

# Inhibition of phagocytic recognition of anti-D opsonized Rh D+ RBC by polymer-mediated immunocamouflage

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The Rh D antigen posed both a significant clinical risk and inventory supply issue in transfusion medicine. The successful development of the immunocamouflaged RBC has the potential to address both the risk of acute anti-D transfusion reactions and to improve D- blood inventory in geographic locations where D- blood is rare (e.g., China). The immunocamouflage of RBC was mediated by the covalent grafting of methoxy(polyethylene glycol) to the cell membrane thereby obscuring the D protein from the immune system. To determine the potential efficacy of mPEG-D+ RBC in D- recipients, anti-D alloantibodies from previously alloimmunized individuals were utilized. The effects of polymer chain size (2–30 kDa) and grafting concentration (0–4 mM) on antibody binding and erythrophagocytosis were determined using the clinically validated monocyte monolayer assay (MMA) and flow cytometry. The immunocamouflage of D was polymer size and grafting concentration dependent as determined using human anti-D alloantibodies (both pooled [RhoGAM] and single donors). Importantly, the 20 kDa polymer provided excellent immunocamouflage of D and reached a clinically significant level of protection, as measured by the MMA, at grafting concentrations of  $\geq 1.5$  mM. These findings further support the potential use of immunocamouflaged RBC to reduce the risk of acute transfusion reactions following administration of D+ blood to D- recipients in situations where D- units are unavailable or supply is geographically constrained.

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## Introduction

Red blood cell (RBC) transfusions are an essential tool in clinical medicine with over 100 million units collected annually worldwide [1–3]. However, despite their extensive use, RBC are immunologically complex and their transfusion can pose a significant risk of alloimmunization [4–10]. The immunologic complexity of RBC arises from the presence of 35 blood group systems consisting of more than 300 unique antigens that vary between different ethnic and racial groups [11–13]. Among the non-ABO blood groups, the Rh blood group is one of the most polymorphic and immunogenic with more than 50 serologically-defined antigens including D, C, c, E, and e [6,12,14,15]. Of these, D is the most immunologically and clinically important.

In North America and Western Europe the supply of D- blood, while often problematic, can typically be met due to the relatively high incidence of D- donors as approximately 15% of Caucasians and 5%–7% of Blacks are D- [16,17]. However, the frequency of D- individuals has significant geographical and racial bias and the adequate supply of D- blood poses a significant challenge within non-European blood collection systems. This is dramatically illustrated in China where D- individuals represent only 0.1%–0.4% of the population [16]. Thus, D presents challenges in the maintenance of blood inventories and of acute transfusion reactions when transfused into a D- individual.

The “immunocamouflage” of D+ RBC may address both the inventory issues and immunologic risks that D+ blood poses to the D- recipient. The immunocamouflage of cells is produced by the covalent grafting of biologically safe and immunologically inert polymers such as methoxypoly(ethylene glycol) (mPEG), polyethoxyazoline (PEOZ), and hyperbranched polyglycerols (HPG) to RBC membrane proteins; though research suggests that mPEG provides superior biologic immunocamouflage [18–28]. Mechanistically, the grafted polymer induces cellular immunocamouflage by both steric hindrance and charge camouflage of surface antigens on the donor RBC [18,22,24,27,28]. Importantly normal cell morphology, structure, and function are not affected adversely by the grafted mPEG polymer at immunoprotective grafting concentrations. Moreover, in murine transfusion models mPEG-modified RBC exhibits normal in vivo survival [18,19,25].

*Additional Supporting Information may be found in the online version of this article.*

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**Conflict of Interest:** If yes, please state: Dr. Scott is a listed inventor on patents related to RBC PEGylation. Canadian Blood Services (CBS), a not-for-profit, blood operator is the assignee of said profit. Neither Dr. Scott nor CBS receives any income or other consideration from these patents.

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To investigate the potential utility of immunocamouflage in preventing D recognition, we analyzed the ability of the immune cells to recognize and phagocytose mPEG-grafted D+ RBC following opsonization with human-sourced anti-D alloantibodies. Immune recognition was assessed using the clinically validated monocyte monolayer assay (MMA) [29–32]. The MMA measures FcγR-mediated phagocytosis of alloantibody-coated donor cells and is a clinical predictor of acute transfusion reactions [29,30,32–36]. Using MMA and flow cytometry, the effects of mPEG polymer size and grafting density on the immunocamouflage of D+ RBC was examined.

