Membrane interactions of hemoglobin variants, HbA, HbE, HbF and globin subunits of HbA: Effects of aminophospholipids and cholesterol

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Abstract

The interaction of hemoglobin with phospholipid bilayer vesicles (liposomes) has been analyzed in several studies to better understand membrane–protein interactions. However, not much is known on hemoglobin interactions with the aminophospholipids, predominantly localized in the inner leaflet of erythrocytes, e.g., phosphatidylserine (PS), phosphatidylethanolamine (PE) in membranes containing phosphatidylcholine (PC). Effects of cholesterol, largely abundant in erythrocytes, have also not been studied in great details in earlier studies. This work therefore describes the study of the interactions of different hemoglobin variants HbA, HbE and HbF and the globin subunits of HbA with the two aminophospholipids in the presence and absence of cholesterol. Absorption measurements indicate preferential oxidative interaction of HbE and alpha-globin subunit with unilamellar vesicles containing PE and PS compared to normal HbA. Cholesterol was found to stabilize such oxidative interactions in membranes containing both the aminophospholipids. HbE and alpha-globin subunits were also found to induce greater leakage of membrane entrapped carboxyfluorescein (CF) using fluorescence measurements. HbE was found to induce fusion of membrane vesicles containing cholesterol and PE when observed under electron microscope. Taken together, these findings might be helpful in understanding the oxidative stress-related mechanism(s) involved in the premature destruction of erythrocytes in peripheral blood, implicated in the hemoglobin disorder, HbE/beta-thalassemia.
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1. Introduction

Hemoglobin interaction of phospholipids of the erythrocyte inner leaflet has been investigated earlier to understand the relationship between hemoglobin and the inner surface of the red blood cell membrane [1–6]. The membrane effects induced by hemoglobin interaction include the increase in osmotic fragility [7], increase in membrane permeability, inactivation of membrane-bound enzymes and cross-linking of membrane constituents [8]. Shaklai and coworkers have demonstrated that the hemoglobin-binding sites on the red blood cell membrane at pH 6 exhibit two different affinities with binding constants differing from each other by two orders of magnitude indicating both protein(s) and membrane lipids to be involved [9]. The majority of binding was identified with an equilibrium dissociation constant, K_d of about 160 nM, while a small portion with a K_d of about 12 nM. Recent studies have shown that phospholipid vesicles promote human hemoglobin oxidation and such oxidative reactions have been studied also with normal and abnormal hemoglobins in the presence of phosphatidylserine (PS) vesicles [10–14].

Hemoglobin variants are abnormal forms of hemoglobin that occur when changes (point mutations, deletions) in the globin

genes cause changes in the amino acids that make up the globin protein. These changes may affect the structure of the hemoglobin, its behavior, its production rate, and/or its stability. Several hundred hemoglobin variants have been documented, however, only a few are common and clinically significant. The majority of these are β-globin variants. Hemoglobin E is the most common hemoglobin variants in the world. It is highly prevalent in Southeast Asia and is due to a mutation in the gene that creates the β-globin (Glu26(B8)→Lys) chain. People homozygous for HbE (have two copies of β^E) have a mild hemolytic anemia, due to premature removal of red blood cells from the circulation, microcytosis, and mild enlargement of the spleen. A single copy of HbE does not cause symptoms unless it is combined with another mutation, such as one for β-thalassemia trait. Hemoglobins A and F are both tetramers made up of four polypeptide subunits: two α-globin subunits and two β-like globin subunits, β-globin and γ-globin respectively [15].

Most mammalian plasma membranes share an asymmetric transbilayer distribution of phospholipids between the inner and outer monolayer, a basic feature of normal cell operations [16]. Generally, PS and phosphatidylethanolamine (PE) are found primarily in the inner leaflet while phosphatidylcholine (PC) and sphingomyelin are found in the outer leaflet. Asymmetric distribution of the aminophospholipids, PS and PE is recognized as very important in vesicular trafficking, molecular recognition and cellular sorting [17]. It has also been proposed that PE and PS are exposed on the cell surface during early stages of apoptosis, resulting in a total loss of aminophospholipid asymmetry in the plasma membrane bilayer [18,19]. Erythrocyte membrane contains larger amounts of 18% PE and 7% PS by weight in human [20], those are almost exclusively localized in the inner leaflet. Cholesterol is another highly abundant lipid in the human erythrocyte membrane, 23% by weight, which has been shown to protect against the changes induced by hemoglobin [21]. Most of the earlier studies involving hemoglobin and membrane systems were done with PS alone and little is known about the hemoglobin interaction of PE and cholesterol. Moreover, there are not many studies on the membrane interaction of hemoglobin variants HbE and HbF in this respect. The present study describes the interaction of hemoglobin variants HbA, HbE and HbF along with the globin subunits of HbA with PC-based phospholipid membranes containing PE and PS in the presence and absence of cholesterol. The tailor-made small unilamellar vesicles (SUVs) have been used as model membranes made of phospholipids with two different fatty acyl chains of myristic (C14:0) and oleic acids (C18:1). DOPC and DMPC favor the formation of lamellar bilayer organization with N-methylated head groups. DMPE alone could form bilayer however, DOPE, containing one double bond in the fatty acid chains, does not favor bilayer organization [22,23].

Efforts have been made to study the effects of the aminophospholipids, DMPS, DMPE and DOPE doped in DMPC and DOPC membranes in the presence and absence of cholesterol on the autoxidation of hemoglobin/globin species using absorption measurements, on the extent of leakage of entrapped CF by fluorescence measurements and electron microscopic observation of membrane vesicles upon treatment with HbE. Results indicated preferential interactions of HbE over HbF and HbA and α-globin chain over that of the β-chain with membranes containing the aminophospholipids, with PE imparting lesser effects than PS. Cholesterol showed some kind of stabilizing effects in both in the autoxidation and CF leakage.

