Simultaneous Camouflage of Major and Minor Antigens on Red Blood Cell Surface With Activated mPEGs

Zahra Gholami [1]; Sameereh Hashemi Najafabadi [1,*]; Masoud Soleimani [2]

[1]Faculty of Chemical Engineering, Tarbiat Modares University, Tehran, I.R. IRAN
[2]Faculty of Medical Sciences, Tarbiat Modares University, Tehran, I.R. IRAN

Corresponding author: Sameereh Hashemi Najafabadi, Faculty of Chemical Engineering, Tarbiat Modares University, Tehran, I.R. IRAN. Tel: +98-2182884384, Fax: +98-2182884931, E-mail: s.hashemi@modares.ac.ir

Background: Host immune system response against blood group antigens is a major problem in blood transfusions, especially for thalassemic patients. Thus, an approach was proposed coating the red blood cell (RBC) surface by polyethylene glycol.

Objectives: This study aimed to obtain the optimal simultaneous camouflage of the major and minor antigens by activated methoxy polyethylene glycol (mPEG) with succinimidyl valerate (SVA) and succinimidyl carbonate (SC), separately.

Materials and Methods: The degree of RBC agglutination by antibodies against the major and minor blood groups was used as a surrogate measurement for quantitative assessment of the effectiveness of the surface coating. Also, the RBC morphology was assessed using scanning electron microscope (SEM). In addition, to evaluate the host immune system response, the PEGylated RBCs were transferred between two different mouse strains.

Results: Statistical analysis of the results demonstrated that the optimal reaction conditions for simultaneous coating of the antigens by mPEG-SVA and mPEG-SC are as $mPEG_{20}$ in the polymer mixture, 91.2 and 90.0%, and polymer concentration, 17.21 and 19.80 $mg.mL^{-1}$, respectively. However, according to the SEM results, the maximum polymer concentration of 14.5 $mg.mL^{-1}$ was suggested as the best condition for mPEG-SVA modified human RBCs.

Conclusions: It is concluded that the membrane PEGylation camouflages the blood group antigens. This effect is observed significantly for non-ABO/Rh(D) antigens. Also, it is found that the mPEG-SVA provide better coverage than mPEG-SC. The results of in vivo analysis showed that the immune reactions against PEGylated RBCs were considerably reduced, so that the levels of the relevant biochemical parameters in serum were similar to those of the normal hosts 24 hours after transfusion.

Keywords: Red Blood Cells; Methoxy Polyethylene Glycol; Carbonates; Transfusion

1. Background

The most important obstacle in organ transplantation and blood transfusion is the immunological response to the transplanted tissues and cells. Red blood cells (RBCs) are the simplest transfused allogeneic cells (1). The membrane of RBCs architecturally contains complex structures that carry defined polymorphic epitopes, identified serologically as blood group antigens.

The first reason of RBC rejection is the immune reaction against the transfusion of mismatched RBCs (2). Following Landsteiner's discovery of ABO blood group antigens, the blood transfusion was changed from a highly risky surgical procedure to a mundane yet clinically crucial procedure (2). Thus, it is essential to identify the appropriate donors, with ABO and D (Rhesus) blood typing, in all transfusions.

However, it is difficult to identify appropriate blood donors (3-5). The chronically transfused patients, who suffer from thalassemia and sickle cell disease encounter the problems of alloimmunization (6, 7). Immunoprotection of RBCs can be achieved by covalent attachment of methoxy polyethylene glycol (mPEG) to the proteins expressed on the cell membrane (PEGylation) (8, 9). Owing to the mPEG flexibility, a large steric exclusion volume surrounding the RBCs inhibits the binding of large molecules (e.g. antibodies) or other cells (e.g. immune cells) to them (8). Several reactive derivatives of mPEG, such as mPEG-succinimidyl valerate (mPEG-SVA) and mPEG-succinimidyl carbonate (mPEG-SC), have been employed to covalently attach mPEG to the surface of RBCs (5, 8, 10-15).

Recently, Wang et al. have investigated the effect of PE-Gylation using mPEG-SVA on reducing the antigenic rec-

ognition of blood group antigens expressed by normal human RBCs (5). Lee and Scott used mPEG-SC and mPEG-SVA for PEGylation of polystyrene beads to evaluate the charge camouflage and protein adsorption (8).

Despite the development of this strategy, optimization of the reaction conditions for simultaneous camouflage of the major and minor RBC antigens has not been thoroughly investigated. In our previous works, we have investigated the attachment of activated mPEG to RBCs in order to optimize the reaction conditions for protection of major antigens on RBCs (16, 17).

2. Objectives

Ongoing work on the modification of RBC suggests that PEGylation may be a practical method for decreasing the risk of transfusional alloimmunization, and treatment of alloimmunized patients (5, 8).

In the present study, RBCs were coated with two derivatives (mPEG-SC and mPEG-SVA) in two different molecular weights to investigate simultaneous camouflage of the major and minor antigens (A, Rh (D) and Kell antigens). Then, the effect of polymer size and linker chemistry on camouflage of these antigens was assessed. Additionally, central composite design (CCD) methods were used to find the optimal conditions for PEGylation of RBCs. Finally, the immunocamouflage and immunogenic potential of unmodified and PEGylated RBCs were evaluated in a murine transfusion model.

3. Materials and Methods

3.1. Materials

In our research, we used several ingredients from different sources, which are listed as follows: mPEG (10 and 20 kDa) purchased from Sigma; mPEG-SVA (10 and 20 kDa) obtained from Laysan Bio, Inc. (Arab, AL, USA); N, N'-Disuccinimidyl carbonate purchased from Aldrich (U.S.A); sodium chloride, isopropanol, dry diethylether, 4-(dimethylamino) pyridine, cyclohexane and dioxin obtained from Merck (Germany); packed Rh-positive RBCs (A+-Kell+) obtained from Iranian Blood Transfusion Organization; anti-D and anti-A sera purchased from Cinna Gen Inc. (Iran); and anti-Kell sera obtained from Diagast (France).

3.2. Preparation of Samples

3.2.1. mPEG-SC Derivatization

We performed derivatization of 10 and 20 kDa mPEGs with SC using a modified method described by Miron and Wilchek (18). Briefly, 1 mmol of vacuum dried mPEG (overnight at 80°C) was dissolved in 100 mL dioxan and warmed slightly to help the dissolution and in the following step, the solution was cooled at room temperature.

Then, threefold excess of N, N'-Disuccinimidyl carbon-

ate was slurried in 10 mL of dry acetone and added to the above solution. Next, 6 -mmol of 4-(dimethylamino) pyridine was dissolved in 20 mL of dry acetone, added to the reaction mixture, and is allowed to proceed at room temperature under dry nitrogen atmosphere for 24 h.

