MODIFICATION OF RED CELL ANTIGENIC CHARACTERISTICS VIA COVALENT MODIFICATION WITH BRANCHED POLY (ETHYLENE GLYCOL)

by

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B.Sc., McGill University, 2004

A THESIS SUMITTED IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF

MASTER OF SCIENCE

in

THE FACULTY OF GRADUATE STUDIES

(Pathology and Laboratory Medicine)

THE UNIVERSITY OF BRITISH COLUMBIA

December 2006

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Abstract

Background: Red Blood Cell (RBC) transfusions are associated with the risk of rejection and sensitization for the recipient. Covalent modification of human RBC using poly (ethylene glycol)) (PEG) is currently being investigated as a method to modify the antigenic characteristics of RBC in order to develop an antigenically silent RBC transfusion product. The focus of this study is to determine whether a branched PEG, 4PEGss [MW 10,000] can concurrently camouflage and stabilize the RBC membrane, giving the red cell higher osmotic resistance.

Study Design and Methods: Human blood was collected from consenting donors into EDTA tubes. RBC were separated, counted, washed and incubated with 4PEGss stock solution for up to 120 minutes at room temperature (RT). Agglutination assays and flow cytometry were used to determine polymer–dependent antigen masking while scanning electron microscopy (SEM), erythrocyte sedimentation rate (ESR) assays, osmotic fragility assays, hemolysis measurements and generation of hemoglobin oxidation species were used to measure the impact of 4PEGss treatment on RBC function.

Results: Agglutination assays and flow cytometry showed that 4PEGss treatment significantly camouflaged RhD and A and B antigens in a dose–dependent manner. However, SEM and ESR assays indicated 4PEGss treatment may cause cell aggregate formation. Osmotic fragility assays and hemolysis measurements showed a 4PEGss–mediated increase of hemolysis.

Conclusions: At all concentrations investigated, 4PEGss modification yielded antigenically camouflaged (both ABO and RhD) yet less stable RBC compared to non–treated controls.
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Acknowledgements

Many people have contributed to my graduate education. Thank you Dr. M. Gyongyossy-Issa and Dr. D. Devine for supervising this project and for being the best teachers and mentors a graduate student could hope to have. Thank you to the Supervisory Committee: Dr. C. Fyfe, Dr. M. Scott and Dr.G. Mauk for contributing your knowledge and expertise to this project. A very special thank you to Dr. C. Carter, also a member of the supervisory committee, who taught me truly inspiring lessons in science, history and life. Thank you to Dr. E. Levin, Dr. E. Maurer, Dr. K. Serrano, Dr. E. Madsen, J. Thon, B. Culibrk, I. Constantinescu, C. Pittendreigh and A. Labrie for all of your wisdom and kindness. Thank you to my dear family and friends. I never could have come this far without all of your support and encouragement. Finally, thank you to Canadian Blood Services Research and Development, for funding this work.
1. Introduction

1.1 Blood Transfusions in Clinical Medicine

Blood transfusions are required in a number of clinical circumstances, for example, emergency traumas, surgery and cancer treatment. Blood transfusions are also used to treat acute episodes of underlying chronic conditions such as sickle cell, thalassemia and chronic anemia. In these cases the patients may receive numerous blood transfusions over time, and are referred to as chronically transfused. The major complication of transfusion therapy is host immune-mediated rejection of transfused blood products, a complication which occurs more frequently in the chronically transfused (Bradley et al. 2001.) The subject of this work was the red blood cell (RBC); focusing on the inherent antigenicity of this cell type, caused by the numerous red cell antigens that can stimulate a host immune response directed against allogenic donor RBC. Therefore, a procedure that would globally minimize the chance of foreign RBC antigen presentation, without affecting normal cell function would have a major impact on the availability and success of RBC transfusion therapies. The primary research objective for this project aimed to develop a method to alter reduce red cell antigenicity and at the same time increase RBC resistance to hemolysis, as a strategy to increase the safety and effectiveness of this procedure.

1.2 Red Cell Surface Antigens and Blood Transfusions

Landsteiner was the first to describe the presence of ABO blood group antigens on the RBC surface (Landsteiner 1901). Since then serologists continue to report new blood group antigen families, characterize new family members and describe the infinite number of ways that these antigenic RBC surface structures differ within and between populations (Telen 2005). It is
largely the population variance of the RBC blood group antigens which has a direct impact on the success of RBC transfusions.

The International Society for Blood Transfusion (ISBT) has recognized 25 different blood group systems (Reid and Yahalom 2000). However current protocol follows that RBC products are generally matched to recipients based on two criteria: (1) ABO and RhD antigen compatibility. (2) A negative result from a screening of the patient’s serum to detect the presence of antibodies directed against the other numerous blood group antigens. Once the above two criteria are satisfied, the blood is classified as compatible (Garratty, Telen and Petz 2002)

However, from an immunological point of view, apart from autologous transfusions, or transfusion from one identical twin to another, transfused blood will always be largely antigenically incompatible, due to the presence of minor red cell antigens which are foreign to the recipient. Cross-matching for all the different RBC antigens is technically difficult and the probability of finding a truly compatible donor is unlikely. Incompatibility between donor and recipient can lead to a delayed transfusion reaction and/or limits the choice of blood products for future transfusions, as over time the recipient can develop antibodies to the foreign minor RBC antigens. The ability of a patient to raise such antibodies is highly variable. Therefore the preferred approach would be to minimize exposure to immunologically incompatible RBC antigens at the time of the initial transfusion.

The current compatibility testing for the ABO blood group system comes from the fact that the corresponding antibodies are commonly found in human sera even without there having been documented exposure to the corresponding human red cell antigens. Moreover, the anti-A and anti-B produced readily react at 37 °C and can lead to hemolytic transfusion reactions in the event that ABO incompatible blood is transfused (Dacie and Lewis 1984). The following discussion focuses on the ABO and Rh antigen systems, as these are initially the two antigen families of clinical importance.
The observations made by Landsteiner, in the early part of the twentieth century, that sera of certain individuals caused the agglutination of RBC from other individuals (Landsteiner 1901) was not only the first recorded description of the ABO blood group system, but is also considered by most to be the foundation for the modern practice of transfusion medicine.

At a phenotypic level, the ABO antigens interact with the Hh, Lewis (Lele) and Secretor (Sese) antigens (Watkins 2001, Oriol 1980). There are two allelic forms of the Lewis antigen: Le and le. ABO and Le^a antigens appear both on the RBC membrane surface and in the exocrine secretions of Se individuals; individuals who lack Se, express ABO and Le^b antigens exclusively on the RBC membrane surface and not in exocrine secretions (Kelly et al. 1995). The le allele represents an inactive form of the Lewis gene and in a homozygous state results in the rare phenotype Le(a-b-) (Watkins 1980 and 2001, Olsson et al. 2004, Morgan and Watkins 2000). While these antigens interact, they remain genetically distinct from each other (Watkins 1980 and 2001).

The protein products of the A, B, H, Se and Le genes are all glycosyltransferases: A = α1,3-N-acetylgalactosaminyltransferase, B = α1,3-galactosylaminyltransferase, H = α1,2-fucosyltransferase, Se = 1,2-fucosyltransferase and Le = α1,3 fucosyltransferase (Morgan and Watkins 2001). The combination of the above glycosyltransferases transfers the immunodominant sugar for H, A, B and Le to acceptor chains on glycoproteins or glycolipids upon the RBC surface (Watkins 2001) (Figure 1.1).

Although the precise physiological function of this blood group antigen family has not been characterized; it has been hypothesized that the ABH determinants function as capping sugars on the oligosaccharide chains, thus inhibiting interactions with lectins and other cell surfaces (Koschielak 2001).
Figure 1.1 Immunodominant Structures of the H, A, B, Le\textsuperscript{a} and Le\textsuperscript{b} Specificities on Glycoproteins and Glycolipids. Shown are the minimal structures necessary for the expression of the blood group H, A, B, Le\textsuperscript{a} and Le\textsuperscript{b} specificities on glycoproteins or glycolipids. The arrows indicate positions where differences occur between the blood group A and B structures. Abbreviations: Fuc, L-fucose; Gal, D-galactose; GlcNAc, N-acetylglucosamine; GalNAc; N-acetylgalactosamine; Glc, D-glucose; AcNH, N-acetylamino group (Morgan and Watkins 2000).
Figure 1.1 Immunodominant Structures of the H, A, B, Le^a and Le^b Specificities on Glycoproteins and Glycolipids

<table>
<thead>
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<th>Specificity</th>
<th>Minimal determinant structure</th>
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<tr>
<td>H</td>
<td>Fucα1→2Gal-β1-R</td>
</tr>
<tr>
<td>A</td>
<td>GalNACα1→3 \ Galβ1-R \ Fucα1→2</td>
</tr>
<tr>
<td>B</td>
<td>Galα1→3 \ Galβ1-R \ Fucα1→2</td>
</tr>
<tr>
<td>Le^a</td>
<td>Galβ1→3 \ GlcNAC-β1-R \ Fucα1→4</td>
</tr>
<tr>
<td>Le^b</td>
<td>Fucα1→2 Galβ1→3 \ GlcNAC-β1-R \ Fucα1→4</td>
</tr>
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The second major RBC antigen family is the Rh group. The Rh antigen is a transmembrane heterodimeric complex composed of 2 RhDCE subunits and 2 Rh–associated glycoprotein (RhAG) subunits (Ridgwell et al. 1992, Ridgwell et al. 1994 and Eyers et al. 1994) (Figure 1.2). The complex is believed to contribute to the maintenance of RBC membrane stability (Nicolas et al. 2003) and has also been reported to function as both an ammonium (Westhoff et al. 2004) and a CO$_2$/O$_2$ transporter (Bruce et al. 2003).

The antigenicity of the Rh complex comes from the protein products of the homologous RhD and RhCE genes which undergo several rearrangements resulting in the many polymorphisms defined for this blood group family (Westhoff 2004). No polymorphisms of the RhAG proteins have been described, and thus it is not considered to be antigenic (Le Van Kim et al. 2006).

The RhD gene codes for the D antigen and the RhCE genes code for the CE antigens. Approximately 80–85% of Caucasians are phenotypically classified as D–positive (Colin et al. 1991). The RhCE genes can be inherited in one of several possible combinations: ce, Ce, cE, CE (Cherif-Zahar et al. 1990). Together there are 12 possible combinations for the Dd, Cc and Ee haplotypes, and in pairs (genotypes) these allow for 78 different combinations (Dacie and Lewis 1984). The most common genotype combinations followed by their frequencies are as follows: CDe/cde (31.7 %), CDe/CDe (16.6 %), cde/cde(15.1 %), CDe/cDE (11.5 %), cDE/cde (11.0 %), cDe/cde (2.0 %) and cDE/cDE (2.0 %) (Race et al. 1948, Dacie and Lewis 1984).
Figure 1.2 Rh and RhAG Proteins

TOP Predicted membrane topology of Rh proteins. Open circles represent amino acid residue differences that distinguish RhD from RHDCcEe proteins. The amino acid residues shown are those for the RhD polypeptide. Positions 103 and 226 determine the C/c (S→P) and E/e (P→A) polymorphisms on the RHDCcEe polypeptide, respectively.

BOTTOM Predicted membrane topology of the RhAG protein. The N-Glycan at position N^{37} carries ABO and Ii specificities. The RhAG protein itself does not represent an Rh blood group antigen (Le Van Kim, Colin and Cartron 2006).
The Rh complex is believed to facilitate RBC transmembrane transport of CO$_2$/O$_2$ (Bruce et al. 2003) and ammonium (Westhoff et al. 2004). The RhAG subunits in particular, are thought to be critical for ammonium transport by acting as a transmembrane NH$_4^+$/$H^+$ exchangers (Westhoff et al. 2004).

RhD is the most highly immunogenic component of the Rh D, C, E unit (Moore et al., 1982 and Colin et al. 1991). Its immunogenicity comes from the fact that phenotypic D-negative individuals, rather than having an allelic variation, completely lack the D polypeptide; and the absence of an entire protein holds a far greater potential for immunologic stimulus (Liu et al. 1999). In addition to that, amongst D-positive individuals there are ~37 serologically defined D antigens that result from amino acid substitutions within the polypeptide (Liu et al. 1999), further adding to the antigenic potential of this blood group.

