INVESTIGATION OF COMPLEMENT SYSTEM ACTIVITY INDUCED BY HYPERBRANCHED POLYGLYCEROL GRAFTING TO RED BLOOD CELL MEMBRANES

by

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Abstract

Repeated transfusion of red blood cells (RBCs) is the only treatment modality currently available for certain blood related genetic disorders such as thalassemia and sickle cell anemia. Due to chronic transfusion of RBCs in these patients, clinical problems surrounding alloimmunization develops in approximately 30% of patients. The pathology arises from adverse immune reactions to minor antigens that are either not routinely typed for, or cannot be readily matched. Hence, the development of donor RBCs that reduces the risk of alloimmunization would be highly beneficial.

An innovative approach to address this problem involves the use of polymers to mask the immunogenic blood group antigens on RBC membranes. Given potential applications of polymer grafted RBCs, non-toxic and non-immunogenic materials are desired. In this research, we have investigated the covalent attachment of hyperbranched polyglycerols (HPG), a highly biocompatible polymer, to red blood cell surfaces. The aim is not only to shield immunogenic blood group antigens, but also to prevent the degradation of biomaterial modified cells by the immune system, particularly by the proteolytic convertases of the complement system.

We investigated the mechanism of complement activation on HPG modified cells, and the influence of various polymer properties, including: grafting concentration, molecular weight, and degree of HPG functionalization in an effort to optimize the grafting process on cells. Traditional assays using antibody sensitized sheep erythrocytes and rabbit erythrocytes were used to assess the overall complement activation. Complement activation products C4a, C3a, Bb, and SC5b – 9 were quantified by ELISAs to determine the specific pathway of complement activation by HPG modified RBCs. Flow cytometry was also performed to demonstrate the effectiveness of antigen protection by the different graft properties.

HPGs with a molecular weight greater than 28 KDa at grafting concentrations greater than 1.0 mM, as well as a high degree of HPG functionalization result in the activation of complement via the alternative pathway. No activation was observed when these threshold levels were not exceeded. These insights may have an impact on devising key strategies in developing novel therapeutics, especially in the fields of both transfusion and transplantation medicine.

Preface

The data presented in chapters 2, 3, and chapter 4 is based on work conducted by Vincent Leung in Kizhakkedathu laboratory at UBC. A manuscript is in preparation based on the results presented in chapter 3. With the exception of synthesizing hyperbranched polyglycerols and performing ^{1}C NMR and ^{1}H NMR analyses, I designed, performed, and analyzed all experiments. The unmodified hyperbranched polyglycerol was synthesized by Irina Chafeeva. Dr. Jayachandran Kizhakkedathu contributed to the design of experiments and the analyses of the data.

Suggestions and guidance were provided by the supervisory committee, which consisted of Dr. Edward Pryzdial (chair), Dr. Edward Conway, Dr. Mark Scott, and Dr. Jayachandran Kizhakkedathu.

Ethics approval for this project is titled “Development of Immunocamouflaged Stealth Erythrocytes via Surface Initiated Controlled/Living Polymerization from Cell Membranes: Towards Universal Donor Red Cells”, which was obtained from the University of British Columbia’s clinical research ethics board. The certification number of the ethics certificate obtained is UBC CREB: H07 – 02198.

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List of Abbreviations

Bovine Serum Albumin	BSA
Clinical Research Ethics Board	CREB
Cobra Venom Factor	CVF
N, N' - diisopropylcarbodiimide	DIC
Degrees Celsius	^{o}C
Dimethylaminopyridine	DMAP
Dimethylformamide	DMF
Sheep erythrocytes sensitized with rabbit anti-sheep IgM antibodies	EA
Ethylene Diamine Tetraaceitc Acid	EDTA
Ethylene Glycol Tetraacetic Acid	EGTA
Enzyme – Linked Immunosorbent Assays	ELISA
Enhanced Optical System	EOS
Fluorescein Isothiocyanate	FITC
Gelatin Veronal Buffered Saline (Without Calcium and Magnesium)	GVB^{o}
Gelatin Veronal Buffered Saline with 0.15 mM Calcium and 1.0 mM Magnesium	GVB^{2+}
Gel Permeation Chromatography	GPC
Hyperbranched Polyglycerols	HPG
Hemolytic Transfusion Reactions	HTR
Integrin – Associated Protein	IAP
Membrane Attack Complex	MAC
Multi – Angle Laser Light Scattering	MALLS

MBL – Associated Serine Proteases	MASP
Mannose Binding Lectins	MBL
Methoxypoly(ethylene glycol)	mPEG
Molecular Weight Cut – Offs	MWCO
N – Hydroxysuccinimide	NHS
Nuclear Magnetic Resonance	NMR
Phosphate Buffered Saline	PBS
Polyethylene Glycol	PEG
Polyethyloxazolines	PEOZ
Polyvinyl Alcohol	PVA
Red Blood Cell	RBC
Terminal Complement Complex	SC5b – 9
Signal Regulatory Protein Alpha	SIRPα
Succunimidyl Succinate	SS
Succinimidyl Succinate Functionalized Hyperbranched Polyglycerols	SS - HPG
Terminal Complement Complex	TCC
Trimethylolpropane	TMP
University of British Columbia	UBC

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Chapter 1 Introduction

Genetic disorders such as sickle cell anemia and thalassemia result in abnormal red blood cell (RBC) physiology, inducing early destruction and removal of RBCs from circulation, and causing anemia[1-3]. Consequently, the quality of life for these patients is affected as insufficient amounts of oxygen are being delivered to their tissues, resulting in fatigue, weakness, pain, frequent infections, and delayed growth[3]. A bone marrow transplant is currently the only cure available for these inherited blood disorders, but not feasible for all the patients due to costs and non-availability of matching donors. The only effective treatment modality available is frequent RBC transfusions to carry out functions that otherwise do not occur by the defective RBCs that are produced. However, due to the frequent transfusions, which may range between 25 to 50 times per year, an estimated 30% of these patients develop alloimmunization due to immune reactions to the multitude of blood group antigens present on RBC membranes[4-6]. Alloimmunization occurs as the RBC recipient develops antibodies against foreign blood group antigens located on the surface of transfused RBCs, resulting in adverse transfusion reactions[7-9]. Several of these blood group antigens are minor and not normally typed prior to transfusion. In addition to the problem of alloimmunization for sickle cell anemia and thalassemic patients, there are also other clinically relevant problems caused by some of the highly immunogenic antigens[7,10]. In circumstances where an exact match between the recipient and donor blood is not achieved, the risk of a transfusion reaction is increased, which may result in several non-infectious serious hazards of transfusions including hemolytic transfusion reactions[8,10].

Clinical interventions to treat for alloimmune hemolytic anemia induced by transfusion of incompatible blood rely on supportive care, and the best treatment options

remains controversial[7]. This approach is problematic as attention to alloimmunization occurs after the adverse reaction occurs. A more active approach could involve extensive testing for antigens with higher risks of causing alloimmunization in both donor and recipient blood[7,11]. However, this may complicate blood banking procedures as identifying appropriate blood donors with ideal combinations of minor antigens would be difficult and not economically feasible[7,11]. Hence, the development of economical and practical methods to reduce the risk of alloimmunization is of significant clinical interest[12,13].

Several immunomodulatory therapies have been investigated to suppress alloimunization, such as blocking the costimulation of immune cells, blocking cytokines, as well as tolerance induction through immunotherapy to prevent the production of alloantibodies[6,14]. Alternatively, decreasing the antigenicity and immunogenicity of RBC antigens by modifying the surface of the membrane has been an emergent cellular therapy that has attracted attention in recent years[10,15,16]. The current state of RBC surface modification techniques involves the covalent attachment of polymers, such as polyethylene glycol (PEG), to membrane proteins of intact RBCs[15,17]. This strategy is termed PEGylation, which increases the hydrophilicity of the cell surface, while concealing surface proteins, carbohydrates, and lipids that may otherwise trigger alloimmunization[10,17-19]. Through PEGylation of the RBCs, interactions with the surrounding environment and host immune system is prevented, thereby increasing the survival of transfused RBCs[20]. The effective action of the attached polymer results in steric exclusion zones, which inhibits protein binding, and mask potential antigenic or epitopes located on the cell membrane, thereby preventing immune recognition and responses[21,22]. This mechanism is possible due to the molecular flexibility, rapid movement, and hydration of the attached PEG chains[23,24]. Such

immunomodulatory therapies suggest that polymer grafting may be a practical approach to prevent alloimmunization resulting from transfusion, and may also be beneficial for patients already alloimmunized.

Polymers have been attractive, as many of them are non-toxic, biocompatible, and highly functional[25-27]. In addition, the properties may be altered in various ways to best achieve its intended use[26]. For example, they may be produced in many different molecular weights and architectures, including linear or branched structures[28,29]. In this thesis, I describe the potential use of hyperbranched polyglycerols (HPG), a functional and biocompatible polymer, to mask the inherent antigenicity of RBCs. Since HPGs are synthetic, the primary focus will be to elucidate the innate immune response to these polymers once they are grafted to RBCs; importantly the influence of polymer properties such as molecular weight, size, graft density and the number of reactive functional groups will be investigated. In hemolytic transfusion reactions, the foreign antigens are recognized by the immune system, which activates the complement system to remove the perceived threat by lysing these cells[30,31]. The knowledge of the mechanism by which HPG modified RBCs activate the complement cascade would help in the design of novel cell-surface modification technologies to address this challenge, or to modulate the immune system response in cell-based therapies.

1.1 Blood Group Antigens of Red Blood Cells, Their Immunogenicity, and Clinical Significance

Biochemical studies have demonstrated that important blood group antigens on the membrane lipid bilayer of RBCs are comprised of complex carbohydrates and proteins[32-34]. There are currently 308 different antigens that have been identified, and each of these

antigens are classified accordingly to one of 30 blood groups systems[35,36]. The combination of blood group antigens located on an individual's RBCs are determined by genetics, and individuals who lack the necessary genes are unable to produce a particular antigen, which is therefore absent from the membrane of their red blood cells[34,37].

Under circumstances where foreign antigens are introduced to circulating blood, this antigenic stimulation will result in the production of a specific IgM or IgG alloantibody against the unknown antigen[21,30,38]. When this occurs for carbohydrate antigens, B lymphocytes are stimulated to produce IgM antibodies, which are able to directly agglutinate RBCs that express the non-self carbohydrate antigen[6,39]. Alternatively, IgM antibodies may also naturally exist in the serum of individuals who lack a particular antigen on their RBCs, despite an absence of prior antigenic stimulation[30,31]. This is believed to be caused by cross-reacting antigens found in the environment, such as those found on gut bacterial surfaces, which stimulate the specific IgM production[30,31]. Because IgM is a pentamer, once bound, it can initiate the classical pathway of the complement system through antigen-antibody complexes formed as a result of specific epitope recognition by the IgM antibodies[31]. In addition, an antibody isotype switch from IgM to IgG may occur in the event of prior immunization from either transfusion or pregnancy[8,31]. This immune response is typically due to protein antigens like the RhD blood group[40]. Similar to IgM antibodies, IgG antibodies that bind incompatible RBCs may activate the complement system, but are considered to be less effective due to their dimeric structure[31].

Under circumstances where the complement cascade has been initiated as a result of transfusing incompatible blood containing foreign antigens, hemolysis of the transfused RBCs may occur. This jeopardizes the health of the blood recipient. An example of IgM

antibody recognition of foreign antigens is when there is a transfusion mismatch involving the ABO blood group system, which is the most clinically important blood group system in clinical transfusion[8,34]. Individuals who lack the corresponding antigen will have anti A or B or both, and antibody – mediated hemolysis may occur when these antibodies recognize the antigen, thereby initiating the complement system, leading to complications known as extravascular hemolytic transfusion reactions (HTR)[8,35]. The fatality rate is estimated to be approximately 10%[8].

Further studies are still required to elucidate the function of the ABO antigens, despite its clinical significance[34,39]. Since the ABO antigens are carbohydrate structures located on glycolipids and glycoproteins, our current understanding is limited to their contribution to the RBC glycocalyx, an extracellular matrix of carbohydrate that surrounds the cell[34,35,41]. When ABO antigens are absent from the glycocalyx, there is no reported disease associated with this, as RBC survival and propensity to infection remain normal in these individuals[34,39].

The ABO antigens are not encoded by particular genes, but rather genes that encode for glycosyltransferases that adds terminal sugar moieties[39]. The A transferase adds a single N-acetylgalactosamine, whereas the B transferase adds a galactose unit. The O antigens refers to an absence of these additional sugars, and therefore terminates the carbohydrate sequence with fucose[34] (Figure 1.1). The genes that code for the A or B glycosyltransferases are inherited in a classical Mendelian fashion, and are both dominant traits compared to the O blood group[41]. Hence, depending on geographical location, there is a difference in the prevalence of the four ABO blood types: A, B, AB, and O. Blood type O is the most

common, followed by group A and then group B. The least common worldwide is group AB.

Due to the antigenicity and subsequent transfusion risk, the second most clinically important blood group system is the Rh blood group system, which consists of over 40 known independent variants of the antigen[40]. Belonging to this group is the D antigen, which is highly immunogenic and induces a strong adverse immune response when transfused to individuals who do not express the D antigen[34,40]. Despite having identified the molecular basis associated with many of the antigens that belong to the Rh blood group system, the actual epitopes that are recognized by antibodies are not well defined[34]. However, it is known that the different Rh antigens depend on different conformations all located on a hydrophobic protein with a molecular mass of approximately 30 – 32 KDa[32,40]. Despite the clinical importance of the Rh blood group system, possible functions and biological role of this protein complex remains unclear[32,34]. It is estimated that an immune reaction is induced in approximately 80% of transfusion mismatches of red blood cell units expressing the D antigen[40]. When transfusion of RhD positive blood to a RhD negative recipient occurs, approximately 50 percent of RhD negative individuals produce anti-D antibodies after the initial exposure and become immunized against it[8,40]. Hence, the D antigen is considered to be one of the most immunogenic antigens that are found on the surface of RBCs[34,40]. The IgG isotype is typically produced during repeated exposures, resulting in complement activation, extravascular hemolysis, and delayed hemolytic transfusion reactions[8,34]. Furthermore, IgG antibodies are able to cross the placenta, and in circumstances where pregnant RhD – negative women are immunized against the D antigen, fetuses who are RhD – positive may develop hemolytic disease of the newborn[8,40].

Given the clinical significance of the ABO and Rh blood group systems, routine typing of the ABO and D antigen is performed on blood products. However, In addition to the ABO and Rh blood group systems, there are several other blood group systems that have varying importance in transfusion medicine, such as the Lutheran, Kell, and Duffy systems[8-10]. Many of these blood group systems are made up of protein complexes associated with the RBC cytoskeleton[32]. Depending on the immunogenicity of these minor blood group antigens, several of these antigens may not be routinely typed prior to transfusion. In individuals who do not express a particular antigen, repeated exposure will result in the development of alloimmunization and production of specific antibodies, which can cause the destruction of these RBCs on subsequent transfusions of antigen positive blood.

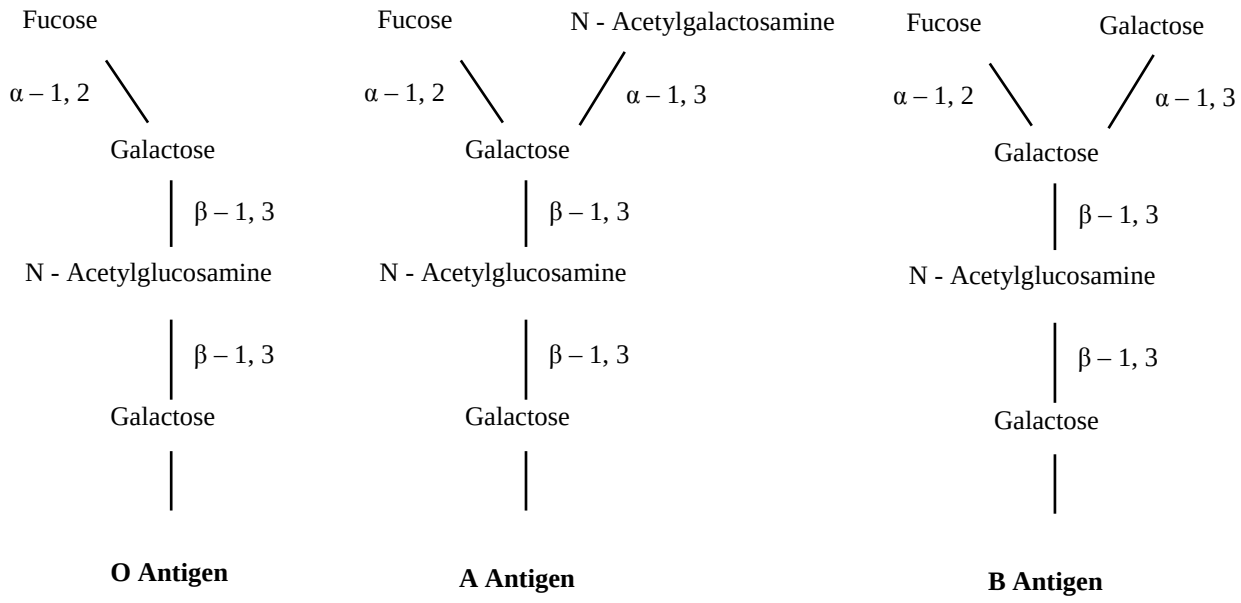


Figure 1.1 Structures of the ABO Blood Groups Antigens.

1.1.1 CD47

Cluster of differentiation 47 (CD47), also known as integrin – associated protein (IAP), was first discovered by scientists who were investigating aspects of the Rh Blood group antigens[42,43]. They observed monoclonal antibodies initially believed to recognize Rh blood group antigens of normal human erythrocytes react to CD47, which is expressed on many cell types belonging to different tissues[43,44]. Following its discovery, investigations into CD47 revealed that it is associated with Rh membrane complex, and is therefore reduced on RBCs that are Rh null[32]. CD47 is a highly glycosylated transmembrane protein of approximately 50 KDa[43]. It belongs to the immunoglobulin superfamily of membrane proteins, and is an integrin associated protein that binds ligands, such as the transmembrane signal-regulatory protein alpha (SIRPα)[43,45]. CD47 interacting with SIRPα has been shown to mediate cell to cell adhesion and migration, along with a key role in immune responses[45]. CD47 binding to SIRPα may regulate Fcγ receptor and complement receptor mediated phagocytosis of opsonized RBCs[43]. Furthermore, CD47 on normal healthy RBCs binding to SIRPα of splenic red pulp macrophages prevents these erythrocytes from being eliminated by providing an inhibitory signal. CD47 deficient cells are known to be phagocytized and removed from the bloodstream by macrophages[42,44]. Hence, CD47 is known as marker of self, allowing RBCs to avoid phagocytosis. This aspect of CD47 is of particular interest in this thesis, and any discussion on CD47 will be limited to its role in immune clearance.

1.2 The Complement System

The complement system is a component of the innate immune system and is comprised of over 30 different fluid-phase and membrane bound proteins[46-48]. Through an intricate cascade of serine protease activation, it functions to remove pathogens such as

bacteria, materials that are foreign to the body, and even host cells that are either damaged or lack appropriate protective mechanisms[30,47,48]. Depending on the initiating factor or surface that is being recognized, the complement system is activated through three different pathways: the classical, lectin, or alternative pathway (Figure 1.2)[30,49]. Once activated, the pathways proceed and converge at the level of C3 convertase, which cleaves C3 into C3a and C3b. C3b is a potent opsonin that binds to target cells allowing for complement to amplify, as well as enhancing phagocytosis by leukocytes. The complement system ends with the terminal pathway resulting in the formation of the membrane attack complex (MAC). This complex forms a pore in the target membrane resulting in an influx of water, causing the cell to lyse. Furthermore, as a result of complement activation, enhanced signalling of other components of the immune system is achieved through the anaphylatoxins C3a, C4a, and C5a[50]. These anaphylatoxins can trigger inflammation by enhancing the release of pro-inflammatory cytokines, thereby resulting in degranulation of inflammatory cells, mediating chemotaxis and activating leukocytes, facilitating histamine release by mast cells, increasing vascular permeability, and causing smooth muscle cells to contract[30,51].

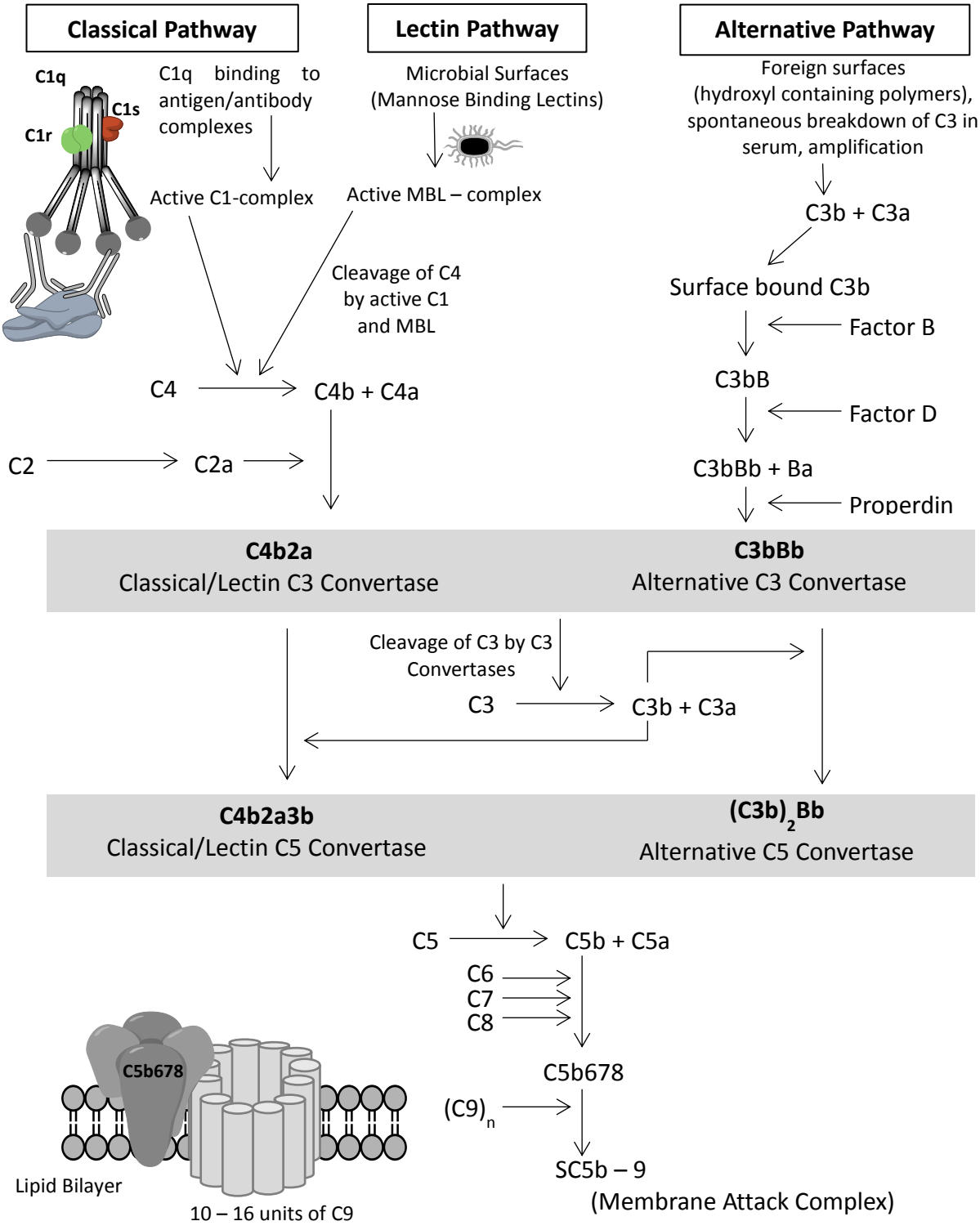


Figure 1.2 Schematic diagram of the complement cascade

1.2.1 The Classical Pathway

IgM and IgG antibodies that opsonize and bind to antigenic epitopes form immune complexes allowing for an initiating surface to which C1q can bind, initiating the classical pathway[30]. Once C1q binds, it undergoes a conformational change, followed by the recruitment of two molecules of serine proteases C1r and C1s to the site of activation. Once C1r binds to C1q, it becomes activated and further activates C1s through proteolytic cleavage, forming an enzymatic pentameric structure known as the C1 complex[30,31]. C1s of this activated complex is then able to cleave C4 into the products C4a and C4b[30]. A highly reactive thioester that was previously hidden becomes exposed, and C4b is covalently deposited onto the activating surface. Surface bound C4b is able to bind C2, thereby facilitating its cleavage by the adjacent C1s molecule into C2a and C2b[30]. C4b and C2a fragments together form the complex C4b2a, known as the classical and lectin C3 convertase[52-54]. Although the classical and lectin pathway C3 convertase differs in its molecular constituents to the alternative pathway C3 convertase, the subsequent activation steps are similar as they both function to cleave C3 into the products C3a and C3b[31,52]. It is at this step that the three pathways converge to fixate and amplify the complement system to eliminate the pathogen or foreign material.

