

DePEGylation Studies: PEG-RBC Stability in Conditions Consistent with Massive Transfusion

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ABSTRACT

Each year the United States population receives an estimated 12 to 14 million units of packed red blood cells (RBCs) and whole blood.^{1,2} It is estimated that 33% of transfusions associated with trauma are with unmatched type O RBCs (UORBC).³ UORBCs have been proven effective and relatively safe³ however, by masking RBC surface antigens the risk of transfusion reaction may be further decreased. It is, therefore, important to evaluate and validate the stability of antigen masked RBCs, which may play a part in avoiding transfusion reactions. These antigen-masked RBCs would be regularly subjected to abnormal *in vivo* conditions commonly associated with massive transfusion such as lactic acidosis, bacteremia, and *in vitro* irradiation, which is frequently used to sterilize and decrease T Lymphocyte counts in RBC units before transfusion. This study compared two methods of masking RBC antigens by PEGylation: maleimide-PEGylation and cyanuric chloride-PEGylation. RBC PEGylation effectively masks the Rh(D) antigen^{4,5} and PEG-RBC bond stability was evaluated by comparison of pre and post exposure agglutination with anti-D sera. While the stability of maleimide-PEG-RBCs remained unaffected, the cyanuric chloride-PEG-RBCs remained stable in the bacteremia and irradiation studies, but critical concentrations of lactic acid caused dePEGylation. Further studies are warranted to ensure *in vivo* stability.

INDEX TERMS: Pegylation, Erythrocytes, Transfusion, Polyethylene Glycol (PEG), Antigen Masking

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INTRODUCTION

Many attempts have been made to create antigen-masked RBCs for transfusion, the most promising of which is PEGylation of RBC antigens.^{5,6} By attaching long polymer chains of polyethylene glycol (PEG) to RBC membranes, researchers have created a product whereon RBC surface antigens remain undetected by recipient antibodies.⁵ PEG-RBCs have been shown to remain fully functional in O₂ transport, pH buffering capacity, and clearance from the body. Furthermore, PEGylated blood products may enhance peripheral circulation and PEGylation of residual lymphocytes in transfused RBC units may decrease the possibility of graft vs. host disease.⁷ Due to their potential utility, PEG-RBCs could provide a supplement to the current blood supply, yield an alternative for transfusion recipients with multiple abnormal antibodies, and could be immediately administered in cases of urgent transfusion.⁸ Trauma, which may be complicated by septicemia or acidosis, may be an indication for emergent administration of PEG-RBC; such conditions may induce instability in the PEG-RBC bond. Patients in shock commonly present with lactic acidosis due to

hypoperfusion.⁹ Traumatic injuries and many life-saving procedures increase the risk of bacterial infection,^{10,11,12} the subsequent bacteremia may weaken the PEG-RBC bond due to bacterial byproducts that decrease blood pH or the enzymatic catabolization of PEG.¹³ As dePEGylation ensues, donor RBC surface antigens would be exposed to host antibodies and hemolytic transfusion reaction could occur. Many, but not all RBC units undergo pre-transfusion irradiation,¹⁴ which may also hydrolyze the PEG-RBC bond.

Two PEGylation methods were also compared to distinguish the most stable product in various conditions: these were maleimide-PEGylation and cyanuric chloride PEGylation. PEGylation effectively masks the Rh(D) antigen,^{5,6} therefore by quantifying agglutination reactions with anti-D sera before, during and after subjecting the PEGylated RBCs to each simulated condition above, a standardized determination of dePEGylation can be established.

MATERIALS AND METHODS

One unit of type O Rh(D) positive whole blood was drawn according to current standards by a licensed phlebotomist in a blood donation center where the plasma was also expressed. The unit of packed RBCs was immediately shipped to the laboratory of Dr. Parimala Nacharaju where it was split into thirds and PEGylated. PEGylation is the addition of polyethylene glycol to the protein amino terminals on the RBC membrane. One third of the RBCs were derivatized by maleimide-PEG-5000 (Laysan Biological) in the presence of 2-iminothiolane for 2 hours in incubation buffer, washed three times and incubated again as previously described.¹⁵ The second portion was derivatized by cyanuric-PEG-5000 (Sigma-Aldrich), incubated for one hour and subsequently washed twice.¹⁶ The third portion was kept as a control. The derivatized and control RBCs were received three days after initial shipment and the maleimide-PEG-RBCs, cyanuric PEG-RBCs, and control RBCs were each assayed (as described below) 10 times in order to determine a baseline value of agglutination with which to compare all other results. All testing was completed within 30 hours after the blood was received so that minimal aberrant results due to sample degradation or RBC metabolism were observed.