As a blood group system, Rh is associated with numerous pathologies including alloimmune transfusion reactions, hemolytic disease of the newborn, auto-immune hemolytic anemia and Rh-deficiency syndrome (Le Van Kim et al. 2006) thus placing further importance on the cross-matching of this antigen between donor and recipient.

1.3 Red Cell Substitutes

Several factors have contributed to an increased demand for donated blood products: increasingly complex surgical procedures, an aging population and individuals with either rare blood types or increased sensitization to minor blood group antigens as a result of repeated transfusions (Chang 2000). Clinical studies investigating the incidence of allosensitization in sickle cell (Vichinsky et al. 1990) and thalassemic (Sirchia et al. 1985) patients, i.e. the chronically transfused, reported an incidence of RBC allosensitization between 5–30 %. This allosensitization limits availability of compatible donated RBC products for future transfusions.
To supplement the supply, blood substitutes are at various stages of clinical development. The following discussion highlights work specifically in the area of RBC substitutes.

The ideal synthetic RBC transfusion product would have the following characteristics: reduced antigenicity, reasonable storage conditions, adequate post transfusion circulation time, lack accumulation in the reticulo–endothelial system (RES) and equivalent or improved oxygen transport capabilities (Chang 2000). A number of approaches have been taken to create such RBC substitutes and these can be broadly categorized into three classifications: bio–artificial oxygen carriers, synthetic oxygen carriers and modifications to human RBC membranes to produce a normally functioning antigenically silent red cell.

Hemoglobin based oxygen carriers (HBOCs) are the prototype bio–artificial oxygen carriers. In this class of RBC substitutes the hemoglobin is either purified directly from human or animal sources, or is a recombinant form of the protein (Kim and Greenburg 2004). However, these products are associated both with short–term and long–term tissue toxicity due to vascular leakage and iron deposition (Scott Murad and Eaton. 1997). Liposome carriers for the purified hemoglobin were tried as a method to avoid these side–effects; however, the encapsulated hemoglobin products continue to present challenges with stability and adequate circulation retention times (Scott, Murad and Eaton 1997).

Synthetic oxygen carriers, the second class of RBC substitutes, mimic the reversible binding characteristics of oxygen to hemoglobin through metal chelators (Monomenteau and Reed 1994) or artificial fluorinated organic fluids capable of carrying large quantities of dissolved oxygen (Riess and Krafft 1998). The disadvantage of these fluids is the high atmospheric oxygen concentration required to achieve sufficient blood oxygen levels in vivo. Patients must be breathing in up to 95 % oxygen, a concentration much higher than that in air (typically 21 % oxygen) (Chang 2000).
The third approach taken in the development of RBC substitutes differs from the above two strategies in that the focus is on direct modification of human RBC rather than trying to mimic RBC function through a synthetic system. To this end, the target is antigenic modification of RBC to limit presentation of foreign red cell antigens (major and minor) thus avoiding immune-mediated transfusion complications, increasing the number of potential recipients eligible to receive the converted blood donation and the overall success of the transfusion therapy.

The first approach taken in RBC antigenic modification was enzymatic cleavage of A and B antigens. Drs. Goldstein and Lenny (Lenny and Goldstein, 1980, Goldstein et al. 1982) used a galactosidase enzyme isolated from green coffee beans (Coffea canephora) to cleave type B immunodominant sugars from the RBC membrane surface (Zarnitz and Kabat 1960, Yatziv and Flowers 1971 and Harpaz and Flowers 1975).

Compared to B, the type A blood group antigen proved more challenging to remove for two reasons: (1) it was more difficult to isolate an effective glycosidase capable of specific type A sugar cleavage and (2) several slightly different isoforms of the A antigen exist, thus requiring several different cleavage enzymes (Donald 1981 and Clausen et al. 1986). However, despite these challenges several type A cleavage enzymes have been identified from several different sources: Clostridium perfringens α-N-acetylgalactosaminidase (Levy and Aminoff, 1980, Calcutt et al., 2002), chicken liver derived α-N-acetylgalactosaminidase (Goldstein 1989, Goldstein 1984) and Ruminococcus torques derived α-N-acetylgalactosaminidase (Falk et al. 1991). All of these appear to decrease type A antigenicity to varying degrees. However, the variable conditions required for optimal enzyme activity combined with highly unpredictable, heterogeneous antigen cleavage has resulted in the limited efficiency of this technique (Olsson et al. 2004). As a strategy, enzymatic cleavage has focused primarily on conversion of type A, B and AB blood to type O blood (Lenny et al. 1991, 1994 and 1995) However, there remain many
different RBC blood group antigens, each of which would require a specific cleavage enzyme in order to create a truly antigenically silent RBC transfusion product.

Covalent modification of RBC surfaces using polyethylene glycol (PEG) polymers has been used in order to produce a less targeted, more global RBC antigenic camouflage (Scott et al. 1997, Armstrong et al. 1997, Nacharaju et al. 2005). This method is advantageous as PEGylation of RBC prior to transfusion would diminish recipient exposure to minor blood group antigens which are not routinely tested for compatibility between donor and recipient, yet still have the potential to initiate transfusion complications. This is particularly important for the chronically transfused, as these patients are repeatedly exposed to foreign RBC antigens over time. When applied to the RBC, PEG imparts the desired qualities of decreased immunogenicity by sterically hindering access of host antibodies to both major (ABO and Rh) and minor antigenic sites on the RBC surface (Scott and Chen 2004).

1.4 PEGylation

The origins of this technology came from the protein pharmaceutical industry where PEG has been applied to a number of protein-based therapies in order to improve pharmacokinetic and pharmacodynamic properties (Roberts, Bently and Harris 2002, Roberts and Harris 1998 and Abuchowski et al. 1977). Peptide/protein PEGylation is useful in that the modified products show camouflaged antigenic and immunogenic epitopes, decreased incidence of receptor mediated uptake by the reticulo-endothelial system (RES), increased apparent size of the peptide/protein, thus reducing renal filtration and subsequent excretion, as well as an increased specificity for target action sites (Roberts, Bentley and Harris 2002 and Roberts and Harris 1998).

PEGylation is also further advantageous in that the polymer is flexible, non-toxic, non-immunogenic and soluble in both organic and aqueous solvents. These characteristics allow for
application to the target surface using relatively mild conditions. Finally, a variety of molecular weights and geometries can be easily synthesized allowing for optimal cell surface coverage (Veronese et al. 1997).

1.5 PEG Properties

As mentioned above, PEG may be either linear or branched. The general polymer structure is: \( \text{HO-}-(\text{CH}_2\text{CH}_2\text{O})_n\text{-CH}_2\text{CH}_2\text{-OH} \) (Harris 1992), and it is synthesized by anionic ring opening polymerization of ethylene oxide initiated by nucleophilic attack of a hydroxide ion on the epoxide ring (Harris 1992). Monomethoxy PEG (mPEG) is the form most commonly used for covalent modification of proteins. This PEG derivative has the general structure \( \text{CH}_3\text{O-CH}_2\text{CH}_2\text{O}_n\text{-CH}_2\text{CH}_2\text{-OH} \), and is synthesized by anionic ring opening polymerization initiated with methoxide ions (Harris 1992).

The high degree of solubility associated with this polymer comes from the approximately 2–3 water molecules which bind to each ethylene oxide unit (Harris 1992). The addition of water as well as polymer backbone flexibility, once conjugated onto the target proteins are believed to be key factors regulating the characteristic changes to antigenicity (Abuchowski 1977), circulatory retention time (Working 1997), solubility (Polson 1977) and altered cell–cell interactions (Gombotz et al. 1992) reported for PEG modified peptides/proteins. In vivo PEG is cleared without any structural changes (Roberts, Bently and Harris 2002). Low molecular weight PEGs (< 20 kDa) are cleared more rapidly through the urine while higher molecular weight PEGs are cleared more slowly in the urine and the feces (Roberts, Bently and Harris 2002).

Whether or not the PEG polymer itself is immunogenic in vivo is an area still being investigated. Roberts (2002) reported that under common clinical administration settings, PEG
modified peptides/proteins have not resulted in PEG antibody production. In line with this, Scott et al (1997) have reported normal in vivo survival of mPEG–modified murine RBC even after repeated transfusion, indicating that the polymer does not stimulate the host’s immune system. However, there have also been reports documenting the production of an anti–PEG IgM generated after repeated exposure to PEG–stabilized liposomes (Semple et al. 2005) and PEG conjugated proteins (Cheng et al. 1999). It is likely that generation of any immune response depends both on the nature of the modified protein/liposome as well as the degree of PEG modification (Richter and Åkerblom 1983).

1.6 PEGylation Chemistry

The first step to the PEG coupling reaction requires preparation of a polymer derivative that contains a functional group at one or both ends. The functional group is chosen based on the available reactive sites on the given peptide, or protein and thus incorporates some degree of specificity into the modification process. For example, lysine, cysteine, histidine, arginine, aspartic acid, glutamic acid, serine, threonine, tyrosine, N-terminal amino groups and the C-terminal carboxylic acid groups are some of the most common PEG conjugation targets. However, lysine, being one of the most prevalent amino acid residue within proteins (> 10% of total amino acid sequence) represents the most commonly substituted amino acid in modified proteins (Roberts, Bentley and Harris 2002).

The general reaction mechanism of protein/peptide PEGylation involves an attack by nucleophilic ε– and α–amino groups on the electrophilically activated (due to the functional group) PEG, thus yielding a substituted amino acid residue. This process occurs at many points along the protein/peptide yielding a “PEGylated” protein.
A succinimidyl succinate (PEGss) reactive group was chosen to provide the coupling reaction in this project. To create this PEG derivative, the first step of PEGss synthesis involves reacting mPEG with succinic anhydride, followed by activation of the carboxylic acid to a succinimidyl ester functional group. The resulting PEGss derivative preferentially reacts with the ε amines of lysine residues on the red cell surface according to the general reaction given below (Figure 1.3).
**Figure 1.3 PEGss Coupling Reaction**

\[
\text{PEG-O-} \begin{array}{c} \text{C} \quad \text{C} \\ \text{C-CH}_2 \text{CH}_2 \quad \text{C-O-N} \end{array} \xrightarrow{\text{R-NH}_2} \text{PEG-O-} \begin{array}{c} \text{C} \quad \text{C} \\ \text{C-CH}_2 \text{CH}_2 \quad \text{C-NH-R} \quad \text{O} \quad \text{N} \end{array}
\]

**Figure 1.3 PEGss Coupling Reaction.** Nucleophilic attack by the amino acid residue onto the electrophilic PEGss yields a modified residue and releases a succinimidyl succinate leaving group (Roberts, Bentley and Harris 2002).

### 1.7 Project Rationale

Red cell transfusions are associated with the risk of allosensitization and rejection, complications that occur with greater frequency in the chronically transfused patient. While this project focused on the major ABO and RhD antigens, PEG modification can also camouflage minor RBC antigens (Scott and Chen 2004) resulting in a more extensively camouflaged red cell. At the same time, PEG modification may increase the stability of the RBC membrane, such that it is more resistant to hemolysis, a feature that would be even more advantageous as this would decrease the frequency of transfusions required to treat chronically transfused individuals, thus limiting their possible exposure to foreign RBC surface antigens. This project aimed to achieve the above goals by using a tetra-functional polyethylene glycol-succinimidyl succinate polymer (4PEGss) (MW 10,000) (Figure 1.4) to modify the antigenic and osmotic characteristics of human RBC.
Figure 1.4 Tetra-functional Polyethylene Glycol Succinimidy succinate MW 10,000
The rationale behind using a branched polymer, rather than the traditional linear polymer, was two-fold. Firstly, the multiple potential attachment points, due to the multiple functional groups located at the polymer ends could impart increased stability onto the RBC membrane, thus increasing osmotic resistance to hemolysis. Secondly, application of a branched polymer to the RBC surface could also increase the apparent "thickness" of the polymer coating, thus providing a more effective immunocamouflage compared to that achieved when cells are treated with linear polymers (Fisher et al. 2000)(Figure 1.5).
Figure 1.5 Proposed Experimental Model. Represented above is a cross section of the RBC membrane studded with Rhesus and ABO blood group antigens. Coupling 4PEGss polymers onto surface proteins could yield an antigenically silent RBC with an increased ability to resist membrane hemolysis.
1.8 Hypothesis

4PEGss covalently attached to the RBC surface can provide the same level of antigenic camouflage as a linear PEG polymer but concurrently can increase the osmotic resistance of the RBC via cell membrane stabilization.

