POLYPHOSPHATE:

A NOVEL NEGATIVE REGULATOR OF COMPLEMENT

AND ITS THERAPEUTIC POTENTIAL

IN AGE-RELATED MACULAR DEGENERATION

by

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Abstract

The innate mammalian response to injury involves the initiation of activation of two major blood-borne proteolytic systems; coagulation and complement. Recent studies have revealed that there is considerable crosstalk and interplay between these two systems. Polyphosphate (polyP) is a naturally occurring inorganic linear polymer that co-regulates these two systems, acting as a promoter of coagulation and an inhibitor of complement. This thesis aims to further characterize the mechanisms by which polyP regulates the complement system, and to test its physiological relevance in a model of human disease, age-related macular degeneration (AMD), the pathogenesis of which involves excess complement activation and oxidative stress. Based on data from our lab and studies in bacteria that polyP dampens complement activation and interferes with oxidative stress, I hypothesized that polyP would protect against AMD. To test this hypothesis, I used hemolytic assays to measure the complement activity in response to polyP, in vitro studies with AMD-associated cell lines to examine protective properties of polyP, and an in vivo model of AMD to evaluate the therapeutic efficacy of polyP. I showed that polyP dampens complement activation by interfering with the terminal pathway of complement, and that it also interferes with oxidative stress-induced cellular damage. The mechanisms by which it exerts this effect have not yet been determined. However, in vivo, in rodent models of AMD, polyP protects against laser-induced choroidal neovascularization (CNV), a feature of AMD, with reduced deposition of complement. An agent such as polyP, that simultaneously suppresses complement activation and protects against oxidative stress, holds potential therapeutic value. The findings in this thesis raise awareness of the potential importance of a ubiquitous, naturally occurring inorganic compound that has largely been overlooked. Most important, the findings reveal a promising use for polyP as a treatment for AMD, a common and devastating disease that affects millions of people worldwide.
Preface

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I conceptualized the study designs (except for the gel-filtration study, which was conceptualized by a previous graduate student), and all experiments were also performed and analyzed by me (except for the rat in vivo study, which was performed by a Research Associate). The cell proliferation studies were performed by a summer student whom I was supervising.

The UBC Animal Care Committee examined and approved the use of animals for experiments as described in this thesis under Application Number: A14-0135. Studies with humans were approved by the UBC Clinical Research Ethics Board (CREB) under protocol number: H12-02508.
# Table of Contents

**Abstract** ........................................................................................................................... ii

**Preface** ............................................................................................................................... iii

**Table of Contents** ............................................................................................................... iv

**List of Figures** ................................................................................................................... vii

**List of Abbreviations** ........................................................................................................ viii

**Acknowledgements** ........................................................................................................... xi

**Dedication** .......................................................................................................................... xii

**Chapter 1: Introduction** .................................................................................................... 1

1.1 The Coagulation Cascade................................................................................................. 2

1.2 Polyphosphate (PolyP) ..................................................................................................... 4

1.2.1 Polyphosphate and Coagulation ................................................................................. 5

1.3 The Complement System ................................................................................................. 8

1.3.1 Regulation of Complement Activation ..................................................................... 11

1.3.2 Role of Polyphosphate in Complement .................................................................... 13

1.4 Age-Related Macular Degeneration (AMD) ................................................................ 14

1.4.1 Structure of the Retina and Choroid ...................................................................... 15

1.4.2 Pathogenesis of AMD ............................................................................................. 18

1.4.3 Role of Complement in AMD .................................................................................. 19

1.4.4 Role of the MAC in AMD ......................................................................................... 21

1.4.5 Role of Oxidative Stress in AMD ............................................................................ 22

1.4.6 Complement and Oxidative Stress ......................................................................... 22

1.4.7 Current Therapies for AMD ..................................................................................... 23

**Chapter 2: Hypothesis** .................................................................................................... 25
Chapter 3: Overall Goal and Objectives ......................................................... 26
  3.1 Overall Goal ......................................................................................... 26
  3.2 Specific Objectives ............................................................................. 26

Chapter 4: Materials and Methods ............................................................... 27
  4.1 Reagents ............................................................................................ 27
  4.2 Cell Culture ......................................................................................... 27
  4.3 Terminal Pathway Hemolytic Assay .................................................. 28
  4.4 Platelet Releasate Hemolytic Assay ................................................... 28
  4.5 Stability of PolyP≥1000 in Serum .................................................... 29
  4.6 Gel Filtration ...................................................................................... 29
  4.7 Measuring Effect of PolyP≥1000 on C5b-7 and C5b-8 Binding to Erythrocyte Membranes .... 30
  4.8 In vivo rodent model of laser-induced choroidal neovascularization (CNV) ................. 31
  4.9 Quantification of C5b-9 (MAC) Deposited on ARPE-19 (RPE) Cells and RF/6A (CEC) .......... 33
  4.10 Quantification of SC5b-9 ................................................................ 34
  4.11 Quantification of Nuclear Integrity .................................................. 34
  4.12 Effect of PolyP on the Integrity of H2O2 .......................................... 35
  4.13 Catalase Assay ................................................................................ 35
  4.14 qRT-PCR Catalase Gene Expression Analysis ................................. 36
  4.15 VE-cadherin Expression Assay ........................................................ 36
  4.16 EA.hy 926 Cell Proliferation Assay ............................................... 37
  4.17 BrdU Assay for Cell Proliferation .................................................. 37
  4.18 Statistics .......................................................................................... 38
  4.19 Ethics ............................................................................................... 38

Chapter 5: Results ......................................................................................... 39
  5.1 Mechanisms by which PolyP Suppresses Complement Activation ............. 39
    5.1.1 PolyP Interacts Directly with C5b,6 ............................................... 40
    5.1.2 PolyP≥1000 Interferes with Binding of C5b-7 and C5b-8 Complexes to Erythrocyte Membranes ........................................................................... 42
    5.1.3 Human Platelet Releasates Suppress Complement Activation via the Terminal Pathway 44
  5.2 In vivo Validation of Therapeutic Utility of PolyP in a Complement-mediated Disease .... 46
5.2.1 Comparing the Effect of PolyP130 and PolyP≥1000 on Complement Activation .......................... 46
5.2.2 PolyP≥1000 Suppresses Complement-mediated Damage in a Rodent Model of Wet AMD .......................... 47
5.2.3 PolyP≥1000 is Functionally Stable for at least 10 days in Serum .............................................. 51
5.3 Cellular Effects of PolyP .................................................................................................................. 52
  5.3.1 PolyP130 Suppresses C5b-9 Deposition on CEC and RPE Cells ...................................................... 53
  5.3.2 Effects of PolyP130 on Release of SC5b-9 from CEC and RPE Cells .............................................. 56
  5.3.3 PolyP130 and Oxidative Stress ..................................................................................................... 58
  5.3.4 Morphologic Evidence that PolyP Protects Cells Against Oxidative stress .................................. 58
  5.3.5 PolyP does not alter the Integrity of H2O2 .................................................................................... 61
  5.3.6 Effect of of PolyP130 and monoP on Cellular Expression of Catalase ............................................ 63
  5.3.7 PolyP Protects Nuclear Integrity of RPE cells and CEC from Oxidative Stress ............................. 65
  5.3.8 Effect of PolyP130 on Oxidative Stress-Induced Changes in Junctional Protein Integrity ............. 66
  5.3.9 Effect of PolyP130 of Endothelial Cell Proliferation .................................................................... 69

Chapter 6: Discussion and Future Directions ......................................................................................... 71
  6.1 PolyP: Complex Roles in Coagulation and Complement ................................................................. 71
  6.2 Mechanisms by Which PolyP Suppresses Complement .................................................................. 72
  6.3 Cellular Models of AMD: Limitations and Advantages ................................................................. 74
  6.4 Role of PolyP and MonoP as an Anti-oxidant ................................................................................ 75
  6.5 Lack of Proliferative Effect on Endothelial Cells ........................................................................... 78
  6.6 Therapeutic Potential for AMD ................................................................................................... 78
  6.7 Strengths and Weaknesses of the AMD Model of Laser-induced CNV ....................................... 79
  6.8 Future In vivo Studies with PolyP ................................................................................................. 80

Chapter 7: Conclusion .......................................................................................................................... 82

Bibliography ........................................................................................................................................ 83
List of Figures

Figure 1: The coagulation cascade ........................................................................................................ 4
Figure 2: Chemical composition of polyphosphate ............................................................................... 5
Figure 3: Function of polyP in the coagulation cascade ......................................................................... 7
Figure 4: The complement cascade. ....................................................................................................... 10
Figure 5: PolyP suppresses complement activation. ............................................................................... 12
Figure 6: Reduced visual acuity in AMD ................................................................................................. 15
Figure 7: Pictorial representation of the normal macula and pathophysiological changes in AMD .......................................................... 17
Figure 8: PolyP suppresses the terminal pathway of complement in a concentration-and size- .............. 40
dependent manner ..............................................................................................................................
Figure 9: Gel filtration of complement proteins to assess interaction with polyP_{≥1000}....................... 41
Figure 10: Effect of PolyP on membrane binding/integration of C5b-7 and C5b-8 ............................... 43
Figure 11: Platelet releasates suppress complement activation via the terminal pathway ...... 45
Figure 12: Terminal pathway hemolytic assay to compare polyP_{130} and polyP_{≥1000} .................... 47
Figure 13: C5b-9 deposition and neovascularization after laser injury in rats ........................................ 49
Figure 14: C5b-9 deposition and neovascularization after laser injury in mice ..................................... 50
Figure 15: Time-dependent change in function of polyP_{≥1000} in serum ............................................ 52
Figure 16: Flow cytometry to detect C5b-9 deposition on CEC and RPE cells ........................................ 55
Figure 17: Measurement of SC5b-9 formation with ELISA .................................................................. 57
Figure 18: Cell morphologic changes in response to oxidative damage and polyP_{130} ....................... 60
Figure 19: Effect of polyP_{130} on functional integrity H_{2}O_{2} .................................................................. 62
Figure 20: Effect of polyP_{130} and monoP on catalase activity ............................................................. 64
Figure 21: Effect of polyP_{130} and monoP on catalase gene expression .............................................. 64
Figure 22: Protective effect of polyP_{130} on nuclear integrity following cell exposure to oxidative .......... 66
stress. ..................................................................................................................................................
Figure 23: Effect of polyP_{130} on VE-cadherin expression. ................................................................. 68
Figure 24: Effect of polyP_{130} and monoP on cell proliferation ............................................................ 70
### List of Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>ADP</td>
<td>adenine diphosphate</td>
</tr>
<tr>
<td>aHUS</td>
<td>atypical hemolytic uremic syndrome</td>
</tr>
<tr>
<td>AMD</td>
<td>age-related macular degeneration</td>
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<tr>
<td>APC</td>
<td>activated protein C</td>
</tr>
<tr>
<td>apoA1/A2/E</td>
<td>apolipoprotein A1/A2/E</td>
</tr>
<tr>
<td>ARVO</td>
<td>The Association for Research in Vision and Ophthalmology</td>
</tr>
<tr>
<td>AT</td>
<td>antithrombin</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
</tr>
<tr>
<td>BCA</td>
<td>bicinchoninic acid</td>
</tr>
<tr>
<td>BrdU</td>
<td>bromodeoxyuridine</td>
</tr>
<tr>
<td>C1INH</td>
<td>C1 esterase inhibitor</td>
</tr>
<tr>
<td>cDNA</td>
<td>complementary deoxyribonucleic acid</td>
</tr>
<tr>
<td>CEC</td>
<td>choroidal endothelial cells</td>
</tr>
<tr>
<td>CFH</td>
<td>complement factor H</td>
</tr>
<tr>
<td>CNV</td>
<td>choroidal neovascularization</td>
</tr>
<tr>
<td>CR1</td>
<td>complement receptor 1</td>
</tr>
<tr>
<td>cRBC</td>
<td>chicken red blood cell</td>
</tr>
<tr>
<td>CRP</td>
<td>C-reactive protein</td>
</tr>
<tr>
<td>DAF</td>
<td>decay accelerating factor</td>
</tr>
<tr>
<td>FACS</td>
<td>fluorescence activated cell sorting</td>
</tr>
<tr>
<td>FB</td>
<td>factor B</td>
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<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>FH</td>
<td>factor H</td>
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<tr>
<td>FI</td>
<td>factor I</td>
</tr>
<tr>
<td>GWAS</td>
<td>genome wide association study</td>
</tr>
<tr>
<td>GVB</td>
<td>gelatin veronal buffer</td>
</tr>
<tr>
<td>HI-FBS</td>
<td>heat inactivated fetal bovine serum</td>
</tr>
<tr>
<td>HMWK</td>
<td>high molecular weight kininogen</td>
</tr>
<tr>
<td>iNKT cells</td>
<td>invariant natural killer T cells</td>
</tr>
<tr>
<td>Kal</td>
<td>Kallikrein</td>
</tr>
<tr>
<td>MAC</td>
<td>membrane attack complex</td>
</tr>
<tr>
<td>MASP</td>
<td>MBL-associated serine protease</td>
</tr>
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<td>MBL</td>
<td>mannose-binding lectin</td>
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<tr>
<td>monoP</td>
<td>monophosphate</td>
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<tr>
<td>Oligo-dT</td>
<td>oligomeric deoxy thymine nucleotides</td>
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<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
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<tr>
<td>PGE</td>
<td>prostaglandin E</td>
</tr>
<tr>
<td>PK</td>
<td>prekallikrein</td>
</tr>
<tr>
<td>PNH</td>
<td>paroxysmal nocturnal hemoglobinuria</td>
</tr>
<tr>
<td>polyP</td>
<td>polyphosphate</td>
</tr>
<tr>
<td>PPK1</td>
<td>polyphosphate kinase 1</td>
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<tr>
<td>PPK2</td>
<td>polyphosphate kinase 2</td>
</tr>
<tr>
<td>qRT-PCR</td>
<td>quantitative real-time polymerase chain reaction</td>
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<tr>
<td>ROS</td>
<td>reactive oxygen species</td>
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<tr>
<td>RPE</td>
<td>retinal pigment epithelial</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
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<tr>
<td>RT-PCR</td>
<td>reverse transcription polymerase chain reaction</td>
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<tr>
<td>SAP-A</td>
<td>serum amyloid A</td>
</tr>
<tr>
<td>SC5b-9</td>
<td>soluble (or S-protein) C5b-9</td>
</tr>
<tr>
<td>TER</td>
<td>transepithelial resistance</td>
</tr>
<tr>
<td>TF</td>
<td>tissue factor</td>
</tr>
<tr>
<td>TFPI</td>
<td>tissue factor pathway inhibitor</td>
</tr>
<tr>
<td>VE-cadherin</td>
<td>vascular endothelial cadherin</td>
</tr>
<tr>
<td>VEGF</td>
<td>vascular endothelial growth factor</td>
</tr>
<tr>
<td>VWF</td>
<td>von Willebrand Factor</td>
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Last but not least, to God, for giving science its meaning and beauty.
Dedication

To my parents, who were the first ones to teach me the value of education.
Chapter 1: Introduction

Organisms have developed means to contain wounds by limiting bleeding and eliminating pathogens and damaged host cells via the recruitment of innate defense mechanisms, particularly within the vascular system [1]. Disease emerges when there is unchecked activation of innate immune and/or coagulation responses. These include, for example, atherosclerosis, stroke, venous thromboembolic disease, arthritis, atypical hemolytic uremic syndrome, and age-related macular degeneration. Understanding the mechanisms that regulate innate immunity and coagulation will uncover strategies to reduce tissue damage in these diseases.

The focus of the Conway lab is to explore molecular interactions between the coagulation cascade, and another major blood-borne proteolytic cascade, complement, which is a key component of innate immunity, in the hopes of gaining novel therapeutic insights. The complement and coagulation systems are highly regulated proteolytic cascades, which result in the containment of infection and bleeding, respectively. This is achieved through the production of final effectors. In the complement system, the final effector is the membrane attack complex (MAC), which causes lysis and opsonization of pathogenic organisms or damaged host cells. Byproducts of the enzymatic reactions are anaphylatoxins, which initiate a range of pro-inflammatory events. Analogous to the complement activation pathway, the final effector in the coagulation system is thrombin, a serine protease that cleaves fibrinogen to fibrin to generate a fibrin clot. Recent studies have revealed significant overlap between these two systems. This is best exemplified by the dramatic success of the inhibitor of complement activation, eculizumab, in preventing the thrombotic manifestations of two diseases that are caused by excess complement activation – atypical hemolytic uremic syndrome (aHUS) and paroxysmal nocturnal hemoglobinuria (PNH) [2, 3].

Work initiated in the Conway lab, prior to my arrival, uncovered a factor - polyphosphate (polyP) - that co-regulates complement and coagulation. This thesis aims to further characterize the mechanisms by which polyP functions, and to test its physiological relevance in a model of human disease.
In this Introduction, I will first provide a brief background of coagulation, highlighting how polyP participates. This will be followed by a discussion of the complement cascade and its regulation. A more detailed description of the origins and functions of polyP will be provided, followed by a review of the findings that led to this thesis, i.e. the discovery of how polyP regulates complement. Finally, I will discuss the clinical course and pathogenesis of a common, complement-mediated cause of blindness - age related macular degeneration – setting the stage for in vivo investigations with polyP.

