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Modulation of oxidative stability of haemoglobin inside liposome-encapsulated haemoglobin

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Abstract

The major hurdle in the formulation of liposome-encapsulated haemoglobin (LEH) is the oxidation of haemoglobin (Hb) into methaemoglobin during storage and after administration. In order to reduce this oxidative degradation, we tested various reducing conditions in the presence of catalase. We found that at 37°C more than 50% of Hb oxidized to methaemoglobin within 24 h, whereas in presence of catalase, the oxidation was significantly reduced. The effect of catalase was further enhanced by a reduction mixture containing β-NAD, d-glucose, adenine, inosine, $MgCl_2$, KCl, KH_2PO_4 and Na_2HPO_{4-}, only 14% methaemoglobin was generated in the presence of catalase and reduction mixture (CRM). Contrary to the expectation, glutathione, deferoxamine and homocysteine enhanced Hb oxidation. The presence of CRM inside liposomes (250 nm) significantly decreased Hb oxidation. The results suggest that catalase and a well-defined mixture of co-factors may help control Hb oxidation for improvement in the functional life of LEH.

Keywords

haemoglobin; transfusion; methaemoglobin; oxygen carriers; liposomes; catalase

Introduction

During the recent past, blood banking and transfusion practices have made significant progress towards achieving a “zero-risk” supply of whole blood as well as its components. However, a majority of the population worldwide still faces the risk of inadequate supply of safe blood, and though the transfusion with whole blood or packed red blood cells (RBCs) is widely practiced as relatively safe in a controlled hospital environment, it may not be regarded as absolutely risk free (Uhl, 2002; Dzik et al., 2003; Goodnough, 2003). By one estimate, by 2030, there will be a shortage of 4 million units of blood per year in the USA (Arnoldo and Minei, 2001). Therefore, development of a readily available universal oxygen-carrying resuscitative fluid for administration in victims of acute haemorrhagic shock has been a historical goal (Awasthi, 2005). The ideal oxygen-carrying resuscitative fluid would emulate as many functions of fresh whole blood as possible. It would have the capacity to

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carry and deliver oxygen as RBCs as well as provide oncotic activity to maintain circulatory filling pressure. Like RBCs, it would remain in the intravascular space for a prolonged period of time without any toxicity or immunogenicity. In addition, the product would be shelf-stable, easy to use and moderate in cost. In order to accomplish these desirable characteristics in an artificial oxygen carrier, we are developing a formulation of liposome-encapsulated haemoglobin (LEH).

Following acute blood loss, the human body takes 5–7 days to replace the lost volume and oxygen-carrying capacity (Hughes et al., 1995). The desired intravascular persistence of an oxygen carrier should be at least equal to the time required to regenerate RBCs (Sehegal et al., 1983). Transfused RBCs remain functional in circulation for many days, but the same cannot be said for other haemoglobin-based oxygen carriers (HBOCs). It is a significant challenge to formulate an HBOC that matches the functional efficacy of RBCs, because after administration, not only is HBOC eliminated from circulation via usual metabolic pathways, but haemoglobin (Hb) also undergoes oxidation into methaemoglobin (metHb) at a rate of >1–2%/h at body temperature (Ogata et al., 1997). The oxidized iron atom (Fe^{3+}) in metHb is unable to bind oxygen. Hb within the RBCs remains in Fe^{2+} state and maintains its functionality by virtue of the enzyme system comprising of catalase, superoxide dismutase, glutathione peroxidase and glutathione. One of the goals of blood substitute research is to imitate this redox ability of RBCs in artificially assembled oxygen carriers.

In theory, a physical mixture of redox enzymes, reducing agents and acellular Hb might improve the stability of Hb molecule; however, the components of redox system will disassociate from Hb, leaving Hb vulnerable to oxidation at body temperature. It is possible to create a complex molecule of Hb linked to catalase and superoxide dismutase (Chang et al., 2000; Powanda and Chang, 2002), but how far this modification fulfils the requirements of prolonged circulation and function, remains unknown. Encapsulation of Hb inside the artificial membranes provides formulators with an opportunity to reconstitute the RBC-like environment inside the nanometre-sized carrier system. The advantages of encapsulated Hb over acellular Hb products have been previously reviewed (Awasthi, 2005). One approach is to encapsulate Hb in biodegradable 80–150 nm nanocapsules of polyethylene glycol-polylactide polymer (Chang et al., 2003; Chang, 2004). These nanocapsules carried co-encapsulated superoxide dismutase, catalase, carbonic anhydrase and other enzymes of Embden-Meyerhof pathway needed for functional stabilization of Hb (Chang et al., 2000). Another elegant approach is based on Hb encapsulation inside liposomes (Takeoka et al., 1998; Phillips et al., 1999; Li et al., 2005; Sakai et al., 2010). Like nanocapsules, LEH also offers an opportunity to co-encapsulate stabilizing molecules along with Hb (Teramura et al., 2003). Recently, we have shown that LEH administration helps recover cerebral oxygen and energy metabolism in a rat model of haemorrhagic shock (Awasthi et al., 2007, 2010). This good news could become even better, if the prolonged circulation persistence of LEH is matched with an enhanced functional life *in vivo*. In this study, we report the effects of various biocompatible reducing agents and their mixtures on their ability to keep Hb in reduced form, with an ultimate goal of simulating the RBC-like conditions inside an LEH preparation. The results suggest that by choosing an appropriate composition of reducing agents and co-factors, it is possible to control the oxidative degradation of Hb.

