted using mild detergents. The proteins were visualized by Western blotting using polyclonal VACV specific antibodies. The extracted proteins were also subjected to mass spectrometry (MS) analysis for further identification. A bioinformatics based search was also conducted to identify HS binding domains in proteins expressed by VACV particles.
6.3 Results

6.3.1 Purification of the EV and MV forms of VACV from the IHD-J strain and extraction of membrane proteins

Much of the research on VACV infections in the past has focused on the MV form of VACV, as this form of the virus is relatively easy to purify and study. Indeed, several studies have already identified key proteins on the surface of the MV form that interact with cell surface HS (Chung et al., 1998; Lin et al., 2000) and CS (Hsiao et al., 1999). However, the results presented in previous Chapters suggest that heparin and other highly sulfated molecules based on the HS polysaccharide structure are unable to have a significant inhibitory effect on MV infectivity, compared with EV infectivity, where heparin acts as a much more potent inhibitor.

The EV form of VACV has a fragile outer membrane around the virus particle (Figure 3.5), which makes it difficult to purify intact EV particles for investigation. The EV form of VACV may only represent a fraction of the total VACV infection, however, it is extremely important biologically as this form of VACV is responsible for long range dissemination of the virus in cell cultures and in vivo (Appleyard G, Hapel, & Boulter, 1971; Payne, 1980; Payne & Kristensson, 1985). Therefore, it is important to characterize the receptors on the surface of the outer envelope of the EV particles that are involved in interacting with HS molecules during an infection. Some studies undertaken previously have demonstrated that the A34R (24-28 kDa) and B5R (42 kDa) glycoproteins on EV particles initiate the dissolution process of the outer membrane of EV particles upon contact with sulfated
Figure 6.1: Flow chart depicting the multi-step process used to obtain EV and MV membrane proteins. BHK-21 cells were infected with VACV-IHD-J at a MOI of 0.1. The culture medium over the infected monolayers (containing EV particles) was gently aspirated and centrifuged at 230xg for 10 min to remove any cell debris. The EV containing culture medium was further centrifuged at 18,000xg for 1 hr to pellet EV particles and resuspended in Tris-HCl buffer. In parallel, adherent cells (containing MV particles) were released by scraping the surface of the flask, centrifuged at 1800xg for 5 min to pellet cells and resuspended in Tris-HCl buffer. Cells were transferred to a dounce homogenizer where they were homogenized with 40 strokes to release MV particles. Once homogenized, the cells were centrifuged at 300xg for 5 min to remove any nuclei and other debris. The supernatant containing MV particles was further centrifuged at 32,000xg for 1 hr to pellet MV particles and resuspended in Tris-HCl. Both MV and EV fractions were detergent treated using a cocktail of Brij-58, β-mercaptoethanol and complete protease inhibitors, to release membrane proteins. The treated fractions of MV and EV particles were subsequently loaded on a caesium chloride (CsCl) gradient and centrifuged at 154,000xg for 30 min to separate solubilized outer membrane proteins from virus cores. The solubilized membrane proteins from MV and EV were carefully removed using a 29-gauge needle and syringe and transferred to respective 10 kDa cutoff ultrafiltration tubes. The ultrafiltration tubes were centrifuged at 3,200xg for 10 min to remove traces of CsCl from MV and EV membrane proteins. Once purified, MV and EV membrane proteins were transferred to respective 1.5 mL tubes.
VACV- IHD-J infected BHK-21 monolayers

Cell monolayer released by scraping. Centrifuged at 1800xg. Cells resuspended in Tris-HCl

Culture media over infected cells gently aspirated. Centrifuged at 230xg to remove nuclei/debris

Dounce homogenizer used to homogenize cells. Centrifuged at 300xg to pellet nuclei/cell debris

EV containing culture medium centrifuged at 18,000xg to pellet EV particles. EV particles resuspended in Tris-HCl

Released MV particles centrifuged at 32,000xg. MV pellet resuspended in Tris-HCl

Purified MV and EV fractions treated with a cocktail of Brij-58, β-mercaptoethanol and protease inhibitor.

Detergent treated mixtures of MV and EV fractions loaded on CsCl gradients. Centrifuged at 154,000xg to separate membrane proteins from core

Layer of purified membrane proteins over CsCl gradient removed using needle and syringe.

Proteins transferred to 10 kDa ultrafiltration tubes. Centrifuged at 3,200xg to remove CsCl.

Purified MV membrane proteins

Purified EV membrane proteins
polyanions (Law et al., 2006). However, assays reported herein strongly indicate that soluble heparin, or other highly sulfated HS structure-based small molecules, inhibit EV particles from infecting cells in vitro, possibly by inhibiting virus particles from interacting with cell surface HS. It is worth noting that results published by Law et al., were mainly obtained using plaque assays performed in the presence of MV neutralizing antibodies, whereas the results presented in this thesis have been obtained using a novel FACS based virus entry assay which detects early events in a VACV infection. The results presented in earlier Chapters strongly indicate that heparin, at least very early during infection, inhibits EV infectivity by potentially interfering with the virus/cell-surface HS interaction. It is likely that later on in the infection process the viral glycoproteins A34R and B5R play a role in the dissolution of the outer EV envelope to initiate virus entry into cells. Therefore, it was important to determine protein(s) on the surface of the EV form of VACV that bind heparin and could be potentially blocked in the presence of soluble heparin.

As a first step towards the identification of protein(s) on the surface of EV that interact with HS, the IHD-J strain of VACV was cultured as described in Chapter 2 to obtain high concentrations of the intact EV form of the virus. This was followed by gentle detergent treatment of both EV and MV particles to release proteins in the membranes of the two forms of VACV as also described in detail in Chapter 2. The process used for EV and MV purification, followed by protein extraction, is summarized in Figure 6.1.
6.3.2 SDS-PAGE and Western blotting of VACV membrane proteins

The extracted and concentrated EV and MV membrane proteins were visualized using Western blotting. However, prior to SDS denaturation, both EV and MV membrane protein extracts were pre-incubated overnight with heparin-Sepharose 6 fast flow beads to separate and enrich heparin-interacting membrane proteins from the rest of the mixture, as described in section 2.2.6. The ‘bound’ fraction represents membrane proteins from the MV and EV extracts that interact with and bind to the heparin-Sepharose beads, whereas the ‘unbound’ fraction represents proteins in the MV and EV extracts that did not interact with the heparin-Sepharose beads. The ‘crude’ fraction represents the total protein mixture extracted from both EV and MV forms of VACV. All the three fractions were subjected to SDS-PAGE and Western blotting, with the VACV proteins being detected using various antibodies.

The use of polyclonal anti-VACV antibodies revealed several VACV membrane proteins that interacted with heparin coupled Sepharose beads (Figure 6.2A). Several proteins appeared in both the ‘bound’ and ‘unbound’ lanes of the SDS-PAGE gel (an example highlighted by purple arrows in Figure 6.2A). It is likely that the proteins appearing in both ‘bound’ and ‘unbound’ fractions may, in fact, be proteins associated with other heparin binding proteins and, consequently, co-purifying as a result of incomplete solubilization of VACV particles. Another explanation for this observation could be that the proteins bind weakly to heparin-Sepharose beads. Saturation of binding sites on heparin-Sepharose beads may also result in some proteins appearing in ‘bound’ and ‘unbound’ fractions, however, this
possibility is unlikely as the binding capacity of heparin-Sepharose beads is extremely high.

There were at least 4 striking protein bands of interest that appeared on the blots (highlighted with red, yellow, blue and green arrows in Figure 6.2). The 150 kDa band (red arrows) appeared to be present in ‘bound’ fractions, but was depleted from the ‘unbound’ fractions. The same 150 kDa band was less intense in the ‘crude’ fractions compared to the respective ‘bound’ fractions. Furthermore, it is worth noting that this band appeared to be more intense in the MV membrane protein extracts compared to the EV membrane protein extracts, despite many other protein bands being of similar intensity in the ‘crude’ extracts from both EV and MV. Hence, it would be safe to presume that the overall concentration of proteins extracted from the MV and EV particles was relatively similar and, therefore, differences in the intensity of the 150 kDa band in the ‘crude’ MV extracts compared to the ‘crude’ EV extracts cannot be explained by there being differences in protein concentration. It is worth highlighting that the relative volumes of material ‘bound’ and eluted from the beads compared to the ‘unbound’ crude mixtures was kept the same (i.e., 100 μL), to ensure that there were no dilution effects.

The second interesting band was an 85 kDa protein band (highlighted with yellow arrows in Figure 6.2A). Similar to the 150 kDa band, the 85 kDa band appeared to be present in the ‘bound’ EV fraction but was depleted from the ‘unbound’ fraction, indicating that this protein bound strongly to heparin- Sepharose beads. A band of similar molecular weight also appeared in the MV extract but the band was not
fully depleted in the ‘unbound’ MV fraction. However, since a polyclonal antibody was used to detect VACV proteins, it is possible that there were multiple 85 kDa MV proteins being detected by the antibody. Nevertheless, it seemed unlikely that this 85 kDa protein was, in fact, the VACV hemagglutinin (HA) (A56R protein), a key component of the EV outer envelope. Molecular characterization of VACV HA revealed that the glycoprotein is present in two distinct forms, an 85 kDa form that appears very early during infection and continues to accumulate throughout the infection, and a smaller 68 kDa form which only appears late during the infection cycle (Brown, Turner, & Moyer, 1991). However, identifying the HA using a VACV HA (A56R)-specific mAb revealed that there was very little, if any, HA in the EV protein extract and heparin-binding fractions (Figure 6.2B). In contrast, the A56R VACV HA appeared to be abundant in the MV protein extract and fractions (green arrows in Figure 6.2B). The ‘crude’ MV protein extract yielded the most intense band, whereas the ‘bound’ fraction contained the least intense band, suggesting that VACV HA binds weakly to heparin-Sepharose beads. Most of the VACV HA did not bind to heparin-Sepharose beads and, therefore, appeared as an intense band in the ‘unbound’ fraction. Based on the Western blotting results obtained using the HA-specific mAb, it can be concluded that the 85 kDa heparin binding EV protein detected using the polyclonal VACV antiserum (yellow arrows in Figure 6.2A) is not the VACV HA.

