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Factorial Design Optimization of Red Blood Cell PEGylation with a Low Molecular Weight Polymer

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ABSTRACT

A proposed method to overcome the donor-recipient blood group incompatibility is to mask the blood group antigens by the covalent attachment of poly(ethylene glycol) (PEG) to the red blood cell (RBC) membrane. Despite much work in the development of PEG-coating of RBCs, there is a paucity of data on the optimization of the PEG-coating technique by using low molecular weight polymers. It is the aim of this study to determine the optimum conditions for PEG-coating using a cyanuric chloride reactive methoxy-PEG derivative as a model polymer. Activated PEG of 2 kDa molecular mass was covalently attached to human RBCs under various reaction conditions. Inhibition of binding of a blood-type specific antiserum (anti-D) was employed to evaluate the effect of the PEG-coating, quantified by flow cytometry. RBC morphology was examined by scanning electron microscopy (SEM). Statistical analysis of experimental design together with SEM results showed that the optimum PEGylation conditions are: temperature 14ºC, reaction time 20 min, and concentration of reactive PEG 10 mg/mL.

Key Words:

red blood cells;
methoxy poly(ethylene glycol);
polymer coating;
flow cytometry;
experimental design.

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INTRODUCTION

Blood transfusion is a widely used therapy in medicine, surgery and trauma conditions involve such transfusion. In blood transfusion, matching the blood group of the donor and patient is a critical step [1]. Elaborate systems of identification and testing are in place to ensure that correctly matched red blood cells (RBCs) are transfused [2]. The matching is done generally by testing for major blood group specific antigens, ABH and D [1,3]. In most transfusions, this simple blood typing is sufficient to identify appropriate donors. More often, problems are encountered in individuals who receive multiple transfusions, such as patients with sickle cell anemia and thalassemia. In such patients, alloimmunization against minor RBC antigens can make it nearly

impossible to identify appropriate blood donors [1,4-6].

More than 20 years ago, Abuchowski et al. developed a technique to modify proteins by the covalent attachment of poly(ethylene glycol) (PEG) molecules [7,8]. The covalent attachment of PEG is now commonly used to modify a variety of proteins, enzymes, drugs, and artificial surfaces that come into contact with human blood [9-14]. But, RBCs have recently been considered for use as substrate for PEG modification [15].

In our previous studies, the process of red blood cell coating was optimized using 5 kDa molecular mass methoxy-PEG (mPEG) activated with cyanuric chloride [16] and succinimidyl carbonate [17]. Optimization of this process, using low molecular weight polymers, has not been presented in the literature. Therefore, in the present study, RBCs were coated with a reactive cyanuric chloride mPEG derivative of 2 kDa molecular mass and the optimum conditions were determined for this process. In this regard, the effects of process variables (e.g., polymer concentration, temperature, and reaction time) were investigated on the extent of PEGylation of RBCs. Full factorial design and Taguchi analysis were employed to identify the optimum reaction conditions of RBCs PEGylation.

To assess the effectiveness of the PEG-coating, the inhibition of agglutination by blood-type specific antisera was measured by flow cytometric analysis of fluorescein-labeled anti-D binding to PEG-coated RBCs. Finally, the morphology of PEG-RBCs was studied by scanning electron microscopy (SEM) technique.

EXPERIMENTAL

Materials

mPEG of 2 kDa molecular mass, triethanolamine (TEA) and fluorescein- isothiocyanate (FITC) were purchased from Sigma (St. Louis, MO). Cyanuric chloride (2,4,6-trichloro-1,3,5-triazine), benzene, anhydrous sodium carbonate, D-glucose, sodium chloride and potassium chloride were obtained from Merck-Schuchardt (Darmstadt, Germany). Cyclohexane was purchased from Roth (Karlsruhe, Germany). Packed Rh-positive RBCs were obtained from Iranian Blood Transfusion Organization. Anti-D was purchased from CinnaGen Inc. (Tehran, Iran).