Material and methods

Isolation of stroma-free Hb from outdated RBCs

The isolation of Hb involved hypotonic lysis of RBCs followed by several tangential-flow filtration steps. Outdated RBC units for Hb isolation were kindly provided by the South Texas Blood and Tissue Center (San Antonio, TX) or Oklahoma Blood Institute (Oklahoma City, OK). For long-term storage, Hb was sometimes carbonylated with filtered carbon

monoxide (CO). The CO-Hb was converted back to oxygenated form (oxy-Hb) for all the experiments. The procedure was performed inside a laminar flow hood using endotoxin-free material and water for injection (Winslow and Chapman, 1994). The lysed blood was sequentially filtered through 0.65 μm, 0.1 μm and 500 KDa hollow fibre filters (Amersham Biosciences, Piscataway, NJ). The bulk stroma-free Hb was concentrated through a 10 KDa hollow fibre filter and stored frozen at −70°C. If carbonylated, the CO-Hb was converted into oxy-Hb by exposing it to a bright visible light from a 500 W halogen lamp under saturating oxygen atmosphere at 4–8°C (Sakai et al., 1996). The conversion was ensured by monitoring a left shift in Hb spectrum upon oxygenation.

Catalase preparation

Catalase (48 000 U/mL) of bovine origin was obtained from Worthington Biochemical Corporation (Lakewood, NJ). The solution of catalase was freeze-dried and reconstituted in water for injection to obtain a concentrated preparation (120 000 U/mL).

Treatment of Hb with reducing agents and catalase

All chemicals were obtained from Sigma-Aldrich (St. Louis, MO). We chose glutathione, deferoxamine and homocysteine as the small molecule reductants, catalase as an enzymatic means of controlling peroxide-based oxidation, and a mixture of co-factors present in RBCs as a reduction mixture (RM) to mimic RBCs. Table 1 presents the concentrations of these compounds which were either used alone, or, where applicable, in a mixture of two or more entities. β-NAD is a co-enzyme involved in intracellular redox reactions; glucose is the energy source; adenine allows RBCs to resynthesize ATP; inosine is a purine nucleoside that affects carbohydrate and phosphate metabolism and helps prolong the viability of stored RBCs. All incubations were performed in a closed tube in a 37°C incubator, unless otherwise specified. No attempt was made to replace room air in the tube with any inert gas. Briefly, 100 μL of oxy-Hb (10 g/dL) was mixed with specified amounts of reductants (Table 1) for 6 or 24 h. At the end of incubation, the samples were assayed for metHb content as described below.

MetHb assay in free Hb

MetHb content was measured in Hb by following the method of Matsuoka (Matsuoka, 1997). The method is based on the measurement of absorbance at 563 nm which is an isobestic point for the treated Hb samples. The isobestic point was obtained by scanning the entire spectra (350–700 nm) of Hb samples (Matsuoka, 1997). Briefly, about 10 μL of Hb sample was placed into two separate plastic cuvettes (labelled A and B). For blank cuvettes, Hb was replaced with equal volume of water (labelled C and D, respectively). The volumes were made up to 2 mL with phosphate-buffered saline, pH 7.4. First, the absorbance of cuvette A was measured (X_1). To the cuvette A, 5 μL of sodium cyanide solution (0.8 M) was added, mixed and absorbance was measured to obtain X_2. To the cuvette B, 50 μL of potassium ferricyanide solution (0.9 M) was added, and after 2 min, the absorbance was measured again to obtain (Y_1). To cuvette B, 5 μL of sodium cyanide solution (0.8 M) was added and absorbance (Y_2) was measured after 30 s. The absorbance readings were applied to calculate the metHb content in the samples using the following equation.

$$\text{MetHb}\% = 100 \times (X_2 - X_1) / 1.05 (Y_2 - Y_1)$$

where ($X_2 - X_1$) is the CNMetHb formed from endogenous MetHb, ($Y_2 - Y_1$) the total Hb estimated as CNMetHb and 1.05 in the denominator the dilution factor.