1.1 The Coagulation Cascade

Coagulation is a major proteolytic cascade in the blood, designed to prevent excess bleeding upon injury. It has two initiating pathways – extrinsic and intrinsic. The extrinsic pathway is triggered by exposure of tissue factor (TF) to circulating blood, either by damage to the endothelium or activation of circulating and/or subendothelial cells (e.g. monocytes, macrophages, pericytes, smooth muscle cells) by inflammatory stimuli [4]. TF forms a complex with small amounts of constitutively circulating factor VIIa, thereby forming the so-called extrinsic factor tenase complex. This complex, situated on cell surfaces at the site of injury, activates coagulation factors IX and X to generate factors IXa and factor Xa, respectively. Factor Xa produced from the tenase complex, cleaves prothrombin to generate the key enzyme in coagulation, factor IIa (thrombin). Like most coagulation steps, this requires the presence of a cofactor – in this case, factor Va – as well as a phospholipid surface and calcium ions [4]. With thrombin generated beyond a threshold, it is believed to feed back to activate factor XI of the intrinsic pathway, thereby amplifying its own production. Thrombin cleaves fibrinogen to yield fibrin monomers. Formation of a stable fibrin clot is achieved by cross-linking of the monomers by factor XIIIa that is itself generated by thrombin-mediated activation of factor XIII [5] (Figure 1).

The intrinsic pathway is initiated by activation of factor XII (Hageman Factor) to factor XIIa upon contact with a negatively charged surface [5, 6]. Factor XIIa promotes clot formation by activating factor XI, which in turn activates factor IX. In the presence of factor VIIIa (carried by
von Willebrand Factor, VWF), factor IXa activates factor X, thereby channeling into the common pathways, with subsequent generation of thrombin. Factor XIIa also cleaves prekallikrein (PK), which is bound to high molecular weight kininogen (HMWK) on the surface of the membrane. Cleavage of PK generates the serine protease kallikrein (Kal), which activates HMWK to HMWKa. Kal is also able to further cleave factor XII bound on the surface of the membrane, resulting in feedback amplification of the pathway [6]. An important negative regulator of factors XIa, XIIa and kallikrein is C1 esterase inhibitor (C1INH) [5, 7, 8].

Patients with factor XII deficiency do not bleed. However, lack of factor XII in rodent models protects against thrombosis and immune defects, and elevated factor XI and factor XII levels in humans increase the risk of atherothrombosis. This relatively recent recognition of the clinical importance of the contact pathway has resulted in increased efforts to understand how it is initiated. In fact, the mechanism of triggering activation of factor XII remained a mystery until approximately 10 years ago, when a physiologic in vivo anionic surface on which factor XII, prekallikrein and HMWK assemble for activation of factor XII, was identified as polyphosphate (polyP) [9].

This discovery of a role for polyP has not only opened the door for the development of novel therapeutic interventions to prevent vascular disease and coagulation abnormalities, but it led to studies in the Conway lab to examine the role of polyP in the other major blood-borne proteolytic cascade, complement, thereby setting the stage for this thesis.
Figure 1: The coagulation cascade. Activation of coagulation is triggered by injury to a cell surface (endothelial in the above example), exposing initiators of the intrinsic (HMWK-PK, factor XII) and extrinsic (TF-VIIa) pathways. These converge with transformation of factor X to Xa, and prothrombin (II) to thrombin (IIa). The cascade culminates with the formation of a stable fibrin clot. Key negative regulators of the cascade that prevent excess thrombin generation and clot formation (not discussed in the thesis text) are shown in red (AT – antithrombin; APC – activated protein C; TFPI – tissue factor pathway inhibitor).

1.2 Polyphosphate (PolyP)

Polyphosphate (polyP) is an inorganic linear polymer comprising orthophosphate monomers which are linked by high-energy phosphoanhydride bonds [10] (Figure 2). PolyP is ubiquitously expressed, found in virtually all organisms, but with variable polymer lengths [11, 12]. Bacteria produce longer lengths of the polymer (>300 to 1000 residues), while mammals produce shorter
forms (from 5-800 residues) [11, 13, 14]. In the last 3-4 decades, bacterial polyP has been the best characterized. It is essential for the pathogenicity of most prokaryotes, playing a crucial role in metabolism, immune protection, as a source of energy, and as an anti-oxidant [15-17]. The major enzyme that synthesizes polyP in bacteria is polyP kinase 1 (PPK1), loss of which results in decreased virulence [15, 18]. PolyP kinase 2 (PPK2) is the other bacterial enzyme that synthesizes polyP, and may also play a role in phosphorylating ADP to ATP, allowing the bacteria to use polyP as an energy reserve [16]. PolyP can also be synthesized in vitro, and is used for a variety of industrial purposes such as flame-retardants, fertilizers, water treatment reagents, and food additives [19].

Figure 2: Chemical composition of polyphosphate. Polyphosphate (polyP) consists of a string of orthophosphate monomers (bracketed). It is found in varying lengths in all living organisms, ranging from tens to more than a thousand units long.

1.2.1 Polyphosphate and Coagulation

Extensive studies in the last decade have revealed that polyP plays an important role in coagulation [20, 21]. Although ubiquitously expressed, polyP is found at high concentrations in the dense granules of platelets, which are analogous to acidocalcisomes of bacteria [9, 22]. When platelets are activated, polyP with a mean chain length of ~70 orthophosphate units (range ~60-100) is released [23]. It has not been established what happens to the polyP, i.e.,
whether it binds to the surface of cells (e.g., platelets, endothelial cells), or if it is cleared by degradation, the kidney or the reticuloendothelial system.

PolyP enhances coagulation at different steps in the cascade [23], and the site of action depends partly on the length of the polymer (Figure 3). For primary hemostasis, the concentration of polyP released from platelets was sufficient to initiate fibrin formation [23]. PolyP of at least 45 orthophosphate units, at a concentration of >1 µg/mL is able to activate factor XII and prekallikrein in human or murine plasma [23]. PolyP reduces TF-induced clotting time almost 6-fold in normal plasma, and accelerates TF-triggered clotting in factor XII-deficient human plasma ~1.3 fold compared to TF alone [23]. PolyP (~25-75 orthophosphate units long) achieves this by abrogating the anti-coagulant effect of tissue factor pathway inhibitor (TFPI) [9]. PolyP also accelerates factor V activation by thrombin [9]. Further downstream, polyP at a concentration of as little as 0.5 µg/mL dramatically accelerates clotting in the presence of exogenous factor Va [23]. In the intrinsic pathway, polyP is able to decrease the time and increase the amount of thrombin generation [23]. In addition, platelet-sized polyP accelerates factor XI activation by thrombin, potentially acting as a cofactor [24]. These pro-coagulant activities are dependent on the presence of factor XII [23]. PolyP also affects fibrin formation, increasing clot integrity, which results in prolonged fibrinolysis time [23, 25]. This function, unlike polyP’s pro-coagulant effects upstream of thrombin generation, is independent of factor XII activation [23]. In platelet-poor plasma, polyP (~75 orthophosphate units) reduces the clotting time more than 5-fold and delays fibrinolysis ~1.6-fold [9].
Figure 3: **Function of polyP in the coagulation cascade.** The role of polyP in the coagulation cascade is partly dependent on the length of the polymer, depicted here simply as “long-chain” (A) or “platelet-size” (B) polyP. Long-chain polyP (up to several thousand units) is most effective at activating factor XII (1). Both long and short polyP chains (~60-100 units long) enhance thrombin-mediated activation of factor V (2), increase clot integrity (3), and accelerate back-activation of factor XI by thrombin (4). Figure adapted from Morrissey et al. 2012, *Blood.* [20]

The molecular mechanisms underlying polyP’s procoagulant properties are not yet well-defined. PolyP binds tightly to thrombin but does not bind to prothrombin [26]. It also binds to fibrinogen, factors XI and XII, prekallikrein, HMWK, and von Willebrand factor [25, 27]. Intravenous infusion of polyP into mice induces thrombosis [23]. Consistent with the biochemical studies, mice lacking factor XII are protected against polyP-induced thrombosis [23, 28].
Overall, there are ample data, *in vitro* and *in vivo* in mouse models, to support the notion that polyP promotes coagulation. With increasing evidence of interplay between coagulation and innate immunity, our lab examined the role of polyP in a key blood-borne component of innate immunity, the complement system.

### 1.3 The Complement System

The complement system is a proteolytic system that comprises over 30 soluble and membrane-bound proteins that contribute to innate and adaptive immunity [29]. Analogous to the coagulation system, complement activation is tightly regulated to ensure a rapid, highly localized and temporally restricted response to promote clearance of the offending pathogen or injured cells, while limiting damage to surrounding tissue, and facilitating healing [1].

There are three pathways by which the complement system is initiated; classical, lectin, and alternative [30] (Figure 4). The classical pathway is triggered by the binding of the C1 complex to a pathogen surface, directly or via an IgG or IgM antibody [31]. The C1 complex comprises a C1q molecule and two each of C1r and C1s [32]. C1q has a collagen tail and 6 globular heads which are responsible for attaching to pathogens [31]. When more than one globular head has bound to a pathogen, the C1r2C1s2 complex undergoes a conformational change, activating each C1r to cleave one C1s. This transforms C1s to a serine protease, which then cleaves C4 to C4a and C4b [31]. C4b attaches to a pathogen, recruiting circulating C2, bringing it into close proximity for cleavage by C1s into C2a and C2b. C4b and C2b form the C4bC2b complex, also known as C3 convertase [33]. (Note that we use current nomenclature for C2a and C2b, the latter being the larger fragment).

The lectin pathway, also known as the mannose-binding lectin (MBL) pathway, is triggered by the interaction of MBL to a mannose-containing polysaccharide on pathogen surfaces. This results in binding of MBL-associated serine protease (MASP) 1 and 2 to the MBL. The MBL-MASP1-MASP2 complex results in activation of the MASPS, cleaving C4 into C4a and C4b, in an identical fashion as for C1s, yielding again the C4b2b C3 convertase [34, 35].
The alternative pathway is unlike the other two pathways in that it does not require a pathogen surface or antibody to be initiated, i.e., it is constitutively active at low levels, but may be rapidly amplified, wherein it does require exposure to non-host surfaces. Moreover, the alternative pathway is highly dependent on the presence of ionic calcium and ionic magnesium, whereas the classical and lectin pathways are only dependent on ionic calcium [36]. The alternative pathway relies on the fact that circulating C3 is constantly being hydrolyzed to a C3b-like moiety known as C3(H2O2), to which the active fragment of factor B, Bb, may bind, thereby forming a solution phase C3(H2O2)Bb C3 convertase. Most of this is inactivated by negative regulators (see below). In a setting requiring recruitment of an innate immune response, this C3 convertase may further cleave C3 to generate C3a + C3b, the latter which binds to a pathogen surface, providing a binding site for factor B. Factor B is cleaved by Factor D (FD), yielding Bb and liberating Ba. Bb binds to C3b, forming C3bBb, the alternative pathway C3 convertase. This is relatively unstable compared to the C3 convertase of the lectin and classical pathway, and is stabilized by properdin (Factor P) [37].

As can be seen from the above, the three pathways converge with formation of a C3 convertase, which cleaves C3 to C3a and C3b. C3a is released into the circulation and is a relatively potent anaphylatoxin, promoting the recruitment of inflammatory cells to the site of infection/injury [38]. As the density of C3b deposited on the target surface increases and binds to the C3 convertases, the substrate specificity of the convertase shifts to C5, and thus, C4b2b(C3b)n and C3bBb(C3b)n become C5 convertases [39]. C5 convertase cleaves C5 into C5a and C5b. C5a is an anaphylatoxin that is more potent that C3a [40]. C5b immediately binds to circulating C6, forming a strong C5b-6 complex. This allows C7 to bind via C6, forming the C5b-7 complex that integrates partially into the membrane of the target cell or pathogen. C8 then binds to C5b-7 and this C5b-8 complex integrates completely into (but not through) the membrane. Numerous C9 molecules then attach (up to ~20 per C5b-8) and extend to form a pore-like complex that inserts into the membrane, causing damage and lysis of the target cell or pathogen [41, 42]. This C5b-9 complex is often referred to as the membrane attack complex (MAC) [43]. Assembly of the MAC from C5b is known as the terminal pathway. It occurs spontaneously, in an ion-
independent manner, and without the necessity of enzymes [43]. Some C5b-9 may not insert into the membrane or may be forced out by membranous negative regulators. This is then referred to as soluble C5b-9, S-protein C5b-9 or SC5b-9 [44]. Unlike membrane-bound C5b-9 which is amphiphilic, SC5b-9 is hydrophilic, which allows it to be measured in the plasma [44, 45]. Although SC5b-9 is non-cytolytic, it is known to have pro-inflammatory and procoagulant properties [44, 46].

![Diagram of the complement cascade](image)

Figure 4: **The complement cascade.** Complement is initiated via the lectin, classical, or alternative pathways. These converge to form a C3 convertase, cleaving C3 to C3a and C3b. With more C3b deposited on the target surface, the specificity of the so-formed C3bBbC3b convertase shifts to C5, thereby resulting in the cleavage of C5 to C5a and C5b. C6 binds to C5b, yielding C5b,6, the starting point of the terminal pathway. With subsequent binding of C7, C8 and multiple C9 molecules, the terminal pathway ends with the assembly of the C5b-9 membrane attack complex (MAC), causing lysis of or damage to the pathogen or target cell. Specific negative regulators of complement activation, discussed in the text, are shown in red. This figure is based on a version provided by Jovian Wat [47].
1.3.1 Regulation of Complement Activation

As with all biological systems, a delicate balance is required to optimize the function of the complement system. Inadequate complement activation results in reduced clearance of pathogens and risk of overwhelming infection, while excess activation may cause tissue damage to the host [48]. Thus, there are several regulatory mechanisms in place at various points in the cascade that keep the system in check, several of which are described in the following.

The need for a membrane surface for most complement proteins localizes complement activation where it is needed. Unbound, most complement components are not stable in their active form. There are therefore many negative regulators that participate to prevent autoimmunity. C1 esterase inhibitor (C1INH) is a circulating serine protease inhibitor (serpin) that dampens activation via the lectin and classical pathways. It dissociates the activated (C1rC1s)2 complex from C1q, rendering it unable to generate the C4bC2b C3 convertase. It also neutralizes C1s and the MASPs, rendering them unable to cleave/activate C2 and C4 [49]. Deficiency of C1INH in humans results in hereditary angioneurotic edema, an inflammatory disease that is associated with excessive swelling around the airways [50].

The plasma serine protease, Factor I (FI) dampens complement activation primarily via the alternative pathway, although it may also affect the other pathways [29]. FI cleaves C3b and C4b bound on host cell membranes, rendering them inactive in terms of forming a C3 convertase. C3b is cleaved to iC3b, and subsequently to C3dg, while C4b is cleaved to C4c and then to C4d [30]. FI requires a cofactor to cleave/inactivate either C3b or C4b. These include soluble factor H (FH) and C4b-binding protein (C4BP), and membrane-bound cofactors CD46 and complement receptor 1 (CR1) [50, 51] (Figure 4).

FH has multiple mechanisms by which it negatively regulates complement. Although a soluble protein, FH attaches to polyanionic-rich host cell surfaces, and there binds most effectively to C3b, where it acts as a cofactor for FI-mediated C3b inactivation [51]. Bacterial cell walls lack polyanion sialic acid, and thus complement activation is allowed to proceed, as effective
inactivation of C3b and C4b by Fl/FH does not occur [50]. FH also interferes with binding of B/Bb to C3b, thereby preventing formation of the alternative pathway C3 convertase [52, 53]. It also destabilizes the C3 convertase. The importance of FH is highlighted by the diseases that result from its deficiency, including atypical hemolytic uremic syndrome (aHUS) and age-related macular degeneration, both of which feature excess complement activation [54].

Similar to FH, Complement receptor 1 (CR1) and decay accelerating factor (DAF, also known as CD55) compete with Bb binding to C3b, and can cause Bb to dissociate from the C3bBb C3 convertase complex [50] (Figure 4). CD59, also referred to as protectin, inhibits the final step of the terminal pathway – the binding of C9 to the C5b-8 complex. CR1, DAF, and CD59 are found almost exclusively on host cells [55, 56]. A deficiency in CD59 and DAF is associated with paroxysmal nocturnal hemoglobinuria (PNH), wherein red blood cells are susceptible to episodes of spontaneous lysis by complement [50]. The diseases that arise from regulatory protein deficiencies emphasize the importance of regulating complement activation, both spatially and temporally.

