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Effect of α-Tocopherol on the Rate of Photohemolysis of Liposome Encapsutated Haemoglobin

MARWAN A. ALFAHHAD
Department of Chemistry, Riyadh College of Technology, P.O. Box-25744, Riyadh-11476, Saudi Arabia
E-mail: alfamar@rct.edu.sa

Deformation of the liposome encapsulates the oxyhemoglobin upon exposure of liposome encapsulated haemoglobin to light wave and oxygen (photooxygenation conditions), due to the production of cholesterol hydroperoxide, can be controled depending on the results of this study. The present study suggests that α-tocopherol prohibits the production of cholesterol hydroperoxide in the liposome capsule, which if produced leads to a weakened capsule observed as a deformed Liposome Encapsutated Haemoglobin under the electron microscope.

Key Words: Liposme encapsulated haemoglobin, Liposome, Haemolysis, Hydroperoxide, Oxyhaemoglobin, α-Tocopherol.

INTRODUCTION

Many studies on photooxygenation conditions of the human erythrocyte membrane have been shown to be susceptible to damage by exposure to long ultraviolet irradiation[1–4]. Some materials have been used to prohibit the production of this problem like β-carotene[5, 3] and α-tocopherol (vitamin E)[4, 6].

Liposome Encapsutated Haemoglobin is the most promising synthetic alternative to the genuine natural human blood[7], it is the predominant one since its chemical structure resembles that of genuine blood. The encapsulated oxygenated haemoglobin is crucial, as it conserves the oxygen carrying reliability and helps the efficiency of oxygen in the blood run. 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 pressure at high oxygen capacity, low toxicity and adequate circulation life times.

In this study, haemoglobin was extracted from fresh human blood RBC. Beissinger, Farmer and Gossage's[6] 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 hydrogenated soy phosphatidyl choline (HSPC), cholesterol (CHOL), ductile phosphate (DCP); dimyristoyl phosphatidylcholine can also be used instead of DCO and α-tocopherol.

The following work is directed toward the determination of the efficiency of α-tocopherol and to estimate its capability to inhibit the LEH budded due exposure to long wavelength ultraviolet irradiation in the presence of oxygen as a function of time.

EXPERIMENTAL

Hydrogenated soy phosphatidylcholine (99%), contains 80–85% distearoyl-PC, 10–15% dipalmitoyl-PC and traces of lysolecithin. About one g of material was used in each 2 g sample. Cholesterol was added at the rate of 0.8 g per sample to protect the LEH against lysis and fusion at the normal conditions. Cholesterol also acts to reduce membrane permeability to small ion and helps bring the erythrocytes osmotic pressure close to that of natural systems. Dicetyl phosphate was added at a rate of about 0.2 g to reduce the aggregation tendency in the liposome. The α-tocopherol was added to inhibit oxidation of both haemoglobin and lipids. The amount used per sample was about 0.04 g.

The components were dissolved in 120 mL of chloroform : ethanol 1 : 1 in a round-bottom flask. The solvent was then evaporated under partial vacuum and slight heating using Buchi rotary evaporator model # RE 111. The glass containing the solution was placed in a constant temperature water bath, supplied by a Buchi # 461 water bath, maintained at 30°C.

A vacuum of 10^{-4} nm Hg was maintained by means of vacuum pump. The evaporated solvent was condensed in a condenser that was cooled by tap water. The samples were evaporated to dryness in about 2 h. The dried material formed a thin film coating the bottom of the flask.

Blood samples were taken from donors and mixed with 20 mL of washing buffer, consisting of 154 mM NaCl in 10 mM, pH 7.4 potassium phosphate buffer, and centrifuged at 3000 rpm for 10 min at 4°C. The supernatant was discarded and the washing process was repeated until the supernatant was entirely clear. The residual RBC material was added to 500 mL of lysis buffer, consisting of 5 mM NaCl and 0.5 mM EDTA in pH 8.0 potassium phosphate buffers and stored at 5°C for 60 min, 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 haemoglobin solution was concentrated by dialysis. Standard dialysis tubing (M.W. cutoff 12,000–14,000 from Fisher Scientific Company) was cut into one-foot long strips and placed in boiling water with small amount of EDTA (anti-coagulation) for 60 min. The prepared hemoglobin was then transferred to the swollen dialysis tubes with both ends tightly clipped and then covered with Aquacide II to absorb the lysis buffer. The wet aquacide layer absorbed on the surface of the tube was removed after 30 min. This process was repeated until the total volume of hemoglobin was reduced to 20 mL, which is the same as the original volume of the blood sample. The optical absorption of the hemoglobin product was measured using integrating sphere spectrophotometer to verify the purity of the sample and to ensure that no oxidation of the oxyhaemoglobin to methemoglobin had occurred, throughout the optical density of the sample.

