International Journal of Urology and Nephrology ISSN 2091-1254 Vol. 6 (2), pp. 001-008, February, 2018. Available online at www.internationalscholarsjournals.org © International Scholars Journals

Full Length Research paper

Effective inhibition of agglutinability of human A and B type erythrocytes by sodium periodate - chloroacetic acid combined treatment

Dipankar Ghosh[1]*, Susmita Santra[1], Sujit Dutta[2] and Panchanan Pramanik[1]

[1]Department of Chemistry, Indian Institute of Technology, Kharagpur 721302, India.

[2]Hematology and Blood Bank Division, Kharagpur Sub-divisional Hospital 721302, India.

Accepted 14 September 2017

The study focused on effective inhibition of the agglutinability of human A and B type erythrocytes by sodium periodate-chloroacetic acid concerted action. Suppression of agglutinability was determined by using anti-A and anti-B antisera. Effective concentrations of sodium periodate were 6.8 and 2.7 mM, respectively for A and B type erythrocytes in presence of 4.5 mg.ml^{-1} chloroacetic acid in the reaction mixture. Some major bio-physiological consequences of human erythrocytes including agglutination index, spontaneous red blood cell lysis percentage, zetapotential, morphology, surface functional groups (- OH, > C = O, -COOH) and semi-quantitative oxygen uptake and phagocytic uptake were also analyzed after the chemical treatments. The future work is directed towards the universal human erythrocytes preparation from A and B type human erythrocytes.

Key words: ABH antibodies, agglutination, concerted action, erythrocyte, medicine, transfusion.

INTRODUCTION

Blood transfusions are crucial components of modern medical care in patients with thalassemia, sickle cell disease (Vichinsky et al., 1990; Sirchia et al., 1985). But the most important technical problems are allosen-sitization. When universal donor is not available then conversion of A or B type to universal type is only alternative to overcome the life threatening situation. Generally, blood typing (A and B) occurs as results of polymorphism of complex carbohydrates structure of glycoproteins and glycolipids expressed on the surface of erythrocyte membrane. The structure of most of the A and B determinants has been established, but the complete structure of the glycoproteins and glycolipids remains to be ascertained (Szulman, 1966). Since 1947, several eminent research groups were involved to identify the structural chemistry of surface antigenic receptors of human erythrocyte by several chemical and enzymatic treatments (Springer, 1963). Amongst these experimen-tations, periodic acid was most effective and used in various biochemical purposes (Gahmberg and Hakomori, 1973; Roberts, 1977). Morgan et al. 1951 (Aminoff et al., 1951) carried out experimentation on oxidation of chemically synthesized human blood group 'A' substances with periodate ions. But there was no strong evidence that periodate oxidation alone can completely oxidize the hydroxyl groups of surface antigenic determinants to carboxylic acids rather aldehyde functional groups attenuate the human surface antigenic determinants and increase negative surface charge to reduce the chances of phagocytosis.

Conceptually, these attempts were only limited towards structural aspects of erythrocyte antigenic determinants. But later on blood chemistry has been shifted towards a new avenue for universal immuno-compromised human erythrocyte preparation. Since 1981, several innovative research works were initiated to mask A and B antigenicity. These differential techniques included perflubron emulsion, polyethylene glycol, methoxy-polyethylene glycol; bissulfosuccinimidyl- substrate and diamide. Still these various methodologies were not quite efficient to solve these problems associated to complete loss of A and B antigenicity (Garratty, 2002).

Presently, rapid development of modern sciences has revealed that reversible disulfide formation of erythrocyte

*Corresponding author. E-mail: dipankarghosh.iitkgp@gmail.com. Tel: + 91-3222-83322, 9732590703. Fax: + 91-3222-255303.

membrane proteins was the major reason for macrophage recognition of periodate- treated erythrocyte compared to lipid or sialyl residues on the surface (Beppu et al., 1989). Even protein carbonyl formation of surface proteins may be an additional problem of macrophage recognition and clearance (Sangeetha et al., 2005). To this end, inhibition of disulfide and protein carbonyl generation may be an outstanding remedy of existing situation regarding periodate oxidations. It has been shown that chloroacetic acid and chloroacetyl amino acids have the ability to alkylate the active SH -group of several proteinase enzymes and prevent the disulfide formations (Gerwin, 1967). Moreover, SH- group alkylation activity of chloroacetic acid also remains active in a vast pH range (Oka et al., 1986).

