Chapter 2

Immunocamouflaged RBC for Alloimmunized Patients

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Additional information is available at the end of the chapter

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Abstract

While ABO/Rh(D) red blood cells (RBC)-matched transfusions are generally considered as safe, a significant risk of alloimmunization to non-A/B blood group antigens exists; especially in chronically transfused patients. Indeed, alloimmunization to non-A/B antigens can be so severe that RBC transfusion can no longer be safely administered without the risk of a potentially deadly immune haemolytic reaction. Currently, no satisfactory solutions exist either to prevent blood group alloimmunization or to cost-effectively treat patients with severe alloimmunization. To address this problem, we have pioneered the *immunocamouflage* of donor RBC. The immunocamouflaged (stealth) RBC is manufactured by the covalent grafting of biologically safe polymers to RBC membrane proteins. As a result of the grafted polymer, non-A/B blood group antigens are biophysically and immunologically masked. Of particular interest is the immunocamouflage of the Rh(D) antigen which could be used to improve blood inventory and transfusion safety. The polymer-modified RBCs are morphologically normal and, in mice, exhibit normal *in vivo* survival at immunoprotective grafting concentration. In this chapter, we explore both the biophysical and immunological consequences of the grafted polymers, explore the conditions in which they might be appropriately used, and describe the technology necessary to manufacture functional transfusable units of these cells within the clinical setting.

Keywords: red blood cell, immunocamouflage, alloimmunization, Rh(D), polymer

1. Introduction

The transfusion of red blood cells (RBC) remains the most common, and best tolerated, form of tissue transplantation. Indeed, an estimated 108 million units of whole blood (~49 million litres) are collected annually worldwide for processing and eventual transfusion [1]. In spite

of this massive collection effort, the need for blood constantly exceeds availability due to a combination of collection, manufacturing, storage and, most important clinically, biological (i.e., immunological) issues. The biological challenges facing successful RBC transfusions are vastly underappreciated, largely because of the long history and ubiquity of blood transfusions in modern medicine. Indeed, the RBC is an immunological complex cell with 35 major blood group systems that give rise to over 300 unique antigens capable of eliciting an immune response. Moreover, this immunological complexity is further exacerbated by the finding that the non-A/B (often referred to as *minor*) blood group antigens exist with varying frequencies among different ethnic and racial groups [2, 3]. Within the non-A/B antigens, Rh(D) deserves special attention.

Among the non-A/B blood groups, the Rh system, and in particular Rh(D), is considered to be the most immunogenic antigen. Indeed, the Rh(D) antigen is highly immunogenic and when $Rh(D)^+$ blood is transfused into an $Rh(D)^-$ individual, there is a 50% risk for the development of anti-Rh(D) antibodies resulting in very high risk of a haemolytic transfusion upon a second $Rh(D)^+$ transfusion. Consequent to its immunogenicity, Rh(D) is always determined simultaneously with ABO type and constitutes the '±' found alongside the ABO phenotype. Consequent to its immunogenicity, Rh(D) poses a significant challenge to blood operators since Type O $Rh(D)^-$ (O^-) blood is the universal donor cell. In Euro-centric populations, 6–7% of the population is O^- making the maintenance of an adequate inventory of this universal donor blood problematic but possible. Indeed, in North America and Europe, virtually all blood service providers experience a chronic shortage of Type O^- blood. However, in other geographic regions, especially Asia, $Rh(D)^-$ individuals are extremely rare. Indeed, in China, only 0.1–0.4% of the population, regardless of ABO type, is $Rh(D)^-$, making the $Rh(D)^-$ individual (especially with the increasing influx of European tourists) an at-risk patient [4]. Thus, within transfusion medicine, Rh(D) remains a significant problem in terms of both supply and its clinical risk.

Despite the immunological complexity of the RBC, simple ABO/Rh(D) matching has been, typically, considered sufficient for most acute transfusion needs. However, even when ABO/Rh(D) are appropriately matched, transfusion reactions still occur as mismatched non-A/B antigens do carry some immunological risks to a patient. While the incidence of clinically noteworthy (i.e., *significant* patient morbidity) transfusion reactions is relatively low (~0.017% of transfused individuals), less severe transfusion reactions (e.g., transient fever, malaise, premature RBC clearance) and alloimmunization are considerably more frequent and increase with the number of transfusions received by an individual [5–9]. Indeed, with approximately 108 million units of whole blood collected worldwide per annum for blood product preparation, the actual numbers of adverse events become quite significant. Of clinical importance, alloimmunization to non-ABO group antigens is significantly exaggerated in individuals (~30%), especially minorities, receiving chronic transfusion therapy as seen in thalassemia and sickle cell anemia. Indeed, alloimmunization to non-ABO blood groups can be so severe that blood transfusion can no longer be safely administered without risk of a potentially deadly immune haemolytic reaction.

Historically, various interventions have been used in an attempt to prevent transfusion reactions arising from alloimmunization. While ABO/Rh(D) typing has been used since the

1940s, the practice of phenotyping some of the more problematic non-A/B/Rh(D) antigens is still uncommon and likely underlies the high frequency of alloimmunization in chronically transfused patients. In studies on β thalassemia, up to 20% of these chronically transfused individuals demonstrate *clinical* evidence (i.e., mild to severe transfusion reactions) of alloimmunization against non-A/B donor blood group antigens [10, 11]. Rates in patients with sickle cell anemia are even higher (>30%) [12]. As a result, many US National Institutes of Health (NIH) funded Sickle Cell Centres now evaluate a blood recipient for high risk (for alloimmunization) blood group antigens and to prophylactically utilize phenotypically matched blood for transfusion in this cohort of patients. However, even antibody screening does not identify patients alloimmunized to less common RBC antigens or, more importantly, prevent primary alloimmunization to non-tested antigens. To date, the only solutions to prevent alloimmunization, or for individuals with very rare blood type, are to store autologous blood (4°C), maintain an inventory of frozen rare blood group units, keep a blood bank registry of potential donors with rare blood types, utilize extensive RBC phenotyping prior to transfusion and/or encourage minority blood donations [7, 12–27]. While all of these steps are prudent and variably effective, situations still arise where an appropriate (or even satisfactory) blood match cannot be made.