2. Materials and methods

Critical considerations in the design of the experiments were to eliminate all oxidants and metal ions other than oxygen during the experiment. All glassware was acid washed before use, and buffers were prepared with de-ionized water doubly distilled on quartz. Finally, all buffers were filtered through 0.2-μm filter (Millipore). All organic solvents used were of HPLC grade or freshly distilled.

2.1. Materials

DMPC, DMPE, DOPE, cholesterol and CF were purchased from Sigma-Aldrich Corporation (St. Louis, MO). DMPS and DOPC were from Avanti Polar Lipids (Alabaster, AL). Cholesterol was re-crystallized from ethanol before use.

2.2. Methods

2.2.1. Isolation and purification of hemoglobin from human blood samples

Human blood samples taken for diagnosis from patients with hemoglobin disorder were characterized by BioRad Variant HPLC system. The hemoglobin variants, HbA, HbE and HbF, were characterized and estimated by the HPLC system [24]. We have purified HbA from normal individuals with HbA ranging from 95% to 97%, HbE from homozygous HbE patients with HbE ranging from 90% to 92% and HbF from patients with β-major thalassemia with 91% HbF, respectively. The blood samples were taken from patients at the time of diagnosis. Human erythrocytes, after removal of the buffy coat and plasma, were extensively washed with phosphate-buffered saline (5 mM phosphate, 0.15 M NaCl, pH 7.4). Hemoglobin was isolated from packed erythrocytes by osmotic lysis using three volumes of 1 mM Tris, pH 8.0, at 4 °C for 1 h. The hemoglobin mixture was purified by gel filtration on Sephadex G-100 column (30 × 1 cm) in a buffer containing 5 mM Tris, 50 mM KCl, pH 8.0. The hemoglobin samples were stored in oxy-form at −70 °C for less than 7 days and characterized by the measurements of absorption at 415 nm and 541 nm, respectively. The purity of the hemoglobin preparations was checked by 15% SDS-PAGE after staining with Coomassie blue. Densitometric analysis (Quantity One software, BioRad USA) indicated the hemoglobin preparations to be >90% pure. The protein concentration was determined spectrophotometrically using a molar extinction coefficient of 125,000 M^{-1} cm^{-1} at 415 nm and 13,500 M^{-1} cm^{-1} at 541, nm respectively [25].

2.2.2. Preparation of human α- and β-globin subunits

The PMB derivatives of HbA were prepared following the method of Bucci and Fronticelli [26]. The α-PMB and β-PMB chains were separated by following a method consisting of two-column selective ion-exchange chromatography as described earlier [27]. To obtain α-PMB, the splitting solution was equilibrated with 0.01 M phosphate buffer at pH 8.0 and passed through a DEAE-cellulose column equilibrated and eluted with the same buffer. To obtain β-PMB, the splitting solution was equilibrated with 0.01 M phosphate buffer at pH 6.6 and applied on a CM-cellulose column, equilibrated and eluted with the same buffer. The PMB was removed from the isolated α-PMB and β-PMB chains by the addition of 50 mM 2-mercaptoethanol in 0.1 M phosphate buffer, pH 7.5. The intact globin chain was purified from the mixture of globin chains and unreacted PMB by gel filtration on a BioGel P2 column. Immediately after separation, the subunits were dialyzed extensively against 0.1 M phosphate buffer, pH 7.5 [28]. The concentrations of the subunits were measured by the method of Lowry et al. [29]. The globin subunits were not stored for more than 48 h at 4 °C and characterized from their spectral characteristics to ascertain their oxidative states.

Hemin (Sigma) was dissolved in a minimal volume of 0.1 N NaOH and diluted with water to a final volume of 1 ml. The resulting solution was centrifuged at 15,000×g for 15 min and the clear supernatant was used for experimentation. Hemin was always freshly prepared and concentrations were determined spectrophotometrically [30].

Table 1
The pseudo-first order rate constant (k in h^{-1}) of the interaction of hemoglobin variants and the globins chains of HbA with DMPC SUVs containing DMPS, DMPE and cholesterol

Hemoglobin	Membrane type			
	DMPC	DMPC: DMPE	DMPC: DMPS	DMPC: DMPS:Chol
HbA	0.03±0.005	0.067±0.008	0.55±0.09	0.583±0.11
HbE	0.13±0.03	0.477±0.135	0.923±0.22	0.79±0.16
HbF	0.116±0.01	0.293±0.07	0.85±0.17	0.78±0.08
α globin	0.16±0.04	1.04±0.2	1.49±0.32	1.27±0.26
β globin	0.133±0.02	0.74±0.13	1.105±0.2	0.82±0.18

2.2.3. Preparation of small unilamellar vesicles (SUV)

The required amount of phospholipid was dissolved in chloroform and the phospholipid film was deposited by removing the solvent under a slow stream of nitrogen and further dried for overnight under high vacuum. The lipid film was finally hydrated with the required buffer and vortexed to disperse the lipids. The dispersion was sonicated for 20 cycles (1-min burst with 10-s interval) maintaining the temperature around 4 °C using a probe sonicator (dr.hielscher, GmbH, UP 200 s). Following probe sonication, SUVs were centrifuged at 12,000×g for 15 min to remove titanium and lipid aggregate. Then the liposomes were allowed to stand for 30 min at ~25 °C and used within 6 h of preparation [31].

Phospholipid SUVs of various compositions were used in the experiments described. We have used the following compositions with the mole percent of the particular lipid given in the parenthesis, e.g., DMPC (100), DMPC/DMPE (80:20), DMPC/DMPS (80:20), DMPC/DMPS/Chol (70:20:10) and DOPC (100), DOPC/DOPE (90:10), DOPC/Chol (90:10) and DOPC/DOPE/Chol (85:10:5).