We, therefore, explore the hypothesis that sodium periodate and chloro acetic acid combined treatment may be a better solution. It not only attenuates the human erythrocyte surface antigenic receptors but also increases the negative surface charge to inhibit the pha-gocytic recognition. Experimentally, to test that combined chem.ical approach, human erythrocyte was treated with sodium periodate and chloroacetate in sodium phosphate buffer (pH7.4) and several biological consequences like zetapotential, surface morphology, agglutination index, spontaneous human erythrocyte lysis percentage, oxygen uptake across the human erythrocyte membrane were investigated.

Zetapotential is the degree of negative charge on the surface of an erythrocyte cell; the potential difference between the negative charges on the erythrocyte cell and the cation in the fluid portion of the blood. Agglutination index is the statistical indicator of the clumping of red blood cells in the presence of an antibody. Here agglutination index has been measured as the percentage ratio in between the slope of optical density of the antibody reacted with erythrocyte and slope of optical density of the unreacted erythrocyte sample in 600 to 1000 nm range. Spontaneous erythrocyte lysis was quantitated by measuring the amount of extracellular hemoglobin against the total hemoglobin concentration.

In this way, the major aim of this study is directed to effectively inhibit the agglutinability of human A and B type erythrocytes by sodium periodate-chloroacetic acid combined treatment and studies on major biophysical consequence of treated A and B type erythrocytes.

MATERIALS AND METHODS

Materials

Sodium periodate ($NaIO_4$), chloroacetic acid ($ClCH_2COOH$), ammonium oxalate, potassium oxalate, acetone, sodium hydroxide, hydrochloric acid, sodium phosphate buffer (pH 7.4) purchased from Sigma-Aldrich, Drabkin's reagent (Span Diagnostics), ABH monoclonal antibody (Span diagnostics). Centrifuge (Eppendorf), UV-Vis Spectrophotometer (Shimadzu), Scanning Electron Micro-Scope (Olympus), FTIR spectrophotometer (Perkins Elmer) and Zeta potentiometer (Zeta plus).

Human erythrocyte collection and chemical oxidation

Erythrocyte rich fraction of healthy human blood (ABH type) containing double oxalate as anticoagulant was obtained from local hospital, stored at 4°C. Erythrocyte enriched fraction was centrifuged twice (3000 g, 15 min) at 4°C to remove the residual plasma and buffy coat. Human erythrocytes were washed with sodium phosphate buffer (pH 7.4). After that, the washed erythrocytes were resuspended to make packed cell volume of ~12% (v/v). Then the erythrocyte suspension, sodium per iodate ($NaIO_4$) and chloro-acetic acid ($Cl\text{-}CH_2COOH$) were mixed and incubated at 37°C for 60 min with gentle shaking. In first set of reactions, reaction mixture contained 5.5% (v/v) of human erythrocyte, 4.5 $mg.ml^{-1}$ of chloroacetic acid and various final concentrations of sodium periodate (5.5, 5.9, 6.4, 6.8, 7.3 mM) for A and (1.4, 1.8, 2.3, 2.7, 3.2 mM) for B blood type, respectively. In second set, the reaction mixture contained 5.5% (v/v) of RBC, 6.8 and 2.7 mM of sodium periodate (for A and B type, respectively) and various final concentrations of chloroacetic acid (0.9, 1.8, 2.3, 2.7, 3.2 $mg.ml^{-1}$). Afterwards the oxidized erythrocyte cells were concomitantly centrifuged (3000 g, 15 min) 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 or sometimes more than three times and results reported as means ± standard deviation.

Analysis on agglutination index on $NaIO_4$ and $Cl\text{-}CH_2COOH$ treated human erythrocyte

Agglutination index of native and modified human erythrocyte cells were determined by using anti-A and anti-B antisera (Span Diagnosis supply). A novel erythrocyte micro agglutination method (Ramasubramanian et al., 2008) was applied to determine the agglutination index. Agglutination index (AI) was formulated from the slope of the absorption spectra in the range 600 - 1000 nm. The threshold value of agglutination index (AI) was fixed at 17 (Narayanan et al., 2002) indicating no agglutination below this value. Human erythrocyte test samples were prepared by adding 100 µl of diluted human erythrocyte (1:8) to 100 µl of prediluted antibodies in sodium phosphate buffer (pH 7.4) either anti-A (1:4), anti-B (1:2) and anti-H (undiluted). Again, 100 µl of reaction mixture was extracted and added to a cleaned quartz cuvette containing 2 ml of sodium phosphate buffer (pH 7.4) and mixed by gentle inversion.