Polymer Derivatization

Derivatization of 2 kDa mPEG with cyanuric chloride was performed using a modification of the method described by Abuchowski et al. [7]. Briefly, 5 g vacuum dried mPEG (overnight at 80°C) was dissolved in 100 mL hot anhydrous benzene, cooled to 15°C and then slowly added to a solution of cyanuric chloride in benzene (5 fold molar excess). 2.5 gram anhydrous sodium carbonate was then added. The mixture was stirred for 48 h at 15°C under an atmosphere of dry nitrogen, and then the sodium carbonate was removed by vacuum filtration. The PEG derivative was precipitated with anhydrous cyclohexane, collected by vacuum filtration, and then re-suspended in benzene. This process was repeated 5 times to remove any unreacted cyanuric chloride [18]. The product was then dried under vacuum, using a freeze dryer system (Zirbus Technology, Vaco 5), and stored at -70°C, under vacuum, until use [19].

Coating of Red Blood Cells with mPEG

Packed Rh-positive RBCs were re-suspended to a 10% hematocrit in TEA buffer (30 mM TEA, 110 mM NaCl, 4 mM KCl, and 5 mM D-glucose; pH=8.7). A fresh cold stock solution of the derivatized polymer was prepared in 0.9% NaCl, containing 1 mM HCl, and appropriate volumes were immediately added to the RBC suspensions to yield final polymer concentration of 2-10 mg/mL; an equivalent volume of buffer was added to the control samples. This acidic solution retards hydrolysis of the reactive PEG derivative prior to exposure to the RBCs; since only small volumes of the stock solution were added, the final pH of the suspensions remained unaltered. The samples were incubated with gentle mixing under various conditions (temperature, reaction time and polymer concentration, defined in subsequent sections). After 2 washes with isotonic phosphate buffer solution (PBS, pH=7.4) at 200 xg for 10 min, packed RBCs were prepared for evaluation of the polymer coating [20].

Flow Cytometry

Labeling of anti-D with FITC was achieved by following the procedure described by Coligan et al. [21]. Fluorescein-labeled anti-D was added to a dilute suspension of RBCs in PBS. The ratio of fluorescein-labeled anti-D to RBCs, obtained by titration, was high to prevent agglutination (60 μL of 0.1% hematocrit

in PBS, added to 200 μL of fluorescein-labeled anti-D solution with a protein concentration of 0.16 mg/mL). The samples were incubated for 30 min at room temperature in the darkness with gentle mixing, centrifuged and washed twice with PBS at 500 xg for 2 min. The labeled cells were then re-suspended in 1 mL of PBS.

The fluorescence intensity of FITC-anti-D labeled PEG-cells was measured using a FACSTAR PLUS flow cytometer (Becton Dickinson, San Jose, CA). Ten thousand cells were counted for each sample [20]. The fluorescence intensity of FITC-anti-D labeled cells was recorded for each sample and expressed as a ratio to the intensity of FITC-anti-D labeled control (uncoated) cells. The lower was this ratio, the greater was the effectiveness of RBC PEGylation.

Scanning Electron Microscopy (SEM)

The morphology of PEG-RBCs and control (uncoated) RBCs at physiological pH, was studied by SEM (XL 30, Philips, Netherlands). In order to prepare the RBCs for imaging, the procedure described by Kayden and Bessis [22] was followed.

DESIGN OF EXPERIMENTS

Statistically designed experiments are highly efficient which they give a fixed amount of information with much less effort than the classical one-variable-at-a-time approach and many of them give additional information about interactions between variables. Factorial and fractional-factorial experiments are the most powerful (statistical) techniques in research and well chosen fractional-factorial are particularly economical in assessing multivariable systems [23].

Full Factorial at Two Levels

The first step (using the Yates table) was to identify which variables have the largest effects on the PEGylation reaction. Selection of these factors was based on previous studies in literature for RBCs PEGylation. According to our previous study [16], we found that pH= 8.7 is an appropriate condition for RBCs PEGylation, using a cyanuric chloride mPEG derivative. Then, the evaluated variables, at present study, were temperature and time of PEGylation reaction and also polymer concentration (Table 1).

