Potent suppression of agglutinability of human A and B type erythrocytes by KMnO_4 -EDTA concerted action

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Abstract

Purpose of the study Study was focused to effectively suppress the agglutinability of human A and B type erythrocyte by concerted chemical oxidation approach. Consequently some major bio-physiological consequences of human erythrocytes were analyzed.

Materials and methods Concerted chemical oxidation of human A and B type erythrocyte were done by potassium permanganate (KMnO_4) in presence of ethylene diamine tetra-acetic acid (EDTA). All the chemical reactions were carried out at 37°C temperature and pH 7.4 in phosphate buffer. Finally, suppression of agglutinability were determined by using anti-A and anti-B antisera.

Results A marked suppression in agglutinability was observed after chemical treatment on human A and B type erythrocytes *in vitro*. Effective suppression of agglutinability was observed in 0.57 mM concentration of KMnO_4 in presence of 0.9 mM EDTA at optimum condition.

Conclusions Results showed that KMnO_4 -EDTA concerted action is feasible. The future work is directed towards

the universal human erythrocytes preparation from A and B type human erythrocytes.

Keywords Erythrocyte · Agglutination · Antibodies · Concerted action · Transfusion medicine

Introduction

Erythrocyte surface antigens are the major obstacles towards the preparation of universal red blood cell (RBC). Chemically blood antigenic determinants are glycoprotein in nature bearing special signature oligosaccharides of *N*-acetylgalactosamine, galactose and fucose, respectively [1]. It has been concluded that these antigens of blood groups are mainly responsible for formation of autoantibodies and alloantibodies in blood transfusion and hemolytic diseases [2, 3]. When universal donor is not available, the conversion of A or B type to universal type is an alternative to overcome the life-threatening situation. For this reason multi-dimensional research works have already been initiated to camouflage the RBCs [4–7]. However, there remain various drawbacks in new techniques including development of neoantigen, formation of PEG antibodies against mPEGylated erythrocyte, high chances of phagocytic recognition, poor oxygen diffusion, incomplete loss of antigenicity of A, B blood types [8]. The oxidation of signature oligosaccharides may be a chemically effective process to solve the problem. However some research groups have shown that carbonyl formation on surface proteins by oxidative processes introduced an additional problem through recognition of oxidized erythrocyte by macrophages [9]. So inhibition of formation of carbonyl groups on cell-surface protein is essential during oxidation. We, therefore, have explored a simple and an effective approach to suppress the agglutinability of human A and B type antigenic

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determinants on human erythrocyte by controlled oxidation by mixture of potassium permanganate (KMnO_4)-ethylene diamine tetra-acetic acid (EDTA) in an ambient condition. We also have studied the biophysical consequences of chemically treated human erythrocytes including zeta-potential, surface morphology, and time course of oxygen uptake, agglutination index (AI) and spontaneous human RBC (hRBC) lysis percentage. This chemical treatment has removed effectively antigenicity of A and B types erythrocytes. Moreover, this combined action distinctively has increased the negative surface charge on hRBC. This enhanced negative charge may reduce the chances of phagocytic recognition and cellular clearance. Experimentally it has been proven that KMnO_4 oxidizes hexahydric alcohol to acidic group $-\text{COOH}$ in neutral and alkaline conditions [10]. We have found experimentally that after oxidative action of permanganate, the products manganese ions (II/III) remain as water soluble complexes in presence of EDTA instead of producing water insoluble MnO_2 as formed normally in various conventional reactions with permanganate.

Materials and methods

Materials

KMnO_4 , EDTA, ammonium oxalate, potassium oxalate, acetone, sodium hydroxide, hydrochloric acid, sodium phosphate buffer (pH 7.4) purchased from Sigma-Aldrich, Drabkin's reagent (Span diagnostics), AB monoclonal antibody kit (Span diagnostics). Centrifuge (Eppendorf), Trypan blue, phosphate buffer saline (pH 7.4), UV-visible spectrophotometer (Shimadzu), scanning electron microscope (Olympus), FTIR spectrophotometer (Perkins Elmer) and zeta potentiometer (Zeta plus).