## ■ Methods and Materials

### Human RBC, PBMC isolation, and plasma collection

All experiments using human blood cells were done following protocol approval by the University of British Columbia Clinical Research Ethics Board and in accordance with the Declaration of Helsinki. Following informed written consent, donor blood was collected in EDTA-Vacutainer® collection tubes (BD, Franklin Lakes, NJ) from normal D+ and D− donors. RBC were washed (3×) in isotonic saline prior to use. De-identified human anti-D alloantibody plasma samples were obtained from volunteer blood donors at Life-Share Blood Centers (Shreveport, LA) and stored at −80°C until used. Peripheral blood mononuclear cells (PBMC) were isolated using histopaque-1077 (Sigma-Aldrich, St. Louis, MO) and washed (2×) with PBS prior to suspension in culture media.

### PEGylation of RBC

Washed RBC were covalently grafted (derivitized) with methoxy-poly(ethylene glycol) succinimidyl valerate (SVA-mPEG; Laysan Bio Inc. Arab, AL) using different polymer size (2, 5, 10, 20, and 30 kDa) as previously described [18–28]. RBC suspensions (12% final hematocrit) were prepared in mPEG buffer (50 mM K<sub>2</sub>HPO<sub>4</sub>, 105 mM NaCl, pH 8.0), mixed with the activated mPEG solution (final grafting concentrations of 0–4 mM) and incubated for 30 min at room temperature followed by washing (3×) with isotonic saline. The activated polymer species used in this study non-specifically targets protein lysine residues on the external surface of the RBC [18–28]. While only small volumes (2–3 mL) of blood were necessary for the described studies, the semi-automated methodology used is fully scalable for full units of RBC.

### Monocyte monolayer assay (MMA)

The MMA, which measures FcγR-mediated adherence and phagocytosis of opsonized RBC by monocytes, was used to assess the efficacy of grafted polymer in preventing immune recognition and phagocytosis [29,30,34,36,37]. Control and polymer grafted D+ RBC (~75 μL of a 3% hematocrit) were opsonized with either approximately 150 μL of undiluted commercial anti-D antibody [Rh<sub>0</sub>(D) Immune Globulin (Human) RhoGAM Ultra-Filtered PLUS; Ortho Clinical Diagnostics] or undiluted anti-D plasma obtained from alloimmunized individuals. Opsonized RBC were then washed, overlaid on the monocyte monolayer, and incubated at 37°C (5% CO<sub>2</sub>) for 60 min. After incubation, the slides were stained with Wright-Giemsa and phagocytosis was assessed via light microscopy. Positive and negative controls consisted of RhoGAM opsonized D+ (positive control; typically yielding an MI between 60% and 100%) and D− (negative control; MI typically <3%) human RBC. The Monocyte Index (MI) represents the percentage of macrophages with adhered or phagocytized RBC divided by the total number of monocytes. MI values of ≤5% indicate that incompatible RBC can be given with minimal risk of an acute hemolytic reaction (although less predictive of long-term circulation) [32,33,35]. The MMA used in this study did

not include complement addition thus there was no lytic component to measure.

### Analysis of D blood group antigen by flow cytometry

The efficacy of mPEG grafting on the immunocamouflage of the D blood group antigen was measured by flow cytometry (FACS CantotII, BD Biosciences, Mississauga, ON, CA). Immunocamouflage of D blood group antigens was evaluated using both total mean cell fluorescence (MCF) and percent positive cell (PPC) values. Control and mPEG-modified RBC were diluted to a 3% hematocrit in isotonic saline and incubated with RhoGAM or human plasma from D-alloimmunized patients for 30 min at 37°C followed by washing (3×) and re-suspension in PBS containing FITC conjugated anti-human IgG-Fc specific secondary antibody (Abdserotec, Oxford, UK). The mixture was incubated for 30 min at 37°C, washed with PBS (3×) and resuspended in 0.5 mL of 1% methanol free formaldehyde (MFF) for analysis via flow cytometry as previously described [25,28]. A minimum of 20,000 cells were counted per sample.