Table 1. Selected experimental variables with corresponding values at two levels for full factorial design.

Variable	Low level (1)	High level (2)
A: Temperature of reaction (°C)	4	25
B: Time of reaction (min)	10	40
C: mPEG concentration (mg/mL)	2	10

Taguchi Design

We have employed Taguchi statistical technique to study the impact of multiple variables on single outputs (prevention of agglutination and antibody binding).

If a systematic, sequential approach to experimental design be adopted, it is usually sufficient to restrict the design to two-level experiments. Sometimes, however, it is advantageous to work at three levels or more. The usual reason for employing either of these techniques is that a maximum or minimum is approached. This design estimates the non-linear (quadratic) effects [24].

To obtain the optimum condition for PEGylation of RBCs, an L_{27} array of Taguchi [24] for three variables at three levels was designed. The variables and their corresponding values are presented in Table 2. All experiments with RBCs were performed in duplicate unless stated otherwise.

RESULTS AND DISCUSSION

PEG Coating

During the incubation period at elevated pH, virtually no lysis (<1%) was observed in either the control

Table 2. Selected experimental variables with corresponding. values at three levels for Taguchi design.

Variable	First level (1)	Second level (2)	Third level (3)
A: Temperature of reaction (°C)	4	14	25
B: Time of reaction (min)	10	20	40
C: mPEG concentration (mg/mL)	2	5	10

or mPEG-modified RBCs. Indeed even mPEG concentration of up to 10 mg/mL yielded no significant lysis during RBC derivatization.

As a result of RBC coating by mPEG, attachment of FITC labeled-anti-D to RBCs decreased and the corresponding ratio of mean fluorescence intensity of the PEG-RBCs versus control (uncoated) RBCs was decreased. It is worth mentioning that inhibition of agglutination does not necessarily indicate that antibody binding was inhibited relative to uncoated cells, and may merely reflect the physical prevention of IGgs bridging between cells. However, inhibition of agglutination does facilitate a way to quantify the PEG-coating of RBCs, and is sufficient to determine the optimum conditions for PEGylating. Direct measurement of inhibition of anti-D binding to PEG-RBCs by flow cytometric analysis, using a FITC-labeled anti-D, demonstrates that the polymer coating does prevent antibody binding.

Experimental Design

The selection of the levels of factors for optimization was based on the ranges given in the literature. In the case of 5 kDa mPEG, we used the range of 30-60 min for reaction time [16], but for 2 kDa mPEG, this range was changed to 10- 40 min, because the shorter PEG may react faster than the longer one. Also the temperature range of 4-25°C was selected for RBC PEGylation.

The results and statistical analysis for full factorial design (using Yates table [23]) at two levels are presented in Table 3. All the responses are presented as the average mean of the fluorescence intensity ratio of the FITC labeled anti-D cells of PEG-RBCs versus control (uncoated) RBCs, with two replicates for each test. A decrease in the intensity ratio shows that the PEG-RBCs were protected against agglutination. By comparison the obtained F-values with a critical $F_{(1,8)}$ value of 11.26 (obtained from the F-Table [23]), it is apparent that three variables (temperature, reaction time, and polymer concentration) and their interactions have F-values greater than critical F. Hence they were selected as important factors for PEGylation of RBCs.

The obtained results, using an L_{27} array of Taguchi design, with flow cytometry method are presented in Table 4. The main effects of the experimental variables on the mean fluorescence intensity ratio of the FITC-anti-D labeled cells of PEG-RBCs versus control (uncoated) RBCs, as the indicators of the extent of PEGylation are shown in Figure 1. This figure shows the effect of one variable, while the others vary. Each point in each curve presents an average of the obtained responses from 9 duplicate experiments (18 experiments). Figure 1 indicates that the optimum temperature and time of the reaction as well as polymer concentration are 14°C, 20 min, and 10 mg/mL, respectively.At 4°C the rate of reaction is low and more time is required for completion of the reaction. At temperatures higher than 20°C, the activated polymer may show a less active form [25] which it suggests a

Table 3. Yates table analysis of a 2^3 full factorial design (3 variables in 2 levels).