2. Bioengineering the red blood cell

Currently, no satisfactory solutions exist to prevent or cost-effectively treat blood group alloimmunization or to improve the inventory of $Rh(D)^-$ blood. To address these unmet needs, the covalent grafting of biocompatible polymers to donor RBC has been proposed to *immunocamouflage* the allogeneic RBC. The immunocamouflaged (stealth) RBC is manufactured by the covalent grafting of methoxypoly(ethylene glycol) [mPEG; PEGylation], as well as other polymers (e.g., polyoxazolines, POZ; and hyperbranched polyglycerols, HPG), to membrane proteins on the surface of allogeneic donor RBC (**Figure 1**).

Most commonly, the chemically activated polymers are covalently grafted to proteins at exposed lysine residues. As a result of the grafted polymer, donor blood group antigens are biophysically and immunologically masked while the modified RBC remaining biologically and functionally viable. To date, most studies have focused on mPEG as the polymer of choice due to its superior ability to both sterically and charge camouflage allogeneic RBC and its well-characterized, and safe, pharmacological profile. The basic chemical structure of mPEG is $HO\text{-}(CH_2CH_2O)_n\text{-}CH_2CH_2CH_3$. mPEG is of low toxicity and is US FDA approved for oral, intravenous, subcutaneous and intramuscular administration [29]. The mPEG polyether polymer is neutrally charged, available in an extraordinarily wide range of molecular weights, and is highly soluble in aqueous-based solutions making it very suitable for pharmacological use. In contrast, both the POZ (e.g., PEOZ) and HPG polymers are poorly soluble in aqueous solutions and only confer weak charge camouflage.

A large number of biological and biophysical studies have been done to characterize the effects of polymer grafting on immune recognition and *in vitro* and *in vivo* viability [28, 30–58]. These studies have demonstrated the significant potential of this immunocamouflage technology in

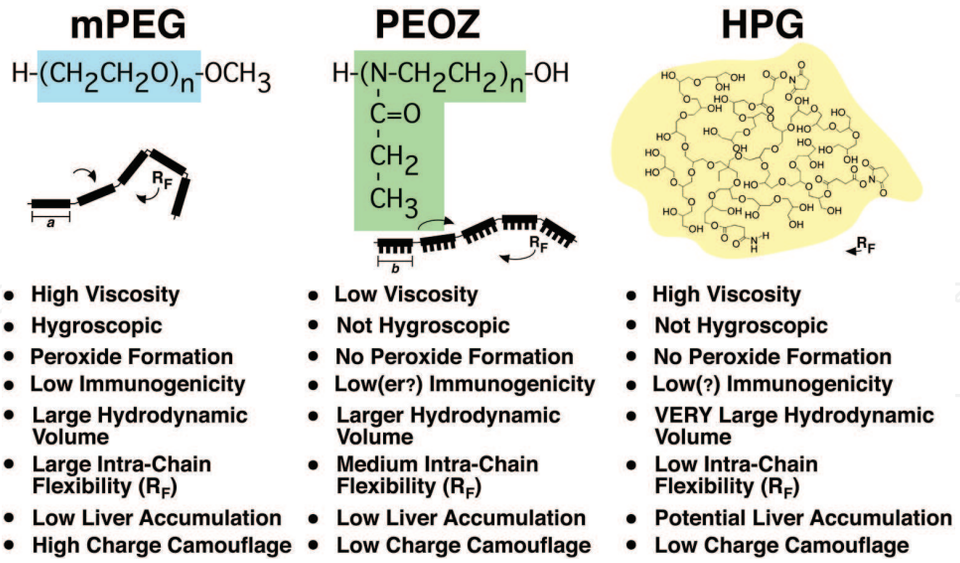

Figure 1. Comparison of mPEG, PEOZ and HPG. The repeating structures of mPEG and PEOZ are denoted by the shaded areas while HPG, consisting of repeating polyglycerols, functions more as a mass. The *(a)* and *(b)* notations denote the independent rotational segments of mPEG and PEOZ (respectively) governing intra-chain mobility. The relative intra-chain mobility of the polymers, coupled with polymer size (e.g., 2 versus 20 kDa), underlies the radius of gyration of the grafted polymer. The relative radii of gyrations for mPEG, PEOZ and HPG are indicated by the arrows denoting the Flory radii (R_F: root mean square of end-to-end length of the polymer chain) of the polymers. As illustrated, the side-branches of PEOZ decrease intra-chain mobility producing a larger hydrodynamic volume relative to mPEG. Similarly, the highly branched HPG has very limited intra-chain mobility but yields a dense steric 'mushroom'. Modified from Kyluik-Price et al. [28].

transfusion medicine. Example uses include but are not limited to: (1) derivitize RBC to diminish transfusion reactions arising from mismatched blood or alloimmunization; (2) utilization by clinical blood banks to camouflage the Rh(D) antigen to improve blood inventories and utilization; (3) use of mPEG-modified RBC as a 'chain-breaker' (i.e., preventing RBC aggregates arising from abnormal cell-cell interaction) in vascular occlusive diseases such as sickle cell anemia; and (4) prevention of transfusion-associated graft-versus-host disease. Other uses outside of transfusion medicine include the implantation of derivitized cells or cell aggregates (e.g., pancreatic islets) to correct enzyme deficiencies; the induction of tolerance via PEGylated leukocytes; and the prevention of viral infections via antiviral gels.

Biophysical and biological characterization of the stealth RBC: The immunocamouflage of cells is a function of the biophysical and biochemical nature of the grafted polymer (**Figure 1**). Biophysically, the grafted polymers confer its immunoprotective effects via both steric hindrance and charge camouflage (**Figure 2**). The efficacy of membrane immunocamouflage is dependent upon both the density (i.e., how much) and depth (i.e., thickness; polymer molecular weight) of the polymer layer. As shown in **Figure 1**, steric hindrance arises from either the rapid mobility arising from intra-molecular flexibility of the polymer (mPEG and PEOZ) and/or polymer density itself (HPG). Perhaps of even more importance is the ability of the polymer to obscure the surface charge (charge camouflage) of the cell. Biophysically, charge camouflage arises from polymer-mediated extension of the shear plane (SP) thereby decreasing the apparent surface charge (**Figure 2A** and **B**). The grafted polymers can give rise to both DIRECT (direct binding to the antigen in question) and INDIRECT (binding to sites other than