Studies on morphology, spontaneous erythrocyte lysis and zetapotential on $NaIO_4$ and $Cl\text{-}CH_2COOH$ treated human erythrocyte

Structural integrity of chemically treated human erythrocytes was tested in terms of morphology, surface functional groups and membrane surface charge. The morphology of both native and modified red blood cells was observed under 4000× magnification (Herbert and Marcel, 1970). Spontaneous human erythrocytes lysis percentage was measured by determining total hemoglobin concentration spectrophotometrically (540 nm) for both total human erythrocytes suspension and cell supernatant using Drabkin's reagent (Rowan, 1996). Zeta plus equipment was used to measure zetapotentials of native and chemically modified human erythrocytes at 25°C temperature. The accepted value of mean of zetapotential of normal erythrocytes (from ten individuals) was 13.07 ± 0.61 mV (mean ± S.D.) (Omi et al., 1994).

Isolation of erythrocyte membrane for FTIR studies

The washed human erythrocyte suspension (20 ml) was suspended in an equivalent volume of glucose (250 mM) solution (Tomoda et al., 1984) . Each sample was allowed to stand at 4°C for 5 min 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 3000 g for 2 min. After that, the washed human erythrocytes membrane was dried at 25°C under vacuum and used for FTIR studies on erythrocyte membrane surface of native and modified human erythrocytes. The FTIR spectra of membrane of washed human erythrocyte were analyzed. Each FTIR spectrum represents 16 scans with 4 cm^{-1} spectral resolution.

Oxygen uptake studies on $NaIO_4$ and $Cl\text{-}CH_2COOH$ 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. Multi-stage gas regulator has also been assembled to control the gas flow rate and gaseous pressure inside the micro reactor. Through out the experiment gas flow rate and gaseous pressure were maintained at 7.5 ml. s^{-1} and 36 p.s.i, respectively. Human erythrocyte suspension was first deoxygenated by sparging with oxygen free argon for 15 min. Then deoxygenated red blood cell suspension was immediately sparged with oxygen (~80% v/v) at 25°C temperature and collected oxygenated sample after 10, 20, 30, 40 min regular intervals for spectrophotometric analysis for absorbance change (at 560 nm) in a controlled environment. Absorbance changes have been taken place due to oxy-hemoglobin formation (Coin et al., 1979).

Phagocytic uptake assay on $NaIO_4$ and $Cl\text{-}CH_2COOH$ treated human erythrocyte

Phagocytic uptake of untreated and treated human erythrocyte by human peripheral blood mononuclear cells (PBMC) was examined. PBMC were prepared by the method of Pommier et al. (Pommier et al., 1984). After that, the washed packed cell volume of human erythrocyte and PBMC (> 95% viable as assessed by trypan blue) (Helinski et al., 1988) were mixed. PBMC concentration was held constant at 2×10^6 per ml. The cell mixture was centrifuged (120 × g for 2 min) to pellet the cell mixture and to initiate cell: cell contact and phagocytosis. Following 30 min incubation at 37°C, volume of deionized (4°C) water was added to lyse non-phagocytosed human erythrocyte. After 30 s 1 volume of 2× PBS was added to restore isotonicity. The total number of monocytes and the number of monocytes that had phagocytosed human erythrocyte were counted microscopically. The Phagocytic uptake was expressed as ingested human erythrocytes per 100 monocytes after treatment with various concentrations of sodium periodate in presence of chloro-acetic acid.

RESULTS

Effect of sodium periodate-chloroacetic acid composite action on agglutination index and spontaneous erythrocytes lysis percentage of A, B type erythrocytes

Concerted chemical action prevented agglutination of A and B type erythrocytes (Figures 1a and b). Indeed, chemical modification was found to consistently result in more efficient antigen masking at pH 7.4 and 37°C for 60 min. Experimentally, it has been found that the AI value was 17.87 ± 0.016 (mean ± S.D.) for A type chemically modified human erythrocyte at 6.8 mM sodium periodate and 4.5 $mg.ml^{-1}$ chloroacetic acid concentrations in reaction mixture. Similarly, AI value for B type modified human erythrocyte was measured 11.13 ± 0.007 (mean ± S.D.) at 2.7 mM sodium periodate and 4.5 $mg.ml^{-1}$ concentrations in reaction mixture. The values were far below the threshold value of agglutination index 17 which means there is no agglutination.

It was found that sodium periodate-chloro acetic acid modified human erythrocyte did lead to a minor increase in percentage of spontaneous hemolysis. However, the spontaneous hemolysis percent was less than 19.11 ± 0.04 and 5.29 ± 0.54 (mean ± S.D.) for A and B type human erythrocyte, respectively at 6.8 and 2.7 mM of sodium periodate concentrations, respectively in presence of 4.5 $mg.ml^{-1}$ chloroacetic acid in reaction mixture. On the other hand, we also estimated the percentage of spontaneous human erythrocyte lysis profile (Figures 1c and d) at previously mentioned optimized condition. Moreover, it had also been shown that the chemical modification by sodium periodate and chloroacetic acid combined action was more effectively working out for modified B blood group compared to the modified A blood group human erythrocyte in terms of spontaneous human erythrocyte lysis percentage.