Figure 5: **PolyP suppresses complement activation.** Hemolytic assays were performed in serum (conducted by Jovian Wat) and show suppression of the terminal pathway (a) by polyP_{≥1000} (○), compared to monophosphate (●). The mechanism was partially elucidated by the discovery that suppression only occurs before the addition of C7 when this assay is conducted in a purified protein system (b). This suggests an interaction of polyP with any one or all of C5b,6, C6, or C7. Figure adapted from Wat et al. 2014, *Blood* [57].
1.3.2 Role of Polyphosphate in Complement

Coagulation and complement are coordinately activated to protect the organism from injury induced bleeding and infection. Although polyP reportedly interferes with complement activation in bacteria [58], its profound pro-coagulant properties in mammalian systems led us to predict that polyP would similarly enhance complement activation. Surprisingly, however, studies from the Conway lab, published in Blood in 2014 [57], revealed that polyP suppresses complement activation. This was determined using well-established in vitro hemolytic assays, measuring complement activation in human serum and then with purified proteins. The assays are based on the increased sensitivity of rabbit, chicken or sheep erythrocytes to human complement, and the ion-dependence of the different pathways. By incubating the erythrocytes with human serum, complement activation proceeds, causing the red blood cells to lyse, releasing hemoglobin which is measurable with a spectrophotometer at an absorbance at 405 nm.

As compared to monoP, polyP caused a concentration-dependent reduction in total complement-mediated hemolysis (Figure 5). The effect was also polymer length-dependent, with increasing lengths eliciting greater suppression. Further studies from the Conway lab showed that polyP suppresses complement most prominently via the terminal pathway, either at the C5b,6, C7, or C5b-7 step of the cascade [57] (Figure 5). This was observed not only in serum, but also in a purified system, indicating that polyP must directly interact with one or more complement components. Notably, once the C5b-7 complex was formed, polyP had no dampening effect.

These unique observations led to further investigations of the mechanisms by which polyP interferes with complement activation via the terminal pathway, allowing me to add new important knowledge and providing a foundation for this thesis.
The finding that this naturally occurring inorganic polymer, polyP, could suppress complement activation, also raised the question as to whether polyP could be used as a therapy. Based on work by other groups, intravenous administration of polyP is expected to be complicated by thrombosis (see above). Therefore, for validation of the therapeutic efficacy of polyP, we identified a complement-mediated disease that was relatively protected from the systemic vasculature, i.e., age-related macular degeneration (AMD).

1.4 Age-Related Macular Degeneration (AMD)

AMD is a world-wide leading cause of impaired vision or blindness for people over the age of 50 [59]. With the steady increase in the aging population, the number of affected individuals is expected to continue to climb. AMD is therefore not only a challenge for the affected patient and his/her family, but also a major public health concern, as well as a huge economic burden. In Canada alone, AMD has an estimated $2.6 billion negative impact on the gross domestic product [60].

AMD affects the macula, the part of the eye that is responsible for detailed central vision. Individuals who suffer from AMD have trouble with common tasks, such as recognizing faces, reading small print and signs, and independently mobilizing (Figure 6). They are more likely to suffer from depression, and have an overall reduced quality of life [60, 61].

Two forms of advanced AMD exist; the Dry or atrophic form accounts for ~90% of cases, while the Wet or exudative or vascular form accounts for the rest. However, it is the Wet form that results in ~90% of cases of blindness [62]. Thus far, the only specific therapies available for AMD are those that target the Wet form, and these are not uniformly effective. Thus, there is an urgent need for new therapies, preferably those that combat both forms. This will require more knowledge of the underlying mechanisms of AMD. Although the pathogenesis of AMD is not entirely understood, oxidative stress and excess activation of the complement system are considered key contributors [63]. Thus, AMD represents a highly localized disorder, in need of new therapies. We predicted that polyP might be such a therapy.
1.4.1 Structure of the Retina and Choroid

AMD primarily affects the retinal and choroid layers of the eye. The retina comprises several layers of neurons. Light enters the eye and the photons are absorbed by the photoreceptor cells, i.e., rods and cones. The macula contains the densest population of photoreceptor cells. The absorbed photons are converted to an electrical signal that is transferred to bipolar cells. The signal is then transmitted to the ganglion cells, which relay an electrical impulse to the optic nerve [65]. A single layer of melanin-containing cells termed the “retinal pigment epithelial (RPE)” cells is positioned posterior to the photoreceptor cells (Figure 7a). These cells support the photoreceptor cells by phagocytosing old photoreceptor disks, recycling photopigment molecules, and supplying nutrients [65, 66]. Adjacent and exterior to the RPE layer, is a barrier known as Bruch’s membrane. This contains the basement membrane of the RPE layer and the underlying choroid layer, as well as an outer collagenous zone, an elastic zone, and an inner collagenous zone [67].
Posterior to Bruch’s membrane is the choroid layer (Figure 7a). The choroid consists of smooth muscle cells, secretory cells, neurons, and an abundance of blood vessels lined by choroidal endothelial cells (CEC). There are various functions of the choroid, including secretion of growth factors, adjusting the position of the retina, supplying the retina with nutrients and oxygen, and removing waste [68]. Growth factors secreted from the choroid participate in altering the shape of the eye in pathologies such as hyperopia and myopia [69]. Adjustment of retinal position is achieved by thickening and thinning of the choroid, and this in turn is accomplished by the smooth muscle cells, although the exact mechanisms are unknown [68]. Of all its properties, the choroid is most important as the major vascular supply for the retina [68].

Lined by choroidal endothelial cells, the choroidal blood vessels form a network of choriocapillaris. These vessels are fenestrated and 20-30 μm in diameter, which is ~75% wider than the capillaries in the retina, thereby allowing for a slower rate of blood flow. Permeability to proteins [62], owing to the fenestrations and slow flow rate, facilitates the exchange of nutrients, oxygen, and wastes with the retina. The choriocapillaries also play a role in thermoregulation of the eye [68].
Figure 7: Pictorial representation of the normal macula and pathophysiological changes in AMD. The normal macula consists of organized layers of photoreceptors, a monolayer of RPE cells, and a choroid vasculature that is separated from the retina by Bruch’s membrane (a). In Dry AMD, drusen accumulate in the sub-retinal space, and this is associated with atrophy of the RPE and photoreceptor cells (b). Wet AMD is characterized by proliferation and migration of choroidal blood vessels into the retinal layers (c). The Dry form of AMD accounts for most cases. However, it is the Wet form that more commonly leads to blindness. The Wet form can develop from the intermediate stage or progress from the Dry form (d). Figures a-c adapted from Brightfocus® Foundation. Illustration by Bob Morreale [70].
1.4.2 Pathogenesis of AMD

AMD is classified into early, intermediate, and late stages, based on specific abnormalities and by the associated symptoms. In the early stage, molecular byproducts deposit at the interface of the RPE layer and Bruch’s membrane. These are termed drusen, and consist of lipids and proteins which are largely immune-related, and include inflammatory proteins, immunoglobulins, and several complement components and complexes [71]. Also present is apolipoprotein E, RPE cell debris, lipofuscin, and melanin which are products of RPE cell injury [72, 73]. Drusen that form in the early stage of AMD are small- to medium-sized (<63-124 μm in diameter), well-marginalized deposits known as “hard drusen”. At this stage, vision may be normal or only minimally affected, with mild blurriness, reduced contrast perception, and/or difficulty adjusting to brightness and darkness [74]. The presence of only a few, small, hard drusen is considered a normal part of aging, and is not diagnostic of AMD [74].

The intermediate stage is distinguished by the presence of at least one larger, more diffuse druse termed “soft drusen”, and an increase in the number of medium-sized drusen [75]. Vision loss is more prominent, and may be associated with a decreased ability to stabilize a focal image [65].

The intermediate stage can lead to the advanced stage of AMD, of which there are two forms. Dry AMD is characterized by atrophy of the retina in the macular area, also known as geographical atrophy (Figure 7b). This is linked to the enlargement and confluence of drusen and consequent thickening of Bruch’s membrane, blocking the flow of oxygen and nutrients from the choroid to the retina, as well as the flow of waste from the retina to the choriocapillaries [76, 77]. The RPE cells are the first to be affected, and their loss of function results in secondary degeneration of photoreceptor cells [78]. Loss of visual function associated with Dry AMD occurs very gradually with symptoms that can progress in a span of years before severe visual impairment manifests. There are no treatments for this form, although a few therapies are currently undergoing clinical trial [79, 80].
The other 10% of advanced AMD cases are classified as Wet AMD. The name derives from the hallmark choroidal neovascularization (CNV) that occurs, as Bruch’s membrane is disrupted [81] (Figure 7c). CNV is believed to be the result of chronic inflammation caused by drusen and a damaged RPE layer, although it is likely that the CEC also participate in a major way [81]. Chronic inflammation causes an influx of macrophages, dendritic cells, and iNKT cells. In addition, normal aging of the retina and choroid alters the function of the retinal and choroidal cells, such that they become activated, and express more complement proteins as well as chemoattractants [82]. The consequence is an influx of immune cells, which amplify and sustain an inflammatory response. This is associated with increased expression of angiogenic factors, most notably, vascular endothelial growth factor (VEGF). Blood vessels that form, emanating primarily from the choroid, initially start as capillaries, and may progress to become irregular, large and leaky blood vessels [83]. The danger in CNV is that the vessels will leak their serous contents into the retina [84]. This damages the RPE cells and photoreceptors, causing them to become dysfunctional, and results in loss of vision [85, 86]. Wet AMD may progress from the Dry form, or arise directly from intermediate AMD (Figure 7d) [87].

1.4.3 Role of Complement in AMD

Several lines of evidence support the notion that heightened activation of complement contributes to the pathogenesis and progression of AMD. Most notably, there are mutations in several genes that encode complement components that confer a higher risk of the development of AMD. The strongest association is with complement factor H (FH) [88, 89]. In a genome-wide association study (GWAS), a single nucleotide polymorphism at amino acid 402 of FH was identified that increases the likelihood of AMD development by 7.4-fold [88, 89]. This Y402H (tyrosine replaced with histidine at amino acid residue 402) mutation occurs at the site at which FH binds to C-reactive protein (CRP) and glycosaminoglycans (e.g., heparin) [52]. The latter localizes FH to the surface of the host cell which, as previously discussed, enhances the capacity of FH to act as a cofactor for FI-mediated inactivation of C3b on host cells. CRP is an inflammatory protein that is deposited in high amounts in the eyes of patients with AMD [90, 91], [92]. In patients with this risk variant of FH, complement component deposition on the
choroid vasculature was significantly increased, and associated with increased leakage of plasma contents into Bruch’s membrane [89]. Although not definitively shown, the data suggest that the mutant form of FH is responsible for excess complement activation that, in these patients, leads to AMD. There are other polymorphisms in genes for complement components that have also been linked to AMD [93]. Some of these are described below.

A rare missense mutation of complement Factor I (FI) that has been implicated in increasing the risk of AMD [94, 95] results in the substitution of glycine to arginine (G119R) [95]. This variant form of FI is able to cleave and inactivate C3b, but to a lesser extent than the wild-type protein, resulting in less complement inhibition [95].

Common variants of C3 have also been linked to AMD. A substitution of lysine by glutamine at amino acid 155 (K155Q) results in a C3/C3b that is relatively resistant to cleavage by FI/FH [94]. In a model of laser induced CNV for Wet AMD, mice with heterozygous defects of C3 develop smaller CNV lesions [96], supporting the notion that complement plays an important role. C9 has also been implicated; a substitution of proline for serine at amino acid 167 (P167S) of C9 is linked to an increased risk of AMD, while a nonsense mutation of arginine at amino acid 95 (R95X) may confer protection against AMD in Japanese carriers [94], [96]. The R95X C9 variant is one of the few complement component mutations that are associated with protection (also known as an ‘inverse association’) against AMD development. A Factor B polymorphism that results in a substitution of arginine for glutamine at amino acid 32 (R32Q) is also associated with decreased susceptibility to AMD, based on 2 separate cohort studies [97, 98]. In addition, a Factor B mutation that substitutes leucine for histidine at amino acid 9 (L9H) confers a similar protective effect [98]. In the same studies, the C2 polymorphism that substitutes glutamic acid for an aspartic acid at amino acid 318 (E318D) was also found to have an inverse association with AMD [45, 98].

The link between complement and AMD is further strengthened by evidence of enhanced activation of complement – particularly the alternative pathway - in the eyes of patients with AMD [99, 100]. This results in the recruitment of inflammatory cells via augmented local release
of inflammatory mediators, with subsequent amplification of complement activation [98]. This cycle is responsible for chronic, local inflammation that is a feature of AMD. Deposits of complement components and inflammatory mediators are often found in AMD affected eyes [88]. Within drusen, C5 and C5b-9 are abundant [48, 72, 101]. C3a, C3d, all of the terminal pathway components, complement downregulators (clusterin and vitronectin), apolipoproteins (apoA1, apoA4, and apoE), serum amyloid A (SAP-A), serum amyloid P (SAP-P), and thrombospondin have also been detected in drusen [88, 102, 103]. There is considerable controversy as to the role of C3a and C5a in the damage associated with AMD. C3a and C5a in drusen may induce expression of VEGF, leukocyte recruitment, and CNV [103]. However, there is evidence that critical amounts of C3a and C5a are required for survival and integrity of the RPE cells [104]. The latter may explain why the complement inhibitor, eculizumab (which interferes with the generation of both the MAC and C5a) was not effective in reducing drusen volume in clinical trials [105].

1.4.4 Role of the MAC in AMD

Formation of the MAC is believed by many to be a key causative factor in AMD, directly damaging the RPE [106]. MAC deposition has been correlated with RPE loss and AMD severity [107]. As previously discussed, formation of a stable and functional MAC is negatively regulated via the terminal pathway by CD59, clusterin and vitronectin, each of which functions by binding to terminal pathway components and interfering with MAC assembly. CD59 is a glycosylphosphatidylinositol-linked protein that prevents C9 molecules from polymerizing. Although no genetic studies have linked CD59 to AMD, drusen components suppress CD59 in RPE [108]. Both clusterin and vitronectin are found in drusen, although their roles there are not known [109]. Identification of polyP as a negative regulator of the terminal pathway is rationale to explore whether it has protective properties against AMD.
1.4.5 Role of Oxidative Stress in AMD

The highly vascular retina is continuously exposed to photons from UV light and it is thus susceptible to chronic oxidative stress [110]. Oxidative stress is defined by an imbalance between reactive oxygen species (ROS) production and the body’s ability to detoxify these ROS. Generation of ROS is a physiologically normal process, formed naturally during the metabolism of oxygen. ROS play important roles in cellular function, but in excess, cause significant damage that may result in cell death, and a pro-inflammatory response, with recruitment of innate and adaptive immune responses. Several natural defense mechanisms therefore exist to neutralize ROS. H$_2$O$_2$-producing enzymes are compartmentalized in specialized lysosomes, referred to as peroxisomes [111]. These also contain catalase, an enzyme that degrades H$_2$O$_2$ into water and oxygen [111]. Glutathione peroxidase and superoxide dismutase are other important antioxidant enzymes. From the perspective of AMD, the most prominent antioxidant in the human retina is believed to be catalase [112], and patients with AMD reportedly have reduced catalase activity in their retinas [113].

The major risk factor for AMD is advanced age, and this is also associated with a diminished capacity to defend against the damaging effects of ROS. This is further exacerbated by exposure to smoking and/or second-hand smoke, which increases the risk of AMD by two- to three-fold [114]. Studies suggest that oxidative stresses in the eye modify otherwise normal proteins and lipids in the retina, and these initiate the formation of drusen [113]. Based on large scale clinical studies, oral antioxidant supplements are therefore recommended for patients with early/intermediate AMD to prevent progression to advanced AMD [115-117]. The data are not strong, but the risk-benefit profile favours taking these medications rather than not.

1.4.6 Complement and Oxidative Stress

Chronic excess complement activation and oxidative stress cooperate to augment the damage associated with AMD [88, 118, 119], and not surprisingly, most often coexist. This likely reflects the fact that AMD occurs in older people who acquire, through exposure to environmental,
genetic and/or epigenetic factors, disruptions in the mechanisms that protect against host cell damage. Moreover, there are positive feedback loops wherein activation of complement leads to increased generation of inflammatory mediators, including C3a and C5a [120], as well as ROS, which in turn further augment the inflammatory response, [98, 120], with further activation of complement. In AMD, these escalating, damaging pathways escape natural negative regulatory mechanisms, resulting in cellular damage, decreasing the capacity of the RPE to maintain the health of photoreceptors [88]. It is reasonable to consider that the most effective therapeutic strategies might include simultaneous suppression of the generation of ROS and complement activation.

1.4.7 Current Therapies for AMD

Current treatments for AMD are suboptimal and costly for the Wet form, and entirely lacking for the Dry form [121-126]. Treatments for the Wet or vascular/exudative form are principally designed to suppress vessel growth by interfering with vascular endothelial growth factor (VEGF)-related pathways. For example, monoclonal antibodies that neutralize VEGF are widely used. Pegaptanib is an aptamer that targets a soluble form of VEGF and has been shown to have efficacy over a 2 year period [127]. Bevacuzimab and ranibizumab are both humanized antibodies against VEGF that are able to inhibit all isoforms of VEGF, with ranibizumab having a 3-6 fold higher affinity [128-130]. VEGF pathway inhibitors are injected into the eye (intravitreous) ~once per month, and overall result in improved vision in ~30% of patients, and arrest progression in ~90% [131-134]. However, up to 20% of patients experience mild-to-serious side effects [135], and long-term use may be complicated by development of widespread atrophy of the RPE cells [136]. There are also concerns about potential detrimental effects on the retinal vasculature [137]. Complement pathway inhibitors have been evaluated in preclinical and early clinical trials, mostly for Wet AMD, and these have targeted factor B, factor D, C3 and C5 (eculizumab). Limited information is available on the progress of most of these trials, although eculizumab failed to reduce drusen size in Dry AMD. We believe that polyP holds greater therapeutic promise, as it dampens generation of the MAC, while sparing generation of C3a and C5a, both of which may be protective to retinal health. Moreover, at least in
prokaryotes, polyP has anti-oxidant properties. This raises the possibility that polyP might be capable of simultaneously suppressing the two major pathways that promote AMD, a strong rationale for further study.

