Surface Coating of Red Blood Cells with Monomethoxy poly(ethylene glycol) Activated with Two Different Reagents

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ABSTRACT: *Methoxy poly(ethylene glycol) (mPEG) with molecular mass of 5 kDa activated with succinimidyl carbonate and cyanuric chloride, separately was covalently attached to human red blood cells (RBCs). Inhibition of agglutination by blood-type specific antisera (anti-D) was employed to evaluate the effect of the polymer coating. The remaining single cells after incubation with anti-D sera were counted using a simple hemocytometer (Improved Neubauer Ruling). The extent of surface coating was evaluated by addition of FITC labeled-anti-D to the cells and recording the fluorescence intensity ratio of FITC-anti-D bound cells of the PEG-RBCs versus control (uncoated) RBCs. The morphology of RBCs was evaluated by scanning electron microscopy (SEM). The effect of polymer coating, based on the immunological response of RBCs, using two kinds of activated mPEG, at optimum conditions of PEGylation was compared. It was found that succinimidyl carbonate at its optimum condition (pH=8.7, temperature =14 ℃ and reaction time =60 min) is more effective than cyanuric chloride at its optimum condition (pH=8.7, temperature =14 ℃ and reaction time =30 min) for RBC coating with mPEG.*

KEY WORDS: *Red blood cells, Methoxy poly(ethylene glycol), Succinimidyl carbonate, Cyanuric chloride, Anti-D sera.*

INTRODUCTION

Immunological recognition of foreign cells is a primary concern in both transfusion and cell transplantation. The cell surface serves as an important biological interface and biochemical barrier. However, upon transfusion or transplantation of cells from one individual to another, the extreme complexity of the proteins, carbohydrates, and lipids comprising the cell surface also serves as the primary loci for tissue rejection [1].

Red blood cell (RBC) membrane is architecturally complex and is characterized by significant biochemical diversity. Protein, lipoprotein, glycoprotein, and carbohydrate-rich structures play an important role in ensuring

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1021-9986/08/3/1 9/$/2.90

the physical integrity of the cell and carrying out its physiologic functions. However, the presence of these diverse RBC-surface molecules can also have adverse consequences. Several of these structures carry defined polymorphic epitopes, recognized serologically as blood group antigens, which can stimulate alloimmune responses (alloimmunization) after RBC transfusion or pregnancy [2].

The first and still the best characterized example of tissue rejection due to antigenic variation of cell surface components is reaction to transfusions of mis-matched RBCs [1]. The transition of transfusion from a highly risky surgical procedure to a mundane yet clinically crucial one, was enabled by the discovery of ABO/RhD blood groups by *Landsteiner* (1900) and *Landsteiner* and *Wiener* (1940) [1,3]. In most transfusions, ABO and D (Rhesus) 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 [4,5].

In the late 1950s, the chemical modification of proteins became commonplace and techniques were developed to facilitate the analysis of the structure-function relationships in the protein molecules. Since the late 1970s, many articles concerning the chemical modification of proteins by conjugation with synthetic macromolecules, such as polyethylene glycol (PEG) derivatives, have been published [1,2,6-9]. PEG is a highly investigated polymer for the covalent modification of biological macromolecules and surfaces for many pharmaceutical and biotechnical applications [10]. The aims of these protein modifications have included the reduction of immunoreactivity or immunogenicity in medical processes [11].

The covalent attachment of PEG is now commonly used to modify a variety of proteins, enzymes, drugs, artificial surfaces and cells, but at first it is necessary to activate PEG by preparing a derivative of PEG with a functional group at one or both termini. The functional group is chosen based on the type of available reactive group on the molecule that will be coupled to PEG. The most common reactive groups on proteins are alpha or epsilon amino groups of lysine. Several reactive derivatives of methoxy-PEG (mPEG) have been used to covalently attach mPEG to the surface of RBCs [12,13].