Human erythrocyte collection and chemical oxidation

Erythrocyte rich fraction of healthy human blood (A, B type) containing double oxalate as anticoagulant stored at 4°C was obtained from local hospital. Erythrocyte enriched fraction was centrifuged twice (3,000 g, 15 minutes) at 4°C to remove the residual plasma and buffy coat. RBCs were washed 3 times with sodium phosphate buffer (pH 7.4) and resuspended in same buffer to make packed cell volume of $\sim 12\%$ (w/v) as stock. Then the reaction mixture was prepared by mixing washed RBC stock suspension, EDTA and KMnO_4 . Reaction mixture contained 5.5% (w/v) of RBC, 0.9 mM of EDTA and various final concentrations of KMnO_4 (0.05, 0.11, 0.23, 0.45, 0.57, 0.68, 0.8, 0.9 mM). The reaction was allowed to continue for 60 minutes at 37°C temperature. Afterward, the oxidized erythrocyte cells were concomitantly centrifuged (3,000 g, 15 minutes) at 4°C and washed thrice with sodium phosphate buffer (pH 7.4) to carry out the morphological and biochemical analysis.

Statistical analysis

All sets of experiments were performed at least twice and results reported as means $\pm$ standard deviation.

Analysis on AI on KMnO_4 and EDTA treated human erythrocyte

The effect of combined chemical modification on the antigenicity and agglutinability of native (untreated) and modified human erythrocyte cells were assessed by agglutination using commercially available anti-A and anti-B antisera (Span diagnosis). It was expressed as AI which was formulated [11] from the slope of the absorption spectra in the range 600–1000 nm. The threshold value of AI was fixed at 17 [12] meaning no agglutination below this value.

Studies on morphology, spontaneous RBC lysis and zeta-potential on KMnO_4 and EDTA treated human erythrocyte

Changes in gross morphology and membrane surface charges were examined to test the structural integrity of treated human erythrocyte. The morphology of both control and modified RBCs were assessed by scanning electron microscopy [13] ($1500\times$ and $4000\times$ magnification). Spontaneous hRBC lysis percentage was measured by determining total hemoglobin concentration spectrophotometrically (540 nm) [14] for both total RBC suspension and cell supernatant using Drabkin's reagent. The accepted value of mean of zeta-potential of normal RBCs (from 10 individuals) was -13.07 ± 0.61 mV (mean $\pm$ SD) [15]. Chemically modified and native hRBCs washed with sodium phosphate buffer were used to assess the zeta-potential using DTS1060C type clear possible zeta cell at 25°C temperature.

Isolation of human erythrocyte membrane for FTIR studies

The washed human erythrocyte suspension (20 ml) was suspended in an equivalent volume of glucose (250 mM) solution [16]. Each sample was allowed to stand at 4°C for 5 minutes after adjusting pH of 6.8 with gradual addition of 0.1 (N) HCl in 0.9% (w/v) of NaCl solution. Then samples were centrifuged at 3,000 g for 2 minutes. After that the washed RBC membrane was vacuum dried at 25°C for overnight incubation and stored to do FTIR studies to detect the presence of functional groups ($-\text{OH}$, $-\text{CHO}$ and $-\text{COOH}$) presence on erythrocyte membrane surface of native and modified hRBCs.

Functional groups detection on RBC membrane surface by FTIR

The FTIR spectra of membrane of washed human erythrocyte were recorded from spectrum RX-1 (Perkins Elmer)

IR spectrophotometer. Each FTIR spectrum represents 16 scans with 4 cm^{-1} spectral resolution.

Oxygen uptake studies on KMnO_4 and EDTA treated human erythrocyte

Oxygen uptake into native and modified human erythrocytes was semi-quantitatively estimated by a modified rapid mixing, continuous flow technique in a designed aero-sealed small microreactor. Gas flow rate and gaseous pressure were maintained at 7.5 ml/sec and 36 p.s.i respectively. Human erythrocyte suspension was first deoxygenated by sparging with oxygen free argon for 15 minutes. Then deoxygenated RBC suspension was sparged immediately with oxygen ($\sim 80\%$ v/v) at 25°C temperature and samples were analyzed at regular intervals of time. The collected oxygenated samples after 10, 20, 30, 40 minutes intervals were analyzed by spectrophotometer for finding the changes in absorbance at 560 nm . Changes of absorbance were due to the formation of oxy-hemoglobin [17].

