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Surface Treatment of Red Blood Cells with Monomethoxypoly(ethylene glycol) Activated by Succinimidyl Carbonate

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A B S T R A C T

In the present study, the optimum conditions were determined for PEGylation of human red blood cells by methoxypoly(ethylene glycol) (mPEG) with molecular mass of 5 kDa, activated by succinimidyl carbonate. Factorial and Taguchi design methods for experiments were used to study the effects of process variables on coating of the cells and determine the optimum conditions for PEGylation. The inhibition of agglutination by a blood-type specific antiserum (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 attachment of cells to FITC labeled-anti-D serum and recording as the fluorescence intensity ratio of FITC-anti-D bound cells of the PEG-RBCs versus control (uncoated) RBCs. The morphology of red blood cells was determined by scanning electron microscopy (SEM) technique. Statistical analysis of experimental design together with SEM results showed that the optimum conditions were pH = 8.7, T=14°C, and t = 60 min while the suitable polymer concentration was found to be 12 mg/mL.

Key Words:

red blood cells;
methoxypoly(ethylene glycol);
disuccinimidyl carbonate;
anti-D sera;
polymer coating.

INTRODUCTION

The immunological response to transplanted allogeneic tissue and cells has been a significant barrier in both organ transplantation and blood transfusion. The most common example of allogeneic cell exposure is the use of red blood cells (RBCs) in transfusion. The red blood cell (RBC) membrane is architecturally complex and is characterized by significant biochemical diversity. Protein, lipoprotein, glycoprotein, and carbohydrate-rich structures play an

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important role in ensuring the physical integrity of the cell and carrying out its physiologic functions. However, presence of these molecules, on the RBC surface, also has adverse consequences. Several of these structures carry defined polymorphic epitopes, recognized serologically as blood group antigens, which can stimulate alloimmune responses (alloimmunization) after red blood cell transfusion or pregnancy.

Alloimmunization is particularly problematic in patient groups who receive multiple transfusions [1]. 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 red blood cell (RBC) [2]. The transition of blood transfusions from a highly risky surgical procedure to a mundane yet clinically crucial procedure was enabled by the discovery of the ABO/RhD blood groups by Landsteiner (1900) and Landsteiner and Wiener (1940) [2]. 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 [3-4].

In the late 1950s, the chemical modification of proteins became common 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, that is polyethylene glycol (PEG) derivatives, have been published [17]. The aims of these protein modifications have included the reduction of immunoreactivity or immunogenicity in medical processes [5]. The covalent attachment of PEG is now commonly used to modify a variety of proteins, enzymes, drugs, and artificial surfaces. The most common reactive groups are the alpha or epsilon amino groups of the lysine [6]. Despite these applications, RBCs have only recently been considered for use as substrate for PEG modification [5]. For proteins, typical reactive amino acids include lysine, cysteine, histidine, arginine , aspartic acid, glutamic acid, serine, threonine, tyrosine, N-terminal amino group and the C-terminal carboxylic acid [6].

Several reactive derivatives of methoxy-PEG (mPEG) have been used to covalently attach mPEG to the surface of RBCs [18,19]. One of the reagents for activating mPEG is succinimidyl carbonate. Despite the development of this technology to PEG-coating of RBCs, optimization of the reaction conditions has not been thoroughly investigated. Hashemi-Najafabadi et al. [17] have recently studied the attachment of mPEG activated with cyanuric chloride to RBCs in order to optimize reaction conditions.

In the peresent study, RBCs were coated with a reactive succinimidyl carbonate mPEG derivative of 5 kDa molecular mass. The effects of process variables (polymer concentration, temperature, time and pH) were investigated. Full factorial and Taguchi designe methods were employed to identify the optimum reaction conditions for PEGylation of RBCs. The inhibition of agglutination by a blood-type specific antiserum (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 method (Improved Neubauer Ruling). The extent of surface coating was evaluated by the attachment of cells to FITC labeled-anti-D and recording as the fluorescence intensity ratio of FITC-anti-D bound cells of the PEG-RBCs versus control (uncoated) RBCs. Finally, the morphology of PEG-RBCs was studied by scanning electron microscopy (SEM) technique.