The mixture was filtered to eliminate any solid sediment and then, was poured slowly under high shear into the diethyl ether to precipitate the derivatized mPEG. The precipitate was then refiltered and washed with diethyl ether, resuspended in isopropanol to remove any unreacted N, N'-disuccinimidyl carbonate, filtered and then washed with isopropanol. Next, the precipitate was resuspended in cyclohexane, filtered and washed with cyclohexane. Finally, the product (mPEG-SC) was dried under a stream of dry nitrogen at room temperature for 12 h.

3.3. Characterization of mPEG-SC

A Fourier transform infrared (FTIR) spectra of mPEG-SC and mPEG samples were recorded using an FTIR spectrophotometer (FTIR, Perkinelmer) at room temperature over the frequency range of 450–4000 cm^{-1} to verify the derivatization of mPEG-SC.

3.4. RBC Coating With Activated mPEGs

Packed Rh-positive RBCs (A+-Kell+) were suspended at 10% hematocrit in phosphate buffered saline (PBS) solution. Fresh cold solutions of the derivatized polymer (mPEG-SC or mPEG- SVA, separately) were prepared in PEG-buffer (50 mmol.L^{-1} dibasic potassium phosphate, 105 mmol.L^{-1} NaCl) to produce polymer concentrations of 7–22 mg.mL^{-1} with different molecular weights and pH, according to the experimental design (presenting in the following sections), and immediately added to the RBC suspensions.

Derivatization reactions were carried out with gentle mixing at room temperature for 45 min. After washing three times with isotonic PBS (pH 7.4) at 200 × g for 10 min, the packed RBCs were prepared for assessment of the polymer coating quality (6).

3.5. RBC Agglutination by Antisera and Cell Counting

RBC agglutination by anti-D, A, and Kell sera was used to assess the effectiveness of RBC PEGylation. Four hundred microliters of uncoated or PEGylated RBC suspension (6% hematocrit in an isotonic saline buffer), was separately mixed with a solution of anti-D, A, or Kell in PBS with a known concentration (antisera/PBS: 1/3) and then, incubated with a gentle mixing at room temperature for 30 min.

Next, the RBCs were centrifuged at 200 ×g for 1 min (19). One microliter of the pellet was resuspended in 1 mL of PBS. Next, by using a dye exclusion test with Trypan blue and light microscopic system (Nikon, E200),

non-agglutinated viable free cells were counted on a hemocytometer (improved Neubauer ruling). Under this condition, the viable cells remained uncolored and the dead cells would turn blue. The higher number of viable free cells, the greater would be the effectiveness of RBC coatings.

3.6. Scanning Electron Microscopy (SEM)

The morphology of both control and mPEG-derivatized RBCs was assessed by a scanning electron microscope (SEM 3200, China) to ensure that they are structurally appropriate for transfusion. For preparation of the samples, the procedure explained by Kayden and Bessis was followed (20).

3.7. Statistical Designs

The first step identifies the most important factors in the PEGylation reaction on human RBCs with mPEG-SVA and mPEG-SC. Selection of these variables and their levels (polymer concentration 7–22 mg.mL^{-1}, polymer molecular weight 10–20 kDa, and reaction pH 8.0–9.2) were based on our preliminary experiments (not presented here), and previous studies in the literature for RBC PEGylation (5, 8, 11, 16, 17, 19).

3.8. Central Composite Design

We employed Central Composite Design (CCD) to investigate the impact of multiple variables on the inhibition of agglutination and antibody binding (as a single response), to estimate the contribution of individual factors, and finally to obtain the optimal conditions for PEGylation reaction.

The total number of experiments in a CCD is calculated by the formula; $2^k + 2k + x_0$, where k and x_0 are the number of factors and central points, respectively (21). This methodology allows the modeling of a second-order polynomial equation that defines the process. The nonlinear model obtained with statistical Design Expert (version 7.0.0, Stat-Ease, Inc.) is as follows:

$$Y = b_0 + \sum b_i X_i + \sum b_{ii} X^2_i + \sum b_{ij} X_{ij} \ (1),$$

where Y is the predicted response, X_i shows the independent variable, b_0 is an intercept, bi is the linear effect, b_{ii} is the squared effect, and b_{ij} shows the interaction effect.

To obtain the optimal conditions for camouflaging Kell antigen on the surface of the human RBCs by mPEG-SVA, a CCD for three variables at five levels was designed. The variables, their corresponding levels and the designed experiments are presented in Table 1.

Based on the obtained results, and previous studies in the literature for RBC PEGylation, pH of the reaction is not significantly effective on antigen coating. Thus, other experiments were carried out at constant pH and another CCD for two variables at five levels was designed to investigate the effect of mPEG-SVA on camouflaging

of Rh (D) and A antigens, (Table 2).

All of the responses were presented as the average number of free cells per 1 mL suspension, obtained by cell counting method. Furthermore, a CCD with two variables at five levels was designed to find the optimal conditions for camouflaging Kell, Rh (D) and A antigens with mPEG-SC,. Table 3 presents the variables, their corresponding values and the designed experiments.

3.9. In Vivo RBC Immunogenicity

For in vivo studies, two genetically distinct mouse strains (C57Bl/6 and BALB/c) were selected to investigate the alloimmunization (5). The selected mice were adult male aged 2.5–3.0 months (25–30 g in weight). The RBCs of C57Bl/6 mice as the donors were collected via cardiac puncture. Mouse RBCs were washed three times in PBS (pH 7.4) and resuspended in a 10% hematocrit.

The RBC suspension was derivatized by mPEG-SVA at the concentration of 10 mg.mL^{-1} for 30 min at 4°C in PBS (pH 8.6) as previously described. After derivatization, the cells were washed three times in PBS and resuspended in a 30% hematocrit before transfusion.

The mice were divided into three groups; a positive control group which are transfused with unmodified RBCs; the negative control group without transfusion; and the PEGylated group which are transfused with PEGylated RBCs.

Recipient mice (BALB/c) were transfused through the tail vein with 300 μL of non-PEGylated or PEGylated RBCs. After 24 hours, two mice in each group (the negative control, the positive control and the PEGylated group) were sacrificed, and their serum was collected for biochemical analysis. The levels of alanine amino transferase (ALT), aspartate amino transferase (AST), lactate dehydrogenase (LDH), and total bilirubin (BLT) in the mouse were measured by an auto analyzer (Selectra XL, The Netherlands).

4. Results

4.1. Characterization of mPEG-SC

Figure 1 shows the FTIR spectra of mPEG and mPEG-SC. The FTIR spectra of mPEG-SC (curve b and c), in comparison with the FTIR spectra of mPEG (curve a), has new peaks appearing at 1744.16 and 1743.96 cm^{-1}, corresponding to the succinimide group in mPEG-SC of 10 and 20 kDa molecular weight, respectively, which approves the successful polymer derivatization.

