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Liposome Encapsulated Hemoglobin Photosensitization by Protoporphyrin

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Liposome encapsulated hemoglobin (LEH) photosensitization by the localized protoporhyrin in the LEH membrane leads to liposome membranes damage of the liposome-encapsulated hemoglobin through out the photooxidation process of the LEH target in the visible electromagnetic wave range. The paper presents an empirical study of the photohemolysis of the LEH in the presence of protoporphyrin. In previous studies, it has been shown that the membrane damage cannot be attributed to photooxidation of unsaturated fatty acid side chains. But recent experiments showed that some amino acid residues of membrane proteins, unsaturated fatty acid side chains of phospholipids and cholesterol are sensitive to photooxidaton, induced by protoporphyrin. The significance of this paper is that cholesterol appeared to be very sensitive to protoporphyrin induces photooxidation in the LEH membrane, not the phospholipids unsaturated fatty acid side chains in the liposomes construction.

Key Words: Lipsome encapsulated hemoglobin, Liposome, Hemolysis, Hydroperoxide.

INTRODUCTION

Liposome encapsulated hemoglobin (LEH) undergo photohemolysis in the presence of protoporphyrin up-on irradiation *in vitro* with visible electromagnetic waves in the region (400-700 nm). The significant importance of this study is the actual reliable enhancement of LEH as a synthetic alternative to the normal blood[1-3]. The LEH response to visible electromagnetic radiation in the presence of protoporphyrin sensitizer as well as normal blood with slightly little difference in the characteristics hemolytic curve, indicate the domination of the same mechanism in both red blood cells and the LEH. The cause of LEH photohemolysis is mainly induced by protophyrin throughout the oxidation of the LEH membrane as a target of the incident radiations. It could be shown that the process cannot attribute to peroxidation of phospholipids unsaturated fatty acid side chains in the liposomes[4]. As prolonged illumination with visible electromagnetic light of LEH in the presence of protoporphyrin led to an almost complete

photooxidation of some amino acid residues in the structure of LEH membrane, in compression, the suggestions that the membrane proteins are presumably the primary target of the protoporphyrin induced photodynamic damage of the red blood cell membrane[5,6], is accepted in this work.

The paper of Lamola *et al.*[7] demonstrated that cholesterol hydroperoxides formation in photophyrin containing red blood cells ghosts, after prolonged irradiation with approximately 400 nm wavelength light. In model, expriments it was shown that cholesterol hydroperoxide in red blood cell may cause hemolysis under certain conditions. On the basis of observations, these authors suggested that photohemolysis of red blood cell from EPP patients might be explained along these lines. Further more in LEH the ghost in the present work undergo photophymolisis uner photooxygenation conditions, explained on the cholesterol hydroperoxide which led to the deformation of the LEH[2].

In the present work, and for the discrimination purpose, investigation for both cholesterol and membrane proteins were done. Studies on protophyrin induced cholesterol peroxidation and posible significance in photohemolysis in LEH are also reported.

EXPERIMENTAL

LEH preparation

Hydrogenated soy phosphatidylcholin (99 %), contains 80-85 % distearoyl-PC, 10-15 % dipalmitoyl-PC and traces of lysolecithin. About 1 g of material was used in each 2 g sample. The cholesterol was added at the rate of 0.8 g per sample to protect the LEH against lyses 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. Dicetyle phosphate was added at a rate of about 0.2 g to reduce the aggregation tendency in the liposome. The α-tocopherol was aded to inhibit oxidation of both hemoglobin and lipids. The amount used per sample was about 0.04 g.

The components were dissolved in 120 mL of 1:1 choloroform-ethanol 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 30ºC.

A vacuum of 10^{-4} mm Hg was maintained by a 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 300 rpm for 10 min at 4ºC. 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 lyses buffer, consisting of 5 mM NaCl and 0.5 mM EDTA in pH 8.0 potassium phosphate buffers and stored at 5ºC for 1 h, swirling gently every 10-min. The suspension was centrifuged at 15000 rpm for 0.5 h at 4ºC and the supernatant, which contained the pure hemoglobin was transferred to a container and kept in an ice bath.

The hemoglobin 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 1 h. The prepared hemoglobin was then transferred to the swollen dialysis tubes with both ends tightly clipped and then covered with Aquacie II to absorb the lyses buffer. The wet aquacade layer adsorbed on the surface of the tube was removed after 0.5 h. 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 oxyhemoglobin to methemoglobin had occured through out the opitcal density of the sample.

LEH was prepared using the film hydration method. Conc. oxyhymoglobin solution was added to the dry lipid film, and the mixture was stored at 4ºC 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 4ºC for 6 min to remove the un-encapsulated material. The supernatant was transferred to a clean centrifuge tube, and centrifuged again at 15000 rpm at 4ºC 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 suspensions according to Shothorst *et al.*[6], LEH lipids were extracted according to Deierkauf and Booij[8]. Cholesterol was measured according to Hanel and Dam[9]. Separaton and determination of phospholipids was done by paper chromatography[7]. Fatty acid analysis was performed with gas-chromatography.