Materials and Methods

Materials

Methoxypoly(ethylene glycol) of 5 kDa molecular mass, triethanolamine and fluorescein- isothiocyanate (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 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 sera was purchased from CinnaGen Inc. (Tehran, Iran).

Polymer Derivatization

Derivatization of 5 kD mPEG with succinimidyl carbonate was performed using a modification of the method described by Mirion et al. [7]. Briefly, 5 g vacuum dried mPEG (overnight under 80°C) was dissolved in 100 mL dioxin (warmed slightly to aid dissolution) and then cooled to room temperature, 3 fold excess of *N,N′*-disuccinimidyle carbonate was then slurried in 10 mL of dry acetone and added to the solution. Six (mmol) of 4-(dimethylamio)pyridine was dissolved in 20 mL of dry acetone, added slowly to the reaction mixture, and the reaction allowed to proceed for 24 h at room temperature under nitrogen. The reaction mixture was filtered to remove any solid precipitate, and the filtrate poured slowly under high shear 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′*-disuccinimidyle carbonate. The precipitate was then resuspended in cyclohexane , filtered and washed with cyclohexane. Finally the mPEG succinimidyl carbonate derivative was dried under a stream of dry nitrogen for 12 h.

Coating of Red Blood Cells with mPEG

Packed Rh-positive RBCs were resuspended in a 10% hematocrit in triethanolamine (TEA) buffer (15 mM triethanolamine, 120 mM NaCl, 4 mM KCl, 7.5 mM D-glucose, and 0.5 mM NaOH). 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 2-15 mg/mL, and equivalent volume of buffer was added to 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 (pH, temperature, time and polymer concentration, defined in subsequent sections). After 3 washes with isotonic phosphate buffer solution (PBS, pH=7.4) at 200 x g for 10 minutes, packed RBCs were prepared for evaluation of the polymer coating [8].

RBC Agglutination by Anti-D sera

Inhibition of anti-D sera mediated agglutination was employed to assess the effectiveness of the polymer coating. Four hundred micro liters of a control or PEGylated RBC (Rh-positive) suspension (6% hematocrit in isotonic saline) [9] was mixed with a solution of anti-D in PBS with a known concentration (anti-D sera/PBS: 1/3) and were incubated with a gentle mixing at room temperature for 30 min. The RBCs were then centrifuged at 200 x g for 1 min.

One microliter of the pellet was resuspended in 1 mL of PBS, and then using a dye exclusion test with Trypan blue (viable cells remained uncolored, dead cells showed blue color) and light microscopic system (Nikon, E200), non-agglutinated viable free cells were counted using a hemocytometer (Improved Neubauer Ruling) which is a 3 by 3 mm (9 mm^2) grid, subdivided into nine secondary squares, each 1 by 1 mm (1 mm^2).

The central square has been subdivided to 25 sections of 16 smaller squares. The smallest squares in the center of the grid have an area of 1/400 mm^2. Single free erythrocytes in the 5 sections of the 25 sections of 16 smaller 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 is the effectiveness of RBC PEGylation.

Flow Cytometry

Labeling of anti-D sera with fluorescein-isothiocyanate (FITC), was achieved by following the procedure described by Coligan et al. [10]. 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 enough to prevent agglutination (60 µL of 0.1% (v/v) hematocrit in PBS, added to 200 µL of fluorescein-labeled anti-D solution with protein concentration of 0.16 mg/mL). The samples were incubated for 30 min at room temperature

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)	30	60
C: mPEG concentration (mg/mL)	2	15
D: pH of TEA buffer	7.8	9.3

in darkness with gentle mixing, centrifuged and washed twice with PBS at 200 x g 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. 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 be this ratio, the greater is the effectiveness of RBC PEGylation.

Scanning Electron Microscopy

The morphology of PEG-RBCs and control (uncoated) RBCs was studied by a scanning electron microscope (XL 30, Philips, Netherlands) to ensure that they are structurally appropriate for transfusion. For preparation of samples, we followed the procedure described by Kayden et al. [20].