Our research group [6,14], recently studied the attachment of mPEG activated with cyanuric chloride and succinimidyl carbonate, separately to RBCs in order to optimize reaction conditions. RBCs were coated with a reactive mPEG derivative of 5 kDa molecular mass.

At present study, RBCs were coated with those reactive mPEG derivatives of 5 kDa molecular mass, separately at their optimum conditions, according to our previous studies [6,14]. The effects of process variables (polymer concentration, temperature, reaction time and pH) were investigated. Full factorial design and Taguchi analysis were employed to identify the optimum reaction conditions for PEGylation of RBCs. Inhibition of agglutination by a blood-type specific antisera (anti-D) was employed to evaluate the effect of the polymer coating. The remaining single cells after incubation with anti-D were counted using a simple method (Improved Neubauer Ruling). The extent of surface coating was evaluated by attachment fluorescein-isothiocyanate (FITC) labeled anti-D to the cells and recording the fluorescence intensity ratio of FITC-anti-D bound cells of PEG-RBCs, versus control (uncoated) RBCs. Finally, the morphology of PEG-RBCs was evaluated by scanning electron microscopy (SEM).

EXPERIMENTAL

Materials

MPEG of 5 kDa molecular mass, triethanolamine (TEA) and FITC were purchased from Sigma. Dioxan was purchased from BDH (England) and N,N'-disuccinimidyl carbonate was purchased from Aldrich (U.S.A). Anhydrous sodium carbonate, D-glucose, sodium chloride, isopropanol, diethylether, 4-(dimethylamio) pyridine, potassium chloride, benzene and cyanuric chloride were obtained from Merck-Schuchardt (Darmstadt, Germany). Cyclohexane was purchased from Roth (Karlsruhe, Germany). Packed Rh-positive RBCs (type B) were obtained from Iranian Blood Transfusion Organization. Anti-D was purchased from CinnaGen Inc. (Tehran, Iran).

Polymer derivatization

Derivatization of 5 kD mPEG with succinimidyl carbonate was performed as follows. Vacuum dried

mPEG was dissolved in dioxin, N,N'-disuccinimidyl carbonate was then slurried in dry acetone and added to the solution. 4-(dimethylamio) pyridine was dissolved in dry acetone, added to the reaction mixture, and the reaction allowed to proceed for 24 h at room temperature. The reaction mixture was filtered to remove any solid precipitate, and the filtrate added into diethyl ether to precipitate the derivatized mPEG. The precipitate was filtered and washed with diethyl ether, resuspended in isopropanol, filtered and washed with isopropanol to remove any unreacted N,N'-disuccinimidyl carbonate. The precipitate was then re-suspended in cyclohexane, filtered and washed with cyclohexane. Finally the mPEG succinimidyl carbonate derivative was dried under a stream of dry nitrogen for 12 h [14,15]. Derivatization of 5 kDa mPEG with cyanuric chloride was conducted as our previous work [6].

Coating of red blood cells with mPEG

Packed Rh-positive RBCs (type B) re-suspended as a 10 % hematocrit in TEA buffer (15 mM TEA, 120 mM NaCl, 4 mM KCl, 7.5mM D-glucose, 0.5 mM NaOH) for succinimidyl carbonate and (30 mM TEA, 110 mM NaCl, 4 mM KCl, 5 mM D-glucose) for cyanuric chloride. A fresh cold stock solution of the derivatized polymer was prepared in 0.9 % (w/v) NaCl, containing 5 mM HCl and appropriate volumes were immediately added to the RBC suspensions to yield final polymer concentration of 12 mg/ml and 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 optimum conditions (pH=8.7 and temperature =14 ºC, as well as reaction time =60 and 30 min for succinimidyl carbonate, and cyanuric chloride, respectively) [6,14]. After 3 washes with isotonic phosphate buffer solution (PBS, pH=7.4) at 200 xg for 10 minutes, packed RBCs were prepared for evaluation of the polymer coating [2]. RBC coating with cyanuric chloride activated mPEG was performed using a method described before [6].