### Indirect antiglobulin test (IAT) and IgG subclassing

IAT and IgG subclassing were done on the human alloantibodies. Washed RBC were incubated with human plasma for 60 min at 37°C, then washed three times in PBS, and resuspended in saline (2%–5%). An aliquot of the RBC suspension was tested by IAT using anti-IgG and anti-C3, to estimate the degree of sensitization. IgG subclassing was done in microtiter plates (0.04% RBC suspension) by doubling dilutions (starting at 1:10) of sheep anti-human IgG1, IgG2, IgG3, and IgG4 (PELICLASS Human IgG Subclass, Sanquin, Amsterdam, The Netherlands). Following overnight incubation at 2°C–8°C, the microtiter plates were placed at an angle of 60° and the agglutination was read macroscopically.

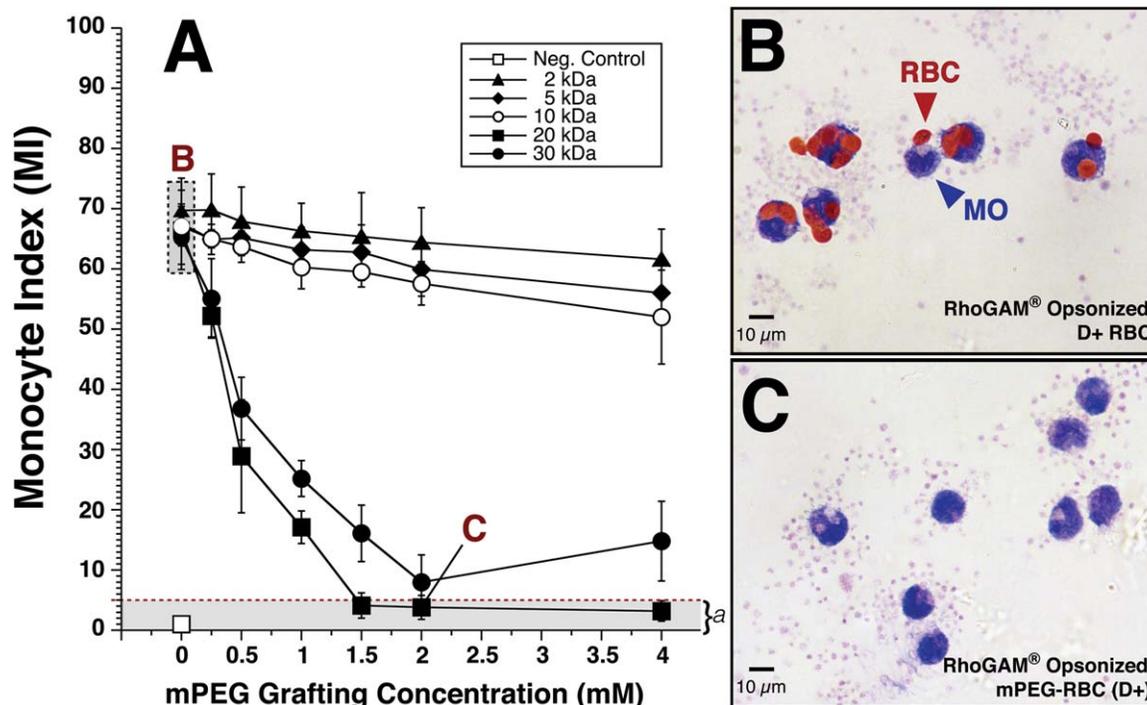
### Statistical analysis

Statistical analysis was conducted using SPSS v.16.0 statistical software (Statistical Products and Services Solutions, Chicago, IL). Student's *t*-test was used for comparison of two mean values. When three or more means were compared, a one-way analysis of variance (ANOVA) was performed followed by a post hoc analyses using the Tukey test. A *P* value of <0.05 was considered statistically significant.

## ■ Results

To assess the effects of grafting concentration and polymer size on the efficacy of immunocamouflage, D+ donor RBC were modified with increasing amounts (0–4 mM) of 2, 5, 10, 20, and 30 kDa mPEG followed by opsonization with RhoGAM anti-D antibody. As shown in Fig. 1, phagocytosis of opsonized D+ mPEG-RBC in the MMA was both size and grafting concentration dependent. While short chain polymers (e.g., 2–10 kDa) were ineffective (*P* > 0.05) at camouflaging D, long chain polymers (20 kDa and 30 kDa) significantly blocked phagocytic recognition of RhoGAM-opsonized D+ RBC (*P* < 0.005 at 0.5 mM and *P* < 0.001 at concentrations > 0.5 mM). Of note, the 20 kDa polymer was actually superior (*P* < 0.05) to the 30 kDa polymer. Importantly, the 20 kDa polymer reduced the MI to ≤5% indicating that the RhoGAM-opsonized D+ mPEG-RBC would be unlikely to cause an acute transfusion reaction.