Apoptotic cells and different types of bacteria, such as streptococcus pneumonia and streptococcus pyogenes, are removed from the host through the activation of the classical pathway[19,32,47]. Hence, a fully functional pathway is important in the ability to prevent pathogens from invading, and maintaining a healthy state[30]. Under circumstances where there is an inability for the classical pathway to proceed, consequences include autoimmune diseases such as systemic lupus erythematosus[30].

1.2.2 The Lectin Pathway

The lectin pathway is similar to the classical pathway as they both have the same C3 convertase, which is formed by the cleaved products C4b and C2a[53,54]. However, the initiation of the lectin pathway differs from that of the classical pathway[53,55,56]. Mannose binding lectins (MBL) that are synthesized in the liver or ficolins recognize mannose residues that are often associated with parasitic and bacterial surfaces[19,30,56]. As a result of this recognition, MBL-associated serine proteases (MASP) including MASP – 1 and MASP – 2 become activated and cleave C4 and C2 into their respective products to allow for the formation of the classical and lectin pathway C3 convertase, C4b2a[30,53,55,56].

1.2.3 The Alternative Pathway

Foreign surfaces such as artificial heart valves and extracorporeal devices are often initiators of the alternative pathway[47,50,52]. However, this pathway is also commonly referred to as being constitutively active as there is a "tickover" effect that involves a low level of circulating C3(H_2O) that is capable of initiating the alternative pathway[49,52,56]. This occurs through spontaneous hydrolysis of the reactive thioester of C3 in the fluid phase, and functionally similar to C3b, C3(H_2O) can bind serum factor B and undergo a conformational change, allowing factor D to cleave Factor B into the Bb and Ba fragments. The Bb fragment is a proteolytic enzyme that remains in complex with C3(H_2O), forming the fluid phase C3-convertase, C3(H_2O)Bb [30,49,56]. C3-convertase can further generate significant amounts of C3b by cleaving C3 into C3a and C3b. The majority of this C3b is inactivated by hydrolysis. Those that remain active covalently bind to amino or hydroxyl groups located on foreign or pathogen surfaces, as well as host cells[48,57,58]. Complement regulatory proteins prevent initiation and amplification on host cell surfaces, but in the absence of these

regulators, amplification of the alternative pathway can occur[30]. Once C3b binds to activating surfaces, Factor B can bind, which is subsequently cleaved by Factor D, resulting in an active proteolytic enzyme, Bb[30]. The C3 convertase of the alternative pathway, C3bBb, is formed as a bimolecular complex that is formed when Bb remains bound to C3b[54,59,60]. Amplification of the complement system occurs as more C3 is cleaved by the C3 convertase to yield C3a and C3b, which is then deposited onto activating surfaces in large amounts.

1.2.4 The Terminal Pathway

At the level of C3 convertase formation and amplification, the three different activation pathways converge despite whether it is the classical and lectin pathway C3 convertase, C4b2a, or the alternative pathway C3 convertase, C3bBb[19,30,61]. As more C3b fragments are generated through amplification, it is capable to bind either classical and lectin pathway, or alternative pathway C3 convertase to form C5 convertases, C4b2a3b, or (C3b)2Bb, respectively[53,62]. This C5 convertase cleaves C5 molecules into C5a and C5b through the serine protease activity of C2a or Bb[30,31]. The early stages of MAC formation occurs as C5b binds one molecule of C6, forming a stable C5b,6 complex, which then binds a single molecule of C7. Insertion of this C5b,6,7 complex into the outer leaflet of lipid bilayers on pathogens or other activating surfaces occurs through a conformational change to expose a hydrophobic region on C7. Once this hydrophobic region allows for the C5b,6,7 complex to attach, C8 can bind to the complex to anchor it as C8 contains similar hydrophobic regions[30,31,63]. Furthermore, C8 induces the polymerization of 10 – 16 molecules of C9, which are combined with the existing C5b,6,7,8 complex to form the MAC or C5b – 9 complex, which is a pore-like structure with a diameter of 100 angstroms. MAC concentrations of approximately 850 on each target cell results in significant passage of

solutes and water into the cell resulting in lysis, and elimination of the pathogen or target cell.

1.3 Biomedical Applications of Polymers and Hyperbranched Polyglycerols

The use of biomaterials in modern medicine is a practical means to improving the therapeutic outcomes of various treatment procedures[19,51,64,65]. Biomaterials have been used in the fields of regenerative medicine, tissue engineering, implantable medical devices, and encapsulation of biopharmaceutical compounds for enhanced drug delivery purposes[47,66]. Many of these approaches involve the use of polymers, and is commonly referred to as polymer therapeutics. The complexity of interactions occurring at the molecular level can be modulated by using polymers to enhance many therapeutic aspects such as: plasma circulation of various polymer – drug conjugates, pharmacokinetic properties, bioavailability, as well as targeting specific tissues for drug delivery systems[28,66,67]. Furthermore, polymers have been used to modify surface properties of cells, while preserving the main biological functions of the cells. This may decrease the immunogenicity and antigenicity of macromolecules and foreign cells to prevent adverse immune responses from occurring, thereby improving the overall biocompatibility.

Initial studies of polymer grafting to RBC surfaces involved the covalent attachment of polyethylene glycol (PEG) to RBCs[16,20,68,69]. This resulted in a reduction in antibody reactivity with several blood group antigens, including antigens of the ABO and Rh blood group systems[10,17]. Many different studies used a linear 5 KDa PEG capped at one end by a methyl group (mPEG), which was then coupled to RBCs using cyanuric chloride[20]. Although in these initial studies, the masking of RBC antigens was shown to be effective, the method used to investigate masking is suboptimal, as direct agglutination tests were used

to evaluate PEGylated RBC agglutination as an endpoint[17]. In addressing this limitation as well as problems related to first generation PEGylated RBCs, modifications to PEG was investigated, which include the use of bifunctional PEG derivatives, branched PEG, as well as cross – linking of PEG[21,22,69,70].

More recently, alternative polymers such as hyperbranched polyglycerols (HPG) and polyethyloxazolines (PEOZ) are being explored for their potential in developing immunocamouflaged cells for application in transfusion and transplantation medicine[13,27,71-73]. The current status of these studies demonstrates efficient masking of minor antigens, with no significant effects on the RBC structure or function. Furthermore, polymer modified murine RBCs demonstrate normal circulation and *in vivo* survival[27,73]. Although polyethylene glycols (PEG) have been traditionally used in the surface conjugation of RBCs, hyperbranched polyglycerols offer many advantages due to their compact and functional nature[27,67,71]. HPGs demonstrate biocompatibility and functionality due to the abundance of hydroxyl groups located on the periphery of HPG structures[29,71]. This structure allows for modification with a large number of different biologically relevant therapeutic agents. This thesis will include discussions on the covalent attachment of hydrophilic HPGs to the surface of RBCs, with the aim to mask surface antigens to sterically block antibodies from epitope recognition and elimination.

1.3.1 Chemical Properties of Hyperbranched Polyglycerols

Hyperbranched polyglycerol is a dense, globular polymer that contains many branches of repeating glycerol units that form a highly flexible polyether backbone structure, with each branch ending with a derivatizable hydroxyl group[27,29,71]. These hydroxyl groups allows for different modification and functionalization strategies that can be achieved

through covalent attachment to biologically important molecules. Depending on the application, the degree of branching, end group functionality, molecular weight, and polydispersity can be manipulated for specific functions to meet current shortcomings or deficits in modern medicine.

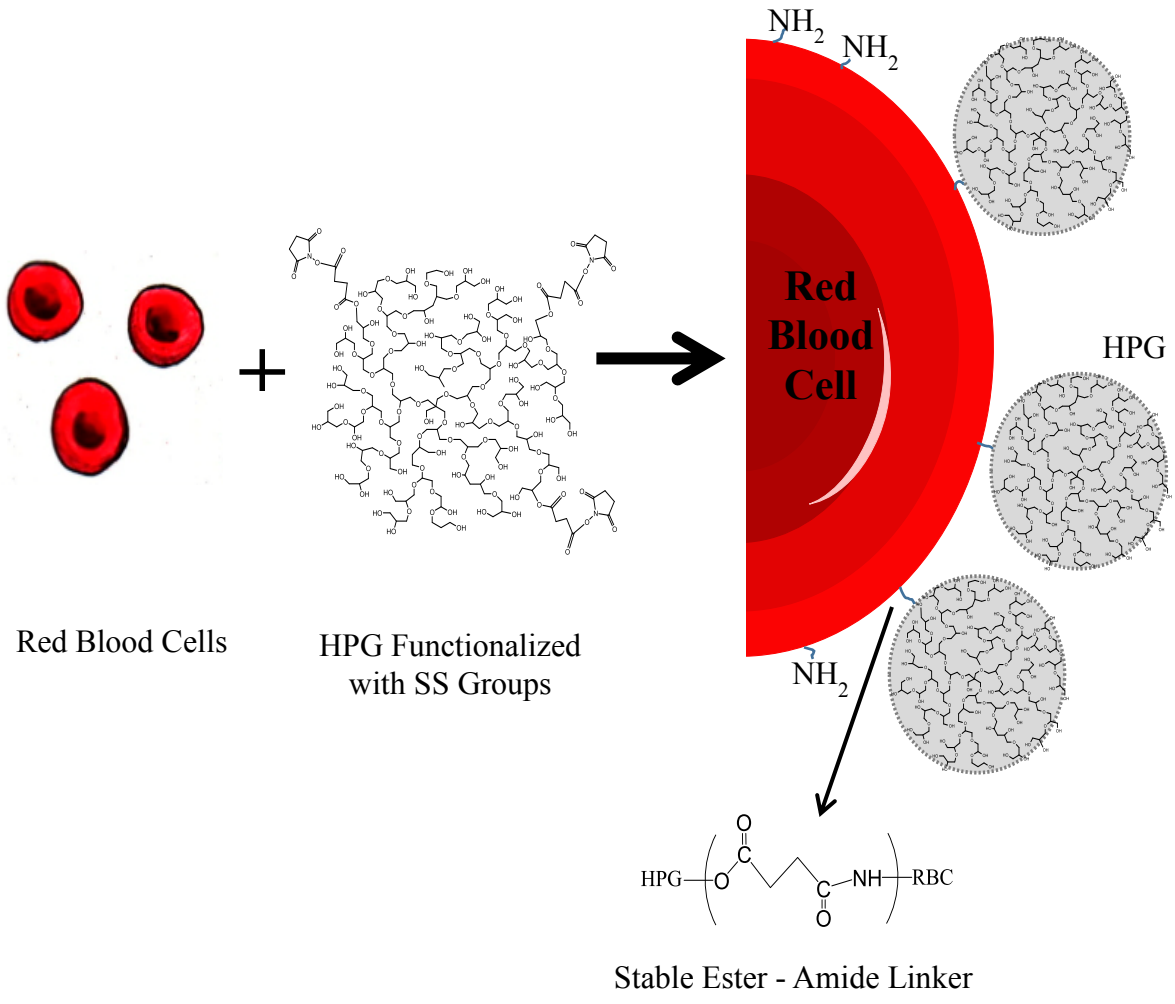
1.3.2 Biocompatibility of Hyperbranched Polyglycerols

The biocompatibility of HPGs has been extensively investigated and has been found to be non – toxic to cells and in blood[27,29,73]. This is a highly desirable feature of HPGs as this is essential for biomedical applications. The hydroxyl end groups and polyether backbone structures have many similarities to that of PEG, which has been approved by the Food and Drug Administration, for a large variety of biomedical applications[10,17,28,66,67].

1.3.3 Conjugation to Red Blood Cell Surfaces

The conjugation of HPG to RBC surfaces has been attempted through covalent attachment to primary lysine residues located on the surface of RBCs using a stable ester-amide linker[27,71] (figure 1.3). This is accomplished by modification of the hydroxyl groups to succinic acid. The carboxyl groups are then activated through N-hydroxysuccinimide (NHS) chemistry, resulting in succinimidyl succinate (SS) groups.

Certain parameters increase the extent of HPG grafting to RBCs, including: reaction time allowed for succinimidyl succinate functionalized HPGs to graft to RBCs, HPG grafting concentrations, molecular weight, as well as the average number of reactive SS groups functionalized to each HPG molecule.



* Schematic not drawn to scale

Figure 1.3 Schematic diagram of HPG conjugation to red blood cells

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1.4 Hypothesis and Objectives

The abundance of antigens located on the RBC membrane requires important consideration in modern medicine as they may elicit strong adverse immune responses in circumstance where incompatible blood is transfused to patients[8,10]. The overall objective of this project is to mask the inherent antigenicity of RBC antigens by modifying the RBC surface through the grafting of a functional and highly biocompatible polymer known as hyperbranched polyglycerols. However, various parameters such as molecular weight, grafting concentration, and degree of succinimidyl succinate functionalization affect the protection of immunological antigens, and under certain conditions, complement mediated lysis of HPG modified RBCs has been observed[27,71]. The exact mechanism of complement activation is not clear, although it is documented that surfaces grafted with biomaterials containing hydroxyl groups activate the alternative pathway of complement[62,74,75]. Given that the branching structure of HPG terminates with hydroxyl groups, **we hypothesize that RBCs conjugated with HPGs may induce complement activation via the alternative pathway, and that the complement activation on RBCs will depend on the molecular properties of HPG grafts and concentrations.** The research reported in this thesis investigates which specific pathways of complement activation is involved, and the significance of various graft polymer properties have on complement activation. Key objectives of this thesis are:

1. Identify the mechanism and pathway of complement activation by HPG modified RBCs.
2. Evaluate and compare the influence of graft polymer properties on complement activation, including studies on grafting concentration, molecular weight, and degree of succinimidyl succinate functionalization.

3. To ascertain the effects of various graft polymer properties on camouflaging antigens that are clinically relevant.

Chapter 2 Preparation and Characterization of Hyperbranched Polyglycerol Grafted Red Blood Cells[1]

The extent of covalent attachment of HPG to RBC membranes is influenced by different polymer properties and grafting conditions[71]. The parameters chosen for further investigation on complement activation include the: influence of grafting concentration, influence of molecular weight, and the influence by the average number of reactive succinimidyl succinate groups on HPG molecules. To investigate whether the extent of HPG grafting affects complement activation, we synthesized various molecular weight HPGs, and the number of SS groups was also varied. The different HPGs were grafted onto RBCs at different grafting concentrations (0.5 mM to 2.5 mM) and characterized by osmotic fragility measurements, and aqueous two phase partitioning.

2.1 Materials

Chemical reagents used for the synthesis and functionalization of succinimidyl succinate hyperbranched polyglycerols were purchased from Sigma – Aldrich (Oakville, Ontario), including: glycidol, potassium methylate, 1, 4 – dioxane, succinic anhydride, 4 – dimethylaminopyridine, N, N' – diisopropylcarbodiimide, and N – hydroxysuccinimide (NHS), pyridine, dimethyl formamide, methanol, and acetone. Trimethylolpropane (TMP) was purchased from Fluka (Ontario, Canada). All reagents were used without further purification, with the exception of glycidol, which was distilled and dried prior to use.

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[1] This chapter is based on work conducted in Dr. Kizhakkedathu Laboratory by Vincent Leung and Irina Chafeeva.

2.2 Experimental Methods

2.2.1 Synthesis of Hyperbranched Polyglycerols (HPGs)

Hyperbranched polyglycerols were synthesized according to the established literature protocols[27,29,71]. TMP was used as an initiator for the synthesis of HPG, which was done through ring opening multi-branching anionic polymerization of glycidol and the polymer was precipitated in acetone prior to dialysis against water for 3 days using a 3.5 – 5 KDa MWCO cellulose membrane (Spectrum Laboratories Inc.). After lyophilisation to extract the purified polymer, fractional precipitation from methanol to acetone was done to yield different molecular weight HPGs. Further dialysis and lyophilisation was done to remove residual solvents and impurities. Four different sizes of HPG were synthesized, and their molecular weights were determined using gel permeation chromatography (GPC) equipped with a DAWN EOS (enhanced optical system) multi-angle laser light scattering (MALLS) detector (Wyatt Technology Inc., Santa Barbara, USA) and Optilab RI detectors in an aqueous 0.1 M $NaNO_3$ solution. Branching of the hyperbranched polyglycerol was determined by ^{13}C NMR. Detailed synthetic procedures as well as the characterization protocols have been described elsewhere[27,71].

2.2.2 Functionalization of HPGs with Succinimidyl Succinate Groups

The functionalization of HPG with succinimidyl succinate groups was done according to established laboratory procedures[27,71]. The reaction scheme for functionalization is given in figure 2.1. For a typical reaction, 0.5 g (0.012 mmoles) of lyophilized, acid functionalized HPG (42 KDa, 7 acid groups per molecule) was dried under vacuum at 90^{o}C overnight before being dissolved in 3 mL of pyridine. As a catalyst, one

drop of dimethylaminopyridine (DMAP) at a concentration of 5 mg/mL dissolved in pyridine was added. The hydroxyl groups of HPG was then reacted with succinic anhydride (8.34 mg dissolved in 0.5 mL of pyridine) to form succinic acid groups. To allow sufficient time for the reaction, the mixture was stirred overnight at room temperature under argon. Cold acetone (40 mL) was used to precipitate the polymer, which was then centrifuged at 15 000 rpm for 20 minutes using a Beckman J2-MC centrifuge. The acetone was decanted and flushed with argon to remove residual acetone prior to dissolving the polymer in 3 mL of dimethylformamide (DMF). To activate the carboxyl groups, a reaction with 9.6 mg of N-hydroxysuccinimide (NHS) in the presence of 10.5 mg of N, N' – diisopropylcarbodiimide (DIC) dissolved in DMF was done at room temperature and stirred overnight under argon. Purification of succinimidyl succinate functionalized HPG is done through precipitation in cold acetone similar to before. Proton NMR analysis was used to characterize the synthesized polymers to assess the purity and degree of functionalization.

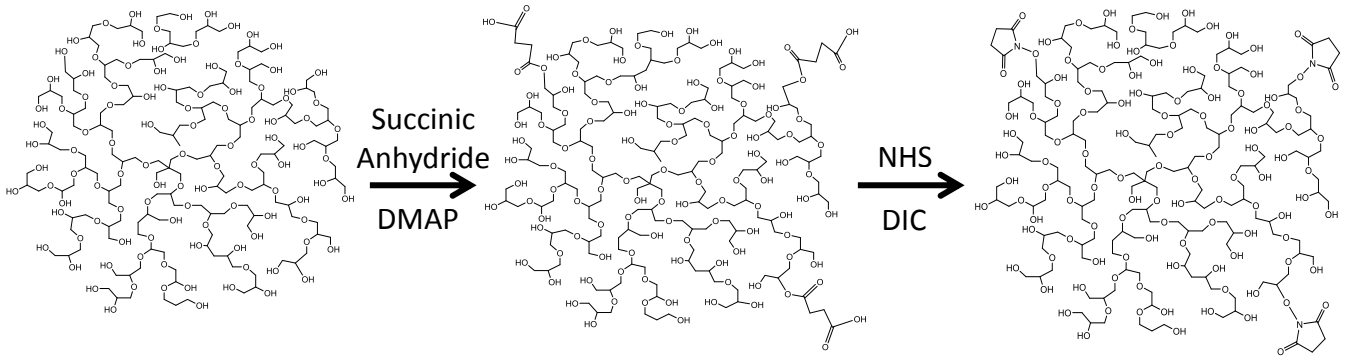

Figure 2.1 Synthesis of succinimidyl succinate functionalized hyperbranched polyglycerols (SS – HPG). To form acid functionalized HPGs, succinic anhydride and dimethylaminopyridine (DMAP) was first used in stoichiometric amounts with HPG. N- hydroxysuccinimide (NHS) and diisopropylcarbodiimide (DIC) was added to form SS – HPG dissolved in DMF.

2.2.3 Membrane Grafting of HPG to Red Blood Cell Surfaces

Ethics approval for collecting human blood from donors was obtained from the University of British Columbia's clinical research ethics board (UBC CREB: H07 – 02198). From consented volunteer donors, whole blood was collected into citrated vacutainer tubes and centrifuged at 1000 xg for 10 minutes. The plasma containing most of the platelets and white blood cells were removed using a Pasteur pipette and discarded. The RBCs were washed with 0.9% saline three times, before being suspended in phosphate buffered saline (PBS, pH 7.0) (Sigma – Aldrich, ON, Canada). Cell concentrations were determined using ADVIA 120 Hematology System (Siemens, Germany), and red blood cell counts were adjusted to a final concentration of $2.25x10^{12}$ cells/L.

For a typical reaction, the RBC suspension (1 mL) was centrifuged at 1000 xg for 3 minutes, and various volumes (0 µL, 50 µL, 100 µL, 150 µL, etc.) of the supernatant was removed. A 10 mM HPG stock solution was prepared by dissolving the purified succinimidyl succinate functionalized 42 KDa HPG (HPG – SS) in PBS (pH 8.0) containing 150 mM NaCl and 50 mM K_2HPO_4. Volumes of HPG-SS solution was added back to each of the respective RBC suspension samples where the supernatant was previously removed. All RBC suspensions containing HPG – SS solutions then contained a total volume of 1 mL, with an approximate RBC concentration of $2.25x10^{12}$ cells/L. To allow for sufficient covalent attachment of HPG to RBC membranes, the samples were gently mixed prior to placing onto an orbital shaker for 1 hour at room temperature. The RBCs were washed twice with PBS buffer and once with 0.9% saline to remove any unreacted polymer, and re-suspended in 0.9% saline to the initial 1 mL volume to obtain a final RBC concentration of $2.25x10^{12}$ cells/L.

2.3 Characterization of HPG Modified RBCs

2.3.1 Aqueous Two Phase Partitioning

Partitioning of control and HPG modified RBCs were performed in an aqueous two – phase system consisting of PEG 8000 (Sigma – Aldrich, Canada), and dextran T500 (Pharmacia, Sweden)[76,77]. The refractive index of the PEG and dextran solutions were measured and diluted with distilled water to make the two – phase system, which consisted of 5% (w/w) dextran, 4% (w/w) PEG, 0.15 M NaCl, 6.84 mM Na_2HPO_4, and 3.16 mM NaH_2PO_4 (pH = 7.4). Phase systems were made up in 15 mL polypropylene tubes and centrifuged at 1500 rpm (433 xg) for 10 minutes to allow for the dextran and PEG layers to separate. The upper PEG phase and lower dextran phase were isolated and transferred to a separate tubes for storage until use. The partitioning experiments were performed in duplicates. Unmodified control RBCs or HPG modified RBCs were added (20 µL of 2.25 x 10^{12} cells/L) to a 1 mL mixture of the two – phase system, which consisted of equal volumes of the dextran and PEG phase. The samples were mixed thoroughly before separation at room temperature. This method was used as a quality control to ensure successful grafting of HPG to RBCs, and the decision was based on visual observations.

2.3.2 Osmotic Fragility Measurements

Osmotic fragility is a common assay used to help diagnose diseases associated with RBC membrane abnormalities[78]. In this thesis, we used this assay to assess the membrane integrity and propensity of HPG modified RBCs to lyse when subjected to osmotic stress[20].