Each of these samples was separated and used to simulate each of the abnormal *in vitro* conditions; 9 tubes were used for each reaction, 3 separate 3 mL aliquots of all 3 RBC types were placed into red top Vacutainer® tubes, where the abnormal *in vitro* conditions were created; lactic acidosis and septicemia (Figure 1). Prior to separation, 25 mL samples of each RBC type were irradiated with 25 Gray¹⁷ according to current standards.

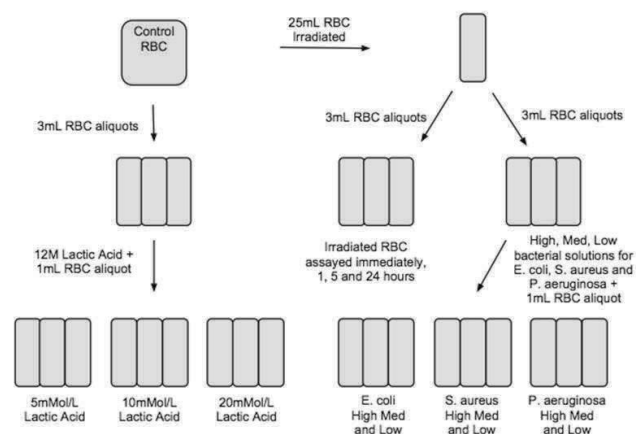


Figure 1: Example flow diagram for control RBCs. This process was repeated for both the Maleimide PEGylated RBCs and Cyanuric PEGylated RBCs.

Lactic Acidosis

Twelve M lactic acid was diluted with Phosphate Buffered Saline (PBS) and added to all nine of the 3 mL aliquots to simulate low (5mMol/L), medium (10mMol/L), and high (20 mMol/L) concentrations in each RBC type. Each tube was assayed initially, and at 30 minutes, 1 hour and 5 hours; serum lactate levels were measured by the enzymatic LDH method with spectrophotometric detection (Cobas chemistry analyzer, Roche Diagnostics) at each interval to track increases in lactate caused by RBC metabolism.

Irradiation

Three mL aliquots of each RBC type that had been irradiated were placed in three separate tubes. Extent of dePEGylation was assessed subsequently, at 1, 5 and 24 hours. The remainder of the irradiated blood was then used for the bacterial portion of the study to ensure complete sterility of the RBCs.

Bacteremia

All three types of irradiated RBCs were immediately cultured on a blood agar plate under aerobic conditions

at 37°C in triplicate to ensure initial sterility; no initial irradiated plates exhibited bacterial growth. After initial sterility was confirmed, strains of *P. aeruginosa*, *S. aureus* and *E. coli* were diluted to low, medium, and high concentrations separately in Phosphate Buffered Saline (PBS) to create 3 bacterial solutions for each species: low (1,000 CFU/mL), medium (10,000 CFU/mL) and high (100,000 CFU/mL). One mL of Control RBCs was added to 500 uL of the high, medium, and low concentrations of *S. aureus*. This pattern was followed for *E. coli* and *P. aeruginosa* as well resulting in 9 samples of control RBCs (Figure 1). Cyanuric PEG-RBCs and maleimide PEG-RBCs were also tested according to the same pattern. Each tube was then assayed initially, at 24, 48 and 72 hours: 1uL of each solution was cultured on a blood agar plate and colonies were counted in CFU/mL.

Assay

To evaluate any change in PEGylation, microscopic agglutination reactions were graded, in quadruplicate, on a hemocytometer for each RBC type and each simulated condition. This protocol used for the agglutination reactions was the same for every assay performed in the study. As anti-D sera is added to Rh(D) RBCs, the antibodies are able to disrupt the RBC membrane which allows trypan blue inside the cell providing visualization and quantification of RBC membrane destruction.

For each tube, a 500 uL aliquot of a 6% hematocrit solution was added to 500 uL of a 1:3 anti-D sera (Ortho) to PBS solution and centrifuged. The centrifugate was poured off and 10 uL of the resulting RBC button were pipetted into a separate tube along with 10 uL of trypan blue, which only stains RBCs lacking an intact membrane and indicates a nonviable cell,¹⁸ and 2.5 mL of PBS were added to each of 4 separate test tubes for testing in quadruplicate. 15 uL of the resulting PBS/trypan blue/RBC solution from each of the 4 tubes were loaded into separate hemocytometer for counting. Five RBC squares were counted in every hemocytometer reading (on average 239 RBCs were counted per five RBC squares). RBCs were counted to determine a ratio of viable (non-agglutinated and unstained) to total RBCs counted. Thus, a decrease in this ratio that did not occur simultaneously in the control RBCs would indicate dePEGylation.