4.2. Central Composite Design

We used Central Composite Design (CCD) to find the suitable variables on camouflaging the human RBCs. Tables 1–3 presents the results of CCD experiments consisted of the predicted and experimental data after PEGylation with mPEG-SVA and mPEG-SC for evaluation of

Table 1. CCD for Independent and Dependent Variables to Evaluate the Camouflaging of Kell Antigen on Human RBCs Using mPEG-SVA

Run No.	X_1	X_2	X_3	Experimental response[b] $\times 10^7$	Predicted Response[c] $\times 10^7$
	Polymer Concentration (mg.mL^{-1})	mPEG$_{20}$ in Mixture[a] (%)	pH		
1	10.04	79.73	8.96	25	26.60
2	14.50	50.00	8.60	30	28.06
3	14.50	50.00	8.00	15	15.21
4	18.96	79.73	8.24	25	26.15
5	7.00	50.00	8.60	10	9.68
6	14.50	50.00	8.60	27	28.06
7	22.00	50.00	8.60	35	33.58
8	14.50	50.00	8.60	28	28.06
9	18.96	20.27	8.24	25	24.63
10	14.50	50.00	8.60	28	18.06
11	10.04	20.27	8.24	5	5.42
12	10.04	79.73	8.24	20	19.44
13	10.04	20.27	8.96	5	5.81
14	14.50	50.00	9.20	25	23.05
15	18.96	79.73	8.96	35	35.81
16	18.96	20.27	8.96	25	26.79
17	14.50	0.00	8.60	15	14.45
18	14.50	100.00	8.60	35	33.81
19	14.50	50.00	8.60	25	28.06

[a] Mixture is a combination of 20 and 10 kDa mPEGs.
[b] Each value is the average number of free cells per 1 mL suspension, following reaction with anti-Kell, for each test with two replicates.
[c] Each value is the predicted response obtained by the model, presenting in the following sections, after reaction with anti-Kell

Table 2. CCD for Independent and Dependent Variables to Evaluate the Camouflaging of Rh (D) and A Antigens on Human RBCs Using mPEG-SVA

Run No.	X_1	X_2	Experimental Response[b] $\times 10^7$	Predicted Response[c] $\times 10^7$	Experimental Response[b] $\times 10^7$	Predicted Response[c] $\times 10^7$
	Polymer Concentration (mg.mL^{-1})	mPEG$_{20}$ in Mixture[a] (%)				
1	7.0	50.00	0	0	0	0
2	19.8	85.36	30	28	25	23
3	9.2	14.64	0	0	0	0
4	22.0	50.00	25	26	20	20
5	14.5	0.00	10	10	5	5
6	19.8	14.64	20	20	15	14
7	14.5	50.00	18	18	15	14
8	14.5	100.00	20	22	15	16
9	9.2	85.36	10	9	5	5
10	14.5	50.00	15	18	15	14
11	14.5	50.00	20	18	15	14
12	14.5	50.00	20	18	15	14
13	14.5	50.00	15	18	10	14

[a] Mixture is a combination of 20 and 10 kDa mPEGs.
[b] Each value is the average number of free cells per 1 mL suspension after reaction with anti-Rh (D) and anti-A, respectively, for each test with two replicates.
[c] Each value is the predicted response obtained by the model, presenting in the following sections, after reaction with anti-Rh (D) and anti-A, respectively.

Table 3. CCD for Independent and Dependent Variables to Evaluate the Camouflaging of Kell, Rh (D) and A Antigens on Human RBCs Using mPEG-SC

Run No.	X1	X2	Experimental response[b] $\times 10^7$	Predicted response[c] $\times 10^7$	Experimental response[b] $\times 10^7$	Predicted response[c] $\times 10^7$	Experimental response[b] $\times 10^7$	Predicted response[c] $\times 10^7$
	Polymer Concentration (mg.mL^{-1})	mPEG$_{20}$ in mixture[a] (%)						
1	7.0	50.00	5	5	0	1	0	0
2	19.8	85.36	36	36	30	30	30	30
3	9.2	14.64	10	5	5	0	0	0
4	22.0	50.00	30	30	30	29	18	19
5	14.5	0.00	5	6	0	0	0	0
6	19.8	14.64	15	15	10	10	15	17
7	14.5	50.00	25	27	15	16	15	15
8	14.5	100.0	25	25	15	16	15	17
9	9.2	85.36	10	10	5	4	5	4
10	14.5	50.00	30	27	21	19	15	15
11	14.5	50.00	25	27	20	19	15	15
12	14.5	50.00	30	27	22	19	15	15
13	14.5	50.00	25	27	20	19	15	15

[a] Mixture is a combination of 20 and 10 kDa mPEGs.
[b] Each value is the average number of free cells per 1 mL suspension, after reaction with anti-kell, anti-Rh(D) and anti-A, respectively, for each test with two replicates.
[c] Each value is the predicted response obtained by the model, presenting in the following sections, after reaction with anti-kell, anti-Rh(D) and anti-A, respectively.

the effects of independent variables on masking the minor and major antigens on human RBCs. All the responses are reported as the number of viable free cells per 1 mL suspension. An increase in the number of free cells for PEGylated versus control (uncoated) RBCs, indicates that PEG-RBCs have been protected against agglutination with antisera.

To calculate the response, Y, we used a mathematical relationship in the form of a second-order polynomial equation. Table 4 shows the coefficients of this model (Equation 1) for camouflaging of Kell, Rh(D), and A antigens on human RBCs using mPEG-SVA and mPEG-SC.

These polynomial equations show the quantitative effect of the process variables and their interactions on the response Y. The values of coefficients of X_1, X_2, and X_3 are related to the effect of these variables on the response. A positive value in Table 4 represents a favorable effect, while a negative value indicates an adverse effect.

In this case, X_1 and X_2 have the main effects on the response. The values of X were substituted in the equation to obtain the theoretical values of Y. The analysis of variance indicated that X_1 is more significant than X_2 and then X_3.

Model F values presented in Table 4 imply that the models are significant. The small difference between the predicted R-squared and adjusted R-squared of the models indicates that they are in good agreement with each other (Table 5).

Figures 2 and 3 present the main interaction effect on the number of free RBCs, as an indicator of the extent of PEGylation. As shown in these Figures, polymer concentration and molecular weight (size) were important in preventing the recognition of A, Rh(D), and Kell antigens on RBCs. In addition, it shows that higher concentration and molecular weight of these polymers have effectively increased the number of free RBCs (P <.05). Higher polymer concentrations even at low molecular weights are more effective and yield the maximum camouflage of minor and major antigens on the RBCs.

The red zones with the maximum number of free cells in Figures 2 and 3 shows that the combination of 20 and 10 kDa polymers with a high percentage of mPEG$_{20}$ is significantly more effective than 20 kDa polymers alone.

In addition, Figure 2 A indicates no significant difference between pH values of 8.0 and 9.2 after derivatization. This finding shows that pH value of the reaction medium is not significantly effective in preventing the antigenic recognition of Kell antigens on RBCs. Thus, 8.6 were selected as the optimal pH value.