Morphological analysis after sodium periodate-chloroacetic acid combined action

The scanning electron microscopy showed that chem.-ically modified human erythrocytes were morphologically normal after sodium periodate-chloro acetic acid composite action at optimized concentration (6.8 and 2.7 mM sodium periodate treatment of A and B type blood, respectively in presence of 4.5 $mg.ml^{-1}$ chloroacetic acid in reaction mixture) (Figure 2) under 4000X magnification in previously mentioned optimized condition.

Effect of sodium periodate-chloroacetic acid action on FTIR and zetapotential

In the present investigation, the FTIR spectroscopy was used to investigate the nature of surface functional groups -OH, -CHO, > C=O and -COOH) on erythrocyte membrane after concerted action of the sodium periodate (6.8 and 2.7 mM for A, B type human erythrocytes) and chloroacetic acid (4.5 $mg.ml^{-1}$). FTIR spectra of washed human erythrocyte membrane (both treated and untreated) were measured at wave numbers ranging from 4500 - 500 cm^{-1}. The FTIR spectra of RBC membrane (Figures 3c and f) showed bands at 3335; 3442 cm^{-1}, which was attributed to -OH group for both A and B type

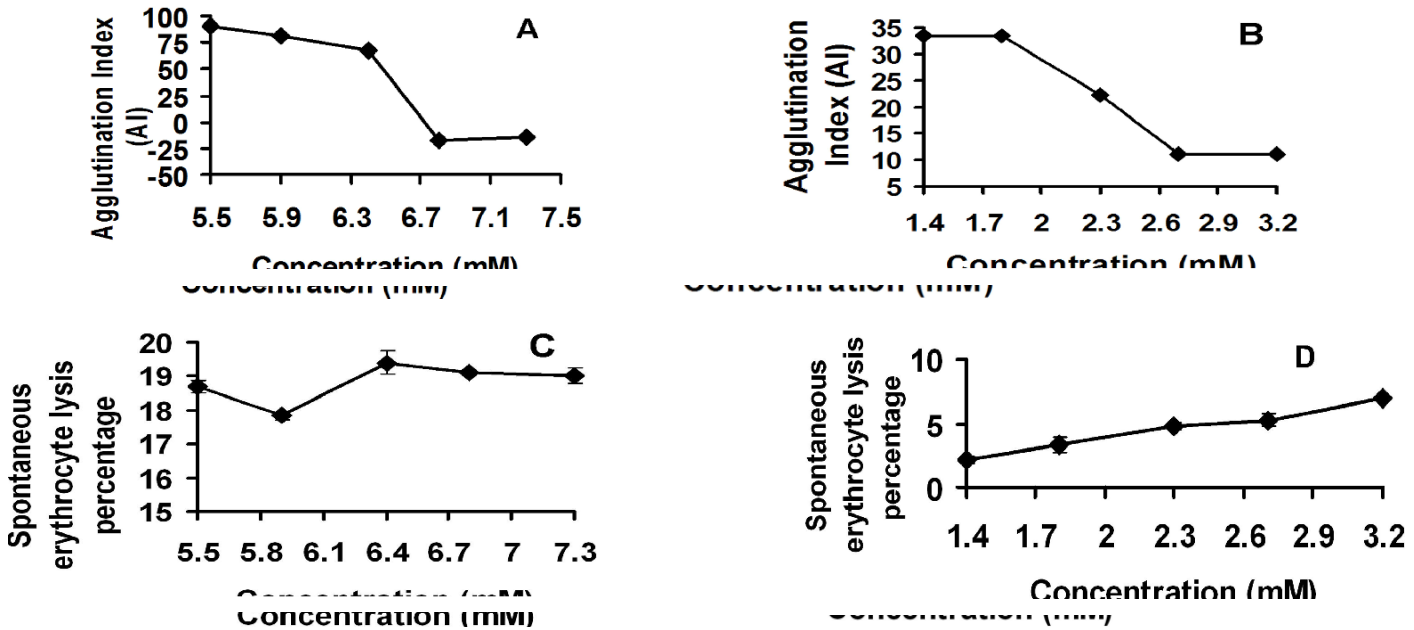

Figure 1. Agglutination index and spontaneous erythrocyte lysis percentage profile on $NaIO_4$-$ClCH_2COOH$ concerted approach. Each data point represents the mean ± S.D. of minimum duplicate. Concentration (mM) in X axis corresponds to $NaIO_4$ concentration. (A) Represents treated A blood type; (B) conveys treated B blood type; (C) corresponds to treated A blood type; (D) represents treated B blood type.