A 60 kDa protein band was also detected (purple arrows in Figure 6.2A), being more abundant in the EV than the MV extract and appearing in both the ‘bound’ and ‘unbound’ fractions, implying that the protein binds weakly to heparin or
**Figure 6.2: Visualization of EV and MV membrane proteins using Western blotting.** The membrane proteins extracted from the EV and MV forms of VACV using gentle detergent treatments ('crude' extracts) were run on SDS-PAGE along with extracted membrane proteins that bound ('bound' fractions) or did not bind ('unbound' fractions) to heparin-Sepharose beads, and Western blotted using VACV specific antibodies. (A) Proteins on a nitrocellulose membrane detected using rabbit anti-VACV polyclonal antibodies. The red arrows indicate a 150 kDa protein that is present in crude EV and MV protein extracts and is enriched (darker band) in fractions binding to heparin-Sepharose beads, the 150 kDa band being depleted in the ‘unbound’ fractions of both EV and MV protein extracts. Yellow arrows indicate an 85 kDa protein in EV extracts that binds to heparin, and is depleted in the ‘unbound’ EV fraction. Purple arrows indicate an ~60 kDa protein that appears in approximately similar amounts in both the ‘bound’ and ‘unbound’ fractions of EV and MV proteins. The blue arrows identify a 25 kDa protein in the EV extract that is also a heparin-binding protein. Thus, similar to the 150 kDa band, the 25 kDa band is depleted in the ‘unbound’ EV protein fraction. (B) An anti-A56R mAb was used to determine if the A56R protein (VACV A56R) interacts with heparin, only the 85 kDa form of A56R being detected using this mAb. The “Control” lanes in both blots represent the protein extracted from uninfected cells.
associates with a heparin-binding protein. Also, there may be multiple VACV proteins in this region of the SDS-PAGE gel.

Both MV and EV derived proteins of <50 kDa appeared as faint smears on the blots and appeared in both the heparin-Sepharose ‘bound’ and ‘unbound’ fractions. However, from previously published studies, the proteins in the smear would likely include the 42 kDa trans-membrane and 35 kDa secreted form of B5R (Martinez-Pomares, Stern, & Moyer, 1993), the 24-28 kDa A34R (Duncan & Smith, 1992), the 35 kDa H3L (Lin et al., 2000) and, possibly, some other proteins that are thought to interact with heparin. Of course, interpretation of these data is difficult as many proteins may not be directly heparin binding but forming multi-molecular complexes with heparin binding proteins. Despite this complexity, a 25 kDa band was observed in the EV extract that bound to heparin (highlighted with blue arrows in Figure 6.2A). Unlike the 150 kDa and 85 kDa bands, the 25 kDa band was not as discrete, but was fully depleted in the ‘unbound’ EV fraction, indicating that this protein may also be another heparin binding protein. It is possible that this 25 kDa protein is also present in the MV extract, however, the band is extremely faint and appears as a smear.

Nevertheless, since the 150 kDa protein (red arrows), the 85 kDa (yellow arrows), the 60 kDa (purple arrows) and the 25 kDa protein (blue arrows) from EV protein extracts all appear to strongly interact with heparin, these four proteins were considered likely candidates to be on the surface of the EV envelope and be targets of heparin mediated inhibition of infection in vitro. Furthermore, it is possible that there are more heparin-binding proteins on the EV envelope that may not have
been detected on the blots using the polyclonal anti-VACV antibodies. Therefore, to further identify the EV protein(s) that appeared to be heparin binding via Western blotting and detect additional proteins on the surface of the EV envelope that could potentially interact with heparin or cell surface HS, a bioinformatics search approach was embarked upon.

6.3.3 Identification of HS binding EV proteins using a bioinformatics approach

There are two known HS binding proteins on the outer membrane of the MV form of VACV, namely the A27L (14 kDa) and the H3L (37.5 kDa) proteins (Chung et al., 1998; Lin et al., 2000). Furthermore, K2 (42.2 kDa), a serine protease inhibitor (SPI)-3 found on the surface of EV virus particles, has been shown to have heparin binding sites (Brum et al., 2003; DeHaven et al., 2011). The K2 protein also forms a complex with the A56R protein of VACV. To identify protein(s) on the outer membrane of EV particles that may interact with cell surface HS, a bioinformatic approach was undertaken to search for characteristic HS binding domains within all VACV proteins. Mr. Cameron Jack, a bioinformatician at the Genome Discovery Unit, ANU, initially characterized the amino acid sequences of known HS binding proteins by calculating the net positive charge of their HS binding motifs. The two HS binding motif templates used were XBBXBX and XBXXXXBX, where X is an uncharged residue and B is a basic amino acid. These two motifs were added to previously known HS binding motifs (Hileman, Fromm, Weiler, & Linhardt, 1998). The HS binding motif of VACV protein H3L has a net charge of +3, A27L has a net charge of +4 and K2 has a net charge of +4. Since A56R exists as a complex with K2, the net charge of A56R (+5) was also determined.
Based on the net charge information, custom designed python scripts were developed and used in Multiple Em for Motif Elicitation (MEME) (Bailey & Elkan, 1994) to restrict search results for protein sequences consisting of <300 residues and >900 residues. This was done to narrow the search results to proteins between 80 kDa and 150 kDa.

An attempt to improve detection through sequence similarity was made by using sequence “spike-ins” from known HS binding VACV proteins (K2 (accession #: YP_232915.1); H3L (accession #: YP_232983.1); A27L (accession #: YP_233032.1)), and VACV HA (accession #: YP_233063.1), to deliberately bias output to include HS binding regions. As a result nucleoside triphosphatase I (72 kDa DNA dependent ATPase; accession #: YP_232998.1) appeared in the search results. This search result was of a particular interest, as this does not have any known functional association with heparin/HS. However, since the triphosphatase must bind to ssDNA to exhibit its ATPase activity and that DNA is negatively charged like heparin, it is likely that the triphosphatase has sequence similarities to heparin binding proteins.

Some of the other search results included three different IL-1 receptor antagonists (accession #: YP_233091.1, YP_232892.1 and YP_232906.1). It is known that VACV encodes IL-1 receptor antagonists that interfere with the host immune response by blocking IL-1 (Alcami & Smith, 1992). This is an interesting finding, however, viruses generally secrete IL-1 receptor antagonists to block IL-1 receptors and evade detection. Therefore, it is unlikely that a soluble IL-1 receptor antagonist would inhibit EV infectivity when in contact with heparin.
Based on this argument the above search results were removed and MEME was subsequently re-run, this time with widened parameters to include shorter protein sequences from 100 residues upwards (to 900 residues maximum), leaving in place true positives and removing known false positives. The output appeared biased towards these newly introduced low molecular weight proteins but did not return any expected motifs.

Restricting the protein search list to higher molecular weight VACV proteins (implying less post-translational modifications) was more productive. Proteins consisting of <450 amino acid residues in length were removed from the search list. These modified scripts, when run again on MEME, identified the 65 kDa VACV ankyrin/F-box B4R protein (accession #: Y233068.1) as containing a possible HS-binding motif (residues: KHRWHNA) with a net positive charge of +4, placing it firmly in the range of likely candidate sites. Another protein that was identified based on this modified search was the 77.6 kDa RNA helicase DExH-NPH-II domain (accession #: YP_232959.1) with a net positive charge of +4 (residues: KHKQHNA).

Since MEME uses the residue frequencies of the supplied sequences as the control for calculating relative entropy scores (Bailey & Elkan, 1994) for short sequence lists, there is always potential for random positives. For instance, a protein hit containing one or more ‘discovered motifs’ may in fact be a protein with well-characterized functions and therefore may not be the protein of interest. However, it is well known that viruses continue to evolve to produce viral proteins which can perform multiple functions (Hasiow-Jaroszewska, Fares, & Elena, 2014). Therefore, the ‘positive’ hits obtained from such a BLAST search cannot be
disregarded as false positive hits just because the identified proteins have another well-characterized function. The bioinformatic search for HS-binding EV membrane proteins was informative, nevertheless, the 150 kDa protein (red arrows in Figure 6.2A) and the 85 kDa protein (yellow arrows in Figure 6.2A) observed in Western blotting analysis of EV membrane proteins could not be identified using this approach.

6.3.4 The use of 1D nanoLC ESI MS/MS to identify EV proteins that interact with heparin/HS

The bioinformatics search performed as part of the identification process of heparin/HS interacting EV membrane protein(s), identified several protein hits with possible heparin binding domains. However, the 150 kDa protein (red arrows in Figure 6.2A) observed on Western blots developed using a polyclonal anti-VACV antiserum, could not clearly be identified using a bioinformatic approach. Therefore, the ~150 kDa protein band was excised from a freshly run SDS-PAGE gel and sent to the Australian Proteome Analysis Facility (APAF) where a team led by Dr. Xiaomin Song performed 1D nano-scale liquid chromatography electrospray ionization tandem mass spectrometry (1D nanoLC ESI MS/MS) on the protein sample. Unfortunately, due to time restrictions, the 85 kDa band (yellow arrows in Figure 6.2A) and the 25 kDa band (blue arrows in Figure 6.2A) could not analyzed using 1D nanoLC ESI MS/MS prior to thesis submission.