Because RhoGAM is a highly purified and concentrated anti-D IgG antibody, it does not fully reflect the biological/clinical heterogeneity of alloimmunized individuals. To better assess the potential utility of mPEG-RBC in alloimmunized individuals, frozen plasma samples from D alloimmunized individuals (*N* = 8) were assessed using the MMA. The strength of serologic activity in these eight samples ranged from weakly macroscopic positive (W+) to very strong (4+) reactions and did not correlate significantly with the MI value



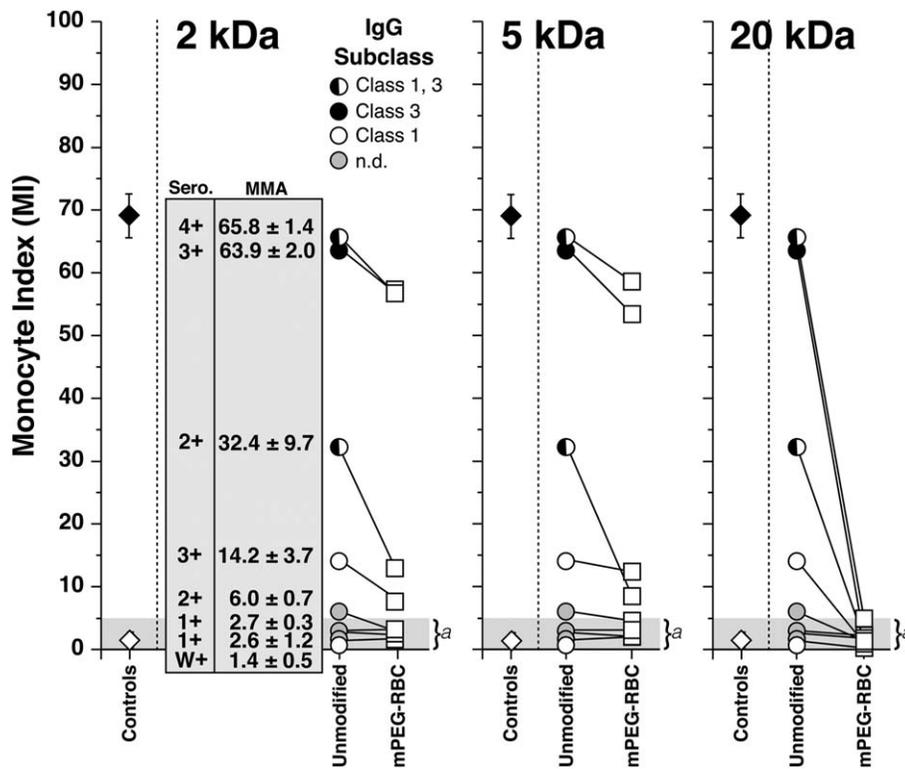
**Figure 1.** Polymer size and grafting concentration governs the efficacy of D immunocamouflage and the inhibition of erythrophagocytosis. **Panel A:** Effect of polymer size and grafting concentration on MMA phagocytosis of RhoGAM opsonized D+ RBC. As shown, short chain polymers (2–10 kDa) were ineffective at inhibiting erythrophagocytosis. In contrast, membrane modification of D+ RBC with both the 20 and 30 kDa mPEG showed a significant ( $P < 0.005$  at  $\geq 0.5$  mM grafting concentration) dose dependent decrease in phagocytosis. Importantly, the 20 kDa polymer effectively reduced the MI value to less than 5% at grafting concentrations  $\geq 1.5$  mM. Interestingly, at equimolar concentration, the 30 kDa polymer was less effective than the 20 kDa polymer. Shown are the mean  $\pm$  SD of a minimum of three independent experiments. Gray zone (a) indicates MI  $\leq 5\%$ , the clinically acceptable range for a non-significant reaction. Also shown are representative photomicrographs of a RhoGAM opsonized D+ MMA experiment. Oil-immersion light microscopy of Wright-Giemsa stained MMA slides. **Panel B:** Positive control; multiple monocytes with phagocytosed RhoGAM-opsonized D+ RBC. **Panel C:** Shown are the same D+ donor RBC as Panel B but modified with mPEG (20 kDa; 2 mM) prior to opsonization. As shown, immunocamouflaged D+ RBC significantly ( $P < 0.001$ ) inhibited erythrophagocytosis.