It is estimated that the prevalence of AMD will exceed 150 million people worldwide by the year 2020 [138]. Although progress has been made in developing effective treatments for Wet AMD, there remains an urgent need for the development of alternative and more effective therapeutic strategies for both Wet and Dry AMD.
Chapter 2: Hypothesis

Based on our findings that polyP dampens activation of the complement system, we hypothesized that polyP will confer protection against AMD-associated stresses.
Chapter 3: Overall Goal and Objectives

3.1 Overall Goal

To increase our understanding of the mechanisms by which polyP regulates complement and cellular function and to apply this new knowledge in a rodent model of age-related macular degeneration (AMD).

3.2 Specific Objectives

1. To uncover the mechanisms by which polyP regulates complement
   a. Hemolytic assays to verify the effect of polyP on complement
   b. Determine the biochemical interaction of polyP with complement components

2. To evaluate the therapeutic efficacy of polyP in a model of AMD
   a. In vivo, with a laser-induced choroidal neovascularization rodent model in
      i. Rats
      ii. Mice
   b. In vitro, using retinal pigmented epithelial and choroidal endothelial cell lines, in response to:
      i. Complement-mediated stress
         • Measure formation of complement final effectors
      ii. Oxidative stress
         • Elucidate possible mechanisms by which polyP may confer protection
            o Assess anti-oxidant enzyme activity and expression in response to polyP
            o Test the effects of polyP on cell-junction integrity in response to oxidative stress
            o Test the effect of polyP on cell-proliferation
Chapter 4: Materials and Methods

4.1 Reagents

Synthesized polyP$_{\geq 1000}$ was a generous gift from Dr. James H. Morrissey (Urbana, Illinois, USA). Synthesized polyP$_{130}$ was from Regenetiss Inc. (Tokyo, Japan). All complement proteins and human sera were purchased from Complement Technology, Inc. (Tyler, Texas, USA). Unless otherwise stated, antibodies were purchased from Life Technologies (Carlsbad, California, USA).

4.2 Cell Culture

Cell culture media and reagents were purchased from Life Technologies (Carlsbad, California, USA). RF/6A cells, a choroidal endothelial cell line derived from monkeys, and ARPE-19, a retinal pigment epithelial cell line derived from humans, were purchased from American Type Culture Collection (Manassas, Virginia, USA). EA.hy 926 cells, a hybridized human umbilical vein cell line, was a gift of Dr. Cora-Jean C. Edgel (University of North Carolina, USA).

EA.hy 926, RF/6A, and ARPE-19 cells were cultured in Dulbecco’s Modified Eagle’s Media, Modified Eagle’s Media, and Dulbecco’s Modified Eagle’s Media-F12 (Sigma-Aldrich Corp., St. Louis, Missouri, USA), respectively, all supplemented with 10% heat-inactivated fetal bovine serum (HI-FBS), L-glutamine (2 mM), Na pyruvate (1 mM), and penicillin and streptomycin (50 U/mL). From frozen aliquots, cells were thawed for 2 minutes in a 37°C water bath and directly added to polystyrene cell-culture dishes (Corning Inc., Corning, NY, USA) at 1:10 dilution in their growth media. They were allowed to adhere overnight in a humidified 37°C incubator with 5% CO2, after which the media was changed to fresh growth media. The cells were subsequently passaged every 3-5 days. Passage number did not exceed 25, after which a fresh aliquot was used. For propagation, cells at low passage were frozen with 50% cryoprotective media (Basal Eagle’s Medium with Hank’s BSS and 15% DMSO (Lonza, Walkersville, Maryland, USA)) and 50% HI-FBS and stored in liquid nitrogen.
### 4.3 Terminal Pathway Hemolytic Assay

Chicken red blood cells (cRBCs) were isolated from chicken whole blood (Colorado Serum Company, Denver, CO) by centrifugation at 1000 x g, and subsequently washed 3 times with gelatin veronal buffer (GVB) (Sigma-Aldrich Corp.) with 10 mM EDTA. The EDTA chelates cations to prevent activation of upstream complement pathways, which are ion-dependent. Prior to activation of the terminal pathway, varying concentrations of polyP are added to 3.33 x 10^7 cells/mL of cRBCs in a 96-well plate. The terminal pathway was activated by the addition of purified C5b,6 (the concentration to achieve ~80% hemolysis was determined on the same day) to 3.33 x 10^7 cells/mL of cRBCs, followed by the addition of normal human serum at 2% final concentration in a final volume of 300 µL. All dilutions were conducted in GVB-EDTA. Reaction mixtures were incubated at 37°C for 30 minutes, causing hemolysis of the cRBCs and consequent release of hemoglobin due to complement-mediated lysis. The intact cRBCs were pelleted by centrifugation at 600 x g for 3 minutes. 100 µL of the supernatant was transferred to a new 96-well plate, and diluted 1:1 with dH2O. The absorbance of the supernatant samples at 405 nm was determined with the Spectramax 384 Plus Microplate Reader (Molecular Devices, Sunnyvale, CA, USA). The absorbance of the sample reflects the amount of hemolysis that occurred due to complement-mediated lysis.

Similarly, hemolytic assays using purified terminal pathway complement proteins were conducted, eliminating the need for normal human serum as well as EDTA, as the terminal pathway is ion-independent. In these purified systems, purified C5b,6 (the concentration to achieve ~80% hemolysis) is combined with a mixture of purified C7, C8, and C9 (final concentrations of 15 nM, 10 nM, and 25 nM, respectively).

### 4.4 Platelet Releasate Hemolytic Assay

Using a 16-gauge needle, blood was drawn from a human volunteer (with Ethics Approval from UBC) using the straight-drip technique into 4.5 mL citrated Vacutainer® tubes (BD, Franklin Lakes, New Jersey, USA) containing 2 µM prostaglandin E (PGE). A total of 12 tubes were
collected, with the first tube being discarded to avoid using blood in which the coagulation and/or complement pathways were activated through the trauma of the needle insertion. Whole blood obtained was centrifuged at 100 x g for 10 minutes. Platelet rich plasma was collected and transferred to 1 mL microcentrifuge tubes. These were centrifuged at 200 x g to obtain a platelet pellet, which was then re-suspended in 1 mL CGS buffer (10 mM trisodium citrate, 30 mM dextrose, 1.2 mM sodium chloride at pH 6.5). The platelets were centrifuged at 200 x g and re-suspended in CGS buffer twice more, followed by another centrifugation at 200 x g. One sample was re-suspended in 200 µL CGS buffer, while another sample was re-suspended in 10 nM thrombin diluted in CGS buffer to activate the platelets. The “activated” samples were mixed on a rocker for 15 minutes, after which platelet aggregates formed. The aggregates were centrifuged at 200 x g for 10 minutes and the supernatant containing the platelet releasate (which would also contain polyP released from the dense granules) was transferred to a clean tube. A terminal pathway hemolytic assay was then performed with the supernatants of the non-activated and activated samples.

4.5 Stability of PolyP≥1000 in Serum

The terminal pathway hemolytic assay was modified to test the stability of polyP≥1000 in terms of its ability to dampen complement activation. 100% normal human serum and 200 µM final concentration of polyP≥1000 were co-incubated at 37°C for varying periods of time, ranging from 30 minutes to 10 days. The serum and polyP≥1000 mixture was then added simultaneously to the hemolytic assay, to a final concentration of 2% serum and 300 µM polyP≥1000. These samples were compared to serum without polyP≥1000 but incubated at the same time points.

4.6 Gel Filtration

C5b,6 was stored in a buffer consisting of 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 120 mM NaCl, pH 7.2 (HBS). C7 was stored in 10 mM Na₃PO₄, 145mM NaCl, pH 7.3 (PBS). The buffers were filtered with a 0.22 µm pore diameter filter (Stericup Millipore Express™PLUS), under a Class 2A Biosafety Cabinet. Proteins were thawed in a 37°C water bath.
for 10 minutes. To exclude protein aggregates from being loaded onto the chromatography column, samples (proteins and polyP/monoP) were centrifuged at 20,800 x g for 15 minutes and the supernatants were removed for incubation with polyP$_{\geq1000}$ or monoP and gel filtration. The following conditions were evaluated:

a. C5b,6 alone
b. C5b,6 with polyP$_{\geq1000}$
c. C5b,6 with monoP (Na$_3$PO$_4$)
d. C7 alone
e. C7 with polyP$_{\geq1000}$

20 µg of each protein in 100 µL of their respective buffers was prepared. polyP$_{\geq1000}$ and monoP were used at a final concentration of 9.71 mM. The gel filtration column (G.E. Superose 6 PC 3.2/30) was equilibrated with filtered HBS for C5b,6 or PBS for C7 for at least 1 hour, at 50 µL per minute. The detector was set for absorbance of 280 nm. After equilibration, samples were loaded onto the column and 50 µL fractions were collected. Data for each curve were normalized to the time point when the peak starts.

4.7 Measuring Effect of PolyP$_{\geq1000}$ on C5b-7 and C5b-8 Binding to Erythrocyte Membranes

Chicken red blood cells (cRBC) were obtained by washing chicken whole blood (Colorado Serum Company, Denver, CO) with gelatin veronal buffer (GVB), followed by centrifugation at 1000 x g for 10 minutes. The cells were washed 4 times. The concentration of the cells was measured using Advia 120 Hematology Analyzer from Bayer (Leverkusen, Germany). The final concentration of cells used in each experiment was 3.00 – 3.15 x 10$^9$ cells/mL.

Varying concentrations of polyP$_{\geq1000}$ (0 µM – 10 mM) were added to the cell suspension and incubated for 5 minutes. C5b,6 was then added to the reaction mixtures (final concentration 2.5 nM) and incubated for 5 minutes. Finally, C7 and C8 or C7 alone were added at final concentrations of 2.5 nM and incubated for a further 5 minutes. The cells were then pelleted by
centrifugation at 300 x g for 3 minutes and 40 μL of the supernatant was transferred to another microfuge tube. This fraction was again centrifuged at 300 x g for 3 minutes to remove any remaining cRBCs. 32 μL of this fraction was mixed with 8 μL of Laemmli loading buffer (with β-mercaptoethanol) for separation by SDS-PAGE using a 10% acrylamide gel. After transfer of the gel, Western blotting was performed using a goat-anti-human C5 primary antibody and 680RD donkey-anti-goat secondary antibody from LI-COR Biosciences. Following detection, densitometry was performed on the scanned blot images using the Odyssey Software from LI-COR Biosciences (Lincoln, Nebraska, USA). Values were normalized to the experimental conditions in which cRBC were incubated with maximal concentrations of polyP≥1000, but without C7 or C8. The amount of unbound C5b under these conditions was considered to be 100%. Three independent experiments were averaged.

4.8 In vivo rodent model of laser-induced choroidal neovascularization (CNV)

In collaboration with Dr. Joanne Matsubara (Eye Care Centre, Vancouver, BC), laser-induced CNV experiments with rats were performed. All animal studies were approved by the UBC Animal Ethics Committee. The methods are as previously described [139]. Briefly, eleven-week-old female Long Even (LE) rats (Charles River Laboratory, Wilmington, MA) weighing 256 to 315 g were used and handled in accordance with institutional guidelines and the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. The LE rats were anesthetized by intramuscular injection of a 1 mL/kg mixture (1:1) of ketamine hydrochloride (40 mg/kg) and xylazine hydrochloride (10 mg/ml), and their pupils were dilated with tropicamide (0.5% Mydrin). The eyes were pressed against a 22 mm x 22 mm glass coverslip to act as a contact lens. A slit-lamp biomicroscope and a diode red laser (OcuLight SLx; Iris Medical Instruments, Mountain View, California, USA) set at 650 nm, 150 mW intensity, 100 ms duration, and 75 μm spot size was used to inflict localized damage to Bruch’s membrane in the retina of the rats. Damage was confirmed by the appearance of a central bubble. 4 roughly equidistant laser injuries around the optic nerve of each eye were administered to each rat.
Rats were administered 5% isoflurane immediately after the laser injury, and maintained at 2% isoflurane for the intravitreal injection (occurring within 10 minutes of the laser injury). Pupils were dilated using eye drops - 0.5% tropicamide and 2.5% phenylephrine hydrochloride. 0.4% benoxinate-HCl was also applied topically to the eyes as an additional local anesthetic, followed by a drop of 0.5% levofloxacin ophthalmic antibiotic solution. Under a Stereo dissection microscope (SMZ 1000; Nikon, Tokyo, Japan), the eye was punctured in the limbus region with a fine 28-gauge needle. This allowed the penetration of a 32-gauge Hamilton syringe needle, used to slowly inject 5 μL of monoP or polyP_{21000} diluted in sterile water into the vitreous of the eye, to yield a final estimated concentration of ~200 μM. After injection, the needle was held in place inside the eye for 30-60 seconds and slowly withdrawn, to minimize fluid leakage. After intravitreal injection, the rats were monitored in an enclosed container to ensure recovery.

5 days post-injury, rats were euthanized with CO2 asphyxiation, and the eyes were harvested and fixed in 4% paraformaldehyde for 24 hours. Each eye was dissected under a dissecting microscope. The anterior segment and crystalline lens were removed, and the retina was detached and cut from the optic nerve using fine curved scissors. The remaining choroidal/RPE eye cups were washed with PBS and subsequently immunostained with a 1:100 dilution of 500 μg/mL solution of FITC-labeled Graffonia lectin (IB4) (Griffonia simplicifolia, Alexa Fluor® 488 Conjugate, Life technology, Burlington, ON) to detect endothelial cells, and a 1:250 dilution of a 1 mg/mL solution of rabbit IgG polyclonal anti-C5b-9 (Bioss, Woburn, MA) to detect deposition of C5b-9 (MAC). With fine curved scissors, radial cuts were made towards the optic nerve, around the lesions. The cut eye cups were flatmounted on glass slides and visualized on a confocal microscope (Zeiss-LSM 510 META, Thornwood, NY, USA), and the images were processed with the ImageJ program (1.47v, National Institute of Health, USA). Each eye was considered as n=1. Lesion size and MAC deposition for each rat eye was determined from the average of the 4 lesions on that eye.

Similar methods were used to study CNV in C57Bl6 wild-type mice (age 6-10 weeks). Equal final concentrations of polyP and monoP were injected intravitreally into eyes. As the total vitreous volume of mice is ~7 μL compared to ~54 μL in rats, 1 μL of solution was injected into the eyes.
of mice. After sacrifice, the eyes were extracted, 14 days post-injury. The same antibodies as with the rats (above) were used to detect the CNV lesions, with the fluorochrome label being changed to Cy3. C5b-9 was detected with a rabbit anti-human polyclonal antibody for SC5b-9, followed by a secondary antibody detected with a FITC-labelled anti-rabbit IgG. Volumes of CNV lesions and C5b-9 deposition were determined following imaging with a Nikon confocal microscope (Nikon Instruments, Melville, NY, USA).

4.9 Quantification of C5b-9 (MAC) Deposited on ARPE-19 (RPE) Cells and RF/6A (CEC)

Cells were grown to 100% confluence in 100 mm cell-culture dishes, and washed with PBS, after which serum-free growth media was added. The cells were incubated overnight (~16 hours) in the serum starvation media, which was then removed. Cells were washed 3x with PBS and were lifted after incubation for 10 minutes with 2.5 mL of StemPro® Accutase® Cell Dissociation Solution. 7.5 mL of their respective growth media were used to neutralize the cell dissociation solution, and the cells were centrifuged at 250 x g for 8 minutes. The supernatant was removed and the cell pellets were resuspended in PBS, transferred into sterile 1.5 ml microfuge tubes, and pelleted at 500 x g for 10 minutes, after which the supernatant was removed. A range of concentrations of polyP130 or monoP diluted in PBS was added to the cell pellets, and incubated for 1 hour at 37°C. Normal human serum was added to the cells at a final concentration of 5% (for CEC) or 25% (for RPE) for another 2 hours to induce complement activation on the cells. The cells were once again pelleted at 500 x g for 10 minutes and the supernatant from each sample was transferred to microfuge tubes and frozen at -80°C for subsequent analysis by ELISA (see below). Cells were washed once, resuspended in 100 µL of FACS buffer (1% BSA in PBS) and centrifuged at 500 x g, removing the supernatant after spinning, before addition of the primary antibody. A rabbit anti-mouse C5b-9 antibody diluted in FACS buffer was added to the tubes at a final concentration of 1 µg/mL and incubated at 4°C for 40 minutes. Excess primary antibody was removed by washing the cells once as previously described. Secondary antibody (goat anti-mouse IgG Alexa Fluor 488) diluted in PBS was added at a final concentration of 5 µg/mL and the cells were incubated for another 30 minutes at 4°C. Cells were washed and each sample was
resuspended in 500 µL of FACS buffer with 1 µg/mL (final concentration) of propidium iodide and transferred into tubes for FACS analysis (BD LSRII, BD Biosciences, San Jose, CA, USA). Data analysis was conducted using FlowJo software (vX.0.7, FlowJo, LLC, Ashland, OR, USA) and GraphPad Prism.