Preparation of LEH

LEH was prepared essentially by the method described previously (Agashe et al., 2010). Oxy-Hb was mixed with catalase (24 kU/mL) and the components of RM (Table 1) before encapsulation was performed in liposomes consisting of dipalmitoylphosphatidylcholine, cholesterol, dimyristoylphosphatidylcholine and poly(ethylene $glycol_{5000}$)-distearoylphosphatidylethanolamine (44:44:10.5:1.5, M ratio). The processing was performed in an aseptic manner at temperatures not exceeding 20°C. The LEH preparation was characterized for [Hb], [phospholipid], %metHb, particle size and zeta potential. The methods pertaining to these assays have been described previously (Awasthi et al., 2004; Agashe et al., 2010).

Microscopy of LEH

Transmission electron micrograph of uranyl acetate-stained LEH was obtained in the Imaging Core Facility of Oklahoma Medical Research Foundation (Oklahoma City, OK) following the method described earlier (Agashe et al., 2010). To further confirm the liposome integrity, the LEH was labelled with a lipid partitioning fluorescent dye DiO (Invitrogen, Carlsbad, CA). The instructions supplied by the manufacturer were followed. Briefly, an eight-chambered tissue culture glass slide was coated with LEH for 16 h at 4°C. The coated wells were washed with PBS (pH 7.4), followed by incubation with DiO stain solution for 4 h. The excess stain was removed by washing with PBS. Finally, the samples slides were observed under the Zeiss LSM-710 multiphoton laser microscope.

MetHb assay in LEH

LEH was incubated for 6 and 24 h at different temperatures as described above. We modified the metHb estimation method using phosphate-buffered (0.1 M, pH 6.8) 25% dimethylsulphoxide (PB-25%DMSO) to quantitatively release Hb from liposomes without changing its oxidation state. Briefly, about 50 μL of LEH was diluted with 200 μL of PB-25%DMSO. The dilution was vortexed vigorously for 1 min, further diluted to 1 mL with PB and probe-sonicated (50 Sonic Dismembrator, Fisher Scientific) for 30 s on ice at 45% power output. The mixture was spun at 14 000 rpm for 10 min to remove any debris and the supernatant was used for metHb estimation by the method described above using 500–700 nm spectra from Biotek's Synergy 2 (Winooski, VT). The absorbance values at isobestic point at 563 nm were used to calculate the metHb content. All estimations were performed in duplicates.

Data analysis

The experiments were performed at least three times and the results were calculated as mean (±standard error of mean). The data were analysed by ANOVA and the differences were considered significant at $p < 0.05$.

Results

MetHb formation during processing, storage and post-administration circulation is a problem in all HBOCs. We investigated the effect of reducing agents, catalase and a mixture of co-factors on metHb formation. The optimal RM was tested in LEH preparations carrying co-encapsulated Hb.

It is clear, from Figure 1, that Hb oxidation is dependent on the temperature and duration of incubation. At 4°C, there was negligible increase in metHb over 24 h. When the incubation temperature was 37°C, the rate of oxidation was significantly increased ($p < 0.05$ compared to control Hb). The effect was moderate, but still significant, at room temperature (25°C).

To decrease the rate of metHb formation, first, we studied the effect of various concentrations of catalase on total metHb after 24 h of incubation at 37°C (Figure 2a). It was found that catalase significantly reduced metHb formation by about 20%. The optimal concentration of catalase was found to be about 25 000 U/mL; increasing the catalase concentration above this optimal level had no additional improvement. Based on these results, we started using about 24 000 U/mL in further experiments. Since enzyme systems work better at optimum temperature, we studied the effect of incubation temperature, as well as duration of incubation, on the activity of catalase to inhibit Hb oxidation (Figure 2b). We found that catalase was effective at all temperatures over a period of 24 h. The effect was not always discernible during shorter incubation time of 6 h. These initial experiments indicated that the presence of catalase in Hb preparations may have beneficial effect on Hb stability both under storage conditions as well as at body temperature.