Phagocytic uptake assay on KMnO_4 and EDTA treated human erythrocyte

Phagocytic uptake of untreated and treated hRBC by human peripheral blood mononuclear cells (PBMC) was examined. PBMC were prepared by the method of Pommier et al. [18]. After that, the washed packed cell volume of hRBC and PBMC ($>95\%$ viable as assessed by Trypan blue) [19] were mixed. PBMC concentration was held constant at 2×10^6 per ml. The cell mixture was centrifuged ($120 \times g$ for 2 minutes) to pellet the cell mixture and to initiate cell:cell contact and phagocytosis. Following 30 minutes incubation at 37°C , volume of deionized (4°C) water added to lyse non-phagocytosed hRBC. After 30 seconds 1 volume of $2\times$ PBS was added to restore isotonicity. The total number of monocytes and the number of monocytes that had phagocytosed hRBC were counted microscopically. The phagocytic uptake was expressed as ingested hRBC per 100 monocytes after treatment with various concentrations of permanganate in presence of EDTA.

Results and discussion

Effect of KMnO_4 and EDTA composite action on AI

The AI of treated hRBC was determined by anti-A and anti-B antisera at pH 7.4 and 37°C for 60 minutes incubation time with reagents (Fig. 1). Experimentally it was found that the AI values were 10.765 ± 0.07 and 11.39 ± 0.39 (mean $\pm$ SD) for A and B type chemically modified hRBC. The values were far below the threshold value of AI (17) at

0.57 mM KMnO_4 in presence of 0.9 mM EDTA in reaction mixture with 5.5% (w/v) RBC, respectively.

Effect of KMnO_4 and EDTA composite action on zetapotential

The maximum zetapotential were found to be -16.8 ± 0.07 and $-16.0 \pm 0.14\text{ mV}$ (mean $\pm$ SD) for A and B type hRBC at optimized concentrations of reagents mentioned above (Fig. 2). The enhanced negative zetapotentials of chemically attenuated hRBC may prevent the phagocytic recognitions.

Effect of KMnO_4 and EDTA composite action on percentage of spontaneous RBC lysis

It was found that KMnO_4 -EDTA modified hRBC did lead to a minor increase in percentage of spontaneous hemolysis. However the amounts were $<8.39 \pm 0.47$ and 6.38 ± 0.47 (mean $\pm$ SD) for A and B type hRBC, respectively. We also estimated the percentage of spontaneous RBC lysis profile (Fig. 3) at 37°C temperature, pH 7.4 for 60 minutes incubation time of reaction.

Fig. 1 Agglutination index profile on KMnO_4 concentration in presence of EDTA (blue line represents treated B blood type and red line indicates treated A blood type human erythrocytes)

Fig. 2 Relationship between zetapotential and KMnO_4 concentration in presence of EDTA (blue line corresponds to treated B blood type and red line represents treated A blood type human erythrocytes)

Morphological analysis for KMnO_4 and EDTA combined action on hRBC

Scanning electron microscopy (SEM) showed that chemically modified human erythrocytes were morphologically normal after KMnO_4 -EDTA composite action at optimized concentration (0.57 mM KMnO_4 and 0.9 mM EDTA) in reaction mixture (Fig. 4). The images from SEM were taken after chemical treatment of KMnO_4 and EDTA in phosphate buffer (pH 7.4) in above mentioned optimized conditions.

Semi-quantitative oxygen uptake assay after KMnO_4 and EDTA combined treatment

The variation of oxygen uptake with time for native and modified hRBC was semi-quantitatively evaluated (Fig. 5).

Fig. 3 Spontaneous RBC lysis percentage versus KMnO_4 concentration with EDTA (blue line expresses treated B blood type and red line conveys treated A blood type human erythrocytes)

It was observed that absorbance at 560 nm decreased gradually decreased in 10, 20, 30, and 40 minutes time intervals due to oxygen binding with hemoglobin within native and treated hRBC. Trends of typical oxygen uptake curves clearly revealed that the ability of oxygen uptake of chemically treated hRBC was almost equivalent to native human erythrocyte.

Fig. 4 Effect of KMnO_4 -EDTA concerted action on human erythrocyte morphology. (A) treated A blood type at 1500 $\times$, (B) treated B blood type 1500 $\times$, (C) treated A blood type 4000 $\times$, (D) treated B blood type 4000 $\times$ magnifications

Fig. 5 Typical semi-quantitative time course of oxygen uptake by KMnO_4 -EDTA treated human erythrocyte (A_{560} is represented as absorbance changes at 560 nm wavelength since absorbance changes due to oxygen binding). (A) Treated A blood type, (B) treated B blood type, (C) untreated A blood type, (D) untreated B blood type of human erythrocytes. Data points presented here without considering light scattering artifact.