Based on these Figures and Models, Tables 6 and 7 present the optimal conditions of PEGylation (pH of the reaction medium, polymer concentration, and percentage of mPEG20 in the polymer mixture) obtained by using free cell counting. The validation experiments were carried out under optimized conditions, showed a good agreement with the statistically predicted values, and confirmed the model's authenticity.

As shown in Figures 2, 3 also Tables 6, 7, significant differences are demonstrated between the linker chemistries

with respect to effective camouflage of blood antigens. Therefore, mPEG-SVA is more efficient than mPEG-SC at all the grafting concentrations and polymer sizes. Furthermore, these results showed that Rh (D) and Kell antigens are efficiently camouflaged by mPEG. In comparison to A or Rh (D) antigens, Kell antigen as a minor antigen, is effectively more camouflaged, as shown by decreasing in antibody binding.
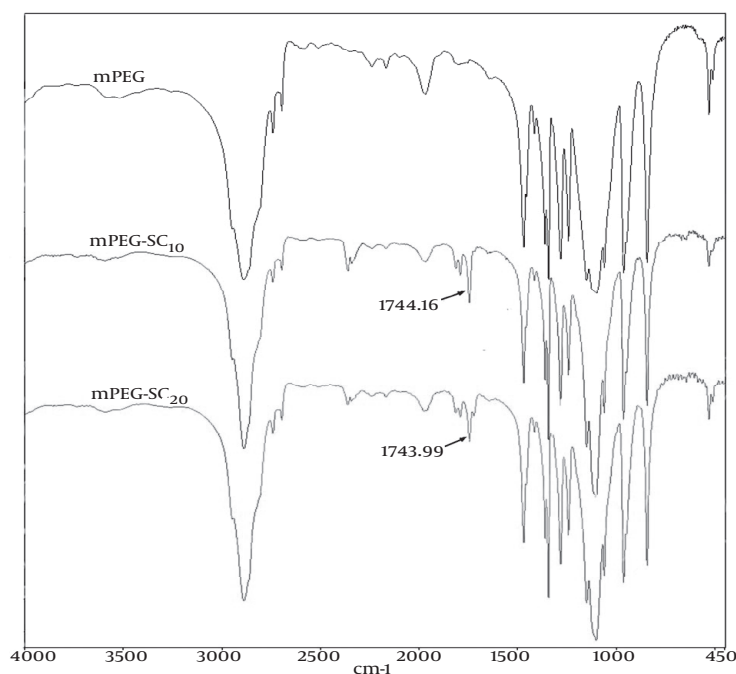

Figure 1. FTIR Spectra of mPEG and mPEG-SC With 10 and 20 kDa Molecular Weight

Table 4. The Coefficients of the Models Obtained According to Equation 1 for Camouflage of Kell, Rh (D), and A antigens Using mPEG-SVA and mPEG-SC

Model Parameters	mPEG Derivative					
	mPEG-SVA			mPEG-SC		-
-	Kell	Rh(D)	A	Kell	Rh(D)	A
Intercept $\times 10^8$	2.81	+1.82	+1.40	+2.70	+1.90	+1.50
$b_1{}^a \times 10^7$	7.11	+9.42	+7.91	+8.92	+9.68	+2.93
$b_2{}^a \times 10^7$	+5.76	+4.27	+3.64	+6.79	+5.78	+6.40
$b_3{}^a \times 10^7$	+2.33	-	-	-	-	-
$b_{12}{}^b \times 10^7$	-3.13	0.00	+1.25	+4.00	+3.75	+5.00
$b_{13}{}^b \times 10^6$	+6.25	-	-	-	-	-
$b_{23}{}^b \times 10^7$	+1.88	-	-	-	-	-
$b_{11}{}^c \times 10^7$	-2.27	-2.54	-1.69	-4.69	-2.00	-2.56
$b_{22}{}^c \times 10^7$	-1.39	-1.29	-1.69	-5.94	-5.75	-3.31
$b_{33}{}^c \times 10^7$	-3.16	-	-	-	-	-
P Value of the model	< 0.0001	< 0.0001	0.0001	< 0.0001	< 0.0001	< 0.0001
F Value of the model	44.44	47.17	31.44	64.59	64.11	70.22

[a] These parameters are the coefficients of: X_1: Polymer concentration, X_2: Percentage of mPEG20 in the polymer mixture (combination of 20 and 10 kDa mPEGs) and X_3: pH in Equation 1, respectively.

[b] These parameters are the coefficients of: X_{12}, X_{13}, and X_{23} (interaction of factors), respectively in Equation 1.

[c] These parameters are the coefficients of: $X^2{}_{11}$, $X^2{}_{22}$ and $X^2{}_{33}$ (squared factors), respectively in Equation 1.

Table 5. Statistical values obtained by CCD to evaluate the camouflage of Kell, Rh (D), and A antigens using mPEG-SVA and mPEG-SC

Statistical values	mPEG derivative					
	mPEG-SVA			mPEG-SC		
R-squared	0.98 [a]	0.97 [b]	0.96 [c]	0.98 [a]	0.97 [b]	0.98 [c]
Adj R-squared	0.96 [a]	0.95 [b]	0.93 [c]	0.96 [a]	0.96 [b]	0.97 [c]
Pred R-squared	0.90 [a]	0.90 [b]	0.90 [c]	0.96 [a]	0.93 [b]	0.86 [c]

[a], [b], [c] Statistical values for coating of Kell, Rh (D), and A antigens, respectively.

4.3. Scanning Electron Microscopy

Figures 4 and 5 present the morphology of control (un-coated) and PEG-coated human RBCs (at 17.21 and 14.50 mg.mL-1 of mPEG-SVA) under optimal conditions (percentage of mPEG20 = 91.2% and pH 8.6). Selection of these concentrations was based on the optimal condition and our pervious results (16, 17).

These Figures display that at polymer concentrations higher than 14.5 mg.mL^{-1}, the human RBCs will deform by increasing RBC echinocytosis. It showed that polymer concentration of mg.mL^{-1} is a useful level for PEGylation of RBCs by 91.2% mPEG20-SVA in the polymer mixture. Echinocytes are not deformable and hence tend to get trapped in the microcirculation (22). Thus, the optimal concentration of 17.21 mg.mL^{-1} (obtained by cell counting method), can-not be recommended as an optimal polymer concentra-tion due to the loss of discocytic morphology.

These results are similar to our previous finding for PEGylation of RBCs by mPEG, activated by cyanuric chloride and succinimidyl carbonate (16, 17). Morever, the cell counting method was used to investigate this lower polymer concentration (14.50 mg.mL^{-1}) on camouflaging of Kell, Rh (D), and A antigens. In this regard, the numbers of obtained viable free cells were 3.2, 2.8, and 2.4 × 108 per 1 mL suspension for Kell, Rh (D), and A antigens, respectively. Thus, there was no significant difference between these results and the results given in Table 6 for polymer concentration of 17.21 mg.mL^{-1}.