Since the use of catalase alone does not seem to be sufficient in controlling Hb formation, we investigated the effect of commonly used biocompatible reducing agents, both alone as well as in combination with catalase (Figure 3). Not only were glutathione and homocysteine ineffective, but the reducing agents were also found to denature Hb, cause precipitation and considerably enhance Hb oxidation. Deferoxamine by itself did not cause any precipitation at the concentrations used, but was unable to influence oxidation reaction in a desirable manner. These results suggested that the use of reducing agents to maintain Fe^{3+} state of iron in Hb may not be a recommended strategy. Even catalase addition (25 000 U/mL) had no effect on Hb oxidation, except in the case of glutathione at 10 mM concentration. It appears that the reductant-induced enhancement in Hb oxidation is not dependent on hydrogen peroxide, and may involve the generation of other highly reactive free radicals during the incubation.

Next, we tested the idea of using small molecule co-factors to influence Hb oxidation (Figure 4). For convenience, we designated the mixture of co-factors as RM. The preliminary composition of the mixture of co-factors was reported by Ogata et al. (1997), and was targeted to mimic the constitution inside the RBCs. This composition was termed 1× in our experiments; for 2× and 3× compositions, we doubled and tripled the concentration of components, respectively. We found that the RM alone had significant impact on Hb oxidation. At 3× concentration of RM, metHb formation was reduced from >50% to about 33% during 24 h incubation at 37°C. The difference between using 1× concentration versus 3× concentration of RM was not significant.

We examined the possibility of using RM and catalase together to manage Hb oxidation. Again, various combinations of the two entities were used (Figure 5). It was found that 24 000 U/mL catalase and 3× amount of RM decreased metHb levels from >50% to about 14% over 24 h incubation at 37°C ($p < 0.05$). With 3× co-factors, the catalase requirement to achieve metHb formation below 20% is considerably lowered only 12 000 U/mL of catalase is required with 3× co-factors (Figure 5). Thus, it appears that with an optimal combination of catalase and co-factors present in RM, it is possible to reduce the rate of Hb oxidation. We designate this combination of co-factors and catalase as complete reduction mixture or CRM (Table 1).

Finally, we co-encapsulated CRM with Hb inside the liposomes and investigated its utility in controlling the metHb formation in LEH stored at various temperatures. Figure 6 shows the change in spectral properties of Hb as it undergoes various treatments. The typical Hb absorbance pattern is gradually lost in LEH samples stored at elevated temperatures. At the same time, the characteristic peak of metHb at ~630 nm appears in a temperature-dependent manner. These spectral changes are minimized in LEH preparations containing CRM. Figure

6 also clearly shows that the presence of CRM with Hb inside the liposomes reduces metHb formation in a time-dependent manner.

The characteristics of the final LEH preparation are shown in Figure 7. The methaemoglobin content of CRM-stabilized LEH was significantly lower than that determined in LEH without CRM (Figure 7a). This effect is also clearly visible in the picture where the darkening of LEH was prevented by the presence of CRM (Figure 7b). The presence of CRM did not negatively influence the formation of liposomes; a typical donut-shaped structure was observed in electron micrographs, which was confirmed by fluorescence micrograph of a bigger LEH particle (Figure 7d and e). For confocal micrograph the large size of LEH was deliberate because of the resolution limitation of the technique.

Discussion

The need for sophisticated blood processing, storage and cross matching as well as the safety concerns has given impetus to the search for safe, shelf-stable and efficacious artificial oxygen-carrying fluids (Goodnough et al., 2003). Even though RBC and whole blood units remain the gold standard in transfusion medicine, serious efforts in developing an artificial oxygen carrier have recently resulted in first generation products that are now being tested in clinical trials (Chang, 2003; Kjellstrom, 2003; Lowe and Ferguson, 2003; Winslow, 2003). The most direct replacements of RBCs are based on the use of stroma-free human or bovine Hb. These products are classified as HBOCs. Whereas transfused RBCs remain in circulation and continue to function for several days, the same could not be said for the artificially formulated HBOCs. A synopsis of circulation half-lives of various HBOCs has been reviewed elsewhere (Chang et al., 1992). It is a significant challenge to formulate an HBOC that matches the functional efficacy of RBCs.

The early developments in HBOC research were primarily focused on prolonging circulation half-life of Hb, essentially ignoring its functional status in circulation. In other words, even if an HBOC emulating circulation persistence of transfused RBCs was assembled, its ability to carry and deliver oxygen to hypoxic tissues during its stay in circulation was not guaranteed. The reason for this mismatch is the propensity of Hb to undergo a rapid conversion from oxygen-carrying ferrous Hb to non-functional ferric Hb at body temperature. The repeated oxygenation–deoxygenation cycles experienced by Hb *in vivo* augment this process. Thus, exogenously administered purified Hb converts to methaemoglobin at a rate of >1–2%/h (Ogata et al., 1997). A relatively short circulation persistence of HBOCs, coupled with their short-functional half-life, necessitates frequent administrations to maintain haemostasis for a time period required to replenish lost RBCs via erythropoiesis.