4.10 Quantification of SC5b-9

The MicroVue SC5b-9 Plus Enzyme Immunoassay Kit (Quidel, San Diego, CA, USA) was used to quantify the amount of SC5b-9 according to the manufacturer’s instructions. Briefly, 96-well plates coated with mouse monoclonal anti-human SC5b-9 antibodies were washed with PBS containing 0.05% Tween-20®, and Proclin® 300 (wash buffer). Samples and controls were incubated in wells at room temperature for one hour. Unbound proteins were removed by washing the plate 5x with wash buffer. Horseradish peroxidase-conjugated polyclonal goat anti-SC5b-9 antibodies were added to each well and incubated at room temperature for 30 minutes. The wells were washed as before, and a peroxide substrate and 3,3’,5,5’-teramethylbenzidene (TMB) – a chromogen – was added. H₂SO₄ was used to stop the reaction after 15 minutes. The absorbance of the samples was read at 450 nm with a Spectramax 384 Plus Microplate Reader, with the level of absorbance correlating with the amount of SC5b-9 in the sample.

4.11 Quantification of Nuclear Integrity

RF/6A and ARPE-19 cells were seeded in Falcon TC-treated black 96-well plates (BD Biosciences, San Jose, California, USA) at a concentration of 104 cells per well and cultured in their respective growth media for 24 hours at 37°C. The cells were then simultaneously treated with varying concentrations and combinations of H₂O₂ and polyP₁₃₀ or monoP diluted in their respective growth media, with final concentrations ranging from 0-1650 µM and 0-2 mM, respectively, at 37°C for 24 hours. An Eclipse TS100 brightfield phase-contrast microscope (Nikon Instruments, Melville, New York, USA) was used to visualize changes in cellular morphology (confluence, intercellular interactions, and opacity of nuclei). Hoechst dye, a nuclear stain, was added to the cells at a final concentration of 333 ng/mL, and incubated for 30 minutes at 37°C. An
ArrayScan™ VTI High-Content System Reader (Thermo Fisher Scientific, Burlington, ON, Canada) was used to quantify the intensity of nuclear staining and the number of cells with detectable nuclei.

4.12 Effect of PolyP on the Integrity of H2O2

The integrity of H₂O₂ was assessed by monitoring changes in absorbance of 1 mM H₂O₂ at 240 nm in the presence of polyP and/or catalase over different time periods as noted. Catalase was used as a positive control to demonstrate that its degradation of H₂O₂ caused a rapid (within 2-3 minutes) decrease in absorbance [140]. In this case, the absorbance of H₂O₂ was followed for 10 minutes after addition of catalase to the cuvette of the spectrophotometer. The addition of 1 mM polyP₁₃₀ instead of catalase had no effect on the baseline absorbance of H₂O₂.

4.13 Catalase Assay

ARPE-19 and RF/6A cells were grown to 90% confluence in 6-well plates in their respective growth media and washed with PBS. Varying concentrations of polyP₁₃₀ or monoP diluted in growth media were added to the cells and incubated for 24 hours. The cells were lysed with RIPA buffer (30mM Tris-HCl, 150mM NaCl, 1% Igepal, 0.5% deoxycholate, 2mM EDTA, 0.1% EDTA, pH 7.4) and centrifuged at 14,000 x g for 15 minutes. The pellets were discarded and supernatant lysates (80-100 µl volumes) were removed and stored at -80°C for subsequent measurement of catalase activity.

Total protein content of each sample (cleared lysate) was measured using the Pierce™ BCA Protein Assay kit (Rockford, Illinois, USA) according to the manufacturer’s instructions. Catalase in each sample was measured using the catalase assay kit from Cell Biolabs, Inc. (San Diego, California, USA) according to the manufacturer’s instructions. Briefly, H₂O₂ was added to the lysates for 3 minutes, allowing the catalase in the samples to degrade H₂O₂ to O₂ and H₂O. The reaction was quenched after 3 minutes with sodium azide. The leftover, undegraded H₂O₂ allows the coupling reaction of 3,5-dichloro-2-hydroxy-benzenesulfonic acid (DHBS) with 4-aminophenazone (4-aminoantipyrene, AAP) to proceed, catalyzed by horseradish peroxidase.
(HRP). After 30 minutes, the coupling reaction produces a quinoneimine dye that was measured at 540 nm with the Spectramax 384 Plus Microplate Reader. The absolute amount of catalase was determined from a standard curve of 0-100 U/mL that was generated with purified catalase, provided in the kit.

4.14 qRT-PCR Catalase Gene Expression Analysis

Treatment of the cells followed an identical procedure as that for the catalase assay. A kit for converting the RNA from cells to cDNA was used, according to the manufacturer’s instructions (RNeasy Mini® Kit, Applied Biosystems, Foster City, California, USA). Briefly, cells were lysed with lysis buffer (provided in kit) and β-mercaptoethanol, followed by addition of 70% ethanol. These were centrifuged in spin columns at ≥8000 x g and underwent multiple washes with the supplied washing buffer. The RNA was finally eluted with RNase-free water. qScript™ cDNA Synthesis Kit from Quanta Biosciences (Gaithersburg, Maryland, USA) was used to synthesize cDNA from the isolated RNA. A master mix consisting of reverse transcriptase, dNTPs, random primers, magnesium, and oligo(dT) was added to 400 ng of the isolated RNA. The samples were run in a thermal cycler program according to the manufacturer’s instructions. A TaqMan® Fast Advanced Master Mix (Applied Biosystems, Carlsbad, California, USA) was added to the synthesized cDNA, with a catalase gene primer from the same company. GAPDH was used as a housekeeping gene control, using primers from the same company. The samples were again run and analyzed using the StepOnePlus System™ (Applied Biosystems) qPCR machine and program.

4.15 VE-cadherin Expression Assay

RF/6A were seeded at 10^4 cells/well in black 96-well plates and grown to 90% confluence. Treatments of varying H_2O_2 and polyP_{130} were added and incubated for 48 hours. Cells were fixed with 2% PFA for 5 minutes and blocked with 5% BSA in PBS for 1 hour. Rabbit anti-human VE-cadherin polyclonal antibody at a final concentration of 2 µg/mL was incubated for 40 minutes, followed by a 20 minute incubation with AlexaFluor 488 nm goat anti-rabbit secondary antibody at a final concentration of 2 µg/mL. Hoechst dye (100 ng/mL) was added and
incubated for 20 minutes at 37°C. The plate was scanned on a Cellomics ArrayScan Target Activation program, at 15 fields per well with a 20x objective lens.

4.16 EA.hy 926 Cell Proliferation Assay

EA.hy 926 cells were seeded at a density of 2 x 10^4 cells/mL in a 96-well plate in the appropriate growth media (see Materials), and were incubated at 37°C and 5% CO₂ overnight. The media was removed and replaced with different dilutions of polyP₁₃₀ and monoP in EA.hy 926 growth media. At 0, 18, 24, 42, 48, 66, 72 hours, media was removed and replaced with 30 µL of 0.5% trypsin to detach the cells from the plates. Trypsinization was stopped with 30 µL growth media. The cell suspension was mixed with Trypan Blue at a 1:1 dilution, and the number of cells was counted with a hemocytometer, as previously described [141]. Averages were obtained from 2 counts of each sample, in triplicate.

4.17 BrdU Assay for Cell Proliferation

EA.hy 926 cells were seeded in 96 well plates at 2 cell densities (2.5 x 10^3 or 5 x 10^3 cells/well). The cells were grown in media containing 1% FBS for 5.5 hours, followed by treatments with varying concentrations of polyP₁₃₀ or monoP in growth media. A (bromodeoxyuridine) BrdU cell proliferation assay kit from Abcam (Toronto, ON, Canada) was used to quantify cell-proliferation, performed according to the manufacturer’s instructions. Briefly, 20 µL/well of the BrdU labelling solution was added at 20 hours following addition of the polyP₁₃₀ or monoP and incubated a further 4 hours, to allow the BrdU to be incorporated into the newly synthesized DNA. Media and labeling solution were removed from the wells, and the cells were fixed (fixative solution provided in kit) for 30 minutes at room temperature. After washing, 100 µL/well of the anti-BrdU antibody was added and incubated for 1 hour at room temperature, followed by addition of a peroxidase goat anti-mouse IgG conjugate (100 µL/well). A 3,3’-5,5”-tetramethylbenzidine (TMB) peroxidase substrate solution was added and incubated in the dark at room temperature. 100 µL of stop solution was added and the absorbance of the samples was measured at 450 nm with the Spectramax 384 Plus Microplate Reader.
4.18 Statistics

Statistical analyses were conducted using GraphPad Prism (version 5.0, La Jolla, CA, USA) and Microsoft Excel. Where appropriate, student’s t-tests and one way analysis of variance (ANOVA) were conducted between samples, with P ≤ 0.05 being considered significant. Unless otherwise noted, standard errors of the mean are shown in all results.

4.19 Ethics

All animal protocols were approved by the UBC Animal Ethics Committee, application number A14-0135. Studies with humans were approved by the UBC Clinical Research Ethics Board (CREB) under protocol number H12-02508.
Chapter 5: Results

5.1 Mechanisms by which PolyP Suppresses Complement Activation

Previous work in our lab revealed that polyP suppresses complement activation via the terminal pathway. Here, I first confirmed those findings, using the terminal pathway hemolytic assay (see Methods) in which cRBCs are incubated with varying concentrations of polyP or monoP, followed by addition of EDTA-treated serum, and a quantity of purified C5b,6 to achieve ~80% red blood cell lysis after a 30 minute incubation. As seen, polyP suppresses complement activation in a concentration-dependent manner (Figure 8a). With the same assay, Jovian Wat (a previous MSc student in the Conway lab) also showed that this suppression is polymer length-dependent, and that monoP has no effect at equivalent orthophosphate concentrations (Figure 8b) [57]. Similar results were obtained with purified complement factors C7, C8 and C9 (replacing serum), indicating that the polyP interacts directly with one or more of the complement proteins, rather than mediating its effects solely through interactions with other serum component(s).
Figure 8: PolyP suppresses the terminal pathway of complement in a concentration-and size-dependent manner. (a) Increasing concentrations of polyP≥1000 resulted in greater suppression of the terminal pathway of complement. This was consistent with what was previously shown by Jovian Wat [57]. Moreover, monoP does not affect the terminal pathway of complement, excluding the possibility that suppression occurs via an ionic effect (b, P1). Rather, with increasing lengths of polyP, a greater suppression is observed (b). The amount of C5b,6 used was selected to result in ~80% hemolysis, and this was designated as the maximum (100% in a, 1.0 in b) hemolysis under these experimental conditions. n=3 for a; n=4 for b. *Comparisons to control sample (no phosphate). *P ≤ 0.001.

5.1.1 PolyP Interacts Directly with C5b,6

The mechanisms by which polyP suppresses the terminal pathway of complement were examined. Evidence provided by Jovian Wat suggested that polyP is active only in early steps in the assembly of the C5b-9 membrane attack complex, i.e. prior to addition of C7 to the C65b,6 complex (Figure 8b). We therefore tested whether polyP≥1000 binds to C5b,6 or C7 by gel filtration. The addition of polyP≥1000 to C7 did not cause any shift in the gel filtration profile of C7 (Figure 9a), suggesting that there was no interaction between polyP≥1000 and C7 under these chromatography conditions. In contrast, pre-incubation of polyP≥1000 with C5b,6 caused the C5b,6 gel filtration profile to shift left, indicating a size increase, and suggesting a direct interaction between polyP≥1000 and C5b,6 (Figure 9b). The broadness of the peak suggested that
polyP may cause C5b,6 to form multimers. These findings were in line with thermal shift assays (aka differential scanning fluorimetry (DSF)) performed by Jovian Wat, which revealed that polyP binds to and destabilizes C5b,6 in a concentration-dependent manner. In the same assay, polyP$_{\geq1000}$ had no effect on C7 [57].

Figure 9: Gel filtration of complement proteins to assess interaction with polyP$_{\geq1000}$. (a) C7 and polyP$_{\geq1000}$ were gel filtered individually or in combination. The retention time of C7 off the gel filtration column did not change with the addition of polyP$_{\geq1000}$. (b) The addition of polyP$_{\geq1000}$ shortened the retention time of C5b,6. The broader peak can be attributed to the heterogeneity of polyP lengths in the sample. These findings indicate that polyP$_{\geq1000}$ binds to C5b,6 and causes it to have a higher oligomeric state. The ion dependence of this effect was excluded by the observation that the retention time of C5b,6 was unchanged by the addition of an equivalent molar concentration of monoP.
5.1.2 PolyP≥1000 Interferes with Binding of C5b-7 and C5b-8 Complexes to Erythrocyte Membranes

The preceding results indicated that polyP binds to C5b,6 and alters its structure. We predicted that the so-formed C5b,6 would alter the ability of downstream terminal complexes, C5b-7 and C5b-8, from binding to and/or integrating into the target membrane, thereby explaining the reduced red cell lysis in the terminal pathway assay (Figure 8). To test that hypothesis, varying concentrations of polyP≥1000 were incubated for 5 minutes with cRBC, followed by a 5 minute incubation with C5b,6, and 5 minutes later by the addition of equimolar concentrations of C7 and C8 (Figure 10a) or C7 alone (Figure 10b) for a further 5 minutes. The amount of unbound C5b-7 or C5b-8 was determined by quantifying the amount of C5b in the supernatant, as detected by Western blot and immunodetection of C5/C5b (Figure 10). As can be seen with the densitometry results, when no C7 or C8 is added (right bars), there is no binding to the membrane, and 100% of the C5b is recovered. polyP≥1000 interferes with binding of C5b-7 (Figure 10a) and C5b-8 (Figure 10b) in a concentration-dependent manner. These findings, reported in the journal Blood [57], support the notion that destabilization of C5b,6 by polyP either reduces formation of the downstream complexes (C5b-7, C5b-8, C5b-9) and/or alters their structures so that binding to the membrane is reduced.
Figure 10: **Effect of PolyP on membrane binding/integration of C5b-7 and C5b-8.** With increasing concentrations of polyP_{≥1000}, the amount of unbound C5b-7 increases (a), indicating that polyP interferes with the binding of this complement protein complex to the membrane. Similarly, when C8 was added to the mixture (b), the reduction of membrane binding/integration was still observed. From these findings we can infer that destabilization of C5b,6 by polyP_{≥1000}, as previously found by Jovian Wat [57], results in the suppression of binding and/or integration of C5b-7 and C5b-8, a key step in formation of a functional MAC. The results are representative of 3 experiments, each performed in triplicate. Error bars represent standard deviation. One way ANOVA was used to assess significance, comparing the results to 0 µM polyP. *P ≤ 0.05.
5.1.3 Human Platelet Releasates Suppress Complement Activation via the Terminal Pathway

Platelets are a major source of polyP, where they are primarily located in the dense granules [22]. Platelet polyP has a size in the range of 60-100 orthophosphate units. PolyP$_{60-100}$ is released into the circulation and/or onto cell surfaces when the platelets are activated [20, 22]. Isolation of polyP from platelets is difficult, as the polyP is prone to degradation (personal communication, Dr. James Morrissey). Nonetheless, we attempted to validate that polyP released from platelets also dampens complement activation. Platelet releasates were therefore prepared from human platelets that were stimulated with thrombin. Aggregation of the platelets provided confirmation that the thrombin was functional. The effect of the supernatants from non-activated and thrombin-activated platelets on terminal pathway complement activation, using purified terminal pathway complement components, was tested. As seen in Figure 11 (published in Blood 2014 123:768-76) [57], platelet releasate from thrombin-activated platelets significantly suppressed complement activation as compared to the supernatant from non-activated platelets. This effect appeared to be concentration-dependent. Since we cannot directly measure polyP in the blood or in the releasates, we cannot be certain that it is the polyP that is the active constituent. However, it is likely that polyP participates, and thus these studies support the notion that polyP released from activated platelets dampens complement activation via the terminal pathway.
Figure 11: **Platelet releasates suppress complement activation via the terminal pathway.** The left side of the figure shows the concentration-dependent suppression of complement activation via the terminal pathway, using synthesized polyP_{≥1000}. 100% lysis was achieved with dH_{2}O. The beige bars serve as a point of comparison for the suppressive effect of the releasates on complement activation via the terminal pathway. On the right side of the figure, the releasate from platelets activated with thrombin (red bars) significantly reduced hemolysis compared to the supernatant from non-activated platelets (grey bars). Two concentrations of releasate/supernatant were used. The findings support the notion that polyP that is released from activated platelets dampens complement activation. n=3 for each condition. Student’s t-test was used to assess significance. *P ≤ 0.05, **P ≤ 0.01.
5.2 In vivo Validation of Therapeutic Utility of PolyP in a Complement-mediated Disease

5.2.1 Comparing the Effect of PolyP130 and PolyP≥1000 on Complement Activation

Prior to initiating in vivo studies to test the clinical efficacy of polyP in a model of human disease, we considered which polyP preparation to use. We appreciated the possibility that different length polymers may have differential effects, but within the limited time of this thesis, we decided to select one size range to evaluate. In the pilot studies in rats (below), studies were performed with polyP≥1000, as this was the only formulation available at that time. However, polyP≥1000 is not normally found in mammals [13, 142]. Moreover, the preparations of polyP≥1000 are highly heterogeneous, and this length is known to have the most profound prothrombotic effects in vivo when administered intravenously [142]. The vitreous is not known to contain procoagulant proteins. In fact, little is known of the composition of the vitreous. Nonetheless, our lab secured a steady source of a homogeneous preparation of polyP130 (a gift from Regenetiss, Inc., Japan). This length is in the range that mammals naturally produce (5-800 orthophosphate units). We therefore verified that this preparation is active and comparable to the polyP≥1000 that was provided by Dr. James Morrissey. This was achieved using the serum-based hemolytic assay to directly compare the effect of polyP130 and polyP≥1000 on the terminal pathway. As seen in Figure 6, polyP130 was very similar to polyP≥1000 in suppressing terminal pathway complement activation. Thus, polyP130 was used for the in vivo studies in mice (see below) and most of the subsequent in vitro experiments, unless otherwise stated.
Figure 12: **Terminal pathway hemolytic assay to compare polyP_{130} and polyP_{\geq 1000}.** The effect of each form of polyP on the terminal pathway of complement was examined in a serum-based hemolytic assay. The fact that the two forms came from different sources and were thus synthesized by slightly varying techniques may account for the slightly different response between the two polyP forms. Nevertheless, polyP_{130} closely mimicked the suppressive effect of polyP_{\geq 1000}. n = 3.