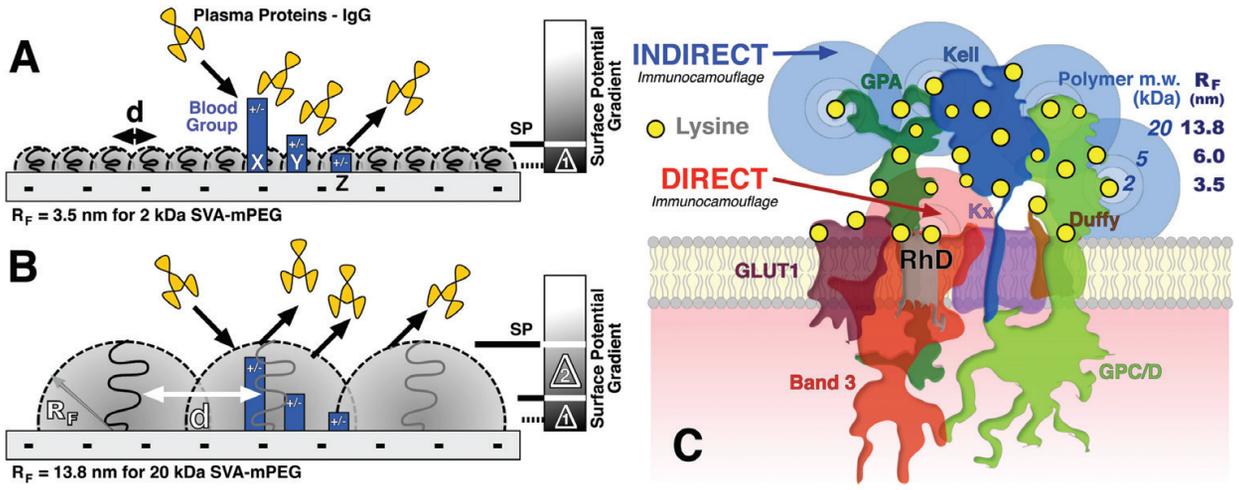

Figure 2. Biophysical mechanisms of immunocamouflage. Panels A and B: Prevention of plasma protein (e.g., immunoglobulins) interaction with the cell membrane is due to both steric exclusion (shaded areas induced by the polymers radius of gyration; R_F: Flory radii is the root mean square of end-to-end length of the polymer chain) and surface charge camouflage. The effects of both short chain (Panel A) and long chain (Panel B) polymers on the immunocamouflage of surface proteins (X, Y, Z) are schematically shown. The steric effect is maximized when chains are grafted at higher density, that is, with small separation between the chains (d). Importantly, antibody-antigen interaction is, biophysically speaking, charge-mediated. Membrane surface charge camouflage is primarily driven by polymer-mediated extension of the shear plane (SP) toward a region of decreased surface potential (Surface Potential Gradient). In the absence of polymer, the inherent shear plane (SP) of a cell is typically located 1–3 nm above the surface. The extension of SP is proportional to the hydrodynamic thickness of the polymer layer, which in turn is governed by the R_F of the grafted polymer. Thus, 20 kDa polymers (large R_F; Panel B) provide improved charge camouflage over 2 kDa polymers (small R_F; Panel A). Delta (Δ) is the difference in the surface potential at the shear plane of a particle modified with the short (Δ1) versus the long polymer (Δ2). The membrane proteins X, Y and Z denote blood group antigens extending different distances from the cell surface. Panel C: Not all proteins in the complex topology of the RBC are equally accessible to grafting by the activated polymer, due to either its location in the protein complex or the paucity of lysines (the grafting site of activated mPEG). For example, Rh(D) is deeply buried in the complex while Kell is easily accessible. Thus, *indirect immunocamouflage* maybe more critical than *direct immunocamouflage* (i.e., direct modification of Rh(D) by mPEG) for many blood group antigens. Modified from Refs. [47, 28].

the antigen but conferring indirect camouflage) immunocamouflage of blood group antigens (**Figure 2C**). The importance of indirect immunocamouflage is significant as blood group antigen proteins do not exist in isolation but are most commonly part of complex protein clusters. As shown, the Rh(D) protein is deeply buried within a large protein complex on the surface of the RBC making the direct camouflage of Rh(D) difficult. However, the indirect immunocamouflage of Rh(D) arising from polymers grafted to surrounding proteins (e.g., Kell protein, Band 3) results in the highly efficient immunocamouflage of RhD [28, 52, 55, 59].

Indeed, one of the most promising prospects of RBC immunocamouflage is in both diminishing the risk of Rh(D) alloimmunization and safely increasing blood inventory during emergency situations or in circumstances where $Rh(D)^-$ blood is unavailable. As shown in **Figure 3A**, immune recognition and phagocytosis of anti-D (RhoGAM®; Rh_o(D) Immune Globulin (Human) RhoGAM Ultra-Filtered PLUS; Ortho Clinical Diagnostics)-opsonized $Rh(D)^+$ RBC are blocked in a grafting concentration-dependent manner by the grafted mPEG polymer. Importantly, RhoGAM® is a highly purified and concentrated human-derived anti-D IgG antibody that is highly effective at RBC opsonization yielding Monocyte Index (MI) scores in the monocyte-monolayer assay (MMA) in the range of 60–100%. The RhoGAM® antibody is used clinically for the prevention of Rh

immunization, including during and after pregnancy and other obstetrical conditions or incompatible transfusion of Rh-positive blood. However, RhoGAM® does not fully reflect the biological/clinical heterogeneity of anti-D alloantibodies arising in alloimmunized individuals. To assess the potential utility of mPEG-RBC in alloimmunized individuals, human-sourced anti-D alloantibodies ($N = 8$) were assessed using the MMA. Of note, the naturally occurring heterogeneity of anti-D responses is demonstrated by the wide range in MI values when used to opsonize Rh(D)$^+$ RBC (**Figure 3B**). Indeed, only five of eight of the alloantibodies exhibited MI values of >5% (i.e., potential for acute haemolytic transfusion reaction). However, as predicted by the RhoGAM findings, grafting of the 20 kDa polymer (2 mM) yielded significant reductions ($p < 0.01$) in the MI values of all samples. Of the five plasma samples with MI values >5%, PEGylation resulted in four having MI values of <5% while the remaining sample had an MI of 5.5 ± 0.9. Moreover, the anti-phagocytic effect of polymer-mediated immunocamouflage of Rh(D)$^+$ RBC was observed regardless of the IgG subclass of the alloantibody. It is also important to note that the serological score (ranging from W+ to 4+) did not correlate significantly with the MI value [59]. These findings are in line with previous studies that 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 [60–63]. In aggregate, these findings suggest that immunocamouflaged Rh(D)$^+$ RBC could be safely transfused into Rh(D)$^-$ patients in an emergency situation.