1.9 Specific Aims

1. Assess the efficiency of RBC antigenic camouflage achieved via 4PEGss modification and compare this to the antigenic camouflage achieved using linear PEGss polymers.

2. Assess the membrane-stabilizing effect of 4PEGss modification.

3. Determine the consequence of 4PEGss modification for normal RBC indices of function.
2. Materials and Methods

2.1 PEG - Modification of Red Blood Cells (RBC)

One tube (4 mL) of whole blood was collected from a consenting donor into EDTA anticoagulant. Fresh blood was used for modification as cell shape and osmotic balance change over time. Immediately following collection, the blood was centrifuged (Beckman GS 6R Centrifuge, Palo Alto CA) at 165 \( \times \) g for 10 minutes. The platelet rich plasma and buffy coat were removed and the remaining RBC pellet was centrifuged at 915 \( \times \) g again to further separate the RBC from the buffy coat layer. Once the buffy–coat and most of the residual plasma were removed the remaining RBC were resuspended in isotonic saline (150 mM NaCl, 308 mOsmol/L), then washed three times with the same solution. The resulting RBC suspension was counted on a Bayer Advia 120 Hematology Analyzer (Bayer Inc., Toronto, OT) and the red cell numbers and indices (mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC) and red cell distribution width (RDW)) were recorded. Red cell samples were then diluted to give a final concentration of 1.0 \( \times \) 10^{12} RBC/L (approximately a 10 % hematocrit) in order to facilitate uniform polymer coating and to avoid RBC aggregate formation.

Polymer solutions in aqueous buffer of 0.5, 1.0, 2.0 or 5 mM were prepared containing polyethylene glycol (PEG) (SunBio PEG-Shop, Korea). Solutions consisted of either linear polymers, PEG–succinimidyl succinate, MW 2000 (PEGss–2000) or linear PEGss MW 5000 (PEGss–5000) or a 4 branched polymer PEGss MW 10,000 with each branch being \( \sim \) 2500 MW (4PEGss). These suspensions were prepared by dissolving the desired PEG in an appropriate volume of \( N\text{-}2\text{-Hydroxyethylpiperazine\textendash}N'\text{-}2\text{-ethanesulfonic acid} \) (Hepes) buffer (HB, 50 mM Hepes, Sigma Aldrich, 110 mM NaCl, Fisher Scientific, pH 8.2;) at room temperature (RT).
Three hundred µL of each PEGss solution were immediately added to 300 µL of washed, diluted red cells. The mixture was incubated with gentle mixing at RT for 40 minutes. This was followed by five sequential additions of 300 µL of PEGss solution to reach a final concentration of 0.5, 1.0, 2.0 or 5.0 mM. Under these conditions the succinimidyl coupling reaction occurred rapidly and spontaneously, primarily targeting ε amino groups of lysine residues. Following the last addition of hydrated PEGss solution, the modified RBC were washed 3 times by centrifugation and resuspended in isotonic saline. Controls consisted of RBC treated in parallel, but with the addition of 5 x 300 µL of HB pH 7.2.

2.2 Agglutination Assay

This assay was used to detect whether at a macroscopic level, the RBC blood group A, B and RhD antigens were camouflaged as a result of PEGylation. In a sterile, non–treated polystyrene 96–well flat bottom plate (Corning Incorporated, Corning, NY), 15 µL of AB+ human RBC PEGylated with one of 4PEGss, PEGss–2000 or PEGss–5000 (final polymer treatment range = 0.5, 1.0, 2.0 or 5.0 mM) or control RBC were added to 15 µL of appropriate typing serum: undiluted murine anti–A IgM; murine monoclonal anti–B IgM (Organon Teknika, Durham, NC); or polyclonal/monoclonal blend anti–human RhD IgG antibody (Gamma Biologicals, Houston, TX). The RBC–antibody mixtures were gently mixed and incubated at RT for 10 minutes. The level of agglutination for each sample was scored on a scale of 1⁺⁺⁺ (w = weak)(lowest) to 4⁺⁺⁺⁺ (highest) by two independent observers. A 4⁺⁺⁺⁺ score was assigned to samples for which one complete mass of agglutinates was easily visible. A 1⁺⁺⁺⁺ score (the lowest possible score that can be given at the macroscopic level) was assigned to samples where RBC appeared free and evenly distributed.
2.3 Flow Cytometry

Flow cytometry was used to measure surface A and RhD blood group antigen levels on PEG-modified (PEGylated) and non-modified RBC. PEGylation can be expected to cause steric hindrance of the interaction between antibodies and their RBC surface antigen targets (Lee, Sehon and Akerblom 1981) thereby decreasing the level of blood group antigens such as RhD or A that would be detected by a double antibody labeling system. Thus, based on the amount of fluorescence, the degree of PEG coverage of the A or RhD antigens can be estimated. Blood group A and RhD antigens were chosen as “representative” antigen markers for the degree of PEG–mediated immunocamouflage due to the availability of the respective corresponding non-agglutinating antibodies.

For this assay, RBCs were modified with either 4PEGss, PEGss–2000 or PEGss–5000 at final treatment doses of 0, 0.5, 1.0, 2.0 or 5 mM. The primary antibody used to detect the RhD surface antigen was a polyclonal/monoclonal blend of anti–human RhD IgG (Gamma Biologicals, Huston, TX) and the secondary antibody was a F(ab’)2 fragment fluorescein isothiocyanate (FITC) conjugated goat–anti human IgG (GaH–IgG–FITC) (Jackson Immunoresearch, West Grove, PA) The primary antibody used to detect the A blood group antigen was a mouse monoclonal IgG directed against human surface antigen A. This antibody was the generous gift of Drs. Real Lemieux and Louis Thibeault (HemaQuebec, Quebec City, Canada). The secondary antibody was also a F(ab’)2 fragment FITC conjugated goat-anti mouse (GaM–IgG–FITC) (Jackson Immunoresearch, Westgrove, PA).

To detect RhD–dependent fluorescence inhibition, samples were assayed in triplicate: 45 µL of filtered Hepes buffered saline (HBS, 20 mM Hepes, 150 mM NaCl, pH 7.4) were added to microcentrifuge tubes, followed by 5 µL of a 1/10 dilution of packed RBC (either PEGylated
or control). Then 5 μL of undiluted anti–RhD were added and incubated with the RBC for 30 min at RT. The samples were then centrifuged in the microcentrifuge at 2000 x g for 5 min and the supernatant fluid was removed. Five μL of ½ diluted GaH–IgG–FITC secondary antibody were added and incubated at RT for 30 minutes. This was followed by the addition of 1 mL of saline containing 2 % formaldehyde (Fisher Chemicals, Fair Lawn, NJ) pH 7.4, to fix the samples. The samples were analyzed on a Beckman Coulter EXEL–MCL flow cytometer (Hialeah, FL) using forward scatter and side scatter parameters to define RBC, then selecting 6,000 cells in the RBC bitmap for fluorescence determination.

The same procedure, as described above, was used to analyze the degree of PEG–mediated blood group A antigen camouflage; the differences being the species and the dilutions of the primary and secondary antibodies used. Five μL of 1/40 diluted primary mouse monoclonal anti-A were added to a 1/10 dilution of packed RBC (either PEGylated or control), followed by a 30 min incubation at RT. Immediately following this incubation, the samples were centrifuged at 2000 x g for 5 min and the supernatant fluid was removed. Five μL of 1/32 diluted GaM–IgG–FITC secondary antibodies were added and the samples were again incubated at RT for 30 min. The fixation and analysis procedure for these samples was exactly as described above for the RhD samples.

2.4 Scanning Electron Microscopy

Scanning electron microscopy (SEM) was used to assess morphological changes of the cells that appeared as a result of 4PEGss modification. RBC were collected and modified according to the procedures described above (section 2.1) and subsequently prepared for SEM analysis according to the standard methods employed at the University of British Columbia Bio Imaging Facility. The following is an outline of this protocol.
Both PEGylated and control RBC samples were immersed in fixative solution (2.5 % glutaraldehyde, 0.1M sodium cacodylated pH 7.3–7.4) for approximately 8 hours at a ratio of 100 :1 fixative: RBC (RBC concentration = 1.0 x 10^{12} RBC/L). The samples were then rinsed twice with washing buffer (0.1 M sodium cacodylate) and stored in this buffer overnight. Post fixation, the samples were washed in a solution composed of 1 % osmium tetroxide and 0.1M cacodylate buffer, and finally rinsed once with distilled water. Next, the samples were dehydrated by repeated washes in ethanol solutions of increasing concentrations: 70 % ethanol, 85 % ethanol, 95 % ethanol and finally 100 % ethanol. The samples were incubated in each of these ethanol solutions for 10 minutes. Following dehydration, the samples were dried on a Balzers CPD020 critical point dryer (Bal Tec, Zurich, Switzerland) and once dried, coated with gold particles using an SEMPREP 2 gold sputter coater (Nanotech Ltd, Manchester, England). Prepared samples were analyzed on a Hitachi S4700 SEM (Hitachi HighTechnologies, Schaumburg, IL) and Quartz PCI imaging software was used to upload images.

2.5 Erythrocyte Sedimentation Rate (ESR) Assay

The ESR assay measures the distance erythrocytes descend during one hour in a vertical column of anticoagulated blood under the influence of gravity. This method was used to analyze the effect of 4PEGss modification on the ability of modified RBC to participate in cell to cell contact. Sedimentation of RBC in solution requires the formation of rouleau– a phenomenon dependent on cell–cell interactions (Hardwicke and Squire 1952). The following procedure is a modified version of the Westergren method commonly used in clinical hematology laboratories (Westergren 1921).

Both control and PEGylated RBC samples (modified at final concentrations of: 0.5, 1.0, 2.0 or 5.0 mM 4PEGss) were counted on the ADVIA 120, and the cell concentrations were
adjusted to match, usually at approximately $1.0 \times 10^{12}$ RBC/L. Capillary tubes with millimeter markings were filled to the same level and changes of the height of the packed RBC (pRBC) in solution were measured over a 60 minute time interval. The ESR was calculated according to the following equation:

$$\text{ESR} = \frac{\Delta \text{Height pRBC (mm)}}{\Delta \text{Time (hour)}}$$

$$\text{ESR} = \frac{\text{Height of pRBC}_{\text{initial}} (\text{mm}) - \text{Height of pRBC}_{\text{final}} (\text{mm})}{\text{Time}_{\text{final}} (\text{hours}) - \text{Time}_{\text{initial}} (\text{hour})}$$

### 2.6 Osmotic Fragility Assay

The osmotic fragility assay was expected to detect alterations of RBC osmotic resistance caused by 4PEGss modification. This procedure was modified from the Dacie method (Dacie and Vaughn 1938) and was done according to the standard operating procedure of the Pediatric Hematology Department of BC Children's Hospital (Vancouver, BC). Fifteen saline solutions were prepared ranging from 0 % NaCl to 0.9 % NaCl. For each osmotic step, 1 mL of salt solution was added to a labeled microcentrifuge tube until the range was covered. Each tube received 25 µL of washed packed RBC (control samples). Parallel assays were run for RBC modified with final concentrations of 0.5, 1.0, 2.0 and 5.0 mM 4PEGss. The samples were vortexed slowly, until an even suspension was achieved, followed by a 30 minute incubation at RT. A 190 µL sample of the suspension was removed and combined with 10 µL of 10 % Triton X–100 detergent (BioRad Laboratories, Richmond, CA) solution in order to lyse the RBC and release the hemoglobin into suspension to establish the maximum amount of hemoglobin in the sample. One hundred fifty µL of this detergent–RBC suspension was transferred to a 96 well microplate and provided the value for the total releasable hemoglobin. The remaining RBCs
were centrifuged at 2000 x g for 5 minutes in a microcentrifuge (Micromax International Equipment Company, Needham Heights, MA)). A 190 μL sample of the resulting supernatant was removed and 10 μL of 10 % Triton X–100 was added to the supernatant to keep the volumes comparable. One hundred fifty μL of this RBC supernatant - detergent solution was subsequently transferred to a 96 well microplate. Absorbance at 540 nm was measured on a plate reader (Spectra Max 190, Molecular Devices, Sunnyvale, CA) and the percent hemolysis was calculated as follows:

\[
\left( \frac{A_{540_{\text{supernatant}}}}{A_{540_{\text{suspension}}}} \right) \times 100 = \text{Percent Hemolysis.}
\]

In a separate experiment both PEG–modified and control samples were stored for 24 hours at 37 °C in isotonic saline solution. Following incubation, the entire procedure was repeated in order to assess any changes to osmotic resistance as a result of the short storage period.