The ESI MS/MS technique of identifying peptides in protease digested complex mixtures of proteins is very well established. Coupling of liquid chromatography to electrospray ionization mass spectrometry allows for highly efficient identification
of peptides and increased sample throughput (Yang et al., 2007). The proteins containing peptide hits obtained from this analysis of the ~150 kDa protein band are presented in Table 6.1. An encouraging aspect of the APAF output was that all of the high scoring hits detected were poxvirus-specific. This indicates that the VACV extracts were of high purity and were not contaminated with proteins derived from the cell line, which the two VACV forms were cultured in.

Similar to the bioinformatic searches, proteins with well-characterized functions appeared to have significant sequence similarity to the 150 kDa unidentified protein. A simple BLAST search of the 147 kDa DNA directed RNA polymerase (third hit in Table 6.1) to identify other proteins with sequence similarity to the RNA polymerase yielded interesting findings. The VACV F12 polypeptide appeared to have 97% sequence similarity to the polymerase, with the expect value (E) being $8 \times 10^{-3}$. A very low E value represents a very highly significant match. VACV F12 is a 65 kDa protein that is involved in export of newly formed virions from the virus factories to the cell periphery via microtubules using kinesin-1 (Zhang, Wilcock, & Smith, 2000). Also, it should be noted that a ~60 kDa band, which could be the F12 protein, was identified by Western blotting in approximately similar amounts in the ‘bound’ and ‘unbound’ heparin-Sepharose fractions for both EV and MV extracts (Figure 6.2A) although, if so, F12 appears to have a low affinity for heparin.

The mass spectrometry search also identified two VACV core proteins P4a (60 kDa) and P4b (62 kDa), as possible heparin-binding proteins. Both proteins localize to viral factories and are responsible for organization of the viral core and
Table 6.1: List of protein hits from 1D nanoLC ESI MS/MS analysis of the 150 kDa unidentified protein.

<table>
<thead>
<tr>
<th>Proteins identified</th>
<th>Accession #:</th>
<th>Score†</th>
<th>Sequences matched*</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA directed RNA polymerase 132 kDa peptide (Cowpox virus)</td>
<td>NP_619941.1</td>
<td>1436</td>
<td>26</td>
</tr>
<tr>
<td>DNA directed RNA polymerase 147 kDa peptide (Variola virus)</td>
<td>ABG44469.1</td>
<td>254</td>
<td>7</td>
</tr>
<tr>
<td>DNA directed RNA polymerase 147 kDa peptide (Vaccinia virus)</td>
<td>AAB96506.1</td>
<td>223</td>
<td>7</td>
</tr>
<tr>
<td>Major core protein 4b (Vaccinia virus)</td>
<td>AAA48118.1</td>
<td>103</td>
<td>2</td>
</tr>
<tr>
<td>Major core protein 4a (Vaccinia virus)</td>
<td>AAA48129.1</td>
<td>72</td>
<td>1</td>
</tr>
</tbody>
</table>

†Ion score is -10*Log(p), where P is the probability that the observed match is a random event. Individual ion scores > 27 indicate identity or extensive homology (p<0.05). Protein scores are derived from ions scores as a non-probabilistic basis for ranking protein hits.

*Number of peptide sequences identified.
DNA encapsulation (Heljasvaara et al., 2001; Resch, Hixson, Moore, Lipton, & Moss, 2007). As described earlier, it is likely that these core proteins that interact with DNA were co-purified with the membrane proteins. Furthermore, since many heparin-binding proteins interact with negatively charged DNA, it is likely that this is the reason why DNA interacting proteins appear in the search to identify heparin-binding proteins.
6.4 Discussion

There is substantial evidence suggesting that viruses can interact with and recognize a wide spectrum of cell surface molecules including glycoproteins, glycolipids and proteoglycans. Results presented in this thesis, along with previously published studies, indicate that cell surface HS plays an important role in VACV infections (Chung et al., 1998; Hsiao et al., 1999; Lin et al., 2000). There are at least two proteins on the surface of MV that have previously been shown to interact with heparin/HS (Chung et al., 1998; Lin et al., 2000). However, since it has already been established in previous Chapters that the EV form of VACV is much more highly dependent on cell surface HS for infectivity than the MV form, it was important to identify protein(s) on the surface of EV that interact with heparin/HS.

Membrane proteins from both MV and EV particles were extracted by following a multi-step extraction procedure (Figure 6.1). Once isolated, the proteins were incubated with heparin-coupled beads to separate heparin interacting VACV proteins from the rest of the protein mixture. Detection of VACV proteins by Western blotting using a polyclonal VACV antiserum, which should react with most VACV proteins, revealed at least four different heparin-binding proteins of 150 kDa, 85 kDa, 60 kDa and 25 kDa (Figure 6.2A), the 85 kDa and 25 kDa proteins appearing to be EV specific.

MS analysis of the 150 kDa band identified the 147 kDa DNA directed RNA polymerase from 3 different poxvirus species, all with a very high expect score. Viruses do pack a high number of multifunctional proteins, however, it is highly
unlikely that a RNA polymerase is present on the outer EV envelope. It is possible that the heparin-binding site of the 147 kDa RNA polymerase could be the site where RNA binds to the polymerase as RNA, like heparin, is also negatively charged. Furthermore, several DNA/RNA interacting proteins have been shown to be heparin-binding as well (Gadgil & Jarrett, 1999). Nevertheless, a BLAST search performed on the RNA polymerase from VACV to identify other proteins similar in sequence to that of the polymerase discovered a 65 kDa VACV F12 protein. An extremely low expect value of $8 \times 10^{-3}$ suggests a strong sequence similarity between the polymerase and the F12 protein. VACV F12 has previously been shown to participate in the export of newly formed MV particles from the virus factories to the cell periphery. A mutant virus lacking F12 produces smaller plaques (Zhang et al., 2000) and F12 has been shown to interact with and form a complex with VACV E2 and A36, both of which contribute towards the transportation of virions to the cell surface (Morgan et al., 2010).

A bioinformatics search approach was also used to identify possible VACV heparin-binding proteins, however, neither the 147 kDa DNA directed RNA polymerase, nor the 65 kDa F12 protein could be detected by the bioinformatics approach. Both the bioinformatics approach and SDS-PAGE separation followed by MS/MS are excellent tools that are widely used in the field of proteomics, however, interpreting interactions of proteins with other molecules in 3D space based on the results from these two techniques may not be as informative. It is plausible that the 147 kDa DNA directed RNA polymerase and the 65 kDa F12 protein are heparin binding in their tertiary conformation, however, the primary amino acid sequence of both proteins may not have obvious HS binding motifs, which would
explain why both molecules were not identified in the bioinformatic searches.

Furthermore, a weakly heparin binding protein of similar molecular weight to F12 was detected by Western blotting (Figure 6.2A). Therefore, in order to determine if F12 is indeed a heparin binding protein, Western blotting analysis using a F12-specific mAb followed by MS analysis, would be required.

The 85 kDa protein of interest resulting from the Western blotting experiments (Figure 6.2A) was initially believed to be the VACV HA, also known as VACV A56R. The use of a VACV HA-specific mAb revealed, however, that the 85 kDa heparin-binding protein (Figure 6.2A) was, in fact, not the 85 kDa HA (Figure 6.2B). Indeed, there was little HA in the EV protein extract and heparin-Sepharose fractions compared to the MV extract and fractions where the HA was abundant. Furthermore, the MV derived VACV HA detected by a HA-specific mAb was observed to interact very weakly with heparin-coupled beads as most of the 85 kDa HA appeared in the ‘unbound’ fraction.

The bioinformatics searches did identify three VACV proteins with potentially strong heparin/HS binding sequences, the strongest hits being the VACV IL-1 receptor antagonist. Many viruses have been shown to have elegant methods to avoid detection by the immune system by mimicking cytokines and chemokine receptors, a mechanism that has been extensively studied (Alcamí, 2003). Indeed, structural studies performed in the past have shown that IL-1 has a heparin binding sites, such that heparin/HS molecules are able to regulate the synthesis of IL-1 (Chowers et al., 2001). However, interaction between the VACV the IL-1 receptor antagonist and HS is unlikely to have a role in EV infectivity as the
receptor antagonist is released from infected cells and does not associate with the virus particle itself.

The second protein identified by the bioinformatic searches with a possible HS-binding motif was the 65 kDa VACV ankyrin/F-box protein, B4R. There is little known about the VACV B4R protein in the literature. Some recent studies have demonstrated that mutant viruses lacking B4R form both MV and EV forms of VACV just like the wild type VACV, however, plaque sizes are reduced considerably with a decreased ability of the virus to spread (Burles et al., 2014). Furthermore, there is an unknown ~60 kDa protein detected by Western blotting that weakly binds to heparin-Sepharose, which could be a candidate B4R protein (Figure 6.2A). Therefore, it is possible that B4R could be playing a role in the interaction of heparin/HS with EV, however, the unavailability of a B4R specific mAb meant that this hypothesis could not be directly tested.

The third protein identified by the bioinformatic searches with a possible HS-binding motif within the 77.6 kDa RNA helicase DExH-NPH-II domain. The helicase superfamily is a class of enzymes involved in almost every aspect of RNA metabolism (Taylor, Solem, Kawaoka, & Pyle, 2010). The VACV NPH-II has been shown to be essential for mRNA transcription by enzymes encapsulated in the virions (Jankowsky, Gross, Shuman, & Pyle, 2001; Schwer, 2001). Furthermore, the helicases have also been speculated to affect RNA-protein interactions and therefore may participate in the structural reorganization of ribonucleoprotein assemblies (Jankowsky et al., 2001; Schwer, 2001). The bioinformatic searches described in this thesis revealed a potential heparin-binding domain, with a net
positive charge of +4, within the VACV DExH RNA helicase, suggesting a new role for the helicase. However, the helicase is unlikely to be expressed on the outer EV membrane, possibly associating with the EV envelope from the inside. Furthermore, it is possible that this apparent heparin-binding site could also bind negatively charged RNA/DNA. It is also possible that the 77.6 kDa DNA helicase is, in fact, the ~85 kDa band that appears in Western blotting using polyclonal antibodies (Figure 6.1A).