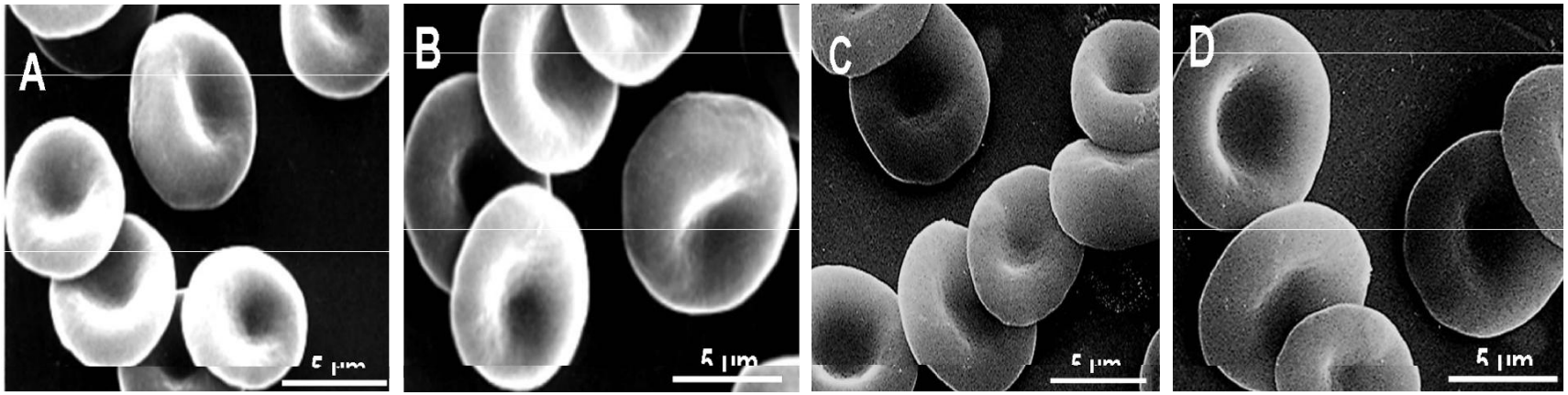

Figure 2. $NaIO_4$-Cl CH_2COOH treated human erythrocytes are morphologically normal. (A) Untreated A blood type at 4000×, (B) Treated A blood type at 4000×, (C) Untreated B blood type at 4000×, (D) Treated B blood type at 4000×.