Hb is a 64 KDa tetramer of two α- and β-chains with four heme prosthetic units, each capable of binding one molecule of oxygen. Earliest attempts to use purified Hb from RBCs demonstrated that it is unstable *in vivo*; it dissociates into constituent chains and have significant toxic reactions upon administration (Savitsky et al., 1978; White et al., 1986; Chang, 1998; Chan et al., 2000). While the dissociation problems could be partially addressed by approaches such as cross linking (Creteur et al., 2000; Gould et al., 2002; Squires, 2002; Stowell, 2002) and/or its conjugation to poly(ethylene glycol) (Conover et al., 1999), oxidative degradation of Hb at body temperature remains an unsolved problem to date. Oxidation of Hb increases the rate of heme loss resulting in its denaturation. The Hb oxidation is not limited to Fe(II) to Fe(III) conversion. Under oxidizing conditions, the Fe(III) Hb may oxidize further to ferryl (Fe(IV)→O) Hb by the spontaneous formation of H_2O_2 from superoxide radical (Alayash, 2001). Auto-oxidation, as shown in Equation (1), occurs inside RBC also, but RBC Hb is maintained functional by a highly specialized and

effective enzymatic system consisting of methaemoglobin reductase, superoxide dismutase, catalase, glutathione peroxidase and glutathione reductase (Akintonwa, 2000). Activities of these enzymes depend on a constant supply of reduced nicotinamide dinucleotides (NADH/NADPH).

$$\begin{aligned} HbFe^{2+}+O_2 \quad &=>HbFe^{2+}-O_2 \iff HbFe^{3+}-O_2^- \\ &=>HbFe^{3+}+O_2^- \end{aligned} \quad (1)$$

Ultrapurified free Hb is not expected to be associated with RBC-like protection, because of the loss of enzymes and co-factors during separation. Reconstituting free Hb preparations with this redox machinery will be futile because of its loss after intravenous administration. Large amounts of oxidized methaemoglobin may contribute to the oxidative injury in the recipients (Alayash et al., 1994; Vercellotti et al., 1994; Dorman et al., 2002). For instance, oxidized Hb serves as a proinflammatory agonist in vascular endothelium and induces recruitment of polymorpho-nuclear cells (Silva et al., 2009). Several investigators have attempted to conjugate free radical scavenging enzymes (catalase and superoxide dismutase) with Hb in order to mitigate oxidative injuries (Chang et al., 2000; Powanda and Chang, 2002; Malhotra et al., 2003). Others have attempted the conjugation of glutathione and adenosine (Simoni et al., 2000), or the use of ascorbate-glutathione antioxidant system to control oxidative degradation of Hb (Simoni et al., 2009).

Encapsulation of Hb compartmentalizes it in a manner similar to that of RBCs. Encapsulation is purported to mitigate several toxicities associated with free and modified Hb preparations (Sakai et al., 2010). In addition, it offers an opportunity to partially reconstitute the redox systems of RBCs with an assurance of its association with Hb through the life of the preparation in circulation (Chang et al., 2000; Teramura et al., 2003). The goal of this research was to stabilize Hb that has been encapsulated inside the liposomes. Co-encapsulation of catalase with Hb to reduce the rate of oxidation *in vivo* has been attempted (Teramura et al., 2003), but systematic preformulation studies addressing this aspect of product design are lacking.

In LEH, it is possible to co-encapsulate ingredients of the enzyme system with Hb. Our report suggests that together with catalase various small molecular co-factors can significantly decrease Hb oxidation at body tempera ture. Over the 24 h incubation period at 37°C, CRM reduced the oxidation of Hb by more than 33%, suggesting that co-encapsulation of CRM with Hb might be a useful strategy in managing the rate of methaemoglobin formation after administration at body temperature. Another related and important factor in the formulation development of oxygen carriers is their shelf-life and storage conditions. Oxidation of Hb is influenced by the temperature and inertness of the storage atmosphere. Since the oxidation reaction is temperature sensitive, HBOCs are stored at 4°C. The reaction is further slowed if the preparation is deoxygenated by purging it with an inert gas during storage (Sakai et al., 2000). Previously, encapsulated Hb containing 0.1 mM β-NAD, 100 mM d-glucose, 2 mM adenine and inosine, 1 mM $MgCl_2$ and KCl, 9 mM KH_2PO_4 and 11 mM Na_2HPO_4 has been reported to reduce methaemoglobin formation from 1%/h to 0.4%/h at 37°C (Ogata et al., 1997). The effectiveness of the simultaneous use of reductants (thiols, ascorbate, glutathione, cysteine, homocysteine and methylene blue, etc.) depends upon their redox potential relative to that of ferrous–ferric system (Takeoka et al., 1998; Teramura et al., 2003). For instance, cysteine is pro-oxidative, but homocysteine in presence of catalase and superoxide dismutase improves the reduction process (Takeoka et al., 1998). The use of glutathione, cysteine, homocysteine and deferoxamine as reducing agents in isolation may be detrimental to the Hb molecule, especially in the concentration used in this study. It must also be noted that pH has a profound effect on the rate of Hb

oxidation, and acidic pH may enhance oxidative damage to the Hb molecule (Mansouri and Winterhalter, 1973; Hoffman and Bull, 1976).

