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Thermal and Optical Radiation Effects on Liposomes Encapsulated Haemoglobin

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Synthetic red blood cells known as liposomes encapsulated haemoglobin (LEH) is a material that offers an excellent alternative to substitution blood since the chemical composition of the liposomes encapsulated oxyhaemoglobin (Oxy LEH), is similar to that of the natural blood cells. Unfortunately, this alternative, like the natural blood, has a limited shelf life at room temperature due to thermal, and / or solar (optical) radiations exposure. In this study experiments were run on LEH assays, with and without protection additives, to weigh up the effect of exposing LEH to thermal effect, using a digital controlled oven, and to optical radiations, using an appropriate apparatus to stimulate the solar optical radiations. Constant evaluation of the acceptability of this artificial blood alternative was done with the help of an advanced computerized technique. The results of the experiment have shown that exposing LEH to thermal and optical radiations leads to a reduction of the concentration of the oxyhaemoglobin and a heightened of the concentration of the methaemoglobin. The rate of LEH degradation in the two treatments was linked to the time in which they were exposed both to the thermal and optical radiations effects. Antioxidants, ascorbic acid and α-tocopherol were used in order to conserve and guard the prepared LEH from the degradation that happens to it due to the effects of both thermal and optical radiations.

Key Words: Hb; Haemoglobin. LEH; Liposomes encapsulated haemoglobin. OxyLEH; Liposomes encapsulated oxyhaemoglobin. HiLEH; Liposomes encapsulated methaemoglobin. HbLEH; Liposomes encapsulated deoxyhaemoglobin. CoLEH; Liposomes encapsulate carboxyhaemoglobin.

INTRODUCTION

There is a growing interest in the development of synthetic alternatives for genuine blood. The three alternatives that we currently have are: perfluoro compound emulsion, oxygen-bonding chelates and solutions of encapsulated haemoglobin LEH[1–12]. The third alternative *i.e.* solutions of encapsulated oxygenated haemoglobin is the predominant one since its chemical structure resembles that of genuine blood. The encapsulation of oxygenated haemoglobin is crucial as it conserves the oxygen-carrying reliability and helps the efficiency

of oxygen in the blood run. It is imperative that the encapsulated material is compatible to the genuine material and it must not result in changing the properties of the oxygen-carrying medium. It has been observed that liposomes encapsulated haemoglobin (LEH) is a promising alternative[13]. Emulsions can be encapsulated in the interior aqueous phase of liposome or dissolved in the lipid membrane, depending on their chemical properties. Moreover, cholesterol can be added to the liposome formulation to increase the fluidity of the membrane. On the other hand, ionic compounds provide a surface charge, and antioxidants inhibit lipid peroxidation. In addition, liposome suspension shows customary colloidal osmotic pressures at high oxygen-carrying capacity, low toxicity and adequate circulation life times.

In this study haemoglobin was extracted from fresh human blood RBC. Beissinger, Farmer and Gossage's[9] formula was used, after making some improvements on it, to prepare the liposome mixture. The molar ratio of the four lipid components is 5 : 4 : 1 : 0.4, consisting of the hydrogenated soy phosphatidylcholine (HSPC), cholesterol (CHOL), dicetyl phosphate (DCP) dimyristoyl phosphadidylcholine can also be used instead of DCP and the α-tocopherol.

EXPERIMENTAL

Liposome Preparation: The hydrogenated soy phosphatidylcholin (99%) was obtained from the American Lecithin Company, (Oxford, Connecticut, U.S.A). It contained 80–85% distearoyl-PC, 10–15% dipalmitoyl-PC and traces of lysolecithin. About 1.0 g of material was used in each 2.0 g sample. Cholesterol, which was obtained from Sigma Company, was also added at the rate of 0.8 g per sample to protect the LEH against lysis and fusion. It reduced membrane permeability to small ions[14] and helped to bring the erythrocytes osmotic pressure close to its natural reading. Likewise, Dicetyl phosphate, also obtained from Sigma Company was added at a rate of about 0.2 g to reduce the aggregation tendency in the liposome. In order to inhibit oxidation of the haemoglobin and lipids, α-tocopherol, was added. The amount used per sample was about 0.04 g.

The components were dissolved in 120 mL of 1 : 1 chloroform-ethanol in a round bottom flask. The solvent was then simultaneously evaporated under partial vacuum and light heating, using Buchi, rotary evaporator model number RE 111. The glass containing the solution was placed in water bath with constant temperature maintained at 30°C. The water bath type was # 461 supplied by Buchi. A vacuum of 10^{-4} mm Hg was maintained by means of a vacuum pump.