Design of Experiments

Full Factorial at Two Levels

The first step (using Yates Table) identifies which variables have the largest effects on the PEGylation reaction. Selection of these factors were based on previous studies in literature for RBC PEGylation [1,2,11]. The evaluated variables were temperature, time of PEGylation reaction, polymer concentration and pH of TEA buffer (Table 1). Each experiment was performed at 2 levels, 4 factors and 3 replications.

Taguchi Design

To obtain the optimum condition for PEGylation of RBCs, an M_{16} array of Taguchi [12] for 4 variables at 3 levels was also designed. Each experiment was performed in 3 replications. The variables and their corresponding values are presented in Table 2. This design was also employed to compare the hemocytometery results with the flow cytometry data.

RESULTS

Full Factorial Design

The results and statistical analysis for full factorial design (using Yates Table) at two levels are presented in Table 3. All responses are presented 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 shows that the PEG-RBCs were protected against agglutination. By comparison of the obtained F-values with a critical $F_{(1,16)}$ value of 8.575 (obtained from F-Table) [13], it is apparent that 4 variables (temperature, polymer concentration, pH, and time) and their interactions have the F-values greater than critical F. Hence they were selected as important factors for PEGylation of RBCs. The effect of reaction time was less important. Similar results

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

Variable	First level (1)	Second level (2)	Third level (3)	Fourth level (4)
A: Temperature of reaction (°C)	4	14	20	25
B: Time of 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 3. Yates Table analysis of a 24 full factorial design with cell counting method (4 variables in 2 levels).

Test	TC[1]	Response[2] × 10^{-7}	F-value[3]
1	1	48	-
2	a	13	9.388
3	b	46	1.844
4	ab	20	1.037
5	c	73	23.139
6	ac	15	6.485
7	bc	33	3.279
8	abc	68	54.124
9	d	10	92.566
10	ad	143	3.323
11	bd	150	2.001
12	abd	28	18.014
13	cd	228	7.378
14	acd	126	2.510
15	bcd	103	18.988
16	abcd	95	19.987

(1) 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.

(2) Each value is the average number of free cells, per 1 mL suspension, for each test with three replicates.

(3) $SS_x = (f_x^2)/N$, 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 (32), $MS_x = SS_x/\varnothing_x$, which $\varnothing_x$ is the degree of freedom corresponding to x, $TSS = \sum y_i^2 - (\sum y_i)^2/N = 7717/916$, y_i is the result, $RSS = TSS - \sum SS_x = 832/693$, Error degree of freedom ($\varnothing$)= 16, Residual error variance = $RSS/\varnothing = 832/693$, $F - value = MS_x/(\text{Residual error variance})$, $F - critical\ (\varnothing_1 = 1,\ \varnothing_2 = 16) = 8.575$ (obtained from standard Table).

were obtained for conjugation of mPEG, activated with cyanuric chloride, to RBCs [17].

Taguchi Design

The obtained results, using an M_{16} array of Taguchi, with both data from cell counting and flow cytometry methods, are presented in Table 4. The main effects of the experimental variables on the number of free single RBCs, and also the mean fluorescence intensity ratio of FITC-anti-D labeled cells of PEG-RBCs versus control (uncoated) RBCs, as two indicators of the extent of PEGylation are shown in Figures 1 and 2, respectively. These figures show that the optimum temperature and pH of the reaction medium, time, and polymer concentration are T=14°C and pH=9.3, t=30 min and c=15 mg/mL by free cell counting. But the optimum of these factors by flow cytometry methods, are T=14°C and pH=8.7, t=60 min and c=15 mg/mL. Since flow cytometry method, is more accurate than free cell counting method, the result will be explained later by the SEM studies.

SEM

The morphology of control (uncoated) and PEG-coated RBCs (2, 7, 12, and 15 mg/mL of activated mPEG) at optimum condition (60 min, 14°C, and pH=8.7) are presented in Figure 3. This figure shows that at polymer concentrations above 12 mg/mL, the morphology of RBCs is abnormal by increasing echinocytosis.

DISCUSSION

The present study was carried out to optimize the reaction conditions for conjugation of mPEG, activated with succinimidyl carbonate to RBCs.