The osmotic fragility of HPG modified RBCs was measured shortly following polymer grafting. Twelve different saline solutions of different concentrations were prepared

in 50 mL aliquots. The weight percentages of NaCl include: 0.900, 0.650, 0.550, 0.475, 0.425, 0.400, 0.375, 0.350, 0.300, 0.250, and 0.000. One millilitre of each saline solution was transferred to microcentrifuge tubes prior to the addition of 20 µL of either unmodified control RBCs or HPG modified RBCs (2.25×10^{12} cells/L). All samples were gently mixed by inversion and incubated at 37ºC for 30 minutes. After the incubation, samples were mixed and 50 µL of each RBC suspension were placed in 1 mL of Drabkin's solution for subsequent quantification of hemoglobin[79]. To prepare supernatant samples for comparison, the incubated RBCs were centrifuged for 3 minutes at 700 xg, and 200 µL of the supernatant was mixed with 1 mL of Drabkin's reagent. The absorbance of hemoglobin in the total suspension samples compared to hemoglobin from lysed cells in the supernatant were determined by measuring the absorbance at 540 nm (SpectraMAX 190, Molecular Devices, USA), while using Drabkin's reagent as a reference.

2.4 Results

2.4.1 Succinimidyl Succinate Functionalization of Hyperbranched Polyglycerols

The primary amines of RBC membrane proteins react with succinimidyl succinate functionalized HPG (SS – HPG) to form stable amide linkages during the grafting process[27,71]. A range of SS – HPG molecules was synthesized with varying molecular weights and average number of succinimidyl succinate groups (Table 2.1). To achieve comparable surface binding properties of the four different molecular weight HPGs, the surface concentration of SS was fixed at one SS group per 20 nm^2 of surface area of HPG. The hydrodynamic size of HPG was used to calculate the surface area. Furthermore, 28 KDa and 42 KDa HPGs were chosen to have additional number of SS groups to study the effects of reactive SS binding groups. The average number of SS groups is determined based on ^1H

NMR analysis of the intensities of SS methylene signals associated with the HPG acid groups at 2.5 ppm, relative to methylene signals of the HPG ether backbone at 3.25 – 4.1 ppm (Figure 2.2). The characteristics, properties, and functionality of each polymer is described in table 2.1.

Table 2.1 Molecular weight characteristics and succinimidyl succinate functionality of hyperbranched polyglycerols

Polymer	Mn	Rh (nm)	PDI	# of OH Groups	Succinimidyl Succinate Groups
HPG 9 KDa	8 700	1.44	1.32	116	2 (± 0.5)
HPG 28 KDa	28 500	3.2	1.23	383	5 and 15 (± 0.5)
HPG 42 KDa	41 700	4.3	1.26	562	4, 7, and 16 (± 0.5)
HPG 100 KDa	98 500	5.03	1.22	1329	24 (± 0.5)

*All molecular weight characteristics were measured by gel permeation chromatography and assumes branching structures. Values are rounded to the nearest whole number.

Mn- Number average molecular weight.

Rh- Hydrodynamic radius measured using QELS detector connected to MALLS detector.

PDI- Polydispersity index.

Sample calculation for the average number of succinimidyl succinate groups on a 28 KDa HPG

*Calculations based on data given in Figure 2.2

1) $\frac{Molecular\ Weight\ of\ HPG}{\frac{g}{mol} of\ glycidol} = \frac{28000\ KDa}{74} = 378.38 = \boldsymbol{x}$

2) $\frac{Integration\ value\ of\ HPG\ Backbone}{5} = \frac{176.63}{5} = 35.33$

3) $\frac{Integration\ value\ of\ Succinimidyl\ Succinate}{4} = \frac{1.95}{4} = 0.488$

4) $\frac{Integration\ Value\ of\ succinimidyl\ succinate\ groups}{Integration\ Value\ of\ HPG\ backbone} = \frac{0.488}{35.33} = 0.0138 = \mathbf{y}$

(x)(y) = average number of succinimidyl succinate groups = (378.38)(0.0138) = 5.23 Groups of Succinimidyl Groups

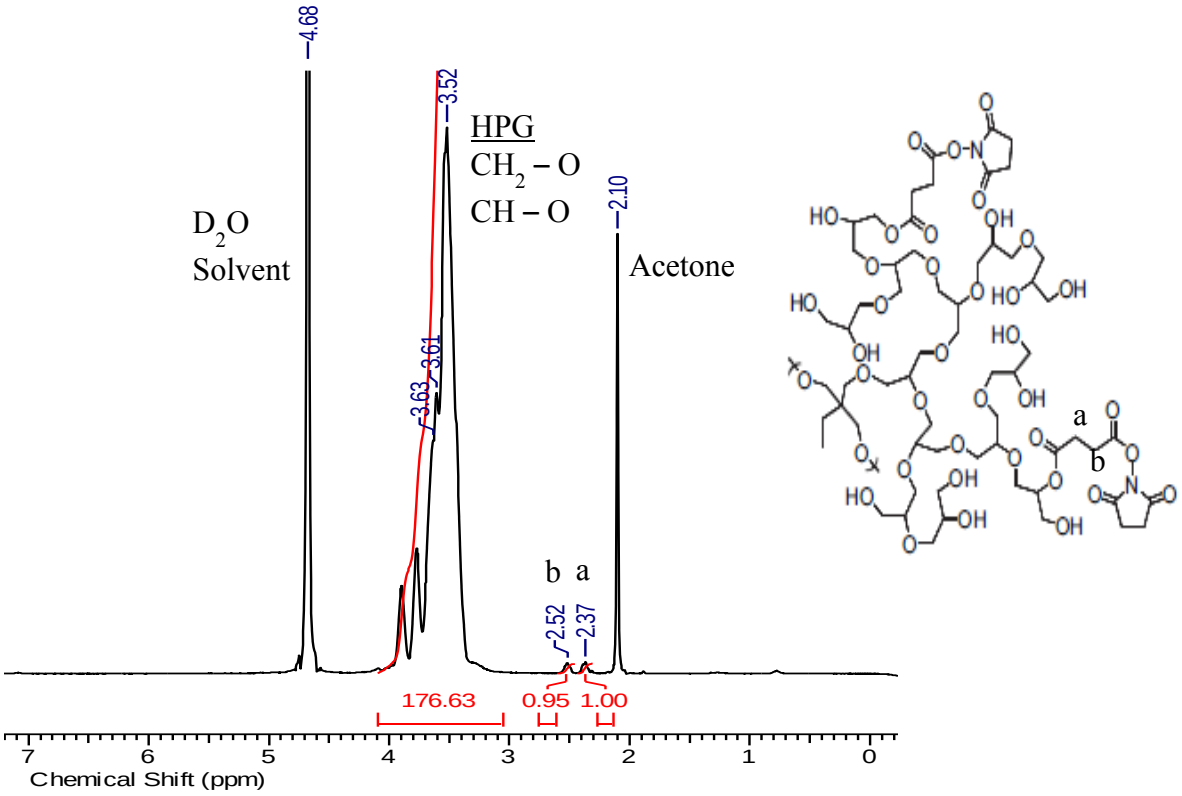

Figure 2.2 ^{1}H - NMR spectrum of a 28 KDa HPG functionalized with succinimidyl succinate

2.4.2 Aqueous Two – Phase Partitioning

Aqueous two – phase partitioning technique was used to measure the changes in the physical properties of RBCs derivatized with HPG. The dextran – PEG aqueous partitioning system provides information on the extent of cell – surface modification and surface properties[80]. Unmodified RBCs separate into the lower dextran phase, whereas RBCs grafted with HPG tends to separate into the upper layer containing PEG[71,80]. Relative to unmodified control RBCs, the modified RBCs demonstrate different properties, which are likely due to the covalent attachment of SS – HPG[71]. The SS – HPG modified RBCs are shown to partition into the upper PEG rich layer, whereas the unmodified RBCs associate with the lower dextran rich layer (figure 2.3). RBCs modified with a 42 KDa HPG containing 7 groups of succinimidyl succinate demonstrate increased partitioning into the upper PEG phase with increasing polymer grafting concentration as shown in figure 2.3. This suggests that with higher grafting concentrations, there are proportionately more HPG macromolecules grafted onto RBC membranes. Comparable experiments using different molecular weight HPGs and different number of SS functionalization showed similar trends (data not shown).

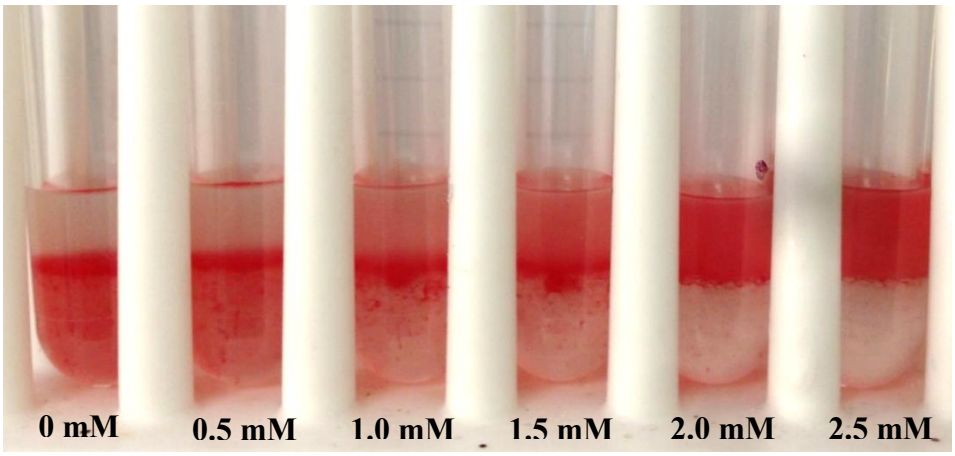

Figure 2.3. Effect of HPG grafting concentration on surface modification of RBC as measured by aqueous two – phase partitioning. HPG 42 KDa with 7 groups of succinimidyl succinate groups was used. The reaction was for 1 hour at room temperature (22ºC). Phase layers: PEG 8 KDa aqueous phase (upper layer) and dextran aqueous phase (lower layer).

2.4.3 Osmotic Fragility Measurements

Normal membrane stability and behaviour of RBCs are important aspects of cellular functions. Hence, the osmotic fragility of HPG modified RBCs was determined to show the membrane integrity and fragility of cells after derivatization by subjecting them to osmotic stress. The osmotic fragility of RBCs modified with a 28 KDa HPG shortly after grafting is shown in figure 2.4. Results show that up to 2.5 mM grafting concentration, there was no significant difference in the osmotic fragility, suggesting that the modification process did not affect the integrity of the cellular membrane.

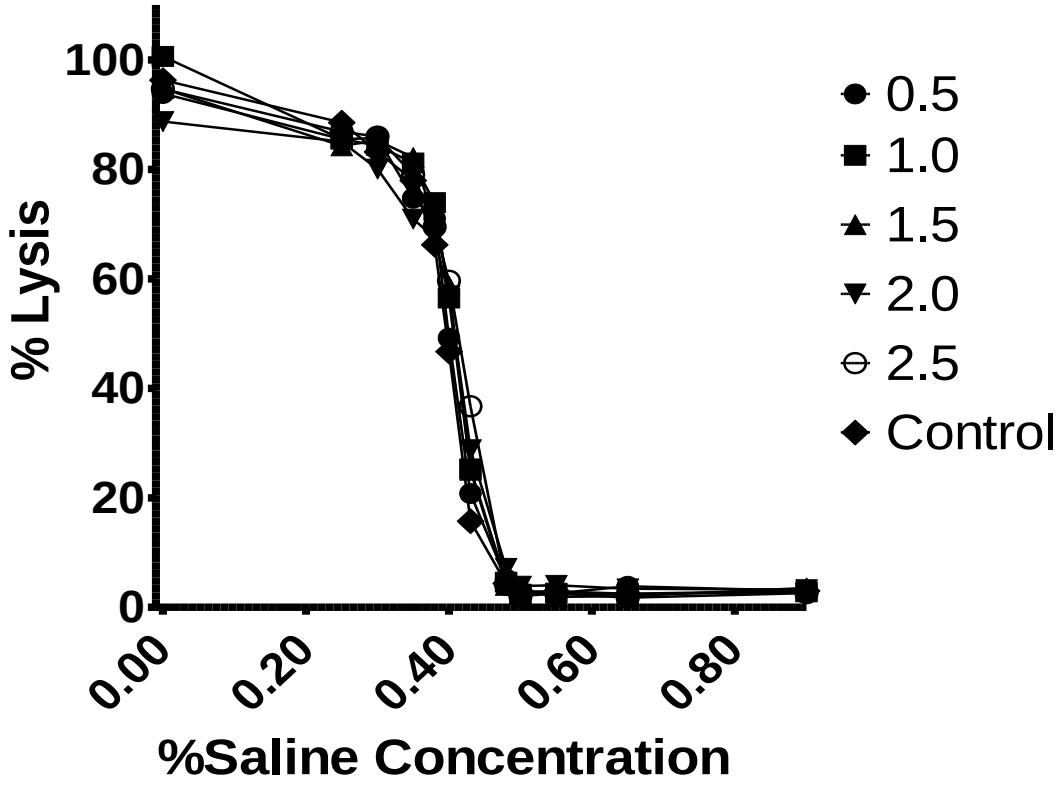

Figure 2.4. Influence of grafting concentration on the osmotic fragility of RBCs modified with a 28 KDa HPG. Measurements were performed shortly following the 1 hour grafting process. Grafting concentrations include: 0, 0.5, 1.0, 1.5, 2.0, and 2.5 mM.

The influence of molecular weight of grafted HPG on the osmotic fragility at identical grafting concentration is demonstrated in figure 2.5. RBCs modified with different molecular weight HPGs including 9 KDa, 28 KDa, 42 KDa, and 100 KDa are investigated for their membrane perturbation using similar methods and conditions as previously mentioned. The osmotic fragility curve was slightly shifted to the right when higher molecular weight HPGs are grafted to RBCs, indicating a modest alteration in the membrane stability compared to control cells.

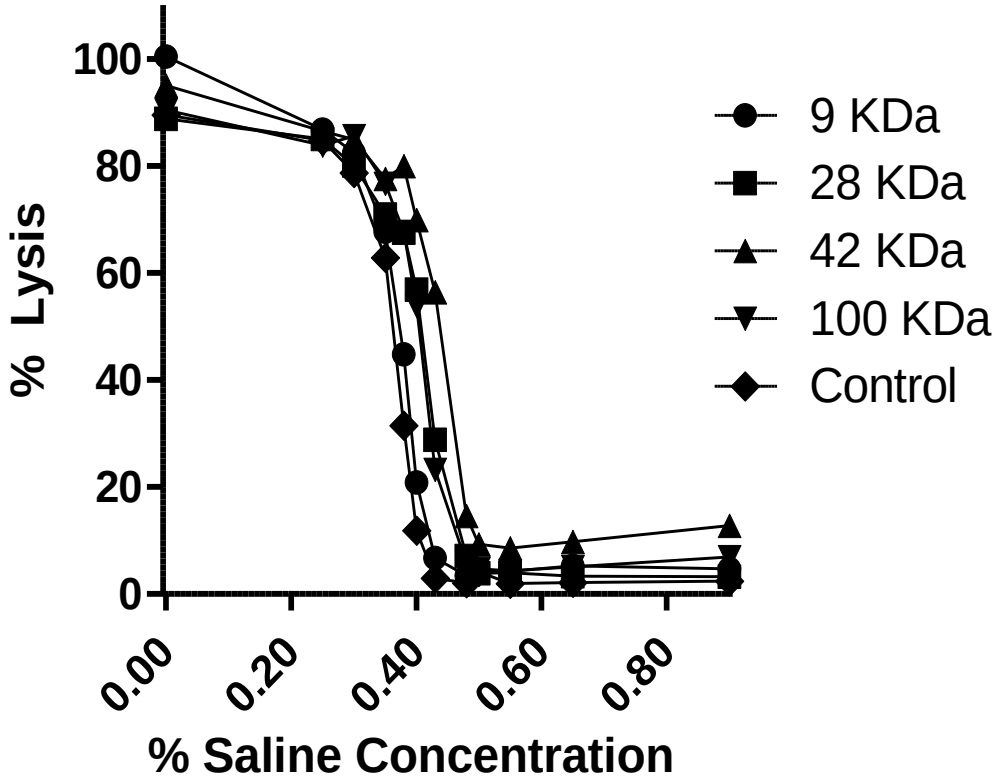

Figure 2.5. Influence of molecular weight on the osmotic fragility of HPG modified RBCs. The RBCs were grafted at 1.0 mM concentrations and measured shortly following the 1 hour grafting process at room temperature (22ºC).

To investigate the influence of the number of reactive succinimidyl succinate groups per HPG molecule on the osmotic fragility, 42KDa HPG functionalized with 4, 7, or 16 SS groups was grafted to RBCs membrane and subjected to osmotic stress similar to previously described methods. As shown in figure 2.6, as RBCs were grafted with varying numbers of SS groups on 42 KDa HPG, the modified cells demonstrate slightly different osmotic fragility curves compared to control cells. When comparing the difference in membrane integrity of RBCs modified with HPGs having a different number of SS groups at similar molecular weights, the effect was minimal.

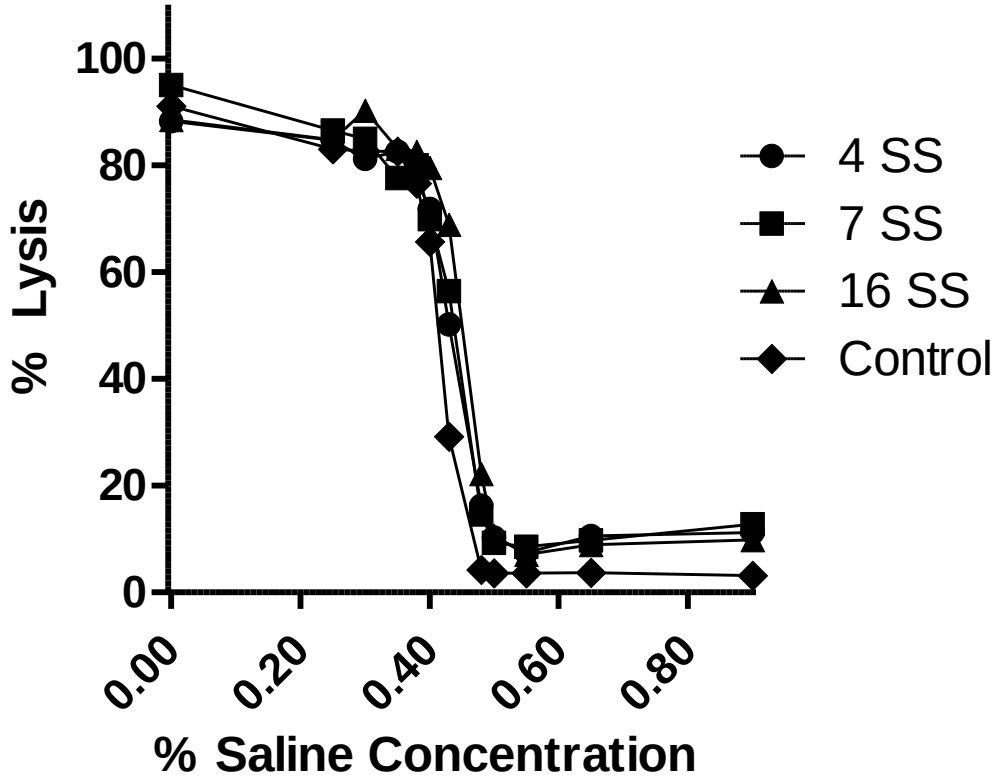

Figure 2.6. Influence of the number of succinimidyl succinate groups on the osmotic fragility of HPG modified RBCs. A 42 KDa HPG grafted at 2.0 mM concentration was used. The reaction was for 1 hour at room temperature (22ºC).

2.5 Discussion

The derivatization of HPG to RBC surfaces may prove to have great potential in preventing alloimmunization, while treating patients who are already alloimmunized. Here we have synthesized and characterized hyperbranched polyglycerols containing different numbers of succinimidyl succinate functional groups. The purity and degree of functionalization of synthesized HPG was determined by proton NMR. To investigate the efficiency of polymer grafting onto RBCs, aqueous two – phase partitioning system was used based on visual qualitative observations to assess the influence of grafting

concentration, molecular weight of HPG, and degree of SS functionalization. Dextran – PEG aqueous two phase system analysis demonstrated that HPG was grafted to RBC membranes. As previously mentioned, unmodified cells associate with the lower dextran layer or remain at the interface, whereas cells that demonstrate sufficient covalent binding of HPG to RBC surfaces partition into the upper PEG layer. As expected, high grafting concentrations, larger molecular weight HPGs, as well as HPGs with greater SS functionalization result in better grafting of RBCs. Despite this assay being a confirmation of the grafting success based on visual qualitative observations, future experiments should include quantitative measurements of the percentage of cells within each of the phases, which would allow us to confirm the optimal grafting conditions for the modification of RBCs. This wasn't included in this thesis work as current methods resulted in inconsistent results, and the development of an efficient, standardized method is needed.

In demonstrating the membrane integrity of HPG modified RBCs, osmotic fragility experiments showed that grafting concentrations 2.5 mM and below, as well as higher numbers of SS groups attached to HPGs do not affect the membrane integrity of modified RBCs. However, the molecular weight of HPG should be considered as large HPGs moderately shifted the curve towards the right, indicating a change in the osmotic fragility of modified RBC membranes.

Chapter 3 Investigation of Complement Activation by HPG Grafted RBCs[1]

The complement system is part of our innate immune system, and is responsible for the elimination of pathogens, foreign materials, and damaged cells[19,30]. In this chapter, the influence of HPG grafting to RBCs on complement activation was investigated. The influence of grafting concentration, molecular weight of grafted HPG, and the number of succinimidyl succinate groups on HPG were characterized. Modified RBCs were incubated with pooled ABO matched human serum. The reacted sera was measured for complement mediated lysis, complement activation by different pathways of the complement system, concentrations of specific complement activation products, as well as the amount of C3 fragments binding onto the surface of modified RBCs.

3.1 Methods and Materials

3.1.1 Pooling of Serum

Pooled serum was prepared by collecting whole blood from 8 healthy human donors with matching ABO blood types into glass serum tubes. To allow for the blood to clot, it was left at room temperature for 30 minutes and then centrifuged at 1500 xg for 15 minutes. The serum was isolated using a Pasteur pipette and pooled in a large beaker prior to freezing in 1 mL aliquots at -80^{0}C. The frozen serum was thawed on ice as needed.

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[1] This chapter is based on work conducted in Dr. Kizhakkedathu Laboratory by Vincent Leung and Dr. Jayachandran Kizhakkedathu. A manuscript is currently being drafted based on the work described.

3.1.2 Complement Mediated Lysis of HPG Modified RBCs

Complement mediated lysis was evaluated by incubating HPG modified RBCs in 20% pooled ABO matched serum for 1 hour at 37^0C. To quantify the amount of modified cells lysed as a result of exposure to ABO matched serum, a sample of cell suspension and supernatant was treated with Drabkin's reagent (Sigma – Aldrich, Ontario), which was prepared according to the standard protocol[79]. Preparation of samples was done by transferring 4.0 µL of the cell suspension into a 96 well plate and 296 µL of Drabkin's reagent was added and mixed. The supernatant was isolated by centrifuging the cell suspensions at 1000 xg for 3 minutes, and 33 µL of the supernatant was pipetted into a 96 well plate, and 267 µL of Drabkin's reagent was added and mixed. The absorbance by hemoglobin and hemoglobin derivatives was measured in triplicates by SpectraMax 190 plate reader (Molecular Devices, USA) at 540 nm. The ratio of hemoglobin concentration found in the supernatant compared to that in the cell suspension was used to calculate the percent of lysed cells.