2.7 Hemolysis Measurements

This assay was used in order to determine the level of hemolysis after 48 hours for PEGylated RBC modified with either linear (PEGss–2000, PEGss–5000) or branched PEG (4PEGss). Samples were modified with the desired PEG polymer as described above (section 2.1) and subsequently washed into saline–adenine–glucose–mannitol (SAGM) RBC storage solution (SAGM, 150 mM NaCl, 45.4 mM dextrose monohydrated, 1.25 mM Adenine, 28.8 mM mannitol, pH 6.4; Baxter, Mississauga, OT) or Hank’s Balanced Salt Solution (HBSS pH 7.2; Invitrogen, Burlington, OT) supplemented with 20 μL of antibiotics to prevent bacterial growth (5000 units penicillin, 5 mg streptomycin and 10 mg neomycin; Sigma–Aldrich, Oakville, OT).

PEGylated RBCs stored in SAGM were held at 4 °C. One hundred and fifty μL were removed from each RBC solution immediately after PEG modification at 0 hours, 24 hours and 48 hours post treatment and the degree of hemolysis was measured.
To assess hemolysis, 12.5 μL of RBC suspension were added to 250 μL Drabkin’s reagent (Sigma Aldrich, Oakville, OT) and incubated for 30 minutes at RT to give the value for the total hemoglobin content of the red cell suspension (RBC suspension). The remaining RBC suspension was centrifuged at 2000 x g on the microcentrifuge. One hundred and twenty five μL of the supernatant were added to 250 μL of Drabkin’s reagent (RBC supernatant) and also incubated for 30 minutes at RT. Two hundred μL of each RBC suspension and each RBC supernatant were placed in a 96 well microcentrifuge plate and absorbance at 540 nm was measured on the Spectra plate reader. Percent hemolysis was calculated according to the following equation:

\[
\text{% Hemolysis} = 100 \times \left[ \frac{\text{OD}_{\text{540 RBC supernatant}} \times (\text{final sample volume/sample volume})/6.8}{\text{OD}_{\text{540 RBC suspension}} \times (\text{final sample volume/sample volume})/6.8} \right]
\]

PEGylated RBC stored in HBSS were incubated at 37 °C over the 48 hour storage period and the degree of hemolysis was calculated exactly as described above.

2.8 Hemoglobin Oxidation

To assess the potential effect of PEGylation on red cell physiology, the degree of hemoglobin oxidation was measured. The method used was from Dr. Scott’s laboratory (University of British Columbia, Canada) and is based on methods originally developed by Winterbourn (Winterbourn 1986).

A hemolysate of RBC was prepared by adding 15 μL of 4PEGss-modified RBC (1.0 x 10^{12} RBC/L) to 5 mL of distilled water. Following this, the hemolysate was scanned spectrophotometrically (Thermo Electron, Madison, WI) from 500 to 701 nm in order to ensure that the characteristic peaks for the major hemoglobin oxidation species were covered (Winterbourn 1990).
The results for the various hemoglobin species were calculated using µM extinction coefficients according to the following equations:

**Oxyhemoglobin (µM)** = 119(OD$_{577}$ - OD$_{700}$) - 39(OD$_{630}$ - OD$_{700}$) - (OD$_{560}$ - OD$_{700}$)

**Methemoglobin (µM)** = 28(OD$_{577}$ - OD$_{700}$) + 307(OD$_{630}$ - OD$_{700}$) - 55(OD$_{560}$ - OD$_{700}$)

**Hemichrome (µM)** = 133(OD$_{577}$ - OD$_{700}$) - 114(OD$_{630}$ - OD$_{700}$) - 233(OD$_{560}$ - OD$_{700}$)

The calculated hemichrome values were negative, and thus the reported values of oxyhemoglobin and methemoglobin were adjusted to equal 100%.

### 2.9 Statistical Analysis

For each assay, data from 3 independent experiments were collected for statistical analysis. Sample averages, standard deviations, one way and two way analysis of variance (ANOVA) were calculated using Microsoft Windows XP Professional version 2002 statistical analysis software. Significance was accepted at $p \leq 0.05$. 


3. Results

3.1 Agglutination Assays

Red cell antigen detection can be accomplished using agglutination with group specific typing sera. The specificity of the antigen detection is directly proportional to the titre of the antibody used. Consequently, agglutination inhibition at a fixed antibody dilution is a direct measure of the level of antigen masking.

Table 3.1 shows that irrespective of the PEG type used, or its concentration, control and PEG-modified AB+ RBC agglutinated with undiluted typing serum A (Table 3.1). Similarly strong agglutination was observed for AB+ RBC modified with all PEG types up to a final polymer concentration of 1 mM using typing serum B. Linear PEGss–2000 modified cells agglutinated with typing serum B even after modification with polymer concentrations of 2 mM and 5 mM. However using higher modification doses, linear PEGss–5000 and branched 4PEGss–10,000 modified RBC agglutination decreased when incubated with typing serum B.

The agglutination scores for PEGss - 5000 modified RBC a 2 mM and 5 mM were reduced from 4++ to 1+w (w=weak). AB+ RBC modified with lower PEGss–5000 concentrations also showed a decreased agglutination score from 4++ to 3+ (0.5 mM and 1 mM) when tested with typing serum B (Table 3.1). Decreased agglutination scores were correspondingly assigned to AB+ 4PEGss modified RBC incubated with typing serum B. Compared to a base-line score of 4++ for non–treated control cells, 0.5 mM and 1 mM 4PEGss modified AB+ RBC received a score of 2+. As expected, 2 mM and 5 mM AB+ 4PEGss modified RBC were even more camouflaged, receiving a score of 1+w (Table 3.1).

The greatest degree of immunocamouflage was achieved for blood group antigen RhD regardless of PEG polymer type used. AB+ non–treated control RBC consistently showed
agglutination with typing serum RhD at the 4++ level. This score was reduced to 1+ for both 0.5 mM and 1 mM treated PEGss–2000 modified RBC, and at concentrations of 2 mM and 5 mM, this score was further reduced to 1++. Thus the RhD antigen was efficiently camouflaged by PEGss–2000.

Linear PEGss–5000 modified RBC also showed a significantly reduced agglutination scores compared to non–modified control cells when treated with typing serum RhD. AB+ RBC treated with 0.5 mM PEGss–5000 received a score of 1+ and AB+ RBC treated with a final concentration of 1, 2 and 5 mM PEGss–5000 each received an agglutination score of 1++. AB+ RBC modified with 4PEGss received a score of 1++ over all concentrations tested (Table 3.2), thus supporting the hypothesis that in comparison to both linear PEG polymers investigated, branched 4PEGss imparts a more effective immunocamouflage onto treated RBC. Taken together, these results offer evidence for both a concentration and PEG geometry–dependent camouflage for ABO and RhD RBC blood group antigens (Table 3.1)
Table 3.1 Macroscopic Agglutination Assay Scores for PEG Modified AB+ RBC

<table>
<thead>
<tr>
<th></th>
<th>A Antigen</th>
<th>B Antigen</th>
<th>Rh D Antigen</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>4++</td>
<td>4++</td>
<td>4++</td>
</tr>
<tr>
<td>0.5 mM PEGss–2000 Modified RBC</td>
<td>4++</td>
<td>4++</td>
<td>1+</td>
</tr>
<tr>
<td>1 mM PEGss–2000 Modified RBC</td>
<td>4++</td>
<td>4++</td>
<td>1+w</td>
</tr>
<tr>
<td>2 mM PEGss–2000 Modified RBC</td>
<td>4++</td>
<td>4++</td>
<td>1+w</td>
</tr>
<tr>
<td>5 mM PEGss–2000 Modified RBC</td>
<td>4++</td>
<td>4++</td>
<td>-j+w</td>
</tr>
<tr>
<td>Control</td>
<td>4++</td>
<td>4++</td>
<td>4++</td>
</tr>
<tr>
<td>0.5 mM PEGss–5000 Modified RBC</td>
<td>4++</td>
<td>3+</td>
<td>1+</td>
</tr>
<tr>
<td>1 mM PEGss–5000 Modified RBC</td>
<td>4++</td>
<td>3+</td>
<td>1+w</td>
</tr>
<tr>
<td>2 mM PEGss–5000 Modified RBC</td>
<td>4++</td>
<td>1+w</td>
<td>1+w</td>
</tr>
<tr>
<td>5 mM PEGss–5000 Modified RBC</td>
<td>-j+w</td>
<td>1+w</td>
<td>1+w</td>
</tr>
<tr>
<td>Control</td>
<td>4++</td>
<td>4++</td>
<td>4++</td>
</tr>
<tr>
<td>0.5 mM 4PEGss Modified RBC</td>
<td>4++</td>
<td>2+</td>
<td>1+w</td>
</tr>
<tr>
<td>1 mM 4PEGss Modified RBC</td>
<td>4++</td>
<td>2+</td>
<td>1+w</td>
</tr>
<tr>
<td>2 mM 4PEGss Modified RBC</td>
<td>4++</td>
<td>1+w</td>
<td>1+w</td>
</tr>
<tr>
<td>5 mM 4PEGss Modified RBC</td>
<td>4++</td>
<td>1+w</td>
<td>1+w</td>
</tr>
</tbody>
</table>

Table 3.1 Macroscopic Agglutination Assay Scores for PEG Modified AB+ RBC.

Agglutination assays show an inverse polymer dose dependence for A, B and RhD antigen-dependent agglutination of PEG modified AB+ RBC. Compared to either linear PEGss, branched 4PEGss modified RBC was assigned lower agglutination scores indicating that 4PEGss treated RBC are more effectively immunocamouflaged. A score of 4++ (highest) were assigned to samples with one complete mass of agglutinates. 1+w (lowest) scores were assigned to samples for which the RBC appeared free and evenly distributed on the slide. Shown is a representative experiment from 1 of 3 independent assays.
3.2 Flow Cytometry

Flow cytometry was used as a quantitative method to assess the effect of polymer type and treatment dose on the efficiency of the immunocamouflage process. Type A+ RBC samples were modified and analyzed to determine the level of surface antigen exposure by using a double antibody labeling system and subsequent flow cytometric analysis. In line with previous agglutination assay data, results of this experiment revealed that the efficiency of antigenic camouflage depends on both the polymer type, i.e. linear vs. branched, as well as the relative amount of polymer grafted onto the red cell surface.

Figures 3.1A and B show that A+ RBC modified with 5 mM 4PEGss exhibited the highest degree of immunocamouflage for both RhD and A antigens. At this concentration 88.2 % of RhD–antibody interactions (Figure 3.1A) and 90.7 % of A antigen–antibody interactions (Figure 3.1B) were masked as compared to the fluorescence level of an unmodified cell population. This level of camouflage is significantly higher than RBC modified, at the same concentration, with either linear PEGss–2000 or linear PEGss–5000 and the difference between them was statistically significant at p<0.05.

Overall, cells treated with PEGss–5000 were more camouflaged than PEGss–2000 modified RBC (Figure 3.1A and 3.1B). Treatment with 5 mM PEGss–5000 gave an average of 73.7 % of blood group RhD antigen masking (Figure 3.1A) and 87.7 % of blood group A antigen (Figure 3.1B) sites were masked.

In comparison, even a high treatment dose could not impart a similarly high level of A antigen camouflage onto PEGss–2000 modified RBC. Following treatment with 5 mM PEGss–2000, 46.9 % of blood group A antigen (Figure 3.1B) still remained available for antibody interaction.
Immunocamouflage of the RhD antigen for PEGss–2000 modified RBC was comparable to that achieved when cells were treated with the larger linear PEGss–5000 polymer: 75.9 % of RhD sites were camouflaged from interactions with their corresponding antibodies following modification with PEGss–2000 versus 73.7 % camouflaged sites measured for PEGss–5000 modified RBC (Figure 3.1B).