Conclusions

We report an optimal mixture of catalase and co-factors for use in controlling undesirable oxidation of Hb in HBOCs during manufacturing and storage as well as after administration. Encapsulated Hb can partially emulate RBCs by providing compartmentalized Hb and co-encapsulated enzymes and co-factors. Using the results described herein, our laboratory is now engaged in formulating an LEH formulation with these substances. Even if the long-term stability of Hb may not be ensured by CRM, any delay in oxidative degradation of Hb is of significance to product development and efficacy.

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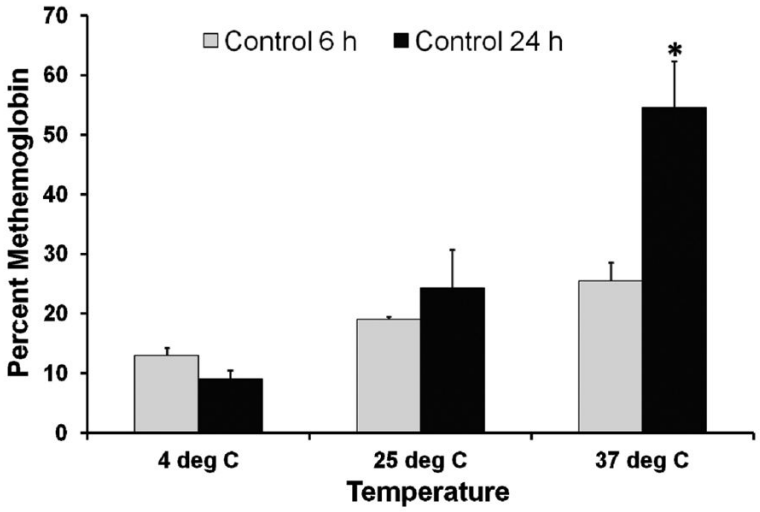

Figure 1.
Effect of temperature and duration of incubation on oxidation of Hb to methaemoglobin (*$p < 0.05$).

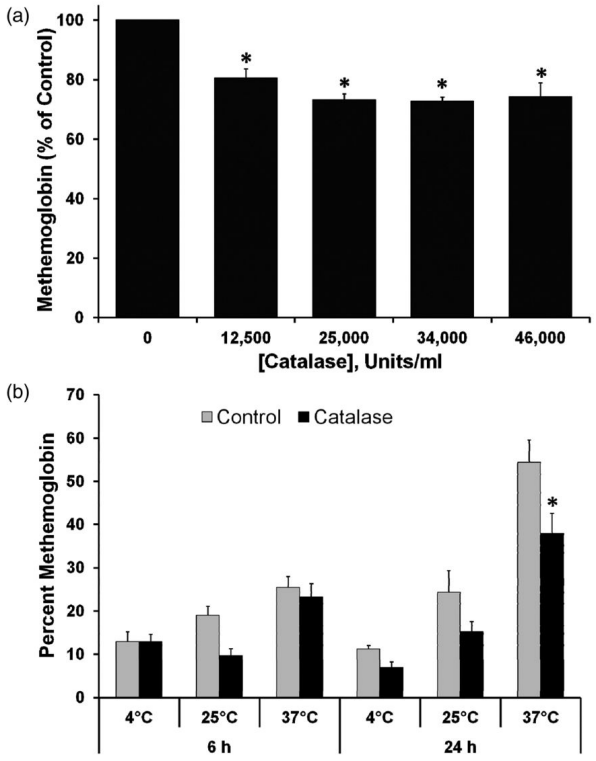

Figure 2.

Presence of catalase reduces the amount of methaemoglobin formation: (a) Hb was incubated for 24 h at 37°C with increasing concentrations of bovine catalase. Approximately, 25 000 U/mL was found to be optimum catalase concentration. The percent methaemoglobin is plotted as percent of control where the control is Hb incubated at 37°C for 24 h without any additions. Asterisk in the figure indicates that the difference from control incubation (0 U/mL) is significant ($p < 0.05$) and (b) temperature dependence of catalase activity in controlling Hb oxidation. In all incubations, 24 000 units of catalase was used per millilitre volume. Asterisk indicates significance ($p < 0.05$) with respect to respective control without catalase.