Trial	TC[a]	Temperature (A) (°C)	Time (B) (min)	Concentration (C) (mg/mL)	Response[b]	F-value[c]
1	1	4	10	2	0.96	-
2	a	25	10	2	0.71	545.40
3	b	4	40	2	0.88	85.58
4	ab	25	40	2	0.77	351.90
5	c	4	10	10	0.91	66.60
6	ac	25	10	10	0.44	4
7	bc	4	40	10	0.82	105.70
8	abc	25	40	10	0.86	118.70

(a) Treatment combination: The low level of any variable is denoted by (1) and the high level of any variable be denoted by its lower-case letter; (b) Each value is the average mean fluorescence intensity ratio of FITC labeled anti-D cells of PEG-RBCs versus control (uncoated) RBCs, with two replicates for each test; (c) $SS_x = (f^2_x)/N$, which f_x is the amount in the final column of Yates analysis corresponding to x (not presented here) and N is the total number of treatment combinations with their replications (16), $MS_x = SS_x / ø_x$, which $ø_x$ is the degree of freedom corresponding to x, $TSS = \sum y^2_i - (\sum y_i)^2/N = 0.377$, y_i is the result, $RSS = TSS - \sum SS_x = 2.3\times10^{-3}$, Error degree of freedom (ø)=8, Residual error variance=RSS/ø $=2.87\times10^{-4}$, F-value=MS_x/(Residual error variance), F-critical ($ø_1$=1, $ø_2$=8) = 11.26 (obtained from standard table).

Table 4. An L_{27} Taguchi array for RBCs PEGylation.

Trial	Temperature (°C)	Time (min)	Concentration (mg/mL)	Response[a]
1	4	10	2	0.94
2	4	10	5	0.81
3	4	10	10	0.91
4	4	20	2	0.90
5	4	20	5	0.92
6	4	20	10	0.81
7	4	40	2	0.88
8	4	40	5	0.87
9	4	40	10	0.82
10	14	10	2	0.89
11	14	10	5	0.92
12	14	10	10	0.83
13	14	20	2	0.76
14	14	20	5	0.69
15	14	20	10	0.78
16	14	40	2	0.86
17	14	40	5	0.76
18	14	40	10	0.83
19	25	10	2	0.88
20	25	10	5	0.89
21	25	10	10	0.76
22	25	20	2	0.94
23	25	20	5	0.76
24	25	20	10	0.78
25	25	40	2	0.77
26	25	40	5	0.84
27	25	40	10	0.79

[(a)] Each value is the average mean fluorescence intensity ratio of FITC labeled anti-D cells of PEG-RBCs versus control (uncoated) RBCs, with two replicates for each test.

moderate temperature is more favorable.