Taken together, these results show that the efficiency of immunocamouflage depends significantly on the relative amount of polymer grafted onto the RBC surface (p<0.05) and that compared to either of the linear PEG polymers investigated, treatment of human RBC with branched 4PEGss imparts a greater degree of antigenic camouflage for both RhD and A antigens and that the increased camouflage was statistically significant at p<0.05. These results also show that in spite of the negative results in the agglutination assay there is a degree of antigenic camouflage of the A antigen that is nonetheless achieved by PEGylation. That camouflage is not detectable by agglutination (previous assay) but is detectable by the more sensitive flow cytometric assay.
Figure 3.1A Effect of PEG Modification on the Level of Blood Group RhD Antigen
Detected by Flow Cytometry.

The degree of immunocamouflage was quantified by PEG mediated inhibition of fluorescence of a double antibody labeling system detected using flow cytometry. PEGss treatment significantly (p < 0.05) reduces the availability of RhD antigen for interaction with its corresponding antibody. Branched 4 PEGss treated RBC show the lowest fluorescence levels indicating that 4PEGss modified RBC have the greatest degree of RhD antigen camouflage. Represented above are averaged data from 3 independent experiments. Error bars represent 1 SD.
Figure 3.1B Effect of PEG Modification on Level of Blood Group A Antigen Detected by Flow Cytometry

Figure 3.1B Effect of PEG Modification on the Level of Blood Group A Antigen Detected by Flow Cytometry. ( ) Linear PEGss–2000 ( —Δ—) Linear PEGss–5000 ( —×—) Branched 4 PEGss–10,000. The degree of immunocamouflage was quantified by PEG mediated inhibition of fluorescence of a double antibody labeling system detected using flow cytometry. PEGss treatment significantly (p < 0.05) reduces the availability of A antigen for interaction with its corresponding antibody. Branched 4PEGss treated RBC show the lowest fluorescence levels indicating that 4PEGss modified RBC have the greatest degree of A antigen camouflage. Represented above are averaged data from 3 independent experiments. Error bars represent 1 SD.
3.3 Scanning Electron Microscopy

Scanning Electron Microscopy (SEM) was used to visualize any morphological changes to the RBC as a result of 4PEGss treatment. Both control non-modified and 4PEGss modified RBC were prepared according to standard SEM protocols. The scanning electron micrographs of control non-modified RBC show an even cellular distribution and lack any gross morphological changes due to sample preparation (Figure 3.2A). However, as the concentration of 4PEGss increased, subtle changes became apparent: an uneven cellular distribution as well as RBC aggregates appeared, and the frequency for both of these increased with increasing polymer concentration (Figure 3.2 B C D & E).

The cellular distribution for 0.5 mM 4PEGss modified RBC was much less even, in contrast to that of unmodified RBC, and there is evidence of RBC aggregate formation (Figure 3.2B). The multiple functional groups located at each end of the four branched polymer may lead to inter-cell cross-linking, as suggested by the presence of small RBC aggregates seen on the scanning electron micrographs.

The rough, textured membrane of 4PEGss treated cells may be caused by intracellular cross-linking between surface membrane proteins (Figure 3.2 B, C, D & E). Again, this change of normal RBC membrane appearance increases with increasing polymer concentration and may be due to the multiple linker moieties promoting both intracellular and intercellular connections.
Figure 3.2 Scanning Electron Micrographs of 4PEGss Modified RBC

Micrographs show that branched 4PEGss treated RBC are less evenly distributed and appear to have an altered membrane appearance. These changes increase in frequency with increasing polymer concentration.
3.4 Erythrocyte Sedimentation Rates (ESR) Assay

Rouleau formation decreases the cell surface area to mass ratio thus promoting the sedimentation of suspended erythrocytes from the solution. However, in order for these rouleaux to form, the negative charge surrounding the individual cells, thus repelling one from the other, must be neutralized.

Control and 4PEGss treated samples were prepared according to a modified version of the Westergren method (Westergren, 1921) and the ESR was monitored over 1 hour. Clinically, the expected ESR for women between the ages 17–50 years is between 3–9 mm/ hour, with the upper limit being 20 mm/hour (Brigden 1999).

This experiment showed that the ESR for both control non-modified and 4PEGss modified RBC, at all concentrations investigated, was within the above stated clinically acceptable range (Figure 3.3). Further statistical analysis revealed no significant polymer concentration dependent effect on the ESR (p = 1).
Figure 3.3 Erythrocyte Sedimentation Rates. (■) Control Non-Modified RBC (□) 0.5 mM 4PEGss Modified RBC (□) 1 mM 4PEGss Modified RBC (□) 2 mM 4PEGss Modified RBC (□) 5 mM 4PEGss Modified RBC. The sedimentation rates for control non-modified and 4PEGss modified RBC were measured over a 1 hour period. All treatment groups show a normal ESR. Represented above are averaged data from 3 independent experiments. Error bars represent 1 SD.
3.5 Erythrocyte Indices

Samples of both control and 4PEGss modified RBC were analyzed on a hematology analyzer immediately following treatment in order to assess the consequences of 4PEGss treatment on normal indices of red cell potential for function.

The mean corpuscular volume (MCV) for control (0 mM), 0.5 and 1.0 mM 4PEGss treated RBC was within normal range (82–98 fL). However, at higher 4PEGss treatment doses the recorded MCV exceeded the upper limit of the clinically normal range indicating that cells treated with higher 4PEGss concentrations are larger compared to either RBC modified with lower polymer treatment doses and non–modified control RBC (Table 3.2).

The mean corpuscular hemoglobin (MCH) levels indicate how much hemoglobin there is per average red cell. The expected MCH should fall between 28–34 pg hemoglobin/ RBC. Overall, the MCH for non–modified control and 4PEGss modified RBC fell within the above range. The exceptions were 0.5 and 1 mM PEGylated samples, which had calculated mean MCH slightly below the expected range, however the calculated standard error was also high and thus the lower than expected MCH is likely a result of experimental variance (Table 3.2).

Mean corpuscular hemoglobin concentration (MCHC) was also measured as an indicator of red cell potential function. For all samples the greatest variation was recorded for this measurement than for any other parameter measured, and for the most part, the failure to meet clinically accepted values can be attributed to experimental variance. However, for 2 and 5 mM 4PEGss modified RBC, the lower than normal MCHC combined with evidence of a greater than normal MCV suggests that these high polymer concentrations have the potential to adversely affect red cell function (Table 3.2). This is further supported by the SEM (Figure 3.2) and ESR data (Figure 3.3).
Finally, the red cell distribution width (RDW) was measured. The RDW is an index of variation for cell volume within a red cell population. In agreement with the MCV and MCHC measurements, the higher than normal RDW measured for 2 and 5 mM 4PEGss modified RBC (Table 3.2) suggest that these higher polymer concentrations may affect red cell size. Branched 4PEGss treatment could potentially result in membrane instability due to polymer attachment onto the RBC membrane protein pumps and channels. This in turn may affect the cell’s ability to properly regulate the osmotic balance across the membrane leading to an influx of fluid and ultimately an increased RBC volume.
Table 3.2 Erythrocyte Indices (N=3)

<table>
<thead>
<tr>
<th>Parameter (range)</th>
<th>MCV (82-98 fL)</th>
<th>MCH (28-34 pg)</th>
<th>MCHC (320-365 g/L)</th>
<th>RDW (11-15%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control Non Modified RBC</td>
<td>97 (2)</td>
<td>30 (0.4)</td>
<td>309 (7)</td>
<td>13 (0.1)</td>
</tr>
<tr>
<td>0.5 mM 4PEGss</td>
<td>91 (1)</td>
<td>26 (5)</td>
<td>281 (57)</td>
<td>14 (0.2)</td>
</tr>
<tr>
<td>1.0 mM 4PEGss</td>
<td>98 (2)</td>
<td>25 (6)</td>
<td>312 (10)</td>
<td>15 (0.2)</td>
</tr>
<tr>
<td>2.0 mM 4PEGss</td>
<td>103 (2)</td>
<td>32 (1)</td>
<td>312 (11)</td>
<td>17 (0.4)</td>
</tr>
<tr>
<td>5.0 mM 4PEGss</td>
<td>100 (1)</td>
<td>31 (1)</td>
<td>312 (6)</td>
<td>18 (1)</td>
</tr>
</tbody>
</table>

Table 3.2 Erythrocyte Indices (N=3). High PEG treatment doses impart changes to red cell volume. Following modification, control and 4PEGss modified RBC were analyzed on the ADVIA 120 Hematology Analyzer and red cell indices were recorded. Data is reported as mean followed by 1 standard error (SE). Reference values are indicated at the head of each column.
3.6 Osmotic Fragility Profiles

The osmotic fragility assay was another method used to investigate the effect of branched 4PEGss grafting on cell membrane integrity. Median corpuscular fragility (MCF), a clinically useful measurement was employed to compare the osmotic fragility profiles amongst the different 4PEGss treated and non–treated control RBC samples. This value is derived from the ionic strength where 50 % hemolysis is achieved. For fresh blood, the expected MCF should occur over the saline concentration range 0.4–0.45 % NaCl. Immediately post treatment, the MCF recorded for control RBC was between 0.5–0.525 % NaCl. In comparison, 4PEGss modified RBC showed a MCF between 0.5–0.6 % NaCl, with the higher PEG concentration treated cells (2 mM and 5 mM) yielding a higher MCF (Figure 3.4) thus these higher polymer treatment doses yield a less osmotically stable RBC.

Following a 24 hour incubation at 37 °C in normal saline, the expected MCF should occur over the saline concentration range 0.465–0.6 % NaCl. Following this incubation period, the recorded MCF for control RBC was approximately 0.525 % NaCl. The MCF for 4PEGss modified cells occurred between 0.525–0.6 % NaCl. Five mM 4PEGss modified RBC had an MCF above this expected range at 0.7 % NaCl (Figure 3.5). Statistical analysis revealed that at both time points, 0 and 24 hours, the 4PEGss treatment dose as well as the extracellular saline concentration were both significant predictors of the degree of membrane lysis (p<0.05) indicating that treatment does affect the ability of modified cell to resist lysis.

All together, the osmotic fragility profiles (Figure 3.4 and 3.5), red cell index measurements (Table 3.2) and the SEM data (Figure 3.2) support that RBC treatment with branched 4PEGss may lead to destabilization of membrane integrity by means of intercellular and intracellular cross–linking events and that this effect is more striking as the branched polymer treatment dose increases.
Figure 3.4 Osmotic Fragility Profile Collected Immediately Post Modification

Control Non-Modified RBC (---) 0.5 mM 4PEGss Modified RBC (---) 1.0 mM 4PEGss Modified RBC (---) 2.0 mM 4PEGss Modified RBC (---) 5.0 mM 4PEGss Modified RBC. Shown are representative osmotic fragility curves for control non-modified and 4PEGss modified RBC from 3 individual experiments. The right-shifted MCF and overall greater level of hemolysis for treated RBC indicates that modified cells are more osmotically fragile compared to non-treated control cells. The above profiles represent 1 of 3 independent assays.
Figure 3.5 Osmotic Fragility Profiles Collected 24 Hours Post Modification

Figure 3.5 Osmotic Fragility Profiles Collected 24 Hours Post Modification. (—) Control Non-Modified RBC, (—) 0.5 mM 4PEGss Modified RBC, (—) 1.0 mM 4PEGss Modified RBC, (—) 2.0 mM 4PEGss Modified RBC, (—) 5.0 mM 4PEGss Modified RBC. Shown above are osmotic fragility curves for control non-modified and 4PEGss modified RBC following a 24 hour storage period at 37 °C in saline. The right-shifted MCF and overall greater level of hemolysis for treated RBC indicate that modified cells are more osmotically fragile compared to non-treated control cells following storage. The above profiles represent 1 of 3 independent assays.
3.7 Hemolysis Measurements

To better understand the effects of 4PEGss modification on RBC membrane stability, the level of red cell hemolysis was measured over a 48 hour storage period. Two storage conditions were chosen: one followed current donated RBC storage conditions of 4 °C in SAGM solution (condition 1) and the second storage condition more closely resembled an in vivo environment at 37 °C in Hanks' Balanced Salt Solution (HBSS) (condition 2).