The evaporated solvent was condensed in a condenser that was cooled by tap water. Then, the samples were evaporated to dryness in about 2 h. Consequently the dried material formed a thin film coating at the bottom of the flask.

LEH Preparation: Sterile defibrinated human blood was obtained from Ravens Wood Hospital in 100 mL bottles. To prevent creating vacuum inside the bottle and to suppress oxidation of the blood, nitrogen gas was injected into the bottles using sterilized syringes. 20 mL of human blood was transferred from the bottle and was mixed with 20 mL of washing buffer, which consisted of 154 mM NaCl in 10 mM, and with pH 7.4 potassium phosphate buffers. It was centrifuged

at 3000 rpm for 10 min at 4°C. The centrifuge that was used was a Sorval RC-5B Refrigerated Centrifuge fitted with an SS-34 rotator. The supernatant was discarded and the washing process was repeated until the supernatant was entirely removed. The residual RBC material was added to 500 mL of lysis buffer, consisting of 5.0 mM NaCl and 0.5 mM EDTA in pH 8.0 potassium phosphates buffer, and stored at 5°C for 1 h, swirling gently every 10 min. The suspension was centrifuged at 15000 rpm for 35 min at 4°C and the supernatant, which contained the pure haemoglobin, was transferred to a container and kept in an ice bath.

The oxygenated haemoglobin solution was concentrated by dialysis. Standard dialysis tubing (M.W. cutoff 12,000–14,000 from Fisher Scientific Company) was cut into 30 cm long strips and located in boiling water with small quantity of EDTA (anti-coagulation) for 1 h. The geared up haemoglobin was then transferred to the swollen dialysis tubes with both ends tightly clipped and then covered with Aquacide II to absorb the lysis buffer. After 30 minutes, the wet aquacide layer adsorbed on the surface of the tube was removed. This process was frequently done and the total volume of oxygenated haemoglobin was reduced to 20 mL, which, as the original volume, did not change.

The optical absorption spectra of the outcome was measured, using advanced computerized techniques, to assure that the oxygenated haemoglobin abundance is in the acceptable percentage, and to validate the cleanliness of the sample and guaranteed that no transformation of the oxyhaemoglobin to methaemoglobin had occurred[14, 15]. The optical density of the sample was measured and compared with a typical absorption spectrum of an authentic blood sample. The peaks of the spectrum at 542 and 576 nm recognized the oxyhaemoglobin. The occurrence of additional peaks at 500 and 630 nm indicated transformation of oxyhaemoglobin to methaemoglobin, and led to the rejection of the sample. The 542 nm absorbance of the sample was 0.432, corresponding to the haemoglobin concentration of 13.2 g/100 mL. The cyanment method, based on the conversion of all haemoglobin to cynmethaemoglobin[16,17] was used to calculate the total haemoglobin concentration in the sample.

After diluting the concentrated material with the reagent solution, the sum Hb concentration C_1, was calculated. The reagent was prepared by dissolving 200 mg of $K_3[Fe(CN)_6]$, 50 mg of KCN, and 140 mg of KH_2PO_4 in distilled deionized water and made up to 1.0 L. The pH was adjusted to 7.4 and the solution was kept in brown borosilicate bottle. Finally, the molar concentration was determined using the equation:

$$Ct = [(OD.F)/(\varepsilon.L)]$$

Where F is the dilution factor (220 : 1); OD is the optical density at 540 nm, ε is the value of the quarter millimolar extinction coefficient of haemoglobin-cyanid at the wave length of 542 nm, which equals 11.51 $mmol^{-1}$ cm^{-1}, and L is the light path equal to 1.00 cm. The dilution factor F was adjusted with the optical density, which was about 0.435 in order to keep the error in C_2 at the smallest quantity.

LEH Types Preparation: The four types of liposome-encapsulated

haemoglobin were prepared using the film hydration technique. These were OxyLEH, HiLEH, HbLEH and CoLEH. The first type OxyLEH was prepared by adding a concentrated oxyhaemoglobin solution to the dry lipid film, and the mixture was stored at 4°C for about 15 h for hydration to take place. The hydrated sample was then bath sonicated for about 4 h, with a constant flow of nitrogen on the surface. The resulting product was then centrifuged at 1500 rpm at 4°C tor 6 min to remove the unencapsulated material. Next, the supernatant was transferred to a clean centrifuge tube, and centrifuged again at 1500 rpm at 4°C for 45 min to isolate the LEH product as a residue at the bottom of the tube. After that, the LEH product was washed three times with washing buffer solution and the resulting pellet was resuspended in the buffer solution. This LEH suspension constituted our final product. Its colour was pinkish-orange. The concentrated LEH preparation was diluted to different levels of concentrations in order to identify the optimal concentration range for spectrophotometric analysis.