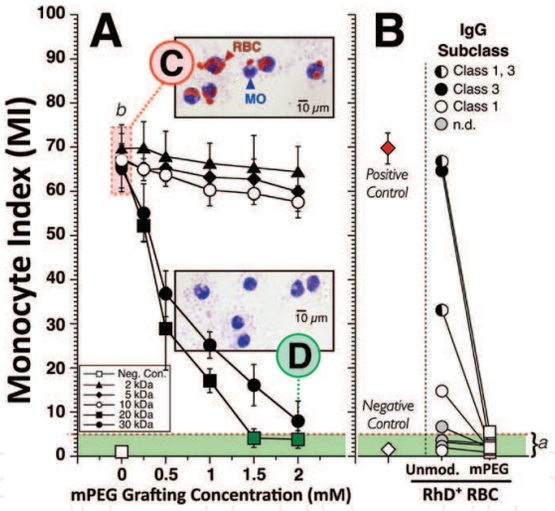

Figure 3. Polymer size and grafting concentration governs the efficacy of D immunocamouflage and the inhibition of erythrophagocytosis. Panel A: Effect of polymer size and grafting concentration on MMA phagocytosis of RhoGAM-opsonized Rh(D)$^+$ RBC. As shown, short chain polymers (2–10 kDa) were ineffective at inhibiting erythrophagocytosis. In contrast, membrane modification of Rh(D)$^+$ RBC with both the 20 and 30 kDa mPEG showed a significant ($p < 0.005$ at ≥0.5 mM grafting concentration) dose dependent decrease in phagocytosis. Importantly, the 20 kDa polymer effectively reduced the MI values to ≤5% at grafting concentrations ≥1.5 mM. Interestingly, at equimolar concentration, the 30 kDa polymer was less effective than the 20 kDa polymer. Shown are the mean ± SD of a minimum of three independent experiments. Also shown are representative photomicrographs of an RhoGAM-opsonized Rh(D)$^+$ MMA experiment. Oil-immersion light microscopy of Wright-Giemsa-stained MMA slides. Panel B: Immunocamouflage inhibits erythrophagocytosis of Rh(D)$^+$ RBC opsonized with a diverse array of human anti-D alloantibodies. Similar 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. Also indicated are the IgG subclass of the alloantibodies. The green zone (*a*) indicates MI ≤ 5%, the clinically acceptable range for a non-significant reaction. Panel C: Positive control; multiple monocytes with phagocytized RhoGAM-opsonized Rh(D)$^+$ RBC. Panel D: Shown are the same Rh(D)$^+$ donor RBC as Panel B but modified with mPEG (20 kDa; 2 mM) prior to opsonization. Data derived from Li et al. [59].

Importantly, at immunologically protective grafting concentrations, the grafted polymer does not affect RBC structure or function as evidenced by normal morphology, O_2 uptake and delivery, cellular deformability or ion transport [30, 33, 34, 37, 41, 52, 55]. While virtually indistinguishable from unmodified cells in most aspects, one interesting difference was noted between unmodified and mPEG-modified RBC. Consequent to the charge camouflage of the RBC, the cell:cell interactions necessary for Rouleaux formation were abrogated (**Figure 4**). Because the grafted polymer camouflages the charge of the cell necessary for cell:cell interaction, Rouleaux formation is inhibited resulting in attenuation of RBC sedimentation and, physiologically, decreased low-shear viscosity (**Figure 4**). Importantly, the decrease in low-shear viscosity may make the use of stealth RBC highly suitable for patients with diseases characterized by RBC-vaso-occlusive events (e.g., sickle cell). Importantly, RBC PEGylation inhibits both non-antibody (e.g., sickle cell self-aggregation) and antibody-mediated aggregation events and donor mPEG-RBC can serve as an efficient chain-breaker in pro-aggregation states [33].

The grafting of immunologically 'inert' polymers to the membrane of allogeneic RBC effectively camouflages multiple non-ABO antigens from immune recognition. The combined actions of both steric and charge camouflage underlie the ability of the grafted polymer to camouflage allogeneic blood group antigens (immunocamouflage) from the recipient's immune system. These immunocamouflaged (i.e., stealth) RBC may be an effective tool in both preventing and treating alloimmunization in the chronically transfused patient; the transfusion of individual patients with rare blood phenotypes; emergency situation or geographic locations (e.g., China) where $Rh(D)^-$ blood is unavailable. Moreover, immunocamouflaged RBCs are inexpensively and easily manufactured in the clinical setting.