The selection of levels of factors for optimization was based on the range given in the literature. Succinimidyl carbonate-mPEG was found to be sufficiently reactive to produce extensively modified proteins under mild conditions, showing the highest reactivity around pH=9.3 [14]. Other researchers used an elevated pH (pH=8-9.2) for coupling an activated PEG with RBC [8,11,15]. Also the ranges of 30-60 min and 4-25°C have been used for the time and temperature of RBC PEGylation, respectively. Fisher [16] in his review noted that at polymer concentrations below 1 mM (5 mg/mL) the 5 kD activated mPEG is not very effective for masking antigens. Higher concentrations up to 50 mg/mL have also been used [15], but some abnormalities in the morphology of RBCs were reported. The in vitro studies [8,11,3] also suggested that in spite of some morphological

Table 4. The obtained results using an M_{16} array of Taguchi with both data from cell counting and flow cytometry methods.

An M_{16} Taguchi array for RBC PEGylation Test	Temperature (A) (°C)	Concentration (B) (mg/mL)	pH (C)	Time (min)	Response[1] $\times 10^{-7}$	Average mean fluorescence of three replications	Response[2]
1	4	2	7.8	30	30	44.09	0.63
2	4	7	8.3	40	52	40.64	0.58
3	4	12	8.7	50	55	34.68	0.49
4	4	15	9.3	60	122	29.82	0.42
5	14	12	8.3	30	107	29.87	0.43
6	14	15	7.8	40	65	28.18	0.40
7	14	2	9.3	50	13	42.19	0.60
8	14	7	8.7	60	27	32.38	0.46
9	20	15	8.7	30	105	34.29	0.49
10	20	12	9.3	40	102	42.90	0.61
11	20	7	7.8	50	37	47.79	0.68
12	20	2	8.3	60	12	51.02	0.71
13	25	7	9.3	30	80	50.24	0.73
14	25	2	8.7	40	17	47.21	0.67
15	25	15	8.3	50	125	35.57	0.50
16	25	12	7.8	60	138	38.82	0.55

(1) Each value is the average number of free cells, per 1 mL suspension.

(2) Each value is the average mean fluorescence intensity ratio of FITC labeled anti-D cells of PEG-RBCs to control RBCs (uncoated, with an intensity of 69.97) for each test.

changes, the mPEG-modified RBCs show normal structure and function.

The optimum temperature, pH of TEA, time of reaction, and polymer concentration determined by flow cytometry method were as follows: T=14°C pH=8.7, t=60 min, and c=15 mg/mL. As mentioned earlier, a pH >7.0 is required for reaction of the PEG derivative with primary amine residues. It can be related to the amino acid type (lysine) that is more reactive with the activated polymer. Also it is well known that a non-physiological pH can be very damaging to cells. A moderate pH of 8.7 was determined as the optimal condition. It should be noted that PEGylation reactions were carried out at different pHs and immediately after that, the cells were returned to the physiological pH (using PBS, pH=7.4).

It can be seen that the optimum conditions at which the number of free cells was maximum, and the fluorescence intensity ratio of FITC-anti-D bound cells of the PEG-RBCs versus control (uncoated) RBCs was minimum, as shown in Table 4, are very similar to those deduced from Figures 1 and 2. These results indicate that as a result of mPEG attachment to RBCs, agglutination between RBCs was decreased and the corresponding free cells number was increased. Also, as a result of RBC coating by mPEG, attachment of FITC labeled-anti-D to RBCs was decreased and the corresponding ratio of the mean fluorescence intensity of the PEG-RBCs versus control (uncoated) RBCs 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