The second type, HiLEH, was prepared by adding 30 mg of potassium ferricyanide to 10 mL of concentrated fresh oxyhaemoglobin. The ferricyanide acts to oxidize the OxyHb to HiHb. The excess ferricyanide was then removed, using a Sephadex G-25 sieving column eluted with washing buffer. The product was collected by an auto fractioner in a test tube and analyzed, using spectrophotometer, to determine the methaemoglobin fraction. The second step was to concentrate the dilute methaemoglobin, using the dialysis method. Finally, the concentrated solution was added to the liposome film. The rate for the process was exactly the same as that used for the preparation of OxyLEH.

The third type, HbLEH, was prepared by two methods. The first method involved mixing 10 mL of freshly prepared OxyLEH with 4.0 mg of sodium dithionite crystals. Reducing the OxyHb to Hb in the LEH oxidized sodium dithionite. The second method involved degassing of the OxyHb under vacuum. Nitrogen was then added to prevent air leakage, which resulted in oxidizing the Hb back to OxyHb.

The fourth type, CoLEH, was prepared by exposing 4.0 mL of and freshly prepared diluted LEH samples to carbon monoxide. The OxyLEH sample was placed in a cuvette equipped with a magnetic stirrer and sealed with an airtight septum. Carbon monoxide was introduced into the cuvette from a cylinder connected with the cuvette, while the OxyLEH was stirred.

Thermal or optical exposure of LEH samples showed a significant decrease in the concentration of OxyHb, accompanied by an increase in the concentration of HiHb. This conversion continued at an appreciable rate for several hours. Under typical summer field conditions (37–40°C) the computerized analysis of many tested samples indicated that significant oxidation, about 50% of the OxyHb to HiHb, occurred approximately during the first 3 h of exposure when no additives were present, as shown in Fig. 1.

The mechanism of the oxidative degradation reaction is not fully understood. However, several investigators have proposed free-radical reactions as the basis for such a mechanism, which contributes to the aging and cellular breakdown[18–21] of the samples. They have hypothesized that the decomposition of lipid hydroperoxides, which is accelerated by exposure to heat and/or light sources

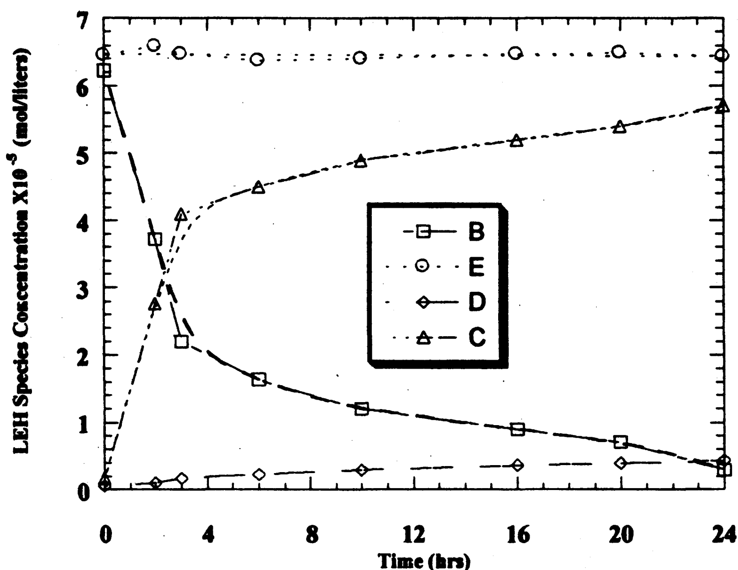

Fig. 1. Incubation of LEH sample at 37°C with no additives. The legend letters represent: B: HbO_2, C: Hi, D: Hb, E: the measured species sum.

especially in the presence of transition metal catalysts, can initiate destructive radical reactions. This hypothesis is substantiated by experimental evidence. Moreover, it is well established that polyunsaturated fatty acids in lipids are susceptible to oxidation. The unsaturated lipid in this study is hydrogenated soy phosphatidylcholine (HSPC), which is known to form lipid hydroperoxides[22–24].