(Fig. 2). Indeed, previous studies have demonstrated that the antiglobulin test does not accurately reflect the amount of IgG bound and is, at best, only a weak predictor of RBC phagocytosis [29,30,32,37]. In contrast, IgG subclass was more suggestive of the MI value with IgG3 being highly predictive of a strong MI value and IgG1 being poorly predictive (Fig. 2). These results are consistent with previous studies demonstrating that phagocytosis is IgG subclass-dependent (IgG3 > IgG1) [32,34,36,38]. The naturally occurring heterogeneity of anti-D responses was also demonstrated by the wide range in MI values of the unmodified RBC (Fig. 2; Supporting Information Table 1) such that only five of eight of the donors exhibited a MI value of greater than 5% (i.e., potential for acute hemolytic transfusion reaction). As predicted by the RhoGAM findings, the 2 and 5 kDa polymers (2 mM) showed minimal reduction of MI (Fig. 2; Supporting Information Table 1). However, in the five plasma samples with a MI value greater than 5% for the unmodified RBC, grafting with the 20 kDa polymer (2 mM) yielded significant reductions ( $P < 0.01$ ) in the MI value such that four of the five anti-D opsonized samples had MI values of less than 5% while the remaining sample had an MI of  $5.5 \pm 0.9$ . The anti-phagocytic effect of polymer-mediated immunocamouflage of D+ RBC was observed regardless of the IgG subclass of the alloantibody.

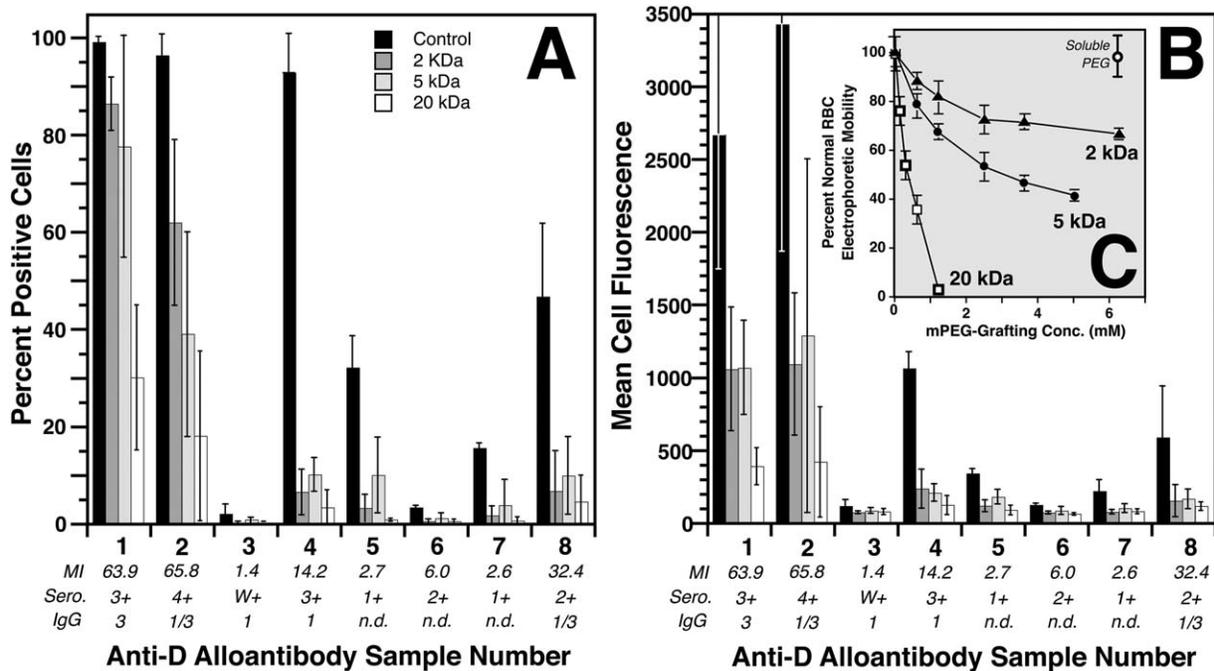
While the MMA is highly reflective of the in vivo biological recognition and clearance of antigen positive donor RBC, we further assessed the effects of immunocamouflage on anti-D binding via flow cytometry. The efficacy of RBC immunocamouflage on D alloantibody recognition was demonstrated by significant reductions in both percent positive cells (PPC; Fig. 3A) and mean cell fluorescence (MCF; i.e., relative amount of bound antibody; Fig. 3B). The PPC