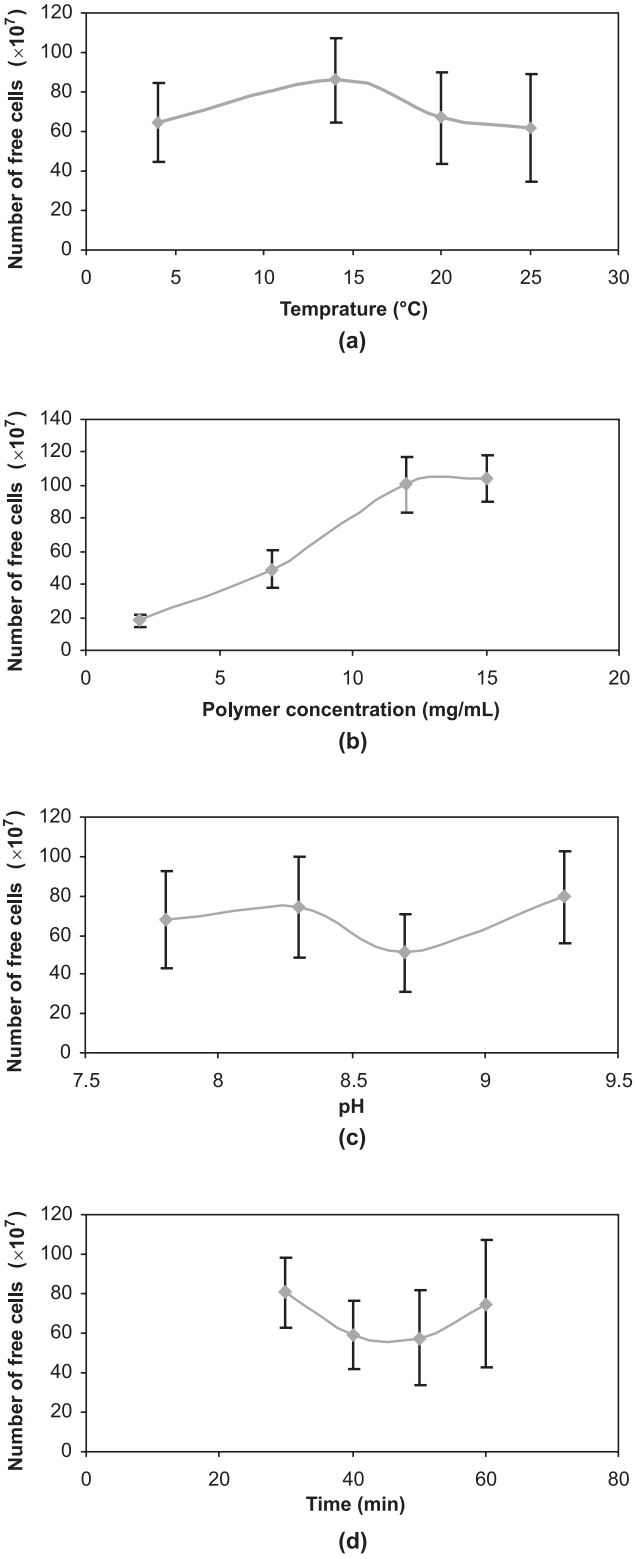

Figure 1. The effect of different factors on free cell (non-agglutinated cell) numbers: (a) temperature, (b) polymer concentration, (c) pH of TEA buffer, and (d) time of reaction.