3.1.3 Classical Pathway of Complement Activation

Sheep erythrocytes sensitized with rabbit anti-sheep IgM antibodies (EA cells) are traditionally used to assess the activity of the Classical pathway of complement[81,82]. We have adopted this assay in such a way that the results are reported as the % complement consumption, and not the minimum serum concentration required to lyse 50% of sheep erythrocytes[83,84]. Pooled ABO matched serum was first diluted with veronal buffered saline (GVB^{2+}) (Comptech, USA) to 40%, and then incubated in equal volumes with the HPG modified RBCs for 1 hour at 37^0C. Following the incubation, the cells were centrifuged at 1000 xg for 3 minutes, and further diluted with GVB^{2+}. Diluted and reacted sera was then

mixed with equal volume of sensitized sheep erythrocytes (Comptech, USA) that have been washed three times and suspended in GVB^{2+} at a cell concentration of 5.0 x 10^8 cells/L. The final serum dilution was 0.067% (1 in 15 dilution). The samples containing diluted serum and sheep erythrocytes were incubated at 37^0C for 1 hour, during which, they were gently mixed every 15 minutes. To quench the reaction, 300 µL of cold 10 mM EDTA dissolved in GVB^0 buffer was added to every sample and mixed after the 1 hour incubation. To determine the amount of lysis, the remaining sheep erythrocytes were pelleted at 1000 xg for 3 minutes, and the absorbance of the supernatant was analyzed at 541 nm. A cell blank sample containing only GVB^0 buffer, as well as a 100% lysis tube containing dH2O and sheep erythrocytes was included for comparison to the test samples. 5 µg/L of heat aggregated IgG (53ºC, 1 hour) was used as a positive control (Sigma – Aldrich, Canada). Because serum was incubated with both sheep and human erythrocytes in this assay, any lysis of human RBCs would have interfered with the interpretation of final result. Hence, the absorbance from human hemoglobin is subtracted from the total absorbance. The percentage of complement consumed is calculated accordingly to the following equations:

$$\% \, EA \, Lysis = \frac{[\text{OD Sample} - \text{OD Cell Blank}]}{[\text{OD 100\% Lysis} - \text{OD Cell Blank}]} \text{ x } 100$$

$$\% \, Complement \, Consumption = 100 - \% \, EA \, Lysis$$

3.1.4 Alternative Pathway of Complement Activation

Rabbit erythrocytes were used to assess the activity of the Alternative pathway of complement[85-87]. Pooled ABO matched serum was first diluted to 50% with GVB^0 containing magnesium chloride and ethylene glycol tetraacetic acid (EGTA) to chelate any calcium ions, as well as to ensure that there is sufficient magnesium concentrations for the alternative pathway C3 convertase to form[60]. Diluted serum was then mixed with equal volumes of HPG modified RBCs, and allowed to react at 37^0C for 1 hour. Similar to the previously described assay to investigate the role of the Classical pathway, the supernatant was extracted and gently mixed with equal volumes of rabbit erythrocytes (Comptech, USA) that have been washed three times and suspended in GVB^0 buffer at a cell concentration of 2.0 $x10^8$ cells/L. Following a 1 hour 37^0C incubation, 300 µL of cold EDTA-GVB^0 was added to the samples and mixed prior to measuring the absorbance at 412 nm and compared relative to the blank control and 100% rabbit erythrocyte lysis sample with H_2O. Cobra venom factor (CVF) was used as a positive control. Absorbance resulting from human hemoglobin in the samples due to lysis during the initial incubation with serum was subtracted from the total hemoglobin absorbance, and the values for percent complement consumption were calculated similarly to the method used for the classical pathway analysis (section 3.1.3).

3.1.5 ELISA Quantification of Complement Products C3a, C4a, Bb, and SC5b – 9

Pooled ABO matched serum (20%) was incubated with HPG modified RBCs at 37^0C for one hour to allow for any complement activation to occur. The samples were then centrifuged so that the reacted serum was isolated and diluted accordingly for each of the assays. Complement activation products within the serum was analyzed using enzyme-

linked immunosorbent assays (ELISA) (Quidel, USA) according to manufacturer's protocols[88].

C3a generated during complement activation through any of the 3 pathways is short lived and quickly cleaved into the more stable C3a-desArg[30,89]. The assay quantifies both products for indications about the complement activation in each of the samples. 5 µg/L of heat aggregated IgG (53ºC, 1 hour) (Sigma – Aldrich, Canada) was used as a positive control.

Activation of the Classical or Lectin pathways of complement results in the cleavage of C4 by the protease C1s into the anaphylotoxin and opsonin products C4a and C4b. C4a-desArg, which is a less active, but more stable form of C4a is also quantified to provide reliable results for any activation of the Classical and Lectin pathways of complement. Heat aggregated IgG (5 µg/L) (Sigma – Aldrich, Canada) was used as a positive control.

Factor B is an important zymogen specific to the Alternative pathway of the complement cascade, and is therefore a suitable marker for any activation through this pathway[90]. Factor B is cleaved through a two-step reaction in either fluid phase or on a membrane, beginning with C3b binding to Factor B, which is subsequently cleaved to yield Ba and a bimolecular complex, also known as the alternative pathway C3 convertase, C3bBb[47,64,91]. Although the Bb fragment may spontaneously dissociate or decay through the action of complement regulators, concentrations of Bb within test samples provide a suitable indicator of the role of the alternative pathway[90]. Cobra venom factor was used as a positive control.

The concentration of the terminal complement complex (TCC) or SC5b – 9 provides insight into complement mediated lysis, the end result of complement activation on target membranes[92]. This complex is generated as the complement fragments C5b, C6, C7, C8, and multiple C9 units assemble together following activation of complement through any of the 3 pathways[30]. The assay detects this complex through the binding of a monoclonal antibody to the C9 ring of SC5b – 9. Heat aggregated IgG (5 µg/L) was used as a positive control.

3.1.6 Flow Cytometric Determination of C3 Fragment Binding to RBC Surfaces

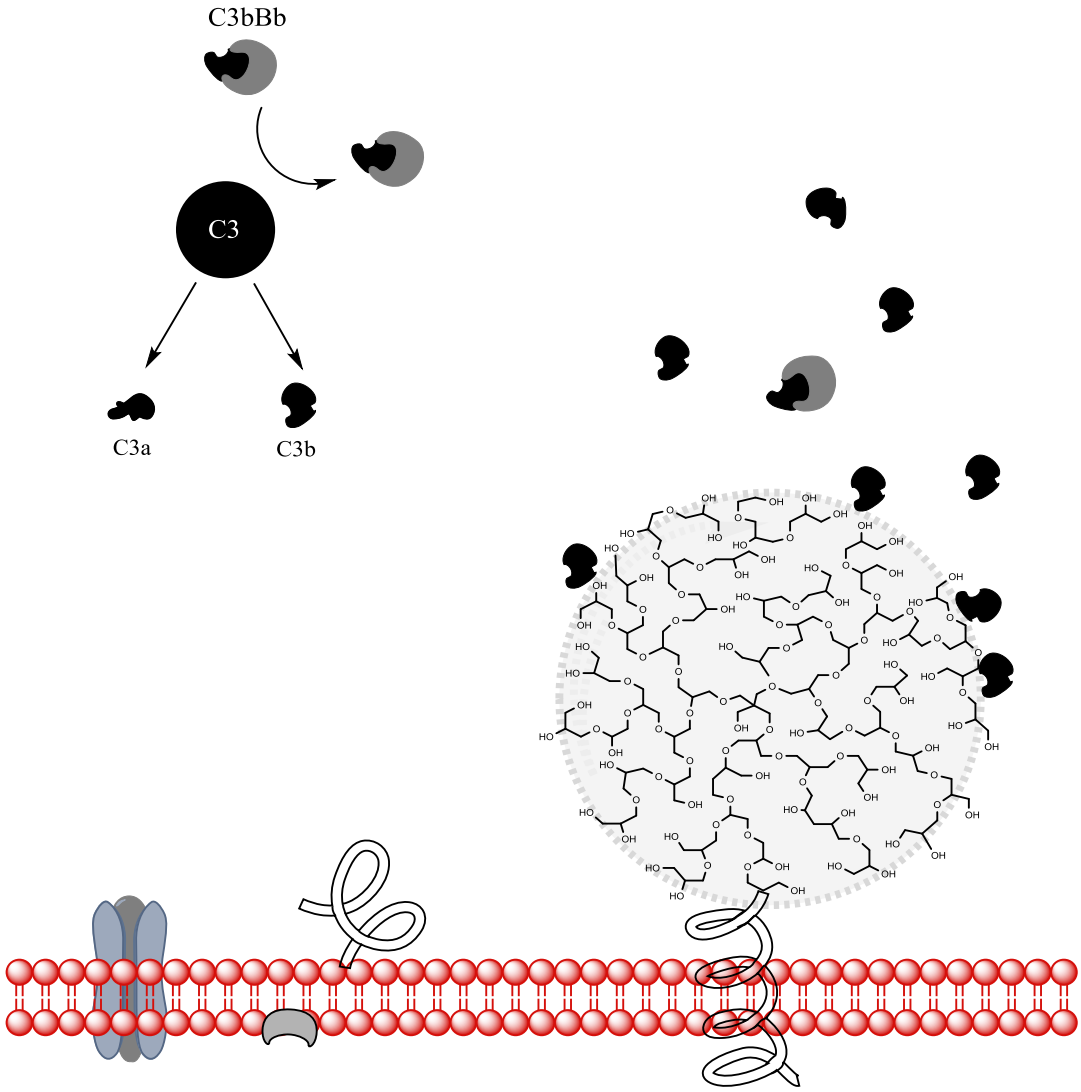
The anti-human/mouse complement component C3 monoclonal antibody recognizes epitopes on C3, as well as different epitopes on C3b and iC3b. Given the major role that C3b plays in forming the C3 and C5 convertases, it functions to promote and amplify the complement cascade[30,93] (Figure 3.1). Increasing concentrations of antibodies bound to cell surfaces relative to the control is indicative of complement activation by HPG modified RBCs.

To investigate whether C3b is bound to HPG modified RBCs, these cells are first treated with pooled ABO matched serum (20%) for 1 hour at 37^0C to allow for any complement fixation. The modified RBCs were isolated through centrifugation at 700 xg for 3 minutes and washed one time with 1% bovine serum albumin (BSA) dissolved in PBS (pH = 8). The modified RBCs were suspended in the same buffer used for the wash step at a concentration of 2.5 x 10^{11} cells/L. The cells were transferred into a 12 x 75 mm flow cytometer tube in duplicates prior to the addition of a FITC labelled anti-human/mouse complement component C3 monoclonal antibody (Cedarlane, Canada) at a concentration of 11 µg/µL. Each sample was mixed gently and incubated at 37^0C for 30 minutes. After

incubation, 1 mL of 1% BSA in PBS buffer was added prior to measuring the mean FITC intensity on the flow cytometer (Beckman Coulter, USA) set at 400 PMT.

3.1.7 Statistical Analysis

One-way ANOVA with Dunnett's comparison tests were performed for all statistical analyses using GraphPad Prism version 5.0 (San Diego, California, USA). A P value < 0.05 is considered as significant unless stated otherwise. All results are presented as the mean ± standard error of the mean.



*Schematic not drawn to scale

Figure 3.1. Schematic diagram of C3b binding to HPG modified RBCs. Diagram is not drawn to scale. Powerful amplification of the alternative pathway of complement can occur following C3b binding and opsonisation of HPG modified RBCs. The C3b can also act as a foundation for new C3 convertase complexes, which is a proteolytic enzyme that is not consumed in the reaction. As more C3 convertases are formed, more C3 is cleaved into C3a and C3b. As this occurs, significant amounts of C3b and its degradation products iC3b and C3dg can be deposited onto HPG surfaces. The formation of the MAC will ensue as C5 convertases are formed to activate C5.

3.2 Results

3.2.1 Influence of HPG Grafting Concentration on Complement Activation

Although the goal is to minimize immune recognition and improve the compatibility of foreign cells in vivo, the polymer is still foreign and can be subjected to removal and clearance by the immune system. If detected by the immune system, these cells would be removed from circulation. To investigate the influence of grafting concentration, a 42 KDa HPG was grafted to RBCs at different grafting concentrations (0.5 to 2.5 mM). The modified RBCs were incubated in 20% pooled serum from several donors with matching ABO blood types. It should be noted that with a higher serum concentration or incubation times, the amount of complement activated may be increased. Following this incubation, experiments were performed to measurement the percent of lysed modified RBCs, the percent of complement activation, relative concentrations of specific complement activation products, as well as the amount of C3 fragments that are bound.

3.2.1.1 Percent Lysis of HPG Modified RBCs in Human Serum

Results are shown in figure 3.2. RBCs grafted with a 42 KDa HPG demonstrated minimal lysis at grafting concentrations below 1.0 mM, whereas concentrations greater than 1.5 mM lysed significantly more compared to the control RBCs. The percentage of lysis was 5.0 ± 1.4 % at 1.5 mM, 10.6 ± 2.8% at 2.0 mM, and 13.1 ± 2.7% at 2.5 mM grafting concentrations, compared to 0.1 ± 0.0% for unmodified control RBCs.

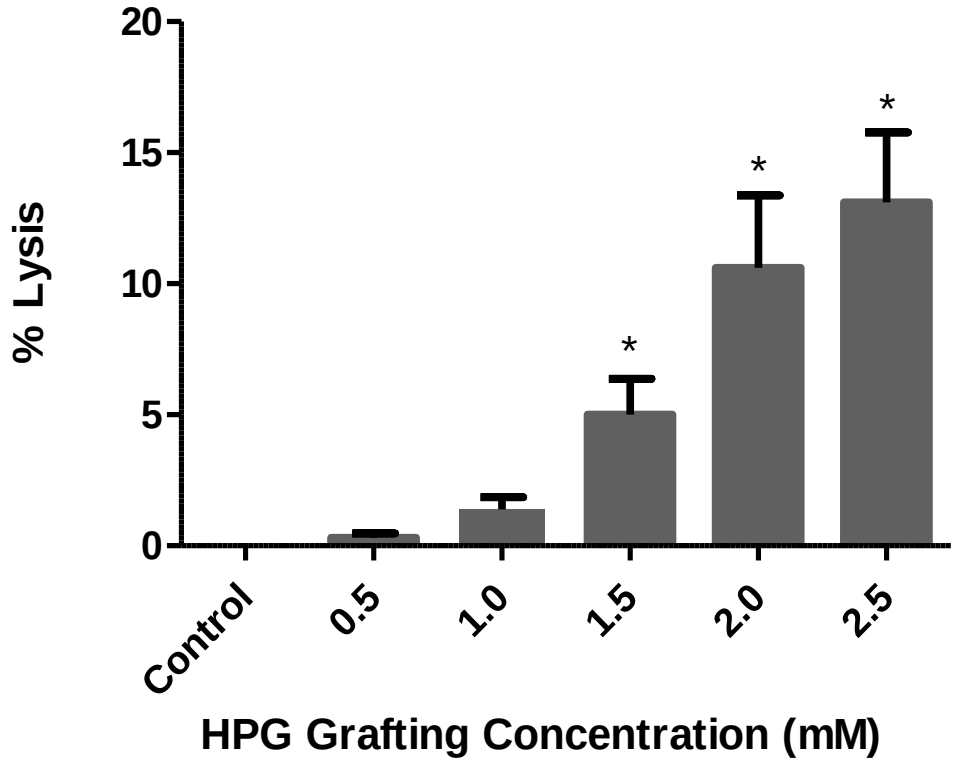


Figure 3.2 Percent lysis of RBCs modified with 42 KDa HPG in human serum. Influence of polymer grafting concentration on the lysis of RBCs modified with a 42 KDa HPG in serum. Prior to measuring the percentage of lysed RBCs, the modified cells were incubated in pooled serum matched to the RBC donor's blood type. * indicates samples that are statistically different from the control, with $P < 0.05$. Experiments were repeated three times ($N = 3$) with technical duplicates.

3.2.1.2 Influence of Grafting Concentration on the Activation of the Classical and Alternative Pathway of Complement by 42 KDa HPG Modified RBCs

During the incubation of HPG modified RBCs with ABO matched pooled human serum, any activation of the complement system will consume complement proteins. Following this, the remaining complement activity in the reacted sera was assessed by the percent lysis of sheep and rabbit erythrocytes. The classical pathway of activation and total complement activation was measured using sheep erythrocytes, whereas activation via the alternative pathway was measured using rabbit erythrocytes. Human serum does not readily lyse sheep erythrocytes through the alternative pathway, and therefore require sensitization using antibodies[87]. Hence, the majority of antibody sensitized sheep erythrocyte lysis is due to the classical pathway. However, the alternative pathway remains functional and exact contribution from the alternative pathway cannot be reliably assessed from this assay. Therefore, the results are reported as the classical pathway and total complement activation. Due to low amounts of sialic acids on rabbit erythrocytes, they spontaneously activate the alternative pathway of complement and have been traditionally used to assess the status of the alternative pathway[87]. Sialic acids, which are abundant on host cells, have a regulatory role in the alternative pathway of the complement system. The mechanism involves the interaction of major complement regulatory protein, factor H, which recognizes the sulfated glycosaminoglycans carrying sialic acids. This is mediated by anionic binding sites. Once factor H is recruited to cell surfaces, it helps to down regulate complement fixation through C3 convertase activity and C3b binding, thereby preventing complement activation via the alternative pathway. However, in the presence of calcium, the Classical and Lectin pathways

both remain functional and must be removed to prevent the C1q complex from forming, which is unique to the Classical and Lectin pathways.

The results for the classical and total complement activation are shown in figure 3.3 A. Relative to unmodified control RBCs, there is minimal activation of complement by 42 KDa modified RBCs up to 1.5 mM grafting concentrations. At 2.0 mM grafting concentration, 18.6 ± 5.3 % of the total complement consumption occurred, which is statistically significant compared to the 1.6 ± 3.7 % activation by the control unmodified cells (Figure 3.3A). Due to higher lysis of modified human RBCs at 2.5 mM grafting concentration in ABO matched serum, human hemoglobin in this sample exceeds 50% of total hemoglobin and is found to have too much interference in this assay. Given the high amount of lysis in human serum, complement is presumably activated and therefore the majority of the complement proteins are depleted and are unable to lyse the antibody sensitized sheep RBCs.

The contribution of the alternative pathway was investigated by the addition of Mg - EGTA, which blocked the classical and lectin pathways. The results are shown in figure 3.3B. The complement activation by 42 KDa HPG modified RBCs at 0.5 mM grafting concentration is measured to be 4.6 ± 6.2%, and 18.0 ± 9.5% at 1.0 mM. Only at 1.0 mM grafting concentration, the activation was significant compared to the 1.9 ± 5.0% activation by unmodified control cells. The results for grafting concentrations 1.5 mM and greater are not included in the graph as these samples contain more human hemoglobin compared to rabbit hemoglobin. Alternative pathway activation measured by the assay using rabbit erythrocytes demonstrates higher activation at all grafting concentrations measured compared to the results of classical and total complement activation. This is most likely due

to the higher magnesium concentration of 7 mM, which would facilitate more alternative C3 convertase formation

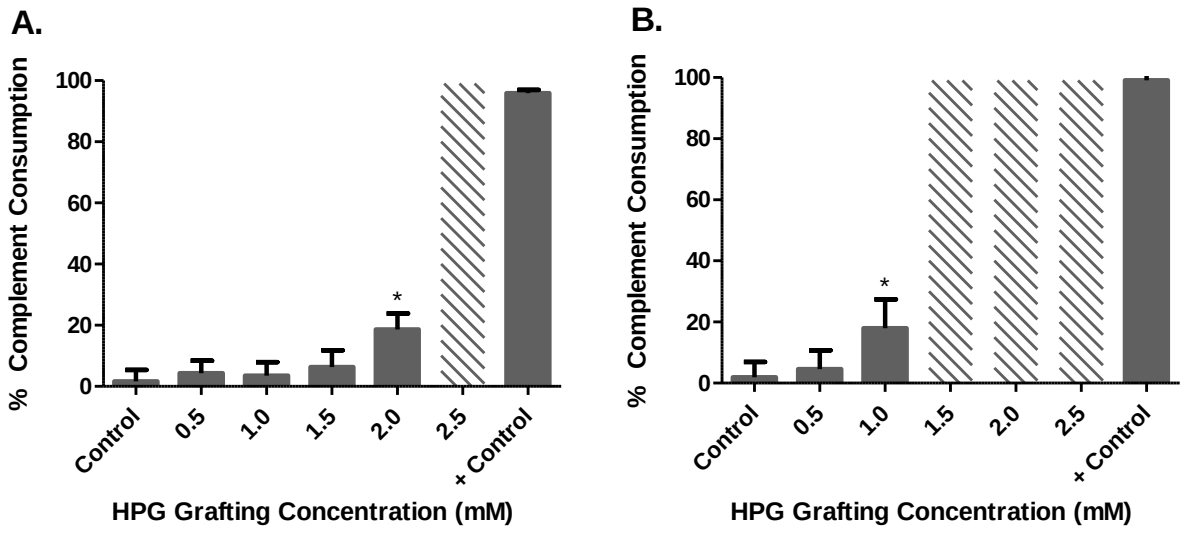


Figure 3.3 Complement consumption in human serum by 42 KDa HPG modified RBCs. Influence of polymer grafting concentration on the complement activation by 42 Kda HPG Modified RBCs. Depletion of serum complement proteins were assessed following incubation of modified RBCs with pooled matched human serum. High levels of HPG modified human RBC lysis in samples are found to interfere with the assay, and the results for these are shown as diagonal patterns indicating 100% complement consumed. **(A)** Antibody-sensitized sheep erythrocytes were incubated with reacted sera to measure the extent of classical pathway and total complement consumption. **(B)** Rabbit erythrocytes were incubated with reacted sera containing Mg-EGTA to determine residual complement activity due to the alternative pathway of activation. * indicates samples that are statistically different from the control, with P value < 0.05. Experiments were repeated three times (N = 3) with technical duplicates.

3.2.1.3 ELISA Quantification of Complement Activation Generated by 42 Kda HPG Modified RBCs

Generation of C3a and the terminal complex SC5b-9 in the complement cascade are downstream from the point at which the classical, lectin, and alternative pathways converge (Figure 1.2). Hence, elevated concentrations of either of these complement activation products provide information regarding the overall complement activation by the test sample.

C3a concentrations increase even at low grafting concentrations. Between 0.5 mM to 1.5 mM grafting concentration, there was a minimal increase in the level of C3a within the different samples (Figure 3.4A). At 2.0 mM, a 3.7 ± 0.3 fold increase of C3a levels compared to the control cells were observed, which was statistically significant.

The terminal complex of complement (SC5b – 9) is generated as C5b, C6, C7, C8, and many C9 molecules assemble together as it is inserted into the cell membrane, forming the membrane attack complex (MAC). This resulting complex is stable and the damage to the cell membrane is irreversible. There is a proportional and steady increase in the concentrations of SC5b – 9 as the HPG grafting concentration increased (Figure 3.4B). Relative to the control cells, there was a significant 2.8 ± 0.7, and 3.6 ± 0.2 fold increase of TCC concentrations at 1.5 mM and 2.0 mM grafting concentrations, respectively.