The factors driving peroxidation reactions are a high degree of unsaturation in the lipid, substrate, a rich supply of oxygen, and a presence of transition metal catalysts, such as Fe^{2+}. Unfortunately LEH has all these characteristics. Therefore, it is essential to protect them from heat and light sources. Tappel[25] has shown that haemoglobin and other haematin compounds lower the activation energy of haemolytic decomposition of lipid hydro peroxide to the corresponding free radicals from 15.2 to 3.3 kcal/mol, and shorten the induction period for this reaction. Exposure to thermal energy or to light radiation could easily provide the reduced activation energy and start a chain reaction.

RESULTS AND DISCUSSION

Thermal Exposure Degradation: The effect of thermal exposure was investigated by incubating samples of varying initial composition at 0, 37 and 40°C for extended periods of time with and without the addition of antioxidants. The results are summarized below:

1. Storage of LEH at 0°C followed by thawing caused only minor changes in the composition of the material. The decrease in the concentration of

oxyhaemoglobin is always less than 6.15%, even after 48 h.

2. When the samples were incubated at 37°C with no additives a harsh degradation occurred at the end of the first 3 h, as shown in Figure 1. The rate of degradation was approximately stopped after the first 7 h, and reduced by about 40%, or more after that. Moreover when ascorbic acid at a concentration of 5.0 mM, with α-tocopherol at a concentration of 0.4 mM, was added, the degradation process was virtually stopped approximately during the first 8 h. It appears that a concentration of 5.0 mM of ascorbic acid is near the optimum, since increasing it further than 5.0 mM resulted in more rapid degradation.
3. When the samples were incubated at 37°C, 100% of the OxyHb was destroyed in about 24 h (Fig. 2).

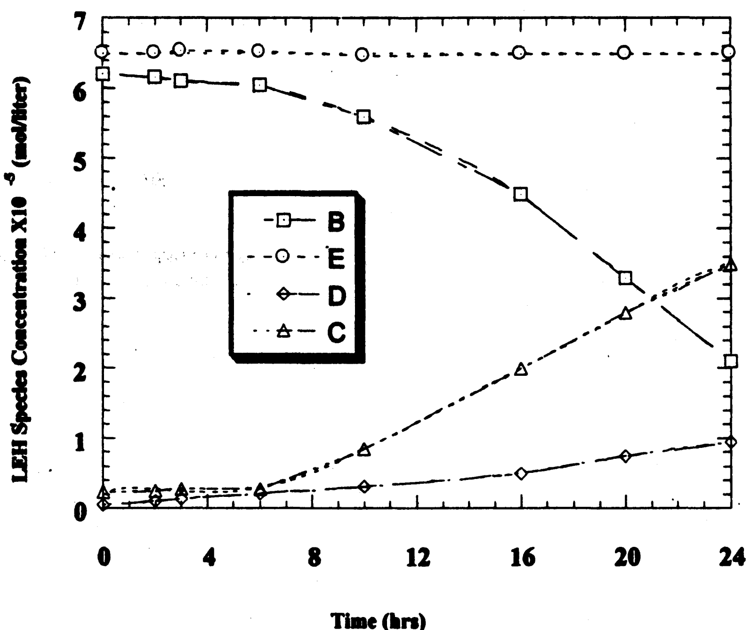

Fig. 2. Incubation of LEH samples at 37°C in presence of ascorbic acid 5 mM, α-tocopherol 1 : 10. The legend letters represent: B: HbO_2, C: Hi, D: Hb and E: the species sum.

Optical Exposure Degradation: The effect of optical exposure was investigated by exposing samples of LEH to optical radiations in the visible range. It was observed to cause significant changes in its composition including the oxidation of the (OxyHb) to (HiHb) and (Hb). Fig. 3. shows the changes in haemoglobin species concentration of LEH during irradiation by an HgXe arc, as stimulator to solar radiations, related to the change in the radiation exposure time. Fig. 4 shows the effect of both the ascorbic acid and α-tocopherol against the optical solar radiation effect. Significant reduction in the degraded rate was obviously achieved as a result of these additives. The results suggest that exposure to sunlight shouldn't exceed 30 minutes even when antioxidants were added.