5.2.2 **PolyP_{\geq 1000} Suppresses Complement-mediated Damage in a Rodent Model of Wet AMD**

A pilot study of the protective effects of polyP against AMD was conducted using a laser-induced choroidal neovascularization (CNV) model in rats. The model mimics the pathology of Wet AMD, in that Bruch’s membrane is disrupted by the laser injury, and this is followed by neovascularization into the sub-retinal space [143]. Laser-induced CNV is the most widely used and accepted research model to test the efficacy of novel AMD therapies and to help elucidate the pathogenesis of AMD [143, 144]. The model may be used in different animals, from rodents to non-human primates. Current therapies for Wet AMD that target VEGF or its receptors, were developed and approved based on findings using this model [143].
While I learned the techniques, the following studies in rats were performed by Dr. Jing Cui, a Research Associate in the lab of Dr. Joanne Matsubara (Eye Care Centre, UBC). I performed the quantitative analyses by confocal microscopy. Lesions in the eyes of the rats were induced by laser, and this was followed by intravitreal injection of either polyP_{≥1000} or monoP (final concentration of ~200 µM). After 5 days, the rats were euthanized, and the choroids were isolated, fixed, whole mounted and stained to detect C5b-9 deposition and lesion vascularity using isolectin B4.

Results from the 4 lesions from each eye were averaged to yield areas of neovascularization and C5b-9 deposition. Overall, there was a trend for less vessel formation in the polyP_{≥1000}-treated eyes. Notably, the CNV lesions from the polyP_{≥1000}-treated eyes had notably less intense C5b-9 staining, findings that are consistent with the suppressive effect of polyP on complement activation (Figure 13). These preliminary results supported our hypothesis that polyP confers protection against AMD by reducing complement-mediated injury.

The experiments set the stage for validating the findings through more extensive in vivo studies. Thus, with the help of Alice O’byrne, the Research Assistant, I performed similar studies using mice and polyP_{130} or monoP at a final concentration of 200 µM. The number of mice was increased (n=17 and 15 for polyP and monoP, respectively), and they were sacrificed for analysis at day 14 after laser injury. Confocal imaging allowed us to measure volumes of stained lesions, rather than areas and fluorescence intensities, thereby providing a more accurate representation of the impact of the therapeutic intervention. As seen in Figure 14, intravitreal injection of polyP_{130} resulted in a significant reduction in the volume of the neovascularity, with a trend for a reduction in C5b-9 deposition. In no case, was the amount of C5b-9 in the monoP-treated eyes, greater than in the polyP_{130}-treated eyes (Figure 14).

Overall, these in vivo studies provide exciting confirmation that polyP exerts protection against laser-induced CNV in rodents, and highlights the importance of further evaluating the underlying mechanisms.
Figure 13: **C5b-9 deposition and neovascularization after laser injury in rats.** The left-side panels in (a) show representative lesions from the monoP-treated eyes, and the right-side panels show representative lesions from the polyP_{≥1000}-treated eyes of rats (a). Red staining reflects C5b-9 deposition. Green staining indicates new vessel formation (CNV). A total of 10 lesions for the monoP group and 8 lesions for the polyP_{≥1000} group were averaged for the fluorescence intensity of C5b-9 deposition (b) and CNV (c). The results from these preliminary studies suggested that polyP dampens complement activation and lesion size in this model, and were rationale for more extensive study.
Figure 14: **C5b-9 deposition and neovascularization after laser injury in mice.** The left side panels of (a) show representative lesions from the monoP-treated eyes, and the right-side panels show representative lesions from the polyP$_{130}$-treated eyes (a). Red-staining indicates new vessel formation (CNV). Green-staining indicates C5b-9 deposition. A total of 15 eyes (3-4 lesions each) for the monoP group and 17 eyes (3-4 lesions each) for the polyP$_{130}$ group were averaged for the volume of C5b-9 deposition (b) and CNV (c). 3D projections of the z-stack images are depicted for both the monoP (d) and polyP$_{130}$ (e) lesions. Student’s t-test was used to assess significance. *P ≤ 0.05.
5.2.3 PolyP≥1000 is Functionally Stable for at least 10 days in Serum

The preceding *in vivo* studies raised the possibility of using polyP as a treatment for AMD. Many challenges remain, of course, before bringing polyP to the clinic. One of these, which we partly address here, involves a question of the stability of polyP. It was previously reported that the half-life of polyP in plasma and serum *ex vivo* is ~90 minutes [9, 142]. However, these studies only examined the stability of polyP in terms of the integrity of the polymer, rather than its function [9]. We therefore used the terminal pathway of complement to measure the residual complement inhibitory function of polyP≥1000 over time, following incubation of the polyP≥1000 in serum *ex vivo*. As seen in Figure 15, only after ~6 hours, the polyP≥1000 appear to lose some of its capacity to suppress complement activation via the terminal pathway. Even at 10 days (240 hours), there was still substantial residual complement inhibiting activity. We did not evaluate the structural integrity of the polyP≥1000 during the course of this study. Nor did we examine the stability of polyP in vitreous, an important step. However, the findings provide an important starting point.
Figure 15: **Time-dependent change in function of polyP_{≥1000} in serum.** After incubation in normal human serum for varying amounts of time, 200 μM of polyP_{≥1000} or buffer alone (no polyP) was added to a hemolytic assay to quantify the effect of any residual polyP on complement activation. When no polyP_{≥1000} was added, lysis in the assay ranged from ~40% to ~25% over the 48 hours of the study. The polyP_{≥1000} that was added to the serum almost totally suppressed complement activation via the terminal pathway, and this suppressive activity was only partly lost over the ensuing 10 days. n=3.

5.3 **Cellular Effects of PolyP**

The preceding *in vivo* pilot studies provided strong evidence that local intravitreal administration of polyP suppresses complement activation in a rodent model of AMD. As discussed in the Introduction, retinal pigment epithelial (RPE) cells and choroid endothelial cells (CEC) are generally accepted to be the major cellular targets involved in the pathogenesis and progression of AMD. We therefore sought to determine whether polyP_{130} protects these cells from stresses known to be associated with AMD, i.e., excess complement activation and exposure to reactive oxygen species. Primary RPE cells and CEC are not easily obtained or cultured. However, human RPE and monkey CEC cell lines (ARPE-19 and RF/6A, respectively)
have been used extensively in several studies that investigate the mechanisms underlying AMD [145-150]. As these cells are commercially available and relatively easy to grow in culture, we used these to evaluate the protective properties of polyP. We herein refer to these cell lines as CEC and RPE.

### 5.3.1 PolyP130 Suppresses C5b-9 Deposition on CEC and RPE Cells

We first assessed whether polyP$_{130}$ could protect the cells from complement-mediated damage. Cells were pre-incubated with varying concentrations of polyP$_{130}$ or equimolar concentrations of monoP (based on orthophosphate units), after which normal human serum was added to the cells in PBS at a final concentration of 5% for CEC and 25% cells for RPE cells to sufficiently induce complement activation. After 1 hour, the cells were washed, and the amount of C5b-9 deposition on the surface of the cells was quantified by flow cytometry.

Figure 16 is representative of several experiments, each performed in triplicate. As can be seen, heat-inactivated serum (HIS) did not induce C5b-9 deposition. When C7-deficient serum was used, no C5b-9 was detected. Additional negative controls included a non-specific isotype-matched primary antibody (Figure 16b, d).

As predicted, polyP$_{130}$ suppressed C5b-9 deposition on the cells in a concentration-dependent manner. MonoP also reduced C5b-9 deposition in a dose-dependent manner, but not to a significant extent. A suppressive effect of monoP on total hemolytic activity would not be surprising, as this was previously observed in our study reported in Blood [57] particularly at concentrations exceeding ~250 µM, at a level where critical complement-dependent cations (Ca$^{2+}$, Mg$^{2+}$) may be chelated. In any case, the suppressive effect of polyP$_{130}$ on C5b-9 deposition was significantly greater than with monoP for the CECs at most concentrations, and at the second highest concentration in the RPE cells. Based on our findings, the protective effect of polyP$_{130}$ in this assay system was more prominent in the CEC than in the RPE cells.
(Legend for above panels of Figure 16a, b on the next page)
Figure 16: Flow cytometry to detect C5b-9 deposition on CEC and RPE cells. C5b-9 deposition on cell membranes was quantified on CEC (a, b) and RPE (c, d) cell lines after pre-incubation with polyP\textsubscript{130} or monoP followed by exposure to human serum. The FACS profiles (a, c) provide a visual representation of the relative abundance of C5b-9 deposition on the cells. Shown here are representative profiles from the highest polyP\textsubscript{130} and monoP concentrations. For both cell lines, treatment with polyP\textsubscript{130} results in a shift to the left compared to the monoP and serum only control samples, indicating that polyP\textsubscript{130} interferes with C5b-9 deposition and thus complement mediated attack. The bar graphs (b, d) show the average intensity of FITC, the marker for C5b-9 on the cell surface, for all the samples tested. Grey bars represent the negative (isotype control, HIS: heat inactivated serum, and C7-depleted serum) and positive (serum only) controls, while coloured bars represent treated samples. Both cell-lines show a concentration-dependent suppression of C5b-9 deposition with polyP\textsubscript{130} and monoP treatment, although polyP\textsubscript{130} treatments result in overall greater suppression for all concentrations. Error bars reflect standard error of the mean, with n=3. One way ANOVA was used to assess significance. * P ≤ 0.05; ** P ≤ 0.01; *** P ≤ 0.001.
5.3.2 Effects of PolyP130 on Release of SC5b-9 from CEC and RPE Cells

Soluble C5b-9 (or SC5b-9) is commonly used as a marker of complement activation and complement-mediated cell damage. Plasma levels of SC5b-9 may be elevated in diseases associated with excess complement activation. SC5b-9 is readily measured by a sensitive commercial sandwich ELISA in which the detecting antibody identifies an epitope that is specific for the SC5b-9 complex. Released SC5b-9 is known to induce membrane changes and intracellular signaling in platelets and endothelial cells that promote inflammation and coagulation.

Based on the preceding results, we predicted that polyP would dampen the generation of SC5b-9 in cells exposed to serum. This was tested as above by pre-incubating the CEC and RPE cells with polyP$_{130}$ or monoP, and then exposing them to serum for 1 hour. Supernatants were collected and SC5b-9 was measured (Figure 17). Release of SC5b-9 into the media was readily detected in cells exposed to normal serum. Exposure of the cells to heat-inactivated serum or serum deficient in C7 resulted in the release of minimal (almost undetectable) amounts of SC5b-9. Pre-incubation of RPE cells with polyP$_{130}$ resulted in only a slight reduction in SC5b-9 that was not different when compared to monoP (Figure 17). The findings were similar with the CEC in that there was no change in the release/generation of SC5b-9 (Figure 17) following pre-incubation with monoP or polyP$_{130}$. Overall, although polyP$_{130}$ caused a significant reduction in serum-induced cell surface deposition of C5b-9, generation and release of SC5b-9 was not affected.
Figure 17: **Measurement of SC5b-9 formation with ELISA.** Following exposure of CEC (a) and RPE cells (b) to serum, SC5b-9 released into the media was quantified. Experiments performed in duplicate, consisted of varying concentrations of polyP_{130} (red) and monoP (beige), as well as negative and positive controls as noted (grey – C7-depleted serum; HIS- heat inactivated serum). From these studies, we could not discern any change in the release of SC5b-9 in response to polyP_{130} or monoP at the concentrations tested.
5.3.3 PolyP130 and Oxidative Stress

In addition to excess complement activation, oxidative stress is believed to play a key role in promoting the cellular damage that is associated with the development of AMD. Although we have evidence that polyP suppresses complement activation and protects against complement mediated cellular (RPE, CEC) damage, we have not excluded the possibility that polyP has other protective effects that may translate into in vivo protection. We therefore explored the possibility that polyP protects CEC and RPE cells against oxidative stress. This was accomplished by exposing cells to different concentrations of H₂O₂ for varying periods of time, in the presence or absence of a range of concentrations of polyP₁₃₀ or monoP. Three independent approaches were used, assessing different endpoints for each. Serum was not present in these experimental setups, thereby excluding the participation of complement.

5.3.4 Morphologic Evidence that PolyP Protects Cells Against Oxidative stress

RPE cells were exposed to a range of concentrations of polyP₁₃₀ for 24 hours, in the presence (Figure 18a, top) or absence (Figure 18a, bottom) of H₂O₂. Changes in morphology were observed under a phase-contrast microscope. In the absence of polyP₁₃₀ or with the lowest concentration of polyP₁₃₀, the cells exhibited major morphologic changes, including cell shrinkage, rounding, loss of intercellular interactions, blebbing of the cytoplasm, and transparent nuclei (Figure 18a, top right-most panel). When the cells were simultaneously treated with polyP₁₃₀, these apparently damaging effects were almost entirely abrogated, an effect that was dose-dependent (Figure 18a, top panels). In the absence of H₂O₂, polyP₁₃₀ had no detectable effects on the cells (Figure 18a, bottom panels). Interestingly, monoP at equivalent concentrations as the polyP₁₃₀ also exhibited a dose-dependent protective effect against H₂O₂-induced oxidative stress (Figure 18b, top).

CEC were also exposed to H₂O₂-induced oxidative stress in the presence or absence of polyP₁₃₀ and monoP (Figure 18d and 18e, top) as with the RPE cells. Although the formation of a precipitate (likely from the concentrated polyP₁₃₀) slightly obscured the view of the cells at the
higher concentrations, we were able to detect a similar response of the CEC to the polyP_{130} and monoP, as with the RPE cells (Figure 18d and 18e, bottom).

Overall, the preceding studies revealed qualitative evidence that both polyP_{130} and monoP protect CEC and RPE cells from H_{2}O_{2}-induced oxidative stress. We further sought to validate the findings via the following more quantitative approaches.

(Legend for above Figure 18a-c on next page)
Figure 18: **Cell morphologic changes in response to oxidative damage and polyP₁₃₀.** Representative images of RPE cells (ARPE-19) (a, b) and CEC (RF/6A) (d, e) that were exposed for 24 hrs to 500 μM H₂O₂ are shown. For both cell lines, H₂O₂ induces dramatic cell morphologic changes, with rounding, less adherence to the surface, and loss of cell-cell interactions (c and f, left panels), compared with the untreated cells (c and f, right panels). With the addition of increasing concentrations of polyP₁₃₀ in the presence of the H₂O₂, these morphologic changes were notably reduced (a, top panels), and cells took on their normal appearance, i.e., similar to without H₂O₂ (a, bottom panels). Neither monoP nor polyP₁₃₀ alone (without H₂O₂) had an effect on the RPE (a, b, bottom panels). The highest polyP₁₃₀ concentrations resulted in the formation of a precipitate on the CEC (d), making it difficult to detect changes in the morphology of the cells. However, all polyP₁₃₀ concentrations equal to or above 500 μM exhibited clear a protective effect (d, top panel). The monoP treatments with the CEC also revealed evidence of protection against H₂O₂-induced cell damage (e, top panels), similar to the effect in RPE. The images are representative of triplicates from each treatment, and are shown at the same magnification.
5.3.5 PolyP does not alter the Integrity of H2O2

The preceding studies suggested that polyP and monoP have anti-oxidant properties. This may be achieved via several mechanisms, including induction of anti-oxidants, such as catalase, glutathione, peroxidase, and/or superoxide dismutase [151, 152]. There is a precedent for this, at least in bacteria, where polyP induces expression of the antioxidant, catalase [17]. However, it is also possible that the polyP and monoP are simply degrading or neutralizing the H2O2 prior to inducing cellular damage.