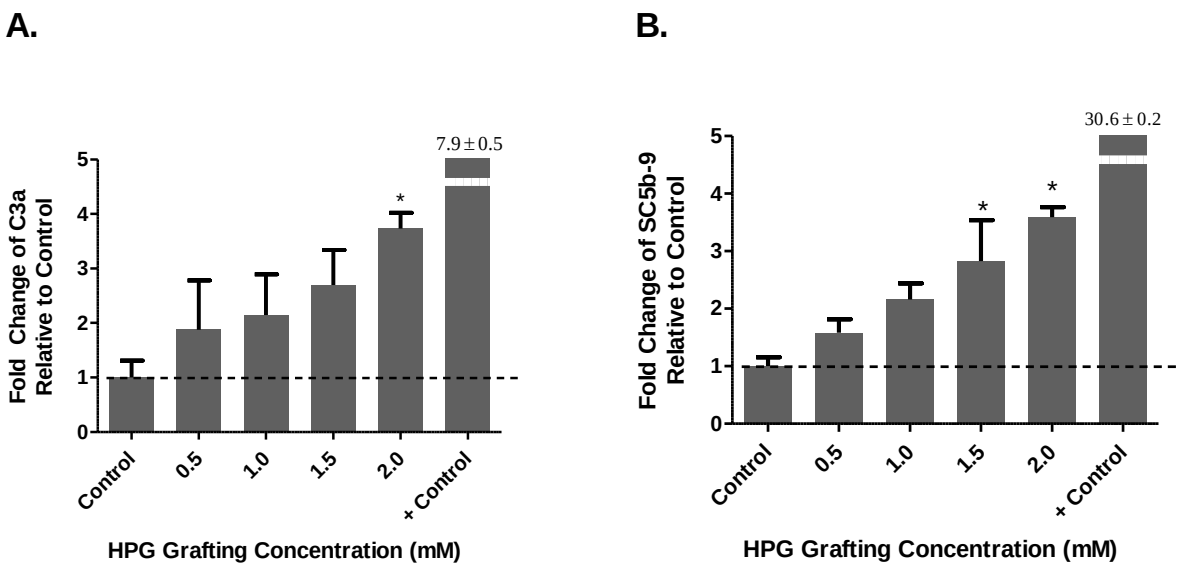


Figure 3.4 Generation of C3a and SC5b – 9 concentrations in human serum by 42 Kda HPG modified RBCs. The complement activation products C3a and SC5b-9 are generated during activation through the classical, lectin, or alternative pathways. The generation of these components in serum was determined using quantitative ELISAs following an incubation of pooled serum with RBCs modified with a 42Kda HPG. **(A)** Influence of grafting concentration on the relative amounts of C3a generated relative to unmodified control RBCs, which showed C3a concentrations of 1218 ± 378 ng/mL **(B)** Influence of grafting concentration on the relative amounts of terminal complex (SC5b – 9) generated in comparison to control unmodified RBCs. The concentration of SC5b – 9 for control samples was 2250 ± 355 ng/mL. * indicates samples that are statistically different from the control, with $P < 0.05$. Experiments were repeated twice (N = 2) with technical duplicates.

The C1s protease cleaves the complement protein C4 into C4a and C4b in circumstances where the classical pathway of complement is activated. Given that C4 is specific to the classical and lectin pathways, any complement activation through these pathways in the test sample can be reliably quantified by measuring the concentrations of C4a and any degraded C4a products. Compared to the serum incubated with control cells, reacted serum incubated with 42 Kda HPG modified RBCs did not show increased levels of C4a at any grafting concentration tested (Figure 3.5A).

Factor B is an important zymogen specific to the alternative pathway of complement. Hydrolyzed C3 ($C3bH_2O$) in fluid phase, or C3b located on membrane surfaces will bind Factor B[49,54,93]. This bound Factor B can then be cleaved by Factor D, resulting in an active proteolytic enzyme, Bb that remains bound to C3b[54,64,93]. The C3bBb bimolecular complex is the alternative pathway C3 convertase, and is capable of further cleaving additional C3 to amplify the complement system, or can form the C5 convertase by binding an additional C3b molecule. Since Factor B is unique to the alternative pathway of complement, Bb is a reliable marker for any complement activation that has occurred via the alternative pathway. Furthermore, it has been reported that ELISA quantification of Bb appears to be the most reliable assay available to assess activation of the alternative pathway[88]. For 42 Kda HPG modified RBCs at grafting concentrations 1.0 mM and below, there was a minimal increase in the generation of Bb (Figure 3.5B). At 1.5 mM, there is an observed increase in the concentration of Bb, but it is not until 2.0 mM grafting concentration that there is a statistically significant 1.7 ± 0.3 fold increase in the Bb concentration relative to the unmodified control cells (Figure 3.5B).

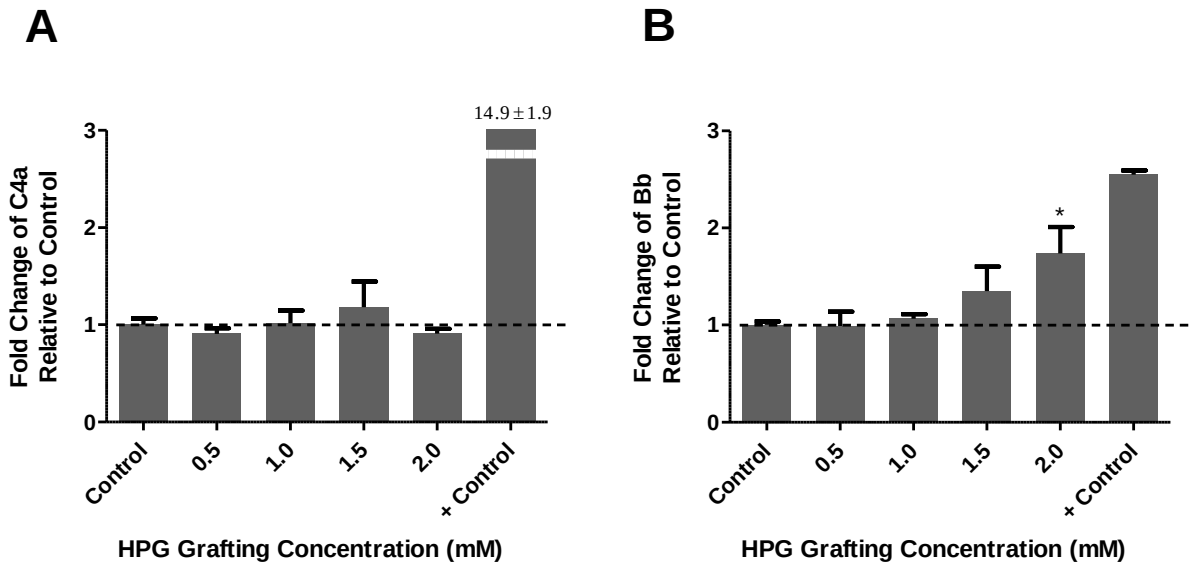


Figure 3.5 Influence of HPG grafting concentration on the generation of C4a and Bb in human serum by 42 Kda HPG Modified RBCs. Classical pathway and alternative pathway of complement activation is determined through the measurement of proteins that are specific to each of these pathways. Classical component C4a and alternative component Bb is quantified by ELISAs in the reacted sera resulting from an incubation of pooled serum with 42Kda modified RBCs at 37ºC. **(A)** Relative amounts of C4a generated upon incubation of HPG modified RBCs with human serum. The concentration of C4a in the control sample was 2092 ± 136 ng/mL **(B)** Relative amounts of Bb generated in comparison to control unmodified RBCs after incubation with human serum. The concentration of Bb for control sample was 7.2 ± 0.3 µg/mL. * indicates samples that were statistically different from the control, with $P < 0.05$. Experiments were repeated twice (N = 2) with technical duplicates.

3.2.1.4 C3b Deposition on 42 Kda HPG modified RBCs

42 Kda HPG modified RBCs demonstrate an exponential increase in the amount of bound C3b as the HPG grafting concentration increased by 0.5 mM increments (Figure 3.6). Beginning at 1.0 mM grafting concentration, and higher, we observe significantly more C3b bound to the modified surfaces compared to the unmodified control cells. This increase C3b

levels was as much as 1.7 ± 0.1, 2.4 ± 0.04, and 3.3 ± 0.15 folds greater at 1.5 mM, 2.0 mM, and 2.5 mM HPG grafting concentrations, respectively.

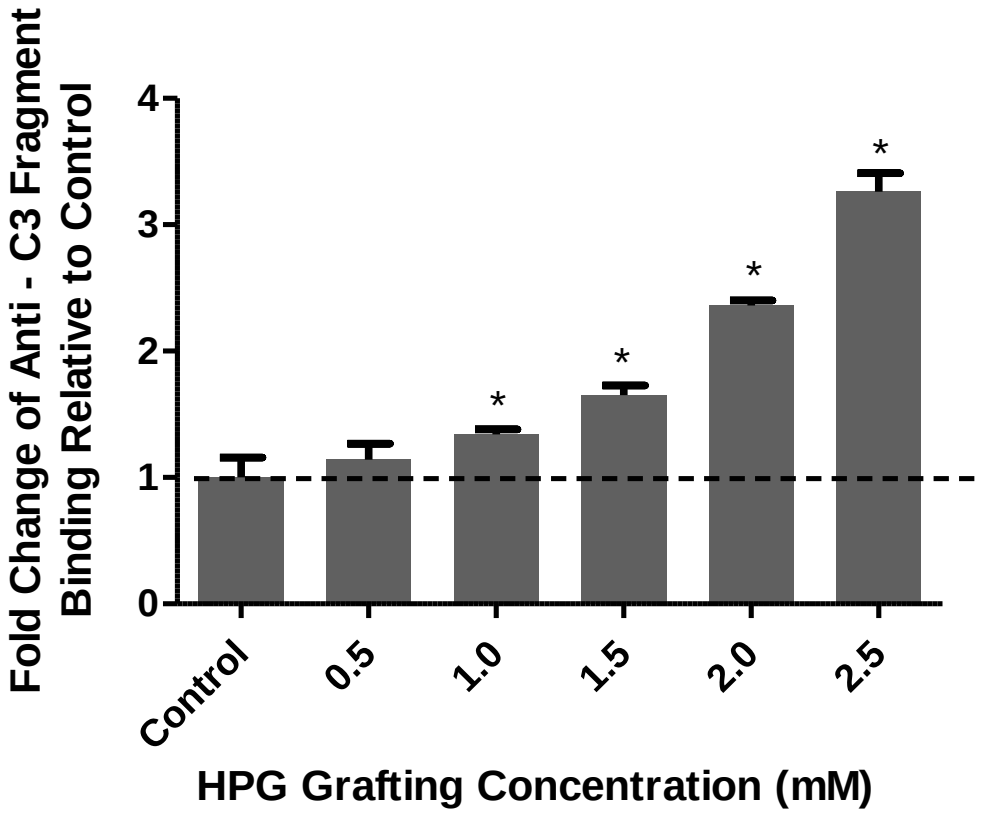


Figure 3.6 Influence of grafting concentration on C3 fragment deposition onto 42 Kda HPG modified RBCs following incubation with human serum as measured by flow cytometry. The cleaved product C3b is an important component of the complement cascade, as its role includes amplifying the overall complement activation. C3b adherence to the surface of modified RBCs with 42 Kda HPG is determined by flow cytometry. The effect of polymer grafting concentration is shown. Isotype control was included for unmodified cells, as well as cells grafted with the highest concentration of polymer. Both showed no non-specific binding.* indicates samples that are statistically different from the control, with $P < 0.05$. Experiments were repeated twice ($N = 2$) with technical duplicates.

3.2.2 Influence of Molecular Weight of HPG on Complement Activation

The number of reactive sites available per molecule of HPG for chemical modification varies depending on the molecular weight of HPG, and a greater number of functional groups may affect the interactions with biological macromolecules or cells. Higher molecular weight HPGs may then be functionalized to have greater biophysical and immunological efficacy, as larger polymers have been reported to camouflage surface antigens more effectively[11,22]. However, a higher molecular weight may have further implications on volume exclusion, cell aggregation, and polymer adsorption. To investigate the influence of molecular weight on complement activation, four different molecular weights of HPG were synthesized and conjugated to RBCs surfaces. Different molecular weight HPGs synthesized include: 9 Kda, 28 Kda, 42 Kda, and 100 Kda. The reactive functional group density on the polymer was fixed at one SS group/20 nm^2 of HPG. This allowed a uniform reactivity to the HPG at different molecular weights. Grafting concentrations of 1.0 mM and 2.0 mM was investigated for each of the four MW HPGs, except for 100 Kda, for which, only 1.0 mM grafting conditions were done as 2.0 mM was not feasible due to solubility issues.

3.2.2.1 Influence of Different Molecular Weight HPG Modified RBCs on Complement Mediated Lysis Following Incubation with Human Serum

The lysis of the HPG modified RBCs in pooled ABO matched serum was measured experimentally. Results are shown in figure 3.7A. At 1.0 mM grafting concentrations, RBCs modified with a MW HPG of 9 Kda and 28 Kda HPG showed minimal lysis (Figure 3.7A). Although not statistically different compared to control RBCs, RBCs grafted with a 42 Kda

HPG showed 1.4 ± 0.5 % lysis. RBCs modified with a 100 Kda HPG showed significantly greater lysis (5.4 ± 1.4%) compared to the control cells (0.02 ± 0.15%).

At 2.0 mM grafting conditions, RBCs modified by either 9 Kda or 28 Kda HPG demonstrate minimal lysis (Figure 3.7B). However, at 2.0 mM compared to 1.0 mM, there is a slight increase in the % lysis. For example, at 1.0 mM, 0.4 ± 0.2% of 28 Kda modified RBC lyse, compared to the 0.6 ± 0.1% at 2.0 mM grafting concentrations. For 42 Kda HPG modified RBCs, there is significantly more lysis at 2.0 mM, which showed that 10.6 ± 2.8% of the cells lysed.

These results suggest that HPGs with MWs 28 Kda and below demonstrate minimal lysis even at high grafting concentrations. Above 28 Kda, the % lysis increases dramatically as the grafting concentration increases.

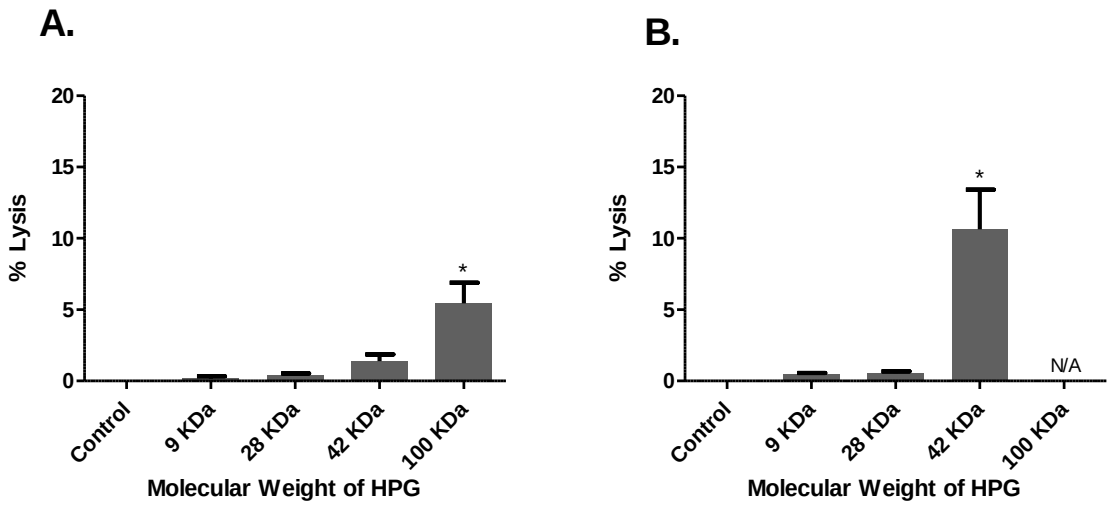


Figure 3.7 Influence of the molecular weight HPG on the lysis of modified RBCs in human serum. The RBC were grafted **at (A)** 1.0 mM and **(B)** 2.0 mM HPG concentrations. Modified RBCs were incubated at 37ºC in pooled serum matched to the RBC donor's blood type. RBCs modified with a 100 KDa HPG at 2.0 mM grafting concentration were not included. * indicates samples that are statistically different from the control, with $P < 0.05$. Experiments were repeated three times (N = 3) with technical duplicates.

3.2.2.2 Influence of Molecular Weight HPG Modified RBCs on Classical and Alternative Pathway of Complement Activation

The classical complement consumption measured using sheep erythrocytes did not increase with molecular weight at 1.0 mM grafting concentration compared to control cells (Figure 3.8A). There was also no increase in the complement consumption for MW HPGs 28 KDa even at 2.0 mM grafting conditions (Figure 3.8B). However, at 2.0 mM grafting concentration, we do observe a significant increase in the total complement activation for 42 KDa HPG modified RBCs, which showed 18.7 ± 5.3% activation compared to the 4.0 ± 3.7% activation by unmodified control RBCs.

The alternative pathway of activation measured by the assay containing rabbit erythrocytes increased for both grafting concentrations (Figure 3.8C and Figure 3.8D). However, consistent with figure 3.8A, at 1.0 mM grafting concentration, RBCs modified with a 28 KDa HPG and below showed no significant increase in the complement activation compared to the 1.3 ± 4.3% activation by unmodified control RBCs (Figure 3.8C). For RBCs modified with a 42 KDa HPG under calcium depleted conditions at 1.0 mM grafting concentrations, there is 18.0 ± 9.5% complement activation. This activation is much greater for 100 KDa modified RBCs, which resulted in the majority of these cells being lysed. Consequently, the human hemoglobin within this sample interferes with the interpretation and the results are not reported. Along with an increase in the grafting concentration, the alternative pathway of activation increased with an increase in molecular weight. Although not significantly different from the 1.3 ± 4.3% activation of the alternative pathway by unmodified control cells, at 2.0 mM grafting concentrations, both 9 KDa and 28 KDa showed similar activation of 17.4 ± 8.6% and 16.3 ± 11.4%, respectively. Since the majority

of cells lysed in the incubation with ABO matched serum, interference from human hemoglobin was high, and the results for the incubation with rabbit erythrocytes are not reported. These results suggest that at 2.0 mM grafting concentration, complement activation is slightly higher compared to 1.0 mM grafting concentrations for lower MW HPGs. Furthermore, given that complement activation was still able to proceed despite the classical and lectin pathways being inhibited by depleting calcium concentrations, this would suggest that any initiation and activation by HPG modified RBCs is achieved through the alternative pathway.

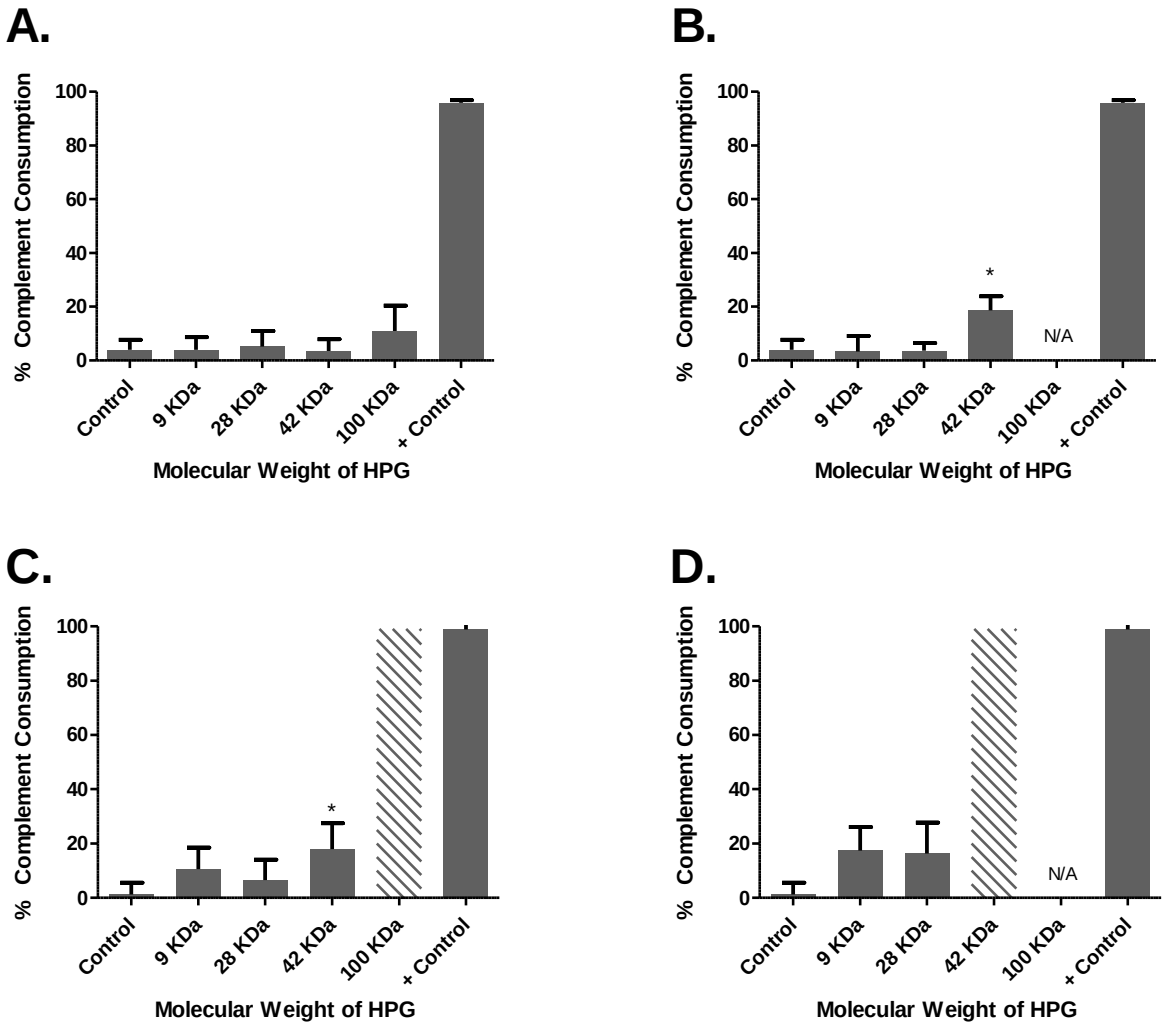


Figure 3.8 Influence of different molecular weight HPG modified RBCs on classical pathway and alternative pathway of complement consumption at 1.0 mM and 2.0 mM grafting concentrations. (A) Influence of molecular weight on classical and total complement activation at 1.0 mM grafting concentration. **(B)** Influence of molecular weight on classical and total complement activation at 2.0 mM grafting concentrations. Due to solubility issues in the grafting of 100 KDa HPG, these samples were not prepared. **(C)** The influence of molecular weight on alternative pathway of complement activation at 1.0 mM grafting concentration. There was significant lysis of 100 KDa HPG modified RBCs when incubated with ABO matched pooled serum. As a result, the human hemoglobin in these samples interfered with the results, and is shown as bars with diagonal patterns indicating 100% complement consumed. **(D)** Influence of molecular weight on the alternative pathway at 2.0 mM grafting concentrations. The results for 42 KDa is shown as bars with diagonal patterns due to significant lysis during incubation of ABO matched serum. Similar to figure 3.8B, preparation of 100 KDa HPG grafted RBCs is not feasible, * indicates samples that are statistically different from the control, with P value < 0.05. Experiments were repeated three times (N = 3) with technical duplicates.

3.2.2.3 SC5b – 9 Terminal Complex Formation on RBCs Modified with Various Molecular Weight HPGs

The influence of molecular weight of HPG grafted onto RBCs on the amounts of terminal complex formation is given in figure 3.9. There is no significant increase in SC5b – 9 deposition on the surface of HPG modified RBCs at 1.0 mM grafting concentrations for all molecular weights. Although there is no statistical difference between the molecular weights that were included in the study, it appears that when molecular weights greater than 42 KDa is grafted, SC5b – 9 formed on cell surfaces increased compared to unmodified cells or cells grafted with low molecular weight HPGs.