The bioinformatic searches provided several hits with possible heparin-binding regions, however, none of the identified protein hits have been previously reported to be part of the EV envelope. Nevertheless, the most likely candidate protein identified from the bioinformatic searches with a possible heparin-binding domain, and also likely to be EV membrane associated, was the 65 kDa protein B4R. None of the protein hits identified by the bioinformatic searches had molecular weights of 150 kDa, and 25 kDa as identified by Western blotting. It is possible, however, that the four heparin-binding proteins identified by Western blotting could have undergone extensive post-translational modifications, which would result in the protein bands appearing at unexpected positions on the Western blots. Therefore, MS analysis of the 85 kDa, 60 kDa and 25 kDa protein bands (Figure 6.2A) is required to conclusively identify the three (or more) heparin binding proteins, with the possible use of F12, DNA helicase and B4R-specific mAbs to determine whether or not VACV F12, DNA helicase and B4R are heparin/HS binding proteins.
Finally, there is increasing evidence suggesting that there are multiple HS binding sites on the surface of viruses like human papillomavirus type 16. Studies have shown that the primary HS interacting site of the capsid protein, following interactions with HS undergoes a conformational change, allowing secondary HS binding sites to be exposed, which then interact with HS (Richards, Bienkowska-Haba, Dasgupta, Chen, & Sapp, 2013). Based on the results reported in this thesis, it could be hypothesized that there are multiple HS binding proteins on the surface of the EV form of VACV, all of which contribute towards the inhibition of EV infectivity by heparin. It is likely that VACV, like human papillomavirus type 16, also has a multi-step HS interaction profile. Further identification of the heparin-binding EV proteins detected by Western blotting is likely to help in the delineation of many of the HS interacting proteins on the surface of the EV envelope.
Demonstration of a role for heparanase in VACV spread post intranasal and intramuscular inoculation

Chapter 7:
7.1 Abstract

VACV, like several other viruses, interacts with cell surface HS prior to interacting with specific cellular receptors to infect cells. The EV form of VACV, when in contact with HS, sheds its outer envelope to release the enclosed MV form of VACV, which is then able to interact with VACV specific cellular receptors. Heparanase is the only known mammalian endoglycosidase that can target and degrade HS. Data presented in this Chapter shows that following intranasal (i.n.) or intramuscular (i.m.) inoculation of VACV, the onset of disease was delayed in heparanase deficient (HPSE -/-) mice, compared to wild type (WT) mice. The onset of disease was measured by weight loss analysis along with virus titres in organs harvested from infected mice at different time points. Comparable virus titres were observed at the primary site of inoculation in both WT and HPSE -/- mice by 48 hr post inoculation regardless of the inoculation route, despite there being a 24-48 hr delay in the spread of the infection to ovarian tissue in HPSE -/- mice receiving VACV by both the i.n. and i.m. routes, and also in the splenic spread of VACV inoculated via the i.m. route. These data suggest that similar to HSV-1, VACV may also rely on heparanase-mediated degradation of HS to migrate to distant sites and establish an infection at the new sites. This conclusion is contrary to the prevailing immunological dogma, which predicts that heparanase deficiency would aid VACV replication and spread by preventing the entry of leukocytes with antiviral activity into sites of infection.
7.2 Introduction and rationale

Pathogens, including viruses, bacteria and parasites, adopt various strategies to overcome host defense responses in order to invade host cells and establish an infection. Several published studies have shown that certain viruses use cell surface GAGs as an interface to interact with and infect the host (Kamhi, Joo, Dordick, & Linhardt, 2013). Results presented in this thesis suggest a dependency of one such virus, VACV, on cell surface HS for infections in vitro, in particular the EV form of VACV. The MV form of VACV also uses cell surface HS for infection, however, unlike EV, MV is able to establish an infection in the absence of HS.

Heparanase, an endo- β-glucuronidase, is the only known mammalian endoglycosidase that can degrade HS in the ECM and BM (Hulett et al., 1999; Vlodavsky et al., 2000; Vlodavsky et al., 1999). During inflammation, in order to traverse the subendothelial BM, leukocytes deploy various proteases and the HS degrading enzyme, heparanase, to solubilize components of basement membranes (Parish, 2006). This heparanase mediated-degradation of HS liberates factors such as cytokines, chemokines, HS-binding growth factors and enzymes, that bind to HS with a high affinity, for physiological processes like angiogenesis and wound healing (Hulett et al., 1999; Parish et al., 2001; Szymczak et al., 2010; Vlodavsky et al., 2000; Vlodavsky et al., 1999). Since heparanase is an important regulator of HS function in vivo, it was of interest to understand VACV spread in vivo in a mouse model that was deficient in heparanase production.

Of particular interest was whether heparanase facilitates the spread of HS-binding viruses, such as VACV, as these viruses would be expected to be trapped in the
ECM, bound to HS. Thus, heparanase would function as a VACV releasing enzyme, analogous to neuraminidase liberating influenza virus by removing cell surface sialic acid residues recognized by the virus hemagglutinin (Gamblin & Skehel, 2010). Of course, this effect of heparanase on virus spread would be counterbalanced by heparanase aiding the entry of leukocytes into sites of infection. Nevertheless, early on in a VACV infection heparanase deficiency may inhibit virus spread rather than prevent virus control by the immune system, a proposition that was investigated in this Chapter.
7.3 Results

7.3.1 Spread of WR strain of VACV following intranasal inoculation of WT and HPSE-/- C57BL/6 mice

Since heparanase is an important regulator of HS degradation \textit{in vivo} and HS plays an important role in VACV infections, it was of interest to determine the effect of heparanase deficiency on the replication and spread of VACV following inoculation at restrictive sites such as i.n. and i.m. Thus, WT and HPSE -/- C57BL/6 mice were initially inoculated i.n. with $10^5$ PFU of the WR strain of VACV, as described in the section 2.3.1. It should be noted that the MV-rich WR strain of VACV was used for these experiments rather than the EV-rich strain as at the time these experiments were performed, it was not realized that the EV form of VACV is much more dependent on HS for its infectivity than the MV form. The animals were monitored over a 5-day period by maintaining a health score for each animal. Body weights were recorded at 24 hr intervals post inoculation. From day 2 post inoculation, 4 animals were ethically euthanized every 24 hr, with lungs, spleens and ovaries harvested from all the animals. Figure 7.1 shows weight loss of animals over the 5-day period as a percentage compared to weight of the respective animals at the time of inoculation, for both WT and HPSE -/- mice. As seen in Figure 7.1, WT mice continued to lose weight from day 2 post inoculation with the average weight loss being approximately 10% by day 3, whereas the weight loss in HPSE -/- mice was significantly less compared to the WT mice up to day 3 post inoculation, with the average weight loss being approximately 4%. However, by days 4 and 5 post infection, HPSE -/- mice had lost a considerable amount of weight, with the weight loss trend of HPSE -/- mice becoming similar to WT mice thereafter (Figure 7.1).
Figure 7.1: Weight loss analysis post intranasal inoculation with VACV WR in WT and HPSE -/- C57BL/6 mice over a course of 5 days. A total of 20 WT and 20 HPSE -/- mice were inoculated with VACV WR i.n. at $10^5$ PFU on day 0. At 24 hr intervals post inoculation, 4 mice from each WT and HPSE -/- group were first weighed and then sacrificed to harvest organs for virus titrations. The % weight loss was calculated based on the weight of each respective mouse at the time of inoculation on day 0. Data presented as mean ± SEM (n=4) and are representative of at least two independent experiments, with statistical analysis performed using an unpaired Student’s t-test.
Weight loss is considered a clinical sign of disease spread (Bouvier & Lowen, 2010; Hayasaka, Ennis, & Terajima, 2007), a uniform weight loss in WT mice post VACV inoculation being indicative of a steady spread of VACV within WT mice. However, based on weight loss, HPSE -/- mice appeared to resist VACV spread until day 3 post inoculation (Figure 7.1).

The weight loss analysis suggested that there was a delay in the spread of VACV in HPSE -/- mice compared to WT animals. VACV spread was evaluated in lungs, spleens and ovaries harvested from euthanised animals. The organs were individually weighed and processed to determine VACV titres in the respective organs as described in section 2.4.2. Lungs served as a positive control for the primary site of VACV replication. Plaque assays performed using lungs harvested at day 2 post inoculation from both WT and HPSE -/- mice indicated that VACV was able to establish an infection equally well in both groups of animals (Figure 7.2). Ovaries served as the distant organ to evaluate VACV spread kinetics in mice as VACV is known to exhibit a strong tropism for ovarian tissue (Zhao, Adams, & Croft, 2011). Spleen virus titres were also determined for all the infected animals.