The morphology of PEGylated murine RBCs (at 14.5 and 10.0 mg.mL^{-1} of mPEG-SVA) under the optimal conditions (percentage of mPEG$_{20}$ = 91.2% and pH 8.6) are shown in Figure 6.
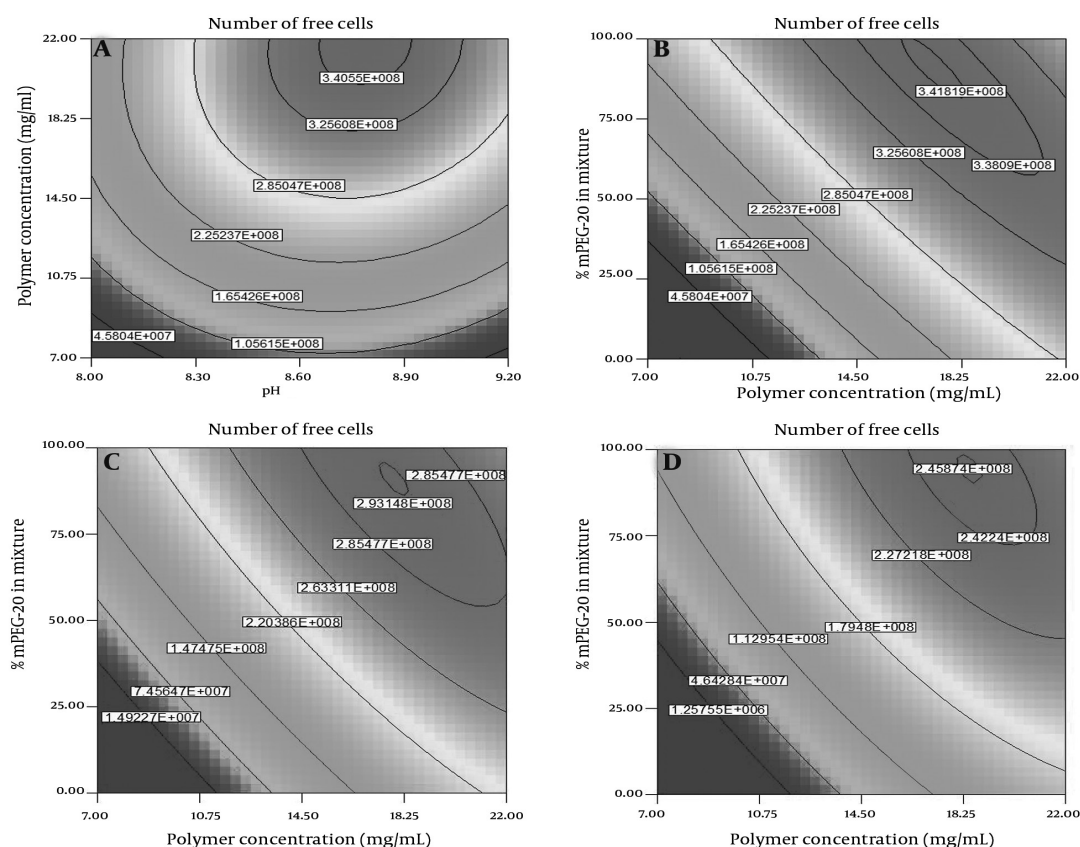

Figure 2. 2D Views of the Response Surface Plots of the Polymer Concentration Effect, Percentage of mPEG20 Activated With SVA and Reaction pH as Independent Variables on the Y Response, and the Effect of Independent Variables on Coating of (A, B) Kell, (C) Rh (D), and (D) A antigens.
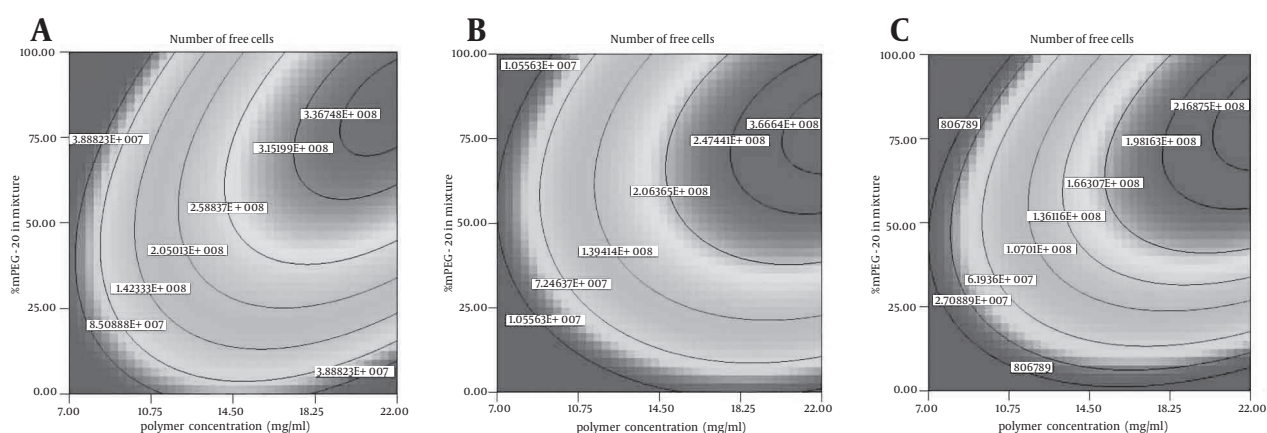
Figure 3. 2D View of the Response Surface Plots of the Polymer Concentration Effect, and Percentage of mPEG$_{20}$ Activated With SC as Independent Variables on the Response Y, and the Effect of Independent Variables on Coating of (A) Kell, (B) Rh (D) and (C) A antigens.

4.4. In Vivo RBC Immunogenicity

We evaluated the effect of PEGylation on *in vivo* RBC survival. In this regard, the PEGylated RBCs and unmodified RBCs (positive control) obtained from donor mice (C57Bl/6) at the optimal conditions according to the SEM results, were transplanted intra the tail of the recipient mice (BALB/c).

Twenty-four hours after transplantation, serum bichemical parameters of mice were measured. Figure 7 shows the changes in the levels of serum bichemical parameters (ALT, AST, LDH and BLT) of the recipients and negative control mice after transplantation. The serum parameters of the modified RBCs group remained at the normal levels. This happened while the increased values of these parameters were detected in positive control mice that were significantly different from negative control.

5. Discussion

Alloimmunization against non-ABO/Rh (D) blood group antigens is a common result of blood transfusion. The immunocamouflaging of RBCs using grafting of mPEG on the RBC membrane is a unique approach in preventing this alloimmunization (12, 23).