LEH was prepared using the film hydration method. Concentration oxyhaemoglobin solution was added 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 using (Ultrasonic FS-14), with a constant flow of nitrogen at the surface. The resulting product was then centrifuged at 1500 rpm at 4°C for 6 min to remove the unencapsulated material. The supernatant was transferred to a clean centrifuge tube and centrifuged again at 15000 rpm at 4°C for 45 min to isolate the LEH product as a residue at the bottom of the tube. The LEH product was washed three times with washing buffer solution and the resulting pellet resuspended in the buffer solution. This LEH suspension constituted the final required product for this work. Its colour was pinkish-orange. The concentrated LEH preparation was diluted to different concentrations in order to identify the optimal concentration range for spectrophotometer analysis.

LEH photooxygenation: Prepared LEH, diluted 1 : 1 with isotonic buffer (0.125 M NaCl, 20 nM phosphate buffer, pH 7.4), placed in Warburg flasks and fixed with 0.2 mL of heparin. The flasks were flushed with an atmosphere of pure oxygen and then a positive oxygen pressure continued during the irradiation. The samples were immersed in a constant temperature bath (25°C) and irradiation with 50 watt sunlamps from below. Samples were removed at periodic times and cooled to 10°C until prepared for electron microscopy. At this stage of work the α-tocopherol as antioxidant is not used in the isotonic buffer formula while it will be an essential component of it in the second stage of this work, used with 0.45 mM concentration, in order to prohibit the budding phenomenon.

The electron micrographic samples were prepared by OsO_4 fixation and capsule fabrication from polymethylmethacrylate[7]. Each sample was thin sectioned and the erythrocytes from 10 representative sections of each capsule counted using electron micrographic pictures. A minimum of 400 LEH were counted per section, meaning each sample had a minimum of 4000 LEH examined in determination of the extent of budded LEH formation expressed in a per cent of total LEH as shown in Fig. 1, in the first stage, with different concentrations of antioxidant, starting from zero, and Fig. 2, represents the ultimate value of antioxidant concentration.

Assay of cholesterol hydroperoxide: Procedure of Marchesi and Palda[8] was used to treat the oxygenated LEH samples in order to prepare hemoglobin free LEH. The liposome was extracted with ten volumes of diethyl ether in dark environment at a temperature of 10°C during 25 h. After that, the ether was decanted away and concentrated using a slow stream of nitrogen. The concentrate was analyzed for the presence of cholesterol hydroperoxide by thin-layer chromatography on silica gel at standard authentic cholesterol hydroperoxide prepared by the method of Bagli[9]. The presence of cholesterol hydroperoxide was indicated by staining with *p*-phenylenediamine dihydrochloride[10] and cholesterol by charring with concentrated sulphuric acid.

RESULTS AND DISCUSSION

The cholesterol material present in the LEH membrane, *i.e.*, in the capsules themselves which are equal to 0.8 g per sample that is to protect the LEH against lysis and fusion at the normal conditions[11], is converted to cholesterol hydroperox-

ide 1 when the LEH stained with sensitizing dye are exposed to light and oxygen. Exposure of LEH to light and oxygen most properly leads to excitation by the capsulated haemoglobin[10]. The excited oxygen would then be capable of attacking the membrane's cholesterol leading to the cholesterol hydroperoxide 1, as awaking of the membrane, and lysis of the LEH at the previous mentioned conditions.

LEH samples were stabilized in the heparinized saline solution and exposed to light and oxygen at room temperature, *i.e.*, 24°C. The suspension was fixed by standard electron micrography techniques and sections examined for visual demonstration of membrane destruction.

The electron micrographs displayed a high percentage of the LEH exhibiting a budded configuration as shown in Fig. 1.