As seen in Figure 7.3, spleen virus titres were observed by day 2 post inoculation in both WT and HPSE -/- animals and were maintained over days 2-5 with virus titres in the spleens from HPSE -/- mice tending to be lower than in WT mice on days 2-4, although this difference was not statistically significant. Furthermore, VACV appeared to accumulate in the ovarian tissue of WT mice from day 2 onwards (Figure 7.3). However, the ovary virus titres decreased dramatically in
Figure 7.2: Measurement of lung VACV titres in WT and HPSE -/- C57BL/6 mice at day 2 post intranasal inoculation of the WR strain of VACV. WT and HPSE -/- mice were weighed on day 2 post i.n. inoculation, followed by harvesting and weighing their lungs prior to measuring lung VACV titres. Lungs from all the mice were homogenized using a micro-homogenizer and used as virus stocks to infect confluent 143B osteosarcoma monolayers in 6-well plates. Culture medium was removed from monolayers and cells stained to identify plaques 48 hr post infection. The PFU/g for all the lungs harvested was calculated and the data are presented as mean PFU/g at the respective time points, along with ± SEM (n=4), for each group of mice. Data presented are representative of at least two independent experiments, with statistical analysis performed using the Wilcoxon Rank-Sum test.
WT mice, by day 4 only one of four mice having detectable VACV in their ovaries and there being no detectable virus on day 5 (Figure 7.3). On the other hand, only one of four HPSE -/- mice had detectable VACV in their ovarian tissue at day 2 post inoculation and by day 3 post inoculation, still only two out of four HPSE -/- animals had detectable VACV. However, by day 4 post inoculation there was ~100-fold increase in ovarian VACV titres over day 3, with the titres starting to decline by day 5. Comparing the trends of VACV spread in the ovaries of WT and HPSE -/- mice strongly highlights a delay in spread of VACV to distant organs in mice deficient in heparanase. In fact, ovary virus titre trends directly correlate with weight loss trends in the two groups of animals, with the delay in VACV spread in HPSE -/- mice resulting in a delay in the onset of sickness as seen by a delay in weight loss and a delay in spread of VACV to distant organs like ovaries. As mentioned above, spleen titres were not significantly different in the two animal groups, with Figure 7.3 clearly depicting splenocytes being infected by day 2 in both WT and HPSE -/- mice, which could be due to the ability of VACV to rapidly reach the spleen once it has entered the circulation.

7.3.2 Spread of WR strain of VACV following intramuscular inoculation of WT and HPSE -/- C57BL/6 mice

To understand the role of heparanase mediated HS degradation in VACV spread, a second restrictive infection strategy was used where animals were inoculated with VACV via the i.m. route. Quadricep muscles of WT and HPSE -/- mice were inoculated with $10^5$ PFU of VACV WR as described in section 2.3.1. Mice were monitored over a 5-day period and weighed every 24 hr as done previously after i.n. inoculation. From day 3 post inoculation, 4 animals/day were ethically
Figure 7.3: Measurement of VACV WR strain spread in WT and HPSE -/- C57BL/6 mice over 5 days post intranasal inoculation. WT and HPSE -/- mice were weighed on days 2, 3, 4 and 5 post i.n. inoculation, followed by harvesting and weighing their ovaries and spleens prior to measuring ovary and spleen VACV titres. To assess VACV titres, all the harvested organs were homogenized using a micro-homogenizer and used as virus stocks to infect confluent 143B osteosarcoma monolayers in 6-well plates. Culture medium was removed from monolayers and cells stained to identify plaques 48 hr post infection. The PFU/g for every organ, harvested at different time points, was calculated and the data are presented as mean PFU/g for every organ at the respective time points, along with ± SEM (n=4), for each group of mice. Data presented are representative of at least two independent experiments, with statistical analysis performed using the Wilcoxon Rank-Sum test.
euthanised and quadricep muscles, spleens and ovaries harvested for viral titre measurements. The change in whole animal weight is depicted in Figure 7.4 as a percentage of the weight of the respective animals at the time of inoculation. Unlike i.n. inoculation however, the i.m. route of inoculation did not result in a significant difference in weight loss between the WT and HPSE -/- animals, in fact there was little weight loss in all the infected animals. A direct comparison between weight loss in WT and HPSE -/- mice following i.m. inoculation, therefore, could not be used to predict the development of a VACV infection or sickness. To ensure VACV delivery to the quadricep muscles was successful, inoculated mice from both WT and HPSE -/- groups were euthanised on day 2 post inoculation, as performed previously for the i.n. inoculation, and the quadriceps were harvested for assessment of virus titres. High organ virus titres in both right and left quadriceps from both WT and HPSE -/- mice indicated successful inoculation and comparable virus replication in WT and HPSE -/- mice (Figure 7.5). Spleens and ovaries harvested from the WT and HPSE -/- mice were processed to determine virus titres and thereby evaluate the VACV infection profile in the two groups of mice. Ovaries and spleen served as the distant organs of interest as described earlier in section 7.3.1 for the i.n. inoculation route.

Since quadriceps, compared to the lungs, are a much more restrictive site for virus replication and for virus spread to distant sites, it was anticipated that organs harvested on days 3, 4 and 5 following i.m. inoculation of VACV would show a delayed time course of virus spread than that seen on days 2, 3 and 4 following i.n. inoculation. As seen in Figure 7.6, spleen virus titres were observed by day 3 post inoculation in WT animals and persisted over the next two days. Furthermore, in
Figure 7.4: Weight loss analysis post intramuscular inoculation with VACV WR in WT and HPSE -/- C57BL/6 mice over a course of 5 days. A total of 20 WT and 20 HPSE -/- mice were inoculated with VACV WR i.m. at 10^5 PFU on day 0. At 24 hr intervals post inoculation, 4 mice from each WT and HPSE -/- group were first weighed and then sacrificed to harvest organs for virus titrations. The % weight loss was calculated based on the weight of each respective mouse at the time of inoculation on day 0. Data presented as mean ± SEM (n=4) and are representative of at least two independent experiments, with statistical analysis performed using an unpaired Student’s t-test.
Figure 7.5: Measurement of VACV titres in the quadricep muscles of WT and HPSE -/- C57BL/6 mice at day 2 post intramuscular inoculation of the WR strain of VACV. WT and HPSE -/- mice were weighed on day 2 post i.m. inoculation, followed by harvesting and weighing their quadricep muscles prior to measuring VACV titres. Left and right quadricep muscles from all the mice were individually homogenized using a micro-homogenizer and used as virus stocks to infect confluent 143B osteosarcoma monolayers in 6-well plates. Culture medium was removed from monolayers and cells stained to identify plaques 48 hr post infection. The PFU/g for all the muscles harvested was calculated and the data are presented as mean PFU/g at the respective time points, along with ± SEM (n=4), for each group of mice. The data presented are representative of at least two independent experiments, with statistical analysis performed using the Wilcoxon Rank-Sum test.
WT mice substantial ovary virus titres also appeared by day 3 post inoculation, but were reduced to below the detection limit by day 5. Contrary to WT mice, VACV was undetectable in the spleens of HPSE-/- mice until day 5 post inoculation. The titres of VACV in the ovaries of HPSE-/- mice followed a similar trend to the spleen, only appearing at day 5 (Figure 7.6).

These results indicate that there was a clear delay in the spread of VACV to distant organs in mice deficient in heparanase. Unlike with the i.n. route of virus delivery, the weight loss trends and titres of virus within distant organs did not correlate when the delivery route of VACV was changed to i.m. This could be due to muscle being a highly restrictive site for VACV replication and trafficking. Nevertheless, the overall results obtained with the i.m. route of inoculation are consistent with the i.n. inoculation route findings, suggesting that lack of heparanase hampers the ability of VACV to spread to distant organs from the primary site of infection.
Figure 7.6: Measurement of VACV WR strain spread in WT and HPSE -/- C57BL/6 mice over 5 days post intramuscular inoculation. WT and HPSE -/- mice were weighed on days 3, 4 and 5 post i.m. inoculation, followed by harvesting and weighing their ovaries and spleens prior to measuring ovary and spleen VACV titres. To assess VACV titres, all the harvested organs were homogenized using a micro-homogenizer and used as virus stocks to infect confluent 143B osteosarcoma monolayers in 6-well plates. Culture medium was removed from monolayers and cells stained to identify plaques 48 hr post infection. The PFU/g for every organ, harvested at different time points, was calculated and the data are presented as mean PFU/g for every organ at the respective time points, along with ± SEM (n=4), for each groups of mice. The data presented are representative of at least two independent experiments, with statistical analysis performed using the Wilcoxon Rank-Sum test.
7.4 Discussion

It is clear from the current study that cell surface HS plays an important role in VACV infectivity and soluble GAG molecules can inhibit VACV infections in vitro. However, the role of HS in the spread of VACV in vivo needs to be addressed. Therefore, prior to progressing with the development of GAG based small molecules that can act as antivirals, it was first important to understand the role of HS in VACV spread in vivo. Of particular interest was whether the HS degrading enzyme, heparanase, aids the spread of HS-binding viruses like VACV, analogous to neuraminidase aiding the spread of sialic acid binding influenza virus.

Two different mouse strains were used to understand VACV spread, namely heparanase deficient HPSE-/- and WT C57BL/6 mice. Heparanase is the only known mammalian endoglycosidase that is able to cleave HS chains to release small saccharide products (Levy-Adam et al., 2010; Parish et al., 2001). In order to understand the role of HS and heparanase in VACV movement from one site to another in vivo, it was important to design an assay that would require VACV to establish a local infection before spreading to distant organs, thus the i.n. and i.m. routes of VACV inoculation were used. Initial studies using the i.n. route of inoculation revealed a 24 hr delay in VACV-induced weight loss in the HPSE-/-mice compared to the WT mice, suggesting a delay in the onset of disease (Figure 7.1). Furthermore, a 24-48 hr delay was also observed in the ability of VACV to reach ovarian tissue in HPSE-/- mice compared to WT mice (Figure 7.3), with VACV titres being detected in the ovaries of WT mice at day 2 post infection, but declining over the next two days and reaching undetectable levels by day 5.
In contrast, ovary titres of VACV were observed to be low on days 2-3 in HPSE -/- mice, but the ovaries of all mice having high viral titres on days 4 and 5 post infection. Furthermore, lungs from both HPSE -/- and WT mice were infected equally well on day 2 post infection (Figure 7.2), indicating that the differences seen in VACV spread were not due to any differences in VACV replication at the inoculation sites of the two mouse strains. Thus, the ovary virus titres clearly indicate that the lack of heparanase in HPSE -/- mice reduces the ability of VACV particles to spread from the primary site of infection.