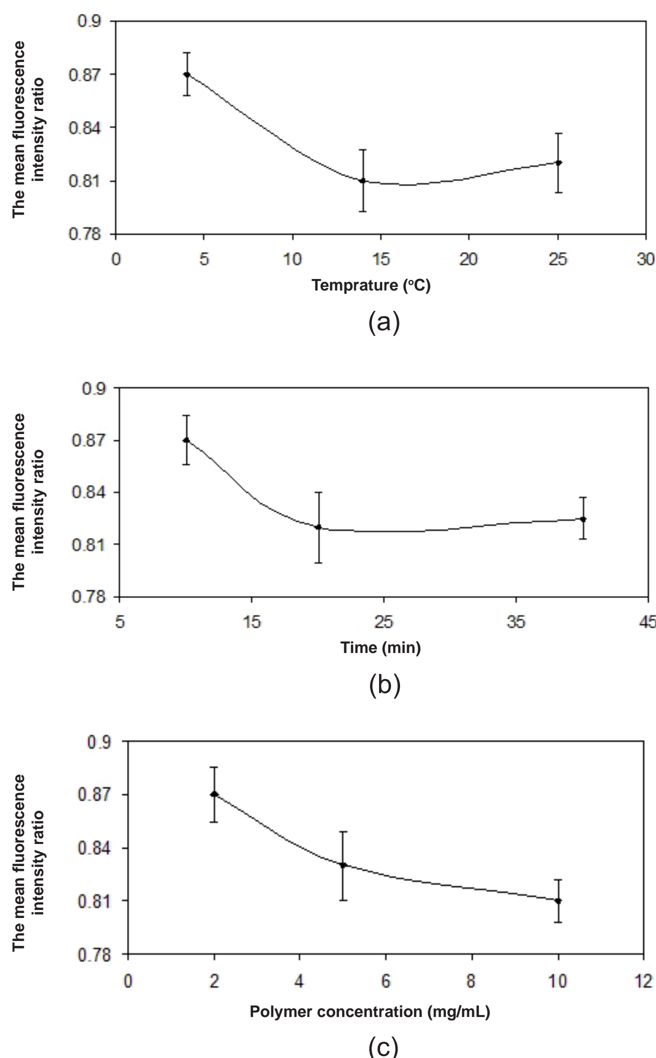

Figure 1. The effect of different factors on the mean fluorescence intensity ratio of PEG-RBCs versus control (uncoated) RBCs incubated with FITC labeled anti-D: The effect of (a) temperature of reaction, (b) reaction time, and (c) polymer concentration.

SEM Results

The morphology of control (uncoated) and PEG-coated RBCs (using 2, 3, 4, 5, and 10 mg/mL of activated mPEG) that were prepared under optimum conditions (20 min, 14°C and pH=8.7) and then returned to a physiological pH with PBS (pH=7.4), are presented in Figure 2. RBC Morphology was classified according to Bessis [26]. At 2 mg/mL, type I echinocytes was observed. At 3 and 4 mg/ mL, type II echinocytes predominate while some cells of type I were still observable. At 5 and 10 mg/mL, an equal amount of types II and III echinocytes and some cells of normal and type I were observed.

Fisher [15] in his review noted that at polymer concentrations below 1 mM (5 mg/mL), the 5 kDa activated mPEG is not very effective for masking