The results of the first part of the present work shown in Fig. 1 strongly indicate

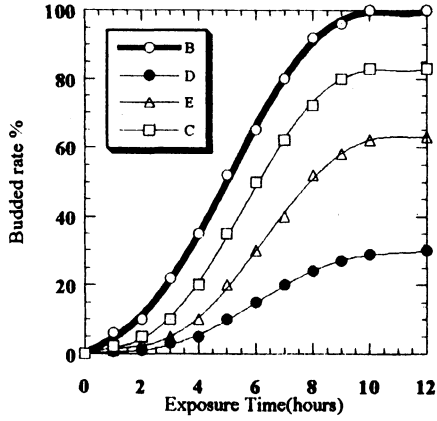

Fig. 1. LEH budded rate with 0 mM(B), 0.1 mM(C), 0.2 mM(E) and 0.3 mM(D) alpha-tocopherol

that the budded rate of LEH, without α-tocopherol, increases with the exposure time, while complete destruction takes place after a period of about nine exposure hours. More than this, Fig. 1 strongly indicates that the budded rate of LEH decreases as the concentration of α-tocopherol increases. It is found as one of the essential results of this study that the LEH photochemolysis at the oxygenation conditions can be described as a sigmoid function.

Obviously, the y-axis indicates the budded rate starting from zero to minimum value to the maximum value hundred per cent, while the x-axis indicates the exposure time.

The results of the second part, after the usage of α-tocopherol, (Fig. 2), strongly indicate that the budded rate of LEH increases at a very slow rate with the exposure time at a concentration equal to 0.45 mM; the maximum destruction rate which equals about 6% is reached after a period of thirteen exposure hours. It is found as one of the essential results of this study that the LEH photohemolysis at the oxygenation conditions in the presence of α-tocopherol, 0.45 mM, can be described as a polynomial function of power nine, with coefficients as follows:

$$y = a_0 + a_1x + a_2x^2 + \ldots + a_9x^9$$

where $a_0 = 0.4567$, $a_1 = 0.7910$, $a_2 = -0.0245$, $R^2 = 0.9778$.

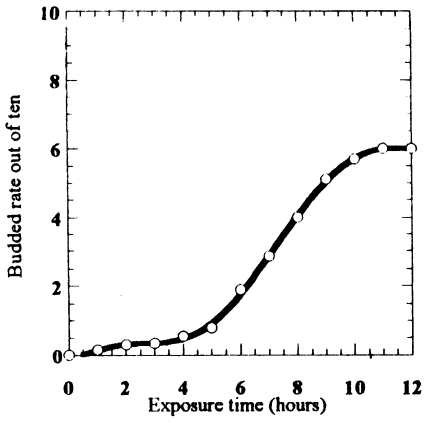

Fig. 2. LEH Budded rate after exposure to oxygenation conditions, with alpha-tocopherol

These results strongly enable us to introduce the above mentioned polynomial function as a new distinguished model describing the LEH photohemolysis process at the previous specific conditions, with α-tocopherol, remembering the polynomial coefficient values.

The effect of α-tocopherol is suggested to be that of chemically trapping the oxidant species before reaction with liposome cholesterol, which results in liposome weakening, evidenced by bedding, accompanied by production of hydroperoxide 1 in the LEH taken before introduction of α-tocopherol regimen.

REFERENCES

1. L.C. Harber, A.S. Fleischer and R.L. Baer, *J. Am. Med. Ass.*, **189**, 191 (1964).
2. M.M. Mathews-Roth, M.A. Pathak, T.B. Fitzpatrick, L.C. Harber and E.H. Kass, *New Engl. J. Med.*, **282**, 123 (1970).
3. C.S. Foote, Y.C. Change and R.W. Denny, *J. Am. Chem. Soc.*, **93**, 5218 (1970).
4. B.D. Goldstein and L.C. Harber, *J. Clin. Invest.*, **51**, 892 (1972).
5. A.A. Lamola, T. Yamani and A.M. Trozzol, *Science*, **179**, 1131 (1973).
6. G.W. Grams, K. Eskins and G.E. Inglett, *J. Am. Chem. Soc.*, **94**, 866 (1972).
7. F.N. Low and J.A. Freeman, in Electron Microscopic Atlas of Normal and Leukemia Human Blood, Mc Graw-Hill Book Co., New York, pp. 2–7 (1958).
8. V.T. Marchesi and G.E. Palade, *J. Chem. Biol.*, **35**, 385 (1967).
9. L. Bagli, *J. Am. Chem. Soc.*, **83**, 1498 (1961).
10. L.L. Smith and F.L. Hill, *J. Chromatogr.*, **66**, 101 (1972).
11. M.A. Alfahhad, *Asian J. Chem.*, **13**, 1500 (2001).

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