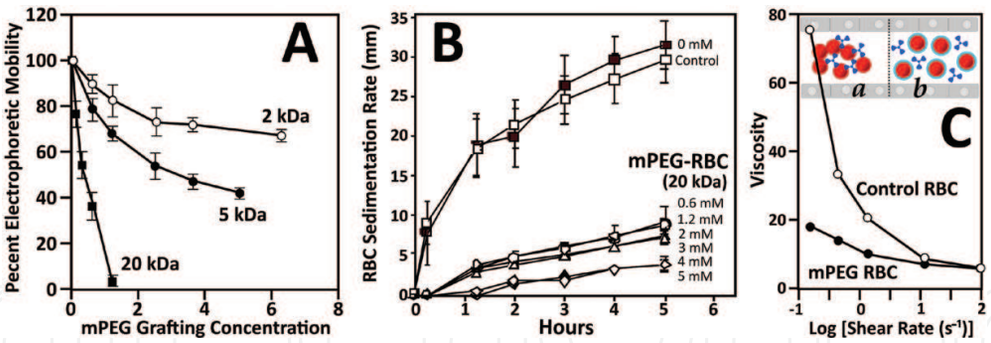

Figure 4. Biophysical consequences of RBC PEGylation. Panel A: Charge camouflage of RBC is readily accomplished by polymer grafting and is a function of both grafting concentration and polymer size. As shown, the electrophoretic mobility of the human RBC was completely abrogated by the 20 kDa-activated mPEG at very low grafting concentrations. Mobility of the unmodified human RBC was −1.18±0.12 (μm/s)/(V/cm). Per cent change in mobility was normalized to the mobility of unmodified RBC. Panel B: Polymer grafting prevents cell:cell interaction (Rouleaux formation) via steric and charge camouflage. The loss of Rouleaux formations leads to a dramatically decreased RBC sedimentation rate. Panel C: Low-shear viscosity of the PEG-RBC is significantly reduced in comparison to normal control cells. Control and PEGylated (5 kDa mPEG) RBCs were resuspended to a 40% hematocrit in autologous plasma. Viscosity was measured over a range of shear rates using a Contraves LS30 low-shear viscometer (Contraves AG, Zurich, Switzerland). Data derived from Armstrong et al. [64]. INSERT: PEGylation of RBC alters many characteristics of the RBC. Shown on side *(a)* is the effect of antibody binding to control cells. The RBC agglutinate resulting in a potentially vaso-occlusive event mediated by both antibody binding and enhanced blood viscosity under low-shear conditions. In contrast, as shown on side *(b)*, antibody-mediated aggregation is suppressed in the PEGylated cells, and low-shear viscosity is actually enhanced due to the loss of aggregation and the neutral surface charge of the modified RBC. In addition, the PEGylated cells do not readily interact with the vascular endothelium.

3. Manufacturing the 'stealth RBC'

The immunocamouflaged RBC is unlikely to be a *mass-market* product but rather a *boutique blood product for the discriminating customer*. Hence, *on demand* manufacturing of the stealth RBC will be the most likely scenario facing the clinician and blood provider. Fortunately, the manufacturing process of the stealth RBC is rapid and straightforward and requires no specialized equipment on part of the blood provider, hospital or clinic. Moreover, current laboratory tests exist that can, relatively rapidly (~48 h), evaluate the potential clinical utility of the stealth RBC in the at-risk alloimmunized patient. Crucial to the clinical use of the stealth RBC is the manufacturing process. The key tenet of the manufacturing process is the maintenance of a constant polymer:cell ratio in order to achieve a homogenous grafting of the polymers to the individual cells within donor RBC unit (**Figure 5**). If cells are *under-PEGylated*, they retain significant immunogenicity/antigenicity; if cells are *over-PEGylated*, the *in vivo* viability (i.e., circulation time) of the cell is compromised.

To achieve the homogeneity necessary for a clinical mPEG-RBC unit (**Figure 5**), two scalable devices (**Figures 6** and **7**) utilizing micro-mixing chambers (alternatively Y-connectors to induce turbulence and mixing) have been designed, constructed and validated to semi-automate the RBC derivatization process and minimize the risk of contamination. Both approaches can be done aseptically using modifications of existing blood bags and sterile docking devices.

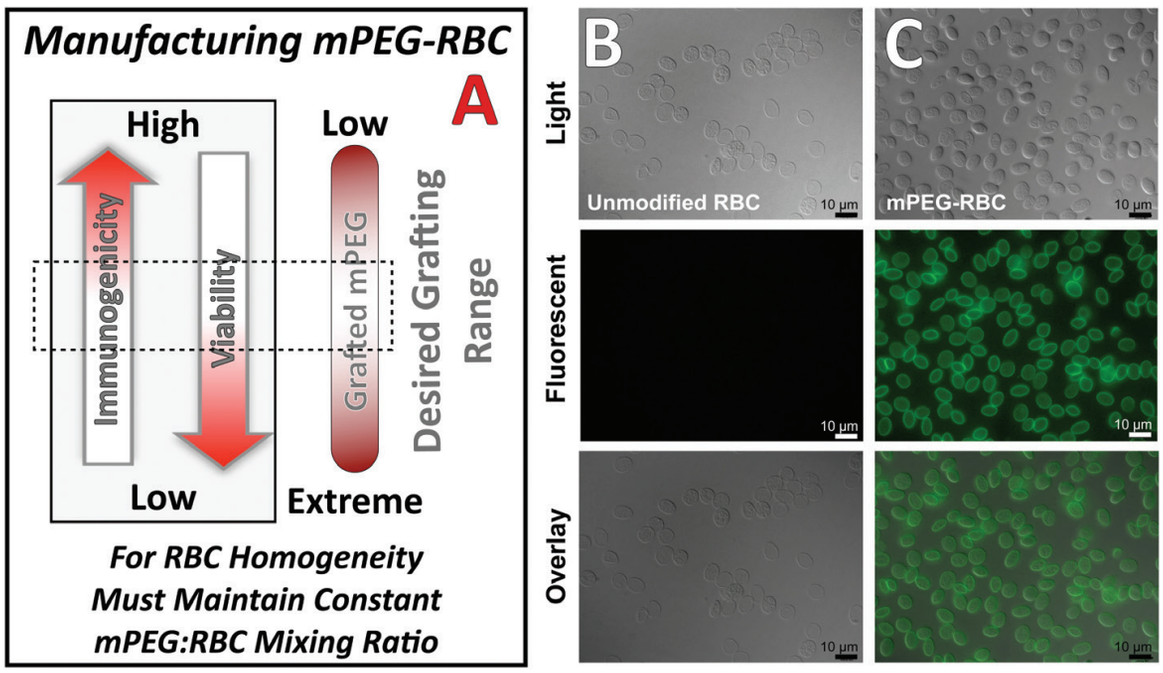

Figure 5. Homogeneity of polymer grafting is a critical concern. Extreme grafting levels yield mechanically unstable RBC, while minimally modified RBC retain significant immunologic recognition. To achieve improved grafting homogeneity, 'manufacturing' processes and devices have been developed by our lab. Panels B and C: Using the semi-automated devices described in this chapter, RBCs are uniformly modified by the activated polymer. The uniformity of grafting was documented using an mPEG polymer formulation containing 1% fluorescent 20 kDa mPEG. As shown, the PEGylated RBCs are all fluorescently labeled showing complete derivatization with the semi-automated devices described in this chapter. Modified from Wang et al. [52].