RBC agglutination by anti-D

Inhibition of anti-D mediated agglutination was employed to assess the effectiveness of the polymer coating. Four hundred micro liters of control or PEGylated RBC suspension (6 % hematocrit in isotonic saline) [16] was mixed with a solution of anti-D in PBS with a known concentration (anti-D:PBS as 1:3) and was incubated with a gentle mixing at room temperature for 30 min. The RBCs were then centrifuged at 200 xg for 1 min. One micro liter of the pellet was re-suspended in 1 ml of PBS.

Using a dye exclusion test with Trypan blue (viable cells remained uncolored and dead cells showed blue color) and light microscopic system (Nikon, E200), non-agglutinated viable free cells were counted using a hemocytometer (Improved Neubauer Ruling).

Improved Neubauer Ruling is a 3 by 3 mm (9 mm^2) grid, subdivided into nine secondary squares, each 1 by 1 mm (1 mm^2). The smallest squares in the center of the grid have an area of 1/400 mm^2 and are arranged in the groups of 16. Single free erythrocytes in 5 of the 25 sections of 16 small squares of the hemocytometer (four corner sections and the center square) were counted. For determination of free cells in 1 ml of RBC suspension, the number of free cells was multiplied by 5×10^7. The higher the number of free cells, the greater the effectiveness of RBC PEGylation.

Flow cytometry

Labeling of anti-D with FITC was achieved by following the procedure described by *Coligan et al.* [17]. FITC-labeled anti-D was added to a dilute suspension of RBCs in PBS. The ratio of FITC-labeled anti-D to RBCs, obtained by titration, was high to prevent agglutination (60 µl of 0.1 % (v/v) hematocrit in PBS, added to 200 µl of FITC-labeled anti-D solution with protein concentration of 0.16 mg/ml). The samples were incubated for 30 min at room temperature in the dark with gentle mixing, centrifuged and washed twice with PBS at 200 xg for 2 min. The labeled cells were then resuspended 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 [2].

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

Table 1: Selected experimental variables and corresponding values at two levels for succinimidyl carbonate (full factorial design).

Variable	Low level (1)	High level (2)
A: Temperature of the reaction (ºC)	4	25
B: Time of the reaction (min)	30	60
C: mPEG concentration (mg/ml)	2	15
D: pH of TEA buffer	7.8	9.3

Table 2: Selected experimental variables and corresponding values at two levels for cyanuric chloride (full factorial design).

Variable	Low level (1)	High level (2)
A: Temperature of the reaction (ºC)	4	25
B: Time of the reaction (min)	30	60
C: mPEG concentration (mg/ml)	5	45
D: pH of TEA buffer	8	9.5

Table 3: Selected experimental variables and corresponding values at four levels for succinimidyl carbonate (Taguchi design).

Variable	First level (1)	Second level (2)	Third level(3)	Fourth level (4)
A: Temperature of the reaction (ºC)	4	14	20	25
B: Time of the reaction (min)	30	40	50	60
C: mPEG concentration (mg/ml)	2	7	12	15
D: pH of TEA buffer	7.8	8.3	8.7	9.3

Table 4: Selected experimental variables and corresponding values at three levels for cyanuric chloride (Taguchi design).

Variable	First level (1)	Second level (2)	Third level (3)
A: Temperature of the reaction (ºC)	4	14	25
C: mPEG concentration (mg/ml)	5	25	45
D: pH of TEA buffer	8	8.7	9.5

(uncoated) cells. The lower this ratio, the greater the effectiveness of RBC PEGylation.

Scanning electron microscopy (SEM)

The morphology of PEG-RBCs and control (uncoated) RBCs was evaluated by SEM (XL 30, Philips, Netherlands) to ensure that are structurally appropriate for transfusion. For sample preparation, the procedure described by *Kayden* and *Bessis* [18] was followed.