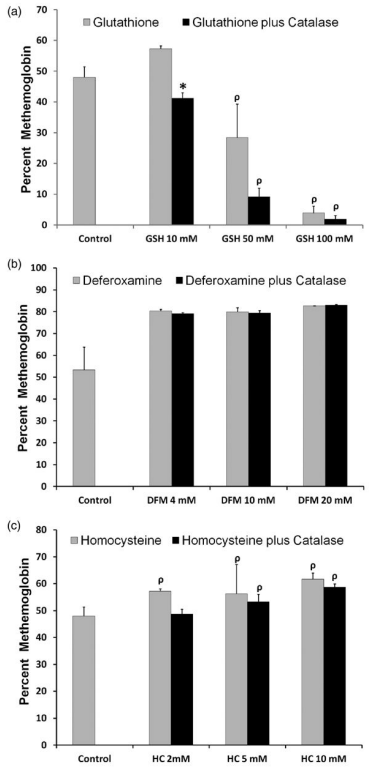

Figure 3.

Effect of reductant and catalase (25 000 U/mL) combination on Hb oxidation for 24 h at 37°C: (a) glutathione (GSH); (b) deferoxamine (DFM); and (c) homocysteine (HC). The reduction in methaemoglobin formation by these reducing agents was because of the remarkable precipitation and denaturation of Hb found in the samples marked ρ. Asterisk indicates significance ($p < 0.05$) with respect to respective control without catalase.

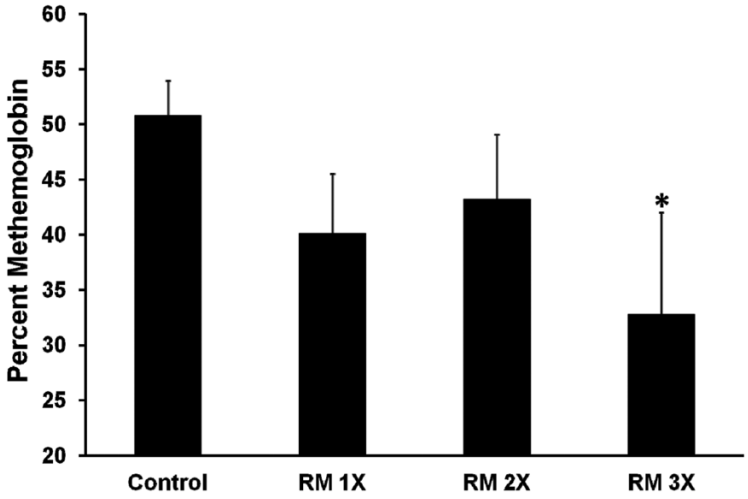

Figure 4.

Addition of RM to the Hb solution decreases methaemoglobin formation at 37°C for 24 h. Asterisk in the figure indicates that the difference from control incubation is significant ($p < 0.05$).

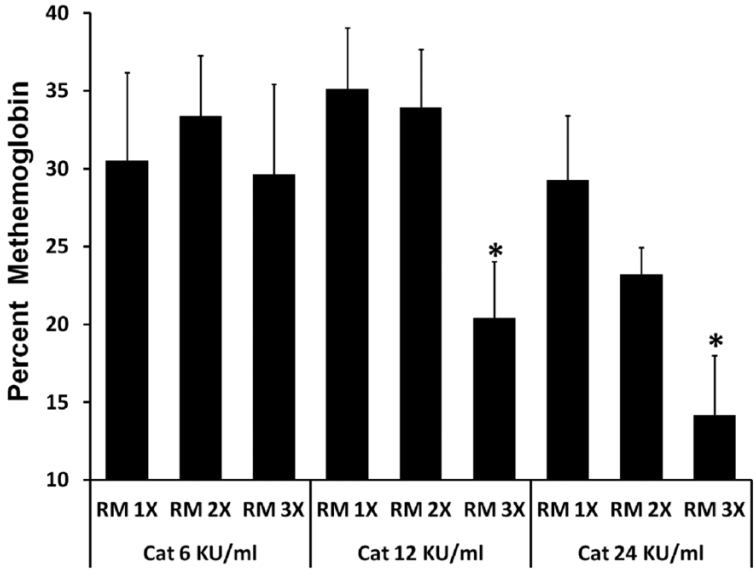

Figure 5.
Catalase (Cat) and RM synergistically decrease Hb oxidation over 24 h at 37°C. Asterisk in the figure indicates that the difference from control incubation is significant ($p < 0.05$).