Although the i.n. route of infection gave informative results regarding a role for heparanase in VACV spread, the nasal vestibule does contain small hairs and a mucus layer covering the entire respiratory area of the nasal cavity. It is possible that the mucociliary clearance of VACV particles could carry the particles to the back of the throat and down the oesophagus and further down the digestive tract (Illum, 2003). This could be the reason why low PFU counts were seen in the lungs of the infected mice (Figure 7.2). Therefore, to confirm the results obtained from the i.n. route of delivery of VACV, the i.m. route of delivery was examined next.

As seen with the i.n. route of delivery by day 2 post inoculation VACV was able to replicate to similar levels at the primary site of inoculation in both the HPSE -/- and WT mice (Figure 7.5). However, unlike the i.n. route of delivery, infected HPSE -/- and WT mice did not lose a significant amount of weight over the 5 day observation period (Figure 7.4). Therefore, weight loss post i.m. inoculation could not be used as an indicator of sickness resulting from VACV infections. Nevertheless, the effects of heparanase deficiency on VACV spread were more
marked following i.m. inoculation. Thus, inhibition of virus spread to the spleen was clearly evident on days 3 and 4 following i.m. inoculation of the HPSE -/- mice, whereas such a difference was not seen in i.n. inoculated mice. On the other hand, the effects of heparanase deficiency on virus spread to the ovaries gave similar results for both routes of inoculation, in both cases there being a 24-48 hr delay in the spread of VACV to the ovaries in the HPSE -/- mice.

Thus the key finding from both the i.n. and i.m. routes of VACV delivery is that heparanase deficiency substantially delays VACV spread from the inoculation site, which implies a direct role for heparanase in enabling the escape of VACV particles from sites of primary infection. It is well documented that HS in the ECM binds to a number of cytokines, chemokines and enzymes with high affinity, which are made available for physiological processes such as angiogenesis and wound healing by the enzymatic action of heparanase (Hulett et al., 1999; Parish et al., 2001; Szymczak et al., 2010). Therefore, to put the findings obtained from the current study in perspective, it is likely that similar to chemokines and cytokines, newly synthesized EV and MV particles exiting primary infected cells become trapped in cell surface and ECM HS. Furthermore, since it is well established that heparanase mediated degradation of HS in the ECM and BM is a prerequisite for leukocytes to invade tissues (Parish et al., 2001; Vlodavsky et al., 2000; Vlodavsky et al., 1999), leukocyte-derived heparanase could possibly allow newly synthesized progeny EV particles to escape from the primary site of infection and establish an infection at a distant site. Another possibility is that platelets are the source of heparanase (Freeman & Parish, 1998) as they are likely to enter and be activated to release heparanase at sites of infection, particularly with cytopathic viruses like VACV.
There is ample evidence suggesting that several viruses, bacteria and parasites use evolutionary conserved glycosaminoglycans, such as HS, as adhesion receptors possibly due to their high abundance (Rostand & Esko, 1997; Wadstrom & Ljungh, 1999). However, a disadvantage of using HS as an adhesion ligand is that its abundance acts as a barrier to virus spread. Having host-derived heparanase to degrade HS is one way in which pathogens can overcome this disadvantage. Therefore, it is likely that similar to VACV, other HS binding pathogens may also rely on heparanase-mediated degradation of ECM HS as a mechanism to aid spread to distant sites. In fact, a recent study of herpes simplex virus-1 (HSV-1) infection of the cornea clearly demonstrated that host-derived heparanase digests cell surface HS, thereby facilitating HSV-1 release in vivo (Hadigal et al., 2015). In this study, knocking down heparanase expression in mouse corneas using shRNA against murine heparanase, followed by infection with HSV-1, decreased virus egress and tissue damage at the site of infection, whereas overexpression of heparanase significantly increased HSV-1 spread and tissue damage (Hadigal et al., 2015). Interestingly, these findings with VACV and HSV-1 are contrary to the immunological dogma that would predict that heparanase deficiency would enhance virus spread by preventing the heparanase dependent entry of leukocytes with antiviral activity into sites of infection.

Collectively, the findings presented in this Chapter suggest that like influenza virus, VACV relies on a glycosidase for virus spread. However, influenza virus encodes within its genome the neuraminidase required for virus escape, whereas VACV relies on host-derived heparanase to aid its exit from the primary site of infection and to spread to other organs. Targeting this heparanase-HS interplay exploited by
VACV, could allow the development of novel therapies for treating HS-dependent viral infections.
Final discussion

Chapter 8:
8.1 Introduction

The expansion of traditional areas of habitation, along with mass long-distance travel, has opened up the possibility of rapid widespread dissemination of new and re-emerging pathogens once considered eradicated and is regarded as a major concern for the medical and public health systems worldwide (Littler & Oberg, 2005; Morens et al., 2004). Moreover, the threat of eradicated viruses like smallpox being used as potential weapons of bioterrorism has attracted a lot of interest in the scientific community and has stimulated the fast-track development of novel antiviral treatments (Hughes et al., 2010). With this increasing disease burden on the public health system, increasing efforts are being made by the scientific community to understand viral pathogenesis and develop new strategies to treat these infections (Finlay et al., 2004).

Research targeted at understanding the molecular pathways controlling virus life cycles is the engine that drives the development of therapies against viral infections (Liang & Ghany, 2013). Furthermore, use of ‘combination therapies’ using disease-modifying treatments acting on hosts, coupled with inhibitors of virus replication, have shown great potential in the treatment of viral infections (Littler & Oberg, 2005). An example of combination therapy is the treatment of hepatitis C virus (HCV) using inhibitors of HCV proteases, along with treatment for fibrosis, which could have a major impact on liver disease (Elbaz, El-Kassas, & Esmat, 2015; Littler & Oberg, 2005). Another example is the treatment of HSV where the use of aciclovir, an inhibitor of HSV replication, combined with an anti-inflammatory agent, has resulted in a significant decrease in HSV-1 infections.
(Hull, Levin, Tyring, & Spruance, 2014; Littler & Oberg, 2005; Opstelten, Neven, & Eekhof, 2008).

8.2 VACV interacts with cell surface HS

The research focus of this thesis was the development of an understanding of the molecular mechanisms used by VACV to enter and infect cells, particularly the role of HS, an understanding that would be valuable for the design of antivirals that could potentially inhibit VACV infections. VACV is a member of the Orthopoxvirus (OPV) genus of the Poxviridae family. VACV was the vaccine strain used to eradicate smallpox, however, it wasn’t until 1939 that scientists realized that the strain of poxviruses being used to vaccinate against smallpox was, in fact, VACV and not the cowpox virus (CPXV) originally used by Edward Jenner in 1796 (Roberts & Smith, 2008). Today, VACV is commonly used as the prototype virus to study poxviruses.

VACV, like many other viruses, interacts with cell surface HS prior to interacting with specific cell surface receptors that mediate its entry into cells (Chung et al., 1998; Lin et al., 2000). ELISA results presented in Chapter 3 of this thesis clearly suggest that the MV form of VACV binds to immobilised HS hi, with only soluble heparin being a potent inhibitor of this binding, suggesting that the MV form of VACV preferentially interacts with GAG molecules with extremely high sulfate content (Figures 3.1 and 3.2). The MV form of VACV also has a known CS binding protein on its outer membrane (Hsiao et al., 1999), however, the unavailability of BSA conjugated CS meant similar ELISA studies could not be performed to determine the strength of interaction between MV particles and immobilised CS.
Nevertheless, an alternative ELISA assay was designed to determine if soluble CS could inhibit MV particles from binding to immobilised HS<sup>hi</sup> in ELISA wells. Only very high concentrations of CS could partially inhibit MV binding, suggesting that soluble CS could indirectly interfere with the interaction between immobilised HS<sup>hi</sup> and proteins on the MV outer membrane (Figure 3.4). Furthermore, there is ample published evidence indicating that soluble CS has little effect on MV infectivity (Carter et al., 2005), therefore, it would be safe to conclude that the GAG molecule that plays a major role in VACV infectivity is cell surface HS (and soluble heparin, a highly sulfated version of HS).

Previously published studies have shown that there are at least two HS-binding proteins on the outer membrane of MV particles, however, due to the fragile nature of the outer EV envelope, similar studies using the EV form of VACV have not been undertaken (Chung et al., 1998; Lin et al., 2000). Nevertheless, plaque assay results presented in Figure 3.5 clearly demonstrate that heparin/HS play a crucial role in the way VACV forms plaques on cell monolayers <i>in vitro</i>. Not only does the presence of heparin in the culture medium reduce the plaque numbers of the WR and IHD-J strains of VACV by 5- to 10-fold, but it also influences the plaque morphology (Figure 3.5). Thus, there was an incremental increase in the diameter of WR strain plaques (MV rich strain of VACV) in the presence of heparin. However, the effect of heparin on IHD-J strain plaques (EV rich strain of VACV) was more dramatic, with the disappearance of the trademark ‘comets’ associated with EV plaques and a similar incremental increase in plaque diameter as seen with the WR strain.
Previously published studies have shown that the VACV A56 protein, also known as the VACV haemagglutinin (HA), binds to and forms a complex with a serine protease inhibitor (K2) (DeHaven et al., 2011; Moss, 2006). Infected cells express A56/K2 complexes on their plasma membranes, which interact with the entry/fusion complex (EFC) on the membrane of new MV particles and inhibit the ability of the EFC to mediate MV entry into cells (DeHaven et al., 2011; Turner & Moyer, 2008). The EFC is composed of at least eight proteins, namely, A16, A21, A28, G3, G9, H2, J5 and L5 (Wagenaar & Moss, 2009). The interaction of the A56/K2 complex with the EFC on the surface of MV particles is a mechanism in place to avoid superinfection of infected cell, resulting in MV particles only being able to infect cells that do not have the A56/K2 complex expressed on their surface (Turner & Moyer, 2008). Antibodies against the A56 or K2 proteins have been shown to decrease the interaction of the A56/K2 complex with EFC on MV and, therefore, increase the amount of superinfection (DeHaven et al., 2011).