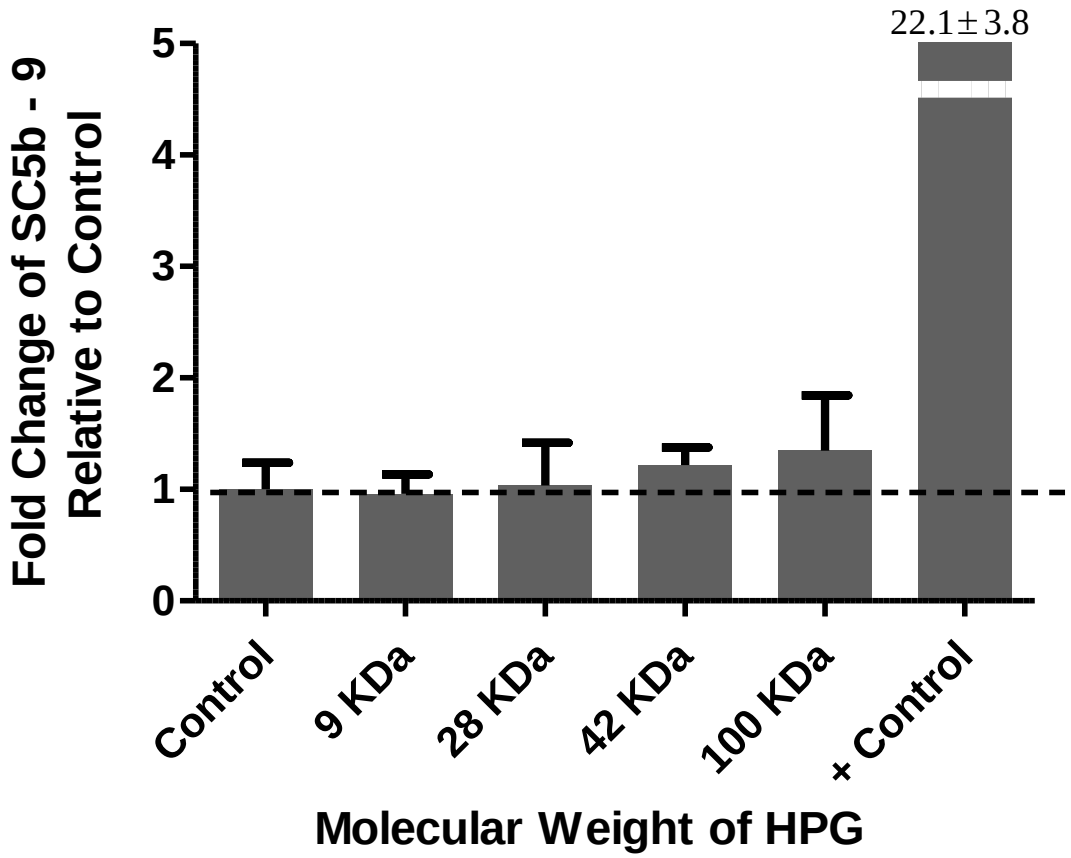


Figure 3.9. Concentration of terminal complex SC5b – 9 generated in human serum following incubation with different molecular weight HPG modified RBCs. All molecular weight HPGs tested were grafted to RBCs at 1.0 mM grafting concentration. The fold change relative to unmodified control RBCs is reported. * indicates samples that are statistically different from the control, with P value < 0.05. Experiments were repeated twice (N = 2) with technical duplicates.

3.2.2.4 C3 Fragment Deposition on RBCs Modified with Various Molecular Weight HPGs

The amount of C3 fragment deposition on RBCs modified with different molecular weight HPGs following incubation with human serum is given in figure 3.10. Relative to control unmodified cells, RBCs grafted with a 9KDa HPG demonstrate comparable levels of bound C3 fragments (0.93 ± 0.04 fold increase) at 1.0 mM grafting concentrations (Figure 3.10A). However, under these same grafting conditions, RBCs modified by HPGs with a molecular weight greater than 28 Kda have statistically more C3 fragments bound to the surfaces. Similar to these findings, at 2.0 mM grafting concentrations, molecular weights greater than 28 Kda show significantly more bound C3 fragments relative to control unmodified RBCs (Figure 3.10B). However at this higher grafting concentration of 2.0 mM, RBCs grafted with molecular weights 28 Kda and higher, show more C3 fragments bound to the surfaces compared to 1.0 mM. There is no difference in the amounts of C3 fragments bound to 9 Kda HPG modified RBC at either of the two grafting concentrations tested.

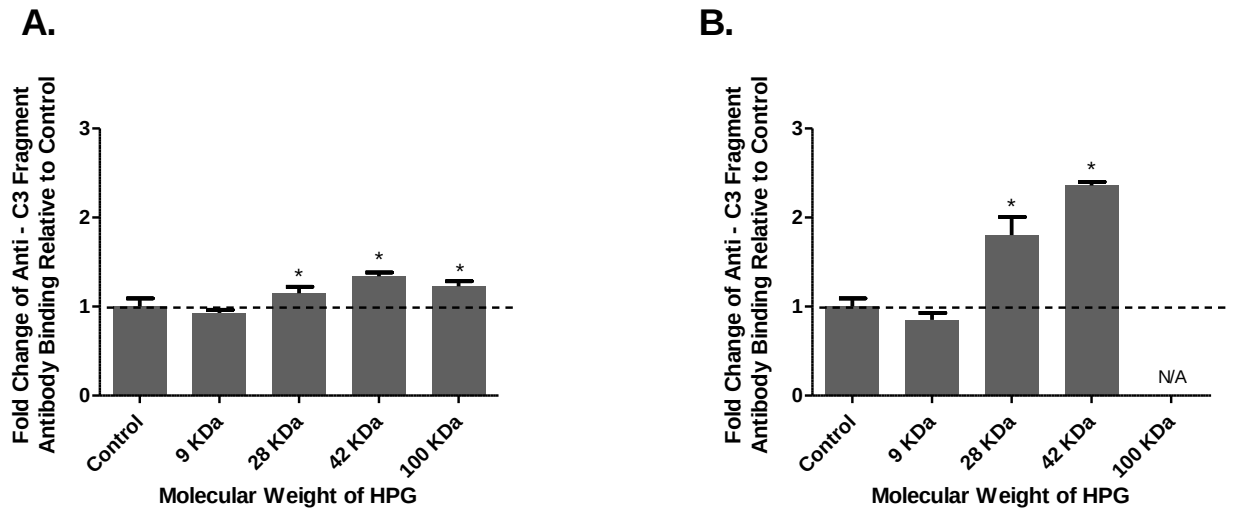


Figure 3.10 Flow cytometric quantification of C3 Fragment deposition on RBCs modified with various molecular weight HPGs following incubation with human serum. RBCs were grafted at **(A)** 1.0 mM and **(B)** 2.0 mM HPG concentrations**.** Isotype control was included for unmodified cells, as well as cells grafted with HPG. Both showed no non-specific binding. The fold change relative to unmodified control RBCs is reported. * indicates samples that are statistically different from the control, with P value < 0.05. Experiments were repeated twice (N = 2) with technical duplicates.

3.2.3 Normalization of Grafting Concentration and Molecular Weight of HPG, and the Influence on Complement Activation

Increasing both the polymer grafting concentration and molecular weight will both increase the amount of HPG present on the RBC membrane. This increase in the amount of HPG is correlated with the amount of exposed surface hydroxyl groups, which may in turn affect the amount of complement activation. In an attempt to normalize the amount of HPG, the results pertaining to the grafting concentration and molecular weight were taken into consideration and analyzed accordingly to the amount of HPG present (figure 3.11). The percentage of lysis, percentage complement consumption, and the amount of anti – C3 fragment antibody binding to the HPG modified RBCs as a function of concentration of SS-HPG used for modification are shown in figure 3.11. The results for percentage of lysis, percentage complement consumption (figure 3.11A, and figure 3.11B, respectively) are very similar and suggest a threshold amount of approximately 1 mg/mL of HPG, as higher amounts of HPG results in significantly higher amounts of lysis and complement activation. Figure 3.11C suggests a relationship between the amount of HPG and the amount of surface bound C3 fragments. With the exception of 100 KDa HPG at 1.0 mM grafting concentration (amount of HPG = 1.35 mg/mL), more HPG resulted in more bound C3 fragments. Despite the greater amount of surface bound C3 fragments when more HPG is present, it is not reflected in the % lysis and % complement consumption, which indicates that all surface bound C3 fragments do not lead to the formation of the convertases and membrane attack complex.

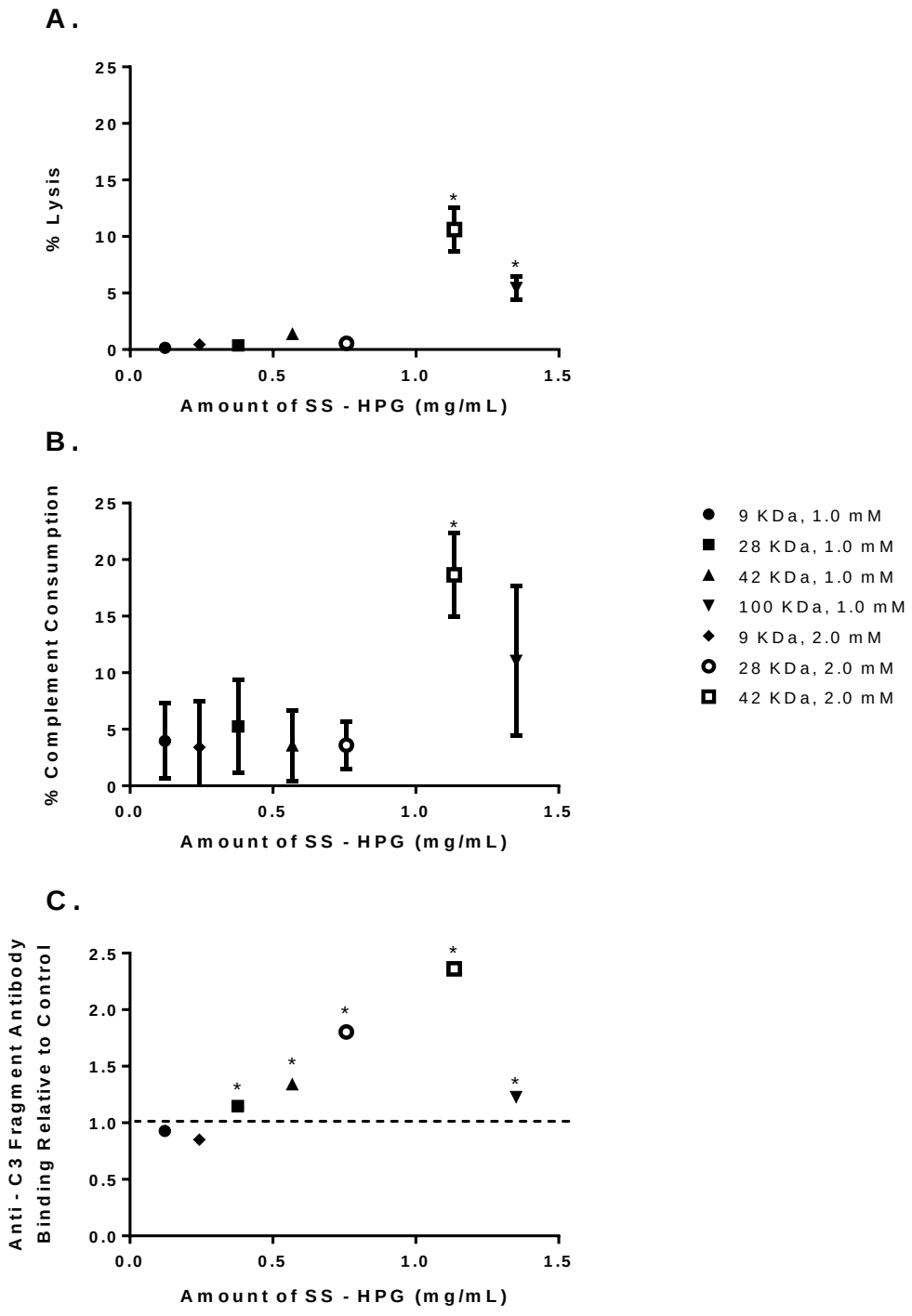


Figure 3.11 Influence of the amounts of HPG on Different Complement Activation Assays. A) % Lysis of modified RBCs following one hour incubation at 37ºC in human serum **B)** % Complement Activation measured by antibody sensitized sheep erythrocytes **C)** Inhibition of Anti - C3 Fragment antibody binding on HPG Modified RBCs. * indicates samples that are statistically different from the control, with P value < 0.05.

3.2.4 Influence of the Number of Reactive Succinimidyl Succinate (SS) Groups on HPGs on Complement Activation

Previous work by our group has shown that the degree of reactive groups (SS) on HPG influences the biophysical and cell surface properties of modified RBCs. A high number of SS groups may result in cross-linking of cell – surface proteins, which would influence the properties of grafted RBCs. We investigated the influence of the number of SS groups on HPG grafted RBCs on the complement activation. For this set of experiments, 42 KDa molecular weight HPG was chosen and functionalized to obtain an average of 4, 7, and 16 groups of SS per HPG molecule, and grafted to RBCs at different grafting concentrations (0.5 mM – 2.5 mM).

3.2.4.1 Influence of SS Groups on HPGs on the % Lysis of Modified RBCs in Human Serum

The % lysis of modified RBCs upon incubation with matched ABO pooled serum at each grafting concentration is quantified. Results are shown in figure 3.12. RBCs modified with 42 KDa HPG functionalized with an average of 4 SS groups per HPG molecule demonstrate no increase in the amount of lysed cells despite significantly increasing the grafting concentration from 0.5 mM to 2.5mM (Figure 3.12). When the degree of functioalization is increased to contain an average of 7 SS groups per HPG, the percent lysis increase with increasing grafting concentration. A statistically significant increase in the lysis was observed when the grafting concentration was greater than 1.5 mM. In the case of higher SS functionalization (16 groups), the lysis was significantly greater than control samples starting from 1.0 mM concentration. The results suggest that the optimization of the

number of reactive functionality is critical to achieve properties similar to the control unmodified RBCs.

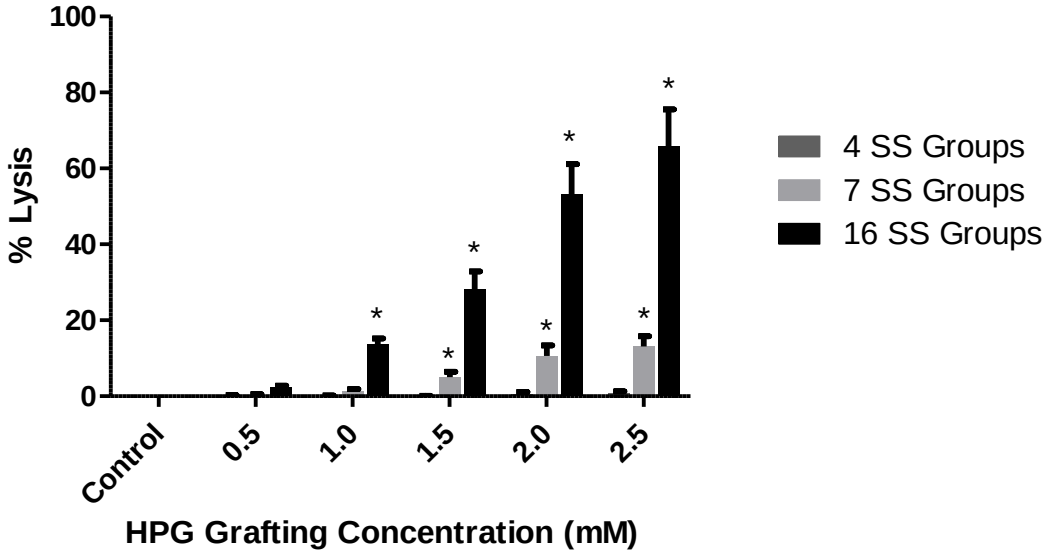


Figure 3.12 Influence of reactive succinimidyl succinate groups (SS) on % lysis of HPG modified RBCs incubated with human serum. HPG (42 KDa) is synthesized to have 4, 7, or 16 SS groups per HPG molecule and grafted to RBCs. The percent lysis of modified RBCs is measured after a 37ºC incubation with pooled ABO matched serum for one hour. * indicates samples that are statistically different from the control, with P value < 0.05. Experiments were repeated three times (N = 3) with technical duplicates.

3.2.4.2 Influence on the Classical Pathway of Complement Activation by the Number of Succinimidyl Succinate Groups on HPG

The degree of complement activation by the modified human RBCs in ABO matched human serum was quantified by the traditional classical pathway activation assay using sensitized sheep erythrocytes. The experiment was performed under conditions where all three complement pathways were functional. The results are given in figure 3.13. Similar to the trends for the % lysis data, there was no increase in the complement activation by HPGs functionalized with 4 SS groups despite increasing the grafting concentration from 0.5 – 2.5 mM (Figure. 3.13). When RBCs were grafted with 42 KDa HPG functionalized with 7 SS groups per molecule, there was no increase in the amount of complement activation at grafting concentrations 1.5 mM and below. For this set of data, the % complement activation for unmodified control cells are 2.7 ± 5.7%, whereas at 1.5 mM, the % activation is at 6.3 ± 5.5%. At a higher grafting concentration of 2.0 mM, there was 18.6 ± 5.3% activation, which is statistically significant compared to the control. When RBCs were grafted at 2.5 mM, the cells lysed significantly after incubation with serum, and this data for complement activation was not reported. Cells modified by a 42 KDa HPG functionalized with 16 SS groups showed similar increase in complement activation at low grafting concentrations. All the grafting concentrations resulted in high complement activation, except for the case of 0.5 mM. At 0.5 mM grafting concentration, the value was similar to control cells.

Complement activation through the alternative pathway by the three different SS group density on HPG grafted RBCs was also tested. However, due to significant lysis of modified cells during the incubation with pooled ABO matched human serum at higher

grafting concentrations, especially for HPG grafted RBCs with higher number of SS groups, the results are not shown.

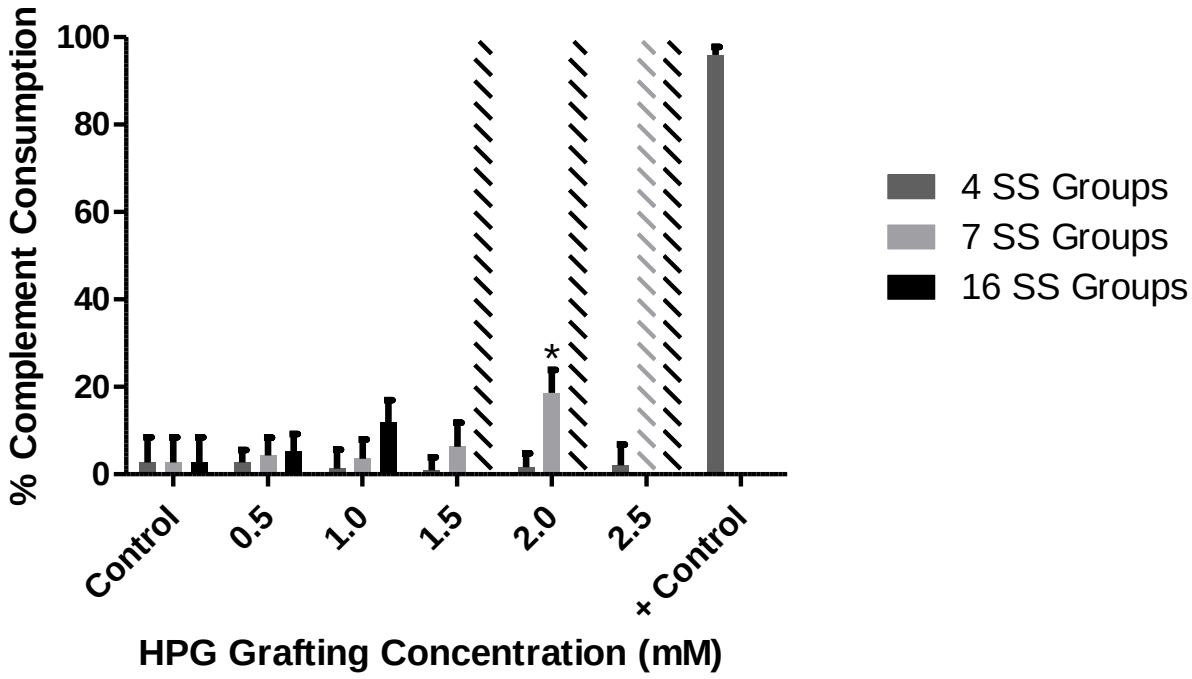


Figure 3.13. Influence of the number of reactive succinimidyl succinate binding groups on HPG on the classical pathway of complement activation. A 42 KDa HPG is synthesized to have 4, 7, or 16 groups per HPG, which was then grafted to RBCs at 0.5 mM – 2.5 mM grafting concentrations. Complement activation by these modified RBCs with different number of groups is assessed by incubating reacted sera with antibody-sensitized sheep erythrocytes. Any residual complement activity in the serum will be activated when incubated with the sheep erythrocytes, resulting in lysis. Since both human and sheep RBCs are used in this assay, and lysis of modified human RBCs may occur, any absorbance from human hemoglobin is accounted for in the results. All samples in which the absorbance by human hemoglobin exceeds 50% of the total hemoglobin are considered to have too much interference with the assay, and not included in the results. * indicates samples that are statistically different from the control, with P value < 0.05. Experiments were repeated three times (N = 3) with technical duplicates.

3.2.4.3 Influence of the Number of Succinimidyl Succinate Groups on Complement Activation Products

HPG modified RBCs with 4, 7, or 16 SS groups were incubated with ABO matched human serum, and the complement activation products C3a, C4a, Bb, and SC5b – 9 were quantifed by ELISA assays. The results are shown in figure 3.14. Although there was a slight increase in the amount of C3a that was generated by 4 SS groups on HPG grafted RBCs at 0.5 mM grafting concentrations (1.3 ± 0.6 fold) compared to unmodified control cells, there was no further change as the grafting concentrations was increased from 0.5 mM to 2.0 mM. When comparing HPG modified RBCs with 7 SS groups to 16 SS groups, the results were similar. For both sets of experiments, as the grafting concentration increased from 0.5 mM to 2.0 mM, there was increasing amounts of C3a generated. The only sample that generated statistically significant C3a levels compared to unmodified control RBCs was by HPG grafted RBCs with 7 SS groups at a grafting concentration of 2.0 mM.

The three polymers with different number of SS groups show similar data for SC5b – 9 compared to the data for C3a. The overall trends suggest that HPG modified RBCs with 4 SS groups did not increase the complement activation at all grafting concentrations tested (Figure 3.14B). As the grafting concentration increased, there was more terminal complex on the surface of HPG modified RBCs with 7 SS groups, with statistically significantly more at grafting concentrations 1.5 mM and greater. For RBCs modified by HPG with 16 SS groups, there was a 2.6 ± 0.5 fold increase even at 0.5 mM, but was not significant until the grafting concentrations reached 1.0 mM. With this high number of SS groups on HPG, very high levels of SC5b – 9 were found at high grafting concentrations, with as much as 8.4 ± 0.9 fold increase relative to the control at 2.0 mM.

The results in figure 3.14C supports the hypothesis that HPG modified RBCs initiate the complement cascade through the alternative pathway, as there was no increase in C4a levels in any of the reacted sera despite increasing the grafting concentration and number of SS groups functionalized on 42 KDa HPG. The observation was completely different in the measurements of the alternative pathway component, Bb (Figure 3.14D). With higher number of SS groups on HPG, there was an increase in the Bb concentration at higher grafting concentrations of HPG. Samples incubated with 7 SS groups show a fold increase of 1.4 ± 0.3 and 1.7 ± 0.3 at 1.5 and 2.0 mM grafting concentrations, respectively. Only at 2.0 mM grafting concentration was the level of Bb statistically significant compared to the unmodified control RBCs. Similar observations was seen for HPG grafted RBCs with 16 SS groups.