2.2.4. Study of autoxidation of hemoglobin and its derivatives in the presence of phospholipid SUVs

The interaction of hemoglobin variants (HbA, HbE, and HbF) and purified globin subunits (both α-globin and β-globin) with SUVs of different phospholipid compositions were studied by monitoring the changes in characteristic absorption spectral properties of hemoglobin and its derivatives.

All experiments were performed with hemoglobin variants mainly in the oxy-form (HbA, HbE, HbF), globin subunits (both α- and β-globin), and SUV mixtures at a phospholipid to hemoglobin molar ratio of 100 with 250 μM phospholipid monomers interacting with 2.5 μM of tetrameric hemoglobin or equivalent globin subunit (10 μM per heme). The reaction mixture containing 20 mM HEPES, 10 mM NaCl, pH 7.0, was incubated at 37 °C and the absorption spectra (250–700 nm) recorded every 15 min for about 2 h. This phospholipids to hemoglobin ratio was chosen at a value so that oxidation occurred in a time period that could be measured reliably by spectrometric methods [10] using a double beam absorption spectrophotometer (Aquarius 7000 series, CeCil Instruments Limited, UK).

Spectral changes from 500 to 700 nm were followed to monitor the loss of oxy-hemoglobin species and generation of met-hemoglobin and hemichrome indicating the interaction between hemoglobin species and the phospholipid components [11]. The determinations of the concentrations of the different oxidized hemoglobin species were done by using a four-component analysis method elaborated before [32]. At definite time intervals absorption spectra were recorded, from which the concentrations of oxy-hemoglobin were calculated following the equations below (Eq. (1)). Care was taken so that no precipitation occurred during the reaction time due to formation of choleglobin.

$$[\text{oxy-Hb}] = 119 \times A_{577} - 39 \times A_{630} - 89 \times A_{560}. \tag{1}$$

To compare the extent of loss of oxy-Hb of different species in the presence of phospholipid membranes, percent of oxy-Hb remaining at a specific time interval was calculated as follows:

$$\text{oxy-Hb}(\%) = \{[\text{oxy-Hb}]_t / [\text{oxy-Hb}]_0\} \times 100 \tag{2}$$

where [oxy-Hb]t is the concentration of oxy-Hb at time 't' in the presence of the membrane SUVs and [oxy-Hb]$_0$ is the initial oxy-Hb concentration at time t=0 min, before the addition of the membrane SUVs.

The pseudo-first-order rate constant (k) were obtained from the slopes of the linear fit of the logarithmic values of concentrations of oxy-hemoglobin vs. time (t) plot for the initial disappearance of the oxy-hemoglobin, from two independent experiments, elaborated in the earlier reports [11]. Errors in the rate constants quoted in Tables 1 and 2 were estimated from the uncertainties in the individual rate constant values emerging from the fitted process. Comparison of the k-values was done to estimate the rate of induced oxidation of hemoglobin species in the presence of different type of phospholipid components.

2.2.5. Preparation of CF entrapped SUVs for leakage experiment

The interaction of hemoglobin and its derivatives with phospholipids SUVs was also studied by measuring the extent of release of SUV entrapped self-quenching dye, 6-CF. SUVs containing 6-CF at its self-quenching concentration were prepared by the method of probe sonication [33–35].

Concentration of 6-CF was determined spectrophotometrically at 492 nm using molar extinction coefficient of 72,000. The phospholipid film was hydrated in HEPES buffer (20 mM HEPES, 10 mM NaCl, pH 7.0) containing 30 mM 6-CF (used as self-quenching concentration). The liposomes entrapped with 6-CF were separated from the free dye by gel permeation on Sephadex G-50. The CF-entrapped SUVs were collected in the void volume and were used immediately for further experimentation within 2 h [35].

The SUVs of different phospholipid composition were treated with hemoglobin samples at a phospholipid to hemoglobin molar ratio of 100 at pH 7.0 and 37 °C. Each experimental set consisted of 500 μM of phospholipid monomers interacting with 5 μM of hemoglobin or globin subunit or hemoglobin sample (20 μM with respect to heme) in the same HEPES buffer and was incubated for 60 min at 37 °C before measuring the fluorescence intensity a in a Jobin-Yvon (Horiba, USA) fluorescence spectrophotometer. The CF fluorescence intensity increased more than 30-fold when treated with 0.1% (w/v) Triton X-100 which was taken as 100% leakage [34,35]. In all CF leakage experiments, hemin was used as a positive control and BSA as a control for non-heme protein. The error bars associated with the percentage of CF leakage are the mean CF leakage (%) with standard error of mean (S.E.M.), presented as mean± S.E.M. values from four independent experiments for each membrane systems and were further subjected to the two-tailed Student's t-test. The changes in the extent of CF leakage (%) values were found to be statistically significant with $P<0.05$.

2.2.6. TEM study of the phospholipids SUVs treated with HbE

DOPC SUVs containing DOPE and cholesterol have been used for TEM (Hitachi H-600 operating at 75 kV) studies after treatment with HbE. The mixture of hemoglobin (5 μM) and the phospholipids SUVs (500 μM) was incubated at 37 °C for 30 min and was placed on formver/carbon-coated copper grids and was negatively stained with 2% phosphotungstic acid for TEM observation. The sizes of the phospholipid vesicles were analyzed counting more than 50 SUVs of different DOPC-based membranes and the diameter was expressed as mean±S.E.M.