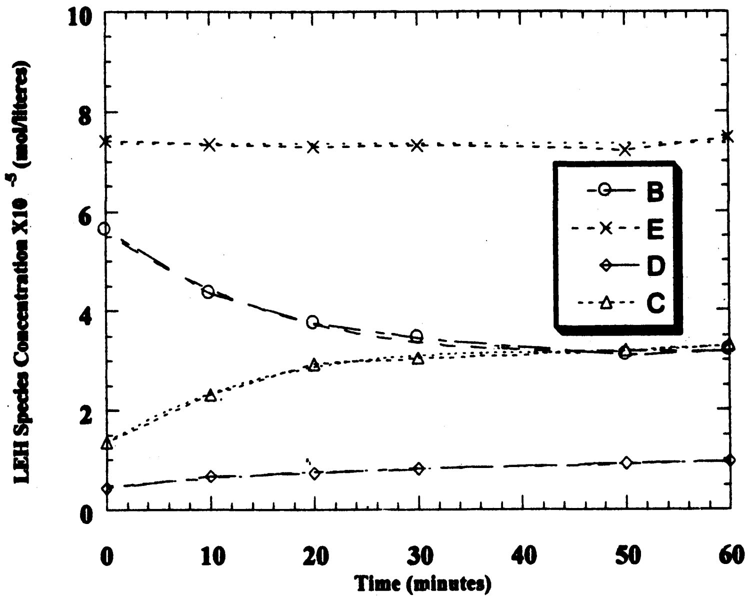

Fig. 3. Changes in concentration of haemoglobin derivatives in LEH during irradiation by HgXe arc. The legend letters represent: B: Hb_2O C: Hi, D: Hb, E: the species sum.

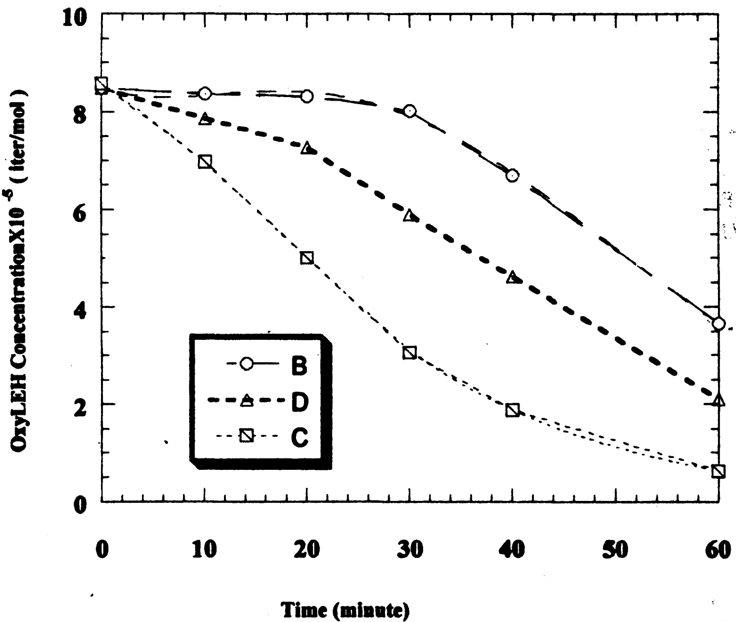

Fig. 4. Changes in HbO_2 concentration as a function of antioxidents, α-tocopherol 1 : 10, for all. Ascorbic acid: B: 5.0 mM, D: 3.0 mM, C: 0.0 mM.

Conclusion

Thermal exposure of the prepared LEH samples at differing length of time, showed a significant decrease in the concentration of oxyhaemoglobin, accompanied by an increase in the concentration of methaemoglobin. This tendency continued at a considerable rate for several hours depending upon the severity of the exposures. Under typical summer field conditions (37–40°C) the computerized optical spectra of many tested samples indicated that, significant oxidation of the OxyLEH to HiLEH, occurred during the first 3 h of exposure with out additives, while with additives, the time was extended to about 8 h. Moreover, to preserve the integrity of the LEH at high temperature and under optical exposure levels, the formula recommended by Beissinger *et al.*[9] for the liposome preparation, which is HSPC = 5, HC = 4, DCP = l, α-tocopherol = 0.2, was improved on to the formula, HSPC = 5, HC = 4, DCP = 1, α-tocopherol = 0.4, adding ascorbic acid, at a concentration of 5.0 mM. The modified LEH using ascorbic acid and α-tocopherol showed increased resistance to thermal degradation mechanisms during the first 9 h, and to optical degradation mechanisms during the first 0.5 h.

Optical exposure showed a fast significant degradation of the OxyLEH in the prepared LEH to HiLEH; and with preserved additives the maximum period is just 0.5 h under the solar radiations exposure (Fig. 3).

Another significant conclusion throughout the comparison of Fig. 3, and Fig. 4, showed that the composition of the OxyLEH started a fast degradation without the presence of the antioxidant materials, and as a result of the sunlight effect at room temperature.

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