As previously demonstrated, covalent attachment of PEG to RBC membrane greatly reduces antigenicity and immunogenicity of RBCs by the inhibition of the antigen detection via antibodies and also by lowering antibody formation in response to allogeneic and xenogeneic transfusion in murine models (5, 17, 23).

This study was performed to optimize the reaction conditions for covalent attachment of mPEG activated with SVA and SC to the RBCs. The choice of linker chemistry is critical for efficient coupling in aqueous solutions. SVA and SC were used to react with primary amines (i.e. lysine) located on the cell membrane proteins.

mPEG-SVA forms an amide linkage while mPEG-SC employs a carbamate bond to the amine groups (8). The choice of factors and their levels for optimization were based on the range given in the literature. Both mPEG-SVA and mPEG-SC were found to be sufficiently reactive to produce extensively coated proteins under moderate conditions, showing the highest reactivity around pH 9.3 (24).

Other researchers utilized an elevated pH (8.0–9.2) for grafting an activated PEG to RBC (5-8, 25, 26). Fisher in his review displayed that at polymer concentrations lower than 1 mM, 5 kDa activated mPEG was not very effective for coating the antigens (27). Higher concentrations up to 50 mg.mL^{-1} had been also employed, but some abnormalities in the morphology of RBCs were observed (25).

The results presented in Figures 2, 3 show that free cell number would go up by increasing the PEG concentration and length due to the blood group antigen masking and the corresponding decrease of agglutination between RBCs. Increasing the percentage of mPEG$_{20}$ in the mixture of mPEG10 and mPEG$_{20}$ caused an initial increase in the free cells with a maximum point at 91.2% of mPEG$_{20}$.
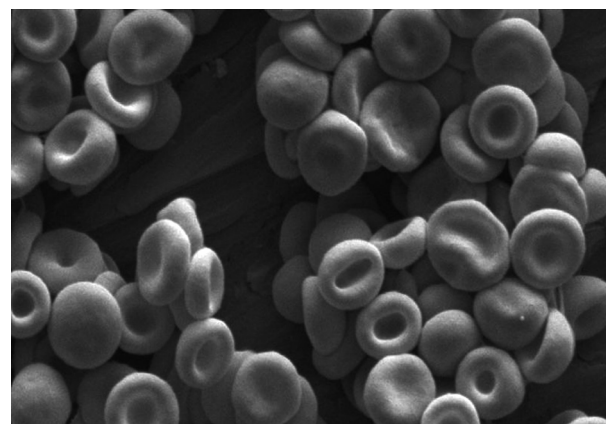

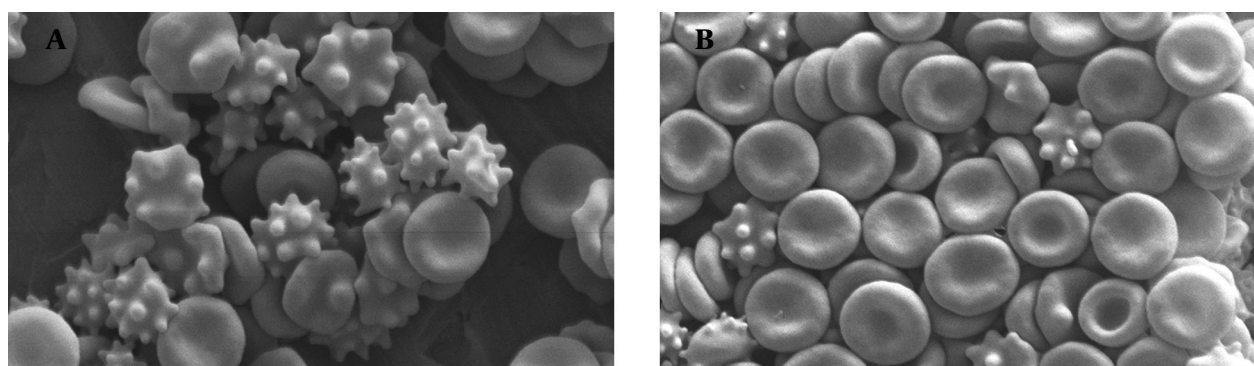**Figure 4.** Morphology of Uncoated Human RBCs, 3000×

Figure 5. Morphology of mPEG-SVA-derivatized Human RBCs (percentage of $mPEG_{20}$ = 91.2 %), 3000×, at Polymer Concentrations of (A) 17.21 and (B) 14.5 mg.mL^{-1}.

Table 6. Optimal Conditions for PEGylation of Human RBCs Using mPEG-SVA

Number of Free Cells After Reaction With Anti-A $\times 10^8$		Number of Free Cells After Reaction With Anti-Rh(D) $\times 10^8$		Number of Free Cells After Reaction With Anti-Kell $\times 10^8$		Polymer concentration (mg.mL^{-1})	$mPEG_{20}$ in mixture (%)	pH
Experimental[a]	Predicted	Experimental[b]	Predicted	Experimental[c]	Predicted			
2.50	2.57	3.00	2.90	3.50	3.40	17.21	91.20	8.60

[a] Confidence interval: (2.1-3.2) $\times 10^8$
[b] Confidence interval: (3.5-4.0) $\times 10^8$
[c] Confidence interval: (2.8-3.5) $\times 10^8$

Table 7. Optimal Conditions for PEGylation of Human RBCs Using mPEG-SC

Number of Free Cells After Reaction With Anti-A $\times 10^8$		Number of Free Cells After Reaction With Anti-Rh (D) $\times 10^8$		Number of Free Cells After Reaction With Anti-Kell $\times 10^8$		Polymer concentration (mg.mL^{-1})	$mPEG_{20}$ in mixture (%)	pH
Experimental[a]	Predicted	Experimental[b]	Predicted	Experimental[c]	Predicted			
2.50	2.80	2.60	3.00	3.00	3.60	19.80	90.00	8.60

[a] Confidence interval: (2.4-3.1) $\times 10^8$
[b] Confidence interval: (2.5-3.4) $\times 10^8$
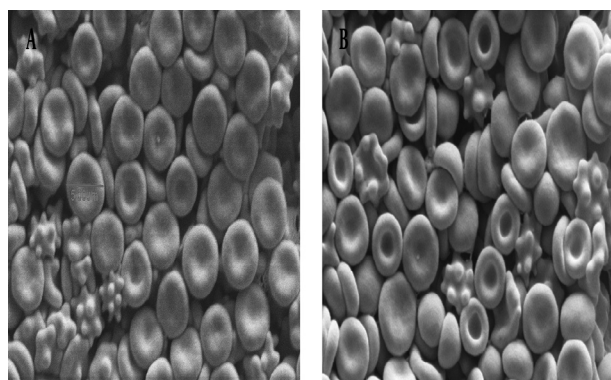[c] Confidence interval: (3.0-4.0) $\times 10^8$

Figure 6. Morphology of mPEG-SVA-Derivatized Murine RBCs (percentage of $mPEG_{20}$ = 91.2 %), 3000X, With Polymer Concentration of (a) 14.5 and (b) 10 mg.mL^{-1}.
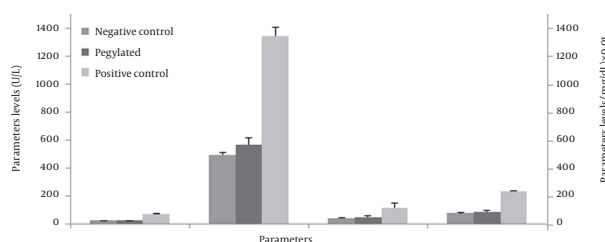

Figure 7. Serum Biochemical Parameters of the Transfused Mice. Right Axis Showing LDH, ALT, and AST levels and Left Axis Showing BLT Value.