Design of experiments

The first step (using Yates table) [19] was to identify which variables have the largest effects on the PEGylation reaction for each reagents. The evaluated variables were temperature, time of the PEGylation reaction, polymer concentration and pH of TEA buffer. Each experiment was performed at 2 levels for 4 factors, using succinimidyl carbonate and cyanuric chloride, separately, as shown in tables 1 and 2, respectively [6,14].

The corresponding experimental variables, based on M_{16} and L_{27} arrays of Taguchi design of experiments [20] for succinimidyl carbonate and cyanuric chloride reagents are given in tables 3 and 4, respectively [6,14].

Also at obtained optimum conditions (temperature, pH and time of the reaction) for cyanuric chloride and succinimidyl carbonate and a similar polymer concentration, a comparison experiment was performed.

RESULTS AND DISCUSSION

In our previous studies [6,14] the optimum conditions for RBC PEGylation, by mPEG activated with succinimidyl carbonate and cyanuric chloride, separately were determined. The selection of the levels of factors for optimization were based on the range given in the literature [2,3,21-24]. The optimum temperature, pH of TEA buffer, reaction time and polymer concentration determined by flow cytometry and cell counting methods.

The corresponding results as shown in Figs. 1 and 2 were as follows. It should be mentioned that these results

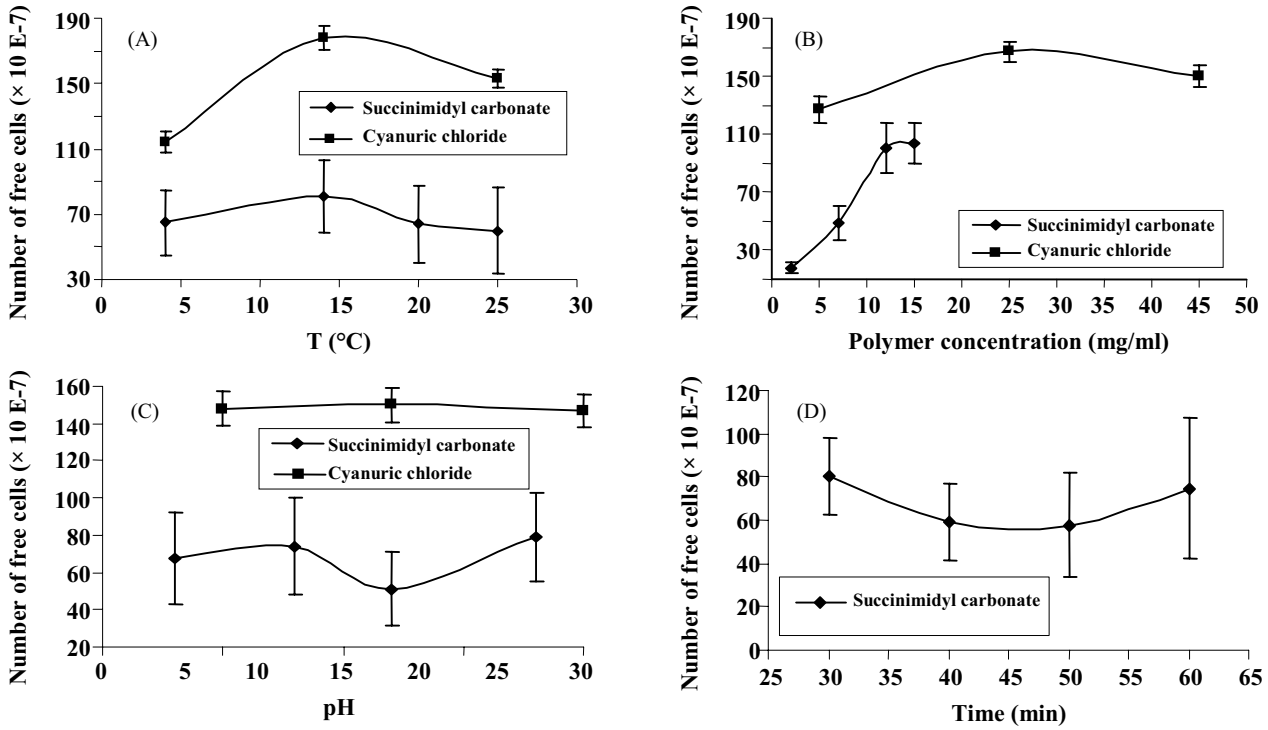

Fig. 1: The effect of different factors on the number of free (non-agglutinated) RBCs: A) Temperature of the reaction, B) Polymer concentration, C) pH of TEA buffer and D) Time of the reaction of RBCs with succinimidyl carbonate and cyanuric chloride activated mPEGs, separately.

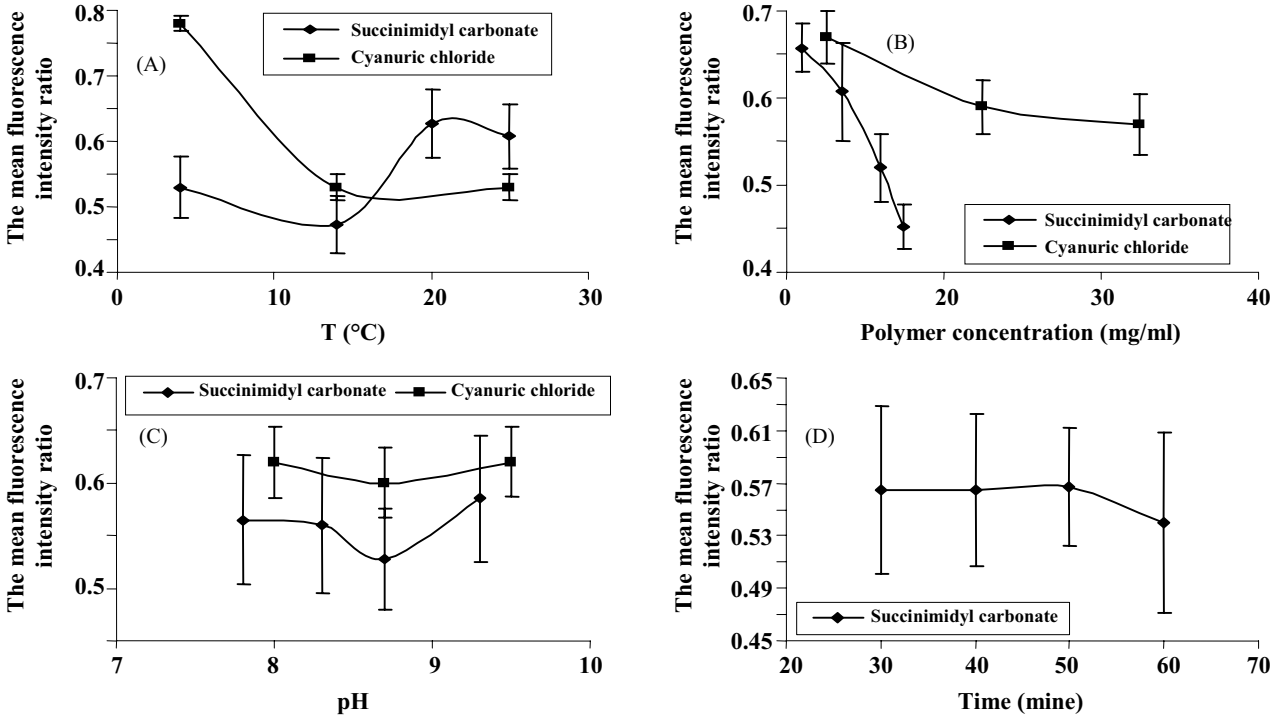

Fig. 2: The effect of different factors on the mean fluorescence intensity ratio of FITC labeled anti-D-cells of PEG-RBCs to control (uncoated) RBCs. A) Temperature of the reaction, B) Polymer concentration, C) pH of TEA buffer and D) Time of the reaction of RBCs with succinimidyl carbonate and cyanuric chloride activated mPEGs, separately.