3. Results

3.1. Study of autoxidation of hemoglobin species by absorption spectroscopy

The relative extent of formation of various hemoglobin oxidation products and loss of oxy-hemoglobin concentration has

Table 2
The pseudo-first order rate constant (k in h^{-1}) of the interaction of hemoglobin variants and the globins chains of HbA with DOPC SUVs containing DOPE and cholesterol

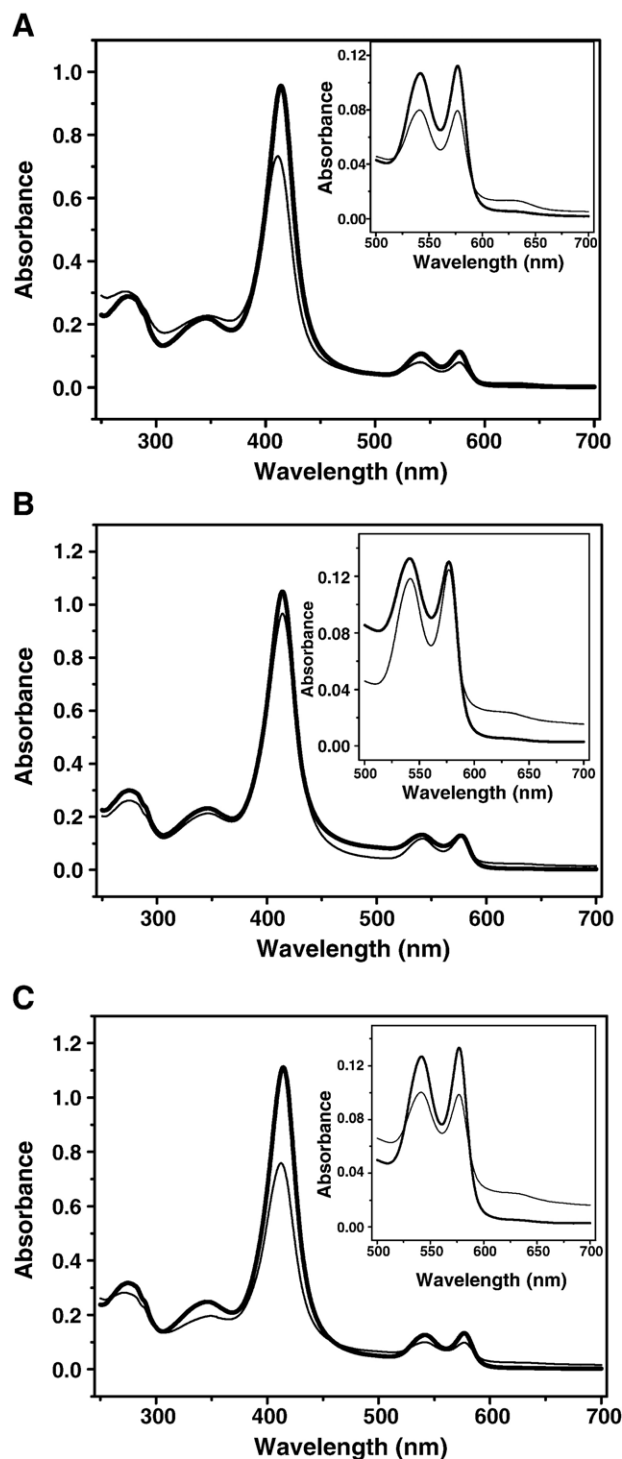| Hemoglobin	Membrane type			
	DOPC	DOPC:DOPE	DOPC:Chol	DOPC:DOPE:Chol
HbA	0.364±0.02	0.437±0.09	0.193±0.02	0.255±0.04
HbE	0.85±0.17	1.02±0.19	0.614±0.12	0.63±0.11
HbF	0.564±0.076	0.617±0.1	0.45±0.08	0.6±0.06
α Globin	1.01±0.18	1.24±0.25	0.675±0.12	0.874±0.2
β Globin	0.666±0.13	0.97±0.22	0.392±0.07	0.644±0.16

Fig. 1. Absorption spectra of (A) HbA (>95% in oxy-form); (B) HbF (>95% in oxy-form) and (C) HbE (>90% in oxy-form) in the presence of DMPC/DMPS SUVs at two different time intervals, in the absence (bold lines) and 15 min after the addition of the SUVs at 37 °C. Inset shows the same spectra in the wavelength range of 500–700 nm showing the new peak at 630 nm.

37 °C, shown in Fig. 1, from which the oxy-hemoglobin concentration was evaluated (Eq. (1)). We have observed decrease in the absorbance at 415 nm and an increase in absorbance at 630 nm indicative of the formation of met-hemoglobin species in the presence of the membrane SUVs [21].