The immunocamouflaging of blood group antigens is directly proportional to the concentration and length of the grafted polymer. This covering was proportional to the hydrodynamic thickness of the polymer layer and

was best achieved by long-chain polymers (8). The Flory radii (RF: root mean square of end to end length of the polymer chain; radius of gyration) of grafted polymers can be estimated as $R_F = aN^{(3/5)}$ (a = 3.5 Å, N = the number of monomers) (8). So, the calculated R_f (in nm) for 10- and 20-kDa polymers are 9.2 and 13.8 nm, respectively. Longer polymers would result in improved efficacy of cell surface charges and antigen masking (Figures 2 B–3C).

The steric exclusion effect of the attached polymer chains primarily inhibits protein adsorption (or antigen-antibody recognition) (8). This effect is maximized when higher density of polymer chains with small separation between the chains are grafted to the cell surface. However, it is difficult to achieve the high density grafting with long polymers that have a large gyration radius. In contrast, the long polymers sterically prevent binding onto the surface, but this inhibition is not against the short polymers (8). Therefore, higher density can be achieved by using long and short polymers together, as shown in Figures 2 B– 3 C.

As mentioned earlier, pH > 7.0 is required for reaction of the activated mPEG with primary amine residues. Also, it is well known that a non-physiological pH can be very damaging to the cells. Thus, a mild pH of 8.6 was found as the optimal condition. The optimal condition, at which the number of free cells is maximal (as presented in Tables 6 and 7) was determined by counting the free cells. It can be observed that the optimal conditions are in agreement with those deduced from Figures 2 and 3.

Linker chemistry is an effective factor in the efficacy of immunocamouflaging by the polymer (8, 12). mPEG-SVA more effectively camouflaged the blood group antigens, as noted by the reduction in antibody adsorption and agglutination formation (Figures 2 and 3 and Tables 6 and 7). This difference is relative to the extended hydrolysis half-life of mPEG-SVA (33.6 min) in comparison to mPEG-SC (20.4 min) in aqueous solution (8).

As shown in this study, PEGylation resulted in a decrease in antibody recognition of A, Rh (D), and Kell antigens. Meanwhile, the data noted that Kell antigen was significantly camouflaged, and also Rh (D) antigen was masked more effectively in comparison to A antigen. This is because there is a large difference between the densities of Kell, Rh (D) and A antigens on the RBC (5). Therefore, as shown in Tables 6 and 7, immunocamouflaging of these antigens is different. Most importantly, antigen masking was attenuated by increasing the number of antigens on the cell surface. The results of SEM showed that polymer concentration of 14.5 mg.mL^{-1} is a useful level for PEGylation of RBCs by 91.2% mPEG20-SVA in the polymer mixture. Echinocytes RBCs are not deformable and hence tend to get trapped in the microcirculation (22). Thus, the optimal concentration of 17.21 mg.mL^{-1}, obtained by cell counting method, cannot be recommended due to the loss of discocytic morphology. These results are similar to our previous finding for PEGylation of RBCs by mPEG, activated by cyanuric chloride and succinimidyl carbon-

ate (16, 17).

Another interesting observation was derived from investigation of murine PEGylated RBCs SEM results. These findings showed that the optimal polymer concentration (14.5 mg.mL^{-1}) for PEGylation of human RBCs is unsuitable for murine RBCs. The density of antigens on the murine RBCs is lower than human ones, so at this concentration, the echinocytosis increases. Thus, mPEG-SVA-coated RBCs with concentration of 10 mg.mL^{-1} have been employed for the mouse model.

The SEM results indicated that the better maintenance of mouse RBCs discocytic morphology was achieved at this polymer concentration. While in vitro analyses indicated the efficacy and potential clinical value of immunocamouflaged RBCs, in vivo efficacy and stability are both crucial for evaluating potential utility. Importantly, immunocamouflaging of RBCs did not have adverse effect on in vivo survival of a murine transfusion model.

Figure 7 shows the biochemistry parameters of positive control, negative control, and polymer-modified (10 mg.mL^{-1} mPEG-SVA) RBCs. As noted, no significant differences were observed between the serum biochemistry parameters of negative control group and PEG-modified RBCs group and both populations fell in the normal murine biochemistry patterns.

Blood biochemistry assays revealed that ALT, AST, LDH, and BLT levels in positive control mice significantly have increased, while these parameters did not significantly increase in the serum of PEGylated group. Elevation of these parameters in the blood serum depends on the donor RBCs lysing by host immune system after recognition of antigens on the donor RBCs. Our results indicated that coated donor RBCs by mPEG-SVA would result in reducing the immune recognition and clearance by the recipient mice.

Based on these results, we concluded that PEGylation of RBCs is an appropriate approach for their immunocamouflaging. Covalent attachment of activated mPEGs to the RBC membrane provides a unique strategy in inhibiting the immunological recognition of allogeneic cells. The aim of the present study was to optimize the covering of minor and major blood group antigens, simultaneously.

The variables of PEGylation reaction were optimized using factorial and CCD methods. The optimal pH of the reaction medium was 8.6. Also, the other optimal conditions determined for mPEG-SVA and mPEG-SC using cell counting method were as follows: mPEG20 in the polymer mixture, 91.2 and 85.36%, and polymer concentration, 17.21 and 19.80 mg.mL^{-1}, respectively. These conditions are similar, but mPEG-SVA is a more effective reagent for RBC coating. It was also concluded that it is not sufficient selecting the optimal polymer concentration only by evaluating the extent of PEGylation as the only criterion.

However, according to the SEM results, the maximum polymer concentration of 14.5 mg.mL^{-1}, as the best condition, was suggested for mPEG-SVA modified human RBCs due to the observation of enhancing the rate of echino-

cytosis by increasing the polymer concentration. By considering the in vitro results, it is obvious that membrane PEGylation camouflages the blood group antigens. This effect is observed dramatically and significantly for non-ABO/Rh (D) antigens.