Illumination was carried out with a 125-watt super high-pressure mercury lamp. K^+ leakage was determined by measuring the K^+ concentration in the medium with a flame photometer. Osmotic fragility of the cell was measured as described by Van Steveninck *et al.*[9], but the incubation period in the hypotonic NaCl solution was two min only.

Peroxide was measured with the KCNS method described by Robey and Wiese[11], with 2-methoxy-methanol, instead of chloroform, as solvent.

Separation of phospholipids and cholesterol photooxidation products was accomplished by thin layer chromatography with the solvent system benzene/acetone (90:10. v/v)[9]. Detection of oxidation products was performed according to Smith and Hill[12]. Subsequent qualitative analysis was performed by applying the sample in the form of a narrow, 15 cm long sterak on the TLC plate. After developing, a 2 cm wide strip of the plate was sprayed with N,N,N′,N′–tetramethyl-*p*-phenylenediamine dihydrochloride, to localized peroxides[11]. Guide by this reference strip the silica gel of the remaining part of the TLC plate was scraped off in small tubes and incubated with the NCNS reagent for quantitative analysis. Other reaction products could be detected after spraying with $2NH_2SO_4$ and heating at 80ºC.

The velocity of the oxidation was studied with a manometric technique, utilizing diffrential respirometer units with air as the gas phase.

RESULTS AND DISCUSSION

To evaluate the experimental results of the photooxidation of the LEH membranes, the lipid composition had to be studied in the normal range with a mean value of 3.04×10^{-1} μmol/cell, in good agreement with a mean value of 3.2×10^{-1} μmol/cell for normal red blood cells[8]. The phospholipids and fatty acid composition of both natural red blood and LEH are almost the same.

The velocity of the sensitizer protoporphyrin induced photooxidation of linoleic acid, oleic acid, dioleoyl lecithin and cholesterol in chloroform is shown in Fig. 1. With or without illumination in the absence of protophyrin no oxygen uptake could be measured.

Fig. 2 shows similarity in the velocity of photooxidation in 33 % alcohol. Fig. 3 shows the formation of KCNS reactive peroxide under the mentioned experimental conditions. The main oxidation products obtained by photooxidation of cholesterol appeared to 3β-hydroxy-5α-choelste-6-ene-5-hydroperoxide, in agreement with the result of Smith and Hill[12] and Lamola *et al.*[7].

Fig. 4 shows measurements fragility of LEH during the illumination in the presence of protoporphyrin. Samples for determination of K^+ leakage and osmotic fragility were taken after illumination periods of 0, 5, 10, 15, 20, 25 and 30 min. During the prelytic phase, with K^+ leakage increasing

from zero to 100 %, there was a gradual increase of osmotic fragility, with no concomitant change of the shape of the curve.

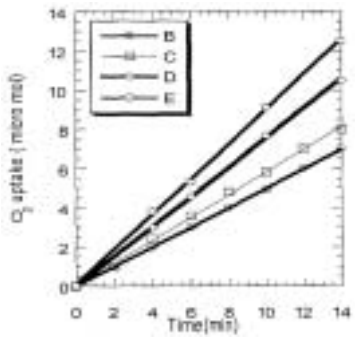

Fig.1 : Photoxidation E-inchloroform 33 mM linolei acid, D-33 mM oleic acid, C-16.5 mM dioleoyl lecithin, B-33 mM cholesterol, in 0.03 mM protoporphyrin at 25ºC

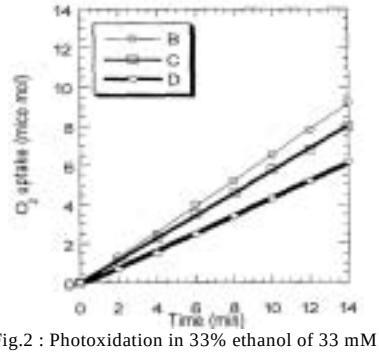

Fig.2 : Photoxidation in 33% ethanol of 33 mM linolec acid, B, 33 mM oleic acid, C, 33 mM cholesterol, D, in the presence of 0.03 mM protoporphyrin at 25ºC

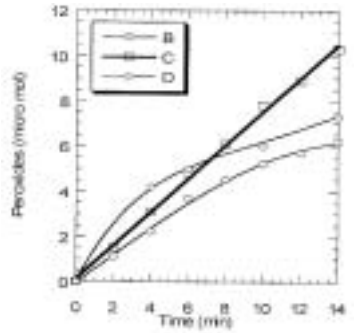

Fig.3 : Formation of KCNS-reactive peroxide during photoxidation in chloroform of 33 mM linoleic acid D, 33 mM oleic acid C and 33 mM choleterol B in the presence of 0.03 mM protoporphyrin at 25ºC

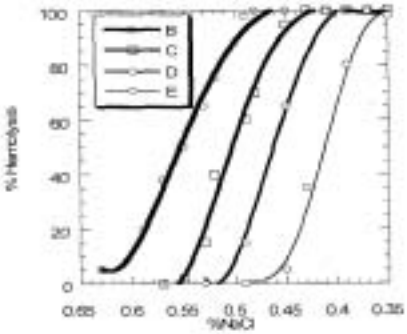

Fig.4 : Osmotic fragility of LEH, illumination period B-25, C-20, D-15, E-5 min.