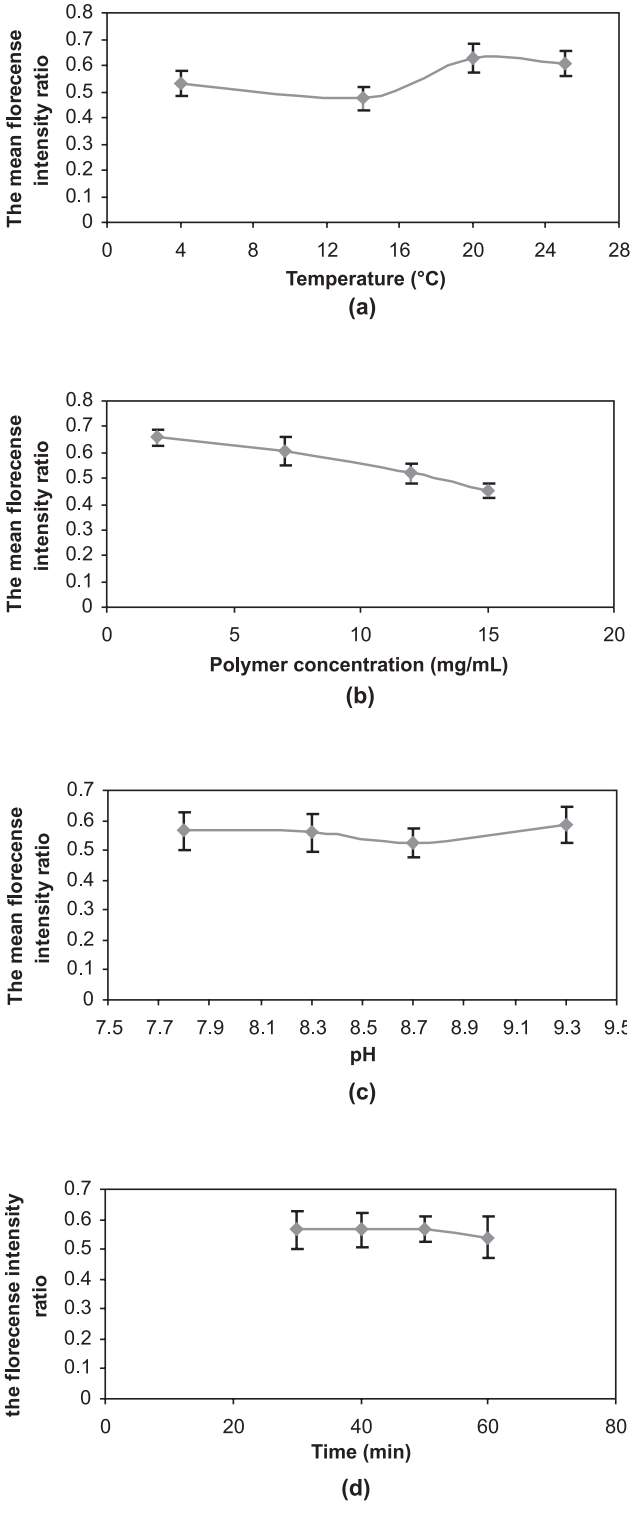

Figure 2. The effect of different factors on the mean fluorescence intensity ratio of FITC labeled anti-D-cells of PEG-RBCs versus control (uncoated) RBCs: (a) temperature of reaction, (b) polymer concentration and (c) pH of TEA buffer and (d) time.