Finally, these results clearly recommend that PEGylation of non-ABO/Rh(D) matched RBCs significantly reduces the risk of alloimmunization in patients requiring chronic blood transfusions. Therefore, application of this technology may be clinically useful in chronically transfused persons with diseases such as sickle-cell anemia and thalassemia because of the increased risk of alloimmunization. Our findings (regarding in vivo murine) with allogeneic donor cells suggest that the actual risk of alloimmunization would be effectively reduced.

Acknowledgements

Authors' Contributions

All authors participated equally in the present study.

Funding/Support

Financial Disclosure

There was no conflict of interest.

References

1. Tan Y, Qiu Y, Xu H, Ji S, Li S, Gong F, et al. Decreased immunorejection in unmatched blood transfusions by attachment of methoxypolyethylene glycol on human red blood cells and the effect on D antigen. *Transfusion.* 2006;**46**(12):2122–7.
2. Scott MD, Murad KL. Cellular camouflage: fooling the immune system with polymers. *Curr Pharm Des.* 1998;**4**(6):423–38.
3. Castro O, Sandler SG, Houston-Yu P, Rana S. Predicting the effect of transfusing only phenotype-matched RBCs to patients with sickle cell disease: theoretical and practical implications. *Transfusion.* 2002;**42**(6):684–90.
4. Vichinsky EP, Earles A, Johnson RA, Hoag MS, Williams A, Lubin B. Alloimmunization in sickle cell anemia and transfusion of racially unmatched blood. *N Engl J Med.* 1990;**322**(23):1617–21.
5. Wang D, Kyluik DL, Murad KL, Toyofuku WM, Scott MD. Polymer-mediated immunocamouflage of red blood cells: effects of polymer size on antigenic and immunogenic recognition of allogeneic donor blood cells. *Sci China Life Sci.* 2011;**54**(7):589–98.
6. Blackall DP, Armstrong JK, Meiselman HJ, Fisher TC. Polyethylene glycol-coated red blood cells fail to bind glycophorin A-specific antibodies and are impervious to invasion by the Plasmodium falciparum malaria parasite. *Blood.* 2001;**97**(2):551–6.
7. Murad KL, Mahany KL, Brugnara C, Kuypers FA, Eaton JW, Scott MD. Structural and functional consequences of antigenic modulation of red blood cells with methoxypoly(ethylene glycol). *Blood.* 1999;**93**(6):2121–7.
8. Le Y, Scott MD. Immunocamouflage: the biophysical basis of immunoprotection by grafted methoxypoly(ethylene glycol) (mPEG). *Acta Biomater.* 2010;**6**(7):2631–41.
9. Sezer A, Yağc A. Overview of peptide and protein PEGylation: properties and general strategies. *Acta Pharm Sci.* 2010;**52**:377–89.
10. Bradley AJ, Test ST, Murad KL, Mitsuyoshi J, Scott MD. Interactions of IgM ABO antibodies and complement with methoxy-PEG-modified human RBCs. *Transfusion.* 2001;**41**(10):1225–33.
11. Bradley AJ, Scott MD. Immune complex binding by immunocamouflaged [poly(ethylene glycol)-grafted] erythrocytes. *Am J Hematol.* 2007;**82**(11):970–5.
12. Scott MD, Chen AM. Beyond the red cell: pegylation of other blood cells and tissues. *Transfus Clin Biol.* 2004;**11**(1):40–6.
13. Armstrong JK, Meiselman HJ, Fisher TC. Covalent binding of poly(ethylene glycol) (PEG) to the surface of red blood cells inhibits aggregation and reduces low shear blood viscosity. *Am J Hematol.* 1997;**56**(1):26–8.
14. Murad KL, Gosselin EJ, Eaton JW, Scott MD. Stealth cells: prevention of major histocompatibility complex class II-mediated T-cell activation by cell surface modification. *Blood.* 1999;**94**(6):2135–41.
15. Chen AM, Scott MD. Immunocamouflage: prevention of transfusion-induced graft-versus-host disease via polymer grafting of donor cells. *J Biomed Mater Res A.* 2003;**67**(2):626–36.
16. Hashemi-Najafabadi S, Vasheghani-Farahani E, Shojaosadati SA, Rasaee MJ, Armstrong JK, Moin M, et al. A method to optimize PEG-coating of red blood cells. *Bioconjug Chem.* 2006;**17**(5):1288–93.
17. Sarvi F, Vasheghani Farahani E, Shojaosadati SA, Hashemi Najafabadi S, Moin M, Pourpak Z. Surface treatment of red blood cells with monomethoxypoly(ethylene glycol) activated by succinimidyl carbonate. *Iran J Polym.* 2006;**15**:525–34.
18. Miron T, Wilchek M. A simplified method for the preparation of succinimidyl carbonate polyethylene glycol for coupling to proteins. *Bioconjug Chem.* 1993;**4**(6):568–9.
19. Bradley AJ, Murad KL, Regan KL, Scott MD. Biophysical consequences of linker chemistry and polymer size on stealth erythrocytes: size does matter. *Biochim Biophys Acta.* 2002;**1561**(2):147–58.
20. Kayden HJ, Bessis M. Morphology of normal erythrocyte and acanthocyte using Nomarski optics and the scanning electron microscope. *Blood.* 1970;**35**(4):427–36.
21. Prakash O, Talat M, Hasan SH, Pandey RK. Factorial design for the optimization of enzymatic detection of cadmium in aqueous solution using immobilized urease from vegetable waste. *Bioresour Technol.* 2008;**99**(16):7565–72.
22. Poraicu D, Sandor S, Menessy I. Decrease of red blood cell filterability seen in intensive care. II. Red blood cell crenelation "in vivo" as morphological evidence of increased red blood cell viscosity in low flow states. *Resuscitation.* 1983;**10**(4):305–16.
23. Bradley AJ, Scott MD. Separation and purification of methoxypoly(ethylene glycol) grafted red blood cells via two-phase partitioning. *J Chromatogr B Analyt Technol Biomed Life Sci.* 2004;**807**(1):163–8.
24. Zalipsky S, Seltzer R, Menon-Rudolph S. Evaluation of a new reagent for covalent attachment of polyethylene glycol to proteins. *Biotechnol Appl Biochem.* 1992;**15**(1):100–14.
25. Scott MD, Murad KL, Koumpouras F, Talbot M, Eaton JW. Chemical camouflage of antigenic determinants: stealth erythrocytes. *Proc Natl Acad Sci U S A.* 1997;**94**(14):7566–71.
26. Neu B, Armstrong JK, Fisher TC, Meiselman HJ. Surface characterization of poly(ethylene glycol) coated human red blood cells by particle electrophoresis. *Biorheology.* 2003;**40**(4):477–87.
27. Fisher TC. PEG-coated red blood cells-simplifying blood transfusion in the new millennium? *Immunohematology.* 2000;**16**(1):37–48.