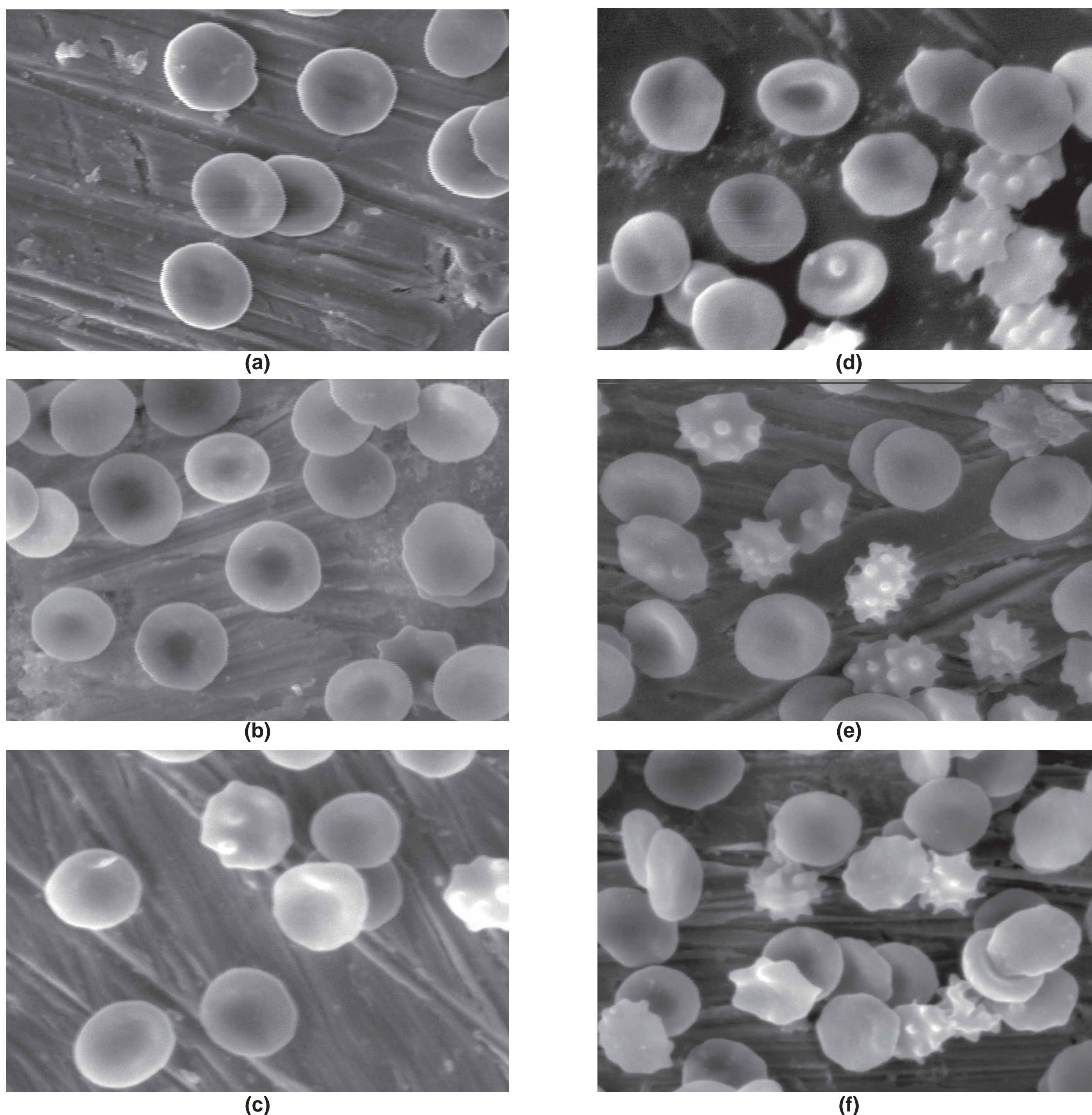

Figure 2. The morphology of mPEG-derivatized RBCs (3000x). The incubation concentrations of the reactive derivative of mPEG 2kDa are (a) 0 mg/mL control (uncoated), (b) 2 mg/mL, (c) 3 mg/mL, (d) 4 mg/mL (e) 5 mg/mL, and (f) 10 mg/mL.

antigens. Higher concentrations up to 50 mg/mL (10 mM) have also been used [27,28] but some abnormalities were reported in the morphology of RBCs. We found [16] that for 5 kDa activated mPEG, it is not recommendable to use polymer concentrations higher than 15 mg/mL, since most of the cells have the shape of types II and III echinocytes. Therefore, for 2 kDa activated mPEG, we used the range of 2-10 mg/mL.

The SEM results from our work that are presented

Table 5. A comparison among the obtained optimum conditions, using different molecular weights of polymer and activating agents, for PEGylation of RBCs with mPEG.

Molecular weight ofmPEG (Dalton)	Activating agent	pH	Temperature (°C)	Reaction time (min)	Polymer concentration (mg/mL)
2000	Cyanuric chloride	8.7	14	20	10
5000	Cyanuric chloride	8.7	14	30	15
5000	Succinimidyl carbonate	8.7	14	60	12

here; show a polymer concentration of 10 mg/mL is a convenient level for PEGylation using a linear PEG of 2 kDa molecular mass. RBCs of echinocyte types I and II may circulate in the host body, but cells of echinocyte type III would not circulate, because such cells are not deformable and hence would tend to get trapped in the microcirculation [29].

The observation of increasing echinocytosis with increasing reactive PEG concentration is solely related to the presence of PEG on the cell surface, and not exposure to high pH, as all control cells showed normal discocytic morphology after returning to the physiological pH. For the purposes of the work undertaken here, it was reasonable to select a linear PEG molecular mass of 2 kDa. It is possible that PEGs of larger molecular mass or of different geometry may sustain discocytic RBCs' morphology, while being effective antigen masking agents [30]. But investigation of these variables was beyond the scope of the current study.