Table 5: The results of the comparison tests, using two methods (flow cytometry and free cell counting), for succinimidyl carbonate-mPEG and cyanuric chloride-mPEG.

Kind of activated mPEG	Response[a] × 10^{-7}	Average mean fluorescense of 3 replications	Response[b]
Succinimidyl carbonate activated mPEG	120	23.153	0.33
Cyanuric chloride activated mPEG	73	26.823	0.38

a) Each value is the average number of free cells, per 1 ml suspension. b) Each value is the average mean fluorescence intensity ratio of FITC labeled anti-D cells of PEG-RBCs to control (uncoated) RBCs, for each test.

have been obtained from 2 completely separated set of cells, for obtaining the optimum conditions, by cyanuric chloride and succinimidyl carbonate and their performances can not be compared, here. For comparison the activities of 2 activators, at the end, we examined on one set of cells. It could be seen that at optimum condition, the number of free cells was maximum, but the fluorescence intensity ratio of FITC-anti-D bound cells of the PEG-RBCs to control (uncoated) RBCs was minimum. It showed that as a result of mPEG attachment to RBCs, agglutination between RBCs was decreased and corresponding free cell number was increased.

Also as a result of RBC coating by mPEG, the attachment of FITC labeled-anti-D to RBCs was decreased and the corresponding ratio of mean fluorescence intensity of the PEG-RBCs to control (uncoated) RBCs was decreased.

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 FITC-labeled anti-D, demonstrates that the polymer coating does prevent antibody binding. At this condition, there are some RBCs that show anti-D binding, but are not agglutinated.

The optimum condition for cyanuric chloride was found to be at pH = 8.7, temperature = 14 ºC and reaction time = 30 min and for succinimidyl carbonate was at pH = 8.7, temperature = 14 ºC and reaction time = 60 min. Also, in our previous studies [6,14], SEM results (not presented here) showed that polymer concentration of 15 mg/ml for cyanuric chloride activated mPEG and 12 mg/ml for succinimidyl carbonate activated mPEG are the highest useful levels for PEGylation. Higher concentrations up to 50 mg/ml have also been used [22,24], but some abnormality in the morphology of RBCs was reported. SEM and light microscopy results from our previous work also showed that a polymer concentration of 15 mg/ml is the highest recommended level for PEGylation using a linear PEG of molecular mass 5 kDa. Above this concentration, only type III echinocytes existed. RBCs of echinocyte type 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 [25].

At present study, the extent of polymer coating, based on immunological response of PEGylated RBCs, using activated mPEGs at a constant polymer concentration of 12 mg/ml were compared. The other variables were kept at their optimum values. The results of these comparison tests, using two methods (flow cytometry and free cell counting), are presented in table 5. Also the morphology of the cells, after reaction with two kinds of activated mPEG, at their optimum condition, is presented in Fig. 3. It shows that the effect of polymer coating, with both kinds of activated mPEGs, on producing echinocytosis shapes is approximately similar.

The results showed that succinimidyl carbonate activated mPEG is more effective than cyanuric chloride activated mPEG for RBC coating, i.e. the number of free cells for succinimidyl carbonate is higher than that of cyanuric chloride. The reduced fluorescence intensity ratio obtained by flow cytometry method, also confirm the above results. Cyanuric chloride activated mPEG has one extra active chloride group that can absorb other amino targets in immunological reactions and according to the literature, even after attachment to the RBC surface, can slowly bind other proteins by a secondary non-specific