We therefore used an in vitro approach to test whether polyP130 disrupts the integrity of H2O2. We first showed that H2O2 has a measurable absorbance at 240 nm that is only minimally altered by the presence of polyP130 (Figure 19a). The integrity of H2O2 could then be assessed by monitoring the reduction in absorbance when it is incubated with catalase, thereby converting it to H2O + O2. We confirmed that this occurs within ~2-3 minutes (Figure 19a). The effect of 1 mM polyP130 on 1 mM H2O2 over 5 minutes was similarly studied by monitoring the change in absorbance. polyP130 has no effect on the absorbance of the H2O2 over a period of 5 minutes, and up to 24 hours (not shown). The residual integrity of the H2O2 after 24 hours was confirmed by incubating the polyP130/H2O2 with purified catalase (b). At that time, there was again a rapid decrease in absorbance (Figure 19b), as the H2O2 was converted to H2O with release of O2. The findings exclude the possibility that polyP130 is directly destabilizing or inactivating the H2O2, and suggested that polyP130 (and monoP) have anti-oxidant properties mediated via other mechanisms.
Figure 19: **Effect of polyP\textsubscript{130} on functional integrity H\textsubscript{2}O\textsubscript{2}**. (a) The absorbance at 240 nM of polyP\textsubscript{130} and H\textsubscript{2}O\textsubscript{2} was measured over time. Co-incubation of H\textsubscript{2}O\textsubscript{2} with polyP\textsubscript{130} over 24 hours did not cause any change in absorbance (only 5 minutes shown). As a positive control, the addition of catalase to the H\textsubscript{2}O\textsubscript{2} (a: red), caused a rapid decrease in absorbance, indicative of the conversion of H\textsubscript{2}O\textsubscript{2} to H\textsubscript{2}O and O\textsubscript{2}. (b) After 24 hours, when the absorbance of the combined H\textsubscript{2}O\textsubscript{2}+polyP\textsubscript{130} had not changed, catalase was added to the mixture. The absorbance again dropped rapidly (b: purple line), indicating that the H\textsubscript{2}O\textsubscript{2} remained intact after 24 hours while in the presence of the polyP\textsubscript{130}.
5.3.6 Effect of PolyP130 and monoP on Cellular Expression of Catalase

We examined the effect of polyP_{130} on the expression of catalase, a major cellular anti-oxidant enzyme that degrades H\textsubscript{2}O\textsubscript{2} [152]. RPE cells were exposed to a range of concentrations of polyP_{130} for 24 hours. Cell lysates were prepared and equal amounts were assayed for catalase activity, quantified from a standard curve of purified catalase. The cells were not exposed to H\textsubscript{2}O\textsubscript{2}, so as to avoid confounding the interpretation of the assays results. There was no consistent change in catalase activity in response to polyP as compared to the untreated control (Figure 20a). MonoP at the highest concentration as that of polyP also had no apparent effect on catalase generation. Similarly, the same treatments for the CEC cells did not induce any change in catalase activity (Figure 20b) for any of the treatment concentrations, compared to the untreated control sample. Unlike the RPE cells, however, the absolute catalase activity level was much lower for the CEC than for the RPE cells by \sim 2.5 fold. Again, monoP also had no effect. These results were verified by measuring catalase gene expression by qRT-PCR (Figure 21). Cells were treated in an identical manner as for catalase activity. The qRT-PCR results revealed that polyP_{130} had no apparent effect on catalase gene expression for either RPE cells or CEC.
Figure 20: Effect of polyP$_{130}$ and monoP on catalase activity (CAT). The values in the above graphs were determined from a standard curve using purified catalase with known catalytic activities, and the samples were normalized for total protein content. There was no consistent polyP-dependent change in catalase activity in either the RPE cells (a) or the CEC (b). A high concentration of monoP (grey bar) does not induce catalase activity in these cells. Experiments were performed in duplicate.

Figure 21: Effect of polyP$_{130}$ and monoP on catalase gene expression. qRT-PCR was used to measure catalase gene expression following exposure of RPE cells (a) and CEC (b) to polyP$_{130}$ and monoP. Values were normalized to samples without polyP$_{130}$ (untreated), designated a relative gene-expression of 1. No significant change in expression was observed for either the polyP$_{130}$ or monoP treated cells. Experiments were performed in duplicate.
5.3.7 PolyP Protects Nuclear Integrity of RPE cells and CEC from Oxidative Stress

Direct visualization of the cells by phase-contrast microscopy provided evidence that polyP and monoP exert a cytoprotective effect that is independent of complement. However, these studies were not quantitative. Due to the apparent difference in nuclear transparency between the treatments, nuclear integrity by nuclear staining was chosen as a means of quantifying the effects of polyP$_{130}$ and monoP on H$_2$O$_2$-induced cellular damage. Hoechst dye was chosen as the stain of choice as it is able to cross the lipid bilayer of cells and to bind to intact DNA in the nuclei. Although the Hoechst dye does not distinguish between live and dead cells, it provides an indirect indication of cell viability, as oxidative stress is known to cause cell death, largely by damaging the DNA via reactive oxygen species [153, 154].

As shown in Figure 22a, increasing concentrations of H$_2$O$_2$ resulted in decreased nuclear staining of CEC, findings that are consistent with decreased cell viability [155]. At all concentrations of H$_2$O$_2$, co-incubation of the cells with polyP$_{130}$ augmented nuclear staining, and this appeared to occur in a dose-dependent manner. The results are in line with what was observed under the phase-contrast microscope. However, in contrast with the morphology findings shown in Figure 18, monoP at the highest concentration (1000 µM) did not protect the cells in terms of nuclear integrity (not shown). Similar findings were obtained with RPE (Figure 22b). Interestingly, nuclear staining with the polyP$_{130}$-exposed cells appeared to be increased, at least for the CEC, suggesting that polyP$_{130}$ may induce cell proliferation and/or prevent cell death. Overall, these data are evidence that polyP$_{130}$ protects the nuclear integrity of the cells from H$_2$O$_2$-induced oxidative damage.
Figure 22: **Protective effect of polyP\textsubscript{130} on nuclear integrity following cell exposure to oxidative stress.**

Cells were exposed to H\textsubscript{2}O\textsubscript{2} and polyP\textsubscript{130} as described in the text. Without polyP\textsubscript{130} treatment (a), nuclear integrity in CEC was reduced with increasing H\textsubscript{2}O\textsubscript{2} concentrations (beige line). The addition of polyP\textsubscript{130} reversed this effect in a concentration-dependent manner, with the higher polyP concentrations resulting in protection of nuclear integrity. The response of RPE cells to polyP\textsubscript{130} (b) was similar. Error bars are not shown. Analyses were performed with a One Way ANOVA (significance not shown for samples treated with ≥ 500 µM H\textsubscript{2}O\textsubscript{2}). n = 3, ** P ≤ 0.01; *** P ≤ 0.001.

### 5.3.8 Effect of PolyP130 on Oxidative Stress-Induced Changes in Junctional Protein Integrity

The integrity of the RPE monolayer is vital to the blood-retinal barrier, as the latter regulates paracellular diffusion of nutrients and waste with the choroid [156, 157]. Disruption of the RPE monolayer due to disruption of intercellular junctions allows CNV to proceed, facilitates an inflammatory response with invasion of macrophages, and allows leakage of choroidal fluids into the sub-retinal space [158, 159]. The choriocapillaries, comprising CEC, are fenestrated to facilitate exchange of nutrients and waste with the photoreceptors via the RPE [160]. Tight intercellular junctions are also present in CEC, and although not as well characterized as those of RPE cells, they are believed to play a role in AMD [160, 161]. Oxidative stress is known to damage intercellular junctions [158, 162]. Our observations of cellular protection with polyP\textsubscript{130}...
provided reason to suspect that it may also protect the intercellular junctions of these cells from H$_2$O$_2$-induced damage.

We tested the response of CEC expression of VE-cadherin to H$_2$O$_2$-induced oxidative stress in the presence and absence of polyP$_{130}$ and monoP. For quantitative purposes, we imaged the cells with the Cellomics ArrayScan high throughput confocal microscope, which was optimized for quantifying VE-cadherin on CEC. As seen in a representative experiment shown in Figure 23, CEC were treated with varying concentrations of polyP$_{130}$ or monoP, in the presence/absence of H$_2$O$_2$, after which the cells were fixed and stained for expression of VE-cadherin using a fluorescent-tagged secondary antibody. The Cellomics ArrayScan high content confocal microscope was thus used to quantify the fluorescence in each sample. All experiments were performed in duplicate, and for each sample 15 fields were quantified, from which an average signal was obtained.

In the absence of H$_2$O$_2$, polyP$_{130}$ induced expression of VE-cadherin at a concentration of 100 µM. With the addition of 10 µM H$_2$O$_2$ and in the absence of polyP, there was no evidence of an effect on VE-cadherin. However, polyP at concentrations of 10 µM, 100 µM and 500 µM, caused an increase in VE-cadherin expression, although it was only significantly augmented at 100 µM. A similar trend was observed with 100 and 500 µM H$_2$O$_2$. At 1 mM H$_2$O$_2$, increasing amounts of polyP$_{130}$ resulted in increased VE-cadherin expression as well. Under all H$_2$O$_2$ conditions, monoP at the high concentration used, had little or no effect on VE-cadherin expression.
Figure 23: Effect of polyP₁₃₀ on VE-cadherin expression. VE-Cadherin immunofluorescent staining on CEC cells was quantified by high content confocal microscopy. polyP₁₃₀ exhibits varying effects on VE-cadherin expression following exposure of cells to oxidative stress. At 1 mM of H₂O₂, increasing concentrations of polyP₁₃₀ (red bars) protects against VE-cadherin loss per cell. At lower concentrations and at no H₂O₂, there is an induction of VE-cadherin expression up to 100µM polyP₁₃₀, and a subsequent decrease in expression at higher concentrations. MonoP (blue bars) at a high concentration (1000 µM) had no apparent effect on VE-cadherin expression, compared to the untreated control (white bar). One Way ANOVA was used to analyze significance per H₂O₂ concentration group. *P ≤ 0.05.
5.3.9 Effect of PolyP130 of Endothelial Cell Proliferation

Although the mechanisms are not fully delineated, the preceding studies provide evidence that polyP (and possibly monoP) exhibit cytoprotective properties under some stress conditions that are linked to AMD. These data suggested that polyP may also induce cell proliferation under non-stress conditions. To test this, we used EA.hy 926 cells, a human cell line that is a hybrid of human umbilical vein endothelial cells and A549 lung cancer epidermal cells. The line exhibits phenotypic features that most closely resemble endothelial cells. They are robust, and grow easily in culture [163]. We selected this cell line, rather than the CEC, as we were having difficulties maintaining the latter cell line at the time of these studies.

We assessed the growth response of these cells to different concentrations of polyP130 and monoP by culturing them from low density and allowing them to grow in growth media for up to 4 days. Cells were directly visually counted with a haemocytometer. Although the cell counts did indeed increase over time, there were no significant differences between those exposed to monoP, polyP130 or media only (Figure 24a). A similar effect was found with CEC in a preliminary study (not shown).

We also quantified EA.hy 926 cell proliferation by measuring BrdU incorporation after 24 hrs exposure to polyP130 (100 µM - 1 mM) or buffer alone. Again, we could not detect a significant effect of polyP130 on cellular DNA incorporation of BrdU (Figure 24b). Overall, under these experimental conditions, polyP130 had no effect on cell proliferation.
Figure 24: **Effect of polyP$_{130}$ and monoP on cell proliferation.** (a) EA.hy 926 cells were cultured in 96-well plates in the presence of varying concentrations of monoP or polyP$_{130}$. Neither polyP$_{130}$ nor monoP had any effect on cell number over time. (b) BrdU incorporation for detection of newly synthesized DNA was measured in cells cultured with varying concentrations of polyP$_{130}$ and seeded at 2 densities. Again, polyP$_{130}$ did not exhibit any effect on cell proliferation. One way ANOVA was used to assess significance, n=3. Error bars not shown for (a).
Chapter 6: Discussion and Future Directions

In this thesis, I describe novel findings on the role of polyphosphate as a negative regulator of complement and an anti-oxidant, and apply this new knowledge in rodent models toward a treatment for a common and serious complement-mediated cause of blindness, age related macular degeneration (AMD).

6.1 PolyP: Complex Roles in Coagulation and Complement

In response to injury, coagulation and complement are simultaneously activated to restrict bleeding and to discard invading pathogens and damaged cells, thereby protecting the organism from death, and facilitating healing. These two systems therefore act in concert, temporally and spatially [1, 164, 165]. The findings presented in my thesis appear to conflict with this paradigm, with polyP acting as a promoter of coagulation and an inhibitor of complement. The explanation for these findings is not clear at this time, and we can only speculate as to why polyP has these diverse properties. However, polyP is not alone in exhibiting complex, and sometimes apparently opposing activities. The best example is thrombin, which is known to be a procoagulant, anti-coagulant, and pro-inflammatory [166-169]. Thrombin achieves this through allosteric modifications in its conformation and distinct interactions with its different substrates, influenced by the demands of homeostasis [166, 167]. Thus, thrombin promotes clotting by activating platelets and endothelial cells, and through cleavage of factor V, factor XI, factor XIII and fibrinogen. It also acts as an anticoagulant by binding to thrombomodulin and activating protein C, as well as promotes inflammation by augmenting expression of adhesion molecules on endothelial cells and inducing vascular permeability. Mast cells, one of the first responders of inflammation, have also been found to switch from an initially fibrinolytic phenotype to a prothrombotic phenotype in response to C5a influx [170]. Overall, the response to injury must be able to adapt dynamically to temporal changes, and this might be most efficiently achieved by cells, proteins and other factors that possess diverse properties.
PolyP exhibits procoagulant and complement inhibitory properties, and that may depend in part on the local concentration and length of the polymer. Its differential function also will likely vary depending on environmental factors, and its particular protein partner (e.g., thrombin versus C5b6). The relative affinities of varying lengths of polyP for C5b,6 versus thrombin have not been studied. We also do not know how the complement inhibitory properties of polyP would be affected in the setting of a procoagulant environment, versus a primarily inflammatory environment. We could imagine, however, that polyP may serve simultaneously as a procoagulant and anti-complement factor: following platelet activation, polyP is released from dense granules, whereupon it may coat the cell, protecting it from complement-mediated destruction. At the same time, on the platelet surface, it may enhance the platelet’s procoagulant effects. Clearly, elucidating the complex role of polyP in vivo will require further study, which will hopefully result in the revelation of new therapeutic targets in the pathways that polyP affects.

### 6.2 Mechanisms by Which PolyP Suppresses Complement

In the past 10 years, major advances have been made in elucidating the mechanisms by which different lengths of polyP promote coagulation. Until the studies in the Conway lab, essentially nothing was known about the role of polyP in complement. Such studies were a challenge, due to polyP being strongly anionic, complicating experiments in the complement system, since many of the steps in the classical, lectin, and alternative pathways (but NOT the terminal pathway) are dependent on calcium and magnesium ions. Thus, it has been difficult to distinguish the effect of polyP due to direct interactions with complement proteins from its chelation effect.

Nonetheless, work by a Research Associate in our lab, Dr. Emilie Lameignere, has resulted in progress in this endeavor as she uncovered an ion-independent mechanism by which polyP modulates the classical pathway of complement (unpublished). PolyP was found to interact directly with C1s and the C1-esterase inhibitor (C1-INH), augmenting the activity of C1-INH, thereby dampening cleavage of C4 and C2, and limiting the formation of the classical pathway
C4bC2a C3 convertase. These studies are ongoing, but further support the conclusion from my thesis that polyP dampens activation of complement and justify continuing investigations to elucidate the mechanisms.

We established that polyP binds directly to C5b,6, and in doing so, reduces binding and/or integration of the downstream complexes, C5b-7 and C5b-8, to the target membrane. This would explain our finding that polyP reduces the generation of the lytic MAC. In the formation of the C5b-7 complex, C7 binds mostly to the C6 component of C5b,6 [171]. We have not determined the mechanism of action of polyP in terms of how it affects C7 interaction with C5b,6. PolyP may reduce C7 binding to C5b,6, or it may simply alter the structure of C5b,6 (without affecting the quantitative binding to C7), rendering the C5b-7 complex (and consequently, C5b-8 and C5b-9 complexes) less functional [171]. Interestingly, polyP had no effect on the formation of SC5b-9. SC5b-9 is considered a product of defective assembly of the MAC, when terminal complement protein complexes take on altered structures and bind to S protein (aka vitronectin) instead of the cell membrane, exposing a heparin-binding site [172]. The S-protein is incorporated into the complement complex as C5b,6 and C7 bind to each other, and is still susceptible to binding of C8 and some C9 molecules [173]. PolyP, which destabilizes C5b,6, may still allow the formation of C5b,6 to C7, making it less membranolytic, but also allowing for the incorporation of S-protein. Thus, although the formation of C5b-9 on membranes is suppressed, the formation of SC5b-9 is largely unaffected.