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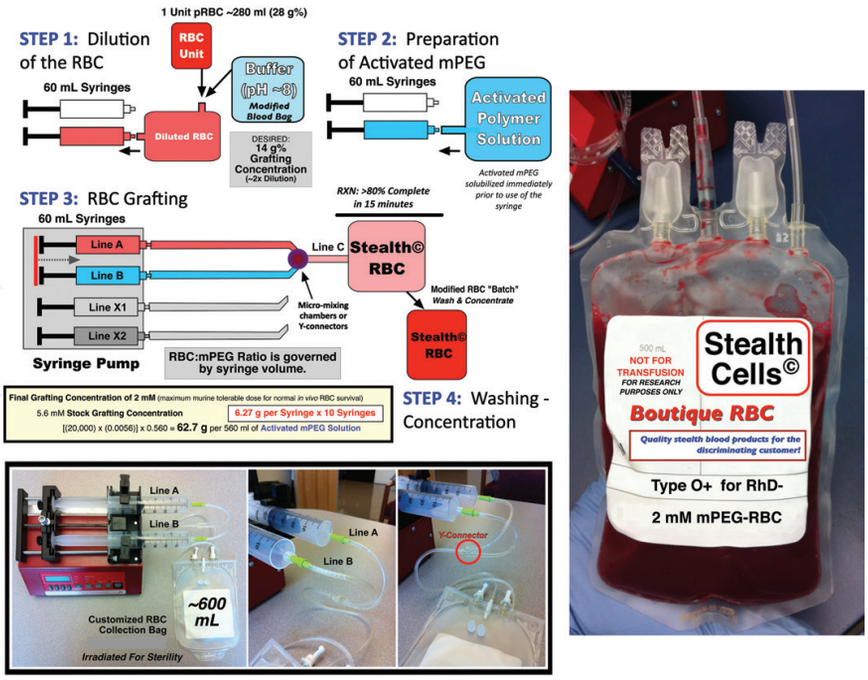

Figure 6. Schematic representation for the clinical PEGylation of a single blood unit using the *Syringe Pump Method*. At a minimum, a two-syringe pump is used. Syringe pumps able to handle four or more syringes are commercially available and would speed up the PEGylation process. The syringes can be of either equal or unequal volumes thereby governing the ratio of RBC to activated polymer. The syringe lines lead to the micro-mixing chamber (or Y-connector which will induce turbulence facilitating mixing). The diluted RBC are collected and re-concentrated prior to transfusion. A RBC washing step could be combined with the centrifugation to concentrate the RBC as desired. If desired, a RBC washing step can be combined with the centrifugation step to remove any unreacted polymer though the mPEG is considered safe for injection. Also shown are photographs of the two-syringe pump method and the final stealth RBC blood bag.

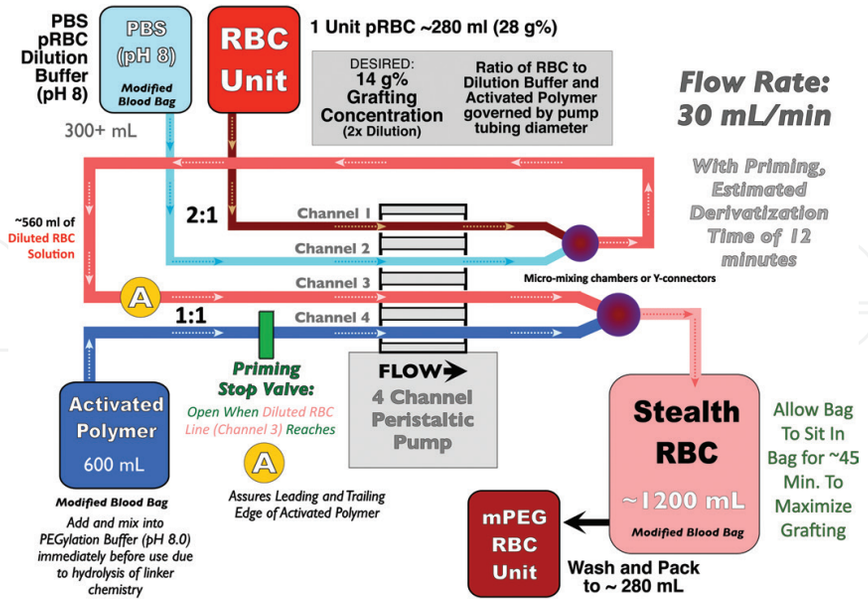

Figure 7. Schematic representation for the clinical PEGylation of a single blood unit using the *Four-Channel Peristaltic Pump Method*. Pump tubing used in Channels 1–4 can be of equal or different diameters to govern RBC dilution and the ratio of diluted RBC to activated polymer ratio. A RBC washing step can be combined with a centrifugation process to remove any unreacted polymer though the mPEG is considered safe for injection.

The *Syringe Pump Method* (**Figure 6**) has an advantage in better controlling the hydrolysis rate (i.e., inactivation rate; $t_{1/2}$ of 33 min for succinimidyl valerate-activated methoxypoly(ethylene glycol) [SVAmPEG]) of the activated mPEG as the activated polymer is prepared in smaller batches. The *Peristaltic Pump Method* (**Figure 7**) has the advantage of being a continuous flow device with fewer points of possible disruptions in blood sterility. As demonstrated in **Figure 6**, the described manufacturing devices are capable of producing a transfusable unit of PEGylated RBC.

RBC manufacturing and product quality control: Quality assurance of the mPEG-RBC to document the reproducibility and homogeneity of SVAmPEG derivatization can be assessed using a two-phase (PEG-Dextran) partitioning system [37, 41]. The partitioning of PEGylated RBC in this system is governed by the ratio of PEG:Dextran, as well as by the density and size (m.w.; number of ethoxy units) of the grafted mPEG. In this simple assay, mPEG-RBC are added to an immiscible PEG:Dextran solution, rapidly mixed and allowed to separate. As shown, unmodified RBC preferentially partition to the Dextran or interface region while the PEGylated RBC preferentially partition to the PEG-rich phase (**Figure 8**). Importantly, both the automated derivatization and the purification methodologies are highly scalable and can be applied to existing blood bank devices and workflows.