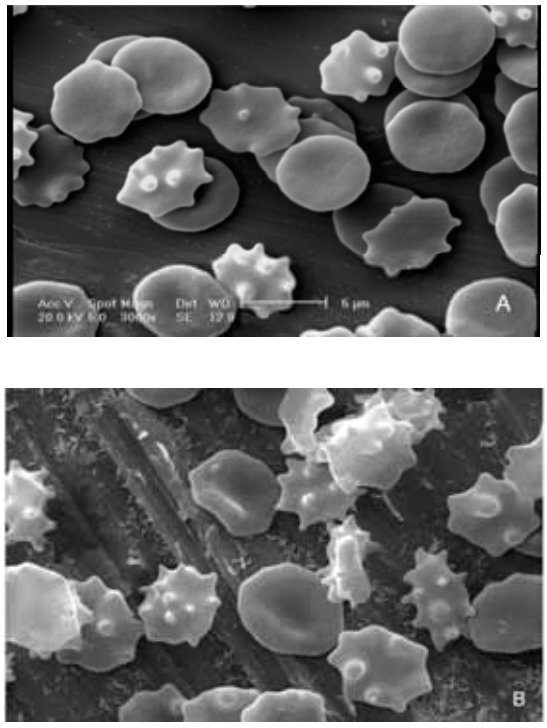

Fig. 3: The morphology of RBCs by 12mg/ml of A) Succinimidyl carbonate and B) Cyanuric chloride activated mPEGs.

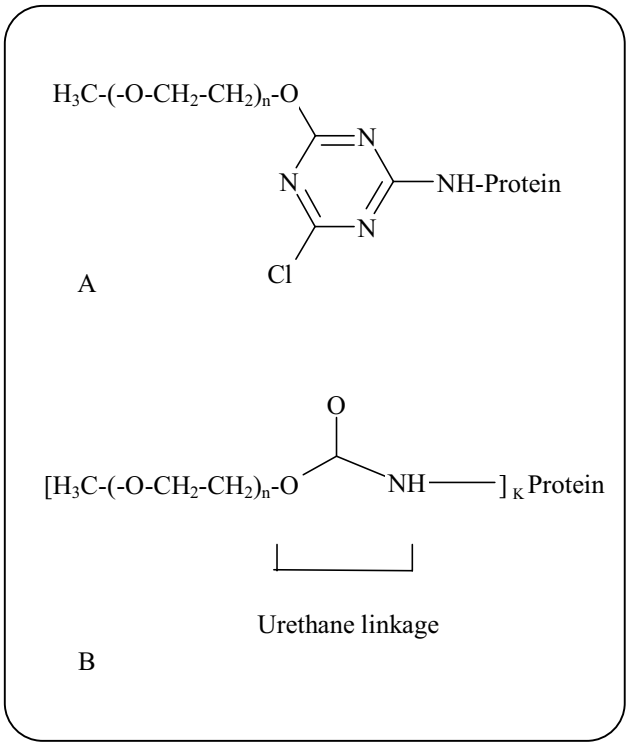

Fig. 4: Chemical structure of A) Cyanuric chloride and B) Succinimidyl carbonate [26].

reaction [23]. But, succinimidyl carbonate, according to its chemical structure, does not have this kind of active group (Fig. 4).

CONCLUSIONS

At this study the optimum conditions for covalent attachment of mPEG with molecular mass of 5 kDa, activated with succinimidyl carbonate and cyanuric chloride, to human RBCs were compared. The optimum condition selected for succinimidyl carbonate, was at pH = 8.7, polymer concentration = 12 mg/ml, temperature= 14 ºC and reaction time = 60 min and that for cyanuric chloride was at pH = 8.7, polymer concentration =15 mg/ml, temperature = 14 ºC and reaction time = 30 min. These conditions are very similar, but succinimidyl carbonate is more effective reagent for PEGyaltion of RBCs with activated mPEG.

Acknowledgments

This work was supported by grants from the Ministry of Science, Research and Technology of Iran and Tehran University of Medical Sciences. The authors also wish to thank Iranian Blood Transfusion Organization for providing the packed Rh-positive RBCs. Thanks are due to Dr. J. K. Armstrong at USC for his fruitful advices during this study.

Received : 3rd April 2007 ; Accepted : 3rd March 2008

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