FTIR studies on KMnO_4 and EDTA treated human erythrocytes

FTIR spectra of washed hRBC membrane (both treated and untreated) was measured at wave numbers ranging from $4500\text{--}500\text{ cm}^{-1}$. The FTIR spectra of RBC membrane showed bands at 3375 and 3442 cm^{-1} , which were attributed to $-\text{OH}$ group for both untreated A (Fig. 6C) and B (Fig. 6F) type hRBC, respectively. Chemically treated A (Fig. 6B) and B (Fig. 6E) type hRBC showed absorption band at 1744 and 1717 cm^{-1} indicating the presence of $-\text{COOH}$ groups. Whereas, chemical treatment with reagents above optimum value of permanganate (0.57 mM) with optimum value of EDTA (0.9 mM) some additional bands representing $-\text{C}=\text{O}$ groups, were appeared at 1637 and 1650 cm^{-1} for A (Fig. 6A) and B (Fig. 6D) type hRBC, respectively. It is expected that initially permanganate will oxidize preferentially signature oligosaccharides of the membrane-surface producing carboxylic acid. After completion of this reaction it starts to form $-\text{C}=\text{O}$ by oxidizing the protein of membrane-surface above the optimum value of permanganate in presence of EDTA.

Phagocytic uptake assay on KMnO_4 and EDTA treated human erythrocyte

Phagocytic uptake assay clearly showed that, permanganate-EDTA treated human A and B type erythrocytes were effectively resistant to phagocytosis. Experimental data showed that the phagocytic ingestion of treated hRBCs per human peripheral blood monocytes were observed $6 (\pm 1.41)$ and $7 (\pm 0.7)$ for treated A and B type hRBCs (Fig. 7A and 7B), respectively at 0.57 mM concentration of permanganate in presence of 0.9 mM EDTA. On the other hand, the phagocytic ingestion of untreated hRBCs per human peripheral blood monocytes were observed $14 (\pm 0.71)$ and $13 (\pm 0.70)$ for untreated A and B type hRBCs, respectively. Therefore, the permanganate-EDTA concerted action was effectively reducing the chances of the phagocytic uptake of treated A and B type human erythrocytes.

Conclusions

In conclusion, antigenically attenuated human erythrocyte cells with normal biophysical consequences (morphology, zetapotential, surface functional groups, AI and spontaneous hRBC lysis percentage and phagocytic uptake assay) *in vitro* have been produced by oxidative modification of antigenic determinants on human erythrocyte cell surface using KMnO_4 -EDTA mixture. The concerted action on hRBC modification removed effectively the antigenicity and agglutinability of hRBC to act as a universal erythrocyte. Zetapotential, FTIR and phagocytic uptake profiles have effectively shown the correlation

Fig. 6 FTIR spectra versus KMnO_4 concentrations with EDTA of washed human erythrocyte membrane. (A) 0.9 mM treated, (B) 0.57 mM treated and (C) untreated A type blood group, respectively. (D) 0.9 mM treated, (E) 0.57 mM treated and (F) untreated B type blood group, respectively

in between the enhanced negative surface charge due to the formation of carboxylic acid on the membrane surface and switching on-off mechanism of phagocytosis. Above the optimum concentrations of KMnO_4 and EDTA in the reaction mixture (0.9 mM KMnO_4 and 0.9 mM EDTA with 5.5% v/v RBC) then formation of $-\text{C}=\text{O}$ group was facilitated due to oxidation of surface proteins. Hence, this optimized chemical treatment by KMnO_4 -EDTA may produce universal erythrocytes from A and B blood groups in near future.

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Fig. 7 Phagocytic uptake of permanganate-EDTA treated human erythrocytes by human peripheral blood monocyte. Permanganate-EDTA treated human erythrocytes were incubated with human peripheral blood monocytic cells for 30 minutes as described. Data were shown without considering the spontaneous ingestion of autologous hRBC. 7A representing for treated A type blood and 7B for treated B type blood group, respectively

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