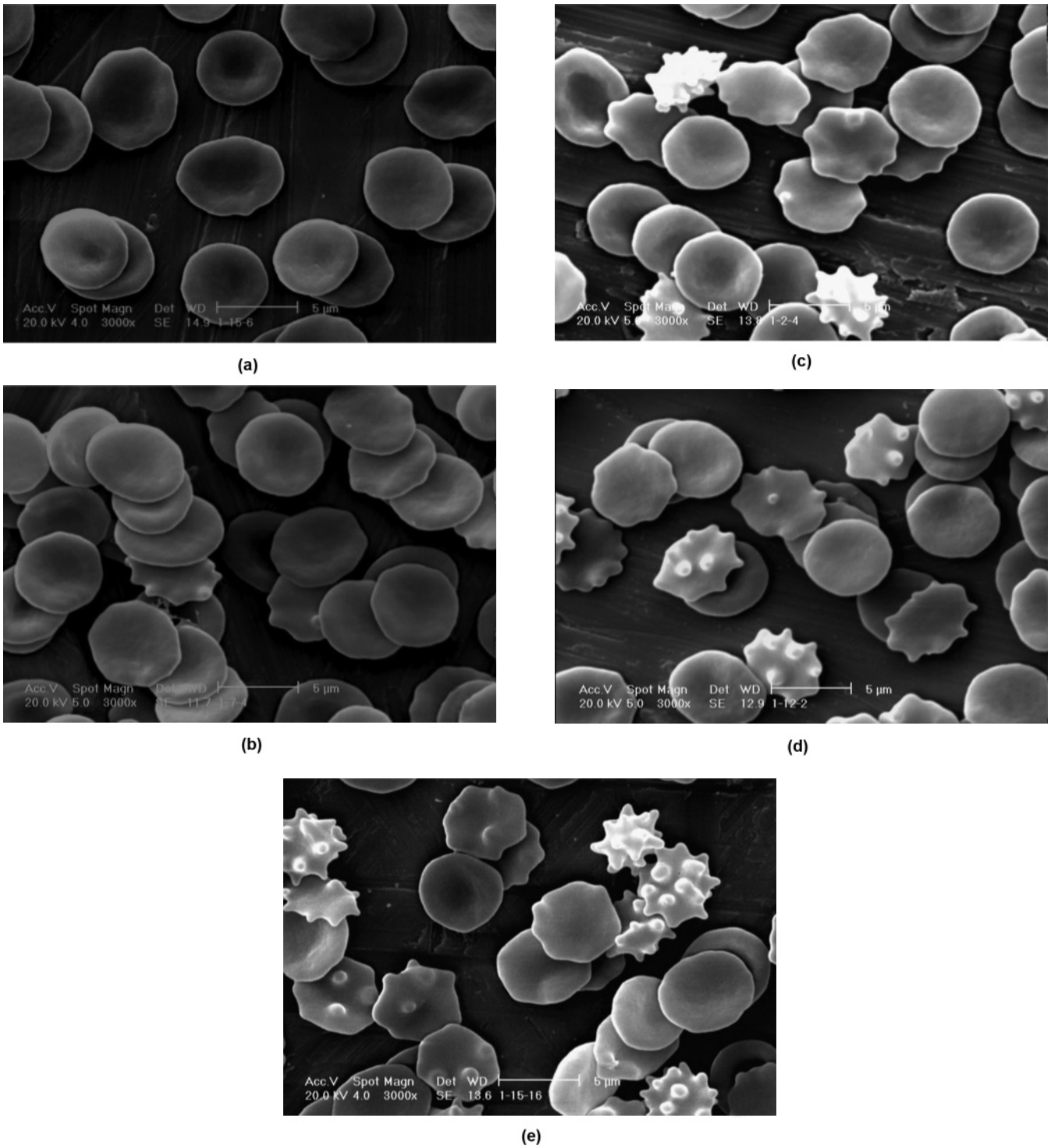

Figure 3. The morphology of mPEG-derivatized RBCs: (a) control RBCs, with polymer concentration of (b): 2 mg/mL, (c): 7 mg/mL (d): 12mg/mL and (e): 15mg/mL (3000x) .

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. At this condition, there are some RBCs that show anti-D binding, but are not agglutinated.

SEM results show that polymer concentration of 12 mg/mL is the highest useful level for PEGylation.

At higher concentrations, RBC loss of discocytic morphology was observed (increasing echinocytosis). Therefore the optimum concentration, 15 mg/mL, obtained by cell counting, and flow cytometry methods, cannot be selected, as an optimum polymer concentration due to the loss of discocytic morphology. It can be concluded that the optimum polymer concentration cannot be determined by considering the extent of PEGylation as a sole criterion. In the light of SEM results, polymer concentration of 12 mg/mL was the highest useful level for PEGylation. Beyond this polymer concentration, the discocytic morphology of PEG-RBCs was lost and echinocytosis enhanced by increasing concentration of mPEG, activated with succinimidyl carbonate.

These results are very similar to those obtained for PEGylation of RBCs with mPEG, activated by cyanuric chloride [17]. Since the optimum conditions for PEGylation of RBCs obtained by both free cell counting and flow cytometry methods, were similar, it can be concluded that the cell counting is a simple and suitable method for primary assessment of RBC PEGylation. However flow cytometry method, which is more accurate than free cell counting method, together with SEM analysis can be used to determine the optimum conditions for PEGylation of red blood cells.

At present study, we also found that more than %99 of cells were viable after the reaction (using dye exclusion test with Trypan blue). This shows that the presence of mPEG does not have adverse effect on cell viability.

CONCLUSSION

In this study, the optimum conditions for covalent attachment of methoxypoly(ethylene glycol) (mPEG) with molecular mass of 5 kDa, activated with succinimidyl carbonate, to human red blood cells were obtained. The optimum condition, determined by Factorial and Taguchi methods, was found to be: pH=8.7, c=15 mg/mL, T=14ºC, and t=60 min. These conditions are similar to those obtained for conjugation of mPEG, activated by cyanuric chloride, to RBCs. It was also found that optimum polymer concentration can not be determined only by considering the extent of PEGylation as a sole criterion. But SEM results showed that polymer concentration of 12 mg/mL was the highest useful level for PEGylation due to the loss of discocytic morphology of RBC at higher concentration. It was also demonstrated that the extent of RBC PEGylation can be determined by simple cell counting method.

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