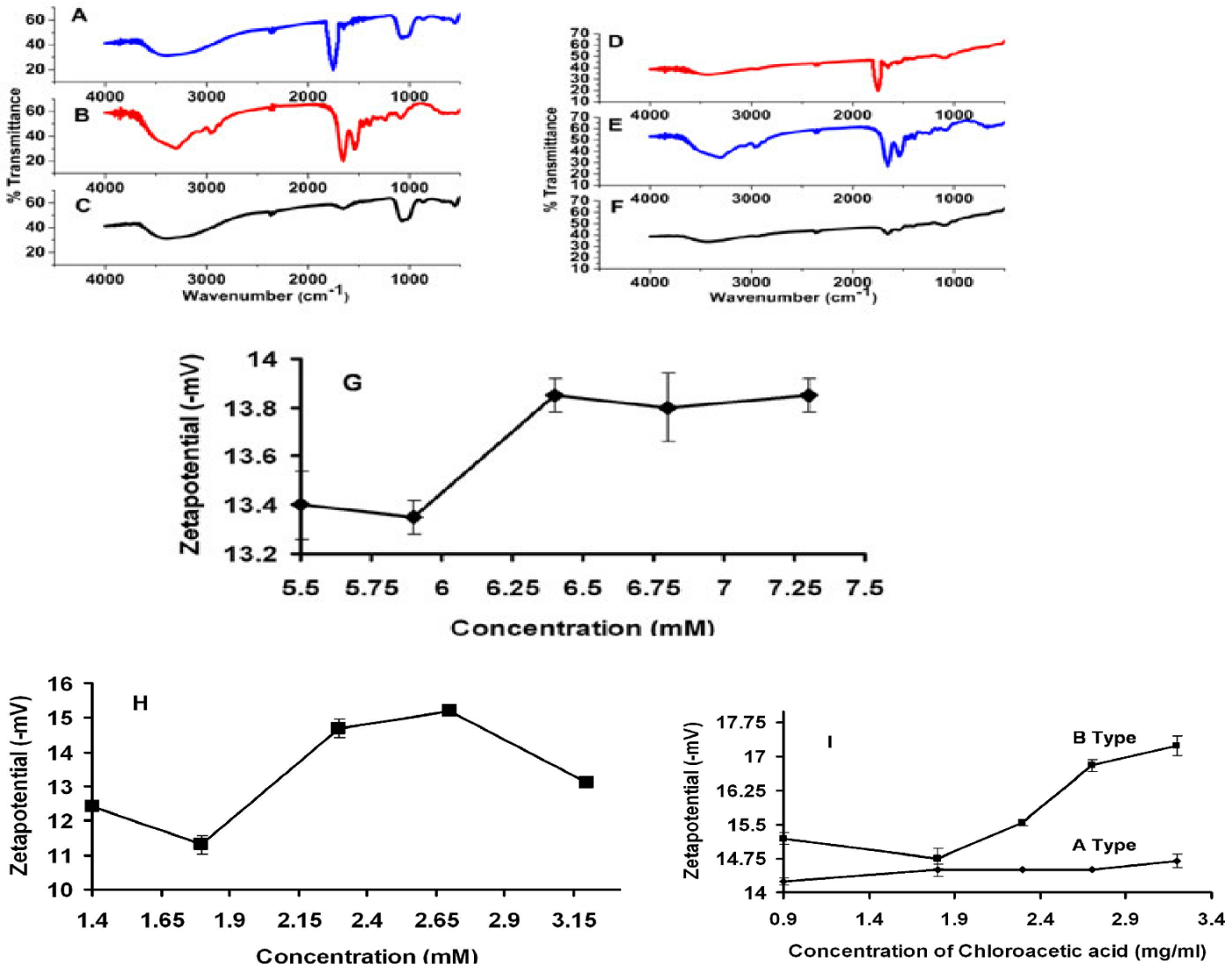

Figure 3. FTIR spectra and zetapotential profiles on $NaIO_4$-$ClCH_2COOH$ treated human erythrocytes. Each data point represents the mean ± S.D. of minimum duplicate. (A) represents A type blood treated with $NaIO_4$ (6.8 mM)-$ClCH_2COOH$ (4.5 mg/ml), (B) corresponds to A type blood treated with $NaIO_4$ (6.8 mM), (C) conveys untreated A blood type, (D) represents B type blood treated with $NaIO_4$ (2.7 mM)-$ClCH_2COOH$ (4.5 mg/ml), (E) corresponds to B type blood treated with $NaIO_4$ (2.7 mM), (F) conveys untreated B blood type. Concentration (mM) along X axis (G, H for A and B blood type, respectively) corresponds to $NaIO_4$ concentration and concentration (mg/ml) along X axis (I) corresponds to chloroacetic acid concentration.