Furthermore, the A56/K2 complex has been shown to inhibit syncytia formation (Moss, 2006) and infected cells are able to fuse with uninfected cells at neutral pH when mutant viruses lacking A56 or K2 are used to infect cells (Turner & Moyer, 2008). There is also evidence that suggests that K2 contains a heparin-binding site similar to that of antithrombin III (Brum et al., 2003; Turner & Moyer, 2006).

Furthermore, Western blotting results presented in Figure 6.2B clearly indicate that the A56 protein binds to heparin-Sepharose beads. Therefore, to put the plaque assay results presented in Figure 3.5 in perspective, a possible reason for the increased plaque diameter is that the A56/K2 complexes expressed on the plasma membrane of infected cells could be blocked by heparin, similar to the effects seen when an antibody against A56 or K2 is used, which then permits
syncytia formation. This would mean that plaque size would grow at a faster rate in the presence of heparin. Apart from interacting with the A56/K2 complex, heparin also directly inhibits EV and MV forms of VACV from interacting with cell surface HS, which would explain the 5-10-fold reduction in plaque numbers observed.

Unlike MV particles, which are generally released from dying cells, EV particles are actively ejected from infected cells (Figure 1.4), a process that results in the infection of distal cells and the formation of the trademark ‘comet’ shaped plaques. However, in the presence of heparin, the comets formed by the IHD-J strain of VACV almost completely disappeared (Figure 3.5A and B). It is likely that heparin inhibits freshly released EV particles from infecting neighbouring cells as heparin is a potent inhibitor of EV infections in vitro as measured by the infections using GFP expressing EV form of VACV (Chapters 4 and 5) and by the IHD-J strain (EV rich) plaque formation (Figure 3.5). Furthermore, newly formed MV particles in an infected cell can directly be pushed into neighbouring cells and escape interaction with heparin (Marsh & Helenius, 2006). Therefore, it is likely that in the presence of heparin, the predominant mode of spread of the IHD-J strain of VACV is via the punching mechanism used by MV particles. This would also explain why the IHD-J strain of VACV forms round plaques in the presence of heparin.

8.3 EV and MV forms of VACV interact differently with cell surface HS

The plaque assay results presented in Figure 3.5 clearly show that heparin affects the EV rich strain of VACV (IHD-J) differently to the MV rich strain (WR).
Therefore, it was important to evaluate the role of heparin/HS in infections caused by the EV and MV forms of VACV.

The outermost envelope of the EV form of VACV is extremely fragile (Ichihashi, 1996). This makes purification of the EV form extremely challenging because an EV particle with a damaged outer membrane behaves like an MV particle (see Figure 3.5F showing broken outer EV envelope). Therefore, to understand the interaction between EV particles and cell surface HS, it was first essential to design protocols to obtain relatively concentrated stocks of EV. Since the IHD-J strain of VACV is constitutively rich in the amount of EV particles it sheds compared to the WR strain of VACV, the IHD-J strain was used to obtain EV stocks for the assays performed in this thesis. The WR strain of VACV was used to obtain MV stocks.

In order to understand the interaction between cell surface HS and VACV during an infection, GFP expressing recombinants of both the WR and IHD-J strains of VACV were created. The kinetics of EV-GFP and MV-GFP infection were monitored by measuring GFP expression in cells at different time-points post infection. Coincubation of heparin with either EV-GFP or MV-GFP during the infection of cell monolayers revealed that heparin was a much more potent inhibitor of EV infections than MV infections (Figures 4.3 and 4.7). Since the EV and MV forms of VACV used in these infection assays were purified from two different strains of VACV, it was important to rule out the possibility that strain differences were the cause of the differences seen in EV and MV infectivity. It was found, however, that the EV particles isolated from the WR strain of VACV could be inhibited by heparin to a similar extent as EV particles isolated from the IHD-J strain (Figure 4.7).
Similarly, MV particles isolated from the IHD-J strain of VACV showed comparable inhibition by heparin to MV particles isolated from the WR strain of VACV (Figure 4.7). These observations clearly suggest that EV particles, regardless of their source, are more susceptible to inhibition by heparin than MV particles. The tightly overlapping inhibition curves observed in these experiments also highlight the reproducibility of the inhibition assay used.

### 8.4 Role of heparanase in VACV spread

Heparanase is the only known mammalian endoglycosidase that is able to cleave HS in ECM and BMs, therefore, it was of interest to understand the effect of heparanase-mediated digestion of HS on EV and MV infectivity in vitro. Digestion of cell surface HS using platelet derived HPSE or commercially available Flavobacterium HPNSES, significantly reduced EV and MV infectivity, however, the reduction in infectivity was more severe for the EV particles than the MV particles (Figure 4.5). Since HPSE and HPNSE digestion of cell surface HS is incomplete, the digestions probably leaving HS stubs on cell monolayers, this could explain why a complete inhibition of the EV and MV infections was not observed. Nevertheless, it was evident that EV infections are more dependent on the presence of cell surface HS than MV infections. Furthermore, infection assays performed with a cell line genetically deficient in the production of HS confirmed the previous finding that EV infections are significantly affected by the unavailability of cell surface HS (Figure 4.6), whereas the MV form is much less dependent on HS to infect cells. Since the EV form of VACV is responsible for long-range dissemination in vivo (Ichihashi, 1996) and the knowledge that heparanase is the only known mammalian endoglycosidase that mediates extracellular HS degradation in vivo
(Levy-Adam et al., 2010; Parish et al., 2001), it was of interest to determine the ability of VACV to spread in mice deficient in heparanase expression.

To perform \textit{in vivo} spreading assays, it was essential to consider the route of inoculation of VACV. Traditionally used routes for infection assays, such as intraperitoneal or intravenous inoculation, allow the systemic spread of VACV particles, which is essential to study immune responses against VACV infections. However, since the research question in this thesis was to understand VACV spread to different organs, it was important to deliver VACV at restrictive sites in order to cause VACV particles to first establish an infection at the primary site of inoculation, before spreading to distant organs. Both i.m. and i.n. routes of delivery where thus used to perform the \textit{in vivo} assays.

Results presented in Chapter 7 clearly suggest that there is a definite delay in disease onset in HPSE-/- mice inoculated with the WR strain of VACV by both the i.n. and i.m. routes, compared to WT C57BL/6 control mice. Plaque assays performed on organs harvested from both the HPSE-/- and WT mice infected with the WR strain of VACV clearly demonstrated that VACV was able to establish an infection at the primary site of inoculation with comparable efficiency in both animal groups, however, VACV dissemination to ovaries, a distal organ, was delayed in the HPSE-/- mice compared to the WT mice. Since the EV form of VACV is mainly responsible for the long-range dissemination of the virus, it is likely that the lack of heparanase in the HPSE-/- mice had its greatest effect on the dissemination of progeny EV particles. The \textit{in vitro} results presented in Chapter 4 showing that EV particles, but not MV particles, much less efficiently infect
heparanase treated cell monolayers, is consistent with the spread of the EV form of VACV being highly heparanase dependent. In fact, it could be argued that the existence of two forms of VACV, namely the EV and MV forms, with substantially different affinities for HS, provides the virus with a diversity of spreading mechanisms that could be advantageous.

There is a large body of evidence indicating that several viruses, bacteria and parasites use evolutionary conserved glycosaminoglycans, including HS, as adhesion receptors during their infection of cells (Rostand & Esko, 1997; Wadstrom & Ljungh, 1999). However, based on the data presented in this thesis, it is highly likely that such pathogens are dependent on heparanase, usually host derived, for their dissemination from their initial site of infection. In fact, a recent publication reported that the spread of HSV-1 is heparanase dependent (Hadigal et al., 2015). Of considerable interest is whether viruses, such as HSV-1, produce progeny virus particles with variability in their affinity for HS as is the case with VACV. It should be noted, however, that heparanase deficiency delays but does not totally prevent virus spread. The heterogeneity in HS binding affinity of viruses could partially explain this observation. An additional possibility is that proteases that degrade ECM and BMs could also aid the spread of HS binding viruses (Levy-Adam et al., 2010).
8.5 Chemically modified heparins and HS mimetics can inhibit VACV infections

Having established that cell surface HS is important for the infection of cells by both the EV and MV forms of VACV, but particularly the EV form, combined with the ability of soluble heparin to interfere with EV and MV infections, it was important to delineate the structural features of heparin/HS that influence the infectivity of the EV and MV forms of VACV.

Initial inhibition assays performed using Fondaparinux, along with other commercially available LMWHs, suggested that these compounds are just as potent as heparin in their ability to inhibit EV and MV infections (Figure 5.1). Since Fondaparinux and other LMWHs contain the antithrombin III binding pentasaccharide of heparin, which allows them to be antithrombotic, it is likely that the EV and MV particles also interact with the same pentasaccharide sequence. Similar to heparin, EV infections are more susceptible to inhibition by Fondaparinux and other LMWHs, compared to MV infections. However, based on these results, it is likely that antithrombotic drugs may be able to act as antivirals by interfering with the interaction of EV and MV particles of VACV with cell surface HS. These results, along with previously published results, raise the notion of a so-called ‘sulfation code’, which entails sulfated sequences along the length of HS chains that permits high avidity binding to certain proteins or VACV, as seen in the current study (Lindahl & Kjellen, 2013).