In another trial (not presented here), we also found that using 5 and 2 kDa mPEGs that reacted at their optimum conditions and a polymer concentration of 15 mg/mL, the 5 kDa mPEG is more effective. It should be noted that at a specific optimum concentration, in terms of mg/mL, the number of 2 kDa PEG molecules is 2.5 times of 5 kDa PEG ones. These results clearly indicate that high molecular weight polymer is more effective for RBC coating, as expected.

Based on the results of the present and previous studies [16,17], a comparison of the resulting optimum conditions for PEGylation of RBCs is presented in Table 5. It is obvious that by changing the molecular weight of mPEG and using different activating agent, different optimum conditions can be obtained for this process. It is worth to mention that among different variables, polymer concentration and reaction time are the most effective.

CONCLUSION

Cyanuric chloride activated 2 kDa mPEG was covalently attached to human RBCs and the optimum conditions for this reaction were determined by different methods of experimental design. The optimum temperature, reaction time, and polymer concentration were found to be 14°C, 20 min, and 10 mg/mL, respectively. Further increase in polymer concentration, may increase the extent of cell coating, but it is not recommended due to increasing the echinocyte type III of red blood cells which is not favorable.

Based on the results of the present and previous studies [16,17], it can be concluded that the optimum conditions for PEGylation of RBCs vary with changing molecular weight of mPEG or using different activating agents.

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REFERENCES

1. Nacharaju P., Boctor F.N., Manjula B.N., Acharya S.A., Surface decoration of red blood cells with maleimidophenyl- polyethylene glycol facilitated by thiolation with iminothiolane: An approach to mask A, B and D antigens to generate universal red blood cells, *Transfusion*, **45**, 374-383, 2005.
2. Methods for preparing antigen masked red blood cells having reduced hemolysis by sera, *WO2004050897*, 2004.
3. Reid M.E., Lomas-Francis C., *The Blood Group Antigen Facts Book, Academic Press*, London, 19-395, 1997.
4. Castro O., Sandler S.G., 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*, **42**, 684-690, 2002.
5. Rosse W.F., Gallagher D., Kinney T.R., Castro O., Dosik H., Moohr J., Wang W., Levy P.S., Transfusion and alloimmunization in sickle cell disease: The cooperative study of sickle cell disease, *Blood*, **76**, 1431-1437, 1990.
6. Vichinsky E.P., Earles A., Johnson R.A., Hoag M.S., Williams A., Lublin B., Alloimmunization in sickle cell anemia and transfusion of racially unmatched blood, New *Engl. J. Med*., **322**, 1617-1621, 1990.
7. Abuchowski A., Es T.V., Palczuk N.C., Davis F.F., Alteration of immunological properties of bovine serum albumin by covalent attachment of polyethylene glycol, J. Biol. *Chem*., **252**, 3578-3581, 1977.
8. Abuchowski A., McCoy J.R., Palczuk N.C., Es T.V., Davis F.F., Effect of covalent attachment of polyethylene glycol on immunogenicity and circulating life of bovine liver catalase, *J. Biol. Chem*., **252**, 3582-3586, 1977.
9. Harris J.M., Zalipsky S., *Poly(ethylene glycol): Chemistry and Biological Applications*, ACS Symposium Series, Washington DC., 1-13, 1997.
10. Greenwald R.B., Choe Y.H., McGuire J., Conover C.D., Effective drug delivery by PEGylated drug conjugates, *Adv. Drug Del. Rev.*, **55**, 217-250, 2003.
11. Hershfield M.S., PEG-ADA replacement therapy for adenosine deaminase deficiency: an update after 8.5 years, *Clin. Immunol. Immunopathol*., **76**, S228-S232, 1995.
12. Keating M.J., Holmes R., Lerner S., Ho D.H., L-asparaginase and PEG asparaginase: Past, present, and future, *Leuk. Lymphoma*, **10**, 153-157, 1993.
13. Thanou M., Duncan R., Polymer-protein and polymer-drug conjugates in cancer therapy, *Curr. Opin. Invest. Drugs*, **4**, 701-709, 2003.
14. Fisher T.C., Armstrong J.K., Red blood cells covalently bound with two different polyethylene glycol derivatives, *U.S. Patent 6,312,685*, 2001.
15. Fisher T.C., PEG-coated red blood cells: Simplifying blood transfusion in the new Millennium?, *Immunohematology*, **16**, 37-46, 2000.
16. Hashemi-Najafabadi S., Vasheghani-Farahani E., Shojaosadati S.A., Rasaee M.J., Armstrong J.K., Moin M., Pourpak Z., A method to optimize PEG-coating of red blood cells, *Bioconjugate Chem*., **17**,1288-1243,2006.
17. Sarvi F., Vasheghani-Farahani E., Shojaosadati S.A., Hashemi-Najafabadi S., Moin M., Pourpak Z., Surface treatment of red blood cells with monomethoxypoly(ethylene glycol) activated by succinimidyl carbonate, *Iran. Polym. J*., **15**, 525-534, 2006.
18. Armstrong J.K., Meiselman H.J., Fisher T.C., Evidence against macromolecular "bridging" as the mechanism of red blood cell aggregation induced by nonionic polymers, *Biorheology*, **36**, 433-437, 1999.
19. Sabolovic D., Sestier C., Perrotin P., Guillet R., Tefit M., Boynard M., Covalent binding of polyethylene glycol to the surface of red blood cells as detected and followed up by cell electrophoresis and rheological methods, *Electrophoresis*, **21**, 301-306, 2000.
20. Blackall D.P., Armstrong J.K., Meiselman H.J., Fisher T.C., 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*, **97**, 551-556, 2001.
21. Coligan J.E., Kruisbeek A.M., Margulies D.H., Shevach E.M., Strober *W., Current Protocols in Immunology*, John Wiley, **1**, 1991.