and MCF of the anti-D opsonized control (i.e., unmodified) D+ RBC were predictive, but not determinative, of both the strength of the antiglobulin test and MI value (Fig. 3). For example, samples 1, 2, 4, and 8 had both high numbers of antibody positive RBC and relatively high MCF values and exhibited antibody reactivity of  $\geq 2+$  and MI values greater than 5%. Importantly, grafting of the 2, 5, and 20 kDa polymers to the D+ RBC significantly reduced both the PPC and MCF values for all alloantibodies; though they did not completely block antibody binding to all D+ cells (PPC). Also of note, the grafted polymer was highly effective in inhibiting IgG binding regardless of subclass.

Of interest was the lack of any consistent differential effects between the 2, 5, and 20 kDa polymer sizes on PPC and MCF across the eight donor alloantibodies (Fig. 3). While the 20 kDa polymer was highly effective in reducing MMA erythrophagocytosis ( $P < 0.001$ ; Fig. 2), for most samples there were no significant differences (aggregate intra-polymer  $P$  value  $> 0.05$ ) between the 2, 5, and 20 kDa polymers in the reduction noted in the PPC and MCF values (Fig. 3). However, as shown in Fig. 3B, the 20 kDa polymer did yield the highest average ( $\sim 70\%$ ) reduction in MCF and, for the strongest alloantibodies (Samples 1, 2, 4, and 8), the average reduction in MCF was greater than 85%. Consequent to the significant decrease in both PPC ( $87.7 \pm 9.5$ ;  $P < 0.001$ ) and MCF ( $P < 0.001$ ), mPEG-modified D+ RBC were poorly opsonized. This is an important consideration, especially as it relates to IgG subclass, as it has been reported that 150–640 molecules of IgG3 per red cell can mediate phagocytosis of RBC, whereas 1,230–4,020 molecules of IgG1 are needed to mediate efficient RBC interaction with monocytes [38]. Also heavily influencing the antiphagocytic effects of the 20 kDa



**Figure 2.** Immunocamouflage inhibits erythrophagocytosis of D+ RBC opsonized with a diverse array of human anti-D alloantibodies. Similarly to the findings with RhoGAM, polymer size is a critical factor in inducing clinically relevant immunocamouflage. Results shown are the mean ± SD for all eight anti-D alloantibodies tested. Gray zone (a) indicates MI ≤ 5%. Also shown in the box insert are the antiglobulin testing results and MMA MI values of the control cells. IgG subclass are also shown.



**Figure 3.** Flow cytometric analysis of human alloantibody opsonized control and immunocamouflaged D+ RBC demonstrate a significant reduction in antibody binding consequent to polymer grafting. The effects of 2, 5, and 20 kDa polymers on alloantibody binding to D blood group antigen are shown by both percent positive cells (PPC; **Panel A**) and mean cell fluorescence (MCF; **Panel B**). Anti-D alloantibody recognition was effectively reduced by 20 kDa polymer immunocamouflage as measured by both the PPC and MCF values when compared with the unmodified RBC. Interestingly, the 2 and 5 kDa polymers often had similar values as the 20 kDa polymer but did not inhibit erythrophagocytosis (Fig. 2). Results shown are the mean ± SD of a minimum of three independent experiments. Also shown to enhance data interpretation is the antiglobulin test findings and mean MI value for unmodified D+ RBC. IgG subclass of the alloantibodies are also shown. **Panel C:** The grafted polymer results in a size dependent decrease in RBC surface charge as measured by particle electrophoresis. Loss of surface charge will inhibit antibody-antigen binding and cell-cell (e.g., RBC-monocyte; RBC Rouleaux formation) interaction [Refs. 24, 28, and 39].

polymer is the observation that, unlike the 2 and 5 kDa chains, the larger polymer is highly effective and obfuscating membrane charges as demonstrated by the loss of electrophoretic mobility of the modified RBC (Fig. 3C). Consequent to the charge camouflage, the multi-focal cell:cell interactions necessary for effective phagocytosis are impaired [24,26,28,39].