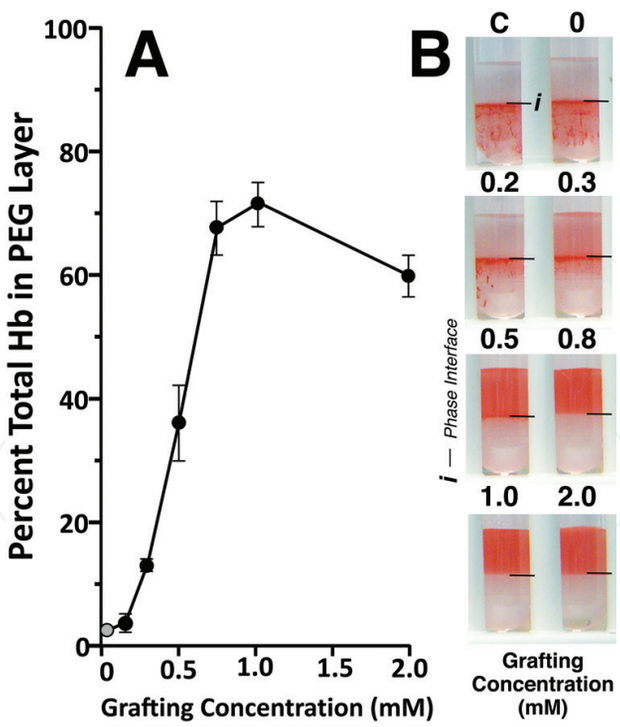

Figure 8. Assessing the efficacy of the derivatization reaction via the two-phase PEG-Dextran partitioning system. Panel A: Quantification of phase separation was done via hemoglobin concentration in the PEG layer. Results are expressed as mean ± standard error mean (SEM). Panel B: Representative photos of phase separation of 20 kDa mPEG-RBC over 0–2 mM grafting concentrations. Reflecting increased grafted polymer, the mPEG-RBCs show increased partitioning into the upper PEG layer. Photos were taken 20 min post mixing. Data derived from Bradley and Scott [41] and Kyluik-Price et al. [28].

The PEGylation Recipe

PEGylation Buffer: While buffer flexibility exists, the chemistry of PEGylation via activated mPEG requires alkaline conditions [28, 30–58]. The formulation of the mPEG-buffer used by our laboratory for the PEGylation of RBC is as follows: 50 mM K_2HPO_4, 105 mM NaCl, pH 8.0.

Activated mPEG: Following extensive analyses of multiple polymer molecular weights and linker chemistries, 20 kDa succinimidyl valerate-activated methoxypoly(ethylene glycol) [SVAmPEG] has been selected as our primary polymer species. While a wide range of polymer molecular weights (e.g., 2–40 kDa) have been tested, *in vitro* (human and mouse) and *in vivo* (mouse) studies demonstrate that the 20 kDa polymer provides the optimal immunological camouflage of the allogeneic cell while maintaining normal *in vivo* survival. Linker chemistry selection was based on the commercial availability of the polymer, the relatively long half-life of the activated polymer and its excellent *in vitro* and *in vivo* findings. Clinically compliant SVAmPEG can be purchased from *Laysan Bio Inc*. (http://laysanbio.com//; Arab, AL, USA). Because the half-life of the activated polymer upon hydration is short ($t_{1/2}$ of 33 min for SVAmPEG), the polymer should only be solubilized immediately before beginning the grafting event. Specific activated mPEG and buffer volumes for the syringe pump and peristaltic pump method are provided within the method schematics.

4. Evaluating the potential clinical utility of the stealth RBC

While multiple studies have demonstrated that RBC immunocamouflage can effectively block immune recognition of multiple blood group antigens, the diversity of alloantibodies produced to a single blood group antigen by humans is staggering. Hence, a crucial step in the clinical use of the stealth RBC should be evaluating the potential efficacy of the stealth cell in the individual patient. While one might assume that standard serological testing techniques would suffice, this is not the case for a variety of reasons. Primary among these reasons, and as shown in **Figure 3C**, is that the laboratory serological score does not correlate significantly with the MI value or the risk of an acute transfusion reaction. Indeed, multiple studies have demonstrated that the antiglobulin test poorly reflects the amount of IgG bound and is, at best, a very weak predictor of RBC phagocytosis [60–63]. This confounding finding is actually by design. Serological testing is 'overly' sensitive in order to detect miniscule amounts of bound antibody to assure the appropriate typing of an individual or to detect the presence of a potentially dangerous alloantibody. Another potential complication is that a large number of commercial testing protocols employ PEG (as either a listed or unlisted ingredient) as a component of the testing reagents. The reagent PEG will cause the mPEG-RBC to segregate as 'PEG likes PEG' (**Figure 8B**). Hence, other predictors of the potential clinical utility of the stealth RBC for an individual patient are needed.

Perhaps the most definitive testing approach for the potential clinical value of the stealth RBC is the monocyte-monolayer assay (MMA). The MMA assesses FcγR-mediated adherence and phagocytosis of alloantibody-opsonized donor RBC by monocytes and has been clinically

correlated with *in vivo* transfusion safety (i.e., prevention of acute transfusion reactions) and efficacy (24 h RBC survival) [60–62, 65–68]. The *in vitro* MMA is reliable, reproducible and, within the transfusion medicine community, is considered to be the best assay currently available for the evaluation of FcγR-mediated phagocytosis of antibody-coated human red cells, having more than 20 years of proven validity for the comparison of *in vitro* phagocytosis to *in vivo* clinical relevance.

Schematically, the MMA is described in **Figure 9** (and experimentally demonstrated in **Figure 3**). The MMA examines FcγR-mediated phagocytosis *in vitro* using adherence-purified monocytes isolated from peripheral blood mononuclear cells obtained from normal volunteers. Blood group antigen positive RBCs are incubated with buffer (negative control) or sera or plasma from alloimmunized patients for opsonization, washed and overlaid on the monocyte monolayer. Both donor-obtained and reagent RBC are suitable for use in the MMA. The MMA uses a visual readout whereby the numbers of adherent and phagocytized control and opsonized RBC are enumerated per 100 monocytes (MI). For validation purposes, consistent positive and negative controls should be used, most commonly anti-Rh(D)-opsonized $Rh(D)^+$ (positive control) and $Rh(D)^-$ (negative control) human RBC (see **Figure 3**). The visual inspection is simplified and enhanced by the use of phase contrast microscopy. Using the MMA, MI values of ≤5% indicate that the donor cells can be given without risk of an overt haemolytic reaction. However, it is worth noting that the MMA is most predictive of acute haemolytic transfusion reactions and is less predictive of long-term survival of donor RBC.