Previously published studies showing inhibition assays performed using HSV, which also interacts with cell surface HS prior to infecting cells, revealed that 2,3-
O-sulfate and 6-O-sulfate groups are critical for heparin to interact with the gB and gC glycoproteins on the surface of HSV-1 (Feyzi et al., 1997; Trybala et al., 2000). Results presented in Chapter 4 indicate that highly sulfated HS (HS^{hi}) inhibits VACV infections much more efficiently than under-sulfated HS (HS^{low}). Previously published HSV infection studies, together with the finding reported in this thesis that key sulfate groups on free heparin/HS chains can inhibit VACV infections, suggest that cell surface HS can act as an important co-receptor for VACV infections. However, to validate the ‘sulfation code’ hypothesis, a series of inhibition assays were performed to extensively analyze key structural features of heparin/HS to which VACV particles bind. Structural analyses using chemically modified heparins revealed that the 2-O-sulfate groups of the iduronic/glucuronic acid residues are not important for the ability of heparin to inhibit EV and MV infections. However, 6-O-sulfate and N-sulfate groups of the D-glucosamine residues are crucial for the ability of heparin to inhibit VACV infections (Figure 5.2). Furthermore, neither N-acetylation of glucosamine residues, nor the removal of carboxyl groups from the iduronic/glucuronic acid residues, had any effect on the ability of heparin to inhibit EV and MV infections (Figure 5.3). These results, therefore, validate the ‘sulfation code’ hypothesis in the context of VACV infections.

In addition to using chemically modified heparins, sulfated di- and oligo-saccharides that differ in their sulfation patterns, monosaccharide constituents and linkages were examined in Chapter 5 for their anti-VACV activity, these molecules being termed HS mimetics. Data obtained from these studies indicate that the linkage of different D-glucose-based sulfated saccharides plays a crucial role in the ability of the molecules to inhibit VACV infections, with the order of
potency being $\beta(1\rightarrow4) > \alpha(1\rightarrow6) > \alpha(1\rightarrow4) > \beta(1\rightarrow3)$, with sulfated pentasaccharides being usually the most effective. These results add a layer of complexity suggesting that the position of negatively charged sulfated groups in 3D space determines the antiviral activity of these molecules and, therefore, builds on the notion of a ‘sulfation code’.

8.6 Anti-cancer drug PI-88 (Muparfostat) can inhibit VACV infections

The anti-cancer drug PI-88 inhibits heparanase-mediated degradation of HS, thereby inhibiting tumour metastasis and reducing tumour growth by inhibiting angiogenesis (Khachigian & Parish, 2004). PI-88 is currently in a Phase III trial as a monotherapy in post-resection hepatocellular carcinoma patients. Since PI-88 is a mixture of highly sulfated oligosaccharides, it was of interest to determine if PI-88 can act as an inhibitor of VACV infections. Indeed PI-88 could inhibit both EV and MV infections, however, it was surprising to note that of all the inhibitors tested in the assays presented in this thesis, PI-88 was the only molecule that was a more potent inhibitor of MV infections than of EV infections. PI-88 does differ from other HS mimetics as PI-88 is based on the monosaccharide D-mannose, whereas the other HS mimetics described in Chapter 5 as inhibitors of VACV are D-glucose-based saccharides. Furthermore, PI-88 is composed of $\alpha(1\rightarrow3)$ and $\alpha(1\rightarrow2)$ linked D-mannose residues (Yu et al., 2002), which also differs from the linkages of the D-glucose-based HS mimetics examined. These data are again consistent with the notion that the ability of HS-based saccharide molecules to inhibit VACV infectivity is dependent on the position of the negatively charged groups of the inhibitor in 3D space.
PI-88 has also been shown to have a significant effect on the disease outcome in dengue virus and flaviviral encephalitis mouse models (Lee et al., 2006). Therefore, based on the results presented in this thesis, mixtures of heparin-/HS-based molecules, an excellent example being a mixture of PI-88 and Fondaparinux that are drugs already used clinically, would likely be effective antiviral agents against VACV as each saccharide molecule targets different VACV forms.

### 8.7 EV surface proteins that interact with HS/heparin

The MV form of VACV has two known HS-binding proteins on its outer membrane (Chung et al., 1998; Lin et al., 2000). Studies published by Law et al., 2006, suggest that the EV outer membrane proteins A34R (24-28 kDa) and B5R (42 kDa) interact with cell surface HS, which results in lysis of the outer EV envelope to release the enclosed MV particles (Law et al., 2006). However, results presented in this thesis clearly suggest that soluble heparin, LMWHs, certain chemically modified heparins and HS-based oligosaccharides, are able to inhibit EV-mediated infectivity with higher affinity than the MV-mediated infectivity. Therefore, it was important to determine whether any other protein(s) on the surface of EV interact with heparin/HS.

Bioinformatic searches were performed to identify, based on sequence homology using known HS-binding protein, the outer EV membrane proteins that would interact with cell surface HS. The EV membrane proteins were also solublised, co-incubated with heparin-coupled beads, and identified via Western blotting using VACV-specific antibodies. A 150 kDa protein band observed on blots was excised and sent to APAF for identification by mass spectrometry (MS) analysis. The MS
analysis identified the 150 kDa band as the 147 kDa DNA directed RNA polymerase with a sequence similarity to 65 kDa VACV F12, a protein involved in the export of newly formed MV particles. Interestingly, Western blotting also identified a ~60 kDa heparin binding protein, however, due to time constrains, the ~60 kDa band could not be sent for MS analysis. Nevertheless, the VACV F12 protein could be a candidate for the unknown ~60 kDa protein identified via Western blotting.

Furthermore, the bioinformatics searches identified a 65 kDa VACV B4R protein that is an important protein responsible for effective plaque formation. Therefore, VACV B4R could also be a candidate protein for the unknown ~60 kDa protein.

Western blotting also identified an 85 kDa heparin-binding protein, which was a different protein to VACV HA (A56R). Bioinformatic searches identified a 77.6 kDa RNA helicase that had a possible heparin-binding motif. Therefore, it is possible that the 77.6 kDa RNA helicase is the ~85 kDa unknown protein (Figure 6.2A). Similar to B4R, an RNA helicase-specific mAb would be required to conclusively identify the ~85 kDa protein. Furthermore, MS analysis could be performed to identify the unknown protein. Lastly, a 25 kDa heparin-binding protein was also observed in blots, however, the identity of this protein is unknown although it could be the previously described 24-28 kDa A34R protein on the EV surface that binds heparin (Law et al., 2006). MS analysis would be required to conclusively identify all the unknown proteins.

Assays performed using heparin/HS mimetics clearly support the notion of the ‘sulfation code’ in 3D space. Therefore, it is likely that there are protein(s) on the surface of the EV envelope, other than A34R and B5R, that based on bioinformatics
analysis are not obvious HS-binding proteins but when folded in their tertiary orientation are able to interact with HS. As an example, human papillomavirus type 16 has multiple HS-binding proteins on its capsid, with the interaction between primary HS-binding sites and HS inducing a conformational change that exposes secondary HS-binding sites, which are then able to interact with cell surface HS (Richards et al., 2013).

8.8 Conclusion and future directions

To summarize, it is clear that VACV, like several other viruses, interacts with cell surface HS to infect cells. However, as described in a recently published study on HSV-1, it is likely that VACV also relies on heparanase-mediated degradation of HS in order to spread to distal sites in vivo. Nevertheless, HS-based molecules could potentially be used to inhibit VACV infections. In fact, extensive studies of different HS mimetics outlined in this thesis suggest that synthetic HS-based molecules could be synthesized that target both EV and MV forms of VACV. Furthermore, many of the HS mimetics with antiviral activity may also act, like PI-88, as heparanase inhibitors and consequently inhibit virus spread.

A number of important unanswered questions arise from the data presented in this thesis. First, what are the key heparin/HS binding molecules on the surface of the MV and EV forms of VACV. Western blotting studies have identified several VACV proteins, based on molecular weight, that bind heparin/HS. MS studies need to be undertaken to identify these molecules, similar to the approach used to identify the 150 kDa VACV protein binding to heparin-Sepharose beads in Chapter 6. Whether the newly identified heparin/HS binding proteins simply aid adhesion
of VACV to cells, activate EV envelope disintegration or prevent superinfection of
cells by VACV also needs to be investigated. Second, although heparanase has been
clearly shown to aid VACV spread, the source of the host-derived heparanase is
unknown. An obvious source is host leukocytes entering sites of infection,
particularly neutrophils and macrophages. Platelets are another source of
heparanase as the platelets carry large amounts of preformed heparanase that is
rapidly released following platelet activation (Freeman & Parish, 1998), a likely
scenario for cytopathic viruses such as VACV. Thrombocytopenic mice, such as the
c-mpl knockout mice (Murone, Carpenter, & de Sauvage, 1998), or platelet
depleting mAbs could be used to establish whether VACV spread is platelet
dependent. If this is the case, use of mice in which platelet heparanase is
conditionally knocked out would conclusively demonstrate that platelet-derived
heparanase aids VACV spread. Conversely, if platelets are not involved, the
conditional knocking out of heparanase in myeloid cells could be used to establish
the leukocyte origin of heparanase.

Finally, it will be of considerable interest whether other pathogens that use HS as a
co-receptor for infecting cells behave in a similar manner as VACV. In particular, do
they use host heparanase to aid pathogen spread and do they produce pathogen
variants with different HS binding properties? Such questions, when answered,
may reveal new approaches to controlling the spread of numerous pathogens.
References:


Neyrinck, Audrey M., Mousson, Ariane, & Delzenne, Nathalie M. (2007). Dietary supplementation with laminarin, a fermentable marine beta (1-3) glucan, protects against hepatotoxicity induced by LPS in rat by modulating immune response in the hepatic tissue. *International Immunopharmacology, 7*(12), 1497-1506. doi: 10.1016/j.intimp.2007.06.011