Fig. 2 shows the decrease in oxy-hemoglobin concentration with time for HbA, HbE, HbF and the two globins chains in the presence of DMPC (Fig. 2A) and DOPC (Fig. 2B) SUVs. The rate of decrease was different depending on the hemoglobin variant or the globin subunits used. Faster rate of disappearance of oxy-hemoglobin was seen for HbE over HbA and α-chain over β-chain. HbF also showed greater oxidative membrane interaction compared to HbA. Both the globin subunits reacted to a much greater extent compared to the intact HbA. With DOPC SUVs, the extent of decrease in total oxy-hemoglobin concentration, comparable for both α-globin and HbE, has been much larger compared to DMPC (Fig. 2B). The hemoglobin preparations, used in the present work were not at the level of all chains 100% oxygenated. The ratio of absorbance at 415 nm to the same at 541 nm was used as the yardstick for hemoglobin in oxy-form (Fig. 1) for
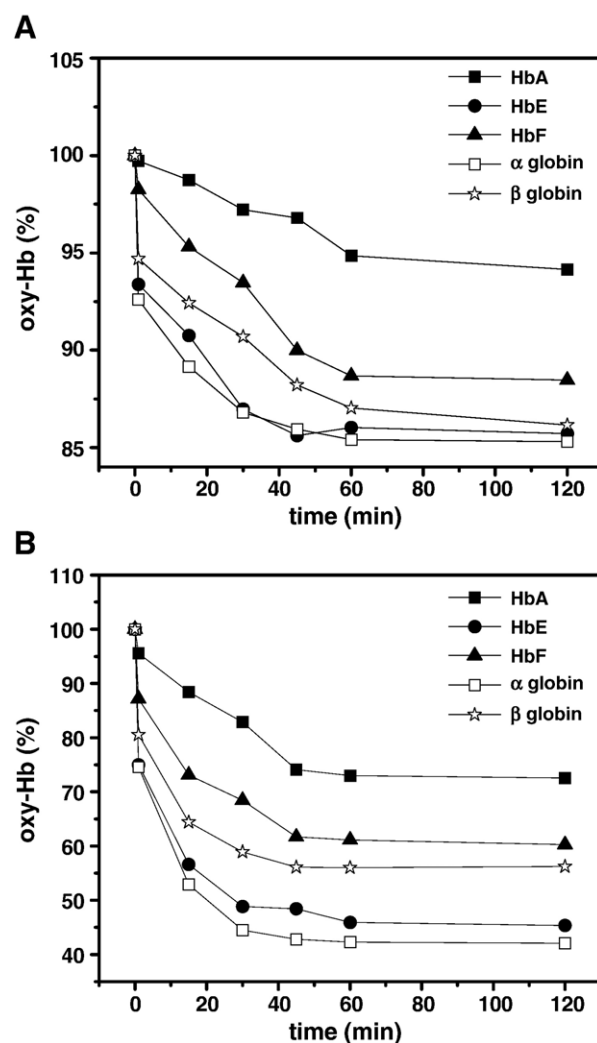

Fig. 2. Plot of percentage of oxy-form of different hemoglobin species (oxy-Hb%) at different time intervals: (A) oxy-Hb (%) in the presence of DMPC SUVs and (B) in the presence of DOPC SUVs.

been considered as an indicator of the oxidative interaction of hemoglobin and globin subunits in the presence of phospholipid SUVs. The representative absorption spectra of HbA, HbF and HbE in the presence of DMPC/DMPS SUVs have been shown just before the addition of the SUVs and after 15-min incubation at
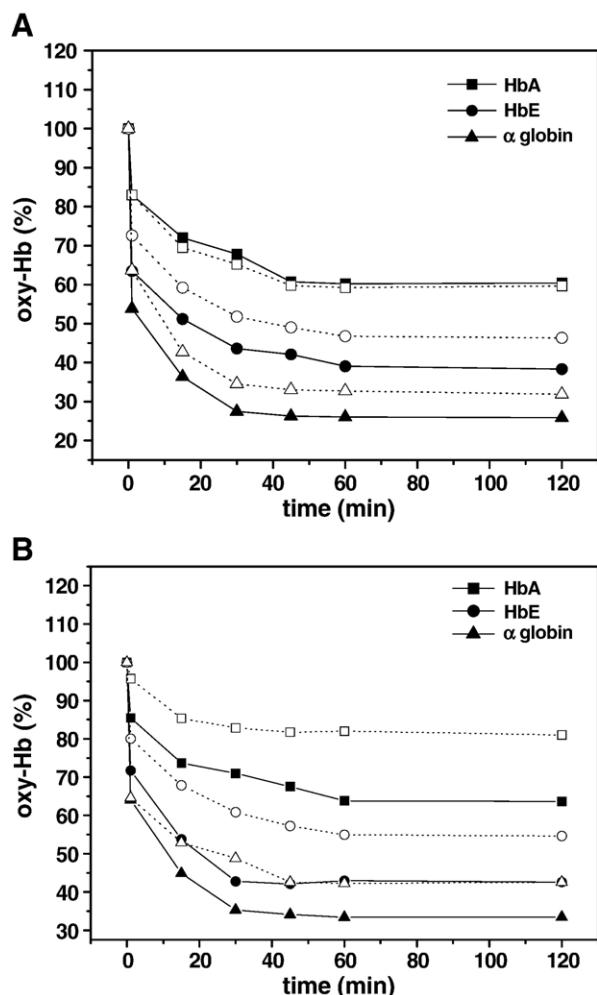
Fig. 3. Plot of percentage of oxy-Hb form of different hemoglobin species at different time intervals: (A) oxy-Hb (%) in the presence of DMPC/DMPS SUVs (filled symbols, solid line) and DMPC/DMPS SUVs containing cholesterol (empty symbols, dotted line) and (B) in the presence of DOPC/DOPE SUVs (filled symbols, solid line) and DOPC/DOPE SUVs containing cholesterol (empty symbols, dotted line).

hemoglobin is much greater in the presence of DOPE compared to control DOPC. Presence of cholesterol showed similar stabilizing effects and inhibited the formation of oxidized products of hemoglobin, e.g., met-hemoglobin (Fig. 3B) compared to pure DOPC or DOPC/DOPE membranes. The pseudo-first-order rate constant of the decrease in concentration of oxy-hemoglobin species has been summarized in Table 1 for DMPC-based membrane systems and Table 2 for the DOPC membrane systems.

3.2. Study of release of 6-CF from phospholipid SUVs

The ability of different hemoglobin variants to interact with the phospholipid SUVs has been measured in terms of the extent of leakage of CF entrapped in the vesicle.