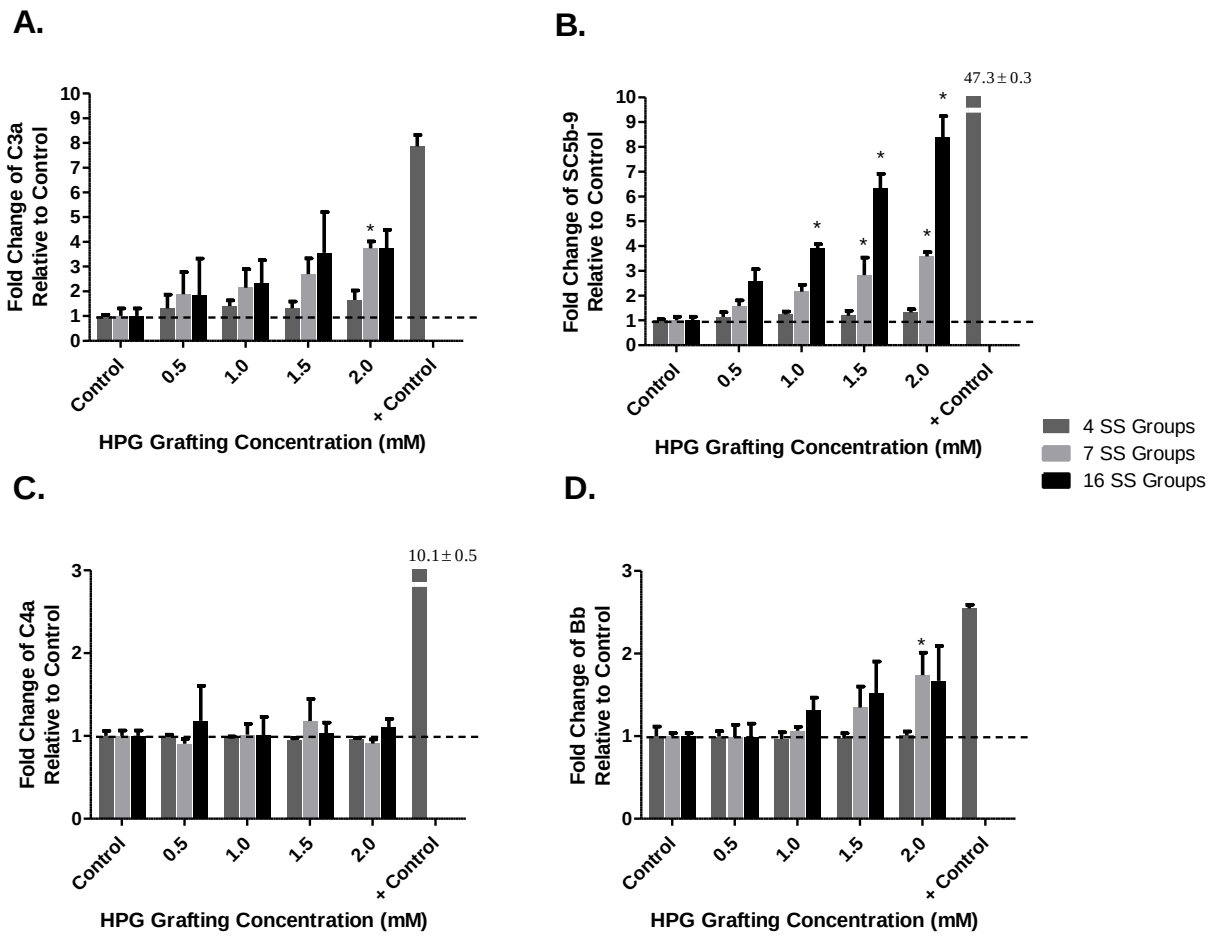


Figure 3.14. Influence of the number of SS groups on HPG modified RBCs on the generation of complement activation products following human serum incubation. The concentrations of C3a, C4a, Bb, and SC5b – 9 in reacted sera is quantified by ELISAs after the HPG modified RBCs were incubated with pooled ABO matched human serum. The results for each of the complement products are shown in different panels **(A)** Complement activation product, C3a **(B)** The terminal pathway/membrane attack complex, SC5b – 9 **(C)** Cleaved product of the classical pathway, C4a **(D)** Proteolytic enzyme of the alternative pathway, Bb. The fold increase relative to unmodified control RBCs is reported.* indicates samples that are statistically different from the control, with P value < 0.05. Experiments were repeated twice (N = 2) with technical duplicates.

3.2.4.4 Influence of the Number of Succinimidyl Succinate Groups on C3 Fragment Deposition on HPG Modified RBCs

Results are shown in figure 3.15. HPG grafting concentration at 2.0 mM was used for the measurements. The amount of C3 deposition increased with an increase in SS groups on HPG. Relative to the control cells, there was a significant increase (17.3 ± 1.2 fold) in the amount of C3 fragments deposited on RBCs modified with 42 KDa HPG containing 16 SS groups (figure 3.15). For RBCs modified with HPGs containing 7 SS groups, there was a 4.1 ± 0.3 fold increase in the levels of C3 fragment binding compared to control cells, but was considerably less compared to fragments that are bound to cells with 16 SS groups functionalized on the HPG. There was no significant change observed in the amounts of C3 fragments bound to HPG grafted RBCs with 4 SS groups.

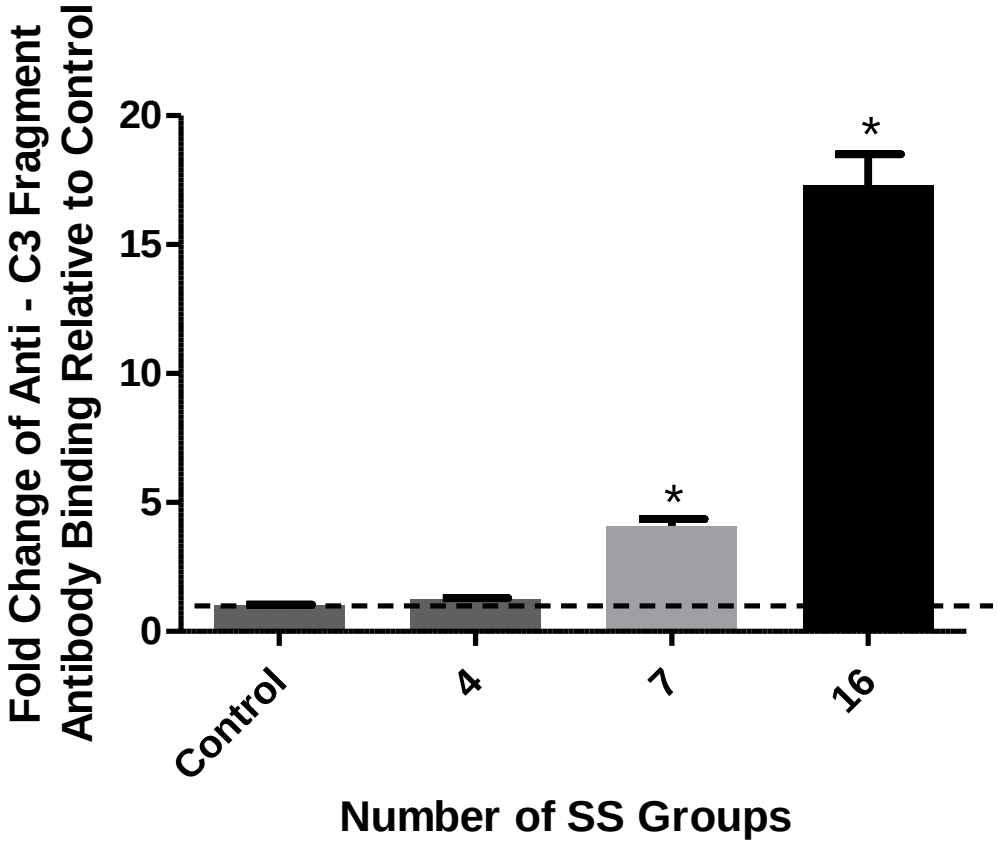


Figure 3.15. Influence of the number of succinimidyl succinate groups on the amount of C3 fragments bound to the surface of 42 KDa HPG modified RBCs following incubation with human serum. The HPG was grafted at a grafting concentration of 2.0 mM. Modified RBCs were incubated with pooled matched serum prior to addition of a FITC labelled antibody reactive to human C3b. Mean FITC intensity is determined by flow cytometry, and the results are reported as fold increase relative to unmodified control RBCs. Isotype controls for unmodified cells, as well as for cells modified with the three different polymers functionalized with varying number of SS groups was included. All of which showed no non-specific binding. * indicates samples that are statistically different from the control, with $P < 0.05$. Experiments were repeated twice ($N = 2$) with technical duplicates.

3.3 Discussion

Optimization of HPG graft properties such as grafting concentration, molecular weight, and number of SS groups on HPG is critical to achieve normal functioning and circulation of modified RBCs. A clinically relevant grafting concentration would minimize complement activation, while maintaining the masking of antigens on the cell surface (see next chapter).

Compared to unmodified control RBCs, both the lysis in serum and SC5b – 9 are observed to have significant differences at 1.5 mM grafting concentration and above for most of the HPGs studied (different molecular weights). At this grafting concentration, there is also significantly more C3 fragment binding to the surfaces of modified cells. Given this, an upper grafting concentration limit of 1.0 mM should be considered for generating viable RBCs. Surface hydroxyl groups have been reported to activate the alternative pathway of complement. Due to self-recognition moieties like sialic acids, which bind factor H in regulating unwanted complement activity, hydroxyl groups on RBCs do not activate the complement system. Since lower molecular weight HPGs have smaller hydrodynamic sizes, and therefore less hydroxyl groups, the influence of molecular weight on complement activation was investigated. We identified a threshold molecular weight and grafting concentration below which complement activation is not induced.

The analysis also gave information regarding the mechanism of complement activation by the surface modified RBCs. Results show that the alternative pathway is active and is influenced by the grafting concentration, molecular weight and number of reactive functionality on the surface of the modified RBCs. The addition of Mg – EGTA to the serum and subsequent measurement of complement activation (Figures 3.3B, 3.8C, and 3.8D),

suggest that complement is being activated via the alternative pathway. Concentrations of the specific complement products C4a and Bb following incubation with of HPG modified RBCs with ABO matched pooled human serum further supports this. There is no increase in the classical pathway product C4a (Figures 3.5A and 3.13C), despite increasing the concentration, molecular weight, and the number of SS groups also supported this hypothesis. Results from the measurements for Bb fragment (Figures 3.5B and 3.13D) also supports this. In addition, the significant increase in C3 fragment binding to RBCs modified with HPG grafted with higher than optimal graft properties and conditions is indicative of amplification through the alternative pathway. Greater amounts of C3b bound to activating surfaces will greatly amplify complement via the alternative pathway. Our conclusion that HPG grafted RBCs activate the alternative pathway of complement under certain graft properties and grafting conditions is also consistent with the literature, as it has been shown that polymers containing a high number of hydroxyl groups, such as polyvinyl alcohol (PVA) and dextran, activate the alternative pathway of complement when bound to surfaces.

Increasing the grafting concentration of HPG increases the amount of surface bound HPG to RBC membranes as there are more HPG molecules available for successful covalent attachment. Alternatively, increasing the molecular weight of HPG does not increase the amount of surface bound HPG, but instead the size and possible immune - modulated zone of inhibition is increased as a large HPG molecule would be able to cover more surface area on the RBC membrane. Due to increased amounts of HPG located on RBC surfaces at high grafting concentrations and high molecular weight HPG, the amount of complement activation is also increased. However, based on osmotic fragility results (figures 2.4 and 2.5), which suggests that molecular weight affects membrane stability more compared to

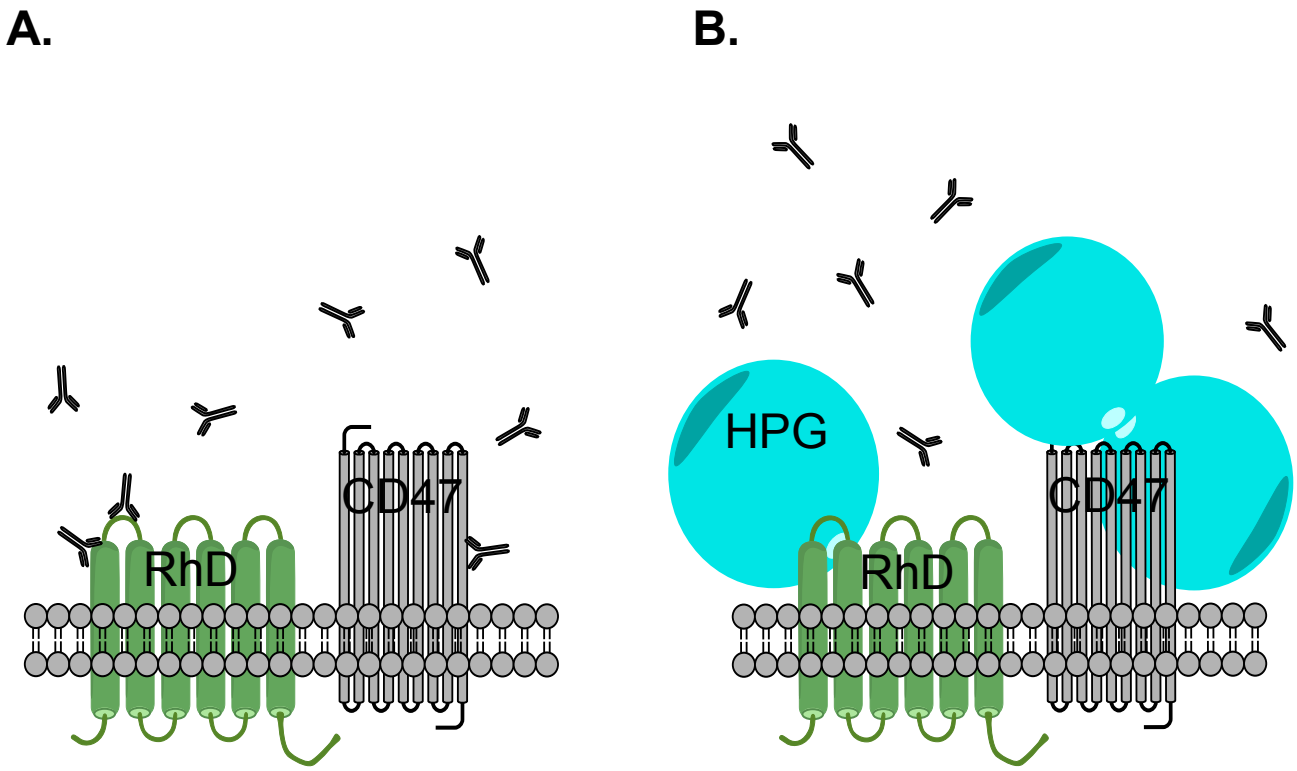
grafting concentration, using a relatively low molecular weight HPG at higher grafting concentrations may be more advantageous in minimizing unwanted complement activation and changes to the RBC membrane. Further optimization between grafting concentration and molecular weight is required.

Surface binding efficiency of HPG molecules is dependent on the degree of SS functionalization of the hydroxyl groups on the HPG structure. With more SS groups, more HPG may be able to bind to RBC membranes effectively compared to HPGs functionalized to have lower number of SS groups. Aqueous two – phase experiments suggest that HPG with an average of 4 SS groups are binding to RBC surfaces, however further experiments should be done to ensure successful binding for HPG functionalized to contain low number of SS groups. Our results from the complement system analysis indicate the need for the optimization of binding of HPG for the normal behaviour of the modified cells. This supported by the fact the HPG molecules (42 KDa) modified 16 SS groups resulted in significant complement activation and the activation level decreased with a reduction in the number of SS groups.

Chapter 4 Inhibition of Antibody Binding to Clinically Relevant Antigens[1]

The previous chapter evaluated the issue of biocompatibility at the level of complement fixation on modified cells. In this chapter, we describe the ability of HPGs to mask the inherent immunogenicity of the D antigen, which is found on the surface of red blood cells. The highly immunogenic D antigen makes it an ideal candidate for investigation into the efficacy of HPGs in preventing recognition by specific antibodies. However, there are other membrane proteins such as CD47 that are important for proper circulation of cells as well as immune response. Given the clinical importance of both the RhD antigen and the role of CD47 in the normal circulation of cells, these two have been chosen to evaluate the extent of camouflaging by HPG grafting.

[1] This chapter is based on work conducted in Dr. Kizhakkedathu Laboratory by Vincent Leung.



*Schematic is not drawn to scale.

Figure 4.1 Schematic Diagram of Antibody Recognition of RhD and CD47 antigens. A. Antigenic recognition of RhD by allo-antibodies. B. Steric hinderance of RhD and CD47 recognition and binding to foreign RhD and CD47 antigens.

4.1 Methods and Materials

4.1.1 Analysis of RhD Camouflage

Properties such as grafting concentration, molecular weight, and number of SS groups on HPG is investigated for its influence on the camouflaging of RhD on modified RBCs. These measurements are performed using flow cytometry. The inhibition of antibody binding to RhD epitopes on RBCs that may remain exposed on the surface of HPG modified cells was measured. Please refer to chapter 2 for the grafting of HPG to RBC protocols. In detecting specific epitopic regions of RhD, a FITC human IgG3 kappa light chain anti-RhD monoclonal antibody (International Blood Group Reference Laboratory, Bristol, UK) was used[94]. Fresh whole blood was collected into citrated vacutainer tubes from O+ blood donors, and the serum and buffy coat were removed following centrifugation at 1000 xg for 10 minutes. The RBCs were then washed with PBS (pH 8.0) three times to remove any residual white blood cells and serum proteins that may interfere with the assay and interpretation of the assay results. To investigate the influence of grafting concentration on preventing antibody binding to RhD, 42 KDa HPG was chosen and grafted at 0.5 mM incremental increases ranging from 0.5 mM to 2.5 mM grafting concentrations. To investigate the influence of molecular weight, 9 KDa, 28 KDa, 42 KDa, and 100 KDa HPG at 1.0 mM and 2.0 mM grafting concentrations were used. The influence by the number of SS groups (4, 7, or 16) at constant molecular weight of HPG was also investigated.

During the HPG grafting process, the RBCs were adjusted to have a final concentration of 2.5×10^{12} cells/L. After one hour at room temperature on an orbital shaker, unreacted polymer was removed through three successive washes using 1% bovine serum albumin (BSA) dissolved in PBS (pH 8.0) at centrifugation speeds of 700 xg for 3 minutes.

The RBC concentration was readjusted to 2.5 x 10^{11} cells/L using the same buffer, followed by transferring 45 µL to a flow cytometer tube in duplicates. A final antibody concentration of 10 µg/mL was added to each of the tubes and gently mixed and incubated at 37°C for 30 minutes. After incubation, 1 mL of 1% BSA in PBS buffer was added prior to analysis of the mean FITC intensity using BD FACS Canto II flow cytometer (Beckman Coulter, USA) set at 400 PMT. 10 000 events were acquired and the results were presented as the means of duplicate measurements.

4.1.2 Analysis of CD47 Camouflage

The protocol for evaluating the masking of CD47 was similar to the protocols used for quantifying the camouflage of RhD antigen. The HPG graft properties were similar to those used for RhD antigen. The accessibility of CD47 on HPG modified RBCs was detected using a PE labelled anti-human mouse IgG kappa chain monoclonal antibody (BD Pharminogen, Ontario, Canada). This antibody reacts with a 42 – 52 KDa N-linked glycan protein of CD47[43,95].

4.2 Results

4.2.1 RhD Camouflage

Results from RhD antibody binding on the modified cell surface is shown in figure 4.2. The antibody binding to RhD antigen decreased with increasing grafting concentration for 42 KDa HPG. There was significantly less RhD antigen recognized by the antibodies starting at 1.0 mM grafting concentration relative to the unmodified control cells (Figure 4.2). This decreasing trend continues as the grafting concentration increases up to 2.0 mM (fold change of 0.58 ± 0.4), above which the decrease was minimal.

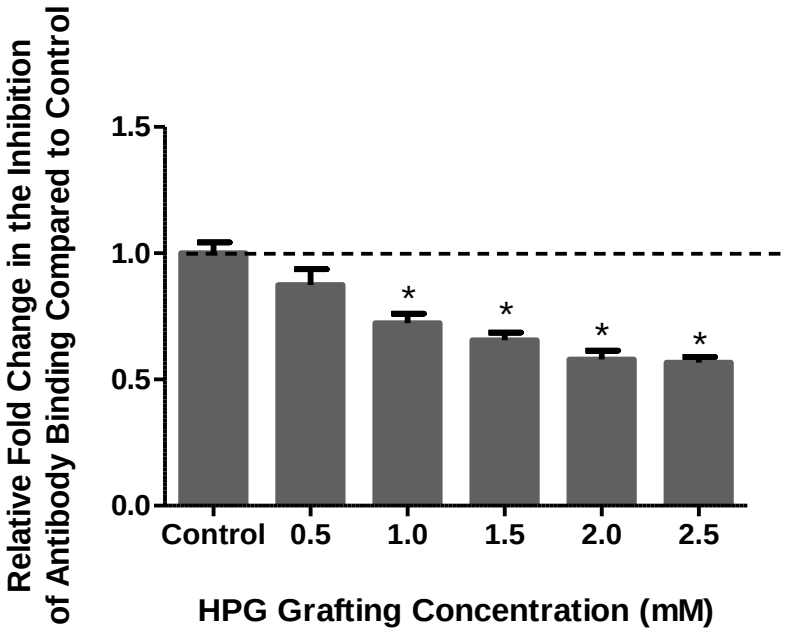


Figure 4.2. Influence of grafting concentration on anti – RhD antibody binding to 42 KDa HPG modified RBCs as measured by flow cytometry. The 42 KDa HPG used was functionalized to have an average of 7 SS groups. Mean FITC intensity was measured. * indicates samples that are statistically different from the control, with $P < 0.05$. Experiments were repeated twice ($N = 2$) with technical duplicates.

To study the influence of molecular weight of grafted HPG on the camouflaging of RhD, four different molecular weight HPGs were chosen, including 9 KDa, 28 KDa, 42 KDa, and 100 KDa. Two different grafting concentrations (1.0 mM and 2.0 mM) were studied for each of the molecular weight HPGs, except for 100 KDa, for which, only 1.0 mM was measured due to solubility issues. Results are shown in figure 4.3. There was less antibody bound to HPG grafted RBC surfaces as the molecular weight increased from 9 KDa to 100 KDa (Figure 4.3A). The trends in the inhibition of antibody binding at 1.0 mM grafting concentration was similar to the trends observed for the same molecular weight HPGs grafted at 2.0 mM (Figure 4.3B). However, compared to control RBCs, at 2.0 mM grafting concentration, the values were statistically different for the molecular weight HPGs

that were tested. Isotype controls were included in these experiments, which demonstrated no non-specific binding to either the RBCs or RBCs modified with HPGs.

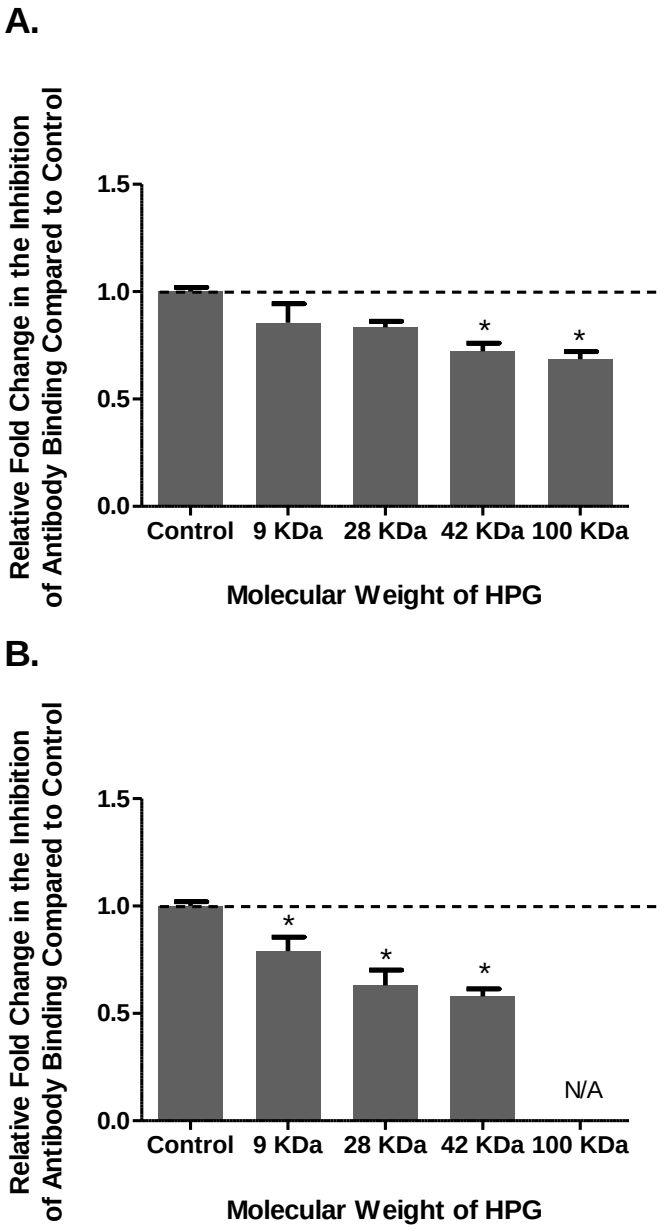


Figure 4.3. Influence of molecular weight on the inhibition of anti – RhD antibody binding to RhD antigens on HPG modified RBCs. HPG grafting concentrations include **A.** 1.0 mM and **B.** 2.0 mM, as measured by flow cytometry. 100 KDa HPG was not measured at 2.0 mM grafting concentration due to solubility issues. The mean FITC intensity was measured. * indicates samples that are statistically different from the control, with $P < 0.05$. Experiments were repeated twice ($N = 2$) with technical duplicates.