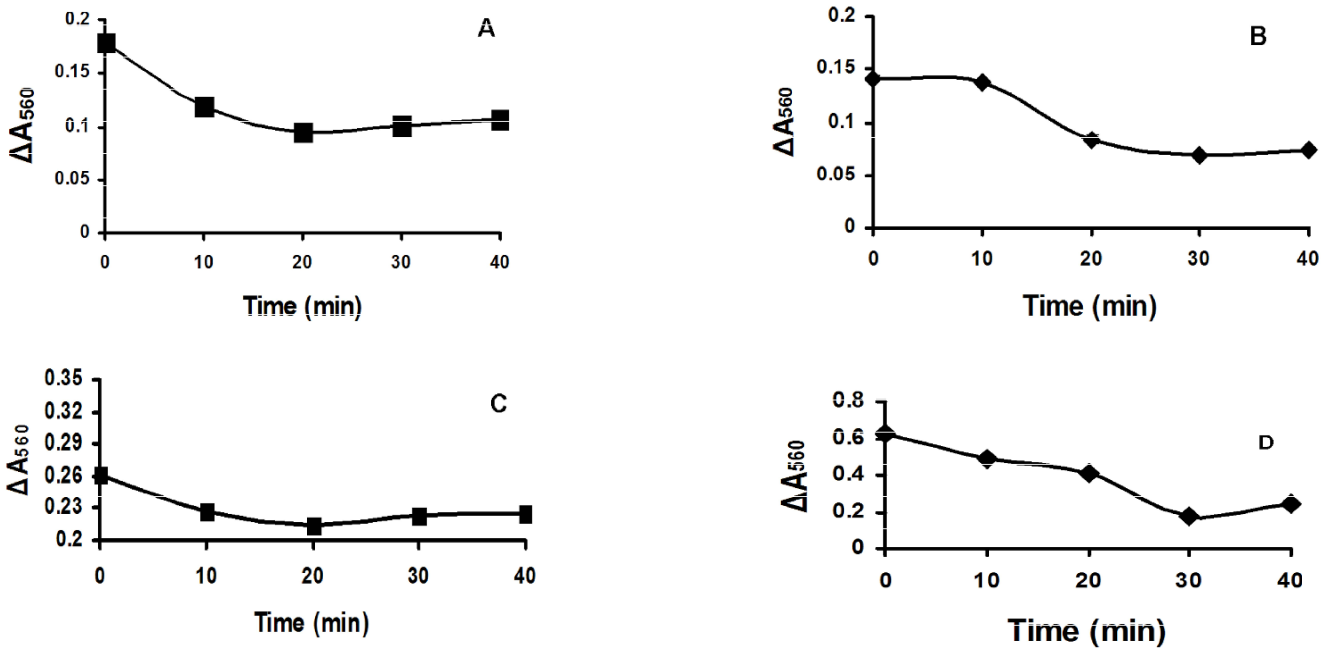

Figure 4. Typical time course for semi -quantitative oxygen uptake detection on $NaIO_4$-$ClCH_2COOH$ treated human erythrocyte. (A), (B), (C) and (D) represent untreated A, treated A, untreated B and treated B blood types, respectively. A_{560} implies the absorbance changes at 560 nm wavelength. Date points presented here with out excluding light scattering (at 570 nm) artifacts. RBC lysis due to oxygen sparging pressure and flow rate applied in micro reactor oxygenation chamber was less than ~10 % for treated hRBCs

untreated hRBC respectively. Whereas, (Figures 3b and e) It also showed bands at 1650, 1664 cm^{-1}, which were projected > C = O or carbonyl groups for only sodium periodate treated both A and B type human erythrocyte. Bands of sodium periodate-chloro acetic acid treated human erythrocyte membrane were obtained at 1744, 1757 cm^{-1}, which (Figures 3a and d) indicated the presence of the -COOH or carboxyl group human erythrocyte membrane surface of both A and B type, respectively. It is expected that sodium periodate-chloro acetic acid composite action completely oxidizes the signature oligosaccharides of the human erythrocytes membrane surface toward carboxylic acid -COOH groups.

To determine the surface charge we measured the zetapotential for both A and B type human erythrocytes. Results showed that the zetapotential or surface charge changes in a dose dependent manner. The maximum zetapotential was -13.8 mV ± 0.14 and -15.2 mV ± 0.14 (mean, ± S.D.) for A (Figure 3g) and B (Figure 3h) type human erythrocytes at 6.8 mM and 2.7 mM of sodium periodate concentrations alone, respectively. Whereas, -14.7 mV ± 0.145 and -17.3 mV ± 0.21 (mean, ± S.D.) zetapotentials were obtained for A and B type human erythrocytes at 6.8 and 2.7 mM of sodium periodate concentrations, respectively in presence of 4.5 $mg.ml^{-1}$ chloroacetic acid. Even chloroacetic acid concentration profile (0.9, 1.8, 2.7, 3.6, 4.5 $mg.ml^{-1}$) on sodium periodate treated (6.8 and 2.7 mM sodium periodate treated A and B blood type, respectively) human erythrocytes had also been estimated (Figure 3i). Thus, it has been cleared that chloroacetic acid and sodium periodate composite chemical action may have a profound effect on increasing negative zetapotentials of chemically modified human erythrocytes. Moreover, increased negative zeta-potentials of chemically attenuated human erythrocytes may prevent the phagocytic recognitions.

Semi-quantitative oxygen uptake assay with chemically treated erythrocytes

Another important physical property of human erythrocyte is the oxygen uptake ability. Oxygen uptake was unaffected by chemically modified human erythrocyte across the membrane of intact human erythrocyte. The time course of oxygen uptake to human erythrocyte was semi-quantitatively evaluated (Figure 4). For this typical time course experiments it has been observed that absorbance changes at 560 nm has been gradually decreased in 10, 20, 30 and 40 min time intervals due to oxygen binding with hemoglobin within human erythrocyte. Even trends of typical oxygen uptake curves clearly revealed that the oxygen uptake ability of chemically treated human erythrocyte is almost equivalent compared to native human erythrocyte.