22. Kayden H.J., Bessis M., Morphology of normal erythrocyte and acanthocyte using nomarski optics and the scanning electron microscope, *J. Hematol*., **35**, 427-436, 1970.
23. Davies L., *Efficiency in Research, Development, and Production: the Statistical Design and Analysis of Chemical Experiments*, Royal Society of Chemistry, Cambridge, 1-88, 1993.
24. Roy R.K., *A Prime on the Taguchi Method*, Van Nostrand Reinhold, New York, 1-155, 1990.
25. Jackson C.C., Charlton J.L., Kuzminski K., Lang G.M., Sehon A.H., Synthesis, isolation and characterization of conjugates of ovalbumin with monomethoxypolyethylene glycol using cyanuric chloride as the coupling agent, *Anal. Biochem*., **165**, 114-127, 1987.
26. Bessis M., *Red Cell Shape: Physiology, Pathology, Ultrastucture, Springer-Verlag*, New York, 1- 25, 1973.
27. Hortin G.L., Lok H.T., Huang S.T., Progress toward preparation of universal donor red cells, *Art. Cells Blood Subs. Immob. Biotech*., **25**, 487-491, 1997.
28. Scott M.D., Murad K.L., Koumpouras F., Talbot M., Eaton J.W., Chemical camouflage of antigenic determinants: Stealth erythrocytes, *Proc. Natl. Acad. Sci. USA*, **94**, 7566-7571, 1997.
29. Poraicu D., Sandor S., Menessy I., Decrease of red blood cell filterability seen in intensive care. II: Red blood cell crenation in-vivo as morphological evidence of increased red cell viscosity in low flow rates, *Resuscitation*, **10**, 305- 316, 1983.
30. Fisher T.C., Armstrong J.K., Meiselman H.J., Leger R.M., Garratty G., Second generation poly(ethylene glycol) surface coatings for red blood cells, *Transfusion*, **40**, 119S, 2000.