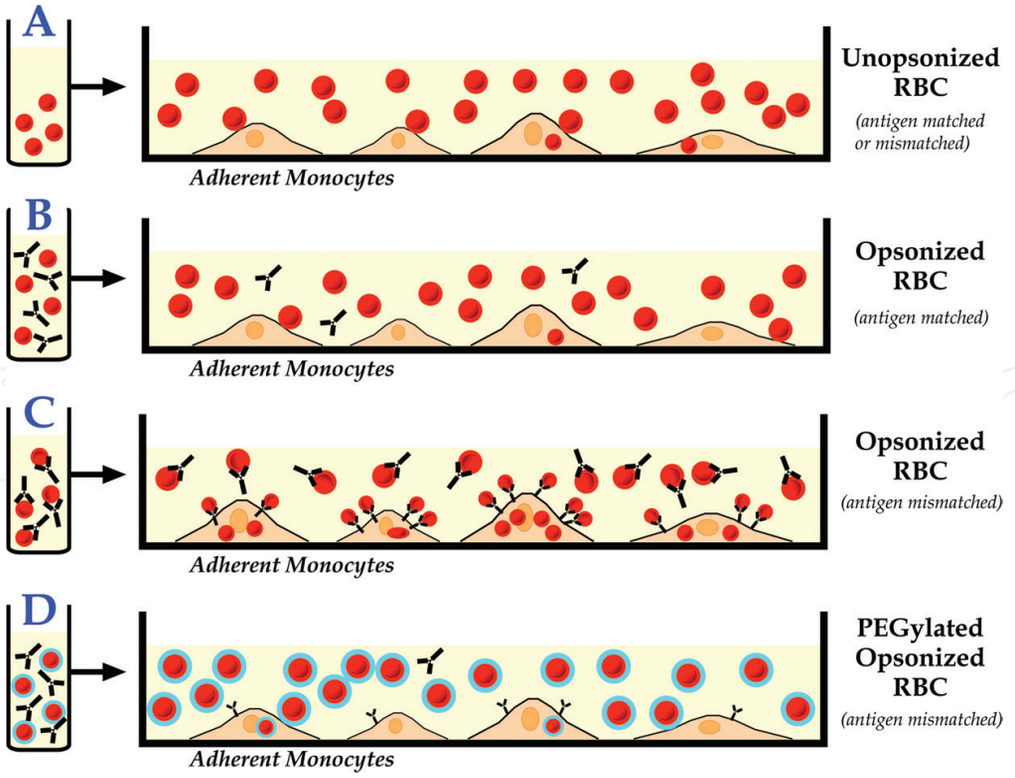

Figure 9. Individualized testing of the potential clinical utility of the stealth RBC. Schematic representation of the monocyte-monolayer assay (MMA). (A) Untreated RBC; (B) RBC treated with control (antigen matched) serum or plasma; (C) RBC treated with alloimmunized serum/plasma; and (D) PEGylated RBC treated with alloimmunized serum/plasma. Representative photomicrographs for panels C and D are shown in **Figure** 3.

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By using the MMA, the potential efficacy of RBC PEGylation can be done on an individualized basis. The probable success of the stealth RBC transfusion can be further enhanced by serologically selecting (or even better MMA testing) the best possible matches from the donor RBC inventory so as to minimize the risks of additional complications. Once the serological or MMA testing of the donor blood unit has been done, the unit can be PEGylated as described then tested against the individual's alloantibodies via the MMA prior to transfusion into the recipient. However, the identification of the donor unit(s), PEGylation and MMA testing does require 48–96 h lead time. Hence, identification of potential patients should be done as early as possible to assure the availability to source appropriate polymer stock, prepare the PEGylation device and identify and test possible donor units. Concurrent with the evaluation of the potential clinical value of the stealth RBC, the physician/transfusion service must also receive institutional and governmental approval for their use.

5. Institutional and governmental approval for patient use

Prior to the actual clinical use of the stealth RBC in a seriously ill patient, compassionate use approval must be obtained from both the hospital Research Ethics Board (REB; or equivalent) and the appropriate governmental agencies (e.g., in Canada, Health Canada). This is likely to be a physician-driven process done in conjunction with the hospital's transfusion service and/or blood provider. Key to these requests is the need to clearly cite the lack of, or very limited availability, of suitable donor RBC. Once institutional approval has been obtained, the hospital REB would likely lead the interaction with the appropriate governmental agency (e.g., Health Canada) regarding an Investigative New Drug (IND) submission. For compassionate use in a single patient who would be likely to die in the absence of a transfusion, a formal IND submission may or may not be necessary. These steps will, obviously, change from country to country.

One question likely to be raised by the REB is whether the proposed mPEG-dosing is safe. The answer to this important question is, at least in part, addressed by recent Phase I–III clinical trials of PEGylated human haemoglobin (PEG-Hb; *Sangart*, San Diego, CA, USA) [69–73]. These clinical trials have infused humans, at the highest dosing schedule, with up to 8.33 ml/kg of PEG-Hb. At this dosing, the typical male volunteers (180 lbs/81.8 kg) received 680 ml of the PEG-Hb solution as a single dose, an infusion of ~25 g of PEG. Importantly, no adverse effects were noted in any of the human volunteers receiving this dose. Furthermore, in animal studies (e.g., rats), PEG-Hb was safely infused at a single adjusted dose exposure of to 46 g of PEG-Hb [74].

6. Conclusion

Grafting of immunologically 'inert' polymers to the membrane of allogeneic RBC can effectively camouflage non-ABO antigens from immune recognition. These immunocamouflaged (i.e., stealth) RBCs may be an effective tool in both preventing and treating alloimmunization in the chronically transfused patient; the transfusion of individual patients with rare blood phenotypes; for emergency situation or geographic locations (e.g., China) where

RhD-negative blood is unavailable. Importantly, several characteristics of the immunocamouflaged RBC may also make them highly suitable in patients/diseases characterized by RBC-mediated vaso-occlusive events (e.g., sickle cell) consequent to the polymer-mediated reduction in low-shear viscosity. For the hospital or clinic, the immunocamouflaged RBCs are inexpensively and easily manufactured using commonly available equipment and existing blood bags. Moreover, the potential clinical utility of the stealth RBC can be evaluated for the individual patient using the clinically validated monocyte-monolayer assay in which antigen-mismatched RBCs are PEGylated and then opsonized with the patient's own alloantibody.

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