LEH photohemolysis can be caused by cholesterol hydroperoxide formation, this fact based on several assumptions and observations. In first place, the formation of cholesterol hydroperoxide in LEH, during prolonged illumination in the presence of excess protoporphyrin demonstrated. These results are confirmed by the present work and agreed with De Goeig and Van Steveninck[5], while contradict with Lamola *et al.*[7]. They assumed cholesterol should be more reactive than the unsaturated fatty acid side chains. In model system it appeared that photooxidation of unsaturated fatty acids produced slightly faster photooxidation of cholesterol, both in chloroform and in 33% alcohol. In illumination, the calculated lipid composition ratio of phospholipids to cholesterol peroxide was about 2.7. For the same ratio, double bonds are also equal to 2.7. These facts indi-

cated clearly that LEH cholesterol is not more sensitive to photooxidation than the unsaturated fatty acid side chains.

Fig. 1 and Fig. 3, both of them show that there is a very close parallel between oxygen uptake and peroxide formation with oleic acid. Both with linoleic acid and with cholesterol the curve of peroxide formation flattens slightly. In the case of linoleic acid, this can be explained by the formation of secondary products from the initial peroxide. For cholesterol, the formation of secondary products from the initial peroxide has been described by Young *et al.*[10] and Robey & Wiese[11]. It is presumed that these products are represented by the spots on TLC not reacting with N,N,N′,N′-tetramethyl-*p*-phenylenediamine dihydrochloride, but detectable after H_2SO_4 treatment.

The slightly slower oxidation of dioleoyl lecithin, as compared to oleic acid at the same effective concentration of these compound, may be attributed to the presence of choline group in the molecule. Quenching of photodynamic reactions by amines has been described by Young *et al.*[10]. Considering modern concepts of membrane architecture it may be assumed that a possible quenching effect of choline group in biological membranes would protect unsaturated fatty acid side chains and cholesterol to about the same extent.

Fig. 4 shows that there is no inclination towards an initial decrease of the fragility, but only a gradual increase during the whole illumination period. Moreover, there is no change in the shape of the curve.

These results clearly indicate that photohemolysis of LEH cannot rather be rationalized based on a cholesterol peroxidation nor on oxidation of unsaturated fatty acid side chains in the membrane. The most likely explanation of protoporphyrin- induced photohemolysis to be the photooxidation of amino acid residue in the membrane proteins. The results in the present work after the usage protoporphyrin, showed in Fig. 4. This indicate strongly that the hemolytic rate of LEH increase in a very slow rate with the exposure time at a concentration equal to 0.03 mM. The maximum destruction rate which equal to about was happened at different periods of exposure. It has found as one the essential results of this study that the LEH hemolysis at the presence of protoporphyrin, 0.03 mM, can described as a polynomial function of the power nine, with coefficients, as the following:

$$y = a_o + a_1x + a_2x^2 + ... + a_9x^9$$

$$a_o = 1.1005E5$$

$$a_1 = -1.007E6$$

$$a_2 = 7.064E7$$

$$R^2 = 0.99032$$

Conclusion

The LEH hemolytic mechanism is almost follows the same function as described by Alfahhad[3], but with different polynomial coefficients values. This can attributed to the effect of protoporphyrin sensitization in hemolytic process.

REFERENCES

1. M.A. Alfahhad, Field Deployable Methods for the Characterization and Preservation of Liposome Encapsulated Hemoglobin, PhD. Theses, IIT, Chicago (1996).
2. M.A. Alfahhad, *Asian J. Chem.*, **13**, 1500 (2001).
3. M.A. Alfahhad, *Asian J. Chem.*, **17**, 1853 (2005).
4. A.F.P.M. de Goeij and J.V. Steveninck, *Clin. Chim. Acta*, **68** (1976).
5. A.A. Schothorst, J.V. Steveninck, L.N. Went and D. Suurmond, *Clin. Chim. Acta*, **39**, 161 (1972).
6. A.F.P.M. de Goeij , P.H.J.Th. Ververgeart and J.V. Steveninck, *Clin. Chim. Acta*, **62**, 287 (1975).
7. A.A. Lamola, T. Yamane and A.M. Trozzolo, *Science*, **179**, 1131 (1973).
8. F.A. Deierkauf and H.L. Booij, *Biochim. Biophys. Acta*, **150**, 214 (1968).
9. H.K. Hanel and H. Dam, *Acta Chem. Scan.*, **9**, 667 (1955).
10. R.H. Young, R.L. Martin, D. Feriozi, D. Brewer and R. Kayser, *Photochem. Photobiol.*, **17**, 233 (1973).
11. R.F. Robey and H.K. Wiese, *Ind. Eng. Chem. Anal. Ed.*, **17**, 425 (1945).
12. L.L. Smith and F.L. Hill, *J. Chromatogr.*, **66**, 101 (1972).

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