The extent of leakage was found to depend on type of hemoglobin species used and the phospholipid composition of the SUVs used. Different hemoglobin variants showed differential
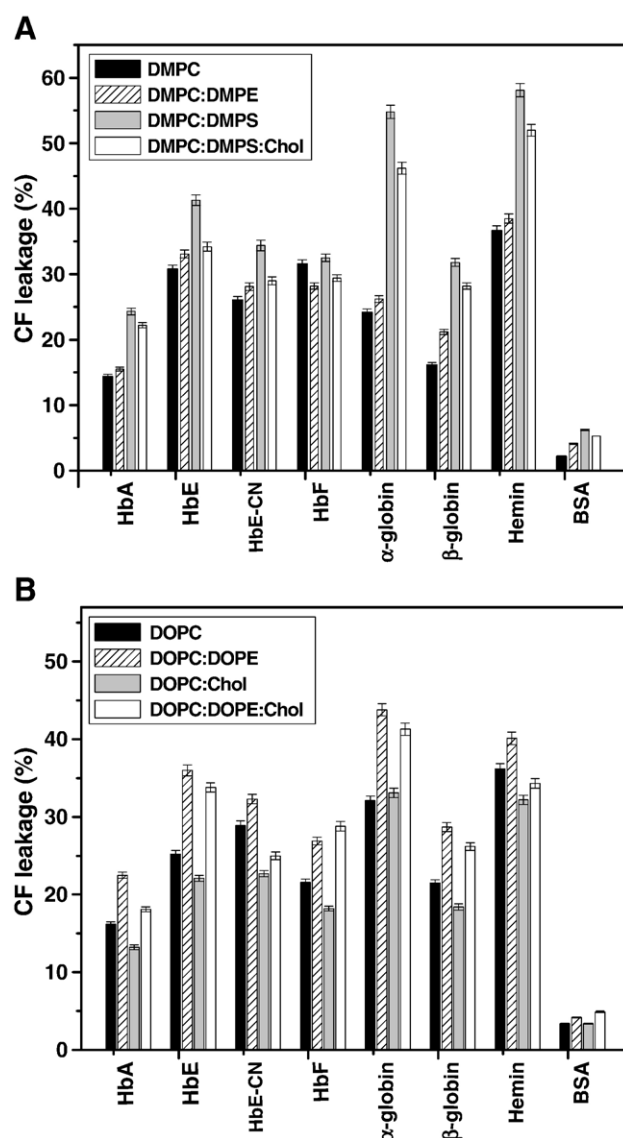
Fig. 4. Histogram representation of the extent of CF leakage induced by HbA, HbE (both in oxy- and cyano-met form), HbF, α-globin and β-globin chains, hemin and BSA in different (A) DMPC- and (B) DOPC-based membrane systems.

all preparations. Fig. 2 indicates that >90% of HbA (95-98%) and HbF (90–98%) were in the oxy-form before undergoing autoxidation in the presence of either of the DMPC and DOPC membranes. More than 90% HbE and both the globin chains were in oxy-form while experimenting in the presence of DMPC SUVs. However, the extent of HbE and the globin chains in oxy-form was about 80%, as reflected in the values at zero time in the presence of DOPC SUVs (Fig. 2).

Presence of 10–20% DMPS in DMPC SUVs showed faster rate and greater oxidative membrane interaction of the hemoglobin species again showing preference for α-globin and HbE (Fig. 3A). In all cases the extent of decrease in oxy-hemoglobin concentration was much greater than in pure DMPC membranes. However, cholesterol showed a distinct inhibitory or stabilizing effect when present in DMPC/DMPS membranes and both the HbE variant and α-globin subunit became substantially stabilized in the presence of cholesterol. The extent of loss of oxy-hemoglobin for HbE remained almost unchanged in DOPC SUVs containing DOPE. However, for globin subunits, the percent loss of oxy-

Table 3
Diameter (Å) of the TEM characterized phospholipid SUVs

Hemoglobin	SUV system used		
	DOPC (Å)	DOPC:Chol (Å)	DOPC:DOPE (Å)
Control	350±60	450±70	490±80
HbE	1100±100	>1500	1600±100

membrane perturbation inducing leakage of CF from the DMPC-based phospholipid systems in combination with DMPE, DMPS and cholesterol. The extent of CF leakage was 14% with HbA, which increased up to 31% with HbE in pure DMPC SUVs. This effect is more pronounced in DMPC SUVs containing the aminophospholipids, DMPE and DMPS. The extent of leakage increased to 15.5% for HbA and to 33.1% for HbE in the presence of DMPE and to 24.3% and 41.3% for HbA and HbE respectively in the presence of DMPS. Presence of 10% cholesterol in DMPC/DMPS SUVs prevented the release of CF to a considerable extent reducing the HbE induced leakage to 34% from 41% in the absence of cholesterol. HbF followed a similar trend with that of HbE inducing 32% leakage of CF from DMPC SUVs (Fig. 4A).

The α-globin subunit induced the largest extent of 55% CF leakage from SUVs of DMPC/DMPS compared to both the DMPC and DMPC/DMPE SUVs which is decreased to 46% in the presence of cholesterol. The effect of β-globin was not so pronounced and was comparable with that of HbA. By examining various membrane systems containing aminophospholipids, it was observed that effect HbE was stronger with DMPC/DMPE membranes compared to the α-globin chain. On the other hand, the effect of α-globin was stronger than both HbE and HbA with DMPC/DMPS membranes. Cholesterol, however, stabilized both the membrane systems towards all the hemoglobin species, HbE and the α-globin chains in particular. The CF leakage data also clearly indicate stronger effects of HbE in DOPC/DOPE SUVs inducing 36% leakage compared to 22.5% by HbA (Fig. 4B). Hemin alone induced 30–40% leakage of CF from almost all types of membranes showing about 60% leakage particularly from DMPC/DMPS SUVs. However, BSA on the other hand induced not more than 5% leakage. Fig. 4 summarizes all CF leakage data in both the DMPC- and DOPC-based membrane systems in combination with the aminophospholipids in the presence and absence of cholesterol. The HbE both in its oxy-form and in its cyano-met form, purified from hemolysates of HbE/β-thalassemia patients, perturbed the phospholipids membrane to a comparable extent (Fig. 4).