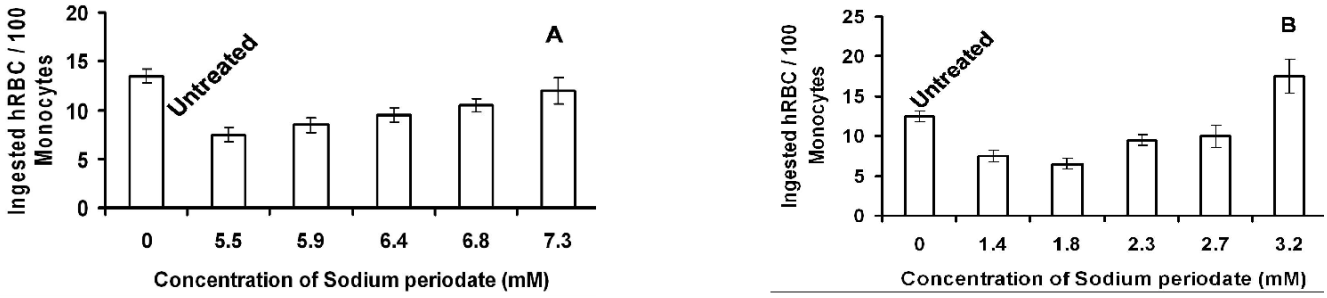

Figure 5. Phagocytic uptake of $NaIO_4$- $ClCH_2COOH$ treated human erythrocytes by human peripheral blood monocyte. $NaIO_4$-$ClCH_2COOH$ treated human erythrocytes were incubated with human peripheral blood monocytic cells for 30 min as described. Data were shown without considering the spontaneous ingestion of autologous hRBC. 7A representing for treated A type blood and 7B for treated B type blood group, respectively.

Phagocytic uptake assay on sodium periodate-chloroacetic acid treated human erythrocyte

Phagocytic uptake assay clearly showed that sodium periodate-chloroacetic acid treated human A and B type erythrocytes were effectively resistant to phagocytosis. Experimental data showed that the phagocytic ingestion of treated human erythrocyte per human peripheral blood monocytes were observed 10 (± 0.70) and 9 (± 1.41) for treated A and B type human erythrocytes (Figures 5a and b) at 6.8 and 2.7 mM concentration of sodium periodate in presence of 4.5 $mg.ml^{-1}$ chloroacetic acid, respectively. On the other hand, the phagocytic ingestion of untreated human erythrocytes per human peripheral blood monocytes were observed 14 (± 0.71) and 13 (± 0.7) for untreated A and B type human erythrocytes, respectively. Therefore, the sodium periodate and chloroacetic acid concerted action was effectively reducing the chances of the phagocytic uptake of treated A and B type human erythrocytes.

DISCUSSION

In brief, sodium periodate- chloroacetic acid composite action in sodium phosphate buffer (pH 7.4) was found to prevent the A and B type blood group recognition and agglutination by anti-A and anti-B anti sera in an optimized condition. The effective concentration of sodium periodate were estimated at 6.8 and 2.7 mM, respectively for A and B type hRBC with 4.5 $mg.ml^{-1}$ chloroacetic acid in the reaction mixture. It is also interesting that the chemical activity does not have any major effect on spontaneous erythrocyte lysis percentage, zetapotential, agglutination index and oxygen uptake ability and cellular morphology.

Considering the existing molecular mechanism of sodium periodate alone on erythrocyte membrane, oxidative stress has mildly occurred, but it is a time, pH and dose dependent fact. Oxidative stress due to periodate mildly increases the membrane permeability for hydrophilic molecule and ions, but reversibly. The leak can easily be solved by N-ethylmaeimide along with periodate treatments (Heller et al., 1984). This is only due to the SH group oxidation of surface protein moiety. Even sodium periodate was unable to stimulate the proteolysis, but it has a tremendous specificity for carbohydrates (Di et al., 1988). To this end, we used the chloroacetic acid which has the ability to inhibit the di- sulfide bond formation which conceptually may reduce the phagocytic recognition and membrane pore leakage by increasing the carboxylic group availability on the erythrocyte membrane surface. In summary, our immediate goal in developing chemically modified RBC is not to necessarily create a universal RBC, but rather to produce antigenically attenuated cells with normal biophysical consequences (morphology, zetapotential, surface functional groups, agglutination index and spontaneous RBC lysis percentage) *in vitro*. Moreover, it has been shown that sodium periodate-chloroacetic acid concerted action on RBC modification that effectively conceals hRBC antigenicity. Thus, use of this “chemically modified human erythrocyte” in the chronically transfused or in patients with rare blood types may be effective at both delivering immediate oxygen as well as attenuating the risk of allosensitization.

ACKNOWLEDGEMENTS

The authors thank Department of Biotechnology (DBT), Government of India for financial support in part and Central Research Facility (CRF), Indian Institute of Technology, Kharagpur for providing the advance instrumental facilities.

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