Baseline control values were obtained before subjecting any of the RBCs to the simulated conditions. 10 aliquots from each blood type were assayed in quadruplicate and an average ratio of unstained to total RBCs was determined for each RBC type. Graphically, these values represented a normal distribution and a +/- 2 standard deviation range was determined. If values remained within this range throughout the course of testing each condition, it was assumed that no significant dePEGylation had occurred. Any decrease in viability 2 SD away from the mean was assumed to be dePEGylation, and there was no observed increase in viability; such variability above the mean could be caused by mistakenly counting non-viable RBCs as viable due to cell overlap on the hemocytometer.

More control RBCs were subjected to the high and low extremes of each condition and the same amount of solution was counted on a hemocytometer without adding anti-D reagent to further ensure that increased levels of agglutination were not a result of each condition's effect on the natural RBC.

RESULTS

Each PEG-RBC type was assayed, the ratios of viable:total RBCs were plotted and the mean value was set as the baseline. The term "did not deviate significantly from the mean" will be used to denote that the values obtained from each assay remained within the predetermined 2 standard deviation range which was calculated individually for each RBC type, indicating that dePEGylation did not occur. (Table 1)

Irradiation

DePEGylation was not observed in either maleimide-PEG or cyanuric-PEG as the ratios of viable cells:total RBCs did not deviate more than 2SD from the mean.

Table 1. Baseline ratio of unstained cells to total cells

| | Maleimide-PEG | Cyanuric-PEG | Control RBC |
|----------------|---------------|--------------|-------------|
| Baseline Ratio | .9847 | .9864 | .2868 |
| +/- 2 SD Range | .9480-1.000 | .9502-1.000 | .2255-.3101 |

Bacteremia

Table 2: Ranges of bacterial counts (in 1,000 CFU/mL units); at all time intervals, ranges indicate the highest

and lowest values seen in all RBC types and in all three species combined. Values from bacteremia assays for both maleimide-PEG and cyanuric-PEG did not deviate significantly from the mean and dePEGylation was not observed after exposure to low, medium, and high CFU/mL concentrations. (Table 2)

Table 2. Range of bacterial counts from *S. aureus*, *E. coli*, and *P. aeruginosa* in both maleimide-PEG and cyanuric-PEG. No bacterial tests deviated significantly from the mean.

| Relative Bacterial Concentration | Time | Range of Bacterial Counts of 500 uL bacterial solution and blood aliquot x 1000CFU/mL |
|----------------------------------|----------|---|
| Low | Initial | <2 |
| Low | 24 Hours | 3-10 |
| Low | 72 Hours | 89-126 |
| Medium | Initial | 3-7 |
| Medium | 24 Hours | 25-37 |
| Medium | 72 Hours | 110-165 |
| High | Initial | 60-80 |
| High | 24 Hours | 100-138 |
| High | 72 Hours | >200 |

Lactic Acidosis

Maleimide-PEG values did not deviate significantly from the mean during the lactic acid study.

Figure 2 is a comparison of maleimide and cyanuric-PEG in lactic acid study. While maleimide-PEG-RBCs were not significantly affected by increased levels of lactic acid, dePEGylation of cyanuric-PEG-RBCs began at a serum lactic acid value between 11.2 – 14.6 mMol/L ($p < 0.0013$). The dashed line shows the lower boundary of the range of significance.

Cyanuric-PEG 5 mMol/L

At 5 hours, significant dePEGylation occurred decreasing the ratio value to .941; this indicates a decrease of 1.01% of PEGylated cells. A serum lactic acid level of 14.8 mMol/L was measured at this time using the LDH method.

Cyanuric-PEG 10 mMol/L

Ratio values of .9039 and .7900 were recorded at 1 hour and 5 hours respectively for a total of 16.1% dePEGylation. Serum lactic acid levels of 14.6 mMol/L and 17.4 mMol/L were measured at the same times using the previously described enzymatic LDH spectrophotometric method.

Cyanuric-PEG 20 mMol/L

DePEGylation immediately occurred upon adding the dilute lactic acid, the viable to total RBC ratio was significantly lower than the mean at .8460; 32.7% dePEGylation had occurred at 5 hours. Serum lactic acid levels above 20 mMol/L are considered higher than critical clinical ranges of significance and were not measured.

DISCUSSION

The consistency in results obtained from every assay of control RBCs verified that any decrease in PEG-RBC viability throughout the experiment was the result of dePEGylation, and not an artifact caused by alteration to the RBC or antibody.