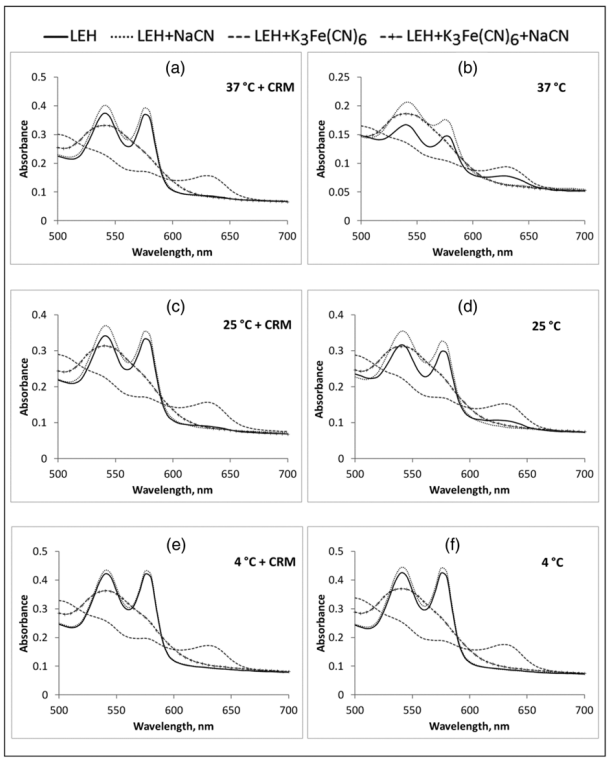

Figure 6.

(a–f) Spectra of Hb extracted from LEH incubated at various temperatures for 24 h. The spectrum of sample without CRM kept at 4°C was similar to that shown for the sample with CRM at 4°C, implying no oxidative degradation. However, the spectrum at 37°C is markedly altered as compared to that at 4°C. The presence of CRM subdues Hb oxidation, as is demonstrated by the relatively unaltered spectrum of 37°C + CRM sample. The results are representative of two separate LEH preparations each run in duplicates.

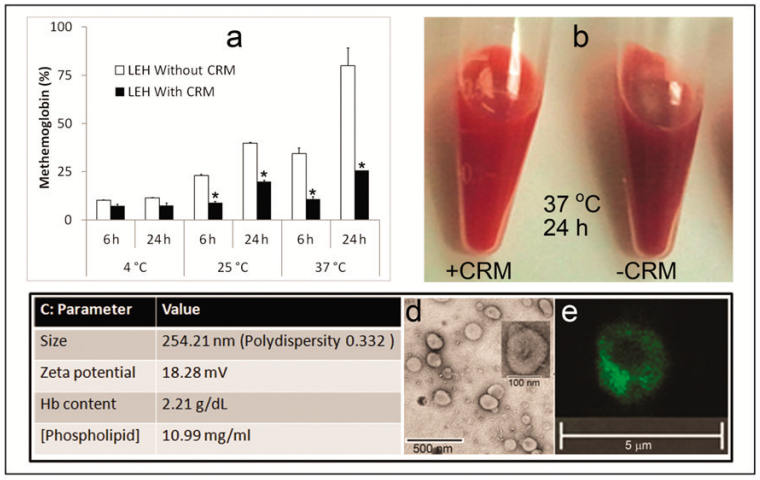

Figure 7.

(a) The absorbance values of variously treated Hb at isobestic point (563 nm from Figure 6) were used to calculate %MetHb in Hb extracted from LEH (*$p < 0.05$); (b) the protective effect of CRM on Hb oxidation is visualized by reduced darkening of Hb in CRM-stabilized LEH (+CRM) as compared to the LEH samples without CRM (−CRM). The LEH samples incubated at 37°C for 24 h are shown; (c) characteristics of LEH preparation carrying co-encapsulated CRM; (d) a transmission electron micrograph of LEH preparation is shown; and (e) A DiO-stained confocal image of an LEH particle. The DiO-stained LEH was prepared at a larger size because the resolution of confocal microscopy does not permit elucidation of structural details of nanometre-sized liposomes.

Table 1

Composition of RMs.

Ingredient	RM (mM)	CRM
Catalase	-	24 000 U/mL
β-NAD	0.01	0.03 mM
d-glucose	100	300 mM
Adenine	2	6mM
Inosine	2	6mM
Magnesium chloride	1	3 mM
Potassium chloride	1	3 mM
Potassium dihydrogen phosphate	9	27 mM
Disodium hydrogen phosphate	11	33 mM