## Discussion

Despite the commonality and clinical success of RBC transfusions into allogeneic recipients, the RBC is an antigenically challenged cell. Of the non-ABO blood group antigens, RhD is the most problematic both clinically and from a blood inventory perspective. While in most European-centric blood systems D- donors relatively abundant (although D- inventory problems persist), in much of the world D- donors are exceedingly rare causing both inventory shortages and clinical complications. While active recruitment of D- donors is done by blood providers, alternative means of providing "D-" inventory would be of value. The immunocamouflage of the D antigen by membrane-grafted polymers may provide a safe and effective means of providing "D safe" blood in the absence of D- donors.

The efficacy of D immunocamouflage arises consequent to polymer grafting to the lysine rich proteins in the protein islands found surrounding the lysine poor D protein (see Supporting Information Fig. 1) [24,26,28]. As demonstrated in this study, the immunocamouflage of D+ donor RBC by grafted mPEG results in a size ( $\geq 20$  kDa) and concentration ( $\geq 0.5$  mM) dependent obfuscation of D such that Fc $\gamma$ R-mediated adherence and phagocytosis of anti-D opsonized D+ RBC is inhibited (Figs. 1 and 2). Indeed at grafting concentration  $\geq 1.5$  mM, the 20 kDa polymer gave rise MI values of  $\leq 5\%$  and would be deemed compatible using the clinically validated MMA. This finding was observed with the highly potent RhoGAM antibody (Fig. 1; used clinically to prevent Hemolytic Disease of the Fetus and Newborn; HDFN) as well as against a diverse panel of heterologous anti-D IgG antibodies (Fig. 2) obtained from alloimmunized D- individuals. Of clinical interest, the polymer-mediated inhibition of erythrophagocytosis may also prevent other immunological sequelae arising from transfusion of D+ cells into D alloimmunized individuals. A recent study by Wang et al. demonstrated that the polymer-mediated immunocamouflage of D peptide, purified D protein and intact D+ RBC effectively prevented all recognition in a human D-sensitized proliferation model as measured by T-cell proliferation and cytokine release assays [26].

Importantly, the grafted polymers are nanoscale structures and, at the immunoprotective grafting concentrations used in this study, do

not alter normal RBC morphology nor impede normal membrane topography or dynamics as evidenced by normal oxygen uptake and delivery, cellular deformability, and ion transport [18–20,40]. Moreover, using an automated grafting technology, RBC exhibit uniform modification and normal murine in vivo survival with no evidence of immunogenicity even after repeated transfusions [19,20,25,41]. Somewhat surprisingly, the 30 kDa polymer resulted in inferior immunoprotection relative to the 20 kDa chain. This in part is derived by the biophysical properties of PEG, in which larger chain polymers effectively impede the binding of other polymer chains within their field of gyration (see Supporting Information Fig. 1) [24,28,39].

In summary, outside of the ABO antigens, D is the most immunogenic antigen on the RBC. The "clinical" significance of the D antigen is most commonly thought of in the context of HDFN and hemolytic transfusion reactions [6,12,14,15,17]. However, D- blood is also a rare blood group in much of the world. In China, and most Asian countries, D- individuals account for less than 1% of the population thus making maintenance of D- blood inventory problematic [16]. With increasing travel of D- individuals to Asia, or in emergency situations (e.g., earthquake or train accident), it can be difficult for blood banks to provide adequate supplies of D- red cells, which puts great strain on the blood supplier and may adversely impact patient care. Therefore, the development of "D safe" blood via the immunocamouflage of D+ RBC could be of significant clinical value. Our data demonstrates that immunocamouflaged donor D+ RBC opsonized by anti-D human alloantibodies effectively prevented immunologic recognition and phagocytosis. Indeed, opsonized D+ RBC modified with the 20 kDa mPEG polymer yielded MMA MI values of less than 5% suggesting that these cells would not cause an acute transfusion reaction. These and previous findings clearly suggest that the immunocamouflaged D+ RBC may be able to increase the availability of "D safe" compatible blood in situations where D- units are unavailable.

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