3.3. TEM observation of HbE-treated DOPC SUVs

DOPC SUVs were subjected to TEM studies indicating large increase in the size of the phospholipid vesicles both in the presence and absence of DOPE and cholesterol upon treatment with HbE. Table 3 summarizes the sizes of the DOPC SUVs containing cholesterol and DOPE in the presence and absence of HbE. The size of DOPC SUVs with a mean vesicle diameter of 350 Å (Fig. 5A) increased to 1100 Å in the presence of HbE (Fig. 5B). The size of DOPC SUVs containing 10% cholesterol, with mean
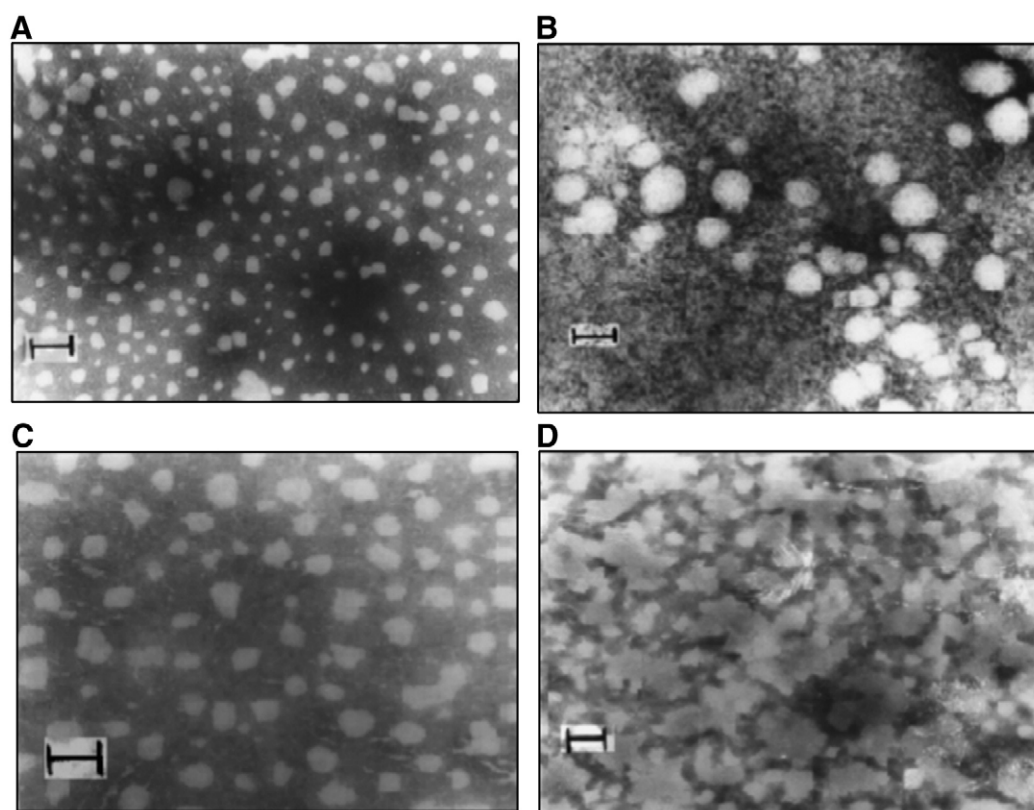

Fig. 5. Transmission electron micrographs of (A) DOPC SUVs; (B) DOPC SUVs treated with HbE; (C) DOPC/Chol SUVs; (D) DOPC/Chol SUVs treated with HbE. The bars represent 1000 Å.

vesicle diameter of 450 Å (Fig. 5C) increased to >1500 Å in the presence of HbE (Fig. 5D). Similar increase was also seen in the size of DOPC SUVs containing 10% DOPE, with mean vesicle diameter of 490 Å increased to >1600 Å in the presence of HbE (not shown). The TEM studies indicated fusion of phospholipid SUVs in the presence of HbE. The effects of HbA on the size of the vesicles were marginal, however, hemin induced similar fusogenic effects on the phospholipids membranes (not shown).

4. Discussion

The oxidative interaction between hemoglobin and the cell membrane is thought to be an important factor in the senescence of red blood cells and also in various hemolytic disorders [36]. Experimental evidence indicated lipid peroxidation and other oxidative membrane alterations or changes in adult hemoglobin as factors responsible for hemolysis. However, such studies on membrane interactions have not been done with abnormal hemoglobins, particularly for HbE which is associated with an important class of anemia, HbE/β-thalassemia. We have studied the oxidative interaction of HbE along with HbA and HbF with DOPC and DMPC-based membranes containing aminophospholipids with and without cholesterol. Membrane interactions of hemoglobin variants were enhanced in the presence of aminophospholipids, e.g., the bilayer forming DOPE in DOPC membranes and DMPS in DMPC membranes. Presence of cholesterol in membranes containing the aminophospholipids decreased the extent of membrane perturbation by all the hemoglobin species used in the present work. Recent studies have indicated influences of the phospholipids head groups, surface charge, asymmetric distribution and the presence of cholesterol to affect the phospholipid peroxidation [37–40]. Membrane cholesterol has been found to have regulatory effects on the aminophospholipid asymmetry in oxidized erythrocytes [41]. The susceptibility of cells to oxidative stress is dependent on the nature and physical state of the membrane lipid bilayer. Cholesterol directly modulates the physical properties of lipid bilayers, altering membrane responses to degenerative process, including lipid peroxidation [42].