As discussed in the Introduction, host mammalian cells are endowed with negative regulators of complement, several of which target the terminal pathway. These include vitronectin, clusterin and CD59. It will be interesting to examine whether polyP interacts with these proteins, thereby further modulating generation of a functional MAC.

Addressing these fundamental questions, and understanding how polyP affects direct interactions with C5b,6 and other complement components (e.g. C1-INH, FH, C1s, vitronectin), will ultimately allow for the design of specific therapies to modulate complement at distinct steps in the cascade. Thus, a modified form of polyP or a compound that binds specifically to
C5b,6, could turn off the terminal pathway and MAC formation, without affecting any other step in the cascade, thereby preserving the generation of other biologically important products (e.g., C5a and C3a, which act as anaphylatoxins). This may be of particular interest for diseases such as AMD, where the MAC is believed to be the most damaging to the RPE and CEC.

6.3 Cellular Models of AMD: Limitations and Advantages

With all models – in vitro and in vivo – there are advantages and disadvantages that must be recognized, so that conclusions from experiments can be appropriately drawn. We used ARPE-19 and RF/6A cell lines that represent, respectively RPE and CEC, to examine the effects of polyP. These cell lines have been well-characterized and are widely used in the field. They are readily cultured, grow steadily, and with little change in morphology over multiple passages [145, 148, 150, 174, 175]. The ARPE-19 cells are particularly well-suited for our studies on AMD. These cells that spontaneously arose from human RPE cells, have a normal karyotype, express RPE-specific markers CRALBP and RPE65, can polarize on laminin-coated transwells, and develop tight junctions with relatively high transepithelial resistance (TER) that is reportedly found in vivo [145]. There are however, some limitations. Depending on the subline of ARPE-19 studied, the TER of the cells gradually decline during passage, with corresponding reduction in the integrity of the tight junctions. We did not test for TER or expression of the specific markers; nor did we grow the cells under conditions in which they would polarize. The latter in particular, may alter their response to the stresses and to polyP.

The RF/6A cells were also derived spontaneously from the choroid endothelial cells, but from a rhesus macaque fetus [150]. These cells exhibit the cobblestone appearance of endothelial cells in culture and have Weibel Palade bodies and express von Willebrand factor (VWF), consistent with them being endothelial in origin. However, the expression of VWF gradually reduces with passage, and we determined by Western blot (not shown) that expression of the endothelial glycoprotein, thrombomodulin, is very low. Thus, there are some limitations to these cells, and they therefore may not be fully representative of their primary counterparts from the choroid endothelium. Finally, for some studies, we also used EA.hy 926, which are phenotypically most
similar to human umbilical vein endothelial cells [163]. These have been widely used, but there are many others that could be tested for validation purposes.

6.4 Role of PolyP and MonoP as an Anti-oxidant

Thus far, studies on the role of polyP in mammalian systems have been limited to the coagulation and complement systems. The anti-oxidant properties of polyP demonstrated in this thesis present a potentially novel mammalian function. This finding is not entirely unprecedented, as polyP is known to play a role in bacterial survival in response to oxidative stress. In studies in prokaryotes, the overproduction of an exopolyphosphatase resulted in decreased levels of polyP, which rendered E. coli significantly more sensitive to H$_2$O$_2$-mediated death [17]. This was attributed at least in part, to enhanced expression of the anti-oxidant catalase HPII, a catalase in E. coli that is reportedly dependent on the presence of polyP, at least in the stationary growth phase. A separate study found that an E. coli mutant lacking the polyphosphate kinase enzyme suffered from decreased viability when challenged with oxidative stress [176]. The mechanism of these anti-oxidant protective properties is believed to involve gene expression regulation of cyclic AMP receptor protein and RNA polymerase, sigma S [177]. Cyclic AMP is an important messenger of many cell-signaling pathways, while RNA polymerase, sigma S is a regulator of transcription of stationary phase bacterial genes. This gene expression regulatory function may be similar to the way inositol polyphosphates play a role in gene regulation and nucleic acid break repair [178-184].

In mammalian cell culture systems, we demonstrated that polyP exhibits protective effects against H$_2$O$_2$-induced oxidative stress, as evidenced by cell morphology, retention of nuclear integrity, and expression of the intercellular junctional protein, VE-cadherin. Interestingly, monoP also exhibited protective properties, but this was only evident in the qualitative, cell morphology studies. MonoP did not appear to protect against oxidative stress-induced changes in nuclear integrity or VE-cadherin expression. This may reflect differential cytoprotective mechanisms of monoP versus polyP, or alternatively, varying sensitivities to the assays. Thus, the stress-induced changes in nuclear integrity and VE-cadherin were not sensitive to monoP,
whereas cytoskeletal changes that may affect morphology, may be more sensitive. Even though the monoP and polyP were compared, based on equivalent molar concentrations of orthophosphate units, differences in response may also be attributed to slightly different ionic strengths, caused partly by folds in polyP, hiding anionic groups from exposure.

The mechanism(s) underlying the cytoprotection of polyP and monoP has not yet been elucidated. In spite of some suggestive data, we have not reliably demonstrated that polyP (or monoP) induces gene or protein expression of catalase, as was found in the bacteria. This is not entirely surprising, as protective mechanisms for mammalian cells and bacteria would be expected to differ. Bacterial expression of catalase was dependent on growth phase, which cannot be extrapolated to mammalian cells, [176, 185]. As noted previously, our cells were not of primary origin, and were not grown to fully represent the in vivo situation, i.e., lacking polarization and a subcellular matrix. We used polyP_{130} to assess catalase expression, which is a much shorter form of the polymer than exists in bacteria. Overall, it is possible that under different conditions, catalase expression might be enhanced by polyP. These theories remain to be determined.

Beyond catalase, it is worth considering that other protective mechanisms may participate to protect RPE cells and CEC from the H_{2}O_{2}-induced oxidative stress. The other key antioxidant enzymes in mammalian cells are superoxide dismutase and glutathione peroxidase, with each one having a distinct mechanism of action, distinguished from that of catalase [152]. Glutathione peroxidase reduces hydroperoxides (including H_{2}O_{2}) to alcohols [186]. This reaction is dependent on the presence of selenium [187]. Unlike catalase, which is not produced in all cell-types and which has specific activity for H_{2}O_{2}, glutathione peroxidase is ubiquitously expressed, and is the main detoxification mechanism to counter low levels of oxidative stress induced by various types of hydroperoxides [152]. Five isoforms of glutathione peroxidase are found in mammalian cells, with expression levels varying with the tissue type [152]. Superoxide dismutase eliminates highly reactive O^{2-} anion radicals by catalyzing their formation into H_{2}O_{2}, which are then hydrolyzed by catalase or glutathione peroxidase [188]. Mice lacking the gene for superoxide dismutase develop multi-organ damage from continuous and unregulated
oxidative stress [189-191]. PolyP more likely affects glutathione peroxidase, which is able to directly degrade \( \text{H}_{2}\text{O}_2 \), the stressor that was used in our studies. It is also possible that polyP alters the manner in which these antioxidant enzymes function in concert. Further studies could include examining the ability of these three enzymes to degrade \( \text{H}_{2}\text{O}_2 \) using purified protein systems in the presence or absence of polyP. Such an approach would allow one to assess whether polyP acts directly on one or more of these enzymes, or is involved in intermediary steps (e.g., gene expression or cellular signaling).

In addition to a direct anti-oxidant enzyme mechanism, the chemical properties of the highly anionic polyP (and monoP) may also explain the protection against \( \text{H}_{2}\text{O}_2 \)-induced cellular damage. Generation of reactive radicals and reactive oxygen species from hydrogen peroxide is catalyzed by the presence of metals, such as iron and copper [192-194]. Through the Fenton reaction, iron accelerates oxidative damage by converting hydrogen peroxide to a hydroxyl radical and ferryl iron [195, 196]. These metals were undoubtedly present in our \textit{in vitro} cell culture models in which we examined the effects of polyP and monoP, raising the possibility that polyP acts at least in part by chelating the free iron or copper, thereby reducing the production of damaging reactive oxygen species. This would be in line with our findings with monoP, the latter of which also provided some protection, at least in the qualitative morphology studies. As already discussed, the monoP, however, had little effect in protecting against \( \text{H}_{2}\text{O}_2 \)-induced changes in nuclear integrity and in VE-cadherin expression, suggesting that chelation of metal ions is not entirely responsible for the protective effects of polyP [145, 197, 198].

Overall, no matter what the mechanism, it is intriguing that polyP exhibits anti-oxidant effects, as well as complement-inhibiting effects. In terms of AMD, the combination of excess complement activation and oxidative stress are considered the key determinants in promoting disease progression. That polyP simultaneously interferes with both, is therefore of potential clinical value from a therapeutic point of view. If indeed, monoP also protects against oxidative stress, this may be of further value, providing longer lasting therapeutic benefit as the polyP is naturally hydrolyzed into the monomeric form.
6.5 Lack of Proliferative Effect on Endothelial Cells

In our *in vitro* studies, data without oxidative stress suggested that polyP may induce cellular proliferation. The implications of such a finding would extend beyond AMD, providing a potential therapeutic target for several conditions, including for example, wound-healing, pathologic angiogenesis, inflammation and cancer. For that reason, we more carefully examined the effect of polyP on endothelial cell growth. Using 2 independent approaches (cell count and BrdU incorporation), we could not discern any significant effects of polyP on the proliferation of EA.hy 926 cells under non-stress conditions. MonoP also had no effect. In smaller scale studies, similar findings were obtained with RPE cells. Further studies are needed to test whether polyP affects the cells when under stress.

Nonetheless, this lack of an effect on endothelial cell proliferation, was in fact reassuring. As previously discussed, a major feature of wet AMD is excess growth of the choroid vasculature, which ultimately leads to leakage and bleeding and damage to the photoreceptors. PolyP-induced proliferation of endothelial cells might therefore be expected to exacerbate the growth of CNV lesions, and that would severely limit the value of polyP as a potential therapeutic agent for AMD. It is intriguing, however, that in spite of extensive studies on the role of polyP on coagulation, little is known about its effect on endothelial cells. Studies to examine that important question are now in progress.

6.6 Therapeutic Potential for AMD

Our *in vivo* studies with rats and mice support the notion that polyP exerts a protective effect against the development of Wet AMD, and provide an exciting novel potential use of polyP in mammalian systems. The two independent experiments indicate that both long-chain polyP (polyP_{21000}) as well as shorter-chain polyP (polyP_{130}) are able to abrogate the progression of AMD-associated damage at different time points (5 days and 14 days). In spite of *in vitro* data suggesting that monoP has anti-oxidant properties, monoP injections had no beneficial effect in the laser-induced CNV model.
6.7 Strengths and Weaknesses of the AMD Model of Laser-induced CNV

We are optimistic of the potential clinical importance of our findings, based on the fact that this model has been widely and repeatedly validated. Laser-induced CNV has been used in rodents, rabbits and non-human primates as the standard model for validating drugs that target the VEGF pathway, currently in use in clinics throughout the world. The advantages of this model are several. It induces a highly reproducible, localized CNV lesion, with which one can measure the efficacy of potential therapies such as polyP. The acute injury provides a rapid turnaround time for results, and the localization of the injury as well as the method of drug delivery allows one to avoid systemic effects that intravenous administration of polyP might otherwise cause.

In spite of the above, observations made with this model should be interpreted with some caution. The physiology of rodent eyes differs from those of humans in that rodents do not possess a macula, which is the structure of the retina that is most affected in AMD [199]. The macula has anatomical features distinct from the rest of the retina, including a greater concentration of photoreceptors. This would suggest that the pathological consequences of the laser-induced injury would differ from the pathology associated with AMD in humans. In turn, the response to polyP administration would likely also differ between the species [200]. From that, it is remarkable (and fortunate) that agents used in mice appear to also be effective in human adults. We do not currently know, of course, if this applies to polyP.

The nature of the injury that results from laser-induced CNV also differs considerably from that found in human AMD. The rodent model is associated with a physical injury to Bruch’s membrane, which then triggers acute inflammation and consequently, the development of CNV lesions [143]. In contrast, AMD in humans is a chronic, progressive multi-stage inflammatory disease. Instead of a single trigger, the progression of AMD is influenced by numerous exogenous factors (e.g., smoking), genetic predisposition (e.g. the genotype CFH Y402H), chronic oxidative stress, age (increased in Alzheimer’s Disease), and a range of other environmental factors (e.g. light exposure), many of which have not been identified yet [59, 113, 201]. These factors are not accounted for in our rodent model. As with most approved
therapeutics, we can only hope that polyP would be effective in treating patients with or without underlying risk factors and varying degrees of pathology.

6.8 Future In vivo Studies with PolyP

Further validation of our in vivo findings is required, with an expansion of the mouse model to include different lengths and concentrations of polyP treatment, and a post-treatment period of 7 days instead of 14 days. The reason for altering this time period is that there have been reports that at 14-21 days, the CNV injury starts to spontaneously regress [143, 144]. Ideally, the most reliable indication of a protective effect will be achieved at the time that the injury is at its worst.

Further analyses will also require histologic studies to assess the localization of the polyP and to examine for toxicity – local and systemic. Techniques to probe for polyP are only recently emerging. Dr. Jim Morrissey is currently using different approaches to develop probes that specifically detect different length polyP. These are based on the existence of exopolyphosphatase binding sites on polyP. Other approaches include DNA-binding probes (i.e., DAPI), due to the similarity of polyP to the DNA sugar-phosphate backbone [202-204]. The development of a sensitive and specific polyP probe will allow for simultaneous imaging or immunohistochemical detection of CNV, complement components, and polyP. This will help determine how and where polyP is acting, and whether it remains intact. In this thesis, I report that long chain polyP retains much of its complement suppressive function even after 10 days in normal human serum. This finding conflicts somewhat with a previous report, but there they measured the stability of polyP75 in terms of its polymer length, and not its function [9]. In any case, the stability of polyP in the vitreous is more relevant for our purposes [205], and these studies will be performed in vivo, with the availability of good detection methods.
One area of concern with administering polyP would naturally be thrombosis. The vitreous is not known to be associated with thrombotic disease, with clots only found there when the retinal vessels bleed. The fluid in the highly viscous vitreous does not turn over, but also has not been well-characterized in terms of coagulation protein constituents. The question as to whether polyP induces thrombosis of the choroid vasculature will be determined histologically. It is not clear whether this would be detrimental, particularly if it occurred only locally at the site of the laser injury. This would not cause loss of vision. Indeed, it may inhibit exudation of serous contents into the retina, which is the effect desired for a drug. In fact, photodynamic therapy, the standard treatment used prior to the introduction of anti-VEGF drugs, exerted its effects by inducing localized thrombosis of the CNV lesions [206]. Only if the polyP caused more widespread thrombosis, would there be concern. Even if the polyP distributed into the systemic circulation, the amounts would be very low, and not likely to cause harm. Indeed, polyP normally circulates in the plasma at micromolar concentrations and apparently does no harm.

We have limited the current studies to the wet form of AMD. As noted, Dry AMD is much more common, and also believed to be mediated by excess complement and exposure to oxidative stress. Unfortunately, in spite of attempts by several groups, validated rodent models to study Dry AMD are lacking. This may be changing. A rodent model of Dry AMD with some promise has recently been reported. This model requires the intravitreal injection of carboxyethylpyrrole adducted proteins, a product of oxidative stress that is found in drusen. Mice do not develop CNV, but do apparently develop features of Dry AMD over many months, and this is associated with increased deposition of complement proteins [207].

In summary, the in vivo findings described in this thesis are rationale for further study of the use of polyP to treat Wet AMD, able to simultaneously target both complement deposition and CNV development. The mechanistic details of this effect as well as potential side-effects warrant further studies, beyond the scope of this thesis.
Chapter 7: Conclusion

In this thesis, I have further clarified the mechanism by which polyP suppresses complement activation via the terminal pathway, an effect that spares generation of C5a, while reducing the production of the damaging membrane attack complex (MAC). These studies have further revealed that polyP not only dampens complement activation, but that it also interferes with oxidative stress-induced cellular damage. The mechanisms by which it exerts this effect have not yet been determined. However, an agent that simultaneously suppresses complement activation and protects against oxidative stress holds potential therapeutic value. Indeed, I have shown that in vivo, in rodent models of AMD, that polyP protects against laser-induced CNV with reduced deposition of complement.

There remains much work to be done. Nonetheless, the findings in this thesis raise awareness of the potential importance of a ubiquitous, naturally occurring inorganic compound that has largely been overlooked. Most important, the findings reveal a promising use for polyP as a treatment for AMD, a common and devastating disease.
Bibliography

64. Institute, N.E. Facts About Age-Related Macular Degeneration. 2015 [cited 2015; This information was developed by the National Eye Institute to help patients and their families search for general information about age-related macular degeneration. An eye care professional who has examined the patient's eyes and is familiar with his or her medical history is the best person to answer specific questions.]. Available from: https://nei.nih.gov/health/maculardegen/armd_facts.


32. *Lucentis Vs Avastin | Macular Degeneration Treatment Options | AMD.* 2015.


141. _Cell Counting with a Hemocytometer: Easy as 1, 2, 3._ 2014.


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