Pre-transfusion radiation of all RBC types was not observed to increase agglutination, which indicates that both PEGylation methods can withstand standard radiation procedures without dePEGylation. Cyanuric-PEG was dePEGylated at a serum lactic acid level between 11.4 and 14.6 mMol/L which is very significant given that *in vivo* serum lactic acid levels above 4 mmol/L are associated with 11% survival in ICU patients if persistent for 24 hours, and the potential complications of a hemolytic transfusion reaction superimposed on severe lactic acidosis would most likely ensure death.¹⁹ Thus, cyanuric-PEG-RBCs are not plausible RBCs in such situations. The lack of reactivity of the remaining results however is encouraging. The maleimide-PEG-RBCs continued to mask the Rh(D) antigen in all simulated conditions.

CONCLUSION

While not confirmatory, this pilot study serves to encourage further testing of PEG-RBCs as a possible method of creating functional antigen-masked RBCs. While masking of the Rh(D) antigen was accomplished in this study, greater utility would be achieved by successful masking of ABO antigens. Testing of only one unit of blood may not accurately represent the entire population and further studies with multiple donors are warranted. The maleimide method of PEGylation demonstrated superior durability in lactic acidosis and should therefore be incorporated into the focus of further studies while the cyanuric method should be re-evaluated. Furthermore, these findings suggest the need to evaluate the stability of PEG-RBCs in a plethora of potential *in vivo* complications given that *in vitro* dePEGylation has occurred.

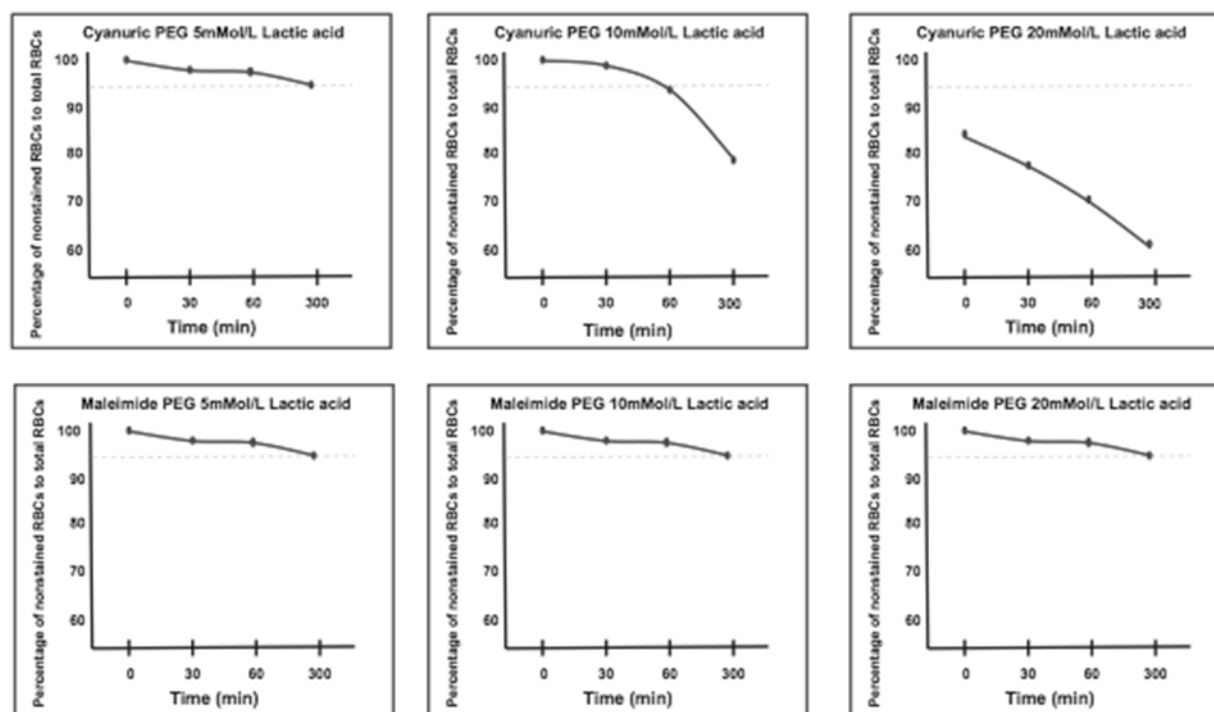


Figure 2. Tracks the percentage of viable PEG-RBC to total cells counted per assay subjected to differing mMol/L concentrations of lactic acid; the 94.82% (Maleimide) and 95.02% (CNCI) lines show the determined 2 S.D. range; a drop below this value indicates significant dePEGylation. Maleimide-PEG was not affected while cyanuric-PEG was significantly dePEGylated by the high lactate concentration ($p < 0.0013$).

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