The pseudo-first-order rate constant of the decrease in concentration of oxy-hemoglobin species for DMPC-based membrane systems with higher phase transition temperatures indicated that in pure DMPC membranes the oxidation rate increased substantially from $0.03 \, h^{-1}$ for HbA to $0.13 \, h^{-1}$ for HbE and $0.116 \, h^{-1}$ for HbF. The hemoglobin preparations used in the present study are >90% pure. However, there could be other redox proteins, e.g., the peroxiredoxins present in the hemoglobin preparations as minor contaminants which could also affect the autoxidation rates. In the presence of 20% DMPE in DMPC, the rate constants were enhanced by 2-fold while about 20-fold in the presence of 20% DMPS in DMPC showing autoxidation of HbA to be favored by the aminophospholipids. Cholesterol, however, decreased the rate constants in all of them. Both the globin subunits showed considerably enhanced oxidation rate with $0.162 \, h^{-1}$ for α-globin and $0.133 \, h^{-1}$ for β-globin compared to $0.03 \, h^{-1}$ for HbA (Table 1). In the DOPC membrane systems, with substantially lower phase transition temperatures, containing DOPE and cholesterol, the rate constant values were increased considerably compared to those in

DMPC membrane systems (Table 2). Presence of HbE favored the oxidation to a large extent, specifically in the presence of DOPC containing unsaturated fatty acyl chains. Also, the presence of aminophospholipids favored the interaction to a larger extent showing significant increase in the rate constants, particularly in the presence of DMPS. Presence of cholesterol in the membrane containing aminophospholipids, however, showed stabilizing effects inhibiting the oxidation process also shown earlier to protect against the changes of hemoglobin [21]. In all DMPC- and DOPC-based membranes, the magnitude of the rate constants was found to increase in HbE over HbA and in α-chain over β-chain (Tables 1 and 2).

Differential effects of HbE and α-globin chains were also revealed from experiments on the CF release from membrane vesicles. An earlier study has indicated that the rate of increase of erythrocyte lipid monolayer surface pressure upon autoxidation of different hemoglobin variants followed the order: HbE>HbF>HbS>HbA and the ability of various hemoglobins to affect lipid peroxidation in the RBC membrane also followed the same order [43]. Autoxidation of different hemoglobin variants in the present work followed the order: α-globin>HbE~HbF>β-globin>HbA in interacting with tailor-made phospholipid SUVs. The CF leakage data also follow a similar trend with phospholipid SUVs. The possible faster rate of heme release could be accounted for greater membrane-mediated autoxidation of HbE as observed in HbS [43,44]. Among the globin subunits, α-globin induced the largest changes in phospholipid SUVs compared to the β-globin subunit or HbA. It has been earlier shown that entrapment of purified α-globin chains within normal erythrocyte significantly enhanced cellular oxidant stress and resulted in changes of thalassemic cells [45]. Previous work on oxidative hemoglobin denaturation by phosphatidylserine liposomes pointed out that oxidation is conditioned by pre-association of hemoglobin with the phospholipid and is dominated by electrostatic forces [46]. Various studies have indicated that the oxidative interaction between hemoglobin and phospholipid occurs via two steps involving both electrostatic as well as hydrophobic interaction, although their relative contributions to the different secondary changes in the protein and the membrane are substantially different. The binding and intrusion of the heme appear to be due mainly to hydrophobic interactions [21]. The released hemin intercalates into the core of the lipid bilayer and also triggers a Fenton-like reaction simultaneously [10,43].

In this study we have used relatively low concentration (2.5 μM) of hemoglobin variants, also used in many other studies on the interaction of hemoglobin with phospholipid membrane vesicles [10,11,46]. It has been earlier shown that at low Hb concentrations, Hb tetramers dissociate to dimers, which exhibit a much higher rate of heme dissociation than the tetramers [47]. Consequently, Hb autoxidation was enhanced by dissociation into dimers [48]. However, it has also been clearly established that at 3 μM Hb tetramer concentration, increased oxidation of Hb was not observed on further decreasing Hb concentration and indicated that the enhanced oxidation of hemoglobin in the presence of phospholipid membrane vesicles is not directly related to the concentrations of oxy-Hb tetramers and dimers and remained linear for about 50 h [11].

The protecting effect of cholesterol has been explained in terms of tighter packing of fatty acyl chains in the presence of cholesterol presenting a steric barrier to the access of hemoglobin and/or heme detached from globin to lipid hydroperoxides [14]. This protecting effect of cholesterol might play a crucial role in maintaining the stability of the inner leaflet of cell membrane when hemoglobin comes in constant contact with the negatively charged PS-rich bilayer and the degree of stabilization is the highest with phospholipids carrying saturated fatty acyl chains. This study also indicates preferential interactions of HbE and the α-globin subunit of HbA with DMPC/DMPS membranes, in particular and in DMPC/DMPE and DOPC/DOPE membranes all of them forming phospholipid bilayers with phase transition temperatures remaining within 24–29 °C [49]. The TEM observation of the membrane vesicles upon treatment with HbE showed large fused membrane structures indicating better fusogenic potential of HbE compared to HbA as observed in HbS at physiological pH and ionic strength conditions [50]. To explain such differential interaction of HbE and α-globin with respect to others, the differential degree of detachment of heme from the protein counterpart might be one of the key reasons. From our crystal structure studies on the HbE it has been shown that the surface charge distribution in HbE is significantly different from that of HbA also indicating the origin of its thermal instability and the heme binding property compared to HbA [51]. Free hemin has been found to induce larger extent of CF leakage from membrane SUVs (Fig. 4) also indicating that HbE could be more susceptible to heme release particularly under oxidative stress and may play a role in the pathophysiology of HbE/β-thalassemia. It has been shown earlier that HbE could release heme faster than HbA upon oxidation [43]. Oxidative denaturation of sickle hemoglobin induced by PS pointed out such oxidations to be conditioned by the pre-association of hemoglobin with the phospholipids and is dominated by electrostatic forces [46,11]. Also an earlier study confirmed stronger affinity of α-globin chains for cell surface lipids and proteins compared to HbA and/or β-globin chains [52]. Taken together, these results might be helpful in understanding the oxidative stress-related mechanism(s) involved in explaining the premature destruction of erythrocytes in thalassemia, and in particular, HbE/β-thalassemia.

Acknowledgments

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