Results show that immediately following modification (time=0), 4PEGss modification does offer some protection against hemolysis for samples stored under condition 1 i.e. 4PEGss modified RBC hemolysed less compared to non–modified control RBC. The average level of hemolysis for control cells was 2.3 % while 4PEGss modified cells, at all concentrations investigated, measured a level of hemolysis under 0.2 % for this time point (Figure 3.6).

However, the 4PEGss–mediated protection against hemolysis did not continue over the remainder of the storage period. Hemolysis increased with storage time for all samples; however, compared to control RBC, 4PEGss modified cells were much more susceptible to hemolysis 24 and 48 hours post modification (Figure 3.6). This observation agrees with previous data that suggests 4PEGss treatment affects RBC membrane stability leading to increased levels of hemolysis.

For storage condition 1 there is a non–linear relationship between polymer dose and hemolysis for 4PEGss modified RBC (Figure 3.7). While hemolysis of control samples was lower than 4PEGss modified RBC, amongst modified RBC samples 1 mM 4PEGss treated RBC consistently outperformed all other treatment doses investigated (Figure 3.7).

Statistical analysis showed that under condition 1, the effect of polymer concentration was a statistically significant predictor for hemolysis (p < 0.05); however, this relationship was not dose–dependent. Storage time did not quite reach the level of statistical significance (p = 0.1) however, the observed trend was that hemolysis increased over the 48 hour storage period.
Overall, there was less hemolysis measured for samples stored under condition 2 (37 °C in HBSS) (both control and 4PEGss modified RBC) compared to condition 1(Figure 3.8). For condition 2, hemolysis of control non-modified RBC remained consistently low over the entire 48 hour storage period (< 0.24 %) (Figure 3.8). In comparison, 4PEGss modified RBC measured higher levels, yet even so, hemolysis remained below 5 % for all concentrations investigated during the 48 hour storage period. Under condition 2 the effect of 4PEGss treatment dose did not quite reach statistical significance (p = 0.1) however the trend showed a more linear relationship between 4PEGss treatment dose and hemolysis (Figure 3.9A & C).

Storage time, for samples stored at 37 °C in HBSS was a statistically significant predictor (p < 0.05) for hemolysis.

The question still remains whether the 4PEGss–mediated increase in hemolysis is consequent to membrane instability resulting from the multiple functional groups per polymer, or the succinimidyl succinate coupling reaction itself. Therefore, to better understand the cause of the membrane instability, the level of hemolysis was measured and compared between RBC modified with either linear PEGss–2000, PEGss–5000 or branched 4PEGss. The 0 mM, 0.5 mM and 2.0 mM PEGss concentrations were chosen in order to make comparisons between RBC which were modified at the same PEG polymer treatment dose (molarity) vs. RBC modified with the same dose (molarity) of reactive groups, i.e. at the same treatment dose, 4PEGss has 4 times as many reactive groups per mole of polymer compared to linear PEGss.

As expected, control non–modified RBC samples showed low levels of hemolysis over the 48 hour storage period (< 2.5 %)(Figure 3.10 A). As storage time progressed and the PEG concentration increased, the effect of polymer type became clearer.

Compared to RBC modified with either the larger linear PEGss–5000 polymer or the branched 4PEGss polymer, the smaller linear PEGss–2000 modified RBC measured the highest levels of hemolysis at both treatment doses investigated (0.5 and 2 mM PEGss–2000) 24 hours
post modification: 8.6 % and 11.2 %, respectively (Figure 3.10B & C). Following an additional 24 hours of storage (t = 48 hours), the levels of hemolysis began to decrease, i.e. following 48 hours of storage, the level of hemolysis for 0.5 mM PEGss-2000 modified RBC came down to similar levels measured immediately following treatment, 0.50 % (Figure 3.10B). At a treatment dose of 2 mM, hemolysis of linear PEGss-2000 modified RBC continued to increase with storage time reaching levels of 8.2 % at 48 hours post modification (Figure 3.10C). Linear PEGss-5000 and branched 4PEGss modified RBC both measured lower levels of hemolysis compared to PEGss-2000 treated RBC both immediately following treatment and after 24 hours of storage (Figure 3.10B & C).

Immediately following treatment was the time point where differences between the level of hemolysis for linear PEGss-5000 and branched 4PEGss modified RBC was the greatest; at a polymer concentration of 0.5 mM, PEGss-5000 modified RBC hemolysed more than 4PEGss-modified RBC: 2.8 % vs. 0.13 % (Figure 3.10B).

However, 24 and 48 hours post modification, linear PEGss-5000 and branched 4PEGss modified RBC measured very similar average levels of hemolysis. The similar levels of average hemolysis were measured at treatment doses 0.5 mM and 2 mM PEG, and this similarity suggests that both polymer size and coupling chemistry contribute to the membrane instability of PEG modified RBC.

Twenty-four hours post treatment the levels of hemolysis for 0.5 mM PEGss-5000 and 0.5 mM 4PEGss modified RBC was 5.2 % and 5.4 %, respectively (Figure 3.10B). Forty-eight hours post modification, the level of hemolysis for 0.5 mM PEGss-5000 modified RBC is again slightly lower than 4PEGss modified RBC at this same treatment dose of 0.5 mM: 7.4 % and 8.8 % respectively (Figure 3.10B). At the 2.0 mM treatment dose, levels of hemolysis for 4PEGss modified RBC and PEGss-5000 modified RBC were again very similar when 24 and 48 hour post modification (Figure 3.10C) measured.
The level of hemolysis of RBC modified with equal molarity of reactive groups suggest that the shape of the polymer may also contribute to the membrane stability of the PEG-modified RBC; however, in these experiments, the effect of polymer shape appeared to be more pronounced immediately following treatment and then again at the end of the 48 hour storage period (Figure 3.11). Immediately following treatment, branched 4PEGss modified RBC had the lowest levels of hemolysis, 0.13 %, followed by 2.9 % and 4.6 % for linear PEGss–5000 and PEGss–2 000 respectively. Twenty–four hours post treatment linear PEGss–5000 and branched 4PEGss modified RBC showed similar levels of hemolysis, 5.2 % and 5.4 %, respectively. Following 48 hours of storage, the level of hemolysis increased slightly. 4PEGss modified RBC showed 8.8 % hemolysis, while the hemolysis levels of linear PEGss–5000 modified RBC increased slightly to 6.4 % (Figure 3.11). Considering that the molar concentration of reactive groups was equal between each PEG modified RBC samples (0.5 mM for each), these data suggest that polymer shape is an important factor which can affect the membrane stability of modified RBC.

Two–way ANOVA analyses showed that polymer type and polymer concentrations are both statistically significant predictors of hemolysis 24 and 48 hours post treatment (p < 0.05). Immediately following treatment polymer type did not reach statistical significance as a predictor of hemolysis (p = 1.0); however, polymer concentration did reach statistical significance as a predictor for hemolysis (p < 0.05). Taken together, the comparative hemolysis data suggest that coupling chemistry, polymer size and polymer shape all contribute to the membrane stability of PEG–modified RBC (Figure 3.11).
Figure 3.6 Hemolysis of 4PEGss Modified RBC Stored in SAGM at 4 °C.

Hemolysis increased over time for both control and 4PEGss modified RBC. However, greater levels of hemolysis measured for 4PEGss treated RBC indicate that modified cells are more susceptible to hemolysis. Represented above are averaged data from 3 independent experiments. Error bars represent 1 SD.
Figure 3.7 Effect of 4PEGss Treatment Dose on RBC Hemolysis of RBC Stored in SAGM at 4 °C. (■) Control Non-Modified RBC (□) 0.5 mM 4PEGss Modified RBC (○) 1.0 mM 4PEGss Modified RBC (◊) 2.0 mM 4PEGss Modified RBC (□) 5.0 mM 4PEGss Modified RBC. A = Immediately post modification (time = 0). B = 24 hours post modification. C = 48 hours post modification. Immediately post modification, 4PEGss treatment dose does not affect the level of RBC hemolysis. Twenty four hours and 48 hours post modification a non-linear relationship between 4PEGss treatment dose and hemolysis becomes apparent. For both these time points, 1 mM 4PEGss modified RBC show the lowest levels of hemolysis compared to all other treatment doses, indicating that 1 mM 4PEGss yields the most stable modified RBC. Represented above are averaged data from 3 independent experiments. Error bars represent 1 SD.
Figure 3.7 Effect of 4PEGss Treatment Dose on Hemolysis of RBC Stored in SAGM at 4°C
Figure 3.8 Hemolysis of 4PEGss Modified RBC Stored in HBSS at 37 °C. (—) Control Non-Modified RBC (— - -) 0.5 mM 4PEGss Modified RBC (— —) 1.0 mM 4PEGss Modified RBC (— — —) 2.0 mM 4PEGss RBC (— - - -) 5.0 mM 4PEGss Modified RBC. RBC samples stored in HBSS at 37 °C hemolysed less compared to RBC stored in SAGM at 4 °C (Figure 3.6). While both control non-modified and 4PEGss modified RBC showed low levels of hemolysis over the 48 hour storage period, 4PEGss treated RBC were more susceptible to hemolysis compared to non-modified RBC. Represented above are averaged data from 3 independent experiments. Error bars represent 1 SD.
Figure 3.9 Effect of 4PEGss Treatment Dose on Hemolysis of RBC Stored in HBSS at 37 °C. (■) Control Non-Modified RBC (□) 0.5 mM 4PEGss Modified RBC (○) 1.0 mM 4PEGss Modified RBC (▲) 2.0 mM 4PEGss Modified RBC (▼) 5.0 mM 4PEGss Modified RBC. A = Immediately post modification (time = 0). B = 24 hours post modification. C = 48 hours post modification. Immediately post modification (A), there is a linear 4PEGss treatment dose dependence for the level of hemolysis. 24 hours (B) post modification, 1 mM 4PEGss modified RBC show the lowest levels of hemolysis compared to all other treatment doses investigated. Forty eight hours (C) post modification the levels of hemolysis for 0.5 mM–2 mM 4PEGss treated RBC are similar. At this time point 5 mM 4PEGss modified RBC continue to show high levels of hemolysis, indicating that this very high PEG concentration results in an unstable modified RBC (C). Represented above are averaged data from 3 independent experiments. Error bars represent 1 SD.
Figure 3.9 Effect of 4PEGss Treatment Dose on Hemolysis of RBC Stored in HBSS at 37 °C

PEG Treatment Groups

PEG Treatment Groups

PEG Treatment Groups
Figure 3.10 Comparison of Hemolysis of Linear PEGss vs. Branched 4PEGss Modified RBC Stored in SAGM at 4 °C. (■) Linear PEGss–2000 (●) Linear PEGss–5000 (□) Branched 4PEGss–10,000. A = 0 mM PEG B = 0.5 mM PEG C = 2.0 mM PEG. Linear PEGss–5000 and branched 4PEGss modified RBC samples give similar levels of hemolysis, at all PEG treatment doses investigated, indicating that coupling chemistry, in addition to polymer size may also be a significant factor affecting the stability of PEG modified RBC. Above are averaged data from 3 independent experiments. Error bars represent 1 SD.
Figure 3.10 Comparison of Hemolysis of Linear PEGs vs. Branched 4PEGs Modified RBC Stored in SAGM at 4 °C
3.11 Comparison of Hemolysis of RBC Modified with Equal Molar Concentrations of Succinimidyl Succinate (ss) Reactive Groups and Stored in SAGM at 4 °C.

Figure 3.11 Comparison of Hemolysis of RBC Modified with Equal Molar Concentrations of Succinimidyl Succinate (ss) Reactive Groups and Stored in SAGM at 4 °C (■) Linear PEGss–2000 (0.5 mM SS reactive group) (□) Linear PEGss–5000 (0.5 mM ss reactive group) (☺) Branched 4PEGss–10,000 (0.5 mM ss reactive group). The effect of polymer shape on hemolysis is more pronounced immediately following (time = 0) and 48 hours post modification, demonstrating that in addition to polymer size and coupling chemistry, polymer shape is an important factor affecting the stability of PEG modified RBC. Represented above are averaged data from 3 independent experiments. Error bars represent 1 SD.
3.8 Hemoglobin Oxidation Species

Hemoglobin oxidation was monitored, over time, in both control and 4PEGss modified cells in order to measure the impact of branched 4PEGss modification on RBC function. The levels of oxyhemoglobin and methemoglobin were measured immediately following, 24 hours and 48 hours treatment.