To determine the influence of the number of SS groups on the inhibition of anti - RhD antibody binding, 42 KDa HPG with 4, 7, or 16 SS groups were used. Each of the three polymers were then grafted onto RBCs at 1.0 mM and 2.0 mM grafting concentrations. Results are shown in figure 4.4. For a 42 KDa HPG, 4 groups of SS do not yield statistically different results relative to control cells at 1.0 mM, whereas increasing the binding properties to 7 SS groups, there was a significant decrease in antibody binding to RBC surface (0.72 ± 0.04 fold) (Figure 4.4A). This decrease was even more pronounced when a HPG with 16 SS groups was used (0.52 ± 0.02 fold). Similar decreasing trends in the amount of antibodies detected for cells grafted at 2.0 mM was observed as the number of SS groups on HPG increased. However, at this higher grafting concentration, each of the three polymers showed greater inhibition of antibody binding compared to 1.0 mM compared to control unmodified RBCs.

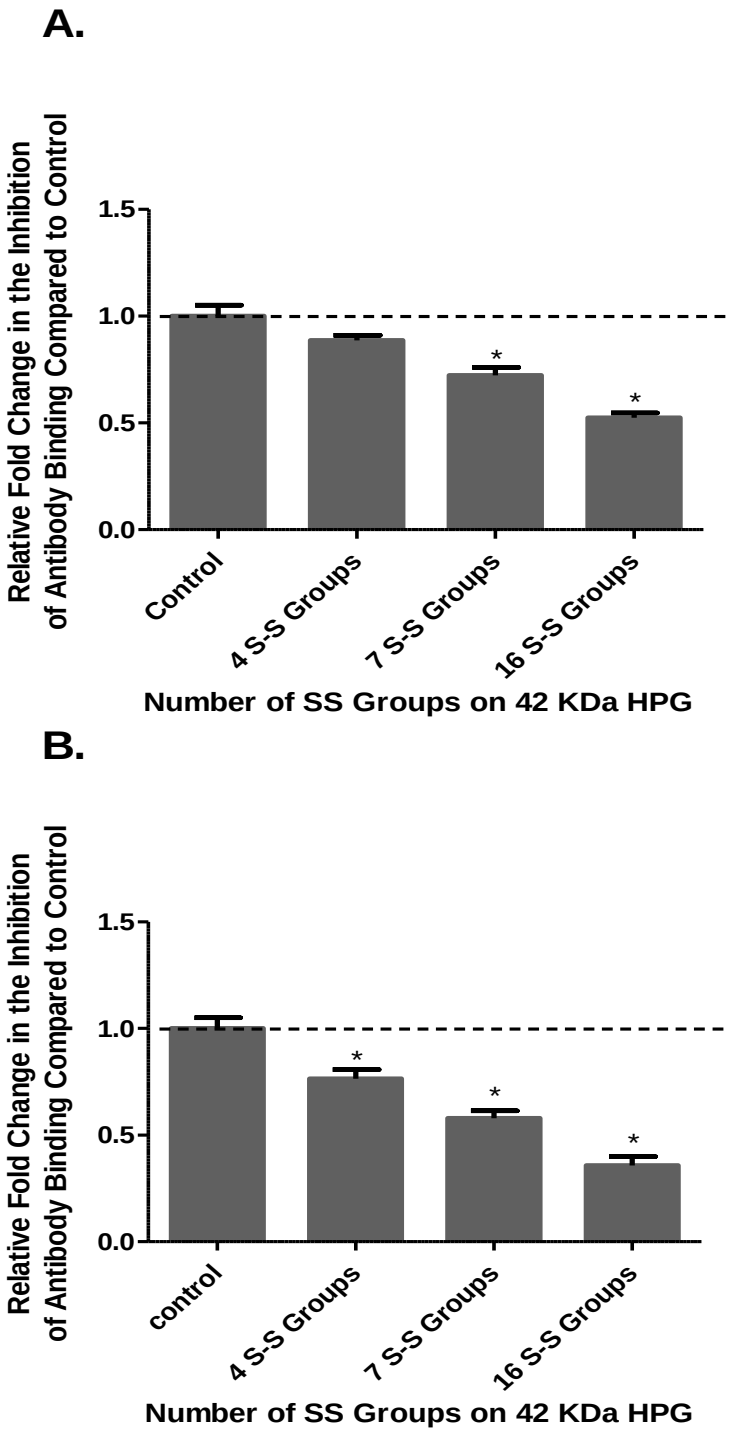


Figure 4.4. Influence of SS Groups on 42 KDa HPG grafted RBCs on the inhibition of anti – RhD antibody binding to RhD antigen. HPG grafting concentrations include **A.** 1.0 mM and **B.** 2.0 mM. Analysis was measured using flow cytometry. Mean FITC intensity was measured. * indicates samples that are statistically different from the control, with $P < 0.05$. Experiments were repeated twice (N = 2) with technical duplicates.

4.2.2 CD47 Camouflage

CD47 is an integrin-associated protein that is expressed on a wide range of different cells[43,45]. This includes hematopoietic cells such as platelets, leukocytes, and erythrocytes, but also other cell types including endothelial cells, epithelial cells, fibroblasts, and even tumor cells. Given the wide expression, it has a fundamental role in chemotactic and adhesive interactions between leukocytes and endothelial cells[44,45]. In addition, it is documented that it functions as a signal transducer as well as having immune functions in blocking phagocytosis by leukocytes found in peripheral blood[42].

The results for the inhibition of antibody binding to CD47 on HPG modified RBCs are shown in figure 4.5. Masking of CD47 was found to decrease significantly upon HPG grafting to RBC membranes, even at low grafting concentrations of 0.5 mM. At this grafting concentration for a 42 KDa HPG, the levels of antibodies binding to CD47 was 0.62 ± 0.13 fold (Figure 4.5) compared to the unmodified cells. Camouflage of CD47 increased with increasing grafting concentration, with 0.22 ± 0.00 fold difference in the amount of bound antibodies at 2.5 mM compared to unmodified cells.

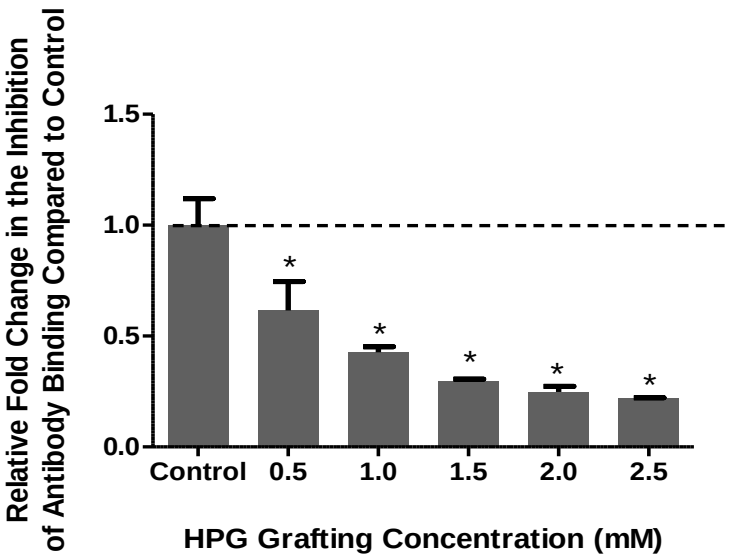


Figure 4.5. Influence of grafting concentration on the inhibition of anti - CD47 antibody binding to HPG Modified RBCs as measured by flow cytometry. The molecular weight HPG used was 42 KDa with 7 SS groups. The mean PE intensity was measured. * indicates samples that are statistically different from the control, with $P < 0.05$. Experiments were repeated twice (N = 2) with technical duplicates.

Compared to RhD, different trends were observed for the influence of molecular weight HPGs on CD47 camouflage (Figure 4.6A and 4.6B). However, similar to low grafting concentrations, a low molecular weight of 9 KDa was shown to have significantly less antibodies binding to CD47 on the surface of RBCs compared to control cells. At 1.0 mM grafting concentration, 9 KDa HPG modified RBCs resulted in CD47 levels of 0.79 ± 0.06 fold difference relative to control unmodified cells. The camouflage was increased when a 28 KDa HPG was used, which gave 0.46 ± 0.04 fold difference in anti – CD47 binding compared to control cells. Interestingly, the difference between 28 KDa and 42 KDa was similar, with only 0.43 ± 0.03 fold. At 1.0 mM grafting concentration, high molecular weight gave higher masking of CD47, presumably due to the higher hydrodynamic size. A similar trend was observed at 2.0 mM grafting concentration for the 3 molecular weights

that were tested (Figure 4.6B). However, greater protection of CD47 was observed at this higher grafting concentration.

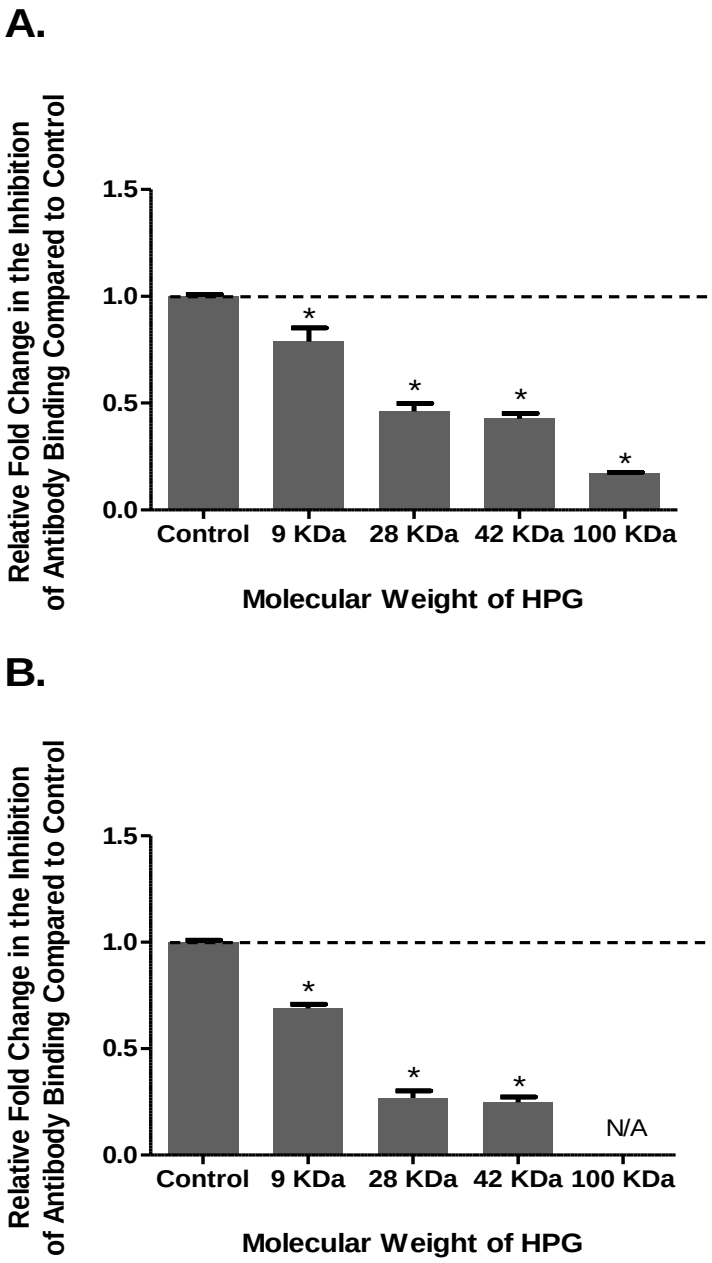


Figure 4.6. Inhibition of anti - CD47 antibody binding by different molecular weight HPGs modified RBCs as measured by flow cytometry. The grafting concentrations used were **A.** 1.0 mM and **B.** 2.0 mM. 100 KDa HPG was not measured at 2.0 mM grafting concentration due to solubility issues. The mean PE intensity was measured. * indicates samples that are statistically different from the control, with $P < 0.05$. $N = 2$.

The influence of the number of reactive binding SS groups on HPG on CD47 camouflage is shown in figure 4.7. At a low degree of functionalization, there was a minimal decrease in the amount of antibodies bound to CD47 on cell surface, whereas a 42 KDa HPG functionalized to have an average of 7 SS groups demonstrated statistically significant results with a 0.43 ± 0.03 fold difference compared to the control (Figure 4.7A). The efficiency of CD47 masking increased with an increase in the number of SS groups on the HPG. When the grafting concentration was increased to 2.0 mM, the trends were similar to 1.0 mM. However, at 2.0 mM grafting concentrations, RBCs modified with HPGs containing a low degree of functionalization showed less masking of CD47 (Figure 4.7B).

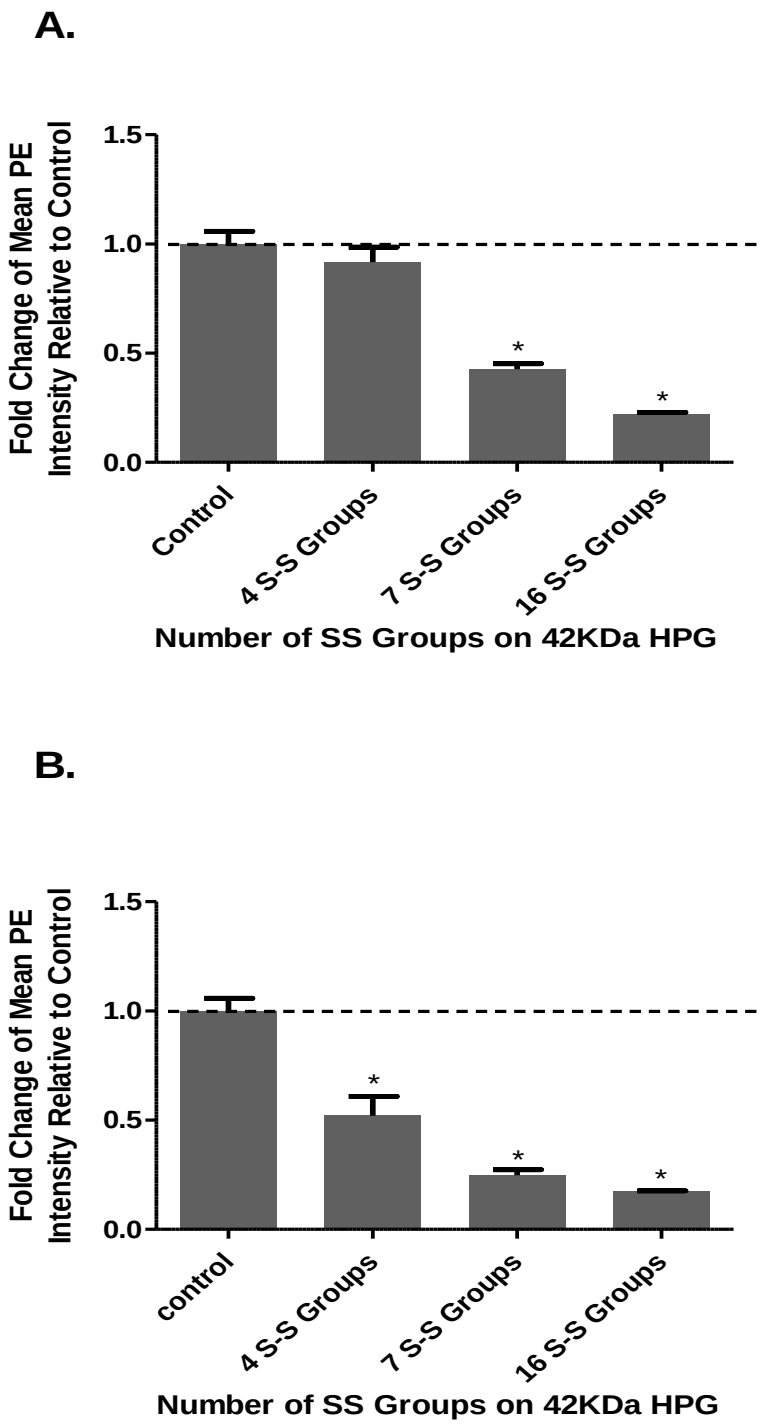


Figure 4.7. Inhibition of anti - CD47 antibody binding to RBCs modified by 42 KDa HPG with different number of succinimidyl succinate groups (SS). RBCs grafted with HPG concentrations of **A.** 1.0 mM and **B.** 2.0 mM were measured using flow cytometry. The mean PE intensity was measured. * indicates samples that are statistically different from the control, with $P < 0.05$. Experiments were repeated twice ($N = 2$) with technical duplicates.

4.3 Discussion

A broad knowledge base pertaining to the nature of blood group antigens has been well established, which allowed for greater transfusion safety[6,8,10,35]. Alloantibodies or naturally occurring autoantibodies that recognize and bind to specific blood group antigens may elicit an adverse reaction, thereby jeopardizing the health of the RBC recipient. This is particularly problematic for individuals requiring chronic RBC transfusions for the treatment of conditions such as sickle cell anemia and thalassemia[4]. Reducing the immunogenicity of major antigens, such as RhD, as well as other minor blood group antigens of clinical significance will increase the transfusion safety. In attenuating the risks caused by various blood group antigens, covalent grafting of polymers to biological surfaces has been shown to reduce the antigenicity of human RBCs and prevent immunological recognition[12,21,71]. In our study, this has been achieved by camouflaging the blood group antigens by the modification of RBC surfaces using HPG. The influence of graft properties/conditions such as grafting concentration, molecular weight, and number of SS groups on HPG has been investigated to determine optimal conditions to achieve immune protection.

Higher grafting concentrations of HPG on RBC surfaces result in better camouflaging of both RhD and CD47. However, CD47 is camouflaged more efficiently compared to RhD at the same grafting concentration of HPG. This may be due to the covalent attachment of HPG to external lysine residues, which the RhD antigen contains low amounts of[40,94]. In addition, the different size of RhD and CD47 may be the reason for this difference. The better camouflaging of CD47 is undesirable given its important cellular functions. Of particular interest, CD47 is a self-marker, and studies have shown that cells that do not express CD47 are cleared by circulating phagocytic cells[42,45]. Hence, a decrease

in CD47 availability following HPG grafting may be a limitation as higher grafting concentrations may be required to achieve immunoprotective levels for highly antigenic blood group antigens. Thus, optimization of grafting concentrations and molecular weight/size of the HPG is paramount to achieve greater immunoprotection and better survival of the RBCs.

At similar grafting concentrations, the influence of 42 KDa and 28 KDa HPG grafted RBCs show comparable CD47 camouflaging. The reason for this may be due to the relatively large size of CD47 (approximately 50 KDa). Perhaps both 28 KDa and 42 KDa HPG is not sufficiently large enough to mask the entire protein, leaving key epitopes exposed where antibodies are able to recognize and bind to CD47. Since the undesired camouflaging of CD47 is similar at the same grafting concentration, it is more strategic to maximize the camouflaging of the immunogenic RhD by using the higher molecular weight 42 KDa HPG.

The camouflaging of RhD antigens by a high molecular weight HPG (100 KDa) relative to a lower molecular weight HPG of 42 KDa is comparable, suggesting that RhD is not camouflaged as efficiently despite the higher molecular weight HPG. This was not expected since the layer of immunoprotection surrounding HPG grafted RBCs would be much larger, thereby able to camouflage most antigens located within this zone. Under these circumstances, antibodies that would otherwise recognize and bind to immunogenic antigens would be sterically hindered. The actual findings may be that with a 100 KDa HPG, the binding efficiency might have been compromised given the globular structure of HPG. Exclusion of the large sized HPGs makes it more difficult to attach on RBC surfaces. With a limited number of HPGs able to successfully attach, it may result in poor masking of the

antigenic epitopes. With smaller molecular weight HPGs, although the binding area available on the RBC membrane remains the same, the smaller size facilitates easier accessibility to the lysine residues on the RBCs resulting in greater efficiency of the grafting.

Increasing the number of SS groups per HPG molecule provides better camouflaging of the RhD antigen. The better protection of antigens may be due to better graft binding efficiencies with more SS groups. However, as previously discussed, complement activation associated with increase in SS groups may hinder the use of polymers that have a high degree of SS functionalization.

Chapter 5 Conclusions and Future Directions

Hyperbranched polyglycerols containing different numbers of succinimidyl succinate functional groups and molecular weights were synthesized and characterized. The reactive HPG was conjugated to RBCs via the primary amines located on membrane proteins to form stable covalent linkages. The *in vitro* measurements for cell membrane fragility and other cell properties suggested that the modification results in normal function of the cells. In general, the effect of HPG grafting was found to increase with an increase in concentration, molecular weight, and the number of reactive succinimidyl succinate groups functionalized on HPG, which confirm the results of other studies[71,73].

Furthermore, this thesis has provided new insights into complement activation by HPG when they are grafted onto red blood cell membranes. Based on our results, we can conclude that RBCs modified by HPG activate the complement system via the alternative pathway at certain grafting concentrations and molecular weights. Due to the observed increase in C3 fragments binding to HPG modified RBCs relative to unmodified control cells, we speculate that the mechanism of complement activation begins with the binding of C3b to exposed hydroxyl groups on HPG. This initiates complement activation via the alternative pathway, and facilitates further amplification as the C3 convertase, C3bBb, is formed on the surface of RBCs[74,91]. High grafting concentrations, high molecular weights, and HPGs with high number of succinimidyl succinate groups all activated the complement system, resulting in the lysis of the HPG modified RBCs. There needs to be a balance between grafting concentration and molecular weight in order to avoid complement activation and the subsequent clearance of modified RBCs, while striving for maximal camouflaging of antigens. Attachment of low molecular weight HPGs at low grafting

concentration did not activate complement, but the degree of camouflaging of antigens was not significant. High molecular weight HPGs and high grafting concentrations activate complement, but antigens are very well camouflaged on the cells that have not been lysed. Thus, the selection of molecular weight, number of reactive groups on the polymer, and graft concentrations is critical to achieve the normal function and survival of the cells while providing sufficient camouflage of major and minor antigens.

5.1 Future Directions

With the knowledge gained from this thesis, we may be able to further devise novel strategies and methods for the development of safe, non – immune reactive red blood cells suitable for transfusion. Modulation or prevention of undesired complement activation to enable the use of higher molecular weight HPGs at high grafting concentrations without activating the alternative pathway of complement could be a suitable next step in this project. A promising study by Wu et al. (2011)[61] describes a method to conjugate smaller molecular weight peptides that immobilize physiological regulators of complement activation to attenuate unwanted complement activation. One such peptide binds naturally occurring complement regulators found in serum, factor H, which has a key role in regulating the alternative pathway of complement activation[30,61]. Given the highly derivatizable hydroxyl groups of HPG, a surface with autoregulatory activity upon exposure to blood may be developed by conjugating these peptides with high affinity for factor H[61,96].

Alternatively, the chemical structure and functional groups on polymers may play a fundamental role in determining the therapeutic potential or toxic characteristics[18]. For example, polyanions prevent complement activation by inactivating C1 or C2 of the classical pathway[18,97]. Inhibition of the alternative pathway by targeting factor B may be

possible by incorporating negatively charged pyran copolymers. Furthermore, other anionic groups such as carboxylate groups and sulfonated polyethylene glycol surfaces have also been demonstrated to inhibit complement activation and improve biocompatibility[98]. Due to the chemical similarities of these polyanions to sialic acids and glycosaminoglycans on human cells and tissues, it is possible that the regulation and prevention of complement fixation and amplification on host cells can be mimicked[97,99,100]. Novel strategies may exploit these inhibitory effects of polyanionic structures by synthesizing HPG to contain polyanionic groups that best prevents complement activation.

By preventing unwanted complement activation by HPG grafted RBCs, the safety of this cellular therapeutic approach can be improved, while allowing for global camouflaging of immunologic antigens. Successful outcomes will have implications not only in transfusion, but also in transplantation medicine.

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