Immediately following treatment (time=0), the oxyhemoglobin levels for control cells measured at 98.4 % (Figure 3.12). 4PEGss modified RBC generally had a comparable level of oxyhemoglobin with the exception being cells treated with 5 mM 4PEGss. At this concentration, oxyhemoglobin levels measured an average of 85.8 % (Figure 3.12). This decreased level of oxyhemoglobin, in comparison to other samples, may be due to high levels of spontaneous lysis (Figure 3.4, 3.5 and 3.6) in this sample consequent to the high PEG treatment dose. Hemolysis leads to the release of intracellular hemoglobin into the extracellular environment where it is rapidly converted to methemoglobin (Winterbourn 1990).

There is a possibility that high PEG treatment dose imparts membrane instability, thus causing hemolysis and ultimately leading to decreased levels of oxyhemoglobin (Figure 3.12) and concurrent increased levels of methemoglobin (Figure 3.12) recorded at this time point. For all other samples (control and PEGylated), the methemoglobin levels were the lowest immediately following treatment, (less than 2.0 %), with the exception of 5 mM 4PEGss modified cells having a methemoglobin concentration of 14.2% (Figure 3.13). The level of oxyhemoglobin remained high 24 hours following treatment. Control non–modified RBC had an average of 98.2 % oxyhemoglobin; this level is similar to that measured for 4PEGss modified RBC at all polymer concentrations investigated (Figure 3.12). At this time point 5 mM 4PEGss modified cells behaved similarly to all other PEG concentrations investigated as well as to control non–modified cells (Figure 3.12). The similar levels of oxyhemoglobin among samples at this time point may result from earlier hemolysis of the most heavily derivatized cells.
Therefore those RBC that remain may be, overall, a less derivatized population and therefore may be less susceptible to hemolysis. Thus the hemoglobin would remain intracellular where methemoglobin reductase would be able to convert any oxidized hemoglobin back to the oxyhemoglobin form.

As expected, 48 hours following treatment, the levels of oxyhemoglobin began to decrease (Figure 3.12) while methemoglobin levels began to increase (Figure 3.13). The oxyhemoglobin and methemoglobin levels measured at 45.6 % and 55.5 % respectively. Similar to the controls, oxyhemoglobin levels for 4PEGss modified RBC fell in the range 36.5–56.6 % and methemoglobin levels measured in the range 49.4–63.5 % (Figure 3.12 and 3.13).

While there were slight differences observed among the levels of hemoglobin oxidation species for the different 4PEGss concentrations investigated, this variation did not reach statistical significance (p ≥ 0.6). Storage time, as expected, was a statistically significant predictor of both oxyhemoglobin (p < 0.05) and methemoglobin (p < 0.05) levels over the 48 hour storage period. Thus, the data suggest that compared to non–modified control RBC, 4PEGss modified RBC (apart from 5 mM 4PEGss modified cells) do not show an altered rate for generation of hemoglobin oxidation species.
Figure 3.12 Oxyhemoglobin Levels Generated Over a 48 Hour Storage Period

Control Non-Modified RBC (→) 0.5 mM 4PEGss Modified RBC (□) 1.0 mM 4PEGss Modified RBC (○) 2.0 mM 4PEGss Modified RBC (□•□•□•) 5.0 mM 4PEGss Modified RBC.

Samples were stored at 4 °C for the duration of the experiment. Immediately following treatment 5 mM 4PEGss modified RBC show much lower oxyhemoglobin levels compared to all other samples, evidence to support that this high PEG dose may cause RBC membrane instability. Twenty four and 48 hours later oxyhemoglobin levels begin to decrease. For both these time points all samples behave similarly, suggesting that the most unstable cells have hemolyzed, leaving behind a more homogeneous RBC population. Represented above are averaged data from three independent experiments. Error bars represent 1 SD.
Figure 3.13 Methemoglobin Levels Generated Over a 48 Hour Storage Period

Samples were stored 4°C for the duration of the experiment. Methemoglobin levels inversely followed oxyhemoglobin levels (Figure 3.12). Immediately post modification, 5mM 4PEGss treated RBC showed the highest methemoglobin levels. This is likely due to membrane instability, as a result of a high PEG treatment dose that may cause the hemolysis followed by leakage of hemoglobin to the extracellular environment and ultimately rapid conversion of released hemoglobin to methemoglobin. At 24 and 48 hours post treatment, all samples behaved similarly suggesting that by these time points the RBC population was more homogeneous.

Represented above are averaged data from 3 independent experiments. Error bars represent 1 SD.
4. Discussion

The complexity of blood transfusion should not be overlooked in spite of its common practice in modern medicine. Current research involving a variety of disciplines continues to work towards both a safer blood supply and decreasing the risk of transfusion related complications. The focus of this study was on RBC antigenicity, a feature that continues to challenge the safety and effectiveness of the red cell transfusion procedure.

The aim of this work was to describe the level of immunocamouflage achieved for RBC the major blood group antigens, as well as to determine the resulting stability of the RBC when modified with a new branched 4PEGss polymer. This study also involved comparison with RBC modification by linear PEG polymers chosen based on their extension from the membrane surface. In addition to creating a potentially more effective antigenic camouflage, the possible advantage of modifying the RBC surface using a branched polymer could be an increased stabilization of the cell membrane, thus increasing the red cell’s resistance to spontaneous hemolysis.

The first specific aim was to compare the level of blood group antigenic camouflage achieved using a four branched PEGss polymer and compare with the degree of immunocamouflage achieved for RBC modified with a linear polymer. This objective was achieved using an agglutination assay followed by a quantitative flow cytometric assay. The total MW of the 4 branches of the PEG molecule is 10,000, but its maximum extension from the membrane would be similar to that of a 5000 MW PEG if 1, 2 or 3 of the 4 branches were coupled, or that of a 2500 MW PEG if all 4 branches were coupled. Linear polymers of these two different molecular weights were used to mimic the maximum radii of the camouflage zone imparted to RBC modified using the branched 4PEGss polymer. Thus linear PEGs of MW 5000 and MW 2000 were chosen for comparison because MW 2500 PEGss is not commercially
available. The level of A, B and RhD antigen camouflage for AB+ PEG modified RBC was first measured qualitatively by an agglutination assay. Recently published data (Nacharaju et al. 2005) demonstrated that modification of human RBC using sulfhydryl chemistry with maleimidophenyl–PEG reagents of different chain lengths, effectively camouflaged both RhD and A blood group antigens as demonstrated by a decreased agglutination assay score of 4+ to 0. Other groups have also reported decreased agglutination scores (4+ reduced to 1+) when modified RBC were tested with anti–RhD serum (Scott et al., 1997). These RBC were treated with a linear PEG (MW 5000) covalently attached to the RBC surface using methoxy chemistry.

In this present study 4PEGss treated RBC gave an agglutination level of at the 1+ indicating that 4PEGss modified RBC agglutinate to a lesser degree when tested with commercial RhD typing serum (Table 3.1). This level of camouflage was not un–expected as this blood group antigen family is a transmembrane surface protein located close to the cell membrane. Being so close to the cell membrane therefore increases the efficiency with which this antigen is camouflaged (Figure 1.6).

Our studies also demonstrate a reduced agglutination score for linear PEG-5000 and branched 4PEGss modified cells incubated with B typing serum (Table 3.1). In comparison however, there was no parallel decrease, for any PEG polymer investigated, of the agglutination scores for PEG modified cells incubated with type A serum (Table 3.1). Initially this was surprising; however, in general, antigens of the ABO blood group family are more challenging to effectively camouflage as this carbohydrate–based antigen family extends further from the membrane surface compared to the transmembrane protein RhD blood group antigen family.

Each of the ABO antigenic sites is the result of specific transferases which apply the respective A and B immunodominant sugars onto the ends of a common precursor protein. Thus there is a level of competition between the “A” and “B” transferases resulting in a difference of copy number between antigens A and B. Generally there is a significantly higher copy number
of blood group antigen A sites on type AB+ adult human RBC, approximately 0.81–1.17 \times 10^6 per cell vs. 0.43 \times 10^6 B sites per cell (Ried and Lomas-Francis 1997). Therefore, the high copy number of blood group antigen A sites likely accounts for both the inability of PEG treatment to reduce the macroscopic agglutination score shown for RBC tested with A typing serum as well as the only slightly reduced score reported for linear PEG–5000 and branched PEG treated erythrocytes incubated with B typing serum. Taken together, these results support a polymer concentration and size dependence for effective immunocamouflage. As well there appears to be a more efficient camouflage achieved via treatment with branched 4PEGss as compared to equivalent concentrations of either linear PEGss–2000 or linear PEGss–5000.

PEG–mediated steric hindrance of the access of antibodies to the blood group antigens, in effect creating a zone of antigenic camouflage surrounding the cell, is the likely mechanism whereby RBC antigen modulation is achieved. As to how the zone of antigen camouflage is achieved, there are two possible scenarios: the first possibility is that all four legs of the branched polymer are bound onto the RBC surface. In this case, the estimated maximum radius of the camouflage zone would be 23.3 nm, which is similar to the radius of the camouflage zone achieved using a linear PEGss–2000 polymer (18.4 nm). The second possibility is that 1, 2 or 3 of the 4 polymer legs do not attach onto the RBC surface. This would thus extend the radius of the immunocamouflage zone from 23.3 nm to about 46.6 nm, which is comparable to the approximately 58.3 nm radius achieved for RBC modified with a linear PEGss–5000 polymer. Therefore the rationale for choosing linear PEGss–2000 and 5000 was to more closely resemble the radii of the camouflage zones that can be created as a result of branched 4PEGss modification, thus providing a relevant comparison.

In agreement with previously published reports (Scott et al. 1997, Murad et al. 1999, Bradley et al. 2002) the efficiency of blood group antigen camouflage increases with increasing polymer concentration. Also in agreement with previous studies (Bradley et al. 2002, Nacharaju
we show that blood group antigen camouflage efficiency is dependent on the size of the polymer. Although A antigen camouflage was not demonstrated by agglutination assays, flow cytometry assays demonstrate that both blood group antigens RhD (Figure 3.1A) and A (Figure 3.1B) are more effectively camouflaged when cells are modified with the branched polymer compared to erythrocytes treated with either of the linear PEG polymers investigated. Moreover, compared to linear PEGss polymer modified RBC, branched 4PEGss modified red cells are also more efficiently immunocamouflaged at lower polymer concentrations.

Eighty seven % of antibody–RhD interactions were blocked by a polymer concentration of 2 mM when RBC were treated with 4PEGss. In comparison, this level of immunocamouflage was achieved with a polymer concentration of 5 mM when cells were modified with either of the linear PEGss–2000 or 5000 (Figure 3.1A). Similarly, an average of 88 % of the blood group antigen A-antibody interactions were blocked as a result of treatment with 4PEGss at a final concentration of 2 mM. Red cells treated with either linear PEGss–2000 or 5000 could not reach this level of immunocamouflage, even at the highest concentration investigated of 5 mM (Figure 3.1B).

Based on both the agglutination and the flow cytometry assays, PEG-mediated blood group antigen camouflage can be achieved and this camouflage is more efficient with the branched 4PEGss than with the comparable linear PEGss polymers.

The second objective of this thesis was to clarify the functional consequences of 4PEGss treatment on normal red cell physiology. The RBC is a cell type which exemplifies a unique interdependent relationship between cell shape and function. Therefore SEM was employed to investigate 4PEGss–mediated changes to RBC morphology and used as an initial indicator for the potential consequences of 4PEGss treatment. The primary observation reported in this study was that inter–cell cross linking was evident as small cell aggregates, present as a result of 4PEGss treatment (Figure 3.2B, C, and D & E). Moreover, the degree of cross-linking appeared
to increase with polymer concentration which supports a PEG-treatment dose dependent effect (Figure 3.2B, C, and D & E). These results agree with previously published data reporting the formation of red cell aggregates for RBC modified with a bifunctional PEG polymer (Bradley et al. 2002). The formation of cross-linked red cell aggregates is a result of the linker groups located on each polymer (Bradley et al. 2002) two linker groups as shown by Bradley et al. and four as shown by this study.

It was also observed that the texture of the cell membrane of 4PEGss modified RBC appeared rougher (Figure 3.2B, C, and D & E). This may be the result of surface protein cross-linking which may occur in addition to the inter-cell cross-linking. Again, this trend was also 4PEGss concentration dependent with 5 mM 4PEGss modified RBC exhibiting the greatest number of RBC aggregates as well as the most visible surface texture changes (Figure 3.2E).

*In vivo,* erythrocytes travel through the vasculature in a rouleau formation. This arrangement requires both surface charge neutralization as well as transient cell–cell interactions (Hardwicke and Squire 1952 and Scott and Chen 2001). Thus ESR can be considered to be a functional assessment of the impact of 4PEGss modification on the red cell’s surface charge as well as on cell-cell interaction. In this study, 4PEGss modified RBC, at all 4PEGss concentrations investigated, measured a normal ESR (Figure 3.3), a finding which contradicts previously published reports which used both monofunctional and bifunctional PEG polymers under similar conditions to those used in this study. Those studies reported a decreased ESR for PEG–treated RBC (Bradley et al. 2002), and explain that PEG molecules covalently grafted onto the RBC surface would sterically hinder cell–cell interactions (Scott et al. 2000, Chen and Scott 2001) and therefore rouleau formation would be affected as shown by a decreased ESR (Bradley et al. 2002).
If the 4PEGss uniformly modified the RBC, they would be no longer able to participate in cell–cell interactions in vitro, and thus any phenomenon dependent on such interaction, such as rouleau formation should be affected. Although the data in figure 3.3 do not reach statistical significance, there appears to be a trend toward reduced rouleau formation with increasing 4PEGss concentration. The lack of achieving statistical significance may be due to small RBC aggregates formed by inter–cell cross–linking which may provide the necessary increased surface area to mass ratio required to maintain an apparently normal velocity ESR. This hypothesis of 4PEGss–mediated inter–cell cross–linking is further supported by the scanning electron micrographs (Figure 3.2).

Red cell indices such as MCV, MCH, MCHC and RDW were also measured for control and 4PEGss modified RBC. These red cell indices show that PEGylation most directly affects cell size (Table 3.2). This may be the result of 4PEGss–mediated inter–cell cross–linking; as the cross–linked RBC move through the hematology analyzer detection lasers, the machine reports “one larger” RBC rather than several aggregated red cells. In line with this, another possibility is that the “texture” seen on SEM could be interpreted by the hematology analyzer as a size increase, rather than a minor change in cell shape. The third possibility is that 4PEGss treatment may affect RBC membrane stability. Osmotic fragility assays (Figures 3.4 & 3.5) and hemolysis measurements (Figures 3.6, 3.7, 3.8 & 3.9) indicate that 4PEGss modified RBC are more susceptible to hemolysis, which may be due to cell membrane instability as a result of 4PEGss treatment. Therefore the increased red cell volume reported for 4PEGss could be consequent to 4PEGss–mediated membrane instability which could ultimately lead to influx of water inside the RBC and thus the red cell volumes would be increased.

The osmotic fragility assay was used to measure how 4PEGss modification affected RBC stability. The osmotic fragility profiles generated both immediately following 4PEG modification and following 24 hour incubation at 37 °C show that 4PEGss treated cells are more
susceptible to hemolysis which may result from 4PEGss–mediated effects on red cell membrane stability. Evidence for this comes from both the increased salt concentration required to reach MCF and the high levels of hemolysis measured for 4PEGss modified cells, even at isotonic conditions (Figure 3.4 and 3.5). Scott et al. (1997) reported no difference between the osmotic fragility profiles of monofunctional linear PEG–treated RBC modified at concentrations comparable to those employed in this study. Thus, the different effect of 4PEGss treatment is likely a result of the membrane instability promoted by RBC treatment with either the branched polymer or the coupling chemistry.

To clarify the consequence of 4PEGss treatment, hemolysis was measured over a 48 hour storage period using two different conditions. Data from this experiment support the association between 4PEGss modification and decreased membrane stability compared to non–modified control RBC (Figure 3.6 & 3.8).

However, the data did not follow a linear polymer concentration dependence. 4PEGss RBC modification using 1 mM PEG showed less hemolysis, when samples were stored in SAGM at 4 °C, than any other 4PEGss concentration investigated (Figure 3.6 and 3.7).

Observations made in numerous reports looking at oxygen–mediated red cell damage showed that in many cases either a small degree or a high degree of damage lead to high levels of hemolysis (Ferrali et al. 1992). Similarly, both low and high concentrations of 4PEGss treatment led to RBC membrane instability that resulted in higher levels of red cell hemolysis than intermediate polymer grafting levels.

Another observation from this experiment was that the red cells appear to be more stable at 37 °C than at 4 °C (Gyongyossy–Issa et al. 2005). The enhanced effect of 4PEGss treatment was accentuated at the lower storage temperature presumably due to the RBCs’ decreased metabolic activity. The decreased metabolic activity may cause less efficient functioning of the RBC membrane ion pumps and therefore the cell would be less able to maintain the ideal
transmembrane gradients necessary to protect the red cell from hemolysis. In addition to this, the surface protein modification reactions that occur with 4PEGss treatment may modify the membrane ion pumps themselves, affecting their ability to function optimally and thus also contributing to the increased level of hemolysis.

Hemolysis of linear PEGss modified RBC stored in SAGM at 4 °C was measured for comparison with branched 4PEGss modified RBC stored under the same conditions. The results of these experiments show that the difference in the degree of hemolysis is more pronounced between linear PEGss–2000 and branched 4PEGss–10,000 modified RBC treated with the same PEG polymer concentration; linear PEGss–2000 modified RBC showed more hemolysis compared to branched 4PEGss modified RBC. Comparatively, the difference in degree of hemolysis for the linear PEGss–5000 and branched 4PEGss modified cells is minimal (modified at equal polymer concentrations (Figure 3.10) and at equal molar concentrations of the succinimidyl succinate reactive group (Figure 3.11), with the most pronounced differences occurring immediately following treatment (time = 0) and 48 hours post modification (Figure 3.10 & 3.11). Taken together these data suggest that polymer size and geometry are important factors that may affect the stability of PEG modified RBC. Also, the observation that branched 4PEGss treatment offers more protection from hemolysis immediately following treatment, combined with the more effective antigenic camouflaged achieved with branched 4PEGss treatment also suggest that this treatment may be most effective if applied immediately prior to transfusion rather than at the time of the initial RBC donation.

Scott et al. (1997) modified RBC using a linear PEG polymer covalently coupled onto the red cell surface via a methoxy functional group (MW 5000). They reported less than 5 % hemolysis for treated RBC 24 hours post treatment for RBC samples modified at PEG treatment doses and storage conditions similar to those used in this study (the Scott group also used two storage condition: 4 °C in SAGM and 37 °C in HBSS). Our study also shows that following 24
hours of storage, linear PEGss–5000 and branched 4PEGss modified RBC show an average hemolysis of 5%. However, while the methoxy–PEG modified RBC continue to show low levels of hemolysis even 48 hours post treatment; PEGss modified RBC samples show a much higher level of hemolysis after 48 hours of storage. Therefore, in addition to polymer size and geometry, the chemistry of the PEG linker group is also important to consider when developing the desired stable antigenically silent red cell for transfusion.

Generation of the hemoglobin oxidation species can be used as an indication of the ability of the red cell to perform its main function; delivering O₂ to the tissues and removing CO₂ from the tissue. Therefore the level of the hemoglobin oxidation species oxyhemoglobin and methemoglobin were monitored over time as another method to measure the impact of 4PEGss modification on potential red cell function. In general, the levels of oxyhemoglobin were higher for both 4PEGss modified and non–modified control RBC samples, especially considering that these are venous blood samples (Figure 3.12). The high oxyhemoglobin levels are most probably an artifact of the treatment protocol which exposes the venous blood samples to oxygen from the atmosphere.

An exception to the high levels of oxyhemoglobin was found for 5 mM 4PEGss modified RBC (Figure 3.12). At this treatment dose, many possible cross–linking events may have occurred, leaving the heavily derivatized RBC membranes unstable and therefore susceptible to hemolysis. Intracellularly, conversion of oxyhemoglobin to methemoglobin is reversible due to the actions of cytochrome b₅ reductase (also referred to as methemoglobin reductase), an enzyme which functions to reduce methemoglobin back to oxyhemoglobin (Winterbourn 1990). Due to treated RBC membrane instability, oxyhemoglobin may leak to the extracellular environment where it will be quickly oxidized to methemoglobin.

However 24 and 48 hours post–modification all samples, 4PEGss modified and non–modified control RBC showed comparable levels of oxyhemoglobin. This may be because
enough time had passed such that the most heavily derivatized 4PEGss treated cells may had already hemolysed, leaving behind a less modified, more stable homogeneous cell population and thus the oxyhemoglobin levels would be very similar between control non-modified and 4PEGss modified RBC, regardless of treatment dose.

The methemoglobin levels followed an inverse pattern to the oxyhemoglobin levels; higher oxyhemoglobin levels occurred with lower methemoglobin levels whereas higher methemoglobin levels resulted in decreased levels of oxyhemoglobin (Figure 3.13). This inverse pattern was expected because methemoglobin is a more oxidized form of oxyhemoglobin. Therefore, as the oxyhemoglobin was oxidized to methemoglobin, we expected the levels of oxyhemoglobin to decrease and the methemoglobin levels to increase concurrently.

The results of this experiment also showed that apart from 5 mM 4PEGss modified RBC, treated red cells behaved similarly to control non-modified RBC, indicating that lower 4PEGss treatment doses do not accelerate the generation of hemoglobin oxidation species and therefore may not alter the normal oxygen carrying capacity of the red cell.
Production of an antigenically modified human red cell transfusion product would be a great achievement in the area of blood transfusion technology. The over-arching intention of this study was to define the effectiveness of using a four branched PEG polymer, 4PEGss, to create an antigenically silent and osmotically resistant modified RBC. 4PEGss modified RBC were more efficiently immunocamouflaged for blood group A, B and RhD antigens than linear PEGss derivatized RBC. This conclusion is supported by both macroscopic agglutination assays (Table 3.1) and flow cytometry (Figures 3.1A & B).

Scanning electron microscopy showed both inter-cell linkages as well as visible surface modification which are believed to be consequences of the functional groups bound at the end of each branched polymer (Figure 3.2 A, B, C, D & E). Absence of obvious changes to the ESR (Figure 3.3) suggest that while red cell charges may be masked, cross-linked small clumps may compensate and thus 4PEGss modified RBC samples showed no apparent 4PEGss-mediated effect on the ESR. Standard red cell physical indices (MCV, MCH, MCHC and RDW) (Table 3.2) as well as more fragile osmotic profiles (Figure 3.4 & 3.5) lend support to the hypothesis that 4PEGss treatment, due to either the succinimidyl succinate coupling chemistry, or the multiple functional groups per polymer (polymer shape), sufficiently alters membrane architecture such that modified cells are more susceptible to hemolysis.

In agreement with the above measurements, hemolysis taken over a 48 hour storage period revealed that a higher percentage of 4PEGss modified RBC hemolysed compared to non-modified control cells (Figures 3.6, 3.7, 3.8 & 3.9). Interestingly however, the degree of hemolysis for linear PEGss–2000 was much greater compared to both linear PEGss–5000 and branched 4PEGss modified red cells (Figure 3.10B & C). Moreover, levels of hemolysis for
linear PEGss–5000 and 4PEGss modified RBC were very similar. Taken together with previously published data which measured the hemolysis for linear methoxy PEG (MW 5000) modified RBC (Scott et al. 1997), this study concludes that polymer size, polymer geometry as well as the polymer coupling chemistry are all factors that have an impact the stability of PEG modified RBC. Therefore, future studies are necessary to define the optimal modification chemistry, polymer size and polymer geometry.

Results from the present work also show that the effects of 4PEGss modification are not strictly dose dependent as 1 mM 4PEGss modified RBC were more stable compared to all other treatment doses investigated i.e. 1 mM 4PEGss modified RBC showed the lowest levels of hemolysis, and at the same time this treatment dose still offered an effective degree of blood group antigen camouflage. Thus a future direction for this project will be to determine if 1 mM 4PEGss RBC modification immediately prior to the transfusion procedure is a practical strategy to prevent immune–mediated transfusion complications. In line with this, another future investigation will be to determine the degree of immunocamouflage achieved for minor blood group antigens— a driving force behind transfusion complications for the chronically transfused.
6. References


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